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Devenir des antibiotiques lors du traitement aérobie et anaérobie des boues de STEPs pour une valorisation agronomique

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Avant propos

Ces travaux de recherche ont été réalisés en cotutelle de thèse dans le cadre d'un projet Franco-marocain (Toubkal : TBK/16/33) entre le Laboratoire Ecologie et Environnement (L2E) de l'université Cadi Ayyad de Marrakech (Maroc) et le Laboratoire Ecologie Fonctionnelle et Environnement (Ecolab) UMR CNRS-UPS-INPT et plus particulièrement au sein de l'équipe Ecolotoxicologie Intégrative (ECI). Les travaux de thèse ont été aussi réalisés en collaboration avec le Laboratoire Biotechnologie de l'Environnement de Narbonne (Unité INRA, France) et l'unité de recherche Innovations thérapeutiques et résistances (InTheRes) de l'Ecole Nationale Vétérinaire de Toulouse (France). Une bourse d'excellence du Centre National de la Recherche Technique et Scientifique (CNRST, Maroc) a été accordée dans le cadre de ce travail de thèse.

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Résumé

L'utilisation massive des antibiotiques contribue à leur accumulation dans les boues des stations d'épurations. L'application directe des boues est parmi les sources de dissémination des antibiotiques et des gènes de résistance aux antibiotiques. Le compostage et la méthanisation sont parmi les bioprocédés de traitement des boues qui permettent d'éliminer ou réduire les teneurs de certains antibiotiques. Dans ce travail, une boue primaire de la STEP de Marrakech a été contaminée par trois familles d'antibiotiques (macrolides, tétracyclines, fluoroquinolones) pour conduire 4 essais de compostage à différentes doses (dont un essai témoin) et un essai de méthanisation en mode semi-continu.

Les résultats du compostage ont montré que l'augmentation des concentrations d'antibiotiques retarde la dégradation de la matière organique et affecte le ratio C/N. De même, la phase thermophile est perturbée, retardée et réduite dans le temps. Pour la méthanisation, une concentration unique et réaliste a été testée. Dans ces conditions, aucun effet sur la production du biogaz ou sur la dégradation de la matière organique n'a été observé.

Afin de suivre la dissipation des trois familles d'antibiotiques utilisées au cours du compostage et de la méthanisation, une approche analytique basée sur l'extraction accélérée par solvant (ASE) suivie par l'application d'une méthode des ajouts dosés avant quantification par chromatographie liquide couplée à de la spectrométrie de masse en tandem (UPLC-MS/MS) a du être mise en point. Le compostage et la méthanisation permettent de réduire significativement les concentrations des molécules parents appartenant à la famille des macrolides et des tétracyclines. Par contre, l'élimination des fluoroquinolones est non-significative et ne dépasse pas 30%. Au cours du compostage, la dissipation des macrolides se fait en phase de stabilisation tandis que la phase de maturation est impliquée dans la dissipation des tétracyclines. Les concentrations en cirprofloxacine (fluoroquinolone) semblent légèrement évoluer au cours du procédé probablement en raison d'une adsorption/désorption sur le co-substrat lignocellulosique utilisé. Concernant la méthanisation, l'élimination des macrolides et des tétracyclines est significative durant la stabilisation du procédé mais n'atteinds pas les rendements observés lors du compostage.

La diminution des concentrations des molécules parents est probablement accompagnée par une biotransformation des antibiotiques sous forme de métabolites qui à ce stade ne sont pas connus. La question de la rémanence de certaines molécules comme les fluoroquinolones, interpelle quand au risque d'antibiorésistance. Ainsi, la valorisation des composts/digestats comme amendements organiques des sols dois à terme conduire à une réflexion concernant la réglementation qui inclus la présence de molécule de la classe des antibiotiques.

Mots clés : Antibiotiques, macrolides, tétracyclines, fluoroquinolones, compostage, méthanisation, ASE, LC-MS-MS

Abstract

The intensive use of antibiotics for human purposes leads to their presence and accumulation in the sludge produced from wastewater treatment plants. The direct application of sludge is among the sources of dissemination of antibiotics and antibiotics resistance genes. Composting and anaerobic digestion are some of the most used bioprocess for sludge treatment, and which allow the removal/decrease of some antibiotics families. In this work, a primary sludge from the wastewater treatment plant of Marrakesh was spiked using 3 families of antibiotics (macrolides, tetracyclines, fluoroquinolones) to conduct (1) 4 composting experiments with various concentrations levels, and (2) an anaerobic digestion experiment in a semi-continuous mode.

Composting results showed that the organic matter degradation was delayed and the C/N ratio was affected by an increase of antibiotics concentrations. Likewise, the thermophilic stage was disturbed, the heat release was affected and the coming of the temperature maxima was delayed. In the other hand, one realistic concentration was used during the anaerobic digestion. In this condition, no effect was observed especially on the biogas production as well as the organic matter degradation.

To assess the fate of antibiotics during composting and anaerobic digestion, an analytical approach based on the accelerated solvent extraction followed by the standard addition method and the UPLC-MS/MS was developed. Composting and anaerobic digestion lead to a significant removal of parent compounds belonging to the family of macrolides and tetracyclines. In contrast, the fluoroquinolones removal is not-significant and has not exceeded 30%. During composting, the thermophilic stage was responsible on macrolides elimination. In contrast, the maturation stage was more implicated on the removal of tetracyclines. Ciprofloxacin (fluoroquinolone) showed some fluctuations in concentrations. The sorption/desorption on palm rachis could probably explain the observed behavior of this molecule during composting. During the anaerobic digestion, the removal of macrolides and tetracyclines was significant, within the stabilization, but still lower than the observed ones during composting.

The decrease of parent compounds of antibiotics is probably accompanied by a biotransformation of these compounds in unknown metabolites. The presence of recalcitrant compounds after the bioprocess could promote the development of resistant bacteria including pathogens. In the future regulations, the valorization of compost/digestate as an amendment of agricultural soil requires taking into account the presence of antibiotics.

Key words: Antibiotics, macrolides, tetracyclines, fluoroquinolones, composting, anaerobic digestion, ASE, LC-MS-MS

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Liste des abbreviations

AC: Ash Content

AD: Anaerobic Digestion

ARB: Antibiotic Resistant Bacteria

ARG: Antibiotic Resistance Genes

ASE: Accelerated Solvent Extraction

$CH_{4:}$ Methane

CIP: Ciprofloxacin

$COD_{s:}$ Soluble Chemical Oxygen Demand

$COD_{t:}$ Total Chemical Oxygen Demand

CTC: Chlortetracyline

DM: Dry Matter

ENR: Enrofloxacin

ESI: electrospray ionization

FQs: Fluoroquinolones

HRT: Hydraulic Retention Time

LC-MS/MS: Liquid Chromatography coupled to tandem Mass Spectrometry

MDs: Macrolides

ME: Matrix Effect

MRM: Multiple Reaction Monitoring

NOR: Norfloxacin

OFL: Ofloxacin

OTC: Oxytetracycline

PLE: Pressurized Liquid Extraction

ROX: Roxithromycine

RSD: Relative Standard Deviation

SAM: Standard Addition Method

SDZ: Sulfadiazine

SMX: Sulfamethaxazole

SMZ: Sulfamethazine

SPE: Solid Phase Extraction

TC: Tetracycline

TCs: Tetracyclines

TN: Total Nitrogen

TOC: Total Organic Carbon

TS: Total solids

TSS: Total Suspended Solidss

TYL: Tylosine

VS: Volatile solid

VSS: Volatile Suspended Solids

WWTP: Waste Water Treatment plant

Introduction Générale

Depuis la découverte de la pénicilline en 1928, le développement de l'utilisation médicale des antibiotiques a permis de réduire considérablement le taux de mortalité des maladies infectieuses dans le monde entier. Au Maroc, comme à l'échelle internationale, les antibiotiques représentent les produits pharmaceutiques les plus utilisés (Klein et al., 2018). Au Maroc, l'exception d'une étude publiée en 2015, sur la consommation des antibiotiques (Inous et al., 2015), on peut souligner l'absence de valeurs officielles et fiables permettant d'estimer la consommation réelle de ces molécules en médecine humaine et vétérinaire. L'étude statistique réalisée était basée sur le système de classification anatomique-thérapeutique-chimique (TAC) et des doses journalières définies (DJD), qui est un système largement utilisé à l'échelle internationale. Les résultats de cette étude ont montré que la consommation des antibiotiques est passée de 9.68 DJD/1000Hab/jour en 2003 à 13.85 DJD/1000Hab/jour en 2012, soit une augmentation de 30% sur 9 ans. En 2012, la classe des pénicillines prédominait avec 59% de la consommation globale suivie par la famille des tétracyclines 11.9%, et la famille des macrolides avec 11.6% (Inous et al., 2015).

Selon une étude réalisée sur 76 pays sur la période allant de 2000 à 2015 (Klein et al., 2018), la Turquie et la Tunisie sont les plus grands consommateurs d'antibiotique au mode, avec une dose journalière de 50 DJD/1000Hab/jour. L'Espagne, la Grèce, l'Algérie et la Romanie se suivent dans ce classement avec environ 40 DJD/1000Hab/jour. La France est le 8ème consommateur d'antibiotique au monde avec 36 DJD/1000Hab/jour. En 2015, 786 tonnes d'antibiotiques destinés à la santé humaine et 514 tonnes d'antibiotiques destinés à la santé animale ont été vendus en France. Pour les grandes villes, la consommation humaine est de 29.9 DJD/1000Hab/jours (ANSES, 2016). Aux Etats Unis et au Canada, la consommation d'antibiotiques est de 28 et 20 DJD/1000Hab/jour respectivement. Selon les résultats de cette étude, la consommation d'antibiotiques au Maroc est faible et est essentiellement liée à l'utilisation des antibiotiques destinés à la santé humaine.

L'utilisation des antibiotiques en médecine humaine est accompagnée par leur élimination vers les stations d'épuration dans les flux d'eaux usées. Les stations d'épuration ne sont pas conçues pour l'élimination spécifique de ces composés chimiques. Ainsi, une partie de ces molécules est dégradée, la partie non dégradée durant le procédé d'épuration est soit concentrée dans les boues résiduaires soit pour les molécules les plus hydrophiles évacuées avec les eaux traitées vers les cours d'eau. En 2010 au Maroc, les systèmes d'épuration des eaux usées ont généré environ 40 000 T de boues. Selon les estimations, la production de boues atteindra 300 000 T/an en 2025 (ONEE, Maroc). Il est important de souligner l'absence de chiffres officiels quant à la valorisation des boues d'épuration au Maroc. En effet, ni le

plan national d'assainissement (PNA), ni le plan national des déchets ménagers (PNDM), ni la loi 28-00 relative à la gestion des déchets ménagers (ou assimilés) ne traitent de la gestion des boues d'épuration. Seule, la nouvelle loi sur l'eau 36-15 stipule dans ces articles 70 et 71 la nécessité de mettre en place des techniques de traitement. Leur utilisation agricole n'est pas officiellement autorisée, de même que leur mise en décharge contrôlée. Autrement dit, actuellement, les solutions appliquées pour éliminer ou valoriser les boues produites à l'échelle nationale sont réalisées sans encadrement institutionnel ou juridique. Dans ce sens, le Ministère de l'Environnement marocain a lancé en 2015 un appel d'offre Recherche et Développement en vue de proposer des solutions innovantes en matière de traitement et valorisation de boues. L'ensemble des résultats obtenus dans le cadre de cet appel d'offre devrait permettre la mise en place de mesures et des normes d'utilisation pour la gestion des boues à l'échelle nationale. Parmi les projets retenus, le Laboratoire Ecologie et Environnement de l'Université Cadi Ayyad a mis en place un projet de traitement et de valorisation industrielle des boues par co-compostage sur la période 2015-2017.

En France, les quantités des boues d'épuration sont encore plus importantes. En 2007, 9 millions de tonnes de boues brutes ont été produites, ce qui représente 22% de la quantité globale des déchets organiques produits (ADEME, 2012). Les boues sont des déchets organiques qui peuvent être valorisés en agriculture comme des fertilisants riches en nutriments et permettent une diminution de l'utilisation d'engrais chimiques. En France, 73% des boues produites sont utilisées en agriculture, 28% seulement sont compostées (Legroux and Trucht, 2009). La valorisation agricole des boues d'épuration est encadrée par l'arrêté du 8 janvier 1998 qui définit les conditions d'épandage.

Il est important de souligner que malgré les efforts déployés à l'échelle internationale, les normes d'épandage existantes ne tiennent toujours pas compte de la présence d'un grand nombre de molécules dans les boues d'épandages et ayant des effets reconnus sur les organismes terrestres et aquatiques. Les teneurs en médicaments comme les antibiotiques ne sont toujours pas règlementées.

L'épandage des boues de station d'épuration est parmi les sources anthropogéniques de dissémination des antibiotiques dans l'environnement la plus importante, avec l'usage des lisiers d'animaux d'élevage (Heuer et al., 2011 ; Mao et al., 2015). Par ailleurs, les antibiotiques sont présents dans la quasi-totalité des compartiments environnementaux, à savoir les sols, les plantes, les sédiments, les eaux de surfaces et souterraines (Hirsch et al.,

1999 ; Michael et al., 2013 ; Rico et al., 2014). La présence des antibiotiques dans tous les compartiments physiques et biologiques est considérée comme une des principales voies de développement de l'antibiorésistance, notamment des bactéries pathogènes pour l'homme (Binh et al., 2016). Selon l'organisation mondiale de la santé, la résistance aux antibiotiques constitue aujourd'hui l'une des plus graves menaces pesant sur la santé à l'échelle mondiale. Pour limiter les risques de dissémination des antibiotiques il devient primordial de s'assurer de l'innocuité des boues avant leur épandage. Pour réaliser cet objectif, des procédés de traitement des boues doivent être mis en place afin de mieux éliminer ce type de molécule. De même, il devient fondamental dans l'attente du développement de ces nouveaux procédés permettant d'éliminer au maximum ce risque, de traiter les boues en combinant élimination des antibiotiques et valorisation.

Le compostage et la méthanisation sont parmi les procédés les plus utilisés pour le traitement des boues d'épuration. Selon un audit réalisé par l'ADEME en 2006, il existe 820 plates-formes de compostage en France, d'une capacité moyenne de 10 000 tonnes par an, qui traitent 6 millions de tonnes de déchets par an dont 1 million de tonnes de boues d'épuration. Du point de vue méthanisation, la France comptait plus de 450 digesteurs en 2016. Le plan Energie Méthanisation Autonomie Azote (EMAA) vise l'implantation de 1000 digesteurs à l'horizon 2020. Au Maroc, la production de compost ou digestat susceptibles d'être commercialisés comme amendements organique est très limité voire inexistante. En effet, durant la période 1964-1980, 5 Unités de compostage des ordures ménagères ont été lancées dans les différents centres urbains (Tétouan, Casablanca, Meknes, Rabat, Marrakech), mais l'éxpéreince a échoué au bout de quelques années en raison de différents problèmes techniques et en autre autre la mauvaise qualité du compost produit, pour celles qui ont pu fonctionner. En 1990, une usine de compostage a été construite à Agadir en 1996, avec la société marseillaise-Canal de Provence, avec un budget de 32 millions dirhams, mais qui a connu le même échec. Actuellement, moins d'une dizaine d'unités de compostage des déchets verts, fumier et certains déchets agricoles existent et fonctionnent, mais le volume des composts produits demeure en-deça des besoins du marché marocain en amendements. En matière de biométhnisation, seule la station d'épuration de la ville de Marrakech et de Fès disposent d'un digesteur permettant la production d'une partrie d'énergie pour les besoins des stations. A ceci, s'ajoute la production de biogaz à partir de certaines décharges contrôlées et/ou réhabilées (Fez, Oujda, Marrakech…). Un travail important reste à faire pour la reconnaissance et le développement de ces filières au Maroc.

La ville de Marrakech compte plus d'un million d'habitant. L'activité touristique de la ville « ocre » a fortement contribué à son développement économique, urbain et démographique. Afin d'accompagner ce développement de mode vie sur le volet environnemental, une nouvelle station d'épuration a été construite en 2009 pour traiter les eaux usées de la ville. Avec une capacité de 120 000 m^3/j traitant 33 millions m^3 d'eau par an pour une charge de pollution journalière de 1.3 millions d'équivalents habitants, la station d'épuration de Marrakech est considérée comme la plus grande station au Maroc. Il s'agit d'un traitement biologique des eaux usées selon le procédé des boues activées suivi par un traitement tertiaire. En parallèle avec la politique nationale quant à la gestion des eaux usées épurées, la station d'épuration traitent les eaux usées pour une réutilisation en irrigation. L'épuration des eaux usées est accompagnée par une production de 140 t/jour de boues qui sont évacuées vers des sites de déchargement sans aucun traitement préalable. Actuellement, un projet ambitieux de séchage solaire (système serre) vient d'être achevé approximité de la décharge contrôlée de Marrakech, bien que la destinée des boues séchées n'est pas encore clairement identifiée.

A travers de multiples travaux de recherche réalisés sur les boues de Marrakech (Amir et al., 2005 ; Hafidi et al., 2008 ; El fels et al., 2015 ; Belloulid et al., 2016), il ressort que les boues contiennent des pathogènes, des parasites, des polluants inorganiques et organiques. Parmi ces polluants, des teneurs de polluants émergents (antibiotiques) ont été observées et varient entre 0.2 et 4.2 µg/kg DM (Khadra, 2015).

Dans ce contexte, les travaux de cette thèse ont porté sur le devenir des antibiotiques au cours de différents procédés de traitement des boues primaires de Marrakech par co-compostage et méthanisation. Afin de réaliser cette étude, les boues primaires ont été inoculées par différentes gammes de concentrations de 5 antibiotiques, les plus utilisés, appartenant à 3 familles, les tetracyclines (TCs), les macrolides (MDs) et les fluoroquinolones (FQs). L'objectif général de ce travail a été d'apporter des réponses quant au devenir de ces trois familles d'antibiotiques au cours des deux différents procédés testés. Afin de s'assurer de l'innocuité ou non des composts et digestats finaux, une approche analytique multi-résidus permettant le dosage des trois familles d'antibiotiques dans différentes matrices, boue, compost et digestat a été développée.

Le manuscrit se divise en six chapitres distincts. Le premier chapitre est constitué de deux revues bibliographiques sur : (1) les sources, le devenir et les effets des antibiotiques dans l'environnement. Une attention particulière a été portée sur le devenir de ces molécules durant

le compostage ainsi que leurs impacts sur l'antibiorésistance. (2) les approches analytiques utilisées pour l'extraction et le dosage des antibiotiques dans les boues et les composts.

Dans un deuxième chapitre, des essais de compostage ont été conduits pour suivre l'effet de doses croissantes d'antibiotiques sur les paramètres physico-chimiques et thermiques du compostage.

Le troisième chapitre est focalisé sur la mise en point d'une méthode analytique multi-résidu pour l'extraction et le dosage des antibiotiques.

Le quatrième chapitre présente l'application de notre méthode analytique pour suivre le devenir des antibiotiques au cours du compostage de boues.

Dans le cinquième chapitre, une synthèse bibliographique du devenir des antibiotiques au cours de la méthanisation a été présentée comme sous chapitre. Par la suite, notre protocole analytique a été utilisé pour suivre les performances de la méthanisation vis-à-vis l'élimination des antibiotiques.

Enfin, l'ensemble des résultats ont été confrontés lors d'une discussion générale, suivie par une conclusion et les perspectives.

Chapitre 1: Synthèse Bibliographique

Contexte :

L'utilisation massive des antibiotiques en médecine humaine ou vétérinaire contribue à leur accumulation dans les boues, issues des stations d'épurations des eaux usées, ou des fumiers après excrétion par les animaux, de 30 à 90% de la dose administrée se retrouve dans les urines et les fèces. L'application directe des boues et des fumiers est parmis les sources de dissémination majeure des antibiotiques. Ceux-ci contribuent grandement au développement de l'antibiorésistance (Binh et al., 2016).

Dans une revue générale soumise à « Journal of Hazardous Materials IF = 6.065 ; article en révision », sont présentés une vue d'ensemble de l'occurrence des antibiotiques dans les boues et les fumiers, ainsi que leur transfert vers les sols agricoles. Par la suite, le devenir des antibiotiques et des gènes de résistance aux antibiotiques au cours du compostage sont abordés en précisant les différents mécanismes d'élimination impliqués. Des pratiques innovantes ont été proposées pour mieux gérer les boues et les fumiers par optimisation des conditions de compostage afin d'assurer l'élimination/réduction des antibiotiques et leurs gènes de résistance.

Le suivi des antibiotiques dans les matrices solides (boues, compost, digestats) est un défi analytique à l'échelle internationale. Jusqu'à présent, il n'existe pas de méthode analytique standard pour une analyse multi-résidus d'antibiotiques dans ces matrices. En effet, la nature de celles-ci conduit à des interactions complexes entre antibiotiques et matière organique rendant leur analyse complexe. La deuxième partie de cette synthèse bibliographique consiste en une revue des méthodes analytiques existantes.

1. Revue générale sur l'occurrence des antibiotiques dans l'environnement et l'efficacité du compostage vis-à-vis l'élimination des antibiotiques et des gènes de résistance aux antibiotiques

Human and veterinary antibiotics during composting of sludge or manure: Global perspectives on persistence, degradation, and resistance genes.

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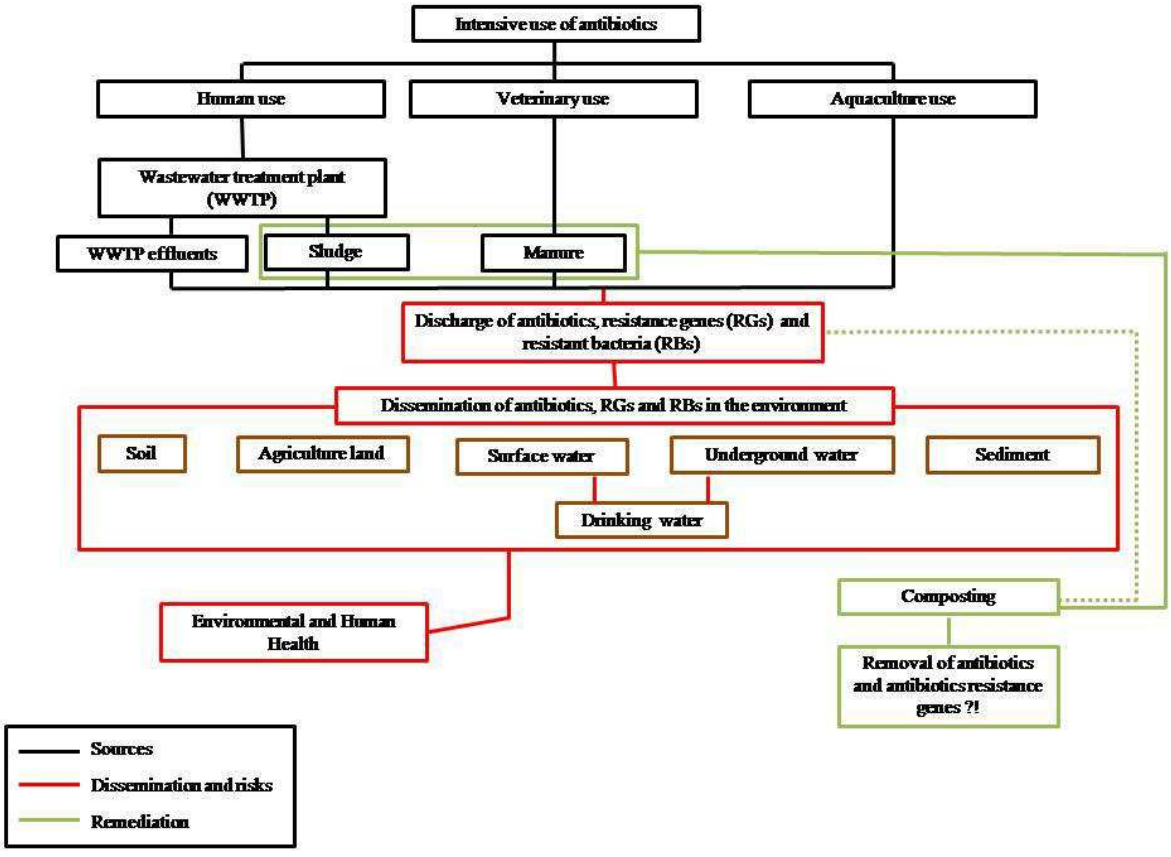
Graphical abstract



Abstract

Wastewater treatment plant effluent, sludge and manure are the main sources of contamination by antibiotics in the whole environment compartments (soil, sediment, surface and underground water). One of the major consequences of the antibiotics discharge into the environment could be the prevalence of a bacterial resistance to antibiotic. In this review, four groups of antibiotics (Tetracyclines, Fluoroquinolones, Macrolides and Sulfonamides) were focused for the background on their wide spread occurrence in sludge and manure and for their effects on several target and non-target species. The antibiotics concentrations range between 1 and 136,000 μg/kg of dry matter in sludge and manure, representing a potential risk for the human health and the environment. Composting of sludge or manure is a well-known and used organic matter stabilization technology, which could be effective in reducing the antibiotics levels as well as the antibiotic resistance genes. During sludge or manure composting, the antibiotics removals range between 17-100%. The deduced calculated half-lives range between 1 to 105 days for most of the studied antibiotics. Nevertheless, these removals are often based on the measurement of concentration without considering the matter removal (lack of matter balance) and very few studies are emphasized on the removal mechanisms (biotic/abiotic, bound residues formation) and the potential presence of more or less hazardous transformation products.

The results from the few studies on the fate of the antibiotic resistance genes during sludge or manure composting are still inconsistent showing either decrease or increase of their concentration in the final product.

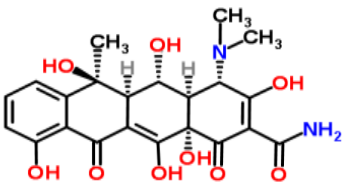
Whether for antibiotic or antibiotic resistance genes, additional researches are needed, gathering chemical, microbiological and toxicological data to better understand the implied removal mechanisms (chemical, physical and biological), the interactions between both components and the environmental matrices (organic, inorganic bearing phases) and how composting process could be optimized to reduce the discharge of antibiotics and antibiotic resistance genes into the environment.

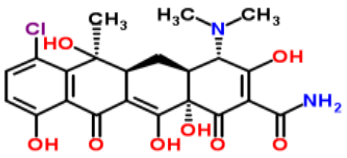
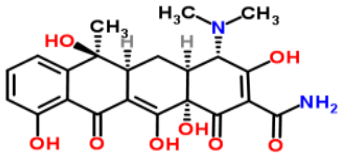
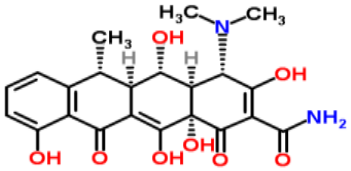
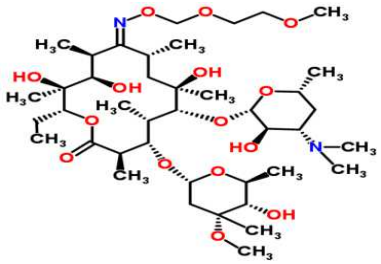
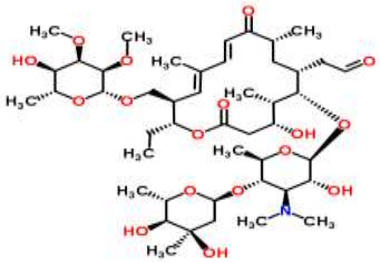
Keywords: Sludge, Manure, Composting, Antibiotics, Antibiotic resistance genes.

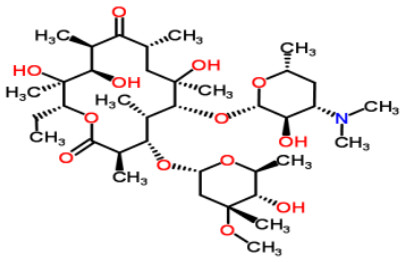
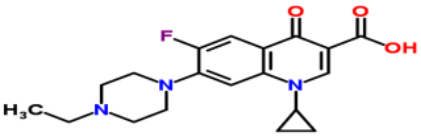
1. Introduction

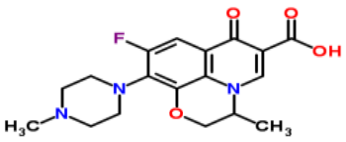
Antibiotics are widely used in human and veterinary medicine [1] (Table 1). The annual use of antibiotics was estimated to be between 100,000 and 200,000 t worldwide [2]. In Europe, 2/3 of antibiotics are used in human medicine and 1/3 for veterinary use [3]. In the United States, 16,000 t of antimicrobial compounds are used annually, 70% are used for non-therapeutic purposes [4]. The intensive use of antibiotics for human purposes leads to their continuous release into the WWTPs. Moreover, most of the WWTPs, based on the biological aerated system for example [5], were not designed to treat such compounds and present limited performances regarding their elimination [6, 7]. So, a part of these molecules is discharged by the WWTPs effluent toward the aquatic environment and the other part, adsorbed on sludge, could reach the soil according to the sludge use state rules [8, 9]. Hence, WWTPs are considered as an anthropogenic source for the environmental contamination by antibiotics [10]. Another hotspot of antibiotic dissemination in the environment is the veterinary use [11, 12]. Very high antibiotic concentrations varying from 91 to 136 mg/kg of dry matter (DM) of sulfonamides [13] and tetracyclines [14], and other molecules were found in manure. As a consequence of these massive discharges into the environment, antibiotics were found in each environmental compartment, in soil [15], sediment [16], surface and groundwater [17]. Such presence is one of the factors contributing to the spread of antibiotic resistance [18]. Thus, it is necessary to propose strategies to manage and reduce the dissemination of antibiotics [19].

Tableau 1: Selected examples of commonly used human and veterinary antibiotics and their physical and chemical properties

Families	**Molecules**	**pKa (25°C)**	**Chemical structure**	**Usage**
Tetracyclines	OTC	4.5		Humans and animals

Families	Molecules	pKa (25°C)	Chemical structure	Usage
	CTC	4.5		Animals
	TC	3.3		Humans and animals
	DOX	-		Humans and animals
Macrolides	ROX	12.45		Humans
	TYL	13		Animals

Families	Molecules	pKa (25°C)	Chemical structure	Usage
	ETM	8.8		Humans and animals
Sulfonamides	SMX	5.7		Humans
Fluoroquinolones	CIP	6.09		Humans
	ENR	2.74		Animals
	NOR	6.34-8.75		Humans

Families	Molecules	pKa (25°C)	Chemical structure	Usage
	OFL	5.97		Humans

OTC: Oxytetracycline; CTC: Chlortetracycline; TC: Tetracycline; DOX: Doxycycline;ROX: Roxithromycin; TYL: Tylosin;ETM: Erythromycin; SMX: Sulfamethaxazole;CIP: Ciprofloxacin; ENR : Enrofloxacin ; NOR: Norfloxacin; OFL: Ofloxacin

Composting is a bioremediation technology able to reduce or eliminate the residual concentrations of antibiotics present in sludge or manure before their application to agricultural fields. The assessment of composting efficiency toward the elimination of antibiotics and their ARGs is still in progress, and needs more knowledge. In the light of the recent researches, providing a global vision and perspectives about the fate of antibiotics and ARGs during composting is highly valuable. In this context, this review explores the sources, the dissemination routes and the impacts of antibiotics and their associated ARGs. In addition, the fate of antibiotics and ARGs during composting of sludge or manure was reviewed to assess the potentiality of composting as a strategy to mitigate the dissemination of antibiotics and ARGs.

2. The occurrence of antibiotics in raw and composted sludge or manure and their impact on the environment after soil application

In the European Union, 53% of the sludge produced by WWTPs is used in agriculture [20]. Many antibiotics end up in sewage sludge as shown on the Figure-1a with concentration varying between few nano-grams and 100 mg/kg DM. The four main families (tetracyclines, sulfonamides, macrolides and fluoroquinolones) have been quantified in raw sludge, including primary, secondary, mixed, dehydrated sludge. However, very few data (none on sulfonamides) are reported on composted sludge.

In raw sludge, CIP, NOR, and TYL are present at the highest concentration with a median value superior to 1000 µg/kg DM. Tetracyclines present a high range of concentrations, particularly TC and OTC, with medians above 10 µg/kg DM. Macrolides present also medians above 10 µg/kg DM but with a lower range of concentrations. Sulfonamides present medians around 10 µg/kg DM. In composted sludge, CIP and NOR present lower medians

than in raw sludge (near 100 µg/kg DM). For the other compounds, reported values are quite similar between raw and composted sludge.

In the United States, 130 million tons of pig, bovine and chicken manure are produced annually. In Canada, 177.5 million tons are produced per year [21], in France in 2012, over 274 million tons of livestock wastes were generated and applied on soil [22]. Considering the high use of antibiotics in veterinary medicine and depending on pharmacokinetics and specific transformation processes in the animals, large proportions of antibiotics are usually excreted (30-90%) by urine or faeces [23]. Therefore, antibiotics could be released into the environment when urine [24] and manure (Fig. 1c) were used directly as fertilizers. A very high range of concentrations was found from µg to g/kg DM due to the various practices in livestock industries, e.g. use of antibiotics as growth promoters in many developing countries [25]. Medians are around 100 and 1000 µg/kg DM. Only one paper reported tetracyclines value on composted manure with also a wide range of concentrations [26].

A high proportion of antibiotics and potentially bioactive transformation products reach agriculture fields or soil throughout the direct discharge or application of sludge and manure [22, 27-31]. Concentrations in soil compartment vary according to the history of the field. In soils amended with raw or composted sludge, Bourdat-Deschamps et al. [22] have measured 4-10 µg/kg DM of fluoroquinolones. Hu et al. [32] mentioned 2.5-105 µg/kg DM for tetracylines and 33.1-1079 µg/kg DM for CTC in soils amended with organic pig manure. Karci and Balcioglu [33] have quantified sulfonamides (40-400 µg/kg DM) in soils amended with cattle or chicken manure. Very few papers studied the long-term impact of several spreading on soil concentration. One recent paper emphasized that even if high concentration of antibiotics were found in amended fertilizers, no accumulation in soil and few transfer to soil leachates were observed [22]. These results may be explained by the various dissipation mechanisms occurring in soils according to the physical and chemical proprieties of antibiotics (Table 1). Indeed, the majority of antibiotics have low to moderate persistency in the soil with half-lives comprised between days and months (Table 3). In addition, low (SMX, SMZ log K_{oc}<3) [34, 35] or high sorption (fluoroquinolones, log K_{oc}>3) [22] contributes in rendering antibiotics more or less available for water and plant transfer. Indeed, under specific conditions plant uptake have been reported but under field conditions the concentration level of antibiotics in crops has been found to be very low [30, 36]. Prosser and Sibley [37] assessing the human health risk of antibiotics in plant tissues due to sludge or manure amendment concluded to a *de minimis* risk to human health for individual compound, but

consider the existence of a risk in the presence of mixtures. [36, 37]. Whatever their behavior (persistence, mobility, transfert), their presence in the soil may impact organisms and biodiversity. Batchelder et al. [38] tested the effect of CTC and OTC on the apricot plants growth in aerated medium. They showed that a low antibiotic concentration can influence the plant growth. Khadra et al. [39] showed an important genotoxic effect of the quinolones and fluoroquinolones on *Vicia faba* root tip, even at low concentrations of antibiotic mixtures. Antibiotics release was also shown to contribute to the dissemination of the antimicrobial resistance thanks to a concomitant release of ARB and ARGs implying an enrichment of the environmental resistome [40].

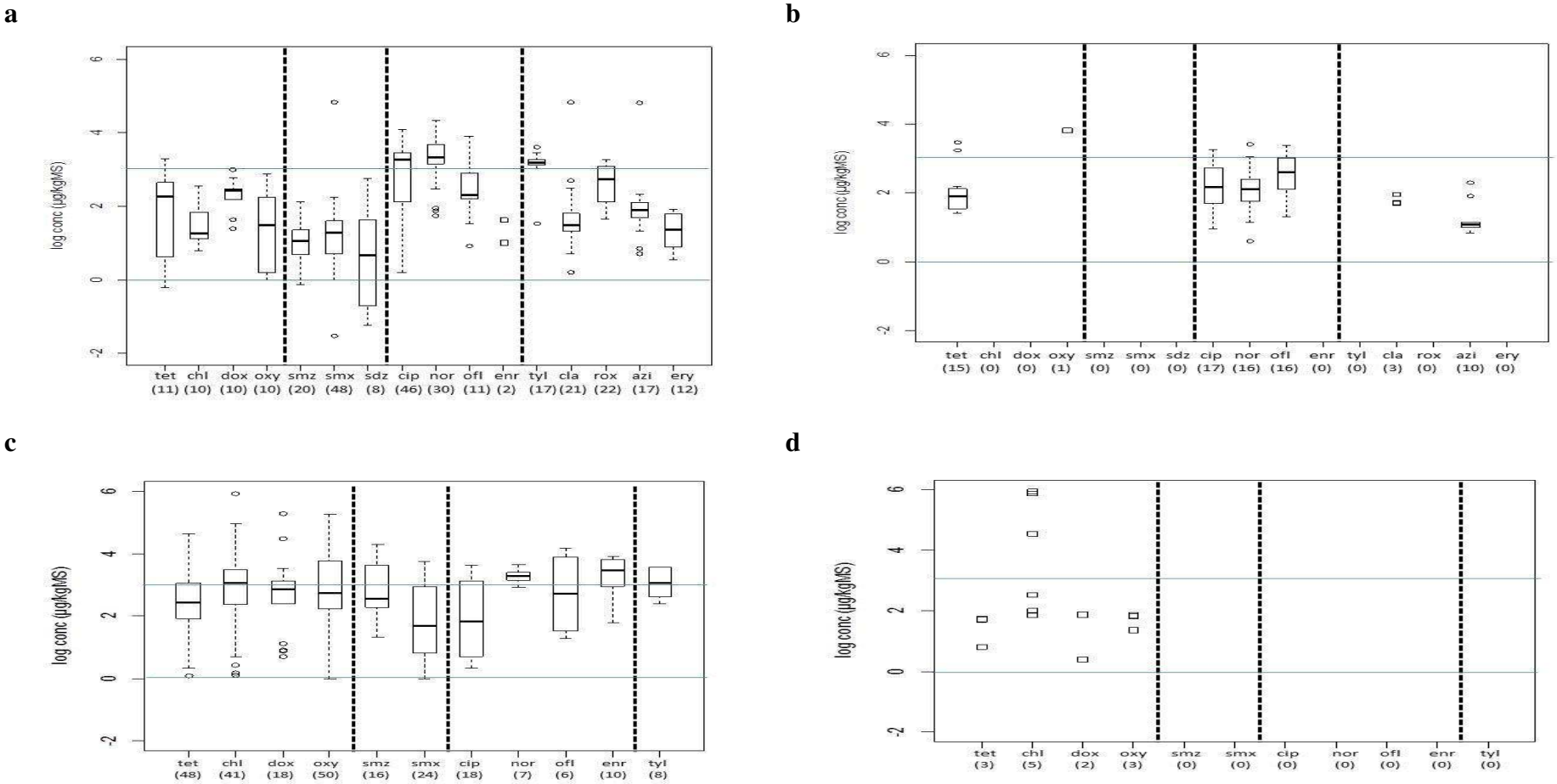


Figure 1: Range of antibiotic concentration in raw sludge (a), composted sludge (b), raw manure (c) and composted manure (d) for the four main families, tetracyclines, sulfonamides, fluoroquinolones and macrolides. Number of data for the boxplots in parentheses. The box corresponds to the 25th and 75th percentiles, the whiskers to the 10th and 90th percentiles, the solid line to the median; outliers are depicted by individual points. Whisker-box plots were drawn if more than 4 values were found. Legend of molecules: tetracycline (tet), chlortetracycline (chl), doxycycline (doxy), oxytetracycline (oxy), sulfamethazine (slfi), sulfamethoxazole (slfo), trimethoprim (tri), ciprofloxacine (cip), norfloxacine (nor), ofloxacine (ofl), tylosine (tyl), clarithromycine (clar), roxythromycine (roxy), azithromycine (azi).

The development of antibiotic resistance still remains a very complex process, and even more with antibiotic concentrations below the inhibitory threshold. Bacteria can be intrinsically resistant to certain antibiotics but can also acquire resistance to antibiotics via horizontal gene transfer [41]. Many researchers have demonstrated that sewage sludge contained large amounts of resistant genes [42, 43] to tetracyclines [44-49], sulfonamides [49], macrolides [48] and ARBs that are usually multi-resistant (*Escherichia coli* or *Enterobacteriaceae* [50-53], Enterococcus [54], *Peudomonas* sp [55], *Staphylococcus aureus* [56] and *Salmonella* sp [57]). ARGs and ARBs are also present in livestock manure [58-62]. ARGs can be readily disseminated via bacterial division and horizontal gene transfer among bacteria in the environment [63]. Therefore, a transfer of antibiotics resistance determinant toward bacteria of human beings including pathogens is established [63]. It has been estimated that antibiotic resistance is responsible for more than 25,000 and 23,000 deaths every year in the European Union and the United States respectively [64]. Infections by ARB have an annual cost of 1.5 billion dollars in the European Union [41]. Antibiotics and ARGs are considered as new contaminants and may pose a potential worldwide human health risk [19, 65, 66]. Therefore, improving the removal of antibiotics and ARGs from sewage sludge or manure before their application to soil is necessary.

3. The fate of antibiotics during composting

Several processes used to treat sludge or manure may impact the fate of antibiotics. Incineration was used to dispose of sludge [67]. However, a large quantity of energy is required. In addition, high amounts of nutrients are transformed into nitrogen oxides, and even dioxins are produced during the process. Pasteurization, thermal hydrolysis, advanced oxidation processes and ammonia treatment can also be used [68]. But the elevated costs of these processes limit their application. To investigate the fate of antibiotics during sludge or manure management, anaerobic digestion was also tested in several researches [69, 70]. On one hand, a significant inhibition of anaerobic digestion was shown at very high antibiotic concentrations (up to 100 mg/L), for example, by affecting the methane production [71-73], and on the other hand, a lower antibiotic degrading ability was also observed [74].

Since aerobic system like composting is one of the promising options to manage contaminated sludge and manure, an overview of the fate of antibiotics and ARGs during this process is presented below.

a. Composting

Composting is an aerobic biological decomposition process where pH, C/N ratio, and moisture are controlled in the initial mixture to be composted. These key factors determine the microbial development and the organic matter stabilization. Microorganisms growing during composting reflect the evolution and the performance of the process. The different communities occurring during composting are: bacteria, actinomycetes, fungi, protozoa or algae [75]. Composting consists of two main stages. During the thermophilic stage, the organic matter undergoes firstly a microbial decomposition which liberates CO_2 and ammonia. The organic matter degradation is accompanied by a heat release involved in the elimination of pathogens [76]. At the end of the thermophilic stage, the easily biodegradable part (sugars, amino acids) is completely mineralized. During the maturation stage, another part of the less biodegradable organic matter such as lignin serves as a new material to build molecules leading to humic substances [77, 78]. Regarding the agronomical value of the final product, composting has been widely used for producing soil amendments [79, 80].

b. The efficiency of antibiotics removal during composting

Composting is an effective approach for stabilizing sludge and manure via the biological degradation of organic matter. It has also been shown to be effective in reducing the levels of persistent organic pollutants [81, 82] and certain antibiotics [83-88]. Indeed, the fate of antibiotics during composting was followed in several studies as summarized in the Table 2. The main findings on recent studies are summarized hereafter:

- The tetracyclines family is the most studied one followed by sulfonamides, macrolides and fluoroquinolones;
- 82% of these studies were conducted at laboratory scale using a reactor and only 18% were carried out by windrowing the compost mixture;
- Only 3 studies were conducted without spiking sludge or manure and the rest was conducted by spiking sludge before composting at high concentrations varying between 1 and 150 mg/kg DM;
- The removal rates were calculated by referring to the initial and final concentrations (expressed in mg or µg per kg of dry matter) without taking into account the compost mass or ash content evolution during composting;
- Composting showed very high removal for the molecules belonging to the tetracyclines, sulfonamides and macrolides families with an elimination rate of 70-

99%. For the fluoroquinolone family, some studies reported an elimination rates up to 99% for the ENR and NOR. In contrast, other studies showed the persistence of NOR, CIP and OFL in the final compost product;

- The half-lives in days, are calculated on the basis of first order kinetic model and they varied according to each antibiotic family from 1-105, 2-105, 1-17 and 200-2500 respectively for tetracyclines, macrolides, sulfonamides and fluoroquinolones;

High antibiotics concentrations are often used during these experiments, showing no inhibition on the organic matter degradation [89, 90] and thus underlying that composting biomasses are either tolerant to such level or develop resistance mechanisms including degradation ability. The antibiotic removal was sometimes well correlated to the removal of several parameters like total organic carbon (TOC), total nitrogen (N), phosphorus (P), TOC/N, TOC/P and metallic trace elements [90]. However, some authors showed also that the heat release and organic matter degradation can be delayed by increasing antibiotic concentration. The effect of antibiotics on composting performances depends on the type of antibiotic and the level of concentrations. At 35 mg/kg DW, erythromycin affected the peak temperature while OTC, NOR and TYL did not [91]. In addition, it was shown that high antibiotics mixture concentrations (50 mg/kg CTC + 10 mg/kg SDZ + 5 mg/kg CIP), delayed the organic matter decomposition and affected the removal of CIP by comparison to the complete CTC and SDZ removals [92]. In a more recent study [93], high antibiotics concentrations (50-250, 20-100 and 10-50 mg/kg DM for tetracyclines, macrolides and fluoroquinolones respectively) decreased the thermophilic stage duration, temperature increase and decrease as well as the heat release. Additionally, the efficiency of the composting system and the compost quality (organic matter degradation and C/N ratio) were altered for the highest antibiotic concentrations.

The thermophilic conditions occurring during composting seemed to have a huge impact on the tetracyclines elimination [94, 95]. Arikan et al. [94] showed a very fast removal of CTC at 55°C by comparison to 25°C. It was confirmed by Wu et al. [95] with CTC, OTC and TC removals of 74%, 92% and 70% respectively during the thermophilic phase. The degradation kinetics of these three antibiotics fitted with a first order model and allowed to calculate half-live for CTC, TC and OTC of 8.2, 11 and 10 days, respectively, indicating that CTC was the more degraded compound [95]. Liu et al. [83] showed similar results for sulfonamides with highest dissipation rate at high temperature.

