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Methylation Profile of 18S rDNA Gene in Brain and Muscle of Tambaqui Exposed to Parasiticide Trichlorfon

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Research Article

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Abstract

Until recently, there were no reports on the methylation profile of Amazonian fish specimens employing restriction enzymes. The 18S rDNA methylation status of samples of captive tambaqui (*Colossoma macropomum*) exposed to the antiparasitic Trichlorfon was examined in the current study. The method performed makes use of the CCGG site's sensitivity to the presence of methylation in palindrome cytosines for the restriction enzymes *Hpa*II and *Msp*I. The cleavage occurs by the detection or non-detection of the methyl radical in the inner ($C^{5m}CGG$) or outer ($5^{m}CCGG$) cytosine, which makes it possible to compare the DNA methylation profile. When compared to the specimens examined as the control group, the results obtained revealed an increase in methylated fragments of the 18S rDNA gene at LC concentrations of 30% and 50%.

Keywords: *Colossoma macropomum*; Enzymatic Digestion; Pisciculture

Introduction

Pesticides are chemical substances currently utilized to minimize the action of parasites and reduce losses in pisciculture. These substances end up contaminating the fish tissues, leading to biochemical, histopathological, genotoxic, and molecular alterations. The direct exposure of animals to toxic chemicals may induce harmful effects by affecting their epigenome and physiology. Extensively used in Amazonian piscicultures, the organophosphate Trichlorfon (dimethyl 2,2,2-trichloro-1 hydroxyethyl phosphonate) is a phosphoric acid currently classified as highly toxic (class II). The levels most commonly used by fish farmers are overestimated, ranging from 0.13 mg/L to 25 g/L of Trichlorfon thrown into the water for immersion baths [1]. This organophosphate is one of the main contaminants found in piscicultures. In this study, it was used a technique to identify the methylation pattern in simple locus in an effort to understand how this contamination affects the tambaqui genome. To achieve this, it was used the 18S rDNA ribosomal gene, which is arranged in tandem into several transcriptional units. It is moderately repetitive; it contains several palindromic CCGG sites. Because it is easily amplified, conserved, and has gene regulation mediated by methylation of the hundreds of copies present in the genome, the 18S rDNA gene exhibits promise and is occasionally employed as

an epigenetic marker for cancer [2]. In this study, an assay using the restriction enzymes *Hpa*II and *Msp*I was developed in order to examine the methylation profile of the 18S rDNA ribosomal gene in tambaqui (*Colossoma macropomum*).

Material and Methods

All procedures performed in this work were approved under protocol number 030/2018 - CEUA/UFAM by the Animal Research Ethics Committee of the Federal University of Amazonas. The animals were exposed to two concentrations of Trichlorfon: 30% and 50% of the LC for 24, 48, 72, and 96 hours as described for the compound [3,4]. A corresponding control group was performed for each sample group. The experimental design is shown in Figure 1, with each treatment being performed in triplicate with eight fish in each tank. Three samples were randomly selected for enzymatic digestion. Each experimental condition involved anesthetizing the animals before collecting their brains and muscles for analysis.

