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Evaluation of toxic effect of monoterpene compounds on anaerobic digestion

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ABSTRACT

Monoterpenes are antimicrobial compounds widely distributed in vegetable biomass, whose inhibitory potential for anaerobic digestion is underestimated. In this research, the toxic effect of limonene and fenchone, two of the main monoterpenes present in vegetable biomasses, and those of 4-terpineol, α-terpineol, and *p*-cymene, compounds described as main metabolites of limonene degradation, have been assessed. Methane production was totally inhibited at dosed of 1000 mg L^{-1} of fenchone and limonene and at 600 mg L^{-1} of *p*-cymene and 4-terpineol. Based on the methane production rate, the inhibition followed the next trend: α-terpineol $<<$ fenchone $<$ limonene $\approx$ *p*-cymene $<$ 4-terpineol. Regardless of dosed concentration, monoterpenes were mostly degraded at the end of the experiment (>85%), except *p*-cymene at 600 mg L^{-1}. Therefore, monoterpenes could entail a high risk of inhibition that can be aggravated by the difficulty to accurately follow their concentration and by the scarce information on their effect on anaerobic process.

1. Introduction

Monoterpenes are volatile organic compounds contained in fruits, vegetables, leaves, and edible aromatic herbs, that contribute to their characteristic aroma and flavour. They are responsible for prolonging shelf life and protecting against microbial invasion [27,43]. Certain monoterpene compounds, such as limonene, carvone, car-3-ene, 4-terpineol, camphor, and *p*-cymene, possess potent antimicrobial properties [7,13,21]. These characteristics can entail a challenge when valorising vegetable biomasses as feedstocks of bioprocesses, such as anaerobic digestion (AD), by hindering the activity of the microbial community [20], which might destabilise the interrelated degradation pathways of the process.

AD is a well-established process. Its response to different inhibitors, such as ammonia, heavy metals or phenols compounds, has been widely reported [9,11,44]. However, research dealing with the toxic effect of monoterpenes during the AD is still scarce [19,39]. In fact, AD inhibition processes described simply as overload could have resulted from the accumulation of undetected terpenes due to their high antimicrobial capacity [39]. The inhibitory mechanism of the monoterpene compounds in AD is associated to the disturbing of the disturbing of the cytoplasmic membrane functionality due to the disrupt the phospholipid bilayer and bind of the monoterpenes to membrane proteins [2]. Limonene is present in citrus peel fruits like lemons and oranges, reaching concentration values of 1.8–6.0% w/w, in dry basis, for orange peels [36]. Limonene has been the main studied monoterpene compound during AD [8,23,25,33,42]. For example, Lotito et al. [23] reported that increasing the added limonene to the reactors from 104 mg L^{-1} to 150 mg L^{-1}, associated to an increment of organic loading rate, entailed the partial inhibition of the AD process. However, the inhibition concentrations reported for limonene in the monitoring of the AD reactors varied in a wide range of several order of magnitude, i.e., from 24 mg L^{-1} up to 2000 mg L^{-1} [8,17,38]. Despite the critical importance of limonene for AD of citrus peels, other vegetable biomasses could present

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other potentially toxic monoterpenes. For instance, fenchone has been identified as one of the main monoterpenes found in fennel (fruit, leaves, and stem) [10,39], as well as in artichoke or carrot leaves [22]. Fenchone concentration in fennel can vary in a range 0.3% up to 11.6% [46,47].

In addition to the toxicity provided by the monoterpenes naturally contained in vegetables, metabolites produced during their anaerobic degradation can be also toxic [34]. Several authors have reported the degradation of limonene to α-terpineol, 4-terpineol and/or *p*-cymene, when using citrus waste as a feedstock for AD process [3,8,23,35]. Ruiz anf Flotats [34] hypothesised that these metabolites might present an even higher toxic effect than limonene. However, their actual inhibition extent has still not been assessed in AD.

The main objective of this research was to assess the toxicity for AD of few monoterpene compounds and that of the metabolites produced during their degradation. Firstly, the toxic effect of limonene and fenchone was determined, as they are common monoterpene compounds found in vegetable biomasses. Subsequently, the toxic effect of 4-terpineol, α-terpineol, and *p*-cymene was evaluated, since they are well known metabolites produced during limonene degradation in anaerobic environments.

2. Material and methods

2.1. Monoterpene compounds

The monoterpenes considered in this research were (R)-(+)-limonene (97%), (+)-fenchone (98%), 4-terpineol (95%), α-terpineol (90%), and p-cymene (99%). All were supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.2. Anaerobic digestion set-up and experimental procedure

Two set of biochemical methane potential (BMP) tests were performed, following the methodology described in Raposo et al. [32]. Five different BMP test were performed: Set 1) limonene and fenchone BMP tests and set 2) 4-terpineol, α-terpineol, and *p*-cymene BMP tests. The first set of BMP tests was carried out by individually dosing limonene and fenchone at the concentrations of 0 (control), 100, 200, 600, and 1000 mg L^{-1}. The second set of BMP tests was carried out by the individual dose of 4-terpineol, α-terpineol, and *p*-cymene at the concentrations of 0 (control), 100, 200, and 600 mg L^{-1}. These concentrations were selected according to the previous inhibition threshold described by Ruiz and Flotats [34] and the concentrations of different monoterpenes in vegetable biomasses [36,47]. Each condition was assessed in triplicate.