Tableau 2: The fate of antibiotics during composting

Molecules	Disposal of composting	Compost mixture	Moisture % (M) and C/N ratio before composting	Compost quantities	Spiking of the initial mixture before composting	Initial antibiotics concentration (mg/kg DW)	Maximal temperature (°C)	Duration of composting (days)	Elimination (%)	Parameters related in antibiotics removal	References
OTC	Self-heating laboratory composting	Beef manure, sawdust	M : 72.4 C:N= 29	3.3 kg	Beefs were medicated by 22mg/kg/day for 5 days	115	60	35	99.8	The basis on the OTC removal is not known. After composting, OTC residues have been rendered biologically inactive or unavailable.	[120]
CTC	Pile and vessel composting	Turkey manure, blend of aspen, shavings and sunflower	M : 40% C:N=12.-12.9		Yes	1.5	60	35	>99	Thermophilic temperature resulted in a reduction in CTC, monensin and TYL concentration. Biotic and abiotic factors contributed to antibiotic degradation during composting.	[126]
MNN						11.9			54		
SMZ						3.7			No degradation		
TYL						10.8			76		
CTC	self-heating laboratory composter	Manure-Straw-Woodchips	M :68.3 C/N=25.5	3.5 kg	Beefs were medicated by 22mg/kg/day for 5 days	-	55	30	98	Abiotic process is responsible of CTC elimination.	[94]
CTC	Lab vessel composter	Broiler and Hog manure	M= 50-60	4 kg	No	94.72 (Broiler Manure) 879.6 (Hug manure)	55 58	42	92.59 27.33	Antibiotics depletion is depending on C, N and P evolution.	[90]
		Layer-Hen manure			Yes	50 100 150	55		100 94.92 94.51		
CTC	Self-heating composter (60L)	Hen manure and straw	M= 65 C/N=13.2	45 kg	Yes	60	55	45	98.5	Not indicated	[89]
		Pig manure and straw	M= 65 C/N=17.7				62		95.7 97.3 96.2		
OTC		Hen manure and straw	M= 65% C/N=13.2				55		97.2		
TC							55		93.8		
CTC	Pilot scale composting	Manure-mushroom residues	M : 56.9 C/N=11.02	5m^3	No	2.9	55-60	52	74	Antibiotics elimination predominately took place in the thermophilic stage. Transformation products indentification	[95]
OTC						1.6			92		
TC	Open-air windrow manually turned					0.4			70		
CTC	Bench-scale composting conducted in computer controlled 20-L reactors	Swine manure-Sawdust	M : 55 C/N=29 Aeration rate: 0.5 L/kg DW/min	7 kg	Yes	50	60-64	56	100	High level of antibiotics delayed the initial decomposition that also affected the nitrogen mineralization	[92]
						5			100		
SDZ						10			100		
						1					
CIP						10			83		
						1			69		
CTC	lab-scale composting apparatus	Manure-Sawdut	M : 70	30 kg	Yes	2	50	40	96	Binding sites resulted in the reduction of residual antibiotic concentrations.	[96]
SMZ						10	55		99		
TYL						20	55		95		
DOX	Plastic containers	Broiler manure	M : 50-60 C/N=8	5 kg	Yes	13.64 30.64 62.57	45.7	40	99.80	pH, compost temperature, TOC, TN, C/N ratio, TP, Na, Cr, Cu, Zn, Fe and Cd are suggested to have a possible effect on the degradation of antibiotics.	[99]
SDZ									>99.99		
TYL									>99.92		

Tableau 2: The fate of antibiotics during composting

Molecules	Disposal of composting	Compost mixture	Moisture % (M) and C/N ratio before composting	Compost quantities	Spiking of the initial mixture before composting	Initial antibiotics concentration (mg/kg DW)	Maximal temperature (°C)	Duration of composting (days)	Elimination (%)	Parameters related in antibiotics removal	References
SMZ and SMX	Composting containers	Manure-straw	M : 50-60 C:N= 22.9	2 kg	Yes	20	55	35	>99.99	Thermophilic stage contributes on reducing sulfonamides.	[83]
CTC	Small-scale composting	Manure	M: 55-65 C:N= 25-30	20-22	Medicated hereford steers	-	Up to 55	42	71-84	Static and turned composting is effective for reducing antibiotics. After composting, some antibiotics apparently more recalcitrant than others.	[85]
TC									66-72		
SMZ									97-98		
PMN									100		
FFC	pilot-scale static pile thermophilic composting	Manure and wastewater biosolids/wood-product (1.3 v/v)	M: 73	1.7 m^3	Yes	0.1	66-73	28	95-99	Thermophilic composting effectively degrades parent antibiotic compounds in manure and biosolids.	[88]
SMX						0.2			98		
SMZ						0.8			97-99		
TYL						0.8			96-98		
CTC	Open-air windrow	Manure from beef cattle	-	-	Medicated beef	-	40	2 years	94.5-99	composting leads to dissipation of antibiotics, the microbial composting process could be inhibited by the presence of antibiotics.	[86]
SMZ									59-93.2		
TYL									93.7		
Sulfamerazine (SM1)	Cylindrical plastic containers	Manure (chicken and hog), sawdust and rice straw	M: 65	-	Yes	-	64	35	100	Adding conditioners improved the removal of 4 kinds of sulfonamides during composting	[121]
Sufachlorpyridazine (SCP)									100		
Sulfadimoxine (SDM')									100		
Sulfaquinoxaline (SQ)									94.7		
CIP	windrow	Anaerobic sludge mixed with peat	-	-	No	1520[ab]	-	12 months	0.3[b]	The decomposition rate of antibiotics depends on the applied sludge treatment technology.	[122]
NOR						580[ab]			0.1[b]		
OFL						134[ab]			0.03[b]		
SDM						73[ab]			Nd		
SMX						22[ab]			0.01[b]		
CIP		Sewage sludge mixed with tree bark				442[ab]			70[b]		
NOR						439[ab]			64[b]		
OFL						157[a]			8[b]		
SDM						32[ab]			nd		
SMX						16[ab]			2[b]		
NOR	Windrow	Sludge-treebark	-	Volume ratio 2:3	No	0.0-110[ab]	-	12 months	0.0-22[b]	The degradation of antibiotics depends on the compost mixture.	[123]
CIP						2.6-111[ab]			0.0-20[b]		
OFL						0.5-39[ab]			0.0-3.2[b]		
SMX						0.0-2.8[ab]			0.0-0.9[b]		
SDM						0.0-7.9[ab]			0.0-4.2[b]		
NOR		Sludge-peat		Volume ratio 4:3		0.0-162[ab]			0.0-5.4[b]		
CIP						0.0-426[ab]			0.0-7.1[b]		
OFL						0.0-38[ab]			0.0-0.5[b]		
SMX						0.0-6.0[ab]			0.0-0.3[b]		
SDM						0.0-20[ab]			0.0-0.2[b]		

Removals are calculated based on the input and output concentrations of antibiotics

OTC: Oxytetracycline; SMZ: Sulfamethazine; SMX: Sulfamethaxazole; CTC: Chlortetracycline; TC: Tetracycline; PMN: Pirlimycin; FFC: Florfenicol;TYL: Tylosin; DOX: Doxycycline; SDZ: Sulfadiazine;CIP: Ciprofloxacin; NOR: Norfloxacin; OFL: Ofloxacin; SDM: Sulfadimethoxine; MNS: Monensin; nd: not determined

[a]: concentrations in sludge before composting; [b]: concentrations expressed by µg/kg DM.

Tableau 3: Antibiotics half-live during composting of sludge or manure

Families	**Molecules**	**Half-live (days)**	**References**
Tetracyclines	CTC	1-86.8	[93, 95, 94]
	OTC	3.2-30	[94, 125]
	TC	4.5-105	[95, 126, 127]
	DOX	<350	[22]
Macrolides	TYL	19	[124]
		2-105	[25, 35, 124]
Sulfonamides	SMZ	2.01 and 2.12	[73]
	Monensin	17	[87]
	SDZ	42-80	[128]
	SMX	64-133	
Fluoroquinolones	NOR, OFL and CIP	217-2500	[22, 129]

CTC: Chlortetracycline;OTC: Oxytetracycline;TC: Tetracycline;DOX: Doxycycline;TYL: Tylosin; SMZ: Sulfamethazine;MNS: Monensin;SDZ: Sulfadiazine;SMX: Sulfamethaxazole;NOR: Norfloxacin;OFL: Ofloxacin;CIP: Ciprofloxacin

The substrates used during composting play an important role regarding antibiotics elimination. The fate of CTC, SMZ and TYL at 3 concentrations (2, 10 and 20 mg/kg DM) showed that CTC and SMZ removal depends on the presence of straw but TYL removal did not [96]. At high temperature, straw provides a wide range of additional biding sites favorable for the CTC and SMZ adsorption. Antibiotics adsorption on co-substrates (palm rachis matrix for example) could reduce the bioavailability of antibiotics towards the microorganism involved during composting, and then explain why composting process was not entirely inhibited by high antibiotic concentration [93].

The coexistence of other pollutants with antibiotics and their influence on the elimination of antibiotics during composting was the object of a study carried out by Liu et al. [83]. Indeed, these authors showed that the dissipation of sulfonamides was slightly delayed in the presence of 2000 mg/kg of copper. This inhibitory effect could be explained by the toxicity of copper to microorganisms involved in the sulfonamides degradation.

c. The mechanisms of elimination of antibiotics during composting

The physicochemical properties of antibiotics (Table 1) drive their fate (persistence, mobility) during composting. Moreover the physicochemical conditions and the degradation of organic matter occurring during the composting process may influence the fate of these molecules by changing their availability, i.e., their chemical form and their interactions with the organo-

mineral matrix. Indeed, several processes could be implied in their removal, i.e., volatilization, abiotic and biotic transformation, mineralization, sequestration (formation of bound residues, non-extractible by the analytical methods). On the described literature in Table 2, very few papers provide information on the mechanisms of antibiotics elimination, for example by using abiotic controlled reactors (to assess abiotic transformation or sequestration), or radiolabeled compounds (mineralization proved by production of radiolabeled carbon dioxide, quantification of non-extractible residues), by quantifying transformation products. For Wu et al. [95], the removal of tetracyclines, which are considered unstable because of their chemical structure, could be linked to abiotic transformations like epimerization or dehydration. In this work, the transformation products (4-epichlortetracycline, 4-epioxytetracycline, 4-epitetracycline, anhydrotetracycline demeclocycline) were quantified and their removal also assessed. In the experiment of Kim et al. [96], the addition of sawdust to manure composting had a significantly positive effect on the removal of CTC; the authors hypothesized that sorption plays a main role in this removal due to great quantity of divalent cations present in these organic matrices and the possible formation of chelates complexes with organic substances. In counterpart, TYL do not form complexes with metal ions, but can form strong chemical bonds with negatively charged compounds in manure [96], accelerating the decline of the extractible antibiotic levels. Paesen et al. [97] described the hydrolysis of A-TYL to B-TYL under acid conditions. Under neutral and alkaline conditions, the compound produces TYL-A-aldol at the same time as other polar decomposition products. The hydrolysis of TYL is reported to be high over the whole pH range from 2 to 13. For the sulfonamides, the decline in concentration of these molecules could be linked to the presence of organic substances, which generate adsorption sites at high temperatures during the composting process rendering them less extractible [98]. Humic substances play also an important role in the elimination of antibiotics. The production of humic substances leads to the sequestration of antibiotics within organic and inorganic matter that render them less extractable as hypothesized by Ho et al. [99] for various antibiotics during 40 days broiler manure composting.

The processes involved in antibiotics removal still remained not elucidated. Biodegradation could be responsible of the antibiotics decrease. Elsewhere, there is no study highlighting the real antibiotics biotransformation as a result of organic matter mineralization. Some abiotic processes could be also implied such as sequestration and/or chemical transformation. Indeed,

biotic and abiotic processes are two concomitant removal pathways that have to be elucidated to gain better understanding and propose process optimization strategies.

4. The fate of antibiotics resistance genes during composting

Several researches investigated the fate of ARGs during composting (Table 4).These studies focused on the most important parameters involved in the ARGs removal during the composting of sludge or manure. A brief resume of the studied conditions is presented hereafter:

- 4 studies were conducted on sewage sludge whereas 20 studies were focused on different types of manure (swine, chicken, cattle, poultry, pig, cow, duck, bovine and dairy manure);
- The composting experiments were mainly conducted with lab-scale reactors and a duration ranging between 4 and 183 days;
- The most used co-substrates during the composting of sludge or manure are mushrooms, composting end-products, sawdust, rice straw, wheat stalks, wheat straw, medicinal herbal residues and cotton stalks;
- ARGs conferring resistance to tetracyclines, sulfonamides, macrolides, fluoroquinolones and β-lactam are the most studied;
- To assess the fate of ARGs during composting, their removals are expressed in percentage (%) or by calculating the absolute or relative abundance (logs units, copies/16S rRNA, copies/g TS or copies/g DM);

The main outcomes are:

- The addition of antibiotics, surfactants (biological or chemical), natural zeolite, biochar or metallic trace-elements are able to control the ARGs abundance and removal during composting;
- The bacterial communities, the abundance of transposons and horizontal gene transfer are some keys parameters implied in the ARGs removal.
- The composting stages (mesophilic, thermophilic and cooling phases) and their duration as well as physicochemical parameters (pH, moisture, ammonium nitrogen, and water-soluble carbon contents) play a crucial role regarding the ARGs removal.

- Some studies showed the effectiveness of composting on the ARGs removal but other ones showed the persistence and the increase of the concentrations of some ARGs after composting.

In the study of Selvam et al. [100], sulfonamides, tetracyclines and fluoroquinolones resistance genes represented respectively 10.28, 1.91 and 0.00022% of the 16S rDNA copies in the initial compost. After 42 days of composting, no ARGs were detected for the studied antibiotics. Yu et al. [101] found also a complete removal of tetracycline ARGs for the ribosomal protection protein groups in swine manure compost, and a reduction of 6 logs for others belonging to the ‘‘efflux” group. In the same way, Chen et al. [40] reported a reduction (7.3 logs) in the abundance of erythromycin ARGs during swine manure. Wang et al. [103] reported a partial removal of the tetracyclines ARGs (2.4 log copies/g DM) especially in the thermophilic stage (1.5 log copies/g DM). In these papers, a positive correlation was found between the ARGs reduction during composting and the temperature [100, 102-106]. Additionally, temperature was identified as an important parameter related to the transfer of a mobile plasmid (pIE732) [107]. In this study, chicken manure and compost were incubated separately at 23 and 50°C. The plasmid transfer from *E. coli* occurred in manure and compost at 23°C. At 50°C, plasmid transfer was not confirmed suggesting that the thermophilic stage is a main contributor in reducing (i) ARGs transfer and (ii) pathogen bacteria’s.

Other studies showed contradictory results. Indeed, while a removal of 100% for OTC and SMZ was reached during swine manure composting, a proliferation of the corresponding ARGs was observed by Wang et al. [108]. Another recent study conducted by Kang et al. [109] showed that the short-term thermophilic compost treatment could not remove the TC resistance genes in pig manure.

The presence of heavy metals in sludge or manure at high level [110, 111] is considered as a co-selection factor for ARGs reduction [112-115]. ARGs come into contact with heavy metals which could drive the selection and the evolution (increase/decrease) of antibiotic resistance bacteria [116]. The bioavailable fraction of heavy metals acts as a selective pressure on microbes [117]. Using a co-substrate such as biochar or superabsorbent polymers (sodium poly-acrylate) during composting could help to adsorb heavy metals, decreasing their availability and then contributing to reduce the selective pressure on antibiotic resistance bacteria [118, 119]. Nevertheless, the mechanisms involved on the dynamics of ARGs in the presence of heavy metals during composting are still unclear.

Tableau 4: Synthesis on recent studies on the fate of antibiotic resistance genes during composting of sludge or manure (AA: absolute abundance; RA: relative abundance)

Antibiotic resistance genes (ARGs)	**Compost mixture**	**Composting conditions**	**Antibiotics Spiking of the initial mixture**	**Initial concentrations of ARGs**	**Fate of ARGs after composting**	**Key parameters implied in the ARGs removal**	**References**
Tetracycline (tetG, tetW and tetX) Sulfonamide (sulI and sulII) Fluoroquinolone (aac(60)-Ib-cr) B-lactam (blaCTX-M and blaTEM) Macrolide (ereA, ermB, ermF and mefA).	-Dewatered sewage sludge and mushrooms -Compost mixture quantity: 45kg (at a ratio of 1 :3 (v/v))	-Lab-scale composting reactor -Three composting experiments: the control (A), natural zeolite addition (B) and nitrification inhibitor addition (C). -Composting duration: 183 days	No	-	-The total ARGs copies were enriched 2.04 and 1.95 times in reactors A and C. -The total ARGs copies were reduced by 1.5% in the reactor B. -Some ARGs were reduced by 0.3-2 logs, while others increased by 0.3-1.3 logs after composting.	-Addition of natural zeolite; -The thermophilic and cooling stages; -The bacterial community structure and changes.	[60]
Tetracycline (*tetA*, *tetB*, *tetC*, *tetG*, *tetL*, *tetM*, *tetQ*, *tetO*, *tetW*, and *tetX*) Macrolide (*ermB*, *ermF*, *ermT*, *ermX*, *mefA*, and *ereA*) Aminoglycoside (*aacA4*, *aadA*, *aadB*, *aadE*, *aphA1*, *strA*, and *strB*) Sulfonamide (*sul1*, *sul2*, and *sul3*).	-Dewatered sewage sludge mixed with composting end-products at a ratio of 1:3 (v/v). -Compost mixture quantity: 200 tons	-Industrial hyperthermophilic and conventional composting in piles. -Composting duration: 45 days	No	-Tetracycline and sulfonamide (the most dominant ARGs): 5.1×10^{11} and 1.1×10^{10} gene copies per gram (dry weight) in the initial raw sludge.	-The RAs of ARGs were of 0.05 and 0.14 copies/16S rRNA genes respectively after huperthermophilic and conventional composting.	-Hyperthermophilic condition.	[103]
23 ARGs encoding tetracyclines, sulfonamides, quinolones, β-lactam antibiotics, macrolides, florfenicol and multidrug resistance	-Sewage sludge	-Lab-scale thermophilic composting reactor. -Composting duration: 15 days	No	-	Removal efficiency (%): **Tetracycline** *tetA*,50.03; *tetB*, 100; *tetD*,92.00; *tetE*,100; *tetG*,85.23; *tetH*,66.41; *tetM*,62.36;*tetQ*,98.10; *tetX*,83.43 ;*tetZ*,63.70; *tetBP*,100 ;*tcrB*,100 **Sulfonamides:** *sul1*,93.93; *sul2*,97.17 **Quinolones:** *qnrS*,N.A; *qnrD*,97.28; *aac (6')-lb-cr*,81.61 **Macrolides**: *ermB*,20.39; *ermC*,N.A. **Beta-lactam:** *blaTEM*,96.18; *blaCTX*,100; *blaSHV*,80.02 **Florfenicol:** *floR* 80.12 **Multidrug:** *oqxA* 63.39	-Thermophilic composting; -Combining thermophilic composting with anaerobic digestion.	[106]
156 ARGs and MGEs conferring resistance to a broad range of antibiotics (tetracyclines, multidrug, macrolide, lincosamide, streptogramin B and Aminoglycosides)	-Dewatered sewage sludge, sawdust and rice straw.	-Lab-scale composting in-vessel. -Composting duration: 50 days	No	-	-The ARGs were significantly higher at the last stage of composting. -The enrichment of ARGs was observed during composting.	-Bacterial phylogenetic; compositions.	[133]
Tetracycline (*tetM, tetO, tetQ, tetS, tetT, tetWtetB/P; tetC, tetE, tetG, tetH, tetY*and*tetZ*) Sulfonamide (*sul1, sul2, sul3, dfrA1, drfA2* and *drfA7*) Fluoroquinolone (*gyrA* and *parC*)	-Swine manure and sawdust mixed at a ratio of 1:1 (v/v).	-Lab-scale reactor -Composting duration: 56 days	Yes (Spiking at two levels)	-Resistance genes of tetracycline, sulfonamide and fluoroquinolone represented 0.02–1.91, 0.67–10.28 and 0.00005–0.0002%, respectively, of the total 16S rDNA copies in the initial compost mass.	No detection of ARGs except *parC*	-Thermophilic stage; -The addition of antibiotics; -The bacterial diversity.	[100]
Tetracycline (*tet*A, *tet*B, *tet*C, *tet*L, *tet*O, *tet*M, *tet*W and *tet*X).	-Swine manure mushroom and red mud. -The windrow size is about 27 m^3.	-Two windrows by adding the red mud (RM) and a control (CK) without adding the red mud. -Composting duration: 53 days	No	-	-ARGs in swine manure were removed after composting (by 2.4 log copies/g TS), especially during the thermophilic stage (by 1.5 log copies/g TS)	-Bacterial community; -The addition of red mud.	[102]
Tetracycline (*tetC, tetG, tetW*, and *tetX*), sulfonamide (*sul1, sul2, dfrA1*, and *dfrA7*) Macrolide (*ermB, ermF, ermQ*, and *ermX*).	-Chicken manure (C), wheat stalks (K) and bamboo charcoal (BC). -Compost mixture quantity: 3 kg	-Lab-scale reactor. -3 different proportions of BC -Composting duration: 26 days	No	-	- RAs of *tetC, tetG, tetW, tetX, sul2, drfA1, drfA7, ermB, ermF, ermQ,* and *ermX* decreased by 21.6-99.5%, while the RA of *sul1* increased by 7.5–17.7 times. - RAs of ARGs (except *sul1*) in the mixture CK showed an elimination of 0.85 logs. -Adding 5, 10 and 20% of BC in the CK mixture increased the average reductions in the RAs of ARGs by 1.05, 1.08, and 1.15 logs, respectively.	-Temperature; -Adding biochar.	[118]
Tetracycline (*tetC, tetG, tetQ, tetW*, and *tetX*), Sulfonamide (*sul1, sul2,* and *dfrA7*) Macrolide (*ermF, ermQ,* and *ermX*) Quinolone (*qnrS, qnrD,* and *aac(6')-ib-cr*).	-Swine manure and wheat straw mixture at a ratio of 1:1 (v/v).	-Lab-scale composting reactor. -Sodium polyacrylate (SP) was added at two levels + control without SP (CK). -Composting duration: 35 days	No	-AAs of ARGs in the initial material were ranged from 10^7 to 10^{10} copies g^{-1} DW.	-The AAs of all the ARGs detected in the CK decreased by 8.12–96.70%. -Greater reductions in the AAs of ARGs were obtained in the H treatment than the other two treatments.	-pH, moisture and bacterial community; -Addition of sodium polyacrylate.	[119]

144 ARGs encoding potential resistance to macrolide, lincosamide, streptogramin B, aminoglycoside, multidrug, tetracycline, and β-lactam.	-Cattle manure and sawdust.	-Conventional composting by windrow.	No	-The average number of ARGs in raw manure : 103.3	-The average number of ARGs in mature compost : 71.3	-The thermophilic stage; -Sawdust addition; -Bacterial community.	[130]
Tetracycline (*tetA, tetB, tetC, tetE, tetG, tetM, tetO, tetQ, tetT, tetW*, and *tetX*) Sulfonamide (*sul1, sul2, sulA, dfrA1*, and *dfrA7*) Macrolide (*ermA, ermB, ermC, ermF, ermQ, ermT,* and *ermX*) Fluoroquinolone (*aac(60)-Ib-cr, gyrA, parC, qnrC*, and *qnrS*).	-Swine manure medicinal herbal residues (MHRs) and wheat straw. -Compost mixture quantity: 5 kg	-Laboratory-scale composting. -Two composting experiment -Composting duration: 40 days	No	-	-The AAs of*aac(60)-Ib-cr* and *tetW* decreased in both of the final composting products by 1.29 and 1.82 logs in SW, respectively, and 2.10 logs and 2.82 logs in SWC. -The AAs of *int1, tetG, qnrA*, and *qnrS* increased by 1.56, 2.08, 3.16, and 3.25 times in SW, respectively, but they decreased by 34.1–84.0% in SWC.	-MHRs; -Changes in the microbial communities.	[131]
ARGs conferring resistance to multidrug, aminoglycoside, betalactam, tetracycline, vancomycin, macrolide, lincosamide and streptogramin B, fluoroquinolone, chloramphenicol and sulfonamides.	-Cattle, poultry, and swine manure mixed with rice straw.	-Large-scale commercial composting.	No	-Abundance of ARGs in raw cattle and poultry manure: 1.9 and 5.5 copies/cell.	-Abundances in the final cattle and poultry mature composts : 0.2 and 1.8 copies	-Thermophilic composting ; -Some ARGs persist after composting; -Optimal conditions and composting duration.	[104]
Tetracycline (*tetA, tetB, tetC, tetE, tetG, tetK, tetL, tetM, tetO, tetQ, tetW* and *tetX*) Sulfonamide (*sulI, sulII* and *sulIII*) Fluoroquinolone (*gyrA, gyrB,parC* and *parE*) Macrolides (*ermB, ermC,ermE*and*ermF*).	-Pig manure and sawdust (ratio of 5 :1 (v/v)). -Compost mixture quantity: 8.5 kg	-Polymethyl methacrylate reactors. -Composting duration: 90 days	Yes Manure was spiked by CTC at two levels	-	-The total ARGs copies number reduction was 0.21, 0.34 and 0.11 logs, respectively, in CK, T1 and T2.	-Microbial community.	[132]
Macrolide (*ermB, ermF, ermQ, ermT*, and *ermX*) Tetracycline (*tetC* and *tetX*) Sulfonamide (*sul1*and *sul2*)	-Cotton stalks and pig manure	-Composting chamber -Two treatment : pig manure with GM or non-GM -Composting duration: 40 days	No	-	-The AAs of ARGs, intI1, and intI2 were reduced by 41.7 and 45.0% in the non-GM and GM experiments respectively.	-Temperature and ammonium nitrogen.	[105]
Tetracyclines (*tetA, tetB, tetL, tetM, tetW, tetQ, tetO* and *tetX*), sulfonamide (*sul1* and *sul2*) and chloramphenicol (*fexA, floR,cmlA, cfr* and *fexB*)	-Rice straw biochar (RSB), mushroom biochar (MB) and chicken manure -Compost mixture quantity: 5 kg	-Lab-scale composting reactor -Three composting experiments were carried out: composting with RSB, MB and a control without biochar (CM) -Composting duration: 42 days	No	-	-The average removal rate of ARGs was 0.86 log units in CM. -The average removal rate was 0.61 and 1.49 log units in (CM + RSB) and (CM + MB) respectively.	-The Microbial community ; -The bio-availability of heavy metals.	[115]
Tetracycline (*tetA, tetB, tetC, tetE, tetG, tetM, tetO, tetQ, tetT, tetW,* and *tetX*) Sulfonamide (*sul1, sul2, sulA, dfrA1,* and *dfrA7*).	-Cow manure and straw mixed at a ratio of 4:1 (v/v). -Compost mixture quantity: 5 kg	-Conventional composting by windrow. -Composting duration: 40 days	Yes The cow manure was spiked by OTC at 3 levels	-	- The RAs of *tetC, tetX, sul1, sul2*, and *intI1* increased 2–43 times, while those of *tetQ, tetM,* and *tetW* decreased by 44–99%. -The OTC addition increased the AAs and RAs of *tetC* and *intI1*, while the highest concentration (200 mg/kg) of OTC also enhanced those of *tetM, tetQ*, and *drfA7*.	-The bacterial community; -Composting was not effective in reducing most of the ARGs.	[134]
Tetracycline (*tetC, tetG, tetM, tetQ, tetW*, and *tetX*), Sulfonamide (*sul1, sul2,* and *dfrA7*) Macrolide (*ermB, ermF*, and *ermX*) Quinolone (*aac(60)-Ib-cr*).	-Wheat straw and chicken manure, bio-surfactant (rhamnolipid, RL) and chemical surfactant (Tween 80, Tw)	-Rectangular bubble boxes. -Composting duration: 30 days	No	-	-The RAs of ARGs and intI1 were reduced by 1–4.7, 0.8–3.7 and 0.3–2.6 logs with the addition of Tw, RL and in the control experiments.	-Temperature and the water-soluble carbon contents; -RL and Tw.	[135]
12 ARGS (*tetB, tetL, tetM, tetW, tetQ, tetX; sul1, sul2,cfr, cmlA, fexA,* and*floR*)	-Pig and duck manure, sawdust, rice straw biochar (RSB) and mushroom biochar (MB).	-Rectangular foam container. -Composting duration: 42 days	Yes Animal feeding operations	-Total abundance of ARGs was 3.75×10^{-1} copies/g (duck manure) which is 3.7 times higher than in pig manure.	-The average removal of ARGS was 2.56 and 2.09 log units in duck and pig manure compost.	- Biochar addition; - The type of biochar and manure.	[136]
Tetracycline (*tetB, tetC, tetM, tetO, tetT*, and *tetZ*)	-Pig manure	-Petri dishes were incubated at 60°C -Thermophilic composting duration: 4 days	Yes Animal feeding operations	-	-	- Thermophilic composting cannot remove tetracycline resistance genes in pig manures.	[109]
bla_{tem}, *tet(M), sul1, sul2, ermB*	-Chicken manure	-Composting heap -Composting duration: 6 weeks	Yes Animal feeding operations	-	-	-Colistin administration had no impact on the ARGs; -Composting is insufficient to eliminate ARGs.	[137]
109 ARGs conferring resistance to aminoglycoside, MLSB, tetracycline, β-lactame, multidrug, vancomycin, chloramphenicol, sulfonamide.	-Bovine, chicken and pig manure	-Industrial composting using 12 different composting mixtures -Composting duration: 15-90 days	-	-The total abundances (copies/16S rRNA) in bovine, chicken, and pig manure were 0.08–0.28, 1.71–3.07, and 0.54–1.49 respectively	-The total abundances (copies/16S rRNA) were 0.06–0.52, 0.08–0.36,and 0.11–1.17 in bovine, chicken, and pig composts respectively	-Animal species, the abundance of transposons, heavy metal concentration, total nitrogen level, dosage and duration of exposure to antibiotics; -Some ARGs were reduced after composting, but other ARGs were inconsistently influenced.	[138]

Tetracycline (*tetA, tetB/P, tetC, tetE, tetG, tetM, tetO, tetQ, tetT, tetW*, and *tetX*) Sulfonamide (*sul1, sul2, sulA, dfrA1*, and *dfrA7*) Fluoroquinolone (*gyrA, parC, qnrA, qnrC, qnrS*, and *aac(6')-ib-cr*) Macrolide (*ermB* and *ermQ*)	-Dairy manure and wheat straw	-Composting incubator -Composting duration: 40 days	-	-	-The abundances of the detected ARGs ranged from 6.2 $\times 10^5$ to 1.1 $\times 10^{11}$ copies per gram dry compost	-Thermophilic and mature phase of composting; -Bacterial succession and horizontal gene transfer.	[139]
Tetracycline (*tetB, tetC, tetL, tetM, tetW*) Erythromycin(*ermA, ermB, ermF, ermX*) Sulfamethazine(*sul1, sul2*)	-Cattle manure	-Lab-scale composter -Composting duration: 30 days	Feed and fortified treatments	-7.39, 8.14, 7.68, 9.23, 9.63, 8.63, 7.42, 8.01, 8.25, 9.31 and 9.38 Log_{10} copies g dry matter respectively for *tetB, tetC, tetL, tetM, tetW, ermA, ermB, ermF, ermX, sul1* and *sul2*. (concentration of 16S rDNA)	-10.58, 7.60, 10.92, 5.43, 8.12, 6.61, 7.95, 10.06, 8.87, 8.80 and 8.64 Log_{10} copies g dry matter respectively for *tetB, tetC, tetL, tetM, tetW, ermA, ermB, ermF, ermX, sul1* and *sul2*. (concentration of 16S rDNA)	-Oral administration; -Antimicrobial concentrations; -Mesophilic and thermophilic temperatures; -Longer thermophilic composting period.	[140]
tetM, tetO, tetQ, tetW, tetA, tetC, tetG, tetL, tetX, sul1 and *sul2*.	-Swine manure	-Composting piles -Composting duration: 32 days	Yes Manure was spiked by SMN and OTC	-	-	-Horizontal gene transfers; -Antibiotics concentrations; -Thermophilic composting failed to prevent the proliferation of ARGs.	[109]
Macrolide (*ermA* and *ermB*)	-Swine manure and wheat straw	-Lab-scale composter -Composting duration: 35 days	No	-	-The AAs were decreased for *ermA* (0.4–1.2 logs) and *ermB* (1.2–1.6 logs).	-Temperature; -Bacterial community; -Copper addition.	[141]

Several studies confirmed that some ARGs were partially eliminated after composting and elucidated the contribution of high temperature, microbial community composition/changes and composting duration. Other ones concluded that the presence and the availability of other pollutants, such as heavy metals, could affect either their increase or their reduction. Hence, the fate of ARGs during sludge or manure composting still remains unclear and needs more detailed researches to determine optimizing removal strategies.

5. Conclusions and perspectives

The use of antibiotics in human or veterinary medicine is continuously increasing and leads to the emergence of these molecules as well as ARGs and ARB on the whole environment compartments. The consequences on human health can involve countless deaths. The research on the mobility, the persistence, and the mechanisms of antibiotics elimination is still in progress. Although, developing common tools to assess the environmental proliferation or attenuation of antibiotic resistances and their impact on the environment and health is recommended.

Composting is a promising solution to eliminate or reduce the discharge of antibiotics and ARGs. However, lot of studies were conducted at lab-scale under controlled conditions with very high antibiotics concentration (often spiked) and none of them elucidate the real mechanisms behind the removal performances, i.e., biotic or abiotic transformations, bound residues formation. This literature review highlighted the importance of process management (temperature, co-composting substrates) regarding the fate of antibiotics and ARGs. More research is still needed to better understand the driving mechanisms during antibiotics and ARGs removal as well as their interactions. This better understanding is a prerequisite for identifying better but compromised process management practices that simultaneously target antibiotics and ARGs in order to reduce their load to the environment. The increase of the composting period can leave more time for antibiotics degradation especially during the maturation stage but may lead to proliferation of ARGs. Regarding the major role of sludge and manure land application in the dissemination of antibiotics and ARGs and the related environment and health issues, the establishment of standards regarding the authorized levels of antibiotics in sludge, manure and compost before the direct land application should be considered.

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2. Extraction and analysis of antibiotics in Compost: a review

1. Sample preparation

After compost sampling, sub-samples were kept usually at -20°C during the storage phase. Before the extraction process, it is necessary that sub-samples be stored at the ambient temperature. To avoid the adsorption of antibiotics onto silanol groups, it is recommended that glasswares were dried or rinsed with Na_2EDTA before use (Hamscher et al., 2002; Yang et al., 2005 ; Castiglioni et al., 2005 ; Batt and Aga, 2005 ; Gros and Petrovic, 2009; Moreno-Bondi et al., 2009; Ho et al., 2012).

Sub-samples require pretreatment to enhance the extraction. Elimination of water can be achieved by drying the solid matrix at 60, 100 or 105°C for 24 or 72 h (Golet et al., 2002; Gobel et al., 2005; Ho et al., 2012; Kim et al., 2012). The lyophilization is also used, but some extraction methods used directly fresh samples (Meesters and Schröder 2002; Moreno-Bondi et al., 2009 ; Li et al., 2013; Pamreddy et al., 2013). The best option is lyophilization because antibiotics are not degraded or evaporated (Meesters and Schröder, 2002). Sample homogenization is obtained by grinding and the fraction that is less than 2 mm, 0.5 mm or 100 μm is used directly during the extraction process (Moreno-Bondi et al., 2009; Andreu et al., 2009; Wu et al., 2011; Li et al., 2013).

In some studies, sub-samples could be spiked before the extraction. The spiking step is conducted using a stock mixture of the target compounds in solvent (methanol or acetonitrile) and then mixing with the sub-samples. To ensure adsorption equilibrium and to allow complete evaporation of the solvent, the mixtures sample-antibiotics are kept under frequent homogenization at room temperature. It is recommended that sub-samples be stored in the dark prior the extraction (Golet et al., 2002; Gobel et al., 2005; Moreno-Bondi et al., 2009).

2. Methods of extraction

Various extraction techniques were developed for solid samples such as soxhlet extraction, shaking extraction, ultrasonic extraction, microwave-assisted extraction and QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) (Zuloaga et al., 2012; Lindholm-Lehto et al., 2016). The extraction of antibiotics from sludge or compost is affected by various adsorption mechanisms such as hydrogen bonding, cationic exchange, hydrophobic interactions and complexation involved during the retention of antibiotics in the solid phase (Pamreddy et al,. 2013). So, the accessibility to antibiotics during their extraction could be more difficult and needs a robust and aggressive technique such as the pressurized-liquid extraction (PLE). PLE, which is commonly known as accelerated solvent extraction (ASE), was already used for the antibiotics extraction from sludge, compost or soil (Golet et al.,

2002; Jacobsen et al., 2004; Stoob et al., 2006; Lillenberg et al., 2009; Nieto et al., 2010; Ding et al., 2010).

To provide good recoveries, PLE is conducted at optimal conditions including temperature, pressure, solvent of extraction and static cycles. Before the extraction process, sub-sample are thoroughly mixed with selected sorbents (quartz sand for example) in order to obtain optimum pressure conditions, to increase contact surface area between compost and solvents and to prevent the overflow of the extraction cell (Pamreddy et al., 2013). The sub-sample and the selected sorbents were placed in a metal cell (6, 11 or 22 ml) lined with glass fiber filters. The cell is transferred to the oven of the apparatus and sealed under pressure before being heated and pressurized. After preheating, the cell is filled with solvent and kept at a set temperature, pressure and static time. High pressure is used to allow the use of solvent at temperature higher than its normal boiling point, and thus improving the extraction rate.

The extraction of antibiotic from complex matrix such as sludge or compost is conducted by using a wide variety of solvent mixtures. Many of those mixtures combine water with other solvent as in the case of water-acetonitrile or water:methanol for the extraction of sulfonamides, tetracyclines and macrolides (Nieto et al., 2007; Ding et al., 2010 ; García-Galán et al., 2013; Pamreddy et al., 2013; Jelic et al., 2009 ; Li et al., 2013). In some studies acidified water was used for the extraction of tetracyclines and sulfonamides (Pamreddy et al., 2013). Other solvents mixtures were used to extract antibiotics such as MeOH-citric acid for tetracyclines and sulfonamides (Pamreddy et al., 2013), MeOH-McIlvaine buffer for quinolones (Dorival-Garcı´a et al., 2013), MeOH-acetone for sulfonamides (Silvia et al., 2006), phosphoric acid-acetonitrile for fluoroquinolones, tetracyclines and sulfonamides (Golet et al., 2002; Lillenberg et al., 2009).

Other particular variables of PLE that are mostly studied are temperature, pressure, number and time of static cycles. In the case of the temperature, the range found in the literature is within room temperature and 100 °C (Golet et al., 2002; Gobel et al., 2005; Silvia et al., 2006; Nieto et al., 2007; Jelic et al., 2009; Ding et al., 2010; García-Galán et al., 2013; N. Dorival-Garcı´a et al., 2013; Li et al., 2013). In order to avoid hydrolysis and thermal degradation of certain analytes, extraction was carried out at room temperature (Pamreddy et al., 2013). In same case, strong interactions with solid matrix and some antibiotics compounds, especially those belonging to the FQs family, lead to use high temperature until 110°C (Lillenberg et al., 2009).

In the case of extraction pressure, this is set around 100 bars, while static extraction time is usually within 1–15 min, although 5 min of extraction time is mostly used. In the case of extraction cycles, from 1 to 5 cycles are mostly used. The combination of 3 extraction cycles of 5 or 15 min is very common (**Table. 5**).

3. Clean-up procedures

Extraction techniques, such as ASE, are not selective and therefore a clean-up step is recommended. Sulfur elimination, solid-phase extraction (SPE), gel permeation extraction (GPC), combined extraction and clean-up in selective pressurized liquid extraction (SPLE) and other clean-up approaches are used.

Generally, all extraction methods of antibiotics that are carried out using the ASE are usually followed by a SPE. This clean-up step is conducted using HLB (Hydrophilic–Lipophilic Balance) and SAX (Strong Anion Exchange) cartridges (Andreu et al., 2009; Akın Karcı et al., 2009; Huang et al., 2010). Oasis HLB is one of the most widely utilized sorbent for pharmaceutical extraction in solid environmental samples. This is mainly due to its hydrophilic–lipophilic balance properties, efficient in the extraction of a wide range of acidic, basic and neutral compounds from various matrices. Some procedures combine two cartridges, one containing SAX to remove organic matter and the other one using HLB (Table 1). The SPE involves conditioning of cartridge with methanol, EDTA buffer or ultra-pure water. The cartridge is eluted by methanol, ethyl acetate, or methanolic oxalic acid, and then the eluate is concentrated under a flow of nitrogen before analysis (Blackwell et al., 2004; Kim et al., 2012; García-Galán et al., 2013; Dorival-Garcı´a et al., 2013; Li 2013).

Tableau 5: Synthesis of the analytical approach based on accelerated solvent extraction for antibiotic determination in sludge

Analyte	**Sample type**	**Extraction conditions**	**Clean-up**	**Analysis conditions**	**Method stability and precision**	**Recovery (%)**	**Ref.**
Sulfonamides	Sewage sludge	-ASE 300 -Sub-sample : 2g -Solvents : ACN-water (25 :75, v/v) -Temperature : 50°C -Pressure : 1500 psi -3 static cycles of 5 min	SPE : Oasis HLB cartridges	-LC-MS/MS -Separation : C_{18} LC-column -Eluent A : Acidified water -Eluent B : ACN with HCOOH -Mass analyses : Tripe-quadruple spectrometer -Ionisation : ESI+ -Acquisition mode : SRM	-RSD : < 23% -MLODs : 0.03-2.23 ng/g	60-130	García-Galán et al., 2013
Tetracyclines Sulfonamides	Sewage sludge	-ASE 150 -Sub-sample : 0.2g -Solvents :ACN-water, MeOH-citric acid, acidified water -Temperature : room temperature -Pressure : 1500 psi -2 static cycles of 13 min	SPE : hydrophilic–lipophilic balance cartridges	-LC-MS/MS -Separation : C_{18} LC-column -Eluent A : 1% acetic acid- water -Eluent B : Methanol -Mass analyses : Tripe-quadruple spectrometer -Ionisation : ESI+ -Acquisition mode : MRM	-RSD : 0.7-18% -MLODs : 0.6-4.2 ng/g (sulfonamides) , 3.2-13 ng/g (tetracyclines)	90.4-99.9 (sulfonamides) 96.2-100.9 (tetracyclines)	Pamreddy et al., 2013
Quinolones	Sewage sludge	-Sub-sample : 0.5 g -Solvents : MeOH-McIlvaine buffer (50:50; v/v, pH=3) -Temperature : 86°C -Pressure : 1000 psi -5 static cycles of 5 min	The extracts were centrifuged and the supernatants were directly injected into the LC	-LC-MS/MS -Separation : C_{18} LC-column -Eluent A : acidified water -Eluent B : Methanol -Mass analyses : Tripe-quadruple spectrometer -Ionisation : ESI+ -Acquisition mode : SRM	-RSD : < 7% -MLODs : 2-5 ng/g	90.4-94.6	Dorival-Garcı´a et al., 2013
Tetracyclines Sulfonamides other pharmaceuticals compound	Sewage sludge	-ASE 200 -Sub-sample : 0.5 g -Solvents : ACN-water (7:3; v/v) -Temperature : 100°C -Pressure : 1000 psi -3 static cycles of 15 min	SPE : HLB cartridge	-LC-MS/MS -Separation : C_{18} LC-column -Eluent A : water -Eluent B : ACN -Mass analyses : Tripe-quadruple spectrometer	LODs : 4.6-146 ng/g (tetracyclines) 0.6-1 ng/g (sulfonamides)	49-68 (tetracyclines) 64-98 (sulfonamides) 77-88 (other pharmaceuticals)	Ding et al., 2010
Fluoroquinolones	Sewage sludge	-Sub-sample : 0.5 g -Solvents : phosphoric acid-ACN (1:1; v/v) -Temperature : 100°C -Pressure : 1000 psi -3 static cycles of 15 min	MPC disk cartridges	Liquid chromatography fluorescence detection (LC-FLD)	-RSD : 8-11% -LOQ : 0.45 µg/g	82-94	Golet et al., 2002
Fluoroquinolones Tetracyclines Sulfonamides	Sewage sludge	-Sub-sample : 9 g -Solvents : 0.35% phosphoric acid and ACN -Temperature : 100-110°C -Pressure : 100-110 atm -5 static cycles of 10 min	SPE : SCX and HLB cartridges	-LC-MS/MS -Separation : C_{18} LC-column -Eluent A : methanol -Eluent B : ammonium buffer	-RSD : < 5.06% -LOQ : 0.1-160 ng/g	59-98 (fluoroquinolones) 95 TC (tetracyclines) 43-96 (sulfonamides)	Lillenberg et al., 2009
Sulfonamides Macroldies	Sewage sludge	-ASE 200 -Sub-sample : 200 mg -Solvents : Water-MeOH (50 :50, v/v) -Temperature : 100°C -Pressure : 100 bar -2 static cycles of 5 min	SPE : Oasis HLB	-LC-MS/MS -Separation : C_{18} LC-column -Eluent : MeOH-acidified water -Mass analyses : Tripe-quadruple spectrometer -Ionisation : ESI+ -Acquisition mode : MRM	LOQ : 3-41 ng/g	78-142	Gobel et al., 2005
Sulfonamides	Sludge	-ASE 200 -Sub-sample : 5 g	SPE : Oasis HLB	-LC-MS/MS -Separation : C_{18} LC-column	LOQ : 5 pg/g – 0.6 ng/g	1-104	Silviaet al., 2006

Tableau 5: Synthesis of the analytical approach based on accelerated solvent extraction for antibiotic determination in sludge

Analyte	**Sample type**	**Extraction conditions**	**Clean-up**	**Analysis conditions**	**Method stability and precision**	**Recovery (%)**	**Ref.**
		-Solvents : Acetone-MeOH (50 :50, v/v) -Temperature : 75°C -Pressure : 150 bar -3 static cycles of 5 min		-Eluent A: Acidified water -Eluent B : ACN-acidified water -Mass analyses : Tripe-quadruple spectrometer -Ionisation : ESI+ -Acquisition mode : SRM			
Sulfonamides Macroldies	Sewage sludge	-ASE 200 -Sub-sample : 5 g -Solvents : Water-MeOH (50 :50, v/v) -Temperature : 80°C -Pressure : 150 bar -1 static cycle of 5 min	Extracts were filtred (0.45 µm microfilter) and analysed directly	-LC-MS/MS -Separation : C_{18} LC-column -Eluent A: Acidified water -Eluent B : ACN -Mass analyses : Tripe-quadruple spectrometer -Ionisation : ESI+ -Acquisition mode : SIM	-RSD : 11-15% -LOQ : 2-11 ng/g	54-over 74	Nieto et al., 2007
43 pharmaceutical compounds	Sewage sludge	-ASE 300 -Sub-sample : 1 g -Solvents : Water-MeOH (2/1, v/v) -Temperature : 100°C -Pressure : 150 bar -3 static cycles of 5 min	SPE : HLB cartridges	-LC-MS/MS -Separation : C_{18} LC-column -Eluent A: ACN-MeOH -Eluent B : Water -Mass analyses : Tripe-quadruple spectrometer -Ionisation : ESI+ -Acquisition mode : SRM	-RSD : 1.2-15% -LOQ : 0.09-22.5 ng/g	40.3-146	Jelic et al., 2009
Quinolones Sulfonamides Macrolides	Sewage sludge	-ASE 350 -Sub-sample : 0.1 g -Solvents : MeOH -Temperature : 70°C -Pressure : 10.34 MPa -2 static cycles of 10 min	SPE : HLB cartridges	HPLC–ESI MS/MS	-RSD : 1.1-14 -LOD : 0.02-0.5	77-122.5	Li et al., 2013

4. Instrumental analysis

Antibiotics, in sludge or sludge compost, are usually analyzed using chromatographic techniques. They were separated with liquid chromatography (LC) coupled with UV (Turiel et al., 2005; Babic et al., 2006), fluorescence (Golet et al., 2002; Prat et al., 2004) or mass spectrometry detection (Moreno-bondi et al., 2009; Kipper et al., 2011). Considering the time required and the consumption of solvents, LC-MS/MS is increasingly popular for the analysis of antibiotics in environmental samples (Zuloaga et al., 2012; Lindholm-Lehto et al., 2016) because of its higher sensitivity and ability to provide compound confirmation compared with conventional liquid chromatography. LC separation is carried out generally in column. Typically, C18 columns are the most used to separate antibiotics from sludge or compost (**Table 5**). The mobile phase is composed of two phases (organic solvents and aqueous phase). Both acetonitrile and methanol are used as organic mobile phases. In order to obtain sufficient and reproducible retention times, the use of acidified water or a buffer (ammonium) was recommended. In general, electrospray ionization in positive mode (ESI+) was selected as the most sensitive mode for ionizing antibiotics compounds before MS detection (Zuloaga et al., 2012; Lindholm-Lehto et al., 2016).

5. Matrix effect

LC-MS/MS is the most used technique for the quantitative determination of antibiotics extracted from sludge or sludge compost. Nevertheless, one of the main drawbacks of the analysis of antibiotics is the matrix effect (ME) (Matuszewski et al ., 2003; Steene and Lambert et al., 2008 ; Stahnke et al., 2009; Tamtam et al., 2009 ; Stahnke et al., 2012) (**Fig. 2**).

