Inhibition of methanogenesis by chlorophenols: a kinetic approach.

D. Puyol[a*,1], J.L. Sanz [b], J.J. Rodriguez[a] and A.F. Mohedano[a]

[a] *Ingenieria Quimica, Facultad de Ciencias, Universidad Autonoma de Madrid, Spain*

daniel.puyol@uam.es, angelf.mohedano@uam.es, juanjo.rodriguez@uam.es,

[b] *Departamento de Biologia Molecular, Facultad de Ciencias, Universidad Autonoma de Madrid, Spain*

joseluis.sanz@uam.es

** Corresponding author. Tf: 0034 914973525; Fax: 0034 914973516*

Abstract

Chlorophenols exert a critical effect on the methanogenesis, considerably reducing both maximum methane potential and methanogenic rates. However, there is not enough information about the kinetic mechanism of chlorophenols toxicity on the methanogenesis, which is a key aspect for the control of the anaerobic digesters because of the sensitivity and the potential for energy recovery derived from methanogenesis. The International Water Association Anaerobic Digestion Model 1 (IWA-ADM1) can be adapted to a wide range of situations by updating or changing the equations in the model. The present study proposes a general kinetic model for methanogenesis. This model has been applied to predict the inhibition of methanogenesis by chlorophenols, and it can be applied for updating the IWA-ADM1 when treating inhibitory compounds. The model was calibrated and validated using a wide broad of experimental sets of data of methane production by granular sludge in the presence of 2,4-dichlorophenol (24DCP), 2,4,6-trichlorophenol (246TCP) and pentachlorophenol (PCP) in batch assays. A *lag phase* of the effect of chlorophenols on the

[1] Present address: Department of Chemical and Environmental Engineering, University of Arizona, Tucson, AR, USA.

methanogenesis by non-adapted sludge was detected and modeled by the kinetic model proposed. In addition, the inhibitory effect of PCP was more pronounced on the acetoclastic methanogenesis than on the hydrogenotrophic one. Non-competitive and uncompetitive inhibition types were detected using 24DCP and 246TCP, whereas a suicide or irreversible inhibition type was observed in the case of PCP. Values of inhibition constants considerably varied depending on the chlorophenol used, between 45 mg 24DCP L^{-1}, 41-51 mg 246TCP L^{-1} and 0.9-7.8 mg PCP L^{-1}. The higher toxicity of PCP is related with its hydrophobicity, which was determined by adsorption tests and using partition coefficients *n*-octanol/water. Modeling was accompanied by high statistical support in all cases, which confirmed the validation of the model proposed.

Key words: Chlorophenols, methanogenesis, kinetics, inhibition.

Introduction

Chlorophenols are toxic compounds to anaerobic microorganisms. Their high hydrophobicity eases the adhesion of these compounds onto the bacterial membranes [1]. This disrupts the gradient of protons along the membranes and interferes with cellular energy transduction, decreasing the cell growth due to uncoupling of catabolic and anabolic cycles [2].

Methanogenesis is the last stage of the complex food chain that occurs in anaerobic environments and is the only stage that performs the elimination of organic matter from water by transforming it into methane gas in the absence of large amounts of nitrate and sulfate. Methanogens are the most sensitive microorganisms involved in the anaerobic digestion process because they can be inhibited by a wide range of chemicals. In addition, methanogenesis can lead to production of valuable energy from the methane release [5]. Thus, the optimization of methanogenesis is a key aspect for

the proper control and design of anaerobic digesters. The International Water Association (IWA) has developed a simulation model applied mainly to anaerobic digesters of waste (anaerobic digestion model no 1, ADM1 [6]). In this model, all stages of anaerobic digestion are governed by differential-algebraic equations that present dynamic state variables, which favor the adaptation of the model to a wide broad of processes by adding inhibitory or limiting terms to the kinetic equations, as well as adding equations describing abiotic reactions. Methanogenesis has been modeled using Monod [7-12], Haldane [13, 14] or Stover-Kincannon [15] models, which can often be simplified to first order equations [16, 17]. Inhibition of methanogenesis is commonly explained by adding inhibitory factors in the kinetic equations, as in the case of inhibition by pH [8, 12] or ammonium [9]. Inhibition of methanogenesis has been recently explained by modifying kinetic equations using exponential terms [18]. Methane production from acetoclastic and hydrogenotrophic pathways has been studied and the growth of the microorganisms responsible of each stage (*Methanosaeta* spp. and *Methanosarcina* spp. for acetoclastic methanogenesis, and *Methanosarcina* spp. and others hydrogenotrophs for hydrogenotrophic methanogenesis) has been modeled independently [7].

The effect of chlorophenols on methanogenesis has been widely studied (Table 1). However, there is not consensual for the specific mechanisms of toxicity. The half maximal effective concentration (EC_{50}) values strongly differ in a wide variety of studies. Thus, EC_{50} values are in the range between 26-410, 80-300, 41-117 and 6.8-76 for monochlorophenols, 24DCP, 246TCP and PCP, respectively. It seems to be clear that PCP is the most toxic chlorophenol [3, 19], and there are evidences indicating that the chlorophenols toxicity is related with the hydrophobicity through a linear relationship between the logarithm of the partition coefficient *n*-octanol/water (log*P*) and the EC_{50} values [19]. In addition, acetoclastic methanogenesis has been reported to be more sensitive to 246TCP toxicity than the hydrogenotrophic one [20]. Also, there are some studies reporting kinetic effects of chlorophenols on methanogenesis, which are commonly described by non-competitive

toxicity mechanisms [4, 21, 22]. In spite of these works, there is a lack of generalization for determining the effect of chlorophenols on kinetics of methanogenesis.

The present study proposed a general kinetic model for methanogenesis. This model has been applied on data from batch assays to predict inhibition of methanogenesis by chlorophenols in a wide range of experimental conditions, and it could be applied for updating the methanogenesis equations of the ADM1 model when treating inhibitory compounds.

Materials and methods

The experiments were performed in batch. Since the main objective of this study is trying to generalize the inhibition effects of chlorophenols on methanogenesis through the application of a general kinetic model, the experiments have no relationship each other. Instead of that, the experimental conditions were somehow as broad as possible.

A set of 6 different experiments were performed. The only experimental conditions shared were the temperature (30 ± 1ºC, a commonly used temperature in mesophilic conditions), the initial pH (7) and the type of sludge (granular). The rest of experimental conditions and a briefing description of each set of experiments are presented in Table 2.

Methane was measured volumetrically by the displacement method for the 250 mL flask serum bottles [27], or using a wet gas-meter for the 2 L anaerobic batch digesters (Schlumberger, Paris, France). Methane production was expressed as g CH_4-COD (that is so, g of CH_4 expressed as COD) L^{-1}, considering 1 g COD = 404 mL CH_4. Batch assays were performed by duplicate and were accompanied by blanks, in which no toxic was added. Adsorption tests were realized using sterile anaerobic granular sludge in the same conditions described above.

Chlorophenols concentration was quantified by HPLC-UV. Details of the method are described elsewhere [23]. Other analyses were performed according to Standard Methods [28].

The EC_{50} values were obtained quantitatively from the analysis of the average rates of methanogenesis, according to a previous work [23].

