Is anaerobic digestion effective for the removal of organic micropollutants and biological activities from sewage sludge?

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ABSTRACT

The occurrence of emerging organic micropollutants (OMPs) in sewage sludge has been widely reported; nevertheless, their fate during sludge treatment remains unclear. The objective of this work was to study the fate of OMPs during mesophilic and thermophilic anaerobic digestion (AD), the most common processes used for sludge stabilization, by using raw sewage sludge without spiking OMPs. Moreover, the results of analytical chemistry were complemented with biological assays in order to verify the possible adverse effects (estrogenic and genotoxic) on the environment and human health in view of an agricultural (re)use of digested sludge. Musk fragrances (AHTN, HHCB), ibuprofen (IBP) and triclosan (TCS) were the most abundant compounds detected in sewage sludge. In general, the efficiency of the AD process was not dependent on operational parameters but compound-specific: some OMPs were highly biotransformed (e.g. sulfamethoxazole and naproxen), while others were only slightly affected (e.g. IBP and TCS) or even unaltered (e.g. AHTN and HHCB). The MCF-7 assay evidenced that estrogenicity removal was driven by temperature. The Ames test did not show point mutation in *S. typhimurium* while the Comet test exhibited a genotoxic effect on human leukocytes attenuated by AD. This study highlights the importance of combining chemical analysis and biological activities in order to establish appropriate operational strategies for a safer disposal of sewage sludge. Actually, it was demonstrated that temperature has an insignificant effect on the disappearance of the parent compounds while it is crucial to decrease estrogenicity.

1 INTRODUCTION

A great number of organic micropollutants (OMPs) enters sewage treatment plants (STPs), including pharmaceuticals, personal care products, steroid hormones, industrial chemicals, pesticides and many others (Luo et al., 2014). Sludge is the endpoint of most hydrophobic pollutants through sorption (Carballa et al., 2008), but also of an important fraction of hydrophilic OMPs not biotransformed during the wastewater treatment. The concentrations of OMPs in sewage sludge are much dependent on their physicochemical characteristics and usage rates, varying strongly among countries or even the STPs. In general, hydrophobic substances, such as triclosan (TCS) and musk fragrances, are detected at important concentrations (up to 10,000 µg/kg), while much lower levels (10 to 100 µg/kg) are measured for hydrophilic pharmaceuticals, as diclofenac (DCF), trimethoprim (TMP), ibuprofen (IBF), naproxen (NPX), carbamazepine (CBZ) or sulfamethoxazole (SMX) (Stasinakis, 2012).

The presence of emerging pollutants in aquatic environments has already been considered by the Water Framework Directive, which establishes a "Watch List" and a priority list of substances. In this sense, some OMPs, such as DCF, 17α-ethinylestradiol (EE2), 17β-estradiol (E2), estrone (E1) and erythromycin (ERY), only have to be monitored, while the concentration of priority pollutants, such as nonylphenol (NP) and octylphenol (OP), is limited. On the contrary, the European regulation on sewage sludge use in agriculture (Directive 86/278/EEC) disregards the presence of most OMPs. Only 7 countries of the EU have included limits for some OMPs in sludge in their national legislation, although the maximum values and the target compounds (usually, halogenated organic compounds, linear alkyl benzene sulphonates, polychlorinated biphenyls, dibenzodioxins/dibenzofurans,

phthalates, NPs and polycyclic aromatic hydrocarbons) vary among countries (Kelessidis and Stasinakis, 2012). Consequently, important environmental and human risks were already reported due to the accumulation of OMPs in biosolid-amended soils (Chen et al., 2014) and due to the leaching of these substances into groundwater after rainfalls (Barron et al., 2010). However, the upcoming regulatory trends for land application of biosolids (Inglezakis et al., 2014) are going to bridge this gap, so that the occurrence of OMPs in sludge is expected to become a "hot topic" for STP managers.

Anaerobic digestion (AD) is the most widely employed process for sludge stabilization. Some studies have already evaluated the fate of OMPs during sludge AD (Carballa et al., 2007; Malmborg and Magnér, 2015; Paterakis et al., 2012; Samaras et al., 2014) but the results are not always conclusive. All of them agreed that the temperature effect (mesophilic and thermophilic conditions) can be neglected, except for NP and their ethoxylates (NPE), and that CBZ is slightly affected by AD, while SMX, NPX, and TMP are highly removed. In contrast, the removal efficiencies of other OMPs are quite controversial; some studies reported low (<25%) or no removal of hormones (E1, E2, EE2) (des Mes et al., 2008; Malmborg and Magnér, 2015), musk fragrances (AHTN, HHCB) (Alvarino et al., 2014; Clara et al., 2011), DCF (Malmborg and Magnér, 2015; Narumiya et al., 2013), IBP (Alvarino et al., 2014; Malmborg and Magnér, 2015) and TCS (Narumiya et al., 2013), while other authors disagree. For instance, Carballa et al. (2007) found that musk fragrances and hormones were removed up to 95% and 70 %, respectively; as well, Samaras et al. (2014) stated eliminations above 90% for DCF and IBP and between 60-80% for TCS. The causes of these discrepancies remain unclear.

In addition, to check the characteristics of the sludge before land-spreading and to set regulatory limits for OMPs, it is not enough to measure the disappearance of the parent pollutant via chemical analysis. In fact, it is essential to perform bioassays that assess the biological effect of the final discharge containing not only the residual parent compound but also the transformation products and other unknown compounds in view of their danger to the ecosystem and to humans (Escher and Leusch, 2011). Once released into the environment, some of them can exert their toxic effect directly interfering with the DNA of living organisms (mutagenic-carcinogenic risks) or with the endocrine system compromising reproductive and development functions in humans and wildlife species (de Jesus Gaffney et al., 2015; World Health Organization, 2006). Chemical analyses reveal adverse impacts with a compound-based approach, while bioassays provide an effect-based view, so both methods are complementary. Notwithstanding, the application of bio-analytical tools to sewage sludge is still very limited and most studies aimed at characterizing the sludge after composting (Kapanen et al., 2013; Patureau et al., 2012). In contrast, there is a very limited experience (Citulski and Farahbakhsh, 2012; Furlong et al., 2010) describing the effect of AD on specific modes of toxic action.

The main aim of this work was to combine chemical and biological methods in order to evaluate the fate of OMPs and the removal of estrogenic and genotoxic activities during mesophilic and thermophilic sludge digestion, at environmentally relevant concentrations (no OMPs spike was performed). To the best of our knowledge, this is the first study conducting an integrated assessment of the biotransformation of OMPs and these specific toxicities to evaluate the effectiveness of different AD strategies.

2 MATERIALS AND METHODS

2.1 Organic micropollutants

20 compounds commonly used in daily life were considered in this study: three musk fragrances, galaxolide (HHCB), tonalide (AHTN) and celestolide (ADBI); three anti-inflammatories, ibuprofen (IBP), naproxen (NPX) and diclofenac (DCF); four antibiotics, sulfamethoxazole (SMX), trimethoprim (TMP), erythromycin (ERY) and roxithromycin (ROX); four neurodrugs, fluoxetine (FLX), carbamazepine (CBZ), diazepam (DZP) and citalopram (CTL); three endocrine disrupting compounds, triclosan (TCS), 4-octylphenol (OP) and 4-nonylphenol (NP); and three hormones, estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2).

2.2 Sewage sludge

A mixture of primary and secondary sludge (70/30, v/v), coming from the thickener and the activated sludge flotator of a nearby STP in Santiago de Compostela (Spain), was used. The STP is designed for 184000 population equivalent with an average wastewater flowrate of approximately 55000 m^3/d, which is mainly composed by domestic wastewater (hospital discharges represent 1-2% of the total flowrate). The characteristics of the mixed sewage sludge were almost stable along the experimental period (330 d). The average pH was 5.4 ± 0.3, the total and soluble chemical oxygen demands (COD) were correspondingly 34.5 ± 5.9 g/L and 2.9 ± 1.0 g/L, the average concentration of total (TS) and volatile (VS) solids were 28.8 ± 5.5 g/L and 22.3 ± 4.1 g/L respectively, and the content of volatile fatty acids (VFA) was 2.1 ± 0.9 g/L. More differences would be expected regarding the season, because of rainfalls, but it seems that the main factor affecting the sludge characteristics was the

operation of the STP. The measured values are in accordance with previously reported data for sewage sludge coming from the same STP (Carballa et al., 2007).