(a) (b)

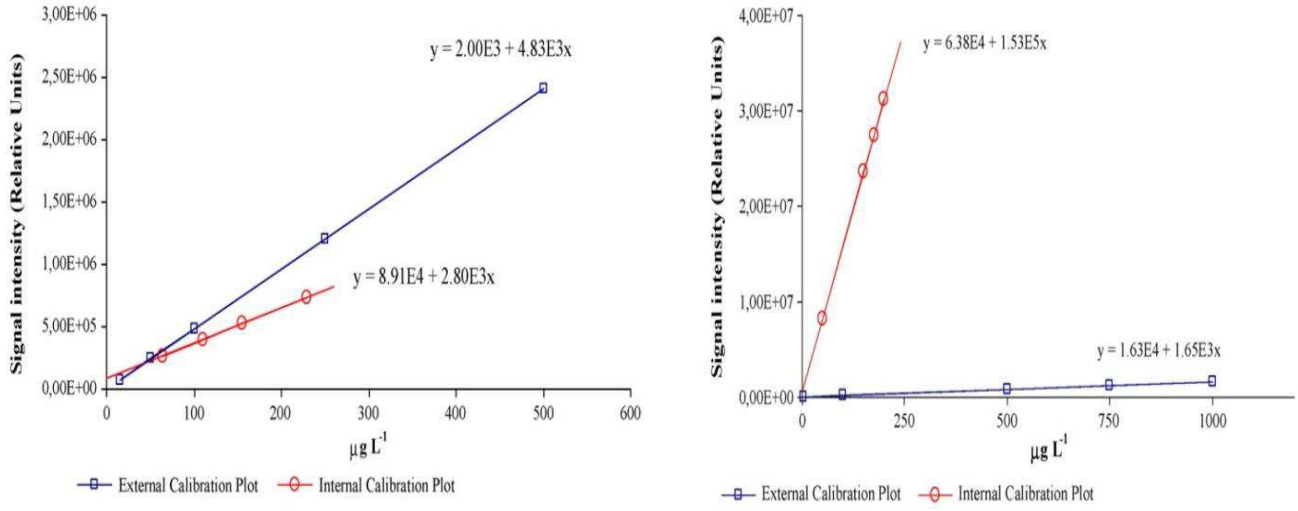

Figure 2: Example of signal suppression (a) and enhancement (b) (Grosetti et al., 2010)

ME effect is the result of a combined effect of all components of the sample, other than analyte on the analytical measurement. If a specific component can be identified to produce an effect on the analytical signal, it is defined as causing interference.

Interferences between antibiotics and all compounds characterized by a chemical structure more or less similar to the target analyte, e.g., ionic species, polar compounds (phenols) and organic molecules (carbohydrates, amines, lipids), organic matter, salts and non-target contaminants, during the evaporation of the target analytes in the electrospray ionization source (ESI) lead to the potential suppression/enhancement of the signal (Gosetti et al., 2010; Cimetiere et al., 2013). Ion suppression/enhancement is induced by the presence of volatile compounds in the matrix that are able to change the efficiency of analyte droplet formation as well as the amount of the analyte ions formed in the gas phase that reaches the detector (Annesley, 2003). The ME is more important in complex matrices such as sludge or compost and affects dramatically the method performance in term of selectivity, repeatability, accuracy, linearity of the response and limit of detection. The extraction process can lead to obtain extracts interfering with the target analytes. Also reagents added to the mobile phase (buffers and organic acids) could be responsible of ion suppression (Annesley, 2003; Matuszewski et al., 2003 ; Mei et al., 2002 ; Mallet et al., 2003 ; Gosetti et al., 2010).

The current regulatory for bioanalytical methods requires the evaluation and correction of matrix effects, because they are considered as a part of the analytical method validation (Matuszewski et al., 2003). The evaluation of the ME consists in comparing the signal

behavior of the target using the matrix-matched calibration curve and the external standard calibration curve. In recent years, approach with isotopically labeled internal standards (deuterium, ^{13}C, ^{15}N) is used to tackle with the ME (García-Galánet al., 2013; Vom Eyser et al., 2015; Aga et al., 2015). Nevertheless, the inconvenient of the isotopically labeled internal standards are their high prices (Punt et al., 2017) and their limited commercial availability (Aga et al. 2015). Several authors reported that ME was negligible for the determination of antibiotics compounds after a clean-up step. The dilution of samples prior to the analysis leads to a decrease in ME but a signal decrease could be observed (Zuloaga et al., 2012). In addition to all previous stated methodologies, the use of calibration based on the standard addition method is employed to correct the ME in LC-MS measurements.

6. Standard addition method

The standard addition method (SAM) is an analytical approach for both inorganic and organic quantification. The use and discovery of the SAM belongs to a chemist called Hohn in 1937 in his book on polarography, *Chemische Analysen mit dem polarographen* (Kelly et al., 2011). The SAM comprises three steps: (1) A measure of the analytical response produced by the test solution; (2) Spike the test solution with one or more known amounts of analyte, and measure the new responses; and (3) From the responses calculate the concentrations of target analyte (Ellisona and Thompson, 2008). Analyte concentration is obtained after plotting response (ordinate) by the amount of standard added (abscissa), and fitting a line to the data and finding the intercept on the abscissa (**Fig. 3**) (Saxberg1 and Kowalski, 1979). The use of several spiking concentrations is justified in the SAM approach by the idea that it helps to check that the calibration is truly linear before calculating concentrations. Linearity is one of the most important criteria that could be checked before using the SAM.

One of the major benefits of the SAM is in mitigating the effects of matrix interferences in the analytical measurement when the matrix composition is unknown or relatively complex (Andersen, 2017). This method assumes that for any analyte there is a specific analytical signal which responds to that analyte and no other unknown sample component leading to correct the ME, recovery loses and quantifies several compounds in the same sample (Saxberg and B R Kowalski, 1979; Punt, 2017)

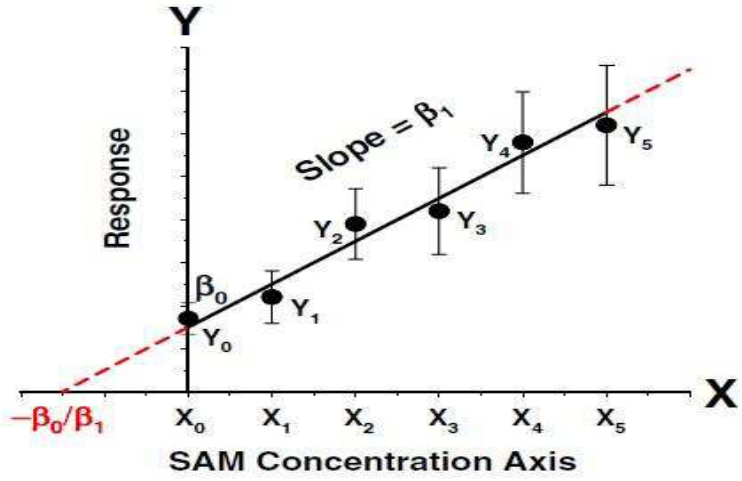

Figure 3: Traditional SAM plot with five additions of increasing amounts (X1 to X5) and one sample without addition at the origin of the abscissa (X0, Y0). (W. Robert Kelly et al., 2011)

The first and original application of the SAM was started since the works of Campbell and Carl (1954) and Tsaihwa and Thompson (1955). Some other studies have been published with a view to estimate the precision (Larsen et al., 1973) or to optimize the SAM approach (Franke and Zeeuw, 1978). After that, the SAM was used successively during the analytical measurements of analytes in water (Santos et al., 2015), urine (Mamian-lopez and Poppi, 2013; Westley et al., 2017), blood (Clarke et al., 2013), tissue (Julshamn and Andersen., 1978), soil (Sadler et al., 1997) or fruit and vegetables (Cesarino et al., 2012).

Antibiotics exhibit a wide range of proprieties, then different behaviors, and their interferences with matrix compounds present substantive analytical challenges. The SAM was applied for the quantification of pharmaceuticals compounds and antibiotics in some matrix such as water (Yanget al., 1996 ; Ide et al., 2016 ; Cimentiere et al., 2013 ; Zhang et al., 2012 ; Zhang et al., 2013), milk (Cañada-Cañada et al., 2009 ; Hajian et al., 2013), urine (Hidi et al., 2016), plasma (Lozano et al., 2009 ; Punt et al., 2017), food (Boscher et al., 2010; Amelin et al., 2016) and solid tissues (Hasegawa and Suzuka, 2014). Nevertheless, the SAM was not used previously as an alternative to correct interferences during antibiotics analysis a complex matrix such as sludge or compost.

In conclusion, this overview allowed us to see the essential points and the limits for the extraction and quantification of antibiotics in these complex matrix. So, all analytical approach on these matrices must take into account the complex interactions between analytes and matrix. Despite the development of increasingly efficient extraction tools (microwaves, ASE, etc..), the SAM remains the best way to increase the reliability of antibiotic analyzes in these matrices.

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Résultats et Discussions

Chapitre 2: Evaluation des effets des antibiotiques sur les paramètres physicochimiques durant le co-compostage des boues avec des déchets de palmier en bioréacteur

Contexte :

Aujourd'hui, une grande quantité d'antibiotiques est utilisée en médecine humaine. Par conséquence, ces molécules peuvent rejoindre l'environnement à travers plusieurs sources, dont l'épandage des boues de stations d'épuration. Le compostage est parmi les technologies les plus utilsées pour le recyclage des boues. Beauccoup d'études ont été focalisées sur le devenir des antibiotiques durant le compostage. En revanche, très peu de travaux s'interessent aux effets des antibiotiques sur les différentes étapes du compostage.

Dans ce travail, le fonctionnement d'un bioréacteur de compostage a été optimisé, en utilisant des boues primaires et secondaires de la station d'épuration de Marrackech, en mélange avec des déchets verts en différentes proportions. Les essais d'optimisation ont permis d'ajuster les paramètres de fonctionnement (débit d'air, fréquence de brassage) et d'identfier le mélange qui permettrait d'avoir une meilleure dégradation de la matiée organique. Les résultats des essais d'optimisation ont été valorisés par un brevet accepté par l'Office Marocain de la Propriété Industrielle et Commerciale (OMPIC). L'OMPIC Maroc a suggèré aussi de déposer le brevet à l'international avec recommandation d'application à l'échelle industrielle.

Suite aux résultats d'optimisation, un mélange a été retenu pour conduire des essais de compostage en présence de trois familles d'antibiotiques à différents niveaux de contaminations. L'effet des antibiotiques sur les paramètres physiques et chimiques a été évalué. En plus, une approche microbiologique sur les interactions entre les antibiotiques et le co-substrat lignocellulosique utilisé a été proposée. Cette étude a permis d'identifier les concentrations critiques capables d'affecter le processus de compostage et d'altérer la qualité du compost final.

Principaux résultats :

Cette étude a mis en évidence l'effet des antibiotiques sur les paramètres thermiques, l'efficacité du processus de compostage ainsi que la qualité du compost. La présence des antibiotiques à des concentrations élevées réduit la durée de la phase thermophile, affecte la montée et la descente de la température ainsi que la libération de chaleur le long de la phase de stabilisation. De plus, la dégradation de la matière organique est retardée dans le temps et le rapport C/N est significativement affecté.

Ces résultats démontrent que l'éfficacité du processus de compostage et la qualtité finale du compost sont affectées par les antibiotiques. La présence et la disparition du rebond de

température dans ces conditions de co-compostage est un critère important permettant de prédire la qualité du compost final. Le modèle proposé pour décrire les interactions dans le système rachis-boue-antibiotiques permet de montrer que l'adsorption de certains antibiotiques sur les rachis débuterait dès la phase de stabilisation. Le niveau de contamination aurait un effet sur le degré de colonisation des rachis par les microorganismes et jouerai un rôle important sur l'érosion biologique. Cet effet se traduit par la conservation de la taille des rachis après la phase de maturation.

Ces travaux, présentant les premiers résultats de la thèse, on fait l'objet d'un article publié dans la revue Waste Management, intitulé :

Evaluation of the antibiotics effects on the physical and chemical parameters during the co-composting of sewage sludge with palm wastes in a bioreactor

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Abstract

The objective of this study was to investigate thermal and physicochemical parameters of sewage sludge-palm waste mixtures contaminated by different families of antibiotics (tetracyclines, macrolides and fluoroquinolones) during co-composting. Sludge was spiked with chlortetracycline (CTC), oxytetracycline (OTC), roxithromycin (RXY), enrofloxacin (ENR) and ciprofloxacin (CIP). Antibiotics were spiked at a low level, medium level, high level and a control without antibiotics was conducted. The results showed that the organic matter degradation was delayed and the carbon/nitrogen (C/N) ratio was affected by an increase of the antibiotics concentration. The presence of antibiotics, especially at high level, delayed the coming of the temperature maxima, and disturbed the thermophilic phase. The calorific model showed that the heat release is affected during the thermophilic phase when high antibiotics concentrations were used. In addition, the microbiological approach showed that the adsorption of antibiotics on the rachis could be probably responsible of the fungi inhibition especially during the maturation phase. Therefore, the medium and high levels of antibiotics affected the thermal, physical and chemical parameters as well as the compost quality.

Keywords: Antibiotics, Sewage sludge, Palm wastes, Bioreactor co-composting, Microbiological approach.

1. Introduction

The annual use of antibiotics is estimated to be between 100,000 t and 200,000 t worldwide (Jeong et al., 2010). In the United States, 16,000 t of antimicrobial compounds are used annually, 70% of them are used for non-therapeutic purposes (UCS, 2001). Although, 2/3 of antibiotics are used in human medicine and 1/3 are used for veterinary purposes in the Europeans countries (Sarmah et al., 2006). For Morocco, the consumption per family of antibiotics was evaluated statistically for human use over a period of ten years (Inouss et al., 2015). The use of antibiotics for human health purpose (domestic and hospital) leads to their continuous release into the environment via different sources, such as the waste water treatment plants (WWTP). Several researches confirm the persistence of antibiotics in the WWTP which can be considered as an anthropogenic source for contamination by antibiotics (Rizzo et al., 2013). WWTP include different processes which are regarded as limiting for antibiotics elimination (Mao et al., 2015; Polesel et al., 2016).So, a part of these molecules are discharged in the environment with the out coming of treated water and the other part is fixed to the sludge process (Michael et al., 2012; Xu et al., 2015). Several studies were reported that the antibiotics concentrations can range of µg/kg to a few mg/kg in sludge (Golet et al, 2002a; Martin et al, 2014; Verclicchi et al., 2015) and their direct application is a source of antibiotics emergence in the environment. The wide occurrence of antibiotics in the environment can affect the aquatic and terrestrial organisms even at low concentrations of a mixture of antibiotics (Khadra et al., 2012), alter the microbial activity and the microbial community composition, and can also lead to the prevalence of a bacterial resistance to antibiotics (Zhou et al., 2013).Composting is a natural process for organic matter conversion by micro-organisms under aerobic conditions and leads to a stable and hygienic fertilizer which can be reused for improving the quantity of humic substances and increasing the aromatization degree in the soil (Amir et al., 2010). Composting is an effective solution to reduce the highest produced quantities of a several type of waste such as palm waste and sludge. For example, the palm groves of Marrakech city in Morocco generate a significant amount of palm waste products. According to the environmental services of the urban commune of Marrakech, the palm waste generated is about 8,000 tons per year (El Fels, 2014). In the other hand, The WWTP of Marrakech produces actually 50,400 kg/d of primary sludge and 89,600 kg/d of secondary sludge (Belloulid et al., 2016).

Although the fate of antibiotics during co-composting has been investigated (Arikan et al., 2009; Selvam et al., 2012;Ho et al., 2013; Liu et al., 2015),but little information is given in the literature about the influence of antibiotics on the thermal and physicochemical

parameters during the sludge co-composting process in controlled conditions. The objectives of this study were (i) to optimize and control the operating parameters of a co-composting bioreactor, (ii) to evaluate the effect of some antibiotics at different concentrations on the thermal, physical and chemical parameters during the co-composting process, (iii) to present a microbiological approach concerning the interactions mechanisms between the antibiotics and the substrates used for the co-composting process and more particularly the lignocellulosic one, and finally (iv) to approach an effective antibiotic concentration that is enables or not to influence the co-composting process and alters or not the compost quality.

2. Materials and Methods

2.1.Bioreactor description and its operating conditions

The co-composting experiments were conducted in the bioreactor described by Viel et al. (1987) with some modifications (Fig. 1). The bioreactor is a cylindrical tank in stainless steel (100 L), surrounded by a 8 cm polyurethane insulating. The tank has been fixed on a swivel stand in order to simplify the handling and the compost loading. The bioreactor is composed of a circular waterproof cover with a hole for samples collection. The fresh air, coming out from a compressor, was injected inside the bioreactor with a constant flow under the control of a rotameter, the compost mixing was conducted by a programed system, the temperature of compost was measured at the three points inside the bioreactor, the exhaust gas was trapped in a concentrated sulfuric acid solution, the condensed water was recovered at the top of the bioreactor and a biological filter was used to trap the upsetting odors. After 12 days of stabilization in the bioreactor, this stage of compost was transferred into the perforated bag to conduct the maturation stage for 6 month.

2.2.Bioreactor optimization and the retained co-composting substrates

Optimization experiments were conducted in order to control the operating parameters of the bioreactor and to choose the good mixture composition in view to perform a reference experiment which could allow the better organic matter degradation and offer the maximum loading of sludge during the co-composting process. 3 substrates of co-composting have been used during the optimization experiments. The palm wastes (palm rachis and leaves separate) and the grass were collected from the Faculty of Science-Marrakech (Morocco). The primary (non-treated) and secondary (biological) sludges were collected from the WWTP of Marrakech. The experiments with secondary sludge were conducted for having a comparison

with the primary sludge which was finally retained for the antibiotic co-composting experiment. The Table 1 presents the physical and chemical characteristics of the substrates used during all optimization experiments, and the Table 2 presents the percentage of each substrate used during the 5 experiments of co-composting (E1-E5) conducted to optimize the bioreactor.

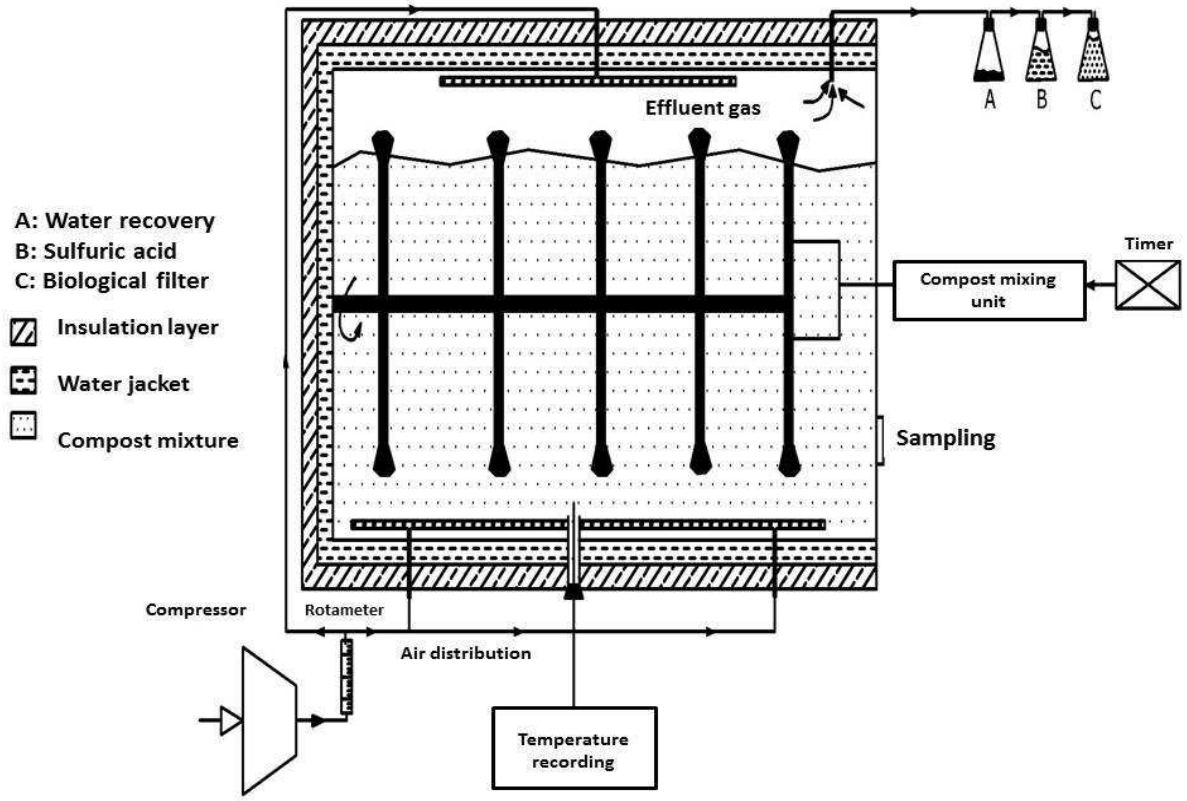

Figure 1: Co-composting bioreactor

Tableau 1: Physical and chemical characteristics of the used substrates during all co-composting experiments

Physical and chemical parameters	**Sludge**		**Green waste**		
	Primary sludge	**Secondary sludge**	**leaves waste** *(Phenix)*	**Palm rachis** *(Phenix)*	**Grass** *(Cynodon dactylon)*
pH	7.1±0.1	7.1±0.1	6.2±0.1	6.5±0.2	6.5±0.2
Moisture[a] (%, FW)	66.8±3.9	56.2±1.3	6.1±0.1	19.7±0.3	67.9±5.6
Ashes content[b] (%, DW)	45.5±0.4	42.9±0.5	11.1±0.1	63.7±0.5	18.4±0.4
TOC[b] (%, DW)	34.5±0.3	36.2±0.3	56.3±0.1	23±0.9	51.7±0.3
TN[b] (%, DW)	3.8±0.1	3.9±0.1	1.2±0.1	0.8±0.3	2.1±0.1
NH_4^{+b} (%, DW)	0.6±0.1	0.4±0.1	0.1±0.2	-	0.3±0.1
C/N	9	9	49	29	24

[a] Results expressed per unit weight fresh matter; [b] Results expressed per unit weight dry matter; TOC: Total Organic Carbon; TN: Total Nitrogen, NH^{4+}: Ammonium

Tableau 2: Substrates proportion (%) for the optimization experiments

Co-composting experiments	**Primary sludge (%)**	**Secondary sludge (%)**	**Rachis (%)**	**Palm leaves** *(Phenix)* **(%)**	**Grass** *(Cynodon dactylon)* **(%)**
E1	40		34	15	11
E2		40	34	15	11
E3	50		32	11	7
E4	66		21	11	2
E5		66	21	11	2

2.3.Antibiotics

The Tetracyclines (chlortetracycline hydrochloride (75% of purity) and oxytetracycline hydrochloride (95%)), a Macrolides (roxithromycin (90%) and the Fluoroquinolones (enrofloxacin (98%) and ciprofloxacin (98%)) were purchased from Sigma-Aldrich-France (F-38297, St-Quentin Fallavier). The stock antibiotics solutions were prepared in methanol (MeOH-HPLC grade, Sigma-Aldrich) and used directly for sludge spiking.

2.4.Co-composting experiments using spiked sludge by antibiotics

The primary retained sludge was spiked by a mixture of 5 molecules of antibiotics according to a low level (AE2), a medium level (AE3) and a high level (AE4) (Table 3). A control test (AE1) without antibiotics was conducted in parallel. For each antibiotic family, the choice of the antibiotics concentrations for the same level was based on the values published in previous studies (Golet al., 2002; Arikan et al., 2009; Sun et al., 2010; Selvam et al., 2012; Ho et al., 2013; Huang et al., 2013; Vom Eyser et al., 2014). The co-composting experiments with antibiotics were conducted under the same conditions as described in the optimization experiments and according to the ratios used in the mixture for the reference experiment which showed good organic matter degradation in presence of a significant amount of primary sludge.

2.5.Sampling and analysis

For the optimization experiments, sampling were carried out at T_0 (before composting), T_2 (two days of co-composting in the bioreactor), T_{12} (after 12 days of co-composting in the bioreactor), T_{30} (30 days of maturation in bags) and T_{90} (90 days of maturation in bags).In view to have an intermediate sample reflecting the stabilization step evolution and to ensure the representativeness of the final compost product under the presence of antibiotics, others

sampling points were added in the co-composting experiments using spiked sludge by antibiotics. So, T_5 (after 5 days of co-composting in the bioreactor), and T_{180} (after 180 days of maturation) were added. In each stage, a homogeneous sample is taken using the quartering method. All samples were kept at -20°C until the physicochemical analysis. Analyses were conducted in triplicate. Different physical and chemical analyses were performed. The pH was measured on aqueous suspension (sample-water; ½; v/v). The moisture was determined by drying the compost at 105°C during 48h (AFNOR, 2000). The total organic carbon (TOC) and the ash contents were determined after calcination in a muffle furnace at 600°C during 6hours. The total nitrogen (TN) was determined according to the Kjeldahl method. Ammonium was determined by a sample distillation in an alkaline medium.

The decomposition rate was calculated according to the following formula (Paredes et al., 1996):

$$\text{Decomposition rate (\%)} = 100\text{-}100[\text{Ashi}(100\text{-Achf})]/[\text{ Achf }(100\text{- Ashi})]$$

Ashi is the initial level of ash and Ashf is the final level of ash.

2.6.Evaluation of calorific value

Among the parameters of the composting process, the temperature is one that influences significantly the final compost stability. However, several mathematical models were developed to assess the temperature or the heat release during the co-composting process. The model developed by Eguchi et al. (2012) was used, with some modifications, to follow the heat release during co-composting. This model is based on the recordings of temperature inside the bioreactor during the aerobic phase. Given our optimized conditions during co-composting, we defined the beginning of the sludge co-composting process when the mixture inside the bioreactor reached a temperature 20°C higher than the room temperature. Then, temperature data was recorded at intervals of 20 min. The assessment of the heat release was done by calculating the following parameters:

Total calorie (TC)	:	$\sum$ (Ti-Tri) ; (Ti-Tri)> 20 Ti: the temperature of the Compost in the time I. Tri: the ambient temperature in the time I.
Calorific Period (CP)	:	The number of the measurement points where (Ti-Tri)> 20 (thermophilic phase duration).
TC/CP ratio	:	A parameter that indicates the average of calorific values and the intensity of the aerobic degradation during thermophilic

phase.

Tableau 3: Antibiotics concentration during co-composting experiments

Family	Molecules	AE1	AE2 (mg/kg DW)	AE3 (mg/kg DW)	AE4 (mg/kg DW)
Tetracyclines	Chlortetracycline	-	10	50	250
	Oxytetracyclines		10	50	250
Macrolides	Roxithromycin		4	20	100
Fluoroquinolones	Enrofloxacin		2	10	50
	Ciprofloxacin		2	10	50

2.7.Evolution of palm rachis remaining weight during co-composting

As it appeared by visual observation that the rachis contributions seemed different in the samples obtained with the different concentrations of antibiotics, an evaluation of the non-attacked part of the rachis was undertaken. The compost samples were sewed using a 10 mm sieve to obtain the final compost homogeneous product (AFNOR, 2004). For each experiment, the refusal from a 100g sub-samples were weighted and estimated taking into account the ash content in percent of the sample. At each sampling time and for each condition, a ratio w/w (g/g) ratio of the remaining rachis present per experiment could be approached versus the ash content.

3. Results and discussion

3.1.Efficiency of a co-composting process using a bioreactor coupled with a perforated bag

5 co-composting experiments were conducted under different mixtures of organic constituents and type of sludge (Table 2) in order to control the stabilization phase inside the bioreactor, then the intermediate product was transferred in a perforated bag for the maturation phase.

Regarding the results shown in Table 4 especially the decomposition rate and the C/N ratio, the degradation rate obtained by using the primary sludge is more effective than the one obtained for the secondary sludge. No significant difference was observed between the final products for the conditions E1 and E4 (E3 being less performant), E4 was retained as the reference condition to conduct the antibiotics experiments since E4 had the maximal load possible for this device (19kg) and was constituted by primary sludge well known for its contain in pharmaceutical compounds and more particularly antibiotics (Martin et al., 2014).

3.2.Sewage sludge co-composting in the presence of antibiotics

According to a previous study on the presence of antibiotics in the WWTP of Marrakech, the antibiotics concentrations observed in the primary sludge were ranging between 0.2 to 1 µg/kg DM for tetracyclines, between 1 and 2 µg/kg DM for the macrolides and between 3 and 5 µg/kg DM for the fluoroquinolones. These concentrations are lower than the ones observed for tetracyclines (Hamsher et al, 2002), for macrolides (Huang et al, 2013; Vom et al, 2015) and for fluoroquinolones (Golet et al., 2002a ; Martin et al, 2014; Verlicchi et al., 2015) due to a lower consumption of antibiotics per capita in Morocco (Inouss et al., 2015). So, the choice of the antibiotics levels used in the spiking protocol was not only depending on the real initial concentration for the primary sludge of Marrakech, but also according to those published elsewhere for the same families of antibiotics in order to assess clearly the effect of the antibiotics on the co-composting process. Co-composting experiments under the E4 condition were conducted by using 3 antibiotics levels. The minimal concentration used in AE2 is a realistic concentration close to the antibiotics concentrations in the environment and more particularly in sludge (Golet et al, 2002a; Martin et al, 2014; Verlicchi et al., 2015). The medium concentration used in AE3 is more generally observed in manure (Hu et al. 2011; Selvam et al. 2012a; Kim et al. 2012). The highest concentration used in AE4 exceeding usual concentrations for sludge or manure co-composting was retained in view to assess the efficiency of the studied co-composting process (Bao et al. 2009; Hu et al. 2011; Kim et al. 2012; Selvam et al. 2012a; Ho et al. 2013).

Tableau 4: Physical and chemical parameters during optimization experiments of co-composting

Experiments		pH	Moisture[a] (%)	Ash content[b] (%)	TOC[b] (%)	TN[b] (%)	NH^{4+} [b] (%)	DR[b] (%)	C/N
E1	T0	5.4±0.1	55.9±1.2	21.3±0.7	49.8±0.4	2.6±0.1	0.2±0.1	-	19
	T12	6.4±0.1	42.4±4.4	24.5±0.5	47.8±0.3	2.5±0.2	0.3±0.3	16.4±2.4	19
	T30	5.9±0.1	35.3±0.4	27.8±0.2	45.7±0.1	2.1±0.2	0.2±0.2	29.6±3.4	17
	T90	6±0.3	43.9±0.2	33.2±0.2	42.3±0.1	3.2±0.1	0.3±0.1	45.4±2.6	14
E2	T0	6.2±0.2	55.1±0.7	28.7±0.5	45.2±0.3	2.6±0.1	0.2±0.4	-	17
	T12	6.6±0.1	53.3±0.9	32±0.3	43.2±0.2	2.4±0.1	0.3±0.1	14.3±3.1	18
	T30	5.8±0.2	48.2±0.5	36.8±0.2	40±0.1	2.8±0.1	0.2±0.1	30.9±1.3	14
	T90	5.7±0.1	51.7±0.3	39.5±0.1	38.3±0.2	3.2±0.2	0.1±0.2	38.4±1.3	12
E3	T0	6.1±0.1	59.4±0.8	28.8±0.3	45.2±0.2	3±0.2	0.3±0.1	-	15
	T12	6.5±0.1	52.3±0.6	33.5±0.2	42.1±0.1	2.6±0.1	0.3±0.2	19.6±1.4	16
	T30	5.9±0.2	38.4±1.2	36.1±0.3	40.5±0.1	3.1±0.2	0.2±0.1	28.3±1.2	13
	T90	6.5±0.1	35.7±0.6	39.7±0.1	38.2±0.2	3±0.1	0.1±0.1	38.6±0.8	13
E4	T0	5.9±0.2	56.1±2.1	29.4±0.1	44.7±0.2	3.9±0.2	0.3±0.1	-	12
	T12	7.3±0.1	38.8±1.8	34.3±0.3	41.6±0.2	3.2±0.2	0.5±0.3	20.4±1.1	15
	T30	6.9±0.1	46.8±0.2	36.1±2.8	40.5±1.7	3.9±0.1	0.5±0.6	26.3±3.1	10
	T90	6.9±0.4	37.1±0.7	41.4±0.2	37.2±0.2	3.5±0.1	0.4±0.3	41.1±2.1	11
E5	T0	5.7±0.2	56.8±2.6	26.6±0.1	46.5±0.1	3.7±0.3	0.3±0.1	-	13
	T12	7.3±0.1	47.7±1.5	32.1±0.3	43±0.2	3.2±0.2	0.5±0.1	23.2±0.5	13
	T30	6.3±0.3	40.2±0.4	35.2±0.3	41.3±0.2	3.8±0.2	0.3±0.1	33.2±0.7	11
	T90	6.5±0.2	42.9±1.2	36.3±0.1	40.3±0.1	3.4±0.1	0.3±0.1	36.5±0.6	12

[a] Results expressed per unit weight fresh matter; [b] Results expressed per unit weight dry matter; TOC: Total Organic Carbon; TN: Total Nitrogen, NH^{4+}: Ammonium; DR: Degradation rate; T0: Before composting; T12: After 12 days of stabilization; T30: After 30 days of maturation; T90: After 90 days of maturation.

3.3.Antibiotics effects on the physical and chemical parameters

Table 5 shows the evolution of the physical and chemical parameters during the co-composting process under antibiotics pressure.

3.3.1. Evolution of pH, Total Organic Carbon (TOC) and Total Nitrogen (TN)

In all experiments, the pH increased rapidly after the 2nd day of the thermophilic phase in the bioreactor (7.6, 7.7, 7.7, and 7.9 respectively for AE1, AE2, AE3 and AE4). This increase of the pH values during the initial stage of co-composting was mainly due to the degradation of organic acids and the release of ammonia in this step. The pH decreased and stabilized toward a value near 7 at the end of all experiments (Amir et al., 2005b; El fels el al., 2014) while the nitrifying process or the synthesis of phenolic compounds took place (He et al., 2009; Lu et al., 2014).

The initial levels of the TOC in the beginning states of the co-composting were similar (46.9, 46.6, 46.3 and 47.0%, respectively for AE1, AE2, AE3 and AE4) and sank to 40.0, 40.5, 37.8 and 42.4% respectively for the 6th month. The decomposition of the TOC in the presence of high antibiotics level especially for AE3 and AE4 is less effective and in agreement with an

inhibition of the microbiological activity under high antibiotics concentrations (Wong et al., 1997).

At the beginning of co-composting process, TN decreased to 3.0, 2.8, 2.7 and 2.8%, respectively for AE1, AE2, AE3 and AE4 after 12 days of co-composting. At the end of the maturation stage, the TN values increased to attempt 3.3, 3.1, 3.1 and 3.1 respectively for AE1, AE2, AE3 and AE4. The decrease of the TN content is mainly due to the loss of ammonia especially during the thermophilic stage (Viel et al., 1987; Eghball et al., 1997). The TN increase is due to the strong degradation of the carbonaceous compounds affecting the total mass of compost but not the nitrogen one (Bernal, et al., 1998).At the end of co-composting, the TN values for AE2, AE3 and AE4 are lower than AE1, in agreement with the proposed vulnerability of the nitrogen transforming microorganisms towards high antibiotics concentrations (Liu et al., 2015).

3.3.2. Degradation rate of organic matter and C/N ratio

At the end of the co-composting process, the decomposition rate of the organic matter is 40.2, 36, 34.9 and 29.8 % respectively for AE1, AE2, AE3 and AE4. Likewise, in the final stage of co-composting the C/N ratio is about 12, 12.7, 13 and 13.6 respectively for AE1, AE2, AE3 and AE4. AE1 conditions showed a very important reduction of the organic matter. Indeed, the presence of the antibiotics especially for the medium and the high level delayed the organic matter degradation in agreement with previous studies (Selvam et al., 2012; Eguchi et al., 2012). In the final stage of co-composting, the C/N ratio for AE1 and AE2 is close to 10 (reference value) indicating the maturity of the final compost (El hajjouji et al., 2007; El fels el al., 2014). Those experiments arrived in a stage of maturity much more advanced than that of AE3 and AE4. The profiles of C/N ratio appeared to be influenced by the changes in the carbon and nitrogen contents, during the co-composting process, especially for AE3 and AE4 (Arikan et al., 2009; Selvam et al., 2012; Liu et al., 2015). As the C/N ratio is a critical factor for the evaluation of the use of the compost as a fertilizer, the difference between C/N ratios reflects the differences in the reaction mechanism in presence of antibiotics or not.

Tableau 5: Physical and chemical parameters during co-composting experiments performed with antibiotics

Experiments		pH	Moisture[a] (%)	Ash content[b] (%)	TOC[b] (%)	TN[b] (%)	DR[b] (%)	C/N
AE1	T0	6.6±0.1	56.1±2.6	25.8±0.4	47.1±0.2	4.1±0.1	-	12
	T2	7.6±0.1	48.7±1.2	28.1±0.8	45.5±0.5	3.2±0.3	11.2±2.7	14
	T5	7.4±0.2	47.3±0.6	28.8±0.2	45.1±0.1	3.1±0.2	14.1±2.3	15
	T12	7.2±0.1	46.1±1.1	29.9±0.1	45.1±0.1	3.1±0.1	14.5±1.7	15
	T30	6.4±0.2	50.3±2.1	30.5±0.3	44.6±0.2	3.5±0.1	16.9±0.9	13
	T90	6.8±0.1	41.9± 0.6	35.6±0.2	40.8±0.1	3.4±0.2	37.2±0.5	12.1
	T180	6.8±0.1	40.1±0.4	36.8±0.2	40.2±0.2	3.3±0.1	40.3±1.5	12
AE2	T0	6.6±0.2	57.2±1.4	26.3±0.2	46.6±0.1	3.8±0.2	-	12
	T2	7.7±0.1	49.1±1.1	27.2±0.7	46.1±0.5	2.8±0.1	4.6±3=2.1	17
	T5	7.5±0.1	51.7±1.3	28.5±0.2	45.2±0.1	2.9±0.1	10.6±1.5	16
	T12	7.4±0.1	48.2±1.6	28.6±0.1	45.2±0.2	2.9±0.2	11.1±1.4	16
	T30	6.9±0.1	43.7±0.9	29.3±0.1	44.7±0.4	3.2±0.1	14.1±2.6	14
	T90	7.1±0.1	35.8±0.2	34.6±0.1	41.4±0.1	3.2±0.1	32.5±1.1	13
	T180	6.9±0.2	35.3±0.9	35.9±0.3	40.5±0.2	3.2±0.2	36.4±0.3	12.7
AE3	T0	6.7±0.1	51.1±1.8	26.7±0.2	46.4±0.1	3.9±0.1	-	12
	T2	7.7±0.1	44.1±0.7	27.8±1.4	45.7±0.9	2.9±0.2	5.6±1.7	16
	T5	7.6±0.1	45.1±1.1	28.1±0.9	45.3±0.6	2.7±0.1	8.6±2.3	17
	T12	8.3±0.1	45.8±3.8	28.1±0.1	44.9±0.1	2.7±0.2	10.8±0.8	17
	T30	7.5±0.1	40.8±1.3	28.9±1.1	44.5±0.6	3.1±0.2	13.9±1.2	14
	T90	7.2±0.2	36.2±0.5	35.8±0.2	40.6±0.1	3±0.1	34.8±0.2	13.6
	T180	7.3±0.1	36.1±2.1	35.9±0.1	40.6±0.1	3.1±0.1	34.9±0.1	13
AE4	T0	6.9±0.3	51.2±1.8	25.7±0.3	47.1±0.2	3.4±0.1	-	14
	T2	7.9±0.2	38.3±1.2	25.9±0.1	46.9±0.1	3.1±0.2	1.2±1.1	16
	T5	7.9±0.1	31.3±0.6	26.1±0.1	46.8±0.1	2.9±0.2	1.7±1.5	16
	T12	8.1±0.1	46.3±1.6	26.3±0.3	46.6±0.2	2.8±0.1	3.4±2.9	17
	T30	7.7±0.2	50.2±1.9	27.8±0.1	45.7±0.1	3.2±0.1	10.2±0.8	14
	T90	7.3±0.1	49.1±2.1	31.9±0.2	43.1±0.1	3.1±0.2	26.1±1.3	13.8
	T180	7.2±0.2	41.3±3.6	33.1±0.1	42.4±0.1	3.1±0.1	29.9±1.5	13.6

[a] Results expressed per unit weight fresh matter; [b] Results expressed per unit weight dry matter; TOC: Total Organic Carbon; TN: Total Nitrogen; DR: Degradation rate; T0: Before composting; T2: After 2 days of stabilization; T5: After 5 days of stabilization; T12: After 12 days of stabilization; T30: After 30 days of maturation; T90: After 90 days of maturation; T180: After 180 days of maturation.

3.4.Antibiotics effects on the thermal parameters

3.4.1. Temperature profiles

Fig. 2 shows the temperature profile during the thermophilic phase in the whole co-composting process. All the co-composting experiments arrived respectively for AE1, AE2, AE3 and AE4 at a maximum temperature of 72.5, 64.5, 71.5 and 66.47°C after 32, 20, 33, and 51 hours after the beginning of the co-composting process. The thermophilic stage began after 13, 13, 18 and 27 hours and its duration was of 64, 37, 53 and 50 hours respectively. During the thermophilic phase, the temperature has remained above 60°C for 48, 20, 33 and 20 hours respectively for AE1, AE2, AE3 and AE4.

Subsequently, the temperature decreased after 5 days of co-composting and stabilized between 25-35°C toward the end of the aerobic digestion for AE2, AE3 and AE4. For AE1, after 5 days of co-composting, we observed a rebound which lasted 3.2 days, according to a potential low level of antibiotics in the primary sludge of the WWTP of Marrakech. The maximum temperature of the rebound was of 49°C and it was not observed for the other experiments under antibiotics. The rebound observed in AE1 is a characteristic temperature shape of a date palm composting process as described by Alkoaik et al. (2011). The appearance of this rebound reflects the development of some fungi or actinomycete species which have the ability to degrade a part of the thermophilic phase residues and it induces an advanced beginning of the maturation phase. This rebound was not observed in the presence of antibiotics for the other experiments. Probably, the fungi or the actinomycete species could be inhibited or these micro-organisms could not find substrates favorable to their apparition and development.

The high temperature recorded and the duration of the thermophilic phase for a long time would destroy most of the pathogens contained in the sludge, leading to obtain compost with a good sanitary standard (Bernal et al., 2009). The thermophilic microorganisms improved the decomposition of organic matter into more stable humic components and inorganic compounds (Hu et al., 2011). The high temperature reached in all co-composting experiments was due to the bioreactor optimization and the continuous air injection. The antibiotic concentrations played on both delay to reach the maximal temperature value and length of the plateau (temperature above 60°C).

3.4.2. Temperature curve analysis for its increase or decrease in the co-composting experiments

The Fig. 1 showed that the temperature shapes are biphasic for all the co-composting experiment. According to the possibilities of the micro-organisms growth models described by Holy and Holzapfel. (1988) and Zwietering et al. (1990); the increase rate of temperature curves can be fitted using a linear function, and the decrease rate of temperature curves can be fitted using an exponential function (Fig. 3). According to the curves of the respective temperature increase (Fig. 3a), there is a decrease in the rise speed depending on antibiotics concentration (reduction of the coefficient ***a***). The temperature increased slowly especially for AE4 for which the coefficient ***a*** was about 0.95 (coefficient ***a*** of the control experiment: 1.70). The curves for the temperature decrease (Fig. 3b) showed that the speed of decreasing is very fast when the antibiotic concentration was increasing (reduction of the coefficient **b**).

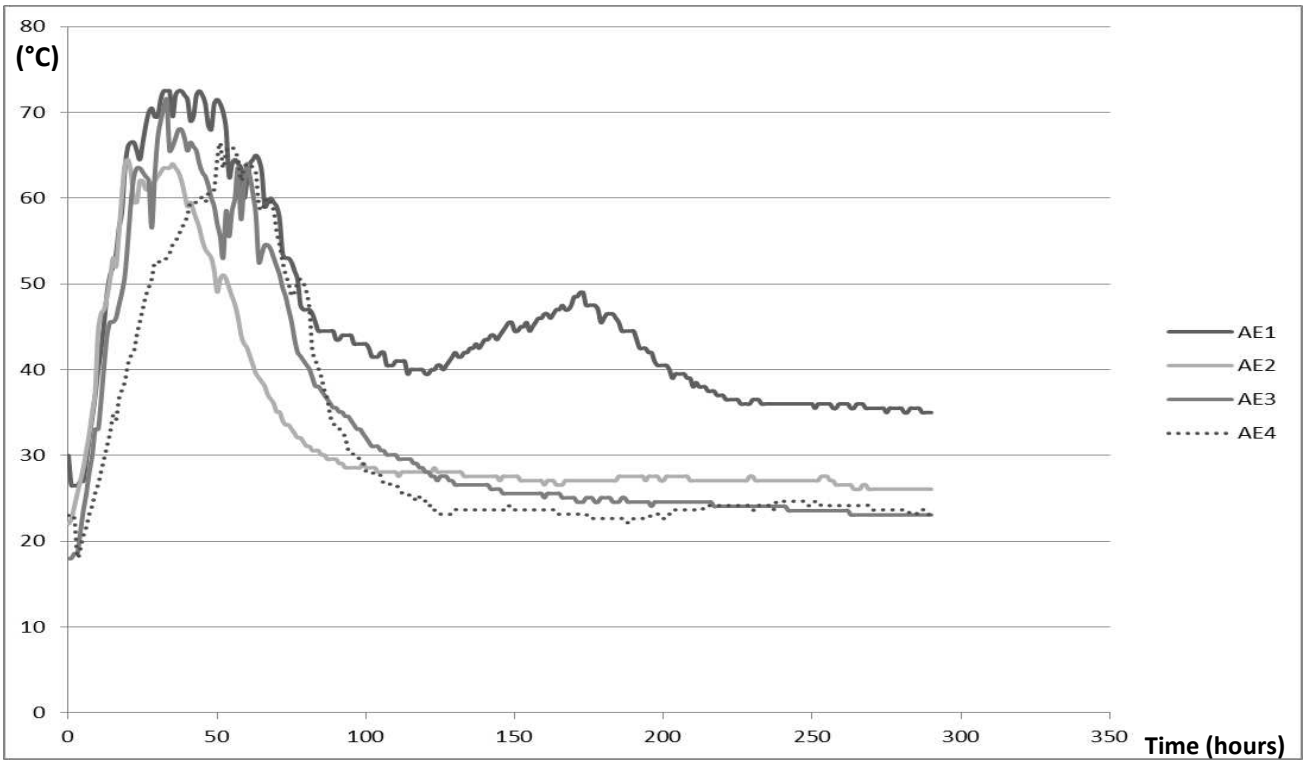

Figure 2: Temperature profiles during co-composting experiments with antibiotics

In addition, the temperature decreased rapidly for AE4 (coefficient ***b***: -0.02). Therefore, the temperature raising and lowering are influenced by increasing antibiotic concentration.

