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Complete List of Authors:	Gonzalez-Gil, Lorena; Universidade de Santiago de Compostela, Department of Chemical Engineering Carballa, Marta; University of Santiago de Compostela, Department of Chemical Engineering Lema, Juan; University of Santiago de Compostela, Department of Chemical Engineering

Cometabolic enzymatic transformation of organic micropollutants under methanogenic conditions

Lorena Gonzalez-Gil[*]*, Marta Carballa, Juan M. Lema*

Department of Chemical Engineering, Institute of Technology, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

[*]Corresponding author.

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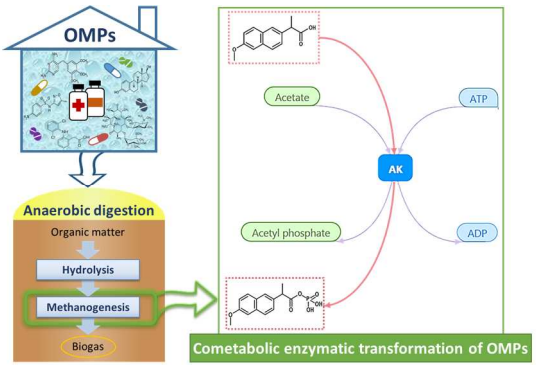
ABSTRACT

Anaerobic digestion (AD) has been shown to have the biological potential to decrease the concentrations of several organic micropollutants (OMPs) from sewage sludge. However, the mechanisms and factors behind these biotransformations, which are essential for elucidating the possible transformation products and to foster the complete removal of OMPs via operational strategies, remain unclear. Therefore, this study investigated the transformation mechanisms of 20 OMPs during the methanogenic step of AD with a focus on the role of acetate kinase (AK), which is a key enzyme in methane production. The results from lab-scale methanogenic reactors showed that this step accounts for much of the reported OMPs biotransformation in AD. Furthermore, enzymatic assays confirmed that AK transforms galaxolide, naproxen, nonylphenol, octylphenol, ibuprofen, diclofenac, bisphenol A and triclosan. Except for galaxolide, for which further studies are required to refine conclusions, the OMP's chemical structure was a determinant for the AK action because only compounds that contain a carboxyl or a hydroxyl group and that have a moderate steric hindrance were enzymatically transformed, likely by phosphorylation. For these 7 compounds, this enzymatic mechanism accounts for 10-90% of the measured methanogenic biotransformation, suggesting that other active enzymes of the AD process are also involved in OMPs biotransformation.

Abstract Art



INTRODUCTION

The increasing use of pharmaceuticals, personal care products, hormones and many other organic compounds in our daily life has resulted in the release of these organic micropollutants (OMPs) into the environment via diverse pathways.[1] There is evidence of negative effects of OMPs on human and ecosystem health,[2] which suggests that reduction measures should be applied, especially for one of the main sources of OMP discharge: effluents of sewage treatment plants (STPs).

Anaerobic digestion (AD) is widely used in STPs for sludge stabilization prior to its application in agricultural soils as a biosolid. Significant quantities of OMPs arrive at the sludge treatment line sorbed onto solids[3] but also solubilized in the water phase of the sludge, achieving concentrations up to 90-140 $\mu g\ L^{-1}$ for musk fragrances (galaxolide and tonalide), 40 $\mu g\ L^{-1}$ for triclosan, 25 $\mu g\ L^{-1}$ for ibuprofen, and 1-10 $\mu g\ L^{-1}$ for hormones (estrone and 17β-estradiol).[4] Most of these OMPs remain in the digested sludge,[4] thus its use as a fertilizer might transfer OMPs to the soil.[5]

Despite the environmental risks associated with biosolid-amended soils,[5] few studies have investigated the fate of OMPs during AD.[4,6–12] They conclude that AD is able to biologically transform OMPs, but the degree of removal of some compounds is still controversial, and few transformation products (TPs) have been identified, which indicates the poor understanding of the microbial mechanisms and factors behind these biotransformations.[3] Therefore, to develop strategies that promote the complete elimination of OMPs and to predict the environmental risks of the TPs that are generated, it is essential to understand the biotransformation pathways involved in AD.

To date, only rare and inconclusive information is available about the relationship between anaerobic populations and the removal of OMPs.[13] This lack of knowledge is justified by the complexity of the biological and chemical processes involved in the four steps of AD (Figure 1): hydrolysis, acidogenesis, acetogenesis and methanogenesis.[14] According to the taxonomic analysis of Guo et al.,[15] *Bacteria* (~93%) was more abundant than *Archaea* (methanogens) (~6%) on sewage sludge AD. Among bacteria, *Proteobacteria* (41%), *Firmicutes* (13%) and *Bacteroidetes* (10%) are the most abundant populations, while *Methanosaeta* (26%), *Methanospirillum* (13%), *Methanosarcina* (13%), *Methanoculleus* (11%) and *Methanoregula* (8%) are the dominant methanogenic genera. During the four AD steps, these microorganisms produce a broad diversity of enzymes to degrade the organic substrates. Depending on their specificity, these enzymes can also modify the structures of OMPs despite being non-growth substrates. This biochemical process is known as cometabolism, and it is considered by many authors to be a major removal mechanism of OMPs during the biological treatment of wastewater.[16–21] The action of enzymes on OMPs is poorly investigated, and most studies have focused on oxygenases.[21–23] A recent study by Krah et al.[24] tested the activity of a cocktail of extracted enzymes from activated sludge towards OMPs. By measuring several TPs and using several enzymatic inhibitors, they concluded that amide hydrolases could be involved in the biotransformation of acetaminophen, acetyl sulfamethoxazole, atenolol and bezafibrate, that oxidoreductases transform 10-OH-carbamazepine and that erythromycin is affected by hydrolases acting on ester bonds. However, the suspected participation of particular enzymes in the cometabolic biotransformation of OMPs was not definitively confirmed because the identification of enzymes by indirect measurements is unreliable, and the isolation of target enzymes has not yet been reported.[21] Although no studies about the enzymatic transformation routes of OMPs during AD have been found, it could be hypothesized that hydrolases from the

two first AD steps, which perform relatively simple reactions, are involved in the biotransformation of some OMPs, as was demonstrated for activated sludge systems.[24] For the more specific acetogenic and methanogenic steps, the removal of polycyclic aromatic hydrocarbons (PAH) has been linked to methanogenesis,[25] but the type of enzymatic biotransformation that could occur is not clear.