Modeling

The data collected from the batch kinetic tests described above are used to model the methane production and thereby determine the kinetic and inhibition parameters. Blanks experiments were used for determining general kinetic parameters in those experiments which was necessary. In order to encompass all the inhibitory effects observed in the experimental results, the following kinetic model explaining the methanogenesis is proposed (Eq. [1]):

$$\frac{dM}{dt}=\underbrace{\frac{V_{\max_1}}{1+\left(\frac{I}{K_{ni_1}}\right)^{m_1}}\cdot\frac{k_1\cdot\left(\frac{M_{\max_1}}{1+\left(\frac{I}{K_{si_1}}\right)^{n_1}}-M_1\right)+(1-k_1)(M_{\max_1}-M_1)}{\frac{K_{S_1}}{1+\left(\frac{I}{K_{ci_1}}\right)^{p_1}}+k_1\cdot\left(\frac{M_{\max_1}}{1+\left(\frac{I}{K_{si_1}}\right)^{n_1}}-M_1\right)+(1-k_1)(M_{\max_1}-M_1)}}_{\text{Acetoclastic}}+\underbrace{\frac{V_{\max_2}}{1+\left(\frac{I}{K_{ni_2}}\right)^{m_2}}\cdot\frac{k_2\cdot\left(\frac{M_{\max_2}}{1+\left(\frac{I}{K_{si_2}}\right)^{n_2}}-M_2\right)+(1-k_2)(M_{\max_2}-M_2)}{\frac{K_{S_2}}{1+\left(\frac{I}{K_{ci_2}}\right)^{p_2}}+k_2\cdot\left(\frac{M_{\max_2}}{1+\left(\frac{I}{K_{si_2}}\right)^{n_2}}-M_2\right)+(1-k_2)(M_{\max_2}-M_2)}}_{\text{Hydrogenotrophic}} \quad [1]$$

where M, M_1 and M_2 represent the total, acetoclastic and hydrogenotrophic quantity of methane produced, respectively (g COD-CH_4 L^{-1}), whereas k_1 and k_2 are the proportion factors for methane produced affected by the inhibitor (dimensionless). Thus, (1-k_1) and (1-k_2) are empirical factors which represent the methane produced before the toxic entering in contact with microorganisms. This approximation simplifies the model by avoiding the use of new timely variables, which would require working with finite difference equations. V_{max1} and V_{max2} are the maximum methanogenic

rate (g COD-CH_4 L^{-1} d^{-1}), K_{s1} and K_{s2} are the Monod constants (g COD-CH_4 L^{-1}), M_{max1} and M_{max2} are the maximum quantity of methane produced (g COD-CH_4 L^{-1}), I is the inhibitor concentration, K_{ni1} and K_{ni2} are the non-competitive inhibition constants, K_{ci1} and K_{ci2} are the competitive inhibition constants, K_{si1} and K_{si2} are the suicide or irreversible inhibition constants (mg inhibitor L^{-1}) and m_1, m_2, n_1, n_2, p_1, and p_2 are the inhibition factors for each type of inhibition (dimensionless), for acetoclastic (1) and hydrogenotrophic (2) methanogenesis, respectively. The use of exponential inhibition factors has been previously described for explaining non-linear inhibition type phenomena [18], and opens considerably possibilities for predicting accurately methane production in toxic environments. A brief description of the model and its derivation are included in Appendix 1. This complex model can be properly simplified for the most of the situations. Thus, this model has been simplified, generating simpler models, and applied to predict inhibitory processes on methanogenesis caused by 24DCP, 246TCP and PCP.

Simplifications for the model have been carried out according to the following assumptions:

(a) Biomass growth is negligible with respect to methane production, so the biomass concentration can be considered constant.

(b) Biological methane production is the limiting step of the methanogenesis, thus external and internal diffusion processes can be neglected in the kinetic models.

(c) There is no competence between microorganisms for methanogenic substrates, so inhibition processes can be exclusively attributed to the concentration of the inhibitor.

The general model proposed takes into account the biphasic production of methane, and all the possible inhibition types: competitive, non-competitive, uncompetitive and irreversible. A brief description of kinetics and inhibition types can be found in [29-32]. In addition, the model can predict the amount of methane produced during the *lag phase* of chlorophenols toxic action, working in non-steady state.

Kinetic fittings were carried out by means of two different techniques:

1) Initial rates method. The initial rates of methanogenesis were calculated by linear regression, and their values were fitted to the proposed models as a function of each selected concentration. The fitting was realized by non-linear regression using the Levemberg-Marquardt method. The goodness of fitting was determined by the R^2 parameter. The software Microcal© Origin 8.0 (Microcal, Northampton, MA, USA) was used for this method.

2) Integral method. Integration of differential equations was accomplished by using the Episode numerical method for Stiff systems. Initial conditions were $t = 0$; $M = 0$. Data from the time evolution of methane produced was fitted to the proposed models by means of a non-linear least squares minimization of the error using a simplex algorithm followed by a Powell minimization algorithm. The goodness of fitting was quantified by the coefficient of correlation. The software Micromath© Scientist 3.0 (Miromath,Saint Louis, MO, USA) was used for this purpose.

Results and discussion

Batch experiments were conducted in order to study the effect of 24DCP and 246TCP on the kinetics of methanogenesis of a non-adapted granular sludge, whose main results are depicted in Fig. 1. Raw data were used for calculating the time evolution of methanogenic rate for the 24DCP (Fig. 1a) and 246TCP (Fig. 1b) experiments. Also, from the adsorption experiments, a maximum biosorption capacity of the granular sludge was determined as 6.7 and 5.3 mg 24DCP and 246TCP g^{-1} VSS, respectively. Final pH in the biotic experiments was around 7.8 in both cases, indicating that the most important form of both chlorophenols is the ionized (chlorophenolates). So, chlorophenols biosorbed onto biomass (predominantly unionized form) can be considered as negligible with respect to effective concentration in liquid phase. As can be seen, in both cases the methane production rate patterns were severely modified by the action of chlorophenols. The maximum methane production rate was diminished severely and displaced timely with increasing initial chlorophenols concentration, suggesting the occurrence of uncompetitive inhibition

phenomena. The initial methane production rates were calculated from these data with an aim towards studying in depth the inhibition phenomena caused by 24DCP (Fig. 2a) and 246TCP (Fig. 2b). In both cases, values diminished in a non-linear way with increasing chlorophenols concentration, which can be explained by exponential-type inhibition phenomena. EC_{50} concentrations were estimated around to 55 and 50 mg L^{-1} for 24DCP and 246TCP, respectively.

The time evolution of methane production using adapted granular sludge in contact with 246TCP is presented in Fig. 2. Maximum values of 246TCP biosorbed onto biomass were calculated from adsorption tests, and they were around 1.7 mg 246TCP g^{-1} VSS. This low value is caused by the origin of the sludge. The adapted sludge was retrieved from an EGSB reactor treating 246TCP [26]. In spite of this sludge was washed with mineral medium, the most of the chemisorption holes were saturated, so the adsorption capacity of the sludge was diminished. Then, adsorption processes can be considered as negligible. The methane production was severely affected by 246TCP despite the pre-adaptation of the sludge, but the methane production was different from the non-adapted sludge data. While 246TCP affected all the methane production from the beginning of the contact between the non-adapted sludge and the toxic, in this case the methane production was biphasic. A molar mass balance of organic acids was performed and concluded that during the first hours of the process the acetate accumulation did not explain the methane produced, so methanogenesis in these hours can be attributed mainly to hydrogenotrophic methanogenesis, as has been previously described in detail [24]. Then, both acetoclastic and hydrogenotrophic methanogenesis occurred simultaneously at different rates. Since the most affected part of the methanogenesis succeeded late, acetoclastic methanogenesis seemed to be strongly inhibited by 246TCP, being the hydrogenotrophic one hardly affected. In addition, no *lag-phases* for the inhibitory action of 246TCP were detected, since the sludge was previously in contact with the chemical. The pre-adaptation of the sludge substantially modified the EC_{50} of methanogenesis, which varied from around 50 (non-adapted sludge) to 75 mg 246TCP L^{-1} (adapted sludge). However, since the biomass

concentration was different in both experiments, the adaptation of the sludge cannot be considered as the only cause for the divergence in the EC_{50} values.