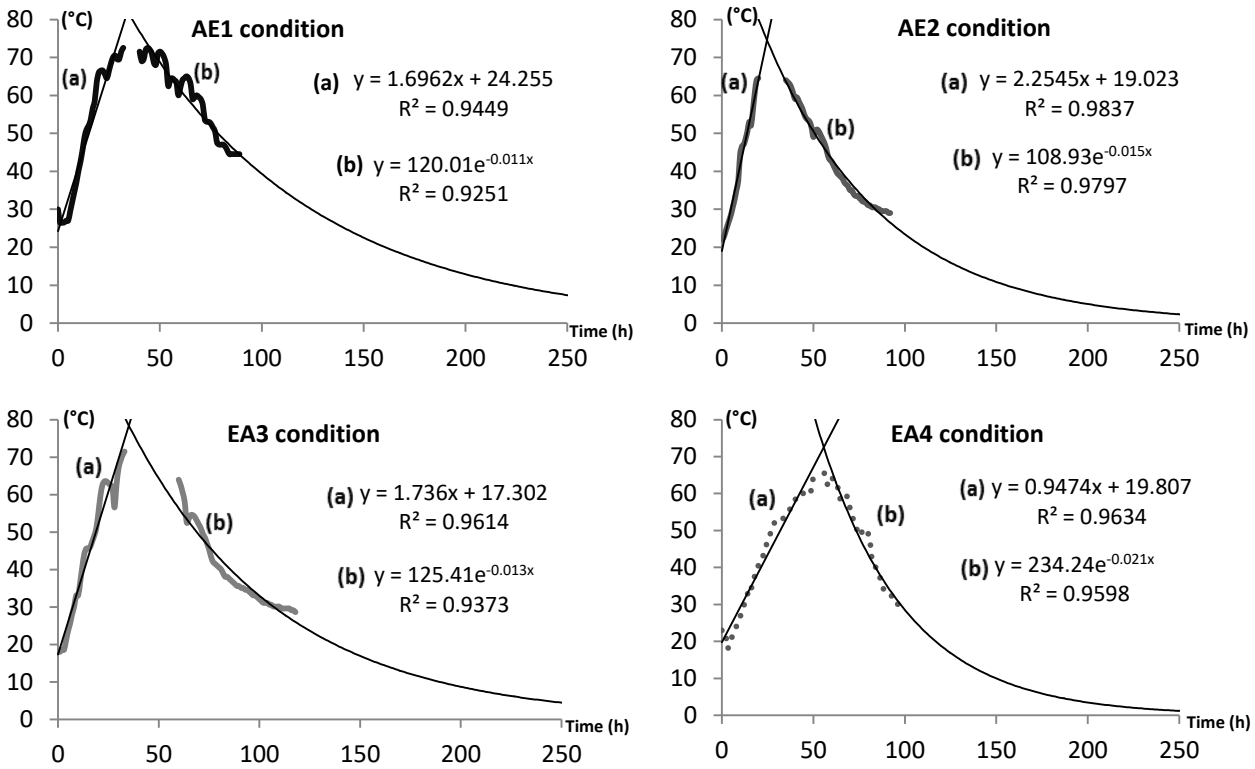

Figure 3: The kinetics parameters of the increase and the decrease of temperature for the condition without antibiotics (AE1) and for the other conditions in the presence of antibiotics (AE2, AE3 and AE4). (a): Curve and equation of temperature increase; (b): Curve and equation of temperature decrease

Tableau 6: Total calorie (TC), calorific period (CP) and TC/CP for whole composting process during thermophilic phase

Thermal parameters	**Co-composting experiments**			
	AE1	**AE2**	**AE3**	**AE4**
TC	8140	4593	7475	6717
CP	247	142	205	181
TC/CP	32.9	32.3	36.4	37.1

3.4.3. Evaluation of calorific value

Table 6 lists the total calories (TC), the calorific period (CP) and the TC/CP ratio for all the co-composting processes during the thermophilic phase. TC and CP values were high for AE1 (TC = 8141; CP = 247). Thereafter, TC decreased especially for AE3 and AE4 (7475 and 6717 respectively for AE3 and AE4). In addition, CP decreased also to 205 and 181 respectively for AE3 and AE4 for which the antibiotics concentrations were important. The intensity of the aerobic degradation is important for AE1 (TC/CP=32.9). Indeed, by increasing antibiotics concentration, epically for AE4 (TC/CP=37.1), the intensity of the aerobic degradation was altered.

Eguchi et al., (2012) studied the effect of certain molecules of antibiotics separately. At the maximum concentration: norfloxacin affects the TC/CP ratio, oxytetracycline and tylosin affect just the maximum temperature and they not had a significant effect on the TC/CP ratio. The effect on the maximum temperature was probably amplified by composting a small quantity of the substrates with an increase of the antibiotics concentration. On the other hand, with the high quantity of the substrates that we used, no significant effect on the maximum temperature was observed even at the maximum concentration. In our study, the overall parameters of the caloric model (TC, CP and TC/CP) were affected by increasing the concentration of the antibiotics mixture. Regarding the effects of the antibiotics that were separately observed by Eguchi et al., (2012), this observation may be due to the persistence of the fluoroquinolones. The effects observed on the model parameters under our conditions began by condition AE3 which is a much lower concentration than the maximal one used by Eguchi et al., (2012) for which the first significant effect. The results obtained for AE2 (Table 6) showed lower TC and CP values than the other conditions, but the TC/CP ratio remained of the same order, leading to the fact that the condition AE2 could not alter the general discussion. Briefly, the heat release, the thermophilic phase duration and the aerobic digestion intensity were influenced by the presence of the antibiotics at high concentration and particularly for EA4 characterized by the highest antibiotics concentration.

4. Microbiological approach

4.1.Microbiological approach during the stabilization phase

As shown in Fig. 2 and by the calorific evaluation, the AE1 experiment condition is dominated by a higher level of the temperature and the greater duration time of the thermophilic phase in relation to a higher microbiological activity which produced a higher plateau of temperature above 60°C. By the end of the aerobic degradation in the bioreactor, a mean difference of 11°C between AE1 and the other experiments in the presence of antibiotics was observed. These conditions are not only favorable to thermophilic bacteria or actinomyctes but also to thermophilic fungi which can develop themselves at high temperature about 50-60°C (Cooney and Emerson 1964).The shortening or the disruption of the stage above 60°C is significant of an inhibitory effect on the development of some species of bacteria or thermophilic fungi by the presence of high antibiotics concentrations (case of AE3 and AE4). Regarding the observed inhibitions, the fluoroquinolones could be responsible

of those behaviors by affecting the microbiological communities during the composting (Eguchi et al., 2012).

4.2.An approach of Rachis-Sludge-Antibiotics interaction mechanisms

Fig. 4 and Table 7 showed the evolution of the Rachis/Ash ratio during the co-composting process. Before co-composting, the differences observed in the Rachis/Ash ratios could be explained by the variability of the sampled sludge used for each experiment. During the stabilization phase in the bioreactor, mechanical erosion due to the mixing system could be involved in the reduction of the initial size of rachis similarly for all experiments. But, after 12 days, for AE1 experiment, a higher erosion of the rachis is observed and could be attributed to the biological erosion. In addition, as the experiments conducted with antibiotics showed a different behavior pattern, the mechanical erosion seeming to be controlled by the biological degradation of the rachis in relation to the antibiotics level spiked in the sludge. Therefore, the antibiotics adsorption on the rachis coming out from the transfer between sludge and rachis could be evoked to control the biological process in the stabilization phase.
During the maturation phase in the perforated bags, the mechanical erosion does not exist and only the biological erosion of rachis can be taken in account. Since this phase is controlled by fungi and actinomycetes, the condition without antibiotics led to a considerable decrease in the rachis size. In contrast, the rachises were almost less attacked in the presence of high antibiotics concentrations (AE3 and AE4) in relation of a potential inhibition in the development of the fungi and actinomycetes communities during the maturation phase. Fig. 5 shows an approach of the proposed Rachis-Sludge-Antibiotics interaction mechanism.

Tableau 7: The evolution of rachis/ash ratio before co-composting, after the stabilization and in the end of the maturation stage

Sampling (days)	**Co-composting experiments**			
	AE1	**AE2**	**AE3**	**AE4**
0	1.2	1.3	1.7	1.6
12	1.4	1.9	2.2	2.0
180	0.5	1.2	2.0	2.1

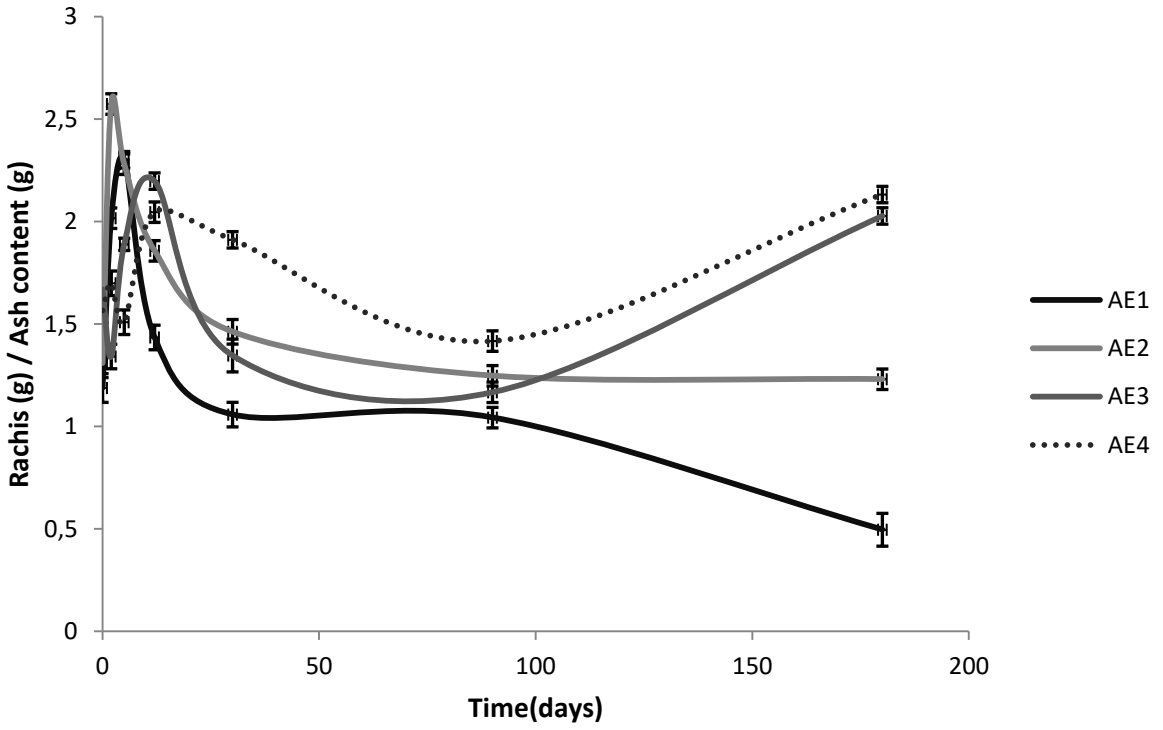

Figure 4: The evolution of Rachis/Ash ratio during co-composting experiments with antibiotics

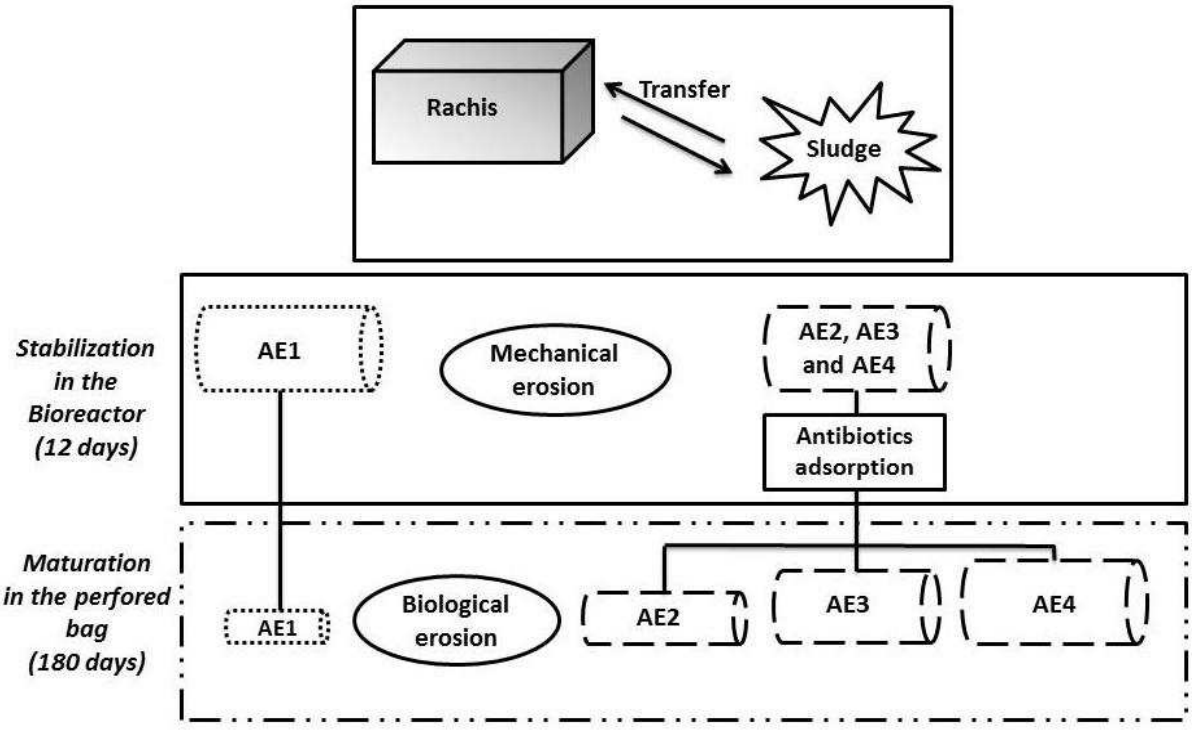

Figure 5: Rachis-Sludge-Antibiotics interaction mechanisms approach

5. Conclusion

The following conclusions can be drawn from the above experiments:

1. Co-composting experiments showed that in the presence of antibiotics at high concentrations, the thermal parameters were affected especially: the thermophilic phase duration, the temperature increase and decrease as well as the heat release. The presence of a rebound under the presented conditions (palm wastes as co-substrate) could be an early criterion of the potential quality of the final end product;
2. The evolution of the organic matter degradation and the C/N ratio are influenced by an increase in the antibiotics concentration. The efficiency of the co-composting system and the compost quality were altered by the way;
3. The adsorption of the antibiotics on the rachis matrix could explain why the co-composting process was not entirely inhibited. However, the microbiological approach showed that rachises could be less attacked and therefore the rachis size remained higher with an increase in the antibiotics concentration. To validate this hypothesis, an assessment of the antibiotics concentrations during co-composting will be done.

Acknowledgements

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Chapitre 3: Mise au point d'une technique analytique multi-résidus des antibiotiques

Contexte :

Le chapitre précédent mettait en évidence l'effet des antibiotiques sur les paramètres physiques et chimiques durant le compostage. Les molécules d'antibiotiques peuvent contribuer aux effets observés différemment en fonction de leurs interactions et comportements dans une matrice complexe comme le compost.

Cependant, le dosage des antibiotiques est indispensable pour répondre à plusieurs hypothèses qui ont été déjà émises dans le chapitre précédent et ainsi suivre le devenir des antibiotiques au cours des procédés de traitement utilisés. Dans ce sens, une méthode analytique non-sélective et multi-classe, adaptée à nos conditions expérimentales a été mise en point et a permis d'extraire et quantifier les antibiotiques dans les boues et les composts à différents stades de compostage.

Principaux résultats :

Dans cette étude, les antibiotiques ciblés font parties de 3 familles avec différentes propriétés physiques et chimiques, et ainsi différents comportements. L'approche analytique multi-résidue choisi est relativement rapide à mettre en oeuvre, non selective et montre que l'utilisation de la méthode des ajouts dosés est une alternative pour palier au problème de l'effet matrice, dans une matrice aussi complexe qu'un compost de boues. Les fortes interactions entre les antibiotiques et la matière oragnique favorise l'utilisation d'une extraction agressive par solvant accélérée (ASE). Les résultats montrent que notre approche d'extraction est reproductible (CV<10%). L'application des ajouts dosés vérifie bien les conditions de linéarité, les rendements de recouverement sont bons et répétables. La stabilité du système d'analyse (LC-MS/MS) a été vérifiée à travers le coefficient de variation relative (RSD%) par utilsation d'une molécule deutérée, les résultats sont inférieurs à 15% malgré la charge organique injectée.

Les résultats de ce chapitre sont présentés sous la forme d'un article soumis à *Chemosphere*, qui s'intitule:

A pressurized liquid extraction approach followed by standard addition method and UPLC-MS/MS for a fast multiclass determination of antibiotics in a complex matrix

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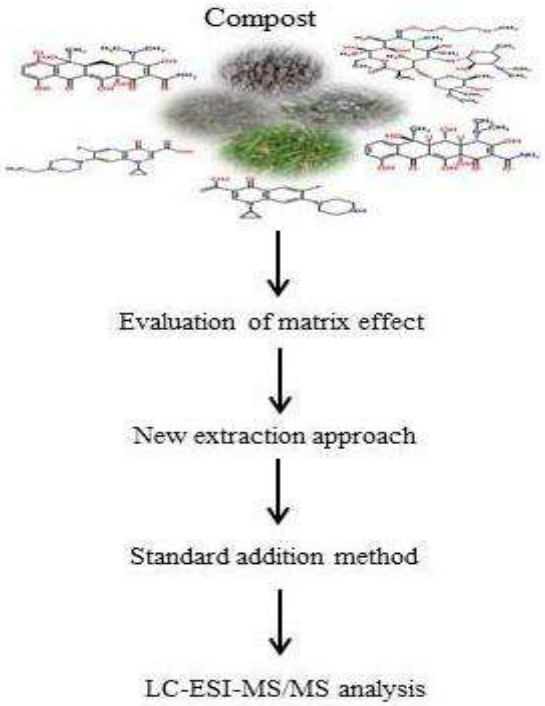
Graphical Abstract



Abstract

In this work a fast analytical method for the determination of macrolides, tetracyclines and fluoroquinolones in a compost originating from a mixture of sewage sludge, palm waste and grass was developed by ultra-high performance liquid chromatography coupled to mass spectrometry (U-HPLC/MS). Antibiotics were extracted from compost by using the accelerated solvent extraction (ASE). The chromatographic separation was carried out on a T3 Cortecs C18 column using a mobile phase gradient mixture of water acidified with 1% of formic acid and acetonitrile. Recoveries of 24-30%, 53-93%, 33-57%, 69-135% and 100-171% were obtained for roxithromycin (ROX), chlortetracycline (CTC), oxytetracycline (OTC), enrofloxacin (ENR) and ciprofloxacin (CIP), respectively. As the most part of antibiotics showed significant matrix effect (ME), the method was validated using the standard addition method (SAM) to correct the observed ME. Instrumental variation, of LC/MS system, showed that 93.75% of the relative standard deviation (RSD %) are below 15%, although the organic load of extracts. This analytical method was applied to assess the fate of antibiotics during composting. Two composting experiments were conducted separately after spiking sludge at 2 different concentration levels. The resulting elimination rates were of 52-76, 69-100, 100 and 24-50 % for ROX, CTC, OTC and CIP, respectively. These results suggest that composting process contributes to the removal of residuals concentrations of macrolides and tetracyclines while the fluoroquinolones persist in the final compost product.

Keywords: Analysis, fluoroquinolone, macrolide, tetracycline, sludge, compost.

1. Introduction

Despite wastewater treatments, most of antibiotics are found in the environment. Their presence is strongly associated with human uptake and excretion but also by the contamination of manures used in agricultural soils. It has been clearly established that wastewater treatment plants (WWTPs) are not designed to remove such kind of compounds and present limited performances regarding their elimination (Rizzo et al., 2013; Polesel et al., 2016). A part of antibiotics is discharged in surface water by the WWTPs liquid effluents and the other part is adsorbed on sludge (Michael et al., 2013; Xu et al., 2015) intended for different treatments or to direct agricultural use (Luque-Munoz et al., 2017). As a consequence of the non-elimination of these organic compounds and the direct application of sludge, antibiotics were found in soil (Michael et al., 2013), sediment (Rico et al., 2014), surface water (Hamscher et al., 2002), underground water (Hirsch et al., 1999) and plants, that can be used for the human consumption (Kumar et al., 2005; Dolliver et al., 2007). This may lead to some human health effects, such as allergic reactions (Kemper, 2008). Moreover, antibiotic emergence and dispersion in the biota are one of the main cause of antibiotic bacterial resistance development (Christian et al., 2003; Costa et al., 2011) and could in part explain the increase of multi resistant pathogens observed by the World Health Organization (WHO, 2016).

Antibiotic residues are often found in sludge (Zhou et al., 2009; Radjenovic et al., 2009; Li et al., 2013; Verlicchi and Zambello, 2015). Although they are present at very low concentrations, their potential long-term effects make it necessary to monitor them in sludge and by-products as compost. In addition, trace screening of antibiotics is essential to limit their impacts and to adopt innovative solutions for their elimination. Composting is a bioremediation technology to reduce or eliminate the residual concentrations of antibiotics (Arikan et al., 2009; Ho et al., 2012; Liu et al., 2015). Nevertheless, some molecules persist in the final product (Lillenberg et al., 2010; Haiba et al., 2013). Composting performances regarding antibiotic elimination are conditioned by the instrumental reliability and the analytical approach efficiency. Liquid chromatography coupled with mass spectrometry (LC-MS) is often used for antibiotics determination in sludge and compost (Ben et al., 2008; Kipper et al., 2017). Using ultra-high performance liquid chromatography (U-HPLC) for antibiotics determination enables to increase the analytical method efficiency while decreasing the analysis duration and solvent consumption (Moreno-González and García-Campaña, 2017). Antibiotics determination involves many extraction techniques such as

ultrasound-assisted, soxhlet, supercritical fluid, microwave or QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) (Zuloaga et al., 2012; Lindholm-lehto et al., 2017; Luque-Munoz et al., 2017). Indeed, several adsorption mechanisms involved during the retention of antibiotics in the solid phase such as hydrogen bonding, cationic exchange, hydrophobic interactions or complexation, make the antibiotics extraction from sludge difficult and tedious (Pamreddy et al., 2013). Therefore, the low accessibility to antibiotics during their extraction needs a robust and aggressive technique such as the pressurized-liquid extraction (PLE). The PLE, also referred as accelerated solvent extraction (ASE), was already used for the antibiotics extraction from sludge, compost or (Golet et al., 2002; Jacobsen et al., 2004; Stoob et al., 2006; Lillenberg et al., 2009; Nieto et al., 2010; Ding et al., 2011). Interferences between antibiotics and the organic matter compounds can occur during the sample storage and/or the extraction process leading to potential suppression/enhancement of the signal in mass spectrometry, especially with electrospray ionization (ESI) (Cimetiere et al., 2013; Vom Eyser et al., 2014). This phenomenon, so-called matrix effect (ME) was characterized by comparing the signal of the target analyte in the matrix-matched calibration curve with the signal of the external standard calibration curve. Recently, using isotopically labeled internal standards (deuterium, ^{13}C, ^{15}N) allow to overcome the ME (García-galán et al., 2013; Vom Eyser et al., 2014; Aga et al., 2016). Nevertheless, the isotopically labeled internal standards are often expensive (Punt et al., 2017) with a poor commercial availability (Aga et al., 2016). Thus, the standard addition method (SAM) is the most often used method for the pesticide analysis (Wang et al., 2005; Andersen, 2017) and has already been applied to the antibiotics determination in water samples (Cimetiere et al., 2013). This method assumes that for any analyte there is a specific analytical signal, which responds to that analyte and no to other unknown sample component. It enables to correct both the ME and the recovery losses and to quantify several compounds in the same sample (Saxberg and Kowalski, 1979; Punt et al., 2017). Thus, the SAM is a good alternative to correct interferences during antibiotics analysis in a complex matrix such as sludge or compost (Vom Eyser et al., 2014). To our knowledge, there are no non-selective studies, i.e. without the use of solid phase extraction, which allows simultaneously extracting and quantifying several classes of antibiotics covering a wide range of polarity, solubility, and thus, different behavior in sludge compost.

The aim of the present study was to propose a rapid and sensitive method for the extraction and the analysis of three extensively used families of antibiotics, potentially present in sludge

and composts: macrolides, tetracyclines and fluoroquinolones. Firstly, the antibiotics were extracted by ASE and analyzed by ultra high performance liquid chromatography coupled to tandem mass spectrometry (U-HPLC-MS/MS). Then, the matrix effect observed for each antibiotic led to validate the method by using standard addition method. Finally, this approach was applied to assess the fate of antibiotics during the sludge composting.

2. Materials and methods

2.1.Chemicals and reagents

Water (18.2MΩ cm) was purified using a Milli-Q system (Millipore, Bedford, MA, USA). LC-MS grade methanol, acetonitrile, formic acid, disodium ethylene diamine tetraacetic (Na_2EDTA), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydrogen phosphate (Na_2HPO_4) and citric acid were purchased from Fisher Chemical (USA).

The tetracyclines, chlortetracycline hydrochloride (CTC, 75% of purity) and oxytetracycline hydrochloride (OTC, 95%); macrolide (roxithromycin (ROX, 90%) and the fluoroquinolones, enrofloxacin (ENR, 98%) and ciprofloxacin (CIP, 98%) were purchased from Sigma-Aldrich-France (F-38297, St-Quentin Fallavier). Ciprofloxacin-d8 (CIP-d8) was selected as a chemical tracer to check the instrumental stability and was purchased from Dr. Ehrenstorfer (Germany). The stock antibiotics solutions were prepared in methanol (HPLC grade, Sigma-Aldrich) and were used directly for spiking.

Sodium phosphate buffer (SPB) was prepared by mixing 10.56 g of NaH_2PO_4 and 0.82 mL of H_3PO_4 in 1 L of water. SPB-EDTA (pH 4) was obtained by dissolving 80.0 g of Na_2EDTA in 1 L of SPB. Phosphoric acid (PA) (50 mM) was prepared and mixed with acetonitrile (ACN) to obtain a solvent mixture of ACN-PA (50:50, v/v). EDTA-McIlvaine buffer (pH 4) was obtained by dissolving 12.9 g citric acid, 27.5 g Na_2HPO_4 and 37.2 g of Na_2EDTA in 1 L of water.

2.2.Sample collection and storage

A composting experiment was conducted in a bioreactor for 180 days by using 3 substrates (primary sludge, palm waste and grass) according to the composting approach described by (Ezzariai et al., 2017). The final compost product, supposed to ensure the absence of antibiotics, was used as a blank sample to establish and validate the analytical protocol.

Before using the blank, it was kept at -20°C, freeze-dried, crushed and stored in amber glass bottles.

2.3. Instrumental extraction and analysis

2.3.1. Extraction

The extraction of antibiotics from the compost samples was performed using an ASE 200 from Dionex (Sunnyvale, CA, USA). The ASE was operated with 22 ml stainless steel extraction cells lined with glass fiber filters from Dionex. Freeze-dried and crushed compost samples (1 g) were thoroughly mixed with 28 g of quartz sand in order to obtain optimum pressure conditions, to increase contact – surface area between compost and solvents and to prevent the overflow of the extraction cell (Pamreddy et al., 2013). The compost sample and quartz sand were added to the extraction cell. The extraction procedure was divided into two extraction stages for the same compost sample. The first extraction is conducted with the SPB-EDTA mixture and the second one with the ACN-PA mixture. For each extraction stage, the operating conditions were : extraction temperature, 100°C; extraction pressure, 1000 psi; preheating period, 5 min; static extraction period, 15 min; extraction cycles number, 3 ; final extraction volume, around 30 mL; solvent flush, 60%; nitrogen purge, 300 s. After the ASE, two extracts were obtained (2 x 30 mL), centrifuged separately (10,000 rpm, 10 min, 4°C), mixed and then centrifuged again. The supernatant was stored at -20°C until the analysis. Between each extraction run, the cells were cleaned by a mixture of Milli-Q water and methanol (50:50, v/v) followed by 30 min of ultrasonication in Milli-Q water. To avoid adsorption of antibiotics onto silanol groups of the glassware surface, all glassware was rinsed with Na_2EDTA and dried at 50 °C before use.

2.3.2. Analysis

Antibiotic analysis was carried out using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). Antibiotics identification and quantification were achieved with an Acquity ultra-performance liquid chromatography (UPLC®) coupled to a Xevo triple quadrupole mass spectrometer (Waters, Milford, MA, USA). Antibiotics were eluted on a T3 Cortecs C18 column (2.1 mm × 100 mm; 1.6 μm; Waters) at 40 °C and a flow rate of 0.3 mL/min with a mobile phase of ultra-pure water acidified with 1% formic acid (solvent A) and acetonitrile (solvent B). The gradient elution program was set at 0 min (A: B) 100: 0, 3.5 min (A:B) 35: 65, 3.6 min (A: B) 100: 0, 4.5 min (A: B) 100: 0. Samples were

ionized in positive electrospray ionization mode (ESI+). The capillary voltage and source temperature were set at 2 kV and 150 °C, respectively. The desolvation temperature and nitrogen flow were 650°C and 800 L/h, respectively. Argon was used as collision gas at a flow rate of 0.12 mL/min. Antibiotics were detected by the multiple reaction monitoring (MRM), MRM transitions and their respective collision energies were reported in **Table 1**. All the chromatographic data were monitored by Masslynx 4.1® software (Waters, Milford, MA, USA).

Tableau 1: MRM parameters of the antibiotics analyzed in this study

Molecule	**t_R(min)**	**Precursor ion**	**Quantification transition**	**Collision energy (eV)**	**Confirmation transition**	**Collision energy (eV)**
ROX	3.28	837,532	679,433	22	158.017	36
OTC	2.00	461,223	426,129	20	200.981	44
CTC	2.47	479,096	443,999	22	153.865	30
ENR	2.18	360,223	245,046	28	203.075	42
CIP	2.05	332,223	245,080	26	231.029	36
CIP-d8	2.05	340,223	245,080	26	234.940	38

ROX, roxithromycin; OTC, oxytetracycline; CTC, chlortetracycline; ENR, enrofloxacin; CIP, ciprofloxacin; CIP-d8, deuterated ciprofloxacin.

2.4.Method suitability

To calculate recoveries on the basis on the SAM, the blank samples (4 g) were spiked with 2 mL of standard solution of antibiotics diluted at 3 different concentration levels (low, medium and high level). The ratio mass/volume was constant. To ensure adsorption equilibrium and to allow complete evaporation of the methanol, the compost-antibiotics mixtures were stirred in a mixer for 24h and left for an additional 24h at room temperature in the dark prior the extraction.

To investigate ME, ASE extracts were spiked at 7 concentrations levels (0, 50, 100, 200, 300, 400 and 500 µg/L) with the standard solution of methanol containing the target antibiotics. Solvent mixtures (SPB-EDTA, ACN-PA) calibration curve were spiked at the same levels and compared with the matrix calibration curve.

Five blank samples were spiked at 200 µg/L of Cip-d8 to check the extraction reproducibility.

3. Results and discussion

3.1.Antibiotics extraction

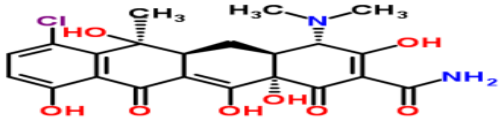
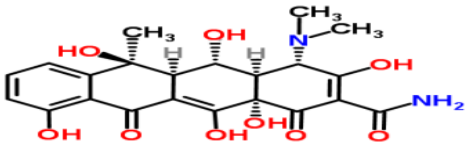
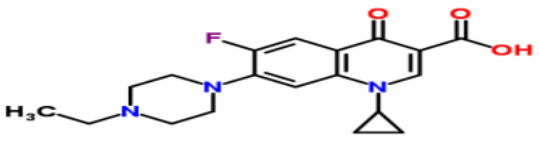
The extraction of chemical compounds in a complex solid matrix requires a methodology able to separate the maximum of target compounds from matrices without chemical or physical alteration of these compounds. Thus, ASE is the method of choice to get good ratio between extraction yield and chemical targets preservation (Zuloaga et al., 2012). However, several parameters such as extraction solvents, extraction duration, pressure, temperature and extraction cycle, need to be optimized during the ASE protocols (Richter et al., 1996). As shown by the **Table 2**, the pKa and Log Kow values are ranged from 2.74 to 12.5 and -0.62 to 2.53 respectively, for all target compounds. Indeed, the 3 families of antibiotics cover a wide range of physical and chemical proprieties, leading to different behaviors. Therefore, the extraction conditions should be a compromise between all these different proprieties.

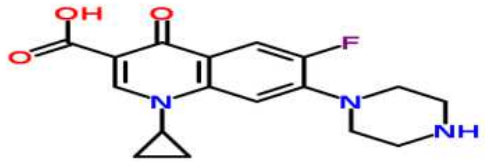
The extraction of tetracyclines, macrolides and fluoroquinolones from several environmental matrixes is known to be difficult (Tong et al., 2009). The tetracyclines can form strong complex with the mineral part of the organic matter requiring the use of buffer agents (citric acid, potassium phosphate) and EDTA. Under EDTA-buffer at pH 4, the tetracyclines are neutral with a high affinity to the hydrophobic extraction conditions. Although the macrolides are positively charged under these conditions, they are enough hydrophobic to be extracted with the same buffer (Jacobsen et al., 2004). Similarly, the fluoroquinolones present strong interactions with the organic matter that should be considered during their extraction (Ho et al., 2012; García-galán et al., 2013; Dorival-García et al., 2015). Since the cationic and amphoteric forms of the quinolones (ENR and CIP) are dominant at low pH value, the electrostatics repulsions between quinolones and sludge or compost could be responsible for the extraction efficiency at acidic pH (Pena et al., 2010). Selecting solvents such as methanol or acetonitrile with the addition of an acid (formic or phosphoric acid) are recommended for the fluoroquinolones extraction (Dorival-Garcı´a et al., 2013). In addition, these conditions are favorable to extract a part of ROX (Huang et al., 2013).

Antibiotics extraction from sludge and compost using the ASE is carried out at a temperature between ambient temperature and 100°C, a pressure from 1000 to 1500 psi and 2 or 4 static cycles from 5 to 15 min has been previously described (Golet et al., 2002; García-galán et al., 2013; Pamreddy et al., 2013).

In the present study, the same compost sample was successively extracted with two solvents mixtures. Each one is specific for the extraction of the tetracyclines, macrolides and fluoroquinolones, and takes into account their physicochemical properties (**Table. 2**). The extraction conditions (temperature, pressure and static cycle) were selected in order to break down the strong interactions between antibiotics and the compost organic matter. Then, matrix effects were evaluated to check the extraction efficiency for each antibiotic.

Tableau 2: Chemical and physical proprieties of antibiotics under study

Families	**Molecules**	**pKa (25°C)**	**Log Kow**	**Chemical structure**
Macrolides	ROX	12.45	-	
Tetracyclines	CTC	4.5	-0.62	
	OTC	4.5	-0.9	
Fluoroquinolones	ENR	2.74	2.53	

	CIP	6.09	0.28	

Compound abbreviations are given in Table 1.

3.2.Matrix effects evaluation

The ME was evaluated by comparing the slopes of the matrix-matched calibration curves with the slope of the external standard calibration curves according to the following equation (Matuszewski et al., 2003; Moreno-González and García-Campaña, 2017):

$$ME\ (\%) = \left(\left(\frac{Slope\ of\ matrix\ matched\ calibration\ curve}{Slope\ of\ external\ calibration\ curve} \right) -1 \right) \times 100$$

The ME is not significant if the ME % value is lower than more or less 20 %. As shown in the **Table 3**, the ME is not significant for ROX, OTC and ENR, and slightly significant for CTC and CIP (ME % = -30 % for both antibiotics). For whole antibiotics ME % is negative, meaning that the matrix effects lead to ion suppression in mass spectrometry. Indeed, the extracts were darker after the ASE process, probably due to the concentration of the organic compounds such as humic acids and lipids occurring during the extraction (Dorival-García et al., 2015). Studies have already reported that humic substances can cause significant ME that can prevent the determination of antibiotics, by inhibiting the signal up to 50% (Gros et al., 2006; López-serna et al., 2010; Pamreddy et al., 2013; Dorival-García et al., 2013; Bourdat-Deschamps et al., 2014;). In LC-ESI-MS/MS, the signal suppression is generally assigned to the interactions between solute-analyte thereby changing the MS response (Furey et al., 2013).

Tableau 3: Matrix effects (%) for the target compounds obtained after the extraction process and the solid phase extraction

Compounds	Calibration curve slope		Matrix effects (%)
	Solvent	Compost extract	Compost extract
ROX	451,81	436,05	-3
OTC	94,934	80,138	-16
CTC	414,83	292,37	-30
ENR	304,37	254,03	-17
CIP	412,03	288,1	-30

Compound abbreviations are given in Table 1.

3.3.Method performance

Extraction repeatability and recovery rates obtained by the SAM were investigated to check the extraction and LC/MS/MS performance.

The extraction reproducibility was evaluated for the fluoroquinolones family by spiking 5 compost samples with CIP-d8 at 200 µg/L. The ASE extraction presents good reproducibility with a coefficient of variation (CV %) lower than 10% (data not shown).

Standard addition is a widely used method when matrix effects are significant or if the analytes are poorly extracted from their matrix. These issues often occur for residual antibiotics in complex matrixes such as compost (Aga et al., 2016). The SAM consists of plotting the responses of the analyte obtained after successive addition of its standards with known concentrations *vs* the amount of standard added. The resulting intercept on the abscissa corresponds to the concentration of the target analyte (Saxberg and Kowalski, 1979). All ASE extracts were spiked using a solution containing the 5 antibiotics at concentrations varying from 50 to 500 µg/L in duplicate (**Table 4**). Linear regressions were good for the most part of antibiotics with a coefficient of determination (R^2) ranging from 0.95 to 0.99, except for CIP with a R^2 lower than 0.95. Thus, the recovery rates for each antibiotic were calculated from duplicated compost samples spiked at 3 concentrations levels before the ASE extraction and using the SAM procedure. The recovery rates are 24-30%, 53-93%, 33-57%, 69-135% and 100-171% for ROX, CTC, OTC, ENR and CIP, respectively (**Fig. 1**). Generally, all spiking levels showed that recovery rates are repeatable. CIP was not detected in the lowest treatment level.

Tableau 4: Range of the added concentration during the SAM validation

Addition points	Concentrations (µg/L)				
	ROX	OTC	CTC	ENR	CIP
1	0	0	0	0	0
2	37,8	87,3	86,6	20,4	20,4
3	75,6	174,5	173,1	40,7	40,7
4	113,5	261,8	259,7	61,1	61,1
5	151,3	349,1	346,2	81,4	81,4
6	189,1	436,4	432,8	101,8	101,8

Compound abbreviations are given in Table 1

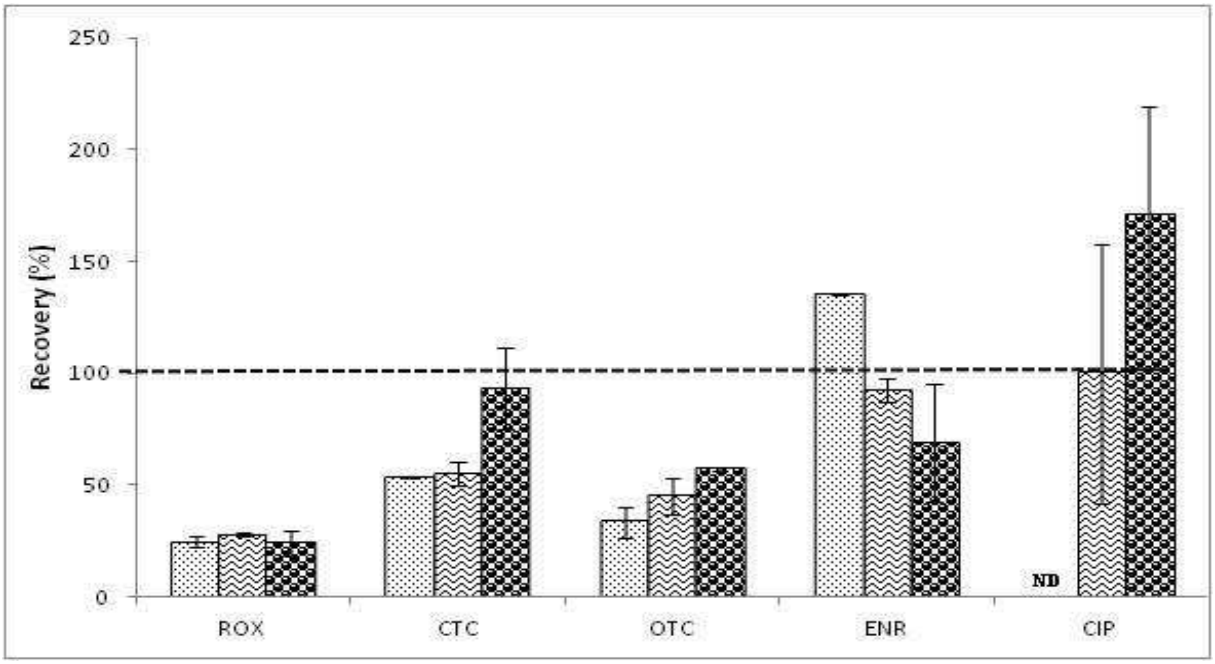


Figure 1: Total recoveries (%) of the target compounds.ND: Non-detectable. Low level, Medium level and High level. ND: Non-detectable.

The ROX showed the lowest recovery rate compared to the other antibiotics, meaning that a part of ROX was lost during the ASE process. Indeed, higher recoveries were obtained when ROX was extracted from sludge with an ultra-sonication extraction using SPB-EDTA solvent (Huang et al., 2013) or by using QuEChERS in presence of EDTA (Peysson and Vulliet, 2013). Moreover, a protocol based on PLE extraction with softer conditions (80°C, 1500 psi, one static cycle of 5 min) enabled to recover 83% of ROX from sewage sludge (Nieto et al., 2010).

For the tetracycline family, recovery rates are in agreement with those reported in previous studies when PLE extraction was used with similar conditions (Ding et al., 2011). Moreover, a recovery rate from 50 to 80% was obtained by using the room temperature and 2 static cycles to extract CTC and OTC from soil (Jacobsen et al., 2004) whereas Lillenberg et al.

(2009) obtained 26% of recovery rate with 5 extraction cycles of 10 min and a temperature of 110°C. These results suggest that high temperature and numerous and long static cycles in ASE extraction can lead to degradation of both macrolides and tetracyclines (Gobel et al., 2005; Arikan et al., 2007; Connor and Aga, 2007; Wu et al., 2011; Mullen et al., 2017). Nevertheless, the recovery rates for tetracyclines with our ASE method are higher than those reported in other methods using the PLE or other types of extractions (recoveries ranged from 26 to 59%) (Lillenberg et al., 2009; Shafrir and Avisar, 2012; Peysson and Vulliet, 2013; Chen et al., 2013; Bourdat-Deschamps et al., 2014).

The highest recovery rates were obtained for ENR (from 69 to 135 %) and for CIP (100%-171%) proving that the use of ACN-PA is a crucial step to extract the FQ correctly (Golet et al., 2002b). Similar results were reported for FQ using the same solvent mixture with recovery rates closed to 100 % (Golet et al., 2002b; Lillenberg et al., 2009). At high concentration, CIP recovery was largely higher than 100%, this phenomenon could be explained by the strong adsorption of CIP to particulate organic matter, which depend on the organic matter composition (Belden, 2007; Uslu and Yediler, 2008). Indeed, during composting, the composition of the organic matter changes modifying continuously the interactions between CIP and the organic matter. Moreover, according to Freundlich isotherms, the sorption of CIP onto sludge is non-linear (Polesel et al., 2015). Therefore, there is no equilibrium sorption of CIP on sludge particles, which may influence sampling homogeneity and increase the recovered CIP levels in the sludge. Recovered levels of CIP could be more important especially when acetonitrile is used under an extraction temperature of 100°C as it was observed by García-galán et al. (2013) for other antibiotics compounds (recovery rate above 200%). Nevertheless, the observed recovery rates in this work for the FQs are more important than those observed in the literature using the PLE or other extraction techniques (Uslu and Yediler, 2008; Dorival-Garcia et al, 2013; Peysson and Vulliet, 2013; Marsoni et al., 2014).

3.4.Instrumental stability

ASE extracts were directly analyzed after centrifugation without additional clean-up step. As high organic load is injected directly onto the LC/MS system, it is crucial to evaluate the stability of the MS signal. Moreover, the SAM approach can be impacted by the stability of the MS signal, particularly when sample batches are large (several hundred of samples). Thus, CIP-d8 regarding its stability and absence in the compost samples was chosen as a chemical tracer to verify MS signal stability during the assays. The instrumental stability in the current

study was evaluated according to Richard B. Cole, (2010). Peak areas of CIP-d8 were used to check the intermediate-term stability (**Fig. 2A**) by calculating RSD% (n^* =6; * same compost extract). Forty-eight series of *6* compost extracts, with different organic matter compositions (approach adapted for a kinetic of organic matter evolution), were used to monitor the instrumental stability. The intermediate-term stability was exploited to establish the long-term stability (**Fig. 2B**) (RSD%) during 24 hours of acquisition. The instrumental variation expressed as RSD% (**Fig.2**) showed that 93.75% of the measures of stability are below 15%, despite the high level of organic matter in the extracts. All these data suggest that our method is suitable to analyze compost extract with various organic matter compositions for at least 24 hours, without using additional clean-up step after ASE extraction. Elsewhere, as the extraction method is not specific to a class of antibiotics, a wide range of antibiotics and their metabolites could be assayed with this approach.

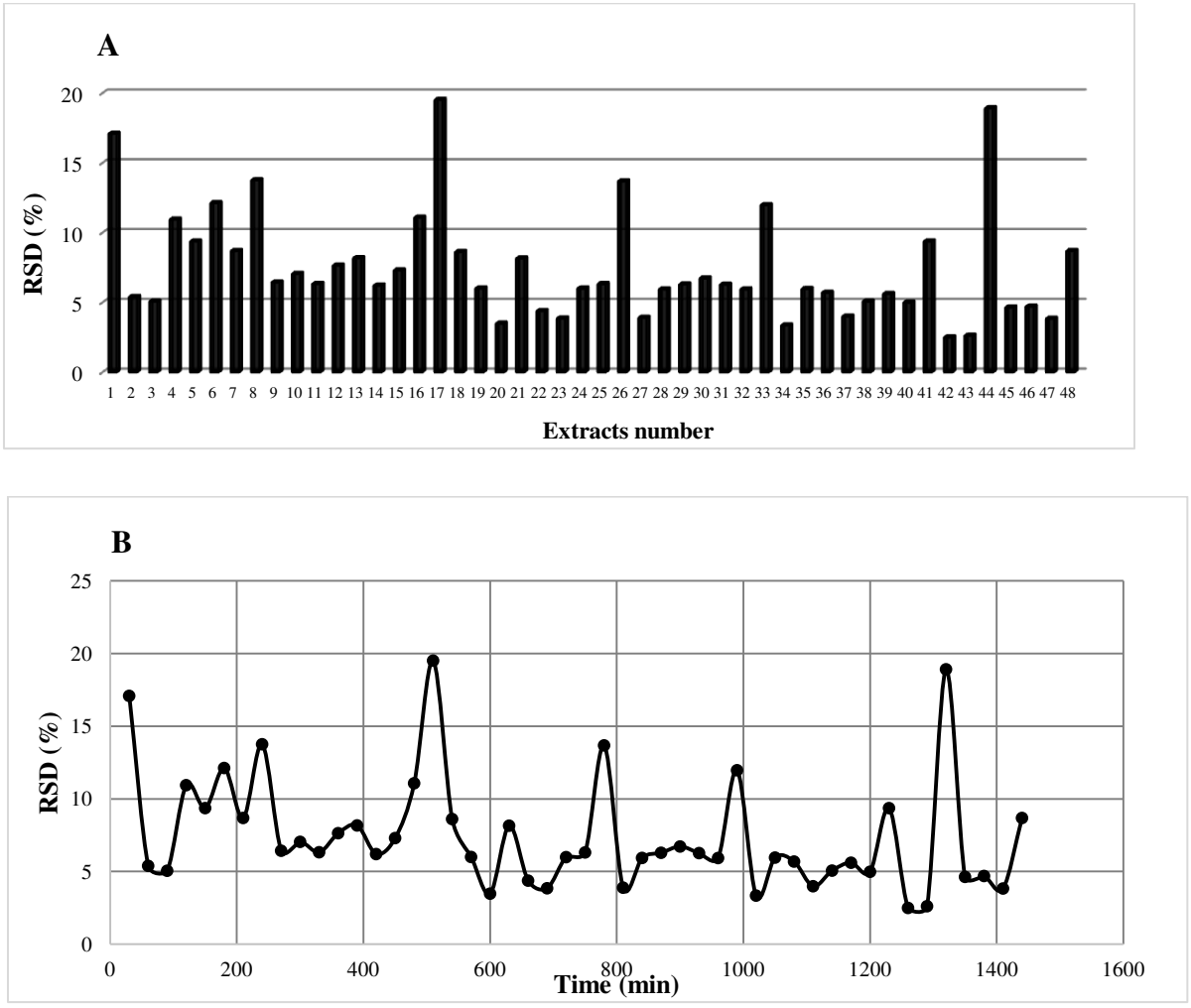


Figure 2: Evaluation of intermediate-term (A) and long-term instrumental stabilities (B). Peak areas of CIP-d8 were used to check the intermediate-term stability (A) by calculating RSD%.

