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Inhibition of biogas production and biodegradability by substituted phenolic compounds in anaerobic sludge

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ABSTRACT

Phenolic compounds are abundant in nature and organic wastes. This biomass may be utilised in biogas generation. Phenolics can inhibit the degradation of readily biodegradable organic fractions and their own biodegradation. In this work, assays were carried out under anaerobic conditions to study the inhibition of both gas production and biodegradability due to seven phenolic compounds and to study their adsorption onto sludge and autoxidation in the aqueous medium. Fifty percent inhibition was in the range of 120 to 594 mg of compound/g VSS. An initial enhancement followed by an inhibition of biogas formation was found. The inhibition by the phenolic compounds was found to be influenced by autoxidation, apolarity, type, size and number of substitutions. Biogas production is influenced by concentration rather than any pH change. The concentration of the phenolic compound was partially biomethanized and the degradation of gallic and caffeic acids by this process is reported here for the first time. The maximum total biodegradation of any phenolic compound was $63.85 \pm 2.73\%$, and remaining non-biodegradable fraction was autoxidized and adsorbed onto the sludge matrix. Inhibition of methanization and partial inhibition of background gas was found at concentrations between 800 and 1600 mg/L organic carbon.

1. Introduction

Aromatic compounds are the second most abundant organic compounds in nature [1]. Their main chemical structure, i.e. the benzene ring, represents 25% of the biomass on earth [2]. Among aromatics are phenols which are found in agricultural and industrial wastes. In this group, caffeic acid, gallic acid, tyrosol, 4-hydroxybenzoic, and protocatechuic acid are found in olive oil, red wine and tanning wastewaters and in the degradation products of lignin [3,4], while phenol and catechol are well known industrial pollutants [5]. All these compounds may inhibit microbial activity [6,7], and therefore cause difficulties in bioremediation and potentially reduce the ability to produce biofuels by biomass fermentation.

There is a lack of information about the toxic effects of gallic acid, 4-hydroxybenzoic acid, protocatechuic acid and tyrosol and, while there are studies of the toxic effects of phenol and catechol on anaerobic microorganisms [6,8–10], these experiments were carried out with different types of inoculum and without standardized assays. One study does report on inhibition of gas production by caffeic acid, 4-hydroxybenzoic acid, and protocatechuic acid [11] but failed to report 50% inhibition.

Data on the methanization of phenolic compounds varies considerably [12–16] while others report the biomethanization to be unsuccessful [16,17]. Biodegradability experiments tend to have been conducted at one specific concentration with relatively short incubation periods. This has led to misinterpretation of recalcitrance due to delays in microbial acclimation. Additionally, no explanation regarding the loss of non-biodegraded mass has been given in previous studies nor have the effects of adsorption onto the sludge, autoxidation and the thermodynamic fixation of CO_2 into carbonate complexes been taken into consideration.

In this work, two standard methods are applied to study the toxicity and biodegradability of a group of phenols: phenol (PHE), catechol (CA) and tyrosol (TY); and a group of phenolic acids: gallic (GA), protocatechuic (PRO), 4-hydroxybenzoic (4HBA) and caffeic (CAF). The effects of the concentration of these compounds on biogas production by anaerobic bacteria are investigated and a novel approach to understanding the effects of molecular size, type, polarity and autoxidation on toxicity is presented. Biomethanization of these phenolic compounds as the sole carbon source is studied taking into consideration adsorption onto

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the sludge matrix, autoxidation and fixation of CO_2 into carbonate complexes.

2. Materials and methods

2.1. Biomass

Active sludge was collected from the Aldwarke waste water treatment plant, Rotherham, UK. The sludge was sieved (1 mm^2) to remove sand and other particles before use and it was not acclimatised. Specific methanogenic activity was carried out by anaerobically incubating triplicates of 1 L bottles with sludge and amending with phenol at concentrations ranging from 100 to 800 mg carbon/L. The production of methane was then recorded daily for 40 days. At 200 mg carbon/L the maximum value of 3.6×10^{-5} [mol CH_4]/[day g VSS] was obtained. The concentrations of biomass in the sludge were 17.6 and 1.3 g/L VSS (volatile suspended solids) in the toxicity and biodegradability experiments, respectively.

2.2. Substrate and chemicals

Toxicity experiments were carried out according to EN ISO 13641-1 [18]. The substrate contained 2 g/L each of glucose, yeast extract and nutrient broth to which was added each phenolic compound as required. The substrate is designed not to be a limiting factor and 3,5-dichlorophenol (150 mg/L) was used as a reference control against every phenolic compound. Biodegradability experiments were developed in accordance with EN ISO 11734 [19] in which phenols are the unique added carbon source. Resazurin was added as redox indicator and the medium was sparged with nitrogen gas (99%) for 20 min per litre of medium immediately before use. Finally, sodium sulphide nonahydrate was added to assure sufficient reductive capacity. This medium was mixed with each phenolic compound. All the chemicals were obtained from Sigma–Aldrich, Co. (UK).

2.3. Equipment

Gas-tight plastic expandable bottles were used for sludge collection and transported in insulated containers. The reactors were 155 mL serum bottles with aluminium caps and butyl rubber septa, which could be pierced to measure the pressure in the headspace with a digital pressure meter fitted with a three-way stopcock and needle. Millipore glass microfibre membrane filters (0.2 μm pore size) were used to sterilise the substrate in the toxicity experiment. An anaerobic chamber containing nitrogen (99%) and an incubator were utilised for setting up and running both experiments. In the adsorption experiment, a distillation unit, as described in standard methods [20], was utilised to recover phenols adsorbed by the sludge matrix.