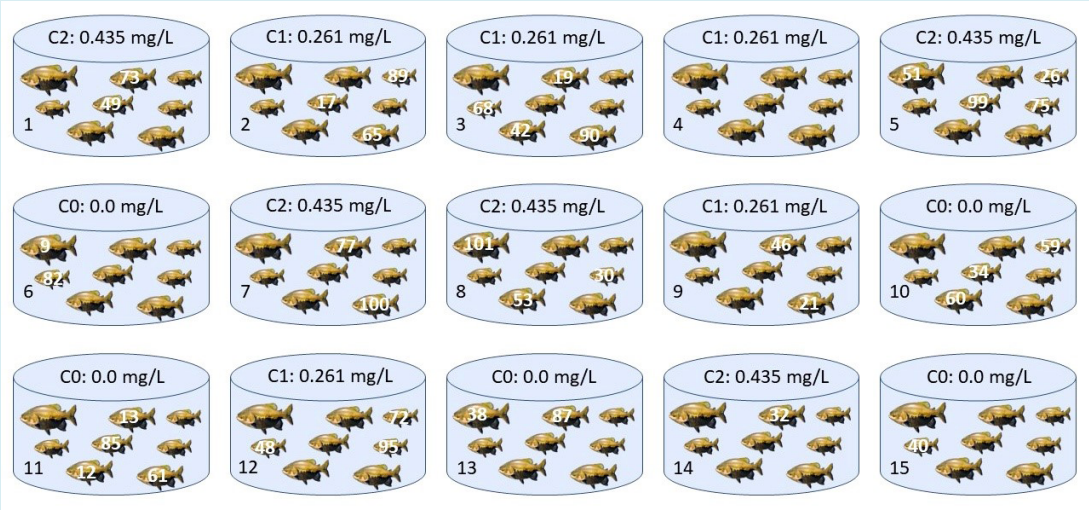

Figure 1: Experimental design performed in the experiment. C0 – control concentration (without the addition of Trichlorfon in the water); C1: concentration 1, 30% of Trichlorfon LC50-96h diluted in water (0.261 mg/L of Trichlorfon); C2: concentration 2, 50% of Trichlorfon LC50-96h diluted in water (0.435 mg/L of Trichlorfon). Numbers of samples are shown for each tank and concentration.

Genomic DNA extraction was performed according to Sambrook, et al. [5]. In accordance with the manufacturer's instructions (Promega™), the genomic DNA (concentration of 0.1g/L) was digested using the restriction enzymes *Hpa*II and *Msp*I. Using the primers and conditions outlined by Gross, et al. [6], the 18S rDNA region was amplified by PCR after the enzyme digestion. Electrophoresis in a 1.5% (m/v) agarose gel, in 1X TBE buffer, labeled with ethidium bromide, and observed in a UV transilluminator was used to verify the reaction products. A methylated fragment ($^{5m}C^{5m}CGG$) was identified by the appearance of bands in the gel of fragments digested by both enzymes; this means that neither the *Hpa*II enzyme nor the *Msp*I enzyme digested the fragment, and it appears intact in the gel. Inner cytosine methylation was hypothesized to be the cause of the fragment's appearance in the gel after *Hpa*II digestion and its absence during *Msp*I digestion ($C^{5m}CGG$). Previously acquired samples of the brain and muscles were used to confirm the methylation profile. In order to better understand the results, they will first be presented for the control condition, C0 (no exposure to Trichlorfon), and then for each exposure time: C1 (concentration 1, 30% of Trichlorfon $LC_{50\text{-}96h}$, 0.261 mg/L) and C2 (concentration 2, 50% of Trichlorfon $LC_{50\text{-}96h}$, 0.435 mg/L). Differences ($p<0.05$) in the frequency of phenotypes (%) were evaluated by the Three-way Analysis of Variance (ANOVA) and the FISHER LSD test (Post - hoc) carried out in the STATISCA demo program (StatSoft®) [7]. The necessary assumptions were evaluated (independence of samples; Homoscedasticity and Normality of data).

Following the manufacturer's instructions, the sequencing reaction was performed using the ABI PRISM® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and the samples were sequenced using an ABI Sequence Analyzer 3500 in an automated four-capillary sequencer (Applied Biosystems).

Results

The samples in the gel are arranged in a triad, with the undigested DNA in the first pit, the DNA being digested with the *Hpa*II enzyme in the second pit, and the DNA being

digested with the *Msp*I enzyme in the third pit.

Figure 2 presents the 18S rDNA sequencing with 1.428pb and eleven sites of CCGG susceptible to methylation. Figure 3 summarizes the methylation profile found in this study. There was no statistically significative difference between methylation profiles in relation to the tissues analyzed (Figure 4).

