Universidad Politécnica de Cartagena

DEPARTAMENTO DE CIENCIA Y TECNOLOGÍA AGRARIA

Pathogen destruction and stabilization of sewage sludge in thermophilic digestion for its agricultural use. Effects on plants and soil microbial community

Eva Lloret Sevilla

2013

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Ph.D. Dissertation
(European Mention Doctorate)

Department of Agricultural Science and Technology
Technical University of Cartagena

Department of Soil and Water Conservation and Organic Waste Management
CEBAS-CSIC

Director

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2013

Esta Tesis Doctoral ha sido realizada en el Departamento de Conservación de Suelos y Agua y Manejo de Residuos Orgánicos del Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), en el grupo de investigación "Enzimología y Biorremediación de Suelos y Residuos Orgánicos". Para la realización del presente trabajo, la Lda. Eva Lloret Sevilla disfrutó de una Beca Predoctoral JAE PreDoc del Consejo Superior de Investigaciones Científicas (CSIC).

Los resultados de esta memoria se presentan como un compendio de trabajos publicados o en fase de revisión, en revistas pertenecientes al Science Citation Index (SCI):

Lloret E, Pastor L, Martínez-Medina A, Blaya J, Pascual JA, **2012**. Evaluation of the removal of pathogens included in the Proposal for a European Directive on spreading of sludge on land during autothermal thermophilic aerobic digestion (ATAD). **Chemical Engineering Journal, 198-199: 171-179**. Factor de impacto: **3,461** (2011 JCR Science Edition).

Lloret E, Pastor L, Pradas P, Pascual JA, **2013**. Semi full-scale thermophilic anaerobic digestion (TAnD) for advanced treatment of sewage sludge: stabilization process and pathogen reduction. **Chemical Engineering Journal, 232: 42-50**. Factor de impacto: **3,473** (2012 JCR Science Edition).

Lloret E, Salar MJ, Blaya J, Pascual JA, **2013**. Two-stage mesophilic anaerobic – thermophilic digestion for sludge sanitation to obtain advanced treated sludge. **Chemical Engineering Journal, 230: 59-63**. Factor de impacto: **3,473** (2012 JCR Science Edition).

Lloret E, Pascual JA, Brodie EL, Bouskill NJ, Insam H, Fernández-Delgado Juárez M, Goberna M, **2013**. Sewage sludge addition modifies soil microbial communities and plant performance depending on the stabilization process. En revisión.

Universidad Politécnica de Cartagena

DT-16

CONFORMIDAD DE SOLICITUD DE AUTORIZACIÓN DE DEPÓSITO DE TESIS DOCTORAL POR EL DIRECTOR DE TESIS

D. José Antonio Pascual Valero, Director de la Tesis doctoral "Pathogen destruction and stabilization of sewage sludge in thermophilic digestion for its agricultural use. Effects on plants and soil microbial community" y D. Ángel Faz Cano, Tutor de la misma,

INFORMAN:

Que la referida Tesis Doctoral, ha sido realizada por Dª. Eva Lloret Sevilla, dentro del programa de doctorado Medio Ambiente y Minería Sostenible, dando mi conformidad para que sea presentada ante la Comisión de Doctorado para ser autorizado su depósito.

La rama de conocimiento en la que esta tesis ha sido desarrollada es:

- ☐ Ciencias
- ☐ Ciencias Sociales y Jurídicas
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En Cartagena, a 16 de Diciembre de 2013

EL DIRECTOR DE LA TESIS

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Dª. María Dolores Gómez López, Miembro de la Comisión Académica del Programa Medio Ambiente y Minería Sostenible.

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Que la Tesis Doctoral titulada, "Pathogen destruction and stabilization of sewage sludge in thermophilic digestion for its agricultural use. Effects on plants and soil microbial community", ha sido realizada, dentro del mencionado programa de doctorado, por Dª. Eva Lloret Sevilla, bajo la dirección y supervisión del Dr. José Antonio Pascual Valero.

En reunión de la Comisión Académica de fecha 13/12/2013 visto que en la misma se acreditan los indicios de calidad correspondientes y la autorización del Director de la misma, se acordó dar la conformidad, con la finalidad de que sea autorizado su depósito por la Comisión de Doctorado.

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Sra. Dña. Eva Lloret Sevilla

Vistos los informes favorables de los Directores de Tesis y el V°B° de la Comisión Académica para la presentación de la Tesis Doctoral titulada: *"Pathogen destruction and stabilization of sewage sludge in thermophilic digestion for its agricultural use. Effects on plants and soil microbial community"* en la modalidad de "compendio de publicaciones" solicitada por Dña. Eva Lloret Sevilla, la Comisión de Doctorado de la Universidad Politécnica de Cartagena, en reunión celebrada el 18 de diciembre de 2013, considerando lo dispuesto en el artículo 33 del Reglamento de Estudios Oficiales de Máster y Doctorado de la UPCT, aprobado en Consejo de Gobierno el 13 de abril de 2011 y modificado el 11 de julio de 2012,

ACUERDA

Autorizar la presentación de la Tesis Doctoral a Dña. Eva Lloret Sevilla en la modalidad de compendio de publicaciones.

Contra el presente acuerdo, que no agota la vía administrativa, podrá formular recurso de alzada ante el Sr. Rector-Magnífico de la Universidad Politécnica de Cartagena, en el plazo de un mes a partir de la notificación de la presente.

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El Presidente de la Comisión de Doctorado

Fdo.: Pablo Fernández Escámez

Agradecimientos

Es tan gratificante como arduo recordar a las numerosas personas que, en el transcurso de estos últimos años, me han acompañado de una manera u otra en el desarrollo de esta Tesis Doctoral, y ante las que me siento profundamente agradecida.

Quisiera comenzar expresando mi agradecimiento a mi director de Tesis, el Dr. José Antonio Pascual, quien decidió abrirme las puertas a su grupo de investigación y sin el cual todo esto no hubiera sido posible. Gracias Beni por la confianza que has depositado en mí. Me gustaría hacer extensible este agradecimiento a aquéllas personas que han formado parte de su grupo y con las que he compartido tantos momentos. A la Dra. Margarita Ros, con la que comencé a trabajar en este grupo y la que me inició en el laboratorio. A Ainhoa, por los momentos compartidos al inicio de esta etapa, a Tina y a Noelia por enseñarme las técnicas de cultivo y por tantos medios vertidos, a Anabel, por su amistad y profesionalidad y por tantos viajes compartidos de Elche a Murcia, a Damián por todas esas risas (y algún que otro susto), a Marieta, gran compañera de piso y de laboratorio, a Jessi y a María José, por su inestimable ayuda en el procesamiento de las muestras, y, como no, especialmente, a mis dos grandes *parteners*, Rubén y Pepa. Gracias por haber sido los mejores compañeros de Tesis que alguien pueda desear; gracias por tantas conversaciones, por vuestro apoyo y por vuestra amistad. Os deseo lo mejor y realmente lo valéis.

Continuando con mis compañer@s del Cebas, también agradecer a todas aquellas personas que amenizaron el paso y el trabajo en la cuarta planta: a Isa, Carlos, Cristina, Sara, César, Carmen, Nuria, Lola, Eli, Keiji, Felipe, Josef, Gonzalo (gracias por tu apoyo estadístico), Anita (gracias por tu alegría), Irene, Pedro, Sara, etc. y como no, al Dr. Carlos García, por su disposición y apoyo.

No quisiera olvidarme de quienes, todavía en la UMH, me mostraron y contagiaron su entusiasmo por este raro mundo de la investigación: Jorge, Raúl, Alicia, Pilar,.. así como de mi tutor en la Universidad Politécnica de Cartagena, el Dr. Ángel Faz, por su ayuda durante la realización del doctorado y del DEA de esta Tesis.

Gracias también a todo el personal de DAM y de la EDAR de Molina de Segura que me atendió siempre tan amablemente y, especialmente, a la jefa de planta, Pilar Pradas.

También quisiera agradecer a José María Marín por los momentos compartidos de trabajo de campo en su finca del Albujón. Gracias por tu contagioso amor a la naturaleza.

Mi más sincera gratitud también a aquéllos que, sin conocerme apenas, me abrieron las puertas de su grupo en países ajenos brindándome la oportunidad de vivir experiencias excepcionales tanto profesional como personalmente. Mi más sincero agradecimiento al Dr. Eoin Brodie del Lawerence Berkeley National Laboratory y a toda la gente que allí me acompañó. A Clark por su alegría, su amistad y la música a últimas horas de la tarde. A Jenny por todo el tiempo robado para enseñarme a usar tanto equipo de última generación, a Krystle y a Ulas por ese 4 de julio y el viaje a Sonoma, a Ryan por su simpatía, a Nick por introducirme en el mundo de la bioinformática, y, especialmente, a Javi y a Eric por apoyarme y escuchar mis avatares con la biología molecular y por ser capaces, juntos, de encontrar las soluciones ante una cerveza en un bar.

También mil gracias a aquella gente externa al laboratorio que iluminó mi estancia de una u otra manera. A Bárbara y a Andy por la maravillosa convivencia, también a Poncho, y, como no a Lucía y a Sonia, a las que todavía echo de menos.

No menos agradecida me siento por las personas que me acompañaron durante mi estancia en el Institut für Mikrobiologie de la Universidad de Innsbruck. Mi profundo agradecimiento al Dr. Heribert Insam, quien me abrió tanto las puertas de su grupo como de su casa sin apenas conocerme, gran profesional y gran persona, fiel reflejo de que el trabajo duro no está reñido con la sonrisa. A María, Sabine y Marina por su gran acogida, su generosidad y por enseñarme a manejarme en Innsbruck tanto dentro como fuera del laboratorio, a Ingrid por esa tarta de cumpleaños, y como no, a mis compañeros de excursiones alpinas e incursiones urbanas, Antigoni y Luis.

Agradecer también a toda la gente que, en Murcia, me hizo sentir como en casa, a Isa (¡cuántos momentos compartidos!), Bea, Aziza, las Marías, Lucía, Domingo, Chules, Teresica, Michela, Eva, Javi, Elena, Esther, Jorge, etc. Gracias por haber estado ahí.

A aquellas maravillosas incondicionales alicantinas que siempre han estado presentes a pesar de mis idas y venidas; mis subidas y bajadas. Gracias a Elena, Berta (¡muchísimas gracias por la ayuda en la maquetación y por nuestras horas de trabajo compartidas!), Laura, Gema, Belén, Nata, Desi, Elen, Alis, Isaac (¡mil gracias por aquellos folios y esa impresora siempre lista!), Jaume, Carlos, gracias por estar ahí, por anclarme al mundo terrenal en los momentos más duros de esta etapa, y por vuestro inconmensurable apoyo en las últimas semanas.

A Marisa Rubio, jefa del Negociado de Postgrado y Doctorado de la Universidad Politécnica de Cartagena, por facilitar los tediosos trámites administrativos que acompañan a la etapa final de la presentación de esta Tesis.

Mi profunda gratitud a David, por su apoyo, por aguantar mis peores momentos y por su gran ayuda.

También gracias a los revisores externos de esta Tesis, cuya implicación a pesar de las prisas exigidas, ha permitido que esta Tesis obtenga la Mención de Doctorado Europeo.

Mi especial agradecimiento a Marta, por ese último aliento imprescindible, por su entusiasmo, apoyo y guía, que han sido el empuje necesario para terminar esta fase.

Muchísimas gracias también a Pablo. Gracias por tu optimismo, tu cariño y por hacer que todo parezca más fácil. Gracias por acompañarme y ayudarme en esas largas noches previas a la entrega de esta Tesis y por haber sido mis segundos ojos en la revisión de su formato.

Y por último, mi eterno agradecimiento a mi familia, a mis padres y a mi hermano, sin los cuales, nunca nada de esto hubiera sido posible. Gracias por vuestro apoyo incondicional. Gracias también a mis abuelos, que ojalá hubieran podido ver materializado este momento. A todos ellos, dedico este trabajo.

¡Mil gracias a tod@s!

Esta Tesis Doctoral ha sido realizada gracias a la financiación del programa "Junta para la Ampliación de Estudios" (Programa JAE) cofinanciado por el Consejo Superior de Investigaciones Científicas y el Fondo Social Europeo y el proyecto 324/pc08/2-04.3 incluido en el Programa Nacional de Ciencias y Tecnologías Medioambientales del Plan Nacional de I+D+i 2008-2011.

A mis padres y a mi hermano

Índice

Abreviaturas / Abbreviations

Principales abreviaturas utilizadas en esta Tesis Doctoral.

Abreviaturas	Inglés	Español
ATAD	Autothermal thermophilic aerobic digestion	Digestión aerobia autotérmica termófila
CEC (CIC)	Cation exchange capacity	Capacidad de intercambio catiónico
CLPP	Community level physiological profile	Perfil fisiológico a nivel de comunidad
C_{mic}	Microbial biomass carbon	Carbono de la biomasa microbiana
CSTR	Continuous stirred-tank reactor	Reactor de tanque con agitación continua
BOD (DBO)	Biochemical oxygen demand	Demanda bioquímica de oxígeno
CFU (UFC)	Colony-forming units	Unidades formadoras de colonias
COD (DQO)	Chemical oxygen demand	Demanda química de oxígeno
DGGE	Denaturing gradient gel electrophoresis	Electroforesis en gel con gradiente de desnaturalización
EC (CE)	Electrical conductivity	Conductividad eléctrica
EPA	Environmental Protection Agency	Agencia de protección medioambiental
EU (UE)	European Union	Unión Europea
LAS	Linear alkylbenzene sulfonate	Sulfonato de alquilbenceno lineal
MAD	Mesophilic anaerobic digestion	Digestión mesófila anaerobia
NPE	Nonylphenol y nonylphenolethoxylate	Nonilfenol y nonilfenol etoxilato
NPQ	Non-photochemical quenching	Quenching no fotoquímico
OLR (VCO)	Organic loading rate	Velocidad de carga orgánica
OTU (UTO)	Operational taxonomic units	Unidades taxonómicas operacionales
PAH	Polycyclic aromatic hydrocarbon	Hidrocarburo aromático policíclico
PCA	Principal component analysis	Análisis de componentes principales
PCB	Polychlorinated biphenyl	Bifenilo policlorado
PCDD	Polychlorinated dibenzodioxin	Dibenzodioxina policlorada
PCDF	Polychlorinated dibenzofuran	Dibenzofurano policlorado
PCR	Polymerase chain reaction	Reacción en cadena de la polimerasa

QIIME	Quantitative insights into microbial ecology	Conocimientos cuantitativos en ecología microbiana
RCC	Relative chlorophyll content	Contenido relativo de clorofila
SRT (TRH)	Sludge retention time	Tiempo de retención hidráulico
TAnD	Thermophilic anaerobic digestion	Digestión anaerobia termófila
TOC (COT)	Total organic carbon	Carbono orgánico total
TOM (MOT)	Total organic matter	Materia orgánica total
TS (ST)	Total solids	Sólidos totales
VFA (AGV)	Volatile fatty acids	Ácidos grasos volátiles
VS (SV)	Volatile solids	Sólidos volátiles
VSD	Volatile solids destruction	Destrucción de sólidos volátiles
WWTP (EDAR)	Waste water treatment plant	Estación depuradora de aguas residuales

I

I. Interés del trabajo, objetivos y estructura de la tesis

Work relevance, objectives and thesis outline

I. Interés del trabajo, objetivos y estructura de la tesis

La producción de lodos de depuradora ha experimentado un elevado incremento en los últimos años debido tanto al aumento del volumen de las aguas depuradas en EDARs, como a la cada vez más restrictiva legislación aplicada a los efluentes (MAPA, 2003). En nuestro país, la generación de lodos se incrementó en un 41,2% en el periodo 2000-2009 con una producción en este último año, de 1.205.124 toneladas de materia seca. El 82,6% de estos lodos fue empleado como enmienda orgánica en agricultura, mientras que el 7,9% se depositó en vertedero y el 5,1% fue incinerado con recuperación de energía (MARM, 2011).

El uso agrícola de los lodos de depuradora, recomendado por la Directiva Europea 91/271/EEC sobre el tratamiento de aguas residuales urbanas, es de especial interés en la región Mediterránea. En esta zona, la acuciante degradación que han venido sufriendo los suelos, reduciendo tanto el contenido en materia orgánica como la fertilidad natural de los mismos, los hace especialmente vulnerables (García et al., 2000).

La aplicación agrícola de lodos de depuradora ha sido intensamente estudiada en los últimos años, demostrando grandes beneficios sobre las propiedades físicas y químicas del suelo (Korentajer, 1991; Barzegar et al., 2002), una mejora en su fertilidad, y un aumento en la producción agrícola (Kelley et al., 1984; Min-Jian, 1997; Singh y Agrawal, 2008). La adición de materia orgánica, también ha demostrado tener efectos sobre las comunidades microbianas del suelo, aumentando, por lo general, su desarrollo y actividad (Bailey y Lazarovits 2003). Estos cambios pueden ser producidos tanto directamente por la adición de microorganismos exógenos procedentes de la materia orgánica añadida, o indirectamente debido a cambios en el ambiente de las comunidades microbianas autóctonas (Perucci, 1992; García et al., 1998; García-Gil et al., 2000).

Sin embargo, el uso agrícola de lodos también puede entrañar riesgos no deseables debido a su potencial contenido de metales pesados, compuestos tóxicos y/o microorganismos patógenos como bacterias, virus, helmintos, etc. (Beuchat, 1996), que pueden suponer un riesgo para la salud humana, animal o medioambiental.

Con el objeto de minimizar estos riesgos, y garantizar la seguridad del uso agrícola de los lodos, la Unión Europea está redactando una nueva legislación sobre aplicación agrícola de lodos de depuradora a través de la "Propuesta de Directiva del Parlamento Europeo y el Consejo sobre el uso agrícola de lodos" (Comisión Europea, 2003, que reemplazará a la legislación vigente; la Directiva 86/278/EEC, relativa a la protección del medio ambiente y en particular de los suelos en la utilización de los lodos con fines agrícolas). Entre las nuevas modificaciones que propone la futura Directiva Europea, uno de los parámetros a destacar es la evaluación del contenido de microorganismos patógenos, distinguiendo entre tratamientos convencionales y avanzados de lodos de depuradora según los niveles de estos microorganismos obtenidos tras el proceso de estabilización. Los tratamientos avanzados, en contraposición con los convencionales, permiten menores restricciones en el uso y manejo de los lodos estabilizados (Comisión Europea, 2003).

En este contexto, el objetivo general de la presente memoria consistió en el estudio de dos de los tratamientos avanzados propuestos en esta futura Directiva Europea, con el fin de evaluar el proceso de estabilización e higienización de los lodos. Los tratamientos fueron: (i) la digestión aerobia autotérmica termófila (ATAD), y (ii) la digestión anaerobia termófila (TAnD). Asimismo, también incluyó el estudio de los efectos de las características del proceso de estabilización en la comunidad microbiana de los lodos. Finalmente, los efectos sobre el suelo y las plantas de la aplicación agrícola de un lodo avanzado, en comparación con uno convencional, fueron estudiados.

Para alcanzar este objetivo general, los objetivos específicos que se plantearon fueron:

i. La puesta en marcha de un digestor ATAD con un volumen efectivo de 15 m^3 con el fin de caracterizar el proceso de estabilización de lodos y evaluar la reducción de los microorganismos patógenos contemplados en la futura Directiva Europea (*Salmonella* spp., *Escherichia coli*, y esporas de *Clostridium perfringens*).

ii. La puesta en marcha de un digestor TAnD con un volumen efectivo de 15 m^3 con el fin de caracterizar el proceso de estabilización de lodos y evaluar la reducción de los microorganismos patógenos contemplados en la futura Directiva Europea (*Salmonella* spp., *Escherichia coli*, y esporas de *Clostridium perfringens*), en relación con una digestión convencional mesófila anaerobia (MAD).

iii. Propuesta de un sistema de digestión en dos etapas con el fin de obtener un lodo avanzado según los criterios microbiológicos de la futura Directiva Europea.

iv. Evaluación de la influencia del proceso de estabilización de los lodos de depuradora sobre la comunidad microbiana de los mismos para determinar la posible incidencia en la microbiota del suelo, comparando un tratamiento de digestión avanzado ATAD con uno convencional MAD.

v. Estudio del efecto de la aplicación agrícola de un lodo avanzado ATAD sobre la estructura y funcionamiento de la comunidad microbiana del suelo, las propiedades químicas del suelo, y la influencia en un cultivo de melón, en comparación con un lodo convencional MAD.

La presente Tesis Doctoral está estructurada en diez capítulos de la siguiente manera: el capítulo I aborda el interés del trabajo y los objetivos, en el capítulo II se describe una introducción general, los cuatro siguientes capítulos componen el cuerpo principal de la memoria y se corresponden a cuatro publicaciones independientes, y, finalmente, los últimos cuatro capítulos corresponden a las conclusiones generales, el resumen, la bibliografía (del interés del trabajo, de la introducción y del resumen) y el apéndice. Los cuatro capítulos presentados como publicaciones, siguen una secuencia lógica para abordar los objetivos específicos descritos anteriormente. En los capítulos III y IV se estudia la puesta marcha y funcionamiento de un digestor ATAD y TAnD, respectivamente. A continuación, en el capítulo V se propone un proceso de digestión de lodos en dos fases para cumplir con los estándares microbiológicos de la futura Directiva Europea. Por último, el capítulo VI aborda el estudio de la

influencia del proceso de estabilización de los lodos sobre la comunidad microbiana de los mismos, y evalúa los efectos de la aplicación agrícola de un lodo avanzado respecto a uno convencional.

I. Work relevance, objectives and thesis outline

The production of sewage sludge has increased significantly during the past few years due to the expansion of wastewater treatment and tougher effluent restrictions (MAPA, 2003). In Spain, sewage sludge production increased by 41.2% in the period 2000-2009, with a production of 1,205,124 tons of dry matter in 2009. 82.6% of the sludge produced was used as an organic fertilizer in agriculture, 7.9 % was sent to landfills and 5.1% was incinerated with energy recovery (MARM, 2011).

Agricultural use of sewage sludge, which is encouraged by the European Directive 91/271/EEC on urban wastewater treatment, is of special interest in the Mediterranean region. In this area, the strong degradation that soils have been subjected to, have reduced both soil organic matter content and natural fertility, turning them particularly vulnerable (García et al., 2000).

Agricultural application of sewage sludge has been widely studied in recent years showing great benefits on physical and chemical properties of soil (Korentajer, 1991; Barzegar et al., 2002), an improvement of soil fertility and an increase in crop yield (Kelley et al., 1984; Jian-Min, 1996, Singh and Agrawal, 2008). The addition of organic matter affects soil microbial communities, generally, by accelerating microbial development and activity (Bailey and Lazarovits 2003). The changes imposed by the organic amendments can be induced either directly through the addition of exogenous microorganisms, or indirectly through changes in the environment of the indigenous communities (Perucci, 1992; García et al., 1998; García-Gil et al., 2000).

However, the agricultural use of sewage sludge may have some undesirable risks, associated with its potential content of heavy metals, toxic compounds and/or pathogens such as bacteria, viruses and parasites (Beuchat, 1996), which may pose risk to human , animal or environmental health.

In order to minimize these risks, and to ensure a safe agricultural use of sewage sludge, the European Union is developing a new legislation regarding land application of sewage sludge through the “Proposal for a Directive of the European Parliament and of the Council on spreading of sludge on land”

(European Commission, 2003), which will replace the existing legislation: Directive 86/278/EEC on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. This provides stricter standards for the content of heavy metals, organic compounds and human pathogens, this latter parameter receiving special attention. In this respect, the Proposal for a European Directive introduces the concept of advanced and conventional treatments, which allow operators to use advanced treated sludge with fewer restrictions compared with a sludge that has been treated conventionally (European Commission, 2003).

In this scenario, the general objective of this thesis consisted of the study of two advanced treatments described in this Proposal for a European Directive, with the aim of assessing the stabilization process and sludge sanitation. The studied treatments were: (i) autothermic thermophilic aerobic digestion (ATAD), and (ii) thermophilic anaerobic digestion (TAnD). Likewise, the effects of the stabilization process on sludge microbial community were assessed. Finally, the effects of the agricultural use on soil and plants of an advanced treated sludge compared to a conventional sludge were evaluated.

To achieve this general objective, the following specific objectives were approached:

i. To start-up an ATAD digester with an effective volume of 15 m^3 with the aim of studying the stabilization process of sewage sludge and of evaluating the removal of pathogens included in the future European Directive (*Salmonella* spp., *Escherichia coli*, and *Clostridium perfringens* spores).

ii. To start-up a TAnD digester with an effective volume of 15 m^3 in order to study the stabilization process of sewage sludge and to evaluate the reduction of pathogens included in the European Directive future (*Salmonella* spp., *Escherichia coli*, and *Clostridium perfringens* spores) compared with a conventional mesophilic anaerobic digestion (MAD).

iii. To propose a 2-stage sludge digestion process with the aim of obtaining an advanced treated sludge according to the microbial requirements of the future European Directive.

iv. To evaluate the influence of the stabilization process of sewage sludge on sludge microbial community to determine its potential influence on soil microbiota, comparing an advanced ATAD treatment with a conventional MAD treatment.

v. To study the effects of agricultural application of an advanced ATAD sludge on the structure and performance of soil microbial communities, soil chemical properties and its influence on a melon crop compared to a conventional MAD sludge.

This Ph.D. dissertation is structured in ten chapters as follows: chapter I refers to the relevance of the work and the aims of the study, chapter II describes the general introduction of this research, and the following four chapters form the main body of the present thesis and correspond to four independent publications. Finally, the four last chapters consist of the general conclusions, the summary, the references (of work relevance, general introduction and summary) and the appendix. The four main chapters written as independent publications follow a logic sequence to achieve the above-mentioned specific objectives. Chapters III and IV, focus on the start-up and stabilization process and sanitation of sewage sludge after ATAD and TAnD digestion, respectively. Chapter V provides a proposal of a 2-stage digestion system to fulfill the microbial standards of the future European Directive. Finally, chapter VI explores the influence of the sludge stabilization process on sludge microbial communities as well as evaluates the agricultural use of an advanced sludge compared to a conventional sludge.

II

II. Introducción general

II. Introducción general

1. Depuración de aguas y lodos de depuradora

1.1. Definición de lodos de depuradora

Los lodos de depuradora son el residuo final que queda tras el proceso de depuración de las aguas y la posterior estabilización del fango. Por tanto, los lodos o fangos de depuradora pueden definirse como cualquier sólido, semisólido o líquido de desecho generado por una planta municipal, comercial o industrial de tratamiento de aguas residuales, de aguas de consumo o instalaciones de control de la contaminación atmosférica u otra clase de desechos de similares características y efectos (Bueno et al., 1997).

1.2. Tratamiento de aguas residuales y tipos de lodos de depuradora

El tratamiento de las aguas residuales, ya sean industriales o urbanas, consiste en la aplicación de unos procesos básicos u operaciones unitarias cuya secuencia y utilización vienen definidas por el grado de depuración a alcanzar, las características del agua a tratar y el coste de las instalaciones. A continuación se describen los procesos básicos de la línea de aguas:

Pretratamiento

El pretratamiento de las aguas residuales consiste en determinadas operaciones físicas y mecánicas con el fin de eliminar los constituyentes de las aguas residuales cuya presencia pueda provocar problemas de mantenimiento y funcionamiento de los diferentes procesos, operaciones y/o sistemas auxiliares. Ejemplos de pretratamiento serían el desbaste y la dilaceración para la eliminación de sólidos gruesos y trapos, la flotación para la eliminación de grasas y aceites, y el desarenado para la eliminación de la materia en suspensión gruesa que pueda causar obstrucciones en los equipos y un desgaste excesivo de los mismos.

Tratamiento primario

En el tratamiento primario se elimina una fracción de sólidos en suspensión y de materia orgánica de las aguas residuales mediante procesos físicos o químicos, con el objetivo de realizar un acondicionamiento previo del agua antes de su entrada al tratamiento secundario. La sedimentación y la

flotación son los procesos más usados en este tratamiento, con las que se obtiene la eliminación del 50-70% y 25-40% de los sólidos en suspensión y de la demanda biológica de oxígeno (DBO) respectivamente. El fango que se acumula en el fondo del decantador primario forma los **lodos de decantación primaria**.

Tratamiento secundario (convencional)

El tratamiento secundario de las aguas residuales consiste en la transformación de la materia orgánica biodegradable, no separable por procesos físico-químicos, en materia celular decantable y productos finales mediante procesos biológicos. Este tratamiento incluye diversos procesos como los fangos activos, los reactores de lecho fijo, los procesos de biofiltración y la sedimentación. Mediante la floculación, se forman flóculos de materia orgánica, materia viva y materia inorgánica que se depositan en el fondo del decantador secundario formando los **lodos de decantación secundaria**. Estos lodos son ricos en microorganismos **(lodos activos**), por lo que parte de ellos se reciclan para activar la degradación microbiana de las aguas residuales después de la decantación primaria (**lodos activos de retorno**). El resto suele mezclarse con los lodos primarios formando los **lodos mixtos frescos**.

Tratamiento terciario o avanzado

Este tratamiento constituye un nivel de tratamiento adicional para la eliminación de constituyentes de las aguas residuales que merecen especial atención (nutrientes, compuestos tóxicos, excesos de materia orgánica o sólidos en suspensión), permitiendo obtener una mayor calidad de efluente que la lograda con los tratamientos primarios y secundarios. Se lleva a cabo mediante procesos biológicos y físico-químicos de alto rendimiento. Además de los procesos de eliminación de nutrientes, se utilizan procesos como la coagulación-floculación, o la sedimentación seguida de filtración. Para la eliminación de iones específicos y para la reducción de sólidos disueltos, se emplean métodos menos comunes, como el intercambio iónico y la ósmosis inversa. Como producto de este tratamiento se obtiene los **lodos terciarios**.

Los lodos generados en las estaciones depuradoras de aguas residuales (EDARs), pueden proceder, pues, tanto de procesos de tratamiento primario, como secundario o terciario. Estos lodos serán posteriormente tratados

obteniendo los **lodos digeridos o estabilizados**. Las características de estos lodos dependerán del uso y tratamiento que se ha dado a las aguas, y de los tratamientos a los que posteriormente hayan sido sometidos (Milieu et al., 2010).

1.3. Tratamiento de lodos de depuradora

Los lodos poseen un elevado contenido en agua, una alta capacidad de fermentación y una elevada carga patógena, por lo que deben ser tratados a fin de poder ser manipulados más fácilmente y de evitar problemas ambientales y para la salud, en su posterior eliminación o utilización. Los objetivos buscados en el tratamiento de lodos son por tanto la **reducción de volumen**, la **reducción de la carga patogénica**, la **estabilización de la materia orgánica** y la **disminución de la generación de olores**. A continuación se describen las diferentes etapas del proceso de tratamiento de los lodos:

Espesamiento

El espesamiento es un procedimiento físico que se emplea para aumentar el contenido de sólidos del lodo mediante la eliminación de parte de la fracción líquida del mismo, consiguiendo reducir entre una y cuatro veces el volumen inicial del lodo. La concentración típica de sólidos secos totales de un lodo primario oscila entre el 2-8%, mientras que la de un lodo secundario presenta un intervalo típico de 0.83-1.16% (Metcalf y Eddy, 1995). La reducción del volumen de fango resulta beneficiosa para los posteriores procesos de tratamiento tales como la digestión, deshidratación, secado y combustión. Esta etapa puede realizarse antes o después de la estabilización. Existen distintos modos de espesado atendiendo al modo de acción. Los más utilizados son: por gravedad, filtros banda por gravedad, por flotación, por centrifugación y por tambor rotativo.

Estabilización

La estabilización del fango se lleva a cabo para reducir la presencia de patógenos, reducir el contenido en materia orgánica, eliminar olores desagradables, e inhibir, reducir o eliminar su potencial de putrefacción (Carballa, 2005). Este proceso consiste en una descomposición rápida que puede desarrollarse mediante diversas técnicas como la estabilización química; entre la

que destaca el uso de cal viva, biológica; que incluye la digestión aerobia, anaerobia y el compostaje, y térmica.

Acondicionamiento

El acondicionamiento es un proceso físico-químico cuyo principal objeto es la ruptura de la estabilidad coloidal para liberar parte del agua ligada a los coloides preparándolos para la fase posterior de deshidratación que ha de permitir obtener un producto relativamente sólido o semisólido. Los procedimientos básicos utilizados son el acondicionamiento químico, térmico o físico (elutriación).

Deshidratación

El objetivo de esta etapa es la obtención de un material semisólido más fácil de manejar en su utilización posterior. Esta operación puede realizarse por métodos naturales (eras de secado y lagunas) o mecánicos (filtración a vacío, filtración con banda prensora, filtración a presión y centrifugación).

Desinfección

La desinfección del fango está adquiriendo gran importancia como proceso adicional debido al desarrollo de normativas cada vez más estrictas respecto a la reutilización del mismo con fines agrícolas. Entre los medios para conseguir la higienización de los lodos figuran algunos de los procesos de estabilización descritos anteriormente (estabilización con cal, tratamiento térmico y digestión termófila tanto anaerobia como aerobia). Asimismo, se puede utilizar la desinfección por radiación de alta energía, la adición de productos químicos (cloro, principalmente), la pasteurización o el compostaje. El Gráfico 1 muestra un esquema de una línea convencional de tratamiento de aguas y lodos.

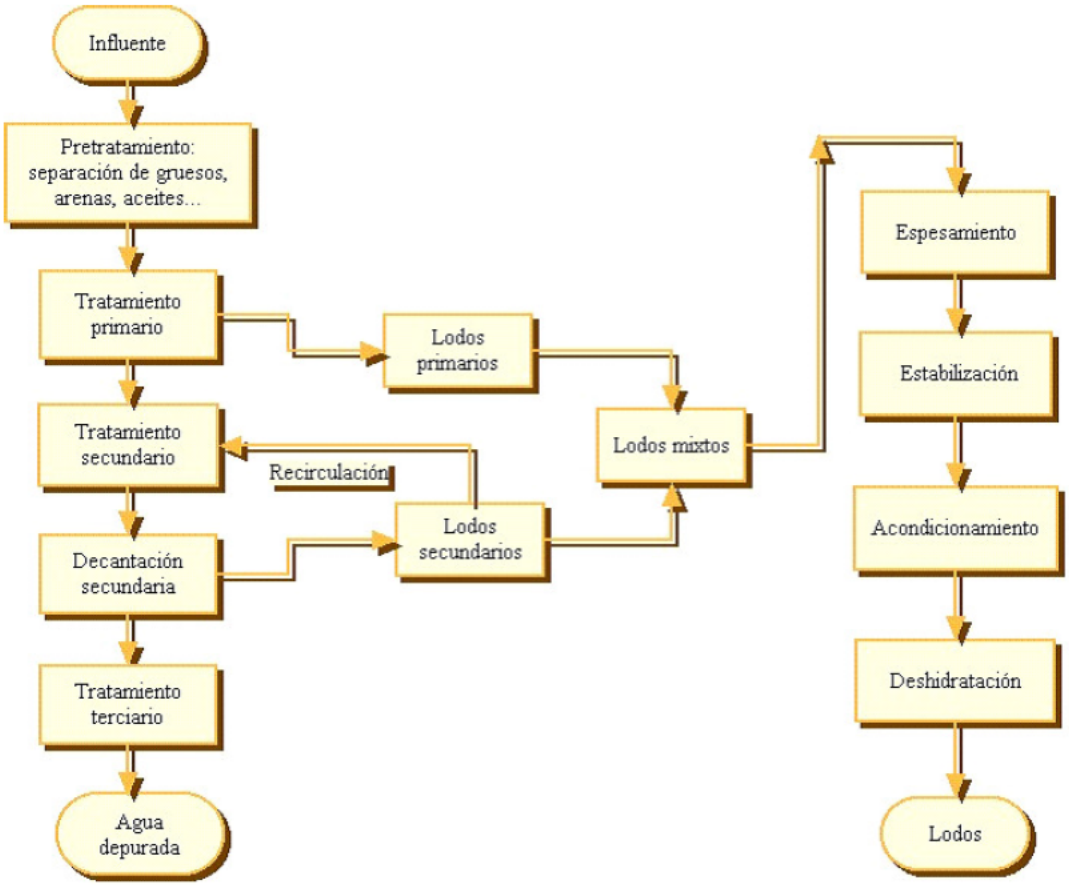

Gráfico 1. Esquema de una línea convencional de tratamiento de aguas y lodos. Adaptado de De San Pedro Manzanera, 2007.

2. Estabilización biológica de lodos de depuradora

A continuación se describen los tratamientos de digestión aerobia y anaerobia por ser los de mayor relevancia en esta Tesis Doctoral.

2.1. Digestión aerobia

La digestión aerobia consiste en la aireación prolongada de los lodos frescos para provocar el desarrollo de microorganismos aerobios hasta sobrepasar el periodo de síntesis celular y llevar a cabo su autooxidación. Durante el proceso de aireación, los microorganismos metabolizan la materia orgánica presente en el lodo convirtiéndola en materia celular. Cuando el sustrato orgánico se agota, los microorganismos empiezan a consumir su propio protoplasma (respiración endógena) a fin de obtener energía para las reacciones de mantenimiento de las células. El tejido celular es oxidado de forma aeróbica a dióxido de carbono (CO_2), agua (H_2O), y amoníaco (NH_3), que será oxidado

posteriormente a nitratos (NO_3^-). A continuación se describen las principales ventajas e inconvenientes de la digestión aerobia:

Tabla 1. Ventajas e inconvenientes de la digestión aerobia. Adaptado de Mahamud et al., 1996.

Ventajas	Incovenientes
Bajo coste inicial, sobre todo para pequeñas instalaciones.	Menor reducción de sólidos volátiles que en la digestión anaerobia.
Sobrenadante menos problemático que el de los procesos anaerobios.	Altos costes energéticos asociados al suministro de oxígeno.
Control de operación simple.	Proceso muy sensible a la temperatura.
Amplio intervalo de aplicación.	Mayor dificultad de deshidratación del fango.
Poca generación de olores con diseño y operación adecuados.	Puede precisar la adición de álcali para reducir la bajada de pH.
Reducción de la masa total del lodo	Pueden producirse espumas.
	Posibilidad de dispersión de patógenos por medio de aerosoles.
	Aplicable generalmente a EDARs de tamaño reducido (< 17.000 m^3/día).

El proceso de digestión aerobia puede representarse por las siguientes reacciones:

Etapa 1. Oxidación

$$COHNS + O_2 + \text{microorganismos} \longrightarrow CO_2 + NH_3 + \text{prod. intermedios} + \text{energía}$$

(materia orgánica)

La energía producida en esta etapa es utilizada por los microorganismos aerobios para la síntesis de nuevos microorganismos.

Etapa 2. Síntesis

$$COHNS + O_2 + \text{microorganismos} + \text{energía} \longrightarrow C_5H_7NO_2$$

(materia orgánica) (nuevos microorganismos)

Estos dos procesos pueden desarrollarse simultáneamente. En ausencia de materia orgánica que degradar, los microorganismos entran en fase de respiración endógena.

Etapa 3. Respiración endógena

$$C_5H_7NO_2 + 5O_2 \longrightarrow 5CO_2 + NH_3 + 2H_2O + \text{energía.}$$

$$NH_3 + 2O_2 \longrightarrow HNO_3 + H_2O \longrightarrow NO_3 + H^+ + H_2O$$

Cabe destacar cuatro procesos de digestión aerobia:

• **Digestión aerobia convencional**: tiene lugar a temperatura ambiente y consiste en airear los lodos en periodos variables de 15 a 50 días obteniéndose así un producto prácticamente estabilizado, con reducciones de sólidos volátiles (VSD) del 35 al 45%.

• **Digestión aerobia con oxígeno puro**: modificación del proceso de digestión aerobia convencional en la que se utiliza oxígeno puro en lugar de aire. El fango resultante y los caudales de sobrenadantes a recircular son similares a los producidos en la digestión aerobia convencional. Está especialmente indicada para climas fríos, debido a su relativa insensibilidad a los cambios de temperatura del aire ambiente gracias al aumento de la actividad biológica y a la naturaleza exotérmica del proceso.

• **Digestión aerobia autotérmica termófila (ATAD)**: este proceso utiliza el calor metabólico producido por la biodegradación aerobia de la materia orgánica del lodo, alcanzándose temperaturas superiores a 50°C (Juteau, 2006). El éxito de este procedimiento depende fundamentalmente de que exista una alta proporción de materia orgánica oxidable y de que se limiten al máximo las posibles pérdidas de calor en el tanque de digestión. Entre las ventajas de la digestión aerobia termófila, se encuentran altas VSD con tiempos de residencia (SRT) muy cortos. Como ventaja adicional, puede citarse la importante destrucción de patógenos producida como consecuencia de las altas temperaturas (Smith, 1996).