The aceticlastic methanogenesis (Figure 1) is performed by the archaea *Methanosaeta* and *Methanosarcina* and it is considered the main pathway in the synthesis of methane in anaerobic digesters.[15,26] The first step in the methanization of acetate by *Methanosarcina* species is the phosphorylation of acetate to acetyl phosphate (Figure 1 and Figure S1), which is performed by the key intracellular enzyme acetate kinase (AK). Then, the acetyl phosphate is further converted to acetyl-CoA by phosphotransacetylase (PTA)[27] and the aceticlastic methanization continues with the action of other enzymes (Figure S1). In addition, AK associated with PTA catalyzes the reverse reaction (conversion of acetyl-CoA to acetate; Figure 1) in fermentative prokaryote bacteria during acetogenesis.[28] AK is a relatively specific enzyme but, in addition to acetate, it is able to phosphorylate other substrates to a lower extent;[29,30] thus, AK might also have effects on some OMPs.

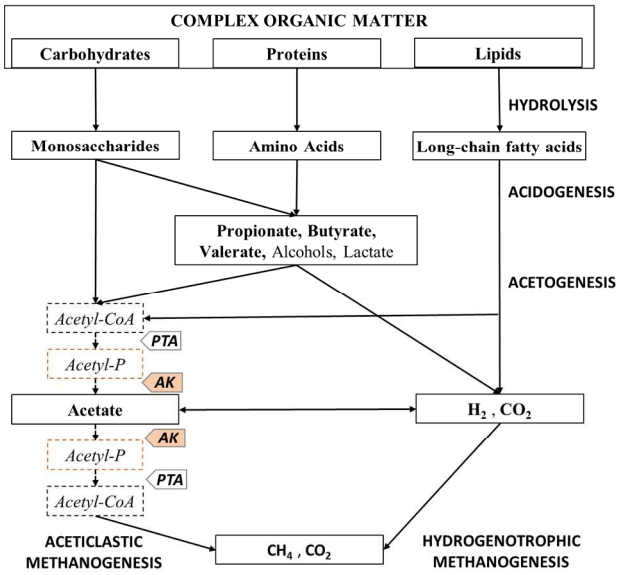


Figure 1. Schematic representation of the steps of anaerobic digestion, including the roles of the enzymes acetate kinase (AK) and phosphotransacetylase (PTA).

The major aim of this work was to gain insight into the biotransformation pathways involved in the removal of OMPs during methanogenesis. In particular, the enzymatic transformation of OMPs was assessed via in vitro assays with AK. Furthermore, the cometabolic impact of AK on the biotransformation of OMPs in methanogenic reactors (MRs) was quantified. To the best of our knowledge, this is the first attempt to clarify the role of AD enzymes on the biotransformation of OMPs.

MATERIALS AND METHODS

Organic micropollutants. This study focuses on 20 compounds that are commonly detected in sewage sludge[3,4,8,9,31–33] and whose chemical structures (Table S1), applications and physicochemical properties (Table S2) are representative of a huge range of OMPs. The selected pollutants are: the musk fragrances galaxolide (HHCB), tonalide (AHTN) and celestolide (ADBI); the anti-inflammatories ibuprofen (IBP), naproxen (NPX) and diclofenac (DCF); the

antibiotics sulfamethoxazole (SMX), trimethoprim (TMP), erythromycin (ERY) and roxithromycin (ROX); the neurodrugs fluoxetine (FLX), carbamazepine (CBZ) and diazepam (DZP); and the endocrine-disrupting compounds triclosan (TCS), bisphenol A (BPA), 4-octylphenol (OP), 4-nonylphenol (NP), estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2). These substances were purchased from Sigma-Aldrich (Steinheim, Germany) except for the fragrances, which were provided by Ventos (Spain). Stock solutions were prepared in HPLC grade methanol or acetone, depending on the compound, and stored at -18 °C.

Methanogenic reactor. Two lab-scale (14 L) continuously stirred (IKA RW20, 150 rpm) MRs were operated under mesophilic (37 °C) conditions. Both reactors were inoculated with sludge from a mesophilic STP anaerobic digester. The feeding consisted of a synthetic mixture of volatile fatty acids (VFA) (acetic:butyric:propionic 50:25:25, %COD) and other trace nutrients (section S4), in order to promote the growth of acetogenic bacteria and aceticlastic methanogenic archaea (i.e. *Methanosaeta* and *Methanosarcina).*[15,34,35] After a start-up period, both MRs reached steady-state operation at a hydraulic retention time (HRT) of 10 d, an organic loading rate (OLR) of 1 g COD L^{-1} d^{-1} and a methanization efficiency above 70%. After 1-2 months under these conditions (Table S4), a pulse of the selected OMPs (100 μg L^{-1} except for the hormones, which were 10 μg L^{-1}, section S4) was added to each MRs, and their concentrations were followed in the liquid (17 samples) and solid (10 samples) phases for 10 d.

