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## **Comparative Study of Benzenediols, Methylphenols and Chloroanilines Isomers on the Inhibition of Acetoclastic Methanogenesis By Digested Pig Manure Methanogenic Archaea**

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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 PCS wrote the protocol; author PTM wrote the first draft of the manuscript, managed the analyses of the study, author KM made the literature searches. All authors read and approved the final manuscript.*

**Research Article**

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### **ABSTRACT**

**Aims:** The present work aims to study the effect of aromatic structure on the inhibition of biogas biosynthesis, and more specifically the comparative study of benzenediols, methylphenols and chloroanilines isomers on the inhibition of acetoclastic methanogenesis by digested pig manure methanogenic archaea. The objective of this study was also to examine the structure-toxicity relationships of aromatic compounds to acetoclastic methanogens. The ultimate goal is the modeling of the influence of the aromatic structure on the inhibition of methane biosynthesis.

**Study Design:** Anaerobic digestion of pig manure, anaerobic toxicity essay, Effect of the isomerism (functional groups position) on the methanogenic toxicity, Correlation of the methanogenic toxicity with aromatic compounds hydrophobicity (logP<sub>oct</sub>),

**Place and Duration of Study:** Department of Chemistry, University of Kinshasa, DR Congo, between January 2011 and March 2012.

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**Methodology:** The toxicity to acetoclastic methanogenic archaea was performed with the standard method of serum bottles, digested pig manure was utilized as inoculums and acetate as substrate. 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 the isomerism of cresols, benzenediols (catechol, resorcinol, and hydroquinone) and chloroanilines; and their inhibitory effects on methanogenic archaea. The toxicity of Cresols, benzenediols and reference compounds increases, respectively, in the following order: Benzenediols < Phenol < Cresols. The grafting of methyl (-CH<sub>3</sub>) at phenol to form cresols, make cresols to be more toxic than phenol. Secondly, by adding a hydroxyl group (-OH) at phenol to form benzenediols, these compounds become less toxic than phenol. The grafting of chlorine (-Cl) at aniline to form chloroanilines, make chloroanilines to be more toxic than aniline. And secondly, by adding an amino group (-NH<sub>2</sub>) at chlorobenzene to form chloroanilines, these compounds become less toxic than Chlorobenzene. For the three isomers studied in this work, ortho is the most toxic followed by meta while para is always the less toxic.

A high significant linear correlation between the toxicity of cresol, benzenediols and chloroanilines isomers and reference aromatic compounds and their hydrophobicity ( $R^2 = 0.9278$ ) was found.

**Conclusion:** The results obtained in this paper indicate that it exist a correlation between the isomerism (chemical structure) of Cresols, benzenediols, chloroanilines and their inhibitory effects on methanogens. Hydrophobicity of a compound as indicated by logP<sub>oct</sub> is directly related to the partition of a compound into archaeal membrane.

**Keywords:** Cresols; benzenediols; chloroamines; acetoclastic methanogenesis; toxicity; biosynthesis; methanogenic archaea; methane.

## 1. INTRODUCTION

Experimental studies have shown that solid waste and wastewater, especially industrial effluents may contain compounds that are toxic to methanogenic bacteria. These compounds are: oxidizing agents, long chain fatty acids, antibiotics, ammonia, detergents, metal ions and especially the aromatic compounds. Anaerobic treatment of effluents may be limited by the methanogenic inhibition exerted by these types of compounds, not only the biogas production is not possible but the organic matter contained in the effluent is not reduced. In the nature, these effluents can be conducted to the pollution [3,4,5,6]. Aromatic compounds are naturally present in the environment as degradation products of lignin, tannins, phenolic amino acids, pigments and other aromatic compounds from plants. Human activity also contributes to the presence of aromatic compounds in the environment: waste incineration, petrochemical effluents, industries of paper manufacturing, pharmaceutical and chemical industries, pesticides etc. are very important sources of aromatic pollution [7,12]. Monomeric tannin compounds (benzenediols) are naturally present in the environment as degradation products of tannin [9]. The cresols are protein-bound uremic retention solutes that have become a frequent subject of both *in vivo* and *in vitro* research. The phenols, as derived from the amino acid tyrosine, are chemically characterized by an aromatic ring structure to which at least one hydroxyl group is linked. The *p*-Cresol is a phenol derived from the tyrosine carrying one methyl group in the para position. Both phenol and *p*-cresol are precursors of uremic retention compounds and are generated by the intestinal flora. Although, environmental factors, herbal substances and alternative or traditional medicines might be additional sources [1,2]. 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. The presence of aromatic xenobiotic in the environment may create serious public health and environmental problems. Some aromatic compounds are mutagenic or carcinogenic and some may bioaccumulate. Additionally, synthetic aromatic compounds are often resistant to biodegradation and toxic to microorganisms [9,10,12].