• **Digestión aerobia criofílica**: se trata de la digestión aerobia de fangos a temperaturas por debajo de 20 °C y es utilizada en plantas prefabricadas de pequeñas dimensiones ubicadas en zonas de clima frío.

2.2. Digestión anaerobia

La digestión anaerobia es una de las tecnologías más ampliamente utilizadas en todo el mundo para la estabilización de lodos (Ray et al., 1990; Metcalf y Eddy, 1991). Las ventajas e inconvenientes de los sistemas de digestión a anaerobia se presentan en la Tabla 2.

Tabla 2. Ventajas e inconvenientes de la digestión anaerobia. Adaptado de Mahamud et al., 1996.

Ventajas	Incovenientes
Importante VSD (entre un 40 y un 60%).	Potencial producción de olores.
Proceso excedentario en energía.	Posibilidad de depósitos minerales en el equipo, dificultades de limpieza y posible formación de espumas.
Bajos costes de operación si se recupera el metano producido.	Puede presentar problemas de "digestión ácida" ya que los microorganismos productores de metano son de crecimiento lento.
Lodos utilizables para la agricultura, pudiendo aplicarse generalmente en mayor cantidad que los obtenidos mediante digestión aerobia.	Peligrosidad de los gases inflamables producidos.
Relativa buena reducción del número de microorganismos patógenos.	Elevado volumen de inversión para llevar a cabo su instalación.
Reducción de la masa total del lodo.	Presenta sobrenadantes con elevadas DBO, DQO, sólidos en suspensión y NH_3.
Es el método más rentable económicamente para plantas que traten por encima de 7.500 m^3/día y se puede aplicar a plantas cuyo intervalo de tamaño abarca más de dos órdenes de magnitud.	

En el proceso de digestión anaerobia, la materia orgánica biodegradable es asimilada en ausencia de oxígeno por un consorcio de microorganismos que forman una cadena trófica compleja y equilibrada operando de forma consecutiva y sinérgica. Los productos finales obtenidos son metano (CH_4), dióxido de carbono (CO_2), ácido sulfhídrico (H_2S) y trazas de otros gases. A este conjunto de gases se le denomina biogás por su origen biológico. La digestión anaerobia consta de las siguientes etapas:

Etapa 1. Hidrólisis

COHNS + microorganismos ⟶ Prod. intermedios + energía
(materia orgánica)

Productos intermedios: aminoácidos, azúcares, ácidos grasos, alcoholes, etc.
Actúan bacterias hidrolíticas.

Etapa 2. Acidogénesis

Prod. intermedios + microorganismos ⟶ Ácidos orgánicos, alcoholes, cetonas, + H_2 + CO_2 + energía

Actúan bacterias fermentativas acidogénicas.

Etapa 3. Acetogénesis

Prod. intermedios + microorganismos ⟶ Ácido acético + H_2 + CO_2 + energía

Actúan bacterias acetogénicas.

Etapa 4. Metanogénesis

$$\text{Ácido acético} + H_2 + CO_2 + \text{microorganismos} \longrightarrow CH_4 + CO_2 + H_2O + \text{energía}$$

Actúan bacterias metanógenas.

La energía obtenida mediante las reacciones anteriores es utilizada para la síntesis celular:

$$\underset{\text{(materia orgánica)}}{CONHS + \text{microorganismos} + \text{energía}} \longrightarrow \underset{\text{(nuevos microorganismos)}}{C_5H_7NO_2}$$

En ausencia de materia orgánica que degradar los microorganismos entran en la fase de respiración endógena:

$$C_5H_7NO_2 \longrightarrow CO_2 + CH_4 + H_2O + NH_3 + \text{energía}$$

El proceso de digestión anaerobia se realiza en tanques cerrados mediante distintos métodos, tales como:

• **Digestión mesófila o convencional**: los microorganismos mesófilos actúan a temperaturas entre 12 °C y 35 °C, optimizándose el proceso entre 29 °C y 33 °C. Se suele llevar a cabo en una sola fase. Las funciones de digestión, espesado de fangos y formación de sobrenadante, se llevan a cabo de forma simultánea.

• **Digestión de una fase y alta carga**: difiere del proceso convencional de una fase en que la carga orgánica es mucho mayor. El fango se mezcla mediante recirculación de gas, mezcladores mecánicos, bombeo, o mezcladores con tubos de aspiración (no se produce la separación de espumas y sobrenadantes). Se calienta para conseguir optimizar la velocidad de destrucción de la materia orgánica.

• **Digestión en dos fases**: en muchas ocasiones, un digestor de alta carga se combina en serie con un segundo tanque de digestión. El primer tanque se utiliza para la digestión y se equipa con dispositivos para el mezclado. El segundo tanque

se utiliza para el almacenamiento y concentración del fango digerido y para la formación de un sobrenadante relativamente clarificado.

• **Digestores independientes**: algunos diseños recientes separan la digestión del fango primario de la del fango biológico y, en algunos casos, la digestión de este último no se realiza por vía anaerobia sino en condiciones aerobias.

• **Digestión termófila (TAnD)**: los microorganismos termófilos trabajan a temperaturas comprendidas entre los 37 °C y 65 °C, con un óptimo en las proximidades de los 55 °C. Entre las ventajas de la digestión termófila se encuentra: una mayor destrucción de patógenos, menores tiempos de retención, una mayor producción de biogás y una mejora de las características de deshidratación del fango (Rimkus et al., 1982; Carrington et al., 1991; Peddie et al., 1996). Los inconvenientes que presentan son mayores necesidades energéticas para el calentamiento, peor calidad de sobrenadante y menor estabilidad del proceso (Bueno et al., 1997).

3. Producción de lodos de depuradora

En los últimos años, el aumento progresivo de la población mundial y de las actividades productivas relacionadas con el agua, han disparado el consumo de ésta. Unido a este aumento del consumo de agua, se encuentra el incremento de las actividades cuyos vertidos y productos residuales provocan la contaminación y degradación de las aguas, lo que ha provocado la necesidad de establecer un tratamiento de las mismas para su posterior reutilización ya que éstas constituyen un recurso natural cada vez más escaso. Este aumento en el tratamiento y depuración de las aguas residuales, y la cada vez más restrictiva legislación aplicable, ha provocado un aumento considerable en la producción de lodos de depuradora (MAPA, 2003).

En la Unión Europea (UE-27), la producción de lodos se sitúa en una media anual en torno a diez millones de toneladas de materia seca (Milieu et al., 2010). Los datos existentes respecto a los Estados Unidos, sitúan la producción de lodos en seis millones y medio de toneladas de materia seca anuales (Kalogo y Monteith, 2008). Por otro lado, debido la construcción de un gran número de nuevas plantas de tratamiento de aguas residuales en China, la producción de

lodos ha superado allí los veintidós millones de toneladas de materia seca anuales (Chinadialogue, 2012).

Respecto a la producción de lodos en España, hay que señalar que ha experimentado un incremento continuo en los últimos años, aumentando su producción en el periodo 2000-2009 en un 41,2%. En el año 2009 se generaron en España 1.205.124 toneladas de lodos procedentes de instalaciones de depuración, un 2,4% más que en 2008. Esta tendencia se prevé que continúe al alza ya que la Directiva 91/271/EEC sobre tratamiento de aguas residuales urbanas, transpuesta a la legislación española a través del Real Decreto-ley 11/1995 de 28 diciembre, obliga a la depuración de aguas urbanas de poblaciones de más de 2.000 habitantes equivalentes que viertan en aguas continentales (MAPA, 2003; MARM, 2011). En el Gráfico 2 se observa la producción de lodos en España durante el periodo 2000-2009.

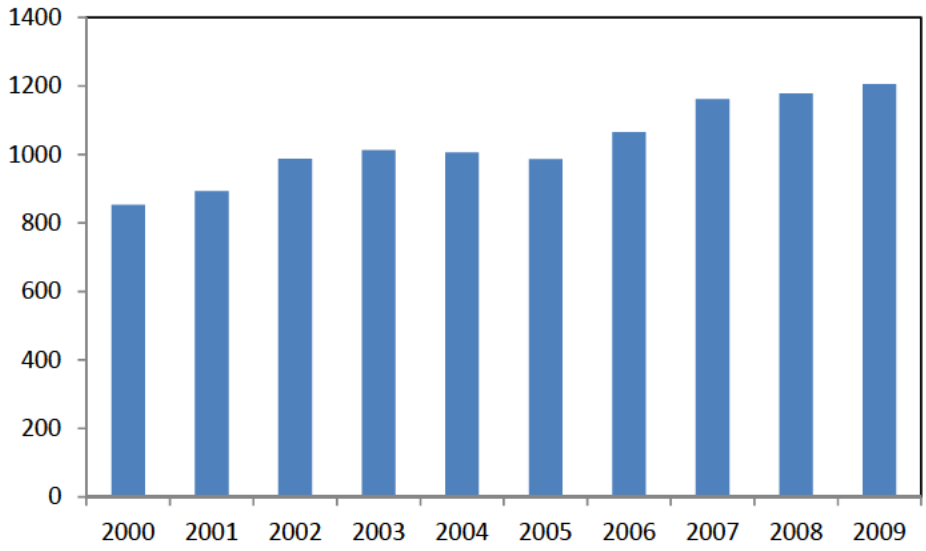

Gráfico 2. Producción de lodos en España (miles de toneladas de materia seca). Fuente: MARM, 2011.

4. Destino final de los lodos de depuradora

El destino final de los lodos de depuradora está estrechamente ligado a las disposiciones legales vigentes cuyo objetivo es minimizar los potenciales impactos medioambientales. Los principales destinos de los lodos de depuradora son:

Uso agrícola

El elevado contenido de materia orgánica que poseen los lodos, así como las considerables cantidades de macronutrientes, especialmente de nitrógeno y fósforo, los convierten en unos valiosos fertilizantes orgánicos en agricultura. Esta opción supone un doble ahorro de energía; por una parte, la que se emplea para eliminar los lodos, y por otra, la necesaria para obtener fertilizantes de síntesis (Wong y Su, 1997), además de transformar un desecho en un nuevo recurso.

Por otro lado, los lodos también pueden utilizarse como acondicionadores de suelos, lo cual permitirá a largo plazo mejorar las propiedades físicas de los mismos, contribuyendo a reducir su erosión, así como para la recuperación de zonas marginales improductivas y la regeneración de suelos forestales (Guidi et al., 1990; Logan y Harrison, 1995). Otra opción es su utilización como substrato para la producción de plantas ornamentales y hortícolas (Pérez, 1999), para el establecimiento de suelos sobre escorias de minas (Moreno, 1997), la recuperación de suelos quemados (Villar et al., 1998) y en silvicultura (Roldán et al., 1996). El marco legal del uso agrícola de los lodos se describe detalladamente en el último apartado de esta Introducción.

Depósito en vertedero

Es un sistema de eliminación definitivo aunque precisa de zonas adecuadas. Existe una tendencia a reducir la cantidad de materia orgánica a introducir en vertederos para promocionar el reciclaje de la misma y minimizar los lixiviados, emisiones de gases contaminantes y los problemas de gestión que estos provocan. El vertido de residuos está regulado por el Real Decreto 1481/2001, de 27 de diciembre, por el que se regula la eliminación de residuos mediante depósito en vertedero, que incorpora al derecho interno la Directiva 1999/31/EEC, relativa al vertido de residuos.

Incineración

La incineración de los lodos resulta poco interesante pues supone un gran desperdicio energético importante y además se debe buscar un destino para las cenizas resultantes y evitar los problemas ocasionados por las emisiones de gases (Wong et al., 2001). Tecnologías que incluyen la oxidación termal, como la

pirólisis están siendo utilizadas como alternativa a la combustión normal (Benabdallah, 2006). La incineración de residuos está regulada por la Directiva 2000/76/EEC, que se traspone al reglamento español a través del Real Decreto 653/2003, de 30 de mayo.

Recuperación de energía

Una alternativa que recibe especial interés, es la consideración de los lodos como punto de partida para la obtención de metano, fuel e hidrocarburos en general. También se está utilizando el lodo como combustible auxiliar en ciertas instalaciones de coincineración como es el caso de las cementeras.

Otros usos

En Europa, se están desarrollando algunas aplicaciones novedosas de los lodos como puede ser la utilización de éstos y de sus cenizas para elaborar piedra artificial como material para asfaltado de carreteras, y la utilización de la mezcla de lodos y arcilla para la confección de ladrillos para la construcción. También se han realizado estudios para utilizar los lodos en la creación de filtros de carbón activo (Pérez, 1999). Así mismo, en Japón, la legislación limita el transporte a vertedero de las cenizas que contengan metales pesados, como la procedente de incineradoras, y ello ha conducido al desarrollo de la tecnología de "fusión de lodos". Este proceso vitrifica el lodo en una cámara de combustión a 1400 °C, lo que estabiliza y disminuye al mínimo el volumen ocupado por el lodo, al tiempo que ofrece posibilidades para reutilizarlo como material de construcción (cemento, cerámica, escoria cristalizada, etc.) (Magoarou, 2000).

En la UE, el destino final de los lodos varía considerablemente entre los distintos Estados Miembros. Sin embargo, la aplicación agrícola de los lodos, constituye uno de los principales destinos, al que se destinaron una media anual de 3,6 toneladas de materia seca (el 37% de la producción) durante el periodo 2003-2006 (Milieu et al., 2010). La incineración y el depósito en vertedero constituyen las principales alternativas a la aplicación agrícolas. La mayoría de los Estados Miembros, tratan una porción de los lodos mediante incineración y depositan las cenizas residuales en vertederos (Milieu et al., 2010). Por otro lado, en los Estados Unidos, la aplicación agrícola de lodos, con unas tres toneladas y

media de materia seca anuales, constituye el 55% de la producción total (USEPA, 2003).

En España, el sector agrario sigue destacando como principal destino de este tipo de residuos. En el año 2009, el 82,6% de los lodos se emplearon como enmienda orgánica, el 7,9% se depositó en vertedero y el 5,1% fue incinerado con recuperación de energía. Con relación al año 2008, el uso agrícola se ha visto incrementado un 22,8%, el depósito en vertedero ha disminuido un 34,9% y la incineración ha aumentado un 40%. En el periodo 2000-2009, la utilización con fines agrícolas fue el único destino que aumentó, pasando a destinarse de 454.000 a 995.000 toneladas de materia seca, mientras que el resto de los destinos han experimentado descensos, con una reducción del depósito en vertedero en dicho periodo del 37,5% y de la incineración con recuperación de energía del 12,3% (MARM, 2011). El Gráfico 3 representa los destinos de los lodos de depuradora en España durante el periodo 2000-2009.

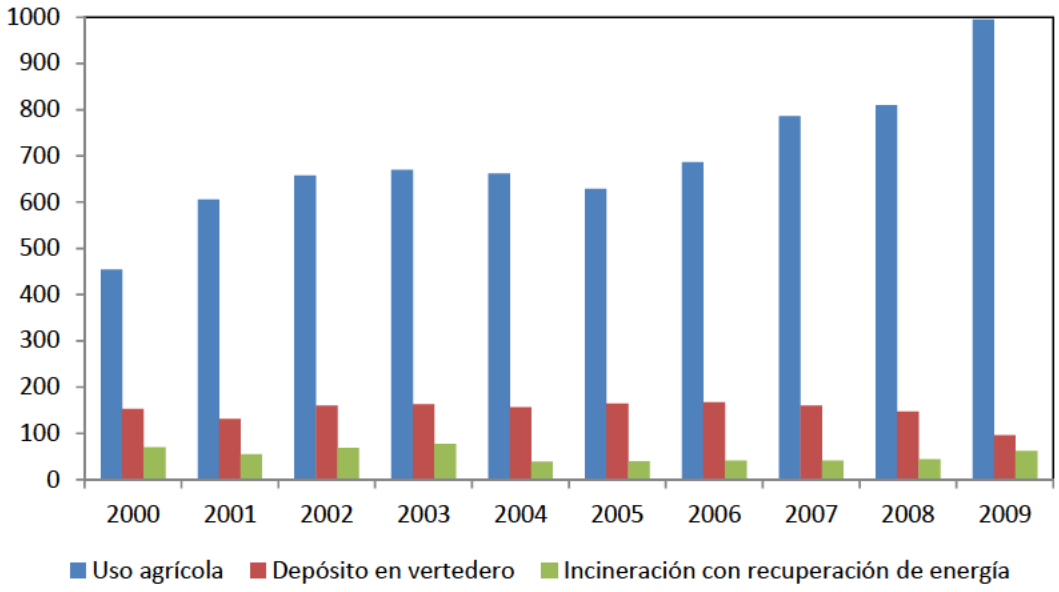

Gráfico 3. Destino de lodos de depuradora en España (miles de toneladas de materia seca). Fuente: MARM, 2011.

5. Aplicación agrícola de lodos de depuradora

Las características de los lodos de depuradora les confieren un gran interés como enmiendas orgánicas. La gran mayoría de los lodos de depuradora, poseen un alto contenido en materia orgánica y nutrientes que pueden contribuir al aumento de la producción de los cultivos y a la mejora de las propiedades físicas, químicas y biológicas de los suelos. En la mayoría de los sistemas de cultivo tradicionales es necesario aportar al suelo materia orgánica procedente de estiércoles u otros residuos orgánicos para mantener en éste unos niveles aceptables de humus. Por otro lado, en regiones áridas y semiáridas, como es el caso del sureste español, no es sólo la mencionada problemática creada en torno a los residuos orgánicos lo que nos debe preocupar, sino también el problema añadido existente que afecta a gran parte de los suelos de estas zonas y que es la degradación que han venido sufriendo, debida fundamentalmente a acciones antrópicas agresivas y a la adversa climatología. Todo ello, ha promovido una paulatina disminución de la fertilidad natural de los suelos y una erosión acelerada, siendo el escaso nivel de materia orgánica, un factor clave en este proceso (García et al., 1996).

En nuestro país, donde existen graves problemas de erosión (en más del 13% de la superficie española este problema está calificado como muy grave), y donde la mayoría de los suelos cultivados presentan serias deficiencias de materia orgánica. Por tanto, por razones tanto económicas como ecológicas, la incorporación de los lodos a los suelos, reintegrando los lodos tratados al ciclo natural, parece el método de gestión más adecuado, siempre que se realice de forma racional y bajo riguroso cumplimiento de la legislación vigente (Costa et al., 1987). La solución pasaría pues, por emplear como fuente de materia orgánica para los suelos aquélla contenida en los lodos de depuradora, consiguiendo de este modo, por una parte, mejorar la fertilidad de estos suelos, y por otra, eliminar racionalmente los residuos mediante su reciclado en los mismos, paliando los problemas de erosión, dependencia de productos químicos y carencias orgánicas y minerales (MAPA, 2003). Además, este tipo de material tiene un potencial intrínseco no explotado como es su carga microbiana, la cual se caracteriza por su elevada diversidad. Parte de esta carga microbiana, puede tener características más que adecuadas para su incorporación en el suelo, como

supresión de enfermedades de los cultivos, pudiendo actuar como biopesticidas, movilización de compuestos orgánicos no asimilables por la planta a nutrientes ya asimilables, e incluso se ha demostrado la producción de compuestos con capacidad estimulante tipo hormona (García et al., 2004).

5.1. Características nutricionales de los lodos

Materia orgánica

Los lodos poseen un alto contenido en materia orgánica, que varía según el tratamiento y acondicionamiento que hayan sufrido. La materia orgánica contenida en los lodos está formada principalmente por materia soluble como hidrocarbonos, amino-ácidos, proteínas pequeñas y lípidos. Al incorporarse al suelo, esta materia sufre un proceso de descomposición hasta la formación de compuestos orgánicos sencillos, que posteriormente se mineralizan de forma más o menos rápida. Durante el proceso de mineralización, la sustancia se transforma desde una forma orgánica a una inorgánica como resultado de la acción de los microorganismos, quedando disponible para las plantas. El carbono orgánico no mineralizado de forma rápida, sigue un proceso de mineralización más lento y tras profundas transformaciones, pasa a formar parte de la biomasa microbiana o queda estabilizado como sustancia húmica.

Nutrientes

Las características fertilizantes de los lodos se podrían expresar en función de sus contenidos en macronutrientes (N, P, K) aunque la presencia de niveles notables de distintos micronutrientes (Fe, B, Cu, Mn, Zn, etc.), es otra fuente de valor fertilizante. La proporción de estos elementos está influenciada por la procedencia, los procesos de depuración y tratamientos finales a los que han sido sometidos (Milieu et al., 2010). El nitrógeno se encuentra principalmente en los lodos en forma orgánica y, en menor medida, en forma amoniacal ($N\text{-}NH_3$), mientras que el fósforo se encuentra mayoritariamente bajo forma inorgánica (Carballa, 2005). En la Tabla 3 se muestran las características químicas de lodos obtenidos de 80 depuradoras distintas del sureste español.

Tabla 3. Características químicas de los lodos del sureste español. Adaptado de Moral et al., 2010.

Parámetro	Rango Total	Valor Medio
pH	3,92-7,73	6,54
CE (dS m^{-1})	0,5-7,15	2,37
MOT (%)	14-74,8	53,03
COT (%)	11,98-46,3	33,0
NT (%)	1,16-8,41	4,74
C/N	2,34-14,3	5,75
P (%)	0,24-2,35	0,94
K (%)	0,08-1,27	0,33
Cd (mg kg^{-1})	1-189	8
Cr (mg kg^{-1})	1-32662	544
Cu (mg kg^{-1})	26-4912	427
Pb (mg kg^{-1})	1-1119	137
Zn (mg kg^{-1})	152-24634	941
Ni (mg kg^{-1})	1-1500	51
Hg (mg kg^{-1})	0,01-7	0,92

5.2. Efectos sobre el suelo

Efectos sobre las propiedades físicas

La adición de enmiendas orgánicas puede influir positivamente en las propiedades físicas del suelo, mejorando su estructura, incrementando la formación y estabilidad de los agregados del suelo, reduciendo su densidad aparente, y aumentando la capacidad de retención hídrica (Caravaca et al., 2002; García-Orenes et al., 2005; Lacherveque et al., 2006; Mabuhay et al., 2006; Amlinger et al., 2007). La actividad microbiana se verá positivamente influenciada por el mayor contenido de materia orgánica energía y nutrientes del suelo, aumentando su desarrollo y actividad, lo que tendrá efectos positivos sobre el crecimiento vegetal (Bailey and Lazarovits 2003).

Efectos sobre las propiedades físico-químicas

En los suelos tratados con materiales orgánicos se produce un aumento de la capacidad de intercambio catiónico (CIC), debido a la presencia de iones calcio y de sales básicas que ejercen un efecto tampón (Hernando, 1988; Weber, 2007). La CIC es una propiedad importante del suelo debido a que controla la disponibilidad de nutrientes para las plantas, interviene en los procesos de floculación-dispersión de las arcillas (y por tanto en el desarrollo de la estructura y estabilidad de agregados), y determina el papel del suelo como depurador

natural, permitiendo la retención de elementos contaminantes incorporados al suelo.

Efectos sobre las propiedades químicas

El efecto más significativo que se produce sobre un suelo cuando se le adicionan residuos orgánicos es la incorporación de materia orgánica. Los ácidos húmicos presentes en el lodo, pueden tener una influencia positiva en la fertilidad de los suelos y, consecuentemente, en el crecimiento de las plantas (Fernández et al., 2007). Además, esto residuos presentan grandes cantidades de macro y micronutrientes (Ayuso et al., 1996; Tejada et al., 2006; O'Dell et al., 2007), que van liberándose de forma gradual durante el proceso de descomposición de la misma incrementando la fertilidad de los suelos (Ayuso et al., 1992).

Efectos sobre las propiedades microbiológicas y bioquímicas

Las poblaciones microbianas del suelo están constituidas por un gran número de animales y de vegetales microscópicos a cuyo conjunto se le denomina microorganismos. El aporte de lodos al suelo favorece el desarrollo y la actividad de las poblaciones microbianas autóctonas del suelo debido a la mejora de las propiedades físicas y a la disponibilidad de una fuente de carbono fácilmente biodegradable (Ros et al., 2003; Tejada et al., 2006). Este aumento se traduce a su vez en un incremento de las enzimas y metabolitos. Las enzimas son las responsables de la mayor parte de reacciones que intervienen en los procesos de mineralización e inmovilización de los nutrientes en el suelo y por tanto están en relación con la disponibilidad de los mismos para la planta (Perucci, 1990). Algunos de los metabolitos liberados por los microorganismos, o moléculas de bajo peso molecular procedentes de la mineralización de la materia orgánica, pueden influir de forma positiva y directa sobre el crecimiento vegetal (Albuzio et al., 1989). También es importante indicar que parte de estas enzimas quedarán protegidas de la degradación e inactivación, al quedar inmovilizadas por la fracción húmica de la materia orgánica incorporada mediante la formación de complejos tipo enzima-humus (Nannipieri et al., 1990; Benítez et al., 2005).

Por otro lado, la adición de materia orgánica al suelo, y especialmente de lodos, que poseen una alta carga microbiológica, también puede producir

cambios en la composición de las comunidades microbianas del suelo debidos a la incorporación de nuevos microorganismos (Marschner et al., 2003).

5.3. Efectos sobre las plantas

Numerosos trabajos de investigación han demostrado que los suelos enmendados con lodos mejoran el crecimiento y producción de cultivos vegetales (Golabi et al., 2007; López-Pineiro et al., 2007). Sin embargo, otros estudios informan de que aplicaciones continuadas de lodos sobre las tierras de cultivo pueden producir una acumulación de metales pesados a niveles tóxicos para las plantas (Sánchez-Martín et al., 2007; Singh y Agrawal, 2008), pudiendo suponer un riesgo para la salud humana, lo que ha llevado a imponer límites sobre la cantidad y frecuencia de la aplicación de los lodos sobre las tierras de cultivo. No obstante, algunos estudios han demostrado una reducción del contenido en metales pesados biodisponibles en el suelo mediante la aplicación de enmiendas orgánicas (O'Dell et al., 2007).

5.4. Riesgos potenciales de la utilización agrícola de lodos

Los lodos pueden contener elementos potencialmente tóxicos tanto para las plantas como para los animales y el ser humano; consumidor de dichas plantas (Krebs et al., 1998). Las principales características de los lodos que pueden actuar como factores limitantes en aplicación en el sector agrícola son:

Contenido en metales pesados

El contenido de metales pesados de los lodos (Cd, Cr, Cu, Hg, Ni, Pb y Zn) se encuentra en concentraciones variables (de 0,3 a 2.000 mg kg^{-1}) según la naturaleza del agua residual y el tratamiento realizado en la estación depuradora. Además, cabe destacar la presencia de otros elementos no metálicos (metaloides) de igual o mayor efecto perjudicial (Ar, Br, Bi, Se, I). La importancia de éstos viene dada por su posible acumulación en el suelo y su absorción y almacenamiento en los tejidos de las plantas, quedando así incluidos en la cadena trófica de los animales y del ser humano. El grado de peligrosidad de los metales pesados va ligado a dos propiedades principales como son su toxicidad y su persistencia (Moreno, 1997). Por otro lado, la incidencia contaminante de estos metales, depende en gran medida de la movilidad que presentan en el mismo. El

pH es uno de los factores primordiales en este sentido ya que la movilidad de los metales aumenta, en general, a medida que el pH disminuye (Shuman, 1986).

Sustancias orgánicas tóxicas

Los hidrocarburos aromáticos policíclicos (PAH_s), los bifenilos policlorados (PCB_s), los nonilfenoles (NPEs), las dibenzodioxinas policloradas (PCDDs), y los dibenzofuranos policlorados (PCDFs), son moléculas orgánicas presentes en plaguicidas, disolventes industriales, colorantes, plastificantes, y agentes tensoactivos que tienden a acumularse en los lodos, aunque generalmente se encuentran en pequeñas cantidades. Las principales razones por la que estos compuestos se consideran tóxicos son debidas a su baja solubilidad en agua, baja biodegradabilidad y alta persistencia. Su bioacumulación dentro de la cadena alimentaria debida a su gran afinidad lipídica, podría causar efectos negativos en animales e incluso en el ser humano. Estos compuestos tienen tres tipos de reacciones por las que pueden salir del medio: biodegradación, degradación química y fotoquímica. La degradación biológica es bastante lenta y se produce muy a largo plazo. En el suelo, se puede llegar a producir una degradación química que implicaría procesos hidrolíticos y oxidativos.

Contenido de nutrientes

Aproximadamente, el 80% del nitrógeno contenido en los lodos estará disponible para las plantas, mientras que la tasa de utilización del fósforo está en un rango de 40-80%. Un aporte demasiado elevado de nitrógeno y de fósforo, puede provocar la contaminación de las aguas subterráneas a través del lixiviado de nutrientes solubles y la eutrofización de las aguas subterráneas y superficiales.

Salinidad

Es un factor a tener muy en cuenta, puesto que la cantidad de aniones y cationes que van a estar solubles en un suelo al que se le ha aplicado lodo puede ser muy importante, sobre todo en aquellos con una limitada capacidad de drenaje. Así, se puede producir una disminución en la germinación de las semillas, inhibición del crecimiento de las plantas y empeoramiento de la estructura del suelo. Cabe destacar, que el ión más perjudicial para la planta es el cloruro (Cl^-), y que la relación entre el contenido de cationes Na^+, Mg^{2+} y Ca^{2+} es un parámetro muy adecuado para evaluar efectos negativos sobre el suelo.

Microorganismos patógenos

Los materiales orgánicos pueden contener desde microorganismos patógenos como bacterias, virus, helmintos, etc. (Beuchat, 1996) no deseables hasta otras sustancias fitotóxicas y materia orgánica lábil, capaz de provocar en el suelo competencia entre microorganismos y planta por algún nutriente, como por ejemplo, el nitrógeno. Algunos de los microorganismos patógenos que puedan contener los residuos orgánicos pueden pasar al suelo y de ahí, incluso a la cadena trófica si inciden en el alimento cultivado sobre ese suelo. Por todo ello, los lodos deben ser estabilizados e higienizados antes de su disposición final o reutilización en el suelo para evitar efectos negativos sobre el medioambiente. Este es un factor importante a tener en cuenta, que insta a conocer la cantidad de microorganismos y su capacidad de supervivencia, con el fin de prever las posibles contaminaciones por ingestión de partes comestibles de plantas que hayan podido estar en contacto con los lodos (Wong y Lai, 1996). Las cantidades de microorganismos presentes en un lodo varían según el grado de tratamiento del agua, así como el nivel alcanzado en las distintas fases de la línea de fangos. En cuanto a su supervivencia en el suelo, ésta puede ser muy variable, alcanzando desde pocos días a varios años, dependiendo de distintos factores como la temperatura, humedad, cantidad de materia orgánica del suelo, luz, y tipo de suelo. En la Tabla 4 se describen los principales organismo patógenos contenidos en los lodos. Anteriormente, en el apartado 1.3., se han descrito las principales metodologías utilizadas para reducir el contenido de patógenos de los lodos mediante los tratamientos de estabilización y desinfección.

Tabla 4. Organismos patógenos presentes en aguas residuales y lodos de origen municipal con mayor repercusión en salud pública. Adaptado de EPA/625/006 en Lue-Hing et al., 1998.

Organismo	**Enfermedad / Síntoma**
Bacterias	
Salmonella spp.	Salmonelosis, fiebre tifoidea
Shigella spp.	Disentería bacilar
Yersinia spp.	Gastroenteritis aguda
Vivrio cholerae	Cólera
Camylobacter jejuni	Gastroenteritis
Escherichia coli	Gastroenteritis
Bacillus anthracis	Ántrax
Leptospira interrogans	Leptospirosis
Virus	
Poliavirus	Poliomelitis
Coxsackievirus	Meningitis, neumonía, hepatitis, fiebre
Echovirus	Meningitis, neumonía, hepatitis, fiebre, resfriado
Reovirus	Hepatitis infecciosa
Rotavirus	Gastroenteritis aguda
Protozoos	
Criptosporidium	Gastroenteritis
Entamoeba *histolytica*	Enteritis aguda
Giardia lambia	Giardiasis
Balantidium gondii	Diarrea, disentería
Toxoplasma gondii	Toxoplasmosis
*Acenthammoeb*a	Meningoencefalitis e infecciones mucosas
Helmintos	
Ascaris lumbricoides	Alteraciones digestivas y nutricionales, dolor abdominal, vómitos
Ascaris suum	Tos, fiebre, dolor de pecho
Toxocara canis	Fiebre, molestias abdominales, dolor muscular, síntomas neurológicos
Trichuris trichiura	Dolor abdominal, diarrea, anemia, pérdida de peso
Taenia saginata	Nerviosismo, insomnio, anorexia, dolor abdominal, molestias digestivas
Taenia solium	Nerviosismo, insomnio, anorexia, dolor abdominal, molestias digestivas
Necator americanus	Anquilostomiasis
Hymenolepis nana	Teniasis

6. Marco legal de la aplicación agrícola de lodos de depuradora

6.1. Legislación actual

Situación en Europa

En junio de 1986, la UE aprobó la Directiva 86/278/EEC de 12 de junio, relativa a la protección del medio ambiente y, en particular, de los suelos y de la utilización de los lodos de depuradora en agricultura. El objeto principal de esta Directiva, es regular la utilización de los lodos de depuradora en agricultura de modo que se eviten efectos nocivos en los suelos, las aguas, la vegetación, los animales y en el ser humano. Al mismo tiempo, se promueve su correcta utilización, permitiendo el empleo de lodos de depuración en suelos agrícolas siempre y cuando la concentración de metales pesados, tanto en los lodos como en los suelos receptores, no supere ciertos límites, estableciendo unos aportes máximos anuales y se controle la acumulación de metales en las parcelas receptoras. Además, esta Directiva, prohíbe el empleo de lodos de depuradora sin tratar, salvo en los casos de inyección directa o enterramiento en el suelo, siempre que lo autoricen los Estados Miembros. Así mismo, prohíbe la aplicación en determinados cultivos, al tiempo que establece plazos para su aplicación en los cultivos autorizados.

Por otro lado, en los casos en que los lodos de depuradora se aporten a zonas vulnerables es también aplicable la Directiva 91/676/EEC, contra la contaminación producida por nitratos procedentes de fuentes agrarias, estableciendo limitaciones en las cantidades anuales de nitrógeno orgánico.

Situación en España

La Directiva Europea 86/278/EEC, se traspone al ordenamiento jurídico español a través del Real Decreto 1310/1990 de 29 de octubre de 1990, por el que se regula la utilización de lodos de depuradora en el sector agrícola. Este Real Decreto impone una serie de disposiciones administrativas sobre el control de la producción y comercialización de los lodos tratados que deberán ser controladas por las CC.AA. Por otro lado, establece unos valores límite para los parámetros mínimos que deberán ser analizados para permitir la aplicación del lodo al suelo. Los parámetros incluidos son: materia seca, materia orgánica, pH, nitrógeno, fósforo, cadmio, cobre, níquel, plomo, zinc, mercurio y cromo.

En los casos en que los lodos de depuradora se aporten a zonas vulnerables, el Real Decreto 261/1996 de 16 de febrero es la trasposición de la Directiva 91/676/EEC.

Paralelamente, siguiendo las directrices de la Directiva Europea 86/278/EEC, se creó el Registro Nacional de Lodos adscrito al antiguo Ministerio de Agricultura, Pesca y Alimentación, siendo todo ello regulado, hasta junio 2013, por la Orden de 26 de octubre de 1993 sobre utilización de lodos de depuración en el sector agrario. Esta orden incorporaba algunos requisitos tales como la obligatoriedad del suministro de información de la estación depuradora al inicio de su funcionamiento y el envío por el responsable de una ficha semestral elaborada por la entidad que gestiona los lodos de uso agrícola de forma que permitiera controlar las cantidades dedicadas a fines agronómicos.

Sin embargo, debido al largo periodo de tiempo transcurrido desde la entrada en vigor de esa orden y teniendo en cuenta los avances técnicos que han ocurrido en materia de producción, tratamiento y aplicación al suelo agrario de los lodos de depuración, desde junio 2013, la nueva Orden de 7 junio sobre utilización de lodos de depuración en el sector agrario, reemplaza a la anterior de 26 de octubre de 1993. En esta nueva Orden Ministerial, se regula la información que deben proporcionar los titulares de las depuradoras de aguas residuales, las instalaciones de tratamiento de lodos de depuración, los gestores que realizan la aplicación en los suelos de los lodos de depuración tratados, así como la información que debe acompañar a todo transporte de lodos destinados a la actividad agraria. También debe tenerse en cuenta que el Plan Nacional Integrado de Residuos para el período 2008-2015, establece en su apartado número trece, objetivos cualitativos con los que se trata de asegurar la correcta gestión de los lodos, desde su origen hasta su destino final, protegiendo el medio ambiente y especialmente el suelo. Entre estos objetivos cualitativos, cabe destacar la mejora del sistema de información sobre la gestión de los lodos y la mejora del control de las aplicaciones agrícolas garantizando el uso adecuado de los lodos de depuración en el suelo. Una de las medidas contempladas en el Plan para la consecución de estos objetivos, es la revisión y modificación de los anexos de la Orden 26 de octubre de 1993.

Carencias de la legislación vigente: el caso americano

Tanto la actual Directiva Europea como su trasposición a la legislación española han demostrado ser eficaces en la prevención de la propagación de microorganismos patógenos en los cultivos, brotes epidémicos en los seres humanos y para mantener la reducción de metales pesados que los lodos aportan al suelo. Sin embargo, presentan las siguientes deficiencias:

• Solo cubren el lodo urbano (procedente de aguas residuales urbanas, domésticas o de composición similar), pero no consideran otros lodos no peligrosos (lodos de la industria textil, papelera o agroalimentaria) que también pueden tener efectos beneficiosos sobre el suelo.

• Regulan la aplicación de lodos de depuradora en terrenos agrícolas pero no prevén ningún acto en relación con otro tipo de usos del suelo. Aunque la protección de los suelos agrícolas es de primordial importancia para la producción de alimentos de buena calidad, los esparcimientos de lodos en tierras no agrícolas (plantaciones de árboles, áreas verdes, etc.) también pueden tener impactos adversos sobre la salud humana y la biodiversidad.

• No tienen en cuenta los efectos de la acumulación en el suelo de metales pesados a largo plazo.

• No regulan el contenido en patógenos de los lodos.

Por el contrario, la legislación vigente en Estados Unidos, regulada a través de la Environmental Protection Agency (EPA) mediante la 40 CFR Part 503 Sludge Rule (USEPA, 2003), presenta algunas diferencias con la legislación europea, entre las que destacan:

• La regulación del contenido en los lodos de cromo, arsénico, molibdeno y selenio, además de los seis metales regulados en la legislación europea.

• La clasificación de los lodos en dos categorías (Clase A y Clase B) según el tratamiento a los que han sido sometidos y el grado de reducción de patógenos obtenido. Los biosólidos (lodos tratados) de Clase A, son considerados seguros para el contacto inmediato o directo con humanos y animales, permitiendo su aplicación directa sin ningún tipo de restricción en cuanto a los cultivos, si

cumplen unos determinados límites en cuanto al contenido de metales pesados. Por otro lado, los biosólidos de Clase B están sujetos a un contenido en patógenos menos restrictivo. En función del uso del lugar donde se apliquen estos lodos, éste puede permanecer con acceso restringido hasta un año para permitir la atenuación natural de los niveles de patógenos.

• El establecimiento de normas específicas para la reducción del poder de atracción de vectores de transmisión de enfermedades (insectos, roedores, pájaros). Entre los procedimientos aprobados para este fin se incluyen la digestión aerobia y anaerobia, la estabilización alcalina, el secado térmico o la inyección en el terreno.

• La comercialización como "Lodo de calidad excepcional" con aquel lodo de Clase A que cumpla con los niveles más restrictivos de metales pesados y haya sido sometido a uno de los procesos aprobados de reducción de vectores de enfermedad. El manejo de este lodo queda exento de muchos de los tediosos procedimientos de control y seguimiento obligatorios para otros tipos de lodos.