2.3 Lab-scale anaerobic digesters and monitoring campaigns

Two continuously stirred (IKA RW20, 150 rpm) tank reactors with a total volume of 15 L (liquid volume of 13 L) were operated in parallel conditions, except for the temperature. One digester was mesophilic (MAD, 37ºC) and the other one thermophilic (TAD, 55ºC). The reactors were inoculated with biomass from a STP mesophilic anaerobic digester (sludge retention time (SRT) of 25-30 d) and operated semi-continuously by feeding the sludge mixture manually every day. The operation of the digesters can be divided into three periods: start-up (days 0-15) with an organic loading rate (OLR) of below 1 g COD/L d and a SRT of 40 d; Period I (days 15-90) was characterized by a SRT of 30 d and an average OLR of 1.1 g COD/L d; and Period II (days 90-330), with a SRT of 20 d and an OLR of around 1.8 g COD/L d. Conventional parameters of raw and digested sludge were analysed twice a week to check the performance of both reactors.

In order to evaluate the fate of OMPs and the estrogenic and the genotoxic activities during different AD conditions, two monitoring campaigns were conducted: one during Period I (days 71-77, spring 2014) and the second one during Period II (days 267-273, autumn 2014). Two samples of sewage sludge and digestates from MAD and TAD were taken in different days of each sampling campaign. The samples were immediately centrifuged at 3,500 rpm for 30 min in order to separate the solid from the liquid phase. The supernatant proceeded preparation for OMPs analysis (see section 2.4), while the sludge phase was frozen. OMPs concentration and estrogenic/genotoxic activities were measured in both liquid and solid fractions of each sample.

2.4 Analytical techniques

2.4.1 Conventional parameters

pH, TS, VS, total and soluble COD, alkalinity and ammonium were determined following the standard methods (APHA, 2005). The concentration of VFA (acetic, propionic, butyric, valeric) was determined using a gas chromatograph (HP 5890A) with a Flame Ionization Detector (HP 7637A). Biogas production was monitored continuously via a Ritter milligascounter (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany) and its composition was measured by gas chromatography (HP 5890 Series II).

2.4.2 OMPs in sludge samples

The liquid phase of sludge was pre-filtered (AP4004705, Millipore) and filtered by 0.45 µm (HAWP04700, Millipore) before performing the solid phase extraction (SPE) with 200 mg OASIS HLB cartridges (Waters, Milford, MA, USA), as described by Fernandez-Fontaina et al. (2013). The quantification of musk fragrances (HHCB, AHTN, ADBI), anti-inflammatories (IBP, NPX, DCF) and endocrine disrupting compounds (TCS, NP, OP) was accomplished using a gas chromatograph (Varian CP-3900) coupled with an ion trap spectrometer (Varian CG-2100). Antibiotics (ERY, ROX, SMX, TMP), neurodrugs (FLX, CBZ, DZP, CTL) and hormones (E1, E2, EE2) were quantified using an Agilent G1312A liquid chromatograph with a binary pump and automatic injector HTC-PAI (CTC Analytics) connected to a mass spectrometer API 4000 triple quadrupole (Applied Biosystems). In the first monitoring campaign (Period I), the sample volume analysed was 100 mL, and the final volume of the extract was 3 mL, leading to an enrichment factor (concentration in the extract compared to the source) of 33 $L_{supernatant}/L_{extract}$. In order to improve the quantification and

181 detection of OMPs, in the second monitoring campaign (Period II) the enrichment factor was
182 increased to 50 $L_{supernatant}$ /$L_{extract}$.

183 The frozen solid phase was lyophilized to afterwards perform ultrasonic solvent extraction
184 (USE), following a procedure based on the one described by Ternes et al. (2005). Three
185 sequential extractions with methanol and two with acetone were performed on the freeze-
186 dried samples (0.5 g). In each extraction, samples were sonicated for 15 min and centrifuged
187 at 3,000 rpm for 5 min. After the addition of the corresponding solvent, samples were
188 ultrasonicated for 15 min and then centrifuged at 1,500 rpm for 5 min. The resulting
189 supernatants were combined, filtered through glass wool, evaporated (R-205, Büchi) under
190 vacuum conditions (150 mbar) at 40ºC and then diluted with distilled water. Finally, SPE and
191 OMPs quantification were performed as previously described for the liquid phase. The
192 enrichment factors during the first and second monitoring campaigns were 166 and 250
193 $g_{sludge}/L_{extract}$, respectively.

194 Limits of quantification (LOQ) for the target OMPs are collected in Table 1. These values
195 refer to the monitoring campaign of Period II, whose values were 1.5 times lower than those
196 of Period I due to increased enrichment factors. The OMPs recoveries were determined in
197 the liquid and solid phases of three different sludge matrixes (sewage sludge, MAD digestate,
198 and TAD digestate) by duplicate. To quantify the recoveries in the liquid phase, a spike of
199 OMPs was added to the filtered (0.45 μm) liquid sample. In order to simulate the losses of
200 OMPs during the filtration process, the spiked liquid samples were again pre-filtered and
201 filtered by 0.45 μm prior to SPE. To quantify the recoveries in the solid phase, a spike of
202 OMPs was added to freeze-dried sludge (2 g). To homogenize the sample, 5 mL of acetone
203 (HPLC) were added and the sample was manually stirred for 5 min. After overnight solvent

evaporation, USE was carried out as previously described. The spike levels of OMPs must be high enough to minimize the effect of background concentrations; thus, 35 μg/g of fragrances and 8 μg/g of the rest OMPs were added in the solid phase, while 40 ppb was the concentration for liquid phase recoveries. Recoveries were calculated dividing the difference between the spiked and non-spiked sample by the measured concentration of the spike (Table S1). The aforementioned procedure enables the calculation of the total or absolute recovery of the method, including losses during sample preparation and measurement deviations during the chromatographic analysis. When not substantial differences were found among matrixes, the mean values were used for all measurements, in other case individual recoveries were applied. In the Supporting Information (S1) a detailed discussion of the recovery results is included.

Table 1. Limits of quantification (LOQ) for the monitoring campaign of Period II. Those of the monitoring campaign of Period I are 1.5 higher.

Compound	LOQ	
	Liquid (ng/L)	Solid (ng/g)
CTL, ERY, FLX, ROX	2	0.4
DZP, CBZ, SMX, TMP	10	2
E1, E2, EE2	20	4
ADBI, AHTN, HHCB, TCS	100	20
IBP, NP, OP	40	8
NPX	50	10
DCF	200	40