BMP tests were carried out using Erlenmeyer flasks of 250 mL, placed in a water bath with a circulation thermostat (*JULABO, Argentina*) to maintain the operating temperature (35 ± 2 ºC). The reactors were hermetically sealed with a rubber stopper after nitrogen flashing to ensure anaerobic conditions. The BMP tests were run with an inoculum to substrate ratio of 2 g of volatile solids (VS) of inoculum per 1 g VS of substrate (initial substrate concentration: 6.25 g L^{-1}). Microcrystalline cellulose, provided by Sigma-Aldrich (St. Louis, MO, USA), was used as a model substrate for all experiments [18]. Each BMP test included blanks (only inoculum) and controls (inoculum and substrate), both in triplicate. The methane volume was measured by liquid displacement using 1-L gasometers submerged in 2 N NaOH solution, to remove the CO_2 from the generated gas. Methane yield coefficients were calculated based on the organic matter, including both cellulose and monoterpene compounds, added to each BMP condition. Ambient temperature and pressure were continuously recorded to normalize the methane volume to normal temperature and pressure conditions, i.e., 25 ºC and 1 atm. The biodegradability was determined by comparing methane production against the theoretical maximum methane production that would be generated stoichiometrically, where 1 g of COD corresponds to 382 mL CH_4 at 25 ºC and 1 atm.

Anaerobic sludge from an industrial sewage anaerobic digester of the wastewater treatment plant "COPERO" from Seville (Spain) was used as the inoculum. The inoculum was collected before each set of BMP tests. The main anaerobic inoculum characteristics were similar in both samples, presenting the following mean values: pH = 7.3 ± 0.1; alkalinity = 7240 ± 155 mg $CaCO_3$ L^{-1}, and VS/TS (total solids) ratio = 56 ± 2.

2.3. Kinetic study

The kinetic parameters and the mathematical adjustment for the AD process were determined from the experimental data obtained through a non-linear regression using the software SigmaPlot (version 14.5). The Transference Function kinetic model (Eq. 1) was used for all conditions tested, which other authors have applied for other organic substrates where a lag phase in the biogas production occurred using the following expression [14,40]:

$$G = G_{max}\left(1 - \exp\left[\frac{R_{max}(\lambda - t)}{G_{max}}\right]\right) \quad (1)$$

where G (mL CH_4 g VS^{-1}) is the cumulative specific methane production, G_{max} (mL CH_4 g VS^{-1}) is the ultimate methane production, R_{max} (mL CH_4 g VS^{-1} d^{-1}) is the maximum methane production rate, t (d) is the time, and λ (d) is the lag time. Additionally, r^2 and error (%) were determined to evaluate the fit and precision of the kinetic results. The error was defined as the difference in percentage between the experimental accumulated final methane production (G_{exp}) and G_{max}.

2.4. Chemical analysis

The pH, alkalinity, solid concentrations (TS and VS), and soluble chemical oxygen demand (sCOD) determinations were performed following the recommendations of the American Public Health Association (APHA) [6].

The volatile fatty acids (VFA) (C_2-C_5) were analysed and quantified by a *Shimadzu* GC-2025 gas chromatograph equipped with a Stabilwax-DA column (30 m x 0.25 mm i.d., film thickness 0.25 μm; RESTEK, Bellefonte, PA, US) and a flame ionization detector (FID) at 250 ºC, as previously described by Trujillo-Reyes et al. [39]. The monoterpene compounds at the end of the BMP tests were determined by gas chromatography. Samples of 0.5 mL were collected, conditioned to room temperature, and placed in a vial heater at 40 ºC. After a 10 min equilibrium time, volatile compounds from headspace were absorbed on an SPME fibre DVB/Carboxen/PDMS50/30 mm (Supelco, Bellafonte, PA, US). The sampling time was 50 min at 40 ºC, and compounds adsorbed in the SPME fibre were desorbed directly into the GC injector. These compounds were analysed and quantified by HP-6890 *Agilent Technologies* gas chromatograph (GC-FID), and compound identity was checked by 78204/GC-5975/MSD system *Agilent Technologies* gas chromatograph equipped with a mass detector (MS) (GC-MS), as previously described by Pérez et al. [29]. The monoterpenes concentrations were quantified through individual calibration curves made using pure commercial standards supplied by Sigma-Aldrich (St. Louis, MO, US).

3. Results and discussion

3.1. Effect of limonene and fenchone concentrations on the AD process

3.1.1. Methane yield coefficient and biodegradability

The variation of the accumulated methane yields (mL CH_4 g VS^{-1}) during the experimental time for both compounds at each tested concentration is shown in Fig. 1. Degradation of cellulose without adding monoterpene compounds, used as a positive control (C0), showed a methane yield coefficient of 352 ± 8 mL CH_4 g VS^{-1} (Fig. 1). Raposo