6.2. Futura Directiva Europea

La UE, consciente del problema que puede ocasionar un uso inadecuado de los lodos, está trabajando en la modificación de la Directiva 86/278/EEC a través del desarrollo de la "Propuesta de Directiva del Parlamento y del Consejo Europeo sobre aplicación agrícola de lodos" (Comisión Europea, 2003). La presente propuesta, tiene como objetivo mejorar la protección de la salud humana, del medio ambiente y de los suelos en los que se apliquen lodos de depuradora tal y como exige el artículo 174 del Tratado Constitutivo de la Comunidad Europea, bajo la presunción de que la utilización de lodos de depuradora como fertilizante en suelos agrícolas, es la mejor opción ambiental, siempre y cuando no represente ninguna amenaza para el medio ambiente, los animales y/o la salud humana. Esta propuesta de Directiva, contempla una estricta definición del uso de los lodos en función de su origen, del sistema de tratamiento de estabilización e higienización al que han sido sometidos, y de la naturaleza y uso de los suelos. Esta nueva normativa, también limita su uso en caso de presencia de metales pesados, compuestos orgánicos y microorganismos patógenos. En la Tabla 5 se muestran los límites de estos compuestos y

microorganismos contemplados en la futura Directiva Europea. Entre ellos, los patógenos humanos que merecen especial atención son:

• *Escherichia coli* habita en los intestinos de los humanos sanos y en la mayoría de los animales de sangre caliente. Esta bacteria ayuda a mantener el equilibrio de la flora intestinal normal y sintetiza algunas vitaminas. No obstante, existen ciertas cepas de *E. coli*, como la *E. coli* 0157:H7, cuyas toxinas pueden causar graves infecciones en humanos, siendo la cadena trófica una de las principales vías de transmisión de este patógeno.

• *Salmonella* spp. es un género de bacterias principal causante de las enfermedades relacionadas con la alimentación en todo el mundo. La trasmisión habitual es través del consumo de comida contaminada de origen animal; principalmente carne, aves, huevos y leche. Los síntomas incluyen fiebre, dolor abdominal, diarrea, nauseas, vómitos y fiebre tifoidea (Gorbach et al., 2004). En 2012, un brote de *Salmonella* Agona generó 160 casos de gastroenteritis en 10 países.

• *Clostridium perfringens* es el patógeno más ampliamente distribuido en la naturaleza (Willies, 1969), y también se encuentra frecuentemente en el intestino de humanos, así como también en el de animales domésticos y salvajes. Sus esporas sobreviven en el suelo, en los sedimentos y en las áreas sujetas a la polución fecal, tanto humana como animal (McClane, 2006). Muchas especies del género *Clostridium* tienen la capacidad de producir exotoxinas, que son las responsables de ocasionar graves cuadros tóxicos. La alfa-toxina (presente en todas la variedades de *Clostridium*) juega un papel primordial en la patogenia de la gangrena gaseosa, la beta-toxina de *C. perfringens* tipo C está implicada en la enteritis necrótica y la enterotoxina de *C. perfringens* tipo A en las intoxicaciones alimentarias.

Tabla 5. Valores límite de metales pesados, compuestos orgánicos y microorganismos patógenos contemplados en la Propuesta de Directiva Europea. Fuente: Comisión Europea, 1986 y 2003.

Elemento / Organismo	Directiva 86/278/EEC	Futura Directiva Europea
Metales pesados (mg kg-1 materia seca)		
Total Cd	20-40	10
Total Cr	-	1000
Total Cr_{VI}	-	10
Total Cu	1000-1750	1000
Total Hg	16-25	10
Total Ni	300-400	300
Total Pb	750-1200	750
Total Zn	2500-4000	2500
Compuestos orgánicos		
PAH (mg kg^{-1} materia seca)	-	6
PCB (mg kg^{-1} materia seca)	-	0,8
PCDD/F (ng ITEQ kg^{-1} materia seca)	-	100
LAS (g kg^{-1} materia seca)	-	5
NPE (mg kg^{-1} materia seca)	-	450
Patógenos. Tratamientos convencionales		
Escherichia coli (UFC g-1 materia húmeda)	-	Reducción del 99% hasta < 5 x 10^5
Patógenos. Tratamientos avanzados		
Escherichia coli (UFC g-1 materia seca)	-	Reducción del 99,99% hasta < 1 x 10^3
Clostridium perfringens (esporas g -1 materia seca)	-	< 3 x 10^3
Salmonella spp. (50 g materia húmeda)	-	No detectable

Al mismo tiempo, y homólogamente a la legislación americana, esta futura Directiva Europea, establece dos grandes grupos de tratamiento y condiciones para los fangos: **convencionales** y **avanzados**, según los cuales tendrán unas limitaciones de uso, permitiendo estos últimos la aplicación directa de los lodos. Los tratamientos que describe como avanzados son:

• Compostaje en hileras garantizando que el material mantenga una temperatura mínima de 55 °C durante al menos cuatro horas entre cada volteo. Las pilas deben ser volteadas al menos tres veces al día y debe obtenerse una completa estabilización del material.

• Compostaje en vasos garantizando que el material mantenga una temperatura mínima de 55 °C durante al menos cuatro horas hasta alcanzar la completa estabilización.

• Secado térmico garantizando que la temperatura de las partículas del lodo alcance una temperatura mínima de 80 °C durante diez minutos y un contenido de humedad menor del 10%.

• **Estabilización termófila aerobia o anaerobia** de los lodos con una temperatura mínima de 55 °C durante al menos un periodo continuado de cuatro horas después de la última alimentación y antes de retirar el lote. El digestor debe ser diseñado para funcionar a temperaturas mínimas de 55 °C con un SRT suficiente para obtener la estabilización del fango.

• Tratamiento térmico del lodo líquido durante al menos diez minutos a 80 °C, veinte minutos 75 °C o treinta minutos a 70 °C, seguido de una digestión anaerobia mesófila (MAD) a 35 °C con un SRT de doce días.

• Acondicionamiento con cal viva (CaO) alcanzando un pH mínimo de 12,6 y manteniendo una temperatura de al menos 55 °C durante dos horas.

Por último, la nueva Directiva, también impondrá un procedimiento de certificación y responsabilidades de la calidad del fango, basada en análisis exhaustivos de los mismos antes de ser ofrecidos al receptor. El productor estará obligado a cumplir con una serie de requisitos de calidad, entre los que destaca el origen del fango, el certificado de auditoría y el tipo de tratamiento de estabilización e higienización. A su vez, el receptor también debe de archivar y/o proveer al productor de la localización y tipo de cultivo donde se va a aplicar el fango, el tipo de fertilización orgánica previa, la dosis aplicada por año de fango, así como otras fertilizaciones e insumos agroquímicos.

III

III. Evaluation of the removal of pathogens included in the Proposal for a European Directive on spreading of sludge on land during autothermal thermophilic aerobic digestion (ATAD)

III. Evaluation of the removal of pathogens included in the Proposal for a European Directive on spreading of sludge on land during autothermal thermophilic aerobic digestion (ATAD)

Evaluación de la eliminación de los patógenos incluidos en la Propuesta de Directiva del Parlamento y del Consejo Europeo sobre aplicación de lodos en campo durante la digestión aerobia autotérmica termófila (ATAD)

Eva Lloret, Laura Pastor, Ainhoa Martínez-Medina, Josefa Blaya, José A. Pascual.

Chemical Engineering Journal (2012) 198-199, 171-179.

Resumen

La Unión Europea está desarrollando una nueva legislación a través de la "Propuesta de Directiva del Parlamento y del Consejo Europeo sobre aplicación agrícola de lodos de depuradora", en la que hace especial hincapié en el contenido de patógenos humanos. En esta propuesta de Directiva Europea, la digestión aerobia autotérmica termófila (ATAD) se describe como un tratamiento avanzado capaz de producir un lodo higienizado. En este trabajo, se estudió la puesta en marcha y funcionamiento de un digestor ATAD de una sola etapa con un volumen efectivo de 15 m^3 durante un periodo de 19 meses para evaluar el proceso de estabilización e higienización de lodos de una EDAR municipal. Para ello, se estudiaron los parámetros físico-químicos del proceso y se analizó el contenido de *Salmonella* spp., *Escherichia coli* y esporas de *Clostridium perfringens* mediante recuento en placa, y a través de la amplificación de los genes de patogenicidad *invA* y *cpa* mediante PCR, tanto a la entrada como en el efluente del sistema. Tras el tratamiento ATAD, la destrucción de sólidos volátiles obtenida fue del 38,0% consiguiendo una alta velocidad de carga orgánica (2,7 kg VS m^{-3} d^{-1}) y un bajo tiempo de retención (14,6 días). Respecto al contenido de microorganismos patógenos, éste descendió significativamente con la completa eliminación de *Salmonella* spp. y de *E. coli*, y la reducción en 2 unidades

logarítmicas del contenido de esporas de *C. perfringens*. Con el objeto de eliminar las esporas de *C. perfringens* para obtener una total higienización de los lodos, se introdujo una etapa mesófila intermedia después del tratamiento ATAD. De este modo, se obtuvo un lodo estabilizado y libre de patógenos, adecuado para su aplicación agrícola. Por último, el análisis de los lodos mediante DGGE, mostró diferencias en las estructuras de las comunidades de hongos y bacterias entre los lodos frescos (influente), mesófilos y termófilos, indicando la relevancia del proceso de estabilización en la alteración de las comunidades microbianas de los lodos.

Chemical Engineering Journal (2012) 198-199, 171-179

Evaluation of the removal of pathogens included in the Proposal for a European Directive on spreading of sludge on land during autothermal thermophilic aerobic digestion (ATAD)

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HIGHLIGHTS

- Pilot-scale authothermal thermophilic aerobic digester was studied over 19 months.
- Removal of pathogens included in the future European legislation was evaluated.
- *Escherichia coli* and *Salmonella* spp. were not detected in ATAD- digested sludge.
- *Clostridium perfringens* spores were eradicated following a mesophilic stage.
- Qualified organic amendment was obtained after mesophilic-thermophilic treatment.

ARTICLE INFO

Article history:
Received 1 March 2012
Received in revised form 11 May 2012
Accepted 21 May 2012
Available online 29 May 2012

Keywords:
Thermophilic aerobic digestion
ATAD
Sewage sludge
Salmonella
Human pathogens
Clostridium perfringens

ABSTRACT

The European Union is promoting a new legislation through the "Proposal for a Directive of the European Parliament and of the Council on spreading of sludge on land", with special interest on human pathogens. Autothermal Thermophilic Aerobic Digestion (ATAD) is described in this Proposal as an advanced treatment capable of producing sanitized sludge. A one-stage ATAD digester with effective volume of 15-m^3 was started up and studied over 19 months for its ability to stabilize and sanitize municipal sludge. *Salmonella* spp., *Escherichia coli*, and *Clostridium perfringens* spores were cultivated and pathogenity genes *invA* and *cpa* PCR-amplified. Volatile solids removal was 38.0% and the pathogen content significantly decreased by completely eliminating *Salmonella* spp. and *E. coli* but not *Clostridium perfringens* spores (9.6 x 10^3 spores mL^{-1}). To completely achieve the disinfection of the sludge, a mesophilic stage was introduced after the ATAD

treatment. Denaturing gradient gel electrophoresis analysis showed differences in the structures of the bacterial and fungal communities between thermophilic, mesophilic and raw sludge. The results demonstrated that the ATAD technology had the capability to produce sludge suitable for agricultural application when the operational parameters were stable and a mesophilic stage was introduced.

1. Introduction

In Spain, the production of sewage sludge has increased significantly during the past few years due to the obligation of wastewater treatment and tougher effluent restrictions [1]. This increase in the production of sewage sludge has created significant pressure concerning the optimal management and disposal of this product. On the other hand, organic wastes have been used to increase crop productivity due to their beneficial effects on the physical, chemical and biological properties of soil resulting in improved soil structure, provision of plant nutrients, increased humus content and cation exchange capacity and enhanced microbiological biomass activity [2,3]. In Spain, 65% of the sludge produced is currently used for land application [4]. However, the agricultural use of sewage sludge has some potential risks, associated with its potential content of heavy metals, toxic compounds, pathogenic bacteria, viruses and parasites [5]. In response, microbiological standards have been developed for the application of sewage sludge to agricultural land. The United States regulations specify treatment goals or post-application measures designed to reduce these risks [6]. In contrast, the European legislation requires only that the sludge be subjected to a process of stabilisation before land application [7]. In order to improve this situation and prevent decreases in soil quality and risks to human health caused by land application of sludge, the European Union (EU) is developing a new regulation through the Proposal for a Directive of the European Parliament and of the Council on spreading of sludge on land [8]. This provides stricter standards for the contents of heavy metals, organic compounds and human pathogens, this latter parameter receiving special attention. In this respect, the Proposal for a European Directive introduces the concept of advanced and conventional treatments, which allows operators to use advanced-treated sludge with fewer restrictions compared with a sludge that has been treated conventionally.

Autothermal thermophilic aerobic digestion (ATAD) is considered as an advanced technology for the treatment of sludge produced by municipal

wastewater treatment plants in the Proposal for a European Directive. In this type of bioreactor, the temperature rises above 50 °C due to the conservation of part of the heat produced by the aerobic metabolism of the microorganisms that consume the abundant organic material present in the sludge [9]. One of the main expected benefits of ATAD is its efficiency in the killing of pathogenic microorganisms, and it has been recognised in the United States as a process capable of fulfilling Class A biosolids pathogen requirements in order to produce a product that can be used as a soil amendment with few restrictions. The pasteurization effect of ATAD processes is due to the temperature but also to the pH, which increases during the treatment [6]. Other claimed benefits of this system are: its high stabilisation rate and volatile solids reduction capability, the reduction in explosive gas emissions, the simplicity and high speed of the process, its robustness, its small volume and land space requirement, its nitrogen conservation and the possibility of heat recovery [9,10]. Furthermore, the aerobic thermophilic bacteria involved in the ATAD treatment have a wide range of potential substrates and therefore complex industrial problems can also be tackled by this method [11]. Recent studies have shown the efficiency of ATAD to eliminate pathogenic bacteria or have evaluated the ATAD sludge as an organic amendment [12,13]. However, there is a lack of available data regarding the microorganisms included in the Proposal for a European Directive.

In view of the above, the aim of this work was to start-up and characterise, over 19 months, a one-stage ATAD digester in order to assess whether the digested sludge achieved acceptable values for microbial parameters related to human pathogens established in the Proposal for a Directive of the European Parliament and of the Council on spreading of sludge on land [10]. The microorganisms analysed were: *Salmonella* spp., *Escherichia coli*, *Clostridium perfringens* spores and total coliforms. Physico-chemical parameters - such as volatile solids, pH, electrical conductivity (EC), macronutrients and heavy metals - were measured to ensure appropriate sludge-digestion conditions. The fungal and bacterial communities from raw and digested sludges were analysed by denaturing gradient gel electrophoresis (DGGE), to assess changes in the microbial community structure.

2. Materials and methods

2.1. Description of the ATAD digester

ATAD reactor consisted of a 15-m3 digester covered by a heat-resistant, 10-cm-thick polyurethane layer, for its insulation and cladding (Fig. 1). Oxygen was supplied by using a Venturi-type aerator that permits the acquisition of external air by pumping sludge through tubes of small cross-sectional area inside the digester that are connected to the exterior with capillaries. Foam production was controlled with the installation of a polyester rack with a 25-mm reticulum. The sludge stream was operated in batch mode. The temperature of incoming and outgoing sludge, the loading rate, pH, EC, and redox potential were measured periodically and registered with a SCADA (supervisory control and data acquisition).

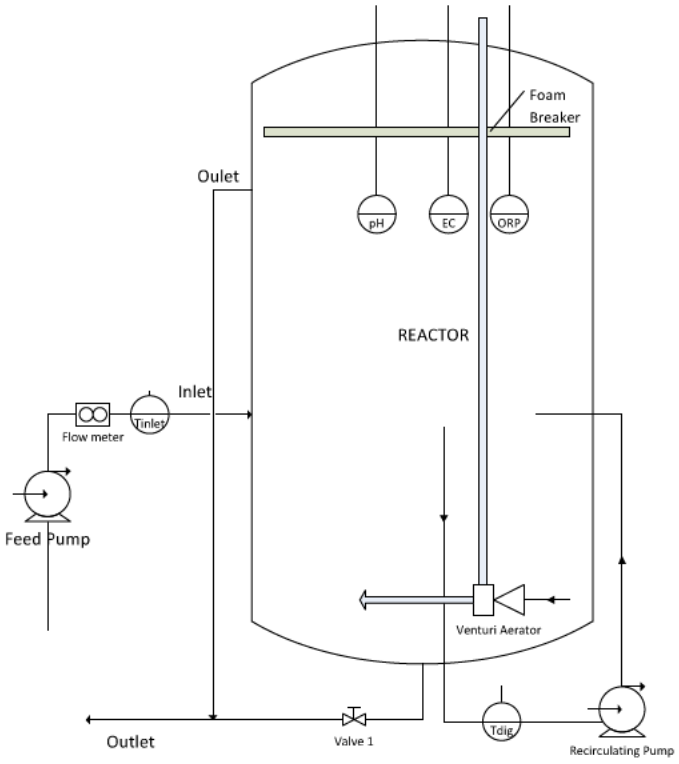

Figure 1. Schematic diagram of the ATAD digester.

2.2. Sludge sampling and physico-chemical analysis

Incoming sludge (mixed primary and secondary sludge, raw sludge hereafter) and digested sludge were sampled weekly during the last 12 months of the operation of the digester, to analyse *Salmonella* spp., *E. coli*, *C. perfringens* spores and total coliforms.

Electrical conductivity and pH were measured in a 1:5 (w/v) aqueous solution in a CM2002 (Crison, Barcelona, Spain) conductivity meter and pH2002 (Crison, Barcelona, Spain) pH meter, respectively. Total organic carbon (TOC) was determined by oxidation with $K_2Cr_2O_7$ in an acid medium evaluating the excess of dichromate with $(NH_4)_2Fe\ (SO_4)_2$ [24]. Total N was determined by the Kjeldahl method modified by Bremner and Mulvaney [15]. Total P and K and heavy metals were determined in nitric-perchloric (1:1) digestion extract; P was determined by colorimetry following the Murphy and Riley [16] method and K and heavy metals by atomic absorption spectrometry (Perkin-Elmer 5500).

Analysis of total solids (TS) and volatile solids (VS) were performed daily according to standard methods [17]. Destruction of volatile solids was calculated as the fraction of volatiles solids destroyed by the constant ash method.

2.3. Determination of pathogens

The detection method of *Salmonella* spp., consisted of four steps. The first step was non-selective enrichment using buffered peptone water (BPW) (Scharlab, Sentmenat, Spain) and 50 mL of sludge sample incubated at 37 °C for 24 h in a rotary shaker at 100 rpm. The second step consisted of a selective enrichment performed using liquid Rappaport-Vassiliadis' soy broth (Condalab, Madrid, Spain), incubated at 42 °C for 24 h in a rotary shaker at 100 rpm. As third step, the obtained suspension was plated on Colorex *Salmonella* Plus agar (VWR International, Barcelona, Spain), and incubated at 37 °C for 24 h. For the last step, the DNA of positive colony-forming units (CFUs) was extracted (Chelex-100 resin solution, Bio-Rad Laboratories, Hercules, CA, USA), subjected to electrophoresis in 1.5% (w/v) agarose gels with 1× Tris–acetate–EDTA (TAE) buffer, stained with ethidium bromide (10 mg mL^{-1}) and visualised under UV light. The DNA concentration was quantified using the Nanodrop ND-1000 (Thermo Fisher Scientific, Rockford, IL, USA). A 284-bp region of the *invA* gene [18] enconding for invasine was amplified in a PCR thermal cycler (PCR Thermal

Cycler, TAKARA, Shiga, Japan) with the set of primers 139 (5´-GTGAAATTATCGCCACGTTCGGGCAA-3´) and 141 (5´-TCATCGCACCGTCAAAGGAACC-3´) designed by Rahn et al. [19]. The PCR reactions were carried out in a 25-µL volume, with final concentration of 1× PCR buffer, 2 mM $MgCl_2$, 200 µM each dNTP, 0.4 µM each primer, 0.1 mg mL^{-1} bovine serum albumin (BSA, Roche, Valencia, Spain, 5 mg mL^{-1}), 0.5 U of DNA polymerase (Biotools, Madrid, Spain) and 100 ng of bacteria DNA. The thermocycling conditions were as follows: an initial denaturation step at 94 °C for 2 min, followed by 35 cycles consisting of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min.

For the quantification of *Escherichia coli* and total coliforms, sludge was suspended in sterilised Ringer solution (1:10 w/v), serially diluted (1:10), plated on Chromocult coliform agar (Merck, Darmstadt, Germany) and incubated at 35 °C for 24 h.

To quantify *C. perfringens* spores, vegetative cells were first eradicated by subjecting the sludge to a thermal shock at 75 °C for 20 min [20]. Sludge was suspended in sterilised Ringer solution (1:10 w/v) and serially diluted (1:10). One mL of each dilution was inoculated on Tryptose sulphite cycloserine (TSC) agar (Condalab, Madrid, Spain) and incubated at 35 °C for 24 h under anaerobic conditions (anaerobic Kit Oxoid, Cambridge, UK). DNA extraction and quantification of positive CFUs was performed as described for *Salmonella* spp. A 900-bp region of the *cpa* gen enconding for the α–toxin [21] was amplified in a PCR thermal cycler (PCR Thermal Cycler, TAKARA, Shiga, Japan) with the set of primers Cpa5f (5´-AGTCTACGCTTGGGATGGAA-3´) and Cpa5r (5´-TTTCCTGGGTTGTCCATTTC-3´) designed by Baums et al. [22]. The PCR reactions were carried out as described for *Salmonella* spp. The thermocycling conditions were as follows: an initial denaturation step at 95 °C for 2 min 30 s, followed by 35 cycles consisting of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min 30 s and a final extension at 72 °C for 10 min.

2.4. Mesophilic incubation of the sludge

Mesophilic incubation of the sludge was carried out both under laboratory conditions and in the ATAD digester. Under laboratory conditions, 500 mL of ATAD-treated sludge was incubated in 1L flasks at 40 °C for 24 h in a rotatory shaker at 60 rpm. Then, the temperature was raised to the thermophilic range (65 °C) again and the sludge was incubated for another 24-hour period. The assay was carried out in triplicate. To performance the incubation in the digester, sludge loading and recirculation were stopped until the temperature fell to 40 °C and this temperature was maintained for 24 hours. Then, ATAD digester was re-started by sludge recirculation and thermophilic conditions were reached again. The experiment was carried out four times.

2.5. Sludge DNA extraction, PCR amplification and DGGE analysis

Total DNA was extracted from freeze-dried sludge with a FastDNA SPIN Kit for Soil (Q-BIOgene, Carlsbad, CA, USA), following the manufacturer's instructions. The extracted DNA was subjected to electrophoresis, and quantified as described in section 2.3.

Ribosomal RNA gene sequences were amplified in a PCR thermal cycler (PCR Thermal Cycler, TAKARA, Shiga, Japan) using the set of primer ITS1F/ITS4 and ITS1F-gc/ITS2 [23,24] for the amplification of fungal sequences and primers sets 338f-gc/907r [25] for the amplification of bacterial sequences. A GC-clamp was added to the forward primer to improve electrophoretic separation of amplicons by DGGE [25]. The PCR reactions were carried out in 25-µL volume and the reaction mixture was the same as described in section 2.3 with the addition of 20 mM tetramethylammonium chloride (TMAC), and 1 µL of a 1:10 dilution of extracted DNA. For fungi, a nested PCR was performed. The first thermocycling program was preceded by an initial denaturation step at 95 °C for 5 min, followed by 30 cycles consisting of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The product of this reaction was then 1:10 diluted with sterile water and used as a template for a subsequent round of a thermocycling program consisting of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 1 min, and a final

extension at 72 °C for 10 min. For bacteria, the thermocycling program was preceded by an initial denaturation step at 95 °C for 3 min, followed by 35 cycles consisting of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

The PCR products from each sample were analysed by DGGE using the DCODE™ Universal Mutation detection System (Bio-Rad Laboratories, Hercules, CA, USA). For fungi, 8% polyacrylamide gels [30% acrylamide/bisacrylamide (37.5:1) (Protogel, National diagnostics, Atlanta, GA, USA)] were prepared with a 30% [2.02 M urea, 12% (v/v) formamide] to 60% [4.04 M urea, 24% (v/v) formamide] vertical denaturing gradient gel. For bacteria, 7% polyacrylamide gels [30% acrylamide/ bis-acrylamide (37.5:1) (Protogel, National diagnostics, Atlanta, GA, USA)] were prepared with a 40% [2.69 M urea, 16% (v/v) formamide] to 70% [4.71 M urea, 28% (v/v) formamide] vertical denaturing gradient gel. 20-μL of the PCR products were loaded into the denaturing gels. The DGGE was performed at 200 V for 10 min, followed by 75 V for 16 h at 60 °C in 1× TAE buffer (40 mM Tris–acetate, 1 mM EDTA). The gels were stained with SYBR Gold (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions, and visualised under UV light.

The DGGE gel images were analysed using Quantity One software (version 4.5, Bio-Rad Laboratories, Hercules, CA, USA). The Dice similarity coefficient [26] was determined for the resulting DNA band profiles, and the clustering algorithm of Ward [27] was used to calculate the dendograms of each DGGE, considering a band as having an intensity >10–15.

2.6. Statistical analysis

For the analysis of the pathogenic microorganisms of the sludge, the data (log-transformed) were submitted to a student's t test for paired samples ($p < 0.05$), preceded by the autocorrelation test to determine whether the samples were independent of each other with time, for 2-week intervals. One-way ANOVA, followed by Tukey's significant difference *post hoc* test ($p < 0.05$), was performed, to compare the temperature and *Clostridium perfringens* spores counts in both laboratory and ATAD digester experiments. The statistical software SPSS 19.0 was used for the analysis (SPSS Inc.).

3. Results and discussion

3.1. ATAD digester start-up and operational parameters

Table 1 shows the monthly average performance parameter data for the 19 months of operation.

The dimensions of the plant were chosen to ensure aerobic conditions, which is one of the most-critical points since oxygen is used by the microorganisms present in the sludge, producing an exothermal reaction that releases heat. Energy conservation was another challenge that was assure with the thermal isolation of the digester and reducing its loss through aeration. Consequently, an efficient aerator was installed in order to minimise the quantity of air that had to be injected into the bioreactor. Nevertheless, during the start-up process, aerator obstruction due to rotosieve blockage took place resulting in inadequate aeration of the sludge, low volatile solids destruction (VSD) and short SRT (months 4 to 7). Therefore, the operational period was divided into two phases: start-up stage and process stabilization. The data obtained once the process was stabilized are discussed below.

The OLR was 2.7 kg VS m^{-3} d^{-1} with a SRT of 14.6 days and an average temperature of 62.0 °C. The OLR was higher than values between 0.7-1.42 kg VS m^{-3} d^{-1} established for conventional aerobic digestion [28] and the SRT was between the rank of 8 and 15 days required for the normal two-stage ATAD [29]. The required temperature was obtained by the supply of fresh raw sludge and also by the degree of sludge recirculation, which is closely related to the quantity of air introduced into the system by a Venturi-type aerator. Temperatures above 65 °C tried to be avoided as they approach the maximum temperature at which thermophilic Bacilli can survive [13].

Table 1. Monthly average performance parameter data.

	Time	Raw sludge						ATAD sludge							
	(months)	OLR	TS	VS	T	pH	EC	TS	VS	T	pH	EC	Redox	SRT	VSD
		(kg VS m^{-3} d^{-1})	(%)	(%TS)	(°C)		(dS m^{-1})	(%)	(%TS)	(°C)		(dS m^{-1})		(d)	(%)
Start-up stage	1	3.4	5.1	69.5	18.5	6.2	4.8	2.8	64.4	57.8	8.1	10.3	-84.2	10.5	20.5
	2	4.5	6.2	69.6	20.4	6.0	5.1	4.0	64.5	59.4	8.1	11.2	-56.0	9.7	20.6
	3	4.3	5.7	73.2	24.8	6.4	4.9	3.3	68.4	61.9	7.7	12.8	-45.3	9.7	20.8
	4	1.0	5.5	74.3	24.7	6.0	4.8	2.8	71.9	57.7	7.0	20.9	5.3	41.8	11.5
	5	5.3	5.3	75.1	30.0	6.4	3.9	2.4	73.8	65.6	7.9	9.0	-46.4	7.6	6.6
	6	3.9	4.0	68.4	30.7	6.3	2.8	2.2	70.8	70.3	7.9	2.3	-49.3	7.0	-12.0
	7	4.8	4.4	67.7	27.1	6.5	2.6	2.2	65.6	69.4	7.6	1.5	-32.0	6.3	9.0
	8	2.6	4.9	74.2	21.3	7.8	3.4	2.5	71.4	67.5	7.1	9.2	-58.3	13.9	13.2
	9	3.0	5.0	74.6	17.9	6.7	2.3	2.1	69.4	68.8	8.5	10.2	-79.4	12.6	22.8
	10	3.0	5.4	76.1	14.7	6.5	2.3	2.6	71.6	67.4	8.5	9.4	-76.3	13.9	20.8
	Average	3.6	5.2	72.3	23.0	6.5	3.7	2.7	69.2	64.6	7.8	9.7	-52.2	13.3	13.4
Process stabilization	11	3.5	5.3	76.2	15.3	6.4	2.3	2.4	66.9	60.0	8.4	10.4	-75.9	11.6	36.9
	12	3.6	6.3	75.9	16.6	6.4	2.5	2.3	65.1	60.2	8.3	11.2	-74.9	13.4	40.8
	13	3.0	5.0	76.3	19.3	6.3	2.6	2.0	65.0	52.6	8.1	9.9	-63.8	12.9	42.3
	14	3.3	6.7	71.4	21.8	6.7	7.1	2.0	62.3	61.6	8.6	9.4	-73.9	14.8	33.8
	15	1.8	4.8	69.3	22.9	6.7	5.2	2.0	60.1	59.3	8.3	10.8	-75.1	18.7	33.4
	16	2.1	5.2	71.1	27.8	6.9	6.9	2.3	62.9	68.4	8.8	10.4	-91.0	17.7	31.2
	17	2.8	5.4	67.5	28.9	7.2	8.6	2.6	56.0	65.3	8.6	12.4	-79.8	13.3	38.7
	18	2.2	4.5	65.6	30.7	7.4	12.6	2.9	55.0	66.4	8.7	12.0	-104.5	13.5	35.9
	19	2.0	4.5	70.1	25.3	7.3	9.0	2.3	54.6	64.6	9.0	11.4	-100.9	15.9	48.7
	Average	2.7	5.3	71.5	23.2	6.8	6.3	2.3	60.9	62.0	8.5	10.9	-82.2	14.6	38.0

OLR Organic loading rate, *TS* Total solids, *VS* Volatile solids, *T* Temperature, *EC* Electrical conductivity, *SRT* Sludge retention time, *VSD* Volatile solids destruction.

3.2. Physico-chemical parameters

A pH increase was produced by the ATAD process, the digested sludge having an average value of 8.5 compared with 6.8 for the raw sludge (Table 1). The pH increase in ATAD systems has been attributed to the stripping of ammonia and carbon dioxide that is converted into carbonate salts, during the degradation of organic matter at high temperatures [10].

An increase in EC from 6.3 dS m^{-1} to 10.9 dS m^{-1} was observed in the digested sludge (Table 1). This increase might have been due to the loss of organic matter and release of different mineral salts in available forms, such as phosphate, ammonium and potassium [30] (Table 1).

The total and volatile solids content in raw sludge were 5.3% and 71.5 TS% respectively. After the digestion process, VS decreased to 60.9 TS% and VSD had mean values of 38.0%, although individual values above 48% occurred (Table 1). This values, achieve the requirements of ATAD processes for municipal sludge, established in a maximum concentration of 7% TS to ensure adequate mixing effectiveness and a minimum concentration of 3% to 4% VS as a surrogate measure for COD [29]. VSD is an important indicator parameter for substrate mineralization and sludge stability that is related to both reactor temperature and sludge retention time [31]. These values are in agreement with minimum VS removal efficiencies of 25-35% [32] and 35-45% [33] for ATAD systems with SRT of 6 or more days and achieved a VSD of 38% established as the sludge vector attraction requirement for Class A biosolids [6]. Figure 2 shows the evolution of the VSD and the temperatures of the inlet stream and the digester with time. It can be observed that the variation of temperature in the digester followed a similar trend than in the inlet stream and that too elevated temperatures in the digester (above 65 °C) produced a slight decrease in the VSD efficiency, coinciding with the maximum survival temperature for thermophilic Bacilli [10] and in accordance with other authors that observed a reduced efficiency of organic material consumption in ATAD reactors at high temperatures and suggested a temperature of 60 °C as a good compromise between COD removal and pathogen destruction [34].

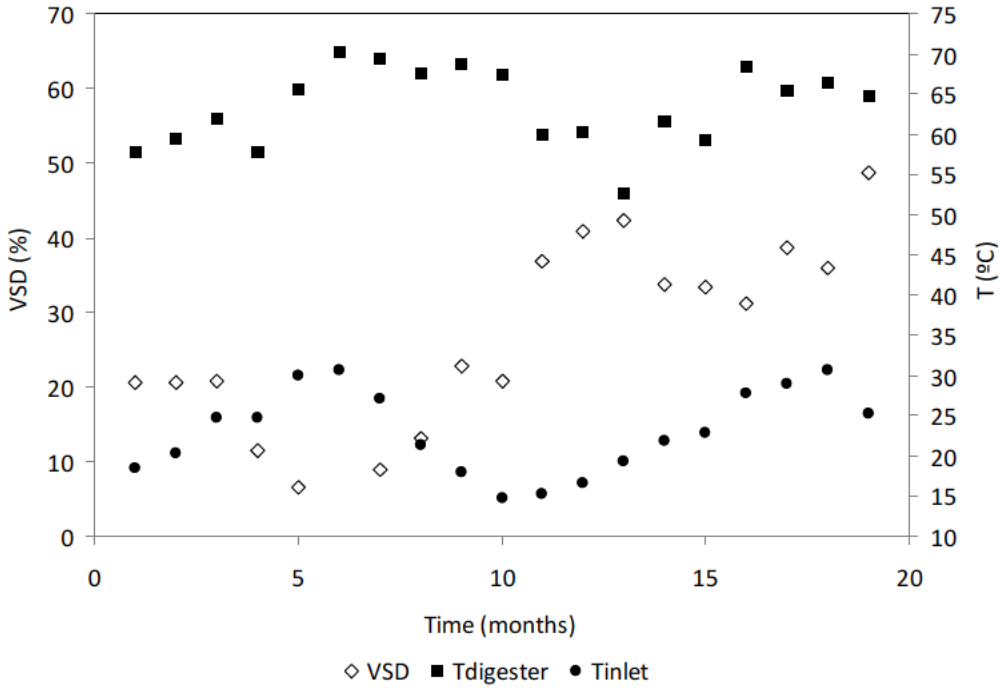

Figure 2. Volatile solids destruction (VSD) and temperatures of the inlet stream and in the ATAD digester.

Table 2 shows the physico-chemical characteristics of raw and digested sludge. The total organic C (TOC) declined from 376 g kg^{-1} in raw sludge to 346 g kg^{-1} in the digested sludge. The loss of TOC in the ATAD reactor is produced by the metabolic activity of aerobic microorganisms that convert soluble organic materials into lower-energy forms, hence reducing the sludge solids content [31]. Therefore, it is expected that ATAD sludge will show a higher degree of stabilisation, as the less-stable organic matter is degraded first, producing a biologically-stable product while reducing both sludge mass and volume [35]. One of the reasons that are often presented to support the use of aerobic thermophilic reactors is their faster specific transformation rate of organic material at high temperature, which would permit the use of smaller bioreactors [36].

Table 2. Physico-chemical sludge characterization (values on a dry weight basis).

Parameter	Raw sludge	ATAD sludge
TOC (g kg^{-1})	376	346
TN (g kg^{-1})	51	65
TP (g kg^{-1})	29	26
TK (g kg^{-1})	3	8
Cu (mg kg^{-1})	221.0	262.5
Fe (mg kg^{-1})	61986	66233
Zn (mg kg^{-1})	633.4	782.3
Ni (mg kg^{-1})	21.5	22.5
Pb (mg kg^{-1})	40.8	41.1
Cd (mg kg^{-1})	1.1	1.7
Cr (mg kg^{-1})	42.0	44.2

TOC: total organic carbon.

The elemental analysis of the raw and digested sludge showed average values for TN, TP and TK of 51, 29 and 3 g kg^{-1} in raw sludge and of 65, 26 and 8 g kg^{-1} in ATAD-digested sludge respectively. The ATAD system increased total N and total K while decreased total P. The increase in these nutrients is the result of organic matter oxidation inside the reactor, for which CO_2, water, heat and nutrients are the final products, reinforcing, therefore, its nutritional value for agricultural application. On the other hand, one characteristic revealed in the early investigations of aerobic thermophilic processes, is the absence of nitrification at temperatures above 40 °C [37]. Consequently, the effluent of aerobic thermophilic bioreactors contains two main forms of N: ammonia and organic N. In addition, an inorganic precipitate like struvite should be present. Decrease in TP may have occurred due to the pH increase that causes the crystallisation of ortho-phosphate and to the thermophilic treatment of sludge that can enhance the release of phosphorous. These results are supported by Riley and Forster [38], who reported that the ATAD process caused an increase in the mean ammoniacal-N concentrations and a decrease in the mean ortho-phosphate concentrations and by Juteau et al. [34] that found better removal of soluble phosphorous at high temperatures and proposed that thermophilic temperatures favoured the crystallisation of soluble phosphorous.

The content of heavy metals was higher in the digested sludge since inorganic elements cannot be decomposed during the digestion process [39]. Nevertheless, in both raw and digested sludge the levels obtained satisfied the

threshold values established in the European Commission Proposal for spreading of sludge on land [8].

3.3. Microbiological characterization

3.3.1. Monitoring of pathogenic microorganisms

The average efficiency of pathogen reduction in the ATAD system is presented in Table 3. The ATAD system significantly decreased the pathogen content for both the start-up and the stabilization process (Table 4).

The presence of *Salmonella* spp. in raw sludge was 100% whereas it was never detected in the digested sludge. These data are comparable to those of Zábranská et al. [40], who also found 100% *Salmonella* spp. detection in raw sludge and 7-27% detection in thermophilic sludge samples. These results satisfy both European and U.S. regulations with regard to obtaining either an advanced sludge or a Class A biosolids (Table 5).

Table 3. Average (CFU ml^{-1}) of pathogenic microorganisms in raw and digested sludge.

		Raw sludge				ATAD sludge			
	Week	Total Coliforms	*E. coli*	*C. perfringens spores*	*Salmonella* spp.	Total Coliforms	*E. coli*	*C. perfringens spores*	*Salmonella* spp.
		(CFU mL^{-1})				(CFU mL^{-1})			
Start-up stage	1	2.7 x 10^7	9.5 x 10^4	4.5 x 10^5	Pres.	1.0 x 10^1	1.0 x 10^1	3,6 x 10^3	Abs.
	2	3.8 x 10^7	8.5 x 10^5	7.0 x 10^5	Pres.	1.0 x 10^1	1.0 x 10^1	3,4 x 10^3	Abs.
	3	5.6 x 10^7	8.9 x 10^5	1.9 x 10^6	Pres.	1.0 x 10^1	1.5 x 10^1	6,0 x 10^3	Abs.
	4	1.3 x 10^8	2.9 x 10^5	1.0 x 10^6	Pres.	3.2 x 10^2	N.D	5,5 x 10^3	Abs.
	5	4.1 x 10^7	1.5 x 10^5	1.6 x 10^6	Pres.	2.4 x 10^2	N.D	9,4 x 10^3	Abs.
	6	6.6 x 10^7	1.6 x 10^6	8.9 x 10^5	Pres.	9.7 x 10^3	N.D	1,7 x 10^4	Abs.
	7	1.7 x 10^8	1.5 x 10^6	5.0 x 10^5	Pres.	2.0 x 10^1	N.D	5,1 x 10^4	Abs.
	9	6.6 x 10^8	1.4 x 10^6	6.0 x 10^5	Pres.	3.0 x 10^1	N.D	6,6 x 10^3	Abs.
	10	4.7 x 10^8	5.4 x 10^6	2.8 x 10^5	Pres.	5.0 x 10^1	N.D	2,2 x 10^4	Abs.
	Average	1.8 x 10^8	1.4 x 10^6	8.8 x 10^5	100% Pres.	1.2 x 10^3	<1.5x10^1	1.4 x 10^4	100% Abs.

Pres.: presence, Abs.: absence, N. D.: not detected.

Table 3. (continued).