4. *Method application*

The primary sludge collected from the WWTPs of Marrakech was spiked by a mixture of the target antibiotics, according to 2 different levels (L1 and L2), and mixed with the palm waste and grass to conduct 3 co-composting experiments separately (Ezzariai et al., 2017). Our analytical methodology based on the SAM was applied to assess the fate of antibiotics during composting. However, the spiked compost samples at T_0 (before composting) and T_{180} (180 days of maturation) were retained to check antibiotic concentrations in each composting experiment. Before the application of our analytical methodology, approximate antibiotic concentrations (C_{ap}) in T_0 and T_{180} was calculated using a matrix matched-calibration curve established at 7 concentration levels (0, 50, 100, 200, 300, 400 and 500 µg/L). The C_{ap} was taken into account for choosing the added concentrations for the SAM application. All samples were spiked in duplicate with a stock solutions containing the target antibiotics (not spiked; $2\times C_{ap}$; $4\times C_{ap}$). The statistical significance difference of antibiotics concentrations between T_0 and T_{180} was investigated by the *t* test (SigmaPlot 12.0).

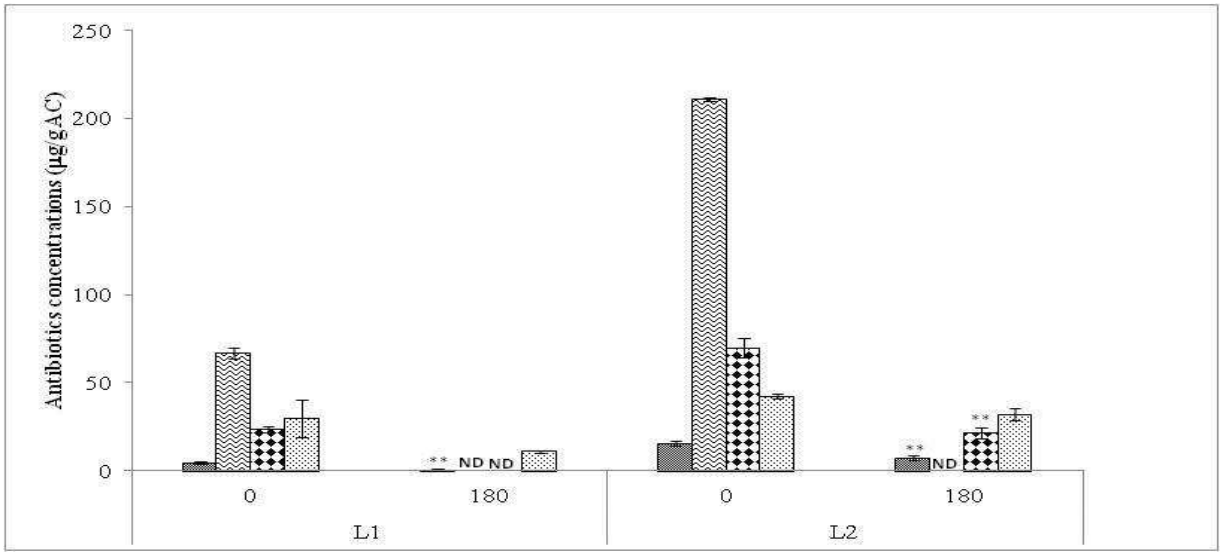


Figure 3: The fate of extractible antibiotics during composting.* and **, probability of significance according to t-test for $p<0.05$ and $p<0.01$ respectively (T0 versus T180 for each condition). T0: initial time of composting; T180: end of composting reached after 180 days. ND: Non-detectable.

ROX, OTC, CTC and CIP. L1: Low level; L2: high level. Concentrations were normalized by referring to the gram of ash content (g AC).

The recovery assay showed a values ranging from 20-29, 40-61, 50-63 and 87-153% for ROX, CTC, OTC and CIP, respectively. The observed recovery rates are close to the observed ones during the protocol validation and repeatable except the case of CIP. In contrast, the ENR data were not interpretable regarding a problem of linearity. Composting

can concentrate recalcitrant compounds which are present in the sludge (Lazzari et al., 2000; Zorpas et al., 2003). The increase of some recalcitrant antibiotics could be due to the organic matter weight loss during composting. In this view, to compensate this loss, all measurements were normalized by referring to the ash content (AC). The evolutions of the extractible concentrations of ROX, OTC, CTC and CIP during composting (between T_0 and T_{180}) are shown in the **Fig. 3**. At the end of composting, an elimination rate of 52-76, 69-100, 100 and 24-50 % respectively for ROX, CTC, OTC and CIP were observed for all treatments. The removal of ROX, OTC and CTC was very significant ($p<0.01$) while CIP removal was not significant ($p>0.05$). In agreement with previous literature, the composting process contributes on the removal of residuals concentrations of ROX, OTC and CTC (Arikan et al., 2009, 2007; Bao et al., 2009; Hu et al., 2011; Wu et al., 2011). In contrast, CIP still persists in the final compost products (Lillenberg et al., 2010; Selvam et al., 2012; Haiba et al., 2013).

5. Conclusion

In the present study, the target compounds belong to 3 different antibiotic families with different physicochemical properties and behavior. The multiclass analytical method developed in this work showed that using the SAM is validated on compost from sewage sludge. The strong interaction created between antibiotics and organic matter during composting required an aggressive extraction. An approach using ASE was proposed to cover a wide range of antibiotic compounds by applying two successive extractions step with two different mixture of solvent. The extracts were analyzed directly by the LC-MS/MS by ensuring the instrumental stability (RSD% < 15%). The proposed study is a simple, fast and relative sensitive method to assess the fate of antibiotics during sludge composting. To be validated, this methodology needs to be performed on different types of matrix such as compost, digester and soil with a wide range of antibiotics concentration.

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Chapitre 4: Devenir des antibiotiques au cours du procédé de compostage

Contexte :

Le chapitre précédent consiste en la mise en point d'une approche analytique dont l'objectif est de suivre le devenir des antibiotiques au cours des bioprocédés utilisés dans cette thèse. L'application de cette approche analytique sur les essais de compostage permettra de mettre en évidence la contribution du compostage vis-à-vis l'élimination des antibiotiques. Dans cette optique, la présente étude s'est intéressée à apporter des informations quant à l'implication de la phase thermophile et la phase de maturation, ainsi que les rachis de palmier envers l'élimination des d'antibiotiques, au cours du compostage.

Principaux résultats :

Cette étude a montré que le compostage contribue à l'élimination de la roxithromycine, la chlortétracycline et l'oxytétracycline à des taux d'élimination de 52 à 100%. En revanche, la ciprofloxacine (fluoroquinolone) persiste après maturation et son élimination est non significative quelque soient les essais de compostage.

La phase thermophile est responsable de l'élimination de la roxithromycine, tandis que la phase de maturation est imliquée dans l'élimination de la chlortétracycline et l'oxytétracycline. A des concentrations élevées d'antibiotiques, les taux d'élimination ont été réduits en phase thermophile. Par conséquence, la phase de maturation joue un rôle complémentaire vis-à-vis de l'élimination des antibiotiques après l'inhibition de la phase thermophile. La sorption/désorption de la ciprofloxacine sur les rachis de palmier pourrait expliquer les fluctuations de concentrations durant les essais de compostage.

Ces résultats suggèrent que la famille des fluroquinolones représente une préoccupation environnementale importante. La présence d'antibiotiques et plus particlièrement les fluroquinolones, doit être prise en considération dans les réglementations futures qui contrôlent la valorisation du compost dans les sols agricoles.

Ce chapitre est rédigé sous la forme d'un article qui s'intitule:

Sequential removal of antibiotics in function of sludge composting stages

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Abstract

The objective of this study was to investigate the fate of antibiotics during the two main stages of sludge composting. The primary sludge, palm waste and grass were used as raw materials. The sludge was spiked with roxithromycine (ROX), chlortetracycline (CTC), oxytetracycline (OTC), and ciprofloxacine (CIP) at a low level (L2), medium level (L3) and high level (L4), a control (L1) without antibiotics was also conducted. All composting experiments were conducted separately during 180 days under optimized conditions in a bioreactor. The target antibiotics were extracted from the compost using the accelerated solvent extraction (ASE). An accurate determination of antibiotics was obtained by the standard addition method (SAM) associated with ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). Physical and chemical results showed that the highest antibiotics levels (L3 and L4) affected the thermophilic stage, the process efficiency and the compost quality. For all treatment levels, ROX, CTC and OTC were removed significantly with an elimination rate of 52-87, 69-95 and 100% respectively. Whereas, CIP was not significantly removed during all composting experiments. The thermophilic stage is responsible of the ROX removal. In contrast, the maturation stage is more implicated on the removal of CTC and OTC. The sorption/desorption of CIP on palm rachis could explain the observed behavior of this molecule during all composting stages. To conclude, composting process could effectively remove parent compounds such as ROX, CTC and OTC spiked even at highest concentration level, but CIP still persists in the final compost. Metabolites levels must be checked and CIP removal needs to be improved to ensure using the compost safely for amendment of agricultural soils.

Key words: Composting, Antibiotics, thermophilic stage, maturation stage, fluoroquinolones

1. Introduction

Marrakesh wastewater treatment plant (WWTP) produces 50,400 kg/day of primary sludge (Belloulid et al., 2016). A large amount of pathogens and unstable organic or inorganic compounds are present in the primary sludge of Marrakesh (Amir et al., 2005; Hafidi et al., 2008; El et al., 2015) which limit their direct application. Composting is an economical and environmentally friendly biotechnology for the sludge treatment. During the stabilization and the maturation stages, the organic matter is decomposed, pathogens are destroyed and humic substances are built (Kögel-Knabner, 2000; Tuomela et al., 2000; Tiquia et al., 2002). The final compost product can be used as a nutrient source for plant growth and to improve the soil structure and its organic matter content (Farrell and Jones, 2009).

Besides pathogens and organic or inorganic compounds, sludge is contaminated by antibiotics (Yang et al., 2011; Li et al., 2013; Michael et al., 2013; Zhang et al., 2013; Xu et al., 2015) since they are widely used in the human medicine, and then excreted in the domestic wastewater stream which arrives until the WWTP. As a consequence of the direct application of sludge, antibiotics were found in soil, sediment, surface and underground water (Hamscher et al., 2002; Rico et al., 2014). However, antibiotics emergence is one of the sources contributing to develop antibiotic resistance in the microbiologic communities including pathogen bacteria (Costa et al., 2011).

Tetracyclines (TCs), macrolides (MDs) and fluoroquinolones (FQs) are the most abounded families in sludge (Shafrir and Avisar, 2012; Li et al., 2013; Zhang et al., 2013). Several studies showed important removals of TCs (Arikan et al., 2007; Bao et al., 2009; Hu et al., 2011; Wu et al., 2011) and MDs (Eguchi et al., 2012; Ho et al., 2012; Kim et al., 2012) after their sludge composting with an elimination rate of 70-99%. In contrast, some studies found that FQs family persist after sludge composting (Lillenberg et al., 2010; Selvam et al., 2012; Haiba et al., 2013). Thermophilic conditions occurring during composting seemed to have a huge impact on antibiotics elimination (Arikan et al., 2009; Wu et al., 2011). In addition, adsorption on the used substrates during composting plays also an important role regarding antibiotics elimination and limited their bioavailability towards microorganism involved during thermophilic or maturation stages (Ezzariai et al., 2017). Composting performances regarding the removal of TCs, MDs and FQs are also conditioned by the analytical approach efficiency. Several extraction techniques were used to extract antibiotics from complex matrix such as sludge or compost. Accelerated solvent extraction (ASE) was already used for the extraction of TCs, MDs and FQs from sludge or compost (Golet et al., 2002; Jacobsen et al.,

2004; Nieto and Pocurull, 2007; Lillenberg et al., 2009; Ding et al., 2011) and recoveries ranged from 40-146% were obtained. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is often used for antibiotics determination in sludge or compost (Ben et al., 2008; Kipper et al., 2017). Nevertheless, interferences between antibiotics and organic matter compounds could be developed in the electrospray source ionization (ESI) of the MS/MS leading to the so-called matrix effect (ME) and a potential suppression/enhancement of the signal is occurred (Cimetiere et al., 2013). The standard addition method (SAM) assumes that for any analyte there is a specific analytical signal which responds to that analyte and no to other unknown sample component. However, this approach could serve as an alternative to remove the ME, recovery loses and quantifies several compounds in a complex matrix such as compost (Vom Eyser et al., 2014).

Little information is given in previous studies about the effects of composting stages on the antibiotic removal. This study aims to explore information about the contribution of the thermophilic and the maturation stages as well the compost substrates, especially the lignocellulosic one, on antibiotics removal.

2. Material and methods

2.1.Chemicals and reagents

Tetracyclines (CTC hydrochloride (75% of purity) and OTC hydrochloride (95%)), a Macrolide (ROX (90%) and Fluoroquinolones (CIP (98%)) were purchased from Sigma-Aldrich-France (F-38297, St-Quentin Fallavier). The stock of antibiotic solution was prepared in methanol (MeOH-HPLC grade, Sigma-Aldrich) and used directly for spiking.

2.2.Composting experiments

Composting experiments were conducted in a bioreactor described by Ezzariai et al. (2017). 3 composting substrates were used during the experiments. The palm wastes, the grass and the primary sludge (from the WWTP of Marrakech). The physical and chemical parameters of composting substrates are presented in the **Table 1.**

The primary sludge was spiked by a mixture of 4 antibiotics according to a low level (L2: MDs, 4 mg/kg DM; TCs, 10 mg/kg DM; FQs, 2 mg/kg DM), a medium level (L3: MDs, 20 mg/kg DM; TCs, 50 mg/kg DM; FQs, 10 mg/kg DM) and a high level (L4: MDs, 100 mg/kg DM; TCs, 250 mg/kg DM; FQs, 50 mg/kg DM). A control (L1) without antibiotics was conducted. For all composting experiments, the stabilization stage was carried out in a

composting bioreactor for 12 days and the maturation stage was conducted in a perforated bag at the ambient temperature for 180 days.

Figure 1: The physical and chemical parameters of the composting substrates

Physical and chemical parameters	**Primary sludge**	**leaves waste**	**Palm rachis**	**Grass**
pH	7.1±0.1	6.2±0.1	6.5±0.2	6.5±0.2
Moisture[a] (%, FW)	66.8±3.9	6.1±0.1	19.7±0.3	67.9±5.6
Ashes content[b] (%, DW)	45.5±0.4	11.1±0.1	63.7±0.5	18.4±0.4
TOC[b] (%, DW)	34.5±0.3	56.3±0.1	23±0.9	51.7±0.3
TN[b] (%, DW)	3.8±0.1	1.2±0.1	0.8±0.3	2.1±0.1
C/N	9	49	29	24

[a] Results expressed per unit weight fresh matter; [b] Results expressed per unit weight dry matter; TOC: Total Organic Carbon; TN: Total Nitrogen.

2.3.Sampling and physicochemical analysis

Sampling was carried out at T_0 (before composting), T_5 (after 5 days of the thermophilic stage), T_{12} (after 12 days of stabilization), T_{90} (90 days of maturation) and T_{180} (after 180 days of maturation). In each point, homogeneous samples were taken using the quartering method. All samples were kept at -20°C, lyophilized, crushed and stored in amber glass bottles until the analysis.

The physical and chemical analyses were conducted in triplicate. The pH was measured on aqueous suspension (sample-water; ½; v/v). The moisture was determined by drying the compost at 105°C during 48 h (AFNOR, 2000). The total organic carbon (TOC) and the ash contents were determined after calcination in a muffle furnace at 600°C during 6 h. The total nitrogen (TN) was determined according to the Kjeldahl method. The decomposition rate was calculated according to the following formula (Paredes et al., 1996):

$$\text{Decomposition rate (\%)} = 100 - 100[\text{Ashi}(100 - \text{Achf})]/[\text{Achf}(100 - \text{Ashi})]$$

Ashi is the initial level of ash and Ashf is the final level of ash.

2.4. Antibiotic extraction and determination

The target antibiotics were extracted from the compost samples using the ASE. The quantification was carried out by the SAM associated with LC-MS/MS. The extraction and the determination of antibiotics were conducted according to the proposed protocol by Ezzariai et al. (2018) (in preparation).

2.4.1. Pressurized liquid extraction (PLE)

Antibiotics extraction from compost samples was performed using an ASE 200 from Dionex (Sunnyvale, CA, USA). The extraction procedure was divided into two main stages for the same compost sample. The first extraction is conducted using a mixture of sodium phosphate buffer and ethylene diamine tetraacetic (SPB-EDTA), and the second one is by using a mixture of acetonitrile and phosphoric acid (ACN-PA).

2.4.2. Standard addition method (SAM)

Extracts were pretreated after the extraction process and then used directly for the SAM application. All samples were spiked using a solution containing the 4 targeted antibiotics at 3 concentrations levels in duplicate (not spiked; 2×C; 4×C).

2.4.3. Liquid chromatography coupled with mass spectrometry in tandem (LC-MS/MS)

Ultra-performance liquid chromatography (UPLC) separations were performed on a T3 Cortecs C18 column (2.1 mm × 100 mm; 1.6 µm; Waters). Mass spectrometry analyses were carried out on an Acquity coupled to a Xevo triple quadrupole mass spectrometer and an Acquity PDA detector (Waters, Milford, MA, USA). The mass-spectrometer was working in ESI (+) under the multiple reaction monitoring (MRM).

Composting can concentrate recalcitrant compounds which are present in the sludge (Lazzari et al., 2000; Zorpas et al., 2003). The increase of some recalcitrant antibiotics can be due to the organic matter weight loss during composting. To compensate this loss, all measurements were normalized by referring to the ash content (AC). In this view, all concentrations were calculated in microgram per gram of the ash content (µg/g AC).

2.5. Kinetic model

Antibiotic degradation was evaluated according to the first-order kinetic equation ($C = C_0\, e^{-kt}$) and the half-lives were calculated using: $t_{1/2} = \ln 2 / k$

2.6.Statistical analyses

The statistical significance difference of antibiotics concentrations between T_0-T_{12} and T_0-T_{180} was investigated by the *t* test ($p<0.05$, significant difference (*); $p<0.01$, very significant difference (**)) for the treatment L2, L3 and L4. The statistical analyses were studied with SigmaPlot 12.0.

3. Results

3.1. Physical and chemical parameters

3.1.1. Temperature

All experiments reached a maximum temperature up to 64 °C after 32, 20, 33, and 51 hours of the beginning of L1, L2, L3 and L4 experiments respectively. The thermophilic stage began after 13, 13, 18 and 27 hours and its duration was of 64, 37, 53 and 50 h respectively for L1, L2, L3 and L4. During the thermophilic stage, the temperature has remained above 60 °C for 48, 20, 33 and 20 h for L1, L2, L3 and L4 respectively. After the thermophilic stage, the temperature decreased and stabilized around 25 °C until the end of composting for all experiments except the case of L1 where a rebound was observed and lasted for 3.2 days with a maximum temperature of 49 °C. After that, the temperature in the L1 stabilized around 35 °C toward the end of composting.

3.1.2. Total organic carbon (TOC), total nitrogen (TN), degradation rate and C/N ratio

The **Table 2** shows the evolution of the physical and chemical parameters during all composting experiments. A similar value of TOC, ranged from 46.3 to 47.0%, was observed before composting. After 180 days of composting, the TOC decreased to 40.2, 40.5, 40.6 and 42.4% respectively for L1, L2, L3 and L4.

After 12 days of stabilization, the TN decreased to 3.0, 2.8, 2.7 and 2.8% days respectively for L1, L2, L3 and L4. At the end of the maturation stage, the TN increased to 3.3, 3.1, 3.1 and 3.1 respectively for L1, L2, L3 and L4.

At the final stage of composting, the organic matter reached a degradation rate of 40.2, 36.0, 34.9 and 29.8% and the C/N ratio is about 12, 12.7, 13 and 13.6 respectively for L1, L2, L3 and L4.

3.2. Removal of antibiotics during composting stages

Fig. 1 showed the evolution of antibiotics concentrations during the composting process.

For L1 condition, all the target compounds were below the limit of detection except the CIP, which showed a slight and non significant ($p>0.05$) decrease around 30% after composting process.

Tableau 2: The evolution of the physical and chemical parameters during all composting experiments

Experiments		pH	Moisture[a] (%)	TOC[b] (%)	TN[b] (%)	DR[b] (%)	C/N
L1	T0	6.6±0.1	56.1±2.6	47.1±0.2	4.1±0.1	-	12
	T12	7.2±0.1	46.1±1.1	45.1±0.1	3.1±0.1	14.5±1.7	15
	T180	6.8±0.1	40.1±0.4	40.2±0.2	3.3±0.1	40.3±1.5	12
L2	T0	6.6±0.2	57.2±1.4	46.6±0.1	3.8±0.2	-	12
	T12	7.4±0.1	48.2±1.6	45.2±0.2	2.9±0.2	11.1±1.4	16
	T180	6.9±0.2	35.3±0.9	40.5±0.2	3.2±0.2	36.4±0.3	12.7
L3	T0	6.7±0.1	51.1±1.8	46.4±0.1	3.9±0.1	-	12
	T12	8.3±0.1	45.8±3.8	44.9±0.1	2.7±0.2	10.8±0.8	17
	T180	7.3±0.1	36.1±2.1	40.6±0.1	3.1±0.1	34.9±0.1	13
L4	T0	6.9±0.3	51.2±1.8	47.1±0.2	3.4±0.1	-	14
	T12	8.1±0.1	46.3±1.6	46.6±0.2	2.8±0.1	3.4±2.9	17
	T180	7.2±0.2	41.3±3.6	42.4±0.1	3.1±0.1	29.9±1.5	13.6

[a] Results expressed per unit weight fresh matter; [b] Results expressed per unit weight dry matter; TOC: Total Organic Carbon; TN: Total Nitrogen; DR: Degradation rate; T0: Before composting; T12: After the stabilization stage; T180: After the maturation stage.

In L2 condition, ROX concentration decreased rapidly and reached an elimination rate of 93% within 5 days of composting. After 12 days of stabilization period, ROX removal was around 100%. In the same treatment, the removal of OTC and CTC was not significant during the stabilization stage (**Fig. 1**). Although, their removal increased significantly after the maturation stage. OTC was removed completely during the first 3 months of maturation and CTC was removed of about 62% after the maturation stage. A slight but no significant ($p>0.05$) decrease of CIP (10% and 35%) was observed during all composting stages.

In the L3 treatment, ROX removal (44%) is lower than observed in the thermophilic stage of L2 condition. Nevertheless, a removal of 83% was observed after the stabilization stage ($p<0.01$). As observed for L2, OTC and CTC were strongly removed during the maturation stage. OTC completely disappeared and CTC reached a decrease of 66%. CIP was not significantly removed; this molecule persisted at a concentration of 32 µg/g AC until the end of composting.

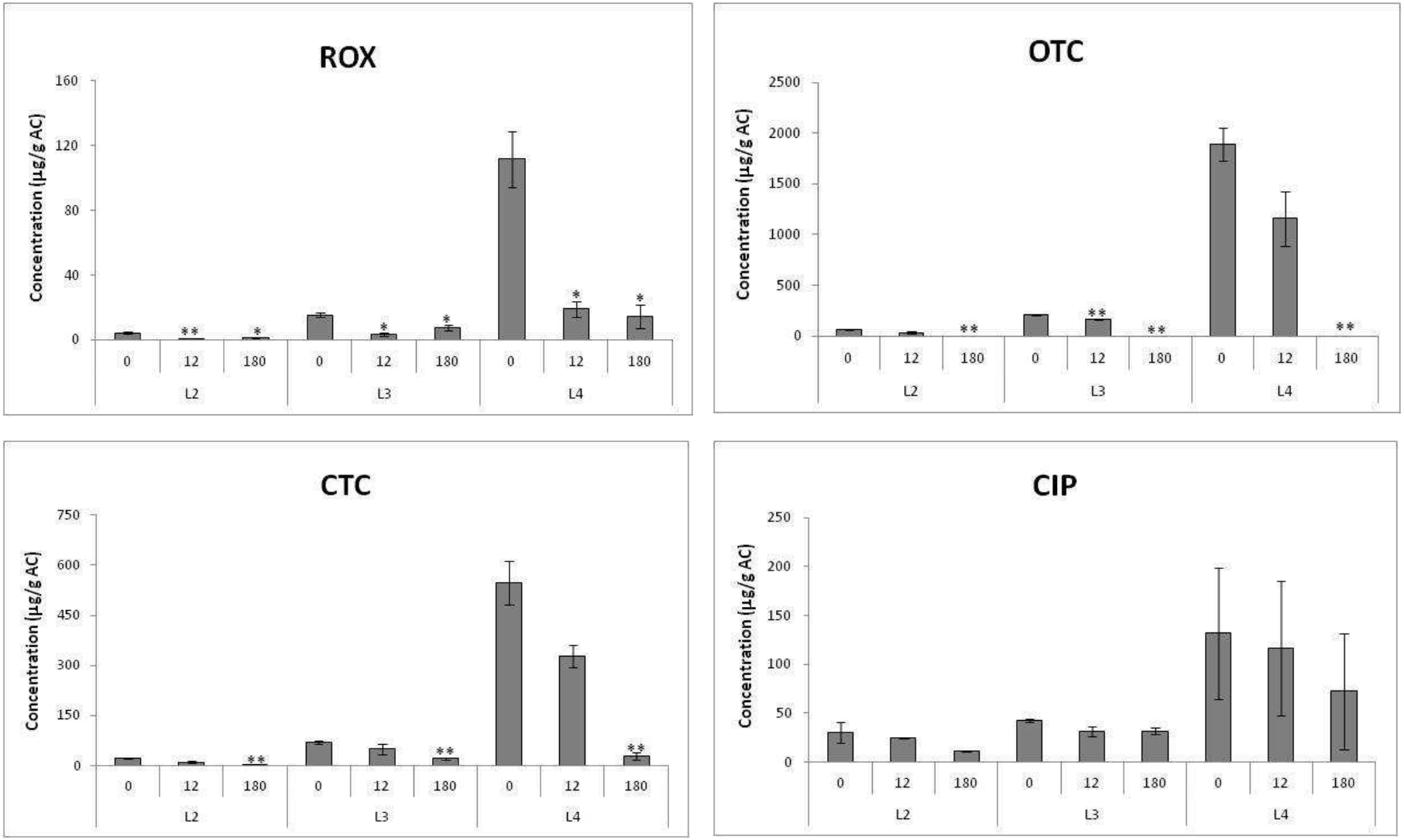
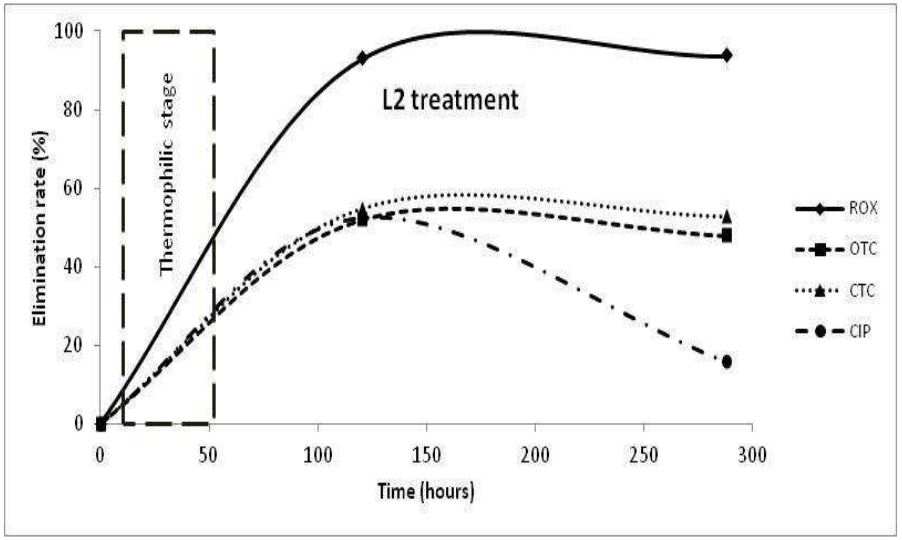
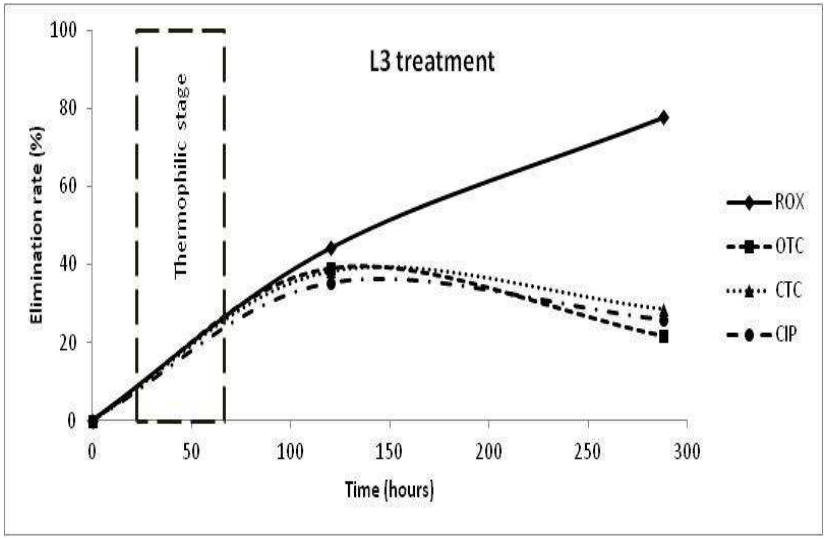


Figure 1: The fate of antibiotics during all composting experiments. Concentrations are expressed referring to ash content (AC). * And **, probability of significance according to t-test for $p<0.05$ and $p<0.001$ respectively (T0 versus T12 or T180, for each condition). T0: initial time of composting; T12: end of stabilization stage reached after 12 days; T180: end of maturation stage reached after 180 days.





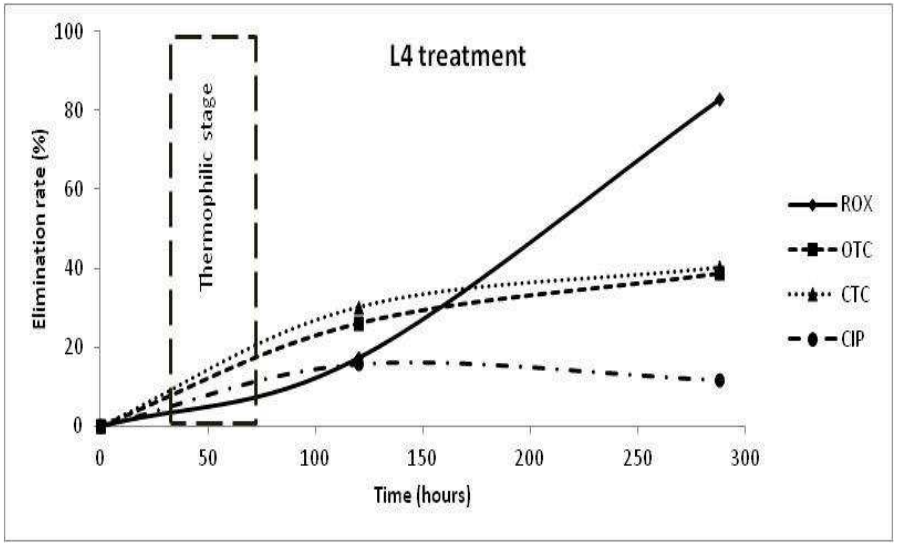


Figure 2: Elimination rates of antibiotics during the stabilization stage indicate in hours. The thermophilic period is indicated in dashed box

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After the thermophilic stage of the L4 condition, ROX removal is only about 17%, which is lower than those observed in L2 and L3 conditions. At the end of the stabilization stage, ROX removal reached 79% between 5 and 12 days. For the first time, ROX removal occurs during the first 3 months of the maturation stage. OTC, CTC and CIP removals were not significant during the stabilization phase. OTC was completely and significantly removed after the maturation stage and the CTC reached a significant removal of 90%. CIP showed a slight but non-significant decrease and a concentration around 72 μg/g AC still persists until the end of composting.

The **Fig. 2** showed the elimination rate of antibiotics during the stabilization stage. The thermophilic stage plays a crucial role on antibiotics removal. For L2, it was observed that ROX was totally removed during the first 5 days of the stabilization phase, including the thermophilic stage. Then, the kinetic of removal between 0 and 5 days, is affected by increasing antibiotics concentrations. Instead of an elimination rate around 100% after 5 days of the stabilization in L2, removals of 40 and only 20% were observed in L3 and L4 respectively. At the end of the stabilization stage, ROX was removed of about 80% in L3 and L4. As it was described above, CTC and OTC were removed significantly in the maturation stage. Nevertheless, a partially decrease (sometimes not significant) of these compounds was observed during the stabilization stage and their removal is also affected by antibiotics concentrations. In L2, an elimination rate of 46% was observed after 5 days of the stabilization for CTC and OTC. This removal decreased until 40 and 25% in L3 and L4 for CTC and OTC. The molecule belonging to the fluoroquinolone family (CIP) showed fluctuations in concentrations and persisted during all composting stages. CIP removal was not statistically significant during all composting stages as it was described above.

4. Discussion

a. Antibiotics effects on physical and chemical parameters during composting

The thermophilic stage is known by their important bacterial activity which leads to the temperature increase (Tiquia, 1996). The high maximal temperature reached in all composting experiments (up to 64 °C) would destroy most of the pathogens in sludge, leading to produce compost that meets sanitary requirements (Bernal et al., 2009). Nevertheless, increasing antibiotics concentrations delayed the coming of the maximal temperature and disturbed the thermophilic stage. The observed effect on thermal parameters is a result of disturbing the microbiological activity and then the organic matter degradation (Eguchi et al., 2012). For L3

and L4 treatments, the organic matter degradation is delayed and the C/N ratio is not close to 10. The delay of the TOC decrease (Wong et al., 2010) and the vulnerability of the nitrogen transforming microorganisms (Liu et al., 2015) in the presence of high antibiotics concentrations could be responsible of the observed effect on the C/N ratio (Selvam et al., 2012; Arikan et al., 2009; Liu et al., 2015). The process efficiency and finally the compost quality are affected by increasing antibiotics concentrations (Ezzariai et al., 2017).

b. Correlation between antibiotics elimination, composting stages and palm rachis evolution during composting

The current study demonstrated a significant decrease in concentrations of ROX, CTC and OTC during laboratory scale composting. These antibiotics compounds were removed by an elimination rate of 52-87, 69-95 and 100% respectively after all composting experiments. A number of previous studies have demonstrated that composting can result significant decrease of antibiotic concentrations. ROX was found at low concentrations of 51.5 and 46.7 μg/kg in a refined commercial and rice husk compost (Zhang et al., 2014). In addition, it was removed completely after a soybean meal composting (Zhang et al., 2014). Several studies reported an elimination rate ranging from 71 to 100% for the TCs after composting (Arikan et al., 2007; Bao et al., 2009; Hu et al., 2011; Wu et al., 2011). However, as observed by numerous authors (Haiba et al., 2013; Lillenberg et al., 2010; Selvam et al., 2012), non-significant removal of CIP was observed in this work.

The effect of composting stages on antibiotics removal was assessed. In most cases, the fate of antibiotics during composting was assessed especially for the compounds from tetracyclines family (Arikan et al., 2009; Bao et al., 2009; Wu et al., 2011). Generally, sorption is responsible of CTC and OTC removal due to the formation of chelates complexes with organic substances (Kim et al., 2012). Previously, it was observed that thermophilic composting was not significant in terms of tetracycline removal (Selvam et al., 2013). In contrast, Arikan, et al. (2008) observed a decrease of 96% within the first 6 days of composting. In the current study, a partial removal was observed for CTC and OTC during the stabilization stage (half-lives varying from 11 to 34 days), but their removals were very significant during the maturation stage. The contribution of fungi and actinomycetes could explain the removal of CTC and OTC during the maturation stage. ROX was generally removed during the stabilization stage with a half-life from 3 to 5.5 days, demonstrating the implication of the thermophilic stage (Arikan et al., 2007; Wu et al., 2011) and more particularly thermophilic bacteria and/or archaea microorganisms.

It was observed that antibiotics removal during the stabilization stage was more effected in the L4 condition, and some compounds were removed significantly during the maturation stage (the case of ROX). In agreement of this study, increasing antibiotics concentrations affected microorganism's activity and then the removal of target compounds during the thermohpilic stage (Eguchi et al., 2012). It could be concluded that composting stages play complementary roles regarding antibiotics removal more particularly at high antibiotics concentrations.

The composting substrates could lead to the sorption of antibiotics (Kim et al., 2012). In this study, a lignocellulosic substrate (rachis palm) was used as a biological structuring agent. It was observed that antibiotics adsorption on rachis is a key element which leads to protect the composting process progress and controls the removal of antibiotics during the stabilization and the maturation stages (Ezzariai et al., 2017). During all composting stages, CIP showed some fluctuations in concentrations. In the other hand, CIP removal was not significant during the process. However, CIP sorption and desorption on palm rachis could explain the obtained results and behavior of this molecule during composting stages. A strong adsorption of CIP to particulate organic matter was reported previously (Belden, 2007; Uslu and Yediler, 2008). In addition, CIP adsorption depends on the organic matter composition (Belden, 2007), which is varying continuously during composting. According to Freundlich isotherms, non linear adsorption of CIP to the organic matter was observed (Polesel et al., 2015). Thus, non equilibrium sorption and desorption could be occurred between CIP and palm rachis leading to the observed fluctuations in concentrations.

5. Conclusion

The present study revealed that composting could remove ROX, CTC and OTC; whereas the CIP could persist even at the end of the maturation stage. The thermophilic stage was responsible on the ROX elimination. In contrast, the maturation stage was more implicated on the removal of CTC and OTC. At high antibiotic concentrations, the thermophilic stage is disturbed and the maturation stage plays a complementary role regarding antibiotics removal especially for ROX. The sorption/desorption of CIP on palm rachis could probably explain the observed behavior of this molecule during composting. These results suggest that ciprofloxacin and perhaps fluoroquinolone family represent an important environmental concern. So, in view of the important consumption of these classes of molecules, their persistence in the environment (Haiba et al., 2013; Lillenberg et al., 2010; Selvam et al.,

2012) and the large use of urban compost in agriculture, fluoroquinolones must be the subject of future regulation.

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Chapitre 5 : Devenir des antibiotiques au cours de la digestion anaérobie

1. Synthèse bibliographique: digestion anaérobie et antibiotiques

1.1.La digestion anaérobie

La digestion anaérobie consiste en une transformation de la matière organique en biogaz, mélange de méthane en de dioxide de carbone, par un consortium microbien et en absence d'oxygène. Trois groupes de microorganismes ont été identifiés durant le processus de digestion anaérobie : (1) les microorganismes d'hydrolyse et (2) d'acidogénèse (3) les bactéries d'acétogènes et (4) les archées méthanogènes. Ces communautés microbiennes définissent les 4 étapes dans le déroulement du processus de méthanisation (l'hydrolyse, l'acidogénèse, l'acétogénèse et la méthanogénèse) comme présenté dans la figure 1. La digestion anaérobie est appliquée principalement pour la production du biogaz à partir des résidus agricoles. En plus, il s'agit d'un outil aussi appliqué pour le traitement de la pollution organique des déchets solides et des effluents agroalimentaires et industriels. La digestion anaérobie est parmi les méthodes les plus utilisées pour la stabilisation de la boue et du fumier soit pour des fins énergétiques ou pour une valorisation agronomique. Du point de vue valorisation, la digestion anaérobie arrive à éliminer des polluants organiques et inorganiques initialement présents dans la boue ou le fumier. Parmi ces polluants, notons les antibiotiques, dont plusieurs travaux portent sur leur occurrence et devenir au cours de la digestion anaérobie.

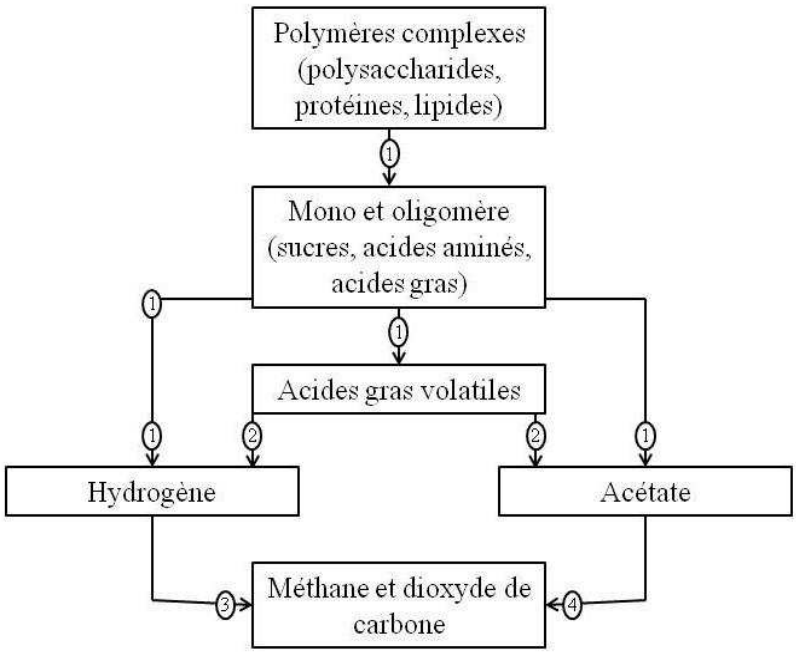

Figure 1: Processus de la digestion anaérobie (1 : l'hydrolyse et l'acidogénèse ; 2 : l'acidogénèse; 3 et 4 : la méthanogénèse)

1.2.La dissipation des antibiotiques au cours de la digestion anaérobie

Le tableau 1 présente une synthèse bibliographique sur le devenir des antibiotiques au cours de la digestion anaérobie.

Les essais de digestion anaérobie ont été généralement lancés en mode batch et peu d'expériences ont été conduites en mode continu, qui est le mode d'opération représentatif de l'échelle industrielle pour les boues. La digestion mésophile (35-37°C) est beaucoup plus utilisée en dopant le substrat utilisé (boue ou fumier) par des concentrations d'antibiotiques allant de 40 µg/L jusqu'au 2000 mg/L avec des durées d'incubation de 3 à 330 jours. Les familles d'antibiotiques les plus étudiées sont les sulfamides suivies par les macrolides et les tétracyclines. Peu de travaux ont été réalisés sur les fluoroquinolones.

Carballa et al. (2007) ont réalisé une étude sur le devenir des sulfamides (SMX) et des macrolides (ROX) après digestion d'une boue mixte en mode continu à deux températures d'incubation (37 et 55°C). Les résultats de cette étude montrent des taux d'abattement important de l'ordre de 94 et 99% pour la ROX et SMX respectivement. Sur le même substrat, Gonzales-Gil et al. (2016) ont trouvé des taux d'élimination similaires pour ces deux molécules (85 et 90% pour la SMX et la ROX respectivement) en utilisant deux températures d'incubation (37 et 55°C). Les taux d'élimination les plus élevés sont obtenus en conditions thermophiles. La TYL qui est une autre molécule appartenant aux macrolides a montré une biodégradabilité significative après la digestion anaérobie mésophile avec des taux d'élimination de l'ordre de 100% (Mitchell et al., 2013). Globalement, les macrolides et les sulfamides sont généralement biodégradables en conditions anaérobies et leur élimination n'est pas conditionnée ni par la température d'incubation ni par le substrat utilisé. Le devenir des tétracyclines au cours de la digestion anaérobie a été suivi principalement en utilisant le fumier comme substrat et en conditions mésophiles (Tableau 1). Arikan et al. (2008) ont trouvé que 75% de la dose administrée de CTC est éliminés après 33 jours d'incubation. A une durée d'incubation encore courte (21 jours), des taux d'éliminations de 53 à 91% ont été observé par Alvarez et al. (2010) pour l'OTC et la CTC. Pour le cas des fluoroquinolnes, Ning et al. (2017) ont enregistrés des taux d'élimination de 60 à 70% pour 4 molécules appartenant à la famille des FQs (OFL, NOR, CIP et LOM). Par contre, d'autres études ont observées que les FQs sont moins affectés par le processus de digestion (Martin et al., 2015) et des taux d'élimination de 30 à 50% ont été observés (Narmunya et al., 2013).

L'élimination des antibiotiques au cours de la digestion anaérobie n'est pas influencée par certains paramètres de fonctionnement, notamment la température et la durée d'incubation (Carballa et al. 2007 ; Gonzalez-Gil et al., 2016). Par contre, même si la température n'est pas impliquée dans leur élimination, elle est associée à la diminution des effets oestrogéniques des antibiotiques au cours de la digestion anaérobie (Gonzalez-Gil et al., 2016).

La digestion anaérobie continue est caractérisée par une phase de stabilisation où le procédé arrive à des conditions performantes et stables en termes de dégradation de la matière organique et de production de biogaz. Dans une étude menée par Carballa et al. (2007), la stabilisation du procédé déclenche le début d'abattement de certains antibiotiques. Deux voies principales sont impliquées dans l'élimination des antibiotiques, l'une est physique et l'autre biologique. La dissipation physique consiste en la sorption sur la matière organique, alors que la dissipation biologique est liée à l'activité microbiologique. La digestion anaérobie modifie les groupements fonctionnels amino-carboxyliques des antibiotiques, ce qui a pour conséquence de changer leur coefficient de dissociation et ainsi leur distribution entre la phase aqueuse et solide. La variation de partage des antibiotiques entre les deux phases contrôle leur dissipation physique ou biologique et ainsi leur élimination au cours de la digestion anaérobie (Narumiya et al., 2013). L'élimination de CTC et OTC est liée à leur forte adsorption sur la fraction solide (Alvarez et al., 2010). Par contre, TYL est un composé qui s'adsorbe faiblement, son élimination se fait plus facilement par voie biologique (Stone et al., 2009). Les deux voies, physique et biologique, peuvent être impliquées en même temps dans la dissipation de certains antibiotiques. L'élimination de TC et SMD est liée à leur adsorption sur la fraction solide, toutefois leur dégradation est liée à l'activitée des microorganismes (Shi et al., 2011). La digestion anaérobie est parfois conduite après un prétraitement des substrats utilisés, ce qui permet d'améliorer la production du biogaz et l'abattement de la matière organique. Néanmoins, Li et al. (2017) ont appliqué l'hydrolyse thermique (160°C) comme prétraitement pour évaluer sa contribution vis-à-vis de la dissipation des antibiotiques, mais aucun effet significatif n'a pu être observé.

