

STUDY OF THE TOXICITY OF PARA SUBSTITUTED ANILINES ON METHANE GAS BIOSYNTHESIS BY METHANOGENIC BACTERIA

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ABSTRACT

Aims: To determine the toxicity of para substituted anilines in anaerobic digestion process and evaluate the effects of the nature of substituents on inhibition of methane production by acetoclastic methanogenic bacteria.

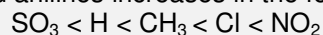
Study design: Anaerobic digestion of pig manure, anaerobic toxicity essay, The effects of functional group nature on inhibition of methane production by acetoclastic methanogenic bacteria. Correlation of the methanogenic toxicity (IC_{50}) with aromatic compounds hydrophobicity ($\log P_{oct}$),

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 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 general relationships exist between para substituted anilines functional groups nature and their inhibitory effects on methanogenic bacteria. The toxicity of para bisubstituted anilines increases in the following order:



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 Aniline and p-Aminosulfonic acid (Sulfanilic acid) with 1407.4 and 2777.8 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 functional groups nature of para substituted anilines compounds and their inhibitory effects in methane biosynthesis by methanogenic bacteria

Keywords: para substituted anilines, methane biosynthesis, anaerobic digestion, toxicity, methanogenic bacteria, methane gas.

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21 **1. INTRODUCTION.**

22

23 Aniline, phenylamine or aminobenzene is an organic compound with the formula $C_6H_5NH_2$.
24 Consisting of a phenyl group attached to an amino group, aniline is the prototypical aromatic
25 amine. Being a precursor to many industrial chemicals, its main use is in the manufacture of
26 precursors to polyurethane.

27 Aniline is mainly produced in industry in two steps from benzene. First, benzene is nitrated
28 using a concentrated mixture of nitric acid and sulfuric acid at 50 to 60 °C, which gives
29 nitrobenzene. In the second step, the nitrobenzene is hydrogenated, typically at 200-300 °C
30 in presence of various metal catalysts.

31 As an alternative, aniline is also prepared from phenol and ammonia, the phenol being
32 derived from the cumene process.

33 Many derivatives of aniline can be prepared in similar process from nitrated aromatic
34 compounds. Nitration followed by reduction of toluene affords toluidines. Nitration of
35 chlorobenzene and related derivatives and reduction of the nitration products gives aniline
36 derivatives, e.g. 4-chloroaniline.

37 Like phenols, aniline derivatives are highly susceptible to electrophilic substitution reactions.
38 Its high reactivity reflects that it is an enamine, which enhances the electron-donating ability
39 of the ring. For example, reaction of aniline with sulfuric acid at 180 °C produces sulfanilic
40 acid, $H_2NC_6H_4SO_3H$, which can be converted to sulfanilamide. Sulfanilamide is one of the
41 sulfa drugs, which were widely used as antibacterials in the early 20th century

42 Aniline is a weak base. Aromatic amines such as aniline are, in general, much weaker bases
43 than aliphatic amines because of the electron-withdrawing effect of the phenyl group. Aniline
44 reacts with strong acids to form anilinium (or phenylammonium) ion ($C_6H_5-NH_3^+$). Although
45 aniline is weakly basic, it precipitates zinc, aluminum and ferric salts, and, on warming,
46 expels ammonia from its salts. The weak basicity is due to a negative inductive effect as the
47 lone pair on the nitrogen is partially delocalized into the pi system of the benzene ring
48 (McMurry, 1992).

49 Aniline is toxic by inhalation of the vapour, ingestion, or percutaneous absorption. The early
50 manufacture of aniline resulted in increased incidents of bladder cancer, but these effects
51 are now attributed to naphthylamines, not anilines.

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53 The influence of substituents on the physical and chemical properties of compounds has
54 been an important focus of interest in chemistry. In general, substituents alter the reactivity,
55 conformations, equilibrium relationships, spectra, and thermal properties of the substituted
56 compounds.

57 Most commonly, studies of substituent effects have focused on the influence of substitution
58 on a single molecular property, and more general studies of substituent effects on a variety
59 of molecular properties have been comparatively rare. In this study, we report an
60 examination of alterations in several representative structural, energetic, and chemical
61 properties induced by substitution in aniline, and an examination of the extent to which these
62 changes correlate with one another. Aniline is an interesting candidate in which the influence
63 of substituents can be studied for several reasons.