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 30°C 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±1°C). 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. But, there almost no literature data concerning the methanogenic inhibition of aromatic compounds determined with digested pig manure as inoculum.

Traditionally, biological treatment of aromatic containing wastewaters was based on aerobic processes. Recently, anaerobic processes are being used more often; however their application is restricted by the limited data available on the behavior of aromatic compounds in anaerobic treatment systems. 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 archaea. 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 [8,7,10].

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 antagonism, synergism, acclimation, nature of inoculum and complexing could significantly affect the inhibition phenomenon [3,4,5].

The present work aims to study the effect of aromatic structure on the inhibition of biogas biosynthesis and more specifically the comparative study of benzenediols, methylphenols and chloroanilines isomers on the inhibition of acetoclastic methanogenesis by digested pig manure methanogenic archaea. The influence of the nature, the number and the position of substituents has to be investigated. The objective of this study was also to examine the structure-toxicity relationships of aromatic compounds to acetoclastic methanogens. The ultimate goal of the work is the modeling of the influence of the aromatic structure on the inhibition of methane biosynthesis.

## 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 163.40 -210.81 mg COD-CH<sub>4</sub>/g VSS .d (27±1°C).

### 2.2 Stock Solutions

#### 2.2.1 Stock substrate solution

The stock solution of the substrate is composed of acetic acid neutralized to pH = 7 with NaOH solution. It is at the concentration of 100 g COD-CH<sub>3</sub>COOH /l (chemical oxygen demand per liter) = 1,562.5 mM of acetic acid.

#### 2.2.2 Stock solution 1

Macro-nutrients: NH<sub>4</sub>Cl (170 g/l); KH<sub>2</sub>PO<sub>4</sub> (37g/l); CaCl<sub>2</sub>. 2H<sub>2</sub>O (10g/l); MgSO<sub>4</sub>.4H<sub>2</sub>O (37g/l);

#### 2.2.3 Stock solution 2

trace elements : FeCl<sub>3</sub>.4H<sub>2</sub>O (2000mg/l); CoCl<sub>2</sub>.6H<sub>2</sub>O (2000 mg/l); MnCl<sub>2</sub>.4H<sub>2</sub>O (500 mg/l); CuCl<sub>2</sub> (50 mg/l); H<sub>3</sub>BO<sub>3</sub> (50 mg/l); (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O (90 mg/l); Na<sub>2</sub>SeO<sub>3</sub>. 5 H<sub>2</sub>O (100 mg/l); NiCl<sub>2</sub>. 6 H<sub>2</sub>O (50 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<sub>2</sub>S (100 g/l) [12,13,14].

### 2.3 Aromatic Compounds

The isomers of benzenediols, cresols (methylphenols) and chloroaniline were all used: ortho cresol, meta cresol, para cresol; ortho benzenediol (catechol), meta benzenediol (resorcinol), para benenediol (hydroquinone), ortho chloroaniline, meta chloroaniline and para chloroaniline. Otherwise, phenol, toluene, chlorobenzene, aniline, and benzene were used as reference aromatic compounds. . In addition, in order to have enough data that can permit to study the structure-toxicity correlation (correlation of the methanogenic toxicity with aromatic compounds hydrophobicity), the following aromatic compounds have also been investigated: m-dichlorobenzene, p-tolualdehyde, p-methoxyphenol and vanillin. All aromatic compounds were pure for analysis products supplied by MERCK.

### 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[12-14].The flask were sealed with rubber septum cap and placed in a reciprocating shaker at  $27\pm 1^{\circ}\text{C}$  (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 % [7,12]. The concentrations of inhibitors used in the anaerobic toxicity assay are given in the Table 1.