1.3. L'effet des antibiotiques sur le procédé de digestion anaérobie

Plusieurs travaux ont étudié l'effet des antibiotiques sur la digestion anaérobie (Tableau 1). Les effets observés concernent plus particulièrement les communautés bactériennes impliquées dans l'abattement de la matière organique et la production du biogaz. Les tétracyclines inhibent la production du biogaz de 20 à 65% après ajout de CTC, TC et OTC à

des concentrations allant de 0,25 à 550 mg/L (Massé et al., 2000 ; Lallai et al., 2002 ; Loftin et al., 2005 ; Arikan et al., 2008 ; Stone et al., 2009 ; Alvarez et al., 2010 ; Shi et al., 2011). La production du biogaz est beaucoup plus affectée par la CTC que l'OTC ou TC. La TYL est la molécule la plus utilisée pour suivre l'effet des macrolides sur la production du biogaz. A des concentrations de 1.1 à 920 mg/L, la TYL affecte la production du biogaz de 28,4 à 40% (Massé et al., 2000 ; Stone et al., 2009 ; Mitchell et al., 2013). L'inhibition de la production du biogaz par les sulfamides diffère selon chaque étude et parfois en utilisant la même molécule. La présence de la SMZ à des concentrations de 0.28 à 280 mg/L n'a pas d'effet sur la production du biogaz (Mitchell et al., 2013). A l'inverse des auteurs ont montré que des doses de 1,5 à 110 mg/L, de SMZ, SMD et SMX inhibent la production du biogaz de 20 à 35% (Massé et al., 2000 ; Loftin et al., 2005 ;Shi et al., 2011). Pour les FQs peu d'études existent et seul l'article de Li et al. (2017) montre un effet sur la réduction de la matière volatile et la production du biogaz à des concentrations de 2 à 100 mg/L de OFL, NOR, CIP et LOM. A la lumière des travaux précédents, plusieurs conclusions ont été faites pour expliquer comment ces antibiotiques arrivent à inhiber le procès de digestion anaérobie. Les effets inhibiteurs sont en fonction du type d'antibiotique utilisé et sa concentration de dopage (Tableau 1). La présence des antibiotiques, à certaines concentrations, empêche l'utilisation de l'acétate par les communautés homo-acétogénique et méthanogènes acétoclastiques, ce qui conduit à l'accumulation des acides gras volatile ou la diminution du pH et ainsi l'inhibition de la production du biogaz (Laillai et al., 2000; Stone et al., 2009). Les antibiotiques ciblent les communautés bactériennes qui sont l'élément clé qui conduit à inhiber la production du biogaz (Shi et al., 2011).

Les travaux bibliographiques montrent que peu d'études ont été focalisées sur les effets et le devenir des antibiotiques durant la digestion anaérobie en conditions réalistes similaires aux conditions industrielles. Les potentialités de la digestion anaérobie en présence d'un mélange d'antibiotiques avec différents comportements sont aussi peu étudiées.

Tableau 1: Devenir des antibiotiques au cours de la digestion anaérobie

Composés	**Concentrations initiales (unité)**	**Mode de fonctionnement du réacteur**	**La température d'incubation (°C)**	**Elimination (%)**	**Temps de rétention (jours)**	**Substrat utilisé**	**Inhibition du procès**	**Références**
SMX ROX	40 µg/L 40 µg/L	Continu	37 55	99 94	10, 20 et 30	Boue mixte	-	Carballa et al., 2007
TYL CTC	1.1 mg/L 28 mg/L	Batch	10-20	-	216	Lisier de porc	Inhibition de la production du CH_4 et CO_2 par 27.8% et 28.4% en présence de CTC	Stone et al., 2009
SMX TMP ERY ROX	8 µg/g	Continu	37 55	85 75 - 90	330	Boue mixte	-	Gonzalez-Gil et al., 2016
Ampicillin Florfenicol SMZ TYL	0.35-350 mg/L 0.36-360 mg/L 0.28-280 mg/L 0.92-920 mg/L	Batch	37	100 100 Non dégradé 100	40	Lisier de bovins	Pas d'effets de SMZ et AMP sur la production du biogaz à 280-350 mg/L. TYL réduit la production du biogaz de 10 à 38% à 130-913 mg/L. FFL réduit la production du biogaz de 5%, 40% et 75% à 6.4, 36 et 210 mg/L.	Mitchell et al., 2013
CTC	Administration d'une dose de 22 mg/kg/jr	Batch	35	75	33	Fumier de bovins	-	Arikan et al., 2008
TC SMD	0.25 mg/L 50 mg/L	Batch	25	-	20	Fumier de porc	Les doses utilisées d'antibiotiques affectent la production du méthane et inhibent l'activité bactérienne.	Shi et al., 2011
TYL LINCO TC SMZ PENIC Carbadox	110 mg/kg 220 mg/kg 550 mg/kg 110 mg/kg 16 mg/kg 55 mg/kg	Batch	20	-	60	Fumier de porc	La présence du pinicilline et TC réduit la production du méthane par 25-35% La présence des antibiotiques n'affecte pas la stabilité du procès et l'efficacité du traitement.	Massé et al., 2000
AMOX OTC Fénicol	60-120 mg/L 125-250 mg/L 80-160 mg/L	Batch	37	-	21	Fumier de porc	Le thiamphénicole affecte la production du méthane à 80-160 mg/L. Pas d'inhibition de la production du méthane à 60-120 mg/L d'AMOX TC n'a pas d'effet sur la production du méthane.	Lallai et al., 2002

Tableau 1: Devenir des antibiotiques au cours de la digestion anaérobie

Composés	**Concentrations initiales (unité)**	**Mode de fonctionnement du réacteur**	**La température d'incubation (°C)**	**Elimination (%)**	**Temps de rétention (jours)**	**Substrat utilisé**	**Inhibition du procès**	**Références**
OTC CTC	10, 50 et 100 mg/L	Batch	35	53-68 90-91	21	Fumier de porc	La production du méthane est réduite à 65, 60 et 62% par ajout d'OTC et CTC à des concentrations de 10, 50 et 100 mg/L	Alvarez et al., 2010
CLX	0, 50, 200, 400, 600, 1000 et 2000 mg/L	Batch	35	-	150	Boue activée	CLX affecte la production de méthane durant les 25 premiers jours d'incubation plus particulièrement pour les doses 1000 et 2000 mg/L.	Lu et al., 2014
Ceftiofur, Danofloxa Micospect	1.7-13.8 mg/L 1.1-8.5 mg/L 14-112 mg/L	Batch	37	70 30 34	60	Fumier de porc	L'association de deux familles d'antibiotiques (Lincomycine et spectinomycine) inhibe la digestion anaérobie. Danofloxacine réduit la production du biogaz par 10-5%.	Panseri et al., 2013
OFL NOR CIP LOM	2, 20 et 100 mg/L	Batch	160 + 35	70.3 69.9 69.4 60.8	28	Boues	Effet sur la réduction de la matière volatile et la production du biogaz à 100 mg/L	Li et al., 2017
STZ SMZ SMX Linco CTC OTC TC	1.5-25mg/L	Microcosme Lagunes	-	-	3-14	Fumier de porc	Inhibition de la production du méthane de 20 à 45% L'effet des antibiotiques a affecté partiellement la DCO, la production des odeurs, température et pH.	Loftin et al., 2005
48 produits pharmaceutiques	-	Digesteur de step	37	90 (SMZ et TMP), 30-50 (OFL)	-	Boue	-	Narumiya et al., 2013

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2. Potentialités de la digestion anaérobie en mode semi-continu vis-à-vis de l'élimination des antibiotiques dans les boues

Contexte :

La digestion anaérobie est parmi les procédés les plus utilisées pour le traitement des boues des stations d'épuration. Les potentialités épuratoires de ce bioprocédé couvrent une large gamme de micropolluants incluant les antibiotiques. Dans se sens, la digestion anaérobie en mode semi-continu a été utilisée pour le traitement des boues primaires en présence d'antibiotiques.

Le devenir des antibiotiques durant la digestion anaérobie en conditions réalistes, similaires aux conditions industrielles, est peu traité dans la littérature. Cependant, la concentration de contamination a été choisie conformément aux teneurs d'antibiotiques réalistes déjà reportées dans les boues. Trois familles d'antibiotiques ont été utilisées en mélange pour conduire l'essai de digestion anaérobie en condition mésophile (37°C). L'objectif de cette étude est de mettre en évidence les performances de la phase de stabilité vis-à-vis l'élimination des antibiotiques.

Principaux résultats :

La présence des antibiotiques à une concentration réaliste de 2.5 mg/g MS n'a pas d'effet sur la production du méthane ainsi que la dégradation de la matière organique. Ces résutats suggèrent que les antibiotiques n'ont pas d'effets notables sur le déroulement normal de la digestion anaérobie à l'échel industriel à une concentration typique à celle utlisée. Durant la phase de stabilisation, l'élimination de la roxithromycine, la chlortétracycline et l'oxytétracycline est significative et elle est de 50, 100 et 59% respectivement. L'élimination de l'enrofloxacine est non significative et elle est estimée vers 30%.

La digestion anaérobie peut éliminer partiellement les molécules parents des antibiotiques. La biotransformantion est une voie principale d'élimination des antibiotiques ce qui donne lieu à l'apparition des métabolites. Cependant, l'application du digestat dans les sols agricoles doit vérifier certains critères à savoir la persistance de quelques familles d'antibiotiques, la présence des métabolites et des gènes de résistance aux antibiotiques.

Ce chapitre est présenté sous la forme d'un article qui s'intitule:

Potentialities of semi-continuous anaerobic digestion for mitigating antibiotics in sludge

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Abstract

The behavior and removal of the antibiotics roxithromycin (ROX), oxytetracycline (OTC), chlortetracycline (CTC) and enrofloxacin (ENR) were investigated during the steady-state of a sludge anaerobic digestion experiment in semi-continuous mode (37°C). A primary sludge was spiked at realistic concentrations (50 μg/L of each antibiotic) and then used to feed the bioreactor for 80 days. The target antibiotics were extracted from the substrate and digested sludge samples by accelerated solvent extraction (ASE). An accurate determination of antibiotics was obtained by the standard addition method (SAM) associated with the liquid chromatography-tandem mass spectrometry (LC-MS/MS). The presence of antibiotics at a concentration of 2.5 μg/g DM had no inhibitory effects on the methane (CH_4) production, the total and volatile solids (TS and VS) removal as well as the chemical oxygen demand (COD) removal. At steady-state, antibiotics were removed significantly by 50, 100 and 59 % respectively for the ROX, OTC and CTC. ENR removal was not statistically significant but could be estimated at 36%. Anaerobic digestion process could partially remove parent compounds, but ROX, CTC and ENR still persisted in the digested sludge. This study highlights the potential of anaerobic digestion as a sludge treatment for mitigating, but not suppressing, the release of antibiotics through sludge application.

Key words: Antibiotics, Sludge, Anaerobic digestion

Nomenclature	
Abbreviation	**Definition**
AD	Anaerobic Digestion
ASE	Accelerated Solvent Extraction
CH_4	Methane
COD_s	Soluble Chemical Oxygen Demand
COD_t	Total Chemical Oxygen Demand
CTC	Chlortetracycline
ENR	Enrofloxacin
FQs	Fluoroquinolones
MDs	Macrolides
OTC	Oxytetracycline
ROX	Roxithromycin
SAM	Standard Addition Method
TCs	Teteracyclines
TS	Total solids
TSS	Total Suspended Solidss
VS	Volatile solid
VSS	Volatile Suspended Solids
WWTPs	Waste Water treatment plants

1. Introduction

In the last decade, antibiotics have been increasingly used worldwide with an annual consumption between 100,000 t and 200,000 t (Jeong et al., 2010). Antibiotics used for human consumption are partly excreted in urine and feces which arrives up to the wastewater treatment plants (WWTPs). WWTPs include different processes which are not specifically designed for antibiotics removal and are thus regarded as limiting for antibiotics elimination (Mao et al., 2015; Polesel et al., 2016). As a result, antibiotics are not efficiently removed by the conventional WWTPs and a part of these molecules is adsorbed in the sludge and the other remains in the treated water, the latter being directly discharged into surface water (Michael et al., 2013; Xu et al., 2015). In raw sludge, macrolides, tetracyclines and fluoroquinolones are present at concentrations varying from 33.8 to 1074 μg/kg dry matter (DM), from 0.12 to 3262 μg/kg DM and from 0.75 to 4328 μg/kg DM respectively (Petrovic, 2009; Zhou et al., 2009; Li et al., 2013; Verlicchi and Zambello, 2015; Ezzariai et al., 2018 (submitted)). As a result, sludge contributes to the release of antibiotics to the environment by their direct application (Luque-Munoz et al., 2017) which could be responsible for the contamination of soil (Rico et al., 2014). Antibiotics could then be transferred and reach sediments (Rico et al., 2014), surface water (Hamsher et al., 2002), underground water (Hirsch et al., 1999) and plants biomass (Kumar et al., 2005; Dolliver et al., 2007; Prosser and Sibley, 2015). Antibiotics dissemination can also alter the microbial communities in the

environment and result in the emergence of antibiotics resistant bacteria and the transfer of antibiotic resistance genes (Costa et al., 2011; Li et al., 2015), and could impact soil organisms and biodiversity (Pino-Otín et al., 2017).

Sludge application could act as a source of nutrients for plant growth and a conditioner to improve the soil structure and its organic matter content. Beyond its agronomic advantages, soil fertilization with sludge is also virtuous in the overall context of waste recycling and resources preservation. However, an effective process must be used to treat sludge and mitigate the antibiotics release into the environment. Anaerobic digestion (AD) is a widely used process for the treatment of sludge generated from WWTPs, with economic and environmental benefits (Stasinakis, 2012). The potentiality of AD for partially removing a wide range of pollutants, including antibiotics, has been demonstrated (Carballa et al., 2007; Bernal-martinez et al., 2009; Álvarez et al., 2010; Barret et al., 2010; Barret et al., 2012; B; Mitchell et al., 2013; Malmborg, 2015; Semblante et al., 2015; Gonzalez-gil et al., 2016; Aemig et al., 2016; Youngquist et al., 2016; Spielmeyer et al., 2017; Ghattas et al., 2017). AD of sludge contaminated with antibiotics questions (i) the efficiency of AD for removing antibiotics and (ii) the potential negative effects of antibiotics on the AD process. Generally, the fate and effects of antibiotics during the AD are investigated separately. The experimental design of studies focused on the fate of antibiotics was generally based on realistic concentrations which are not able to inhibit the process. Using concentrations from 0.4 to 1.1 µg/g (10 to 40 µg/L) (ROX, ERY, SMX, SMZ) showed that AD of sludge in continuous mode removed sulfonamides and macrolides from 75 to 99% (Carballa et al., 2007; Gonzalez-Gil et al., 2016). Ciprofloxacin and norfloxacin were found at concentrations of 2660 and 4328 µg/kg DM respectively from a full-scale digester (Martín et al., 2014), demonstrating that the AD process is partially effective regarding FQs removal. Antibiotics removal varies according to antibiotics properties, AD mode, used substrates (sludge or manure) and incubation time. Besides, the potential inhibitory effect of antibiotics on the AD process by increasing antibiotics concentrations was investigated. The decreases of biogas production and organic matter removal rates as well as the effects on methanogenesis are the most affected. At initial concentrations from 20 to 4580 µg/g (0.35 to 920 mg/L), macrolides, tetracyclines and fluoroquinolones have been shown to inhibit the biogas productions from 8 to 64.1% during the mesophilic AD in batch mode (Arikan et al., 2006; Stone et al., 2009; Álvarez et al., 2010; Chelliapan et al., 2011; Mitchell et al., 2013; Li et al., 2017). These inhibitory effects could be a result of using antibiotic mixtures which may affect propionate

and butyrate-oxidizing syntrophic bacteria, leading in unfavorable effects on methanogenesis (Aydin et al., 2015).

Literature is still scarce about the fate of antibiotics in realistic conditions. Indeed, very few studies were setup with realistic antibiotic concentration, taking into account potential mixture effects, and in operating conditions that are representative of the full scale anaerobic digestion. In this study, sludge was spiked with four extensively used antibiotics (ROX, CTC, OTC and ENR) with demonstrated occurrence in sludge. An AD experiment was conducted in mesophilic condition (37°C) and in semi-continuous mode. The objective of this work was to study the fate of antibiotics in realistic conditions during the steady-state.

2. Materials and methods

2.1. Antibiotics

The Tetracyclines (CTC hydrochloride (75% of purity) and OTC hydrochloride (95%)), a Macrolide (ROX (90%)) and a Fluoroquinolone (ENR (98%)) were purchased from Sigma-Aldrich-France (F-38297, St-Quentin Fallavier). The stock antibiotics solutions were prepared in methanol (HPLC grade, Sigma-Aldrich) and used directly for spiking sludge at a concentration of 50 µg/L/Molecule.

2.2. Chemical analyses

Total Solid (TS) and Volatile Solid (VS) contents were determined after desiccation (24h at 105°C) and calcination (4h at 550°C) respectively. The same procedures were followed to measure the Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) starting with the pellet of a known volume of centrifuged sample (18,600 × g, 20 min, 4°C). The chemical oxygen demand (COD) was measured on the total sludge (CODt) and the supernatant after centrifugation (CODs) according to the international standard ISO 15705:2002 using a Spectroquant kit (Merk, range 0-1500 mgO_2/L) with an optical density measurement (HACH LANGE DR5000 spectrophotometer). The biogas content (CH_4, CO_2, N_2, O_2, and H_2S) was analyzed using the GC Clarus 480 Gas Chromatography system (Perkin Elmer, USA) after injection of 200 µl of the gas sample. The volatile fatty acids (VFAs) i.e. acetate, propionate, butyrate, isobutyrate, valerate and isovalerate were quantified using a gas chromatograph (Perkin Clarus 580) with capillary column [Elite-FFAP crossbond®carbowax® (15 m), 130 °C, N2 as gas vector with flow rate of 3 mL/min] and equipped with a flame ionization

detector maintained at 250°C. Before use, 500 µL of centrifuged bulk sludge (20 min., 18600 G) were mixed with 500 µL of internal standard (ethyl-2-butyric acid, 1 g/L).

2.3. Sludge characteristics

A primary sludge was collected from the WWTP of Marrakech (Morocco), treating domestic and industrial wastewater using an activated sludge process including a tertiary treatment. Marrakech WWTP treats the waste water of a population equivalent about 1,300,000 with a capacity of 120,000 m^3/day (Belloulid et al., 2016). After sampling, sludge was stored at -20°C. This primary sludge was diluted at 32.15±2.1 gTS/L in order to adjust the organic loading in the AD experiment (1.4 gCOD/day/L). The diluted sludge was spiked by a mixture of 4 molecules of antibiotics (ROX, CTC, OTC and ENR) at a concentration of 50 µg/L/molecule (2.5µg/gDM).

The anaerobic inoculum for the AD experiment was collected from a full-scale reactor in Limoges (France). The main characteristics of the substrate, the inoculum and the digested sludge are shown in **Table 2**.

Tableau 2: The main characteristics of the substrate, the inoculum and the digested sludge

	TS (g/l)	**VS (g/l)**	**TSS (g/l)**	**VSS (g/l)**	**CODt (g/l)**	**CODs (g/l)**
Inoculum	37±*0,1*	26±*0,1*	31±*0,1*	22±*0,1*	40±*4,9*	1±*0,1*
Susbtrate	32 ±*2.1*	21 ±*3.3*	30 ±*4.4*	26±*2.1*	45±*10.02*	2±*0.1*
Digested sludge* (Steady-state)	20±*0.3*	14±*0.3*	19±*0.8*	12±*1.1*	23±*0.9*	0.8±*0.1*
Removal (%)	38	36	38	51	46	62

*These results are a mean values for the five retained samples during the stady-state (after 1.6, 2, 2.4, 3 and 3.7 HRT).

2.4. Anaerobic digestion

The AD of the primary sludge was performed using a cylindrical reactor working under mesophilic temperature (37°C) in semi-continuous mode (**Fig. 2**). The total volume of the reactor was 6 L and the working volume was 5 L. The hydraulic retention time was fixed at 20 days. The reactor was equipped with 4 connections to feed substrate, withdraw digester and measure the produced biogas (quantity and quality). A mechanic stirring system, operated continuously at a rate of 60 rpm, was used to improve the mass transfer in the reactor. The temperature was maintained at 37°C by a thermostatic double jacket containing water. The volume of biogas produced was measured on-line by Milligascounter MGC-1 flow meters

(Ritter gas meters) fitted with 4-20 mA output. The outlet of this ritter was connected to a closed bubbler containing a NaOH solution (1N) for CO_2 trapping. The volume of methane was measured at the outlet of the bubbler using another ritter. The ‘’Modular SPC’’ software developed at the INRA Narbonne laboratory (Torrijos et al., 2014) was used to log the online data.

Feeding and withdrawing were carried out manually five times per week. The semi-continuous reactor has been operated for 4 hydraulic retention times (80 days). The TS, VS, TSS, VSS, CODt and CODs analyses were carried out 3 times per week. In order to analyze the antibiotics, 3 homogenous samples of digested sludge and one homogenous sample of the inlet substrate were constituted from day 30 to day 80. For the digested sludge, the retained points correspond to average outlet samples at 1.6, 2.4 and 3.7 (HRT). After freeze-drying and grinding, the samples were stored in amber glass until antibiotics extraction.

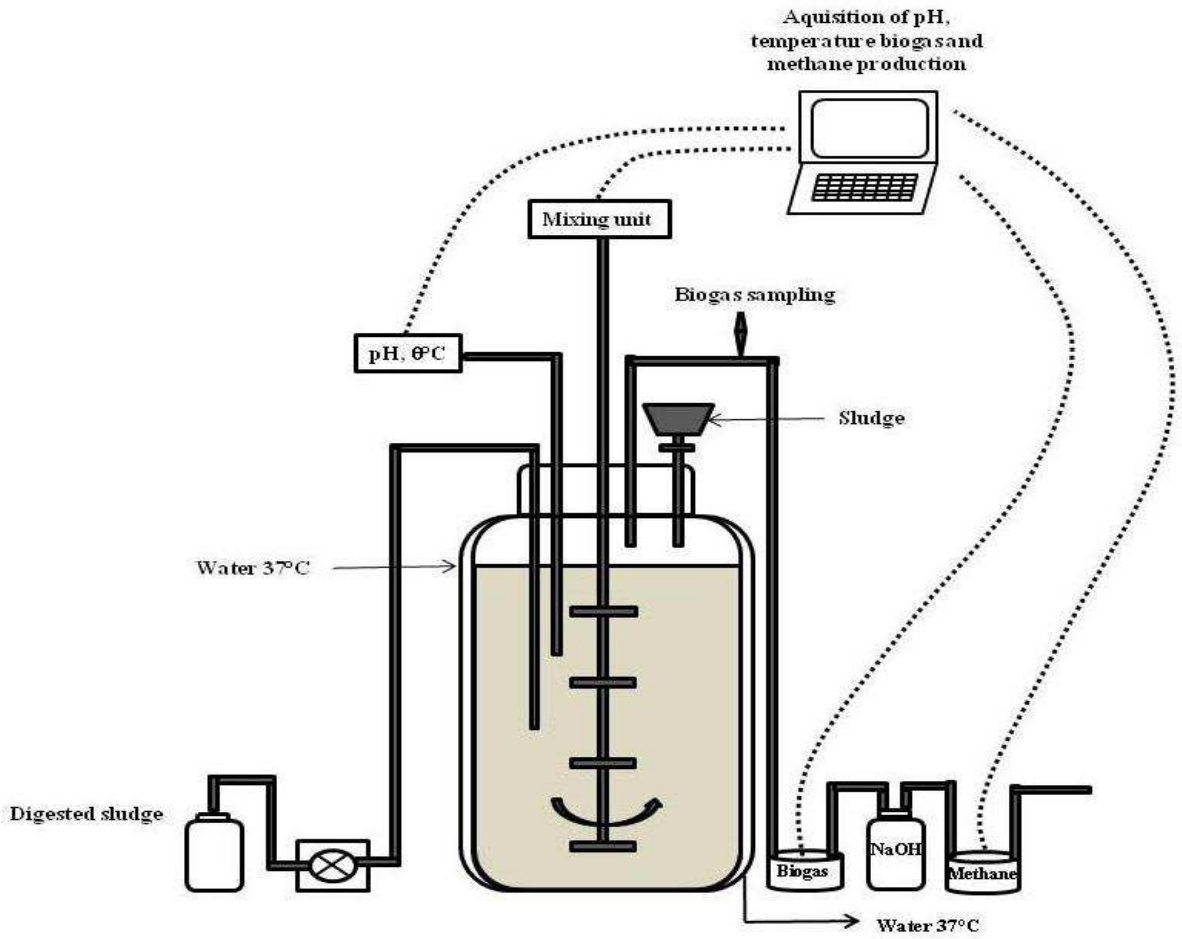

Figure 2: Schematic representation of the semi-continuous reactor

2.5. Antibiotics extraction and quantification

The target antibiotics were extracted from the digested sludge samples using the ASE and an accurate determination by the standard addition method (SAM) associated with LC-MS/MS according to the protocol proposed by Ezzariai et al. (2018) (submitted) that was adapted in this case for the digested sludge samples.

Antibiotics were extracted using an ASE 200 from Dionex (Sunnyvale, CA, USA). The extraction approach consisted of using two solvent mixtures to extract antibiotics from the same digested sludge. The first extraction step was carried out using sodium phosphate buffer and ethylene diamine tetraacetic (SPB-EDTA), and the second one was conducted using acetonitrile and phosphoric acid (ACN-PA, 50:50, v/v).

The standard addition method was applied directly on the ASE extracts. All samples were spiked using a solution containing the 4 targeted antibiotics at 3 concentrations levels in duplicate (not spiked; 2×C; 4×C).

For antibiotic analysis, ultra-performance liquid chromatography (UPLC) separations were performed on a T3 Cortecs C18 column. Mass spectrometry analyses were carried out on an Acquity coupled to a Xevo triple quadrupole mass spectrometer and an Acquity PDA detector (Waters, Milford, MA, USA). The mass-spectrometer was working in ESI (+) under the multiple reaction monitoring (MRM).

2.6. Validation of antibiotics quantification method

The analytical method was validated in term of linearity and trueness.

Linearity. Linear correlation coefficient (R^2) was evaluated after the SAM. The results showed that the R^2 coefficient ranged from 93% to 99% in all cases which indicates a good linearity.

Trueness (recovery assays). A digested sludge sample was spiked in duplicate at a concentration of 50 µg/L using a standard solution containing the 4 target compounds. The results showed a recovery rate of 55, 25, 38 and 102% respectively for ROX, OTC, CTC and ENR. These recovery rates are approximately close to those obtained during the protocol validation (Ezzariai et al. (2018) (in preparation) where more detailed explanations about the recovered concentrations, impacts of extraction conditions and antibiotics stability were discussed.

2.7. Statistical analyses

The statistical significance of antibiotics concentrations difference between substrate and digested sample was assessed using the *t* test ($p<0.05$, significant difference (*); $p<0.01$, very significant difference (**)). The significance tests were performed using SigmaPlot 12.0.

3. Results and discussion

3.1. Anaerobic digestion performances

During all the experiment, the pH values remained between 6.90 and 7.35 and therefore in the range reported in the literature for standard process conditions (Gil et al., 2017).During the transitory phase, the TS, VS, TSS and VSS decreased and stabilized after 30 days (**Fig. 3**). A summary of feeding and digested sludge characteristics at steady state is provided in **Table 2**. At steady state, TS, VS, TSS and VSS were of 20±0.3, 14±0.3, 19±0.8, 12±0.9 g/L respectively. The recorded elimination rates were about 38, 36, 38 and 51 % for TS, VS, TSS and VSS respectively.

The CODt and CODs exhibited the same pattern (**Fig. 3**) and stabilized at 23±0.9 and 0.8±0.1 respectively. During the AD, the CODt and CODs elimination rates were 46 and 62% respectively.

The **Fig. 4** shows the biogas production rate and composition during the AD operation. After 20 days, the methane content reached 62% and remained stable until the end of the operating period. The methane production efficiency varied from 12 to 87 mL/g COD added (mean value of 32 ml/ g COD added).

Besides, the VFA concentrations were about 0.03-0.116 g/L and 0.03-0.079 g/L respectively for the acetic and the butyric acid during the process. Propionate, isobutyrate, valerate and isovalerate acids were not detected during all the AD process. The highest values of the VFA were measured in the transitory stage. Generally, low VFA concentrations were detected which demonstrate the absence of accumulation of intermediary metabolites in the digester.

Taken as a whole, TS removal, COD removal, biogas yield and methane content were in the range of process performances usually measured (Carballa et al., 2007; Gonzalez-Gil et al., 2018, 2016), i.e. in absence of any inhibitory effect. This observation suggests that the cocktail of antibiotics (ROX, OTC, CTC and ENR at 28±3.8, 12.4±2.7, 19.1±3.8 and 51.2±24 μg/L respectively) used in this study had no marked inhibitory effects on all studied

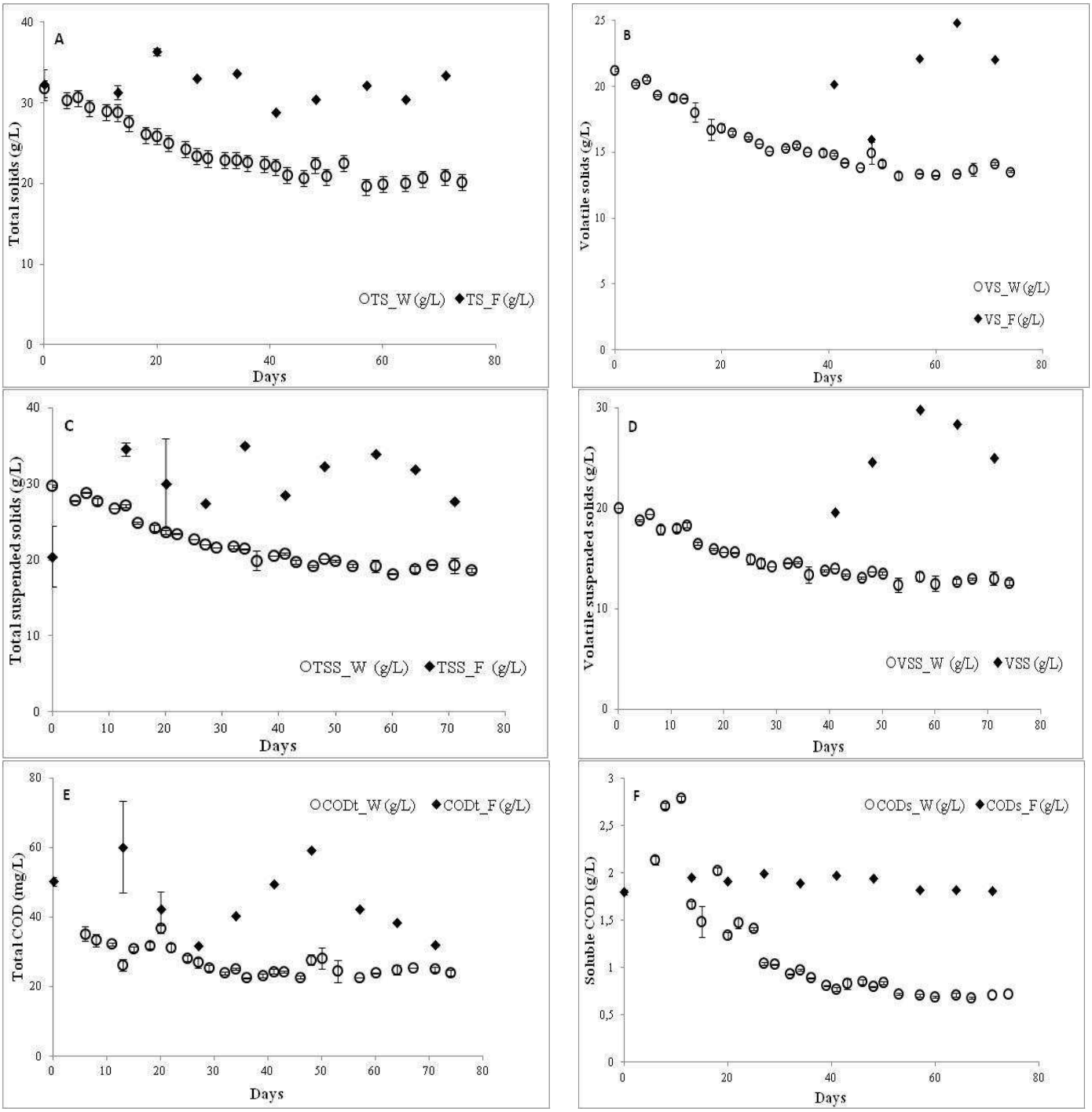

Figure 3: Dynamics of TS (panel A), VS (B), TSS (C), VSS (D), CODt (E) and CODs (F) during the anaerobic digestion of sludge spiked with antibiotics. (F), feeding sample; (W) withdrawing samples.

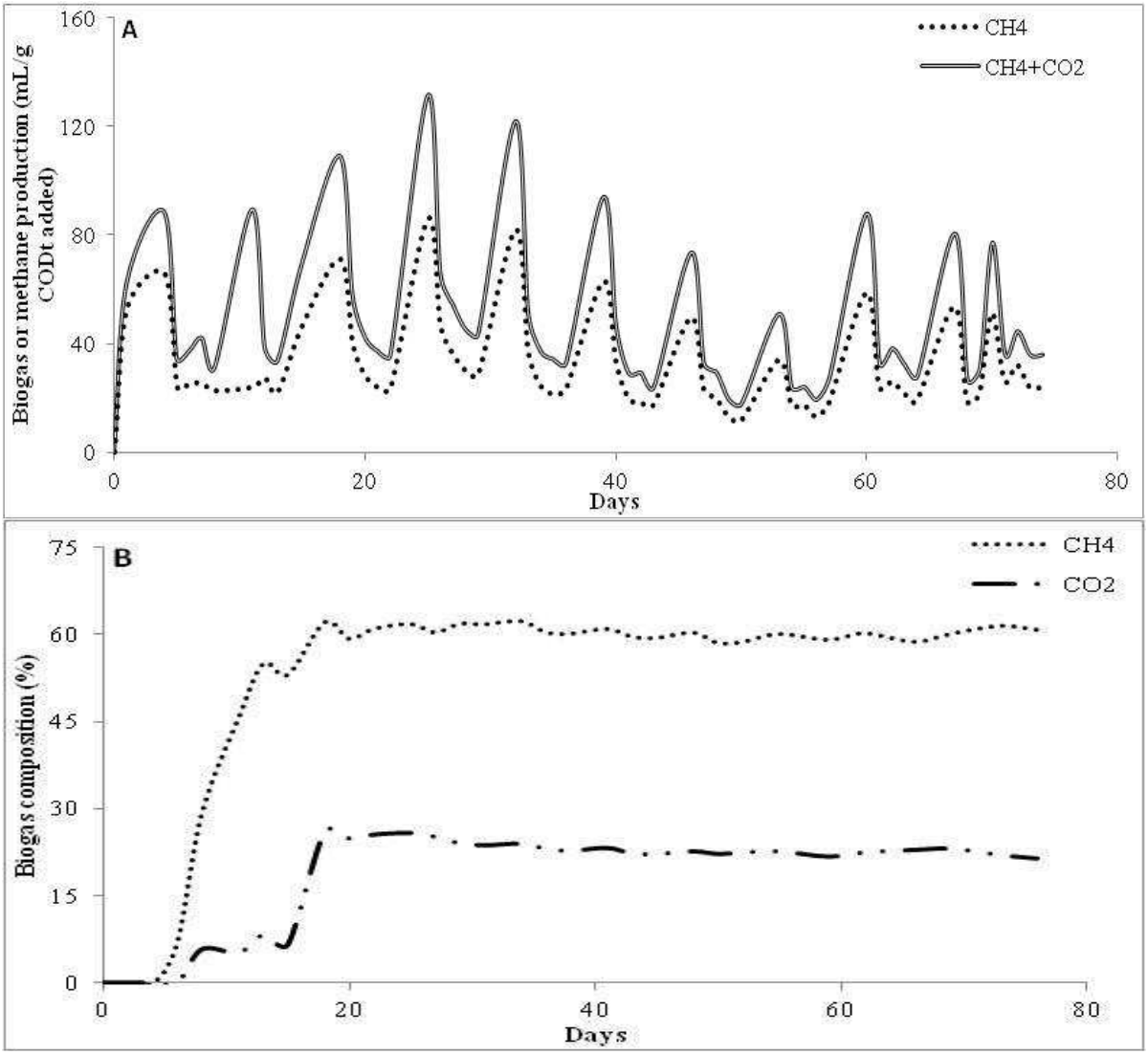

Figure 4: Dynamics of the volumetric biogas / methane production rates (A) and of the biogas composition (B) during the anaerobic digestion

parameters (especially biogas production or the VS removal). This concentration is realistic and corresponds to the antibiotics concentrations range reported in sludge (Narumiya et al., 2013, Martín et al., 2014; Verlicchi and Zambello, 2015). On the other hand, this absence of inhibitory effect is consistent with literature. Indeed, the lowest concentrations for which an inhibition was reported in previous studies are from 0.25 to 28 mg/L (Arikan et al., 2006; Stone et al., 2009; Álvarez et al., 2010; Chelliapan et al., 2011; Mitchell et al., 2013; Li et al., 2017). In addition, the spiked concentration is not effectively reached in digesters operated in semi-continuous or continuous mode (unless antibiotics are not removed at all) whereas it is reached at time 0 in batch mode. These intrinsic properties of operating modes might also contribute to explain gaps between different experimental setups.

3.2. The fate of antibiotics during anaerobic digestion

As described above, 3 sampling dates during the steady-state (after 1.6, 2.4 and 3.7 HRT) were chosen to quantify the antibiotics in sludge and calculate the mean concentrations at steady state. The **Fig. 5** shows these concentrations in the substrate and digested sludge.

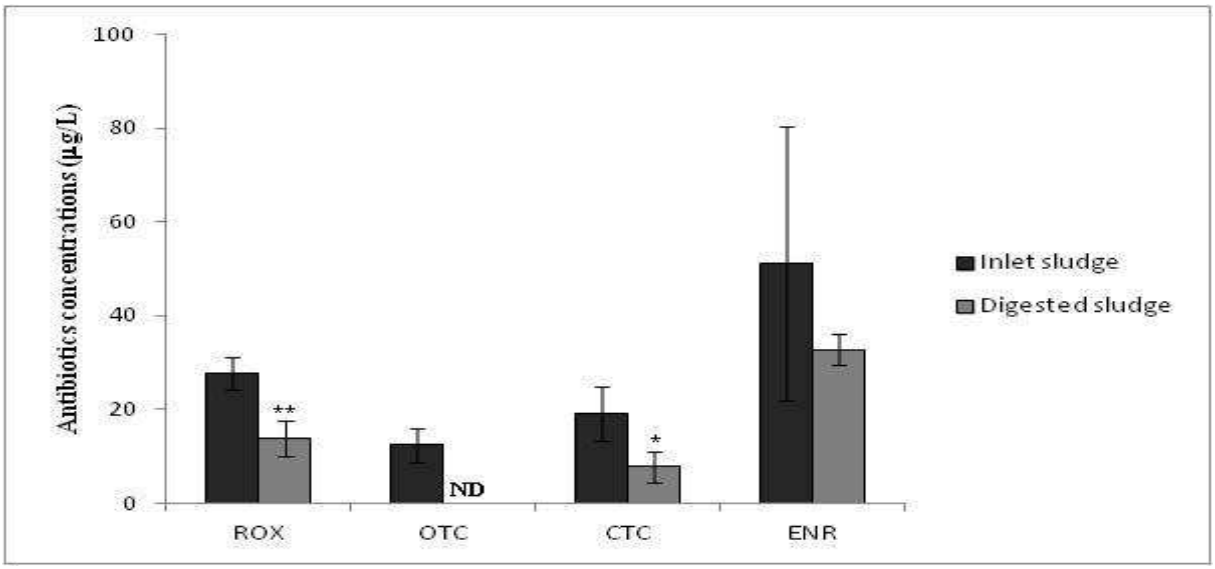

Figure 5: Concentrations of antibiotics in sludge before and after AD.* and **, probability of significance according to t-test for $p<0.05$ and $p<0.01$ respectively (substrate versus digested sludge). ND: not detected.

The concentrations of antibiotics in spiked sludge are in agreement with the concentrations occurring in sudge (Ezzariai et al., Submitted) and with the concentrations used in other reported studies focused on AD (Carballa et al., 2007; Gonzalez-Gil et al., 2016). Nevertheless, some recovered concentrations are lower than the spiked one. More detailed explanations about the impacts of extraction conditions and antibiotics stability during the analytical approach were reported previously by Ezzariai et al. (Submitted).

The concentrations in digested sludge were about 14±3.8, 7.8±3.3 and 32.8±3.4 µg/L for ROX, CTC and ENR respectively. OTC was not detected in the digested sludge. The difference between inlet and outlet concentrations was very significant for ROX and OTC (p value of 0.02 and <0.001 respectively) and significant for CTC (p=0.015). In the case of ENR, the difference was not statistically significant (p=0.177). The yields of ROX, OTC and CTC removal were about 50, 100 and 59% respectively.

The ROX removal after the AD of sludge, in a semi-continuous mode, was estimated between 60 and 94% (Carballa et al., 2007; Narumiya et al., 2013; Gonzalez-Gil et al., 2016). The removal rate measured in our study (**Fig. 5**) was thus in the lower range of the values

previously reported. In batch mode, an enhancement of the ROX removal rate was observed between the AD of sludge and wastewater (Alvarino et al., 2016a). The authors concluded that sludge represents an external carbon source leading to increased antibiotics removals. This "cometabolism" effect had also been reported previously for other xenobiotics (Barret et al., 2012, 2010). In our experiment, the cometabolic potential is might be lower than the other studies, regarding the low content of organic matter in the used substrate (45.5±0.4% of Ash content in the raw sluge).

The tetracyclines removal rates observed in the current study (100 and 59% respectively for OTC and CTC) are consistent with other published studies, despite they were carried out in batch mode. For example, CTC removal of 75 and 64% was achieved after 33 and 216 days of incubation respectively (Arikan, 2008; Stone et al., 2009). During a short incubation (21 days), a removal of 53-91% was observed for OTC and CTC, depending on the spiked concentration of antibiotics (Álvarez et al., 2010). However, no influence of HRT on antibiotics removal was observed in continous mode (Carballa et al., 2007; Gonzalez-Gil et al., 2016).

On the other hand, a removal of 36% for the molecule belonging to the fluoroquinolone family (ENR) was observed in this study. In several works, the fate of fluoroquinolones was investigated during sludge AD in batch mode. The reported results showed that fluoroquinolones were not efficiently removed by the AD process (Golet et al., 2003; Martín et al., 2014) and removal rates of 30-50% for norfloxacin and ofloxacin were previously observed (Narumiya et al., 2013). Fluoroquinolones behavior could explain the lowest removal rate that was observed after the AD process when compared to other antibiotics. Fluoroquinolones tend to adsorb to particulate organic matter (Otker and Balcioğlu, 2005; Belden et al., 2007) mainly via electrostatic interactions with negatively charged particles (Golet et al., 2003). In addition, non-linear sorption of FQs onto sludge particles was demonstrated (Polesel et al., 2015) which may decrease their bio-availability. On the other hand, the functional groups of FQs can decrease their affinity with the microorganisms during the AD (Zhang et al., 2015). Hence, fluoroquinolones sorption and their interaction with microorganisms could be the main contributors which lead to the lowest observed removal rate.

Little is known about organisms and processes involved during the removal of antibiotics (Ghattas et al., 2017). A decrease of the parent compounds is not necessarily related to a

complete mineralization (Carballa et al., 2004; Göbel et al., 2007; Xu et al., 2007). ^{14}C labeling is a powerful tool to investigate their fate. For example, Alvarino et al. (2016a) used this approach and demonstrated that complete mineralization to CO_2 accounted for less than 5% of sulfamethoxazole removal, while biotransformation was the main process resulting in the presence of metabolites compounds. In addition, minor structural modifications of the transformation products of tetracyclines, such as O-demethylation and hydrogenation, did not considerably reduce their antimicrobial activity (Spielmeyer et al., 2017). Moreover, in some cases, it was reported that digested sludge still presented genotoxic affects (Mitchell et al., 2013; Gonzalez-Gil et al., 2016). For a lot of antibiotics classes, biotransformation was the main removal mechanism compared to mineralization and sorption (Alvarino et al., 2016a, 2016b, Gonzalez-gil et al., 2018, 2016; Spielmeyer et al., 2017).

2. Conclusion

In the realistic conditions of this study, the antibiotics ROX, CTC, OTC and ENR had no inhibitory effects on the AD of sludge. This result suggests that antibiotics might not inhibit AD process in full-scale digesters fed with sludge containing typical antibiotics concentrations. Among the four molecules, ROX, CTC and OTC exhibited the highest removal. In contrast, the molecule belonging to the FQs family was not removed significantly. The AD is more effective for macrolides and tetracyclines removal. Even if we demonstrated the potential of AD for partially removing these antibiotics, ROX, CTC and ENR persisted in digested sludge, as parent compounds. In addition, biotransformation mechanisms could be involved during parent compounds removal, thus questioning the suitability of digested sludge for application and its implication in terms of toxicity, antibiotics and antibiotics resistance dissemination in the environment.

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Discussion générale, Conclusions et Perspectives

1. Effets des antibiotiques sur les procédés de compostage et de méthanisation

Le compostage et la méthanisation sont parmi les procédés les plus utilisés pour le traitement des boues d'épuration en vue de leurs valorisations. Ces procédés sont aussi très étudiés en raison de leurs capacités à réduire les teneurs en contaminants dont les antibiotiques (Arikan et al., 2007 ; Carballa et al., 2007 ; Bao et al., 2009 ; Gonzalez-Gil et al., 2016). Toutefois, la présence des antibiotiques à des concentrations élevées peut engendrer des effets notables sur les performances épuratoires de chaque procédé.

Dans ce travail, une boue primaire de Marrakech a été dopée avec 3 familles d'antibiotiques (macrolides (ROX), tétracyclines (CTC et OTC), fluoroquinolones (ENR et CIP)) à 3 doses différentes (faible, moyenne, élevée) pour conduire des essais de compostage durant 180 jours. Un essai témoin a été lancé dans les mêmes conditions (Chapitre 2, Tableau 3). Les familles d'antibiotiques ont été retenues en raison de leur utilisation importante et de leur présence dans les boues (Martin et al., 2014 ; Verlicchi et al., 2015). Les résultats des essais de compostage ont montrés que l'augmentation des concentrations en antibiotiques retarde la dégradation de la matière organique et affecte le ratio C/N. De même, la phase thermophile est retardée et réduite dans le temps. Les doses moyennes et élevées écrasent le plateau des températures et réduisent la chaleur libérée durant toute la phase de stabilisation comme déjà observé par Eguchi et al. (2012). La présence des antibiotiques à des concentrations élevées engendre des effets négatifs sur la croissance et la diversité bactérienne, ainsi que sur les activités enzymatiques associés à ces germes (Ding and He, 2010; Kotzerke et al, 2008; Sukul and Spiteller, 2006; Eguchi et al, 2012). Les bactéries étant principalement responsables de la montée en température durant la première partie de la phase de stabilisation, toute perturbation de la croissance ou de l'activité bactérienne engendrera des perturbations thermiques. De même, l'inhibition de la minéralisation de l'azote et la faible décomposition de carbone organique durant cette phase de stabilisation (Wong et al, 2010), conduisent à des modifications du profil C/N. Les modifications de ce ratio étant les plus élevées aux plus fortes concentrations d'antibiotiques. L'effet des antibiotiques se traduit aussi par la disparition du rebond en température observé dans l'essai témoin. Ce rebond est une caractéristique du palmier dattier (Alkoaik et al., 2011) lié au développement des champignons et des actynomycètes responsables de la dégradation des résidus après la phase thermophile, au début de la phase de maturation. Ce rebond n'est plus observé dans les essais de compostage en présence d'antibiotiques. Les rachis de palmier dattier sont parmi les substrats utilisés durant les essais de compostage. Un suivi de l'évolution du poids des rachis

par rapport au taux de cendres a été réalisé pour chaque essai de compostage. Il s'est avéré que ce ratio était impacté en fonction de la dose d'antibiotique. Durant la phase de stabilisation, l'érosion mécanique des rachis se fait presque de la même façon, qu'elle que soient les concentrations en antibiotiques. Nous avions émis l'hypothèse que certaines molécules pouvaient s'adsorber sur les rachis dès le début de la phase de stabilisation. Cette adsorption pouvant limiter leur accessibilité. Au cours de la maturation, l'érosion biologique du substrat lignocellulosique par les champignons et les actinomycètes est fortement inhibée pour les fortes doses d'antibiotiques. Ce qui se traduit par une conservation de la taille des rachis. Dans nos conditions expérimentales, le suivi de l'évolution des rachis de palmier peut servir d'outil simple de contrôle de maturation des composts.