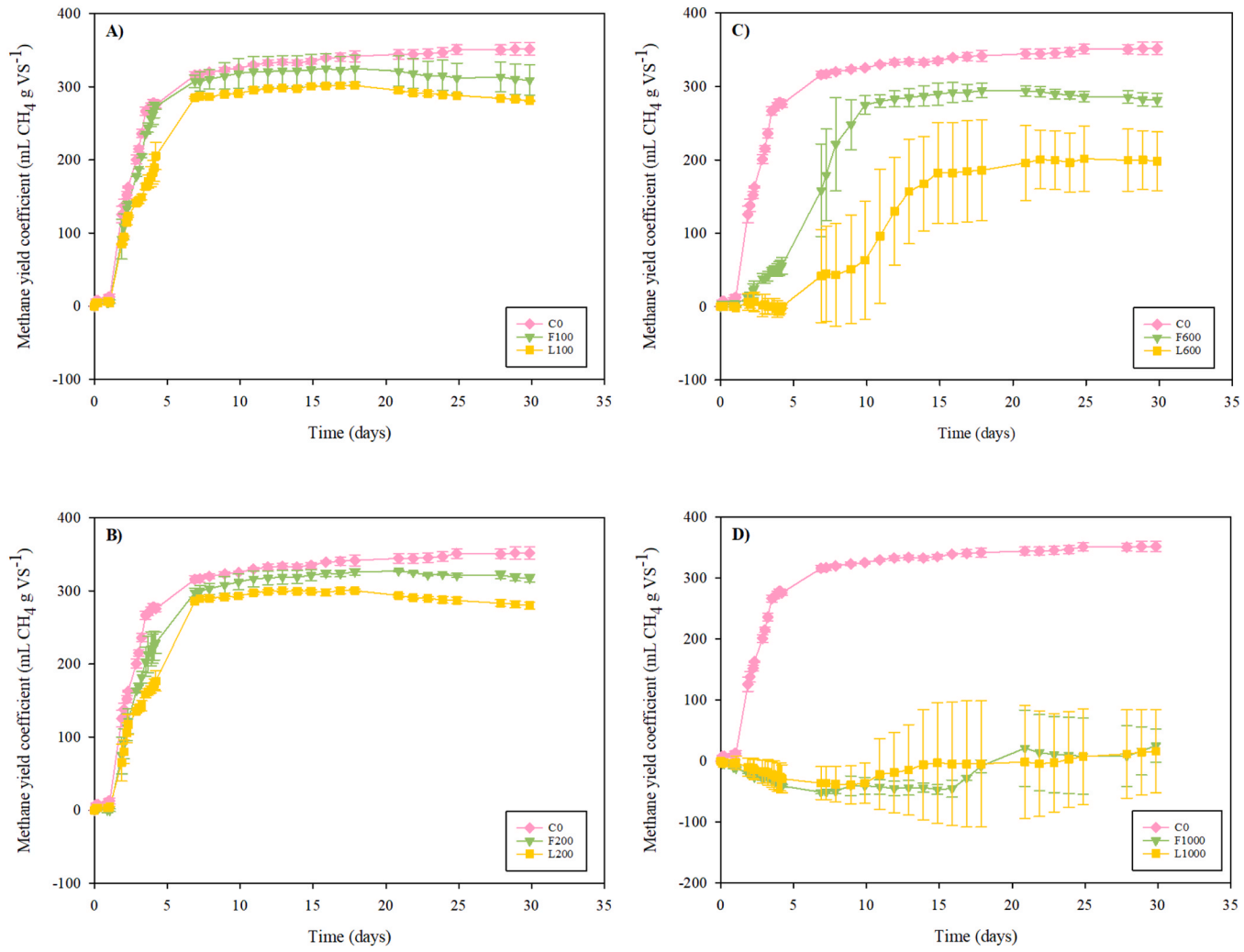

Fig. 1. Accumulated methane yields during AD of microcrystalline cellulose, supplemented with the indicated concentrations of limonene (L) and fenchone (F) A) 100 mg L^{-1}, B) 200 mg L^{-1}, C) 600 mg L^{-1}, and D) 1000 mg L^{-1}.

et al. [32] reported methane yield coefficients in the same range, i.e., between 302 and 412 mL CH_4 g VS^{-1}. The toxic effect of the fenchone on the methane yield coefficient was only evident at a dosed of 1000 mg L^{-1} (Fig. 1). In contrast, limonene showed a gradual toxic effect on the methane yield coefficient at increasing dosed concentrations (Fig. 1). When applying limonene concentrations of 100 and 200 mg L^{-1}, the methane yield coefficient was reduced by around 14.5%, in comparison with the control (C0) (Fig. 1A and 1B). Ruiz and Flotats [34] similarly reported that a concentration of 200 mg L^{-1} of limonene reduced the methane yield coefficient around 8.2%, in a toxicity test using also microcrystalline cellulose as substrate. The addition limonene at 600 mg L^{-1} reduced the methane yield coefficient by 43%, whereas the same dose of fenchone resulted in only 16.8% reduction (Fig. 1C). The toxic effect was markedly incremented by increasing the dosed limonene and fenchone concentrations up to 1000 mg L^{-1}, resulting in almost total exhaust of the methane production for both. At that condition, methane yield coefficients were reduced by 95% and 93% compared to the control for limonene and fenchone, respectively (Fig. 1D). Previous studies have reported presence of fenchone at periods of process destabilisation, involving decreases in methane production up to 75% [39]. Similarly, other monoterpenes such as car-3-ene, myrcene, and α-pinene have shown toxic potential for AD. Concretely, concentrations around 5000 mg L^{-1} of car-3-ene, myrcene, and α-pinene resulted in methane yield coefficient reductions of 95%, 75%, and 77%, respectively [43].

The biodegradability of cellulose, used as a control substrate (C0), reached 80% (Table 1). Although, it is worth noting that its degradation into methane is not fully achieved due to approximately 10–15% of the degradable substrate components being utilized for microbial growth and cell maintenance [31]. At increasing dosed concentrations of limonene, biodegradability values gradually decreased, whereas for fenchone a big drop in the biodegradability was only observed at the concentration of 1000 mg L^{-1} (Table 1).

3.1.2. *Process stability*

The characterization of the BMP test at the end of the experimental time is shown in Tables 1 and 2. pH values ranged from 7.0 to 7.5 for all the tested conditions (Table 1), despite the 95% and 93% in the methane yield coefficient reduction at dosed of 1000 mg L^{-1} of limonene and fenchone, respectively (Fig. 1-D). The maintenance of the pH at neutral values might be favoured by the high alkalinity concentration in the reactors, i.e., higher than 4000 mg $CaCO_3$ L^{-1} in all the cases (Table 1).

A similar sCOD concentration, below 900 mg O_2 L^{-1}, was determined at the end of the experimental time for C0 and for the reactors dosed with fenchone and limonene at 100 and 200 mg L^{-1} (Table 1). The increment of limonene and fenchone concentration up to 600 mg L^{-1} entailed an sCOD accumulation of 50% and 83% with respect to C0, respectively (Table 1). Likewise, the highest sCOD accumulation occurred at 1000 mg L^{-1} doses of limonene and fenchone, reaching 3281 ± 871 and 3565 ± 801 mg O_2 L^{-1}, respectively

Table 1
Physicochemical characterization of the effluents of the anaerobic digestion process at the end of the limonene and fenchone biochemical methane potential tests.