**Table 1. The inhibitory concentrations of aromatic compounds used in anaerobic toxicity assay**

N°	Compounds	Concentrations (mg/l)					
		1	2	3	4	5	6
1	Toluene	0	80	200	300	400	600
2	Benzene	0	150	300	450	600	750
3	o-Cresol	0	400	1,200	1,800	2,500	3,500
4	m-Cresol	0	400	1,200	1,800	2,500	3,500
5	p-Cresol	0	400	1,200	1,800	2,500	3,500
6	Phenol	0	500	1,220	2,500	3,500	4,500
7	Catechol	0	1,000	3,000	5,000	8,000	10,000
8	Resorcinol	0	1,000	3,000	5,000	8,000	10,000
9	hydroquinone	0	1,000	3,000	5,000	8,000	10,000
10	Chlorobenzene	0	15	50	100	150	200
11	o-Chloroaniline	0	15	30	60	90	120
12	m-Chloroaniline	0	300	700	1,500	2,000	2,500
13	p-Chloroaniline	0	500	1,500	2,000	2,500	3,000
14	Aniline	0	1,000	2,000	3,000	4,000	4,500

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 [11,12,14].

## 2.5 Methane Gas Measurement

The methane volume produced was measured by serum bottle liquid displacement systems (Mariotte flask system) as previously described [12,13,14].

The liquid in the displacement serum bottle should contain a concentrated solution of NaOH or KOH in order to rapidly convert CO<sub>2</sub> to carbonate and dissolve it into the NaOH solution [12].

### 3. RESULTS AND DISCUSSION

#### 3.1 Inhibition of Specific Methanogenic Activity

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

Fig. 1 shows the decrease in specific methanogenic activity with increasing the concentration of p-Cresol and the IC<sub>50</sub> values determination.

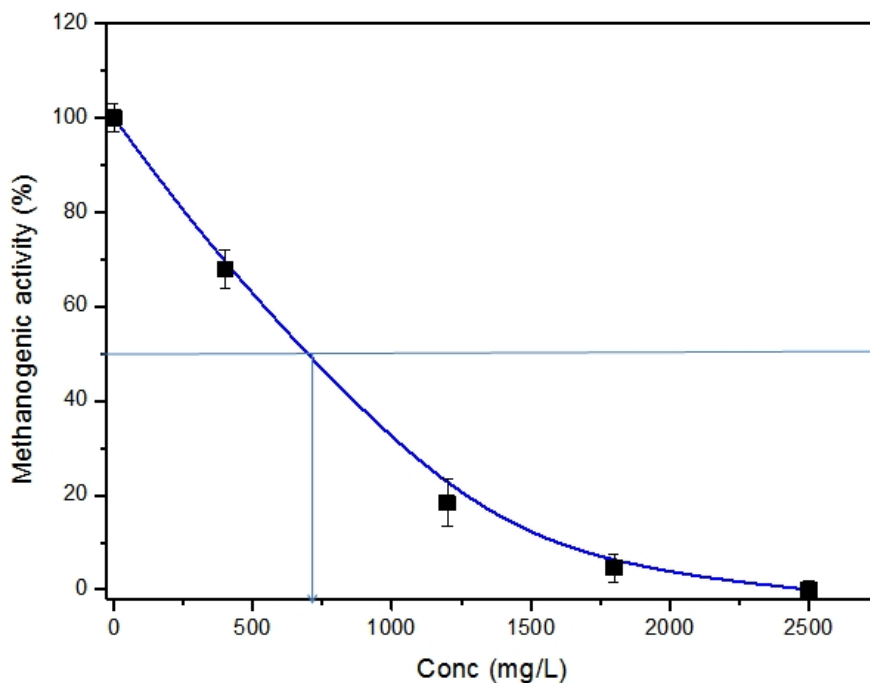


Fig. 1. Methanogenic activity of digested pig manure exposed to p-Cresol as a function of the p-Cresol concentration and IC<sub>50</sub> determination