Finally, the effect of PCP on the methanogenesis kinetics was studied using non-adapted granular sludge. Adsorption tests showed that the maximum adsorption capacity of the granular sludge strongly depends on the PCP concentration. At the higher concentration tested (50 mg PCP L^{-1}), the adsorption capacity was around 37 mg PCP g^{-1} VSS. This implies that PCP has a strong capacity of adhesion onto the bacterial surface. Acetoclastic and hydrogenotrophic methanogenesis were studied independently (Fig. 3) and jointly (Fig. 4). The EC_{50} calculated for acetoclastic methanogenesis resulted much smaller than that for the hydrogenotrophic one (3 versus 20 mg PCP L^{-1}), which means that PCP exerts an effect more severe on the first one. In addition, the PCP effect was noted after a *lag-phase*, typical in non-steady state when non-adapted sludge is used. Fig. 4 shows the time evolution of methane production in the global methanogenesis study, using a complex medium. As can be seen, PCP exerted a critical effect on methanogenesis, presumably due to the strong effect over acetoclastic methanogens. In this case, the EC_{50} for global methanogenesis was estimated to around 5 mg PCP L^{-1}. In all the experiments, the maximum quantity of methane produced strongly depends on PCP initial concentration, suggesting that PCP acted as an irreversible or suicide inhibitor for any type of methanogenesis, decreasing substantially the methanogenic potential.

The first approximation to the Eq. [1] is the 24DCP experiment using non-adapted granular sludge. The data to fit are represented by initial rates of methane production. Taking into account that, (a) at $t = 0$, the methane produced is 0, and (b) K_s can be considered as negligible with respect to M_{max}, the model is simplified to zero order. Then, only the inhibition factor affecting V_{max} (non-

competitive) is modifying the values of initial methanogenic rates in function of 24DCP concentration. Thus, the Eq. [1] can be simplified to Eq. [2] (Table 3). The values of the parameters are included in Table 3, whereas the kinetic fitting is represented in Fig. 1b.

The kinetic study of the effect of 246TCP using non-adapted sludge was performed using the initial rates of methanogenesis, so the previous argumentation for the 24DCP experiment is useful here too. The experimental data was fitted to Eq. [2], being the resulted parameters collected in Table 3, and the fitting located at Fig. 1d. The kinetic study using adapted granular sludge was accomplished with the original data from methane production in function of time. Since zero order is not possible here, the model cannot be simplified in the same way. However, as the sludge is adapted to 246TCP, *lag-phases* of the toxic action of 246TCP on methanogenesis can be neglected, so k_1 and k_2 are close to 1. In addition, as previously pointed, hydrogenotrophic methanogenesis seems to be unaffected by the 246TCP concentration, so inhibition only modified the acetoclastic production of methane. As the relation substrate to biomass is relatively low (around 1:2.5 g COD g^{-1} VSS), saturation constants are much higher than methane production, so the model can be simplified to first order. Then, V_{max} and K_s can be lumping to k_M (d^{-1}). Working with the experimental data, the best fit was found when non-competitive inhibition factors were taken into account. Then, the Eq. [1] can be simplified to Eq. [3] (Table 3). The experimental data was fitted to this equation. The values of the fitting parameters are collected in Table 3, and the experimental fitting, in Fig. 2.

Studying the effect of PCP on methanogenesis using non-adapted granular sludge, *lag-phases* for the toxic effect should be taken into account in the kinetic models, and k_1 and k_2 could be different from 1. Data to fit were the methane production in function of time, so zero order simplification is not possible here. As the relation substrate to biomass is considerable (around 3:1 g COD g^{-1} VSS), saturation constants could be comparable to methane production, so the model cannot be simplified to first order. In the case of acetoclastic methanogenesis, the *lag-phase* was detected, implying that

k_2 is not closed to 1 (Fig. 3a). The best fit of the data was found considering only PCP as an irreversible inhibitor, affecting only the M_{max}. So, the Eq. [1] can be simplified to Eq. [4] (Table 3). In contrast, in the case of hydrogenotrophic methanogenesis (Fig. 3b) no *lag-phase* for the toxic action of PCP was detected. Then, k_1 is closed to 1. Again, the fitting study resulted that PCP acted as an irreversible inhibitor. Then, the Eq. [1] can be simplified to Eq. [5] (Table 3). The values of the parameters of both studies are collected in Table 3, and the fittings, in Fig. 3.

The kinetic analysis about the PCP effect on methanogenesis using adapted sludge was performed for data from Fig. 4. As in the case of the non-adapted granular sludge study, zero order simplification is not suitable here. A divergence in the parameters estimation avoided to use a Monod model, so in this case a first order simplification had to be used in order to a correct estimation of the inhibitory parameters. A *lag-phase* was detected in this case, so k is different from 1. As can be seen in Fig. 4, the PCP considerable reduces the maximum quantity of methane produced (irreversible inhibition). Fitting data separately, it has been found that the basal production of methane during the *lag-phase* is much slower than the rest of the methanogenesis. For this reason, the first order constant varies, and the model should be separated into two terms, explaining independently the basal and the rest of the methane produced. In this way, Eq. [1] is simplified to Eq. [6] (Table 3), where k_B represents the first order constant for the basal methane production (d^{-1}). The parameter values are resumed in Table 3, and the resulting fitting is depicted in Fig. 6.

The parameter values are comparable with those reported in the literature [7, 13, 16], which provides a clear physical sense to the fittings. In addition, the high correlation obtained between experimental and predicted data and the low standard errors calculated for the parameters values gives the data a strong statistical support. According to the parameters values, it seems that there are no strong difference between the toxicity of 24DCP and 246TCP, since values of EC_{50} and

inhibition constants are quite similar. However, PCP is presented as the most toxic chlorophenol, not only because of the different values of its model parameters, but also because this compound exerts an irreversible inhibitory effect not detected for less-chlorinated chlorophenols. Intending to explain the values of the inhibition parameters obtained based on the hydrophobicity of chlorophenols, values of inhibition constants (K_i) for each chlorophenol were compared with the log$P$. The higher hydrophobicity of PCP (log$P$=5.01, [19]) can explain the higher toxicity of PCP with respect to 24DCP and 246TCP. However, 246TCP has a higher hydrophobicity than 24DCP (log$P$=3.38 versus 3.15 for 246TCP and 24DCP, respectively, [19]) and, as has been demonstrated in this work, the inhibitory effect is of both chlorophenols is quite similar or even slightly higher for 24DCP. Other authors have obtained the same results about 24DCP and 246TCP [19]. A more in depth study is needed to elucidate this usual divergence.

As a recommendation, it could be useful if the model described by Eq. [1] is not directly used at first. Previous simplifications to the model (similar to Eqs. [2]-[6]) could be applied with an aim towards improving the initial estimation of the parameter values for limiting the time required by the mathematical software to converge.

Conclusions

Chlorophenols exert a deep impact on kinetics of methanogenesis, reducing the maximum methanogenic rate (in the case of 24DCP and 246TCP) or even the methanogenic potential of an anaerobic sludge (using PCP). The inhibition effects of 24DCP and 246TCP are non-competitive and uncompetitive, whereas PCP exerts an irreversible or suicide inhibition effect on methanogenesis. PCP seems to be the most toxic chlorophenol studied, and this toxicity is directly related with its high hydrophobicity, which strongly affects its adsorption capacity.

A general kinetic model is proposed to predict the inhibition of the methanogenesis caused by chlorophenols. The model accurately predicts any inhibition type caused by chlorophenols in a wide broad of different experimental conditions and it even lets to simulate the *lag-phase* of the inhibitory effect of chlorophenols observed using non-adapted sludge. This complex model can be simplified in the most of the situations for the correct estimation of the kinetic parameters, and it can be considered as a useful tool to improve methanogenesis modeling by the IWA-ADM1 when treating inhibitory and/or toxic compounds with anaerobic technologies.