2.5 Biological assays

2.5.1 Estrogenic activity

Human breast cancer cell line MCF-7 was selected to measure the estrogenic activity. The extracts produced for chemical analyses were dried under nitrogen flow and resuspended in 1 mL dimethyl sulfoxide (DMSO), leading to an initial enrichment factor of 500 $g_{sludge}/L_{extract}$ and 250 $L_{supernatant}/L_{extract}$ for solid and liquid phases, respectively. MCF-7 stably transfected with the ERE-tK-LUC construct (kindly supplied by Mikko Unkila, Hormos Medical Ltd, Turku, Finland) was maintained in DMEM (Modified Dulbecco's Medium, Milan, Italy), supplemented with 10% fetal bovine serum, at 37°C and 5% CO_2. Cells were plated at a density of $2.5 \cdot 10^5$ cells/cm^2 in several plates containing 1 mL of culture medium (phenol red-free DMEM and 5% charcoal-stripped serum). 24 h later, 1 µL of each DMSO extract (dilution factor of 1,000) was added by triplicate and dishes were kept at 37°C for 24 h. As controls without extracts, one cell-plate was supplemented with DMSO solvent, another with ethanol and a last one only with cells. After incubation, cells were harvested and lysed in passive lysis buffer (Promega, Italy). Lysate was spun for 15 s at 12,000 *g* and supernatant submitted to luciferase activity quantification (Luciferase Assay System, Promega, Italy), by means of a luminometer (GloMAx, Promega, Italy) over 10 s (De Wet et al., 1987), and expressed as RLU (relative light units) normalized towards protein content (Bradford assay, Biorad, Italy). The latter value was then expressed as estradiol equivalent concentration (ng EEQ/$L_{bioassay}$), based on the calibration curve, and finally in ng EEQ/L_{sample} using the relative enrichment factor (REF). For calibration curve definition, reference estrogen (E2 dissolved in ethanol) was employed, at concentrations corresponding to physiological/sub-physiological doses, i.e. from 10^{-15} to 10^{-8} M. The resulting curve (sigmoïdal function) was

241 fitted using Graphpad Prism 6.0 software (GraphPad Software, Inc., USA). Details of cell
242 response to the reference estrogen, together with the calibration curve, are reported in Figure
243 S2 of Supporting Information. REF represents the combination of the initial enrichment
244 factor and the dilution factor in the bioassay plates, as explained by Escher and Leusch
245 (2011). REF was equal to 0.5 $g_{sludge}/L_{bioassay}$ and 0.25 $L_{supernatant}/L_{bioassay}$ for solid and liquid
246 phases, respectively.

247 *2.5.2 Genotoxic activity*

248 In order to assess the ability to induce genetic damage in target cells of different organisms,
249 two different genotoxicity tests were performed: Ames test on bacteria and Comet test on
250 human leukocytes.

251 *Ames test*. This mutagenicity test evidences point mutations in bacteria, specifically TA98
252 strain of *Salmonella typhimurium* detecting frameshift mutagens. The TA98 strain was
253 selected on the basis of extensive research carried out on wastewater showing that this strain
254 is more sensitive to substances present in this matrix (Ohe et al., 2004). The test was
255 performed with and without exogenous metabolic activation (mixed function oxidase
256 enzymes, S9 fraction), to detect promutagens and direct-acting mutagens, respectively.
257 Bacteria were exposed to increasing doses of the same organic extracts used for chemical
258 analyses: 1, 10, 25, 50 mg equivalent of sludge/plate for the solid samples and 1, 5, 10, 25,
259 50 mL equivalents of supernatant/plate for the liquid phase. The experimental procedure was
260 the standard preincubation method (APHA, 2005) and all assays were conducted in duplicate.
261 Positive (10 μg/plate of 2-nitrofluorene without S9 and 20 μg/plate of 2-aminofluorene with
262 S9) and negative (DMSO solvent) controls were included in each assay. The average data
263 were expressed as mutagenicity ratio (MR), dividing the revertants/plate by the spontaneous

mutation rate derived from the negative control. Results were considered positive if two consecutive dose levels produced a MR > 2 (a response at least twice the negative control) and these two consecutive dose levels showed a dose-response relationship (APHA, 2005).

Comet test. The single cell gel electrophoresis (SCGE) assay, or Comet assay, detects the primary DNA damage. Human leukocytes were treated with the extracts of the solid phase chemical analysis at 37°C for 1 h at increasing doses (0.5-50 mg equivalent of sludge). After treatment, only those doses with viability >70% were submitted to the assay, as recommended by the International Workshop on Genotoxicity Test Procedures (Tice et al., 2000). Negative (distilled water) and positive (ethyl methanesulfonate, EMS 2 mM) controls were included, and the test was conducted in duplicate. The extent of DNA migration was evaluated by both "visual score" (based on the visual classification of DNA damage) and the comet parameter "tail intensity" (percentage of DNA migrated in the tail) detected by an automatic imaging system (Komet 5, Kinetic Imaging Ltd, UK). Statistical differences between each dose and the negative control were verified.

2.6 Statistical analysis

The differences between performance and OMP removals obtained in the two digesters (MAD and TAD) and in the two monitoring campaigns (Period I and Period II) were statistically evaluated by the analysis of variance ANOVA followed by the Dunnett T3 test for multiple comparisons. Likewise, in the Comet test, the significance of the effect of each dose against the negative control was determined using Dunnett's test. The normal data distribution and the variance homogeneity were analysed with the Shapiro-Wilk test and the Levene test, respectively. All statistical tests were performed at a 5% significance level ($p<0.05$) using the IBM SPSS statistics® software 20.0.

3 RESULTS AND DISCUSSION

3.1 Occurrence of OMPs in sewage sludge

The total concentrations (µg/L) of the 20 selected OMPs are reported in Table 2, together with the concentrations in the liquid (µg/L) and solid phase (ng/g dw) of the sludge. As few studies reported the concentration of OMPs in the liquid phase of sludge, the comparison of results is mainly focused on the solid phase. The presence of ADBI, ERY, DCF, OP and NP could not be confirmed since their concentrations were always under the quantification or the detection limit (data not shown).

The higher concentrations of OMPs were observed for the musk fragrances HHCB and AHTN in the solid phase (1.2 to 5.0 µg/g), with no significant differences between the two sampling campaigns. These substances are highly hydrophobic (Stasinakis, 2012), which hinders their quantification in the liquid phase. Both fragrances were also measured at important quantities (0.5-21 µg/g) by Carballa et al. (2007) and Clara et al. (2011). The latter identified HHCB followed by AHTN as the principal polycyclic musks getting into STP from households, while ADBI is usually present in much lower concentrations (LOQ-0.04 µg/g); in fact, ADBI was not detected in our study.

Regarding the anti-inflammatories, IBP was present in both sludge phases resulting in a high total concentration (12-26 µg/L) in both periods, while NPX was only measured in the liquid phase at low quantities during Period II (0.6 µg/L) and DCF was never detected. Carballa et al. (2007) and Radjenović et al. (2009) reported similar concentrations of IBP (12-31 µg/L). In the case of NPX, Carballa et al. (2007) measured 11 µg/L, while Radjenović et al. (2009) reported 0.1-0.7 µg/L in primary sludge. DCF was not detected by Carballa et al. (2007) but Radjenović et al. (2009) measured around 1.3 µg/L in primary sludge.

Table 2. Average concentrations of OMPs (n=4) in sewage sludge during both monitoring campaigns (Period I and Period II).

	Period I			Period II		
	Cw (µg/L)	Cs (ng/g)	**Ct (µg/L)**	Cw (µg/L)	Cs (ng/g)	**Ct (µg/L)**
HHCB	9.20 ± 0.62	4055 ± 275	**141 ± 9**	<LOQ	4975 ± 574	**141 ± 16**
AHTN	<LOQ	2814 ± 330	**91.3 ± 10.7**	<LOQ	1272 ± 87	**36.1 ± 2.5**
CBZ	0.176 ± 0.004	7.62 ± 0.79	**0.423 ± 0.026**	0.318 ± 0.039	158 ± 24	**4.80 ± 0.67**
DZP	<LOQ	<LOQ	**<LOQ**	0.434 ± 0.054	131 ± 105	**4.16 ± 2.99**
CTL	0.719 ± 0.070	55.2 ± 8.6	**2.51 ± 0.29**	<LOQ	122 ± 13	**3.48 ± 0.38**
FLX	0.126 ± 0.012	74.3 ± 6.9	**2.53 ± 0.22**	0.062 ± 0.024	426 ± 318	**12.2 ± 9.0**
IBP	9.84 ± 0.96	487 ± 12	**25.6 ± 1.0**	4.33 ± 0.29	264 ± 97	**11.8 ± 2.8**
NPX	<LOQ	<LOQ	**<LOQ**	0.586 ± 0.191	<LOQ	**0.586 ± 0.191**
ROX	0.006 ± 0.002	1.81 ± 0.46	**0.065 ± 0.015**	<LOQ	64.8 ± 35.6	**1.84 ± 1.01**
SMX	<LOQ	<LOQ	**<LOQ**	<LOQ	626 ± 160	**17.8 ± 4.6**
TMP	0.264 ± 0.016	12.2 ± 2.4	**0.661 ± 0.079**	0.273 ± 0.004	235 ± 15	**6.94 ± 0.42**
TCS	<LOQ	n.a.	**n.a.**	<LOQ	1418 ± 181	**38.1 ± 5.1**
E1	1.04 ± 0.02	230 ± 144	**8.50 ± 4.67**	0.934 ± 0.116	128 ± 2	**4.57 ± 0.16**
E2	0.056 ± 0.011	18.0 ± 4.3	**0.640 ± 0.140**	0.097 ± 0.004	40.1 ± 20.1	**1.24 ± 0.57**
EE2	0.617 ± 0.095	14 ± 1	**1.07 ± 0.11**	0.602 ± 0.007	38.1 ± 1.6	**1.69 ± 0.05**