#### 3.2 Effect of the Isomerism (Functional Groups Position) on the Methanogenic Toxicity

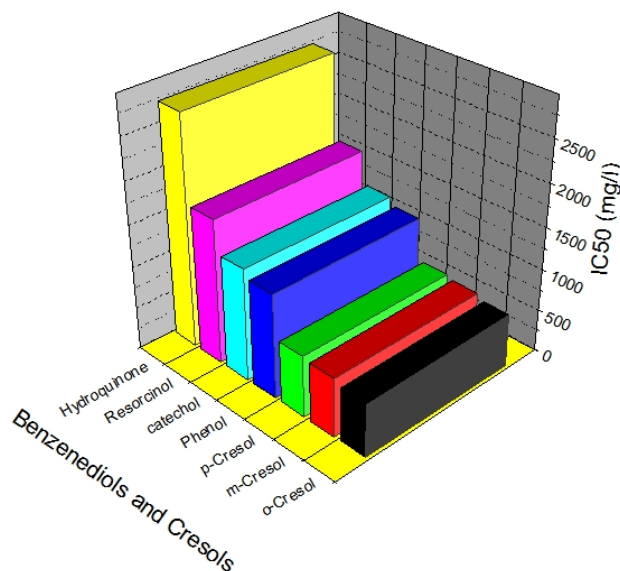
The inhibitory effects of the benzenediols, cresols and chloroanilines on the activity of methanogens were evaluated. The inhibition caused by each compound was tested at various levels, from concentrations that were nontoxic to those that were completely inhibitory to acetoclastic methanogenic activity, as seen in a typical experiment with phenol

in Fig. 1. Table 2 summarizes the 50% inhibiting concentrations (IC<sub>50</sub>) of the aromatic compounds evaluated in this study, ranked in decreasing order of toxicity.

**Table 2. The IC<sub>50</sub> values observed in the study for the cresols, benzediols, chloroanilines and reference aromatics**

Nº	Compounds	IC <sub>50</sub> (mg/l)	logPoct
1	Chlorobenzene	30.08±2.01	2.84
2	o-Chloroaniline	51.00 ±3.26	1.90
3	m-Dichlobenzene	90.13±6.52	3.53
4	Toluene	146.65±6.28	2.73
5	Benzene	208.78±6.32	2.13
6	p-Tolualdehyde	503.95±15.95	2.26
7	m-Chloroaniline	539.78±6.32	1.90
8	o-Cresol	636.14±2.97	1.98
9	m-Cresol	696.06±8.17	1.98
10	p-Cresol	737.26±12.31	1.96
11	p-Methoxyphenol	761.27±8.71	1.58
12	p-Chloroaniline	787.47±38.80	1.87
13	Vanillin	871.09±29.22	1.37
14	Phenol	1248.90±29.59	1.47
15	Aniline	1407.39±22.91	0.90
16	Catechol	1436.38±20.11	0.88
17	Resorcinol	1725.04±5.27	0.80
18	hydroquinone	2744.97±97.80	0.59

The influence of the isomerism on the methanogenic toxicity exhibited by benzenediols and cresols and reference compound are illustrated in Fig. 2.



**Fig. 2. Variation of the methanogenic toxicity as a function of isomerism of benzenediols and cresols**

The isomers of aromatic compounds can give information on the influence of the substituents position on the methanogenic toxicity. Indeed; aromatic isomers have two identical substituents but differ only by their positions on the aromatic ring. The obtained results indicate that relationships exist between the isomerism of Cresols and benzediols (catechol, resorcinol, and hydroquinone); and their inhibitory effects on methanogens. According to the Fig. 2, the toxicity of Cresols, benzediols and reference compound increases, respectively, in the following order:

Benediols < Phenol < Cresols

The grafting of methyl (-CH<sub>3</sub>) at phenol to form bifunctional compounds that are cresols, make cresols to be more toxic than phenol. Secondly, by adding a hydroxyl group (-OH) at phenol to form benzenediols, these compounds become less toxic than phenol. The grafting of chlorine (-Cl) at aniline to form bifunctional compounds that are chloroanilines, make chloroanilines to be more toxic than aniline. And secondly, by adding an amino group (-NH<sub>2</sub>) at chlorobenzene to form chloroanilines, these compounds become less toxic than Chlorobenzene. It can be noticed that o-Chloroaniline is more toxic than benzene.

With some exception, the results obtained in this work are compared quite well with those reported in our previous works [18,19] and by SIERRA and LETTINGA, 1989 [7] for aromatic compounds, as far as acetate was the substrate used in the bioassay. The addition of a functional group containing an oxygen, sulfur and nitrogen heteroatom to aromatic ring decreased the aromatic compounds toxicity as the case of -OH and -NH<sub>2</sub> substitution. However, the addition of -CH<sub>3</sub> and Cl to aromatic ring was associated with an increase in compound toxicity.

