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Effect of Para Substituted Anilines Chemical Structure on Methane Biosynthesis by the Methanogens

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Authors' contributions

This work was carried out in collaboration between all authors. Author KK designed the study and performed the experimental work. Author LB performed the statistical analysis. Author K. Kalala wrote the protocol; Author PTM wrote the first draft of the manuscript. Author CPS managed the analyses of the study and author AKK made the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The present paper aims to study the effect of aromatic structure on the inhibition of biogas production and more specifically the effect of para substituted anilines functional groups (chemical structure) on methane biosynthesis by the digested pig manure methanogens. The objective of this study was also to examine the structure-toxicity relationships of aromatic compounds to acetoclastic methanogens.
Study Design: Anaerobic digestion of pig manure, anaerobic toxicity essay, The effects of functional group nature on inhibition of methane production by acetoclastic methanogens. Correlation of the methanogenic toxicity (IC_{50}) with aromatic compounds hydrophobicity (logPoct).
Place and Duration of Study: Department of Chemistry, University of Kinshasa (DR Congo), between August 2011 and May 2012.
Methodology: The toxicity to acetoclastic methanogenic bacteria was performed with the standard method of serum bottles, digested pig manure was utilized as inoculums, acetate

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as substrate and the methane gas volume produced was measured by serum bottles liquid displacement systems (Mariotte flask system).
Results: The obtained results indicate that relationships exist between para substituted anilines functional groups nature (chemical structure) and their inhibitory effects on methanogens. The toxicity of para bisubstituted anilines increases in the following order:

$$SO_3 < OH < H < CH_3 < Cl < NO_2$$

From this sequence of increasing toxicity, it can be seen that the methanogenic toxicity varies with the functional group nature which is in the para position of the main function. Indeed, p-Nitroaniline and benzene with 45.76 and 208.78 mg/l as IC_{50} values respectively were the most toxic compounds, while p-Aminophenol and p-Aminosulfonic acid (Sulfanilic acid) with 1800.39 and 2777.82 mg/l IC_{50} values were the less toxic.
A very significant negative linear correlation between the toxicity of para substituted anilines compounds and their hydrophobicity was found.
Conclusion: The results of this study indicate that relationships exist between para substituted anilines functional groups nature and their inhibitory effects in methane biosynthesis by the methanogens.

Keywords: Para substituted anilines; methane biosynthesis; chemical structure; toxicity; methanogens; digested pig manure.

1. INTRODUCTION

Nitro-, azo- and amino-substituted aromatics play an important role in the production of explosives, dyes, pesticides, polymers, pharmaceuticals etc. and consequently, appear in the wastes generated by the corresponding industries. They are very dangerous for the environment owing to their mutagenic and carcinogenic influence on all the living organisms and some of them are quite persistent to aerobic biodegradation. Many N-Substituted aromatics have also been shown to be toxic or mutagenic to microorganisms. The presence of these aromatic xenobiotic in the environment may create public health and environmental problems [1-4].

Aromatic compounds are inhibitors of methane biosynthesis in anaerobic treatment of solid wastes and industrial effluents. Anaerobic treatment of solid wastes and industrial effluents may be limited by the methanogenic bacteria inhibition exerted by these types of compounds, the production of biogas is not possible and the organic matter contained in the effluent is not reduced. These effluents poured in the nature can be the basis of the pollution [3-5].

At our knowledge, few works are published on the methanogenic inhibition of aromatic compounds. Most of these works were performed with the granular sludge as inoculums from an industrial anaerobic reactor called "Upflow Anaerobic Sludge Bed" (UASB reactor). Generally, the digesters are heated to about 30ºC and more, but in this work digested pig manure from a laboratory scale digester was used as inoculum and the experiment was conducted at room temperature of an African tropical country (27±1ºC). Unlike granular sludge, digested pig manure is a natural inoculum and has never been in contact with aromatic compounds and thus, not acclimated to them.