LOQ, limit of quantification; Cw, concentration in the liquid phase; Cs, concentration in the solid phase; Ct, total concentration.

313 All neurodrugs (CBZ, DZP, CTL, FLX) were present in sewage sludge in both periods,
314 except DZP which was only detected in the second monitoring campaign. Due to their
315 tendency to sorb (Langford et al., 2011), they were mainly found in the solid phase. The
316 values of CBZ (0.01-0.16 µg/g) agree with previously reported concentrations (Carballa et
317 al., 2007; Narumiya et al., 2013; Radjenović et al., 2009). Few studies investigated the
318 occurrence of DZP, CTL, and FLX in sewage sludge. Carballa et al. (2007) did not detect
319 DZP, Langford et al. (2011) measured CTL at similar concentrations (0.05-0.32 µg/g) than
320 those of this study (0.06-0.12 µg/g), and Radjenović et al. (2009) detected around 0.15 µg/g
321 of FLX in the primary and secondary sludge.

322 An increase in the concentration of neurodrugs and antibiotics was noticed in Period II. This
323 fact may be related to the higher consumption of these pharmaceuticals during winter, as
324 observed by Nieto et al. (2010). The concentration range in the solid phase was 2 to 65 ng/g
325 for ROX, 0.01-0.24 µg/g for TMP and up to 0.63 µg/g for SMX, being the concentrations in
326 the liquid phase negligible except for TMP. ERY was never detected. The measured
327 concentrations of ROX are in accordance with the data gathered by Narumiya et al. (2013);
328 while the reported values for TMP and SMX varied widely, from 0.01 µg/g (Narumiya et al.,
329 2013; Radjenović et al., 2009) to 40-70 µg/g (Göbel et al., 2005).

330 Regarding endocrine disrupting compounds, OP and NP were not detected, even though
331 Paterakis et al. (2012) found 0.23 µg/g of NP and Bolz et al. (2001) around 3 µg/g of NP and
332 0.1 µg/g of OP. TCS was detected in the solid phase of sewage sludge at 1.4 µg/g (Period
333 II), which is in the lower range of the reported values (1-15 µg/g) (Stasinakis, 2012).

334 The concentration range of hormones reported for sewage sludge is quite wide (0.002-0.300
335 µg/g) (Stasinakis, 2012). Consequently, our values are out of the range of some references

but agree with others. Actually, the average solid concentration of E1 (0.18 µg/g) and EE2 (0.03 µg/g) were near the maximum values (0.16 and 0.02 µg/g, respectively) found by Paterakis et al. (2012), while the total concentration of E2 (0.6-1.2 µg/L) agrees with the values of Carballa et al. (2007). An important fraction of these hormones was sorbed to the sludge solids, but its concentration in the liquid phase was also relevant.

To sum up, most of the selected OMPs were found in sewage sludge at expected concentrations according to the bibliography. However, the variation among countries, the seasonal consumption of some OMPs and especially the operational conditions of the STP explain the divergences observed in the measured and reported levels (Paterakis et al., 2012; Stasinakis, 2012).

3.2 Anaerobic digesters performance

Figure S4 in Supporting Information shows the operation of the two digesters (MAD and TAD) during the whole experimental period; Table 3 displays the average operational parameters during both monitoring campaigns (Period I and Period II). The changes in the characteristics of the raw mixture of sewage sludge were responsible for the OLR fluctuations observed in Figure S4. Despite these oscillations and the SRT reduction after day 90, the operation of the reactors remained stable during 330 d with an average methanization above 50% under mesophilic and thermophilic conditions.

pH values were in the neutral range throughout the experiment, no accumulation of VFAs was observed and the mean ratio of VFAs to alkalinity was almost constant and lower than 0.3, although a bit higher in TAD than in MAD (Table 3). As expected (Zábranská et al., 2000), on average the biogas quality was slightly upgraded ($p<0.05$) under thermophilic conditions (CH_4 > 60%). The mean COD_t removalsignificantly ($p<0.05$) increased during

Period II in both MAD and TAD. This improvement in the COD removal and the higher OLR (2.3 g COD/L d) lead to a significant ($p<0.05$) increase in the biogas production rates during Period II. The effect of temperature was only statistically relevant in Period I, where MAD presented a higher COD removal and biogas production than TAD, likely due to a slower adaptation of the inoculum to thermophilic conditions. In any case, the methanization efficiencies had the same order of magnitude (50-65%) and were in the expected range (Song et al., 2004). Therefore, AD was successfully applied to stabilize sewage sludge under mesophilic and thermophilic conditions in the lab-scale reactors during both monitoring campaigns.

Table 3. Average operational and performance parameters of the mesophilic (MAD) and the thermophilic (TAD) digesters during both monitoring campaigns of Period I and Period II (MAD and TAD operation lasted 330 d).

	MAD		TAD	
	Period I	Period II	Period I	Period II
Monitoring campaign (d)	71-77	267-273	71-77	267-273
Temperature (ºC)	37.0 ± 0.5	37.0 ± 0.5	55.0 ± 0.5	55.0 ± 0.5
SRT (d)	30	20	30	20
OLR (g COD/L d)	1.3 ± 0.1	2.3 ± 0.1	1.3 ± 0.1	2.3 ± 0.1
pH	7.5 ± 0.3	7.4 ± 0.1	7.9 ± 0.1	7.7 ± 0.1
VFA (g/L)	<0.01	<0.01	<0.01	<0.01
Intermediate/total alkalinity	0.19 ± 0.02	0.18 ± 0.01	0.23 ± 0.03	0.19 ± 0.02
Ammonium (g N-NH_4/L)	0.77 ± 0.01	1.0 ± 0.1	0.83 ± 0.04	1.3 ± 0.1
COD_t (g /L)	14.7 ± 1.2	13.9 ± 0.9	18.9 ± 0.7	16.4 ± 1.9
COD_s (g /L)	1.2 ± 0.3	0.62 ± 0.23	3.6 ± 1.5	1.8 ± 0.3
COD_t removal (%)	60.6 ± 3.1	67.5 ± 3.3	47.6 ± 2.8	60.6 ± 4.0
TS (g/L)	25.5 ± 3.7	13.8 ± 1.4	31.6 ± 2.1	16.2 ± 1.5
VS (g/L)	11.8 ± 0.8	8.8 ± 0.7	13.9 ± 0.4	10.6 ± 0.7
VS removal (%)	57.9 ± 5.3	62.1 ± 3.6	50.4 ± 4.7	54.3 ± 3.9
Biogas production (L/L·d)	0.55 ± 0.10	1.05 ± 0.11	0.41 ± 0.03	0.91 ± 0.09
Biogas composition (% CH_4)	61.5 ± 0.5	58.9 ± 1.1	66.0 ± 1.0	61.8 ± 1.2

pH, VFA, alkalinity, ammonium, COD, TS, and VS are referred to MAD and TAD digestates.

