Science of the Total Environment 789 (2021) 147869

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Insights into the removal of pharmaceutically active compounds from sewage sludge by two-stage mesophilic anaerobic digestion

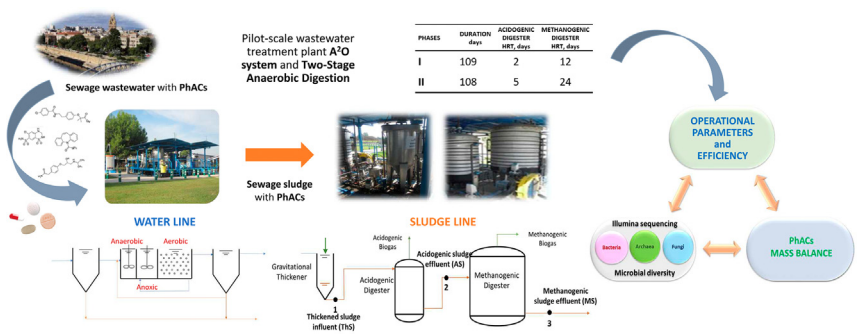
M.J. Gallardo-Altamirano [a,b], P. Maza-Márquez [a,c], N. Montemurro [d], S. Pérez [d], B. Rodelas [a,c,*], F. Osorio [a,b], C. Pozo [a,c]

[a] Environmental Microbiology Group, Institute of Water Research, University of Granada, Granada, Spain
[b] Department of Civil Engineering, University of Granada, Granada, Spain
[c] Department of Microbiology, University of Granada, Granada, Spain
[d] Water, Environmental and Food Chemistry (ENFOCHEM), Institute of Environmental Assessment and Water Research (IDAEA-CSIC), Barcelona, Spain

HIGHLIGHTS

- The REs of 27 PhACs in a two-stage MAD plant treating sewage sludge are reported.
- An SRT ≤ 2 d in the AcD was required to separate acidogenic and methanogenic stages.
- Longer SRT in the MD stage (24 d) correlated with an improved removal of six PhACs.
- SRT, ORL and VFA/ALK influenced the prokaryotic communities' structure in both stages.
- Both MAD performance and REs of PhACs were linked to shifts of community structure.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 30 October 2020
Received in revised form 22 March 2021
Accepted 14 May 2021
Available online 21 May 2021

Editor: Paola Verlicchi

Keywords:
Two-stage mesophilic anaerobic digestion
Sewage sludge
Pharmaceutically active compounds
Biogas
Microbial diversity

ABSTRACT

The removal efficiencies (REs) of twenty-seven pharmaceutically active compounds (PhACs) (eight analgesic/anti-inflammatories, six antibiotics, four β-blockers, two antihypertensives/diuretics, three lipid regulators and four psychiatric drugs) were evaluated in a pilot-scale two-stage mesophilic anaerobic digestion (MAD) system treating thickened sewage sludge from a pilot-scale A^2O™ wastewater treatment plant (WWTP) which was fed with wastewater from the pre-treatment of the full-scale WWTP Murcia Este (Murcia, Spain). The MAD system was long-term operated using two different sets of sludge retention times (SRTs) for the acidogenic (AcD) and methanogenic (MD) digesters (phase I, 2 and 12 days; and phase II, 5 and 24 days, in AcD and MD, respectively). Quantitative PCR (qPCR) and Illumina MiSeq sequencing were used to estimate the absolute abundance of Bacteria, Archaea, and Fungi and investigate the structure, diversity and population dynamics of their communities in the AcD and MD effluents. The extension of the SRT from 12 (phase I) to 24 days (phase II) in the MD was significantly linked with an improved removal of carbamazepine, clarithromycin, codeine, gemfibrozil, ibuprofen, lorazepam, and propranolol. The absolute abundances of total Bacteria and Archaea were higher in the MD regardless of the phase, while the diversity of bacterial and archaeal communities was lower in phase II, in both digesters. Non-metric multidimensional scaling (MDS) plots showed strong negative correlations among phyla *Proteobacteria* and *Firmicutes* and between genera *Methanosaeta* and *Methanosarcina* throughout the full experimental period. Strong positive correlations were revealed between the relative abundances of *Methanospirillum* and *Methanoculleus* and the methanogenesis performance parameters (volatile solids removal, CH_4 recovery rate

* Corresponding author at: Environmental Microbiology Group, Department of Microbiology, University of Granada, 18071 Granada, Spain.
E-mail address: mrodelas@ugr.es (B. Rodelas).

https://doi.org/10.1016/j.scitotenv.2021.147869
0048-9697/© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

and %CH_4 in the biogas), which were also related to longer SRT. The REs of several PhACs (naproxen, ketoprofen, ofloxacin, fenofibrate, trimethoprim, and atenolol) correlated positively ($r > 0.75$) with the relative abundances of specific bacterial and archaeal groups, suggesting their participation in biodegradation/biotransformation pathways.

© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Urban wastewater is the main sink of pharmaceutically active compounds (PhACs) after their consumption by human populations. During urban wastewater treatment, some of these substances are efficiently removed from raw wastewater, but most of them remain in the treated water, sewage sludge, or both (Angeles et al., 2020; Jelić et al., 2012; Martín et al., 2012; Radjenović et al., 2009; Tran et al., 2018; Valdez-Carrillo et al., 2020; Yan et al., 2014).

The development of analytical techniques for the extraction, clean-up and quantification of PhACs from environmental samples has contributed to increase the knowledge about the occurrence and fate of these substances not only in the dissolved phase of raw and treated wastewater, but also in sewage and treated sludge (Gago-Ferrero et al., 2015; Martín-Pozo et al., 2019; Paíga et al., 2019; Pérez-Lemus and López-Serna, 2019). For a given PhAC, the concentrations detected in sewage sludge usually range from tens to several hundred μg kg^{-1} dry weight (dw), and are often related to those measured in the raw wastewater; however, other factors influence the amounts occurring in this complex matrix, such as the physical-chemical properties of each particular compound, their resistance to biodegradation, or the sludge characteristics and operating parameters of each wastewater treatment plant (WWTP) (Carballa et al., 2008; Dubey et al., 2021; Radjenović et al., 2009; Samaras et al., 2014; Stasinakis, 2012).

Anaerobic digestion (AD) is one of the most effective methods for sewage sludge stabilization, as well as an efficient and environmentally friendly technology for bio-natural gas production (Tezel et al., 2011; Xu et al., 2020; Zhang et al., 2016). In particular, the two-stage AD configuration, which relies on the separation of the hydrolysis/acidification and acetogenesis/methanogenesis processes in two different bioreactors, in order to provide optimal conditions for the main microbial groups involved in each of these processes (acid-producing bacteria and methanogenic archaea, respectively), offers some advantages for the stabilization of sewage sludge compared to the single-stage AD configuration, such as improved biogas production, pH self-adjusting capacity, reduction of pathogens and chemical oxygen demand (COD) concentrations, ability to work at shorter sludge retention times (SRT), and improved microbiota stabilization, among others (Maspolim et al., 2015a; Wang et al., 2018).

In Europe, almost half of the sludge generated in WWTPs and stabilized by AD processes is used in agriculture, either directly or after composting (Kelessidis and Stasinakis, 2012). The entry of PhACs into the environment through the application of stabilized sludge as soil fertilizer/conditioner implies a clear risk of contamination not only for environmental compartments (soil and water), but also for human health, particularly due to the spread and maintenance of antibiotic-resistant genes (ARG) and antibiotic resistant bacteria (aus der Beek et al., 2016; Bisognin et al., 2020; Urra et al., 2019; Wu et al., 2016). However, only few countries of the EU set in their national legislation limit values of concentrations for some target compounds in stabilized sludge as a requisite for land spreading, and to date no PhACs have been included among these (Christodoulou and Stamatelatou, 2016; Martín-Pozo et al., 2019).

The removal of PhACs such as non-steroidal anti-inflammatory drugs, antibiotics, or the antiepileptic drug carbamazepine from sewage sludge in laboratory or full-scale AD systems have been previously reported (Carballa et al., 2007; Dubey et al., 2021; Martín et al., 2015; Narumiya et al., 2013; Samaras et al., 2014, 2013; Stasinakis, 2012; Zhou et al., 2018, 2017, 2015). At the same time, several investigations based on molecular methods have been carried out to provide an insight of the community structure and populations' diversity of the microbiota involved in the processes (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) that are accomplished in sewage sludge AD working under several configurations and operation conditions (Hao et al., 2016; Ma et al., 2019; Maspolim et al., 2015a). Nevertheless, no clear conclusions can be drawn regarding the ranges of PhACs removal efficiencies and the way these are influenced by operation parameters, since the results widely vary among the different studies, and reports providing links between microbial diversity shifts and removal efficiencies are limited, particularly for the two-stage AD systems.

The aim of the present work was twofold: i) to study the occurrence and fate of twenty-seven PhACs belonging to six therapeutic groups (antibiotics, anti-inflammatory/analgesic compounds, β-blockers, anti-hypertensives/diuretics, lipid regulators, and psychiatric drugs) in sludge sewage samples undergoing two-stage mesophilic AD (MAD) treatment, in order to ascertain its effectiveness to remove these emerging contaminants, and ii) to characterize the microbial populations present in the sludge samples under the acidogenic and methanogenic conditions, to gain insight into their community structure and diversity, as well as to show by statistical tools the existing relationships between the shifts of the microbial populations' diversity, the two-stage MAD performance, and the removal efficiency of PhACs.

2. Materials and methods

2.1. Description of the pilot-scale two-stage MAD plant and operational conditions

The two-stage MAD plant consisted of two stainless-steel cylindrical digesters (acidogenic and methanogenic, connected in series) with a volume of 640 L and 2340 L, respectively, and operated under mesophilic conditions at different SRTs (Fig. S1). The external surface of the digesters was wrapped with a thermal jacket to maintain constant mesophilic conditions (35 ± 0.5 °C) and a recirculation pump was operated in each digester to maintain the homogeneity of the sludge. A drum-type gas flow meter (TG3 plastic, Ritter Company) and a pH meter (GRLP 22, Crison) were installed to monitor these variables in the digesters.

The two-stage MAD plant was designed for processing the primary sludge (PS) and waste activated sludge (WAS) generated in a pilot-scale anaerobic-anoxic-aerobic (A^2O) system (Fig. S2) installed at the facilities of WWTP *Murcia Este* (EMUASA, Murcia, Spain), which was fed with wastewater from the pre-treatment of the full-scale WWTP. The operation parameters of the A^2O bioreactor and the characteristics of the activated sludge have been previously described in full detail by Gallardo-Altamirano et al. (2018). Both the A^2O and the two-stage MAD were equipped with a supervisory control and data acquisition (SCADA) system to control the operational parameters.

2.2. Inoculation and experimental set-up of the two-stage MAD plant

Digested sludge (200 L) from WWTP *Murcia Este* (EMUASA, Murcia, Spain) was used as biomass inoculum for acidogenic and methanogenic digesters of the two-stage MAD plant. The primary and secondary

sludge generated in the water treatment line of the A^2O system were mixed and thickened in a gravitational thickener (216 L volume, 1.45 m high and 0.5 m diameter, Fig. S2) and then used as feeding for the two-stage MAD plant. Since June 1st, 2016, the A^2O system was working under steady-state conditions, producing a regularly thickened sludge (ThS) at a flow of 41 ± 9 L d^{-1}. Thereby, the acidogenic and methanogenic digesters (AcD and MD, respectively) started up operating at the SRTs selected for phase I (2 days for the AcD and 12 days for the MD) and at a constant organic loading rate (OLR) (when the water line was producing a regularly thickened sludge (41 ± 9 L d^{-1}) with a regularly volatile solid flow (VS flow = 869 ± 150 g d^{-1}, Table 1B), the AcD and MD started up operating at a constant OLR of 11.1 ± 2.1 kg VS m^{-3} d^{-1} and 1.6 ± 0.4 kg VS m^{-3} d^{-1}, respectively). Both digesters were operated until steady-state conditions were reached, before experimental phase I started, which lasted 104 days (July 27th–November 13th, 2016). Then, operating conditions for the experimental phase II were fixed at SRTs of 5 and 24 days for AcD and MD, respectively. Phase II lasted 105 days (December 14th, 2016–March 31st, 2017).

2.3. Physicochemical parameters and monitoring of the two-stage MAD plant performance

Temperature, pH, and biogas production were measured daily in each digester during both experimental phases. Biogas composition was characterized with a gas analyzer (Siemens Ultramat 23) and was verified once per month with a portable gas analyzer (Geotechnical Instrument Ltd., GA5000).

Sludge samples were taken three times a week from the sampling points 1, 2, and 3 (Fig. S2) in order to evaluate the two-stage MAD plant performance. The parameters analyzed were: total solids (TS), volatile solids (VS), volatile fatty acids (VFA), and alkalinity (ALK). TS and VS were measured according to Standard Methods (Baird et al., 2017); volatile solids removal (VSR) in each digester (AcD or MD) was calculated as follows: %VSR = [(VS influent − VS effluent) / VS influent] × 100 (Bhattacharya et al., 1996); and volatile fatty acids (VFA) and alkalinity (ALK) concentrations were measured by titration, according to the method proposed by DiLallo and Albertson (1961). Table 1A and B summarizes the operational conditions of the digesters and physicochemical parameters analyzed in each experimental phase, respectively.

2.4. Sample collection for PhACs analysis

A total of 27 sludge samples were collected for PhACs analysis from sample points 1, 2 and 3 (Fig. S2): 12 samples in phase I and 15 samples in phase II. From each point, integrated samples (6 L) were taken throughout the day and thoroughly mixed before retrieving a 500 mL homogeneous sample for the PhACs analyses. Samples were collected both midweek and at the end of the weekend, stored in amber PET bottles, and frozen at −20 °C until their analysis. All samples were collected under dry weather conditions to eliminate sample dilution effect.

2.5. Analytical methods for PhACs

2.5.1. Chemicals, reagents and solutions

High-purity (mostly 90%) analytical reference standards (acetaminophen, atenolol, bezafibrate, carbamazepine, clarithromycin, codeine, diazepam, diclofenac, fenofibrate, furosemide, gemfibrozil, hydrochlorothiazide, ibuprofen, indomethacin, ketoprofen, lorazepam, metoprolol, naproxen, ofloxacin, paroxetine, propanolol, propyphenazone, sotalol, sulfadiazine, sulfamethazine, sulfamethoxazole, trimethoprim)

Table 1
A. Operational conditions and performance of the acidogenic and methanogenic digesters (AcD and MD, respectively) and the global two-stage mesophilic anaerobic digestion (MAD) process, in the experimental phases I and II. B. Characterization of the thickened sludge (ThS), acidogenic digester (AcD) effluent, and methanogenic digester (MD) effluent in the experimental phases I and II. Values shown are means ± SD. Data marked with an asterisk (*) were significantly different between the two experimental phases (I and II) in each digestion stage (AcD, MD, or global two-stage MAD), according to the Kruskal-Wallis test ($p < 0.05$). TS: total solids; VS: volatile solids. Volumes of biogas were converted to standard conditions.

A.

Parameter		AcD		MD		Global two-stage MAD	
		Phase I	Phase II	Phase I	Phase II	Phase I	Phase II
SRT, d		2.0 ± 0.6	5.2 ± 1.1	12.3 ± 1.4	24.8 ± 1.4	14	29
T, °C		35 ± 0.6	35 ± 0.7	35 ± 0.5	35 ± 0.5	35	35
pH		6.7 ± 0.1*	7.1 ± 0.2*	7.3 ± 0.2*	8.0 ± 0.2*	–	–
OLR, kg VS m^{-3} d^{-1}		11.1 ± 2.1*	4.6 ± 0.6*	1.6 ± 0.4*	0.8 ± 0.4*	1.5 ± 0.3*	0.9 ± 0.3*
% PS, %TS influent		67 ± 15*	57 ± 16*	–	–	67 ± 15*	57 ± 16*
% WAS, % TS influent		33 ± 15*	43 ± 16*	–	–	33 ± 15*	43 ± 16*
VSR, %		9.6 ± 3.9*	25.5 ± 9.6*	25.7 ± 9.0*	33.7 ± 14*	32.8 ± 8.9*	50.5 ± 14.6*
Biogas production, L d^{-1}		71 ± 19*	224 ± 81*	188 ± 38	211 ± 63	258 ± 44*	434 ± 114*
Biogas composition	% CH_4	48.2 ± 7.6*	59.5 ± 3.3*	65.6 ± 2.6*	54.9 ± 10*	–	–
	% CO_2	40.6 ± 6.4*	39.1 ± 3.0*	31.5 ± 1.8	32.5 ± 2.3	–	–
CH_4 production, L d^{-1}		34 ± 10*	131 ± 46*	122 ± 24	119 ± 40	154 ± 28*	247 ± 67*
Biogas production rate, L L^{-1} d^{-1}		0.86 ± 0.23*	1.10 ± 0.40*	0.38 ± 0.09*	0.26 ± 0.10*	0.45 ± 0.09	0.41 ± 0.12
Biogas recovery rate, m^{-3} kg VS $removed^{-1}$		0.788 ± 0.111*	0.819 ± 0.007*	0.909 ± 0.075*	0. 871 ± 0.013*	0.873 ± 0.056	0.858 ± 0.021
CH_4 recovery rate, m^3 kg VS $removed^{-1}$		0.380 ± 0.079*	0.487 ± 0.016*	0. 596 ± 0.047*	0.483 ± 0.043*	0.520 ± 0.051*	0.483 ± 0.042*
Biogas yield, m^3 kg VS fed^{-1}		0.084 ± 0.021*	0.214 ± 0.056*	0. 241 ± 0.049*	0.297 ± 0.067*	0.337 ± 0.053*	0.433 ± 0.066*
CH_4 yield, m^3 kg VS fed^{-1}		0.041 ± 0.010*	0.126 ± 0.032*	0.157 ± 0.031*	0.167 ± 0.043*	0.178 ± 0.030*	0.246 ± 0.042*
VFA effluent, mg CH_3COOH L^{-1}		795 ± 243*	565 ± 168*	257 ± 140*	157 ± 50*	–	–
ALK effluent, mg $CaCO_3$ L^{-1}		2053 ± 321*	2826 ± 377*	2883 ± 101*	3602 ± 329*	–	–
VFA/ALK		0.39 ± 0.11*	0.20 ± 0.06*	0.08 ± 0.04*	0.04 ± 0.01*	–	–

B.