Un essai de méthanisation mésophile en mode semi-continu a été conduit pendant 80 jours. La concentration de dopage testée (2.5 µg/g DM pour la somme des différentes molécules) est plus faible que celles utilisées pour le compostage, mais reste réaliste (Narumiya et al, 2013; Martin et al, 2014; Verlicchi and Zambello, 2015). Les résultats observés en termes de production du biogaz, d'abattement de matière sèche ou volatile, ainsi que d'abattement de la demande chimique d'oxygène ne sont pas affectés et correspondent généralement aux conditions standards de déroulement de la méthanisation (Carballa et al, 2007 ; Gonzalez-Gil et al., 2018, 2016). Autrement dit, la présence des antibiotiques aux concentrations utilisées n'a pas d'effet sur la digestion anaérobie d'une façon générale ainsi que sur la phase de stabilisation.

Les conditions de déroulement de la méthanisation restent standards et non pas critiques comme celles du compostage. A la lumière des résultats bibliographiques, il a été montré que les antibiotiques à partir de 0.25 mg/L peuvent affecter la production du biogaz (20% de réduction) et l'abattement de la matière organique, au cours de la méthanisation (Shi et al, 2011) ce qui limite fortement les performances de méthanisation. Les concentrations utilisées lors de nos essais de compostage dépassent celles utilisées en méthanisation de 1.4 à 4 fois (selon la molécule d'antibiotiques) pour la dose faible où les effets observés sur le processus ne sont pas significatifs. En revanche, les effets des antibiotiques deviennent significatifs à partir des doses moyennes et élevées où les concentrations d'antibiotiques dépassent celles utilisées pour la méthanisation de 4 à 20 et 20 à 100 fois respectivements. Des expérimentations devraient être conduites en méthanisation avec des doses plus élevées d'antibiotiques de manière à chercher les conditions limites de notre mélange et étudier leurs effets sur l'ensemble des paramètres.

2. Devenir des antibiotiques au cours du compostage et de la méthanisation

Pour mieux comprendre le comportement de chaque bioprocédé vis-à-vis de la présence de plusieurs familles d'antibiotiques à différentes concentrations, ainsi que le devenir de ces molécules durant les différentes phases de ces procédés, une mise au point analytique a dû être développée. La méthodologie retenue repose sur une extraction accélérée par solvant (ASE), suivie de la mise en place d'ajouts dosés. Les antibiotiques sont ensuite séparés et quantifiés par chromatographie liquide couplée à de la spectrométrie de masse en tandem (LC-MS/MS).

La *figure 1* présente les taux d'élimination des antibiotiques après compostage ou méthanisation. Quel que soit le procédé utilisé, les résultats montrent que l'OTC est éliminé à 100%, et souligne l'efficacité de ces deux bioprocédés vis-à-vis de l'élimination de cette molécule. Ces résultats sont en accord avec ceux de la littérature qui témoignent de la biodégradabilité de l'OTC (Arikan et al., 2007 ; Arikan et al., 2008 ; Bao et al., 2009 ; Stone et al., 2009 ; Hu et al., 2011 ; Wu et al., 2011). Concernant La ROX et la CTC, le compostage semble plus efficace que la méthanisation. Ces résultats sont en accord avec un certain nombre de travaux de la littérature (Arikan et al., 2007 ; Selvam et al., 2012 ; Zhang et al., 2014). En effet, les résultats de compostage montrent que les teneurs en ROX et CTC ont été réduites significativement de 52 à 87% et 69 à 95% respectivement, même pour les plus fortes concentrations (conditions L3 et L4). Pour la méthanisation, la ROX et la CTC ne sont éliminés que de 50 et 59% respectivement. Il est important de souligner que concernant la ROX, des abattements de 60 à 94% après méthanisation en mode semi-continu ont été décrit (Carballa et al., 2007 ; Narumiya et al., 2013 ; Gonzalez-Gil et al., 2016). Ces résultats suggèrent que les conditions expérimentales peuvent influencer l'élimination des antibiotiques (Arikan et al., 2007).

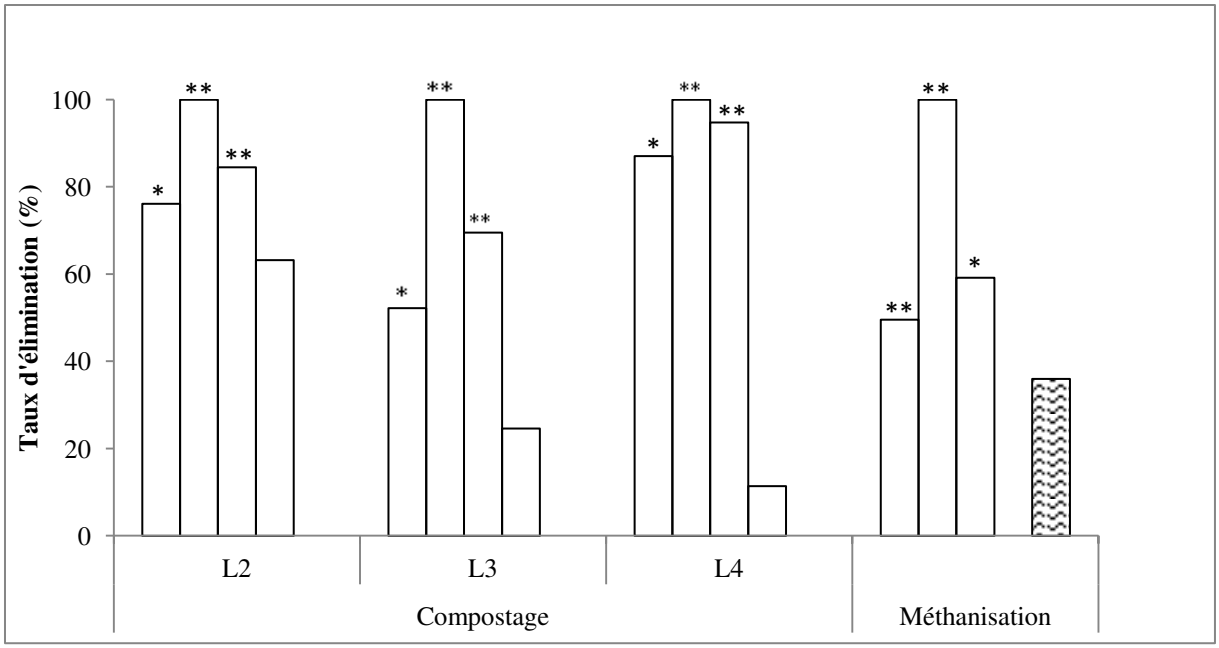


Figure 1: Les taux d'élimination des antibiotiques après compostage et méthanisation (2.5 µg/g MS).L2, dose faible; L3, dose moyenne; L4, dose élevée. .* et * probabilité de significativité selon le t-test pour $p<0.05$ et $p<0.01$ respectivement. ROX ; OTC ; CTC ; CIP ; ENR

Enfin, nos travaux nous ont permis d'observer que les fluoroquinolones qui sont des molécules persistantes dans les boues ne sont pas éliminés, quels que soient les procédés et les conditions utilisées. De nombreux auteurs suggèrent la difficulté d'élimination de ces molécules (Lillenberg et al., 2010 ; Selvam et al., 2012 ; Haiba et al., 2013 ; Narumiya et al., 2013 ; Martin et al., 2014), et l'attribuent à leurs fortes sorption par des interaction électrostatiques sur les particules de matière organique qui sont chargées négativement (Golet et al., 2003 ; Belden et al., 2007).

Dans ce travail de thèse, le suivi des antibiotiques au cours du compostage et de la méthanisation a permis de mettre en évidence la contribution des phases de chaque bioprocédé vis-à-vis de l'abattement des antibiotiques. La contribution des deux grandes phases de compostage (stabilisation et maturation) varie selon la famille étudiée. La phase thermophile semble fortement impliquée dans l'élimination de la ROX. Le rôle important de cette phase et déjà été décrit par Arikan et al., (2008) qui montre qu'un certain nombre de molécules (chlorotétracycline) sont éliminées durant cette phase. Par ailleurs, la phase de maturation semble plus impliquée dans l'élimination de la CTC et de l'OTC comme déjà observé par Selvam et al., (2013). Ce qui permet de souligner le rôle potentiel des espèces fongiques et des *actinobacteria* dans l'élimination de ces deux composés. Le suivi des antibiotiques après la phase de stabilisation du procédé de méthanisation montre l'efficacité

de cette phase vis-à-vis de l'élimination de ROX, CTC et OTC. Carballa et al. (2007) ont montré que c'est durant la phase de stabilisation que débute l'abattement de produits pharmaceutiques dont les antibiotiques.

Les rachis de palmier dattier sont parmi les substrats utilisés durant les essais de compostage. Les antibiotiques peuvent s'adsorber sur les substrats de compostage (Kim et al., 2012). Cependant, nous avions émis précédemment l'hypothèse que certaines molécules pouvaient s'adsorber sur les rachis dès le début de la phase de stabilisation. Après le dosage des antibiotiques, la CIP a montré une fluctuation de concentrations au cours du compostage. Etant donné qu'elle n'est pas eliminée, l'adsorption/désorption de la CIP sur les rachis pourrait expliquer le comportement de cette molécule. Les fluoroquinolones sont des antibiotiques qui inhibent la réplication de l'ADN en agissant sur les gyrases bactériennes (Hooper and Jacoby, 2016). En s'adsorbant sur les rachis, ces molécules peuvent limiter leur dégradation et ainsi permettre la conservation de leur taille, même au cours de la phase de maturation (Ezzariai et al., 2017). La présence de ces molécules aux forts pouvoirs d'adsorption expliquerait donc en partie la conservation des rachis. L'élimination de cette famille d'antibiotique, si elle est possible par voie biologique, demande encore beaucoup d'études d'optimisation.

3. Optimisation du compostage et de méthanisation pour mieux réduire les teneurs d'antibiotiques

Comme nous venons de le voir, le compostage et la méthanisation permettent de réduire les teneurs d'antibiotiques présents avec différents taux d'élimination. La complémentarité entre ces deux procédés est une alternative pour optimiser l'élimination des micropolluants (Semblante et al., 2014 ; Alvarino et al., 2016) et elle pourrait être une option afin d'une part, valoriser les boues pour de la production d'énergie et d'autre part, grâce au compostage, stabiliser et hygiéniser ces même boues et en faire des amendements de meilleure qualité. Cette combinaison de procédés permettrait aussi d'augmenter les abattements d'antibiotiques notamment la famille des fluoroquinolones.

Dans ce sens, un couplage anaérobie-aérobie a été conduit en utilisant le digestat issu de la méthanisation pour le composter à l'aide d'un mini composteur (*figure 2*) avec les mêmes proportions de déchets verts que pour le compostage de boue (résultats non présenté dans la thèse). Les échantillons générés ont été stockés pour suivre le devenir des antibiotiques.

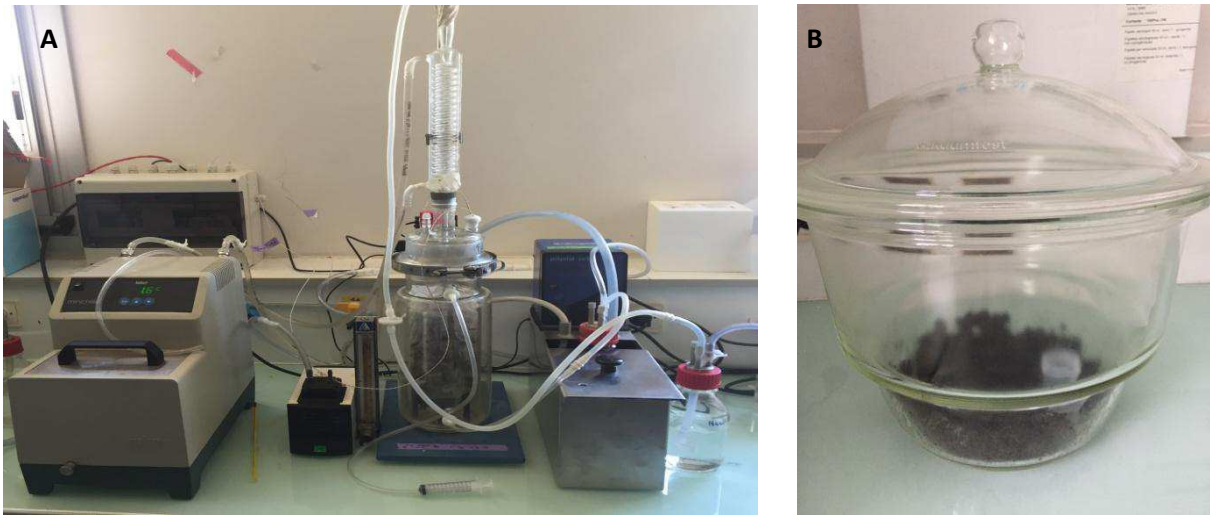


Figure 2: Stabilisation du digestat dans un mini-composteur (A) suivi de la maturation en dessiccateur (B)

Afin d'optimiser les conditions expérimentales au cours des procédés de traitement de boues en présence d'antibiotiques, un protocole de fractionnement de la matière organique (Jimenez et al., 2015) a été appliqué aux échantillons de compost et digestat. Le fractionnement de la matière organique particulaire consiste à diviser la matière organique en plusieurs compartiments de moins en moins accessibles par l'utilisation de solutions de plus en plus agressives (*Tableau 1)*. L'accessibilité chimique est réalisée par des mesures de la DCO et la complexité de la matière organique par des mesures de fluorescence 3D.

Cette approche sert à l'évaluation de chaque compartiment. Le suivi de la localisation des antibiotiques dans les compartiments matière est un point fort du protocole qui permet à la fois d'avoir une idée sur l'affinité des antibiotiques pour un compartiment donné et par la suite optimiser les conditions expérimentales pour rendre la molécule plus accessible en vue d'une meilleure élimination.

Tableau 1: Les compartiments et les conditions de fractionnement de la matière organique

	Compartiment	Nombre d'extraction	Solution d'extraction	Temps d'extraction
SPOM	Matière organique particulaire soluble	2	CaCl2 10 mM	1 h
REOM	Matière organique facilement extractible	4	NaCl 10mM NaOH 10 mM	15 min
SEOM	Matière organique lenetemnt extractible	4	NaOH 0.1 M Sous atmosphère N2	1 h
PEOM	Matière organiquedifficilement extractible	2	H2SO2 72%	3 h
NEOM	Matitère organqiue non extractible	/	Solide restant	/

Les résultats préliminaires sont encourageants et permettent de suivre l'accessibilité à la matière organique en fonction des doses d'antibiotiques (*figure 3.a*), ainsi que l'affinité de chaque molécule d'antibiotique aux fractions de la matière organique (*figure 3.b*). Le suivi de l'accessibilité chimique montre que la phase de maturation est retardée à la dose moyenne. De plus, la localisation des antibiotiques dans les compartiments matière montre l'affinité de l'OTC, ENR et CIP pour les pH basiques, la ROX, l'OTC et CTC pour les pH neutres et l'OTC pour les pH acides.

Le bilan matière pour les antibiotiques dans ces compartiments, montre que la somme des concentrations est inférieure à la dose de contamination. Les conditions d'extraction utilisées lors du fractionnement peuvent donner lieu à une modification des molécules et plusieurs formes, qui ne sont pas prises en compte, peuvent apparaître selon le pH de la solution d'extraction (*figure 4*), rendant leur analyse et quantification extrêmement difficile. De ces résultats nous pouvons dire que la mise en point d'un protocole analytique spécifique à chaque fraction est nécessaire.

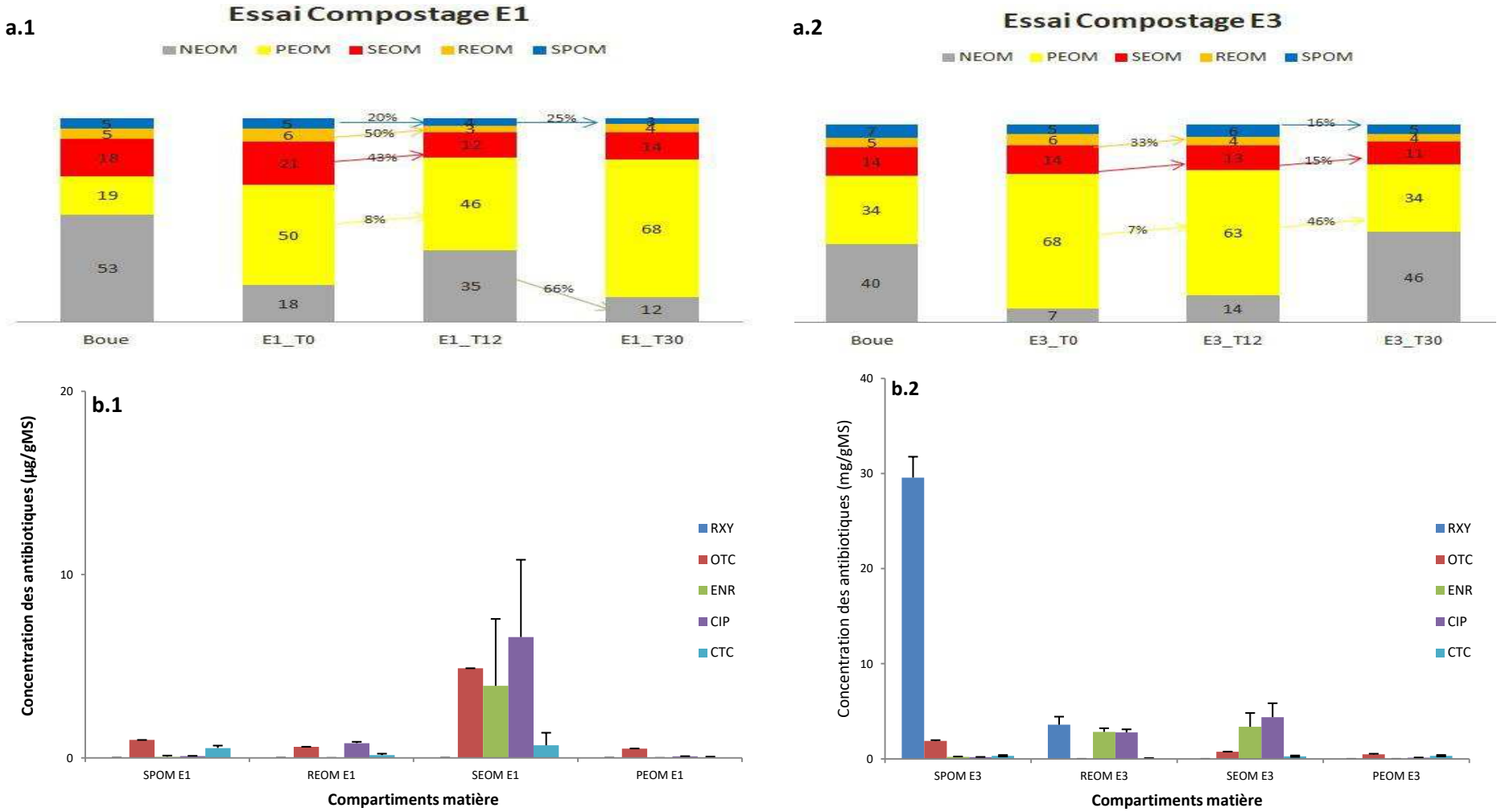
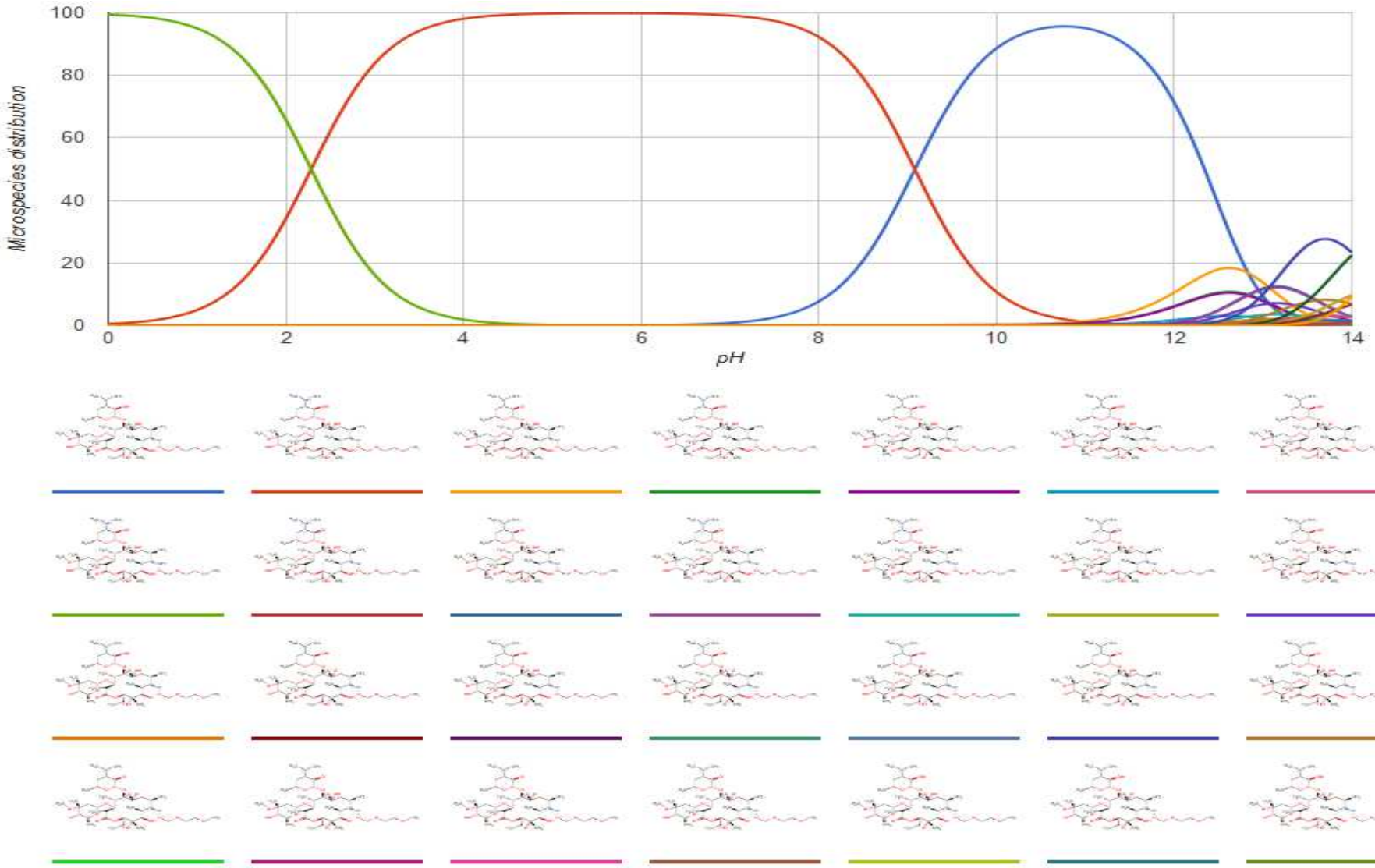


Figure 3: Suivi de l'accessibilité chimique au cours du compostage (a) et la localisation des antibiotiques dans les compartiments matière (b). E1, essai témoin; E3, essai avec dose moyenne. SPOM, Soluble particulate organic matter (OM) ; REOM, Readily extractable OM; SEOM, Slowly extractable OM ; PEOM, Pourly extractable OM ; NEOM, Non extractable OM.

A



B

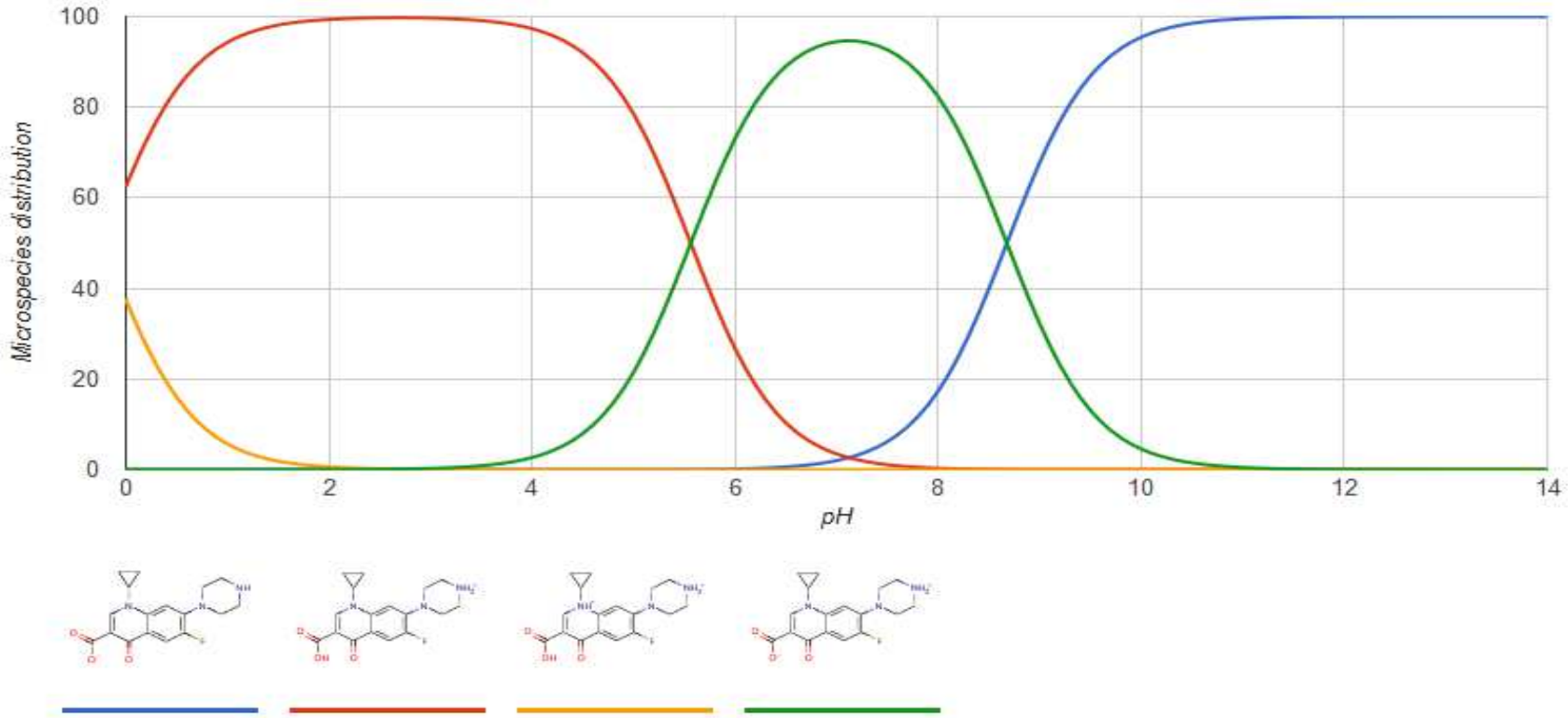


Figure 4 : Les formes de la Roxithromycine (A) et la Ciprofloxacine (B) en fonction du pH

L'optimisation des bioprocédés est aussi conditionnée par la fiabilité instrumentale et l'efficacité de l'approche analytique utilisée. Pendant le développement de la méthode analytique et ainsi son application, nous avons pu observer dans certaines conditions des taux de recouvrement qui ne dépassaient pas 50%. Des explications ont été données dans le chapitre 3 en faisant le lien entre les conditions d'extraction, le comportement de chaque molécule et les taux de recouvrement observés. Pour encore mieux comprendre le comportement de ces molécules et ainsi améliorer leur analyse, des extraits de compost ont été analysés par Orbitrap (voir conditions analytiques en annexes). Les résultats ont montré que les tétracyclines sont caractérisées par des chromatogrammes d'ions avec 3 pics de surface pour la CTC (*figure 5.A*) et 2 pics pour l'OTC (*figure 5.B*). Il s'agit d'une caractéristique de ces deux molécules qui dépendent de la matrice étudiée et des conditions d'extraction (température, pression, pH). Cependant, le choix d'une transition d'acquisition en LC/MS-MS qui tienne compte de tous les pics de surface est important pour ne pas avoir des pertes de signal. Par ailleurs, la masse moléculaire élevée de la ROX peut entrainer plusieurs sites d'ionisation quelque soit le mode d'ionisation choisi. Avec l'Orbitrap, nous avons observé que la molécule pouvait se protoner deux fois (*figure 5.C*). Les approches analytiques basées sur un étalonnage externe réalisé dans le même solvant doivent vérifier cette caractéristique pour avoir un dosage exact. Par ailleurs, le choix de l'ion parent en LC/MS-MS doit prendre en compte cette caractéristique pour obtenir la meilleure sensibilité.

Grace à l'approche des ajouts dosés que nous avons utilisés, le problème d'exactitude évoqué plus haut est corrigé, puisque les antibiotiques étalons sont ajoutés dans la matrice avant l'injection et vont subir le même état d'ionisation. Cependant, les pertes de rendements obtenus pour cette molécule sont liés à une dégradation thermique (Gobel et al., 2005 ; Connor and Aga, 2007 ; Mullen et al., 2017). Pour les fluoroquinolones, plusieurs travaux de la littérature ont signalé les difficultés analytiques liées à cette famille d'antibiotique et plus particulièrement à la CIP (De Witte et al., 2007 ; Polesel et al., 2015). En revanche, plusieurs explications ont été données pour expliquer les résultats trouvés (chapitre 3). Malgré cela, nous avons envisagé d'étudier la précision et la sélectivité analytique pour quantifier les teneurs des fluoroquinolones à base des ajouts dosés.

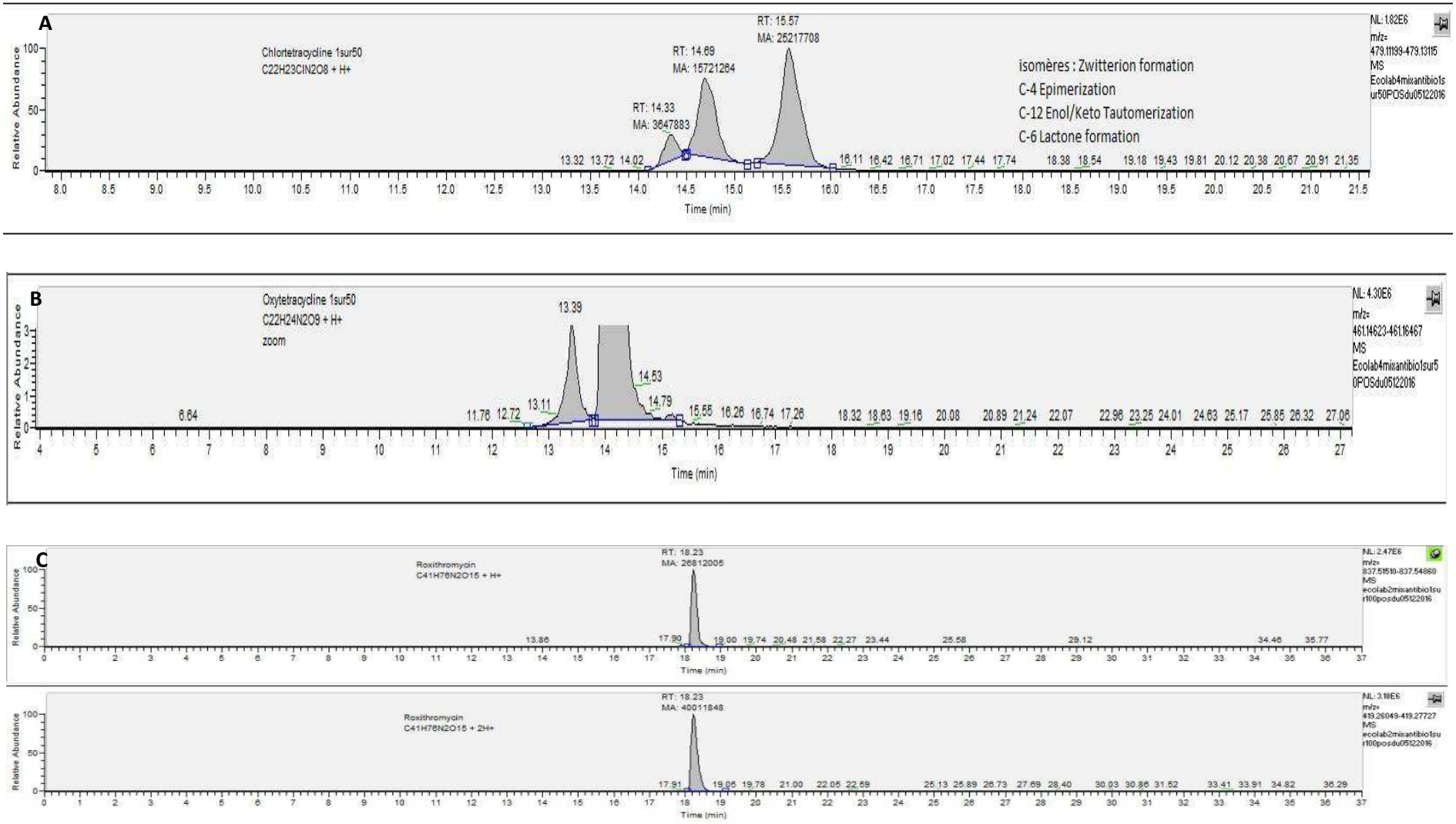


Figure 5: Chromatogrammes obtenus par Orbitrap (LC-HRMS) pour la chlortétracycline (A), l'oxytétracyclines (B) et la roxithromycine (C) dans un extrait de compost

4. Quelques éléments critiques limitant la valorisation du compost et du digestat

Les résultats de cette thèse montrent qu'après compostage ou méthanisation des boues, les molécules parents d'antibiotiques ont été abattues avec des taux d'élimination variables pour l'ensemble des antibiotiques analysés. Les fluoroquinolones sont les molécules les plus récalcitrantes par rapport aux autres familles. Ainsi, des taux d'élimination importants ont été observés pour les tetracyclines et les macrolides. Malgré cela, leur élimination ne reflète pas une minéralisation totale. La biotransformation et la sorption sont d'autres voies qui peuvent expliquer la diminution des teneurs d'antibiotiques. Des études ont montrés que la biotransformation est la voie principale qui peut expliquer la disparition des molécules parents d'antibiotiques au cours du compostage et de la méthanisation (Arikan et al., 2007 ; Alvarino et al., 2016 ; Gonzalez-Gil et al., 2018). Cependant, des métabolites (4-epi-chlorotétracycline, iso-chlorotétracycline) encore plus toxiques que les molécules parents (chlorotétracycline) ont été quantifiés dans des composts et des digestats (Arikan et al., 2007 ; Mitchell et al., 2013 ; Spielmeyer et al., 2017). Il serait donc très interressant dans le cadre d'un approfondissement de cette étude, d'analyser les métabolites résiduels en fin de procédé pour l'ensemble des molécules testés dans nos conditions expérimentales.

La dissémination des antibiotiques, surtout des formes les plus récalcitrantes à la dégradation, et leurs métabolites ont certainement des impacts sur les communautés microbiennes en terme de biodiversité et d'antibiorésistance (Chen et al., 2007), ce dernier point interpelle sur le risque pour l'homme, face à la recrudescence des bactéries pathogènes multi-résistantes observés par l'OMS au niveau mondial (WHO, 2016). Plusieurs études ont montré que les boues contiennent des teneurs variables de gènes de résistance mais aussi des bactéries résistantes aux antibiotiques (Guillaume et al., 2000 ; Munir et al., 2010). Cependant, l'application directe des boues est non seulement une source de dissémination des antibiotiques et de leurs métabolites, mais aussi une voie d'émergence aussi bien des gènes de résistance que des bactéries résistantes. Aujourd'hui, le développement de la résistance bactérienne aux antibiotiques représente une grave menace pour la santé mondiale et la sécurité des aliments. Il a été estimé que la résistance bactérienne est responsable de 25 000 à 23 000 morts par an en Europe et aux Etats unies respectivement (Berglund, 2015). Ce problème de résistance est aussi compliqué pour les chercheurs en médecine puisqu'on ne trouve plus actuellement de traitements appropriés à certains pathogènes multirésistants comme *Staphyloccocus aureus*, *Pseudomonas aeruginosa,* ou encore *Mycobacterium tuberculosis* (Borjesson et al., 2009 ; Kaszab et al., 2010). Ainsi, la résistance aux

antibiotiques entraine une prolongation des hospitalisations et une augmentation des dépenses médicales. Les infections par les bactéries résistantes aux antibiotiques engendrent des dépenses estimées à 1.5 billion de dollars par an pour l'Union Européenne (Wright, 2011).

5. Conclusion

En conclusion, ce travail de thèse a permis de mettre en évidence :

- Le compostage à des capacités épuratoires importantes, par rapport à la méthanisation, même à des concentrations élevées d'antibiotiques, qui en font un procédé robuste ;
- Les conditions analytiques utilisées nécessitent encore de l'optimisation ;
- L'utilisation du compost/digestat de boues urbaines doit être réglementée vis-à-vis la présence des antibiotiques et plus particulièrement les fluoroquinolones.

Les résultats de cette thèse ont contribués au dépôt et l'acceptation d'un projet de recherche en continuité avec ce travail, le projet ''MAD Sludge'' pour lequel j'ai obtenu un stage post doctoral. Il s'agit d'un projet de l'Agence Française Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES), financé par l'Agence Française de l'environnement et de la maîtrise de l'énergie (ADEME), et impliquant toutes les équipes ayant contribué à la réalisation de ce travail de thèse. Le projet MAD Sludge consiste en l'optimisation de la filière de traitement des boues pour limiter la dissémination d'antibiotiques/antibiorésistances. Ce projet vise à évaluer la dynamique de l'antibiorésistance (gènes, intégrons, bactéries) le long des filières de traitement menant au retour au sol des boues d'épuration.

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Annexes

Annexe 1 :

Conditions analytiques par Orbitrap

Les séparations chromatographiques ont été réalisées sur une chaîne HPLC U3000 (Thermo Fisher), constituée d'une pompe DGP-3600SD, d'un injecteur automatique thermostaté WPS-3000TSL, d'une enceinte thermostatée TCC-3000SD et d'un détecteur UV-Visible RSLC VWD-3400RS.

Les séparations ont été effectuées à 40°C sur une colonne Luna PFP 2 (150 x 2,00mm, 3µm) obtenue auprès de la société Phenomenex.

La phase mobile, délivrée à 200µl/min, consiste en un mélange d'eau (0,1% V/V d'acide formique) (éluant A) et d'acétonitrile (éluant B) selon le gradient suivant :

0-5 min, 3% de B ; 5-25 min, 3-95% de B ; 25-30 min, 95% de B ; 30-31 min, 95-3% de B ; 31-37 min, 3% de B.

La détection des composés est réalisée à une longueur d'onde de 254 nm.

Les analyses MS ont été réalisées en mode positif et négatif sur un spectromètre de masse de type Exactive provenant de la société Thermo Fisher. Ce spectromètre comprend une source électrospray (ESI), une cellule de collision HCD et un analyseur Orbitrap à transformée de Fourier.

Les paramètres principaux les plus courants étaient :

- Tension du cône : 3,00 KV (mode positif) ; - 3,00 KV (mode négatif)
- Température du capillaire d'entrée : 350°C
- Débit du gaz principal de désolvatation : 50 (unités arbitraires)
- Débit du gaz auxiliaire de désolvatation : 20 (unités arbitraires)
- Gamme de masses : 50 à 1000m/z
- **Résolution : 100 000** (à la masse m/z 200)
- Fréquence d'acquisition : 1 Hz
- Temps de remplissage maximum de la trappe : 100 ms
- Nombre maximum d'ions dans la trappe : 3.10^6 ions

Les spectres de masses ont été acquis et traités (prédiction des formules brutes) à l'aide du logiciel Xcalibur (version 2.0, Thermo Fisher) et du logiciel MetAlign (version 041011, Arjen Lommen) pour l'élimination du bruit et l'extraction des ions.

Article en co-auteur

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RESEARCH ARTICLE

Effect of dewatering and composting on helminth eggs removal from lagooning sludge under semi-arid climate

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Abstract
In this work, we assessed the drying and composting effectiveness of helminth eggs removal from sewage sludge of a lagoon wastewater treatment plant located in Chichaoua city. The composting was run after mixing sludge with green waste in different proportions: M1 (½ sludge + ½ green waste), M2 ($^2/_3$ sludge + $^1/_3$ green waste), and M3 ($^1/_3$ sludge + $^2/_3$ green waste) for 105 days. The analysis of the dewatered sewage sludge showed a load of 8–24 helminth eggs/g of fresh matter identified as *Ascaris* spp. eggs (5–19 eggs/g) followed by *Toxocara* spp. (0.2 to 2.4 eggs/g); *Hookworm* spp. and *Capillaria* spp. (0.4–1 egg/g); *Trichuris* spp., *Taenia* spp., and *Shistosoma* spp. (< 1 egg/g) in the untreated sludge. After 105 days of treatment by composting, we noted a total reduction of helminth eggs in the order of 97.5, 97.83, and 98.37% for mixtures M1, M2, and M3, respectively. The *Ascaris* spp. eggs were reduced by 98% for M1 and M3 treatments and by 97% for M_2 Treatment. *Toxocara* spp., *Hookworm* spp., *Trichuris* spp., *Capillaria* spp., and *Shistosoma* spp. eggs were totally eliminated (100% decrease) and the *Taenia* spp. was absent from the first stage of composting. These results confirm the effectiveness of both dehydrating and composting processes on the removal of helminth eggs.

Keywords Lagooning sludge · Composting · Dewatering process · Helminth eggs

Introduction

Wastewater treatment produces significant quantities of sewage sludge. According to the national report of the Sludge Step Management Strategy in Morocco in 2009, the sludge quantity is estimated at 98,000 t/year in 2015, and the forecasts are 300,000 t/year in 2025. In general, the sludge that comes from wastewater treatment systems is known for its potential fertilizing characteristics (Hartenstein 1981; El Fels et al. 2014a; Rocha et al. 2016). However, sludge presents evident contents of a large quantity of enteric viruses, pathogenic bacteria, and helminth eggs of human or animal origin in several taxa. We distinguish Platyhelminthes (flatworms) as *Schistosoma* spp. and Nemathelminthes (roundworms) as *Ascaris* spp., *Trichuris* spp., *Capillaria* spp., *Hookworm* spp., and *Toxocara* spp.

Toxocara canis, commonly known as dog roundworm, can affect humans and cause human toxocariasis (Keffala et al. 2012). Hookworm eggs causing human hookworm occur in the stool of infected person; infection is through skin penetration by infective larvae. *Schistosoma* spp. is a parasite responsible for schistosomiasis, *Schistosoma* eggs released into the external environment through feces or urine of infected people; furthermore, *Schistosoma* can be observed in other mammals as mice and wistar rats (Sene 1994; Wang et al. 2016). *Taenia* spp. is a tapeworm of the class of Cestoda, responsible for Taeniasis disease; the usual hosts for this parasite are cattle

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and swine, from which humans become infected with the adult tapeworm (Parkhouse and Harrison 2014). *Ascaris* spp. is the most common intestinal parasites in the world; it is responsible for the human Ascariasis. The natural habitats of adult larvae is the small intestine (Stephenson 2009), the infected individuals excrete up to 200,000 eggs per day (Bethony et al. 2006; Karkashan et al. 2015). *Capillaria* is responsible of Capillariasis disease, and *Trichuris trichiura* eggs are responsible of Trichuriasis disease, which is among the common human parasitic infection (Pullan et al. 2014).

Amahmid et al. (2002) attributed the reduction of parasite eggs during the treatment of wastewater to their accumulation in the solid phase (sludge). The sedimentation speed is estimated by Shuval (1977) at 0.65 m/h for *Ascaris* spp. eggs, 0.39 m/h of *Ankylostome*, 0.26 m/h of *Taenia*, 1.53 m/h of *Trichuris*, and 12.55 m/h of *Schistosoma*. Several authors have showed that wastewater and sewage sludge contain a large quantity of *Ascaris* spp. eggs (Amahmid et al. 2002; Sylla and Belghyti 2008; El Fels et al. 2014b), the abundance of *Ascaris* spp. eggs is explained by their resistance and their transmission mode (direct cycle). The helminth eggs contain a thick outer layer that acts as protection barriers to various environmental conditions, which explain their high resistance to inactivation (Maya et al. 2012).

According to the literature, helminth eggs can survive until 10–12 months in excretion in tropical climate (Sanguinetti et al. 2005; Koné et al. 2007). Feachem et al. (1983) explained the predominance of *Ascaris* spp. in the environment by their extremely abundant egg production and their ability to survive. 0.8 to 1.2 billion people are globally affected by Ascariasis and the most affected populations are in Sub-Saharan Africa, Latin America, and Asia (Lozano 2012). Children between 5 and 15 years old are the most vulnerable (Bundy 2004).

One of the most attractive options for sludge disposal is its use in agriculture and then specific guidelines regarding hygienic quality must be fulfilled. So, as to avert the risk of contamination, the sludge must be properly sanitized (Reilly 2001; Arthurson 2008; Rocha et al. 2016). Dehydration techniques and sludge liming are often used as ways to reduce and/or eliminate their parasite loads. Numerous studies have shown that the basic pH (higher than 10) influences the disintegration of helminth eggs in biosolids (Gaspard and Schwartzbrod 2003; Cappizzi-Banas et al. 2004). Nevertheless, treating sludge by dehydration process on drying beds was not efficient enough to eliminate all helminth eggs. The high concentration of helminth eggs contained in the fresh sludge could not be inactivated in drying beds. El Fels et al. (2014b) and Koné et al. (2007) showed the existence of significant amount of helminth eggs (*Ascaris* and *Trichuris*) after sludge dewatering. Furthermore, Gantzer et al. (2001) detected viable nematode eggs in dehydrated sludge stored for 8 months. Treatment of sludge by anaerobic or aerobic digestion and/or dewatering will reduce the number of pathogens, but significant numbers will remain (Straub et al. 1993). Several sludge treatment procedures include composting were applied; and most factors involved in pathogen inactivation are controlled by composting process (Koné et al. 2007; El Fels et al. 2014b). Haug (1993) showed that pathogen survival could occur in low temperature zones. However, Wichuk and Mc Cartney (2007) demonstrated that pathogen inactivation is expected to occur if all particles of compost maintain temperatures greater than 55 °C for at least 3 days. El Fels et al. (2016) showed that pathogens should be reduced to non-detectable levels during composting of organic matter.

Composting is a process with a low-cost and easy-to-operate, it is a widespread process used for the treatment of wastes in middle-income countries (WHO 2006; Koné et al. 2007; El Fels et al. 2014b). This aerobic biological degradation process of organic matter has developed in Morocco in recent years especially the sector of composting of green waste and sludge treatment plants. Nevertheless, composting has the benefits of reducing the environmental risks associated with waste management by reducing the volume and the destruction of pathogenic organisms (Saeba and Ferrini 2006; El Fels et al. 2014b). Moreover, composting provides a stable, humified and hygienic amendment containing nutrients.

Many studies were interested in the effect of composting on helminthes eggs inactivation. Szabová et al. (2010) studied the influence of composting organic wastes (straw, sawdust, wood brash, and sludge) during the winter and summer seasons on the survival of non-embryonated *Ascaris suum* eggs. Other studies were conducted on activated municipal sludge (Yanko 1988; El Fels et al. 2014b). Helminth eggs inactivation in lagooning sludge is not widely discussed in the composting literature.

The objectives of this study were to assess the quantities of helminth eggs in fresh and dehydrated lagooning sludge under semi-arid climate (case of lagooning wastewater treatment plant of Chichaoua city), and then follow their inactivation during sludge composting with green waste. Moreover, to identify factors involved in the effectiveness of helminth eggs inactivation by the co-composting process of dewatered sludge mixed with green waste.