2.4. Analysis

Biomass concentration was assessed as volatile suspended solids (VSS) by the drying and ignition method [20]. Methane and carbon dioxide were analysed in a chromatograph (GC, Varian 3400) fitted with a methanizer, flame ionization detector (FID) and injector. The chromatographic column (30 m × 0.530 mm GS-Q) was packed with 10% nickel nitrate on Chromosorb GAW 100/120. This column operated at 60 °C and an inlet pressure of 5.7 psi. The injector and the FID operated at 350 and 280 °C, respectively. In this analysis, nitrogen gas was used as a carrier gas at a flow rate of 19.0 cm/s. Phenolics were analysed in a high performance liquid chromatograph coupled to a UV detector at 230 nm (HPLC, PerkinElmer, Co., USA), using 0.1% H_3PO_4 and 70/30 (v/v) acetonitrile/water as the mobile phase at 1 mL/min. The injection volume was 20 μl and separation was carried out in a column (C18, type zorbax ODS, 4.6 mm × 250 mm, 5 μm, Agilent Technologies, Co. (UK) at 25 °C). Interferences, e.g. phenol-decomposing bacteria, extracellular polymeric substances (EPS) and inorganic particles were eliminated by filtering samples through 0.45 μm micro filters. Inorganic carbon (IC) was determined in a total inorganic/organic carbon (TIC/TOC) analyser (Shimadzu, Co.) coupled to a non-dispersive infrared (NDIR) gas analyser.

2.5. Experimental procedures

2.5.1. Toxicity

Sludge and substrate were simultaneously incubated with (test) and without (controls) one of seven phenolic compounds in sealed serum bottles (140 mL of liquid) for 4 days at 35 ± 1 °C. The concentration of the test compound was 500, 1000, 2000, 4000 and 8000 mg/L. Triplicate toxicity assays were carried out for tests, controls, and reference (3,5-dichlorophenol) sets. The amount of biogas produced was determined by recording the pressure in the headspace every 24 h. Pressures were summed and averaged at each interval for each set of bottles and the cumulative mean biogas production was plotted against time.

Percentage inhibition was calculated after 48 h incubation (before the plateau) for each mass concentration of test material and reference substance. This operation was carried out against the control according to the calculations in ISO 13641-1 [18]. Toxicity of the chemicals was expressed as EC%I, i.e. the quantity in milligrams of toxic chemical that inhibits, at certain percentage (%I), the gas produced by one gram of biomass (g VSS). It is calculated by plotting the percentage inhibition against the log of mass concentration of test compound (mg/L). From the plots, the concentrations of toxic chemicals inhibiting 20, 50 and 80% of gas production relative to the blanks can be determined and these are then divided by the concentration of biomass (g VSS/L).

2.5.2. Biodegradability

This experiment was carried out with each phenolic compound as the only carbon source as described in standard methods [19]. CAF was added as a solid owing to its low solubility in water and allowed to dissolve in the final aqueous reaction volume. The inoculum was prepared as in the toxicity experiment and pre-digested anaerobically at 35 ± 0.5 °C for 7 days. This operation was performed without the addition of nutrients or substrate, aiming for the reduction of background gas production and thus decreasing the influence of the blanks. Biomass was cleared of inorganic carbon (IC) by washing with medium and centrifuging at 3000 × *g* for 5 min. The resultant pellet was finally suspended in fresh medium. Preliminary experiments were carried out to determine a range of concentrations of each phenolic compound. These concentrations are expressed as carbon (100, 200, 400, 800 and 1600 mg carbon/L) for convenience in calculating the carbon mass balance. Triplicate experiments were prepared for blank, control and test. Blank and control bottles contained all the components except the phenolic compound or the sludge, respectively. pH was measured in all bottles before being incubated at 35 ± 0.5 °C for 250 days. Cumulative gas production was calculated by averaging pressure readings, which were measured every 5 days. The percentage of organic matter converted to gas in the headspace could then be calculated [19]. The fraction of dissolved CO_2 forming carbonate complexes was determined by measuring aqueous IC and then the percentage of total degradation could be assessed. All parameters were calculated by subtracting values obtained for the blanks. The standard method, EN ISO 11734 [19], was coupled with CH_4 and CO_2 analysis to deter-

mine the effect of concentration on methanogenesis. Dissolved CH_4 and CO_2 were determined from the partial pressure of both gases and the application of Henry's Law [21], from which the following equations were obtained:

$$N_{liq} = K_{Hi} V_{liq} y_i \Delta P$$

$$N_{gas} = (V_{gas}/RT)\Delta P$$

where: i Methane or carbon dioxide. N_{gas} Number of mol of gas in the gas phase. N_{liq} Number of mol of gas in the liquid phase. K_{Hi} Henry's constant for the compound (mol_i/L)/bar. ΔP Cumulative pressure, bar. R Universal constant of ideal gases, 0.082 (L atm)/(mol K), T Temperature, K. V Volume, L. y_i Mol fraction of gas, mol_i/(mol of gas phase).

2.5.3. Polymerization and adsorption

An experiment to detect products of polymerization was performed in bottles containing only medium and 100 mg/L carbon of each phenolic. When the exponential phase of cumulative gas production was reached, samples were taken from the bottles, and filtered and unfiltered fractions were analysed by high performance liquid chromatography (HPLC).

Adsorption was measured by extracting phenols adsorbed into the sludge. These compounds were evaporated and condensed in a distillation unit [20]. A sample was loaded and its boiling point was reduced by the addition of diluted H_3PO_4. Glass beads were then introduced to control the violent formation of steam. The system was operated until the last drop of condensate was received in a graduated cylinder. Turbidity of the condensate was treated with a filtration step (0.45 μm pore-size) before spectrophotometric analysis. This filtrate has an acidic character (pH 1.5–4) and the pH was adjusted as required. Sample losses occur as only 90% of the sample volume can be recovered as a liquid; the missing fraction is probably retained in a black crust inside the flask.