Phase	Phase I			Phase II		
Sludge sampling point	ThS	AcD	MD	ThS	AcD	MD
TS flow, g d^{-1}	1195 ± 213	1111 ± 187	895 ± 185*	1261 ± 187	1010 ± 224	714 ± 168*
VS flow, g d^{-1}	869 ± 150	780 ± 131	581 ± 118*	949 ± 131	680 ± 145	680 ± 131*
% TS	2.96 ± 0.74*	2.72 ± 0.57	2.20 ± 0.32	2.88 ± 0.59*	2.39 ± 0.37	1.95 ± 0.34
% VS/TS	72 ± 2.3*	69.8 ± 1.5	65.3 ± 1.7	74.6 ± 3.7*	67.2 ± 3.0	61.5 ± 3.1

were acquired from Sigma Aldrich (St. Luis, MO, US). Isotope-labelled compounds (acetaminophen-d4, bezafibrate-d4, carbamazepine-d10, codeine-d3, diazepam-d5, diclofenac-d4, furosemide-d5, fenofibrate-d6, gemfibrozil-d6, ketoprofen-d3, indomethacin-d4, lorazepam-d4, ofloxacine-d3, metoprolol-d7, naproxen-d3, paroxetine-d4, propranolol-d7, sotalol-d6, sulfamethoxazole-d4, sulfadiazine-d4, sulfamethazine-d4) were purchased from Cerilliant, Alsachim (Illkirch-Graffenstaden, France) or Santa Cruz Biotechnology (Dallas, TX, USA). CAS numbers, molecular formulas, molecular weight, and other relevant properties of all target compounds are reported in Gallardo-Altamirano et al. (2019).

LC-MS grade acetonitrile (ACN) (≥99.9%), methanol (MeOH) (≥99.9%), ethyl acetate (EtAc) (≥99.9%), dimethyl sulfoxide (DMSO) (≥99.9%), and HPLC water were purchased from Merck (Darmstadt, Germany). Formic acid (≥96%, ACS reagent) and ammonium acetate were supplied by Sigma-Aldrich.

Individual stock standard solutions (concentration of 1000 μg mL^{-1}) were prepared in either 100% methanol or 100% DMSO, depending on the solubility of each compound. Working solution mixtures containing all the aforementioned analytes and the isotopically labelled compounds (1 μg mL^{-1}), for analysis and calibration purposes, were prepared by diluting adequate volumes of the individual stock solutions with MeOH. All the solutions were stored at −20 °C.

2.5.2. Sample preparation

The extraction and analysis of sludge samples were based on methods previously published by Jelić et al. (2009) and Gago-Ferrero et al. (2015) respectively, with some modifications. Briefly, about 500 mL of sewage sludge were lyophilized for about 10 days until constant weight. Then, 0.5 g freeze-dried and sieved sludge was placed in a 50-mL falcon tube and spiked with 50 ng of the corresponding surrogate mixture, and kept in contact overnight into a fume hood. Five mL of extraction solvent (MeOH–HPLC water 1:2 (v/v)) were added and the tube was vortexed for 1 min. The sample was then extracted by ultrasonic bath for 10 min. After the first extraction, the tube was centrifuged for 10 min (4000 rpm, 4 °C) and the supernatant was collected in a 16-mL glass test tube. This procedure was repeated two more times but the last time, 5 mL of 0.1% formic acid in MeOH/water 1:1 (v/v) were used as extraction solvent. In total about 15 mL of supernatant were collected. Then, the extract was evaporated to less than 10 mL under a gentle steam of nitrogen at 24 °C using a TurboVap® LV (Biotage AB, Uppsala, Sweden) to reduce the amount of methanol for the following SPE step. The extract was diluted in 100 mL of HPLC water to ensure a solvent concentration less than 1%. The whole volume of the aqueous solutions was filtered through a 0.45-μm pore size nylon membrane, 47 mm diameter, and transferred to the SPE procedure using Oasis® HLB cartridges (60 mg, 3 mL, Waters Corporation — Milford, MA, USA). For the SPE, the cartridges were pre-conditioned with 5 mL of EtAc and 5 mL of MeOH, and equilibrated with 5 mL of ultrapure water, and afterwards the sample was loaded onto the cartridges at a flow rate of 1 mL min^{-1}. A washing step was conducted with 2 × 3 mL of ultrapure water and, before elution, the cartridges were dried under vacuum aspiration for 30 min. Analytes were eluted with 3 × 3 mL of EtAc:MeOH (1:1, v/v). The extract was evaporated to dryness under a gentle stream of nitrogen and reconstituted with 1 mL of H_2O/MeOH (90:10) and then filtered to 2-mL vials using 0.22 μm polytetrafluoroethylene (PTFE) syringe filters for UPLC-MS/MS analysis.

2.5.3. Liquid-chromatography (LC) separation

LC separation was performed using a SCIEX ExionLC™ AD system (Sciex, Redwood City, CA, U.S.). Chromatographic separation was achieved on a Hibar® HR Purospher® STAR RP-C18 column (100 mm × 2.1 mm i.d., 2 μm particle size, Merck, Darmstadt, Germany), maintained at 40 °C in the column oven. The mobile phase for the positive electrospray ionization consisted of 5 mM ammonium acetate and 0.1% of formic acid in water (A) and ACN (B), while for the negative electrospray ionization it consisted of 10 mM ammonium acetate in water (A) and MeOH:ACN (1:1, v/v) (B). The total run time for each injection was 24 min for positive and 18 min for negative acquisition with a flow rate of 0.4 mL min^{-1}. The injection volume was 5 μL, and the auto-sampler temperature was maintained at 8 °C. The elution gradient for ESI(+) mode started with 5% of B for 30 s, then increasing to 98% in 16 min, held at 98% for 3 min, and finally returning to the initial conditions in the next 20 s. The necessary time for the re-equilibration of the analytical column was 5 min. The elution gradient for ESI(−) started with 5% B. the organic phase was then increased to 95% in 12 min and kept constant for 1 min, before a return to the initial conditions over the next 30 s.

2.5.4. Mass spectrometry (MS) and MS/MS conditions

The SCIEX X500R QTOF system (Sciex, Redwood City, CA, U.S.) with Turbo V™ source and Electrospray Ionization (ESI) and operating in negative/positive polarity was used for the detection of the compounds of interest. Any drift in the mass accuracy of the SCIEX Q-TOF was automatically corrected and maintained throughout batch acquisition by infusion of reserpine reference standard ($C_{33}H_{40}N_2O_9$, *m/z* 609.28066) for positive ionization, and a cluster of trifluoroacetic acid (5(TFA-Na) + TFA-, *m/z* 792.85963) for negative mode. Calibration was running every 5 samples during the batch acquisition making use of the Calibrant Delivery System (CDS).

High resolution data were acquired using a multiple reaction monitoring (MRM^{HR}) workflow consisting of a TOF-MS survey (100–850 Da for 80 ms of Accumulation Time (AT); Declustering Potential (DP) and Collision Energy (CE) were set to 80 V and 10 V and −80 V and −10 V, for positive and negative, respectively. The MRM^{HR} scanning mode was used for accurate quantification of product ion transitions. The Guided MRM^{HR} tool from SCIEX was used for the optimization of high-resolution transitions (Tables S1 and S2A). The optimized ionization mode, fragmentation voltages, and collision energies for each compound have been supplied as Supplementary material (Tables S1, S2A; Figs. S3, S4, S5 and S6). The source conditions for the system were optimized as follow. Ion Spray Voltage was set to 5500 V (−4500 V for negative); source temperature and nitrogen gas flows (Atomizing gas, GS1 and Auxiliary gas, GS2) were set to 550 °C, 50, and 70 psi, respectively. Curtain gas was set to 35 psi.

2.5.5. Method performance

Qualitative and quantitative analyses were performed using SCIEX OS™ Software version 1.6 (Sciex, Redwood City, CA, U.S.). Two high resolution product ions were used for each compound, the most abundant for the quantification and the second most abundant for the confirmation. Linearity of the method was evaluated using the internal standard calibration approach with a calibration curve constructed between 0.05 and 300 ng mL^{-1} corresponding to 0.1–600 ng g^{-1} d.w. Calibration curves were constructed considering a minimum of eight calibration points using linear weighted least-squares regression (1/x as weighting factor) by plotting the ratio of the analyte signal with its corresponding IS and presenting coefficients of determination (R^2) above 0.99 for most compounds. Regarding sensitivity, Method Detection Limits (MDLs) and Method Quantification Limits (MQLs) were estimated from the calibration curves using linear regression analysis and a signal-to-noise ratio of 3.3 and 10, respectively. All information on method performance parameters including recoveries % and method precision (RSD%) (obtained in sludge spiked at concentration of 100 ng g^{-1}), MDLs and MQLs, and R^2, is reported in Table S2B.

2.6. DNA extraction, qPCR, massive parallel sequencing, and data analysis

Environmental DNA was extracted from sludge samples (4 mL) following already described protocols (Gallardo-Altamirano et al., 2018). The absolute abundances of total Bacteria, Archaea, and Fungi were quantified in each of the samples by real-time quantitative PCR

(qPCR), using a QuantStudio-3 Real Time PCR system (Applied Biosystems). The Domain and Phylum-level universal primer pairs used, quantitative amplification reactions and cycling conditions were already described elsewhere (Gallardo-Altamirano et al., 2018).

High-throughput sequencing of the small-subunit rRNA genes of the mixed microbial populations was achieved using the Illumina MiSeq platform (Illumina, Hayward, CA, USA) at the facilities of RTL Genomics (Lubbock, TX, USA, http://www.researchandtesting.com). The primer pairs 28F-519R (Fan et al., 2012) and 517F/909R (Maspolim et al., 2015a) were used to amplify the 16S rRNA genes of Bacteria and Archaea, respectively, while the 18S rRNA gene of Fungi was amplified using the FungiQuantF/FungiQuantR primer set (Liu et al., 2012).

Raw sequencing data were processed using the QIIME software, v. 1.9.1, following the pipeline protocol described by Caporaso et al. (2012). The sequences were filtered, and taxonomic assignments were made as described in full detail by Gallardo-Altamirano et al. (2019). Alpha-diversity indices (Chao-1, Shannon-Wiener H', and Simpson) were calculated using PAST v. 3.25 (Hammer et al., 2001). The 'iNEXT' free online tool (https://chao.shinyapps.io/iNEXTOnline, Hsieh et al., 2016) was used to construct sample-size-based rarefaction and extrapolation (R/E) sampling curves with 95% confidence intervals. Heatmaps displaying the relative abundances of Bacteria, Archaea, and Fungi in the samples were generated using Microsoft Excel.

The Primer software (PRIMER-E v. 6.1.18, Plymouth, UK) was used to construct an UPGMA dendrogram with 95% confidence intervals, and to estimate the contribution of individual PHYs to the dis(similarity) between groups of samples, as already described by Maza-Márquez et al. (2016), by using the SIMPROF and SIMPER commands, respectively.

2.7. Statistical analyses

The Kruskal-Wallis non-parametric test was selected to find significant differences among the different groups of samples in the analyzed data sets, using a 95% significance level ($p < 0.05$). When required, Spearman's rank correlation coefficients (ρ) among the different variables were calculated. All these procedures were done using IBM SPSS Statistics v. 19 (SPSS Inc., IBM, USA). The multivariate statistical analyses (Non-metric multidimensional scaling, MDS; and analysis of similarity, ANOSIM) were performed using the Primer software PRIMER-E v. 6.1.18, Plymouth, UK). Vectors representing the trends through the MDS ordinations of either biotic (absolute or relative abundances of bacterial populations) or abiotic variables (operational parameters or indicators of methanogenesis efficiency) were generated by multiple partial correlation algorithms and represented over the plots. All the aforementioned procedures were described in full detail elsewhere (Maza-Márquez et al., 2016).

3. Results and discussion

3.1. Performance of the pilot-scale two-stage (MAD) plant

Table 1A shows the operational conditions and performance of the AcD and MD of the two-stage MAD plant during the two experimental phases of the study. The characterization of the sludge samples (ThS, AcD effluent, and MD effluent) is summarized in Table 1B.

The two digesters were operated under mesophilic conditions (35 °C) in both phases I and II (Table 1A), with similar influent mass flow (1195 ± 213 and 1261 ± 187 g TS d^{-1} ThS, respectively, Table 1B). However, the ThS had significantly different characteristics in phases I and II, regarding the % TS and % VS/TS ratio (2.96 ± 0.74 vs. 2.88 ± 0.59 and 72.0 ± 2.3 vs. 74.6 ± 3.7%, respectively, Table 1B) and the contribution of primary sludge (PS) and waste sludge (WAS) (67 ± 15 vs. 57 ± 16% and 33 ± 15 vs. 43 ± 16%, respectively, Table 1A). The observed differences could be related to the fact that the water treatment line (A^2O bioreactor) worked under different operational conditions in each experimental phase, particularly with regard to the mixed liquor suspended solids (MLSS) concentration, food-to-microorganism (F/M) ratio, and OLR, as previously reported by Gallardo-Altamirano et al. (2018).

In both experimental phases, the average % TS in the ThS (Table 1B) were lower than those usually measured in mixed ThS in full-scale WWTPs (Appels et al., 2008; Metcalf and Eddy, 2003; Ponsá et al., 2008). Thereby, the global OLR applied to the two-stage MAD plant in both experimental phases (1.5 and 0.9 kg VS m^{-3} d^{-1} in phase I and II, respectively, Table 1A) was low compared with a typical high-rate wastewater sludge AD (1.6–4.8 kg VS m^{-3} d^{-1}, Appels et al., 2008).

The values of the performance parameters VSR, biogas production, biogas composition, biogas recovery rate, biogas production rate, biogas yield, VFA, and ALK recorded in the AcD and MD were statistically different between phases I and II (Table 1A). Lower VSR and biogas production values were detected in phase I in both digesters, possibly in relation with the shorter SRTs selected (2 days and 12 days for the acidogenic and methanogenic digesters, respectively), while in the MD, the biogas and methane recovery rates and the biogas production rate were greater in phase I than in phase II.

In phase II, the AcD (operating at SRT = 5 days) consumed a higher amount of biodegradable substrates, resulting in higher VSR (25.5%), biogas production (224 L d^{-1}) and biogas production rate (1.10 L L^{-1} sludge d^{-1}), and lower VFA concentration (565 mg CH_3COOH L^{-1}), compared to the values obtained in the same digester in phase I (Table 1A). In both experimental phases, the global performance of the mesophilic two-stage MAD plant was in the range of values reported for other similar processes applied to sewage sludge in the earlier literature (Martín-Pascual et al., 2017; Maspolim et al., 2015a; Ponsá et al., 2008; Smith et al., 2017).

The separation of acidogenic and methanogenic microorganisms in the two different digesters was only effective in phase I, while in phase II both digesters yielded similar values of % CH_4 in the biogas and CH_4 production (Table 1A). In phase II, lower VFA/ALK ratio and higher pH were measured in the AcD (Table 1A), compared with data of previous studies in two-stage systems (Ghosh, 1987; Martín-Pascual et al., 2017; Maspolim et al., 2015b; Ponsá et al., 2008). Thereby, our results show that an SRT shorter than 5 days is required for an optimal separation between the acidogenic and methanogenic stages when operating at low OLR. In this sense, Ponsá et al. (2008) optimized the AcD stage of a thermophilic (55 °C) sludge digestion process, concluding that the critical step was the shortening of the SRT from 5 to 4 days, in order to digest a mixed ThS with high concentration of solids (%TS > 4), while the optimal SRT was 1 day for sludge with lower concentration of solids (%TS < 3). Ghosh (1987) reported that the hydrolysis and acidification of the sewage sludge were more efficient at pH 6 and using a SRT of 2 days in the AcD of a mesophilic two-stage digestor. Martín-Pascual et al. (2017), working with digesters of different volumes (20 and 60 L), obtained maximum methane production in the MD of a two-stage system when the SRT of the AcD was 2.18 days, reaching the maximum value of VFA and complete separation of the stages with the lowest digester volume. Otherwise, Maspolim et al. (2015b) operated 3 mesophilic AcDs at HRTs of 5, 3 and 2 days, achieving the highest VFA production at the longest SRT, although reaching the same % CH_4 in the biogas (48%) in the three AcDs when working at a constant pH value of 5.5.

3.2. Occurrence of PhACs in sludge samples of the two-stage MAD plant

Table 2 displays the mean concentration, standard deviation, and detection frequency of 27 targeted PhACs in the ThS, AcD effluent and MD effluent samples of the two-stage MAD plant during the experimental phases I and II.

The detection frequency value in the ThS samples was 100% for 19 and 17 compounds in phase I and II, respectively (Table 2). Among them, 13 compounds (atenolol, carbamazepine, codeine, diclofenac, fenofibrate, gemfibrozil, ibuprofen, ketoprofen, lorazepam, naproxen,

Table 2
Concentration (ng g^{-1} dry solid) of PhACs in the thickened sludge (ThS), acidogenic digester (AcD) effluent, and methanogenic digester (MD) effluent in the experimental phases I (n = 4) and II (n = 5). Values shown are means ± SD; detection frequencies of each compound in the sludge samples are shown in brackets. Data marked with an asterisk (*) were significantly different between the two experimental phases (I and II) in each type of sample (ThS, AcD, or MD), according to the Kruskal-Wallis test ($p < 0.05$). BLD: below detection limit.