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References

[1] Liu Y, Tay JH. Strategy for minimization of excess sludge production from the activated sludge process. Biotechnol Adv 2001;19:97-107.
[2] Chen Y, Cheng JJ, Creamer KS. Inhibition of anaerobic digestion process: a review. Bioresource Technology 2008;99:4044-4064.
[3] Patel GB, Agnew BJ, Dicaire CJ. Inhibition of pure cultures of methanogens by benzene ring compounds. Appl Environ Microbiol 1991;57:2969-2974.
[4] Yi-Tin Wang HDG, Pin-Chieh Pai. Inhibition of Acetate Methanogenesis by Phenols. Journal of Environmental Engineering 1991;117:487-500.
[5] Speece RE. Anaerobic biotechnology for industrial wastewaters. Nashville, TN, USA: Archae Press; 1996.
[6] Batstone DJ, Keller J, Angelidaki I, Kalyuzhnyi SV, Pavlostathis SG, Rozzi A, et al. The IWA Anaerobic Digestion Model No 1 (ADM1). Water Sci Technol 2002;45:65-73.
[7] Qu X, Vavilin VA, Mazeas L, Lemunier M, Duquennoi C, He PJ, et al. Anaerobic biodegradation of cellulosic material: batch experiments and modelling based on isotopic data and focusing on aceticlastic and non-aceticlastic methanogenesis. Waste Manag 2009;29:1828-1837.
[8] Saravanan V, Sreekrishnan TR. A mathematical model for a hybrid anaerobic reactor. J Environ Manage 2008;88:136-146.
[9] Fountoulakis MS, Stamatelatou K, Lyberatos G. The effect of pharmaceuticals on the kinetics of methanogenesis and acetogenesis. Bioresour Technol 2008;99:7083-7090.
[10] Raposo F, Borja R, Sanchez E, Martin MA, Martin A. Performance and kinetic evaluation of the anaerobic digestion of two-phase olive mill effluents in reactors with suspended and immobilized biomass. Water Res 2004;38:2017-2026.

[11] Lokshina LY, Vavilin VA. Kinetic analysis of the key stages of low temperature methanogenesis. Ecological Modelling 1999;117:285-303.
[12] Kalyuzhnyi SV. Batch anaerobic digestion of glucose and its mathematical modeling. II. Description, verification and application of model. Bioresource Technology 59:249-258.
[13] Pereira MA, Roest K, Stams AJ, Mota M, Alves M, Akkermans AD. Molecular monitoring of microbial diversity in expanded granular sludge bed (EGSB) reactors treating oleic acid. FEMS Microbiol Ecol 2002;41:95-103.
[14] Lokshina LY, Vavilin VA, Kettunen RH, Rintala JA, Holliger C, Nozhevnikova AN. Evaluation of kinetic coefficients using integrated monod and haldane models for low-temperature acetoclastic methanogenesis. Water Research 2001;35:2913-2922.
[15] Kuscu OS, Sponza DT. Kinetics of para-nitrophenol and chemical oxygen demand removal from synthetic wastewater in an anaerobic migrating blanket reactor. J Hazard Mater 2009;161:787-799.
[16] Rodriguez-Martinez J, Rodriguez-Garza I, Pedraza-Flores E, Balagurusamy N, Sosa-Santillan G, Garza-Garcia Y. Kinetics of anaerobic treatment of slaughterhouse wastewater in batch and upflow anaerobic sludge blanket reactor. Bioresour Technol 2002;85:235-241.
[17] García-Ochoa F, Santos VE, Naval L, Guardiola E, López B. Kinetic Model for Anaerobic Digestion of Livestock Manure. Enzyme and Microbial Technology 1999;25:55-60.
[18] Puyol D, Monsalvo VM, Mohedano AF, Sanz JL, Rodriguez JJ. Cosmetic wastewater treatment by upflow anaerobic sludge blanket reactor. J Hazard Mater 2011;185:1059-1065.
[19] Sierra-Alvarez R, Lettinga G. The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. Applied Microbiology and Biotechnology 1991;34:544-550.
[20] Collins G, Foy C, McHugh S, O'Flaherty V. Anaerobic treatment of 2,4,6-trichlorophenol in an expanded granular sludge bed-anaerobic filter (EGSB-AF) bioreactor at 15 degrees C. FEMS Microbiol Ecol 2005;53:167-178.
[21] Kim IS, Tabak HH, Young JC. Modeling of the fate and effect of chlorinated phenols in anaerobic treatment processes. Water Science and Technology 1997;36:287-294.
[22] Eker S, Kargi F. Kinetic modeling and parameter estimation in biological treatment of 2,4-dichlorophenol containing wastewater using rotating perforated tubes biofilm reactor. Enzyme and Microbial Technology 2006;38:860-866.
[23] Puyol D, Mohedano AF, Sanz JL, Rodriguez JJ. Anaerobic biodegradation of 2,4,6-trichlorophenol by methanogenic granular sludge: role of co-substrates and methanogenic inhibition. Water Sci Technol 2009;59:1449-1456.
[24] Puyol D, Mohedano AF, Rodriguez JJ, Sanz JL. Effect of 2,4,6-trichlorophenol on the microbial activity of adapted anaerobic granular sludge bioaugmented with Desulfitobacterium strains. N Biotechnol 2011;29:79-89.
[25] Puyol D, Mohedano AF, Sanz JL, Rodriguez JJ. Comparison of UASB and EGSB performance on the anaerobic biodegradation of 2,4-dichlorophenol. Chemosphere 2009;76:1192-1198.
[26] Puyol D, Rajhi H, Mohedano A, Rodríguez J, Sanz J. Anaerobic biodegradation of 2,4,6-trichlorophenol in expanded granular sludge bed and fluidized bed biofilm reactors bioaugmented with Desulfitobacterium spp. Water Science and Technology 2011;64:293-299.
[27] James A, Chernicharo CAL, Campos CMM. The development of a new methodology for the assessment of specific methanogenic activity. Water Research 1990;24:813-825.
[28] APHA, AWWA, WEF. Standard methods for the examination of water & wastewater. 21th ed. Washington: American Public Health Association; 2005.
[29] Gavala HN, Angelidaki I, Ahring BK. Kinetics and modeling of anaerobic digestion process. Adv Biochem Eng Biotechnol 2003;81:57-93.
[30] Okpokwasili GC, Nweke CO. Microbial growth and substrate utilization kinetics. African Journal of Biotechnology 2006;5:305-317.
[31] Mulchandani A, Luong JHT. Microbial inhibition kinetics revisited. Enzyme and Microbial Technology 1989;11:66-73.
[32] Pavlostathis SG. 6.31 - Kinetics and Modeling of Anaerobic Treatment and Biotransformation Processes, In: Editor-in-Chief: Murray M-Y, editor,^editors. Comprehensive Biotechnology (Second Edition), Burlington: Academic Press; 2011, p.^ps. 385-397.
[33] Bajaj M, Gallert C, Winter J. Anaerobic biodegradation of high strength 2-chlorophenol-containing synthetic wastewater in a fixed bed reactor. Chemosphere 2008;73:705-710.

[34] Majumder PS, Gupta SK. Degradation of 4-chlorophenol in UASB reactor under methanogenic conditions. Bioresource Technology 2008;99:4169-4177.
[35] Ennik-Maarsen KA, Louwerse A, Roelofsen W, Stams AJM. Influence of monochlorophenols on methanogenic activity in granular sludge. Water Research 1998;32:2977-2982.
[36] Bali U, Sengül F. The fate and effect of 4-chlorophenol in an upflow anaerobic fixed-bed reactor. Process Biochemistry 2003;38:1201-1208.
[37] Sponza DT, Uluköy A. Treatment of 2,4-dichlorophenol (DCP) in a sequential anaerobic (upflow anaerobic sludge blanket) aerobic (completely stirred tank) reactor system. Process Biochemistry 2005;40:3419-3428.

Nomenclature and notation table (for reviewers only, or at your convenience).

Instant methanogenic rate: calculated by the slope of three consecutive points of cumulative methane production.

Initial methanogenic rate: calculated as the slope of the first 5 points of cumulative methane production.