	C0 1	Limonene				Fenchone			
Dosed monoterpene concentration (mg L^{-1})	0	100	200	600	1000	100	200	600	1000
Biodegradability (%; based on COD)	80	58	51	23	1	64	58	37	3
pH	7.5 ± 0.1	7.2 ± 0.1	7.2 ± 0.1	7.5 ± 0.1	7.0 ± 0.1	7.1 ± 0.1	7.2 ± 0.1	7.4 ± 0.1	7.5 ± 0.1
Total alkalinity (mg $CaCO_3$ L^{-1})	8575 ± 170	5575 ± 205	5315 ± 270	5390 ± 150	4070 ± 360	7010 ± 710	7270 ± 4	7630 ± 110	7605 ± 495
[b]sCOD (mg O_2 L^{-1})	711 ± 46	621 ± 5	824 ± 12	1050 ± 196	3281 ± 871	770 ± 21	880 ± 20	1302 ± 175	3565 ± 801
Total[c]VFAs (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	1699 ± 45	n.d.	n.d.	n.d.	1714 ± 5
Acetic acid (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	1367 ± 28	n.d.	n.d.	n.d.	901 ± 45
Propionic acid (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	333 ± 18	n.d.	n.d.	n.d.	583 ± 55
Butyric acid (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Iso-buryric acid (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	86 ± 2
Valeric acid (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Iso-valeric acid (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	144 ± 7

[a]n.d.: not detected; [b]sCOD: soluble chemical oxygen demand; [c]VFAs: volatile fatty acids.

Table 2
Monoterpene compounds identified and quantified of the effluents of the anaerobic digestion process at the end of the limonene and fenchone biochemical methane potential tests.

		Monoterpene compounds (mg L^{-1})					
	Dosed concentration (mg L^{-1})	Limonene	Fenchone	o-Cymene	α-Terpineol	4-Terpineol	p-Cymene
C0 1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Limonene [a]BMP test	100	n.d.	n.d.	[c]traces	n.d.	n.d.	n.d.
	200	n.d.	n.d.	6.2 ± 1.1	n.d.	n.d.	n.d.
	600	n.d.	n.d.	125.1 ± 23.6	n.d.	n.d.	n.d.
	1000	n.d.	n.d.	126.6 ± 55.9	n.d.	n.d.	n.d.
Fenchone [a]BMP test	100	n.d.	19.8 ± 1.1	n.d.	n.d.	n.d.	n.d.
	200	n.d.	26.8 ± 3.1	n.d.	n.d.	n.d.	n.d.
	600	n.d.	48.1 ± 0.6	n.d.	n.d.	n.d.	n.d.
	1000	n.d.	167.9 ± 2.5	n.d.	n.d.	n.d.	n.d.

a BMP: Biochemical methane potential test; bn.d.: not detected; ctraces: concentration below 0.07 mg L−1

(Table 1). Despite the accumulation of sCOD observed at monoterpene dosage of 600 mg L^{-1}, VFA accumulation was only observed in the reactors with dosed of 1000 mg L^{-1} (Table 1). Concretely, 1367 ± 28 mg O_2 L^{-1} of acetic acid and 333 ± 18 mg O_2 L^{-1} of propionic acid were quantified for reactor dosed with limonene. 901 ± 45 mg O_2 L^{-1} and 583 ± 55 mg O_2 L^{-1} of acetic and propionic acids, respectively, where determined for the reactors dosed with 1000 mg L^{-1} fenchone (Table 1). These results suggest that at a dosed concentration of 600 mg L^{-1} acidogenic activity was hindered, limiting the transformation from sCOD into simple VFAs. At the same time, the methanogenic activity was enough to avoid the VFA accumulation. In contrast, at limonene and fenchone concentration of 1000 mg L^{-1}, AD metabolism imbalanced, mainly affecting the methanogenic step [26]. It is important to highlight that around 50% of the accumulated sCOD corresponded to total VFAs for the dosed concentrations of 1000 mg L^{-1} (Table 1). The remaining 50% could be attributed to the non-degraded monoterpenes and/or other undetermined cellulose metabolites. This accumulation of sCOD, which was not degraded into simpler VFA compounds, would indicate that the acidogenic activity was also partially affected, although not as much as the methanogenic step [26,48].

Monoterpene compounds at the end of the BMP test were also identified and quantified (Table 2). Fenchone concentrations were quantified in the reactors, determining a removal of around 85% of the initial dosed fenchone at all the tested conditions (Table 2). For limonene BMP tests, limonene was not detected at the end of the experimental time despite the initial dose (Table 2, Supplementary material, Fig. S1A). The observed removal of both monoterpenes, even at the concentrations that hindered the methanogenic step, would be explained because the microorganisms involved in their degradation were mainly fermentative bacteria [10,24]. In addition to the dosed monoterpenes, identification of possible secondary metabolites from enzymatic biodegradation of limonene or fenchone was assessed (Table 2, Supplementary material, Fig. 1S). By the end of the limonene BMP test dosed with 1000 mg L^{-1}, the limonene metabolite *o*-cymene reached a concentration of 126.6 ± 55.9 mg L^{-1} (Table 2). Moreover, phellandrene, an intermediate compound of the degradation of limonene to *o*-cymene, was also identified (Supplementary material, Fig. S1A). A degradation pathway of limonene was previously described by Calabró et al. (2016), who proposed a degradation to *p*-cymene based on Wagner-Meerwein rearrangements with phellandrene as an intermediate compound. These authors reported a final accumulation of 120 mg L^{-1} *p*-cymene, considering a limonene feeding of approximately 1600 mg L^{-1} as the primary constituent of the essential oil derived from orange peel waste used as feedstock in AD [8]. The degradation of limonene into a specific isomeric form, e.g., *p*-cymene or *o*-cymene, may be influenced by various factors, including the initial substrate. In the same line, when citrus waste was used as substrate, limonene was degraded to different metabolites, including cymene, α-terpineol and 4-terpineol [3,23,35]. Ruiz and Flotats [34] hypothesized that the difference in the generated metabolites could be due to the different composition of citrus waste compared to the commercial (R)-limonene used in the experiments. Despite of that, these results would indicate that the observed inhibition in the limonene tests would be related to the appearance of toxic metabolites, instead of only limonene itself. In contrast, for fenchone tests, no meaningful concentration of metabolites was observed, being only detected almost negligible peaks of camphor and fenchol (Table 2, Supplementary material, Fig. S1B). So, it might be hypothesised that the toxicity to anaerobic microorganisms would be directly attributed to fenchone. It is worth noting that the inhibition of the AD occurred even when the concentration of the monoterpenes at the end of the experimental time was low or even negligible. Therefore,