Acetate kinase activity. Acetate kinase (AK, EC 2.7.2.1) is a homodimer with two active sites that catalyze the reversible Mg-dependent transfer of the ϒ-phosphoryl group from adenosine triphosphate (ATP) to acetate.[27] AK from *Methanosarcina thermophila* was chosen for this study because it is a well characterized and investigated enzyme[29] and because *Methanosarcina* species appear to be key organisms in AD.[36] AK from *M. thermophila*

recombinant, expressed in *E. coli*, was purchased from Sigma-Aldrich (USA) as lyophilized powder (885 U mg^{-1} protein (Bradford) and 6.94 U mg^{-1} solid). One unit (U) phosphorylates 1.0 μmol of acetate to acetyl phosphate per min at pH 7.6 and 25 °C. The AK powder was stored at -20 °C. Immediately before use, a solution of AK (288 μg mL^{-1}, 2.0 U mL^{-1}) in a potassium phosphate buffer (0.1 M, pH 7.4) was prepared.

Hydroxamate assay. The hydroxamate assay[37,38] is the simplest and most convenient method to determine the AK activity in the direction of acetyl phosphate synthesis.[29] This standard assay measures the rate of the forward Reaction 1 in the presence of hydroxylamine, which reacts with acetyl phosphate (Reaction 2) to form a colored complex in the presence of trivalent iron (Reaction 3). Under standard conditions, dephosphorylate acetyl phosphate is thermodynamically more favorable (Reaction 1); therefore, hydroxylamine is required to shift the reaction equilibrium to the right through the removal of acetyl phosphate.[37]

$$\text{Acetate} + \text{ATP} \overset{AK}{\longleftrightarrow} \text{Acetyl-P} + \text{ADP} \qquad \text{(Reaction 1)}$$

$$\text{Acetyl-P} + \text{Hydroxylamine} \longrightarrow \text{Acetyl hydroxamate} + HPO_4^{2-} \qquad \text{(Reaction 2)}$$

$$\text{Acetyl hydroxamate} + FeCl_3 \longrightarrow \text{Ferric acetylhydroxamate} \qquad \text{(Reaction 3)}$$

An adaptation of the hydroxamate assay[30] was used in this study. A stock solution was prepared with the following components: 290 mM of Tris-HCl (pH 7.4, neutralized with KOH), 400 mM of potassium acetate, 20 mM of $MgCl_2 \cdot 6H_2O$, 20 mM of ATP and 1410 mM of hydroxylamine hydrochloride (pH 7.4, neutralized with KOH). This reaction mixture was prepared immediately before use, since the aqueous solution of ATP at room temperature and the neutralized hydroxylamine solution have a limited stability. The reaction started by adding 1.0 mL of the enzyme solution over 1.0 mL of the pre-warmed (25 °C) reaction mixture. After

incubation for 12 min at 25 °C, the reaction was stopped by the addition of 2.0 mL of 10% trichloroacetic acid. Then, the color reaction was initiated by adding 2.0 mL of $FeCl_3$ (2.5% in 2 N HCl). After 5-30 min of incubation to allow for the formation of the colored complex,[38] the absorbance was recorded spectrophotometrically (Cecil CE-7200, UK) at 540 nm.

A standard curve was prepared by adding 0-1.7 U mL^{-1} of AK to the reaction mixture following the previously described methodology (Figure S2). Furthermore, because 1 U of enzyme dephosphorylates 1 μmol of ATP per minute, the consumption of ATP (μmol mL^{-1}) can also be correlated with the measured absorbance (Equation S1).

AK activity in the methanogenic reactor. The AK activity inside the reactor was determined without special precautions to avoid the presence of air (AK is not sensitive to oxygen)[30,39] following a methodology adapted from Mu et al.[40] Three samples 25 mL were withdrawn at different time points after the OMPs spike (10 min, 3 d and 10 d) and then centrifuged at 3107 *g* for 15 min. The supernatant was discarded, and the biomass was washed and resuspended in 25 mL of 0.1 M sodium phosphate buffer (pH 7.4). This procedure was repeated three times. The last resuspended mixture was sonicated at 20 kHz and 4 °C for 10 min to break down the cell walls and release the intracellular AK. The sample was then centrifuged at 15344 *g* and 4 °C for 30 min to remove the waste debris. When needed, the extracts were stored at -20 °C before measuring the enzyme activity assay via the hydroxamate assay.

Experiments with commercial AK. *Preliminary assays.* The relevance of several key parameters (temperature, pH and OMPs concentration) on the AK activity was first evaluated because they could impair the possible action of AK over the OMPs. Studies with AK from *M. thermophila* have typically been performed at 37 °C,[27,30,41,42] but the product information from SIGMA recommends a temperature of 25 °C for the enzymatic assay. Therefore, several

178 theoretical AK activities (0.01, 0.05, 0.2 U mL^{-1}) were evaluated via the hydroxamate assay at
179 both temperatures. The results did not show significant differences (Table S5); thus, the lower
180 temperature (25 °C) was chosen for the next assays to minimize possible activity losses by
181 enzyme denaturation in long-term experiments.[43]

182 A similar procedure was followed for the pH selection. According to Aceti and Ferry[30] and
183 the product information from SIGMA, the maximum activity of AK appears between pH 7.0-7.6.
184 Both extreme pHs were tested and, as expected, the effect on the AK activity (0.02 U mL^{-1}) was
185 negligible (Table S5). To prevent the pH from decreasing below 7.0 during the OMP assays due
186 to the consumption of acetate (weak base), the initial pH was adjusted with KOH to 7.6.