Therapeutic groups		ThS		AcD		MD	
		Phase I	Phase II	Phase I	Phase II	Phase I	Phase II
AIADs	Acetaminophen	111 ± 222 (25)	68 ± 114 (100)	12 ± 25 (25)*	238 ± 203 (100)*	0.0 ± 0.0 (0)	5.9 ± 0.0 (40)
	Codeine	20 ± 14 (100)*	98 ± 93 (100)*	14 ± 17 (50)*	88 ± 90 (100)*	6.6 ± 6.3 (75)	10 ± 0.9 (60)
	Diclofenac	134 ± 48 (100)	166 ± 141 (100)	401 ± 475 (100)	212 ± 190 (100)	236 ± 166 (100)	214 ± 212 (100)
	Ibuprofen	137 ± 254 (100)	1280 ± 1454 (100)	184 ± 267 (100)*	1125 ± 757 (100)*	433 ± 213 (100)*	1595 ± 14,029 (100)*
	Indomethacin	2.3 ± 1 (100)	3.9 ± 3.7 (80)	2.2 ± 1.0 (100)	3.6 ± 3.6 (80)	2.2 ± 1.1 (100)	2.5 ± 0.0 (40)
	Ketoprofen	28 ± 36 (100)	165 ± 161 (100)	24 ± 30 (100)*	105 ± 71 (100)*	27 ± 42 (75)	103 ± 65 (100)
	Naproxen	57 ± 60 (100)	507 ± 657 (100)	11 ± 19 (100)	65 ± 60 (100)	4.2 ± 8.3 (25)	17 ± 2.6 (100)
	Propyphenazone	12 ± 18 (75)	1.0 ± 1.2 (60)	4.7 ± 4 (75)	0.2 ± 0.5 (60)	11 ± 12 (100)*	0.7 ± 0.0 (60)*
Antibiotics	Clarithromycin	34 ± 32 (100)	10 ± 9.2 (80)	9.1 ± 9 (75)	5.6 ± 8.5 (40)	5.7 ± 4.2 (75)	1.1 ± 0.0 (40)
	Ofloxacin	4922 ± 4843 (100)	475 ± 377 (100)	32,637 ± 59,608 (100)	329 ± 299 (100)	3324 ± 3336 (100)	265 ± 413 (100)
	Sulfadiazine	31 ± 38 (50)	BLD	7.1 ± 9 (50)	BLD	6.7 ± 12 (75)	2.1 ± 0.0 (20)
	Sulfamethazine	30 ± 35 (50)	0.6 ± 1.2 (20)	1.1 ± 2 (50)	1.0 ± 2.3 (20)	0.6 ± 0.7 (50)	0.0 ± 0.0 (0)
	Sulfamethoxazole	55 ± 41 (100)	22.2 ± 30 (60)	19 ± 19 (75)*	0.0 ± 0.0 (0)*	8.6 ± 10 (50)	0.0 ± 0.0 (0)
	Trimethoprim	19 ± 12 (100)	27.9 ± 7.8 (100)	2.0 ± 2 (75)*	6.6 ± 2.8 (100)*	0.4 ± 0.5 (50)	1.5 ± 0.0 (40)
Beta-blockers	Atenolol	24 ± 3 (100)	49 ± 54 (100)	17 ± 7 (100)	25 ± 21 (100)	7.1 ± 8.9 (50)	4.8 ± 0.1 (80)
	Metoprolol	122 ± 82 (75)	1.8 ± 2.8 (40)	14 ± 9 (100)*	0.3 ± 0.6 (20)*	38 ± 35 (75)*	0.0 ± 0.0 (0)*
	Propranolol	86 ± 67 (100)*	5.4 ± 5.1 (80)*	77 ± 54 (100)*	2.1 ± 3.8 (60)*	100 ± 101 (75)*	0.0 ± 0.0 (0)*
	Sotalol	78 ± 52 (100)	12 ± 12 (100)	44 ± 40 (100)	6.7 ± 7.1(100)	48 ± 47 (100)	7.2 ± 0.5 (100)
Diuretics	Furosemide	26 ± 35 (50)*	236 ± 247 (100)*	46 ± 43 (100)*	214 ± 206 (100)*	40 ± 45 (75)*	212 ± 216 (100)*
	Hydrochlorothiazide	33 ± 52 (50)*	356 ± 373 (100)*	23 ± 35 (50)*	193 ± 218 (100)*	9.6 ± 19 (25)	60 ± 16 (100)
Lipid-regulators	Bezafibrate	0.8 ± 2 (25)	11.6 ± 14 (100)	0.7 ± 1 (25)*	4.1 ± 2.1 (100)*	0.4 ± 0.8 (25)*	2.8 ± 0.1 (100)*
	Fenofibrate	2623 ± 2020 (100)*	302 ± 232 (100)*	2678 ± 1863 (100)*	168 ± 189 (100)*	3456 ± 3848 (100)	61 ± 46 (80)
	Gemfibrozil	24 ± 29 (100)	91 ± 86 (100)	17 ± 25 (100)*	101 ± 88 (100)*	28 ± 26 (100)	132 ± 137 (100)
Psychiatric medications	Carbamazepine	15 ± 15 (100)	16 ± 19 (100)	13 ± 7 (100)	19 ± 18 (100)	25 ± 13 (100)	25 ± 3.3 (100)
	Diazepam	22 ± 24 (100)*	0.5 ± 0.7 (40)*	22 ± 25 (100)*	2.3 ± 4.1 (40)*	34 ± 36 (100)*	1.1 ± 0.0 (40)*
	Lorazepam	57 ± 40 (100)	53 ± 36 (100)	39 ± 8 (100)	71 ± 78 (100)	44 ± 33 (100)	20 ± 2.8 (100)
	Paroxetine	835 ± 1604 (100)*	26 ± 34 (80)*	3264 ± 4391 (100)	17 ± 33 (80)	143 ± 153 (100)	55 ± 2.8 (60)

ofloxacin, sotalol, and trimethoprim) were detected with the highest frequency value (100% of samples) in both phases. Similar trends were recorded in the AcD effluent samples, with 17 compounds in phase I and 16 compounds in phase II displaying 100% detection frequency. However, a lower number of PhACs with 100% detection frequency occurred in the MD effluent samples (12 in both phases).

According to the Kruskal-Wallis test, the mean concentrations of codeine, diazepam, fenofibrate, furosemide, hydrochlorothiazide, paroxetine, and propranolol in the ThS samples were statistically different between phases I and II, probably due to the diverse operational conditions also used in the water treatment line (A^2O bioreactor) (Gallardo-Altamirano et al., 2018), which determined different PhACs concentrations in the sludge treatment line. The statistical analyses also revealed significant differences of the average concentrations of several PhACs measured in the effluents of both digesters between the two experimental phases, particularly in the case of the AcD (Table 2).

The highest mean concentration values in the ThS were observed for ofloxacin (4922 ng g^{-1}), fenofibrate (2623 ng g^{-1}), and paroxetine (835 ng g^{-1}) in phase I, and ibuprofen (1280 ng g^{-1}), naproxen (507 ng g^{-1}), and ofloxacin (475 ng g^{-1}) in phase II. Several PhACs were detected at mean concentration values ≤15 ng g^{-1} in both experimental phases (bezafibrate, indomethacin, and propyphenazone) or in phase II samples only (diazepam, metoprolol, and sulfamethazine). The sulfadiazine concentration was below the limit of detection (BLD) only in phase II samples.

Ofloxacin was the PhACs detected at highest concentrations in the three types of sludge samples, although subjected to a high variability (Table S3). Such variability in sewage sludge was previously reported by Narumiya et al. (2013) and Verlicchi and Zambello (2015), reflecting the use of this fluoroquinolone antibiotic to treat outbreaks of infection diseases; in this sense, Coutu et al. (2013) previously reported a clear seasonality in the pattern of detection of ofloxacin concentrations in wastewater.

Otherwise, the highest PhACs mean values in MD effluent samples were measured for fenofibrate (3456 ng g^{-1}) and ofloxacin (3324 ng g^{-1}) in phase I, and ibuprofen in phases I and II (403 and 1595 ng g^{-1}, respectively), while the values of the remaining PhACs were < 300 ng g^{-1} in both experimental phases (Table 2).

The mean concentrations of the targeted PhACs in the three types of sludge samples fell within the ranges measured earlier by several authors (Martín et al., 2012; Narumiya et al., 2013; Phan et al., 2018; Radjenović et al., 2009; Verlicchi and Zambello, 2015; Yang et al., 2016), except for two compounds which were detected at concentrations above (clarithromycin) or below (fenofibrate) those previously reported in the available literature.

As shown in Table 2, the concentrations of most PhACs gradually decreased throughout the two-stage MAD process, except for carbamazepine, diazepam, diclofenac, gemfibrozil, and ibuprofen (in both phases), fenofibrate, furosemide, and propranolol (only in phase I), and paroxetine (only in phase II). Analogous trends were previously reported by several authors for some of the aforementioned PhACs (Narumiya et al., 2013; Phan et al., 2018; Radjenović et al., 2009; Yang et al., 2016), except for diazepam, fenofibrate, furosemide, and paroxetine, of which very little information is actually available.

Table 3 shows the daily measured mass loads (ML, mg day^{-1} 1000 inh^{-1}) of the 27 targeted PhACs in the three sludge flows. The total ML of the ThS was more than twofold in phase I (553 mg day^{-1} 1000 inh^{-1}) compared to phase II (254 mg day^{-1} 1000 inh^{-1}). Antibiotics and lipid-regulators displayed the highest ML percentages in phase I (53 and 28% respectively), while anti-inflammatory and/or analgesic pharmaceuticals (AIADs) and diuretics had highest ML percentages in phase II (58 and 15% respectively). Throughout the full

Table 3
Daily mass load per 1000 inhabitants (ML, mg day^{-1} 1000 inh^{-1}) of pharmaceutically active compounds (PhACs) and percentages of each therapeutic group in the thickened sludge (ThS), acidogenic digester (AcD) effluent, and methanogenic digester (MD) effluent of the pilot-scale two-stage mesophilic anaerobic digestion (MAD) during the experimental phases I and II.

Analytes	ThS		AcS		MS	
	ML Phase I	ML Phase II	ML Phase I	ML Phase II	ML Phase I	ML Phase II
AIADs	26.5	146.0	32.3	91.7	28.3	61.2
Acetaminophen	5.6	4.4	0.6	11.7	0.0	0.2
Codeine	1.1	6.3	0.8	4.3	0.3	0.3
Diclofenac	7.6	10.6	20.0	10.4	10.1	6.7
Ibuprofen	7.0	81.6	9.0	56.7	16.3	50.1
Indomethacin	0.1	0.3	0.1	0.2	0.1	0.1
Ketoprofen	1.5	10.5	1.2	5.3	0.9	3.3
Naproxen	3.0	32.4	0.5	3.2	0.1	0.5
Propyphenazone	0.6	0.1	0.3	0.0	0.5	0.0
Antibiotics	293.1	34.2	1570.7	16.9	127.5	8.4
Clarithromycin	1.8	0.6	0.5	0.3	0.2	0.0
Ofloxacin	283.7	30.4	1568.6	16.2	126.7	8.3
Sulfadiazine	1.8	0.0	0.4	0.0	0.2	0.1
Sulfamethazine	1.7	0.0	0.1	0.1	0.0	0.0
Sulfamethoxazole	3.1	1.4	1.1	0.0	0.3	0.0
Trimethoprim	1.0	1.8	0.1	0.4	0.0	0.0
Beta-blocker	17.6	4.4	8.2	1.7	8.1	0.4
Atenolol	1.3	3.2	0.9	1.3	0.3	0.2
Metoprolol	7.1	0.1	0.8	0.0	1.6	0.0
Propranolol	4.8	0.3	4.2	0.1	4.2	0.0
Sotalol	4.4	0.8	2.3	0.3	2.1	0.2
Diuretics	3.0	37.8	3.4	19.5	1.9	8.5
Furosemide	1.4	15.1	2.3	10.3	1.5	6.7
Hydrochlorothiazide	1.7	22.7	1.1	9.2	0.3	1.8
Lipid-regulators	156.3	25.4	146.1	13.2	130.2	6.2
Bezafibrate	0.0	0.7	0.0	0.2	0.0	0.1
Fenofibrate	155.0	18.9	145.2	8.0	129.2	2.0
Gemfibrozil	1.3	5.8	0.8	4.9	1.0	4.1
Psychiatric medications	56.4	6.1	182.0	5.5	9.6	3.2
Carbamazepine	0.8	1.0	0.7	0.9	1.0	0.8
Diazepam	1.2	0.0	1.2	0.1	1.4	0.0
Lorazepam	3.1	3.3	2.1	3.4	1.9	0.6
Paroxetine	51.3	1.6	178.0	1.0	5.3	1.8
Total	553.0	253.9	1942.6	148.5	305.6	88.0
% AIADs	4.8	58.0	1.7	62.0	9.3	70.0
% Antibiotics	53.0	13.0	81.0	11.0	42.0	10.0
% Beta-blocker	3.2	1.7	0.4	1.2	2.7	0.5
% Diuretics	0.6	15.0	0.2	13.0	0.6	9.7
% Lipid-regulators	28.0	10.0	7.5	8.9	43.0	7.0
% Psychiatric medications	10.0	2.0	9.0	4.0	3.0	4.0

two-stage MAD process, the total ML of the final effluent sludge was reduced to 306 mg day^{-1} 1000 inh^{-1} in phase I and 88 mg day^{-1} 1000 inh^{-1} in phase II (44.7 and 65.3%, respectively). The increased reduction observed in phase II was connected to the highest removal efficiencies (REs) achieved for several of the analyzed PhACs compared to phase I, as it will be further discussed in Section 3.3.

ML values for PhACs are well characterized in the wastewater treatment lines of WWTPs (Papageorgiou et al., 2016; Park et al., 2017; Verlicchi et al., 2012; Zorita et al., 2009). However, to the best of the authors' knowledge, the present study is the first reporting ML values for PhACs in a sewage sludge treatment line. The data reported here should be taken into consideration, since treated sewage sludges are commonly applied as organic amendments to agricultural lands in many countries.

3.3. Removal efficiencies (REs) of PhACs in the two-stage MAD plant

Table 4 shows the REs (mean + SD) calculated for the targeted PhACs in the AcD and MD, as well as the overall REs for the global two-stage MAD process, during the experimental phases I and II. The REs were calculated using mass balance as detailed in Supplementary materials. Those compounds with average concentration values <10 ng g^{-1} and a frequency of detection <50% in both phases (bezafibrate, indomethacin, propyphenazone, sulfadiazine, and sulfamethazine, Table 2) were excluded from the calculations.

According to the Kruskal-Wallis test, statistically significant differences ($p < 0.05$ or $p < 0.10$) were observed between phases I and II for the REs of 2 PhACs in the AcD, 7 PhACs in the MD, and 4 PhACs in the global MAD process (Table 4). In phase I, the REs achieved for the majority of PhACs were similar in both digesters except for gemfibrozil, which showed a significantly higher RE in the AcD. In contrast, significantly higher REs of acetaminophen, atenolol, codeine, hydrochlorothiazide, and lorazepam were measured in the MD compared to the AcD in phase II (REs > 70%, Table 4).

Mean RE values were statistically higher in the MD in phase II compared to phase I for carbamazepine (13 vs. −56%), clarithromycin (88 vs. 39%), codeine (91 vs. 70%, respectively), gemfibrozil (16 vs. −141%), ibuprofen (17 vs. −306%), lorazepam (79 vs. 15%), and propranolol (100 vs. 18%). The Spearman rank-correlation test (Table S4) showed that the SRT was the only operational/environmental variable displaying a strong correlation ($p < 0.01$) with the REs of the aforementioned PhACs in the MD, with the only exception of codeine and clarithromycin. Consequently, the extension of the SRT from 12 (phase I) to 24 days (phase II) was significantly connected with an improved removal of most PhACs in the MD.

The influence of SRT on the efficiency of removal of PhACs in anaerobic digesters has been seldom addressed in previous studies, leading to some contradictory conclusions. Samaras et al. (2014) and Gonzalez-Gil et al. (2016) reported increases of the mean REs of some organic micropollutants when the SRT was extended from 8 to 20 days and from 10 to 30 days, respectively, in line with the results presented here, while Carballa et al. (2007) and Yang et al. (2016) did not observe a significant influence of SRT on the REs of PhACs and personal care products.

High mean REs (>80%) with low standard deviations (<10%) were achieved throughout the global two-stage MAD process at least in one of the experimental phases for acetaminophen, clarithromycin, hydrochlorothiazide, naproxen, sulfamethoxazole, and trimethoprim (Table 4). Similar REs have been found in the literature for these PhACs in sludge AD processes operating under different conditions (Gonzalez-Gil et al., 2016; Martín et al., 2012; Narumiya et al., 2013; Phan et al., 2018; Radjenović et al., 2009; Samaras et al., 2013, 2014; Yang et al., 2016, 2017), with the exception of hydrochlorothiazide, for which various authors (Jelić et al., 2012; Radjenović et al., 2009) have observed lower average RE values (71% and 55%, respectively). Very low (<30%) or negative mean REs were measured for the global two-stage MAD process in both experimental phases for carbamazepine, diazepam, diclofenac, gemfibrozil, ibuprofen, and paroxetine (Table 4), in accordance with previous reports (Carballa et al., 2007; Gonzalez-Gil et al., 2019; Narumiya et al., 2013; Phan et al., 2018; Radjenović et al., 2009; Yang et al., 2016, 2017), except for the AIAD ibuprofen, for which medium-to-high REs (ranging 30 to 95%) (Carballa et al., 2007; Samaras et al., 2013, 2014) or very low REs (ranging from negative RE values to 20%) have been previously measured (Gonzalez-Gil et al., 2016; Phan et al., 2018; Radjenović et al., 2009; Yang et al., 2016, 2017).

Several authors have proposed that the molecular structure of trace organic compounds is the major factor governing their removal from wastewater (Phan et al., 2018; Tadkaew et al., 2011; Wijekoon et al., 2015; Yang et al., 2016, 2017). Hydrophobic PhACs or hydrophilic PhACs with strong electron-donating functional groups (EDGs) are more easily removed under anaerobic conditions, while those carrying at least one strong electron-withdrawing functional group (EWG) were more resistant to removal (Wijekoon et al., 2015; Yang et al., 2016).

In the present study, among those hydrophilic PhACs with strong EDGs previously referenced in literature (Wijekoon et al., 2015; Yang

Table 4
Mass removal efficiencies (REs, %) of the pharmaceutically active compounds (PhACs) in the acidogenic digestor (AcD), methanogenic digestor (MD), and global two-stage mesophilic anaerobic digestion (MAD) process, measured in the experimental phases I (n = 4) and II (n = 5). Values shown are means ± SD. According to the Kruskal-Wallis test, values marked with asterisks ($p < 0.05^*$ and $p < 0.1^{**}$) were significantly different between the two experimental phases (I and II) in the same stage (AcD, MD, or full MAD process), and values marked with ^{x} were significantly different ($p < 0.05$) comparing between digesters (AcD and MD) in the same experimental phase (I or II).