it would be necessary to evaluate the toxicity of the metabolites generated during the anaerobic biodegradation of limonene under the same experimental conditions. Their toxicity potential would explain the destabilisation of the AD process despite limonene degradation.

3.2. Effect of α-terpineol, 4-terpineol and p-cymene concentrations on the AD process

3.2.1. Methane yield coefficient and biodegradability

During this BMP test degradation of cellulose without any monoterpene compound (control, C0) showed a methane yield coefficient and biodegradability values of 393 ± 19 mL CH_4 g VS^{-1} (Fig. 2) and 88% (Table 3) respectively, similar to the control in limonene and fenchone BMP tests (Fig. 1). The accumulated methane yields (mL CH_4 g VS^{-1}) during the experimental time for each tested concentration of α-terpineol, 4-terpineol and *p*-cymene are shown in Fig. 2. 4-terpineol affected methane yield coefficient at concentrations of 200 mg L^{-1}, decreasing by around 33% with respect to C0 (Fig. 2B). On the opposite, α-terpineol and *p*-cymene at 200 mg L^{-1} resulted in methane yield coefficients very similar to C0 (Fig. 2B). Increasing dosed concentrations up to 600 mg L^{-1} entailed a marked reduction of the methane yield coefficients for 4-terpineol and *p*-cymene, i.e., 56% and 74%, respectively with respect to C0 (Fig. 2C). However, the same dose of 600 mg L^{-1} of α-terpineol only showed a reduction of 17% with respect to C0 (Fig. 2C). Notably, the methane yield coefficient reductions of 4-terpineol and *p*-cymene at 600 mg L^{-1} were significantly higher than those observed for limonene and fenchone at the same concentration (Figs. 1C and 2C). This would indicate that the toxicity observed for limonene resulted from the anaerobic toxicity of some of the limonene degradation metabolites, as previously hypothesised by Ruiz & Flotats [34]. The higher impact of 4-terpineol and *p*-cymene respect limonene and fenchone indicates that the toxic effect of these compounds may potentially impact the survival of the microorganisms involved in the process [28]. However, not all tested limonene degradation metabolites presented the previous observed inhibition effects, as α-terpineol under identical experimental conditions has been shown to have a lower toxic effect than limonene (Figs. 1C and 2C).

3.2.2. Process stability

The results obtained after the characterization of the final effluents of the BMP test are shown in Tables 3 and 4. Neither pH nor alkalinity showed values outside the optimal range for methanogenic activity despite of the dosed monoterpene concentration (Table 3) [15,45]. This indicate that the observed reductions in methane yield coefficient due to monoterpene addition are not attributable to acidification.

As it can be expected from the observation of methane yields coefficients, low sCOD concentrations were determined at the end of the experimental time for C0 and for the reactors dosed with α-terpineol at any concentrations (Table 3). Similarly, addition of 200 mg L^{-1} and 600 mg L^{-1} of 4-terpineol and 600 mg L^{-1} of *p*-cymene resulted in sCOD accumulations of 312%, 341%, and 103%, respectively, in comparison to C0 (Table 3). Unlike limonene and fenchone tests, reactors dosed with concentrations below 1000 mg L^{-1} of 4-terpineol and *p*-cymene showed not only increases in sCOD but also VFA accumulation (Table 3). Concretely, 200 mg L^{-1} of 4-terpineol resulted in a total VFA accumulation of 916 ± 138 mg O_2 L^{-1}, whereas 600 mg L^{-1} of *p*-cymene accumulated 802 ± 154 mg O_2 L^{-1} (Table 3). Furthermore, the VFA profile identified in the reactors dosed with 4-terpineol and *p*-cymene was slightly more complex than with limonene, where only acetic and propionic acids were quantified (Tables 1 and 3). For reactors dosed with 600 mg L^{-1} of 4-terpineol, acetic (246 ± 18 mg O_2 L^{-1}), propionic (514 ± 58 mg O_2 L^{-1}), butyric (84 ± 1 mg O_2 L^{-1}), and iso-valeric (106 ± 29 mg O_2 L^{-1}) acids were quantified. Whereas for reactors dosed with 600 mg L^{-1} of *p*-cymene it was quantified acetic (128 ± 23 mg O_2 L^{-1}), propionic (495 ± 114 mg O_2 L^{-1}) and iso-valeric (119 ± 10 mg O_2 L^{-1}) acids (Table 3). The low concentration of acetic acid and the accumulation of VFA with a longer number of carbons (C3-C5) would suggest that the dosed monoterpene compounds might affect not only the methanogenic activity but also the previous acetogenic stage [1,12,41].