187 Finally, a possible inhibition of the AK activity by the OMPs was examined at 3 OMPs/AK
188 ratios: 0.35 μg mg^{-1} (50 μg OMPs L^{-1} and 144 mg AK L^{-1}), 3.5 μg mg^{-1} (100 μg OMPs L^{-1} and
189 28.8 mg AK L^{-1}) and 69 μg mg^{-1} (100 μg OMPs L^{-1} and 1.44 mg AK L^{-1}). In all cases, a negative
190 control without OMPs was included. The results (Table S5) indicated that AK activity was not
191 altered by any OMPs/AK ratio tested, and thus neither by the corresponding solvents added with
192 the OMPs pulse. The OMPs/AK ratio employed in the OMPs assays was 35 μg mg^{-1} (based on
193 the theoretical AK activity of a mesophilic AD operating at an OLR of 2 g COD L^{-1} d^{-1} with a
194 70% methanization efficiency).

195 *Enzymatic transformation of OMPs.* Once the operating parameters were selected (25 °C,
196 pH 7.6, 100 μg L^{-1} of OMPs and 2.9 mg L^{-1} of AK), the enzymatic transformation of the OMPs
197 was evaluated at two reaction times (1 d and 5 d) with and without the primary substrate (acetate)
198 in duplicate in 100 mL Erlenmeyer flasks. The final reaction media contained 145 mM of Tris-
199 HCl, 10 mM of $MgCl_2 \cdot 6H_2O$, 705 mM of hydroxylamine hydrochloride, 200 mM of potassium
200 acetate (in excess) and 50 mM of ATP. The acetate concentration, ATP consumption (Equation

S1), AK activity, pH and temperature were monitored (n=2) at different reaction times (5-11 time points) and the concentration of OMPs was measured in replicate at the beginning (n=4) and end (n=4) of each experiment.

Abiotic disappearance of OMPs. The disappearance of the parent micropollutants by adsorption was evaluated by comparing the concentration of OMPs (100 $\mu g\ L^{-1}$ in distilled water) after 1 d at 25 °C in contact with AK (2.9 $mg\ L^{-1}$) and without the enzyme. No representative differences were found (data not shown), so the adsorption of OMPs during the enzymatic assays with commercial AK was dismissed. Furthermore, to discard possible losses of the parent compound by evaporation, analytical difficulties or chemical reaction with the media (145 mM of Tris-HCl, 10 mM of $MgCl_2$, 705 mM of hydroxylamine hydrochloride, 50 mM of ATP, 200 mM of potassium acetate), duplicated negative controls (without AK) were incubated under the same conditions as the AK assays specified in the previous section. Trichloroacetic acid was not used to stop the enzymatic activity at the end of the assays with OMPs because reductions of the concentrations of ERY, ROX and SMX were observed when it was added. The enzymatic reaction was assumed to stop once the solid phase extraction (SPE) was performed.

Analytical methods. *Conventional parameters.* The operation of the methanogenic digesters was monitored in terms of the temperature, pH, total suspended solids (TSS), volatile suspended solids (VSS), alkalinity, ammonium, and total and soluble COD.[44] Biogas production was recorded using Ritter milligas counters (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany), and its composition was determined through gas chromatography (HP 5890 Series II). Volatile fatty acids (VFA) were measured individually in a gas chromatograph (HP 5890A) equipped with a flame ionization detector (HP 7637A).

223 *Organic micropollutants analysis.* Samples from the MRs were centrifuged at 1880 *g* for
224 15 min. The supernatant was pre-filtered (AP4004705, Millipore) and filtered at 0.45 µm
225 (HAWP04700, Millipore) before performing the SPE with 100 mL samples and 60 mg OASIS
226 HLB cartridges (Waters, Milford, MA, USA).[4,16] Samples from the enzymatic assays (50 mL)
227 did not require any pre-treatment prior to SPE. To quantify the OMPs sorbed onto the
228 methanogenic sludge, ultrasonic solvent extraction (USE) was conducted before SPE, as
229 described by Gonzalez-Gil et al.[4] The limits of quantification (LOQ) and recoveries applied to
230 the MRs and the AK assays are shown in Table S3.

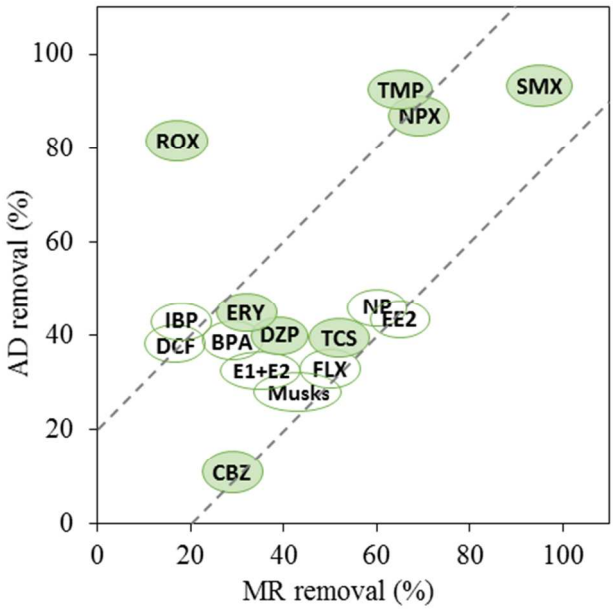
231 **Statistical analysis.** All of the enzymatic assays with OMPs were conducted in duplicate,
232 and each micropollutant concentration was measured twice (n=4). The results are expressed as
233 mean ± standard deviation. The significant differences between the enzymatic results were
234 statistically tested by analysis of variance (ANOVA) followed by the Dunnett T3 test for
235 multiple comparisons. The normal data distribution was analyzed with the Shapiro-Wilk test, and
236 the variance homogeneity was analyzed with the Levene test. When the variances were not
237 homogeneous, Brown-Forysthe analysis was used to assess the significant differences. All of the
238 statistical tests were performed at a 5% significance level using the IBM SPSS statistics®
239 software 20.0.