Therapeutic groups		AcD		MD		Full two-stage MAD	
		Phase I	Phase II	Phase I	Phase II	Phase I	Phase II
AIADs	Acetaminophen	90 ± 0	−8167 ± 16770^x	100 ± 0	98 ± 3.3^x	100 ± 0	93 ± 13
	Codeine	−41 ± 225	23 ± 26^x	70 ± 7.8*	91 ± 10^{*x}	47 ± 79	92 ± 8.4
	Diclofenac	−184 ± 319	−15 ± 41	8.4 ± 72	36 ± 4.3	−40 ± 130	27 ± 23
	Ibuprofen	−294 ± 254	−5.8 ± 42	−306 ± 232*	17 ± 14*	−1714 ± 1249**	13 ± 39**
	Ketoprofen	15 ± 35	16 ± 51	35 ± 60	38 ± 16	52 ± 48	53 ± 22
	Naproxen	90 ± 11	85 ± 5.2	93 ± 14	80 ± 17	98 ± 3.7	97 ± 3.5
Antibiotics	Clarithromycin	42 ± 97	68 ± 40	39 ± 32 **	88 ± 6.2**	81 ± 23	96 ± 6.5
	Ofloxacin	−390 ± 784	39 ± 28	64 ± 25	50 ± 12	50 ± 40	71 ± 10
	Sulfamethoxazole	67 ± 32	100 ± 0	60 ± 50	–	93 ± 7.9	100 ± 0
	Trimethoprim	85 ± 20	80 ± 6.9	90 ± 8.9	89 ± 15	98 ± 3.5	97 ± 3.8
Beta-blockers	Atenolol	31 ± 31	42 ± 27^x	72 ± 36	77 ± 25^x	75 ± 33	82 ± 25
	Metoprolol	90 ± 2.0	90 ± 14	−76 ± 185	100 ± 0	77 ± 14	100 ± 0
	Propranolol	20 ± 65	53 ± 86	18 ± 90**	100 ± 0**	13 ± 121*	100 ± 0*
	Sotalol	43 ± 27	36 ± 46	−60 ± 243	−2.2 ± 76	57 ± 31	53 ± 24
Diuretics	Furosemide	−26 ± 13	−2.9 ± 46	28 ± 59	35 ± 14	−23 ± 60	37 ± 23
	Hydrochlorothiazide	34 ± 4.6	44 ± 23^x	82 ± 25	79 ± 5.6^x	89 ± 16	89 ± 4.7
Lipid-regulators	Fenofibrate	10 ± 37**	57 ± 29**	18 ± 83	69 ± 32	4.6 ± 123**	91 ± 10**
	Gemfibrozil	46 ± 30^{*x}	−20 ± 52*	−141 ± 160^{*x}	16 ± 29*	0.1 ± 35	4.6 ± 46
Psychiatric medications	Carbamazepine	−29 ± 72	−136 ± 260	−56 ± 60 *	13 ± 25*	−81 ± 105	−84 ± 189
	Diazepam	−36 ± 105	–	−69 ± 164	–	−86 ± 262	–
	Lorazepam	20 ± 39	−5.0 ± 66^x	15 ± 63*	79 ± 13^{*x}	32 ± 58*	80 ± 13*
	Paroxetine	−2241 ± 4278	−242 ± 647	19 ± 106	−911 ± 1626	−157 ± 253	−12 ± 150

et al., 2016) only acetaminophen, atenolol, hydrochlorothiazide, naproxen, sulfamethoxazole, and trimethoprim showed high REs (>70%) in both experimental phases of the global two-phase MAD process (Table 4). Otherwise, clarithromycin, codeine, fenofibrate, metoprolol, and propranolol were highly removed (>80%, Table 4) only under the operating conditions fixed for the experimental phase II (SRTs of 5 and 24 days for AcD and MD, respectively). However, the mean REs for gemfibrozil were very low (0.1 and 4.6% in phases I and II, respectively). Tadkaew et al. (2011) and Yang et al. (2016) classified this compound as a hydrophilic PhAC with EWG, and as such more resistant to anaerobic removal.

PhACs with EWGs like carbamazepine, diazepam, diclofenac and ibuprofen were not easily removed under the anaerobic conditions, in accordance with previous studies (Wijekoon et al., 2015; Yang et al., 2016), while others like ketoprofen, lorazepam and ofloxacin showed mean REs around 50% in at least one experimental phase of the global two-stage MAD process (Table 4). Even though ibuprofen and ketoprofen are hydrophilic PhACs (logD < 3.2) with strong EDGs (Tadkaew et al., 2011) and therefore likely to be removed under anaerobic conditions, observed RE values were only remarkable for ketoprofen (Table 4).

It is important to note that the highest REs for lorazepam and ofloxacin were achieved only under the operating conditions fixed for the experimental phase II, as it was remarked previously for several hydrophilic PhACs with strong EDGs (clarithromycin, codeine, fenofibrate, metoprolol, and propranolol). These results show that additional factors other than the chemical structure of PhACs (for example the operational/environmental conditions) could be decisive for their removal.

3.4. Quantification of Bacteria, Archaea and Fungi

Table S5 shows the absolute abundances of Bacteria, Archaea and Fungi in the AcD and MD effluent samples, measured by qPCR throughout the experimental phases I and II. The absolute abundances of Bacteria were in the range of 10^8–10^9 copies L^{-1} sludge in the AcD effluent, while they were kept around 10^{10} copies L^{-1} sludge in the MD effluent. The abundance of Archaea was ca. 10^8 copies L^{-1} sludge in the AcD effluent and ranged between 10^8 and 10^9 copies L^{-1} sludge in the MD effluent. According to the Kruskal-Wallis and Conover-Iman tests, there were only significant differences of the numbers of both Bacteria and Archaea markers between the two experimental phases in the MD effluent samples (Table S5). The higher counts of Archaea in the MD in phase I agree with increased CH_4 recovery rates (Table 1). The average abundances observed here were below the ranges previously reported in different types of anaerobic digesters treating solid wastes, high-strength wastewaters, or municipal sewage sludge, measured by qPCR and digital droplet PCR approaches (10^{13}–10^{14} and 10^{10}–10^{13} copies of 16S rRNA genes of Bacteria and Archaea per L sludge, respectively; Jang et al., 2014; Kim et al., 2015; Nettmann et al., 2010).

Regarding fungal populations, amplification products could not be detected in samples of AcD and MD effluents in phase II, with any of the two primer pairs used, in agreement with the previous analyses conducted in the water line of the A^2O process (Gallardo-Altamirano et al., 2018). In addition, quantifications based on the FungiQuant primer set, which was selected because of its higher specificity for fungi compared to other commonly used primers (Maza-Márquez et al., 2020), were below the limit of detection in several phase I samples. Nonetheless, average quantifications were 10^6–10^7 and 10^9 copies L^{-1} sludge for the FungiQuant and LR primers sets, respectively, indicating that, when detectable, fungal populations in the two-stage MAD represented an important fraction of the total microbial community.

3.5. Structure of bacterial, archaeal, and fungal communities in the two-stage MAD plant

The diversity of Bacteria, Archaea, and Fungi in the two-stage MAD plant was assessed by means of massive parallel sequencing of partial small-subunit rRNA genes. A total of 16 samples were analyzed (four effluent samples from each stage, AcD and MD, collected through each of the two experimental phases). Fungal diversity was not investigated in phase II samples, which failed to render any amplification products, in agreement with the results of the qPCR experiments. Following quality filtering of the raw sequencing data, 152,052 and 261,977 16S rRNA reads were kept for Bacteria and Archaea, respectively, and 18,452 18S

rRNA reads for Fungi. The number of sequencing reads per sample ranged 3998–19,967 for Bacteria, 7653–50,243 for Archaea, and 659–4171 for Fungi. In spite of these differences among the samples analyzed, the R/E curves (Fig. S7) indicated that enough sequencing depth was achieved to appropriately describe the diversity of the three targeted microbial groups in the two-stage MAD system. Applying a 97% sequence identity cut-off, a total of 1341 Bacteria PHYs, 108 Archaea PHYs, and 246 Fungi PHYs were identified, with the numbers of PHYs per sample ranging 68–785, 10–70, and 51–126, respectively.

Table S6 summarizes the numbers of average reads of Bacteria, Archaea, and Fungi per digester and experimental phase, as well as the average values of Richness (S), Shannon-Wiener (H'), Simpson's (1-D), and Chao-1 indices of diversity. For both Bacteria and Fungi, the indices described communities with medium-to-high species diversity and low functional organization. In comparison, the average values of H' and 1-D indices indicated a much lower diversity and community evenness of Archaea in both digesters, particularly in phase II samples. The diversity of the Bacteria communities was also lower in the samples retrieved throughout phase II, in both digesters.

The dendrograms in Fig. S8 show the clustering of the AcD and MD samples according to the dis(similarity) of their bacterial, archaeal, and fungal communities (relative abundances of PHYs detected by Illumina sequencing). The global similarity of both Bacteria and Archaea communities among samples was <20%, and ANOSIM analyses showed that there were significant differences among the community profiles of either Bacteria or Archaea in samples retrieved from the same digester ($R = 0.43$ and 0.81, respectively, $p < 0.05$) or throughout the same experimental phase ($R = 0.72$ and 0.63, respectively, $p < 0.05$).

3.5.1. Diversity of Bacteria communities

Taking the 16 samples altogether, Bacterial PHYs with >0.1% average relative abundance were classified in 16 different Phyla, with *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* cumulatively comprising nearly 80% of the community (29.74, 25.18 and 24.02%, respectively), while 29 Phyla had a relative abundance >0.1% in at least one sample (Fig. 1). The average relative abundances of bacterial clades at the Order and Family levels in the global two-stage MAD process are summarized in Table S7.

The dominant Phyla within the bacterial communities varied depending on the digester and experimental phase. In the AcD effluent, *Proteobacteria* dominated in phase I samples (46.61% average relative abundance) followed by *Bacteroidetes*, *Chloroflexi*, and *Firmicutes* (25.59, 11.36 and 7.66%, respectively), while *Firmicutes* were prevalent in phase II (62.05% average relative abundance) followed by *Bacteroidetes* and *Proteobacteria* (12.85 and 12.54%, respectively). In the MD effluent, *Bacteroidetes* and *Proteobacteria* codominated in both experimental phases (38.26% and 31.23% in phase I, 24.02% and 28.60% in phase II, respectively), followed by *Chloroflexi* and *Firmicutes* (13.21% and 7.44% in phase I, 13.76% and 18.94% in phase II, respectively). The top four aforementioned bacterial phyla (*Bacteroidetes*, *Chloroflexi*, *Firmicutes*, and *Proteobacteria*) are often detected as major components of the bacterial community in mesophilic sewage sludge AD systems by metagenomic approaches based on high-throughput sequencing (McIlroy et al., 2017; Nelson et al., 2011; Wu et al., 2017; Yang et al., 2014), and comprise microorganisms that are known to participate in one or more of the stages of the AD process (hydrolysis, acidogenesis and acetogenesis) (Westerholm and Schnürer, 2019; Xia et al., 2016).

Members of recently proposed Candidate Phyla were detected in most samples, some of which reached average relative abundances >1% within the bacterial communities. *Ca.* Saccharibacteria (TM7) were detected at relative abundances ranging 0.07–4.87% in the AcD effluent samples of phases I and II, while *Ca.* Cloacimonetes (WWE1) were particularly abundant in phase II samples, in both the AcD and MD effluents (1.85–8.66% and 0.40–5.52%, respectively), and *Ca.* Atribacteria (OP9/JS1) were mostly detected in MD samples of phase II (0.15–4.32% relative abundance). The roles of these new Phyla in WWTPs or AD processes are just starting to be elucidated, but there is evidence of their involvement in organic matter depolymerization, fermentation, and syntrophic associations with methanogens (Kindaichi et al., 2016; Nobu et al., 2016; Westerholm et al., 2020).

Differences of the community composition among the two digesters and the experimental phases were also detected at the Class, Order, and Family levels. *Bacteroidia*/*Bacteroidales* displayed the highest relative abundances in the AcD effluent in phase I samples and in the MD effluent in both experimental phases, while *Clostridia* and *Bacilli*/*Clostridiales* and *Turicibacterales* codominated the community in the AcD effluent in phase II samples (data not shown). Fig. S9 displays a heatmap of the relative abundances of bacterial PHYs clustered at the Family level, including only those with ≥1% abundance in at least one sample. The most abundant families in the AcD effluent community were *Rikenellaceae* in phase I (11.13%) and *Turicibacteraceae* in phase II (25.00%), while *Rikenellaceae* were prevalent in the MD effluent in both experimental phases (33.89 in phase I and 18.33% in phase II).

SIMPER analyses were carried out aiming to evaluate which PHYs contributed most to the dis(similarity) of the bacterial communities in the different groups of samples analyzed (Table S8). The overall similarity was lower among the AcD effluent samples (28.09%) compared to the MD (39.95%). When samples were grouped by experimental phases, the overall similarity was higher in phase I (55.94%) than in phase II (25.73%). SIMPER results also allowed to conclude that the bacterial community was highly dissimilar when comparing among the two digesters (69.08%) or the two experimental phases (75.05%). Five PHYs (PHY0384, PHY0142, PHY0527, PHY0453 and PHY0321, related to the families *Turicibacteraceae*, *Rikenellaceae*, *Peptostreptococcaceae*, *Clostridiaceae* and *Caldilineaceae,* respectively) were among those making the highest contributions to explain such dissimilarities, either among the two digesters or the experimental phases; however, the contributions were always <2% for each individual PHY (Table S8E and F).

To better illustrate the observed differences of the bacterial community structure in the AcD and MD effluents during the two experimental phases, the samples were ordinated by MDS based on the relative abundances of the PHYs identified by Illumina sequencing, clustered at the Phyla and Family levels (Figs. 2A, B). At both taxonomic levels, the ordinations show that the community structure was more similar between the two digesters in phase I than in phase II, as also observed with SIMPER analysis. Remarkably, a strong negative correlation occurred among *Proteobacteria* and *Firmicutes*, two of the most abundant Phyla in the samples (Fig. 2A). *Comamonadaceae* and *Xanthomonadaceae* were the main families of the *Proteobacteria* increasing their relative abundances in the samples of the AcD during phase I, while *Clostridia* families (*Christensenellaceae*, *Clostridiaceae*, *Halanaerobiaceae*, *Peptostreptococcaceae*, and *Ruminococcaceae*) together with *Turicibacteraceae* prevailed in the AcD throughout phase II. Samples of the MD had a more similar community in the two phases, sharing 7 of the 10 families with the highest relative abundances: *Anaerolinaceae*, *Caldilineaceae* (*Chloroflexi*), *Rikenellaceae* (*Bacteroidetes*), *Comamonadaceae*, *Hyphomicrobiaceae*, *Oxalobacteraceae*, and *Rhodobacteraceae* (*Proteobacteria*).

Overall, moderate-to-strong positive correlations ($r = 0.5$–0.75) were found among the indicators of the efficiency of methanogenesis (VSR, CH_4 in biogas, or CH_4 recovery rate) and the relative abundances of *Dethiosulfovibrionaceae*, *Rikenellaceae*, *Syntrophaceae*, and *Turicibacteraceae* (Fig. 2B and C, Table S9). Contrariwise, the relative abundances of *Christensenellacae*, *Cytophagaceae*, *Lachnospiraceae*, *Moraxellaceae*, *Porphyrimonadaceae*, *Rhodocyclaceae*, *Saprospiraceae*, and *Xanthomonadaceae* displayed strong negative correlations ($r < -0.7$) with VSR and CH_4 recovery rate. The abovementioned families plus *Ruminococcaceae* and *Propionibacteriaceae* were correlated positively ($r = 0.5$–1.0) with the values of the VFA/ALK ratio and OLR (Fig. 2B and C, Table S9).

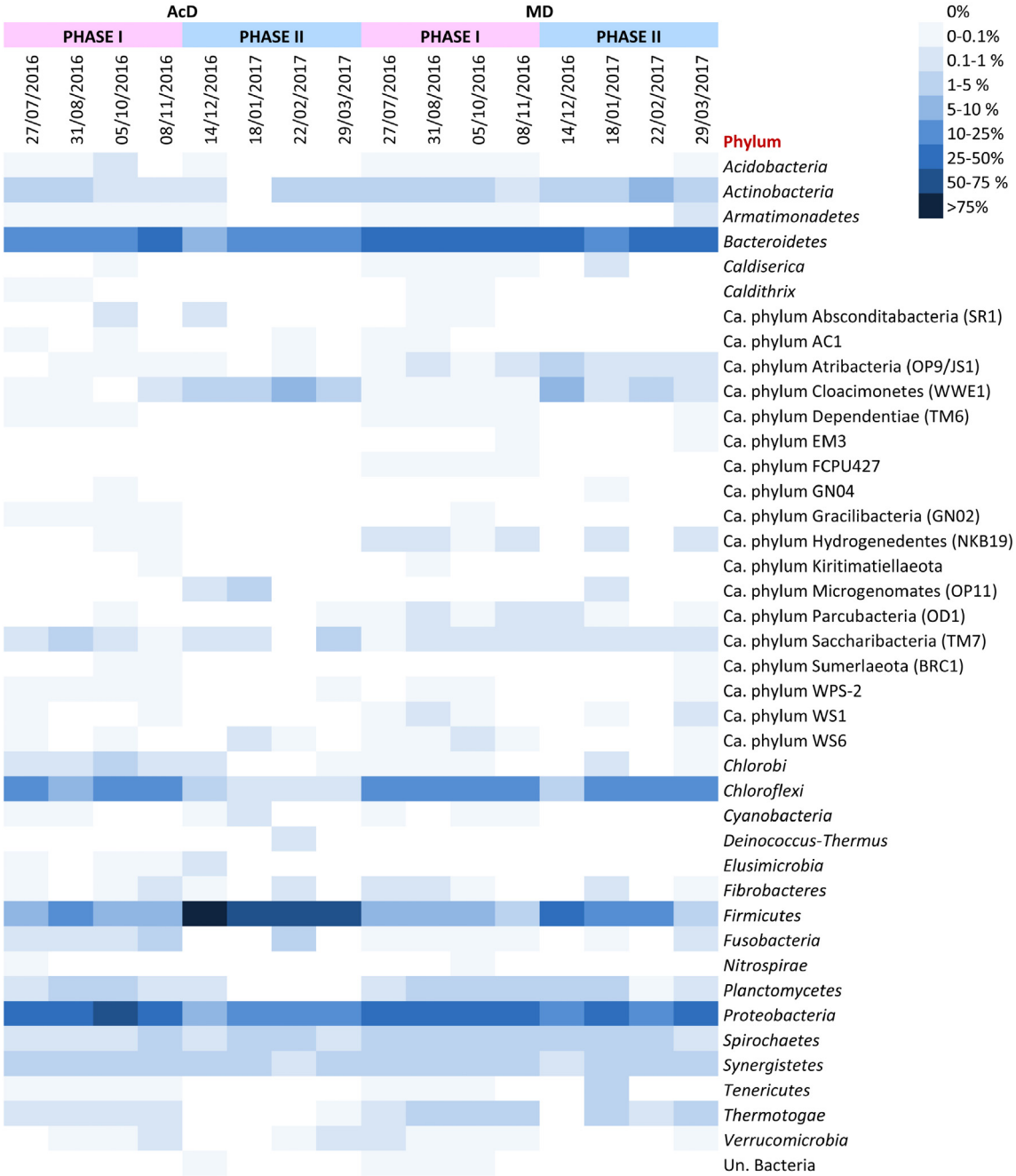


Fig. 1. Heatmap displaying the relative abundances of Bacteria phylotypes (Phylum level), detected by Illumina sequencing in the acidogenic digestor (AcD) and the methanogenic digestor (MD) effluent samples in the experimental phases I and II.