3. Results and discussion

3.1. Toxicity of phenolic compounds

3.1.1. Dose response features

The increments of concentration have different effects on the inhibition of biogas production. In the case of TY, a direct relationship between concentration and inhibition was observed, i.e. monotonic dose-response (Fig. 1, plot 1). A similar pattern was observed in each experiment with PHE, CA, and CAF. Such a dose response has already been observed in anaerobic metabolism of organosulphur compounds [23] and N-substitute aromatics [9]. However, this does not hold for HBA, PRO and GA, and therefore an increment in concentration is not always associated with an increment in toxicity. 4HBA and PRO enhanced bacterial activity to produce more biogas between 500 and 2000 mg/L, and were inhibitory at higher concentrations (Fig. 1, plot 2). In these cases, increasing concentration leads to enhancement of biomethanization (first phase: negative slope) but at higher concentrations biogas production is inhibited (second phase: positive slope). This non-monotonic (biphasic) low-dose high-response adaptative phenomenon is called hormesis and has been observed in other environmental matrixes containing aromatic compounds [22].

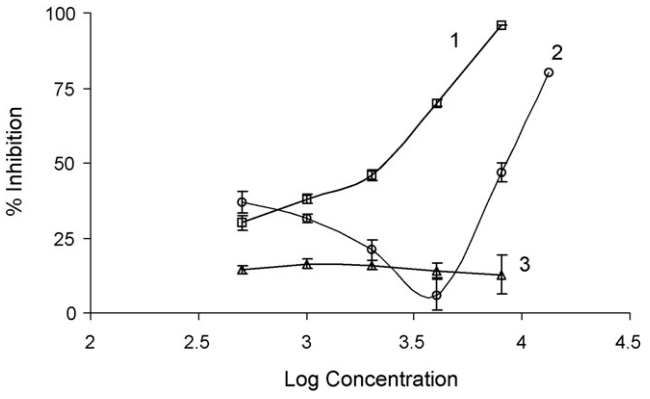


Fig. 1. The effect of concentration on the inhibition of biogas production caused by phenols and phenolic acids. TY followed pattern one (□). A similar pattern was independently described by PHE, CA, and CAF. Pattern two was shown by 4HBA (○) and similarly by PRO. Gallic acid depicted the third pattern (△). The error bars represent the confidence values of replicates for TY, 4HBA and GA.

Hormesis displays a J-shaped dose response curve, which depicts two phases featured by the change of slope sign or enhancement and then toxicity of methanization. In this phenomenon, a very low dose of a chemical, radiation or any stimulus may trigger opposite response to a very high dose over an organism, population or any physiological system. The physiological system is reestablished and adapted directly, via a regulatory process (direct stimulation), or enhanced due to low levels of stress or damage (overcompensation) [22].

The third pattern was that of GA which does not give any changes in inhibition of biogas with increments in concentration (Fig. 1, plot 3).

After 48 h, each concentration giving 20, 50 and 80% of inhibition on biogas production was calculated (Table 1). In the case of HBA and PRO, the concentrations taken were those on the second phase (positive slope) of the inhibition plot. Unlike the other phenolics, GA gave a maximum inhibition of 14.7% at all concentrations. This low toxicity might be due to instantaneous polymerization of GA into carboxy purpurogallin [17] rather than any problems of insolubility.

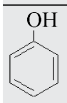
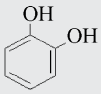
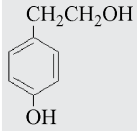
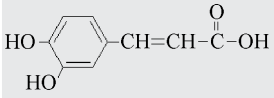
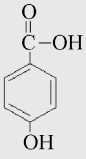
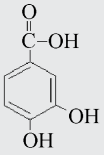
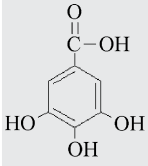
The relationships between chemical properties and EC50% values in Table 1 are discussed in the following subsections.

3.1.2. Number of substitutes and apolarity versus toxicity

Phenolic compounds inhibit the electrochemical proton gradient developed across cell membranes resulting in a reduction of both electron transport and energy production [24]. This toxicity is thought to be due to molecular size [25], substitution in the aromatic ring [26] and apolarity of the molecule [5,10]. Phenols thus disturb the trophic chain established between microorganisms by affecting the production of fatty acids, hydrogen and, consequently, methane [26,27].

The number of substitutes modifies the apolarity of the molecule and consequently its toxicity. A comparison of this apolarity/toxicity can be made by taking the EC50 values as a reference level where concentration and inhibition are directly correlated. Among phenolic acids, the toxicity of the aromatic compound decreases as the number of OH substitutes increases (Table 1). This can be shown by taking 4HBA as a starting model, and adding two OH substitutes in the -*meta* position on both sides of the aromatic ring. This leads to the formation of PRO and GA coupled with a reduction of apolarity (Log P), which diminishes the lipid solubility (lipophilicity or hydrophobicity) and therefore decreases disturbance in the cell membrane, which has a lipid character. This toxicity/apolarity relationship is also valid if PHE, CA and TY are analysed separately (Fig. 2). A linear toxicity/apolarity relationship may be suggested since it has also been observed with different chemicals [5,10]. However, Caffeic acid does not follow this behaviour due to the double bond substitution (—CH=CH—COOH).

Table 1
Apolarity–toxicity relationship of seven phenolic compounds inhibiting the anaerobic biogas production from the digestion of D-glucose, yeast extract and nutrient broth

Test compound[a]	Log *P*[b]	EC mg chemical/[g $VSS_{biomass}$]		
		20%	50%	80%
 Phenol (94)	1.482 ± 0.185	24.64 ± 2.21	120.63 ± 7.03	309.21 ± 5.03
 Catechol (138)	0.880 ± 0.205	64.88 ± 34.99	327.56 ± 14.08	768.15 ± 101.01
 Tyrosol (138)	0.623 ± 0.203	60.15 ± 30.04	411.90 ± 13.13	1040.81 ± 106.52
 Caffeic acid (180)	1.424 ± 0.360	48.36 ± 5.32	206.06 ± 6.97	388.58 ± 5.76
 4-Hydroxybenzoic acid (138)	1.419 ± 0.221	123.15 ± 27.61	271.03 ± 19.74	432.88 ± 12.22
 Protocatechuic acid (154)	1.157 ± 0.238	205.64 ± 4.18	594.01 ± 55.40	1085.67 ± 147.55
 Gallic acid (170)	0.911 ± 0.327	N/A	N/A	N/A

[a] Molecular weight is presented in parenthesis after the compound name.
[b] Apolarities values (Log *P*) were obtained from SciFinder Scholar® 2006.