3.3 Fate of OMPs during MAD and TAD

A general overview of the changes in the concentration of the 15 detected OMPs after mesophilic and thermophilic anaerobic digestion is shown in Figure 1 (note that data is depicted in logarithmic scale). More detailed information about their fate in the liquid and solid phase of sludge is gathered in Table S3a/b of Supporting Information. Average removal efficiencies and partitioning coefficients are summarized in Figure 2.

3.3.1 Biotransformation of OMPs during AD

Four groups of OMPs can be differentiated according to their average biotransformation in AD (Figure 2). The first group contains the musk fragrances HHCB and AHTN, the hormones E1 and E2 and the antiseptic TCS, which were not eliminated (<20%) during AD. Clara et al. (2011) observed the same behaviour of HHCB and AHTN during raw sewage sludge digestion. Oppositely, Carballa et al. (2007) reported removals of 60-70 % for HHCB and AHTN in spiked sludge. Likewise, the removal of TCS is lower (25%, Period II) in raw sewage sludge (Narumiya et al., 2013) than in spiked sludge (75%) (Samaras et al., 2014). The fate of hormones during AD is a controversial topic in literature. It is usually evaluated the sum of E1+E2, since under anaerobic conditions E1 can be reduced to E2 until the equilibrium is achieved (des Mes et al., 2008; Paterakis et al., 2012). For spiked sewage sludge, Carballa et al. (2007) observed a removal of E1+E2 around 85%, while no clear disappearance was found in spiked and unspiked sludge by Malmborg and Magnér (2015) and Paterakis et al. (2012), respectively. The observed increase in the concentrations of E1+E2, particularly after MAD (Figure 1), could be due to the deconjugation of E1 and E2 conjugates present in the sewage sludge. However, these conjugates were not monitored and little information is available about the deconjugation of estrogens in STP (des Mes et al.,

2008). The second group includes IBP, CBZ, and DZP, which presented a medium-low biotransformation (25-50%), supporting previous findings (Alvarino et al., 2014; Carballa et al., 2007; Malmborg and Magnér, 2015). Conversely, Samaras et al. (2014) reported almost complete removal of IBP during AD. The third group with higher biotransformation efficiencies (50-75%) contains CTL, FLX, TMP, and EE2. The studies conducted by Malmborg and Magnér (2015) and Bergersen et al. (2012) showed a lower removal of FLX (0-32%) and controversial results for CTL (23-85%). Likewise, EE2 was highly removed in the study of Carballa et al. (2007), while it was almost stable during the experiments of Malmborg and Magnér (2015) and Paterakis et al. (2012). Regarding TMP, literature agrees that it is highly biotransformed under anaerobic conditions (Alvarino et al., 2014; Malmborg and Magnér, 2015; Narumiya et al., 2013). The fourth group includes the compounds most efficiently removed (75-100%), i.e. SMX, ROX and NPX, as expected according to the bibliography (Carballa et al., 2007; Narumiya et al., 2013; Samaras et al., 2014). Overall, no relationship could be established between sorption and biotransformation efficiency of OMPs (Figure 2).

The controversial results reported for some OMPs reveal a poor understanding of the mechanisms and factors behind their biotransformations. The use of spiked or unspiked sludge is likely to influence these divergences. In fact, it has been proved that the behaviour of freshly added and aged compounds is different; the latter are strongly linked to the matrix, thus they are less bioavailable and biodegradable (Dictor et al., 2003). Therefore, despite the risk of not detecting/quantifying some compounds, the use of unspiked sludge has been a strong core of this research.

3.3.2 Influence of operational parameters

The effect of temperature was only relevant (removal difference ≥ 20% and $p<0.05$) in the case of EE2 (Figure 1 and Figure S5a), which presented an average biotransformation (Period I and II) of 55% in MAD and 88% in TAD. This favourable impact of thermophilic conditions was also stated by Paterakis et al. (2012). As previously reported (Carballa et al., 2007; Malmborg and Magnér, 2015; Samaras et al., 2014), the influence of temperature on the other detected OMPs was negligible. The effect of sludge retention time (SRT) and/or OLR, was only significant (removal difference ≥ 20% and $p<0.05$) for some OMPs in both MAD and TAD (Figure S5b). During Period I (higher SRT), the average biotransformation (MAD and TAD) of TMP was superior (86%) than in Period II (60%). In contrast, ROX was only removed during Period II (higher OLR) and CBZ increased its biotransformation from 23% to 47%. However, the responsible parameter of these trends cannot be ascertained, since SRT and OLR are interrelated variables in our reactors. So far, few and contradictory results are available regarding the role of OLR and SRT on OMPs removal (Barret et al., 2010; Carballa et al., 2007; Samaras et al., 2014). In general, it can be stated that the SRT, in the typical range of 10-30 d, has a slight effect on the biotransformation of pharmaceuticals, musk fragrances, and estrogens, while the influence of the OLR was only proved for polycyclic aromatic hydrocarbons (PAHs).

3.3.3 Distribution of OMPs between the liquid and solid phases of sludge

The partitioning of OMPs (summarized in Figure 2) in raw and digested sludge was evaluated and compared with literature, by calculating the solid-liquid distribution coefficients (Kd) displayed in Figure S6. Results evidenced that, except for IBP, EE2 and NPX, all the detected compounds were mainly present in the solid phase (>50%, log Kd>1.5). Particularly, 90% of HHCB, AHTN, TCS, CTL, FLX and SMX was associated to the solids. Most partitioning results agree with literature (Carballa et al., 2008; Malmborg and Magnér, 2015; Narumiya et al., 2013), although a higher affinity of EE2 for sludge and a higher solubility of SMX was expected.

In accordance with Carballa et al. (2008), a relevant effect of the type of sludge or the AD operational conditions on the Kd values was not observed. Only CTL (pKa=9.8) and DZP (pKa=3.4) increased and decreased, respectively, their Kd value after AD (Figure S6). Narumiya et al. (2013) postulated that the hydrophobicity of OMPs is directly related to the concentration of neutral species, which can vary due to pH shifts during AD (pH of 5-6 in sewage sludge and 7-8 in the digestates). Actually, in agreement with our results, Narumiya et al. (2013) found that OMPs with a pKa around 9 increased significantly the fraction of neutral species during AD and consequently its Kd values; on the contrary, deprotonated species became dominant when the compound has a pKa around 4, decreasing its hydrophobicity.

3.4 Estrogenic activity

The values of estrogenic activity, expressed as estradiol equivalent (EEQ), in the liquid and solid phases of raw and digested sludges are reported in Figure S7 of Supporting Information.

458 The estrogenicity was in the range of ng EEQ/L and the major concentration was present in
459 the solid phase, rather than in the liquid one. This is an expected finding, due to the
460 hydrophobic nature of estrogenic compounds. Figure 3 reports the effect of the AD on the
461 removal of the total estrogenic activity from sewage sludge, expressed as the ratio between
462 outlet and inlet EEQ (C_{OUT}/C_{IN}). Surprisingly, two opposite results were observed for the
463 mesophilic and the thermophilic conditions; the former led to an increase of estrogenic
464 activity ($C_{OUT}/C_{IN} > 1$), whilst the latter to a strong decrease ($C_{OUT}/C_{IN} > 1$). These findings
465 were confirmed by both monitoring campaigns (Period I and II).