In the present study, the dominant phylotypes in the AcD shifted from *Proteobacteria* in phase I to *Firmicutes* in phase II, showing that different groups of microorganisms acting as drivers of the hydrolysis and acidogenesis processes were selected under the different operating conditions applied. Among the families whose relative abundances displayed a positive correlation with the VFA/ALK ratio (Fig. 2 and Table S9), *Cytophagaceae*, *Lachnospiraceae*, and *Xanthomonadaceae* were more abundant in phase I (Fig. S9), when the AcD was subjected to the highest OLR, leading to a fast generation and accumulation of VFAs and low methane production (Table 1A), thus enabling an efficient separation of the AD process in two different stages. Members of the *Cytophagaceae* and *Lachnospiraceae* are both well-known for their key roles in the hydrolysis of several types of macromolecules, and *Lachnospiraceae* generate VFAs such as acetate, propionate, and butyrate through fermentation (Biddle et al., 2013; McBride et al., 2014). On the other hand, *Xanthomonadaceae* are mostly strictly aerobic bacteria; however, they have been often found in significative relative abundances in AD bioreactors, being their roles in these systems still not well defined (Hao et al., 2016).

As previously stated, *Clostridiales* and *Turicibacterales* codominated the bacterial community in the AcD during phase II. Several families comprising well-characterized anaerobic acidogenic bacteria of the *Clostridiales* reached their highest relative abundances in the AcD (Fig. S9), being consistently favored by higher %VS/TS (Fig. 2, Table S9B). Of these, only the *Christensenellaceae* and *Ruminococcaceae* were found positively correlated to the VFA/ALK ratio, together with

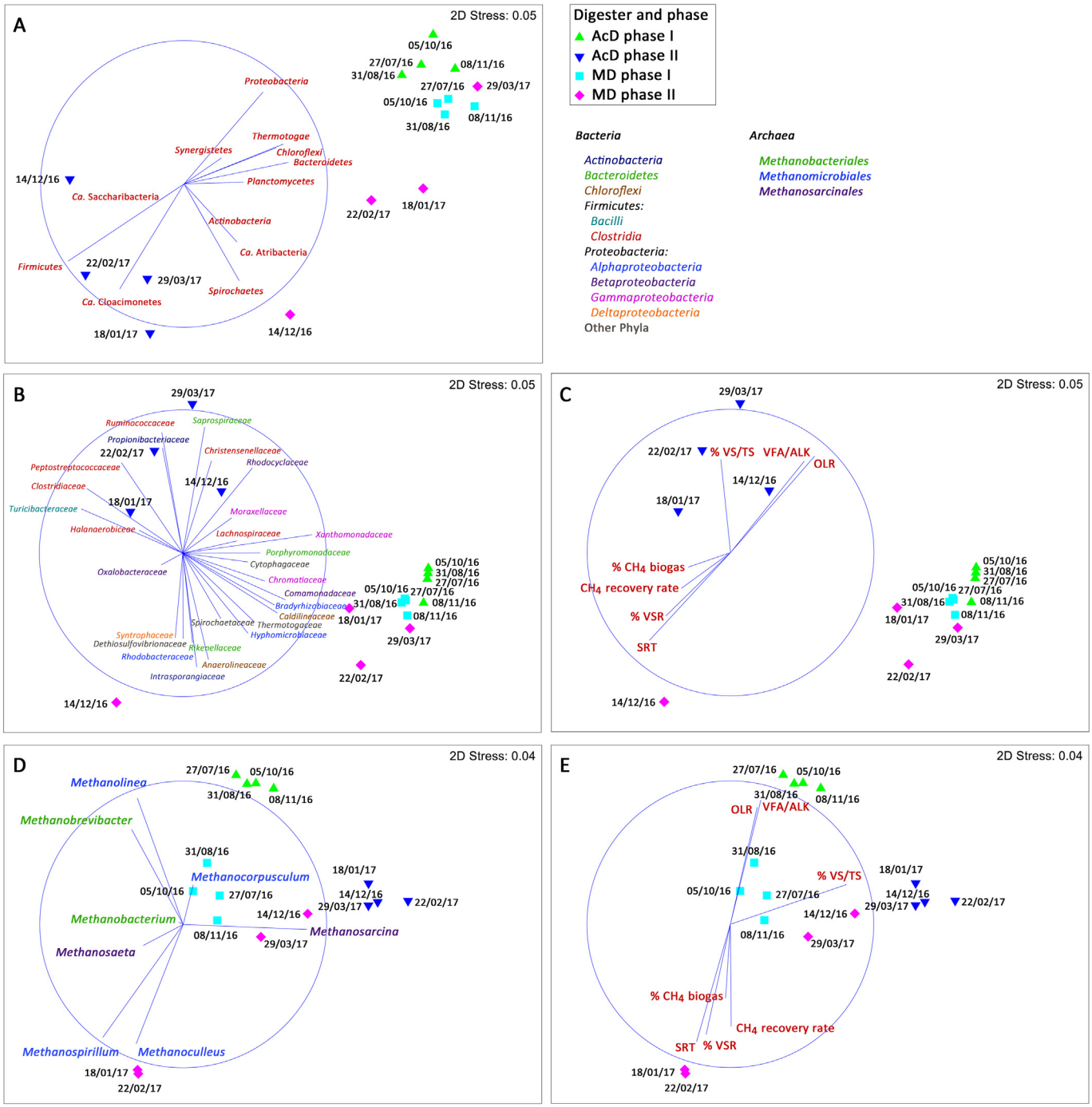


Fig. 2. Non-metric multidimensional scaling (MDS) plots, illustrating the ordinations of the samples retrieved from the acidogenic digestor (AcD) and the methanogenic digestor (MD) in phases I and II, according to the relative abundances of the Bacteria and Archaea phylotypes identified by Illumina sequencing. A. Ordination based on the relative abundance of Bacteria at the Phylum level. B. Ordination based on the relative abundance of Bacteria at the Family level. C. Correlations among operational parameters and the indicators of the efficiency of the AD process and the ordination shown in plot B. D. Ordination based on the relative abundance of Archaea at the Genus level. E. Correlations among the operational parameters and the indicators of the efficiency of the AD process and the ordination shown in plot D. Vectors in plots A, B and D represent the direction throughout the ordination of the relative abundances of the prokaryotic taxa with an average relative abundance ≥1% in a set of samples retrieved from the same digester and phase. Vectors in plots C and E represent the direction throughout the ordination of the abiotic variables % CH_4 in biogas, CH_4 recovery rate, volatile solids/total solids percentage (%VS/TS), volatile fatty acids to alkalinity ratio (VFA/ALK), sludge retention time (SRT), organic loading rate (OLR), and % of volatile solids removal (%VSR). The stress level of the MDS plots (<0.1) validates the 2D-representation of the biotic data distribution (Clarke and Warwick, 2001). Vectors with a length shorter than 0.2 had negligible influence on the ordination and are not shown.

Propionibacteriaceae (*Actinobacteria*) (Table S9B), which also occurred at high relative abundances in two samples of this experimental period (Fig. S9). The *Turicibacteraceae* is a recently proposed family including only the Genus *Turicibacter*, described as anaerobic fermenting organisms generating lactate as the major end-product (Bosshard et al., 2002; Verbarg et al., 2014). Although lactate is a common product of fermentation, its relevance as an intermediate of AD processes has been seldom addressed; however, it has been reported that lactate is efficiently oxidized to acetate in anaerobiosis during the acetogenic step, and the genes encoding this trait are widespread among Bacteria

(Detman et al., 2018). Overall, *Clostridiceae* and *Halanaerobicaeae* together with *Turicibacteraceae* displayed positive correlations among them, and also with increasing %CH_4 in the biogas (Fig. 2B and Table S9).

In the next step of the two-stage MAD, acetate, hydrogen and CO_2 are produced from VFAs in the MD prior to methanogenesis, through the activity of the homoacetogens and syntrophic acetogens which mostly belong to the *Firmicutes* and *Proteobacteria*, although members of the phyla *Actinobacteria*, *Bacteroidetes*, *Ca.* Cloacimonetes, *Chloroflexi*, and *Synergistetes* have been also postulated as partners or supporters of methanogens in syntrophic metabolic reactions (Narihiro et al., 2015; Westerholm and Schnürer, 2019). *Clostridiaceae*, *Halanaerobiaceae*, *Peptostreptococcaceae*, and *Ruminococcaceae* (*Firmicutes*) were common to all samples of the two-stage MAD (Fig. S9) and comprise bacteria able to provide methanogens with either hydrogen or acetate through homoacetogenesis (Drake et al., 2014; La Reau and Suen, 2018; Oren, 2014; Slobodkin, 2014); however, none of these families showed a strong positive correlation with CH_4 recovery rates (Table S9), suggesting that this step was carried out by a functionally redundant community and did not rely on the relative abundance of particular populations. In contrast, the relative abundances of the *Dethiosulfovibrionaceae* (*Synergistetes*), *Syntrophaceae* (*Deltaproteobacteria*), and *Rikenellaceae* (*Bacteroidetes*) correlated positively with both VSR and CH_4 recovery rates, and at the same time were favored by longer SRTs (Fig. 2B and Table S9). *Dethiosulfovibrionaceae* encompasses moderately halophilic anaerobic bacteria able to ferment peptides and amino acids generating substrates for methanogenesis such as acetate, H_2 and CO_2, among other end-products (Magot et al., 1997), and have been described as important players in the anaerobic treatment of fish-canning wastewaters (Militon et al., 2015). The Family *Syntrophaceae* were the only clade of well-known syntrophic acetogens reaching an average relative abundance >1% in the two-stage MAD, more specifically in samples of the MD during phase II. Finally, the species of the *Rikenellaceae* are regarded as anaerobic, mesophilic bacteria able to ferment carbohydrates or proteins, generating propionate, succinate, acetate, alcohols, H_2 or CO_2 as end products (Graf, 2014; Su et al., 2014). *Rikenellaceae* was shared by all samples (Fig. S9) and was the most abundant taxa at the family level in the MD in both experimental phases, pointing to a key role of their interactions with methanogenic archaea for the performance of the two-stage MAD plant. Members of this clade have been found in high relative abundances in anaerobic WWTPs and AD reactors previously (Hao et al., 2016; Yi et al., 2014). *Rikenellaceae* relative abundance was negatively correlated with OLR, in agreement with previous reports (Hao et al., 2016).

3.5.2. Diversity of Archaea communities

The heatmaps in Fig. 3 display the relative abundances of archaeal PHYs in the two-stage MAD samples, at the Phylum, Family, and Genus levels. On average, 99.90% of the Archaea characterized in the samples belonged to *Euryarchaeota*, while PHYs classified within the *Thaumarchaeota* and the miscellaneous *Crenarchaeota* group (MCG) were a minority. *Euryarchaeota* encompasses most of the methanogens, together with sulfate-reducing, extreme thermophilic, and halophilic archaea (Oren, 2019).

Methanomicrobia were the dominant Class in either the AcD or the MD effluent samples, and throughout both experimental phases (75.00–100.00% relative abundance), followed by *Methanobacteria* (0.00–23.72%). *Methanomicrobiales* were also the prevalent Order in most samples, except those retrieved in phase II from the AcD effluent and one sample from the MD effluent, which were dominated by *Methanosarcinales* (59.73–92.10%). The analyses at the Family and Genus levels revealed that the prevalent members of the archaeal community differed among both the digesters and the experimental phases (Fig. 3). The genera *Methanolinea* (*Methanoregulaceae*) (81.15%) and *Methanosarcina* (*Methanosarcinaceae*) (86.99%) displayed the highest average relative abundances in the samples of the AcD effluent during phases I and II, respectively. In the MD effluent, the community was codominated by *Methanospirillum* (*Methanospirillaceae*) and *Methanolinea* in phase I (30.80 and 29.85%, respectively), while *Methanospirillum* prevailed in phase II (57.81%). The specialist acetoclastic *Methanosaeta* (*Methanotrix*), was only detected in higher relative abundances than *Methanosarcina* (6.96–18.25%) in the samples from the MD effluent in phase I.

According to SIMPER analysis (Table S10), the global similarities of the Archaea communities among samples of the AcD and MD effluents were 51.55% and 43.55%, respectively, while phase I and II samples displayed 56.59% and 40.45% similarities, respectively. As observed for Bacteria, the archaeal communities were highly dissimilar when compared either among the two digesters (68.56%) or the experimental phases (69.41%). PHY045 and PHY048 (*Methanolinea*), PHY061 (*Methanospirillum*), and PHY080 (*Methanosarcina*) were confirmed altogether as the major contributors to the observed dissimilarities, either among the digesters (37.77%) or the experimental phases (41.52%) (Table S10E and F).

Fig. 2D displays the MDS ordination of the samples according to relative abundances of the archaeal PHYs identified by Illumina sequencing clustered at the Genus level, and their relationships with the shifts of the operational parameters and the indicators of the efficiency of methanogenesis are depicted in Fig. 2E and Table S11. The relative abundances of *Methanospirillum* and *Methanoculleus* correlated strongly and positively with VSR, CH_4 recovery rates and %CH_4 in the biogas ($r \geq 0.8$), while the prevalence of these two genera was favored by longer SRTs ($r \geq 0.9$) and lower ORL and VFA/ALK ratios ($r \leq -0.9$). In contrast, higher ORL and VFA/ALK ratios ($r \geq 0.7$) and shorter SRTs ($r \leq -0.7$) favored the dominance of *Methanolinea* and *Methanobrevibacter*, leading to a less efficient methanogenesis (Table S11).

Antagonistic trends were detected among the relative abundances of *Methanosaeta* and *Methanosarcina* throughout the experimental period (Fig. 2D). Competition among these two genera has been thoroughly reported in previous studies (Castellano-Hinojosa et al., 2018) and is explained by differences in their respective affinities for acetate and utilization rates, which favor the growth of *Methanosaeta* in environments with low concentrations of acetate or VFAs, or low OLR (Xu et al., 2018; Yang et al., 2014). This is consistent with the correlations observed here among the relative abundances of *Methanosaeta* and the operational parameters %VS/TS, VFA/ALK ratio, and OLR ($r = -0.99$, -0.66 and -0.65, respectively, Fig. 2E, Table S11).

In the AcD, *Methanolinea* were prevalent in phase I, but were displaced by *Methanosarcina* in phase II (Fig. 3), and this shift was connected with higher biogas and CH_4 production (Table 1A), making the separation of hydrolysis/acidogenesis from acetogenesis/methanogenesis only partial during phase II, as already addressed in Section 3.1. In addition, Fountoulakis et al. (2004) revealed the inhibitory effect of some pharmaceuticals (i.e., diclofenac, carbamazepine, or ofloxacin) on methanogens, and Symsaris et al. (2015) showed that these negative effects were more evident on acetoclastic methanogens than on hydrogenotrophic methanogens. The high ML of ofloxacin detected in the AcD effluent in phase I (1568.6 mg day^{-1} 1000 inh^{-1}, Table 3) could explain the under-representation of acetoclastic methanogens.

To sum up, the results of Illumina sequencing showed that methane generation in the two-stage MAD was mostly driven by the biological activity of carbon-dioxide reducing methanogens which use hydrogen or formate as primary electron donors (*Methanolinea*, *Methanospirillum*), or "versatile" methanogens such as *Methanosarcina*, able to generate CH_4 by acetoclastic, hydrogenoclastic, and/or methylotrophic pathways (Oren, 2019). Steady-state in MAD processes has been classically connected to the prevalence of acetoclastic methanogens; however, stable operation has been also thoroughly reported in systems dominated by *Methanospirillum* (reviewed by Saha et al., 2020).

3.5.3. Diversity of Fungi communities

Fig. S10 shows the heatmap of the relative abundances of fungal PHYs clustered at the Phylum and Order levels in both digesters of the two-stage MAD system during phase I. PHYs were affiliated to 7 different

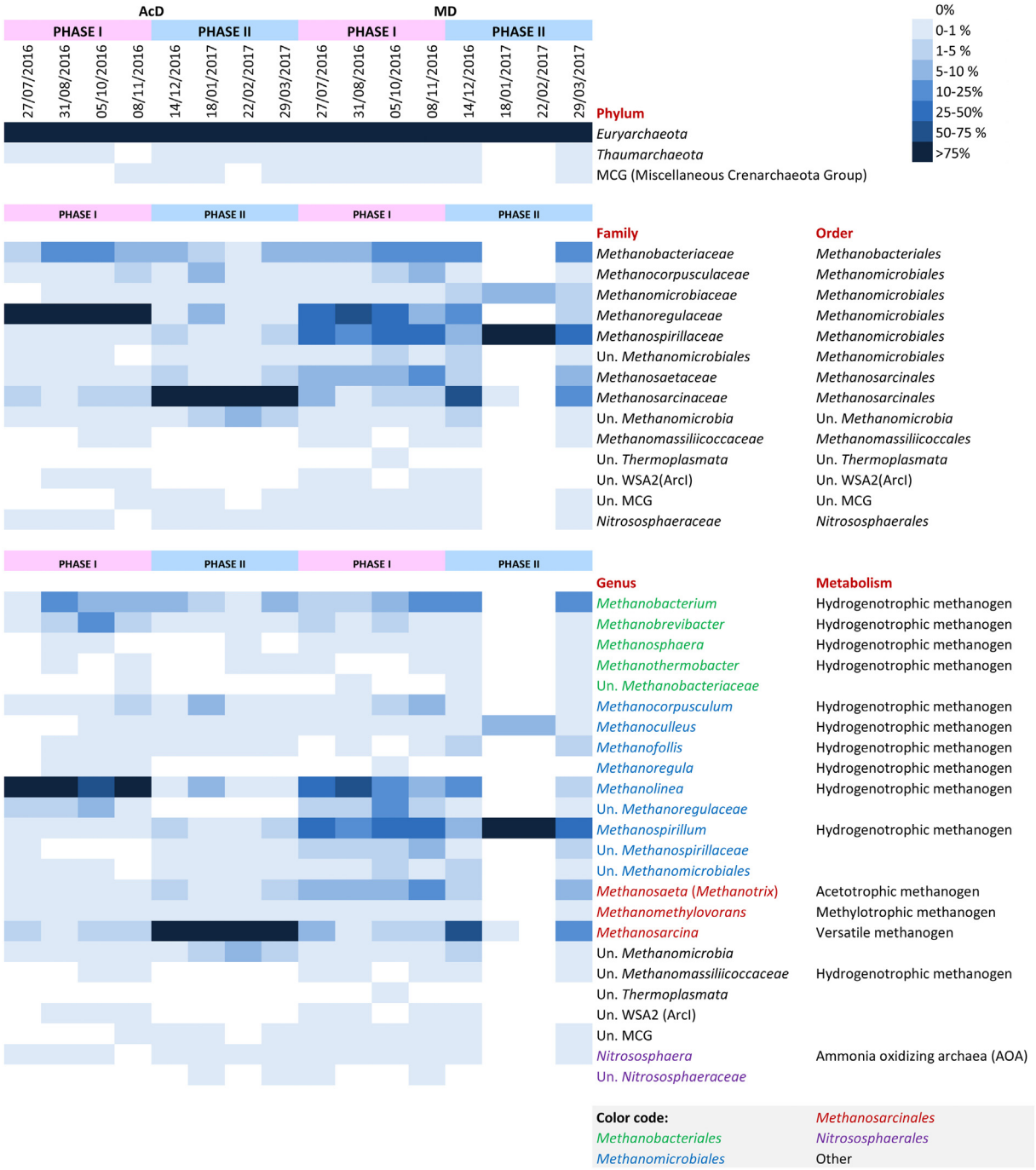


Fig. 3. Heatmap displaying the relative abundances of Archaeal phylotypes (Phylum, Family and Genus levels), detected by Illumina sequencing in the acidogenic digestor (AcD) and the methanogenic digestor (MD) effluent samples in the experimental phases I and II.