Maximum methanogenic rate: calculated as the maximum of the instant methanogenic rates.

246TCP	2,4,6-trichlorophenol
24DCP	2,4-dichlorophenol
2CP	2-chlorophenol
3CP	3-chlorophenol
4CP	4-cholophenol
COD	Chemical Oxygen Demand (g L^{-1})
dM/dt	Methane production rate (g CH_4-COD L^{-1} d^{-1})
dM_1/dt	Acetoclastic methane production rate (g CH4-COD L^{-1} d^{-1})
dM_2/dt	Hydrogenotrophic methane production rate (g CH_4-COD L^{-1} d^{-1})
dM_o/dt_o	Initial methane production rate (g CH_4-COD L L^{-1} d^{-1})
EC_{50}	Half maximal effective concentration
$EC_{50}a$	EC50 for acetoclastic methanogenesis
$EC_{50}h$	EC50 for hydrogenotrophic methanogenesis
$EC_{50}m$	EC50 for global methanogenesis
EGSB	Expanded granular sludge bed
FBBR	Fluidized bed biofilm reactor
FFR	Fixed-film reactor
GB	Granular biomass

I	Inhibitor concentration (mg L^{-1})
IWA-ADM1	International Water Association Anaerobic Digestion Model no. 1
k	Empirical factor for methane production affected by inhibitors
k_1	k for acetoclastic methanogenesis
k_2	k for hydrogenotrophic methanogenesis
k_B	First order constant for basal methane production (d^{-1})
K_{ci}	Competitive inhibition constant (mg L^{-1})
K_{ci1}	Competitive inhibition constant for acetoclastic methanogenesis (mg L^{-1})
K_{ci2}	Competitive inhibition constant for hydrogenotrophic methanogenesis (mg L^{-1})
K_i	Inhibition constant (mg L^{-1})
k_{M1}	First order constant for acetoclastic methanogenesis (d^{-1})
k_{M2}	First order constant for hydrogenotrophic methanogenesis (d^{-1})
K_{ni}	Non-competitive inhibition constant (mg L^{-1})
K_{ni1}	Non-competitive inhibition constant for acetoclastic methanogenesis (mg L^{-1})
K_{ni2}	Non-competitive inhibition constant for hydrogenotrophic methanogenesis (mg L^{-1})
K_s	Half-saturation constant (g CH_4-COD L^{-1})
K_{s1}	Half-saturation constant for acetoclastic methanogenesis (g CH_4-COD L^{-1})
K_{s2}	Half-saturation constant for hydrogenotrophic methanogenesis (g CH_4-COD L^{-1})
K_{si}	Irreversible inhibition constant (mg L^{-1})
K_{si1}	Irreversible inhibition constant for acetoclastic methanogenesis (mg L^{-1})
K_{si2}	Irreversible inhibition constant for hydrogenotrophic methanogenesis (mg L^{-1})
log$P$	logarithm of the partition coefficient *n*-octanol/water
M	Cumulative methane production (g CH_4-COD L^{-1})
m	Non-competitive inhibition order (dimensionless)
M_1	Cumulative acetoclastic methane production (g CH_4-COD L^{-1})
m_1	Non-competitive inhibition order for acetoclastic methanogenesis (dimensionless)
M_2	Cumulative hydrogenotrophic methane production (g CH_4-COD L^{-1})
m_2	Non-competitive inhibition order for hydrogenotrophic methanogenesis (dimensionless)
M_{max}	Cumulative maximum methane production for an infinite time of digestion (g CH_4-COD L^{-1} d^{-1})
M_{max1}	Cumulative maximum acetoclastic methane production for an infinite time of digestion (g CH_4-COD L^{-1} d^{-1})
M_{max2}	Cumulative maximum hydrogenotrophic methane production for an infinite time of digestion (g CH_4-COD L^{-1} d^{-1})
n	Irreversible inhibition order (dimensionless)
n_1	Irreversible inhibition order for acetoclastic methanogenesis (dimensionless)
n_2	Irreversible inhibition order for hydrogenotrophic methanogenesis (dimensionless)
ni	Inhibition order (dimensionless)
p	Competitive inhibition order (dimensionless)
p_1	Competitive inhibition order for acetoclastic methanogenesis (dimensionless)
p_2	Competitive inhibition order for hydrogenotrophic methanogenesis (dimensionless)
PCP	Pentachlorophenol
RTBR	Rotating tube biofilm reactor
SB	Suspended biomass
UASB	Upflow anaerobic sludge blanket
$Vmax$	Maximum methane production (g CH_4-COD L^{-1} d^{-1})

V_{max1}	Maximum acetoclastic methane production (g CH_4-COD L^{-1} d^{-1})
V_{max2}	Maximum hydrogenotrophic methane production (g CH_4-COD L^{-1} d^{-1})
VSS	Biomass expressed as volatile suspended solids (mg L^{-1})

1

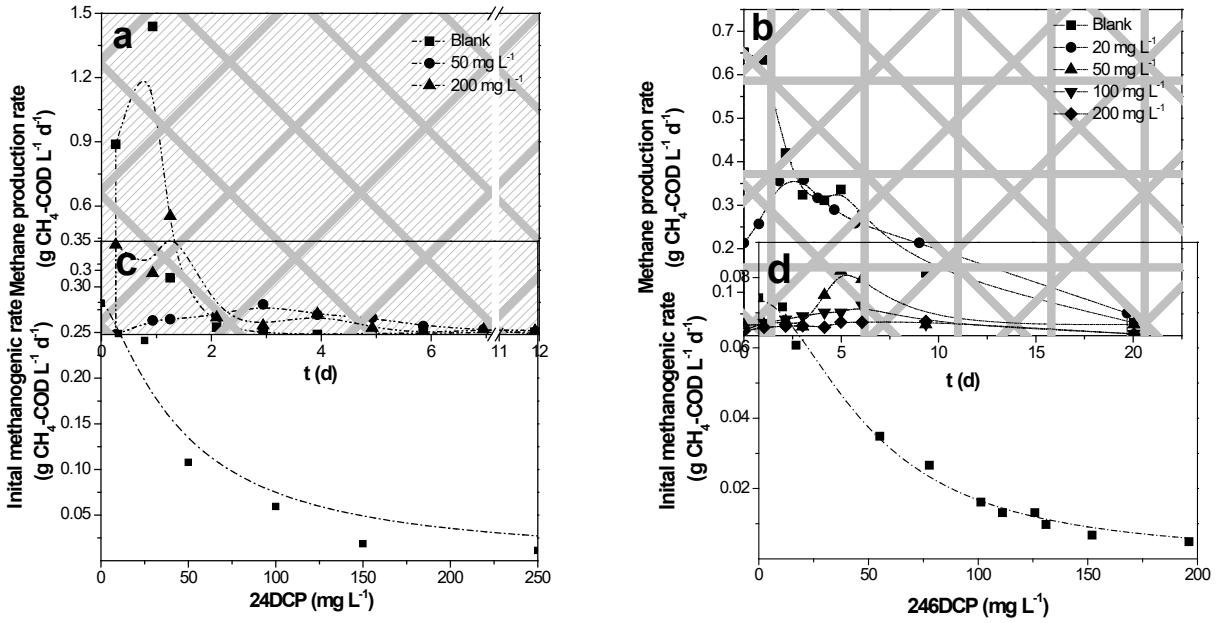

Figure 1: Time patterns of methane production rate using different initial concentrations of 24DCP (a) and 246TCP (b), and effect of 24DCP (c) and 246TCP (d) concentration on initial methanogenic rates (squares) using non-adapted sludge. Dash-dot lines are model fittings to Eq. [2].