3.1.3. Effect over acetogenesis and acetoclastic methanogenesis

It was found that acetogenic and acetoclastic methanogenic bacteria are inhibited to different extents. For example, PHE and CA were more toxic to the overall digestion process (Fig. 2, markers: 7 and 8) than to the methanogenesis from acetate (Fig. 2, markers: 1 and 3) studied by Sierra-Alvarez and Lettinga [10]. This indicates that aromatic compounds disturb the anaerobic digestion of organic matter by affecting the tropic-chain processes preceding methanogenesis and thus biogas production. It can therefore be concluded that hydrolysis and acetogenesis are inhibited to a higher extent than methanogenesis.

3.1.4. Type and size of substitute versus toxicity

Although a direct relationship between apolarity/toxicity was observed in the case of phenols (Table 1), it is unclear if such a relationship is due to the contribution of the type, position or size of substitutions on the aromatic ring, e.g. OH in *-meta* position (catechol) or —CH2—CH2—OH in *-para* position (tyrosol). Type of substitute can explain the non-linearity of caffeic acid (—CH=CH—COOH) among the phenolic acids. This phenomenon can be observed when comparing similar molecules. If the phenol molecule is taken as the start point, and one COOH is introduced in *-para* position, the resulting structure is 4HBA. Both, phenol

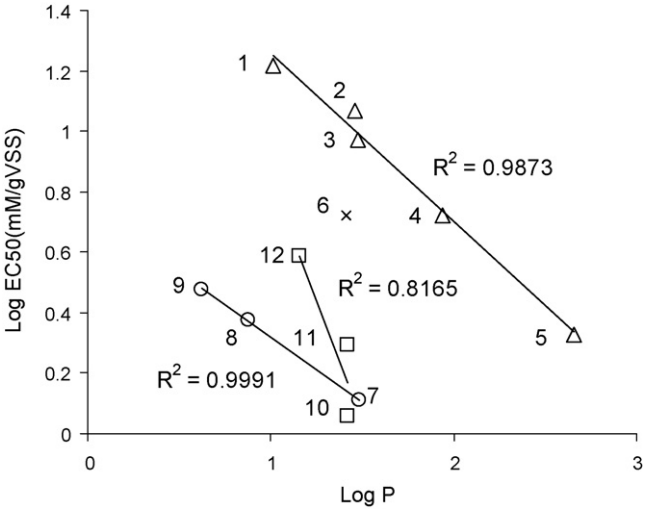


Fig. 2. Toxicity/apolarity relationship of phenols and phenolic acids. Two processes are represented. The upper straight line corresponds to inhibition of acetoclastic methanogenesis (△: 1. Catechol, 2. 2-Methoxyphenol, 3. Phenol, 4. 4-Methylphenol, 5. 4-Ethylphenol, 6. Phenylacetic acid) studied by Sierra-Alvarez and Lettinga (1991), the lower part lines correspond to inhibition of overall digestion studied in this work (◯: 7. Phenol, 8. Catechol and 9. Tyrosol. □: 10. Caffeic acid, 11. 4-Hydroxybenzoic acid and 12. Protocatechuic acid).

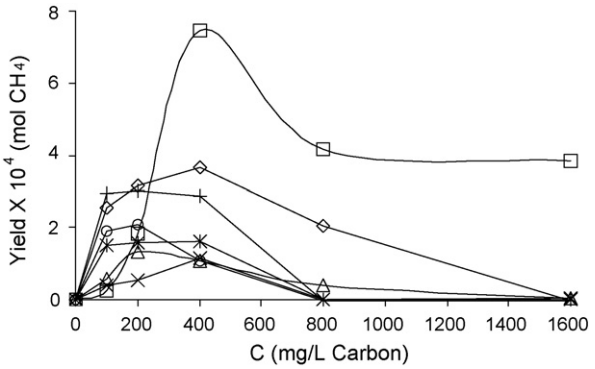


Fig. 3. Yield of methane for phenol (◊), tyrosol (□), catechol (△), 4-hydroxybenzoic acid (×), protocatechuic acid (✳), gallic acid (◯) and caffeic acid (+). This is the total amount found in both aqueous and gas phases. Tyrosol, phenol and catechol partially inhibited methanization themselves (800 mg/L). Partial inhibition of background production was experienced at 1600 mg/L except for tyrosol.

and 4HBA, have similar apolarity (approx. 1.4) but the introduction of the COOH allows microbes to deal with a twofold increase in concentration (Table 1). In contrast, if either OH (–*meta*) or CH2—CH2—OH (-*para*) is introduced, to form catechol or tyrosol, the linear toxicity/apolarity relationship is fulfilled and microbes are capable of dealing with three or four times the concentration compared to phenol. Therefore, the toxicity of phenol is reduced by the introduction of substitutes in the following order: COOH>OH>CH2—CH2—OH. This effect of substitution is also valid in other molecules, e.g. notwithstanding that TY had the lowest apolarity; it is still more toxic than PRO and GA. This finding supports the importance of the substitution group reported by Wang et al. [26]. The low toxicity of GA (up to 14.7%) is therefore a result of the contribution of the COOH and three OH substitutes.

The size of the substitute structure does not affect toxicity of phenolic molecules since the molecular weights of HBA and TY are practically the same but TY is twofold less toxic than 4HBA. This contrasts with the general trend reported in a previous study by O'Connor and Young [25] and indicates that the type of substitution is more important than apolarity and molecule size.