Phyla, and the community was dominated by *Ascomycota* in both AcD and MD effluent samples (87.69 and 81.60% average relative abundances, respectively), followed by *Basidiomycota* (7.07 and 10.23%, respectively), *Mortierellomycota* (4.29 and 6.13%), *Mucoromycota* (0.54 and 1.17%), *Chytridiomycota* (0.05 and 0.37%), *Blastocladiomycota* (0.12 and 0.32%), and *Kickxellomycota* (0.23 and 0.17%).

At the Order level, 41 different clades were detected, of which *Saccharomycetales* comprised nearly half of the identified reads (48.05%) and were followed in descending order of relative abundance by *Eurotiales*, *Hypocreales*, *Mortierellales*, *Trichosporonales*, and *Microascales* (15.91, 6.47, 5.12, 3.93 and 3.76%, respectively). Comparing among the two digesters (Fig. S10), *Saccharomycetales* displayed >55% relative abundance in all the samples of the AcD, while in the MD the fungal community was codominated by both *Saccharomycetales* and *Eurotiales* (35.03 and 21.85% average relative abundances, respectively). SIMPER analysis (Table S12) showed that the global similarities among the samples of the AcD and MD effluents were 42.54 and 45.37%, respectively. The fungal community was 61.89% dissimilar when comparing both digesters, and 15 PHYs mostly affiliated to the *Ascomycota* were required to explain >25% of such dissimilarity. Overall, the structure and diversity of the fungal communities in the AcD and MD effluents were similar to those previously characterized in the water line of the A^2O

system during phase I (Gallardo-Altamirano et al., 2019), except for an increase of the relative abundance of *Ascomycota* and a reduction of the relative abundance of *Blastocladiomycota*.

Not many studies have been devoted to identifying the fungal communities in anaerobic reactors, but those available demonstrate that fungi are viable and active in such environments and highlight relevant roles for these eukaryotic microorganisms in the hydrolysis of carbohydrates, lipids and proteins, and the generation of VFAs (Langer et al., 2019; Vinzelj et al., 2020). The dominance of *Ascomycota* and *Basidiomycota* in the fungal communities was previously observed in full-scale AD systems digesting or co-digesting feedstocks derived from plant, animal, or food sources (Bücker et al., 2020; Langer et al., 2019; Young et al., 2018). Several genera of the *Ascomycota* are facultatively anaerobic yeasts with fermentative metabolism which display a variety of traits potentially contributing to the AD process, such as lipolytic and proteolytic activities and the ability to produce VFAs (Bücker et al., 2020).

Strictly anaerobic fungi of the *Neocallimastigomycota* were not detected in the two-stage MAD plant analyzed in this study, nor in the water line of the associated A^2O system (Gallardo-Altamirano et al., 2019). Members of this Phylum inhabit the gut of herbivores and establish mutualistic symbiosis with methanogens, enhancing the digestion of plant material (Hooker et al., 2019). These interactions are postulated to occur also in engineered AD processes, since *Neocallimastigomycota* have been detected by cultivation-independent methods in biogas reactors fed with mixtures of lignocellulose-rich plant materials and animal wastes (Dollhofer et al., 2017; Kazda et al., 2014). However, absence of strictly anaerobic fungi was reported in analogous AD systems (Langer et al., 2019; Young et al., 2018), in agreement with the results presented here. Besides the nature of the feedstock, several other factors are reported to strongly influence the occurrence and activity of *Neocallimastigomycota* in biogas reactors, including temperature and SRT (Vinzelj et al., 2020).

3.6. Linking population dynamics of Bacteria and Archaea to the REs of PhACs in the two-stage MAD plant

Fig. 4. shows the MDS ordination of AcD and MD effluent samples based on the REs of 6 PhACs which were effectively removed in both digesters in the two experimental phases. Vectors representing the trends through the ordination of either the REs (Fig. 4A) or the relative abundances of bacterial (Fig. 4B, C) and archaeal PHYs (Fig. 4D) were overlapped on the plots, and the Pearson's product-moment correlation coefficients among the vectors are displayed in Table S13. Only strong positive correlations ($r > 0.75$) among REs and the relative abundances of bacterial and archaeal groups will be discussed here.

The REs of naproxen and ketoprofen correlated strongly with the relative abundances of several families of *Firmicutes* of the class *Clostridia*, plus the families *Propionibacteriaceae* (*Actinobacteria*) and *Cytophagaceae* (*Bacteroidetes*), and the versatile methanogenic genus *Methanosarcina*. Similar trends were observed for trimethoprim, although the correlations were weaker ($r < 0.75$) for some of the abovementioned groups. The REs of ofloxacin and fenofibrate displayed strong correlations with the relative abundances of 8 bacterial families each, which belonged to the *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* in the case of ofloxacin, and to the *Chloroflexi*, *Alpha-* and *Betaproteobacteria*, *Synergistetes*, and *Thermotoga* in the case of fenofibrate. Strong correlations were also found among ofloxacin REs and the relative abundances of 4 genera of methanogens, including the acetoclastic specialist *Methanosaeta*, while the REs of fenofibrate correlated with the relative abundances of *Methanobacterium* and *Methanocorpusculum*. Finally, the REs of atenolol increased concomitantly with the relative abundances of three bacterial families (*Intrasporangiaceae*, *Oxalobacteraceae*, and *Syntrophaceae*; *Actinobacteria*, *Beta-* and *Deltaproteobacteria*, respectively) plus the Candidate phyla Atribacteria and Cloacimonetes, and two genera of hydrogenotrophic methanogens (*Methanoculleus* and *Methanospirillum*).

According to the literature, atenolol, naproxen, and trimethoprim are usually removed very efficiently (>70%) in anaerobic WWT systems, while ofloxacin and ketoprofen are 30–70% removed (Lim et al., 2020), and few data are available for fenofibrate (Kujawa-Roeleveld et al., 2008). The biotransformation or biodegradation pathways followed by these and other PhACs in AD sludge remain largely unexplored to date; however, some findings reported in earlier studies which are in accordance with the observations presented in this work will be discussed below.

The biotransformation of naproxen and trimethoprim under anaerobic conditions is well characterized and takes place via *O*-demethylation of phenyl-methyl-ether groups (Ghattas et al., 2017). Wolfson et al. (2018, 2019) characterized the diversity of two consortia able to *O*-demethylate naproxen under methanogenic and sulfate-reducing conditions, observing an enrichment of fermentative and acetogenic bacteria, mostly belonging to the Phylum *Bacteroidetes* and/or the Class *Clostridia*; in addition, these authors postulated that both acetoclastic and hydrogenotrophic methanogens could contribute to convert the methyl groups of naproxen into methane, either by readily using acetate or through an association with syntrophic acetate oxidizers, respectively. Interestingly, no overlap of the bacterial community structure was found among the two consortia, since the dominating families differed depending on the source of the inoculum (AD sludge or anoxic marine sediments), supporting that biotransformation of naproxen should rely on functionally redundant taxa. Similarly, Liang et al. (2019) reported the biotransformation of trimethoprim under sulfate-reducing conditions through *O*-demethylation and further degradation of the hydroxylated derivate, by consortia dominated (>60% cumulative abundance) by Classes *Bacteroidia*, *Sphingobacteriia*, (*Bacteroidetes*), *Clostridia* (*Firmicutes*), and *Deltaproteobacteria* (*Proteobacteria*). The positive correlations found here among the REs of naproxen and trimethoprim and the relative abundances of *Cytophagaceae* (*Bacteroidetes*), the acetogens of the Class *Clostridia* (ie. *Christensenellaceae*, *Halanaerobiaceae*, *Peptostreptococcaceae*, *Ruminococcacee*) and the Genus *Methanosarcina* (Table S13) are in accordance with the aforementioned studies and suggest that analogous patterns of biotransformation could take place in the two-stage MAD, although confirming this hypothesis will require additional research efforts.

Fluoroquinolone antibiotics tend to adsorb to sludge in WWTPs, hence reaching significant concentrations in AD systems, and are regarded as not easily biodegradable (Rusch et al., 2019). Nonetheless, these PhACs undergo a variety of biological transformations in the environment, mediated by both fungi and bacteria. Over 20 transformation products have been identified to date for ciprofloxacin, either generated by modification reactions (ie., acetylation, formylation, nitrosation, or succinylation); cleavage of the piperazine ring; or through the decarboxylation, defluorination, or hydroxylation of the fluoroquinolone core structure (Rusch et al., 2019). Six of the characterized transformation products were identified in a lab-scale sulfate-reducing up-flow sludge bed reactor, and transformations involving hydroxylation and cleavage of the piperazine ring were postulated (Jia et al., 2019). It is assumed that, due to the large number of possible reactions, consortia of mixed species are more likely to be involved with biodegradation of fluoroquinolones in the environment (Rusch et al., 2019). Accordingly, in the present study, ofloxacin displayed strong positive correlations with a large number of evolutionarily dispersed bacterial families (Fig. 4, Table S13).

Fenofibrate is a highly hydrophobic compound which mostly adsorbs onto sludge of WWT systems (Faria et al., 2020). To the best of the authors' knowledge, no published studies are available concerning neither the possible biodegradation/biotransformation pathways nor the resulting metabolites of fenofibrate and the structurally related compounds bezafibrate and gemfibrozil under anaerobic conditions. Since fenofibrate is an ester and bezafibrate an amide, their structures are prone to hydrolysis reactions (de Oliveira et al., 2016), and indeed,

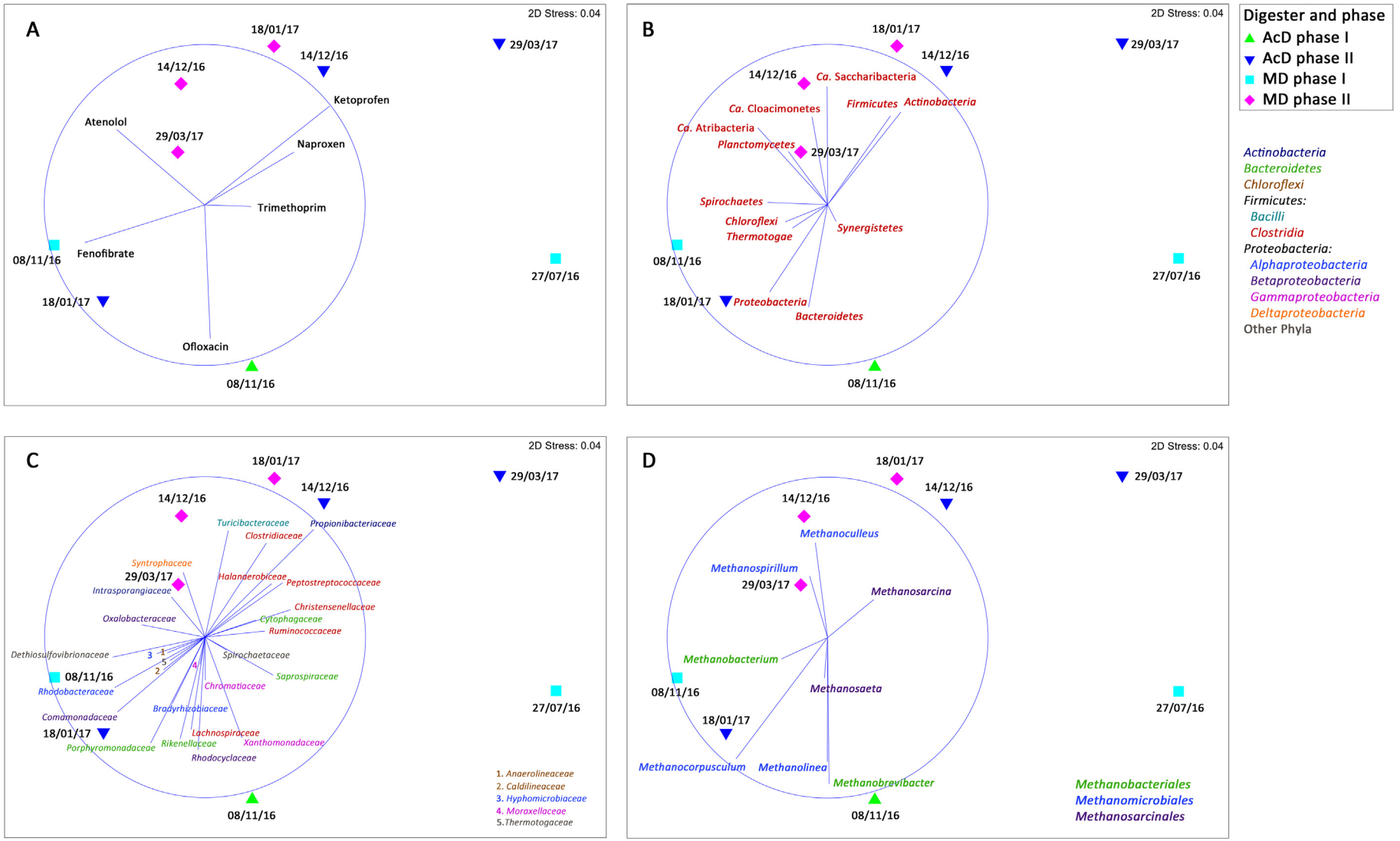


Fig. 4. A. Non-metric multidimensional scaling (MDS) plots, illustrating the ordinations of the samples retrieved from the acidogenic digestor (AcD) and the methanogenic digestor (MD) in the experimental phases I and II, according to the removal efficiencies of the PhACs atenolol, fenofibrate, ketoprofen, naproxen, ofloxacin, and trimethoprim, which are represented by vectors. B. Correlations among the relative abundances of Bacteria at the Phylum level (represented by vectors) and the ordination shown in plot A. C. Correlations among the relative abundances of Bacteria at the Family level (represented by vectors) and the ordination shown in plot A. C. Correlations among the relative abundances of Archaea at the Genus level (represented by vectors) and the ordination shown in plot A. Only the prokaryotic taxa with an average relative abundance ≥1% in a set of samples retrieved from the same digester and phase are represented. The stress level of the MDS plots (<0.1) validates the 2D-representation of the biotic data distribution (Clarke and Warwick, 2001).

biodegradation of bezafibrate to 4-chlorobenzoic acid through hydrolysis of the amide bond was reported by Quintana et al. (2005) in a membrane bioreactor system operated aerobically. Interestingly, in this study the relative abundance of the *Rhodobacteraceae* showed a strong positive correlation with the REs of fenofibrate ($r = 0.98$), and similar patterns were previously observed for bezafibrate and gemfibrozil in the water line of the A^2O system associated to the two stage-MAD (Gallardo-Altamirano et al., 2019). The Family *Rhodobacteraceae* comprises phenotypically, metabolically, and ecologically diverse species, widely distributed in aquatic environments, including WWTPs (Pujalte et al., 2014). Species of the *Rhodobacteraceae* often carry genes for the aerobic and anaerobic degradation of aromatic compounds (Carmona et al., 2009; Pérez-Pantoja et al., 2016); however, the possible involvement of these bacteria in the degradation of PhACs in AD systems has not been explored.

4. Conclusions

The results presented here provide new insights about the mass balance and REs of 27 PhACs in a two-stage MAD plant treating sewage sludge, also revealing robust interlinkages among operational parameters, performance of methanogenesis, shifts of the microbial communities' structure, and REs of PhACs. From the operational/performance point of view, it is concluded that an SRT of ≤2 days in the AcD is required to ensure effective separation of the acidogenesis and methanogenesis processes in two-stage MAD systems treating sewage sludge with %TS < 3%. SRT was also the most significant operational parameter influencing PhACs removal; in particular, the longer SRT used in the MD (24 d) significantly improved the REs of carbamazepine, clarithromycin, gemfibrozil, ibuprofen, lorazepam, and propranolol. Besides SRT, ORL and VFA/ALK were also linked to shifts of the Bacteria and Archaea communities' structure in the AcD and MD sludges, which were subsequently correlated with differences in the efficiency of methanogenesis (VSR, CH_4 in biogas, and CH_4 recovery rate) among the experimental phases. Robust correlations were also detected among the REs of atenolol, fenofibrate, ketoprofen, naproxen, ofloxacin, and trimethoprim, and the relative abundances of several bacterial and archaeal groups, pointing out to their potential involvement in the biodegradation/biotransformation of these xenobiotics under AD conditions.

CRediT authorship contribution statement

M.J. Gallardo-Altamirano: Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft. **P. Maza-Márquez:** Investigation, Formal analysis, Visualization. **N. Montemurro:** Methodology, Formal analysis. **S. Pérez:** Methodology, Formal analysis. **B. Rodelas:** Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **F. Osorio:** Conceptualization, Project administration, Funding acquisition. **C. Pozo:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was funded by Spanish Ministerio de Economía y Competitividad (MINECO) and Fondo Europeo de Desarrollo Regional (FEDER) (CTM2014-60131-P). MINECO is also acknowledged for personal grants to P. Maza-Márquez (postdoctoral fellow) and M.J. Gallardo-Altamirano (FPI program, BES-2015-073595). Regional Goverment of Murcia, Spain (ESAMUR) and Murcia Water Works (EMUASA) are acknowledged for their collaboration in this research project and provision of facilities at Murcia Este Wastewater Treatment Plant (Murcia, Spain). The authors also acknowledge SCIEX for providing the loan of instrument LC/HRMS QToF X500R.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147869.

References

Angeles, L.F., Mullen, R.A., Huang, I.J., Wilson, C., Khunjar, W., Sirotkin, H.I., McElroy, A.E., Aga, D.S., 2020. Assessing pharmaceutical removal and reduction in toxicity provided by advanced wastewater treatment systems. Environ. Sci. Water Res. Technol. 6, 62–77. https://doi.org/10.1039/c9ew00559e.