		Raw sludge				ATAD sludge			
	Week	Total Coliforms	*E. coli*	*C. perfringens spores*	*Salmonella* spp.	Total Coliforms	*E. coli*	*C. perfringens spores*	*Salmonella* spp.
		(CFU mL^{-1})				(CFU mL^{-1})			
Process stabilization	11	4.4×10^7	1.2×10^6	3.0×10^5	Pres.	N.D	N.D	8.7×10^3	Abs.
	12	1.8×10^8	1.7×10^6	4.1×10^5	Pres.	N.D	N.D	1.2×10^4	Abs.
	13	8.8×10^7	4.9×10^6	9.4×10^5	Pres.	N.D	N.D	1.7×10^4	Abs.
	14	1.5×10^7	1.4×10^6	6.5×10^5	Pres.	N.D	N.D	7.1×10^3	Abs.
	18	6.4×10^7	4.0×10^7	1.7×10^6	Pres.	N.D	N.D	5.4×10^3	Abs.
	19	7.2×10^6	4.0×10^5	1.4×10^6	Pres.	N.D	N.D	7.3×10^3	Abs.
	20	5.4×10^5	4.9×10^5	3.5×10^5	Pres.	N.D	N.D	3.6×10^3	Abs.
	21	1.2×10^7	4.8×10^6	3.0×10^5	Pres.	N.D	N.D	3.4×10^4	Abs.
	26	1.2×10^6	4.5×10^4	1.1×10^6	Pres.	N.D	N.D	9.0×10^3	Abs.
	27	3.2×10^6	1.2×10^5	1.1×10^6	Pres.	N.D	N.D	5.5×10^3	Abs.
	28	1.4×10^6	2.8×10^5	1.4×10^6	Pres.	N.D	N.D	9.4×10^3	Abs.
	30	2.0×10^7	2.0×10^6	9.3×10^5	Pres.	N.D	N.D	1.7×10^4	Abs.
	31	2.0×10^7	9.0×10^4	4.0×10^5	Pres.	N.D	N.D	5.1×10^3	Abs.
	33	5.7×10^6	2.5×10^6	5.0×10^5	Pres.	N.D	N.D	6.6×10^3	Abs.
	34	1.8×10^8	1.0×10^6	2.6×10^5	Pres.	N.D	N.D	2.2×10^4	Abs.
	35	1.2×10^7	2.9×10^5	2.0×10^5	Pres.	N.D	N.D	9.5×10^2	Abs.
	36	1.5×10^7	7.0×10^4	3.8×10^5	Pres.	N.D	N.D	2.6×10^3	Abs.
	37	1.3×10^8	3.3×10^6	8.8×10^5	Pres.	N.D	N.D	3.7×10^3	Abs.
	38	9.0×10^7	4.5×10^4	5.2×10^5	Pres.	N.D	N.D	1.1×10^3	Abs.
	39	3.8×10^8	1.4×10^6	1.5×10^6	Pres.	N.D	N.D	9.5×10^3	Abs.
	40	4.1×10^8	6.0×10^6	1.1×10^6	Pres.	N.D	N.D	2.4×10^3	Abs.
	41	1.0×10^8	6.0×10^5	2.7×10^6	Pres.	N.D	N.D	2.4×10^4	Abs.
	42	2.7×10^8	2.2×10^6	1.3×10^6	Pres.	N.D	N.D	5.2×10^3	Abs.
	43	3.9×10^8	3.4×10^6	8.9×10^5	Pres.	N.D	N.D	2.5×10^3	Abs.
	44	1.4×10^8	9.0×10^5	9.1×10^5	Pres.	N.D	N.D	1.4×10^4	Abs.
	45	1.1×10^8	1.7×10^6	1.1×10^6	Pres.	N.D	N.D	1.4×10^4	Abs.
	Average	1.0×10^8	3.4×10^6	8.9×10^5	100% Pres.	N.D	N.D	9.6×10^3	100% Abs.

Table 4. Results of student´s t test for microorganisms in raw and digested sludge.

	Start-up stage		Process stabilization	
	T	*P*	T	*P*
Total coliforms	20.0	0.000	6.6	0.000
Escherichia coli	10.9	0.000	6.5	0.000
C. perfringens spores	5.8	0.004	6.2	0.000

The average value of *E. coli* in raw sludge was 3.4 x 10^6 CFUs mL^{-1}, and it was never detected in the digested sludge. This represents a die-off rate of 6 logs. *Escherichia coli* is one of the human pathogens included in the Proposal for a Directive, since during the last decade the food chain has been identified as a major route of transmission of some of the more-recently-acknowledged pathogens, like *E. coli* type O157 [8]. The requirements of future European legislation are again met, whereas the U.S. regulations provide for fecal coliforms requirements (Table 5). Fecal coliforms are intended to be an indicator of fecal contamination, and more specifically of *E. coli* which is an indicator microorganism for other pathogens that may be present in faeces. The presence of fecal coliforms in water may not be directly harmful, and does not necessarily indicate the presence of faeces [41]. Furthermore, *E. coli* represents approximately 90% of the fecal coliforms and scientific studies suggest that *E. coli* is the preferred indicator of fecal contamination. Thus, the Council of European Communities and the World Health Organization have replaced fecal coliforms by *E. coli* in their standards and guidelines [42,43].

Table 5. Pathogen requirements in the Proposal for a European Directive and in the U.S. Environmental Protection Agency for sludge application on land.

European Proposal for a Directive. Sludge advanced treatment status requirements [a]
- 99.99% (4 Log) reduction in *Escherichia coli* to less than 1 x 10^3 colony forming units per gram (dry weight)
- No more than 3 x 10^3 spores of *Clostridium perfringens* per gram (dry weight)
- No detectable *Salmonella* spp. in 50 grams (wet weight)
Environmental Protection Agency. Class A Biosolids requirements [b]
- Either the density of fecal coliforms be less than 1,000 MPN[2] per gram (dry weight)
- Or the density of *Salmonella* spp. be less than 3 MPN per 4 grams (dry weight)

[a] European Commission (2003). Proposal for a Directive of the European parliament and of the council on spreading of sludge on land (Annex I); [b] USEPA (2003). Environmental Regulations and Technology: Control of Pathogens and Vector Attraction in Sewage Sludge (Chapter 4).

The mean count of total coliforms in raw sludge was 1.0 x 10^8 CFUs mL^{-1}, and were not detected in the digested sludge representing a die-off rate of 8 logs.

Total coliforms were also analysed since, historically, they have been used as an indicator of the presence of pathogens in water and wastewater [44], and include both fecal coliforms and *E. coli*, and therefore are always more numerous.

With mean values of 8.9 x 10^5 spores mL^{-1} in the raw sludge and 9.6 x 10^3 spores mL^{-1} in the digested sludge, *C. perfringens* spores showed the lowest reduction rate (2 log spore units) of the pathogens analysed and did not achieve the European Commission microbial standards for sludge application on land. This may be due to the ability of *Clostridium* species to form metabolically-dormant spores that are extremely resistant to environmental stresses such as heat, radiation and toxic chemicals [45]. Besides the environment in the ATAD system is often classified as microaerobic, since the oxygen demand of the biomass is greater than the oxygen supply and the detection of sequences of anaerobic and facultative thermophiles suggests the presence of anaerobic microenvironments in ATAD reactors [46,47]. This is supported by Han et al. [12], who studied the autothermal thermophilic aerobic treatment of swine manure and found that Clostridia was the major bacterial class in the ATAD-treated manure. However, ATAD-digested sludge still fulfilled Class A Biosolids requirements and could be applied to land without any pathogen-related restrictions in the U.S. (Table 5). In this respect, it should be pointed out that *C. perfringens* is the most-widely-distributed pathogen in nature and a normal component of decaying vegetation, marine sediment, the intestinal tract of humans and other vertebrates, insects, soil and sludge [48]. Smith and Gardner [49] determined the relative numbers of both forms of C. *perfringens*, vegetative cells and spores, present in soil that had not been exposed to known fecal contamination for some years. The values varied from 10^2 to 10^4 *C. perfringens* per gram (on a dry weight basis) and they concluded that it is not necessary to postulate fecal contamination in order to account for its presence in soil. Therefore, the aim of the Proposal for a European Directive of subjecting the sludge to a more-stringent treatment processes so that the level of pathogens applied to land does not increase the numbers already present in the environment, would not take place under these premises.

3.3.2. Clostridium perfringens spores reduction to achieve pathogen requirements of the Proposal for a Directive of the European Commission on spreading of sludge on land

ATAD-digested sludge (DS), sludge after the mesophilic incubation (MS) and sludge after both mesophilic and thermophilic incubations (dual-temperature-treated sludge) (MTS) were analysed in order to determine the survival of *C. perfringens* spores. The spores were not detected in MTS, which showed a die-off rate of 3-4 logarithmic units (ANOVA, $F = 1042.91$, $P < 0.05$) (Fig. 3a).

When the experiment was developed in the ATAD digester, spores of *C. perfringens* were present in the MTS at a mean rate of 1.2×10^{1} mL^{-1} (ANOVA, $F = 38.29$, $P < 0.05$) (Fig. 3b), which represents a die-off rate of 4-5 log spores compared to raw sludge and of 2-3 log spores compared to the previously-tested ATAD system (Table 3). Therefore, European legislation pathogen requirements were achieved this time since the legislation establishes a maximum value of 3.3×10^{3} spores per gram (dry weight basis) (Table 5) and it was obtained 4.8×10^{2} spores per gram (dry weight basis). Hence, this dual-temperature treatment of sludge, from mesophilic to thermophilic conditions, is able to provide a final product that fulfils completely the microbiological parameter requirements of the Proposal for a Directive regarding advanced treatments.

The goal of the mesophilic incubation was to trigger the germination of the spores to produce vegetative cells which could be killed through a sudden temperature change or damaged an unable to produce a viable spore again. The optimum temperature for the germination of vegetative *C. perfringens* cells is 43-45 °C [50]; nevertheless, 40 °C were chosen since it is closer to the conventional operational temperature of mesophilic anaerobic digesters (35-38 °C).

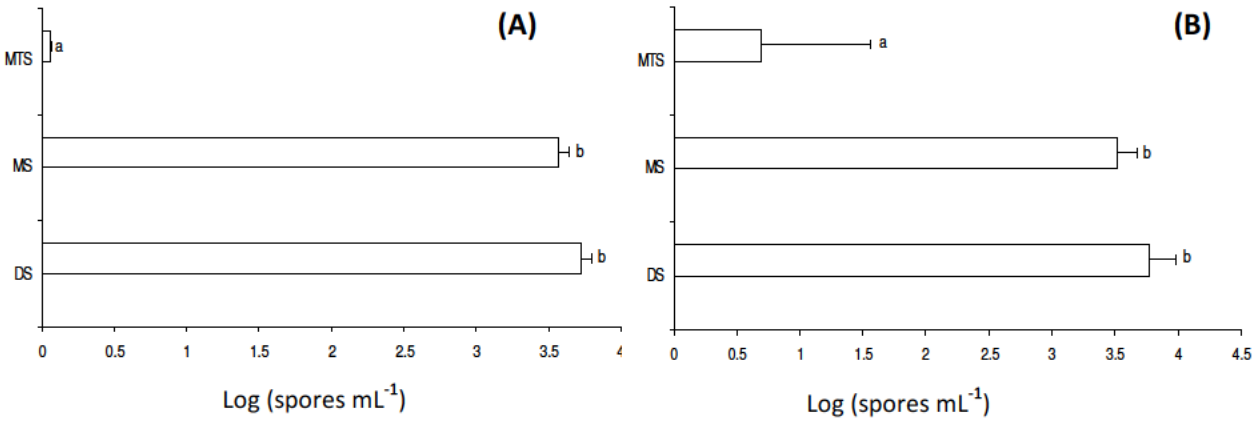

Figure 3. The average reduction in *Clostridium perfringens* spores after dual-temperature treatment under laboratory conditions. The samples shown are ATAD-digested sludge (DS), the same sludge after mesophilic incubation (MS) and sludge after mesophilic incubation followed by thermophilic incubation (MTS) (A). The average reduction in *C. perfringens* spores after four trials of dual-temperature treatment at the pilot-scale plant. The samples shown are ATAD-digested sludge (DS), the same sludge after mesophilic incubation (MS) and sludge after mesophilic incubation followed by thermophilic incubation (MTS) (B). Error bars indicate standard errors. Data followed by the same letter are not significantly different according to Tukey's post hoc test ($P < 0.05$).

3.3.3. Analysis of microbial structure by DGGE

Raw sludge (RS), ATAD-digested sludge (DS), sludge after the mesophilic incubation (MS) and sludge after both mesophilic and thermophilic incubations (dual-temperature-treated sludge) (MTS) were analysed by DGGE in order to assess the changes in the structures of the fungal and bacterial communities (Figs. 4 and 5). The DGGE profile of the fungal community revealed a banding pattern that remained quite similar among the different incubation stages (Fig. 4a). The cluster analysis of this profile showed one main cluster, in which samples RS and DS were grouped together (Fig. 4b) and where the sample obtained at the final stage (MTS) showed the biggest difference among the others. This suggests that the fungal community of the samples changed according to the incubation time, although it remained quite even along the digestion process.

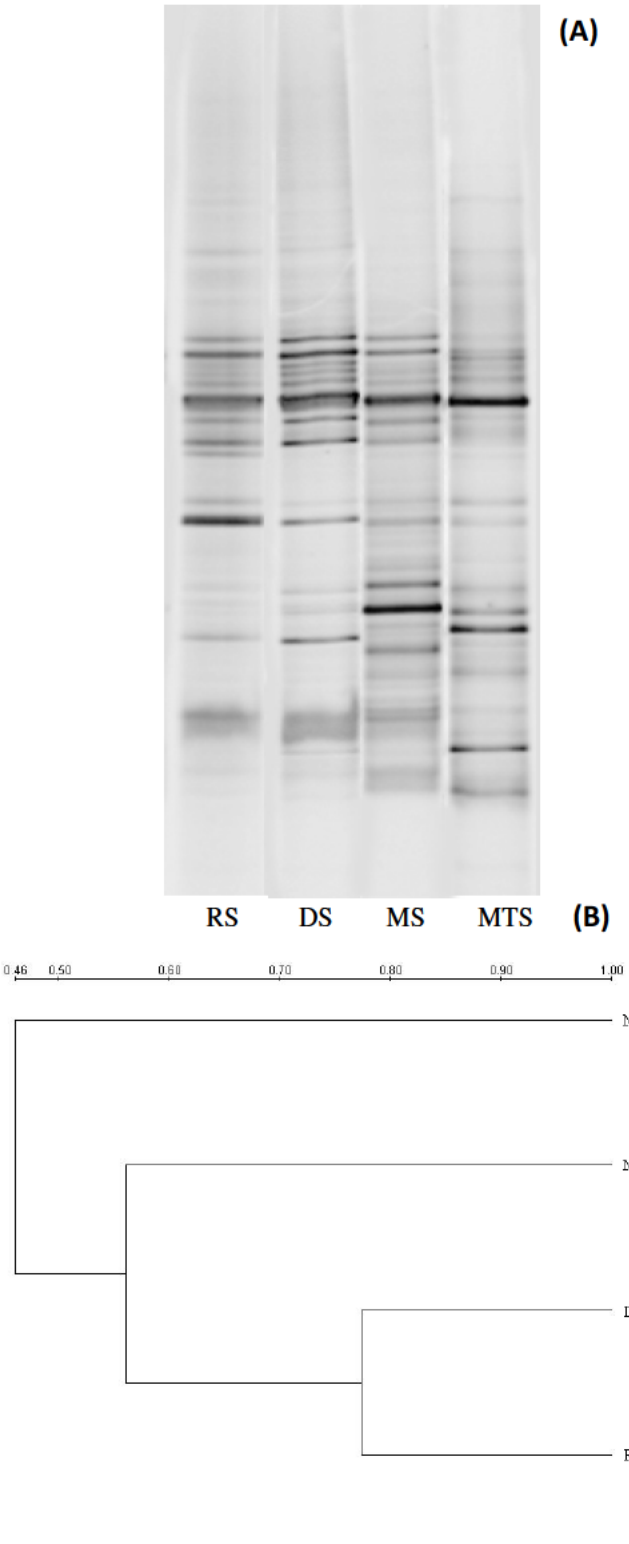

Figure 4. DGGE fingerprint of fungal 18S rRNA gene fragments amplified from sludge samples. Samples were taken from raw sludge (RS), digested sludge (DS), digested sludge after mesophilic incubation (MS) and mesophilic-thermophilic treated sludge (MTS) (A). Ward dendogram of the fungal community generated from the DGGE profile (Ward method, Dice coefficients of similarity) (B).

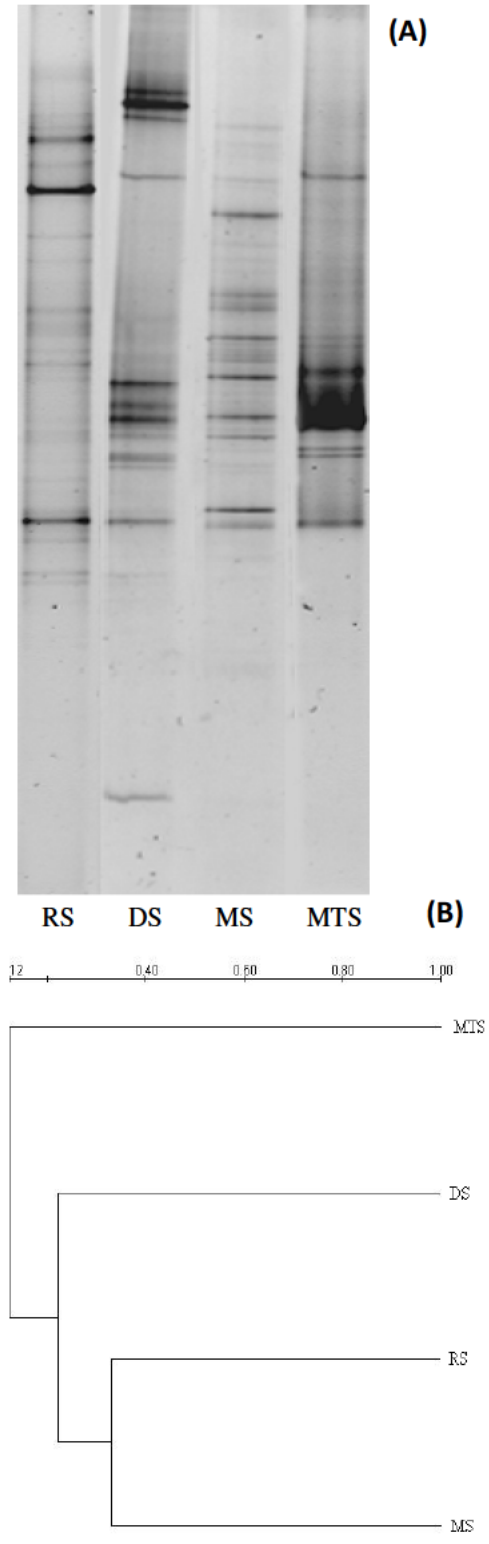

Figure 5. DGGE fingerprint of bacterial 16S rRNA gene fragments amplified from sludge samples. Samples were taken from raw sludge (RS), digested sludge (DS), digested sludge after mesophilic incubation (MS) and mesophilic-thermophilic sludge (MTS) (A). Ward dendogram of the bacterial community generated from the DGGE profile (Ward method, Dice coefficients of similarity) (B).

The DGGE profile for the bacterial community presented a quite-different banding pattern for all the samples (Fig. 5a) and the cluster analysis of this banding pattern showed that all the samples were grouped far from each other, demonstrating a large difference in the bacterial community (Fig. 5b). Similarly to the fungal community, the samples are distributed in one main cluster but with one sub-group composed of samples MS and RS, and MTS showed the biggest difference among the others. This highlights how high temperature was a factor that greatly affected the survival of bacterial microorganisms, producing a decrease in their diversity - especially when incubation occurred under thermophilic conditions. Fig. 5a showed that the number of bands decreased in sample MTS. Likewise, Han et al. [12] found that ATAD treatment of swine manure resulted in a reduction of microbial population richness, diversity and evenness, while Liu et al. [51] showed a banding pattern in which the number of bands decreased and different populations appeared during thermophilic aerobic digestion. These results support the previous experiment in which *C. perfringens* spores were eradicated by means of dual-temperature incubation.

4. Conclusions

Autothermal thermophilic digestion resulted in a suitable technology for sludge stabilization when all operational parameters were stable. Volatile solids destruction in the one-stage digester was 38% and *Salmonella* spp. and *E. coli* were completely eliminated. Consequently, Class A biosolids requirements were fulfilled for both VS reduction and vector attraction. The presence of *Clostridium perfringens* spores prevented the obtaining of advanced sludge. A dual-temperature treatment was proposed to achieve a fully sanitized sludge. The final product was a valuable fertilizer, stable, with high nutrient content and with safe levels of pathogens and heavy metals.

Acknowledgments

This work was supported by the JAE Programme from the Consejo Superior de Investigaciones Científicas (CSIC), Spain, and the project 324/pc08/2-04.3 included in the Plan Nacional de I+D+i 2008-2011. We thank Dr. David Walter for language comments on the manuscript.

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IV

IV. Semi full-scale thermophilic anaerobic digestion (TAnD) for advanced treatment of sewage sludge: stabilization process and pathogen reduction

IV. Semi full-scale thermophilic anaerobic digestion (TAnD) for advanced treatment of sewage sludge: stabilization process and pathogen reduction

Digestión termófila anaerobia (TAnD) a mediana escala para el tratamiento avanzado de lodos de depuradora: estabilización del proceso y eliminación de patógenos

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Chemical Engineering Journal (2013) 232: 42-50.

Resumen

En este trabajo se evaluó la puesta en marcha y funcionamiento de un digestor termófilo anaerobio (TAnD) de mediana escala (15 m^3) durante un periodo de 18 meses. El objetivo de este estudio fue evaluar el proceso de estabilización de lodos de depuradora y los microorganismos patógenos incluidos en la "Propuesta de Directiva del Parlamento y del Consejo Europeo sobre aplicación agrícola de lodos" mediante un tratamiento TAnD, en comparación con una digestión mesófila anaerobia (MAD). La estrategia escogida para la conversión de temperatura desde un inóculo mesófilo a las condiciones termófilas finales, fue el de un rápido y único incremento de temperatura. Para evaluar la estabilidad del proceso, se realizaron medidas de parámetros físico-químicos como la destrucción de sólidos volátiles (VSD), ácidos grasos volátiles (VFA), producción de biogás, macronutrientes y metales pesados. Para evaluar la destrucción de microorganismos patógenos, se cultivaron *Salmonella* spp., *Escherichia coli* y las esporas de *Clostridium perfringens* y se amplificaron los genes de patogenicidad *invA* y *cpa* mediante PCR. El reactor funcionó con tiempos de retención hidráulicos (SRT) de 28, 20, 18 y 16 días, y con una velocidad de carga orgánica (VCO) que osciló entre 1,5 y 2,5 kg VS m^{-3} d^{-1}. En todos los periodos de operación se obtuvo un funcionamiento adecuado del digestor alcanzando valores de VSD superiores al 40% y una producción de biogás media situada en 0,64 Nm^3 kg^{-1} VS^{-1}. El sistema TAnD, admitió mayores VCO y menores SRT que la digestión MAD. Respecto al contenido de microorganismos patógenos, redujo las

poblaciones de *Salmonella* spp. y *E. coli* por debajo de los límites de detección, pero no consiguió eliminar las esporas de *C. perfringens* (4,63 $\log_{10}$ esporas mL^{-1}). Por tanto, el producto final cumplió con los límites establecidos en la legislación americana para alcanzar la clasificación de biosólidos de Clase A, pero no logró satisfacer los límites establecidos en la futura legislación europea.

Chemical Engineering Journal (2013) 232: 42-50

Semi full-scale thermophilic anaerobic digestion (TAnD) for advanced treatment of sewage sludge: stabilization process and pathogen reduction

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HIGHLIGHTS

- A semi full- scale thermophilic anaerobic digester was studied over 18 months.
- Both the start-up and the stabilization process were evaluated.
- The removal of pathogens included in the future European legislation was assessed.
- Volatile solids destruction and biogas production were 40% and 0.64 $Nm^3\ kg^{-1}\ VS^{-1}$.
- *Escherichia coli* and *Salmonella* spp. were not detected after TAnD digestion.

ARTICLE INFO

Article history:
Received 29 December 2012
Received in revised form 9 July 2013
Accepted 18 July 2013
Available online 26 July 2013

Keywords:
Thermophilic anaerobic digestion
TAnD
Sewage sludge
Biogas
Human pathogens
Clostridium perfringens

ABSTRACT

A semi full-scale (15-m^3) one-stage thermophilic anaerobic digester (TAnD) was started-up and studied over a 18-months period. The aim of this work was to evaluate the stabilization process of wastewater sludge and assess the pathogen standards included in the "Proposal for a Directive of the European Parliament and of the Council on spreading of sludge on land". The conversion strategy from mesophilic to thermophilic conditions was performed by a rapid and single temperature increase. Parameters such as volatile solids destruction (VSD), total volatile fatty acids (VFA), biogas production, macronutrients and heavy metals were measured. *Salmonella* spp., *Escherichia coli*, and *C. perfringens spores* were cultivated, and pathogenity genes *invA* and *cpa* PCR-amplified. The reactor was operated over a range of sludge retention times (SRT) of 28, 20, 18, and 16 days and organic loading rates (OLR) ranging from 1.5 to 2.5 kg VS $m^{-3}\ d^{-1}$. Adequate process performance was obtained in all the stable periods reaching

VSD values over 40% and an average biogas production of 0.64 $Nm^3\ kg^{-1}\ VS^{-1}$ fed. Thermophilic anaerobic digestion successfully reduced *Salmonella* spp., and *Escherichia coli* below detection limits but not *Clostridium perfringens* spores (4.63 log_{10} spores mL^{-1}). Thus, the final product met Class A biosolids final disposal regulations, but further investigation is needed in order to satisfy the future European legislation.

1. Introduction

The production of sewage sludge generated in wastewater treatment plants (WWTPs) has significantly increased during the past few years due to the obligation of wastewater treatment, tougher effluent restrictions, and a growing population connected to sewage networks [1]. Hence, this increase in the production of sewage sludge has created significant pressure concerning the optimal management and disposal of this product.

On the other hand, most sewage wastes contain valuable nutrients that could be used to improve soil fertility, crop production, and some soil physical and chemical properties such as soil structure, soil moisture and porosity, provision of plant nutrients, humus content and cation exchange capacity as well as promoting its biological activity [2-5].

In Spain, 65% of the sludge produced is currently used for land application [6]. However, the agricultural use of sewage sludge has some potential risks, associated with its potential content of heavy metals, toxic compounds, pathogenic bacteria, viruses and parasites [7]. In response, sludge reuse and disposal regulations have been developed. In the United States, regulation 40 CFR Part 503 B [8] specifies treatment goals or post-application measures designed to reduce these risks and requires a high degree of pathogen reduction in sewage sludge to achieve Class A biosolids requirements. On the contrary, the current European legislation requires only that the sludge be subjected to a process of stabilisation before land application and only establishes threshold values for heavy metals concentration [9]. In order to improve this situation and prevent decreases in soil quality and risks to human health, the European Union is developing a new regulation through the Proposal for a Directive of the European Parliament and of the Council on spreading of sludge on land [10]. This provides stricter standards regarding the content of heavy metals, organic compounds and human pathogens, this latter parameter receiving special

attention. In this respect, the Proposal for a European Directive introduces the concept of advanced and conventional treatments, which allows operators to use advanced-treated sludge with fewer restrictions compared with a sludge that has been treated conventionally.

Thermophilic anaerobic digestion (TAnD) is considered an advanced technology for the treatment of sludge in the future European legislation and, as a thermal treatment, as an alternative method for demonstrating Class A pathogen reduction [8,10]. Among the benefits of the TAnD process are: its efficiency in the killing of pathogenic microorganisms, reduced sludge retention time, higher biogas production rate, less foaming and better dewaterability [11-13]. In this respect, thermophilic anaerobic digestion is experiencing a renascence from the past interest due to both the public concern about potentially negative impacts of pathogens and pollutants from biosolids on the environment and human health, and the growing interest in renewable energy. The need to reduce the emissions of greenhouse gases, especially carbon dioxide, and to develop a reliable alternative to the fossil fuel economy has led to an increased attention on renewable energy.

On the other hand, mesophilic anaerobic digestion (MAD) is one of the most widely used process for the treatment and stabilization of sludge in wastewater treatment plants, being considered as a conventional treatment in the Proposal for a European Directive and a process capable of producing Class B biosolids in the United States [8,10]. A wide range of existing literature focuses on MAD [14-16] and other studies have shown the efficiency of TAnD processes to achieve Class A biosolids requirements [17,18]. However, there is a lack of available data regarding the microorganisms included in the Proposal for a European Directive, especially in respect of the content of *Clostridium perfringens* spores.

In view of the above, the aim of this work was to start-up, characterise and assess the stabilization process and the pathogen requirements for advanced-treated sludge in a one-stage thermophilic anaerobic digester, evaluating its performance relative to the operation of the full-scale mesophilic anaerobic digester installed in Molina de Segura WWTP. The effect of an increase in the organic loading rate (OLR) -with a subsequent reduction in the sludge retention time (SRT)- on the operation and treatment efficiency (based on volatile solids

destruction and biogas production) of sludge digestion was investigated. *Salmonella* spp., *Escherichia coli*, *C. perfringens* spores, and total coliforms were analyzed to evaluate the hygienization requirements established in the Proposal for a European Directive on spreading of sludge on land.

2. Material and methods

2.1. Description of the TAnD digester designed for this study

The semi full-scale one-stage TAnD digester was installed in Molina de Segura WWTP (Murcia, Spain) and consisted of a 15-m^3 continuously stirred tank reactor (CSTR) covered by a heat-resistant, 10-cm-thick polyurethane layer, for its thermal insulation and cladding. The heating system consisted of a coil heat exchanger of 75.5 kW in which the circulation of the sludge and temperature-controlled water from the primary cooling circuit occurred. The biogas output from the TAnD digester was passed through a stainless steel pipe of 32-mm diameter and was burnt-off in a torch. An over-pressure relief valve was installed to release gas whenever the pressure exceeded 20 kPa. The foam production was controlled with the installation of a polyester rack of a 25-mm reticulum. The sludge stream was operated in batch mode. The temperature of incoming and outgoing sludge, pH, electrical conductivity (EC), and oxidation-reduction potential (ORP) were measured continuously with a set of probes installed in the digester and registered with a SCADA (supervisory control and data acquisition). Figure 1 shows a schematic diagram of the TAnD digester.

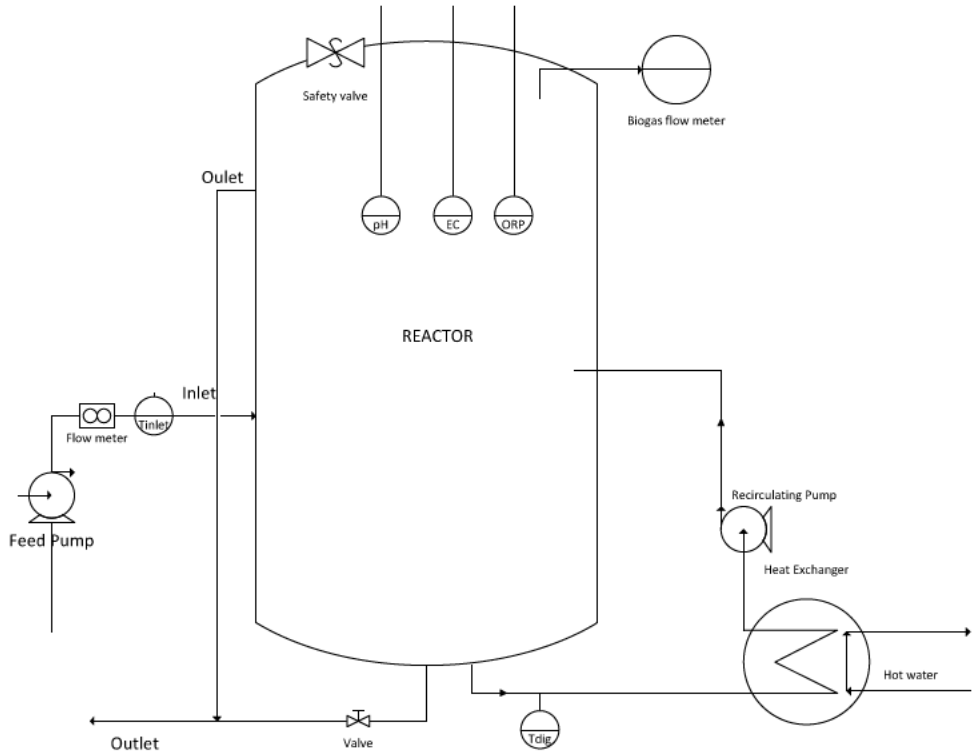

Figure 1. Schematic diagram of the TAnD digester.

2.2 Mesophilic anaerobic digestion

Molina de Segura WWTP consists of a double-stage biological treatment followed by a coagulation-flocculation tertiary system, lamellar settling, filtering, and ultraviolet radiation disinfection, with an average flow of 25,000 m^3 d^{-1}. Primary and secondary sludge produced in the WWTP are first thickened separately and then mixed prior to the introduction into the mesophilic anaerobic digester. The mesophilic anaerobic digester has an effective volume of 7,612-m^3 and serves a population equivalent of 290,000 p.e. The required temperature of the process (around 35 °C) is maintained by the Heatamix system by using hot water from the refrigerating circuit of the motogenerator GUASCOR SFGLD 480/55 of 760 kW. The biogas generated during the digestion process is stored in a double-membrane gasometer of 1,410-m^3 volume to be utilized by the motogenerator. If the storage capacity of the gasometer is surpassed, the excess of biogas will burnt-off in an automatic ignition torch.

2.3. Physical and chemical analysis

Analysis of total solids (TS), volatile solids (VS), and alkalinity were performed daily according to the Standard Methods [19]. Destruction of volatile solids (VSD) was calculated as the fraction of volatile solids destroyed by the constant ash method. The biogas flow rate was measured with a T-Mass AT-70 Thermal Mass Flow meter (Endress+Hauser, IN, USA). The biogas composition and volatile fatty acids (VFA) were analyzed by gas chromatography. Total organic carbon (TOC) was determined by oxidation with $K_2Cr_2O_7$ in an acid medium (H_2SO_4) evaluating the excess of dichromate with $(NH_4)_2Fe(SO_4)_2$ [20]. Total nitrogen (N) was determined by the Kjeldahl method modified by Bremner and Mulvaney [21]. Total phosphorus (P), total potassium (K), and heavy metals were determined in nitric-perchloric (1:1) digestion extract; P was determined by colorimetry following the Murphy and Riley method [22] and K and heavy metals by atomic absorption spectrometry (Perkin-Elmer 5500).

2.4. Dewaterability

To evaluate the dewatering characteristics of the sludges, two laboratory tests were performed: a dynamic jar test and a drainage test. The dynamic jar test was used for the determination of the optimum type and polymer dose (OPD) for an accurate dehydration. This test consisted of the mixing of the sludge and the polymer by a determined number of sludge transfers made from one jar to another. Then, the sludge was mixed with its appropriate type and amount of polymer and the number of necessary transfers to determine the following parameters was calculated: number of transfers needed to achieve flocculation (T_F), floc breakage (T_R), re-flocculation (T_{FR}) and breakage of re-flocculated floc (T_{RR}). The drainage test was used to determine the volume and rate of water released from sludge.

2.5. Cultivation and molecular detection of pathogens

Raw sludge (mixed primary and secondary sludge), sludge produced after mesophilic anaerobic digestion (MAD sludge) and sludge produced after thermophilic anaerobic digestion (TAnD sludge) were sampled weekly during the stable operational periods of the thermophilic digester. Total coliforms, *Escherichia coli*, *Clostridium perfringens* spores, and *Salmonella* spp. were

cultivated and pathogenity genes *invA* and *cpa* PCR-amplified as described by Lloret et al. [23].

2.6. Statistical analysis

For the analysis of chemical parameters, one-way ANOVA was performed with type of sludge as factor followed by Tukey's HSD as a *post hoc* test ($p < 0.05$) to control for multiple testing. For the analysis of pathogenic microorganisms the data (log-transformed) were submitted to a non-parametric test for several independent samples (Kruskal–Wallis test). Pair-wise comparisons between different types of sludge were performed using the Mann–Whitney *U* test ($p < 0.05$).The statistical software SPSS 20.0 was used for the analysis (SPSS Inc.).

3. Results and discussion

3.1. TAnD digester start-up and operational periods

The start-up of this type of bioreactor is a challenging task that can be accomplished in two different ways: by using an inoculum from another thermophilic anaerobic reactor, or by using a mesophilic inoculum. In this study, due to the absence of a thermophilic inoculum, the second option was chosen. In this case, the conversion strategy from mesophilic to thermophilic temperatures may be performed either: a) by a sequential and gradual temperature rise, or b) by a rapid and unique change from mesophilic to thermophilic temperatures. The conversion strategy chosen in this work was to rapidly increase the temperature. In this respect, previous contradictory observations have been reported describing this strategy as not successful for mesophilic-thermophilic temperature conversions [13,24] or as the favourable option to first establish a thermophilic culture when converting from mesophilic operation [25-26]. Consequently, the TAnD digester was filled with mesophilic sludge from Molina de Segura WWTP, sealed, the temperature was increased up to 55 °C and the reactor was maintained without any feed during the following 15 days. Then, the digester was fed with raw sludge with an OLR of 0.7 kg VS m^{-3} d^{-1} that was progressively increased up to 1.0 kg VS m^{-3} d^{-1} within the following 40 days, with the aim of controlling the production of VFA. Figure 2 shows the evolution of the concentration of VFA in the digester during the start-up stage. It can be observed how the concentration of VFA started to increase during the first days of

operation. An initial accumulation of VFA, typical of hydrolysis and fermentation of the easily degradable substrates, is common during start-up process, as the activity of methanogens is exceeded [27].

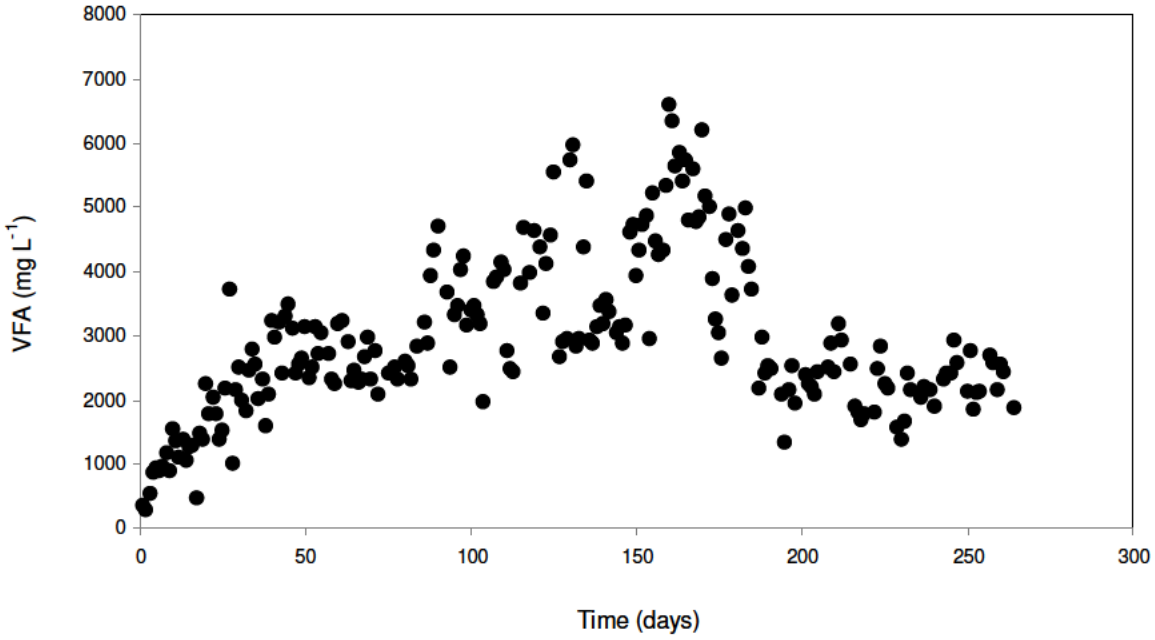

Figure 2. Concentration of volatile fatty acids (VFA) during the start-up of the TAnD digester.

After approximately 50 days, the concentration of VFA began to decrease, which led to an increase of the OLR up to 1.2 kg VS m^{-3} d^{-1}. Nevertheless, as the concentration of VFA started to increase again, suggesting that the system was overloaded, the OLR was lowered to 1.0 kg VS m^{-3} d^{-1} once again and it was not increased until the stabilization of the digestion process occurred. It should be pointed out that the population of methanogenic microorganisms capable of adjusting from a mesophilic to a thermophilic condition is relatively small and needs a long period of time to acclimatize [28]. At the same time, this new thermophilic anaerobic consortia established in the digester will be determinant for its stable and efficient operation [29].