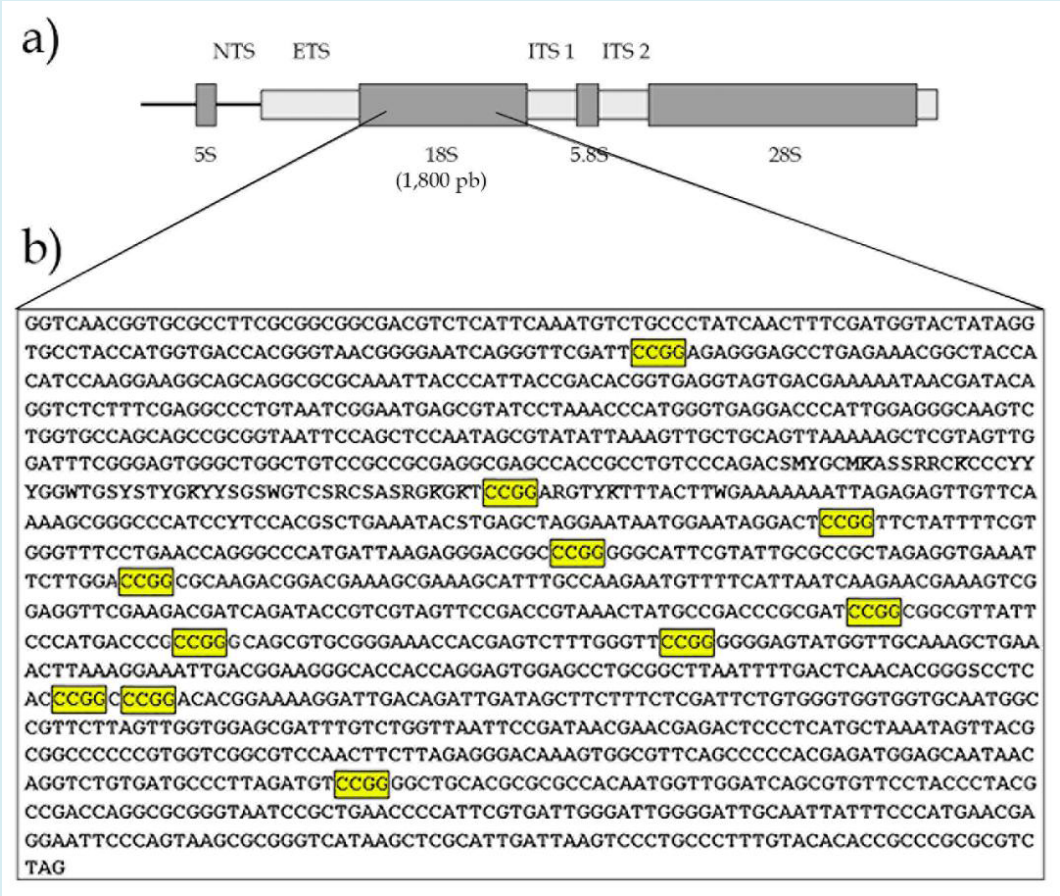

Figure 2: a) Diagram of major subunit of 45S rDNA. b) Restriction map of tambaqui 18S rDNA gene. It was observed that the *Hpa*II and *Msp*I enzymes could cut eleven CCGG restriction sites.

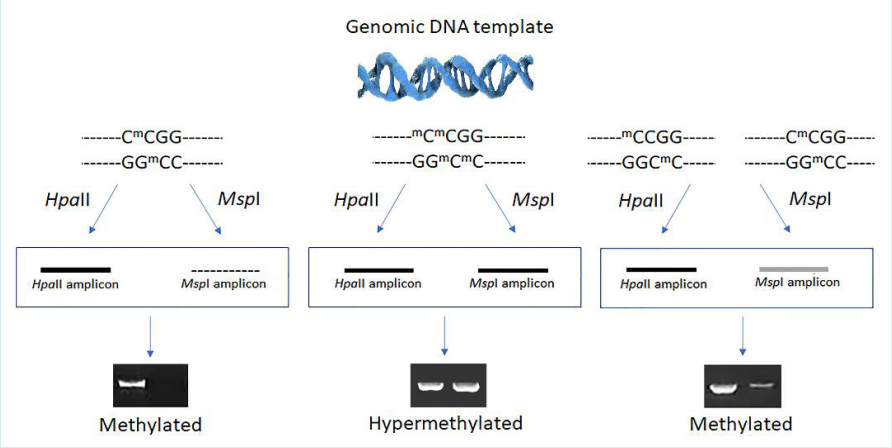

Figure 3: Schematic design showing the phenotypes of methylation found in this study. The observed band phenotypes were transformed into 0, 1, 2, respectively, for the variance analysis.