Indeed, although the anaerobic biodegradability of aromatic compounds has been extensively studied, less attention has been given to the correlation of aromatic compounds

structure and their toxic effects on the community of anaerobic bacteria. The knowledge of the effect of aromatic structure on the inhibition of biogas biosynthesis is essential in predicting the impact of these xenobiotics on anaerobic waste and wastewater treatments, thereby preventing potentially costly upsets of treatment plant operations. A better understanding of the structure-toxicity relationships will make feasible the application of anaerobic technologies to waste and wastewater containing aromatic compounds [4,6].

Literature on anaerobic digestion shows considerable variation in the inhibition/toxicity levels reported for most substances. The major reason for these variations is the complexity of anaerobic digestion process where mechanism such as nature of inoculum and substrate, antagonism, synergism, acclimation and complexing could significantly affect the inhibition phenomenon [3,5].

The present paper aims to study the effect of aromatic structure on the inhibition of biogas biosynthesis and more specifically the effect of para substituted anilines functional groups nature on methane biosynthesis by the digested pig manure methanogens. The influence of the nature, the number and the position of substituents has to be investigated. The objective of this study is also to examine the structure-toxicity relationships of aromatic compounds to acetoclastic methanogens.

2. MATERIALS AND METHODS

2.1 Biomass

Pig manure from DAIPN farm of Nsele /KINSHASA (DR CONGO) was digested in laboratory scale digester during about six months. The digested pig manure (sludge) was utilized as inoculums in our anaerobic toxicity tests. The digested pig manure was not previously acclimated to any aromatic compounds.

Characteristic of inoculums: total suspended solids (TSS) concentration 91.10 g/l, volatile suspended (VSS) concentration 56.59 g/l, specific acetoclastic methanogenic activity 0.163 - 0.211 g COD-CH_4/g VSS .d. (27±1°C).

2.2 Stock Solutions

2.2.1 Stock substrate solution

The stock solution of the substrate is a volatile fatty acid solution (VFA) composed of acetic acid neutralized to pH = 7 with NaOH solution. It is at the concentration of 100 g COD-CH_3COOH /l (chemical oxygen demand per liter).

2.2.2 Stock solution 1

Macro-nutrients: NH_4Cl (170g/l); KH_2PO_4 (37 g/l); $CaCl_2$. $2H_2O$ (10 g/l); $MgSO_4.4H_2O$ (37 g/l).

2.2.3 Stock solution 2

Trace elements: $FeCl_3.4H_2O$ (2000mg/l); $CoCl_2.6H_2O$ (2000 mg/l); $MnCl_2.4H_2O$ (500 mg/l); $CuCl_2$ (50 mg/l); H_3BO_3 (50 mg/l); $(NH_4)6Mo7O_2.4H_2O$ (90 mg/l); Na_2SeO_3. 5 H_2O (100 mg/l);

$NiCl_2$. 6 H_2O (50 mg/l mg/l) ; EDTA (1000 mg/l) ; HCl 36% (1 mg/l) ; yeast extract(200 mg/l) resazurin (500 mg/l).

2.2.4 Stock solution 3

Sulfide Na_2S (100 g/l) [4,7,8].

2.3 Aromatic Compounds

The used of para substituted anilines included: p-Nitroaniline, p-Chloroaniline, p-Toluidine, p-Aminosulfonic acid (Sulfanilic acid), p-aminophenol and Aniline. Otherwise benzene was utilized as reference aromatic compounds. All aromatic compounds were of high purity available, pure for analysis supplied by MERCK. The chemical structures of these aromatic compounds are given in the Fig. 1.