466 There is not a well-established knowledge on the effect of AD on estrogenicity of sludge, as
467 few studies have been attempted to evaluate it. However, the available literature supports the
468 outcomes of the present study. Citulski and Farahbakhsh (2012) and Furlong et al. (2010)
469 reported the substantial net production of estrogenicity during MAD of sludge, while a
470 decrease was achieved after TAD. This behaviour may be ascribed to the bio-activation
471 exerted by bacteria under mesophilic anaerobic conditions: temperature around 37°C and pH
472 around neutrality might encourage the formation of metabolites/by-products exerting
473 estrogenic potencies even higher (about the double in the current study: $C_{out}/C_{in} \approx 2$) than
474 those measured in the sewage sludge. On the contrary, thermophilic biomass demonstrated
475 the capability to reduce estrogenicity, up to 80%. This means that biotransformations during
476 TAD are more favourable for estrogenicity reduction than those occurring during MAD. A
477 clear example of these different metabolic pathways is reported for alkylphenols (Furlong et
478 al., 2010; Samaras et al., 2014). NP, which has a stronger estrogenic potency than
479 nonylphenols polyethoxylates, is produced under MAD because of the breakdown of its
480 longer chain parent compounds. Conversely, TAD is able to decrease the concentration of

NP. These results are consistent with the observed fate of hormones (E1, E2, EE2, Figure 1), which can be considered the main contributors to estrogenicity. In fact, TAD was more effective than MAD in terms of EE2 removal and E1+E2 accumulation, especially during Period II.

3.5 Genotoxic activity

Ames test. The results of Ames test (Table S8) clearly evidences the lack of mutagenic activity in solid and liquid phase, being the mutagenicity ratio (MR = revertants number in the sample/revertants number in negative control) always below 2, which represents the threshold for the mutagenicity onset. As expected, S9 enzymes revealed its detoxifying effect. Indeed, the highest doses without S9 caused general toxicity (death) of *S. typhimurium*, probably due to the significant concentrations of antibiotics and drugs observed in sludge which inhibit bacterial growth. This result agrees well with other studies performed on sludge with mutagenicity test on bacteria (Kapanen et al., 2013).

Comet test. The results of Comet test carried out with the solid phase are graphically summarized in Figure 4 and deeply detailed in terms of tail intensity and visual score in Table S9. Conversely to Ames, a partial genotoxic effect was detected on human leukocytes. Tail intensity values highlighted a clear dose-dependent activity, and up to 30% DNA migration was recorded for TAD at the highest dose (50 mg_{eq}). The AD process seems to exert a positive effect on DNA damage reduction, although a complete and wider comparison is hampered by the onset of general toxicity (cell mortality in sewage sludge was observed at doses > 1 mg_{eq}). Indeed, MAD and TAD reduced the toxicity, allowing the determination of the genotoxic effect of digested sludge at doses higher than 1 mg_{eq}. However, these results did not show a clear influence of AD temperature on the reduction of DNA damage.

3.6 Relevance of combining OMPs and effect-based analyses for a holistic assessment of AD processes

The integrated analyses carried out in this research revealed that treatment technologies should be assessed not only on the basis of conventional parameters but also in terms of emerging pollutants (i.e. OMPs); especially when this resource is released into the environment for agricultural purposes, as frequently happens in European Countries (Kelessidis and Stasinakis, 2012). In order to have a complete approach regarding OMPs, it is necessary to combine chemical analysis to monitor the biotransformation of OMPs with bioassays revealing the biological effect due to the residual parent compound but also to the transformation products and other unknown substances present in the effluent. As summarized in the qualitative heat-map (Figure 5) the combination of both analyses could determine the technology selection. Indeed, the currently widespread applied mesophilic AD process showed strong weaknesses in the removal of estrogens and estrogenic activity, which conversely are mitigated in the thermophilic process. It is well-known that TAD presents advantages over MAD, such as a better hygienization and an increase in the biogas quality and yield. However, TAD displays also operational drawbacks as an elevated energy requirement for heating the digester, a higher risk of process destabilization and a poorer sludge dewaterability (Appels et al., 2008). The better performance of TAD in terms of some OMPs removal and estrogenicity decrease could also mark a strong difference when assessing the feasibility/suitability of sludge stabilization treatments, especially if these bio-analytical tools will be promoted at legislative level in future. Nevertheless, the application of bio-analytical tools to sludge matrices is still very limited; in the present study estrogenicity and genotoxicity were measured, but other biological effects as antibiotic

resistance and oxidative stress (Escher and Leusch, 2011) could complement a holistic assessment of AD processes.

4 CONCLUSIONS

In this study, the potential of AD to remove several OMPs and specific toxicities (namely, estrogenicity and genotoxicity) from sewage sludge was assessed. The chemical analyses evidence the presence of a wide range of organic trace pollutants, mainly in the solid phase of sludge, with the highest concentrations belonging to HHCB, AHTN, IBP, and TCS. Approximately half of the compounds detected are persistent during AD, while NPX, ROX, SMX, TMP, EE2, FLX, CTL suffer a biotransformation above 50%. This elimination is generally not affected by the operational conditions (temperature, OLR, SRT) or the OMPs partitioning, but could differ if the OMPs are spiked or not.

Regarding the biological activities, the temperature is a key factor to reduce estrogenicity, since only thermophilic conditions guarantee estrogenicity decrease. No mutagenic activity was detected in sludge samples by Ames test, while the Comet test revealed that, despite AD decreases the damage on human leukocytes, the digested sludge still presents genotoxic effects. To the best of our knowledge, this is one of the first studies combining chemical and biological methods to characterize the quality of digested sludge in terms of emerging micropollutants. Results reveal that this combination is essential to settle operational strategies, such as thermophilic digestion of sludge, that really promote a safer disposal.

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REFERENCES

Alvarino, T., Suarez, S., Lema, J.M., Omil, F., 2014. Understanding the removal mechanisms of PPCPs and the influence of main technological parameters in anaerobic UASB and aerobic CAS reactors. J. Hazard. Mater. 278, 506–513.

APHA, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

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.

Barret, M., Barcia, G.C., Guillon, a., Carrère, H., Patureau, D., 2010. Influence of feed characteristics on the removal of micropollutants during the anaerobic digestion of contaminated sludge. J. Hazard. Mater. 181, 241–247.

Barron, L., Nesterenko, E., Hart, K., Power, E., Quinn, B., Kelleher, B., Paull, B., 2010. Holistic visualisation of the multimodal transport and fate of twelve pharmaceuticals in biosolid enriched topsoils. Anal. Bioanal. Chem. 397, 287–296.

Bergersen, O., Østnes, K., Vasskog, T., 2012. Bioresource Technology Anaerobic treatment of sewage sludge containing selective serotonin reuptake inhibitors. Bioresour. Technol. 117, 325–332.

Bolz, U., Hagenmaier, H., Körner, W., 2001. Phenolic xenoestrogens in surface water, sediments, and sewage sludge from Baden-Württemberg, south-west Germany. Environ. Pollut. 115, 291–301.

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.

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, 2139–50.

Chen, F., Ying, G.-G., Ma, Y.-B., Chen, Z.-F., Lai, H.-J., Peng, F.-J., 2014. Field dissipation and risk assessment of typical personal care products TCC, TCS, AHTN and HHCB in biosolid-amended soils. Sci. Total Environ. 470-471, 1078–86.

Citulski, J., Farahbakhsh, K., 2012. Overcoming the toxicity effects of municipal wastewater sludge and biosolid extracts in the Yeast Estrogen Screen (YES) assay. Chemosphere 87, 498–503.

Clara, M., Gans, O., Windhofer, G., Krenn, U., Hartl, W., Braun, K., Scharf, S., Scheffknecht, C., 2011. Occurrence of polycyclic musks in wastewater and receiving water bodies and fate during wastewater treatment. Chemosphere 82, 1116–1123.

de Jesus Gaffney, V., Almeida, C.M.M., Rodrigues, A., Ferreira, E., Benoliel, M.J., Cardoso, V.V., 2015. Occurrence of pharmaceuticals in a water supply system and related human health risk assessment. Water Res. 72, 199–208.

De Wet, J.R., Wood, K. V, DeLuca, M., Helinski, D.R., Subramani, S., 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. 7, 725–737.

des Mes, T.Z.D., Kujawa-Roeleveld, K., Zeeman, G., Lettinga, G., 2008. Anaerobic biodegradation of estrogens - Hard to digest. Water Sci. Technol. 57, 1177–1182.