240 **RESULTS AND DISCUSSION**

241 **Biotransformation of OMPs during methanogenesis.** Figure 2 aims to highlight, in a
242 semi-quantitative representation, the relevance of the methanogenic step on the overall removal
243 efficiencies reported for OMPs during AD of sewage sludge. The methanogenic biomass showed
244 the capacity to biotransform all of the tested OMPs, although the efficiencies varied depending

on the compound. For example, SMX, NPX, TMP, OP, NP, FLX, EE2, TCS and the musk fragrances were significantly removed during methanogenesis, while the other compounds had biotransformations of less than 50%.

It was not easy to get a single value for the removal of OMPs during AD process due to the divergences on the reported values. Trying to narrow these differences, the average AD removals depicted on Figure 2 correspond to studies that used mainly continuous mesophilic digesters, treating sewage sludge at a HRT between 10 and 30 d (Table S6). Even though, IBP, DCF, BPA, NP, FLX, the hormones and the musk fragrances still presented high deviations, so it was not possible to accurately determine the influence of the methanogenesis on their disappearance. Most of the OMPs appear near the diagonal (between the two dashed lines) of Figure 2, suggesting that the methanogenic biomass is the main responsible for their biotransformation during AD. Therefore, the action of key enzymes participating in the methanogenic route, particularly AK, were further investigated in the next sections. ROX and TMP are the only compounds that clearly showed a higher removal during the overall AD, which indicates that other anaerobic communities (i.e., hydrolytic and acidogenic) widely participate on their removal.



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262 **Figure 2.** Semi-quantitative representation of the removal efficiencies of OMPs in the
263 methanogenic reactors (MR, x-axis) versus the average removal during AD of sewage sludge[4,8–
264 12,45–47] (y-axis). Compounds with large divergences on the reported AD removals (standard
265 deviations above 25%) are depicted by open ellipses. No data about the removal of OP in AD
266 was found (60-70% in the MRs). The two dashed lines delimit the region where methanogenesis
267 explains to a high extent the overall AD removal of the OMPs.

268 **Transformation of OMPs in the AK assays.** The experiments were performed at two
269 reaction times (1 and 5 days) and, with the exception of the negative control in the absence of
270 AK, consisted of two assays with and without acetate, which were intended to assess the role of
271 the main substrate on the possible AK catalytic transformation of OMPs. No significant
272 differences were found between the negative control and the two AK assays in the 1-day
273 experiment (data not shown), likely because the reaction time was not sufficient to illustrate the
274 action of AK on OMPs. Hence, this section focuses on the 5-day assays.

Performance of assays. The ATP and acetate concentrations during the 5-day assay are shown in Figure 3. The AK activity during the first 2 d was 4.8 ± 0.2 U L^{-1}, which resulted in a moderate conversion of acetate and ATP (approximately 30%). To increase the enzymatic activity, a second pulse of AK (8.6 mg L^{-1}) and ATP (40 mM) was added to the reaction media after 52 h. As a result, more rapid acetate and ATP consumption was achieved (Figure 3), which led to an average AK activity of 13.4 ± 1.5 U L^{-1}. After approximately 4 d, the depletion of both reagents almost stopped, which suggests that the phosphorylation of acetate (Reaction 1) reached an equilibrium. This is likely due to the decomposition of hydroxylamine at room temperature in neutralized solutions,[38,48] which is required to perform Reaction 2 and thus to avoid equilibrium of Reaction 1. This hypothesis is also supported by the fact that the acetate concentration on day 5 was higher than the value that was estimated stoichiometrically from the ATP consumption rates (Figure 3). The average AK activity during the 5-day assay with acetate was much higher (8.0 U L^{-1}) than in the assay without acetate (0.1 U L^{-1}), where a considerably lower conversion of ATP (2% after 5 d) was also achieved because the only potential substrates for AK are OMPs at a much lower concentration than the acetate.

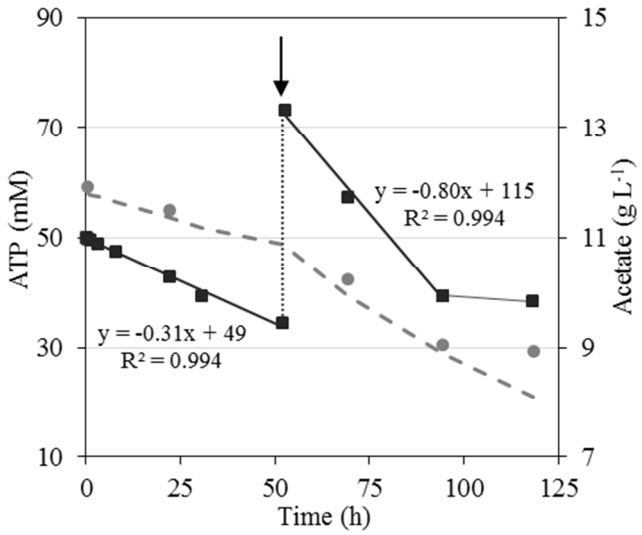


Figure 3. ATP (limiting reagent; squares) and acetate (dots) concentrations in the 5-day AK assay with acetate. The arrow indicates the second addition of ATP and AK. The dashed line represents the stoichiometric acetate concentration considering the consumption rates of ATP.