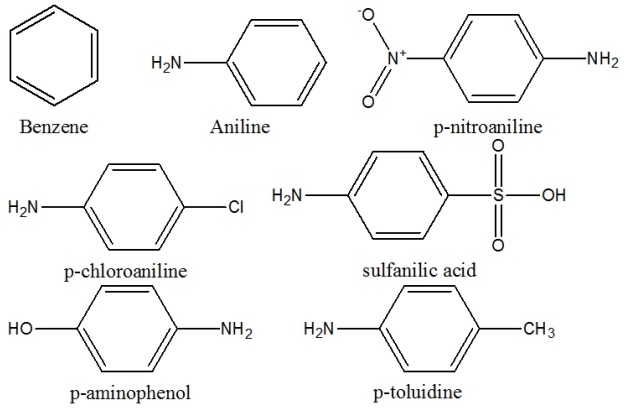

Fig. 1. The structures of para substituted anilines and reference aromatic compounds

2.4 Anaerobic toxicity assay

Specific acetoclastic methanogenic activity measurements were performed with 1l glass serum bottles sealed with butyl rubber septa. Add to each serum bottle from the scale laboratory digester 1.5 g VSS of digested pig manure and add to this:

- Two ml stock solution 1;
- 1 ml stock solution 2;
- 2 drops stock solution 3 ;
- 40 ml stock substrate solution.

Fill the serum bottle to about 1000 ml with oxygen free tap water which is flushed with nitrogen gas for at least 15 minutes [7-10]. The flask were sealed with rubber septum cap and placed in a reciprocating shaker at 27±1°C (room temperature).

The required quantity of inhibitory compound was added to each flask to provide the toxic concentration to be investigated. No toxicant was added to the controls. The toxicant concentrations had chosen as to cause an inhibition of the acetoclastic methanogenic activity ranging from 0-100 % [5,7,8].

The specific methanogenic activity was calculated from the slope of the cumulative methane production versus time curve and the quantity of VSS. The compound concentration that caused 50% inhibition of the methanogenic activity had referred to as “50% IC”. All specific methanogenic activity measurements were conducted in duplicate. To determine the degree of inhibition, the specific methanogenic activities of the control and samples containing inhibitory compounds were determined [5,9,10].

2.5 Methane Gas Measurement

The methane gas volume produced was measured by serum bottle liquid displacement systems (Mariotte flask system) as previously described [5,10,11].

The liquid in the displacement serum bottle should contain a concentrated solution of NaOH or KOH in order to rapidly convert CO_2 gas to carbonate and dissolve it into the NaOH solution [5].

3. RESULTS AND DISCUSSION

3.1 Inhibition of Specific Methanogenic Activity

All concentrations of aromatic compounds examined exerted an inhibitory effect on the specific acetoclastic methanogenic activity. This implies that these aromatic compounds are inhibitory to methane biosynthesis and some of them are toxics even in very small quantity.

Fig. 2 shows the decrease in specific methanogenic activity with the increasing of the concentration of Aniline. The IC_{50} is calculated as the concentration of Aniline corresponding to 50% of inhibition.

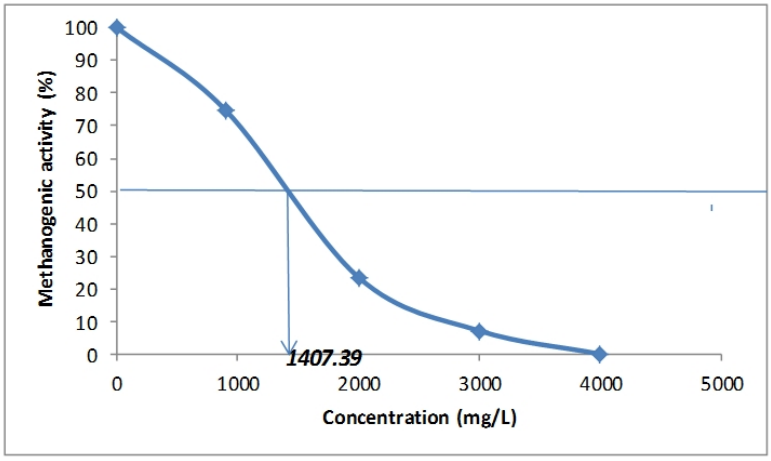

Fig. 2. Methanogenic activity of digested pig manure exposed to Aniline versus Aniline concentration