64 On the basis of microwave, ultraviolet absorption, vibrational and theoretical studies, it is
65 known that the NH_2 plane of the amino group lies at an angle of between 37° and 46° to the
66 plane of the phenyl ring.

67 Substituents can be expected to alter the out-of-plane (OOP) angle θ in a characteristic
68 manner depending on their electron-donating and -withdrawing influences. On the basis of
69 resonance theory, electron-withdrawing substituents, especially at the para position, should
70 favor quinoid resonance structures, which are more planar than aniline itself, decreasing
71 both θ and the C—N bond length (Sudlow and Woolf, 1998).

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Para substituted anilines compound as the aromatic compounds can be biodegraded in anaerobic digestion process but they can also be toxic to methanogenic bacteria (Field and Sierra, 1990; Lettinga and Hulshoff Pol, 1990; Chen et al., 2008). The present paper aims to determine the toxicity of para substituted anilines in anaerobic digestion process and evaluate the effects of substituents nature on inhibition of methane production by acetoclastic methanogenic bacteria.

2. MATERIAL 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 g COD-CH₄/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-CH₃COOH /l (chemical oxygen demand per liter);

2.2.2. Stock solution 1: macro-nutrients : NH₄Cl (170g/l); KH₂PO₄ (37 g/l); CaCl₂. 2H₂O (10 g/l); MgSO₄.4H₂O (37 g/l).

2.2.3. Stock solution 2: trace elements : FeCl₃.4H₂O (2000mg/l); CoCl₂.6H₂O (2000 mg/l); MnCl₂.4H₂O (500 mg/l); CuCl₂ (50 mg/l); H₃BO₃ (50 mg/l); (NH₄)₆Mo₇O₂.4H₂O (90 mg/l); Na₂SeO₃. 5 H₂O (100 mg/l) ; NiCl₂. 6 H₂O (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 Na₂S (100 g/l) (; Field and Sierra,1990; Mambanzulua et al,1999; Mbuyu and Kayembe, 2000a).

2.3. Aromatic compounds

The used para substituted anilines included: p-Nitroaniline, p-Chloroaniline, p-Toluidine, p-Aminosulfonic acid (Sulfanilic acid) and Aniline. Otherwise benzene was utilized as reference aromatic compounds. All aromatic compounds were p.a. products.

2.4. Anaerobic toxicity assay

Specific acetoclastic methanogenic activity measurements were performed with 1l glass serum bottles sealed with butyl rubber septa. Two ml stock solution 1; 1 ml stock solution 2, 2 drops stock solution 3 and 40 ml stock substrate solution were added to each serum bottle from the stock sludge. The serum bottle was filled to 1000 ml with oxygen free tap water which is flushed with nitrogen gas for at least 15 minutes. 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 % (Sierra and Lettinga, 1989; Field and Sierra,1990; Mpiana, 2010).

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 "IC₅₀". All specific methanogenic activity measurements were conducted in triplicate. To determine the degree of inhibition, the specific methanogenic activities of the control and samples containing inhibitory compounds were determined (Field and Sierra,1990; Mambanzulua et al, 1999; Kalyuzhnyi et al.,2000).

2.5. Methane gas measurement

The methane gas volume produced was measured by serum bottle liquid displacement systems (Mariotte flask system) as previously described (Field and Sierra, 1990; Mambanzulua et al, 1999; Mbuyu and Kayembe 2000b).

The liquid in the displacement serum bottle should contain a concentrated solution of NaOH or KOH in order to rapidly convert CO₂ gas to carbonate and dissolve it into the NaOH solution (Field and Sierra, 1990).

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.

Figure 1 shows the decrease in specific methanogenic activity with the increasing of the concentration of Aniline. The IC₅₀ is calculated as the concentration of Aniline corresponding to 50% of inhibition.