3.2. Degradation of phenolic compounds into methane and carbon dioxide

Biomethanization of phenolics is affected by concentration. In this process, the lag phase was a function of physicochemical properties and concentration of the substrate (Table 2). At 100–400 mg carbon/L, the biodegradation of gallic acid started immediately. The ease with which trihydroxylated phenols undergo methanization was also observed by Field and Lettinga [17]. In contrast, mono and di- hydroxyphenols require longer acclimatization times. For instance, at 400 mg carbon/L catechol and caffeic acid showed total inhibition of methanization and partial inhibition of background gas production relative to the blanks for a lag period of 103 and 131 days, respectively. This lag phase might therefore be an effect of selective forces developing aromatic-degrading volatile fatty acid-forming populations. The microbial community then switched to an activated state and the exponential phase began. Biogas formation was then observed in all cases from 100 to 400 mg carbon/L or up to 1600 mg carbon/L for tyrosol. At 800 mg carbon/L methanization of phenol and catechol was only detectable by determining the methane in both aqueous and gas phase (Fig. 3). This gas formation was not detected by direct pressure measurements probably because CO_2 is thermodynamically retained (up to 10%) in aqueous solutions by carbonate complexes, e.g. $NaHCO_3$, $CaHCO_3$ and $MgHCO_3$ [15].

All the phenolic compounds were partially converted into gasified carbon in the gas headspace (Dh) (Table 2). In the present study, Dh of phenol was lower (from 21.67 to 32.21%) than values previously reported (45 to 99%) [15,28,29]. In the case of catechol, its Dh values were also lower than the 67 to 85% obtained in previous studies [12,15]. Similarly, 4-hydroxybenzoic acid and protocatechuic acid developed Dh values lower than the respective values of 80 and 63% previously reported [15].

In this work, total biodegradation (Dt) includes the mass of carbon converted into inorganic carbon (IC) which is trapped in the aqueous phase as carbonate complexes. This consideration is not addressed in some previous investigations, where only gas-production was recognised as a common variable for degradation by digestion, i.e. Dh. The maximum Dt values observed among the phenolic compounds were not greater than 55% (Table 2) and lower than the Dh values reported in the literature. Thus, not even the addition of IC to calculate Dt was sufficient to reach the values reported in the past. These discrepancies may be due to intrinsic differences of inoculum utilised, e.g. its origin, washing of sludge, concentrations of sludge and aromatic compound.

Dt was enhanced or inhibited with increasing concentration depending on the compound. An increase of 100 to 200 mg carbon/L resulted in an enhancement of Dt for phenol, tyrosol, 4HBA, and GA. Dt of the latter was even more enhanced when the concentration increased to 400 mg carbon/L. This is probably due to overcompensation by the microbes to recover disrupted homeostasis [22]. Higher concentrations resulted in a decrease of the Dt value until negative results were obtained (Table 2). Negative values indicate that even the background gas production relative to blanks is inhibited by high levels of the compounds. This was not observed in the case of tyrosol for which biodegradation occurred at all concentrations. It is known that tyrosol undergoes methanization but the percentages of degradation have not previously been calculated [16].

Table 2
Biodegradability parameters and carbon mass balance of biological and non-biological transformations of phenolics (one fraction was converted to biogas and the rest was adsorbed onto the matrix or diluted in the aqueous phase)

Compound	mg C/L × 102			Biodegradability			mg C/L	
		Lag (days)	End (days)	pH[a] ± 0.06	Dh (%)	Dt (%)	Recovered from	
							Aqueous phase	Sludge
Phenol	1	17	38	6.7	21.7 ± 6.7	35.5 ± 9.1	ND	51.0 ± 10.3
	2	24	45	6.5	27.3 ± 5.1	46.2 ± 11.3	ND	90.3 ± 9.0
	4	31	103	6.4	32.2 ± 2.4	45.1 ± 2.6	ND	203.5 ± 10.1
	8	I	I	7.1	−1.1 ± 0.4	−1.9 ± 0.6	57.4 ± 26.1	713.5 ± 8.7
	16	I	I	7.4	−1.7 ± 0.1	−3.9 ± 0.1	889.0 ± 268.4	695.9 ± 9.7
Catechol	1	24	45	6.6	35.4 ± 6.7	52.2 ± 7.2	ND	38.8 ± 11.2
	2	31	82	6.5	35.1 ± 3.6	50.4 ± 4.0	ND	89.9 ± 9.6
	4	103	194	6.4	31.3 ± 6.8	45.2 ± 6.9	80.0 ± 135.2	114.0 ± 21.3
	8	I	I	6.9	−1.7 ± 0.5	−3.0 ± 0.5	632.7 ± 113.1	161.2 ± 15.6
	16	I	I	6.9	−1.7 ± 0.3	−3.0 ± 0.4	1386.1 ± 92.5	209.1 ± 18.5
Tyrosol	1	17	31	6.6	31.0 ± 3.2	46.5 ± 4.4	ND	49.5 ± 12.9
	2	17	55	6.4	39.2 ± 5.6	51.9 ± 5.6	ND	87.2 ± 15.4
	4	17	103	6.2	38.7 ± 7.6	50.2 ± 7.6	ND	189.1 ± 8.8
	8	24	45	6.6	5.9 ± 0.7	7.8 ± 0.9	ND	732.2 ± 15.2
	16	31	82	5.5	2.5 ± 1.2	0.3 ± 1.2	37.4 ± 57.1	1549.0 ± 13.9
4-Hydroxy benzoic acid	1	17	31	6.7	29.1 ± 13.3	49.7 ± 13.7	ND	46.7 ± 9.8
	2	17	45	6.5	40.0 ± 11.9	58.1 ± 12.1	ND	79.1 ± 11.8
	4	45	131	6.2	31.7 ± 1.1	50.2 ± 2.3	ND	188.3 ± 10.2
	8	I	I	5.0	−3.2 ± 0.2	−7.6 ± 0.4	79.3 ± 9.1	701.5 ± 6.5
	16	I	I	4.3	−1.8 ± 0.1	−4.2 ± 0.1	316.3 ± 31.1	1275.7 ± 9.1
Caffeic acid	1	24	45	6.6	30.8 ± 12.1	52.2 ± 12.2	ND	41.2 ± 10.0
	2	31	89	6.4	32.7 ± 9.1	52.2 ± 9.2	ND	83.5 ± 7.1
	4	131	215	6.2	32.2 ± 6.8	43.8 ± 6.8	ND	218.1 ± 4.9
	8	I	I	6.0	−3.1 ± 0.2	−5.7 ± 0.4	411.93 ± 95.5	379.4 ± 11.5
	16	I	I	5.0	−1.3 ± 0.1	−2.7 ± 0.1	1659.24 ± 191.5	ND
Gallic acid	1	0	17	6.5	26.8 ± 6.5	47.6 ± 7.8	ND	48.0 ± 2.5
	2	0	24	6.4	31.2 ± 5.8	50.2 ± 6.2	ND	87.7 ± 10.5
	4	0	131	6.2	35.6 ± 6.5	54.5 ± 6.6	ND	178.8 ± 8.4
	8	I	I	4.9	−3.0 ± 0.3	−7.1 ± 0.5	109.8 ± 28.2	678.2 ± 11.9
	16	I	I	4.1	−1.7 ± 0.2	−1.8 ± 0.3	251.5 ± 92.5	1340.0 ± 9.7
Protocatechuic acid	1	24	38	6.6	36.9 ± 2.6	63.9 ± 2.7	ND	31.3 ± 2.8
	2	24	45	6.4	39.0 ± 7.6	62.4 ± 7.7	ND	66.4 ± 7.9
	4	31	117	6.2	31.4 ± 3.4	52.6 ± 3.7	ND	188.6 ± 5.5
	8	I	I	6.1	−2.8 ± 0.4	0.4 ± 5.2	381.2 ± 105.4	411.9 ± 10.0
	16	I	I	4.2	−1.4 ± 0.1	−3.7 ± 0.1	1073.8 ± 382.7	516.9 ± 9.6