Dictor, M.C., Berne, N., Mathieu, O., Moussay, a., Saada, a., 2003. Influence of Ageing of Polluted Soils on Bioavailability of Phenanthrene. Oil Gas Sci. Technol. 58, 481–488.

Escher, B., Leusch, F., 2011. Bioanalytical tools in water quality assessment. IWA publishing, London.

Fernandez-Fontaina, E., Pinho, I., Carballa, M., Omil, F., Lema, J.M., 2013. Biodegradation kinetic constants and sorption coefficients of micropollutants in membrane bioreactors. Biodegradation 24, 165–77.

Furlong, E.T., Quanrud, D., Stinson, B.M., 2010. Fate of estrogenic compounds during municipal sludge stabilization and dewatering. Water Environment Research Foundation (WERF) and IWA Publishing.

Göbel, A., Thomsen, A., McArdell, C.S., Joss, A., Giger, W., 2005. Occurrence and Sorption Behavior of Sulfonamides, Macrolides, and Trimethoprim in Activated Sludge Treatment. Environ. Sci. Technol. 39, 3981–3989.

Inglezakis, V., Zorpas, A.A., Karagiannidis, A., Samaras, P., Voukkali, I., Sklari, S., 2014. European Union legislation on sewage sludge management. Fresenius Environ. Bull. 23, 635–639.

Kapanen, A., Vikman, M., Rajasärkkä, J., Virta, M., Itävaara, M., 2013. Biotests for environmental quality assessment of composted sewage sludge. Waste Manag. 33, 1451–1460.

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.

Langford, K.H., Reid, M., Thomas, K. V, 2011. Multi-residue screening of prioritised human pharmaceuticals, illicit drugs and bactericides in sediments and sludge. J. Environ. Monit. 13, 2284–2291.

Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci. Total Environ. 473-474,619-41.

629 Malmborg, J., Magnér, J., 2015. Pharmaceutical residues in sewage sludge: Effect of
630 sanitization and anaerobic digestion. J. Environ. Manage. 153, 1–10.

631 Narumiya, M., Nakada, N., Yamashita, N., Tanaka, H., 2013. Phase distribution and removal
632 of pharmaceuticals and personal care products during anaerobic sludge digestion. J.
633 Hazard. Mater. 260, 305–12.

634 Nieto, A., Borrull, F., Pocurull, E., Marcé, R.M., 2010. Occurrence of pharmaceuticals and
635 hormones in sewage sludge. Environ. Toxicol. Chem. 29, 1484–1489.

636 Ohe, T., Watanabe, T., Wakabayashi, K., 2004. Mutagens in surface waters: a review. Mutat.
637 Res. 567, 109–149.

638 Paterakis, N., Chiu, T.Y., Koh, Y.K.K., Lester, J.N., McAdam, E.J., Scrimshaw, M.D.,
639 Soares, A., Cartmell, E., 2012. The effectiveness of anaerobic digestion in removing
640 estrogens and nonylphenol ethoxylates. J. Hazard. Mater. 199-200, 88–95.

641 Patureau, D., Delgenes, N., Muller, M., Dagnino, S., Lhoutellier, C., Delgenes, J.P.,
642 Balaguer, P., Hernandez-Raquet, G., 2012. Chemical and toxicological assessment of a
643 full-scale biosolid compost. Environ. Toxicol. Chem. 31, 2748–2756.

644 Radjenović, J., Petrović, M., Barceló, D., 2009. Fate and distribution of pharmaceuticals in
645 wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced
646 membrane bioreactor (MBR) treatment. Water Res. 43, 831–841.

647 Samaras, V.G., Stasinakis, A.S., Thomaidis, N.S., Mamais, D., Lekkas, T.D., 2014. Fate of
648 selected emerging micropollutants during mesophilic, thermophilic and temperature co-
649 phased anaerobic digestion of sewage sludge. Bioresour. Technol. 162, 365–72.

650 Song, Y.-C., Kwon, S.-J., Woo, J.-H., 2004. Mesophilic and thermophilic temperature co-
651 phase anaerobic digestion compared with single-stage mesophilic- and thermophilic
652 digestion of sewage sludge. Water Res. 38, 1653–1662.

653 Stasinakis, A.S., 2012. Review on the fate of emerging contaminants during sludge anaerobic
654 digestion. Bioresour. Technol. 121, 432–40.

655 Ternes, T.A., Bonerz, M., Herrmann, N., Löffler, D., Keller, E., Lacida, B.B., Alder, A.C.,
656 2005. Determination of pharmaceuticals, iodinated contrast media and musk fragrances
657 in sludge by LC tandem MS and GC/MS. J. Chromatogr. A 1067, 213–223.

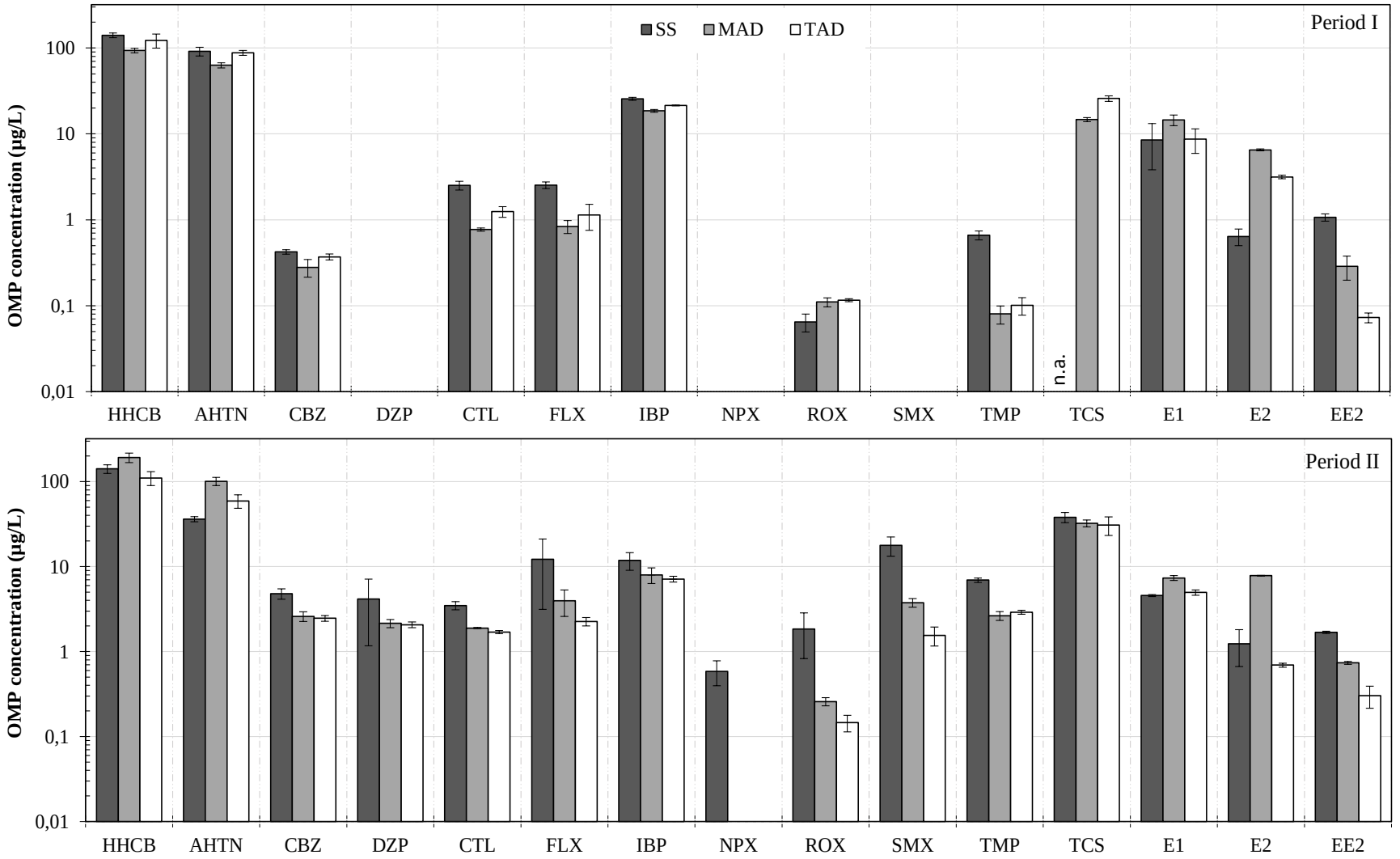
658 Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae,
659 Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for
660 in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–221.