After 250 days, the VFA concentration decreased and stabilized in a range between 2,000 and 3,000 mg L^{-1}, considering this period as the start-up phase. These results are in accordance to other studies that have described the experimental start-up period for a stable thermophilic operation as long -even up to one year- before gas production reaches an acceptable level [30]. Nevertheless,

results from Iranpour et al. [27] that also chose rapid heating as the start-up strategy, show that a shorter process stabilization could be achieved, being their feeding plan proportionally lower [27].

3.2. TAnD digester operational parameters

The stable period in this study had lasted for 300 days, and consisted of four consecutive periods (periods A, B, C, and D) with a duration of 116, 52, 70, and 62 days respectively. The OLR was maintained constant during each operational period.

Both the experimental TAnD digester and the full-scale mesophilic anaerobic digester were fed with the same mixed primary and secondary sludge produced in the WWTP. The main characteristics of the feed sludge used in each period as well as the average performance parameter data of the mesophilic and thermophilic digesters during the process stabilization, are summarized in Table 1. The values presented for the mesophilic reactor are the average values during the experimental period of the thermophilic process.

Table 1. Average performance parameter data of MAD and TAnD digestion processes. Data for TAnD sludge are presented for four different stabilization periods (A, B, C, and D).

		Mesophilic operation	Thermophilic operation			
	Parameter [a]	MAD	TAnD A	TAnD B	TAnD C	TAnD D
Influent	Flow ($m^3\ d^{-1}$)	151.0	0.4	0.6	0.7	0.8
	TS (%)	5.6	5.5	5.3	5.1	5.1
	VS (%TS)	75.3	75.8	75.6	79.3	78.0
	OLR ($kg\ VS\ m^{-3}\ d^{-1}$)	0.8	1.5	2.0	2.2	2.5
Effluent	TS (%)	3.2	2.4	2.7	2.1	1.9
	VS (%TS)	62.8	65.2	62.5	69.4	68.2
	VSD (%)	44.7	40.1	46.1	40.7	39.4
	SRT (d)	50	28	20	18	16
	T (ºC)	37.8	52.3	53.5	53.6	53.9
	ORP	-385	--	--	-382	-365
	ALK ($mg\ CaCO_3\ L^{-1}$)	3114	3302	3852	3366	3056
	VFA ($mg\ L^{-1}$)	227	2030	2022	1705	1104
	VFA/ALK	0.07	0.61	0.52	0.51	0.36
Biogas	Flow ($m^3\ CH_4\ d^{-1}$)	2945	7.6	9.8	10.8	10.6
	$Nm^3\ kg^{-1}\ VS^{-1}_{fed}$	0.46	0.41	0.40	0.39	0.34
	$Nm^3\ kg^{-1}\ VS^{-1}_{destroyed}$	1.04	1.02	0.86	0.96	0.86

[a] TS: Total solids, VS: Volatile solids, OLR: Organic loading rate, VSD: Volatile solids destruction, SRT: Sludge retention time, T: Temperature, ORP: Oxidation-reduction potential, ALK: Alkalinity, VFA: Volatile fatty acids.

In the present study, steady-state conditions were achieved after three times the sludge retention time (SRT).

The OLR of the TAnD digester was gradually increased from 1.5 to 2.5 kg VS m^{-3} d^{-1} while the SRT was reduced from to 28 to 16 days. The OLR of the thermophilic digester showed, therefore, values two to three times higher than the mesophilic digester of Molina de Segura WWTP (0.8 kg VS m^{-3} d^{-1}). Moreover, the increase of the OLR only produced a slight decrease in the volatile solids destruction (VSD) and in the biogas production, suggesting that both the increase of the OLR and the reduction of the SRT did not have a significant negative effect in the efficiency of the process.

The removal efficiency of VS is a significant indicator parameter for substrate mineralization and sludge stability that is related to both reactor temperature and sludge retention time [31] and is commonly used to measure the performance of anaerobic digestion processes. The average VSD achieved in the experimental TAnD digester was 41.5%, achieving values over 46% in period B (SRT of 20 days). These values can be considered adequate since under mesophilic conditions on the industrial scale, a volatile solids reduction of 40% is considered satisfactory [32]. In fact, the VSD achieved in the mesophilic reactor with a SRT of 50 days (considerably higher than the SRT in the thermophilic process) was 44.7%, suggesting that the one-stage TAnD system had a desirable removal efficiency of VS and could achieve the same favorable effect of stabilization as the MAD process. On the other hand, it should be pointed out that both reactors achieved a minimum VDS of 38% established as the sludge vector attraction requirement for Class A biosolids [8].

The temperature in the TAnD digester was maintained within the thermophilic range during the four periods of operation (between 52.3 and 53.9 °C).

Mean concentration of bicarbonate alkalinity (ALK) was maintained above 3,000 mg $CaCO_3$ L^{-1} in both the mesophilic and thermophilic digesters. This alkalinity was enough to maintain an adequate pH in the reactors and is common in anaerobic processes [17,33-34]. On the contrary, with a volatile fatty acids (VFA) concentration that decreased from values of 2030 to 1104 mg L^{-1} during periods A to D, the effluent of the TAnD digester presented higher values compared to the mesophilic anaerobic digestion (227 mg L^{-1}). This is probably due to a high hydrolysis rate coupled with a poor ability of methanogens to consume the resulting VFA. However, much higher VFA concentrations have

been reported in the effluent of thermophilic anaerobic processes [17,32,34]. Besides, in view of the fact that the VFA concentration decreased along the experimental stable period and that the operational parameters were not significantly affected, it can be postulated that the thermophilic digestion supported the organic load applied while achieving a more stable performance. The high concentration of VFA in the final effluents of thermophilic digestion has been indicated as one of the major drawbacks of the use of this sort of process. However, previous studies have reported that thermophilic digestion has an advantage in treating raw sludge under high loading rates compared with the mesophilic process and that the activity of thermophilic bacteria was higher than that of mesophilic bacteria -though thermophilic bacteria tend to remove propionic acid more slowly than mesophilic bacteria- [35].

The VFA to alkalinity ratio also decreased with SRT to a value of 0.36, which is high compared with the value reached at mesophilic digestion (0.07). The higher VFA-to-alkalinity ratio of the thermophilic process is probably due to the higher VFA concentration, indicating that mesophilic anaerobic digestion obtained better buffer capabilities. Nevertheless, the relatively low VFA-to-alkalinity ratio obtained at the end of the experimental period indicates that a healthy microbial population had developed.

Regarding the biogas production, the TAnD digester showed average values from 0.86 to 1.02 $Nm^3\ kg^{-1}\ VS^{-1}$ destroyed, which is within the expected range for anaerobic digestion of wastewater sludge [36] and comparable to those obtained by other authors during thermophilic anaerobic digestion [11,17]. Biogas composition of the TAnD digester showed an average methane content of 62% being similar or even higher, than concentrations previously reported in other studies [17,32], while the mesophilic anaerobic digester of Molina de Segura WWTP had a methane concentration around 60% (data not shown).

3.3. Chemical characteristics of the sludge

Table 2 shows the chemical characteristics of raw, MAD and TAnD sludge. A significant pH increase was produced after the anaerobic digestion in both processes, having average values of 7.7 and 7.5 after mesophilic and thermophilic digestion, respectively compared with 6.1 in the raw sludge. The pH in the TAnD reactor was kept at a constant level without the addition of any reagent. Similarly,

a significant increase in EC values from 6.5 to 13.4 and 12.7 dS m^{-1} after mesophilic and thermophilic digestion respectively, was observed. This increase may have been due to the loss of organic matter and release of different mineral salts in available forms, such as phosphate, ammonium and potassium [37].

Regarding TOC content, its content declined from 523 g kg^{-1} to 365 and 360 g kg^{-1} in MAD and TAnD sludge respectively. The loss of organic matter during the anaerobic digestion is produced by the indigenous microbial communities established in the digesters that consume part of this organic matter during conversions reducing thereby both sludge mass and volume while producing a more stable by-product. In this respect, anaerobic digestion has been described as a process that produces between 70 and 90% less biomass than aerobic digestion, hence favouring the final disposal of the sludge.

The elemental analysis of the different sludges, presented average values for total N, total P, and total K, of 64.9, 26.1, and 3.9 g kg^{-1} in raw sludge, 89.4, 52.3, and 5.6 g kg^{-1} in MAD sludge and 79.7, 40.7, and 4.7 g kg^{-1} in TAnD sludge, respectively.

The sludge digestion increased total N, P, and K content in both mesophilic and thermophilic digestion as a result of the hydrolysis of organic compounds that causes the release of ammonia, nitrogen and phosphate in the bulk and a subsequent increment of their concentration. The increase in these three main nutrients, together with the fact that sludge can also supply many other micronutrients, reinforces its nutritional value for agricultural application.

In regards of the content of heavy metals, a significantly higher amount was observed after both MAD and TAnD digestion in all cases except for chromium (Cr). This increase was higher in the mesophilic effluent for all the cases. These results were expected as inorganic elements cannot be decomposed during the digestion process. Nevertheless, none of the levels obtained for any of the heavy metals exceeded the limits established in the European Commission Proposal for spreading of sludge on land, making them valuable as organic amendments. Furthermore, micronutrient availability has been described as essential for the methanogenic hydrogenothropic population that participate in anaerobic digestion processes [38] and its deficiency, as a likely reason for unstable behaviour and VFA accumulation [39].

Table 2. Chemical characteristics of raw, MAD and TAnD sludge (values on dry weight basis). Data are given for n=5 (standard deviation in brackets).

Parameter [a]	Raw Sludge	MAD sludge	TAnD sludge [b]	UE limits [c]
Dry matter (%)	4.2 (1.4) a	3.2 (0.3) a	3.1 (0.8) a	
pH	6.1 (0.3) a	7.7 (0.1) b	7.5 (0.5) b	
EC (dS m^{-1})	6.5 (1.5) a	13.4 (0.9) b	12.7 (2.0) b	
TOC (g kg^{-1}) [d]	523 (185) a	365 (50) a	360 (52) a	
Total N (g kg^{-1})	64.9 (11.8) a	89.4 (9.2) a	79.7 (14.7) a	
Total P (g kg^{-1})	26.1 (16.8) a	52.3 (1.8) b	40.7 (12.8) ab	
Total K (g kg^{-1})	3.9 (0.9) a	5.6 (1.5) a	4.7 (1.6) a	
Total Cd (mg kg^{-1})	BDL	BDL	BDL	10
Total Cr (mg kg^{-1})	30.2 (13.7) a	46.8 (16.9) a	36.0 (14.5) a	1000
Total Cr_{VI} (mg kg^{-1})	BDL	BDL	BDL	10
Total Cu (mg kg^{-1})	135 (56) a	281 (74) b	206 (47) ab	1000
Total Fe (g kg^{-1})	16.9 (5.3) a	40.5 (7.6) c	28.9 (7.2) b	
Total Hg (mg kg^{-1})	BDL	BDL	BDL	10
Total Ni (mg kg^{-1})	14.9 (4.4) a	38.1 (11.8) c	21.9 (7.3) ab	300
Total Pb (mg kg^{-1})	21.2 (7.6) a	39.4 (7.6) c	38.8 (6.7) bc	750
Total Zn (mg kg^{-1})	376 (134) a	804 (225) b	766 (139) b	2500

[a] EC: Electrical conductivity; TOC: total organic carbon.
[b] BDL: Below detection limits.
[c] Heavy metal limits for soils (European Commission, 2003).
Different letters indicate significant differences between each type of sludge after Tukey HSD test ($p < 0.05$).
[d] Marginally significant differences: Differences were found after ANOVA ($p < 0.05$) but not after Tukey HSD test ($p > 0.05$).

3.4. Reduction of pathogens included in the Proposal for a Directive on spreading of sludge on land

Figure 3 depicts the average concentration of total coliforms, *Escherichia coli*, and *Clostridium perfringens* spores in raw, MAD and TAnD sludge during the stable period in a box-plot format. It should be pointed out that neither severe outliers nor differences between the four stable periods were found in any of the biosolids.

Regarding the densities of total coliforms and *E. coli* found after MAD digestion, it can be observed that the same trend as in the feed sludge was obtained undergoing a significant die-off rate of approximately 2 log units for both microorganism densities ($U = 120$, $p < 0.001$ and $U = 102$, $p < 0.001$, respectively). Nevertheless, a greater dispersion in the distribution of the pathogen content can be observed after MAD digestion, indicating possible fluctuations in the efficiency of the process. The MAD system, with densities of *E. coli* of 3.46 $\log_{10}$ CFU mL^{-1} (2.9 x 10^3 CFU mL^{-1}), achieved the standards for conventionally-treated sludge (2 log reduction to less than 5 x 10^5 CFU per gram -wet weight-). Comparisons between raw sludge and the thermophilic effluent

showed that the TAnD process significantly decreased the pathogen content for both total coliforms and *E. coli* ($U < 0.001$, $p < 0.001$ for both pathogens), which were never detected in the outflow. This system therefore met well, the requirements for advanced treatment status for *E. coli* established in the Proposal for a European Directive (4 log reduction to less than 1 x 10^3 CFU per gram -dry weight-).

Regarding the content of *C. perfringens* spores, average values of 5.28, 5.40, and 4.63 $\log_{10}$ spores mL^{-1} were found in the incoming sludge and after mesophilic and thermophilic digestion respectively. In this case, no reduction was found after the MAD process ($U = 489$, $p = 0.37$), their presence being expected in the high concentrations obtained since clostridia are known to grow well under anaerobic conditions and mesophilic temperatures. On the other hand, despite the significant reduction of 0.65 log units that took place after TAnD digestion ($U = 73$, $p < 0.001$) the densities found after this treatment prevented the thermophilic sludge to fulfilled, as regards *C. perfringens* spores, the requirements of the future European legislation for advanced treated sludge (less than 3 x 10^3 spores per gram -dry weight-).

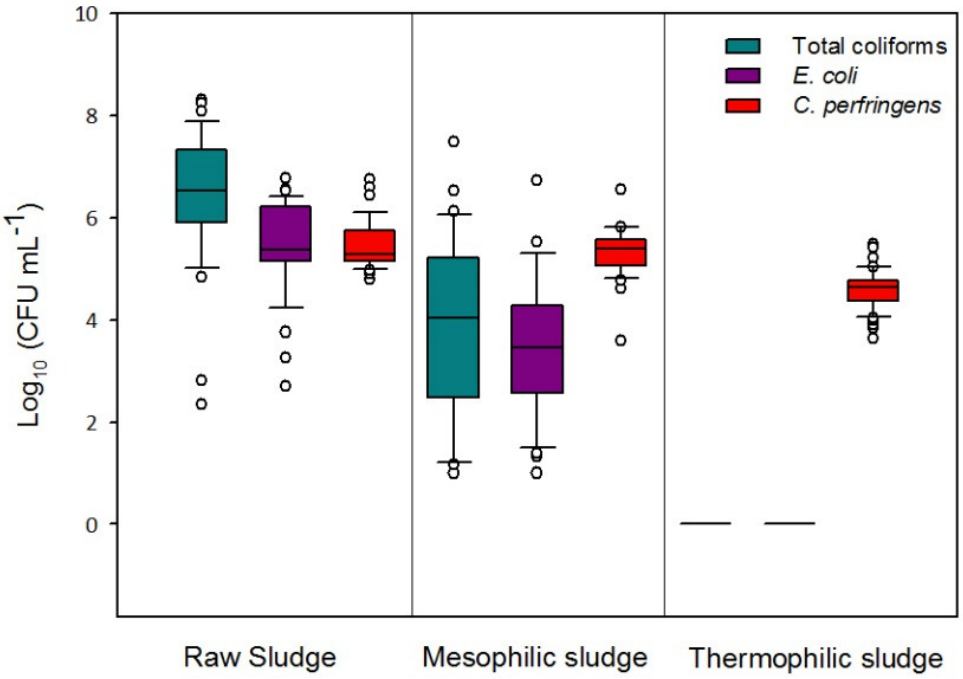

Figure 3. Box-plot of average concentrations of total coliforms, *Escherichia coli*, and *Clostridium perfringens* spores in raw, MAD, and TAnD sludge. The inner box lines represent the geometric medians, while the outer box lines represent the 25th and the 75th data percentiles (inner quartile range [IQR]), the whiskers extend to 1.5 times the IQR, indicanting 10th and 90th percentiles, and the open circles represent data outliers.

In relation to the content of *Salmonell*a spp., the *invA* gene was detected in 100% of raw sludge samples, in about half of the samples of MAD sludge (45.5%) and it was never detected in the TAnD effluent (data not shown). Consequently, the pathogen limits for the advanced treatment of sludge were satisfied in the TAnD process (no detection of *Salmonella* spp. in a sample of 50 grams -wet weight- of treated sludge).

Although total coliforms are not among the pathogens included in the future European legislation for land application of sludge, these microorganisms were analyzed in this study, since they have been historically used as an indicator of the presence of pathogens in water and wastewater. Total coliforms include both fecal coliforms and *E. coli*, being always therefore more numerous, which enables the development of a rough outlook of the degree of disinfection taking place inside the digesters.

The TAnD digester tested in this study successfully sanitized the sludge regarding the content of *E. coli* and *Salmonella* spp. Similar results have been previously reported by other studies. For instance, Coelho et al. [40] obtained a

removal of total coliforms below detection limits after a thermophilic anaerobic digestion in bench-scale reactors when a SRT of 15 or 20 days was performed, but not for 5 or 10 days, suggesting that a minimum of more than 10 days under thermophilic conditions is needed to achieve appropriate sanitation of sludge. Consequently, the SRT obtained in the last stable period of this study (16 days) seemed to be a good time-temperature compromise for sludge hygienization. Thus, Iranpour and Cox [33] reported the presence of fecal coliforms after thermophilic anaerobic digestion, the most likely reason being the relatively short SRT (about 10 days) used in their study. Although, the authors studied the content of fecal coliforms, these results can be compared since fecal coliforms are intended to be an indicator of fecal contamination, and more specifically of *E. coli*, which represents approximately 90% of the fecal coliforms being suggested as the preferred indicator of fecal contamination [41].

The same behavior was observed for the content of *Salmonella* spp. While Foster-Carneiro et al. [16] obtained high densities of *Salmonella* spp. in raw and MAD sludge and Aitken et al. [17] did not detect *Salmonella* spp. in the effluent of laboratory-scale TAnD reactors, Zábranská et al. [42]), still found between 7 and 27% detection of this pathogen in their thermophilic sludge samples. The SRT of approximately 10 days of operation, was pointed out as the major cause of these results, reinforcing again, the hypothesis of that a SRT of a minimum of 15 days is needed for successful *E. coli* and *Salmonella* spp. removal in this type of reactors.

Regarding the content of *C. perfringens* spores, the high levels reported in the present study, are probably due to the ability of *Clostridium* species to form metabolically-dormant spores that are extremely resistant to environmental stresses such as heat, radiation and toxic chemicals [43] and are, consequently, very difficult to eradicate through thermal treatments such as thermophilic anaerobic digestion. These results are in accordance with those found by Guzmán et al. [44], who detected values of *C. perfringens* spores of 5.1×10^7, 4.6×10^7 and 1.3×10^5 CFUs in raw, anaerobic mesophilic and after a thermophilic treatment (composting), respectively. Similarly, Aitken et al. [17] found no removal of *C. perfringens* spores in a lab-scale thermophilic anaerobic reactor, and only a 2 log reduction was observed after more adverse growth conditions such as thermophilic aerobic digestion [23].

In view of the results obtained in this study, the conclusion that both high temperatures as well as SRT are the main factors contributing to sludge disinfection, must be drawn. Other factors reported as influencing pathogen removal, such as pH, matrix components, or high gas production [17,45] should not be taken into consideration since both mesophilic and thermophilic digesters had the same fed sludge and similar values regarding pH and gas production. However, the high concentration of VFA in TAnD sludge should be singled out as an alleged factor influencing pathogen destruction.

As a conclusion, and although TAnD digestion produced a higher sanitation rate compared to mesophilic digestion, fulfilling U.S. EPA Class A pathogen requirements that allow direct land-application of sludge [8], the more restrictive forthcoming European legislation will consider a potential risk in the spreading of TAnD-digested sludge on agricultural soil due to the presence of *C. perfringens* spores.

3.5. Comparative study of dewaterability

Dewatering characteristics of sludge have always attracted interest as an end management property of biosolids because it reduces sludge volume and, consequently,
the cost of transporting sludge to its final disposal site.

Table 3 shows the results of the dynamic jar test in which 13 different flocculants with both high and low cationicity and molecular weight, and both branched and linear, were tested. The optimal polymer type for both MAD and TAnD sludge was SNF IBERICA 4440 SSH. Once the most suitable polymer was determined, the optimal polymer dose (OPD) to achieve flocculation was calculated. The OPD in mesophilic sludge was almost 3 times lower than in thermophilic sludge. Nevertheless, it is expected to have smaller differences when a full-scale TAnD digester is set-up [33]. Sludge-polymer mixing (TF) was more difficult in TAnD sludge, although the thermophilic floc was more resistant (TR). The same behavior was observed in the re-flocculation process, which for thermophilic sludge took longer to re-flocculate (TFR), but also had a better breakage resistance (TRR). These results could be and indicator that a higher level of dryness in the thermophilic sludge after the centrifuge dewatering process

could be achieved, since allegedly, the engine par could be increased while maintaining normal values of the filtrate.

Table 3. Optimum polymer dose (OPD), flocculation and breakage properties of MAD and TAnD sludge.

	MAD sludge	TAnD sludge
Optimum polymer dose (OPD)	25 mL (250 ppm)	70 mL (700 ppm)
T_F	6	14
Score	5	5
T_R	15	26
T_{FR}	5	6
Score	4	5
T_{RR}	17	33

T_F: number of transfers needed to achieve flocculation, T_R: number of transfers for floc breakage, T_{FR}: number of transfers to achieve re-flocculation, T_{RR}: number of transfers for the re-flocculated floc to break again.

Figure 4 plots the volume of drained water versus time. As can be observed, the filtered volume of the thermophilic sludge was considerably higher than that of mesophilic sludge (170 and 120 mL respectively, at 120 s), representing a remarkable advantage for the dewatering process, since there was less water retained in the thermophilic sludge. It is generally believed that the presence of extracellular polymeric substances (EPS) causes the difficulty in sludge dewatering. Previous work has reported that advanced sludge treatment like thermal treatments contribute to the degradation of EPS, thus facilitating sludge dewatering [46]. This test was repeated twice with highly reproducible results (data not shown).

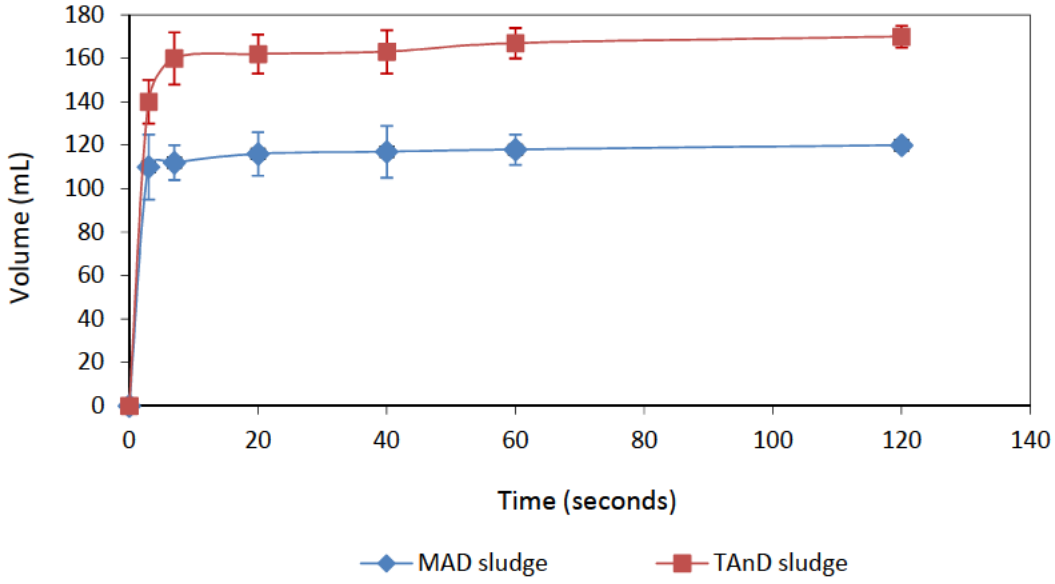

Figure 4. Dewatering characteristics of MAD and TAnD sludge represented as the volume of drained water against time. Bars indicate standard errors (n = 3).

Finally, with the aim of completing the dewaterability study, the particle size of raw, MAD, and TAnD sludge was analyzed. As can be observed in Figure 5, the number of small particles was higher in both digested sludges, and also in TAnD sludge than in MAD sludge. Regarding the content of large particles, raw and mesophilic sludge presented similar values whereas thermophilic sludge had a smaller content of this type of particles. These results are consistent with the data obtained in the dewaterability assays, as smaller particles would demand a greater polymer dose since there are a higher number of them per unit volume, and therefore, more links between particles and the polymeric chain should be performed. This finding also explains why the floc from the thermophilic sludge was more resistant as well as presenting better dewaterability; small particles increased the polyelectrolyte union, enhancing both sludge resistance and dewatering. This test was repeated twice obtaining highly reproducible results (data not shown).

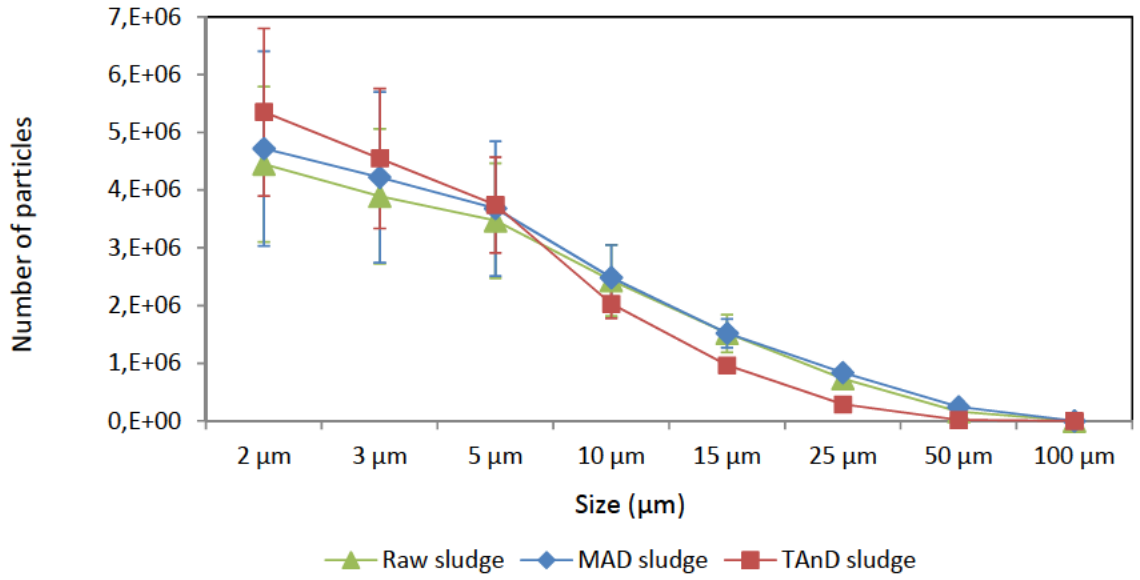

Figure 5. Particle size distribution in raw, MAD and TAnD sludge. Bars indicate standard errors (n = 4).

4. Conclusions

Both mesophilic and thermophilic processes resulted in efficient sewage sludge digestion in terms of volatile solids destruction (> 40%) and biogas production. Nevertheless, thermophilic anaerobic digestion allowed higher organic loading rates and reduced sludge retention times while maintaining the process stabilization. High nutrient content and heavy metal concentrations below the established limits in the future European Directive for spreading of sludge on land, make the final product a valuable organic amendment. Pathogen removal was significantly reduced after the thermophilic process with no detection of *E. coli* and *Salmonella* spp., obtaining a product that met Class A biosolids requirements. However, the presence of *C. perfringens* spores prevented the thermophilic sludge from fulfilling the microbial standards of the future European legislation. Dewatering characteristics of the final products showed a lower polymer dose requirement for mesophilic sludge, whereas thermophilic sludge was more resistant and with a better dewatering capability.

Acknowledgments

This work was supported by the JAE Programme from the Consejo Superior de Investigaciones Científicas (CSIC), Spain, and the project

324/pc08/2-04.3 included in the Plan Nacional de I+D+i 2008-2011. We thank Eur. Ing. Stephen Pearson for language comments on the manuscript and D. Beltrán for graphs assistance.

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V

V. Two-stage mesophilic anaerobic – thermophilic digestion for sludge sanitation to obtain advanced treated sludge

V. Two-stage mesophilic anaerobic – thermophilic digestion for sludge sanitation to obtain advanced treated sludge

Digestión en dos etapas mesófila anaerobia y termófila para la higienización de lodos para obtener un lodo avanzado

Eva Lloret, María J. Salar, Josefa Blaya, José A. Pascual.

Chemical Engineering Journal (2013) 230: 59-63.

Resumen

En el presente estudio se analizaron tres tipos de lodos de depuradora (fresco, mesófilo anaerobio y termófilo anaerobio) con el fin de evaluar si cumplían los límites establecidos en la "Propuesta de Directiva del Parlamento y del Consejo Europeo sobre aplicación agrícola de lodos" respecto al contenido de microorganismos patógenos. Para ello, se procedió al cultivo de *Salmonella* spp., *Escherichia coli* y de las esporas de *Clostridium perfringens* y a la amplificación mediante PCR de los genes de patogenicidad *invA* y *cpa*. La digestión termófila anaerobia (TAnD) produjo biosólidos de Clase A según los criterios de la legislación americana al producir la eliminación de *E. coli* y *Salmonella* spp. Sin embargo, no consiguió cumplir los requisitos establecidos en la futura Directiva debido al contenido de esporas de *C. perfringens* (9,6 x 10^4 esporas mL^{-1}). Por consiguiente, el objetivo último de este estudio consistió en proponer un proceso de digestión en dos etapas capaz de eliminar las esporas de *C. perfringens* para obtener un lodo avanzado adecuado para su directa aplicación a suelo sin riesgos ambientales ni sobre la salud humana. La primera etapa del sistema propuesto, consiste en un proceso de digestión mesófila anaerobia, mientras que la segunda etapa comprende la digestión termófila de los lodos; aerobia o anaerobia indistintamente. La hipótesis de este estudio consiste en provocar en la primera fase la germinación de las esporas de *C. perfringens*, para, en una subsecuente etapa termófila, poder erradicar o dañar estas nuevas células vegetativas, obteniendo así un producto final libre de patógenos.

Chemical Engineering Journal (2013) 230: 59-63

Two-stage mesophilic anaerobic - thermophilic digestion for sludge sanitation to obtain advanced treated sludge

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HIGHLIGHTS

- Pathogen content was analysed in three different types of sludge.
- Mesophilic and thermophilic processes produced Class B and Class A Biosolids.
- The removal of pathogens included in the future European legislation was assessed.
- The presence of C. perfringens spores prevented the obtaining of advanced treated sludge.
- A two-stage digestion process is suggested for fully sanitation of the sludge.

ARTICLE INFO

Article history:
Received 7 February 2013
Received in revised form 30 May 2013
Accepted 16 June 2013
Available online 27 June 2013

Keywords:
Mesophilic anaerobic digestion
Thermophilic digestion
Sewage sludge
Biosolids
Human pathogens
Clostridium perfringens

ABSTRACT

In the present study, raw, mesophilic anaerobic, and thermophilic anaerobic sludge were analysed to evaluate whether the pathogen content limits established in the "Proposal for a Directive of the European Parliament and of the Council on spreading of sludge on land" were satisfied. *Salmonella* spp., *Escherichia coli*, and *Clostridium perfringens* spores were cultivated and pathogenity genes *invA* and *cpa* PCR-amplified. Thermophilic anaerobic digestion produced Class A biosolids by eliminating *E. coli* and *Salmonella* spp. but did not accomplished the microbial requirements of the future Directive due to the presence of *C. perfringens* spores (9.6×10^4 CFUs mL^{-1}). Hence, the final goal of this work was to propose a two-stage process capable of removing the spores of *C. perfringens* to obtain an advanced treated sludge that could be land-applied with no environmental risks. The first stage of the process suggested in this study involved the mesophilic anaerobic digestion of the sludge while the second stage of operation consisted of an aerobic or anaerobic thermophilic digestion.

1. Introduction

The addition of organic matter has proven to be a suitable technique for soil restoration or conservation [1]. Among organic amendments, the use of sewage sludge as soil fertilizer is gaining importance for both soil properties improvement and waste disposal. Most sewage wastes contain valuable nutrients that could be used to improve soil fertility, crop production, and some soil physical and chemical properties as well as promoting its biological activity [2,3]. However, the agricultural use of sewage sludge may introduce some risks associated with its potential content of heavy metals, toxic compounds, pathogenic bacteria, viruses and parasites that pose direct or indirect hazards to human, animal and plant health [4]. In this respect, the Environmental Protection Agency of the United States establishes treatment goals or post-application measures designed to reduce these risks and defines Class A and Class B biosolids with different pathogen content limits and use restrictions [5]. In contrast, the current European legislation only requires that the sludge is subjected to a process of stabilisation before land application and only establishes limits regarding the content of heavy metals [6]. In order to improve this situation, the European Union is promoting a new regulation through the Proposal for a Directive of the European Parliament and of the Council on spreading of sludge on land [7]. This provides stricter parameters for the content of pathogenic microorganisms and introduces the concept of advanced and conventional treatments. In the future legislation, limits for *Escherichia coli*, *Clostridium perfringens* spores, and *Salmonella* spp. are established for the sludge to achieve an advanced treated status.

Many studies have focused on sludge digestion processes with the aim to fulfill Class A biosolids conditions [8,9]. However, there is a lack in the existing literature regarding the requirements to achieve an advanced treated sludge.

In view of the above, in the present work, raw sludge (mixed primary and secondary sludge), sludge produced after a mesophilic anaerobic digestion (MAD sludge) and sludge produced after a thermophilic anaerobic digestion (TAnD sludge), were analysed to assess whether they fulfilled the microbiological standards established for advanced treated sludge in the future European legislation. Mesophilic anaerobic digestion is one of the most widely used processes for the stabilization of sludge in wastewater treatment plants and is

defined as a conventional treatment in the future European Directive. On the other hand, thermophilic anaerobic digestion is considered an advanced treatment of sewage sludge in the future European Directive.

2. Material and methods

2.1. Sewage sludge sampling

Sewage sludge was collected from a waste water treatment plant (WWTP) in Molina de Segura (Murcia, Spain) with a population equivalent of 290,000 p.e. The main characteristics of the MAD process were: a digester effective volume of 7,612-m^3, an organic loading rate (OLR) of 0.8 kg m^{-3} d^{-1} volatile solids, an average temperature of 38.1 °C, a sludge retention time (SRT) of 59.3 days and a volatile solids destruction (VSD) of 45.9%. On the other hand, the principal characteristics of the TAnD process were: a digester effective volume of 15-m^3, an OLR of 1.6 kg m^{-3} d^{-1} volatile solids, an average temperature of 53.9 °C, a SRT of 20.3 d and a VSD of 42.0%. Both the mesophilic and the thermophilic anaerobic digesters were fed with the same raw sludge. The main chemical characteristics of raw, MAD and TAnD sludge are described in Table 1.

2.2. Batch laboratory incubations of sewage sludge

500 mL of raw, MAD and TAnD sludge were placed in 1 L flasks in a rotatory shaker both at 55 and 65 °C and under aerobic and anaerobic conditions. The incubations were performed to determine the time needed to eradicate *C. perfringens* spores for each combination of type of sludge, temperature and atmosphere. Raw and TAnD sludge were previously incubated at 40 °C during 24 h. The experiment was carried out per triplicate. Anaerobic conditions were achieved by introducing each flask in a thermo-sealed bag containing anaerobic sachets (AnaeroGen Compact, Cambridge, UK). Aerobic treatments were sampled on days 1, 2, 3, 5, 7, 9, 11, 14, 18, 21 and 22 and anaerobic treatments were sampled weekly on days 7, 14, 21 and 28.

Table 1. Chemical characteristics of raw, MAD and TAnD sludge (values on dry weight basis).

Parameter [a]	Raw sludge	MAD sludge	TAnD sludge	UE limits [b]
Dry matter content (%)	5.9	3.5	2.9	
pH	6.3	7.6	8.0	
EC (dS m^{-1})	5.3	13.6	13.7	
TOC (g kg^{-1})	801	621	690	
Total N (g kg^{-1})	117	93.1	89.7	
Total P (g kg^{-1})	62.1	55.2	55.2	
Total K (g kg^{-1})	3.4	3.4	3.4	
Total Cd (mg kg^{-1})	BDL [c]	BDL	BDL	10
Total Cr (mg kg^{-1})	24	59	55	1000
Total Cr_{VI} (mg kg^{-1})	BDL	BDL	BDL	10
Total Cu (mg kg^{-1})	108	266	197	1000
Total Fe (g kg^{-1})	17.1	46.0	34.09	
Total Hg (mg kg^{-1})	BDL	BDL	BDL	10
Total Ni (mg kg^{-1})	15	31	24	300
Total Pb (mg kg^{-1})	21	45	38	750
Total Zn (mg kg^{-1})	376	738	821	2500

[a] EC: Electrical conductivity; TOC: total organic carbon.
[b] Limits for advanced treated sludge (European Commission, 2003).
[c] BDL: Below Detection Limits.

2.3. Cultivation and molecular detection of pathogens

After sampling, sludge was immediately cooled (4 °C) and microbial analysis were performed within the following 24 h. Total coliforms, *Escherichia coli*, *Salmonella* spp. and *C. perfringens* spores were cultivated and pathogenity genes *invA* and *cpa* PCR-amplified as described by Lloret et al. [10]. Briefly, the presence/absence detection method of *Salmonella* spp., consisted of four steps: 1) non-selective enrichment of sludge using buffered peptone water, 2) selective enrichment with Rappaport-Vassiliadis' soy broth, 3) plating in the chromogenic media Colorex Salmonella Plus, and 4) DNA extraction of positive colony-forming units (CFUs) and PCR-amplification of the *invA* gene enconding for invasine. The quantification of Escherichia coli and total coliforms was performed by plating 1:10 (w/v) serial dilutions of sludge in sterile Ringer solution in Chromocult coliform agar. To quantify *C. perfringens* spores, vegetative cells were first eradicated subjecting the sludge to a thermal shock at 75 °C for 20 min. Then, 1:10 (w/v) serial dilutions of sludge in sterile Ringer solution were inoculated into Tryptose sulphite cycloserine agar, and DNA extraction and PCR-amplification of the *cpa* gen enconding for the α–toxin were performed. All sludge suspensions were mixed by shaking for 15 min and a minimum of three replicates per dilution

were assayed. The detection limit for both culture-dependent and molecular methods was 1 CFU mL^{-1}.

2.4. Statistical analysis

For the analysis of pathogens in raw, MAD and TAnD sludge, data (log-transformed) were subjected to one-way ANOVA with type of sludge as factor, followed by Tukey's HSD as a *post hoc* test ($p < 0.05$) to control for multiple testing. For the analysis of *C. perfringens* spores inactivation after batch laboratory incubations, three-way ANOVA was performed (log-transformed data) with type of sludge, temperature and atmosphere as factors and with the initial concentration of each pathogen as covariate. Further analysis with Tukey's HSD comparison procedure was performed as a *post hoc* test ($p < 0.05$) to control for multiple testing. The statistical software SPSS 19.0 was used for the analysis (SPSS Inc.).

3. Results and discussion

The average content of pathogens in the different types of sludge used in this study is shown in Table 2. These results are in accordance with the values found for the same pathogenic microorganisms in raw, MAD and TAnD sludge, after a weekly sampling throughout one year in the same WWTP (Lloret et al., unpublished results). In raw sludge, the content of total coliforms, *E. coli* and *C. perfringens* spores, presented average values of 2.1 x 10^6, 2.4 x 10^5 and 8.2 x 10^5 colony-forming units mL^{-1} respectively, and the *invA* gene specific for *Salmonella* spp. was always detected. In MAD sludge, counts for total coliforms and *E. coli* followed the same trend as in raw sludge with a significant reduction of 1.91 and 1.69 log units, respectively. On the other hand, *C. perfringens* spores, with values of 2.7 x 10^5 CFUs mL^{-1}, were only 0.48 log unit lower than in raw sludge and were not significantly reduced. Similarly to raw sludge, the *invA* gene was detected in all samples. Unlike raw and MAD sludge, in TAnD sludge neither total coliforms nor *E. coli* nor *Salmonella* spp. were detected. Nevertheless, *C. perfringens* spores content showed only a 0.93 and a 0.45 log unit reduction compared to raw and MAD sludge respectively.