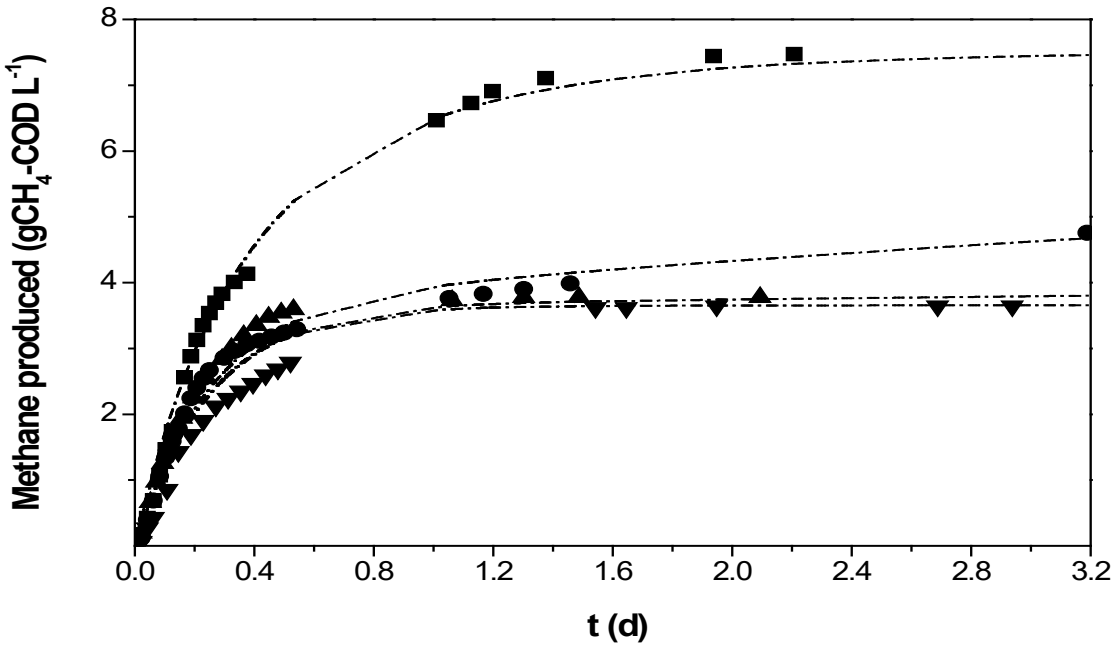

Figure 2: Time evolution of methane produced at initial concentrations of 246TCP of 50 (squares), 75 (circles), 100 (up triangles) and 150 (down triangles) mg L^{-1} using adapted sludge. Dash-dot lines are model fittings to Eq. [3].

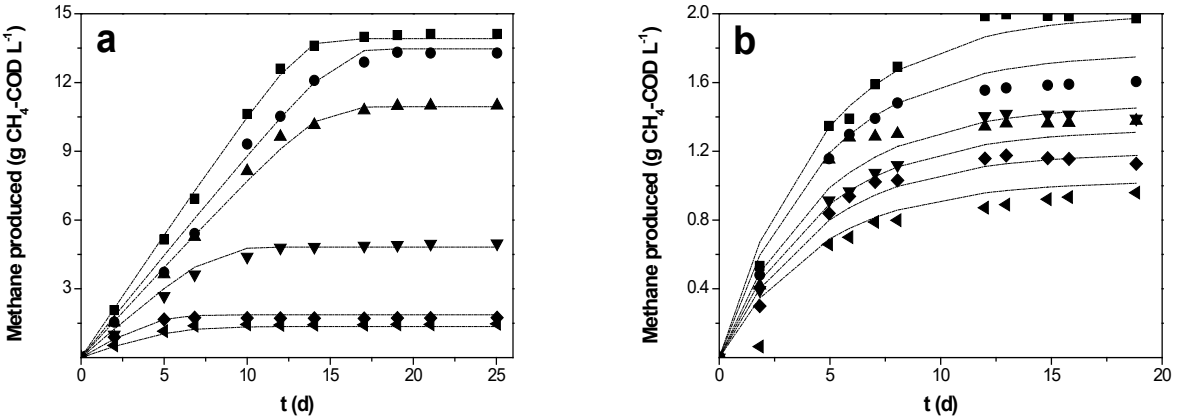

Figure 3: Time evolution of methane produced in acetoclastic (a) and hydrogenotrophic (b) experiments using non-adapted granular sludge at initial PCP concentrations of 0 (squares), 1 (circles), 5 (up triangles), 10 (down triangles), 25 (diamonds) and 50 (left triangles) mg PCP L^{-1}. Dash-dot lines are model fittings to Eqs. [5] (a) and [4] (b).

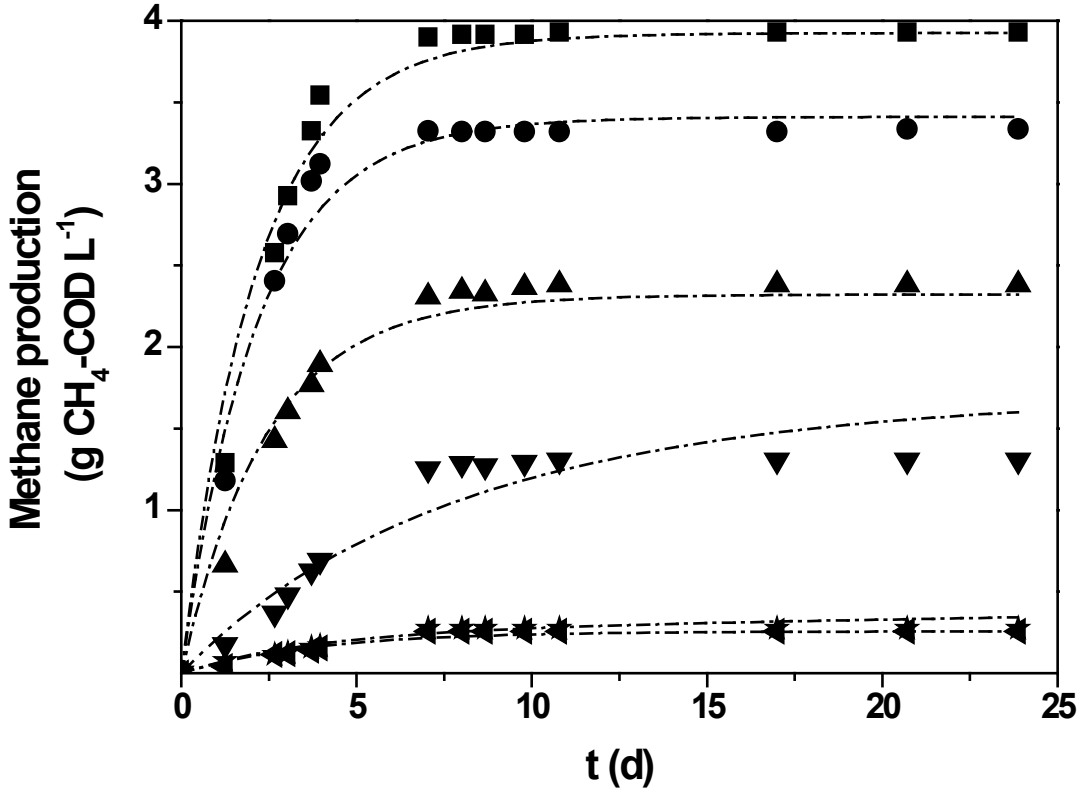

Figure 4: Time evolution of methane production using adapted granular sludge at initial PCP concentrations of 0 (squares), 1 (circles), 5 (up triangles), 10 (down triangles), 25 (stars) and 50 (left triangles) mg L^{-1}. Dash-dot lines are model fittings to Eq. [6].

Table 1: Review of effects of chlorophenols on methanogenesis.