3.2 Toxicity of Para Substituted Anilines in Anaerobic Digestion Process

The inhibitory effect of para substituted anilines compounds: p-Nitroaniline, p-Chloroaniline, p-Toluidine, p-Aminosulfonic acid (Sulfanilic acid), Aniline, p-Aminophenol and reference compound (benzene) on the activity of acetoclastic methanogenic bacteria was studied at various levels, from concentrations that were nontoxic to those that were completely inhibitory concentration to acetoclastic methanogenic activity, as typified by the experiment with Aniline in Fig. 1. The Table 1 summarizes the 50% inhibiting concentrations (IC_{50}) of para substituted anilines compounds evaluated in this study, ranked in decreasing order of toxicity.

Table 1. The IC_{50} values observed in the study for the para substituted anilines compounds and reference aromatic

N°	Compound	IC_{50} (mg/l)	$logP_{oct}$
1	p-Nitroaniline	45.76±2.82	1.39
2	Benzene	208.78±6.32	2.13
3	p-Chloroaniline	787.47±20.43	1.87
4	p-Toluidine	1260.47±38.80	1.39
5	Aniline	1407.39±22.90	0.90
6	p-aminophenol	1800.39±3.39	0.40
7	Sulfanilic acid	2777.82±71.33	-0.512

The methanogenic toxicity exhibited by the para substituted anilines compounds is illustrated in Fig. 3.

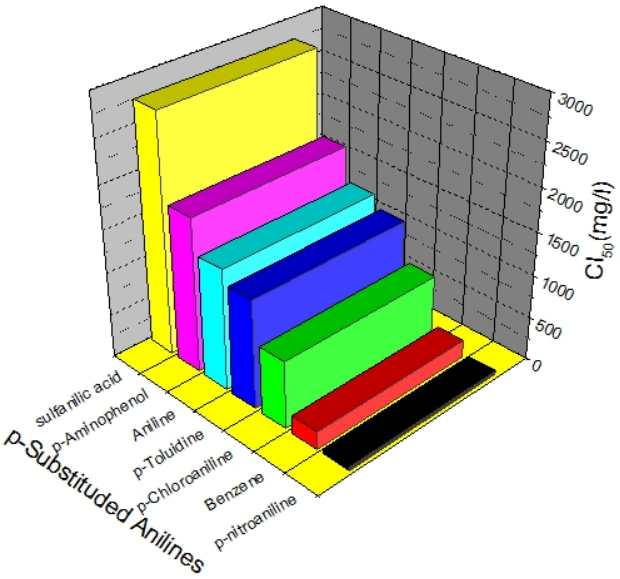

Fig. 3.The methanogenic toxicity as exhibited by para substituted anilines compounds

According to Field and Sierra [5], the IC_{50} values depend of substrate nature, type of inoculum, pH, experimental temperature, etc. Literature on anaerobic digestion shows considerable variation in the inhibition/toxicity levels reported for most substances. The

major reason for these variations is the complexity of anaerobic digestion process where mechanism such as nature of inoculum and substrate, antagonism, synergism, acclimation and complexing could significantly affect the inhibition phenomenon [3,5].