I Complete inhibition of the digestion process.
ND Not detected.
[a] The confidence values of pH in all compounds have insignificant variability and therefore an average is reported herein.

3.3. Spontaneity of methanization

The stoichiometry of methane formation from phenolic compounds is presented in Table 3, showing that approximately 50% of theoretical carbon availability was not utilised in methanization. This process was thermodynamically favourable (exergonic) for phenol and 4HBA. The likelihood of methanization (ΔG) from most compounds cannot be calculated as there is no thermo-

Table 3
Thermochemistry of the global reactions that phenolic compounds undergo under fermentative methanogenesis

Compound	Reactions	CO_2/CH_4		ΔH_r^0 KJ	ΔG_r^0 KJ/mol	ΔS_r^0 KJ/K	K	Notes
		Theoretic	Experimental (Max.)					
Phenol	$C_6H_6O(l) + 4H_2O(l) \rightarrow 2.5CO_2(g) + 3.5CH_4(g)$	0.72	0.33	55.8	−167.12	0.75	3.923×10^{29}	a, b, c, d, e
Tyrosol	$C_8H_{10}O_2(s) + 4.5H_2O(l) \rightarrow 3.25CO_2(g) + 4.75CH_4(g)$	0.69	0.36					
Catechol	$C_6H_6O_2(s) + 3.5H_2O(l) \rightarrow 2.75CO_2(g) + 3.25CH_4(g)$	0.85	0.44					
4-HBA	$C_7H_6O_3(s) + 4H_2O(l) \rightarrow 3.5CO_2(g) + 3.5CH_4(g)$	1	0.58	104.6	−176.64	0.94	1.523×10^{33}	a, b, e, f, g
Protocatechuic a.	$C_7H_6O_4(s) + 3.5H_2O(l) \rightarrow 3.75CO_2(g) + 3.25CH_4(g)$	1.15	0.74					
Gallic a.	$C_7H_6O5(s) + 3H_2O(l) \rightarrow 4CO_2(g) + 3CH_4(g)$	1.33	0.73					
Caffeic a.	$C_9H_8O_4(s) + 5H_2O(l) \rightarrow 4.5CO_2(g) + 4.5CH_4(g)$	1	0.52					

The stoichiometric reactions were obtained by using the equation of Symons and Buswell (1933). (a) Values were calculated from online thermochemical data at standard conditions (1 atm) and 298 K, published in the National Institute of Standards and Technology web site, accessed on 16 February 2006. (b) ΔH_r^0 was calculated by the definition $\Delta H_{r,298}^0 = \sum_i^{products} n_i \cdot \Delta H_{f,i,298}^0 - \sum_j^{reactants} n_j \cdot \Delta H_{f,j,298}^0$. (c) ΔG_r^0 was calculated by the definition $\Delta G_{r,298}^0 = \sum_i^{products} n_i \cdot \Delta \mu_{f,i,298}^0 - \sum_j^{reactants} n_j \cdot \Delta \mu_{f,j,298}^0$. (d) ΔS_r^0 was calculated applying the following relationship $\Delta S_{r,298}^0 = (\Delta H_{r,298}^0 - \Delta G_{r,298}^0)/298$. (e) The reaction constant was calculated with the following equation $K = \exp(-\Delta G_{r.298}^0 / R \cdot T)$. (f) ΔG_r^0 was calculated by the definition $\Delta G_{r,298}^0 = \Delta H_{r,298}^0 - T \cdot \Delta S_{r,298}^0$. (g) ΔS_r^0 was calculated applying the following relationship $\Delta S_{r,298}^0 = \sum_i^{products} n_i \cdot S_{f,i,298}^0 - \sum_j^{reactants} n_j \cdot S_{f,j,298}^0$.