Chemical OMP transformation. The initial concentration of OMPs in distilled water is compared to the concentration of OMPs after 5 d in the negative control (reaction media without AK) in Figure 4. DZP, AHTN, ADBI, ERY, E1, EE2 and CBZ nearly completely disappear, likely due to the reaction between the ketone group of these compounds (except EE2) with the hydroxylamine required to perform the enzymatic assays. This hypothesis was confirmed experimentally because the OMPs concentration did not decrease in a reaction media without this amine. However, hydroxylamine was included in the reaction media to shift the action of AK towards acetate phosphorylation (Reaction 1). Obviously, this decision masks any possible effect of AK on the biotransformation of these seven OMPs.

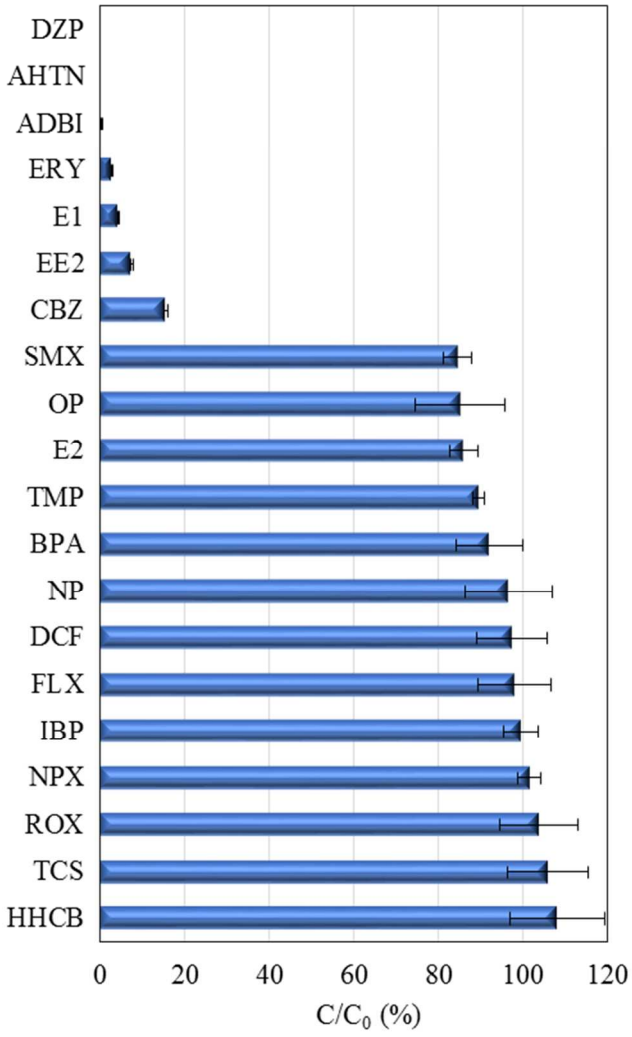


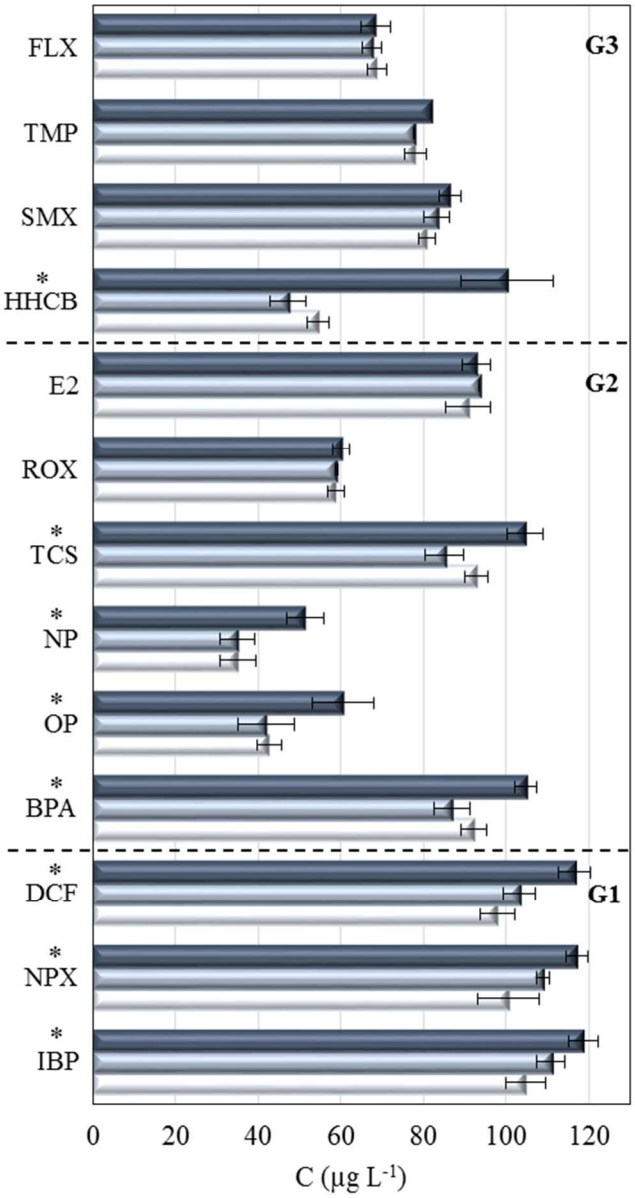
Figure 4. Chemical transformation of OMPs expressed as the ratio between the concentration in the negative control (reaction media without enzyme) after 5 d (C) to the initial concentration in distilled water (C_0).