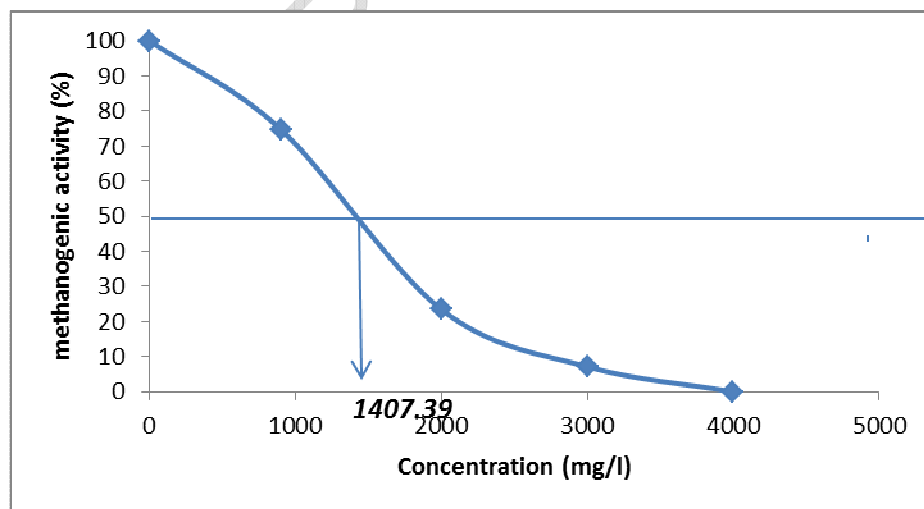


Fig 1: Methanogenic activity of digested pig manure exposed to Aniline versus Aniline concentration.

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3.2. Toxicity of para substituted anilines in anaerobic digestion process

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

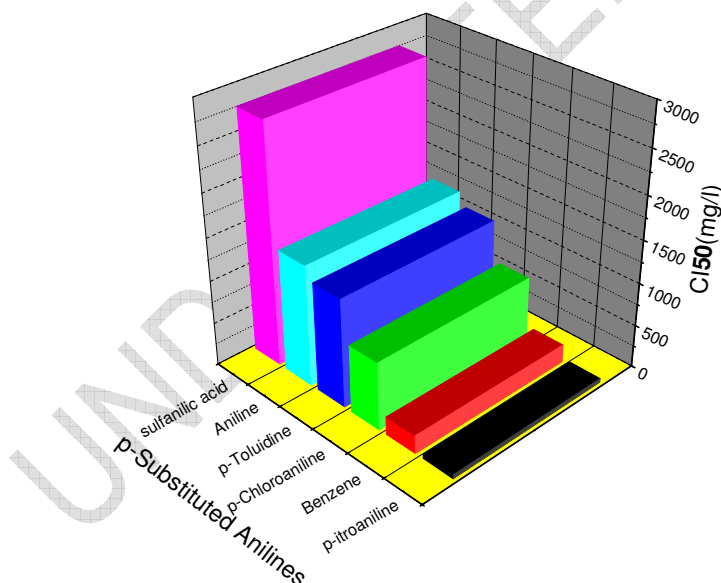
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Table 1: IC_{50} values of para substituted anilines compounds and reference aromatic.

N°	Compound	IC_{50} (mg/l)	logPoct
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	Sulfanilic acid	2777.82±71.33	-0.512

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The methanogenic toxicity exhibited by the para substituted anilines compounds is illustrated in Figure 2.



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Fig. 2: The methanogenic toxicity as exhibited by para substituted anilines compounds.

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

p-Aminosulfonic acid < 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 IC₅₀ values respectively are the most toxic compounds, while Aniline and p-Aminosulfonic acid (Sulfanilic acid) with 1407.39 and 2777.82 mg/l IC₅₀ 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 realize the p-nitroaniline highly toxic. Thus, the substitution of a nitro group (-NO₂) on the Aniline giving the p-Nitroaniline, has the effect of increasing the toxicity of the aromatic ring.

This demonstrates sufficiently that the grafting of hydrophilic substituent on the benzene ring, make the obtained compound less toxic and the number effects of hydrophilic (electron donors) groups such as the hydroxyl group are additives (Debord,2012).