Appels, L., Baeyens, J., Degrève, J., Dewil, R., 2008. Principles and potential of the anaerobic digestion of waste-activated sludge. Prog. Energy Combust. Sci. 34, 755–781. https://doi.org/10.1016/j.pecs.2008.06.002.

aus der Beek, T., Weber, F.A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., Küster, A., 2016. Pharmaceuticals in the environment—global occurrences and perspectives. Environ. Toxicol. Chem. 35, 823–835. https://doi.org/10.1002/etc.3339.

Baird, R., Bridgewater, L., American Public Health Association, American Water Works Association, Water Environment Federation, 2017. Standard Methods for the Examination of Water and Wastewater. 23rd ed. American Public Health Association (APHA), Washington, D.C.

Bhattacharya, S.K., Madura, R.L., Walling, D.A., Farrell, J.B., 1996. Volatile solids reduction in two-phase and conventional anaerobic sludge digestion. Water Res. 30, 1041–1048. https://doi.org/10.1016/0043-1354(95)00252-9.

Biddle, A., Stewart, L., Blanchard, J., Leschine, S., 2013. Untangling the genetic basis of fibrolytic specialization by lachnospiraceae and ruminococcaceae in diverse gut communities. Diversity 5, 627–640. https://doi.org/10.3390/d5030627.

Bisognin, R.P., Wolff, D.B., Carissimi, E., Prestes, O.D., Zanella, R., Storck, T.R., Clasen, B., 2020. Potential environmental toxicity of sewage effluent with pharmaceuticals. Ecotoxicology 29, 1315–1326. https://doi.org/10.1007/s10646-020-02264-7.

Bosshard, P.P., Zbinden, R., Altwegg, M., 2002. *Turicibacter sanguinis* gen. nov., sp. nov., a novel anaerobic, Gram-positive bacterium. Int. J. Syst. Evol. Microbiol. 52, 1263–1266. https://doi.org/10.1099/00207713-52-4-1263.

Bücker, F., Marder, M., Peiter, M.R., Lehn, D.N., Esquerdo, V.M., Antonio de Almeida Pinto, L., Konrad, O., 2020. Fish waste: an efficient alternative to biogas and methane production in an anaerobic mono-digestion system. Renew. Energy 147, 798–805. https://doi.org/10.1016/j.renene.2019.08.140.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 6, 1621–1624. https://doi.org/10.1038/ismej.2012.8.

Carballa, M., Fink, G., Omil, F., Lema, J.M., Ternes, T., 2008. Determination of the solid-water distribution coefficient (Kd) for pharmaceuticals, estrogens and musk fragrances in digested sludge. Water Res. 42, 287–295. https://doi.org/10.1016/j.watres.2007.07.012.

Carballa, M., Omil, F., Ternes, T., Lema, J.M., 2007. Fate of pharmaceutical and personal care products (PPCPs) during anaerobic digestion of sewage sludge. Water Res. 41, 2139–2150. https://doi.org/10.1016/j.watres.2007.02.012.

Carmona, M., Zamarro, M.T., Blázquez, B., Durante-Rodríguez, G., Juárez, J.F., Valderrama, J.A., Barragán, M.J., García, J.L., Díaz, E., 2009. Anaerobic catabolism of aromatic compounds: a genetic and genomic view. Microbiol. Mol. Biol. Rev. 73, 71–133. https://doi.org/10.1128/mmbr.00021-08.

Castellano-Hinojosa, A., Armato, C., Pozo, C., González-Martínez, A., González-López, J., 2018. New concepts in anaerobic digestion processes: recent advances and biological aspects. Appl. Microbiol. Biotechnol. 102, 5065–5076. https://doi.org/10.1007/s00253-018-9039-9.

Christodoulou, A., Stamatelatou, K., 2016. Overview of legislation on sewage sludge management in developed countries worldwide. Water Sci. Technol. 73, 453–462. https://doi.org/10.2166/wst.2015.521.

Clarke, K.R., Warwick, R., 2001. Change in Marine Communities: an Approach to Statistical Analysis and Interpretation.. Plymouth Marine Laboratory, Plymouth, UK.

Coutu, S., Wyrsch, V., Wynn, H.K., Rossi, L., Barry, D.A., 2013. Temporal dynamics of antibiotics in wastewater treatment plant influent. Sci. Total Environ. 458–460, 20–26. https://doi.org/10.1016/j.scitotenv.2013.04.017.

de Oliveira, M.A., da Silva, G.D., Campos, M.S.T., 2016. Chemical degradation kinetics of fibrates: bezafibrate, ciprofibrate and fenofibrate. Braz. J. Pharm. Sci. 52, 545–553. https://doi.org/10.1590/s1984-82502016000300019.

Detman, A., Mielecki, D., Pleśniak, Ł., Bucha, M., Janiga, M., Matyasik, I., Chojnacka, A., Jędrysek, M.O., Błaszczyk, M.K., Sikora, A., 2018. Methane-yielding microbial communities processing lactate-rich substrates: a piece of the anaerobic digestion puzzle. Biotechnol. Biofuels 11, 116. https://doi.org/10.1186/s13068-018-1106-z.

DiLallo, R., Albertson, O.E., 1961. Volatile acids by direct titration. J. Water Pollut. Control Fed. 33, 356–365. https://doi.org/10.2307/25034391.

Dollhofer, V., Callaghan, T.M., Griffith, G.W., Lebuhn, M., Bauer, J., 2017. Presence and transcriptional activity of anaerobic fungi in agricultural biogas plants. Bioresour. Technol. 235, 131–139. https://doi.org/10.1016/j.biortech.2017.03.116.

Drake, H.L., Küsel, K., Matthies, C., 2014. Acetogenic prokaryotes. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes 4t Ed: Prokaryotic Physiology and Biochemistry. Springer-Verlag, Berlin Heidelberg, pp. 3–60 https://doi.org/10.1007/978-3-642-30141-4_61.

Dubey, M., Mohapatra, S., Tyagi, V.K., Suthar, S., Kazmi, A.A., 2021. Occurrence, fate, and persistence of emerging micropollutants in sewage sludge treatment. Environ. Pollut. 273, 116515. https://doi.org/10.1016/j.envpol.2021.116515.

Fan, L., McElroy, K., Thomas, T., 2012. Reconstruction of ribosomal RNA genes from metagenomic data. PLoS One 7, e39948. https://doi.org/10.1371/journal.pone.0039948.

Faria, C.V., Ricci, B.C., Silva, A.F.R., Amaral, M.C.S., Fonseca, F.V., 2020. Removal of micropollutants in domestic wastewater by expanded granular sludge bed membrane bioreactor. Process. Saf. Environ. Prot. 136, 223–233. https://doi.org/10.1016/j.psep.2020.01.033.

Fountoulakis, M., Drilla, P., Stamatelatou, K., Lyberatos, G., 2004. Toxic effect of pharmaceuticals on methanogenesis. Water Sci. Technol. 50, 335–340. https://doi.org/10.2166/wst.2004.0346.

Gago-Ferrero, P., Borova, V., Dasenaki, M.E., Thomaidis, N.S., 2015. Simultaneous determination of 148 pharmaceuticals and illicit drugs in sewage sludge based on ultrasound-assisted extraction and liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 407, 4287–4297. https://doi.org/10.1007/s00216-015-8540-6.

Gallardo-Altamirano, M.J., Maza-Márquez, P., Montemurro, N., Rodelas, B., Osorio, F., Pozo, C., 2019. Linking microbial diversity and population dynamics to the removal efficiency of pharmaceutically active compounds (PhACs) in an anaerobic/anoxic/aerobic (A^2O) system. Chemosphere 233, 828–842. https://doi.org/10.1016/j.chemosphere.2019.06.017.

Gallardo-Altamirano, M.J.J., Maza-Márquez, P., Peña-Herrera, J.M.M., Rodelas, B., Osorio, F., Pozo, C., 2018. Removal of anti-inflammatory/analgesic pharmaceuticals from urban wastewater in a pilot-scale A^2O system: linking performance and microbial population dynamics to operating variables. Sci. Total Environ. 643, 1481–1492. https://doi.org/10.1016/j.scitotenv.2018.06.284.

Ghattas, A.K., Fischer, F., Wick, A., Ternes, T.A., 2017. Anaerobic biodegradation of (emerging) organic contaminants in the aquatic environment. Water Res. 116, 268–295. https://doi.org/10.1016/j.watres.2017.02.001.

Ghosh, S., 1987. Improved sludge gasification by two-phase anaerobic digestion. J. Environ. Eng. 113, 1265–1284. https://doi.org/10.1061/(asce)0733-9372(1987)113:6(1265.

Gonzalez-Gil, L., Krah, D., Ghattas, A.K., Carballa, M., Wick, A., Helmholz, L., Lema, J.M., Ternes, T.A., 2019. Biotransformation of organic micropollutants by anaerobic sludge enzymes. Water Res. 152, 202–214. https://doi.org/10.1016/j.watres.2018.12.064.

Gonzalez-Gil, L., Papa, M., Feretti, D., Ceretti, E., Mazzoleni, G., Steimberg, N., Pedrazzani, R., Bertanza, G., Lema, J.M.M., Carballa, M., 2016. Is anaerobic digestion effective for the removal of organic micropollutants and biological activities from sewage sludge? Water Res. 102, 211–220. https://doi.org/10.1016/j.watres.2016.06.025.

Graf, J., 2014. The family *Rikenellaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes 4t Ed: Other Major Lineages of Bacteria and the Archaea. Springer-Verlag, Berlin Heidelberg, pp. 857–859 https://doi.org/10.1007/978-3-642-38954-2_134.

Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. Past: paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4, 178.

Hao, L., Bize, A., Conteau, D., Chapleur, O., Courtois, S., Kroff, P., Desmond-Le Quéméner, E., Bouchez, T., Mazéas, L., 2016. New insights into the key microbial phylotypes of anaerobic sludge digesters under different operational conditions. Water Res. 102, 158–169. https://doi.org/10.1016/j.watres.2016.06.014.

Hooker, C.A., Lee, K.Z., Solomon, K.V., 2019. Leveraging anaerobic fungi for biotechnology. Curr. Opin. Biotechnol. 59, 103–110. https://doi.org/10.1016/j.copbio.2019.03.013.

Hsieh, T.C., Ma, K.H., Chao, A., 2016. iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). Methods Ecol. Evol. 7, 1451–1456. https://doi.org/10.1111/2041-210X.12613.

Jang, H.M., Kim, J.H., Ha, J.H., Park, J.M., 2014. Bacterial and methanogenic archaeal communities during the single-stage anaerobic digestion of high-strength food wastewater. Bioresour. Technol. 165, 174–182. https://doi.org/10.1016/j.biortech.2014.02.028.

Jelić, A., Fatone, F., Di Fabio, S., Petrovic, M., Cecchi, F., Barcelo, D., 2012. Tracing pharmaceuticals in a municipal plant for integrated wastewater and organic solid waste treatment. Sci. Total Environ. 433, 352–361. https://doi.org/10.1016/j.scitotenv.2012.06.059.

Jelić, A., Petrović, M., Barceló, D., 2009. Multi-residue method for trace level determination of pharmaceuticals in solid samples using pressurized liquid extraction followed by liquid chromatography/quadrupole-linear ion trap mass spectrometry. Talanta 80, 363–371. https://doi.org/10.1016/j.talanta.2009.06.077.

Jia, Y., Zhang, H., Khanal, S.K., Yin, L., Lu, H., 2019. Insights into pharmaceuticals removal in an anaerobic sulfate-reducing bacteria sludge system. Water Res. 161, 191–201. https://doi.org/10.1016/j.watres.2019.06.010.

Kazda, M., Langer, S., Bengelsdorf, F.R., 2014. Fungi open new possibilities for anaerobic fermentation of organic residues. Energy. Sustain. Soc. 4, 1–9. https://doi.org/10.1186/2192-0567-4-6.

Kelessidis, A., Stasinakis, A.S., 2012. Comparative study of the methods used for treatment and final disposal of sewage sludge in European countries. Waste Manag. 32, 1186–1195. https://doi.org/10.1016/j.wasman.2012.01.012.

Kim, Y.M., Jang, H.M., Lee, K., Chantrasakdakul, P., Kim, D., Park, K.Y., 2015. Changes in bacterial and archaeal communities in anaerobic digesters treating different organic wastes. Chemosphere 141, 134–137. https://doi.org/10.1016/j.chemosphere.2015.06.086.

Kindaichi, T., Yamaoka, S., Uehara, R., Ozaki, N., Ohashi, A., Albertsen, M., Nielsen, P.H., Nielsen, J.L., 2016. Phylogenetic diversity and ecophysiology of *Candidate* phylum Saccharibacteria in activated sludge. FEMS Microbiol. Ecol. 92, 1–11. https://doi.org/10.1093/femsec/fiw078.

Kujawa-Roeleveld, K., Els, S., Grotenhuis, T., Kragić, D., Mels, A., Zeeman, G., 2008. Biodegradability of human pharmaceutically active compounds (PhAC) in biological system treating source separated wastewater streams. Proceedings of the "Third SWITCH Scientific Meeting." Belo Horizonte, Brazil.

La Reau, A.J., Suen, G., 2018. The Ruminococci: key symbionts of the gut ecosystem. J. Microbiol. 56, 199–208. https://doi.org/10.1007/s12275-018-8024-4.

Langer, S.G., Gabris, C., Einfalt, D., Wemheuer, B., Kazda, M., Bengelsdorf, F.R., 2019. Different response of bacteria, archaea and fungi to process parameters in nine full-scale anaerobic digesters. Microb. Biotechnol. 12, 1210–1225. https://doi.org/10.1111/1751-7915.13409.

Liang, B., Kong, D., Qi, M., Yun, H., Li, Z., Shi, K., Chen, E., Vangnai, A.S., Wang, A., 2019. Anaerobic biodegradation of trimethoprim with sulfate as an electron acceptor. Front. Environ. Sci. Eng. 13, 1–10. https://doi.org/10.1007/s11783-019-1168-6.

Lim, M., Patureau, D., Heran, M., Lesage, G., Kim, J., 2020. Removal of organic micropollutants in anaerobic membrane bioreactors in wastewater treatment: critical review. Environ. Sci. Water Res. Technol. 6, 1230–1243. https://doi.org/10.1039/c9ew01058k.

Liu, C.M., Kachur, S., Dwan, M.G., Abraham, A.G., Aziz, M., Hsueh, P.-R.R., Huang, Y.-T.T., Busch, J.D., Lamit, L.J., Gehring, C.A., Keim, P., Price, L.B., 2012. FungiQuant: a broad-coverage fungal quantitative real-time PCR assay. BMC Microbiol. 12, 255. https://doi.org/10.1186/1471-2180-12-255.

Ma, S. Jia, Ma, H. Jun, Hu, H. Dong, Ren, H. Qiang, 2019. Effect of mixing intensity on hydrolysis and acidification of sewage sludge in two-stage anaerobic digestion: characteristics of dissolved organic matter and the key microorganisms. Water Res. 148, 359–367. https://doi.org/10.1016/j.watres.2018.10.058.

Magot, M., Ravot, G., Campaignolle, X., Ollivier, B., Patel, B.K.C., Fardeau, M.L., Thomas, P., Crolet, J.L., Garcia, J.L., 1997. *Dethiosulfovibrio peptidovorans* gen. nov., sp. nov., a new anaerobic, slightly halophilic, thiosulfate-reducing bacterium from corroding offshore oil wells. Int. J. Syst. Bacteriol. 47, 818–824. https://doi.org/10.1099/00207713-47-3-818.

Martín, J., Camacho-Muñoz, D., Santos, J.L., Aparicio, I., Alonso, E., 2012. Occurrence of pharmaceutical compounds in wastewater and sludge from wastewater treatment plants: removal and ecotoxicological impact of wastewater discharges and sludge disposal. J. Hazard. Mater. 239–240, 40–47. https://doi.org/10.1016/j.jhazmat.2012.04.068.

Martín, J., Santos, J.L., Aparicio, I., Alonso, E., 2015. Pharmaceutically active compounds in sludge stabilization treatments: anaerobic and aerobic digestion, wastewater stabilization ponds and composting. Sci. Total Environ. 503–504, 97–104. https://doi.org/10.1016/j.scitotenv.2014.05.089.

Martín-Pascual, J., Rueda-Pérez, J.J., Jóver, M., Hontoria, E., Poyatos, J.M., 2017. Optimization of the acid stage of a two-stage anaerobic digestion process to treat urban wastewater sludge. J. Environ. Eng. 143, 04017038. https://doi.org/10.1061/(asce)ee.1943-7870.0001237.

Martín-Pozo, L., de Alarcón-Gómez, B., Rodríguez-Gómez, R., García-Córcoles, M.T., Çipa, M., Zafra-Gómez, A., 2019. Analytical methods for the determination of emerging contaminants in sewage sludge samples. A review. Talanta 192, 508–533. https://doi.org/10.1016/j.talanta.2018.09.056.

Maspolim, Y., Zhou, Y., Guo, C., Xiao, K., Ng, W.J., 2015a. Determination of the archaeal and bacterial communities in two-phase and single-stage anaerobic systems by 454 pyrosequencing. J. Environ. Sci. (China) 36, 121–129. https://doi.org/10.1016/j.jes.2015.02.017.

Maspolim, Y., Zhou, Y., Guo, C., Xiao, K., Ng, W.J., 2015b. Comparison of single-stage and two-phase anaerobic sludge digestion systems — performance and microbial community dynamics. Chemosphere 140, 54–62. https://doi.org/10.1016/j.chemosphere.2014.07.028.

Maza-Márquez, P., Aranda, E., González-López, J., Rodelas, B., 2020. Evaluation of the abundance of fungi in wastewater treatment plants using quantitative PCR (qPCR). Methods Mol. Biol. 2065, 79–94. https://doi.org/10.1007/978-1-4939-9833-3_7.

Maza-Márquez, P., Vilchez-Vargas, R., Kerckhof, F.M.M., Aranda, E., González-López, J., Rodelas, B., 2016. Community structure, population dynamics and diversity of fungi in a full-scale membrane bioreactor (MBR) for urban wastewater treatment. Water Res. 105, 507–519. https://doi.org/10.1016/j.watres.2016.09.021.

McBride, M.J., Liu, W., Lu, X., Zhu, Y., Zhang, W., 2014. The family *Cytophagaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes 4t Ed: Other Major Lineages of Bacteria and the Archaea. Springer-Verlag, Berlin Heidelberg, pp. 577–593 https://doi.org/10.1007/978-3-642-38954-2_382.