Enzymatic transformation. The concentrations of the 13 OMPs that were not chemically transformed in the negative control after the 5-day assays with AK are shown in Figure 5. Based on their chemical structures (Table S1), these compounds are classified into three groups. The first group includes carboxylic compounds (IBP, NPX, DCF), the second group are OMPs with hydroxyl groups (NP, OP, BPA, TCS, ROX, E2), and the third group comprises compounds with

other functional groups (HHCB, SMX, TMP, FLX). All data were normally distributed and, except for NPX, OP and HHCB, their variances were homogeneous. FLX, TMP, SMX, E2 and ROX were not affected by AK, since no significant differences were found between the negative control and both enzymatic assays (with and without acetate). In contrast, this difference was statistically significant ($p<0.05$) for IBP, NPX, DCF, NP, OP, BPA, TCS and HHCB proving that these 8 compounds are transformed by the action of the enzyme. No statistical differences were found between the concentrations of these 8 OMPs in the AK assays performed in the presence and absence of acetate, which suggests that no competitive inhibition occurred between the primary substrate (acetate) and the cometabolic substrates (OMPs).[19,20] Moreover, these results suggest that once AK is available it can directly transform OMPs in the absence of acetate. However, the presence of the primary substrate is needed to trigger the synthesis of AK during the AD process; therefore, according to the definition of cometabolism,[17,20,21] the transformation of OMPs (non-growth substrates) is fortuitous and inherently linked to acetate.

To understand how AK can transform these compounds, it is necessary to look into the AK specificity. The action of AK from *M. thermophila* is quite restrictive because the size of the hydrophobic pocket and its affinity to the methyl group of acetate are determinant for substrate specificity.[42] However, this enzyme still shows slight activity over larger substrates with carboxyl groups (propionate, butyrate), alcohols (ethanol) and even over compounds without hydrophobic groups (formate, glycerol, glycine, glycolic acid).[29,30] For that reason, IBP, NPX and DCF, which have a carboxyl group (Group 1, Table S1) and are relatively small (1-2 benzene rings), were significantly ($p<0.05$) biotransformed by AK (10-15%). In addition, compounds with hydroxyl groups in their chemical structure (Group 2, Table S1) could also be appropriate substrates for AK. Nevertheless, the molecular size appears to be determinant

because only the smaller compounds (NP, OP, BPA and TCS) were biotransformed (15-32%). The steric hindrance of ROX and E2 could hinder the formation of the enzyme-substrate complex and their further reaction. Similarly, ERY, EE2 and E1, which reacted chemically with the hydroxylamine, would not be affected by AK due to their large molecular size. As expected, the concentrations of the rest of the OMPs (Group 3, Table S1) did not decrease during the enzymatic assays, excluding HHCB (45-50%), for which a clear explanation was not found. It is hypothesized that AK could attack the ether group of this compound, but no references were found to support this enzymatic transformation.



344

345 **Figure 5.** Transformation of OMPs by AK after 5 days of reaction time. The dark bars represent
346 the concentrations of OMPs in the negative control (reaction media without enzyme), the light
347 bars refer to the assay with acetate, and the white bars refer to the assay without acetate. The
348 asterisks indicate the statistical differences ($p<0.05$) of both AK assays with respect to the
349 control. The compounds are sorted according to their chemical structures in three groups: (G1)
350 OMPs with a carboxyl group, (G2) OMPs with a hydroxyl group and (G3) OMPs with other
351 functional groups.

The results of these experiments demonstrate the importance of combining the specificity of the enzymes and the chemical structure of OMPs to understand the biotransformation mechanisms. Accordingly, we hypothesized that some of the evaluated OMPs (Figure 5) are enzymatically transformed by the cometabolic action of AK when ATP is available in the media and disregarding the presence of the primary substrate (acetate). The proposed pathway predicts the formation of phosphorylated TPs. For example, the carboxyl group of NPX could act as the acceptor of a phosphoryl group, as occurs with acetate (Figure 6). Because the detection and identification of TPs is very challenging, a deep understanding of the cometabolic enzymatic biotransformation of OMPs allows TPs to be predicted, as was revealed in this study.

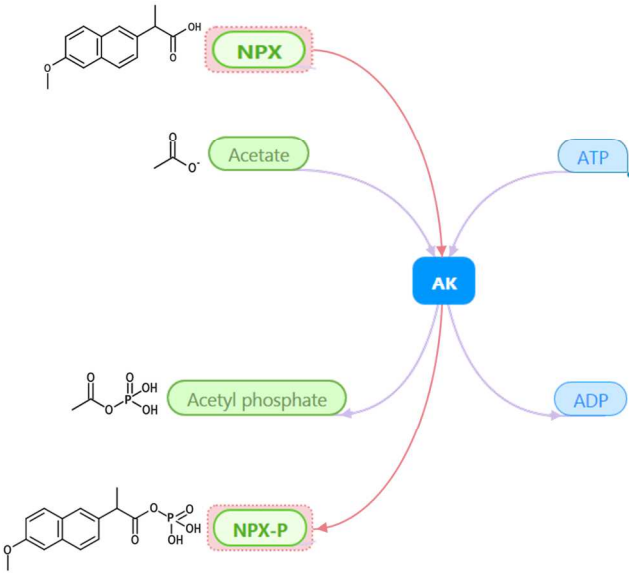


Figure 6. Proposed cometabolic pathway for the biotransformation of some OMPs, such as NPX, by the enzyme AK during methanogenesis.

