**MARCELL VALANDRO SOARES**

# EXPOSIÇÃO NO AR AO TOLUENO PROMOVE APOPTOSE DA LINHAGEM GERMINATIVA, ALTERAÇÕES NEUROCOMPORTAMENTAIS E DANO NEURONAL EM *Caenorhabditis elegans.*

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Dissertação apresentada ao programa de Pós-graduação em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Bioquímica.

Orientador (a): Profa. Dra. Daiana S. Ávila

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Área de concentração: Bioprospecção molecular.

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# RESUMO

Solvente orgânico é uma denominação para um grupo de substâncias químicas orgânicas que são muito utilizadas em processos industriais. Dentre estes produtos, destaca-se a gasolina, que apresenta uma composição variável de compostos e solventes, mas consiste basicamente de hidrocarbonetos aromáticos. O tolueno é um dos solventes orgânicos presentes na gasolina, o qual possui elevado grau de volatilidade, sendo absorvido principalmente por via pulmonar, conseguindo atingir o sistema nervoso central e promover ação tóxica, entretanto, a especificidade dos mecanismos envolvidos é pouco explorada. O presente trabalho buscou avaliar o efeito da exposição por via aérea ao tolueno no modelo alternativo *Caenorhabditis elegans*, a fim de validar o modelo e elucidar mecanismos de toxicidade deste solvente valendo-se das vantagens deste modelo animal. Foram usados nematoides do tipo selvagem N2 e as cepas mutantes EG1285, LX929 e MD701. Vermes no primeiro ou quarto estágio larval foram expostos ao tolueno por 24-48 horas em uma câmara fechada, desenvolvida pelo laboratório, nas concentrações de 450, 850, 1.250 e 1.800 ppm. Após 24-48 horas de exposição foram efetuadas as análises, visando avaliar os parâmetros de letalidade, desenvolvimento, reprodução, fertilidade e neurotoxicidade. Observamos um aumento na mortalidade, sendo a $CL_{50}$ calculada para a exposição volátil 1.340 ppm; também evidenciamos um atraso no desenvolvimento significativo à medida que a concentração de tolueno aumentou. Foi evidenciado um aumento significativo nos eventos apoptóticos nas células de linhagem germinativa nas concentrações de 450 e 850 ppm, o que corroborou com a redução da taxa reprodutiva causada pelo tolueno. Avaliando parâmetros neurocomportamentais, encontramos que o tolueno promoveu uma diminuição significativa nos movimentos de locomoção em todas as concentrações utilizadas. Para caracterizar o efeito neurotóxico do tolueno, comprovamos que nas concentrações de 1.250 e 1.800 ppm o tolueno promoveu uma redução na intensidade da fluorescência de neurônios GABAérgicos e colinérgicos, sugerindo dano nestes neurônios. Com base nos resultados obtidos, podemos concluir que o tolueno apresentou uma significativa toxicidade em *C. elegans*, através da indução de apoptose e neurotoxicidade.

Palavras chave: *C.elegans*, Solvente orgânico, Tolueno, Neurotoxicidade.

# ABSTRACT

Organic solvent is a name for a group of organic chemicals that are widely used in industrial processes. Among these products, gasoline has a variable composition of compounds and solvents, but basically consists of aromatic hydrocarbons. Toluene is one of the organic solvents present in gasoline, which has a high degree of volatility, being absorbed mainly by the pulmonary route and reaches the central nervous system promoting toxic action; however, the specificity of the mechanisms involved is little explored. The present work aimed to evaluate the effect of inhalation exposure to toluene in the alternative model *Caenorhabditis elegans*, in order to validate the model and elucidate the mechanisms of toxicity of this solvent using the advantages of the animal model. N2 wild-type nematodes and the mutant strains EG1285, LX929 and MD701 were used. Worms in the first or fourth larval stage were exposed to toluene for 24-48 hours in a laboratory-developed closed chamber at concentrations of 450, 850, 1,250 and 1,800 ppm. After 24-48 hours of exposure, the analyses were carried out to evaluate the parameters of lethality, development, reproduction, fertility and neurotoxicity. We observed an increase in mortality, the $LC_{50}$ being calculated for the volatile exposure 1,340 ppm; we also showed a significant developmental delay as we increased the concentration of exposure to toluene. A significant increase in the apoptotic events in germline cells was evidenced, only at the concentrations of 450 and 850 ppm, which corroborated with the reduction of the reproductive rate caused by toluene. Evaluating neurobehavioral parameters, it was found that toluene promoted a significant decrease in locomotion movements at all concentrations used. To characterize the neurotoxic effect of toluene, we found that at concentrations of 1,250 and 1,800 ppm toluene promoted a reduction in the fluorescence intensity of GABAergic and cholinergic neurons, suggesting neuronal damage. Based on the results obtained, we can conclude that toluene presented a significant toxicity in C. elegans through the induction of apoptosis and neurotoxicity.