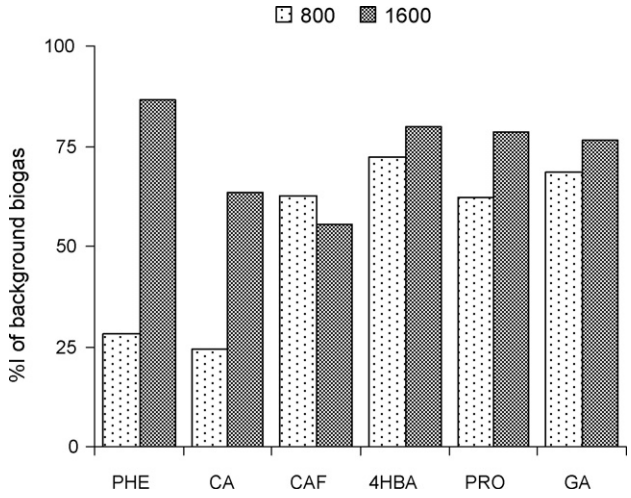


Fig. 4. Percent of inhibition on background gas produced in the blanks at 800 and 1600 mg/L carbon. Tyrosol does not inhibit background biogas at these concentrations.

chemical data available. However, it is valid to assume that all the reactions are exergonic since all the compounds were converted to methane and carbon dioxide. This contradicts previous reports of unsuccessful biomethanization of catechol and caffeic acid [16,17]. These experiments probably failed due to short experimental periods or concentration ratios, rather than thermodynamic limitations.

3.4. Inhibition of biomethanization of the compound and background biogas

The carbon gasified into CH_4 and CO_2 was directly proportional to the stoichiometric quantity of chemical used from 100 to 400 mg carbon/L. In contrast, at 800 and 1600 mg carbon/L, both complete inhibition of methanization of the chemical and partial inhibition of background gas (blanks) production were observed (Fig. and negative values in Table 2). The experiments using tyrosol at inhibitory concentrations resulted in partial inhibition of tyrosol methanization (Fig. 3 and Table 2). In the experiments using phenol and catechol, partial inhibition of their methanization and partial inhibition of background gas production relative to the blanks were observed (Fig. 4, Table 2). Phenolic acids exerted complete inhibition of their methanization. Indeed, these compounds partially inhibited the background gas production relative to the blanks (Fig. 4, Table 2).

3.5. Acidity and inhibition of methane formation

Relating concentration and pH at inhibitory concentrations in Table 2, it can be seen that an increment of concentration is not compulsorily related with an increment in inhibition or acidity. An increment in concentration of phenolic acids and tyrosol leads to acidification of the aqueous medium but a contrary or insignificant effect is observed for phenol and catechol, respectively (Table 2).

Inhibition of background gas production was greater for the phenolic acids (more than 50%) at 800 mg carbon/L (Fig. 4). Increasing the concentration to 1600 mg carbon/L resulted in an increase in inhibition of all phenolics, except caffeic acid. The rates of increment in inhibition for phenol and catechol (0.07 and 0.05) were higher than those experienced by 4HBA, PRO and GA (0.01–0.02) without significant changes in pH (Fig. 4 and Table 2). Therefore, the inhibitory effects of phenolic compounds are due to the highly reactive anionic molecule formed by an inductive effect [30] rather than the ionic hydrogen produced by their dissociation and thus pH is not an indicator of inhibition.

In some cases, increasing the concentration may lead to insolubility of the compound or to chemical polymerization. The decrease of caffeic acid inhibition at incrementing concentration was probably associated with the poor solubility of this compound in water at 35 °C. Insolubility may reduce the interaction of the compound with cells and therefore diminishes its inhibitory capacity. Here again pH was more acidic and failed to predict an increment in inhibition.

Inhibition of bacterial activity might be due to interaction between phenolic compounds and proteins embedded in the cell membrane since phenols can negatively affect enzymatic activity. The charge distribution of proteins allows them to interact with positively and negatively charged substances. For instance, in the aqueous environment, carboxylic acids of polyphenols are ionized and interact with the protonated aminogroup of proteins (phenol-enzyme complexation), conferring a more hydrophobic character which can lead to protein precipitation and thus decreases the enzymatic hydrolytic lysis [31,32]. This inhibition may be increased by natural autoxidation of some phenols leading to the formation of more toxic compounds [33]. For instance, polymerization of catechol leads to the formation of a more toxic product but gallic acid does not [17].

3.6. Polymerization and adsorption onto the sludge

All the compounds exhibit transformations due to biological, chemical and physical interactions (Table 2). Partial conversion into methane and carbon dioxide was found for all the phenolic compounds, however, the non-biomethanized fraction could be utilised for other processes, i.e. cell growth (5–10%) [15], adsorption [31] and autoxidation [17].

The presence of products, other than the tested compounds, due to chemical transformation was analysed in control bottles by HPLC. In the bottles only containing medium and phenolic compounds, the formation of an unstable aggregate was observed (except for phenol). This indicates possible autoxidation has taken place due to diffusion of trace amounts of oxygen [17] into the bottles with colourless liquid. Oxygen diffuses through the septa as confirmed by the pink coloration of the resazurin of discarded bottles. Such polymerization is natural and of vital importance in soil formation [4]. However, no new products were detected by HPLC probably because the aggregates derived from autoxidation [17] were so unstable that they might reversibly dissociate in the aqueous phase during the analysis.

Phenolic compounds undergo autoxidation leading to the formation of coloured products that may differ in toxicity and biodegradability to their precursors [17]. Phenol was herein the only compound that did not polymerize in the control bottles. This was also observed by Field and Lettinga [17] who found that autoxidation of catechol leads to a more toxic compound. They observed that trihydroxyphenols (e.g. GA) were more autoxidized than dihydroxyphenols (e.g. CA) and speculated that carboxylic acid substitution might reduce toxicity of polymerized trihydroxyphenols. This assumption is proved here since GA was not toxic in the range of concentration utilised (up to 14.7%). Such behaviour cannot be explained by the apolarity hypothesis because GA undergoes immediate autoxidation forming a less toxic polymer, i.e. carboxy purpurogallin, which is less likely to interact with microbes and thus reduces toxic effects.