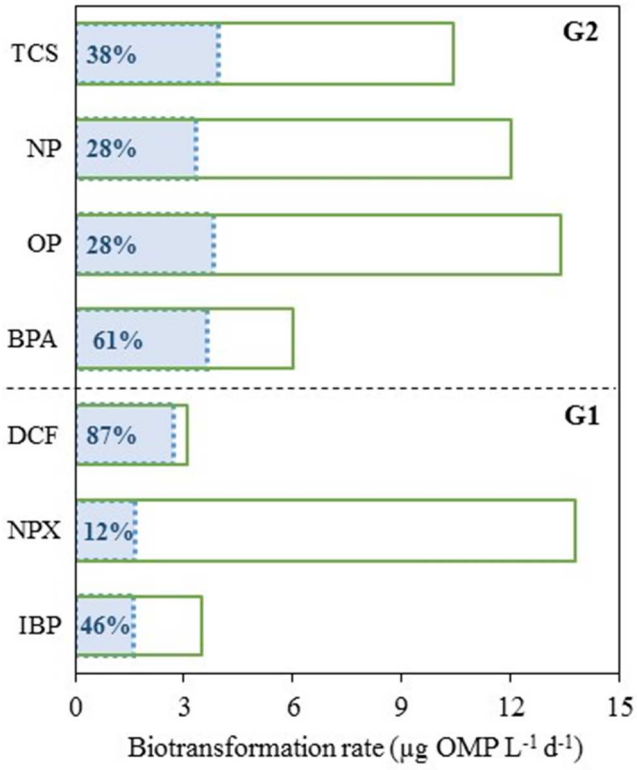
Relevance of AK to the biotransformation of OMPs during methanogenesis. The previous section suggests that when sufficient ATP is present in the media, AK could cometabolically phosphorylate compounds with carboxyl or hydroxyl groups and relatively low steric hindrance, such as IBP, NPX, DCF, NP, OP, BPA and TCS. Therefore, the next step is to

368 determine the relevance of this mechanism to the overall biotransformation that was observed for
369 the aforementioned OMPs in the MRs. Figure 7 compares the biotransformation rates ($\mu g\ L^{-1}\ d^{-1}$)
370 that were obtained in the AK assays with acetate and in the MRs at the same reaction time (5 d).
371 It is important to note that the AK activities in the MRs and the enzymatic assay are quite similar
372 (7.0 and 8.0 U L^{-1}, respectively). Assuming that 2/3 of the total CH_4 is produced from acetate by
373 aceticlastic archaea,[26] the theoretical AK activity in the MRs (OLR of 1 g COD $L^{-1}\ d^{-1}$ and 70%
374 methanization would be 10 U L^{-1}, which is similar to the measured value.

375 Depending on the compound, the biotransformation rates in the MRs varied from 3.0 to
376 14 μg OMP $L^{-1}\ d^{-1}$, while this range is narrower in the AK assays (1.5–4.0 μg OMP $L^{-1}\ d^{-1}$). IBP
377 and DCF are hardly biotransformed during methanogenesis (3.0–3.5 μg OMP $L^{-1}\ d^{-1}$), although
378 an important removal mechanism (>45%) is related to AK action. The AK cometabolic
379 mechanism is also relevant (61%) in the methanogenic biotransformation of BPA (6 μg OMP $L^{-1}\ d^{-1}$). In contrast, the transformation of NPX by AK (12%) does not explain its high depletion
381 during methanogenesis (14 μg OMP $L^{-1}\ d^{-1}$). Likewise, AK accounts for only 28-38% of the
382 overall biotransformation observed for OP, NP and TCS in the MRs (10–13 μg OMP $L^{-1}\ d^{-1}$). On
383 the other hand, AK would explain the whole biotransformation rate of HHCB in the MR
384 (9 μg OMP $L^{-1}\ d^{-1}$); what is a quite surprising fact, especially because it cannot be ascertain that
385 this transformation occurs via the proposed cometabolic phosphorylation pathway.

386 Based on these results, no direct relationship was found between the degree of
387 biotransformation during methanogenesis and the contribution of the AK cometabolic
388 mechanism. Moreover, the biotransformation rate was always higher in the MRs, which suggests
389 that other active enzymes in addition to AK could further transform them. Some candidate
390 enzymes could be those that continue the aceticlastic methanogenesis (Figure S1), those involved

391 in the hydrogenotrophic methanogenesis[49] or other kinases, such as propionate and butyrate
392 kinase. These two kinases have a larger hydrophobic pocket than AK to directly phosphorylate
393 propionate and butyrate, respectively;[50,51] therefore, they could be involved in the enzymatic
394 transformation of the same OMPs as AK, but they could also promote the transformation of
395 larger OMPs with hydroxyl groups, such as ERY, ROX, E1, E2 and EE2, which were not
396 affected by AK. Otherwise, the high methanogenic biotransformations of TMP and SMX (Figure
397 2) should be caused by other types of enzymes because kinases will not modify their chemical
398 structure. In summary, by combining enzyme action and the OMP's chemical structure, new
399 insights are provided to understand the transformation mechanisms of OMPs during AD, which
400 is useful for developing new strategies to maximize the elimination of OMPs from sewage
401 sludge and to predict the TPs that form and their potential risks.



402

403 **Figure 7**. Biotransformation rates of the OMPs prone to be cometabolically phosphorylated by
404 AK in the MRs (empty green bars) and in the AK assay with acetate (blue-pointed bars) after 5
405 days. The percentages indicate the contribution of AK to the methanogenic biotransformation.
406 G1 refers to OMPs with carboxylic compounds, and G2 refers to OMPs with hydroxyl groups.
407 HHCB was excluded from this figure due to the lack of information regarding the
408 phosphorylation action of AK on ether groups.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Enzymatic pathways of the aceticlastic methanogenesis (Figure S1); chemical structures (Table S1) and physicochemical properties (Table S2) of the OMPs; limits of quantification and recoveries of the analytical methods (Table S3); details of the MRs performance (section S4, Table S4); details of the acetate kinase activity determination (section S5, Figure S2); results from the preliminary assays with AK (Table S5) and summary of the removal of OMPs during AD (Table S6).

AUTHOR INFORMATION

Corresponding Author

Lorena Gonzalez-Gil

Telephone: 0034881816739. E-mail address: lorena.gonzalez@usc.es

Notes

The authors declare no competing financial interest.

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