The obtained results indicate that relationships exist between the nature of para substituted anilines compounds and their inhibitory effects on methanogenic bacteria. According to the Fig. 2, the toxicity of para substituted anilines compounds increase in the following order:

p-Aminosulfonic < p-Aminophenol < Aniline < p-Toluidine < p-Chloroaniline < benzene <p-Nitroaniline

In this sequence of toxicity, p-Nitroaniline and benzene with 45.76 and 208.78 mg/l as IC_{50} values respectively are the most toxic compounds, while p-Aminophenol and p-Aminosulfonic acid (Sulfanilic acid) with 1800.39 and 2777.82 mg/l IC_{50} values are the less toxic. In addition, it can be seen in this sequence of toxicity as p-nitroaniline is the only aniline derivative which is more toxic than benzene. This can be explained by the fact that the nitro group is a powerful electron-withdrawing therefore very hydrophilic and nitroaniline have the highest dipole moment of the tested compounds, making him the most chemically reactive compound. Indeed, the nitroaromatics have been reported to be reactive toxicants and when present at similar concentration in the bacterial membrane exert a much higher toxic effect than that which can be accounted for by membrane toxicity alone. The reactivity with microbial subcellular components realize the p-nitroaniline highly toxic [3,6,12] Thus, the substitution of a nitro group ($-NO_2$) on the Aniline giving the p-Nitroaniline, has the effect of increasing the toxicity of the aromatic ring.

The substitution of hydrophilic substituent, as NH_2, OH, SO_3, on the benzene ring, make the obtained compound less toxic. In addition, this decrease in toxicity is also a function of the number of hydrophilic groups on aromatic ring [4,13].

However, this behavior is valid only when the two substituents have no electronic and steric interactions and when there is not the formation of intramolecular hydrogen bonds. This is possible, when the two substituents are in the para position relative to each other. Indeed, when the substituents are in ortho or meta position, interactions change the order of toxicity in one direction or another. In this case, resorcinol (meta isomer) is more toxic than hydroquinone (para isomer). This phenomenon can be interpreted by the fact that the toxicity of isomers varies with the position of functional groups that result in steric and electronic interactions, and also with the formation of intramolecular hydrogen bonds [4,13,14].

3.3 Effect of the Para Substituted Anilines Functional Groups on the Methanogenic Toxicity

The influence of the functional groups nature on the methanogenic toxicity exhibited by the para substituted anilines compounds is illustrated in Fig. 4.

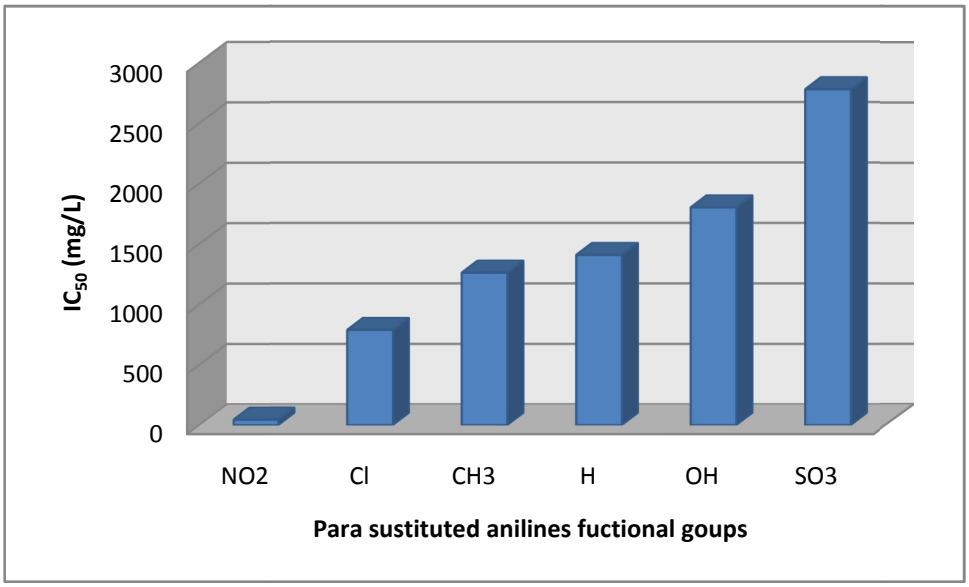

Fig. 4. Variation of the methanogenic toxicity as a function of the para-substituted Anilines functional groups nature