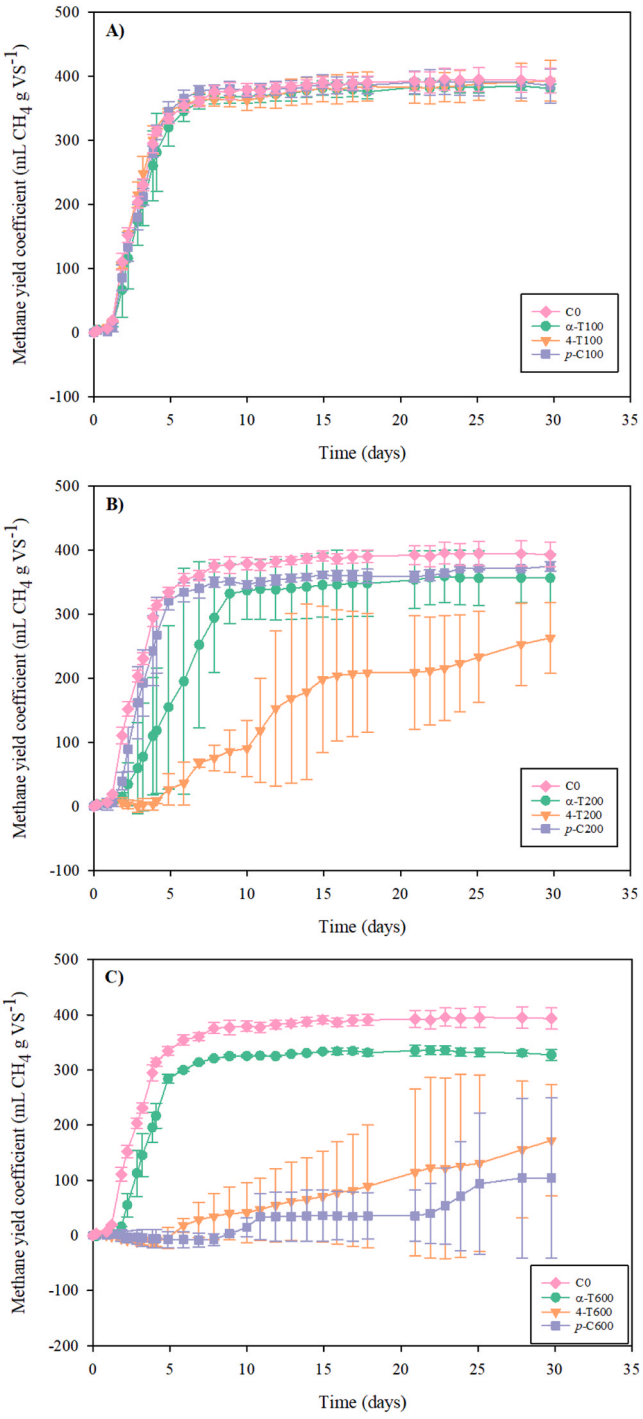

Fig. 2. Accumulated methane yields during AD of microcrystalline cellulose, supplemented with the indicated concentrations of α-terpineol (α-T), 4-terpineol (4-T), and *p*-cymene (*p*-C), A) 100 mg L^{-1}, B) 200 mg L^{-1}, and C) 600 mg L^{-1}.

Monoterpene compounds of the final effluents of the BMP test were also identified and quantified (Table 4). For the α-terpineol test, only traces of α-terpineol were quantified at the different tested concentrations (Table 4). For the 4-terpineol tests, the increment of the initial dose of 4-terpineol resulted in gradually higher concentrations of this

Table 3
Physicochemical characterization of the effluents of the anaerobic digestion process at the end of the α-terpineol, 4-terpineol, and p-cymene biochemical methane potential tests.

	C0 2	α-terpineol			4-terpineol			*p*-cymene		
Dosed monoterpene concentration (mg L^{-1})	0	100	200	600	100	200	600	100	200	600
Biodegradability (%; based on COD)	88	74	61	38	76	45	20	73	62	11
pH	7.5 ± 02	7.2 ± 0.1	7.4 ± 0.1	7.3 ± 0.1	7.2 ± 0.1	7.3 ± 0.2	7.1 ± 0.1	7.4 ± 0.2	7.4 ± 0.1	7.2 ± 0.1
Total alkalinity (mg $CaCO_3$ L^{-1})	4718 ± 24	4903 ± 31	5012 ± 23	4700 ± 23	4772 ± 88	4748 ± 289	4151 ± 244	4553 ± 79	4648 ± 3	4718 ± 205
[b]sCOD (mg O_2 L^{-1})	562 ± 45	587 ± 24	994 ± 181	390 ± 25	370 ± 27	2316 ± 857	2479 ± 1149	467 ± 81	485 ± 18	1142 ± 399
Total[c]VFAs (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	916 ± 138	995 ± 38	n.d.	n.d.	802 ± 154
Acetic acid (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	102 ± 2	246 ± 18	n.d.	n.d.	128 ± 23
Propionic acid (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	469 ± 141	514 ± 58	n.d.	n.d.	495 ± 114
Butyric acid (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	86 ± 6	84 ± 1	n.d.	n.d.	n.d.
Iso-buryric acid (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	118 ± 10	44 ± 9	n.d.	n.d.	60 ± 8
Valeric acid (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Iso-valeric acid (mg O_2 L^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	141 ± 3	106 ± 29	n.d.	n.d.	119 ± 10

[a]n.d.: not detected; [b]sCOD: soluble chemical oxygen demand; [c]VFAs: volatile fatty acids

Table 4
Monoterpene compounds identified and quantified of the effluents of the anaerobic digestion process at the end of the α-terpineol, 4-terpineol, and *p*-cymene biochemical methane potential tests.

		Monoterpene compounds (mg L^{-1})		
	Dosed concentration (mg L^{-1})	α-Terpineol	4-Terpineol	*p*-cymene
C0 2	0	n.d.	n.d.	n.d.
α-terpineol[a]BMP test	100	3.6 ± 0.5	n.d.	n.d.
	200	30.0 ± 0.1	n.d.	n.d.
	600	11.6 ± 0.3	n.d.	n.d.
4-terpineol[a]BMP test	100	n.d.	1.5 ± 0.3	n.d.
	200	n.d.	74.0 ± 6.8	n.d.
	600	n.d.	126.7 ± 18.2	n.d.
p-cymene[a]BMP test	100	n.d.	n.d.	n.d.
	200	n.d.	n.d.	4.4 ± 1.8
	600	n.d.	n.d.	666.8 ± 37.5

[a] BMP: Biochemical methane potential test; [b]n.d.: not detected.

compound in the final effluent. Concretely, the highest concentration was 126.7 ± 18.2 mg L^{-1} for reactors dosed with 600 mg L^{-1} (Table 4). On another hand, trace amounts of *p*-cymene were quantified in all tested conditions, except for the 600 mg L^{-1} addition where no *p*-cymene degradation was observed (Table 4). No new degradation metabolites from these monoterpene compounds were identified in the final effluents (Supplementary material, Fig. S1C-E).