McIlroy, S.J., Kirkegaard, R.H., Dueholm, M.S., Fernando, E., Karst, S.M., Albertsen, M., Nielsen, P.H., 2017. Culture-independent analyses reveal novel Anaerolineaceae as abundant primary fermenters in anaerobic digesters treating waste activated sludge. Front. Microbiol. 8, 1134. https://doi.org/10.3389/fmicb.2017.01134.

Metcalf, Eddy, I., 2003. Wastewater Engineering: Treatment and Reuse. 4th ed. McGraw-Hill, New York.

Militon, C., Hamdi, O., Michotey, V., Fardeau, M.L., Ollivier, B., Bouallagui, H., Hamdi, M., Bonin, P., 2015. Ecological significance of *Synergistetes* in the biological treatment of tuna cooking wastewater by an anaerobic sequencing batch reactor. Environ. Sci. Pollut. Res. 22, 18230–18238. https://doi.org/10.1007/s11356-015-4973-x.

Narihiro, T., Nobu, M.K., Kim, N.K., Kamagata, Y., Liu, W.T., 2015. The nexus of syntrophy-associated microbiota in anaerobic digestion revealed by long-term enrichment and community survey. Environ. Microbiol. 17, 1707–1720. https://doi.org/10.1111/1462-2920.12616.

Narumiya, M., Nakada, N., Yamashita, N., Tanaka, H., 2013. Phase distribution and removal of pharmaceuticals and personal care products during anaerobic sludge digestion. J. Hazard. Mater. 260, 305–312. https://doi.org/10.1016/j.jhazmat.2013.05.032.

Nelson, M.C., Morrison, M., Yu, Z., 2011. A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresour. Technol. 102, 3730–3739. https://doi.org/10.1016/j.biortech.2010.11.119.

Nettmann, E., Bergmann, I., Pramschüfer, S., Mundt, K., Plogsties, V., Herrmann, C., Klocke, M., 2010. Polyphasic analyses of methanogenic archaeal communities in agricultural biogas plants. Appl. Environ. Microbiol. 76, 2540–2548. https://doi.org/10.1128/AEM.01423-09.

Nobu, M.K., Dodsworth, J.A., Murugapiran, S.K., Rinke, C., Gies, E.A., Webster, G., Schwientek, P., Kille, P., Parkes, R.J., Sass, H., Jørgensen, B.B., Weightman, A.J., Liu, W.T., Hallam, S.J., Tsiamis, G., Woyke, T., Hedlund, B.P., 2016. Phylogeny and physiology of candidate phylum "Atribacteria" (OP9/JS1) inferred from cultivation-independent genomics. ISME J. 10, 273–286. https://doi.org/10.1038/ismej.2015.97.

Oren, A., 2014. The order *Halanaerobiales*, and the families *Halanaerobiaceae* and *Halobacteroidaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes 4t Ed: *Firmicutes* and *Tenericutes*. Springer-Verlag, Berlin Heidelberg, pp. 153–177 https://doi.org/10.1007/978-3-642-30120-9_218.

Oren, A., 2019. Euryarchaeota. Encyclopedia of Life Sciences. Wiley Online Library https://doi.org/10.1002/9780470015902.a0004243.pub3.

Paíga, P., Correia, M., Fernandes, M.J., Silva, A., Carvalho, M., Vieira, J., Jorge, S., Silva, J.G., Freire, C., Delerue-Matos, C., 2019. Assessment of 83 pharmaceuticals in WWTP influent and effluent samples by UHPLC-MS/MS: hourly variation. Sci. Total Environ. 648, 582–600. https://doi.org/10.1016/j.scitotenv.2018.08.129.

Papageorgiou, M., Kosma, C., Lambropoulou, D., 2016. Seasonal occurrence, removal, mass loading and environmental risk assessment of 55 pharmaceuticals and personal care products in a municipal wastewater treatment plant in Central Greece. Sci. Total Environ. 543, 547–569. https://doi.org/10.1016/j.scitotenv.2015.11.047.

Park, J., Yamashita, N., Park, C., Shimono, T., Takeuchi, D.M., Tanaka, H., 2017. Removal characteristics of pharmaceuticals and personal care products: comparison between membrane bioreactor and various biological treatment processes. Chemosphere 179, 347–358. https://doi.org/10.1016/j.chemosphere.2017.03.135.

Pérez-Lemus, N., López-Serna, R., Pérez-Elvira, S.I., Barrado, E., 2019. Analytical methodologies for the determination of pharmaceuticals and personal care products (PPCPs) in sewage sludge: a critical review. Anal. Chim. Acta 1083, 19–40. https://doi.org/10.1016/j.aca.2019.06.044.

Pérez-Pantoja, D., Donoso, R., Junca, H., González, B., Pieper, D.H., 2016. Phylogenomics of aerobic bacterial degradation of aromatics. Aerobic Utilization of Hydrocarbons, Oils and Lipids. Springer International Publishing, pp. 1–48 https://doi.org/10.1007/978-3-319-39782-5_33-1.

Phan, H.V., Wickham, R., Xie, S., McDonald, J.A., Khan, S.J., Ngo, H.H., Guo, W., Nghiem, L.D., 2018. The fate of trace organic contaminants during anaerobic digestion of primary sludge: a pilot scale study. Bioresour. Technol. 256, 384–390. https://doi.org/10.1016/j.biortech.2018.02.040.

Ponsá, S., Ferrer, I., Vázquez, F., Font, X., 2008. Optimization of the hydrolytic-acidogenic anaerobic digestion stage (55 °C) of sewage sludge: influence of pH and solid content. Water Res. 42, 3972–3980. https://doi.org/10.1016/j.watres.2008.07.002.

Pujalte, M.J., Lucena, T., Ruvira, M.A., Arahal, D.R., Macián, M.C., 2014. The family *Rhodobacteraceae*. The Prokaryotes: *Alphaproteobacteria* and *Betaproteobacteria* 9783642301, pp. 439–512 https://doi.org/10.1007/978-3-642-30197-1_377.

Quintana, J.B., Weiss, S., Reemtsma, T., 2005. Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. Water Res. 39, 2654–2664. https://doi.org/10.1016/j.watres.2005.04.068.

Radjenović, J., Petrović, M., Barceló, D., 2009. Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. Water Res. 43, 831–841. https://doi.org/10.1016/j.watres.2008.11.043.

Rusch, M., Spielmeyer, A., Zorn, H., Hamscher, G., 2019. Degradation and transformation of fluoroquinolones by microorganisms with special emphasis on ciprofloxacin. Appl. Microbiol. Biotechnol. 103, 6933–6948. https://doi.org/10.1007/s00253-019-10017-8.

Saha, S., Basak, B., Hwang, J.H., Salama, E.S., Chatterjee, P.K., Jeon, B.H., 2020. Microbial symbiosis: a network towards biomethanation. Trends Microbiol. 17. https://doi.org/10.1016/j.tim.2020.03.012.

Samaras, V.G., Stasinakis, A.S., Mamais, D., Thomaidis, N.S., Lekkas, T.D., 2013. Fate of selected pharmaceuticals and synthetic endocrine disrupting compounds during wastewater treatment and sludge anaerobic digestion. J. Hazard. Mater. 244–245, 259–267. https://doi.org/10.1016/J.JHAZMAT.2012.11.039.

Samaras, V.G., Stasinakis, A.S., Thomaidis, N.S., Mamais, D., Lekkas, T.D., 2014. Fate of selected emerging micropollutants during mesophilic, thermophilic and temperature co-phased anaerobic digestion of sewage sludge. Bioresour. Technol. 162, 365–372. https://doi.org/10.1016/j.biortech.2014.03.154.

Slobodkin, A., 2014. The family *Peptostreptococcaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes 4t Ed: *Firmicutes* and *Tenericutes*. Springer-Verlag, Berlin Heidelberg, pp. 291–302 https://doi.org/10.1007/978-3-642-30120-9_217.

Smith, A.L., Shimada, T., Raskin, L., 2017. A comparative evaluation of community structure in full-scale digesters indicates that two-phase digesters exhibit greater microbial diversity than single-phase digesters. Environ. Sci. Water Res. Technol. 3, 304–311. https://doi.org/10.1039/c6ew00320f.

Stasinakis, A.S., 2012. Review on the fate of emerging contaminants during sludge anaerobic digestion. Bioresour. Technol. 121, 432–440. https://doi.org/10.1016/j.biortech.2012.06.074.

Su, X.L., Tian, Q., Zhang, J., Yuan, X.Z., Shi, X.S., Guo, R.B., Qiu, Y.L., 2014. *Acetobacteroides hydrogenigenes* gen. nov., sp. nov., an anaerobic hydrogen-producing bacterium in the Family *Rikenellaceae* isolated from a reed swamp. Int. J. Syst. Evol. Microbiol. 64, 2986–2991. https://doi.org/10.1099/ijs.0.063917-0.

Symsaris, E.C., Fotidis, I.A., Stasinakis, A.S., Angelidaki, I., 2015. Effects of triclosan, diclofenac, and nonylphenol on mesophilic and thermophilic methanogenic activity and on the methanogenic communities. J. Hazard. Mater. 291, 45–51. https://doi.org/10.1016/j.jhazmat.2015.03.002.

Tadkaew, N., Hai, F.I., McDonald, J.A., Khan, S.J., Nghiem, L.D., 2011. Removal of trace organics by MBR treatment: the role of molecular properties. Water Res. 45, 2439–2451. https://doi.org/10.1016/J.WATRES.2011.01.023.

Tezel, U., Tandukar, M., Pavlostathis, S.G., 2011. Anaerobic biotreatment of municipal sewage sludge. Comprehensive Biotechnology, Second edition Elsevier Inc., pp. 447–461 https://doi.org/10.1016/B978-0-08-088504-9.00329-9.

Tran, N.H., Reinhard, M., Gin, K.Y.H., 2018. Occurrence and fate of emerging contaminants in municipal wastewater treatment plants from different geographical regions—a review. Water Res. 133, 182–207. https://doi.org/10.1016/j.watres.2017.12.029.

Urra, J., Alkorta, I., Mijangos, I., Epelde, L., Garbisu, C., 2019. Application of sewage sludge to agricultural soil increases the abundance of antibiotic resistance genes without altering the composition of prokaryotic communities. Sci. Total Environ. 647, 1410–1420. https://doi.org/10.1016/j.scitotenv.2018.08.092.

Valdez-Carrillo, M., Abrell, L., Ramírez-Hernández, J., Reyes-López, J.A., Carreón-Diazconti, C., 2020. Pharmaceuticals as emerging contaminants in the aquatic environment of Latin America: a review. Environ. Sci. Pollut. Res. 27, 44863–44891. https://doi.org/10.1007/s11356-020-10842-9.

Verbarg, S., Göker, M., Scheuner, C., Schumann, P., Stackebrandt, E., 2014. The families *Erysipelotrichaceae* emend., *Coprobacillaceae* fam. nov., and *Turicibacteraceae* fam. nov. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes 4t Ed: Firmicutes and Tenericutes. Springer-Verlag, Berlin Heidelberg, pp. 79–105 https://doi.org/10.1007/978-3-642-30120-9_205.

Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment—a review. Sci. Total Environ. 429, 123–155. https://doi.org/10.1016/j.scitotenv.2012.04.028.

Verlicchi, P., Zambello, E., 2015. Pharmaceuticals and personal care products in untreated and treated sewage sludge: occurrence and environmental risk in the case of application on soil — a critical review. Sci. Total Environ. 538, 750–767. https://doi.org/10.1016/j.scitotenv.2015.08.108.

Vinzelj, J., Joshi, A., Insam, H., Podmirseg, S.M., 2020. Employing anaerobic fungi in biogas production: challenges & opportunities. Bioresour. Technol. 300, 122687. https://doi.org/10.1016/j.biortech.2019.122687.

Wang, G., Dai, X., Zhang, D., He, Q., Dong, B., Li, N., Ye, N., 2018. Two-phase high solid anaerobic digestion with dewatered sludge: improved volatile solid degradation and specific methane generation by temperature and pH regulation. Bioresour. Technol. 259, 253–258. https://doi.org/10.1016/j.biortech.2018.03.074.

Westerholm, M., Liu, T., Schnürer, A., 2020. Comparative study of industrial-scale high-solid biogas production from food waste: process operation and microbiology. Bioresour. Technol. 304, 122981. https://doi.org/10.1016/j.biortech.2020.122981.

Westerholm, M., Schnürer, A., 2019. Microbial responses to different operating practices for biogas production systems. In: Banu, R., Kannah, Y. (Eds.), Anaerobic Digestion. IntechOpen, pp. 1–36 https://doi.org/10.5772/intechopen.82815.

Wijekoon, K.C., McDonald, J.A., Khan, S.J., Hai, F.I., Price, W.E., Nghiem, L.D., 2015. Development of a predictive framework to assess the removal of trace organic chemicals by anaerobic membrane bioreactor. Bioresour. Technol. 189, 391–398. https://doi.org/10.1016/j.biortech.2015.04.034.

Wolfson, S.J., Porter, A.W., Campbell, J.K., Young, L.Y., 2018. Naproxen is transformed via acetogenesis and syntrophic acetate oxidation by a methanogenic wastewater consortium. Microb. Ecol. 76, 362–371. https://doi.org/10.1007/s00248-017-1136-2.

Wolfson, S.J., Porter, A.W., Villani, T.S., Simon, J.E., Young, L.Y., 2019. Pharmaceuticals and personal care products can be transformed by anaerobic microbiomes in the environment and in waste-treatment processes. Environ. Toxicol. Chem. 38, 1585–1593. https://doi.org/10.1002/etc.4406.

Wu, Y., Cui, E., Zuo, Y., Cheng, W., Rensing, C., Chen, H., 2016. Influence of two-phase anaerobic digestion on fate of selected antibiotic resistance genes and class I integrons in municipal wastewater sludge. Bioresour. Technol. 211, 414–421. https://doi.org/10.1016/j.biortech.2016.03.086.

Wu, B., Li, Y., Lim, W., Lee, S.L., Guo, Q., Fane, A.G., Liu, Y., 2017. Single-stage versus two-stage anaerobic fluidized bed bioreactors in treating municipal wastewater: performance, foulant characteristics, and microbial community. Chemosphere 171, 158–167. https://doi.org/10.1016/j.chemosphere.2016.12.069.

Xia, Y., Wang, Yubo, Wang, Yi, Chin, F.Y.L., Zhang, T., 2016. Cellular adhesiveness and cellulolytic capacity in *Anaerolineae* revealed by omics-based genome interpretation. Biotechnol. Biofuels 9, 111. https://doi.org/10.1186/s13068-016-0524-z.

Xu, Y., Lu, Y., Zheng, L., Wang, Z., Dai, X., 2020. Perspective on enhancing the anaerobic digestion of waste activated sludge. J. Hazard. Mater. 389, 121–847. https://doi.org/10.1016/j.jhazmat.2019.121847.

Xu, R., Zhang, K., Liu, P., Khan, A., Xiong, J., Tian, F., Li, X., 2018. A critical review on the interaction of substrate nutrient balance and microbial community structure and function in anaerobic co-digestion. Bioresour. Technol. 247, 1119–1127. https://doi.org/10.1016/j.biortech.2017.09.095.

Yan, Q., Gao, X., Chen, Y.P., Peng, X.Y., Zhang, Y.X., Gan, X.M., Zi, C.F., Guo, J.S., 2014. Occurrence, fate and ecotoxicological assessment of pharmaceutically active compounds in wastewater and sludge from wastewater treatment plants in Chongqing, the Three Gorges Reservoir Area. Sci. Total Environ. 470–471, 618–630. https://doi.org/10.1016/j.scitotenv.2013.09.032.

Yang, S., Hai, F.I., Price, W.E., McDonald, J., Khan, S.J., Nghiem, L.D., 2016. Occurrence of trace organic contaminants in wastewater sludge and their removals by

anaerobic digestion. Bioresour. Technol. 210, 153–159. https://doi.org/10.1016/j.biortech.2015.12.080.

Yang, S., McDonald, J., Hai, F.I., Price, W.E., Khan, S.J., Nghiem, L.D., 2017. Effects of thermal pre-treatment and recuperative thickening on the fate of trace organic contaminants during anaerobic digestion of sewage sludge. Int. Biodeterior. Biodegrad. 124, 146–154. https://doi.org/10.1016/j.ibiod.2017.06.002.

Yang, Y., Yu, K., Xia, Y., Lau, F.T.K., Tang, D.T.W., Fung, W.C., Fang, H.H.P., Zhang, T., 2014. Metagenomic analysis of sludge from full-scale anaerobic digesters operated in municipal wastewater treatment plants. Appl. Microbiol. Biotechnol. 98, 5709–5718. https://doi.org/10.1007/s00253-014-5648-0.

Yi, J., Dong, B., Jin, J., Dai, X., 2014. Effect of increasing total solids contents on anaerobic digestion of food waste under mesophilic conditions: performance and microbial characteristics analysis. PLoS One 9, e102548. https://doi.org/10.1371/journal.pone.0102548.

Young, D., Dollhofer, V., Callaghan, T.M., Reitberger, S., Lebuhn, M., Benz, J.P., 2018. Isolation, identification and characterization of lignocellulolytic aerobic and anaerobic fungi in one- and two-phase biogas plants. Bioresour. Technol. 268, 470–479. https://doi.org/10.1016/j.biortech.2018.07.103.

Zhang, Q., Hu, J., Lee, D.J., 2016. Biogas from anaerobic digestion processes: research updates. Renew. Energy 98, 108–119. https://doi.org/10.1016/j.renene.2016.02.029.

Zhou, H., Liu, J., Chen, X., Ying, Z., Zhang, Z., Wang, M., 2018. Fate of pharmaceutically active compounds in sewage sludge during anaerobic digestions integrated with enzymes and physicochemical treatments. Waste Manag. 78, 911–916. https://doi.org/10.1016/j.wasman.2018.07.018.

Zhou, H., Zhang, Z., Wang, M., Hu, T., Wang, Z., 2017. Enhancement with physicochemical and biological treatments in the removal of pharmaceutically active compounds during sewage sludge anaerobic digestion processes. Chem. Eng. J. 316, 361–369. https://doi.org/10.1016/j.cej.2017.01.104.

Zhou, H., Zhou, J., Wang, M., Wang, X., Zhang, Qianqian, Zhang, Qingjun, Zhan, Y., 2015. Removal of typical pharmaceutically active compounds in sewage sludge using mesophilic and thermophilic anaerobic digestion processes. Int. J. Environ. Sci. Technol. 12, 2169–2178. https://doi.org/10.1007/s13762-014-0627-7.

Zorita, S., Mårtensson, L., Mathiasson, L., 2009. Occurrence and removal of pharmaceuticals in a municipal sewage treatment system in the south of Sweden. Sci. Total Environ. 407, 2760–2770. https://doi.org/10.1016/j.scitotenv.2008.12.030.