The results obtained indicate that relationships exist between para substituted anilines compounds functional groups (chemical structure) and their inhibitory effects on methanogenic bacteria. According to the Fig. 4, the toxicity of para bisubstituted anilines increases in the following order:

$$SO_3 < OH < H < CH_3 < Cl < NO_2$$

The nature of aromatic functional groups is observed to have a profound effect on the toxicity of the para bisubstituted anilines. From this sequence of increasing toxicity, it can be seen that the methanogenic toxicity varies with the substituents nature which is in the para position of the main function (NH_2). With some exceptions, the obtained results are comparable to those reported in our previous works [7-10,14-16]; by Donlon et al. [4] and by Sierra and Lettinga [17] for monosubstituted aromatic compounds as far as acetate was used in the bioassay. The addition of a functional group containing an oxygen or sulfur heteroatom to aniline, our reference compound, decreased the para substituted aromatic compound toxicity as in the case of OH and SO_3 substitution. However, the addition of CH_3, Cl and NO_2 to aniline was associated with an increase in a compound toxicity. Indeed, the results obtained with digested pig manure are in complete agreement with the above theory, which is not the case of the granular sludge.

This implies that the substitution of hydrophobic or hydrophilic substituent on the benzene or monofunctional aromatic compound, make the obtained compound more or less toxic. In fact, it is known that a substitution on the aromatic ring that enhances the hydrophobicity render the molecule more toxic and that enhances the hydrophobicity of aromatic ring causes the molecule to be less toxic. According to Hansch and Leo works [13]:

- hydrocarbon or halogenated substituents on the benzene ring are lipophilic in the case of CH_3, F, Cl, Br, I .
- The substituents containing electronegative atoms such as O and N are generally hydrophilic (OH, SO_3, SH, NH_2, CHO, COOH, $CONH_2$, $OCOCH_3$).

However, this behavior is only valid when the two substituents have no electronic and steric interactions and no intramolecular hydrogen bonds formation. This is possible, when the two substituents are in the para position relative to each other. Indeed, when the substituents are ortho or meta, steric and electronic interactions change the order of toxicity in one direction or an another.

In our previous work [7] the general relationships between the aromatic functional groups nature and their inhibitory effects on methanogenic bacteria for the para-tolyles and para-bisubstituted (thio) phenols increases, respectively, in the following order:

$$NH_2 < OH < CHO < H \quad \text{and} \quad OH < NH_2 < H < CH_3O < CH_3 < Cl^* < CH_3^* < NO_2$$

The star (*) indicates the thiophenols functional groups.

3.4 Correlation of the Methanogenic Toxicity with Aromatic Compounds Hydrophobicity

Correlations between toxicity and partition coefficient within series of organic contaminants structurally related have been reported by a number of research groups using fish, ciliate or microorganisms as tests organisms. Therefore, when comparing compounds that possess different types of substitutions, a perfect correlation with $\log P_{oct}$ of the compound cannot be expected. A higher correlation could potentially be obtained by comparing compounds in homologues series [4,17].

To determine if the lipophilic character of para substituted anilines compounds and reference aromatic compounds (benzene) tested could be correlated with their methanogenic toxicity, the logarithm of the IC_{50} values of these aromatics were plotted against the logarithm of the octanol-water partition coefficient ($\log P_{oct}$) of the aromatic compounds. The p –Nitroaniline was not included in $\log P_{oct}$ correlation because the nitro-aromatics when present at similar concentration in the bacterial membrane exert a much higher toxic effect than that which can be accounted for by membrane toxicity alone [3,4].

Fig. 5 shows the correlation line between the methanogenic toxicity and partition coefficient $\log P_{oct}$ for para substituted anilines compounds.

It can be noticed that there is a significant correlation between the toxicity of these aromatic compounds and their hydrophobicity ($R^2 = 0.9676$). This causes that the more hydrophobic molecule is, the more readily it crosses the cell membrane and becomes highly toxic and inversely [4,18-20].