Compound	**Operation type***	**Chlorophenol concentration**	**Effect on methanogenesis****	**Effects on kinetics**	**Ref.**
2CP	FBBR	51-193	Reduction of 46.7% SMA	Monod with 2CP as the sole substrate. (C)	[33]
4CP	UASB	40	Reduction of 16.6% SMA.	N/A	[34]
2CP 3CP 4CP	Batch, FB	50-1200	$EC_{50}a$=150 $EC_{50}a$=250 $EC_{50}a$=550	Non-competitive inhibition. K_i=86 (2CP), 97 (3CP) and 419 (4CP). *ni*=1 in all cases.	[4]
2CP 3CP 4CP	Batch, GB.	64-643 64-383	$EC_{50}m$ = 77 $EC_{50}m$ = 26 $EC_{50}m$ = 26	N/A N/A N/A	[35]
4CP	FFR	10-200	Desestabilization of biomass at concentrations above 100.	N/A	[36]
24DCP	Batch, GB	25-400	$EC_{50}m$ = 300	Linear inhibition of SMA.	[37]
2CP	Batch, SB	300, 600	Inhibition of acetoclastic	Non competitive	[21]

3CP			methanogenesis.	inhibition. K_i = 157	
4CP		50-300		(2CP), 240 (3CP) and 104 (4CP)	
24DCP	RTBR	50-400	EC_{50}m=250	Non-competitive inhibition. K_i = 26; *ni*=1.	[22]
246TCP	Batch, GB	1-100	EC_{50}a=41-79 (15 ºC), 52-90 (37 ºC). EC_{50}h=52-91 (15 ºC), 66-103 (37 ºC).	N/A	[20]
246TCP	Batch, GB	10-165	EC_{50}a=113	Inhibition of methanogenesis by 246TCP is accumulative	[23]
PCP	Batch, SB	1-100	EC_{50}m=6.8-76	N/A	[3]
2CP	Batch, GB	10-1000	EC_{50}a=410	N/A	[19]
24DCP		10-1000	EC_{50}a=80		
246TCP		10-1000	EC_{50}a=117		
PCP		1-100	EC_{50}a = 8		

* UASB: Upflow anaerobic sludge blanket; FBBR: Fluidized bed biofilm reactor; FFR: Fixed-film reactor; RTBR: Rotating tube biofilm reactor; GB: granular biomass; SB: suspended biomass;

** If nothing is mentioned, effects correspond only to the maximum concentration of chlorophenol applied. EC_{50}m: EC_{50} for global methanogenesis. EC_{50}a: EC_{50} for acetoclastic methanogenesis. EC_{50}h: EC_{50} for hydrogenotrophic methanogenesis.

(C): Not explained in the reference, own calculation from the original data.

N/A: Not applicable or not enough data.

All concentrations are in mg L^{-1}.

Table 2: Experimental conditions of the anaerobic batch experiments.

Compound	Concentration range (mg L^{-1})	Volume (L)	Source of biomass	Biomass concentration (g VSS L^{-1})	Biomass type	Main substrate and concentration (g COD L^{-1})	Methanogenesis target	Main aim
24DCP	10-250	0.25	Lab-scale EGSB [25]	1.5	Non-adapted	Glucose (4)	Global	Determining type and extent of 24DCP and 246TCP inhibition
246TCP	10-200	0.25	Lab scale EGSB [25]	1.5	Non-adapted	Sucrose (2), ethanol (1.3) and yeast extract (0.7).	Global	
246TCP	50-150	2	Lab-scale EGSB [26]	10	Adapted	Sucrose (2), ethanol (1.3) and yeast extract (0.7).	Global	Modeling methanogenesis at similar conditions than continuous reactors
PCP	1-50	0.25	Lab-scale EGSB [26]	1.5	Non-adapted	Formiate (2)	Hydrogenotrophic	Comparing methanogenesis inhibition by PCP in function of electron donor
PCP	1-50	0.25	Lab-scale EGSB [26]	1.5	Non-adapted	Acetate (4)	Acetoclastic	
PCP	1-50	0.25	Lab-scale EGSB	1.5	Non-adapted	Sucrose (2), ethanol (1.3) and yeast extract (0.7).	Global	Study PCP effect on kinetics of global methanogenesis

Table 3: Fitting equations simplified from Eq. [1] used in the kinetic study and values of the parameters estimated.

Compound	Sludge type	Equation		Parameter values*
24DCP	Non-adapted	$\frac{dM_o}{dt_o} = \frac{V_{\max}}{\left[1+\left(\frac{I}{K_{ni}}\right)^m\right]}$	[2]	K_{ni} = 45.1 ± 6.3; V_{max} = 0.286 ± 0.146; m = 1.33 ± 0.17; R^2 = 0.986.
	Non-adapted			K_{ni} = 50.9 ± 3.5; V_{max} = 0.073 ± 0.001; m = 1.81 ± 0.15; R^2 = 0.994.
246TCP	Adapted	$\frac{dM}{dt} = \frac{k_{M_1}\cdot(M_{\max_1} - M_1)}{1+\left(\frac{I}{K_{ni}}\right)^{m_1}} + k_{M_2}\cdot(M_{\max_2} - M_2)$	[3]	k_{M1} = 6.86 ± 4.32; k_{M2} = 4.08 ± 1.2; M_{max1} = 3.85 ± 1.20; M_{max2} = 3.65 ± 0.02; K_{ni1} = 41.4 ± 12.5; m_1 = 7.1 ± 0.76; R^2 = 0.994.
PCP	Non-adapted (hydrogenotrophic)	$\frac{dM_1}{dt} = V_{\max_1}\cdot\frac{\frac{M_{\max 1}}{1+\left(\frac{I}{K_{si_1}}\right)^{n_1}} - M_1}{K_{S_1} + \frac{M_{\max_1}}{1+\left(\frac{I}{K_{si_1}}\right)^{n_1}} - M_1}$	[4]	V_{max1} = 0.251 ± 0.014; K_{S1} = 0.334 ± 0.18; M_{max1} = 2.004 ± 0.042; K_{si1}=0.942± 0.326; n_1 = 0.17 ± 0.01; R^2= 0.996.
	Non-adapted (acetoclastic)	$\frac{dM_2}{dt} = V_{\max_2}\cdot\frac{k_2\cdot\left(\frac{M_{\max_2}}{1+\left(\frac{1}{K_{si_2}}\right)^{n_2}} - M_2\right) + (1-k_2)(M_{\max_2} - M_2)}{K_{S_2} + k_2\cdot\left(\frac{M_{\max_2}}{1+\left(\frac{1}{K_{si_2}}\right)^{n_2}} - M_2\right) + (1-k_2)(M_{\max_2} - M_2}$	[5]	V_{max2} = 1.115 ± 0.034; K_{S2} = 0.469 ± 0.206; M_{max2} = 13.901 ± 0.0761; K_{si2} = 7.3 ±

			0.08; n_2 = 3.1 ± 0.1; k_2 = 0.905 ± 0.005; R^2= 0.999.
Non-adapted	$\frac{dM}{dt} = k_b \cdot (1-k) \cdot \left(\frac{M_{\max}}{1+\left(\frac{K_{si}}{I}\right)^n} - M \right) + \frac{k_M \cdot k \cdot \left(\frac{M_{\max}}{1+\left(\frac{K_{si}}{I}\right)^n} - M \right)}{1+\left(\frac{K_{ni}}{I}\right)^m}$	[6]	M_{max} = 3.669 ± 0.048; K_{si} = 6.495 ± 0.427; n = 0.970 ± 0.071; k_M = 0.478 ± 0.022; K_{ni} = 7.827 ± 0.560; m = 5.055 ± 1.065; k_b = 0.282 ± 0.024; k = 0.944 ± 0.009; R^2 = 0.990.

* Units are in the text.

APPENDIX 1

Derivation of the model.