Adsorption of phenolic compounds onto the sludge occurs to some extent, except for caffeic acid in which a concentration of 1659.24 ± 191.5 mg carbon/L was measured in the aqueous phase (Table 2). A saturation level for adsorption onto the matrix was observed for phenol, catechol and protocatechuic acid when the

concentration increased from 800 to 1600 mg carbon/L. This was deduced since the uptake of chemical from the aqueous phase into the sludge matrix did not significantly increase as the concentration increased. This saturation level was not reached in the case of tyrosol, 4-hydroxybenzoic acid, caffeic and gallic acids. The quantity of the chemical adsorbed onto the sludge (Table 2) was probably enhanced by autoxidation, a natural process responsible for the formation of humic acids, which themselves are capable of adsorbing phenolic compounds. Humic acids, of which 4-Hydroxybenzioc acid is a primitive model compound [34], and enzymes are part of extracellular polymeric substances (EPS) and bind phenols onto them [35,36].

Caffeic acid was not adsorbed when its concentration increases to 1600 mgC/L. Due to this increment, the inhibition over the background gas production was diminished and therefore the physiological system was less disturbed (Fig. 4). At this concentration, pellets of caffeic acid were observed in the blanks. The low solubility of this compound therefore limits its availability to participate in adsorption and cell interaction.

3.7. The need for standardized data

There is large divergence among published values of biodegradation and toxicity; and a lack of information about the anaerobic toxicity of phenolics. So far, inhibitory concentrations are only available for phenol and catechol. For phenol, values of 50% inhibition on acetoclastic methanogenesis range from 1099 to 4596 mg phenol/g VSS [6,9,10,37] whereas for catechol Wang et al. [6] reported a value sevenfold higher (8272 mg catechol/g VSS) than a value formerly published [17].

The information available on fermentative methanogenesis of phenolic compounds is fragmented and sometimes contradictory. It lacks consistent use of units for reporting biomass concentration and making comparisons unfeasible. Thus, while fermentative methanogenic degradation of catechol has been confirmed, the lag phase and end of biodegradation differs enormously, e.g. 0–32 and 6–42 days, respectively [13–15]. In other cases, such biodegradation was unsuccessful [17,38]. The lag and end of exponential phases for the other phenolics have also been reported, with the exception of caffeic and gallic acids whose fermentative methanogenesis is given here. For instance, the lag and total biodegradation periods of phenol were 0–6 and 3–49 days, respectively [14,15,29]; for tyrosol 18–28 and 40 days, respectively [16]; for 4-hydroxybenazoic acid 12 and 14 days, respectively [15] and for protocatechuic acid 13 and 14 days, respectively [15].

Even though previous experiments were carried out anaerobically, they were not strictly under the same conditions due to intrinsic differences in the microbial diversity of the utilised inoculum. This observation is consistent with the findings of previous investigations dealing with the biodegradation of a specific chemical while utilizing sludge from different locations [29,39]. In the experiments reported here an active sludge was utilised and the washing process might have modified the original properties of this sludge by eliminating useful exoenzymes attached to the extracellular polymeric substance surface (EPS). Washing eliminates the enzymes needed for the detoxification/excretory goal and probably their synthesis from gene expression is at the same time inhibited by phenolic compounds due to complexation.

The international standard utilised might lead to confusion between recalcitrance and inhibition by aromatic compounds due to insufficient acclimation periods depending on concentration. These standards also suggest carrying out experiments at low concentration, e.g. 20–100 mg/L organic carbon. However, this is a limitation because the concentration proposed could produce complete inhibition of its own biodegradation or even partial inhibition of background gas from blanks. The experiment can therefore be biased because such a concentration could lead to lower values of maximum biomethanization. Therefore, methods such as EN ISO 11734 [19] and ASTM E 2170-01 [40] should recommend longer periods of time and a geometric series of concentrations that allow the assessment of biodegradability, inhibition and recalcitrance. A standard way to report biodegradability in terms of the percent of degradation exerted by a certain mass of compound per mass of inoculum, e.g. 50% mg phenol/g VSS, is recommended. So far, the utilization of a unique source of inoculum is unfeasible and biodegradation results are still particular to the sludge obtained from a specific location. On the other hand, the applications of non-sludge based methods are not comparable with the diversity of the microbial community and the functionality of the EPS forming the sludge matrix.

4. Conclusions

These findings reveal that autoxidation, apolarity, type and number of substitutions influence toxicity effects in anaerobic biodegradation. Therefore, no single effect should be considered in isolation. Non-monotonic low-dose high-response adaptative phenomena can be produced by increasing the concentration of a chemical which enhances and then inhibits biogas formation.

Evidence for the methanization of GA and CAF is presented here for the first time. The maximum total biodegradation of all compounds was $63.85 \pm 2.73\%$ and the non-biodegradable matter was autoxidized and adsorbed onto the sludge matrix. Complete inhibition of methanization of phenols and partial inhibition of background gas from blanks were developed at concentrations of 800 and 1 600 mg carbon/L of phenolic compounds, except for tyrosol for which methanization was only partially inhibited.

It is recommended that EN ISO 11734 [19] and ASTM E 2170-01 [40] should give ratios of carbon to biomass and should specify longer retention times that allow better assessment of biodegradation, inhibition and recalcitrance of aromatic compounds. It is also recommended that consistent units be used for reporting results relative to the concentration of biomass utilised.

The information reported here is valuable in determining the maximum allowable levels of phenols in complex microbial systems aiming at bioremediation of contaminated sites or biofuels production from biomass stocks.

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