Hydrophobicity of a compound as indicated by $\log P_{oct}$ has directly related to the partition of a compound into bacterial membrane. Compounds of great hydrophobicity are expected to accumulate more efficiently in membranes, causing a greater disturbance to the membrane structure and consequently, they would be responsible of high toxicity [6,17].

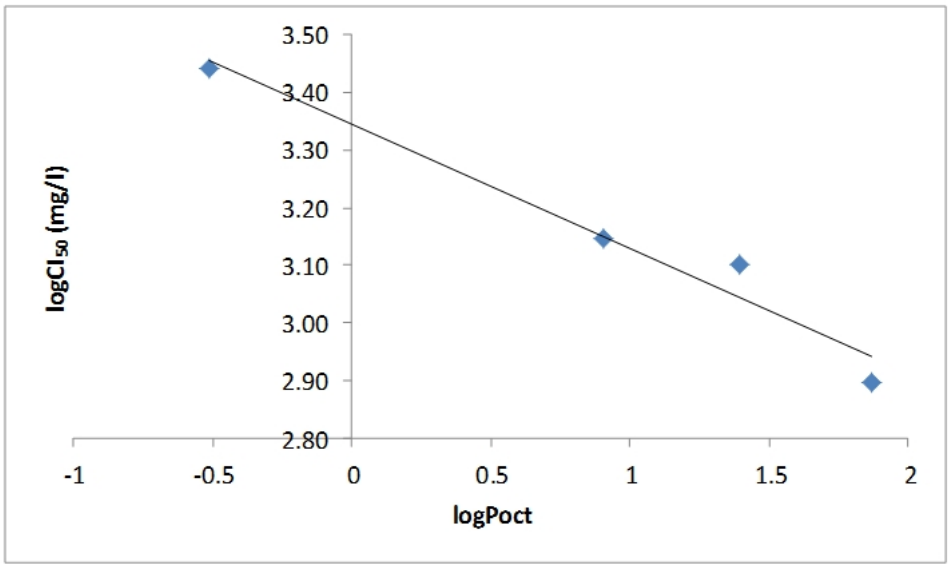

Fig. 5. Effect of hydrophobicity on methanogenic toxicity: methanogenic toxicity (IC_{50}) and partition coefficient (logPoct) correlation ($R^{2 =}$ 0.9676)

The diffusion of a molecule across a membrane depends on the permeability of the membrane. However, the membrane permeability is a function of the partition coefficient $logP_{oct.}$ So the more hydrophobic a molecule is, the higher is its membrane permeability and the greater is its toxicity [10,18].

Indeed, a substitution on the aromatic ring which tends to make the molecule lipophilic (hydrophobic) increases the affinity for membrane phase therefore the permeability of the membrane to this compound. The massive compound diffusion in methanogenic bacteria thus increases the toxicity for these microorganisms causing damage to subcellular components. This contributes to the decrease in methanogenic activity [4,10,17].

4. CONCLUSION

The results obtained indicate that relationships exist between the para substituted anilines compounds functional groups (chemical structure) and their inhibitory effects on methanogenic bacteria. According to the Fig. 4, the toxicity of para bisubstituted anilines increases in the following order:

$$SO_3 < OH < H < CH_3 < Cl < NO_2$$

The addition of a functional group containing an oxygen or sulfur heteroatom to aniline, our reference compound, decreased the para substituted aromatic compound toxicity as in the case of OH and SO_3 substitution. However, the addition of CH_3, Cl and NO_2 to aniline was associated with an increase in a compound toxicity.

A very significant negative linear correlation between the toxicity of para substituted anilines compounds with the reference aromatic compound (benzene) and their hydrophobicity was found.

This causes that the more hydrophobic molecule is, the more readily it crosses the cell membrane and becomes highly toxic and inversely.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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