Key words: *C.elegans*, Organic solvent, Toluene, Neurotoxicity.

# APRESENTAÇÃO

A presente dissertação foi desenvolvida em três partes:

## CAPÍTULO 1

Contém as seções Introdução, Revisão Bibliográfica, Justificativa e Objetivos.

## CAPÍTULO 2

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo científico. As seções Materiais e Métodos, Resultados e Discussão encontram-se no próprio manuscrito. O manuscrito está apresentado da mesma forma em que foi submetido à revista ***Environmental Pollution.***

## CAPÍTULO 3

Contêm as conclusões, perspectivas e referências bibliográficas.

# LISTA DE FIGURAS

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# Sumário

## INTRODUÇÃO

Carlini, Carlini-Cotrim e Monteiro (1988), destacam que a produção e uso generalizado de solventes em âmbito industrial se iniciou em 1940, sendo utilizados para a dissolução, dispersão e diluição de vários materiais. Porém, somente muitos anos após foi evidenciado que os solventes seriam responsáveis por gerar problemas de saúde de origem ocupacional. No Brasil, há algum tempo diversos trabalhadores vem sendo expostos de maneira perigosa a solventes orgânicos em seus locais de trabalho, uma vez em que não há utilização de equipamentos de proteção adequados para uso, fato bastante negligenciado pelos donos das empresas, em especial, dos postos de gasolina (PIVETTA et al., 2001).

A gasolina apresenta composição variável de compostos e solventes, mas consiste basicamente de hidrocarbonetos aromáticos, sendo um destes o tolueno. Exposições ao tolueno em altas concentrações podem ocasionar desorientação, tremores, alucinações e também problemas motores (EISENBERG, 2003). No caso de exposições crônicas, os sujeitos expostos podem apresentar sintomas de hepatotoxicidade e nefrotoxicidade, caracterizados por hipertrofia hepática e lesões nos glomérulos e túbulos renais (AHMADIZADEH; AMIRMOEZY; POLE, 2014; ANA-LILIA et al., 2006; TAS et al., 2011). O principal alvo de ação do tolueno é o sistema nervoso central (SNC), cujos efeitos são amplamente relatados na literatura, como atrofia cerebral, alterações neurocomportamentais, perda de memória e danos neuronais (AYDIN et al., 2002; KANG et al., 2005; OSHIRO et al., 2011; THETKATHUEK et al., 2015; YUCEL et al., 2008).

Um grande impacto em nosso organismo após exposição ao tolueno é em relação a um possível efeito tóxico na reprodução. Plenge-Bonig e Karmaus (1999), investigaram alterações em trabalhadores expostos ao tolueno, em ambos os sexos, utilizando um estudo transversal, no qual trabalhadores foram entrevistados. Foi observado que mulheres expostas ao tolueno apresentavam uma redução em sua fecundidade. Além disso, Xiao et al., (2001), avaliaram a qualidade do esperma de trabalhadores expostos a tolueno, benzeno e xileno os quais apresentaram solventes, tanto no sangue quanto no sêmen, sendo evidenciados parâmetros de vitalidade e motilidade do esperma diminuídos, o que pode causar uma redução na fertilidade.

Visto que o metabolismo do tolueno pode gerar compostos intermediários altamente reativos, como derivados epóxido, e assim lesando moléculas como o Ácido desoxirribonucleico (DNA). Apesar do tolueno não ser classificado como sendo carcinogênico, pelo IARC (*International Agency for Research on Cancer*), muitos estudos

têm demonstrado o contrário. Moro et al., (2012), observaram níveis séricos e urinários do tolueno dentro dos valores de referência, no entanto, um significativo aumento de danos oxidativos aos tecidos e ao DNA foi evidenciado. Além disso, outra pesquisa demonstrou que um grupo de trabalhadores expostos aos derivados do petróleo apresentaram um significativo aumento no nível de aberrações cromossômicas e micronúcleos (ARAUJO et al., 2010).

Por estes motivos, faz-se necessário avaliar e evidenciar os efeitos do tolueno para assim compreender seus mecanismos de ação. Uma vez que a maioria dos estudos com humanos são baseados em questionários e ensaios *Ex vivo*, isto torna por certas vezes os resultados controversos pelas limitações metodológicas. Diante disso, a utilização de modelos alternativos como uma ferramenta de trabalho inovadora, econômica e fácil pode ser uma solução viável para estudos toxicológicos. Uma grande vantagem é que dependendo do tipo de modelo a ser usado podemos encontrar homologias genéticas com humanos e que os resultados poderiam predizer o que ocorreria em humanos. Dentre tantas outras vantagens, podemos encontrar a facilidade em conseguir indivíduos para os estudos, obter resultados mais rápidos e com menor custo (MORALES, 2008).