Table 2. Average pathogen content in raw, MAD and TAnD sludge (values on fresh weight basis).

Parameter	Raw sludge	MAD sludge	TAnD sludge	UE limits [a]
Total coliforms (CFU mL^{-1})	2.1 x 10^6 a	2.6 x 10^4 b	BDL [b]	
E. coli log (CFU mL^{-1})	2.4 x 10^5 a	4.9 x 10^3 b	BDL	1.0 x 10^3 (CFU g^{-1})
C. perfringens spores (CFU mL^{-1})	8.2 x 10^5 a	2.7 x 10^5 a	9.6 x 10^4 a	3.0 x 10^3 (CFU g^{-1})
Salmonella spp.	Positive	Positive	BDL	BDL

[a] Limits for advanced treated sludge (European Commission, 2003); *E. coli* and *C. perfringens* spores limits are given on dry weight basis. *Salmonella* spp. limit is given for 50 grams on fresh weight basis.
Different letters indicate significant differences between each type of sludge for each microorganism after Tukey HSD test ($p < 0.05$).
[b] BDL: Below Detection Limits.

Regarding the content of *E. coli*, these results are in accordance with those found by Iranpour and Cox [8], who despite detecting high levels of fecal coliforms in the incoming sludge, could never detect them in the outcoming sludge after a thermophilic anaerobic digestion. Although, the authors studied the content of fecal coliforms, these results can be compared since fecal coliforms are intended to be an indicator of fecal contamination, and more specifically of *E. coli*, which represents approximately 90% of the fecal coliforms [11]. Although total coliforms are not included in the American or in the European legislation for land application of sludge, this microorganisms were analysed since, historically, they have been used as an indicator of the presence of pathogens in water and wastewater, and include both fecal coliforms and *E. coli*. Concerning the detection of *Salmonell*a spp., the data are comparable to those of Zábranská et al. [12] who found 100% *Salmonella* spp. detection in raw sludge and around 94% detection in anaerobic mesophilic sludge and are also in accordance with Iranpour and Cox [8] who never detected *Salmonella* spp. after different thermophilic anaerobic digestion processes. In relation to the content of *C. perfringens* spores, several authors have found similar values with high densities of spores after different treatments. Guzmán et al. [13] detected values of *C. perfringens* spores of 5.1 x 10^7, 4.6 x 10^7 and 1.3 x 10^5 CFUs in raw, anaerobic mesophilic and composted sludge respectively. Similarly, Aitken et al. [14] found no removal of *C. perfringens* spores in a laboratory-scale thermophilic anaerobic reactor.

Consequently, among the three types of sludge that were assessed, the only one that fulfilled the pathogen requirements established by the EPA to obtained

Class A biosolids was the TAnD sludge [5]. These results are in agreement with Iranpour and Cox [8] and Aitken et al. [14] who studied thermophilic anaerobic digestion for Class A biosolids disinfection in full-scale and laboratory-scale respectively. However, none of the sludges satisfied the microbial standards included in the future European Directive due to presence of *C. perfringens* spores. This may be due to the ability of *Clostridium* species to form metabolically-dormant spores that are extremely resistant to environmental stresses such as heat, radiation and toxic chemicals [15] and are, consequently, very difficult to eradicate through thermal treatments such as thermophilic anaerobic digestion. In fact *C. perfringens* food poisoning currently ranks as the third most common cause of food-borne illness in the United Kingdom and the United States [16]. Thus, for being the most resistant microorganism to treatments, *C. perfringens* spores have been postulated as a good alternative indicator of protozoan oocysts in water treatments [17], as an excellent surrogate for the eggs of *Ascaris* [18], and as a good candidate indicator for treated sludge [13]. In view of the above results, the second part of this study consisted of batch laboratory incubations with the aim of eradicating the spores of *C. perfringens* to fulfill the microbial standards established in the future European Directive.

Figures 1A and 1B show the reduction kinetics of *C. perfringens* spores in each type of sludge at 55 °C and at 65 °C, during aerobic and anaerobic incubations respectively. All factors and their interactions significantly affected the inactivation of *C. perfringens* spores (Table 3). The most successful combinations -in terms of time- of type of sludge, temperature and atmosphere for the reduction of *C. perfringens* spores, were incubations of TAnD sludge at 65 °C both under aerobic or anaerobic conditions, and incubation of MAD sludge at 65 °C under aerobic conditions. All these three incubations achieved fully sanitation of sludge in only 7 days with a reduction of 3.82, 4.98, and 4.53 log CFU mL^{-1} after 3, 7, and 2 days, respectively. On the other hand, incubations of TAnD sludge at 55 °C both under aerobic and anaerobic conditions and of raw sludge at 65 °C under aerobic conditions produced a sanitized sludge in 14 days, whereas MAD and raw sludge incubated at 55 °C under aerobic conditions and at 65 °C under anaerobic conditions destroyed *C. perfringens* spores after 21 and 22 days, respectively. Finally, MAD and raw sludge incubated at 55 °C under anaerobic conditions achieved the disinfection of the sludge after 28 days.

Generally, the inactivation of spores was more rapid at 65 °C, under aerobic conditions, and in TanD sludge. This is probably due to the adverse conditions for cell growth and to the fact that different matrixes influence spores germination [19], suggesting that the exposure of the sludge to high temperatures inside the TAnD digester may have damaged the spores, making them more sensitive to a subsequent thermal treatment. Similarly, studies of Popat et al. [20] have found that microbial inactivation may be a strong function of the composition of the sludge, suggesting the need of composition-dependent time-temperature relationships. The introduction of an initial mesophilic stage at 40 °C during 24 h before the thermophilic incubations of raw and TAnD sludge was based on triggering the germination of the spores to produce vegetative cells. This step was omitted for MAD sludge in which the germination of cells was assumed due to the optimum conditions inside the reactor. The hypothesis of this work relies on the idea that after triggering the germination of spores, a subsequent sudden increase of temperature to a thermophilic rank would kill or damage the newborn vegetative cells producing either their death or inability to produce viable spores again. This could be explained by the fact that the process of sporulation is slow and furthermore, several cycles of germination-sporulation may produce lethal damage in cells. Spore germination has been shown as a possible method to eradicate spores from food products and from room air in hospitals [21,22]. In this study, it is presented as a suitable method for the elimination of *C. perfringens* spores by heat in sludge, representing a good method for sludge sanitation.

Table 3. Three-way ANOVA results of *C. perfringens* spores content after batch laboratory incubations.

	F	*p*
Sludge	3093.25	0.000
Temperature	5402.25	0.000
Atmosphere	1722.25	0.000
Sludge x Temperature	204.75	0.000
Sludge x Atmosphere	529.75	0.000
Temperature x Atmosphere	506.25	0.000
Sludge x Temperature x Atmosphere	383.25	0.000

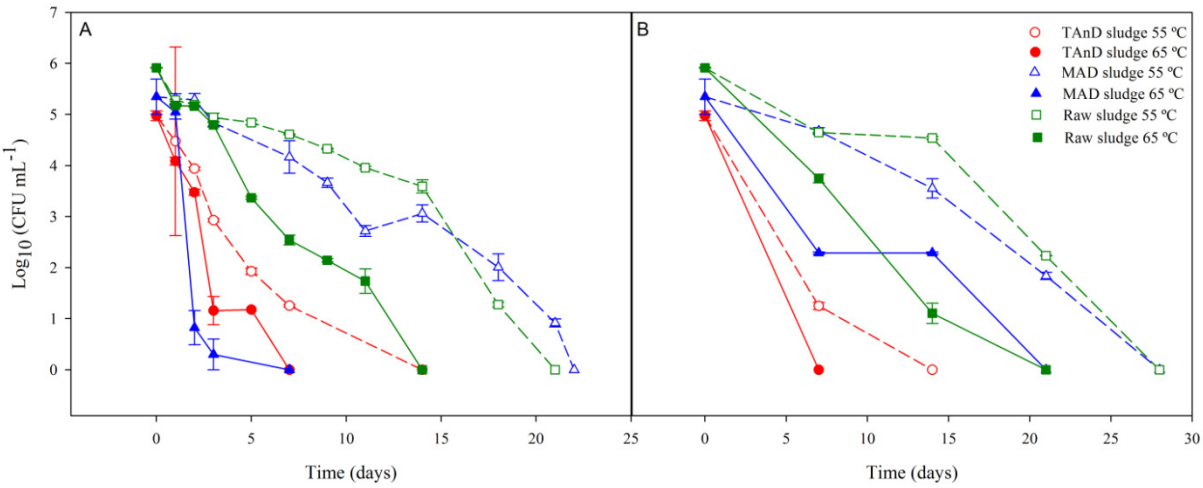

Figure 1. Reduction kinetics of *Clostridium perfringens* spores in batch laboratory incubations under aerobic (A) and anaerobic (B) conditions at 55 ºC (dashed line and open symbols) and at 65ºC (solid line and closed symbols), in raw, MAD and TAnD sludge (values on fresh weight basis). Bars indicate standard deviation.

Regarding the content of total coliforms, *E. coli* and *Salmonella* spp. in raw and MAD sludge, neither of them were detected after an incubation time of 24 h at 55 or 65 °C. These results are in accordance with the time-temperature requirements to achieve Class A biosolids [5] and for thermophilic anaerobic digestion according to the future European legislation [7].

In view of the obtained results and with the aim of fulfilling not only Class A biosolids requirements but also the microbial standards of the future European legislation for advanced treated sludge, this study proposes the setup of a 2-stage mesophilic anaerobic - thermophilic sludge digestion to achieve both sludge stabilization and pathogen control. This dual-digestion system could both consist of the mesophilic anaerobic digestion of sludge followed by either an aerobic or anaerobic thermophilic digestion. In the first scenario, the benefits of conventional mesophilic anaerobic digestion -such as high methane production, reduction of 30 to 50% of volatile solids [23]- would be combined with those of an autothermal thermophilic aerobic digestion (ATAD). The advantages of an ATAD system include high efficiency in the killing of pathogenic microorganisms, high stabilisation rate and volatile solids reduction capability, short SRT, simplicity and high speed of the process, robustness, small volume, nitrogen conservation and the possibility of heat recovery [24,10]. On the other hand, under the second circumstances, the advantages of mesophilic anaerobic digestion would be added to those of thermophilic anaerobic digestion (TAnD)

which includes higher solids destruction efficiency, higher biogas production rate, less foaming, better dewaterability and high pathogen removal efficiency [25]. In both systems, the thermophilic digestion of sludge will require auxiliary heating of the digestion tanks that can be obtained from the released energy by the microorganisms in the ATAD system [5] and by the biogas production in the TAnD process. In both cases, extra energy can be obtained from the biogas production of the preceding mesophilic digestion. At the same time, in light of the obtained results, a minimum of 7 and 28 days SRT would be required to achieve complete sludge sanitation in the ATAD and TAnD system respectively. A SRT of 7 days falls within the average values for ATAD digestion [5, 24] whereas a SRT of 28 is longer than usual TAnD systems [8,23]. Likewise, the sequential digestion process suggested in this study, could also consist of a continuous stirred-tank reactor (CSTR) maintained under mesophilic conditions followed by an ATAD or a TAnD digester, so that the installation of two digesters would not be necessary. Nevertheless, the utilization of a dual digestion system can present extra benefits.The use of 2-stage digestion processes is a widespread practise that up to now has only been focused on fulfilling Class A biosolids requirements or in the reduction of the organic material. To achieve these goals, a thermophilic step followed by a mesophilic step have been suggested [9,26] and the combination of aerobic thermophilic pre-treatment with anaerobic mesophilic digestion is commonly used for the treatment of municipal sludge [27,28]. At the same time, the ATAD system is commonly used as a two-stage process [5,24]. However, these systems do not eradicate *C. perfringens* spores and therefore are unable to meet the future European legislation standards.

4. Conclusions

Thermophilic anaerobic digestion and mesophilic anaerobic digestion resulted in suitable technologies for sludge sanitation to obtain Class A and Class B biosolids respectively. Nevertheless, the presence of *C. perfringens* spores prevented the obtaining of advanced sludge according to the future European regulations. Consequently, a 2-stage mesophilic anaerobic - thermophilic sludge digestion process is suggested to achieve an utterly sanitized sludge. The final product is a potential valuable organic amendment, stable, with high nutrient

content and with safe levels of both pathogens and heavy metals which makes it suitable for land application.

Acknowledgments

This work was supported by the JAE Programme from the Consejo Superior de Investigaciones Científicas (CSIC), Spain, and the project 324/pc08/2-04.3 included in the Plan Nacional de I+D+i 2008-2011.

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VI

VI. Sewage sludge addition modifies soil microbial communities and plant performance depending on the stabilization process

VI. Sewage sludge addition modifies soil microbial communities and plant performance depending on the stabilization process

La aplicación de lodos de depuradora modifica las comunidades microbianas del suelo y el desarrollo de las plantas en función del proceso de estabilización de los lodos

Eva Lloret, José A. Pascual, Eoin L. Brodie, Nicholas J. Bouskill, Marina Fernández Delgado-Juárez, Heribert Insam, Marta Goberna.

Este artículo se encuentra en revisión.

Resumen

A pesar de que la aplicación agrícola de lodos es una práctica ampliamente extendida, el conocimiento de cómo los procesos de estabilización de los lodos afectan a la composición y diversidad de su comunidad microbiana, es relativamente bajo. En este trabajo, se pretende evaluar por un lado, los efectos del proceso de estabilización de los lodos sobre la comunidad microbiana de los mismos, y por otro, los efectos de la aplicación agrícola de lodos que difieren en sus respectivos procesos de estabilización, sobre la diversidad y estructura de la comunidad microbiana del suelo, las propiedades químicas del suelo, así como en el crecimiento y desarrollo de las plantas. El estudio de la comunidad microbiana de los lodos mediante pirosecuenciación de la región SSU V9 ARNr, demostró que el proceso de estabilización al que habían sido sometidos, modificó sus comunidades microbianas. Por otro lado, la aplicación de un lodo avanzado ATAD alteró en menor medida la microbiota y la química del suelo, a la par que estimuló la actividad microbiana y el crecimiento y desarrollo de las plantas de un cultivo de melón. La aplicación de un lodo convencional MAD, sin embargo, no tuvo efectos sobre la actividad de los microorganismos, aunque sí mejoró el crecimiento y desarrollo de las plantas. Los resultados obtenidos sugieren que las variaciones de las poblaciones microbianas del suelo tras la aplicación de lodos, son debidas con mayor probabilidad a los cambios producidos en la química del suelo, que a la adición de una nueva comunidad de microorganismos incorporada

con los lodos. Estos resultados también mostraron que alteraciones en un solo parámetro químico del suelo (p.e. CE), son susceptibles de producir una gran variación en la estructura y activad de la comunidad microbiana, así como en el desarrollo y estado fisiológico de las plantas.

En revisión

Sewage sludge addition modifies soil microbial communities and plant performance depending on the stabilization process

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Running title: S*ludge stabilization determines soil community changes*

ABSTRACT

Despite the widespread use of sludge as an organic amendment, relatively little is known about how different stabilization processes affect the microbial composition and diversity of sewage sludge as well as of sludge-amended soils. The aim of this work was, on the one hand, to evaluate the effects of the stabilization processes on sludge microbial community, and, on the other hand, to assess the effects of landspreading of two sewage sludges differing in their stabilization process on soil microbial community structure and activity, soil chemical properties, as well as on plant growth and performance, were evaluated. Here, pyrosequencing of SSU rRNA V9 region showed that sludge stabilization process shaped sludge microbial community structure. Regarding land application, ATAD advanced sludge addition produced smaller changes on soil microbial community structure and soil chemistry while enhancing soil microbial activity and growth and performance of a melon crop. On the other hand, land application of conventional sludge had no effects over soil microbial activity although it did enhanced plant growth and performance. These results suggest that sludge application-derived changes in soil microbial community are due to changes in soil chemical environment rather than to the addition of sludge-borne microorganisms. They also show that changes in a single chemical parameter (i.e. changes in EC) may produce a big shift in microbial community structure and activity, as well as in plant growth and physiological state.

Introduction

More than ten million tons of sewage sludge (dry matter) is yearly produced in Europe (EU-27) of which approximately 37% are recycled in agriculture (Milieu et al., 2010). Agricultural disposal of sewage sludge, which is encouraged by the EU Directive 91/271/EEC (European Commission, 1991), is of special interest in the Mediterranean region due to the widespread degradation that reduces the organic matter content of soils (García et al., 2000). In Spain, up to 83% of the sludge produced, over one million ton per year (dry matter), is applied to land (MARM, 2011; Milieu et al., 2010).

The quality of sewage sludge is strongly dependent on the original inputs to sewers, the wastewater treatment, and the subsequent sludge stabilization process (Milieu et al., 2010). Mesophilic anaerobic digestion (MAD) is one of the most widely used processes for the stabilization of sludge in wastewater treatment plants (Ahn and Forster, 2000) and is defined as a conventional treatment in the Proposal for a Directive of the European Parliament and of the Council on spreading of sludge on land (European Commission, 2003). On the other hand, autothermal thermophilic aerobic digestion (ATAD) is considered an advanced treatment in this Proposal allowing operators to use advanced-treated sludge with fewer restrictions as well as a technology capable of producing Class A biosolids (European Commission, 2003; USEPA, 2003).

The presence of oxygen in ATAD produces a more efficient mineralization which determines lower amounts of labile carbon and nutrients (Bernal et al., 1998; Odlare et al., 2011). Likewise, increasing process temperatures result in higher stabilization rates and higher solids destruction efficiency (Juteau, 2006), substrate utilization rates being 3-10 times greater in thermophilic processes with a reduced sludge retention time and a more efficient pathogen inactivation compared to mesophilic digestion (Lapara and Alleman, 1999). Other characteristics of the ATAD processes are an increase in pH, total nitrogen, NH_4^+-N and phosphorus crystallization (Juteau et al., 2004; Piterina et al., 2009; Liu et al., 2012). All aerobiosis, temperature and physical-chemical parameters are main factors controlling the microbial communities in organic residues (van Lier et al., 1993; Kim et al., 2002; Piterina et al., 2006; Han et al., 2010; Insam et al., 2010; Liu etal., 2010; Hayes, 2011), and thus it is to be expected that the sludge microbiota is ultimately determined by the sludge stabilization process.

The application of sewage sludge to land improves physical and chemical properties of soils due to the addition of organic matter. Improved properties are soil structure, increased soil moisture and porosity, enhanced humus content and cation exchange capacity (Korentajer, 1991; Barzegar et al., 2002). At the same time, the content of valuable plant nutrients –especially nitrogen- in sludge, and its positive effects on soil biological and biochemical properties, enhances soil fertility and crop production (Kelley et al., 1984; Min-Jian, 1997; Singh and Agrawal, 2008). Both the reduction in pH and the increase in electrical conductivity (EC) have also frequently been reported after sludge addition (Antolín et al., 2005; Pascual et al., 2009). The addition of organic amendments affects soil microbial communities, generally, by accelerating microbial development and activity (Bailey and Lazarovits 2003), although changes in the composition of microbial communities as a result of incorporating inorganic or organic amendments have also been observed (Marschner et al., 2003).

Previous literature has focused on the separate effects of sludge application to land on soil chemical properties (Ros et al., 2003; Speir et al., 2003), crops (Antolín et al., 2005; Pascual et al., 2009; Pascual et al., 2010), or soil microbial activity and genetic fingerprint (Pascual et at., 2007; Bastida et al., 2008). A few studies have assessed the combined effect of sludge on soil chemistry and crops (Fernández et al., 2009), soil chemistry and soil microbial community (Kelly et al., 1999) or crops and soil microbial community (Pascual et al., 2008).

Here, we have holistically studied the effects of landspreading of two sewage sludges differing in their stabilization process. The specific aims of this study were: i) to evaluate the influence of two types of stabilization process on the sludge microbial community, and ii) to compare the effects of land application of both stabilized sludges on the soil environment, soil microbial community structure and performance, as well as plant fitness. The stabilized sludges were produced from the same original sewage sludge after: 1) autothermal thermohilic aerobic digestion (ATAD sludge) and 2) mesophilic anaerobic digestion (MAD sludge; Table 1). Sludge application was performed at the microcosm level on an agricultural soil on which melon was grown.

We postulated that the type of stabilization determines the sludge chemical properties and consequently its microbial community. In addition, we hypothesized that due to the nature of the different stabilization processes,

amending soils with ATAD sludge will result in fewer changes on soil chemical properties, and thus will cause a smaller impact on the soil microbiota, but will result in a decreased crop production due to a more recalcitrant carbon fraction compared to MAD sludge.

Table 1. Chemical characteristics of ATAD and MAD sewage sludge (values on dry weight basis).

Parameter [a]	ATAD sludge	MAD sludge	UE limits [b]
Dry matter (%)	1.2	1.4	
pH	9.5	7.8	
EC (dS m^{-1})	8.9	14.0	
TOC (g kg^{-1})	346	315	
Total N (g kg^{-1})	183	129	
Total P (g kg^{-1})	16.7	14.3	
Total K (g kg^{-1})	8.0	8.0	
Total Cd (mg kg^{-1})	0.80	2.20	10
Total Cr (mg kg^{-1})	25.0	57.1	1000
Total Cu (mg kg^{-1})	208	314	1000
Total Fe (g kg^{-1})	14.8	29.1	
Total Ni (mg kg^{-1})	41.7	157	300
Total Pb (mg kg^{-1})	33.3	96.3	750
Total Zn (mg kg^{-1})	825	550	2500

[a] EC: electrical conductivity; TOC: total organic carbon.
[b] Heavy metal limits for soils (European Commission, 2003).

Results

Soil chemical and microbiological properties

Both ATAD and MAD sludges significantly increased soil EC, total organic carbon (TOC), N, P, and Na compared to control soils (Table 2). This increase took place immediately after sludge addition and was still evident after a 2-month incubation period (start and end of the experiment, respectively). The increase in these parameters was especially remarkable after the addition of MAD sludge. Conversely, a slight but still significant reduction of soil pH was observed in sludge-amended pots (Table 2). Mineral fertilized soils followed a similar trend than sludge-amended soils, although these effects were only statistically significant for pH and P (Table 2). Concentrations of several micronutrients and heavy metals such as Mo, S, Cu, Pb, and Zn, were also altered by sludge addition, presenting significantly higher values in sludge-amended pots than in control soils (Table 2). Primary macronutrients N, P, and K significantly decreased at the end of the study in most of the treatments (Table 2).

Microbial biomass C (C_{mic}) and the ratio C_{mic}/C_{org} were strongly stimulated by the addition of mineral fertilizer and ATAD sludge (Table 3). Basal respiration tended to increase in all treatments compared to control soils, but differences were only statistically significant in ATAD-amended soils (Table 3).

Table 2. Chemical characteristics of control, mineral fertilized, ATAD and MAD sludge-amended soils at the beginning and at the end of the experiment (values on dry weight basis). Standard deviation is given in parentheses. Letters indicate significant differences between treatments and asterisks indicate significant differences between each sampling time ($p < 0.05$).

Parameters [a]	Sampling time	Control soil	Mineral fertilized soil	ATAD-amended soil	MAD-amended soil
pH	Start [b]	8.0 (0.1) b	8.0 (0.1) b	7.8 (0.1) a	7.9 (0.1) ab
	End	8.2 (0.2) c	7.9 (0.0) b*	7.8 (0.1) ab	7.7 (0.1) a*
EC (dS m $^{-1}$)	Start	0.48 (0.06) a	0.48 (0.06) a	2.03 (0.15) b	2.82 (0.11) c
	End	0.88 (0.11) a*	1.09 (0.08) ab*	1.37 (0.010) b*	2.53 (0.41) c
TOC (g kg^{-1})	Start	7.2 (0.9) a	7.2 (0.9) a	8.4 (0.4) b	9.2 (1.1) c
	End	6.6 (1.0) a	7.5 (0.7) a	8.8 (1.0) b	10.2 (0.3) c
N (g kg^{-1})	Start	0.7 (0.1) a	0.7 (0.1) a	1.5 (0.1) b	1.6 (0.0) b
	End	0.4 (0.1) a*	0.5 (0.2) a	0.7 (0.1) b*	1.1 (0.1) c*
P (g kg^{-1})	Start	0.3 (0.0) a	0.3 (0.0) a	0.5 (0.0) b	0.6 (0.1) b
	End	0.2 (0.0) a	0.3 (0.0) b	0.4 (0.1) c	0.5 (0.0) c*
K (g kg^{-1})	Start	8.5 (0.7) a	8.5 (0.7) a	8.2 (0.4) a	8.5 (0.6) a
	End	7.7 (0.4) a*	8.0 (0.3) a*	7.7 (1.0) a*	7.2 (0.2) a*
Cd (mg kg^{-1})	Start	BDL [c]	BDL	BDL	BDL
	End	BDL	BDL	BDL	BDL
Cr (mg kg^{-1})	Start	67.5 (4.4) b	67.5 (4.4) a	59.7 (7.3) a	57.8 (4.7) a
	End	81.7 (12.7) b	62.1 (11.2) a	61.2 (7.5) a	58.5 (6.5) a
Cu (mg kg^{-1})	Start	16.4 (0.9) a	16.4 (0.9) a	23.0 (0.8) b	26.0 (1.0) c
	End	16.5 (2.7) a	17.7 (1.1) a	21.6 (2.0) b	22.2 (0.6) b*
Fe (g kg^{-1})	Start	18.4 (1.9) a	18.4 (1.9) a	20.0 (1.0) a	19.6 (1.0) a
	End	18.6 (1.0) a	17.3 (1.8) a	19.2 (1.5) a	18.5 (1.3) a
Ni (mg kg^{-1})	Start	30.3 (2.5) b	30.3 (2.5) a	30.5 (3.7) ab	29.8 (2.4) ab
	End	37.0 (4.5) b	28.6 (4.8) a	29.9 (3.5) ab	29.1 (2.6) ab
Pb (mg kg^{-1})	Start	11.2 (0.9) a	11.2 (0.9) ab	11.5 (0.6) ab	13.1 (0.7) b
	End	10.6 (0.9) a	11.6 (0.6) ab	11.8 (1.3) ab	11.6 (0.3) b
Zn (mg kg^{-1})	Start	28.4 (3.9) a	28.4 (3.9) a	32.0 (2.2) b	36.5 (2.0) b
	End	28.2 (2.4) a	26.9 (1.7) a	31.8 (2.5) b	31.6 (1.2) b

[a] EC: electrical conductivity; TOC: total organic carbon.
[b] Marginally significant differences: differences were found after ANOVA ($p < 0.05$) but not after Tukey HSD test ($p \geq 0.05$).
[c] BDL: below Detection Limits.

Table 3. Soil microbiological parameters and plant fitness in control, mineral fertilized, ATAD and MAD sludge-amended soils at the end of the experiment. Standard deviation is given in parentheses. Letters indicate significant differences between treatments ($p < 0.05$).

		Control soil	Mineral fertilized soil	ATAD-amended soil	MAD-amended soil
Soil biological parameters [a]					
C_{mic}	(µg C g^{-1}soil)	109 (13) a	288 (30) b	336 (32) b	177 (23) a
C_{mic}/C_{org}	(%)	16.5 (4.2) a	39.4 (10.6) b	38.0 (7.9) b	17.4 (5.0) a
Basal respiration	(µg CO_2-C g^{-1} soil h^{-1})	0.46 (0.21) a	1.32 (0.21) ab	2.04 (0.41) b	1.48 (0.40) ab
qCO_2	(mg CO2-C g^{-1} C_{mic} h^{-1})	3.87 (1.67) a	4.58 (0.64) a	5.92 (0.77) a	8.05 (1.85) a
Plant growth and photochemical efficiency					
Plant weight (g)		22.0 (4.07) a	206 (12.70) c	206 (12.29) c	94.6 (7.99) b
*F*v/*F*m		0.34 (0.081) a	0.69 (0.077) b	0.78 (0.019) c	0.78 (0.013) c
NPQ		0.39 (0.111) c	0.13 (0.039) a	0.16 (0.059) a	0.21 (0.086) b
RCC		0.21 (0.086) a	16.4 (4.4) b	33.6 (7.5) d	26.7 (5.8) c

[a] C_{mic}: microbial biomass; C_{mic}/Corg: microbial quotient; qCO_2: metabolic quotient; *F*v/*F*m: maximum photochemical efficiency of photosystem II; NPQ: non-photochemical quenching; RCC: relative chlorophyll content.

Community-level physiological profiles (CLPPs)

The CLPPS of the soil-borne microorganisms of the different treatments were performed in MicroResp plates and analysed by principal component analysis (PCA), which provides a visual representation of the carbon source utilization patterns of each microbial community. Figure 1 depicts the first two PCs of the overall analysis, which explained 58.7% and 12.1% of the variance, respectively. The effect of sludge was evident, resulting in a unique cluster for both types of sludge-amended soils and indicating different substrate utilization patterns than those of control and mineral fertilized soils. Treatments were significantly separated along PC1 (p = 0.003). Most of the C sources (11 out of 15) were strongly positively correlated with PC1, their loadings ranging from 0.71 to 0.92, whereas only one C source (malic acid) was weakly negatively correlated with PC1 with a loading of -0.12. Substrate utilization of each carbon source across the different treatments is shown in Figure 2. The strongest effect was observed after the addition of ATAD sludge. Substrate utilization of the microbial community of sludge-amended soils differed from that of the control group in its ability to catabolize 8 and 5 carbon sources for ATAD and MAD sludge respectively.

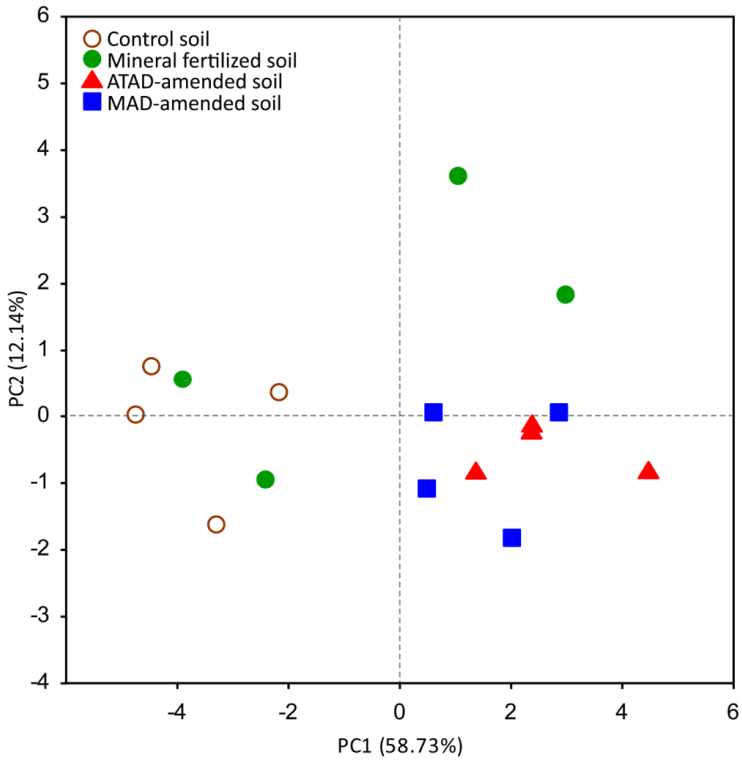

Fig. 1. PCA plot showing community-level physiological profiles across treatments (MicroResp™).

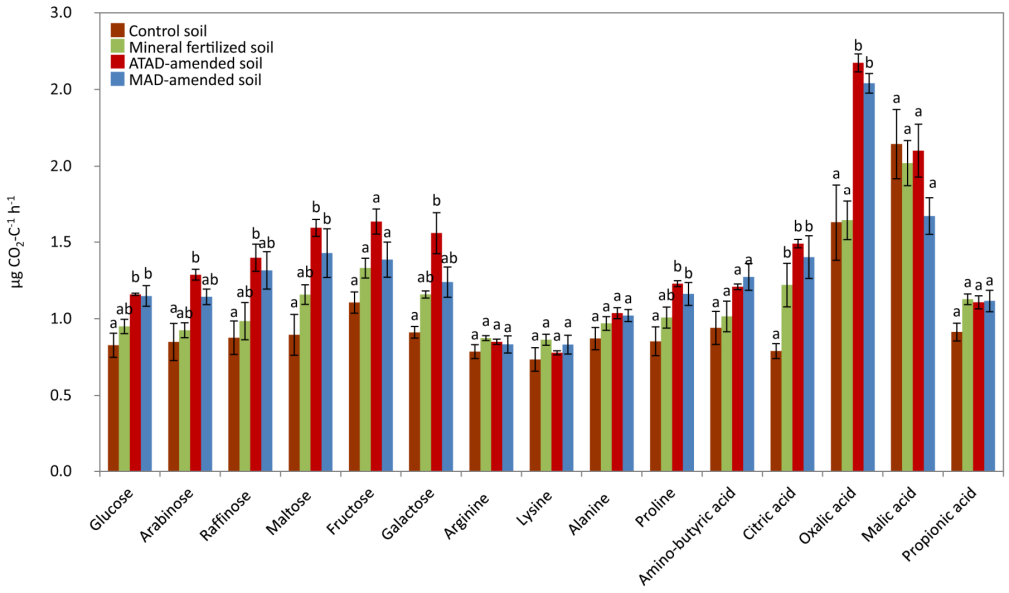

Fig. 2. Substrate utilization for 15 carbon sources (MicroResp™) in control, mineral fertilized, ATAD and MAD-sludge amended soils at the end of the experiment. Bars represent standard errors. Letters indicate significant differences between treatments ($p < 0.05$).

Sewage sludge microbial community structure

The prokaryotic community structure was significantly different depending on the sludge stabilization process ($r^2_{1,8} = 0.518$; $p = 0.006$). This result held when bacterial ($r^2_{1,8} = 0.518$; $p = 0.006$) and archeal communities ($r^2_{1,8} = 0.398$; $p = 0.01$) were analysed independently. Fungi were below detection limits in both cases. Differences in the microbial community structure between ATAD and MAD sludges are depicted in a principal coordinates analysis (PCoA) of the bacterial and archaeal operational taxonomic units (OTU) abundance matrices, with PC1 explaining 80.90% of the variance (Figure 3).

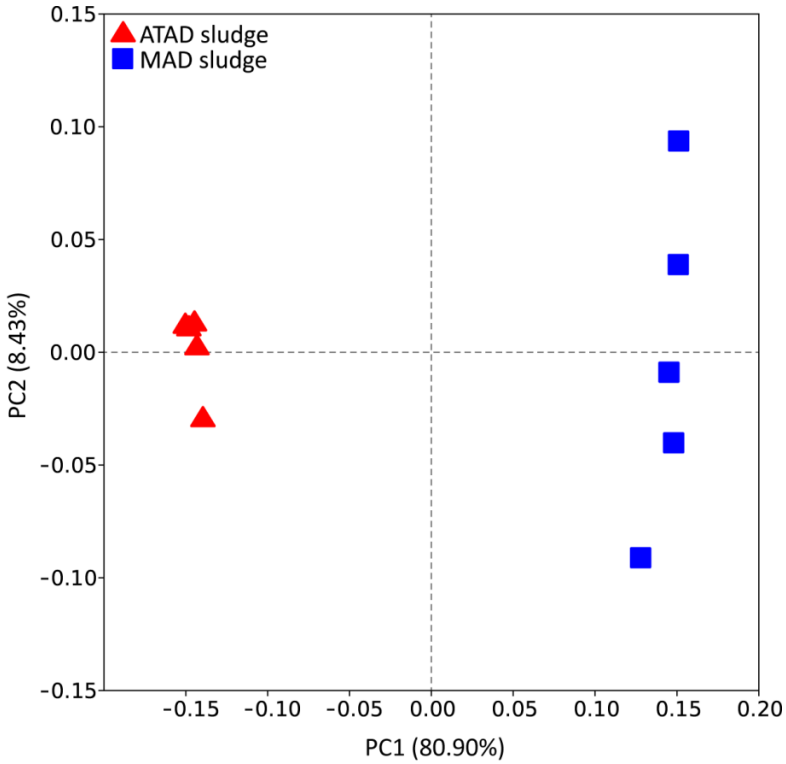

Fig. 3. Weighted principal coordinates analysis of the overall microbial community of ATAD and MAD sludge.

Microbial communities in MAD sludge were significantly more diverse than in ATAD, both in terms of species richness (Chao1) and in phylogenetic diversity (Faith's PD) (Suppl. Table S2). As regards the bacterial composition, ATAD sludge was largely dominated by members of the Firmicutes followed by far by Actinobacteria and Thermotogae (Figure 4A). MAD sludge showed a much more diverse population formed, nearly equitably, by members of the Firmicutes, Actinobacteria, Thermotogae, Synergistetes, Proteobacteria, Chloroflexi and Bacteriodetes (Figure 4A). Archaeal composition of ATAD sludge consisted of one major dominant taxon (Thermoplasmatales), whereas MAD sludge had a population formed almost equally by members of the Thermoplasmata and Halobacteriales (Figure 5A).

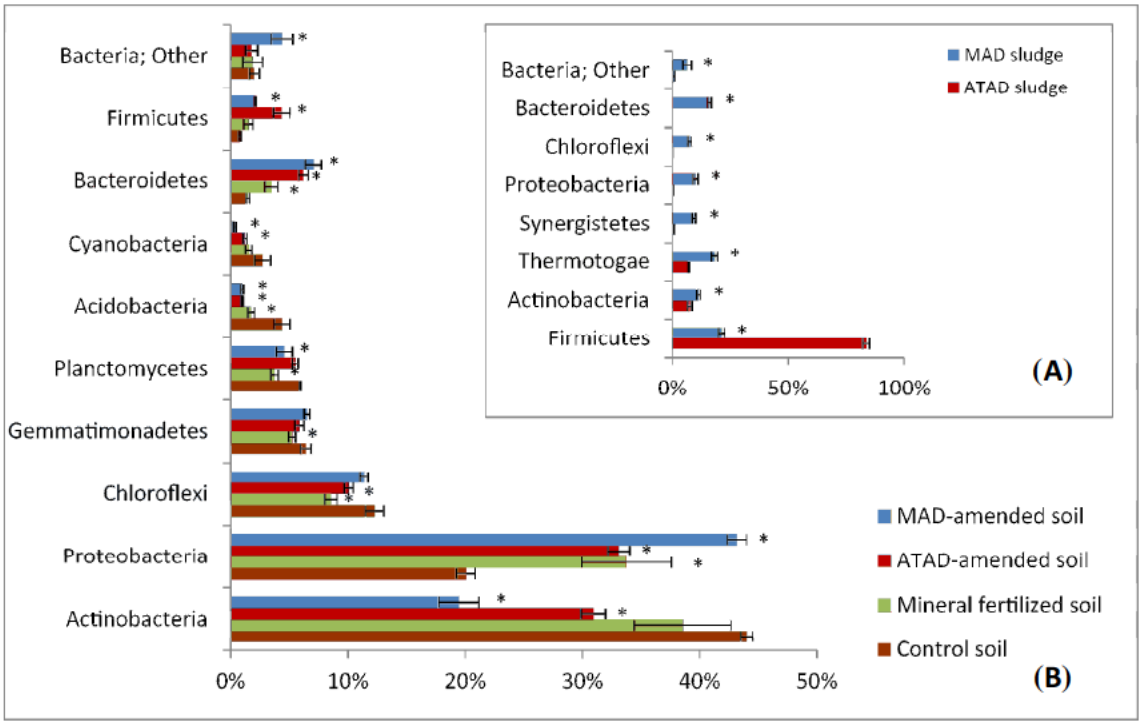

Fig. 4. Relative abundance of bacterial phyla in ATAD and MAD sludge (A) and in control, mineral fertilized and sludge-amended soils (B). Bars represent standard errors. Asterisks indicate significant differences between treatments relative to the control or within type of sludge ($p < 0.05$).