This demonstrates that the grafting of hydrophobic or hydrophilic substituent on the aromatic ring, make the obtained compound more or less toxic as the case and that in the same order of toxicity [16]. However, this behavior is only valid when the two substituents have no electronic and steric interactions and 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 ortho or meta, interactions change the order of toxicity in one direction or another. In fact, the formation of intramolecular hydrogen bonds and steric hindrance are often observed when the substituents are in the ortho position. In the first case there is an increase in the hydrophobicity of the isomer therefore its toxicity, while in the second case there is an increase of hydrophilicity, therefore the reduction of the isomer toxicity.

In fact, according to the results reported in Figs. 3, 4 and 5, the toxicity of various positional isomers of Cresol, benzenediol, and chloroaniline are decreasing, respectively, in the following order:

Ortho > Meta > Para

For the three isomers studied in this work, ortho is the most toxic followed by meta while para is always the less toxic.

In Fig. 5, it can be observed that the various isomers of chloroaniline have toxicities which are very different from 51.00, 539.78 and 787.47 mg / l respectively for the ortho, meta and para-Chloroaniline. One can notice that the o-Chloroaniline IC<sub>50</sub> value is 10.43 and 15.44 times more toxic than meta and para chloroaniline respectively. Contrary to all predictions, it



is more toxic than benzene. This is the consequence of the introduction of the chlorine atom (-Cl), electron-withdrawing (inductive), in ortho position to the amino group (-NH<sub>2</sub>) of aniline and the possibility of forming a very strong intramolecular hydrogen bond, significantly reduces the "hydrogen bond basicity (HHB)" of aniline. The aromatic compound so formed (o-chloroaniline) becomes very toxic (lipophilic) than predicated by his aromatic structure.

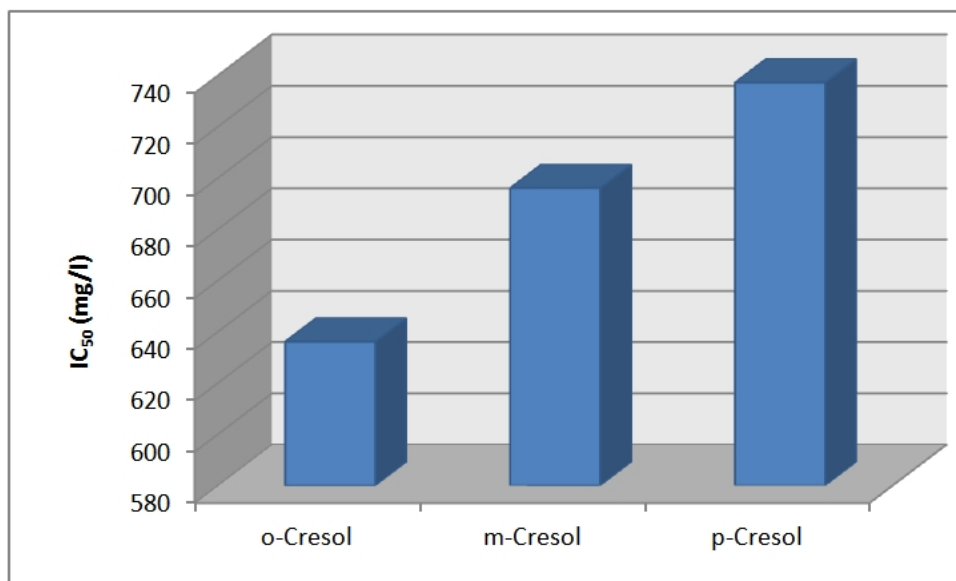


Fig. 3. Influence of functional groups position on the methanogenic toxicity of cresols