Methanogenesis can be described by a pseudo-Monod model as follows:

$$\frac{dM}{dt} = \mu_{max} \cdot X \cdot \frac{1}{Y_{X/S}} \cdot \frac{M_{max}-M}{K_S+M_{max}-M} \quad [A1]$$

Where M and M_{max} are the methane produced and the maximum methane produced for an infinite time of digestion (g CH_4-COD L^{-1}), μ_{max} are the maximum specific growing rate (d^{-1}), $Y_{X/S}$ is the biomass yield factor (g biomass expressed as VSS g^{-1} CH_4-COD), X is the biomass concentration (g VSS L^{-1}) and K_S is the half-saturation constant (g CH_4-COD L^{-1}). However, in some cases the methanogenic biomass growth can be considered as negligible with respect to methane production. Thus, the Eq. [A1] can be reduced as follows:

$$\frac{dM}{dt} = V_{max} \cdot \frac{M_{max}-M}{K_S+M_{max}-M} \quad [A2]$$

where V_{max} is a constant describing the maximum methanogenic rate (g CH_4-COD L^{-1} d^{-1}) of an anaerobic sludge, and it encompasses the parameters μ_{max}, X and $Y_{X/S}$.

In an anaerobic reactor, methanogenesis can be mainly produced from acetate (acetoclastic methanogenesis) or from hydrogen and hydrogenocarbonate (hydrogenotrophic methanogenesis). These two processes occurred simultaneously during the digestion of the organic matter in an anaerobic environment. In an anaerobic reactor, acetoclastic methanogenesis is often predominant, producing around 70% of the total methane production. So, methane production can be separated in two terms by differentiating the acetoclastic and hydrogenotrophic contributions to methanogenesis, leading to:

$$\frac{dM}{dt} = \frac{dM_1}{dt} + \frac{dM_2}{dt} \quad [A3]$$

Where M_1 and M_2 are the methane production derived from acetoclastic and hydrogenotrophic processes, respectively (g CH_4-COD L^{-1}). Combining Eqs. [A2] and [A3] leads to:

$$\frac{dM}{dt} = V_{max_1} \cdot \frac{M_{max_1} - M_1}{K_{S_1} + M_{max_1} - M_1} + V_{max_2} \cdot \frac{M_{max_2} - M_2}{K_{S_2} + M_{max_2} - M_2} \qquad [A4]$$

Inhibition processes are commonly divided into 3 main types: competitive, non-competitive and uncompetitive. Competitive inhibitors affect the half-saturation constant, whereas non-competitive ones modify the maximum rate. Uncompetitive inhibition processes occur when both parameters are affected by the presence of the inhibitor. It can be reflected in the following equations, which are modifications from the Eq. [A2]:

$$\frac{dM}{dt} = V_{max} \cdot \frac{M_{max} - M}{\frac{K_S}{1 + \left(\frac{I}{K_{ci}}\right)^p} + M_{max} - M} \qquad [A5]$$

$$\frac{dM}{dt} = \frac{V_{max}}{1 + \left(\frac{I}{K_{ni}}\right)^m} \cdot \frac{M_{max} - M}{K_S + M_{max} - M} \qquad [A6]$$

$$\frac{dM}{dt} = \frac{V_{max}}{1 + \left(\frac{I}{K_{ni}}\right)^m} \cdot \frac{M_{max} - M}{\frac{K_S}{1 + \left(\frac{I}{K_{ci}}\right)^p} + M_{max} - M} \qquad [A7]$$

Where K_{ci} and K_{ni} are the competitive and non-competitive inhibition constants (mg inhibitor L^{-1}), and p and m are the competitive and non-competitive inhibition factors (dimensionless). Eq. [A7] represents the uncompetitive inhibition type. In some cases, the inhibition constants and factors for this kind of inhibition are considered to have the same values, thus Eq. [A7] can be resumed as follows:

$$\frac{dM}{dt} = V_{max} \cdot \frac{M_{max} - M}{K_S + (M_{max} - M) + (M_{max} - M) \cdot \left(\frac{I}{K_i}\right)^n} \qquad [A8]$$

, which represents, indeed, the Andrews model.

Inhibitors can affect not only the methanogenesis kinetic parameters, but also the methanogenic potential. This could be due to deactivation of part of the methanogenic biomass, or can be attributed to some inhibition processes affecting previous steps in the anaerobic digestion. In both

cases, this leads to a reduction of the maximum methane production. This type of inhibition has been named as irreversible or suicide inhibition and it could conduce to destabilization of the anaerobic sludge by the accumulation of some organic acids, as propionic, lactic or butyric, and can finally end in process failure. Using the IWA ADM-1 can serve to detect this type of inhibition, which would be reflected in the variation of the Monod parameters of the early anaerobic stages. However, this lengthy modeling approach can be simplified by just modifying the methanogenesis equation [A2] as follows:

$$\frac{dM}{dt} = V_{max} \cdot \frac{\frac{M_{max}}{1+\left(\frac{I}{K_{si}}\right)^n} - M}{K_S + \frac{M_{max}}{1+\left(\frac{I}{K_{si}}\right)^n} - M} \quad [A9]$$

Where I and K_{si} are the inhibitor concentration and the "suicide" inhibition constant, respectively (mg inhibitor L^{-1}), and n is the inhibition factor (dimensionless).

Also, it is well-known that the inhibitors take a time to affect every biochemical reaction working at non-steady state. This time is often referred as "lag-phase". Several attempts have been made for trying to modeling this lag-phase, almost all of them based on including a new timely independent variable which represents the time the toxic takes to make the inhibitory effect. However, this approach considerably complicates the modeling, and we think that it is not handy. Instead of this approach, we propose the separation of the methane production in two terms, one of them affected by the inhibitory effect and the other representing the methane production before the inhibitor enters in contact with the biomass. Then, Eq. [A2] can be modifying following this suggestion in this way:

$$\frac{dM}{dt} = V_{max} \cdot \frac{k \cdot (M_{max} - M) + (1-k) \cdot (M_{max} - M)}{K_S + k \cdot (M_{max} - M) + (1-k) \cdot (M_{max} - M)} \quad [A10]$$

Where k is an empirical factor ranging between 0 and 1, and it represents the methane production affected by the toxic effect of the inhibitor. So, (1-k) represents, indeed, the methane production before the toxic enters in contact with microorganisms. This approach avoids the use of a new

independent variable in modeling. Anyway, it is only useful when working at non-steady state using biomass not previously exposed to the toxicant. Once the toxicant has exerted its inhibitory effect, this modification could be rejected.

With an aim towards encompassing all of the inhibitory and non-steady state effects previously mentioned, a general model is proposed by combining the Eqs. [A4], [A5], [A6], [A7], [A9] and [A10], resulting in:

$$\frac{dM}{dt} = \frac{V_{max_1}}{1+\left(\frac{I}{K_{ni_1}}\right)^{m_1}} \cdot \frac{k_1 \cdot \left(\frac{M_{max_1}}{1+\left(\frac{I}{K_{si_1}}\right)^{n_1}} - M_1\right) + (1-k_1)\cdot\left(M_{max_1} - M_1\right)}{\frac{K_{S_1}}{1+\left(\frac{I}{K_{ci_1}}\right)^{p_1}} + k_1 \cdot \left(\frac{M_{max_1}}{1+\left(\frac{I}{K_{si_1}}\right)^{n_1}} - M_1\right) + (1-k_1)\cdot\left(M_{max_1} - M_1\right)}$$

$$+ \frac{V_{max_2}}{1+\left(\frac{I}{K_{ni_2}}\right)^{m_2}} \cdot \frac{k_2 \cdot \left(\frac{M_{max_2}}{1+\left(\frac{I}{K_{si_2}}\right)^{n_2}} - M_2\right) + (1-k_2)\cdot\left(M_{max_2} - M_2\right)}{\frac{K_{S_2}}{1+\left(\frac{I}{K_{ci_2}}\right)^{p_2}} + k_2 \cdot \left(\frac{M_{max_2}}{1+\left(\frac{I}{K_{si_2}}\right)^{n_2}} - M_2\right) + (1-k_2)\cdot\left(M_{max_2} - M_2\right)}$$

[A11]