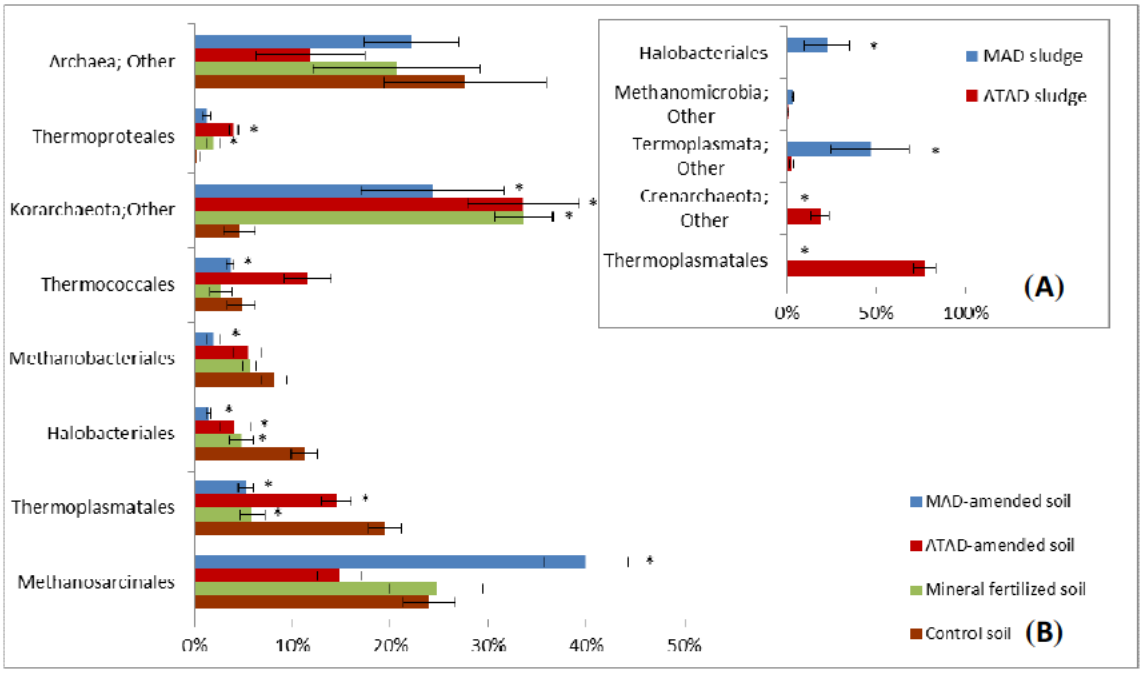

Fig. 5. Relative abundances of archaeal orders in ATAD and MAD sludge (A) and in control, mineral fertilized and sludge-amended soils (B). Bars represent standard errors. Asterisks indicate significant differences between treatments relative to the control or within type of sludge ($p < 0.05$).

Soil microbial community structure

The mineral and organic amendments had a strong influence on the composition of soil microbial communities, which significantly differed across treatments after two months of incubation ($r^2_{1,18}$ = 0.347; p = 0.001). This was confirmed separately for bacterial ($r^2_{1,18}$ = 0.349; p = 0.001), archaeal ($r^2_{1,18}$ = 0.289; p = 0.001) and fungal communities ($r^2_{1,18}$ = 0.264; p = 0.001). In a PCoA representing the overall soil microbial community structure, four different small clusters can be observed corresponding to each treatment (Figure 6), with PC1 explaining 59.01% of the variance. Addition of MAD sludge provoked the strongest shift in the microbial community of soils. The highest resemblance was found between the microbial communities in ATAD-amended and mineral fertilized soils.

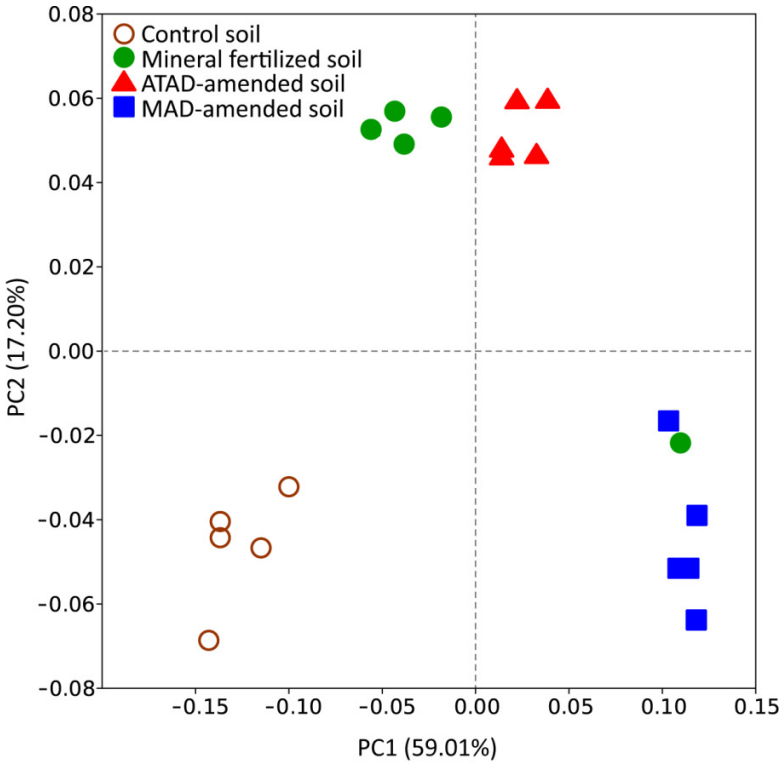

Fig. 6. Weighted principal coordinates analysis of the overall microbial community of control, mineral fertilized and sludge amended soils.

Soil microbial diversity significantly decreased in mineral fertilized and sludge-amended soils compared to control soils, both regarding species richness (Chao1), and phylogenetic diversity (Faith's PD) (Suppl. Table S2). The indigenous bacterial community of the control soil was largely dominated by

Actinobacteria (44% of the total bacteria in control soils) and Proteobacteria (20% of control soils), followed by Chloroflexi, Gemmatimonadetes, Planctomycetes, and Acidobacteria (Figure 4B). Sludge addition resulted in a significant decrease in the Actinobacteria, Acidobacteria and Cyanobacteria whereas the population of Proteobacteria, Bacteriodetes and Firmicutes significantly increased. These changes were especially prominent after the addition of MAD sludge (Figure 4B). Archaeal communities were dominated by Methanosarcinales, Thermoplasmatales, and Halobacteriales in control soils (Figure 5B). The addition of either mineral fertilizer or sludge significantly increased the numbers of organisms belonging to the candidate division Korarchaeota, which became the predominant taxon in mineral fertilized and ATAD-amended soils, while significantly decreasing the abundances of Thermoplasmatales and Halobacteriales. ATAD and MAD amendments individually, resulted in a significant increase of members of the Thermococcales and Methanosarcinales, respectively (Figure 5B). Among fungi, Basidiomycota prevailed in control soils followed by Ascomycota, while Glomeromycota significantly increased after the addition of mineral fertilizer and both sludges (Figure 7).

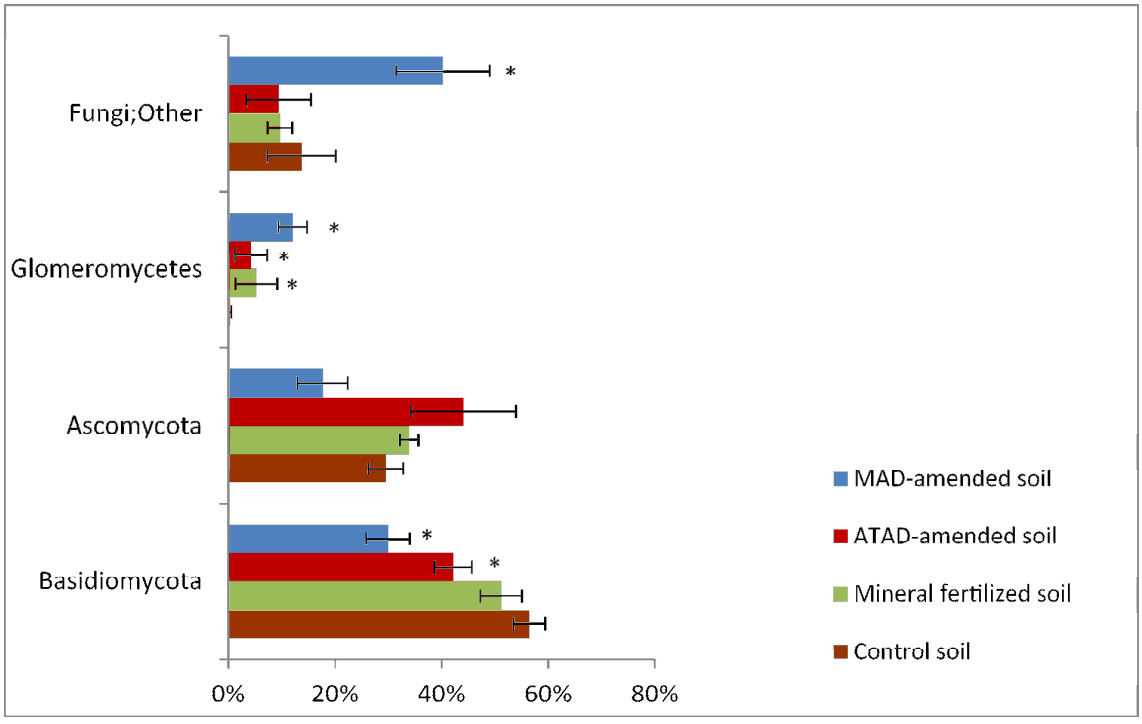

Fig. 7. Relative abundances of fungal phyla in control, mineral fertilized and sludge amended soils. Bars represent standard errors. Asterisks indicate significant differences between treatments relative to the control or within type of sludge ($p < 0.05$).

Plant growth, photochemical efficiency and relative chlorophyll content

Plant performance was substantially altered by all treatments when compared with the control pots (Table 3). Plant growth was strongly stimulated by the addition of mineral fertilization and ATAD sludge, and to a lesser extent by MAD sludge. Maximum photochemical efficiency of photosystem II (*F*v/*F*m) was significantly enhanced by the addition of both sludges followed by mineral fertilization. The non-photochemical quenching (NPQ) response was significantly higher in control soil and lower in mineral fertilized and ATAD sludge-amended pots. Finally, relative chlorophyll content (RCC) was significantly affected by all treatments, the highest increase being produced by ATAD sludge addition, followed by MAD sludge and mineral fertilizer.

Relationships between the soil environment, microbial communities and plant performance

Similarities in community structure in ATAD sludge did not correlate to similarities in community structure in ATAD-amended soils, and the same result was found for MAD sludge and MAD-amended soils (Suppl. Table S3). On the contrary, similarities in soil chemical composition (TOC, pH, EC, N, P and K) were significantly related to similarities in soil microbial community structure two months after the amendments and considering all treatments (r_{mantel} = 0.662, p = 0.001). EC was the single chemical variable that better explained the differences in the overall community structure (r_{mantel} = 0.652, p < 0.001), followed by pH (r_{mantel} = 0.404, p < 0.001) and TOC (r_{mantel} = 0.374, p = 0.004). Nevertheless, the single chemical variable that was most highly correlated to each of the domains was EC for bacteria (r_{mantel} = 0.652, p < 0.001) and archaea (r_{mantel} = 0.508, p = 0.015), and pH for fungi (r_{mantel} = 0.548, p = 0.003). Soils that were chemically similar also resembled in plant performance (growth and physiological state) (r_{mantel} = 0.18, p = 0.026). Finally, plant performance significantly correlated with soil microbial community structure (r_{mantel} = 0.233, p = 0.01) and microbial performance (microbial biomass, metabolic and microbial quotients and CLLPs; r_{mantel} = 0.72, p = 0.001) whereas a marginally significant difference was found between plant weight and EC (r_{mantel} = 0.126, p = 0.069).

Discussion

Land application of sewage sludge modified the soil chemical environment influencing subsequently the community structure of soil prokaryotes and microbial eukaryotes, the metabolic abilities of heterotrophs as well as plant performance. The magnitude of these changes was intimately associated to the stabilization process of sewage sludge, as we proved by landspreading sludges stabilized either through aerobic thermophilic or anaerobic mesophilic digestion from the same original residue. As we discuss below, this shows that the biotreatment technology used for processing sewage sludge with an agricultural fate eventually determines not only shifts in soil chemistry but also in soil microbial diversity and performance, and crop productivity.

1. Sludge stabilization process determined sludge microbial community structure

The microbial communities of sewage sludge were directly determined by the stabilization process, as proved by the significantly different community structure, diversity and composition found in ATAD and MAD sludges. Aerobic thermophilic digestion generated a low diverse sludge by fostering spore-forming (Firmicutes and Actinobacteria) and thermophilic bacterial and archaeal taxa (Thermotogae, Thermoplasmatales and Crenarcheota). Decreased diversity in thermophilic sludge leading to the prevalence of thermophiles has been previously observed by other authors (Piterina et al., 2006; Liu et al., 2010; Hayes et al., 2011). ATAD sludge was dominated by members of the Firmicutes, what has been reported before in thermophilic digestions (Goberna et al., 2009). On the other hand, archaeal population was ruled by Thermoplasmatales and Crenarcheota, both considered to be thermophilic (Blochl et al., 1997; Huber and Stetter, 2006). On the contrary, the anaerobic mesophilic stabilization process generated a highly diverse MAD sludge, with an even presence of up to seven bacterial phyla. This indicates a favorable environment for the growth of a wider range of microbes probably triggered by the presence of easily degradable carbon. The presence of Halobacteriales among the archaeal population of MAD sludge is likely due to the presence of a saline environment (Garrity and Boone, 2001).

2. Sludge application altered the soil microbial community depending on the stabilization process

Organic and mineral amendments altered the soil microbial community structure, reduced the taxonomic and functional diversity, and fostered the microbial activities, in line with previous reports (Borrero et al., 2004; Pascual et al., 2008). Furthermore, we proved that the degree of disturbance of soil microbial communities receiving sludge amendments depended on the sludge stabilization process. As expected, ATAD-amended soils resembled the control soils to a greater extent compared to MAD-amended soils. The changes imposed by the organic amendments can be induced either directly through the addition of exogenous microorganisms, or indirectly through changes in the environment of the indigenous communities (Perucci, 1993; García et al., 1998; García-Gil et al., 2000). Our results indicated that sludge-borne microorganisms did not displace significantly the soil indigenous microbiota, since we found no correlation between the community structure of sludge and sludge-amended soils. Alternatively, we showed that the shifts in the soil chemical variables - mainly EC, pH and total organic C - were responsible for the alteration of soil microbial communities. These abiotic variables, which are common determinants of the microbial genetic fingerprint and functioning (Fierer and Jackson 2006; Louzupone and Knight 2007; Goberna et al., 2012), were significantly modified by the addition of sludge as has been previously reported (Antolín et al., 2005; Pascual et al., 2009).

EC was the most relevant factor in altering the soil microbial community, likely by selecting salinity-tolerant microbes while having a deleterious effect over physiologically less adapted organisms (Simek et al., 1999; Thirukkumaran and Parkinson, 2000). This rationale was confirmed by the lower microbial activity and performance found in MAD-amended soils, whose microbes proved less efficient in using organic substrates as indicated by the C_{mic}/C_{org} ratio (Anderson, 2003; Rietz and Hayes, 2003). This was particularly evident as regards as the consumption of carbohydrates, which were reduced in MAD compared to ATAD-amended soils, confirming the results by Pascual et al. (2008). Another main factor determining soil microbial community was pH. As expected, more similar microbial communities were found at a similar pH probably due to the narrow pH ranges for optimal growth of bacteria (Rousk et al., 2010). As previously

reported by Marschner et al. (2003), it was the factor more strongly correlated factor with the fungal community. Finally, the changes in the levels of oxidizable substances also correlated to shifts in community structure. Soil microbial communities are frequently carbon - limited (Zak et al., 1994), the organic substrates added with sludge becoming the main nutrient source for soil microbes. As a consequence, soil microorganisms undergo a selective enrichment in response to carbon additions, and this may account for the reduced taxonomic and functional diversity of amended soils compared to control soils (Goldfarb et al., 2011).The shifts in composition of the soil bacterial communities were majorly produced by the decrease in the numbers of the dominant Actinobacteria and the increase of the sub-dominant Proteobacteria. In particular, the naturally Actinobacteria-dominated soils shifted to soils co-dominated by Actinobacteria and Proteobacteria when receiving mineral fertilization or ATAD amendments or even to Proteobacteria-dominated soils after MAD amendments. Both Actinobacteria and Proteobacteria encompass an extraordinary diversity of heterotrophic bacteria that commonly dominate soils worldwide (Janssen 2006). Actinobacteria are among the most important litter decomposers in soil (Kopecky et al., 2011), which reach high abundance in arid soils with low carbon contents and show an adaptive resistance to drought (Klevenskaya, 1960; Okoro et al., 2009; Bachar et al., 2010). Proteobacteria become superior competitors, able to displace other soil bacteria, under carbon-enriched conditions (Goldfarb et al., 2011). Thus, artificial carbon amendments typically increase the Proteobacterial abundance (Fierer et al., 2007; Goldfarb et al., 2011). Regarding the archaeal population, the shift in the composition was majorly produced by the increase of Korarchaeota after the addition of both types of sludge. Meanwhile, in ATAD-amended soils it was observed an increase of the representatives of the Thermococcales, a thermophilic heterotrophic taxon, whereas the addition of MAD sludge increased the member of methanogens such as Methanosarcinales, which catalyze the terminal step in the degradation of organic matter in anoxic environments. Finally, concerning the fungal population, all amendments characterized by the increase of members of the Glomeromycota. Since this group is known to be mutualistic symbionts between land plants that form arbuscular mycorrhiza (Smith, 2008) an improved supply of water and nutrients, could be expected in amended plants.

3. Sludge application influenced plant growth and efficiency depending on the stabilization process

Addition of sludge or mineral fertilizer resulted in more vigorous and healthy plants, with an enhanced efficiency of the photosystem II (*F*v/*F*m), lower non-photochemical quenching (NPQ) and higher relative chlorophyll content (RCC). Our data suggest that differences in plant performance across treatments were related both to the direct changes in the soil properties, and to the environmentally-mediated shifts in soil microbial community structure as well as in the metabolic abilities of decomposers. Improved plant growth and efficiency was most remarkable in ATAD-amended treatments, opposed to our predictions based on the highest degree of mineralization expected under aerobic thermophilic conditions (Bernal et al., 1998). Recalling that N was applied at the same concentration with both types of sludge, plant growth and N assimilation were lower in MAD-amended soils. Our results suggest that the increased EC in MAD-amended soils was the most likely reason for their poorer plant performance. It is known that a high EC affects plant growth, nutrient cycling and biological activity and that a too high osmotic pressure around the roots reduces water potential preventing an efficient water absorption by the plant causing water stress (Smith and Doran, 1996; Munns, 2002). In fact, MAD-amended soils may have been turned too saline for an agricultural use of sensitive or even moderately sensitive crops (Richards, 1954). In particular, melon is considered to be moderately salt tolerant (USDA, 1954), but some authors have described it as a sensitive crop with 1.5 dS m^{-1} being the most suitable EC for its growth and yield (Mota-Cadenas, 2010; Zulkarami, 2010).

The significantly lower value of *F*v/*F*m in control soils indicates a harmed photochemical activity in these treatments, suggesting photoinhibition and a subsequent photodamage of the PSII (Maxwell and Johnson, 2000), possibly as a consequence of the effect of oxidative processes on chlorophyll. In fact, *F*v/*F*m levels of melon crops in ATAD and MAD-amended soils are within the values estimated for a healthy plant (Björkman y Demming, 1987). These data are in agreement with Pascual et al. (2008), who found an increased photosynthesis in pepper plants amended with ATAD and MAD sludge compared to control plants. Although heat dissipation was promoted in control plants (higher NPQ), it was not sufficient to prevent damage to the leaves. An increase in NPQ is described as

being the result of the plant developing a system of protection against light excess or intrinsic damage in the leaf. RCC differed across all treatments, with the highest values in ATAD-amended soils, probably due to the fact that synthesis of chlorophyll is known to be highly dependent on N availability (van der Meer and de Jong, 2006) and to a more efficient microbial community.

In conclusion, we demonstrated that the stabilization process of sewage sludge stands out as a pivotal agent determining the feasibility of the final by-product as an organic amendment, shaping the sludge microbial community structure while altering in a different manner the soil microbial community. ATAD sludge produced smaller changes in the soil microbiota whilst enhancing soil microbial activity and plant growth and performance. This is consist with the consideration of ATAD sludge as having an *advanced status* (European Commission, 2003), and indicates that it could be used as an excellent surrogate to inorganic fertilization. Since this study was carried out during a 2-month period in a microcosm experiment, the question that now arises is whether the changes in the studied parameters are permanent or ephemeral. Hence, further investigation is needed in a longer field experiment to assess the pulse between the original and the newly established community, as well as to evaluate the prevalence of the induced chemical changes in order to determine the sludge application frequency to assure enhancement of plant growth.

Experimental Procedures

Soils and organic amendments

Soils were Haplic Calcisols (FAO-ISRIC and ISSS, 1998) collected from an agricultural land in Murcia (Southeast Spain; 38º11´53.84´´ N. 1º02´33.34´´ W). The topsoil layer (0-20 cm) was sampled and sieved (< 3 mm). Both types of sewage sludge were collected from Molina de Segura wastewater treatment plant (WWTP) (Murcia, Spain), treating municipal sludge. The sludges were produced after: 1) an autothermal thermophilic aerobic digestion (ATAD) -heat-treated according to Lloret et al., 2012-, and 2) a mesophilic anaerobic digestion (MAD). The main characteristics of the ATAD process were: a semi full-scale digester with an effective volume of 15-m^3 and an organic loading rate (OLR) of 2.7 kg volatile solids (VS) m^{-3} d^{-1}, a VS removal of 38 %, 62 ºC average temperature, and 15 days sludge retention time (SRT). The main characteristics of the MAD process were: a

full-scale 7,612-m^3 effective volume digester, an OLR of 0.8 kg VS m^{-3} d^{-1}, 45 % VS removal, 35 ºC average temperature and 50 days SRT. Both the ATAD and MAD digesters were feed with the same mixed primary and secondary sludge generated in the WWTP. Both sludges and soils were collected and stored at 4 ºC until the set-up of the microcosm experiment within the following week.

Experimental layout and growth conditions

A soil/vermiculite mixture (1:1 v/v) was placed into 2.8 L pots. Four experimental amendments were performed: none (control soil); mineral fertilizer, ATAD sludge and MAD sludge. The mineral fertilizer (NH_4NO_3 and KH_2PO_4) and sewage sludges were applied at a rate of 1.8 g N kg^{-1} soil (dry weight). Mineral fertilization was performed by irrigation twice a week during 59 days (until one week before harvesting) to avoid over-nitrification. Sludges were mixed with soil by turning at the beginning of the experiment in a single event. All treatments were replicated eight times and pots were arranged in a completely randomised design. After an equilibration period of 24 h, the experiment started and one melon seedling (*Cucumis melo* L., cv. Giotto) (2 or 3-leaf stage) was transplanted into each pot. Melon was chosen as the reference crop due to its economic value in the Mediterranean area. All plants were grown in a controlled environment greenhouse (28 ºC) and harvested after 66 days when fructification became the predominant process. Pots were irrigated as needed to adjust soil water content to 60% WHC throughout the experiment (100% WHC = 0.42 mL H_2O g^{-1} soil). Soil samples were taken both at the beginning and end of the experiment (before planting and after harvesting, respectively). One subsample was air-dried and used for chemical analysis, another subsample was stored at 4ºC and used for soil microbiological parameters and another subsample stored at -80 ºC until molecular analysis.

Soil chemical analysis

Soil chemical analyses were performed at the start and at the end of the experiment (n = 5). EC and pH were measured in a 1:5 (w/v) aqueous solution in a CM2002 conductivity meter (Crison, Barcelona, Spain) and pH2002 meter (Crison, Barcelona, Spain), respectively. C and N content were determined using a LECO TrusSpec CN analyzer (LECO Corp., St. Joseph, MI, USA). All other

macro- and micronutrients, and heavy metals were determined by digestion with HNO_3 and H_2O_2, using an Ultraclave microwave digestion system (Milestone S.R.L., Milan, Italy) followed by analysis by ICP (ICAP 6500 ICP Spectrometer, Thermo Fischer Scientific, Waltham, MA, USA).

Soil microbiological parameters

Soil basal respiration was measured as CO_2 evolution from moist (45% WHC) soil samples at 22 ºC, using continuous flow infrared gas analysis (IRGA) (Heinemeyer et al., 1989). Microbial biomass carbon (C_{mic}) was determined by substrate-induced respiration (SIR) after the addition of 1% glucose (dry matter basis) (Anderson and Domsch, 1978), using the IRGA as above. From basal respiration, organic C and C_{mic}, the microbial quotient (C_{mic}/C_{org}) and the metabolic quotient (qCO_2, μg CO_2-C g^{-1} C_{mic} h^{-1}) were calculated (n = 4).

Community-level physiological profiles (CLPPs)

The CLPPs were assessed by using a micro-respiration technique (MicroResp™) as described by Campbell et al. (2003) but adjusting the moisture content to 45 % WHC when mixed with the C source. The different carbon sources consisted of six carbohydrates (glucose, arabinose, raffinose, maltose, fructose and galactose), five amino acids (arginine, lysine, alanine, proline and amino-butyric acid), and four carboxylic acids (citric acid, oxalic acid, malic acid and propionic acid). A volume of 25 μl of carbon substrate solutions were pipetted into the different wells to a final concentration of 11 mg C mL^{-1} H_2O soil. Deionized water was added to additional samples to determine the respiration responses of non-substrate amended soils. The detection plates were prepared in 150 μl noble agar (1%) with cresol red (12.5 ppm, wt/wt), 150 mM potassium chloride and 2.5 mM sodium bicarbonate, and stored (4 ºC) in plastic bags with wet paper towels and soda lime to ensure no desiccation or reaction with atmospheric CO_2. Carbon sources in solution were adjusted to pH 5.5–6.0 and a calibration curve of absorbance versus headspace equilibrium CO_2 concentration was done according to Lalor et al. (2007). The microplates were read before and after a 6 h incubation period at 25 ºC at an absorbance wavelength of 570 nm (Zenyth 3100, Anthos, Eugendorf, Austria). Four replicates were performed for each treatment.

DNA Extraction

Total nucleic acids were extracted from 0.1 g of ATAD and MAD sludges and from 0.5 g of soil samples taken at the end of the experiment (n = 5). Sludges were freeze-dried and soils ground prior DNA extraction. DNA extraction was performed according to the protocol described by Ivanov et al. (2009) including some modifications. Samples were transferred to a 2 mL Lysing Matrix E tube (MP Biomedicals, Illkirch, France) containing 50 µL of 0.1M aluminum ammonium sulphate. Equal volumes (500 µL) of modified CTAB buffer (10% CTAB, 500 mM phosphate, 300 mM NaCl) and phenol:chloroform:isoamylalcohol (25:24:1) were added. Samples were agitated using a FastPrep instrument (MP Biomedicals, Illkirch, France; 2 x 20 s, 5.5. m s^{-1}) and centrifuged (16,000 x g, 5 min, 4 °C). The aqueous phase was recovered and an equivalent volume of chlorform:isoamylalcohol (24:1) added to yield a crude nucleic acid extract. The solution was centrifuged again and the aqueous phase transferred to a new tube containing 30 % w/v polyethylene glycol and incubated overnight. This solution was centrifuged, the supernatant discarded and the crude nucleic acid pellet washed in 70 % ethanol. The pellet was dissolved in nuclease-free (DEPC-treated) water and purified using the DNA/RNA AllPrep kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Purified DNA was eluted in EB buffer (2 x 30 µl) and quantified in triplicate using the Quant-iT Picogreen assay (Invitrogen, Carlsbad, CA, USA).

Pyrosequencing of SSU rRNA gene

Soil DNA samples were PCR amplified using a universal primer pair targeting the SSU rRNA V9 region. The forward primer was 515F ('5-GTGCCAGCMGCCGCGGTAA-'3; Tanner et al., 1999) and the reverse primer was 907R ('5-CCGTCAATTCCTTTRAGTTT-'3; Lane et al., 1991). Both primers were downstream of the FLX-454 primer adapters and the reverse primer also contained a 12-bp barcode unique to each sample (Hamady et al., 2008). Samples were normalized to 10 ng $µl^{-1}$ prior to PCR. The PCR reactions were carried out in a 25-µL volume, with final concentrations of 1× PCR buffer (20 mM Tris-HCl pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Nonidet P-40, 50% Glycerol), 2 mM $MgCl_2$, 200 µM each dNTP, 1 µM each primer, 0.56 mg mL^{-1} bovine serum albumin and 0.025 U Takara Ex Taq Hot Start polymerase

(Takara, Madison, WI, USA). The thermocycling conditions were as follows: an initial denaturation step at 95 ºC for 1 min, followed by 25 cycles consisting of denaturation at 95 ºC for 20 sec, annealing at 66 ºC for 30 sec and extension at 72 ºC for 1 min, and a final extension at 72 ºC for 10 min. Reaction primer dimers were removed from the PCR products via SPRI bead purification (AMPure XP, Beckman Coulter Genomics, Danvers, MA) according to the manufacturers protocol before being checked for quality and quantity on a Bioanalyzer 2100 using a DNA 7500 chip (Agilent Technologies, Santa Clara, CA, USA). Each PCR sample was normalized to 30 ng and combined together for multiplex sequencing. Sequencing libraries were created using the SV em PCR kit (Lib-A, Roche, Indianapolis, IN, USA) and sequenced on a 454 GS-FLX sequencer (Roche, Indianapolis, IN, USA) at the Veterans Medical Research Foundation (La Jolla, CA, USA).

Pyrosequencing data processing

Raw 454 sequences were uploaded, denoised and processed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010) and chimeric sequence detection performed using ChimeraSlayer (Haas et al., 2011). Low quality reads were filtered out and sequences assigned back to the original samples by 12 bp barcodes. Default QIIME parameters were used for processing the pyrosequencing data. OTUs were defined at 97 % via UCLUST (Edgar, 2010), and a representative sequence of each OTU used for alignment selected via PyNAST (Caporaso et al., 2010). Taxonomy assignment was performed using the RDP (Cole et al., 2009) and Silva (Quast et al., 2013) databases for prokaryotes and eukaryotes, respectively. Phylogenetic trees were created using FastTree (Price et al., 2009) under default parameters and used for the derivation of alpha and beta diversity metrics, including weighted and unweighted Unifrac distance matrices that calculate phylogenetic distance between communities using the degree of overlap between branch lengths in a phylogenetic tree (Hamady et al., 2010).

Plant growth, photochemical efficiency and relative chlorophyll content

Plant growth was determined as the plant fresh weight recorded immediately after plants were harvested.

Modulated chlorophyll fluorescence and relative chlorophyll content (RCC) were measured in all plants shortly before harvesting. Modulated chlorophyll fluorescence was measured in dark-adapted (30 min) leaves using a chlorophyll fluorometer OS-30 (OptiSciences, Herts, UK) with an excitation source intensity of 3000 μM m^{-2} s^{-1}. The minimal fluorescence intensity (F_0) in a dark-adapted state was measured in the presence of a background far-red light to favour rapid oxidation of intersystem electron carriers. The maximal fluorescence intensities in the dark-adapted state (Fm) and after adaptation to white actinic light (Fm´) were measured by 0.8 s saturating pulses (3000 μM m^{-2} s^{-1}). After the Fm´ measurement, the actinic light (400 μM m^{-2} s^{-1}) was switched off, and the far-red light was applied for 3 s in order to measure the minimal fluorescence intensity in the light-adapted state (F_0´). The maximum photochemical efficiency of photosystem II (PSII; Fv/Fm) and the non-photochemical quenching (NPQ) were calculated as (Fm– F_0)/Fm and (Fm – Fm´)/ Fm´, respectively (Maxwell and Johnson, 2000).

RCC was estimated using a SPAD-502 chlorophyll meter (Minolta Camera Co., Ltd, Osaka, Japan). This determines the relative amount of chlorophyll in the leaves by measuring the transmission of light at red (650 nm) and infrared (940 nm) wavelengths, and the difference in transmission at these two wavelengths is an indicator of chlorophyll content per leaf unit area.

Plant growth and efficiency was calculated for eight plants per treatment.

Statistical analysis

To test the effect of treatment and incubation time on soil chemical parameters, data were subjected to two-way ANOVAs. Data were tested for normality and transformed when necessary. When the interaction between factors was not significant, it was removed from the analysis. When the interaction between factors was significant, one-way ANOVAs were performed between treatments at each sampling time and within each treatment at both sampling times to determine pairwise differences among the groups. The effect of treatments on soil microbiological parameters, CLPPs and plant fitness was tested by one-way ANOVAs. The overall variations in catabolic activity of the CLPPs were analysed with principal component analysis (PCA) and PCA scores were analysed by one-way ANOVA. When significant F values were obtained,

further analysis with Tukey's HSD (honestly significant difference) comparison procedure was performed as a *post hoc* test ($p < 0.05$) to control for multiple testing. Non-normal data were subjected to non-parametric tests for several independent samples (Kruskal–Wallis test) and pair-wise comparisons between different types of sludge were performed using the Mann–Whitney U test ($p < 0.05$). The effect of treatments on the community structure of bacteria, archaea and fungi was assessed, both collectively and separately, through permutational multivariate analysis of variance using the vegan package for R (Oksanen et al., 2013). The effect of the treatment on the relative abundance of bacterial, archaeal and fungal taxa was tested by generalized linear models, either based on a Gaussian or a Poisson error distribution. The effect of sludge stabilization process on sludge diversity as well as the effect of treatments on the soil microbial community structure was tested by Student's *t*-Test and ANOVAs, respectively. To test for similarities between the microbial community structure in ATAD or MAD sludge and the corresponding sludge-amended soils we computed matrix correlations between OTU abundance distance matrices with 999 iterations by using the vegan package for R (Mantel, 1967; Oksanen et al., 2013). Finally, whether the similarity of the prokaryotic and the fungal community structure in soils submitted to different treatments was related to the similarity in their chemical and microbiological properties as well as in their plant production was examined. This was performed by calculating Mantel correlations between the occurrence (overall, bacterial, archaeal or fungal OTU abundance), environmental (chemical variables), microbial (microbiological parameters and CLPPs) and plant fitness (growth and photochemical analysis) distance matrices as above. Weighted unifrac distance matrices were used to visualize the soil community composition through ordination in a correspondence analysis (Okansen et al., 2013). All statistical analyses were performed in R (R Core Team, 2012).

Acknowledgments

This work was supported by the JAE Programme (co-funded by the Consejo Superior de Investigaciones Científicas, Spain, and the European Social Fund) and the project 324/pc08/2-04.3 included in the Plan Nacional de I+D+i 2008-2011. MG acknowledges support by the EU Marie Curie Programme (FP7-

PEOPLE-2009-RG-248155). We thank D. Beltrán for his support and encouragement on this work.

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Lloret et al. Supplemental Information for

Sewage sludge addition modifies soil microbial communities and plant performance depending on the stabilization process

Table S1. Macro and micronutrients and heavy metals of control, mineral fertilized, ATAD and MAD sludge-amended soils at the beginning and at the end of the experiment (values on dry weight basis). Standard deviation is given in parentheses. Letters indicate significant differences between treatments and asterisks indicate significant differences between each sampling time ($p < 0.05$).

Parameters	Sampling time	Control soil	Mineral fertilized soil	ATAD-amended soil	MAD-amended soil
Al (g kg^{-1})	Start	26.5 (2.4) a	26.5 (2.4) a	27.0 (2.2) a	26.9 (1.5) a
	End	25.6 (1.3) a*	24.3 (2.5) a*	26.7 (2.2) a*	24.5 (1.3) a*
B (mg kg^{-1})	Start	19.3 (2.8) a	19.3 (2.8) a	20.3 (0.7) a	21.8 (1.3) a
	End	17.6 (1.7) a	20.1 (1.4) a	20.1 (3.1) a	18.4 (0.8) a
Ca (g kg^{-1})	Start	156 (13) a	156 (13) a	145 (7) a	153 (6) a
	End	138 (7) b*	147 (7) b	150 (9) b	128 (4) a*
Mg (g kg^{-1})	Start	17.6 (03) a	17.6 (03) a	20.1 (2.8) a	18.4 (1.2) a
	End	23.1 (2.3) b*	16.5 (3.4) a	19.4 (3.0) ab	18.7 (1.9) ab
Mn (mg kg^{-1})	Start	336 (25) a	336 (25) a	302 (22) a	317 (25) a
	End	308 (25) a*	304 (28) a*	297 (15) a*	285 (10) a*
Mo (mg kg^{-1})	Start	0.50 (0.00) a	0.50 (0.00) a	0.76 (0.07) b	0.90 (0.07) c
	End	0.50 (0.00) a	0.53 (0.01) a	0.73 (0.10) b	0.73 (0.02) b*
Na (g kg^{-1})	Start	0.5 (0.0) a	0.5 (0.0) a	0.6 (0.0) b	0.8 (0.1) b
	End	0.7 (0.1) a*	0.7 (0.1) a*	0.8 (0.0) b*	0.8 (0.1) b*
S (g kg^{-1})	Start	1.3 (0.1) a	1.3 (0.1) ab	1.6 (0.1) b	1.8 (0.1) b
	End	1.7 (0.2) a*	2.0 (0.1) ab*	2.0 (0.1) b*	2.1 (0.3) b*

Tabla S2. Diversity indices of phylogenetic distance (Faith's PD) and species richness (Chao1) of ATAD and MAD sludge, and of control, mineral fertilized, ATAD and MAD sludge-amended soils. Standard deviation is given in parentheses. Asterisks indicate significant differences between types of sludge and letters between soil amendments (p< 0.05).

Diversity Index	Faith's PD	Chao1
ATAD sludge	2.76 (0.43)	31.3 (7.4)
MAD sludge	5.51 (0.51) *	31.3 (7.4) *
Control soil	53.5 (2.3) b	1156 (60) b
Mineral fertilized soil	44.0 (2.5) a	872 (126) a
ATAD-amended soil	42.6 (0.6) a	796 (57) a
MAD-amended soil	44.1 (2.6) a	710 (74) a

Table S3. Mantel test results between microbial communities of ATAD sludge and ATAD-amended soils, and of MAD sludge and MAD-amended soils.

	ATAD sludge x ATAD-amended soils	
	r	*p*
Bacterial community	0.314	0.251
Archaeal community	-0.038	0.438
Fungal community	-0.437	0.898
Overall community	0.284	0.264
	MAD sludge x MAD-amended soil	
	r	*p*
Bacterial community	-0.510	0.982
Archaeal community	0.6304	0.116
Fungal community	-0.026	0.494
Overall community	-0.524	0.937

VII

VII. Conclusiones generales
General conclusions

VII. Conclusiones generales

1. La digestión ATAD de lodos de depuradora realizada en un digestor con un volumen efectivo de 15 m^3, constituyó un tratamiento adecuado de estabilización de lodos una vez se alcanzó la estabilidad de los parámetros del proceso. Esto permitió operar con una alta velocidad de carga orgánica (2,7 kg VS m^{-3} d^{-1}) y un bajo tiempo de retención (14,6 días), y alcanzar los valores de destrucción de sólidos volátiles de un 38% requeridos para la obtención de un biosólido de Clase A, según la legislación americana. Uno de los factores claves en la consecución de esta estabilidad, fue la obtención de una aeración apropiada, por lo que el adecuado funcionamiento del rototamiz fue crucial.

2. El sistema ATAD redujo significativamente el contenido de patógenos humanos del efluente, consiguiendo la completa eliminación de *Salmonella* spp. y *E. coli* y produciendo, por tanto, un biosólido de Clase A según la legislación americana. Sin embargo, aunque produjo una reducción en el contenido de esporas de *C. perfringens*, ésta no fue suficiente para satisfacer los estándares microbiológicos de la futura Directiva Europea respecto a los tratamientos avanzados, que, a diferencia de la legislación americana, sí incluye este patógeno.

3. Los altos niveles de higienización obtenidos en el proceso ATAD, pueden atribuirse tanto a su elevada temperatura como a su elevado pH. Las temperaturas superiores a 65 °C incidieron negativamente en la VSD, por lo que deben tratar de evitarse.

4. La digestión del lodo ATAD en un rango de temperatura mesófilo, seguido de una nueva fase termófila, produjo la reducción de *C. perfringens* a niveles aceptables por la futura Directiva Europea tanto en condiciones de laboratorio como en el propio digestor. El alto grado de saneamiento conseguido en el lodo ATAD, su elevado contenido en nutrientes y materia orgánica, así como su bajo nivel de metales pesados, lo convierten en un fertilizante agrícola potencialmente valioso.

5. El análisis de la comunidad microbiana de diferentes lodos (lodo mixto fresco, ATAD, mesófilo, y tratado con una un doble digestión mesófila-termófila), mediante PCR-DGGE 16S ARNr, mostró un patrón de bandas muy diferentes para las poblaciones bacterianas. Sin embargo, las poblaciones de hongos se mantuvieron mucho más uniformes. En ambos casos, su diversidad se vio reducida tras la incubación o digestión bajo condiciones termófilas.