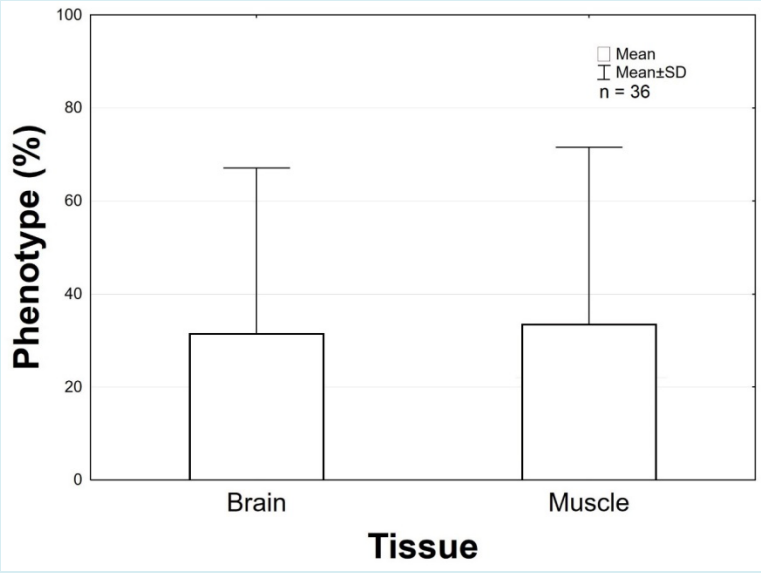

Figure 4: Comparison graph of methylation profiles between brain and muscle. No statistical differences observed.

Figure 5 displays the methylation profile of the 18S rDNA gene in the control condition (C0), condition 1 (C1), and condition 2 (C2) in the brain. Of the 12 samples analyzed as a control group, for all the times, eight presented methylation in the inner cytosine and profile $C^{5m}CGG$, four samples showed methylation in the internal and external cytosine, but the bands appeared faint in the *Msp*I digestion.

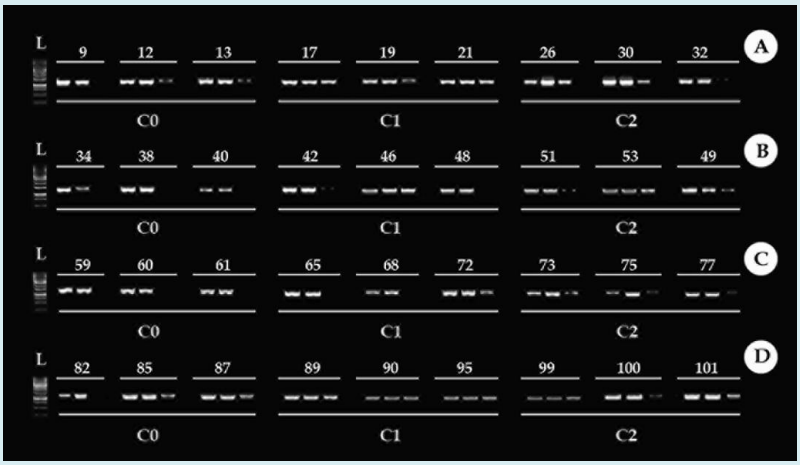

Figure 5: Methylation profile of the 18S ribosomal DNA gene (18S rDNA), in the brain. Times used in the experiment 24h, 48h, 72h, and 96h (A, B, C D, respectively). C0: control condition. C1: concentration 1, 30% of Trichlorfon $LC^{50\text{-}96h}$ diluted in water (0.261 mg/L). C2: concentration 2, 50% of Trichlorfon $LC^{50\text{-}96h}$ diluted in water (0.435 mg/L). First column: ladder.

Figure 6 presents the methylation profile of the 18S rDNA gene in the control condition (C0), condition 1 (C1), and condition 2 (C2) in muscle. For most of the samples in the C0 is observed a $C^{5m}CGG$ methylation profile. In this condition, some individuals presented a $^{5m}C^{5m}CGG$ methylation type, due to the presence of the band in the *Msp*I digestion. However, that bands are weak when compared to bands of *Hpa*II digestion. This occurred by the presence of a few methylated fragments in the outer cytosine. For the other times, was observed an increase in the methylation of $C^{5m}CGG$ for $^{5m}C^{5m}CGG$ (band presence in the *Msp*I digestion). The analysis of the three-way ANOVA showed that there was a significative increase in methylation mainly in the C1 in the times 24h and 96h (Figures 7 & 8), $^{5m}C^{5m}CGG$ (band presence in the *Msp*I digestion).