Material and methods

Sampling of sewage sludge

The sewage sludge comes from natural lagooning wastewater treatment plant of Chichaoua city. A total of 3609 m^3 of lagooning sludge was recovered by a pumping unit set on the banks of each pond. This is the first total cleaning done after 7 years of operation.

Dewatering is one of the primary processes of sludge management. A small decrease in the amount of water in the sewage sludge produces a significant reduction in its volume, which is very necessary for the following stage of sludge recycling (Kolecka et al. 2017). In our case, the sludge was dehydrated on drying beds to facilitate the preparation of mixtures for an industrial scale composting. The dehydrated sludge with 37% of water and a pH of about 7.2 was stored for about 4 months at room temperature before composting. However, samples of fresh sludge (moisture content of about 64% and a pH value of 7.80) were stored at −20 °C until analysis.

Composting trials

Dehydrated sludge and green waste (mixed of blend of leaves, grass clippings, and shrub clipping) from the garden city were the two substrates used in composting.

The main characteristics of the different composting substrate are presented in Table 1.

The composting process was carried out as windrows for 105 days on a composting platform located in Chichaoua city. Three trials with different proportions were conducted:

- Mixture 1 (M1), 1/2 sludge + 1/2 green waste, (total volume of 4 m^3).
- Mixture 2 (M2), 2/3 sludge + 1/3 green waste, (total volume of 4 m^3).
- Mixture 3 (M3), 1/3 sludge + 2/3 green waste, (total volume of 4 m^3).

During the first 3 months of composting, a regular brewing was achieved manually and weekly to ensure aeration of windrows and to moderate the moisture by adding water to 60%. The temperature of the windrows during the process of co-composting was measured daily at different levels with special chips (PH07001 15 Version 1.20 Ector Traceability).

Homogeneous composite samples were taken at T_0 (first day of composting) and during each stage of composting (T_{21}, T_{63}, and T_{105} day). Subsamples of 1 kg were taken at different points along the length and height of the windrow followed by quartering and carefully mixed. The composite samples were kept at −20 °C until analysis.

Table 1 Main features of the substrates used for composting

Parameters	Green waste	Dehydrated sludge	Fresh sludge
pH	7.30 ± 0.18	7.22 ± 0.34	7.80 ± 0.31
Moisture (%)(FW)	35.23 ± 0.16	37 ± 0.4	64.14 ± 0.11
Ash content (%) (DW)	18.73 ± 0.04	60.28 ± 0.1	44.47 ± 0.08
TOC (%) (DW)	51.44 ± 0.35	22.06 ± 0.18	30.85 ± 0.12
TKN (%) (DW)	1.04 ± 0.028	1.06 ± 0.15	1.10 ± 0.1
C/N	49.46	20.82	28.04

FW fresh weight, *DW* dry weight, *TOC* total organic carbon, *TKN* total kjeldahl nitrogen

Physico-chemical analyses

The pH was measured on an aqueous extract of the compost as indicated in the French standard procedure AFNOR NF T90-008. The total kjeldahl nitrogen (TKN) was assayed by distillation according to the French standard procedure AFNOR T90-110 and ammonium ion content was determined by alkaline distillation. Total organic carbon (TOC) was determined according to El Fels et al.'s (2014a) procedure. Moisture contents were determined by drying a fresh sample of compost and sludge at 105 °C for 48 h (AFNOR 2000).

Decomposition rate and ash content were calculated after calcination in a muffle furnace at 600 °C for 6 h according the equation below:

Decomposition rate (%) = 100−100 [initial ash (100−final ash))/[final ash (100−initial ash)].

Identification and quantification of helminth eggs

The analytical technique for the identification of helminth eggs was carried out on 10 g of fresh sludge and of each co-composting stage. The first step was the separation of eggs helminth from the particles by ammonium bicarbonate (11.9%); after centrifugation, the pellet was recovered and washed with water to remove the rest of ammonium bicarbonate.

The second step was the concentration of eggs based on flotation method (Bailenger method) with the presence of zinc sulfate (56.81%, specific gravity, 1.29) (Bowman et al. 2003; Schwartzbrod 2003; Koné et al. 2007; El Fels et al. 2014b). After centrifugation, the liquid supernatant was recovered.

After being located at × 100 magnification, the helminth eggs were identified at × 400 magnification using a Mac Master blade. Photomicrographs were made using a binocular microscope (camera: Moticam 1000, 1.3 M pixel USB 2.0, lens 16 MM, ø28). The total number of helminth eggs was expressed per gram of fresh sample.

Statistical analyses

Principal components analysis (PCA) was applied to the correlation matrix between physical–chemical variables and abatement rate of helminth eggs for the three mixtures. The statistical treatments were studied with the software SPSS Statistics 24 Win version 10.

Results and discussion

In order to follow the evolution of composting process, the physico-chemical parameters were analyzed. Figure 1 presents temperature versus time during composting period for the three mixtures. The graphic has a classic look composting evolution; it has two main phases of temperature changes (thermophilic phase and maturation). In thermophilic phase, the temperature increases to 45, 44.6, and 50 °C, respectively, for mixtures M1, M2, and M3 resulting from the intense microbial activity by degradation of simple molecules in the substrate (Khalil et al. 2001; El Fels et al. 2016). Thereafter, the temperature decreases to reach room temperature; this second phase called maturation phase during which only compounds resistant to degradation are remaining.

Table 2 presents the physicochemical parameters values after 105 days of composting. The pH value of the three mixtures tended to stabilize at around neutrality (7.22, 7.14, and 7.09, respectively, for mixtures M1, M2, and M3) because of buffer power of humus at the maturation phase. The increasing of Carbon/Nitrogen ratio (C/N) is related to the loss of organic carbon due to the biological oxidation of organic matter, and to the augmentation of total kjeldahl nitrogen (TKN) concentration by degradation of carbon compounds and the mineralization of nitrogen-containing molecules. The decomposition rate also increased after 105 days of composting to 33.63, 39.24, and 36.41%, respectively, for mixtures M1, M2, and M3. According to the literature, the results of the physico-chemical parameters after 105 days indicated that the final compost is mature and stable.

Identification and quantification of helminth eggs of raw sludge before and after dewatering

The microscopic examination of sludge samples shows a presence of parasites belonging to Nemathelminthes groups (*Ascaris* spp., *Trichuris* spp., *Capillaria* spp., *Hookworm* spp., and *Toxocara* spp.) and to Plathelminthes groups (*Taenia* spp. and *Schistosoma* spp.), a predominance of *Ascaris* spp. eggs compared to other types of helminths was noted (Fig. 2). The identified Nemathelminthes and Plathelminthes species originated from human and animal intestinal parasites.

The concentration and distribution of nematode eggs (*Ascaris* specifically) in sewage sludge have been studied (Koné et al. 2007; Konaté et al. 2013; El Fels et al. 2014b). It has been shown that the density of the helminth eggs is greater than that of the water (1.056 to 1.237), which favors their settling in sludge (Koné et al. 2007; Gantzer et al. 2001). El Fels et al. (2014b) and Koné et al. (2007) have reported that eggs of intestinal nematodes are stronger than those of tapeworms in wastewater and, therefore, in sludge. Konaté et al. (2013) have examined the vertical distribution of helminth eggs in the sewage sludge and showed that the upper layer of sludge, which is the most recently deposited, contains most of helminth eggs (3100 helminth eggs/g DW) and has the highest percentage of viability (57.2%).

The fresh sludge contains 15.4 to 37 eggs/g, with a predominance of *Ascaris* spp. (10.4–16.6 eggs/g) followed by *Capillaria* spp. (2.4–11 eggs/g), *Trichuris* spp. (0.8–6 eggs/g), *Toxocara* spp. (0.2 to 2.4 eggs/g), *Hookworm* spp. (1.2–1.4 eggs/g), and *Taenia* spp. and *Schistosoma* spp. (< 1 egg/g). After dehydrating the sludge, the concentration of helminth eggs decreased with an abatement rate of about 35 to 48%. We counted from 8 to 24 eggs/g, with a dominance of *Ascaris* spp. (5–19 eggs/g) followed by *Toxocara* spp. (0.2–2.4 eggs/g); *Hookworm* spp. and *Capillaria* spp. (0.4–1 egg/g); *Trichuris*, *Taenia*, and *Schistosoma* (< 1 egg/g). The treatment of sludge by drying beds allows the inactivation of a part of helminth eggs through the ultraviolet rays and leaching, Ayres et al. (1993) have determined that 9 to 41.5 eggs/g of nematode eggs were presented in dewatered sludge. Koné et al. (2007) showed that dewatering process on the drying beds was not efficient enough to inactivate all the helminth eggs. They isolated up to 22–38 helminth eggs/g (*Ascaris* and *Trichuris*), of which 25 to 50% are viable. Furthermore, El Fels et al. (2014b) showed that the dehydration process did not appear to be effective for the complete elimination of helminth eggs; they have counted from 4 to 27 eggs/g (*Ascaris* spp., *Trichuris* spp., and *Capillaria* spp.) in dehydrated sludge.

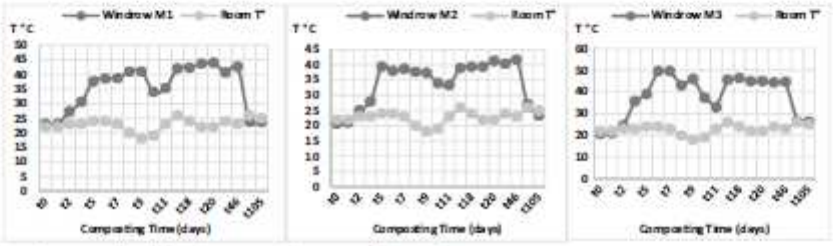

Fig. 1 Temperature versus time during the co-composting process of three mixtures

Table 2 Evolution of physicochemical parameters during composting

Mixtures	Time (days)	pH	C/N	DEC (%)	TKN (%)
1	0	7.49 ± 0.01	29	0	0.84 ± 0.028
	105	7.22 ± 0	9.47	33.63 ± 2.6	1.36 ± 0.1
2	0	7.74 ± 0.19	27.61	0	0.95 ± 0.012
	105	7.14 ± 0.075	9.24	39.24 ± 0.13	1.18 ± 0.13
3	0	*8.16 ± 0.24*	26.49	0	0.88 ± 0.008
	105	*7.09* ± 0.006	10.1	36.41 ± 2.5	1.30 ± 0.12

TKN total kjeldahl nitrogen, *DEC* decomposition rate

In accordance with the recommendations of the WHO (2006) for the reuse of sludge (1 egg/g or less), the concentration of helminth eggs remains high and exceeds the standards. Hence, the treatment of sludge by dehydration alone is insufficient.

Abatement of helminth eggs by composting process

During the co-composting, the identified helminth eggs were the same as those found initially in dehydrated sludge, such as *Ascaris* spp., *Trichuris* spp., *Capillaria* spp., *Toxocara* spp., *Hookworm* spp., and *Schistosoma* spp. At the initial stages of co-composting, we counted a lower concentration of the total helminths eggs than in the sludge only that is of about 16, 18.4, and 12.2 of the total helminth eggs/g, respectively, for mixtures M1, M2, and M3 (Fig.2). High concentration and diversity of helminth eggs was observed in mixture 2 (Fig. 2, Fig. 3a–c).

A significant difference in helminth egg numbers as well as the absence of some species at the initial stage was revealed (Fig. 3a–c). We noted the absence of *Trichuris* spp. and *Schistosoma* spp. at the initial stage of composting of mixture M1, and *Trichuris* spp., *Toxocara* spp., and *Schistosoma* spp. for mixture M3. However, the *Taenia* spp. was absent in all mixtures M1, M2, and M3.

The variability in the numbers of the helminth eggs observed at T_0 in the mixtures M1, M2, and M3 is due to the different proportions of mixed sludge with the green waste. El Fels et al. (2014b) showed that the structure of the lignocellulosic matrix onto which the helminth eggs can be adsorbed, partially explains the differences noted in helminth egg numbers during composting. Similarly, Koné et al. (2007) showed that when the material is digested and the particles are finer, the variability of egg numbers decreased because of the greater consistency of the sample. Beside the substrate structure, the initial load of helminth eggs of sludge could explain the absence of *Trichuris* spp., *Toxocara* spp., *Schistosoma* spp., and *Taenia* spp.

During composting, we observed a decrease in the concentration of total helminth eggs (Fig. 3a–c). This corresponds to a reduction of 72.5, 72.8, and 67.3%, respectively, for mixtures M1, M2, and M3 during thermophilic phase (27 days). However, after 105 days of composting, the reduction rate reached 97.5, 97.8, and 98.4%, respectively, for mixtures M1, M2, and M3.

At final stage of process, the reduction in the numbers of *Ascaris* spp. eggs was about 97.7, 97.4, and 97.9%, respectively, for mixtures M1, M2, and M3. However, a 100% of reduction was obtained for *Toxocara* spp., *Hookworm* spp., *Schistosoma* spp., *Trichuris* spp., and *Capillaria* spp.

A difference of a kinetic abatement of all identified species was noted. This abatement is linked to the variation degree of physico-chemical parameters of each mixture such as temperature. The time necessary to attain high levels of helminth inactivation (> 95%) is variable in the literature, reports varying from 2 h to 180 days, based on the temperature (Gantzer et al. 2001; Ayres et al. 1993; Ghiglietti et al. 1997).

The dominance of *Ascaris* spp. eggs in the three mixtures with low reduction rate is justified by the resistance of *Ascaris*

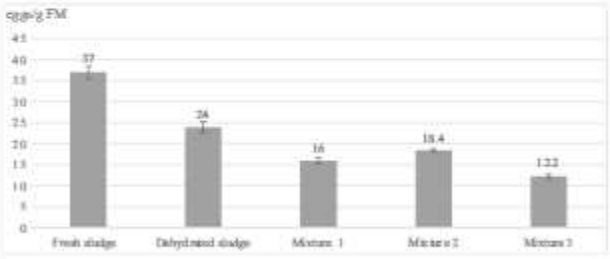

Fig. 2 Total helminth eggs per g of fresh and dewatering sludge material and initial stages (T_0) of composting of mixtures M1, M2, and M3

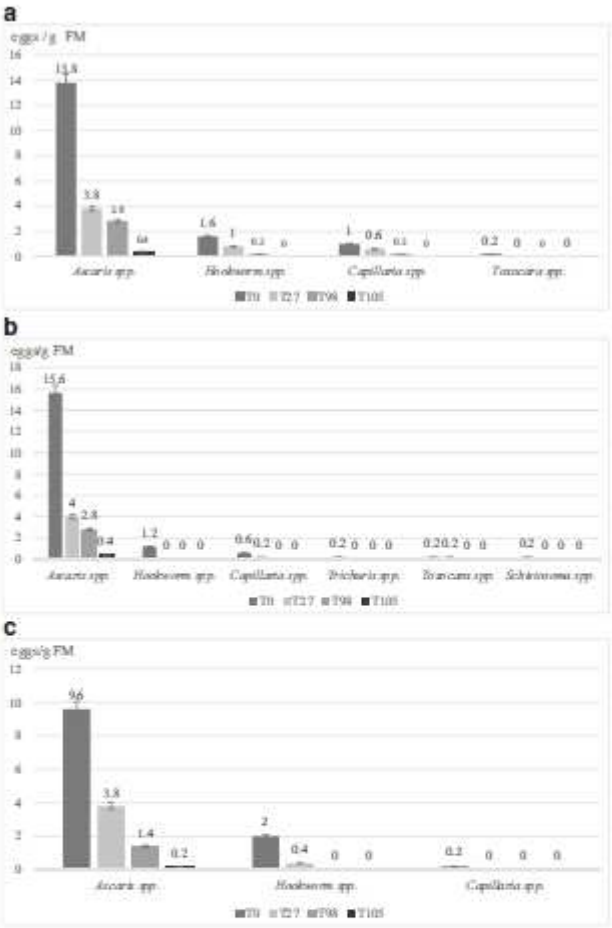

Fig. 3 **a** Evolution of helminth eggs in the mixture M1 during composting. **b** Evolution of helminth eggs in the mixture M2 during composting. **c** Evolution of helminth eggs in the mixture M3 during composting

spp. eggs compared to other helminth eggs. The helminth eggs are more resistant to attack by the environmental factors.

This resistance is due to the presence of thick cuticles that prevent the passage of certain substances (strong acids and bases, oxidants, reducing agents and detergents…etc.). The permeability of the helminth eggs is limited to the passage of water, some solvents and some gas (Keffala et al. 2012).

In our case, the helminth eggs reduction is explained by the temperature increasing which reached 45, 44.6, and 50 °C, respectively, for mixtures M1, M2, and M3. Gaspard and Schwartzbrod (2003), and Cappizzi-Banas et al. (2004) showed that high temperatures accelerate the rate of desiccation of *Ascaris* cells. El Fels et al. (2014b) showed that a high temperature is damaging the cells of the helminths and their capacity to survive after their exposal for several days. The temperature increase to 45 °C can enhance to increase the rate of chemical reactions and enzymatic processes, which involve an increase of the eggs membrane permeability (Maya et al. 2012). Vinneras et al. (2003) showed that the inactivation of all *Ascaris* spp. eggs will take place if the temperature in the composting windrows remains above 45 °C for at least 5 days. The same result can be obtained with 8 days at 44 °C.

Furthermore, other factors may contribute to the inactivation of helminth eggs such as the duration of exposure and the concentration of ammonia (El Fels et al. 2014b). The production of ammonia in the thermophilic phase of composting contributes to

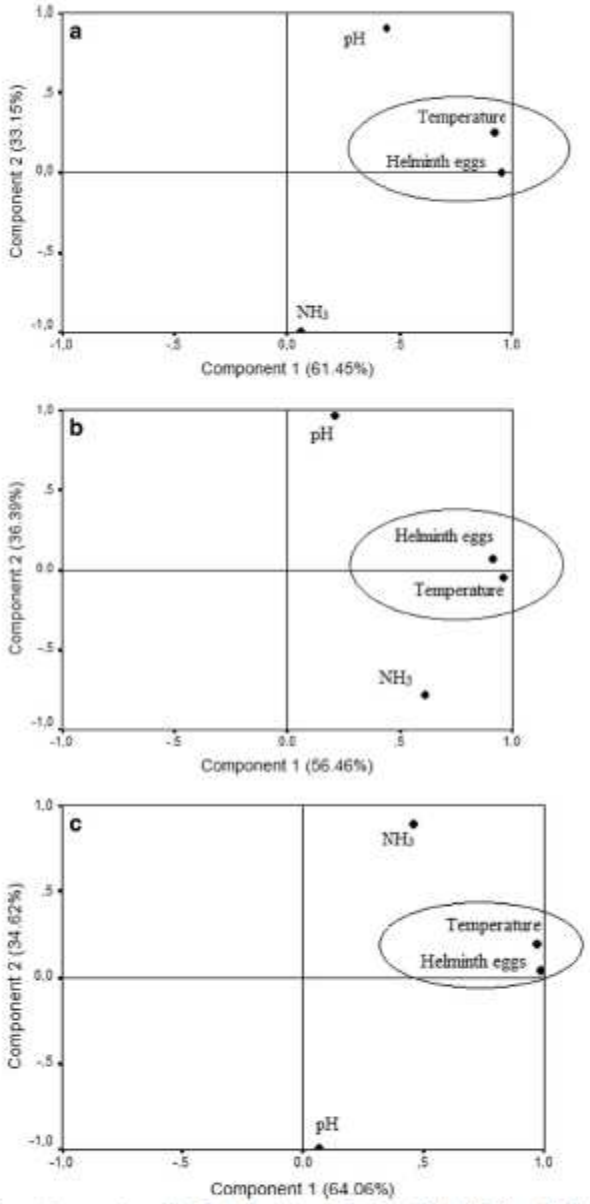

Fig. 4 PCA components of composting parameters and helminth eggs abatement of mixtures M1 (a), M2 (b), and M3 (c)

the reduction of helminth eggs. Ammonia exists naturally in wastewater from the hydrolysis of urea and the degradation of proteins and other compounds containing nitrogen. Thus, the inactivation mechanism of pathogenic microorganisms by ammonia may be due to an alteration of the membrane potential, to the cytoplasmic pH increase and to the loss of potassium ions (El Fels et al. 2014b; Pecson et al. 2007).

Carbonate ions and volatile fatty acids are also known for their ability to inactivate pathogens. Sanguinetti et al. (2005) showed that the decrease in the concentration of water in the environment could promote the inactivation of helminth eggs.

Other authors have studied the pH factor in biosolids disinfection; they showed that pH between 12 and 12.6 for 20 to 60 days is effective for nematode egg inactivation (Gaspard et al. 1997; Reimers et al. 1998). Schuh et al. (1985) have observed inactivation of *Ascaris suum* eggs after 2–4 months of sewage sludge storage at pH 12.5. Gantzer et al. (2001) showed significant influence of temperature and pH on the inactivation of parasite eggs during sludge treatment.

The results obtained in our study reveal that the combination of dewatering of sludge and physico-chemical factors during composting (combination of temperature pH, NH_3, and moisture) provides hygienic compost safe for agricultural reuse.

Statistical analysis

The results of the PCA of composting parameters and reduction rate of helminth eggs for the three mixtures are reported in Fig. 4.

The projection on the plane of variables composting parameters (temperature, pH, and NH_3) and Helminth eggs removal for the three mixtures in terms of two main components (I and II).

Figure 4a–c shows the strong correlation between temperature and the reduction of helminth eggs for mixtures M1, M2, and M3. These results confirm that the temperature is the main factor in the removal of helminth eggs. Ammonia and pH, which are positively correlated with helminth eggs abatement, could also contribute to the helminth inactivation during composting.

Conclusion

The sludge was initially loaded with various species (*Ascaris* spp., *Hookworm* spp., *Capillaria* spp., *Trichuris* spp., *Toxocara* spp., *Taenia* spp., and *Schistosomas* spp.). The concentration of identified helminth eggs decreased after sludge dewatering reduction rate were 35 to 48%. The helminth eggs concentration remained high and exceeded the standards.

Nevertheless, the stabilization of dewatered lagooning sludge by composting during 105 days led to reductions of helminth eggs by approximately 98%. A strong correlation was shown between temperature and helminth eggs abatement. Besides, various parameters were involved in helminth eggs inactivation such as substrate structure, moisture, ammonium content, and pH.

The concentration of helminth eggs decreased to levels meeting the WHO (2006) guidelines, set at 1 egg/g or less.

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ORIGINAL PAPER

Physico-chemical and Spectroscopy Assessment of Sludge Biodegradation During Semi-industrial Composting Under Semi-arid Climate

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Abstract
The purpose of this study is to assess the maturity and stability of the lagooning sludge (LS) with green waste (GW) during semi-industrial composting. Physico-chemical parameters, spectroscopy analysis and germination test were monitored. After 105 days, the decomposition rate of about 33.6; 39 and 36%, a stabilization of pH around neutrality; NH_4^+/NO_3^- ratio decreased to 0.922, 0.974 and 1.038; the C/N ratio decreased to 9.47, 9.24 and 10.10 respectively for mixture 1 (M1), mixture 2 (M2) and mixture 3 (M3). This reduction is explained by the bio-oxidation of organic matter. The intense microbial activity was characterized by the rise of temperature (between 40 and 50 °C) during the first weeks. Humification process was characterized by the increase of humification index (HI), which was approximately 62.1%; 73.6%; 61.5% respectively for M1, M2 and M3. The progress of humification process was determined by the decrease of aliphatic absorbance bands and the increase of aromatic absorbance bands. The increase in germination index (GI) (> 50%) for the four seeds: cress, alfalfa, turnip and radish, at the end of composting indicated that the composts are phytotoxic-free and are rich in stable organic compounds and nutrients. The results show that all three composts are mature, which opens the way for their application in agriculture without risk for the soil–plant system.

Keywords Composting · Lagooning sludge · Semi-arid climate · Maturity indices · Humic substances · FTIR

Novelty Statement

High amounts of sludge are produced after the water-purification using lagooning systems. Nevertheless, no industrial study of lagooning sludge was founded in semi-arid region. The composting performances regarding the pollutant removal are conditioned by the instrumental reliability and the analytical approach efficiency. The process aims to assess the compostability of lagonning sludge, where biodegradability remains little studied unlike the activated sludge. A fast and sensitive approach spectroscopy-based tool was used to give the wide information on organic matter decomposition. In addition, the biological index using the seeds germinations especially, which is adapted to the local climatic, leads to point out to new compositional maturity index likely to be extrapolated on an industrial scale.

Introduction

In Morocco the quantity of sludge from sewage systems varies from 40,000 tons per year in 2010 and is projected to reach 300,000 by 2025. Different types of treatment plants exist in Morocco [1] with the activated sludge and lagooning system being the most used. Handling and disposal of large quantities

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of sludge is one of the major problems. To avoid their negative impacts on the environment, it is required to reduce the amount of sludge waste and reuse this source of organic matter as fertilizer. Several options exist for the elimination of sewage sludge, but the choice must be dependent on the cost of installation, the origin of the sludge, the added value of the resulting product and their impact. Landfill has turned out a bit rewarding and also a prohibited technique in many countries [2]. Incineration of sludge has a prohibitive cost beside their risk linked to toxic gases emission in the environment such as dioxins, and acid gases [3]. Composting is commonly considered as one of the most effective ways for recycling organic waste producing a stable, pathogen-free final product [4–6]. This process is an exothermic aerobic process of degradation of organic matter of different origins by a successive group of microorganisms; this material is transformed into a stable product rich in minerals and humic compounds [7–10].

Humus formed during composting may act as a chelating agent for heavy metals (HM), the resulting effect is a decrease in the free HM concentration in soil and also a controlled provision of nutrient to plants [6, 11].

Land application of compost allows the enhancement of the organic matter content, the humidity and the cation exchange capacity of the soil [12, 13]. Composting can be considered as an alternative to chemical fertilizers; moreover, using compost would permit cash savings to farmers [10, 14].

During composting, the activity of indigenous microorganisms is enhanced by the physico-chemical environmental parameters of the composted solid matrix. When environmental conditions are optimal for microbial activity, mesophilic and thermophilic microorganisms rapidly decompose the substrate, increasing temperature through metabolic heat release. Compost stability refers to the resistance of composted organic matter to further rapid biodegradation [15, 16]. Respirometric methods; principal component physico-chemical analyses C/N and NH_4^+/NO_3^- ratios and humification indices [6, 17–19]; measurement of carbon [20]; spectroscopic methods (UV visible, fluorescence, FTIR) [6, 21] and germination testing are the most used tests to evaluate the maturity and stability of composting products.

The objective of the present study was to evaluate the organic matter breakdown occurring in the lagooning sludge green waste mixture during the semi-industrial composting process, under arid climate, by physico-chemical and infrared spectroscopy (FTIR) analysis to confirm the substrate stability by the germination test.

Materials and Methods

Experimental Site and Composting Test

The present experimental study focuses on valorization of lagooning sludge (LS) from the wastewater treatment plant of the Chichaoua city (Morocco). Because of its high moisture content and small particle size; sludge was mixed with green waste (GW) as bulking agents which, permit adequate gas exchange and prevent excessive compaction of the composting substrate by providing the structural support [22]. The main characteristics of the substrates are presented in Table 1.

Composting was carried out in municipal nursery gardens in 2016. Three mixtures, M1, M2 and M3 were prepared as a windrow with the following proportions:

- Mixture 1 (M_1): 1/2 sludge + 1/2 green waste, for a total volume of 4 m^3.
- Mixture 2 (M_2): 2/3 sludge + 1/3 green waste, for a total volume of 4 m^3.
- Mixture 3 (M_3): 1/3 sludge + 2/3 green waste, for a total volume of 4 m^3.

The composting was carried out during 105 days during which, the temperature was measured every day at different levels of the windrow with sensors with data memory (PH0700115 model 1.20, Ector-Traçability software, ECTOR France). The windrow was turned over by hand weekly for aeration. Homogenous samples were taken at T_0 (first day of composting) and after aeration of the mixture. Homogenous samples of 1 kg at (T_0), 1 month (T_1), 2 months (T_2), 3 months (T_3), 4 months (T_4) were obtained by careful mixing of several subsamples taken at different points (height and length) of windrow, and quartering them. The samples were kept at 20 °C before analysis.

Physico-chemical Analysis

The pH was measured using an aqueous extract of the compost at room temperature (1 g/10 mL of distilled water) after 30 min of agitation at ambient temperature according to NF

Table 1 Main features of the substrates used for composting

Parameters	Sewage sludge	Green waste
Moisture content (%)	16.40 ± 0.021	35.23 ± 0.009
pH	7.22 ± 0.06	7.30 ± 0.015
TKN* (%)	1.06 ± 0.014	1.51 ± 0.116
COT* (%)	25.21 ± 0.171	51.44 ± 0.016
C/N	23.78	34.29
Ashes rate* (%)	60.18 ± 0.163	18.73 ± 0.017

*Results expressed per unit weight dry matter

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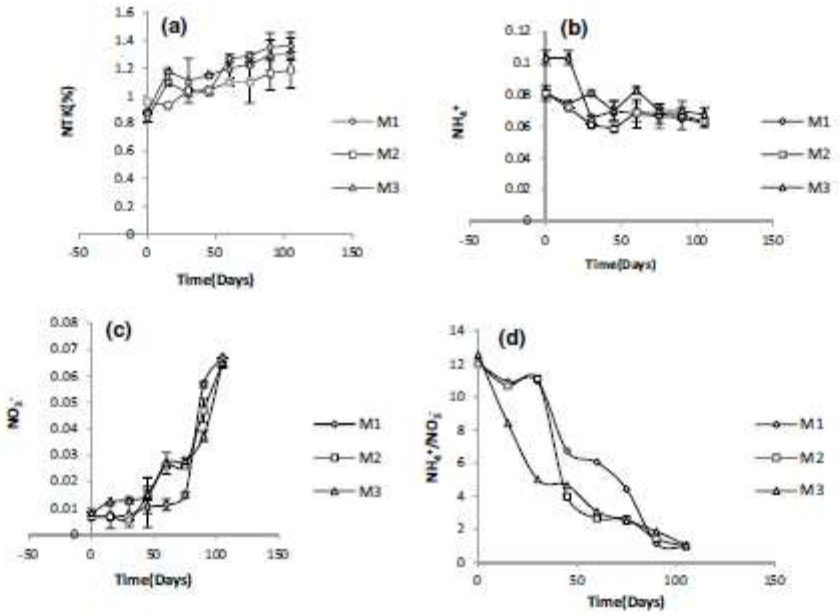

Fig. 2 Changes of total Kjeldahl nitrogen (a), ammonium (b), nitrate (c) and NH_4^+/NO_3^- (d) of different windrows during composting process

mixture M3 the initial value of NH_4^+ was about 0.103% then decreased to 0.067% after 105 day of composting. However, in the M2 mixture, the percentages of NH_4^+ regressed from 0.081 to 0.063% after 105 days (Fig. 2b).

The decrease of NH_4^+ is related to increased ammonia volatilization, especially during the thermophilic phase because of higher temperature and pH value [45]. Monedero et al. [46] and Huang et al. [18] explained that NH_4^+ decrease is related to an increase in ammonia emissions, immobilization by microorganisms and nitrification.

After 13 weeks, the percentage of NO_3^- has increased from 0.00825 to 0.065%, from 0.00825 to 0.0647% and from 0.00825 to 0.065% respectively for mixture M1, M2 and M3 (Fig. 2c). During composting the percentage of NH_4^+ and NO_3^- vary inversely owing to the conversion of NH_4^+ to NO_3^-. The index of nitrification calculated as NH_4^+/NO_3^- (Fig. 2d) varies from 12 to 0.92; from 12 to 0.97 and from 12.55 to1.04 for mixture M1, M2 and M3 respectively. According to Mustin [47], Benhassou, [48], Barje et al. [21] and El Fels et al. [6], the enrichment of the final compost by NO_3^- by contribution of NH_4^+ translate the evolution of the substrate to a compost free of phytotoxicity. These authors explain that the increase in nitrates during the maturation phase, causing the reduction in NH_4^+/NO_3^- ratio; is attributed to the good conditions during this phase that allow the development of the nitrifying bacteria. The NH_4^+/NO_3^- lower than 1 (Fig. 2d) is considered as an indication of the transformation of substrate to yield a compost free of phytotoxicity [6, 49]. Similarly, Apaolaza et al. [50] attributed the change in the index of nitrification in composted sludge to the ammonifying organism and nitrifying bacteria, environmental conditions and the characteristics of the substrates.

As shown in Table 2, the evolution of TOC percentage during the composting process for all treatment is reduced steadily. The TOC values dropped from 17.40, 20.96, and 18.23 to 12.88%, 10.97%, and 13.14% for M1, M2 and M3 respectively. TOC loss has been reported as an important parameter that may serve as an indicator of the degree of ease in organic matter oxidation [42, 51, 52]. Nevertheless, during the maturation phase, the carbon content relatively stabilized. Indeed, in this phase carbon losses are slowed down and there has been a redistribution of carbon by the mechanisms of the humification [6, 47].

The C/N ratio is one of the most important factors influencing compost quality [53] and one of the best indices to

evaluate the maturity of compost [6, 54]. After 105 days of composting the C/N ratio dropped from 20.52 to 9.63; from 21.90 to 9.24 and from 20.72 to 10.11 for M1, M2 and M3 respectively (Fig. 3). The decrease of C/N ratio is mainly due to the carbon losses through organic carbon oxidation of organic matter. Carbon is used as energy source while nitrogen is used for building cell structure [55, 56]. Carbon loss is a result of bacteria activity; therefore the CO_2 emissions and water evaporation during thermophilic stage are produced during this process. Hanajima et al. [57] has stated that the 70–85% of the total loss of the carbon during composting is in the form of CO_2.

Carbon oxidation and CO_2 loss is leading to an increase in the proportion of total nitrogen of the medium [6]. Bernai et al. [58] suggested that a C/N ratio below 12 reflects an advanced degree of organic matter stabilization and a satisfactory degree of maturity in sludge composting.

Table 2 illustrates the concentration of phosphorus (Olsen phosphorus) during composting process. From day 9 of composting the concentration of phosphorus was increased rapidly to reach a maximum value of 0.9476 mg/g for M1, 0.9600 mg/g for M2 and 0.9053 mg/g for M3 at the 86th day. The concentration of phosphorus variation may be explained by the influence of organic matter decomposition on the availability of the phosphorus. The organic acids released after OM mineralization bind to the ligand forming stable complexes which favors the maintenance of the P in solution [59–61]. Some organic acids from the humus and lignin compounds can be substituted for P to form stable complexes. In sum, the decomposition of organic matter favors a competition between organic acids and P for fixing sites [62]. Moreover, there is an increase of the negative charges of the compost with the organic matter (humus) content.

Humic substances are highly abundant organic compounds formed by the microbial activity. During composting the organic matter present in the initial mixture is mineralized, while organic residual matter are transformed into new organic materials such as humic substances produced by humification process [63]. Several authors Tomati et al. [64]; Amir et al. [65] and El Fels et al. [6] showed that the microbial activity and enzymatic reactions of polymerization/repolymerization lead to the formation of aromatic compounds. It was noted that the products formed during composting are characterized by a strong dominance of the carbon of the humic acids for three mixtures and their proportion tends to increase with the development of composts and reached 7.992%, 8.075% and 8.075% respectively for mixture M1, M2 and M3. Nevertheless the carbon of fulvic acid decreased with 0.108%, 0.084% and 0.025% respectively for mixture M1, M2 and M3 after 105 days of composting (Table 3).

During composting organic matter such as reduced sugars and amino acids produced in abundance as by-products of microbial metabolism may undergo non-enzymatic polymerization to form brown containing polymers. Furthermore the catalytic properties of the mineral matter can facilitate their condensation [66]. We observed a significant increase of polymerization degree (DP) 74%, 96.13% and 323%, humification index (HI) 62.050%, 73.610% and 61.454%, humification report (HR) 62.888%, 74.376% and 61.644% and percentage of humic acid (PHA) 98.667%, 98.970% and 99.691% after 105 days of composting respectively for mixture M1, M2 and M3. This increase can be explained by the formation of complex molecules (humic acids), by the polymerization of simple molecules (fulvic acids), or by the biodegradation of non-humic compounds [6].

A strong increase of the DP index was observed in M3. This increase can be due to the composition of this mixture, which contains a large amount of green waste rich in lignin. The humification rate (HR) characterizes the chemical reactivity of substances [67] and expresses the relative proportion of the aromatic and carboxylic groups and varies in proportion to the reactivity of the humic molecules. The degree of humification is high when the molecules have

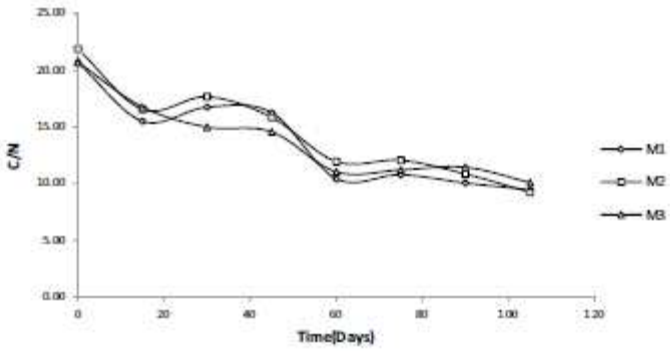

Fig. 3 Evolution of the C/N ratio during composting of M1, M2 and M3

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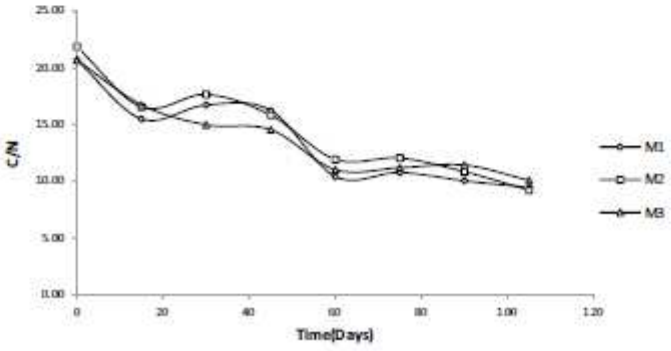

Fig. 3 Evolution of the C/N ratio during composting of M1, M2 and M3

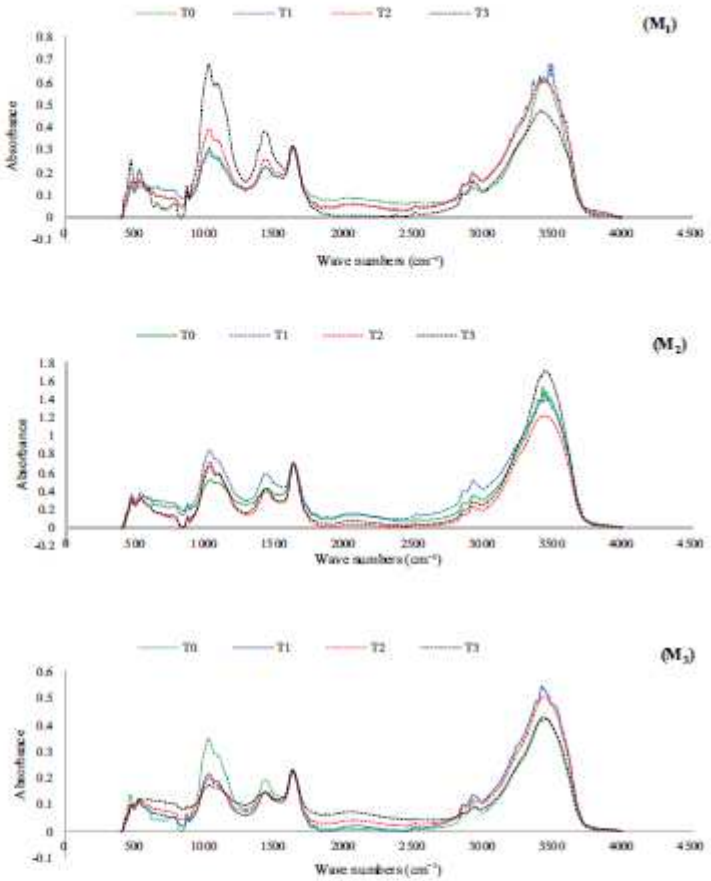

Fig. 4 FT IR spectra of different mixtures (M_1, M_2 and M_3) at various stage of composting. T0 : 0 day; T1: 30th day ; T2: 90th day ; T3: 105th day

could reflect the quality of the environment of the plants. Levi-Minzi et al. [76] indicated that wheat seed germination is influenced by a number of hydroxy and methoxy groups of the different acids, molecular position of single and double hydroxy groups, length of the aliphatic chain and the pKa of the acids within the homogeneous series of isomers.

Aggelis et al. [77] and Moharana et al. [78] suggested intervals of classification of the degree of phytotoxicity according to the value of the indices of germination of the substrates. If the GI value is < 25, the substrate is very phytotoxic; if GI value is 26 < GI < 65, then the substrate is characterized as phytotoxic; if GI value is 66 < GI < 100; then the substrate is characterized as non-phytotoxic; and if GI > 101 the substrate is stable and can be used as fertilizer and phyto stimulant.

In contrast during the maturation phase, there was a significant increase in the germination index, reaching their maximum of about 124.67%, 113.60% and 158.31% for turnip; and 126.51%, 130.62% and 108.79% for radish; and 108.37%, 105.06% and 109.30% for cress; and 139.44%, 154.49% and 143.48% for alfalfa, respectively for M1, M2 and M3 at 78th day. The GI values that exceeded 100% can generally be explained by a great reduction of phytotoxic substances and the enrichment of the compost by the stable organic matter and humic substances during maturation phase [6]. These results confirm the findings of Tiquia et al. [79], El Fels et al. [6] and Yuan et al. [80] who reported that a GI of more than 80% indicates that a compost was free of phytotoxic substances and mature. At the end of composting for especially radish species the GI underwent a slight

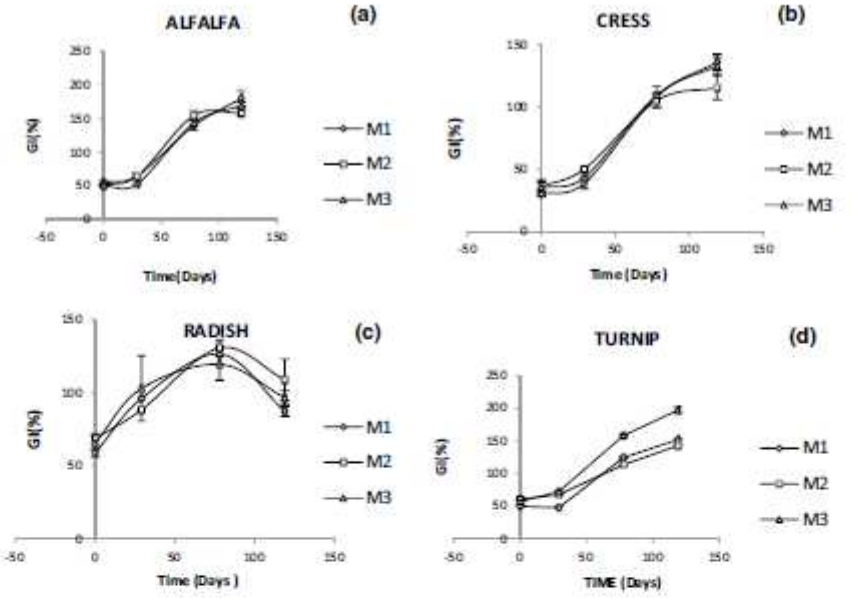

Fig. 5 Changes of GI during composting process of mixture M1, M2 and M3

depression. This result can be explained by the strong ionic charge of water-soluble extracts determined by the negative correlation between the GI and the electrical conductivity, causing a possible osmotic effect [6]. In addition, the concentration of sodium and certain elements (e.g., copper, zinc, manganese and boron) can inhibit the germination and growth of plants [26, 72, 81, 82].

Correlation Analysis

Principal components analysis (PCA) is a statistical method showing the relationship between responses of all experimental variables that vary simultaneously. On the basis of this analysis, the study of physicochemical variables: temperature, TOC, C/N, TKN, NH_4^+/NO_3^-, Olsen P, HI and germination tests, showed distinct behaviors according to the time. Indeed, the two components (Fig. 6) explain respectively 68.01%, 30.98% for M1, 67.32%, 30.68% for M2 and 52.69%, 46.31% for M3 of total variance between the diverse parameters while, in Fig. 6, the first component explains 57.06% and the second component explains 42.94% of the variability between the temperature and time. Each figure of the three mixtures: M1, M2 and M3 (Fig. 6a–c) is formed by two clustering of which the first is formed by TKN, Olsen P, HI, GI (radish, cress, turnip and alfalfa) are correlated positively with the time whereas the second grouping, C/N, TOC and NH_4^+/NO_3^- are inversely correlated with time. Figure 6d shows that the temperature is negatively correlated with time. The PCA results confirm those already determined by studying the evolution of these parameters during composting. The PCA also showed a good correlation between the parameters studied in the composting which indicates that composting M1, M2 and M3 developed correctly.

Conclusion

The use of sewage sludge, compost and other different organic wastes as organic soil amendments is a very important strategy to comply with the Landfill Directives. This study showed that the evolution of a lagooning sludge from wastewater treatment plant of Chichaoua city mixed with green waste during composting substantiated the maturity and stability of the three mixtures M1, M2 and M3. The results meet the thresholds of using compost as an amendment based on the following parameters: the C/N ratio < 10; NH_4^+/NO_3^- <1, enrichment with the nutrient content (N, P), indicating organic matter stabilization, HI increase of about

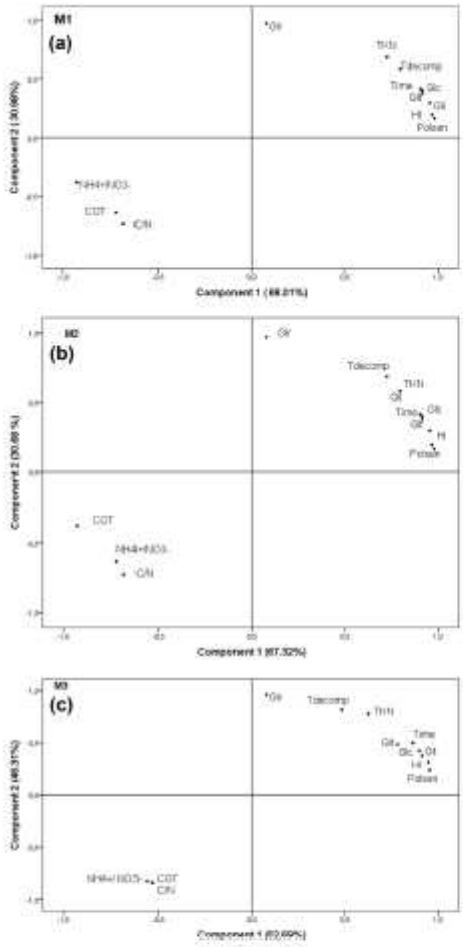

Fig. 6 PCA between the various parameters physico-chemical, humic index (HI) and germination index for alfalfa (GII), cress (GIc), radish (GIr) and turnip (GIr) and the time of composting of mixtures M1 M2 and M3

62.05%, 73.61% and 61.45%, respectively for M1, M2 and M3.

The FTIR spectroscopic analyses recorded a drop in aliphatic chains (peptide, (CH4) and methylene C–H aliphatic groups) due to the degradation of organic matter by microorganisms and an increase in aromatic chains confirmed by the improvement of HI% and also the evolution of the humification process during composting of the three mixtures M1, M2 and M3 and GI> 50%. These observations indicate that the final composted product has reached the maturity and subsequently can be used as a fertilizer for the soil. Moreover, the comparison of the results indicates that the most efficient compost for land application is the compost resulting from the mixture M1 (Higher NTK, NO_3^- and phosphorus content). The mixture M1 (1/2 sewage sludge + 1/2 green waste) permits the recycling of more sewage sludge than the other mixtures.

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