6. La puesta en marcha de un digestor TAnD de 15 m^3, partiendo de un inóculo mesófilo mediante un único y rápido aumento de temperatura, produjo una acumulación de AGVs inicial, determinando un periodo de arranque de 250 días, establecido dentro del rango normal de estos sistemas.

7. El sistema TAnD operó a mayores velocidades de carga orgánica (de 1,5 a 2,5 kg VS m^{-3} d^{-1}) y menores tiempos de retención (de 16 a 28 días) que el sistema MAD mostrando una eficiencia similar respecto a la destrucción de sólidos volátiles (> 40%) y a la producción de biogás (0,64 Nm^3 kg^{-1} VS^{-1} alimentados) y de metano (62%).

8. Los lodos provenientes de una digestión TAnD presentaron una mayor resistencia y mejor capacidad de deshidratación, mientras que los lodos mesófilos requirieron una menor dosis de polímero.

9. Análogamente al sistema ATAD, la digestión TAnD resultó eficaz en la eliminación de *Salmonella* spp. y *E. coli* pero no así de las esporas de *C. perfringens*. El efluente del digestor TAnD cumplió, por tanto, con la legislación americana para biosólidos de Clase A pero no con la futura norma europea para tratamientos avanzados.

10. Como consecuencia de los resultados obtenidos en los dos primeros trabajos de esta Tesis Doctoral y con el fin de dar cumplimiento a los estándares de la nueva Directiva Europea respecto a los patógenos humanos, un lodo no tratado, un lodo mesófilo y un lodo TAnD se sometieron a diversas incubaciones termófilas tanto en condiciones

aerobias como anaerobias. El resultado en el que derivó este ensayo fue la propuesta de un proceso de digestión en dos fases mesófila-anaerobia seguida de una termófila (aerobia o anaerobia indistintamente) para la obtención de un lodo totalmente higienizado. Esta primera fase mesófila, con una duración de 24 horas, puede llevarse a cabo en un tanque de pretratamiento no siendo necesaria la instalación de dos digestores.

11. El precedente de la legislación americana, que ha permitido la aplicación directa de biosólidos de Clase A durante las últimas décadas, la capacidad de las especies de *Clostridium* de formar esporas extremadamente resistentes a condiciones ambientales adversas tales como calor, radiación y compuestos tóxicos, su enorme ubicuidad (es el patógeno más ampliamente distribuido en la naturaleza, constituyente habitual de la vegetación, suelo, lodos, sedimentos marinos y tractos intestinales de humano y animales), así como la ineficacia de su eliminación mediante tratamientos avanzados tales como las digestiones ATAD y TAnD, conlleva necesariamente a una reflexión en torno a los parámetros, umbrales y tratamientos, descritos en el borrador de la futura Directiva Europea relativa a la aplicación agrícola de lodos.

12. Las condiciones bajo las que tiene lugar el proceso de estabilización de los lodos (ausencia/presencia de oxígeno y rango de temperatura), determinó tanto las características químicas de los lodos como su comunidad microbiana.

13. La aplicación agrícola de lodos procedentes de distintos tipos de digestión (ATAD y MAD), alteró en distinto modo las comunidades microbianas del suelo. La fuerte correlación obtenida entre la microbiota de los suelos enmendados y las características químicas de los mismos, sugieren que los cambios en la estructura, diversidad y actividad de la comunidad microbiana se deben principalmente al efecto de la aplicación de los lodos sobre las características químicas del suelo, así como a la adición de distintos tipos de compuestos (más oxidados en los suelos enmendados con lodo ATAD y más reducidos en los enmendados con lodo MAD), más

que a la acción directa del aporte de microorganismos exógenos contenidos en ellos.

14. La aplicación agrícola de lodos procedentes de un tratamiento avanzado como el ATAD, produjo menores cambios en la microbiota del suelo, a la vez que fomentó más intensamente su actividad microbiana, produciendo un mayor crecimiento de las plantas así como la mejora de su estado fisiológico, en comparación con un tratamiento convencional como el MAD. En consecuencia, y teniendo en consideración que el material de partida era el mismo, el tratamiento de estabilización de los lodos determina, en última estancia, la idoneidad de este subproducto como enmienda orgánica, siendo los provenientes de un tratamiento avanzado como el ATAD, un excelente sustituto de la fertilización inorgánica.

Finalmente, con el objeto de evaluar el sistema de digestión en dos fases propuesto en Tesis Doctoral, así como de garantizar la nutrición y el crecimiento de las plantas tras la aplicación agrícola de lodos, y de comprender los cambios experimentados en las comunidades microbianas de los suelos, sería interesante continuar con la investigación de: (i) la digestión en dos fases mesófila-anaerobio seguida de digestión termófila en digestores a gran escala para ratificar la eliminación de patógenos obtenida en esta tesis doctoral, (ii) ensayos en campo a largo plazo con el fin de evaluar el pulso establecido entre la comunidad microbiana autóctona de los suelos y la nueva comunidad formada tras la aplicación de lodos, y (iii) ensayos en campo a largo plazo para evaluar la permanencia de los cambios químicos producidos en el suelo tras la aplicación de los lodos, con el propósito de determinar la frecuencia de aplicación de los mismos para garantizar así el crecimiento de las plantas.

VII. General conclusions

1. ATAD sewage sludge digestion in a reactor with effective volume of 15 m^3 resulted in a suitable technology for sludge stabilization once all the operational parameters were stable. This allowed the use of a high organic loading rate (2.7 kg VS m^{-3} d^{-1}) and a short sludge retention time (14.6 days), reaching the volatile solids destruction of 38% established as the sludge vector attraction requirement for Class A biosolids according to the American regulation. A key factor to achieve the process stability was the obtaining of an adequate aeration. Consequently, adequate rotosieve functioning was crucial.

2. The ATAD system significantly reduced the content of human pathogens, achieving the complete removal of *Salmonella* spp. and *E. coli* and producing, therefore, a Class A biosolid according to the American regulation. However, despite the reduction of the content of *C. perfringens* spores after ATAD digestion, it was not sufficient to meet the microbial standards of the future European Directive regarding advanced treatments which, unlike the American legislation, does include this pathogen.

3. The high sanitation levels obtained after ATAD digestion could be attributed to both the thermophilic temperatures and raised pH. Temperatures above 65 °C should be avoided, since they produced a slight decrease in the VSD efficiency.

4. Mesophilic digestion of ATAD sludge followed by a new thermophilic stage reduced the population of *C. perfringens* spores to levels within the threshold values of the future European Directive. The high disinfection levels obtained after ATAD digestion, its high nutrient and organic matter content, as well as its safe levels of heavy metals, turned it a valuable agricultural fertilizer.

5. Community fingerprinting of raw mixed sludge, ATAD, mesophilic, and dual-temperature treated sludge by PCR-DGGE 16S rRNA, showed a very different banding pattern for bacterial population. On the other hand,

fungal population revealed a much more even profile. In both cases, microbial diversity was reduced after thermophilic digestion.

6. The start-up of a TAnD digester with effective volume of 15 m^3 by using a mesophilic inoculum and through a rapid and unique temperature rise, led to an initial accumulation of VFAs establishing a start-up stage of 250 days. This period is established within the normal range for this type of treatments.

7. The TAnD system operated at higher organic loading rates (de 1.5 a 2.5 kg VS m^{-3} d^{-1}) and shorter sludge retention times (16 to 28 days) than the anaerobic mesophilic digester while maintaining a similar VSD (> 40%) and biogas (0.64 Nm^3 kg^{-1} VS^{-1} fed) and methane (62%) production.

8. TAnD sludge showed a more resistant floc and better dewatering capability whereas mesophilic sludge required a lower polymer dose.

9. Similarly to the ATAD system, TAnD digestion successfully eradicated *Salmonella* spp. and *E. coli*. Nevertheless, *C. perfringens* spores were still present. The TAnD effluent fulfilled, therefore, the pathogen requirements for the US legislation, but not the forthcoming European regulation.

10. As a consequence of the results obtained in the first two studies of this Ph.D. dissertation, and with the aim of achieving the microbial standards established in the future European Directive, raw, mesophilic and TAnD sludge were incubated under thermophilic conditions and both under aerobic and anaerobic conditions. As a result, a 2-stage mesophilic anaerobic–thermophilic (either aerobic or anaerobic) sludge digestion process is suggested to achieve an utterly sanitized sludge. The first mesophilic stage, with a length of 24 hours, could also be performed in a continuous-stirred tank reactor (CSTR) so that the installation of two digesters would not be necessary.

11. The preceding US legislation, which has allowed direct application of Class A biosolids during the last decades, the ability of species of *Clostridium* to form metabolically-dormant spores that are extremely resistant to

environmental stresses such as heat, radiation and toxic chemicals, its remarkable ubiquity (being the most-widely distributed pathogen in nature, a normal component of decaying vegetation, marine sediment, the intestinal tract of humans and other vertebrates, soil and sludge), together with the inefficiency of advanced treatments such as ATAD and TAnD digestion to achieve their removal, leads, necessarily, to a reflection on the parameters, threshold values and treatments described in the Proposal for an European Directive on spreading of sludge on land.

12. Sludge stabilization process (presence/absence of oxygen and temperature range) determined sludge chemical properties as well as shaped the sludge microbial community.

13. Sludge application altered soil microbial communities in a different manner, depending on the stabilization process (ATAD and MAD). The strong correlation observed between the soil microbiota of amended soils and the soil chemical environment suggests that changes in the structure, diversity and activity of soil microbial community are due to changes in the soil chemical environment as a consequence of sludge application as well as to the addition of different compounds (more oxidized in ATAD-amended soils and more reduced in MAD-amended soils) rather than to the addition of sludge-borne microorganisms.

14. Landspreading of sludge from an advanced treatment such as ATAD produced smaller changes in soil microbiota whilst enhancing soil microbial activity and plant growth and performance compared with a conventional treatment such as MAD. Consequently, and taking into account that both systems had the same incoming material, the stabilization process of sewage sludge stands out as a pivotal agent determining the feasibility of the final by-product as an organic amendment, ATAD sludge being an excellent surrogate to inorganic fertilization.

Finally, in order to evaluate the 2-stage digestion system suggested in this Thesis, to assure enhancement of plant growth and nutrition, and to fully understand changes in the soil microbial community after sludge addition, further investigation is required in: (i) 2-stage mesophilic-anaerobic followed by thermophilic digestion in full-scale reactors to assure the pathogen removal obtained in this doctoral research, (ii) long-term field experiments to assess the pulse between the original and the newly established community after sludge addition, and (iii) long-term field experiments to evaluate the permanence of the induced chemical changes after sludge application in order to determine the sludge application frequency to provide enhancement of plant growth.

VIII

VIII. Resumen
Summary

VIII. Resumen

La producción de lodos de depuradora ha experimentado un elevado incremento en los últimos años debido tanto al aumento del volumen de las aguas depuradas en EDARs, como a la cada vez más restrictiva legislación aplicada a los efluentes (MAPA, 2003). En nuestro país, la generación de lodos se incrementó en un 41,2% en el periodo 2000-2009 con una producción en este último año, de 1.205.124 toneladas de materia seca. El 82,6% de estos lodos fue empleado como enmienda orgánica en agricultura, mientras que el 7,9% se depositó en vertedero y el 5,1% fue incinerado con recuperación de energía (MARM, 2011).

El uso agrícola de los lodos de depuradora, recomendado por la Directiva Europea 91/271/EEC sobre el tratamiento de aguas residuales urbanas (Comisión Europea, 1991), es de especial interés en la región Mediterránea. En esta zona, la acuciante degradación que han venido sufriendo los suelos, reduciendo tanto el contenido en materia orgánica como la fertilidad natural de los mismos, los hace especialmente vulnerables (García et al., 2000).

La aplicación agrícola de lodos de depuradora ha sido intensamente estudiada en los últimos años, demostrando grandes beneficios sobre las propiedades físicas y químicas del suelo (Korentajer, 1991; Barzegar et al., 2002), una mejora en su fertilidad, y un aumento en la producción agrícola (Kelley et al., 1984; Min-Jian, 1997; Singh y Agrawal, 2008). La adición de materia orgánica, también ha demostrado tener efectos sobre las comunidades microbianas del suelo, aumentando, por lo general, su desarrollo y actividad (Bailey y Lazarovits 2003). Estos cambios pueden ser producidos tanto directamente por la adición de microorganismos exógenos procedentes de la materia orgánica añadida, o indirectamente debido a cambios en el ambiente de las comunidades microbianas autóctonas (Perucci, 1993; García et al., 1998; García-Gil et al., 2000).

Sin embargo, el uso agrícola de lodos también puede entrañar riesgos no deseables debido a su potencial contenido de metales pesados, compuestos tóxicos y/o microorganismos patógenos como bacterias, virus, helmintos, etc. (Beuchat, 1996), que pueden suponer un riesgo para la salud humana, animal o medioambiental.

Con el objeto de minimizar estos riesgos, y garantizar la seguridad del uso agrícola de los lodos, la Unión Europea está redactando una nueva legislación sobre aplicación agrícola de lodos de depuradora a través de la "Propuesta de Directiva del Parlamento Europeo y el Consejo sobre el uso agrícola de lodos" (Comisión Europea, 2003), que reemplazará a la legislación vigente (Directiva 86/278/EEC, relativa a la protección del medio ambiente y en particular de los suelos en la utilización de los lodos con fines agrícolas). Entre las nuevas modificaciones que propone la futura Directiva, uno de los parámetros a destacar es la evaluación del contenido de microorganismos patógenos, distinguiendo entre tratamientos convencionales y avanzados de lodos de depuradora, según los niveles de estos microorganismos obtenidos tras el proceso de estabilización. Los tratamientos avanzados, en contraposición con los convencionales, permiten menores restricciones en el uso y manejo de los lodos estabilizados (Comisión Europea, 2003).

En este contexto, el objetivo general de la presente memoria consistió en el estudio de dos de los tratamientos avanzados propuestos en esta futura Directiva Europea, con el fin de evaluar el proceso de estabilización e higienización de los lodos. Los tratamiento fueron: (i) digestión aerobia autotérmica termófila (ATAD), y (ii) digestión anaerobia termófila (TAnD). Así mismo, también incluyó el estudio de los efectos de las características del proceso de estabilización en la comunidad microbiana de los lodos. Finalmente, comprendió el estudio de la aplicación agrícola de un lodo avanzado en comparación con uno convencional, evaluando los efectos sobre el suelo y las plantas.

Para alcanzar este objetivo general, los objetivos específicos que se plantearon fueron:

i. La puesta en marcha de un digestor ATAD con un volumen efectivo de 15 m^3 con el fin de caracterizar el proceso de estabilización de lodos y evaluar la reducción de los microorganismos patógenos contemplados en la futura Directiva Europea (*Salmonella* spp., *Escherichia coli*, y esporas de *Clostridium perfringens*).

ii. La puesta en marcha de un digestor TAnD con un volumen efectivo de 15 m^3 con el fin de caracterizar el proceso de estabilización de lodos y evaluar la reducción de los microorganismos patógenos contemplados en la futura Directiva Europea (*Salmonella* spp., *E. coli*, y esporas de *C. perfringens*), en relación con una digestión convencional mesófila anaerobia (MAD).

iii. Propuesta de un sistema de digestión en dos etapas con el fin de obtener un lodo avanzado según los criterios microbiológicos de la futura Directiva Europea.

iv. Evaluación de la influencia del proceso de estabilización de los lodos de depuradora sobre la comunidad microbiana de los mismos para determinar la posible incidencia en la microbiota del suelo, comparando un tratamiento de digestión avanzado ATAD con uno convencional MAD.

v. Estudio del efecto de la aplicación agrícola de un lodo avanzado ATAD sobre la estructura y funcionamiento de la comunidad microbiana del suelo, las propiedades químicas del suelo, y la influencia en un cultivo de melón, en comparación con un lodo convencional MAD.

El cuerpo principal de esta Tesis Doctoral se compone de cuatro capítulos donde se estudian los objetivos mencionados anteriormente:

Capítulo III. Lloret E, Pastor L, Martínez-Medina A, Blaya J, Pascual JA, 2012. Evaluation of the removal of pathogens included in the Proposal for a European Directive on spreading of sludge on land during autothermal thermophilic aerobic digestion (ATAD). Chemical Engineering Journal, 198-199, 171-179.

En este capítulo, que engloba el primer objetivo, se estudió el arranque y funcionamiento de un digestor aerobio autotérmico termófilo (ATAD) con un volumen efectivo de 15 m^3 durante un periodo de 19 meses para evaluar el proceso de estabilización e higienización de lodos de una EDAR municipal. Para ello, se estudiaron los parámetros físico-químicos del proceso y se analizó el contenido de *Salmonella* spp., *E. coli* y esporas de *C. perfringens* mediante

recuento en placa, y a través de la amplificación de los genes de patogenicidad *invA* y *cpa* mediante reacción en cadena de la polimerasa (PCR), tanto a la entrada como en el efluente del sistema. Mediante el tratamiento ATAD, la destrucción de sólidos volátiles obtenida fue del 38,0% consiguiendo una alta velocidad de carga orgánica (2,7 kg VS m^{-3} d^{-1}) y un bajo tiempo de retención (14,6 días). Respecto al contenido de microorganismos patógenos, éste descendió significativamente con la completa eliminación de *Salmonella* spp. y de *E. coli*, y la reducción en 2 unidades logarítmicas del contenido de esporas de *C. perfringens*. Con el objeto de eliminar las esporas de *C. perfringens* para obtener una total higienización de los lodos, se introdujo una etapa mesófila intermedia después del tratamiento ATAD. De este modo, se obtuvo un lodo estabilizado y libre de patógenos, adecuado para su aplicación agrícola. Por último, el análisis de los lodos mediante electroforesis en gel con gradiente de desnaturalización (DGGE), mostró diferencias en las estructuras de las comunidades de hongos y bacterias entre los lodos frescos (influente), mesófilos y termófilos, indicando la relevancia de la temperatura del proceso de digestión en la alteración de las comunidades microbianas de los lodos. Los resultados obtenidos demostraron que la tecnología ATAD produjo un lodo adecuado para su aplicación agrícola una vez se alcanzó la estabilidad de los parámetros del proceso y se introdujo una etapa mesófila intermedia.

Capítulo IV. Lloret E, Pastor L, Pradas P, Pascual JA, 2013. Semi full-scale thermophilic anaerobic digestion (TAnD) for advanced treatment of sewage sludge: stabilization process and pathogen reduction. Chemical Engineering Journal, 232: 42-50.

Este trabajo engloba el segundo objetivo, y consistió en la puesta en marcha y estudio de un digestor termófilo anaerobio (TAnD) de 15 m^3 durante un periodo de 18 meses, con el fin de evaluar el proceso de estabilización de los lodos de depuradora y los microorganismos patógenos incluidos en la futura Directiva Europea sobre aplicación agrícola de lodos. La estrategia escogida para la conversión de temperatura desde un rango mesófilo al termófilo final, fue el de un rápido y único incremento de temperatura. Para evaluar la estabilidad del

proceso, se realizaron medidas de parámetros físico-químicos como la destrucción de sólidos volátiles (VSD), ácidos grasos volátiles (VFA), producción de biogás, macronutrientes y metales pesados. Para evaluar la destrucción de microorganismos patógenos, se cultivaron *Salmonella* spp., *E. coli* y las esporas de *C. perfringens* y se amplificaron los genes de patogenicidad *invA* y *cpa* mediante reacción en cadena de la polimerasa (PCR). El reactor funcionó con tiempos de retención hidráulicos (SRT) de 28, 20, 18 y 16 días, y con una velocidad de carga orgánica (VCO) que osciló entre 1,5 y 2,5 kg VS m^{-3} d^{-1}. En todos los periodos de operación se obtuvo un funcionamiento adecuado del digestor alcanzando valores de VSD superiores al 40% y una producción de biogás media situada en 0,64 Nm^3 kg^{-1} VS^{-1}. El sistema TAnD, admitió mayores VCO y menores SRT que la digestión MAD. Respecto al contenido de microorganismos patógenos, redujo las poblaciones de *Salmonella* spp. y *E. coli* por debajo de los límites de detección, pero no consiguió eliminar de las esporas de *C. perfringens* esporas (4,63 $\log_{10}$ esporas mL^{-1}). Por lo tanto, el producto final cumplió con los límites establecidos en la legislación americana (USEPA, 2003) para alcanzar la clasificación de biosólidos de Clase A, pero no logró satisfacer los límites establecidos en la futura legislación europea.

Capítulo V. Lloret E, Salar MJ, Blaya J, Pascual JA, 2013. Two-stage mesophilic anaerobic – thermophilic digestion for sludge sanitation to obtain advanced treated sludge. Chemical Engineering Journal, 230: 59-63.

El presente estudio, aunque no contemplado inicialmente el los objetivos principales, surge de la necesidad de alcanzar una higienización de los lodos acorde con la exigida en la futura Directa. En el presente estudio se analizaron tres tipos de lodos de depuradora distintos (fresco, mesófilo anaerobio y termófilo anaerobio) con el fin de evaluar si cumplían los límites establecidos en la "Propuesta de Directiva del Parlamento y del Consejo Europeo sobre aplicación agrícola de lodos" respecto al contenido de microorganismos patógenos. Para ello, se procedió al cultivo de *Salmonella* spp., *E. coli* y de las esporas de *C. perfringens* y a la amplificación mediante reacción en cadena de la polimerasa (PCR) de los genes de patogenicidad *invA* y *cpa*. La digestión termófila anaerobia

(TAnD) produjo biosólidos de Clase A según los criterios de la legislación americana (USEPA, 2003) al producir la eliminación de *E. coli* y *Salmonella* spp. Sin embargo, no consiguió cumplir los requisitos establecidos en la futura Directiva debido al contenido de esporas de *C. perfringens* (9,6 x 10^4 esporas mL^{-1}). Por consiguiente, el objetivo último de este estudio consistió en proponer un proceso de digestión en dos etapas capaz de eliminar las esporas de *C. perfringens* para obtener un lodo avanzado adecuado para su directa aplicación a suelo sin riesgos ambientales ni sobre la salud humana. La primera etapa del sistema propuesto, consiste en la digestión mesófila anaerobia, mientras que la segunda etapa comprende la digestión termófila de los lodos; aerobia o anaerobia indistintamente. De esta manera, al provocar en la primera fase la germinación de las esporas de *C. perfringens*, estas nuevas células vegetativas pueden ser erradicadas o dañadas en una subsecuente etapa termófila, obteniéndose así, un producto final libre de patógenos.

Capítulo VI. Lloret E, Pascual JA, Brodie EL, Bouskill NJ, Fernández Delgado-Juárez M, Insam H, Goberna M. Sewage sludge addition modifies soil microbial communities and plant performance depending on the stabilization process. En revisión.

Este último trabajo engloba el cuarto y quinto objetivo específico. En él se analiza por un lado la influencia del proceso de estabilización de los lodos sobre la comunidad microbiana de los mismos, y por otro, los efectos de la aplicación agrícola de los lodos de depuradora que difieren en sus respectivos procesos de estabilización, sobre la diversidad y estructura de la comunidad microbiana del suelo, las propiedades químicas del suelo, así como en el crecimiento y desarrollo de las plantas. El estudio de la comunidad microbiana de los lodos mediante pirosecuenciación de la región SSU V9 ARNr, demostró que el proceso de estabilización al que se habían sometido previamente los lodos, modificó sus comunidades microbianas. Por otro lado, la aplicación de un lodo avanzado ATAD alteró en menor medida la microbiota y la química del suelo, a la par que estimuló la actividad microbiana y el crecimiento y desarrollo de las plantas de un cultivo de melón. La aplicación de un lodo convencional MAD, sin embargo, no

tuvo efectos sobre la actividad de los microorganismos, aunque sí mejoró el crecimiento y desarrollo de las plantas comparado con el control. Los resultados obtenidos sugieren que las variaciones de las poblaciones microbianas del suelo tras la aplicación de lodos, son debidas con mayor probabilidad a los cambios producidos en la química del suelo, que a la adición de una nueva comunidad de microorganismos incorporada con los lodos. Estos resultados también mostraron que alteraciones en un solo parámetro químico del suelo (p.e. CE), son susceptibles de producir una gran variación en la estructura y activad de su comunidad microbiana, así como en el desarrollo y estado fisiológico de las plantas.

De esta memoria, se pueden extraer las siguientes conclusiones generales:

• La digestión ATAD de lodos de depuradora resultó ser un tratamiento adecuado de estabilización de lodos una vez se alcanzó la estabilidad del proceso operando con una alta velocidad de carga orgánica (2,7 kg VS m^{-3} d^{-1}) y un bajo tiempo de retención (14,6 días), y alcanzar los valores de destrucción de sólidos volátiles de un 38% requeridos para la obtención de un biosólido de Clase A, según la legislación americana. A su vez, el sistema ATAD redujo significativamente el contenido de patógenos humanos del efluente, alcanzando la completa eliminación de *Salmonella* spp., *E. coli* y coliformes totales, produciendo, por tanto, un biosólido de Clase A. Sin embargo, aunque produjo una reducción de las poblaciones de esporas de *C. perfringens*, ésta no fue suficiente para satisfacer los estándares microbiológicos de la futura Directiva Europea respecto a los tratamientos avanzados.

• La puesta en marcha de un digestor TAnD partiendo de un inóculo mesófilo mediante un aumento único y rápido de temperatura, determinó un periodo de arranque de 250 días. El sistema TAnD operó a mayores velocidades de carga orgánica (de 1,5 a 2,5 kg VS m^{-3} d^{-1}) y menores tiempos de retención (de 16 a 28 días) que el sistema MAD mostrando una eficiencia similar respecto a la destrucción de sólidos volátiles (> 40%) y a la producción de biogás (0,64 Nm^3 kg^{-1} VS^{-1} alimentados) y de metano (62%). Análogamente al sistema ATAD, la digestión termófila anaerobia resultó eficaz en la eliminación de *Salmonella* spp., *E. coli* y coliformes totales, pero no así de las esporas de *C. perfringens*. El

efluente del digestor TAnD cumplió, por tanto, con la legislación americana para biosólidos de Clase A pero no con la futura norma europea para tratamientos avanzados.

• En esta Tesis Doctoral se propone un proceso de digestión en dos fases, mesófila-anaerobia seguida de una termófila (aerobia o anaerobia indistintamente) para la obtención de un lodo totalmente higienizado. Esta primera fase mesófila, con una duración de 24 horas, puede llevarse a cabo en un tanque de pretratamiento, no siendo necesaria la instalación de dos digestores.

• Las condiciones bajo las que tiene lugar el proceso de estabilización de los lodos (ausencia/presencia de oxígeno y rango de temperatura), determinó tanto las características químicas de los lodos como su comunidad microbiana, que vio disminuida su diversidad tras la digestión termófila.

• La aplicación agrícola de lodos procedentes de distintos tipos de digestiónalteró en distinto modo las comunidades microbianas del suelo. La aplicación agrícola de lodos procedentes de un tratamiento avanzado como el ATAD, produjo menores cambios en la microbiota del suelo, a la vez que fomentó más intensamente su actividad microbiana, produciendo un mayor crecimiento de las plantas así como la mejora de su estado fisiológico, en comparación con un tratamiento convencional como el MAD. En consecuencia, y teniendo en consideración que el material de partida era el mismo, el tratamiento de estabilización de los lodos determina, en última estancia, la idoneidad de este subproducto como enmienda orgánica, siendo los provenientes de un tratamiento avanzado como el ATAD, un excelente sustituto de la fertilización inorgánica.

VIII. Summary

The production of sewage sludge has increased significantly during the past few years due to the expansion of wastewater treatment and tougher effluent restrictions (MAPA, 2003). In Spain, sewage sludge production increased by 41.2% in the period 2000-2009, with a production of 1,205,124 tons of dry matter in 2009. 82.6% of the sludge produced was used as an organic fertilizer in agriculture, 7.9 % was sent to landfills and 5.1% was incinerated with energy recovery (MARM, 2011).

Agricultural use of sewage sludge, which is encouraged by the European Directive 91/271/EEC on urban wastewater treatment (European Commission, 1991), is of special interest in the Mediterranean region. In this area, the strong degradation that soils have been subjected to, have reduced both soil organic matter content and natural fertility, turning them particularly vulnerable (García et al., 2000).

Agricultural application of sewage sludge has been widely studied in recent years showing great benefits on physical and chemical properties of soil (Korentajer, 1991; Barzegar et al., 2002), an improvement of soil fertility and an increase in crop yield (Kelley et al., 1984; Jian-Min, 1996, Singh and Agrawal, 2008). The addition of organic matter affects soil microbial communities, generally, by accelerating microbial development and activity (Bailey and Lazarovits 2003). The changes imposed by the organic amendments can be induced either directly through the addition of exogenous microorganisms, or indirectly through changes in the environment of the indigenous communities (Perucci, 1993; García et al., 1998; García-Gil et al., 2000).

However, the agricultural use of sewage sludge may have some undesirable risks, associated with its potential content of heavy metals, toxic compounds and/or pathogens such as bacteria, viruses and parasites (Beuchat, 1996), which may pose risk to human , animal or environmental health.

In order to minimize these risks, and to ensure a safe agricultural use of sewage sludge, the European Union is developing a new legislation regarding land application of sewage sludge through the “Proposal for a Directive of the

European Parliament and of the Council on spreading of sludge on land" (European Commission, 2003), which will replace the existing legislation (Directive 86/278/EEC on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture). This provides stricter standards for the content of heavy metals, organic compounds and human pathogens, this latter parameter receiving special attention. In this respect, the Proposal for a European Directive introduces the concept of advanced and conventional treatments, which allow operators to use advanced treated sludge with fewer restrictions compared with a sludge that has been treated conventionally (European Commission, 2003).

In this scenario, the general objective of this thesis consisted of the study of two advanced treatments described in this Proposal for a European Directive, with the aim of assessing the stabilization process and sludge sanitation. The studied treatments were: (i) autothermic thermophilic aerobic digestion (ATAD), and (ii) thermophilic anaerobic digestion (TAnD). Likewise, the effects of the stabilization process on sludge microbial community were assessed. Finally, the effects of the agricultural use on soil and plants of an advanced treated sludge compared to a conventional sludge were evaluated.

To achieve this general objective, the following specific objectives were approached:

i. To start-up an ATAD digester with an effective volume of 15 m^3 with the aim of studying the stabilization process of sewage sludge and of evaluating the removal of pathogens included in the future European Directive (*Salmonella* spp., *Escherichia coli*, and *Clostridium perfringens* spores).

ii. To start-up a TAnD digester with an effective volume of 15 m^3 in order to study the stabilization process of sewage sludge and to evaluate the reduction of pathogens included in the European Directive future (*Salmonella* spp., *E. coli*, and *C. perfringens* spores) compared with a conventional mesophilic anaerobic digestion (MAD).

iii. To propose a 2-stage sludge digestion process with the aim of obtaining an advanced treated sludge according to the microbial requirements of the future European Directive.

iv. To assess the influence of the stabilization process of sewage sludge on sludge microbial community to determine its potential influence on soil microbiota, comparing an advanced ATAD treatment with a conventional MAD treatment.

v. To study the effects of agricultural application of an advanced ATAD sludge on the structure and performance of soil microbial communities, soil chemical properties and its influence on a melon crop compared to a conventional MAD sludge.

The main body of this Ph.D. dissertation consists of four chapters corresponding to the above-mentioned objectives:

Chapter III. Lloret E, Pastor L, Martínez-Medina A, Blaya J, Pascual JA, 2012. Evaluation of the removal of pathogens included in the Proposal for a European Directive on spreading of sludge on land during autothermal thermophilic aerobic digestion (ATAD). Chemical Engineering Journal, 198-199, 171-179.

This chapter focuses on the first objective. Here, a one-stage autothermal thermophilic aerobic digester (ATAD) with effective volume of 15-m^3 was started up and studied over 19 months to assess the stabilization process and sanitation of municipal sludge. To do so, physico-chemical performance parameters were monitored and *Salmonella* spp., *E. coli*, and *C. perfringens* spores were cultivated as well as pathogenity genes *invA* and *cpa* PCR-amplified. Volatile solids removal was 38.0% achieving a high organic loading rate (2.7 kg VS m^{-3} d^{-1}) and a short sludge retention time (14.6 days). Regarding the pathogen content, it significantly decreased by completely eliminating *Salmonella* spp. and *E. coli*, and reducing *C. perfringens* spores content in 2-log units. To achieve a complete disinfection of the sludge, a mesophilic stage was introduced after the ATAD treatment obtaining a pathogen free sludge. Finally, denaturing gradient gel electrophoresis

(DGGE) analysis showed differences in the structures of the bacterial and fungal communities between raw (effluent), mesophilic and thermophilic sludge, indicating, indicating the relevance of digestion temperature on shaping the microbial community of sludge. The results demonstrated that the ATAD technology had the capability to produce sludge suitable for agricultural application when the operational parameters were stable and a mesophilic stage was introduced.

Chapter IV. Lloret E, Pastor L, Pradas P, Pascual JA, 2013. Semi full-scale thermophilic anaerobic digestion (TAnD) for advanced treatment of sewage sludge: stabilization process and pathogen reduction. Chemical Engineering Journal, 232: 42-50.

This work develops the second objective. Here, a 15-m^3 one-stage thermophilic anaerobic digester (TAnD) was started-up and studied during a 18-months period. The aim of this work was to evaluate the stabilization process of municipal sludge and assess the pathogen standards included in the "Proposal for a Directive of the European Parliament and of the Council on spreading of sludge on land". The conversion strategy from mesophilic to thermophilic conditions was performed by a rapid and single temperature increase. Parameters such as volatile solids destruction (VSD), total volatile fatty acids (VFA), biogas production, macronutrients and heavy metals were measured. *Salmonella* spp., *E. coli*, and *C. perfringens spores* were cultivated, and pathogenity genes *invA* and *cpa* PCR-amplified. The reactor was operated over a range of sludge retention times (SRT) of 28, 20, 18, and 16 days and organic loading rates (OLR) ranging from 1.5 to 2.5 kg VS m^{-3} d^{-1}. Adequate process performance was obtained in all the stable periods reaching VSD values over 40% and an average biogas production of 0.64 Nm^3 kg^{-1} VS^{-1} fed. The TAnD system was capable of operating with higher OLR and more reduced SRT than MAD digestion. Regarding the pathogen content, TAnD digestion successfully reduced *Salmonella* spp. and *E. coli* below detection limits but not *C. perfringens* spores (4.63 $\log_{10}$ spores mL^{-1}). Thus, the final product met Class A biosolids final disposal regulations, but further investigation is needed in order to satisfy the future European legislation.

Chapter V. Lloret E, Salar MJ, Blaya J, Pascual JA, 2013. Two-stage mesophilic anaerobic – thermophilic digestion for sludge sanitation to obtain advanced treated sludge. Chemical Engineering Journal, 230: 59-63.

The present study, although it was not included in the original specific aims of this thesis, is a necessary consequence of the two previous studies, in which fully sanitation of sewage sludge was not achieve either by thermophilic aerobic or anaerobic digestion. In this work, three types of sludge (raw, mesophilic anaerobic, and thermophilic anaerobic) were analysed to evaluate whether the pathogen content limits established in future European legislation were satisfied. To do so, *Salmonella* spp., *E. coli*, and *C. perfringens* spores were cultivated and pathogenity genes *invA* and *cpa* PCR-amplified. Thermophilic anaerobic digestion produced Class A biosolids according to the American legislation (USEPA, 2003) by eliminating *E. coli* and *Salmonella* spp. but did not accomplished the microbial requirements of the future Directive due to the presence of *C. perfringens* spores (9.6×10^4 CFUs mL^{-1}). Hence, the final goal of this work was to propose a two-stage process capable of removing the spores of *C. perfringens* to obtain an advanced treated sludge that could be land-applied with no environmental risks. The first stage of the process suggested in this study involved the mesophilic anaerobic digestion of the sludge while the second stage of operation consisted of a thermophilic digestion (either aerobic or anaerobic). Thus, by triggering in the first stage the germination of *C. perfringens* spores, these newborn vegetative cells could be eradicated or damaged by a subsequent thermophilic phase, obtaining a pathogen free product.

Chapter VI. Lloret E, Pascual JA, Brodie EL, Bouskill NJ, Fernández Delgado-Juárez M, Insam H, Goberna M. Sewage sludge addition modifies soil microbial communities and plant performance depending on the stabilization process. Under review.

This work approaches both the fourth and fifth objectives with the aim of assessing the influence of the stabilization process on sludge microbial community, and the effects of landspreading of two sewage sludges differing in

their stabilization process (a conventional and an advanced treatment) on the soil environment, soil microbial community structure and activity, as well as on plant growth and performance. Here, pyrosequencing of SSU RNAr V9 region showed that sludge stabilization process shaped sludge microbial community structure. On the other hand, advanced ATAD sludge addition produced smaller changes in soil chemistry and soil microbial community structure while enhancing soil microbial activity and plant growth and performance. Land application of conventional MAD sludge, however, had no effects over soil microbial activity although it did enhanced plant growth and performance. Our results suggest that sludge application-derived changes in soil microbial community are due to changes in soil chemical environment rather than to the addition of sludge-borne microorganisms. These results also showed that changes in a single chemical parameter (i.e. changes in EC) may produce a big shift in microbial community structure and activity, as well as in plant growth and physiological state.

From this memory, the following main conclusions can be inferred:

• ATAD digestion resulted in a suitable technology for sludge stabilization, once all the operational parameters were stable with a high organic loading rate (2.7 kg VS m^{-3} d^{-1}) and a short sludge retention time (14.6 days) and achieving a VSD of 38% established in the American legislation for Class A biosolids. Likewise, the ATAD system significantly reduced the content of human pathogens, achieving the complete removal of *Salmonella* spp., *E. coli* and total coliforms producing, therefore, Class A biosolids. However, the reduction of *C. perfringens* spores found after ATAD digestion was not sufficient to meet the microbial standards of the future European Directive for advanced treatments.

• The start-up of a TAnD digester by using a mesophilic inoculum and through a rapid and unique temperature rise, determined a start-up stage of 250 days. This reactor operated at higher OLRs (1.5 to 2.5 kg VS m^{-3} d^{-1}) and shorter SRTs (16 to 28 days) than the MAD system with a similar VSD (>40%) and biogas (0.64 Nm^3 kg^{-1} VS^{-1} fed) and methane production (62%). Similarly to ATAD system, thermophilic anaerobic digestion successfully eradicated *Salmonella* spp., *E. coli* and total coliforms. Nevertheless, *C. perfringens* spores were still present. The

TAnD effluent fulfilled, therefore, the pathogen requirements for the US legislation, but not the forthcoming European regulation.

• This Ph.D. dissertation suggests a 2-stage mesophilic anaerobic–thermophilic sludge digestion (either aerobic or anaerobic) to achieve an utterly sanitized sludge. The first mesophilic stage could also consist of a continuous stirred-tank reactor (CSTR) so that the installation of two digesters would not be necessary.

• Sludge stabilization process (presence/absence of oxygen and temperature range) determined sludge chemical properties as well as shaped sludge microbial community, whose diversity was reduced after thermophilic anaerobic digestion.

• Sludge application altered soil microbial communities in a different manner, depending on the stabilization process. Land spreading of sludge from an advanced treatment such as ATAD produced smaller changes in soil microbiota whilst enhancing soil microbial activity and plant growth and performance compared with a conventional treatment such as MAD. Consequently, and taking into account that the original material was the same, the stabilization process of sewage sludge stands out as a pivotal agent determining the feasibility of the final by-product as an organic amendment, ATAD sludge being an excellent surrogate to inorganic fertilization.

IX

IX. Bibliografía
References

IX. Bibliografía / References

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X

X. Apéndice
Appendix

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Además de los cuatro artículos que constituyen el cuerpo de esta memoria, y no incluidos en esta Tesis Doctoral pero fruto del trabajo de investigación que en ella se desarrolla, se derivan las siguientes publicaciones y/o comunicaciones a congresos:

Publicaciones en revistas científicas (Science Citation Index):

En preparación:

Lloret E, Goberna M, Brodie EL, Insam H, Pascual JA. Sewage sludge addition modifies soil microbial communities and plant performance depending on process temperature.

Lloret E, Pascual JA. Performance of *Fusarium*-inoculated melon plants and soil pathogen content after ATAD and TAnD sewage sludge addition.

Lloret E, Podmirseg S, Brodie EL, Insam H, Pascual JA. Bacterial and archaeal communities in ATAD and TAnD sludge and amended soils with emphasis on the ammonia-oxidizing population.

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Lloret E, Goberna M, Blaya J, Bouskill NJ, Karaoz U, Pascual JA, Brodie EL. Autothermal thermophilic sludge addition modifies soil bacterial communities and promotes plant development. 14th International Symposium on Microbial Ecology (ISME14). Copenhagen, Dinamarca, 2012. (Póster).

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