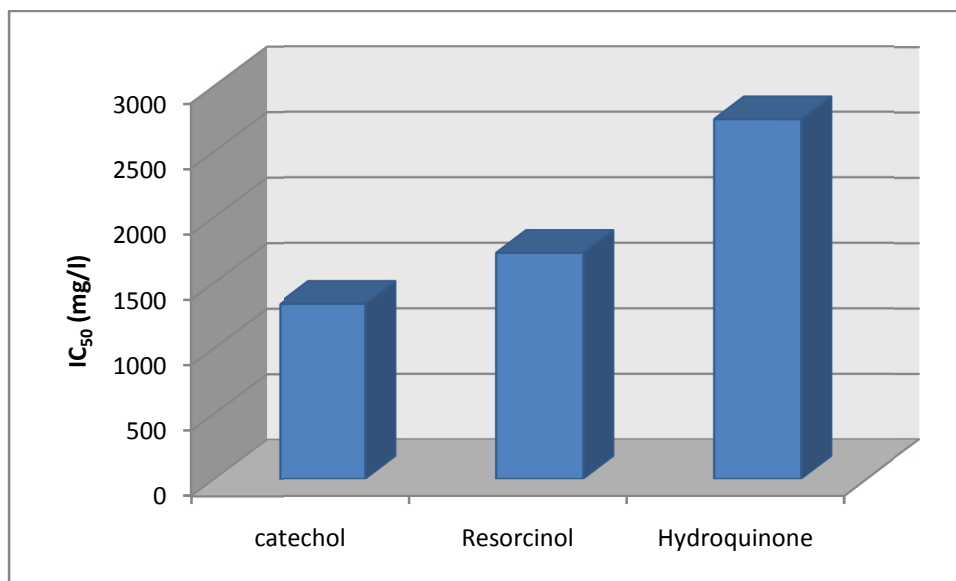
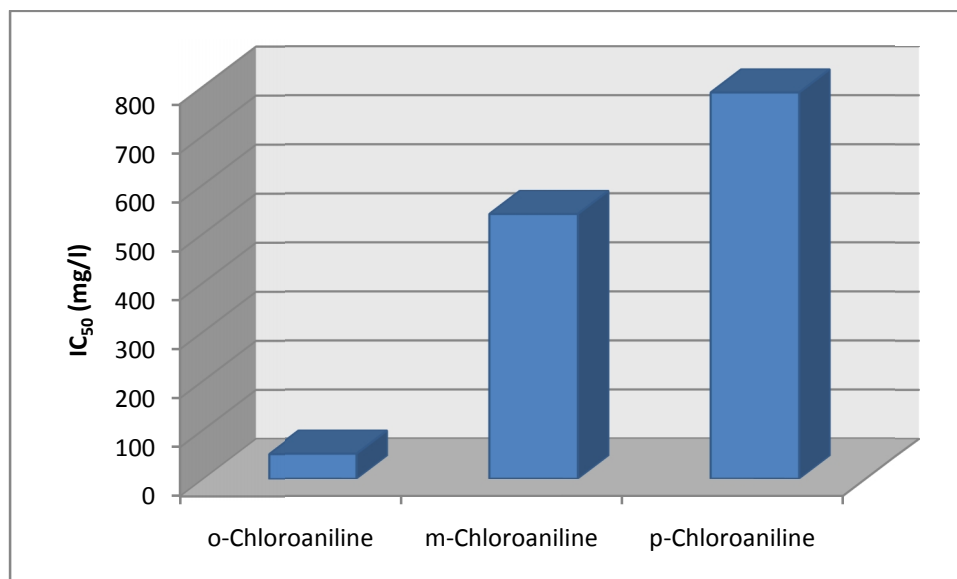


Fig. 4. Influence of functional groups position on the methanogenic toxicity of benzenediols



**Fig. 5. Influence of functional groups position on the methanogenic toxicity of chloroanilines**

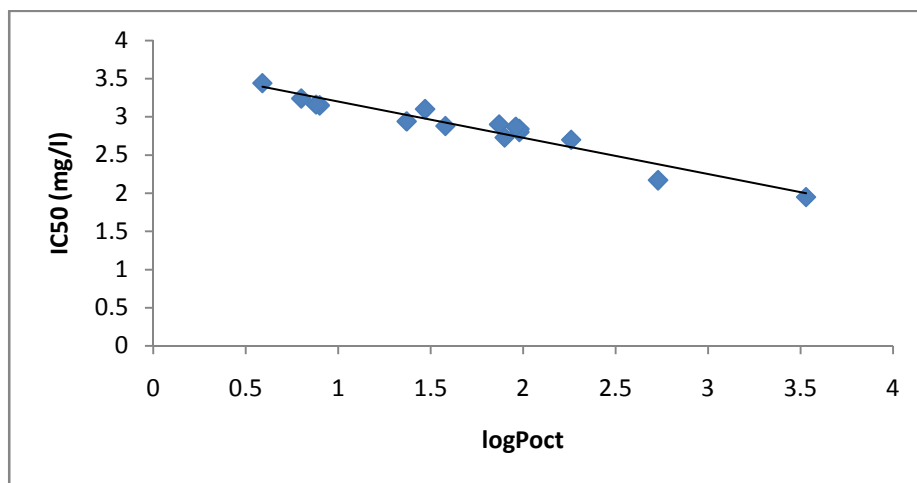
This phenomenon can be interpreted by the fact that the toxicity of isomers varies with the position of functional groups that result in steric and electronic interactions, and with the formation of intramolecular hydrogen bonds [16].