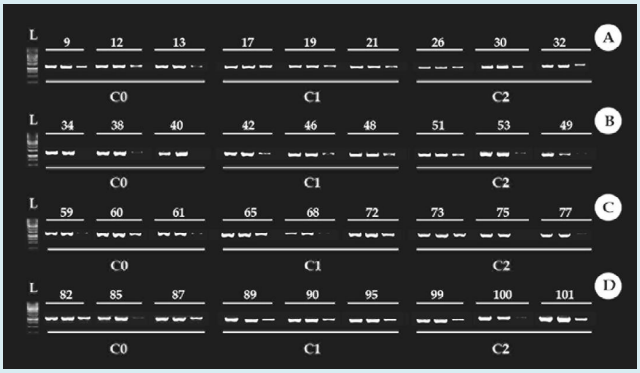

Figure 6: Methylation profile of the 18S ribosomal DNA gene (18S rDNA), in the muscle. Times used in the experiment 24h, 48h, 72h, and 96h (A, B, C, D, respectively). C0: control condition. C1: concentration 1, 30% of Trichlorfon $LC^{50\text{-}96h}$ diluted in water (0.261 mg/L). C2: concentration 2, 50% of Trichlorfon $LC^{50\text{-}96h}$ diluted in water (0.435 mg/L). First column: ladder.

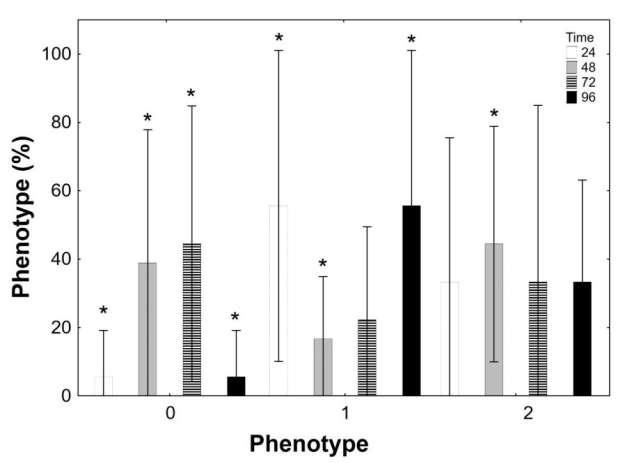

Figure 7: Three-way ANOVA graph showing interaction between phenotypes (0, 1, 2) and times, 24h, 48h, 72h and 96h. Phenotype 1 corresponding to hypermethylated is significantly larger at 24h and 96h times. Asterisk indicates statistical significance ($p<0.05$).

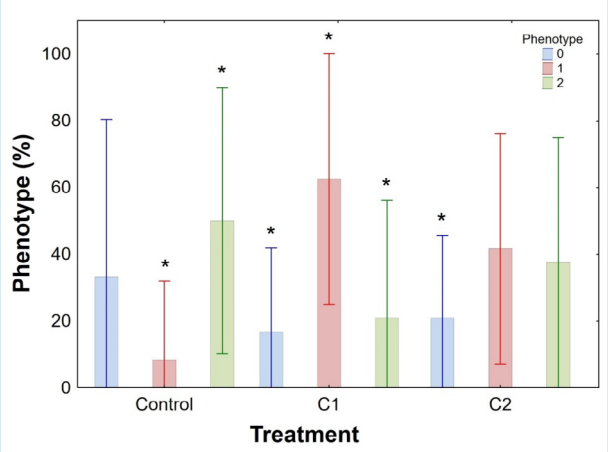

Figure 8: Three-way ANOVA graph showing interaction between phenotypes (0, 1, 2) and treatment (C0, C1 and C2). Phenotype 1 (red color) corresponding to hypermethylated is significantly larger in the C1 concentration. Asterisk indicates statistical significance ($p<0.05$).