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 most toxic than hydroquinone (para isomer). Our previous data in the field of aromatic toxicity show that para substituted isomers are always less toxic than other isomers (Kayembe et al, unpublished data)..

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 (Debord, 2012).

3.3. Effect of the para substituted Aniline functional groups on the methanogenic toxicity

The influence of the functional group on the methanogenic toxicity exhibited by the para substituted anilines compounds is illustrated in Figure 3

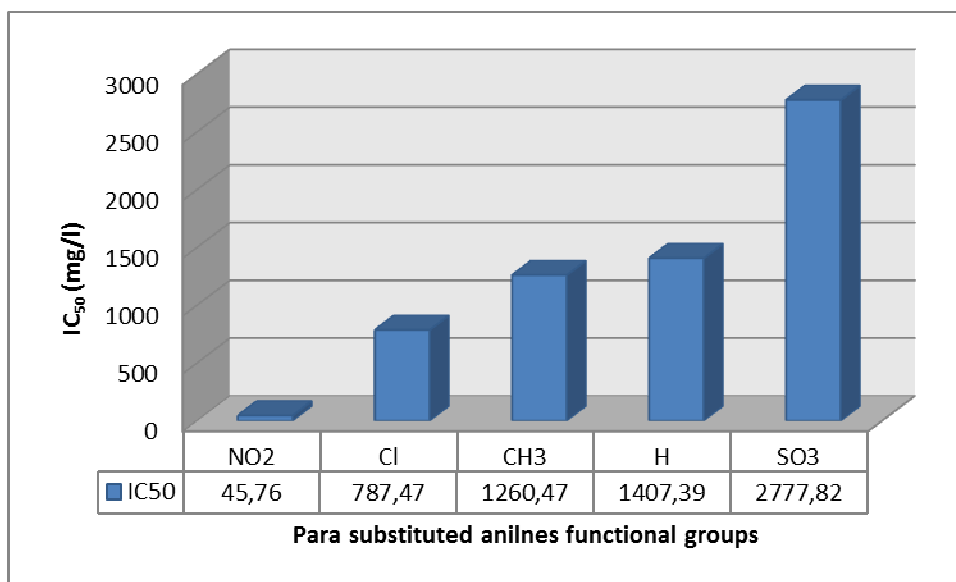


Fig. 3: Variation of the methanogenic toxicity as a function of the para-substituted Anilines functional groups.

The results obtained indicate that general relationships exist between the nature of functional group of para substituted anilines compounds and their inhibitory effects on methanogenic bacteria. According to the figure 3, the toxicity of para bisubstituted anilines increases in the following order:

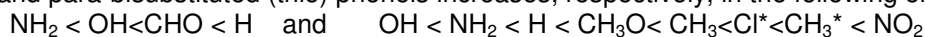


From this sequence of increasing toxicity, it can be seen that the methanogenic toxicity varies with the functional group nature which is in the para position of the main function. The obtained results are comparable to those reported in our previous works (Kayembe et al, in press), by Donlon et al (1995) and by Sierra and Lettinga (1989) for monosubstituted aromatic compounds. The addition of a functional group containing an oxygen or sulfur heteroatom to aniline, the reference compound, decreased the para substituted aromatic compound toxicity as in the case of SO₃ substitution. However, the addition of CH₃, Cl and NO₂ to aniline was associated with an increase in a compound toxicity.

This sufficiently demonstrates that the grafting of hydrophobic or hydrophilic substituent on the benzene or monofunctional aromatic compound, make the obtained compound more or less toxic.

However, this behavior is valid only 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 another.

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



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 logP_{oct} of the compound cannot be expected. A higher correlation could potentially be obtained by comparing compounds in homologues series (Sierra and Lettinga, 1989; Donlon, 1995).

To determine if the lipophilic character of monomeric tannin compounds and reference aromatic compounds (benzene) tested could be correlated with their methanogenic toxicity, the logarithm of the IC₅₀ values of these aromatics were plotted against the logarithm of the octanol-water partition coefficient (logP_{oct}) of the aromatic compounds. Figure 4 shows the correlation line between the methanogenic toxicity and partition coefficient logP_{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 ($R^2 = 0.965$). This causes that the more hydrophobic molecule is, the more readily it crosses the cell membrane and becomes highly toxic and inversely (Mpiana, 2010; Debord, 2012).