Indeed, despite the fact that the cresols, benzenediols and chloroanilines have identical substituents and the same molecular weights, they have different IC<sub>50</sub> and logP<sub>oct</sub>. This implies that the isomerism (chemical structure) has an influence on the physico-chemical properties of aromatic compounds, as it is the case of cresols, benzenediols and chloroanilines.

### 3.3 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 logP<sub>oct</sub> of the compound cannot be expected. A higher correlation could potentially be obtained by comparing compounds in homologue series [7,10,15,18,19].

To determine if the lipophilic character of cresols, benzenediols, chloroanilines isomers and others aromatic compounds tested could be correlated with their methanogenic toxicity, the logarithm of the IC<sub>50</sub> values of these aromatics were plotted against the logarithm of the octanol-water partition coefficient (logP<sub>oct</sub>) of the aromatic compounds. The benzene, chlorobenzene and o-chloroaniline were not included in logP<sub>oct</sub> correlation because they are not structurally related or homologous to the others studied aromatics compounds. Fig. 6 shows the correlation line between the methanogenic toxicity (IC<sub>50</sub>) and partition coefficient (logP<sub>oct</sub>) for the studied aromatic compounds.



**Fig. 6. Effect of hydrophobicity on methanogenic toxicity: methanogenic toxicity (IC<sub>50</sub>) and partition coefficient (logPoct) correlation (R<sup>2</sup> = 0.9278)**

It can be noticed that there is a significant linear correlation between the toxicity of these aromatic compounds and their hydrophobicity (R<sup>2</sup> = 0.9278). The correlation line between the methanogenic toxicity (IC<sub>50</sub>) and partition coefficient (logPoct) is represented by the following equation:  $\log IC_{50} = 0.4744 \log P_{oct} + 3.6739$ .

This equation indicates that the IC<sub>50</sub> is a function of logPoct and it can be used to roughly estimate the IC<sub>50</sub> values of aromatic compounds homologous to the studied compounds such as phenol derivatives. This causes that the more hydrophobic molecule is, the more it readily crosses the cell membrane and becomes highly toxic and inversely [16,17]. Hydrophobicity of a compound as indicated by logPoct is directly related to the partition of a compound into microbial membranes. 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 for high toxicity [10,20].

In fact, it is known that a substitution on the aromatic ring that enhances the hydrophobicity renders the molecule more toxic and that enhances the hydrophobicity of an aromatic ring causes the molecule to be less toxic. 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 logPoct. So the more hydrophobic a molecule is, the higher is its membrane permeability and the greater is its toxicity [15,17].

Indeed, a substitution on the aromatic ring which tends to make the molecule lipophilic (hydrophobic) increases the affinity for the membrane phase, therefore the permeability of the membrane to this compound. The massive compound diffusion in methanogenic archaea thus increases the toxicity for these microorganisms, causing damage to subcellular components. This contributes to the decrease in methanogenic activity [15].

#### 4. CONCLUSION

The obtained results indicate that relationships exist between the isomerism of Cresols, benzenediols (catechol, resorcinol, and hydroquinone) and chloroanilines; and their inhibitory

effects on methanogens. The toxicity of Cresols, benzenediols and reference compounds increases, respectively, in the following order: Benzenediols < Phenol < Cresols.

The grafting of methyl (-CH<sub>3</sub>) at phenol to form bifunctional compounds that are cresols, make cresols to be more toxic than phenol. Secondly, by adding a hydroxyl group (-OH) at phenol to form benzenediols, these compounds become less toxic than phenol. A high negative linear correlation between the toxicity of cresol isomers and reference aromatic compounds and their hydrophobicity ( $R^2 = 0.9278$ ) was found. Hydrophobicity of a compound as indicated by logP<sub>oct</sub> is directly related to the partition of a compound into archaeal membrane.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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