Discussion

Despite recognizing the identical restriction site, the cutoff sensitivity of the *Hpa*II and *Msp*I enzymes differs [3,8,9]. The digestions generated by each enzyme are distinct as a result of their different cutting patterns. Because the frequency of $C^{5m}CGG$ palindromes in the tambaqui genome is higher and as a result, these fragments are more cleaved (resulting in a continuous pattern visible with a trace in the gel), a more effective digestion by the *Msp*I enzyme was expected. Fulneček, et al. [10] claim that the MSAP (Methylation Sensitive Amplified Polymorphism) methodology is a reliable, low-cost, and simple way to find genomic sites where environmental and developmental stressors have altered the cytosine methylation. Additionally, according to these authors, the methylation pattern in plant genomes follows the given order: $C^{5m}CGG$ > $^{5m}C^{5m}CGG$ > $^{5m}CCGG$. In this study it assumed that the tambaqui genome followed a similar pattern.

Several studies have demonstrated that external influences, such as interactions with chemicals, can alter DNA methylation patterns [10-12]. According to Kamstra, et al. [13], who used the zebrafish (*Danio rerio*) as an experimental model for epigenetic and ecotoxicological studies, epigenetic processes in fish generate a pattern of DNA methylation that is sensitive to stressors.

The 18S rDNA gene is present in the genome in many copies, with methylation inactivating half of them [14]. This gene presents many CCGG sites, making it an excellent marker for research on DNA methylation. Therefore, even though the control samples showed some methylation, this result was anticipated because they represent the copies that are typically methylated by conventional epigenetic pathways [14,15].

Even though a methylation profile was observed at the control concentration (C0), it was still possible to detect an increase in methylation in the 18S rDNA gene in samples from concentrations C1 (30% of $LC_{50\text{-}96h}$, 0.261 mg/L), and C2 (50% of $LC_{50\text{-}96h}$, 0.435 mg/L) at the same exposure times. Since the tambaqui 18S rDNA gene may be associated with transposable elements that are methylation targets, this result can be explained by the methylation process in this region. Studies conducted in *Hoplosternum litoralle* by da Silva, et al. [15] found co-localization of the transposable element *Rex*3 and 18S rDNA in this species, supporting this hypothesis. Another theory is that the methylation-friendly 18S rDNA regions are typical heterochromatin areas. Additionally, the method might be more effective in heterochromatic regions where cytosine methylation is concentrated. Furthermore, according to Burt, et al. [16], some transposable elements, like LINE *R1* from *Drosophila melanogaster*, have a particular insertion site in the genome of this species and are typically inserted into the 28S rRNA genes. The rDNA gene is turned nonfunctional by any insertion and hosts can only survive because each individual usually possesses hundreds of copies of the rDNA gene, only a small percentage of which are disrupted (in most species, 5-20%, in certain species up to 50%) [17].

Therefore, other studies, such as that of Li, et al. [18], found that increased methylation in CCGG palindromes for the 18S rDNA gene in *Arabidopsis* samples after nickel exposure can support the idea of a connection between transposable elements and the 18S rDNA gene. Increased methylation in tambaqui exposure to Trichlorfon at concentrations C1 and C2 is consistent with the findings of Costa, et al. [19], who used tambaqui as an experimental model for Trichlorfon exposure and found that the retrotransposable element *Rex*3 had significantly increased in the species' genome. In the present study, the 18S rDNA gene's increased methylation may be directly linked to Rex's inactivation. This is a hypothesis that must be investigated in further analysis. Other studies of these regions, mainly related to cancerous tissues, support the hypermethylation condition of the 18S rDNA gene transcript regions. According to Shao, et al. [2] and Chan, et al. [20], rDNA methylation is positively correlated with ovarian and breast cancer, respectively. The hypermethylation status of rDNA sequences, according to those authors, may be employed as a potential biomarker in the diagnosis and prognosis of these cancers. Additionally, it is important to underline the crucial function that DNA methylation plays in the epigenetic control of the genome and in gene expression, where variations in the methylation profile can lead to variant phenotypes that can be targeted for selection and responsible for epigenomic diversity [21]. Therefore, it is important to investigate how methylation affects species diversity and evolution, given its involvement in the development of diseases [9,22].

Conclusion

The analysis made in this study showed that there was sensitivity to Trichlorfon and an increase in 18S rDNA methylated sites when tambaqui specimens were exposed to 30% and 50% of the $LC^{50\text{-}96h}$. The presence of hypermethylated phenotypes was more significant at C1 concentration at 24h and 96h times. This suggests that Trichlorfon has a meaningful effect on the tambaqui genome, by inactivation of genes through methylation.

Acknowledgement

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