Hydrophobicity of a compound as indicated by logP_{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 (Donlon, 1995; Mpiana, 2010).

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

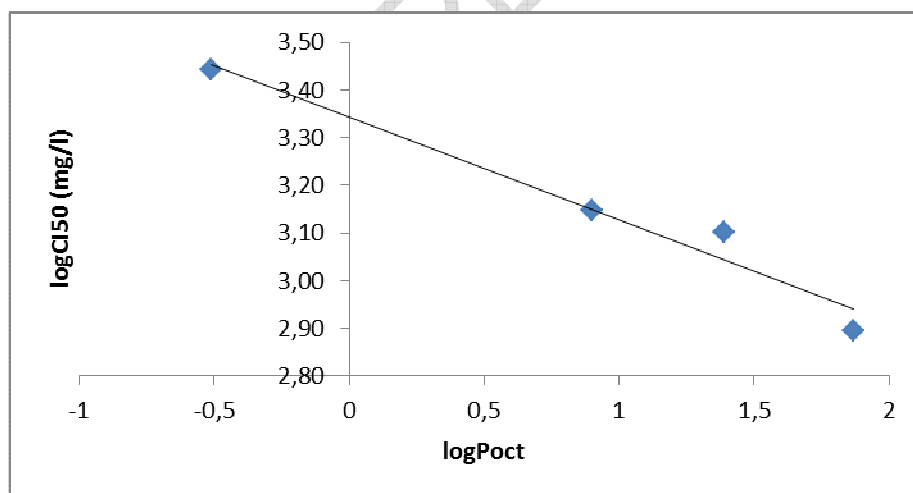


Fig. 4: Effect of hydrophobicity on methanogenic toxicity: methanogenic toxicity (IC₅₀) and partition coefficient (logP_{oct}) correlation ($R^2 = 0.965$).

295 The diffusion of a molecule across a membrane depends on the permeability of the
296 membrane. However, the membrane permeability is a function of the partition coefficient
297 logP_{oct}. So the more hydrophobic a molecule is, the higher is its membrane permeability
298 and the greater is its toxicity (Kobayashi, 1979; Mpiana, 2010).

299 Indeed, a substitution on the aromatic ring which tends to make the molecule lipophilic
300 (hydrophobic) increases the affinity for membrane phase therefore the permeability of the
301 membrane to this compound. The massive compound diffusion in methanogenic bacteria
302 thus increases the toxicity for these microorganisms causing damage to subcellular
303 components. This contributes to the decrease in methanogenic activity (Kobayashi, 1979).

304 4. CONCLUSION

305
306 The results obtained indicate that some general relationships exist between the nature of
307 functional groups of para substituted anilines compounds and their inhibitory effects on
308 methanogenic bacteria. According to the figure 3, the toxicity of para bisubstituted anilines
309 increases in the following order:

310 $SO_3 < H < CH_3 < Cl < NO_2$

311
312 The addition of a functional group containing an oxygen or sulfur heteroatom to aniline, our
313 reference compound, decreased the para substituted aromatic compound toxicity as in the
314 case of SO_3 substitution. However, the addition of CH_3 , Cl and NO_2 to aniline was
315 associated with an increase in a compound toxicity. This confirms the fact that para
316 substituted isomers are less toxic than other isomers.

317
318 A very significant negative linear correlation between the toxicity of compounds with the
319 reference aromatic compound (benzene) and their hydrophobicity was found.
320 This causes that the more hydrophobic molecule is, the more readily it crosses the cell
321 membrane and becomes highly toxic and inversely

322

323 COMPETING INTERESTS

324

325 Authors have declared that no competing interests exist.

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327 AUTHORS' CONTRIBUTIONS

328

329 K. Kayembe designed the study and performed the experimental work, L. Basosila
330 performed the statistical analysis, K. Mbuyu wrote the protocol; C.P. Sikulisimwa managed
331 the analyses of the study and P.T. Mpiana wrote the first draft of the manuscript and
332 managed the literature searches. All authors read and approved the final manuscript.

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