3.3. Kinetic study and IC_{50} assessment for the tested monoterpene compounds

The kinetics parameter values for all BMP tests are shown in Table 5. The high r^2 values, i.e., higher than 0.93, and low values of errors in most cases indicated a good fit of the experimental data to the proposed model (Table 5). The model was not applied to the data obtained for the experiments with a dosed concentration of 1000 mg L^{-1} of limonene and fenchone and 600 mg L^{-1} of 4-terpineol and *p*-cymene because of the almost negligible methane production. The dosed concentration of limonene and fenchone of 600 mg L^{-1} resulted in the highest observed lag phase (λ) values, suggesting a possible inhibitory effect. At this concentration, λ values were 2.09 ± 0.39 and 1.18 ± 0.23 d for limonene and fenchone, respectively. However, for 4-terpineol at a lower concentration of 200 mg L^{-1}, similar λ values were observed, i.e., 1.70 ± 0.40 d, which shows more clearly the toxic effect of this compound

Table 5
Kinetic parameters values obtained after application of the Transference Function model to the results of each biochemical methane potential test.

Type of inhibitor	Dosed monoterpene concentration (mg L^{-1})	[b]G_{max} (mL CH_4 gVS^{-1})	[c]R_{max} (mL CH_4 $g^{d}VS^{-1}$ d^{-1})	[e]λ (d)	r^2	Error (%)
C0 1	0	346 ± 6	122 ± 7	0.35 ± 0.08	0.9648	1.5
Limonene	100	302 ± 5	81 ± 5	0.43 ± 0.09	0.9721	7.4
	200	303 ± 6	77 ± 5	0.49 ± 0.10	0.9669	8.1
	600	328 ± 67	14 ± 2	2.09 ± 0.39	0.9270	66.6
	1000	n.a.	n.a.	n.a.	n.a.	n.a.
Fenchone	100	325 ± 6	112 ± 7	0.41 ± 0.09	0.9572	5.3
	200	329 ± 5	93 ± 5	0.47 ± 0.08	0.9735	3.7
	600	324 ± 17	41 ± 4	1.18 ± 0.23	0.9348	15.1
	1000	n.a.	n.a.	n.a.	n.a.	n.a.
C0 2	0	397 ± 7	126 ± 9	0.41 ± 0.10	0.9678	1.1
α-terpineol	100	390 ± 8	112 ± 9	0.49 ± 0.12	0.9615	2.3
	200	381 ± 13	60 ± 5	0.90 ± 0.20	0.9579	69
	600	344 ± 9	88 ± 8	0.66 ± 0.15	0.9435	5.2
4-terpineol	100	388 ± 7	131 ± 10	0.40 ± 0.11	0.9596	1.2
	200	399 ± 72	16 ± 2	1.70 ± 0.40	0.9568	50.9
	600	n.a.	n.a.	n.a.	n.a.	n.a.
p-cymene	100	397 ± 8	123 ± 10	0.48 ± 0.12	0.9547	3.1
	200	373 ± 9	105 ± 9	0.54 ± 0.13	0.9509	0.5
	600	n.a.	n.a.	n.a.	n.a.	n.a.

[a]n.a.: Not applicable; [b]G_{max}: ultimate methane production; [c]R_{max}: maximum methane production rate; [d]VS: volatile solids; [e]λ: lag time.

(Table 5). Other authors have suggested that the appearance of a lag time is an attempt by microorganisms to adapt to environmental conditions in the presence of toxic compounds [20,43].

R_{max} was affected by all the dosed monoterpene compounds at all the tested concentrations although to a different extent (Table 5, Fig. 3). It is worth to note that R_{max} was impacted even at concentrations where the methane yield coefficient was not impacted (Figs. 1 and 2, Table 5). In the limonene and fenchone BMP tests, the sharpest decrease in R_{max} was observed at 600 mg L^{-1}, reaching 88% and 66% reduction compared to C0, respectively (Table 5). However, in the metabolites BMP tests, the highest toxicity was determined for 4-terpineol, where the addition of 200 mg L^{-1} reduced R_{max} to 87%. This drastic drop corroborates that 4-terpineol would have a greater toxic effect than limonene, whose reduction of R_{max} at this concentration was only 37% (Table 5). Contrarily to 4-terpineol, a concentration of 200 mg L^{-1} of *p*-cymene showed a minimal toxic effect, i.e., only 17% reduction, despite the total inhibition determined at a concentration of 600 mg L^{-1}. The gradual decrease in R_{max} observed with increasing concentrations of all monoterpene compounds tested can be attributed to inhibition of bacterial enzyme activity and suppression of translation of specific regulatory gene products [4,28]. The inhibition mechanism at the highest added concentrations could be related to their accumulation in the cell membrane and other fat structures of cells, causing changes in the membrane structure as reported in several studies [7,30,33]. These membrane changes would affect different membrane functions, hindering the activity of the microorganisms and even entailing the cellular lysis [7,16,33]. Contrary to the other tested monoterpene compounds, α-terpineol slightly affected the AD process as the R_{max} was minimally altered in its presence (Table 5), and, thus, a trend between dosed concentration of α-terpineol and R_{max} reduction could not be observed (Fig. 3B).