661 World Health Organization, 2006. Guidelines for the Safe Use of Wastewater, Excreta and
662 Greywater: Policy and regulatory aspects. World Health Organization.

663 Zábranská, J., Štěpová, J., Wachtl, R., Jenlček, P., Dohányos, M., 2000. The activity of

anaerobic biomass in thermophilic and mesophilic digesters at different loading rates. Water Sci. Technol. 42, 49–56.

667



668 **Figure 1.** Total concentration of OMPs in sewage sludge (SS), MAD and TAD during both monitoring campaigns (Period I and Period
669 II). n.a. refers to not available data.

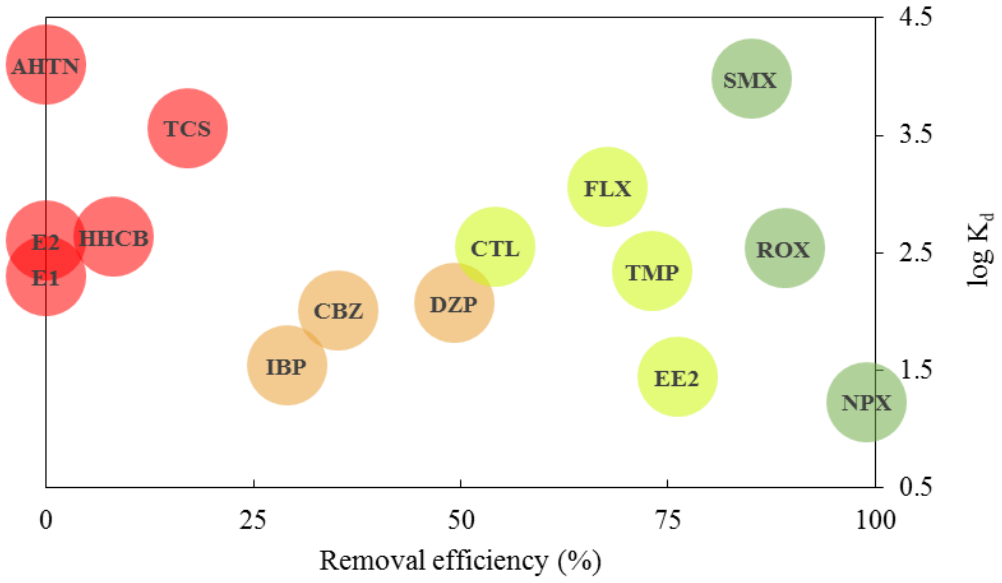


Figure 2. Semi-quantitative representation of the fate of different OMPs after AD process in both monitoring campaigns: removal efficiencies (average of MAD and TAD) versus solid-liquid distribution coefficient (average log Kd of sewage sludge, MAD, and TAD). Transitions from green to red indicate a decrease in removal efficiencies.

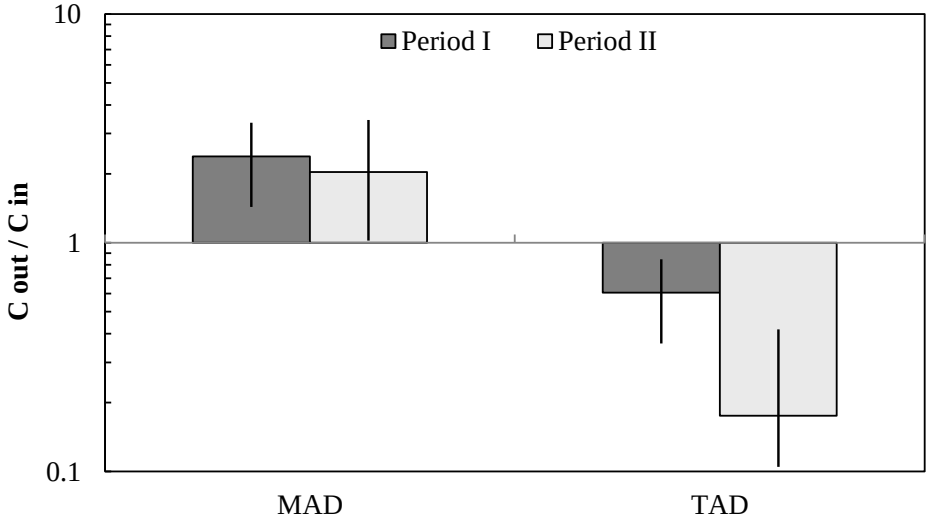
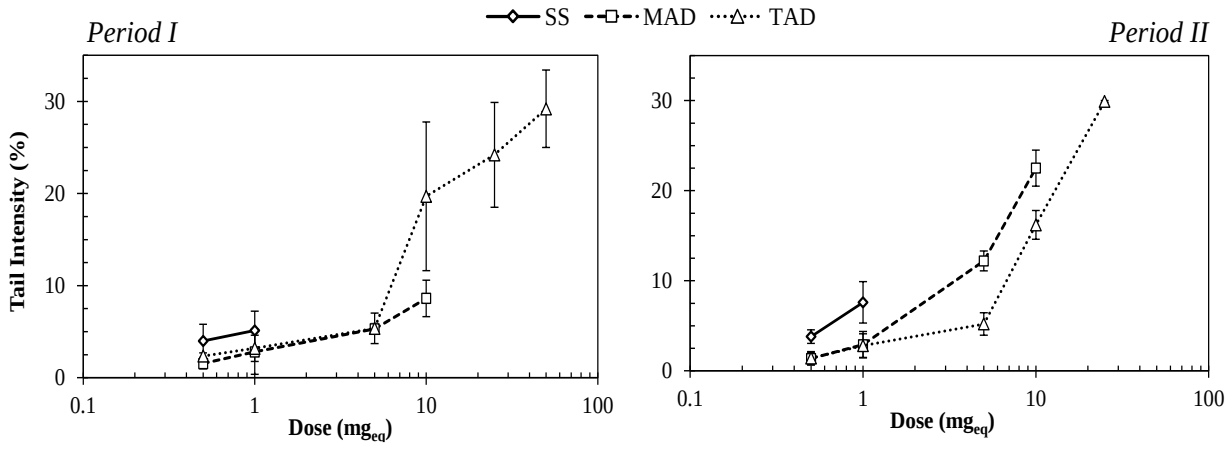


Figure 3. Effect of mesophilic (MAD) and thermophilic (TAD) digestion on the removal of estrogenic activity from sewage sludge.



679

680 **Figure 4.** DNA damage status of leukocytes (Comet test) in sewage sludge (SS), MAD and

681 TAD digestates. Results are referred to the sludge solid phase.

682

		MAD	TAD
Conventional parameters	Hygienization	*M*	**H**
	Biogas (yield & quality)	*M*	**H**
	Operation (cost & stability)	**H**	*M*
OMPs	Musks, drugs, TCS	L – **H**	L – **H**
	EE2	*M*	**H**
	E1+E2	L	*M*
Biological activities	Estrogenicity	L	**H**
	Genotoxicity	*M*	*M*

683

684 **Figure 5.** Qualitative heat-map comparing the efficiency of MAD and TAD towards

685 conventional and innovative parameters. Colors (and letters) encode for the magnitude of the

686 efficiency: green stands for high (**H**), yellow for medium (*M*) and red for low (L).