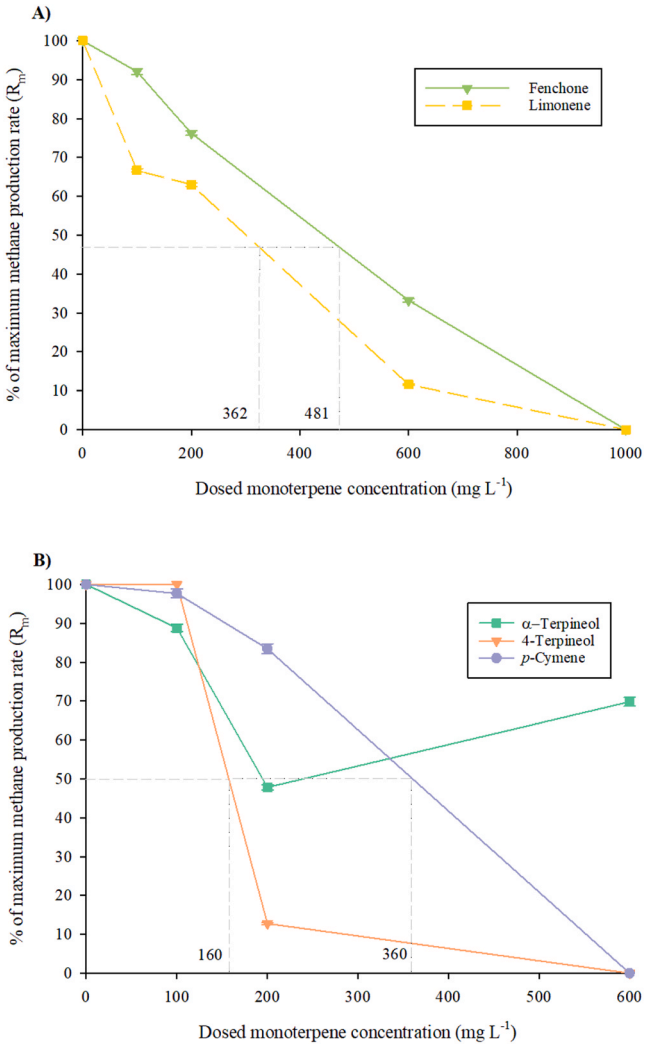

Fig. 3. Percentages of the maximum methane production rate (R_{max}) from microcrystalline cellulose with different dosed concentrations of A) limonene and fenchone and B) α-terpineol, 4-terpineol, and p-cymene in the reactors. Vertical lines indicate IC_{50} values expressed in mg L^{-1}.

To provide a numerical quantification of the inhibition effect, IC_{50} values were calculated based on R_{max} according to the methodology described by Ruiz & Flotats [34] for limonene. This methodology relates the values of R_{max} and the monoterpene concentration extrapolating the monoterpene concentration value at which the R_{max} decreases to 50% (IC_{50}). IC_{50} were calculated to be 160, 360, 362, and 481 mg L^{-1} for 4-terpineol, *p*-cymene, limonene, and fenchone, respectively (Fig. 3). The IC_{50} of limonene was 25% lower than fenchone. Despite using a different inoculum, the IC_{50} value for limonene was similar to the one described in a similar study with microcrystalline cellulose as substrate, reporting limonene IC_{50} value of 423 mg L^{-1} [34]. In the case of fenchone, to the best of our knowledge, IC_{50} has not been previously calculated for AD, although its antimicrobial properties against a wide range of microbes are well-known [5,7,21]. The most noticeable results were that the IC_{50} of 4-terpineol and *p*-cymene metabolites were 56% and 1% lower than that of limonene (Fig. 3), reinforcing the results that suggested that 4-terpineol is more inhibitory than the limonene itself. In a previous study, Sierra-Alvarez and Lettinga [37] evaluated the inhibitory effect of limonene, *p*-cymene and 4-terpineol on AD. However, their study focussed only on the methanogenic activity using a much simpler substrate than ours, i.e., a neutralised mixture of VFAs, obtaining IC_{50} values with inverse trends to those of this study. This might indicate that the inhibition related to limonene degradation metabolites would affect the whole anaerobic consortium, instead of mainly only the methanogenic archaea. However, it is important to note that a second feeding in the same reactors showed that there was no adaptation of the microbial population to 4-terpineol, as its IC_{50} value decreased, indicating a higher inhibition of 4-terpineol like the present study. As observed in the present study, limonene degradation metabolites led to faster and more severe inhibition compared to limonene. Although it would be highly interesting to follow their accumulation in continuous anaerobic digesters fed with monoterpenoid sources. It is worth noting that a high degradation of the dosed monoterpenes, excluding *p*-cymene, occurred even when the AD process is being affected (Tables 2 and 4, Fig. 3). This fact entails a challenge in the operation of the AD process since the monitoring of monoterpenes might not allow anticipating a potential destabilization. It would be necessary to expand the knowledge about the inhibitory potential of other monoterpenes and metabolites to define a degradation model that could help to identify and to prevent destabilization episodes.

4. Conclusions

Based on the IC_{50} values calculated from the methane production rate, the inhibition potential of all assessed monoterpenes followed the next trend: α-terpineol $<<$ fenchone $<$ limonene $\approx$ *p*-cymene $<$ 4-terpineol. Thus, limonene degradation metabolites were potentially more toxic than limonene itself. Despite of AD inhibition, monoterpenes were mostly degraded at the end of the experimental time. That fact entails a challenge in the operation of the AD process because monitoring monoterpenes might not allow anticipating a potential destabilization.

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CRediT authorship contribution statement

Serrano Antonio: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Cubero-Cardoso Juan:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Jeison David:** Supervision, Writing – review & editing. **Fermoso Fernando G.:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Trujillo-Reyes Ángeles:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Pérez Ana G.:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Cuéllar Sofía G.:** Data curation, Formal analysis, Investigation, Methodology.

Declaration of Competing Interest

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Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2024.112035.

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