Dentre os modelos alternativos atuais destacamos o nematoide *Caenorhabditis elegans* que tem sido utilizado como organismo modelo devido à grande homologia genética que existe entre estes nematoides e os mamíferos, bem como a praticidade em sua manipulação e a complexidade celular de vias metabólicas presentes também em organismos superiores (KALETTA; HENGARTNER, 2006). Além disso, possui um ciclo de vido curto, de aproximadamente 21 dias, o que facilita obter resultados mais rápidos; transparência do corpo, que permite que possamos realizar ensaios para estudar vias e mecanismos de ação marcando os alvos com proteína fluorescente, além de sistemas reprodutivo e nervoso, bem estabelecidos (RIDDLE et al., 1997). O sistema nervoso de *C. elegans* é composto de 302 neurônios que podem ser marcados com uma proteína verde fluorescente (GFP) e observados por microscopia de fluorescência e isso permite a observação de processos de degeneração neuronal bem como a elucidação de vias de mecanismo de ação (WHITE et al., 1986).

Diante do exposto, buscamos avaliar os efeitos da exposição por via aérea ao tolueno no modelo alternativo *Caenorhabditis elegans* e confrontá-los com os efeitos encontrados na literatura com outros modelos, a fim de validá-lo. Almejamos elucidar o possível mecanismo de ação envolvido na neurotoxicidade do tolueno, bem como avaliar a possibilidade de sua ação genotóxica ainda não evidenciada por meio da utilização de modelo *in vivo.*

# 1 JUSTIFICATIVA

Tendo em vista a problemática abordada sobre o tolueno em relação a seus efeitos reprodutivos e neurotóxicos, faz-se importante investigar e evidenciar novos alvos de ação do tolueno. Desenvolver metodologias que mimetizem as exposições pelo ar ao tolueno e outros VOCs presentes no ar, assim alertando para uma melhor promoção da segurança de trabalhadores e população em geral.

Ainda cabe ressaltar a importância dos estudos com o tolueno, visto que seus mecanismos de ação no SNC não são bem compreendidos e os resultados encontrados às vezes mostram-se controversos. Sabendo que o tolueno não é classificado como carcinogênico, investigar seu possível efeito genotóxico é essencial para que se possam descobrir os perigos da exposição ao solvente, assim estimulando medidas de fiscalização mais rigorosas.

# 2 OBJETIVOS

## 2.1 Objetivo Geral

Investigar os efeitos tóxicos da exposição aérea com tolueno utilizando o modelo experimental *Caenorhabditis elegans*.

## 2.2 Objetivos Específicos

a) Avaliar os efeitos da exposição inalatória com tolueno em diferentes concentrações frente à sobrevivência dos vermes;

b) Avaliar o método de exposição;

c) Determinar a $CL_{50}$ da exposição no ar para *C.elegans*;

d) Avaliar o impacto da exposição com tolueno frente ao parâmetro de desenvolvimento nos vermes;

f) Avaliar o impacto da exposição com tolueno frente à reprodução dos vermes;

g) Verificar alterações no comportamento dos vermes expostos ao tolueno;

h) Avaliar se a exposição com tolueno promove alterações em diferentes neurônios buscando elucidar alvos de ação.

# 3 FUNDAMENTAÇÃO TEÓRICA

## 3.1 Compostos Orgânicos Voláteis (VOCs)

Atualmente, o processo de industrialização é o principal contribuinte para a poluição atmosférica, por gerar um desequilíbrio no ambiente, ocasionando um grande problema de saúde pública (HELMIG et al., 2014). Tal mudança tem nos mostrado que esta mistura de componentes no ar tem um forte impacto à saúde humana. Como exemplo disso, destacam-se os compostos voláteis presentes na gasolina, que são indicados como contribuintes para poluição do ar, dentre os quais muitos são considerados poluentes com potencial carcinogênico (CARLSEN; BRUGGEMANN; KENESSOV, 2018).

Sabe-se que estes compostos orgânicos voláteis estão envolvidos também em poluição do ar em ambientes fechados, em empresas que trabalham manipulando e produzindo produtos com solventes orgânicos, como por exemplo, indústria de sapato, borracha, cola e combustíveis (ORECCHIO et al., 2017). Tais locais apresentam um elevado risco ocupacional aos trabalhadores, particularmente quando não há ventilação adequada em conformidade com a legislação ou o não uso de equipamentos de proteção (CINCINELLI; MARTELLINI, 2017).

Diante do envolvimento dos solventes orgânicos na poluição ambiental, se faz necessário o monitoramento dos ambientes para avaliação dos limites da exposição. Por exemplo, a nível internacional são publicados anualmente OELs (*occupational exposure limit*), sob responsabilidade da American Conference of Governamental Industrial Hygienists (ACGIH), que são denominados *threshold limit values* (TLVs) ou limiar de valores limites. No Brasil, os níveis aceitáveis de concentração ambiental estão dispostos na Norma Regulamentadora nº 15, anexo 11 da Secretaria de Segurança e Medicina do Trabalho do Ministério do trabalho, sendo aceitável a presença de até 78 ppm.

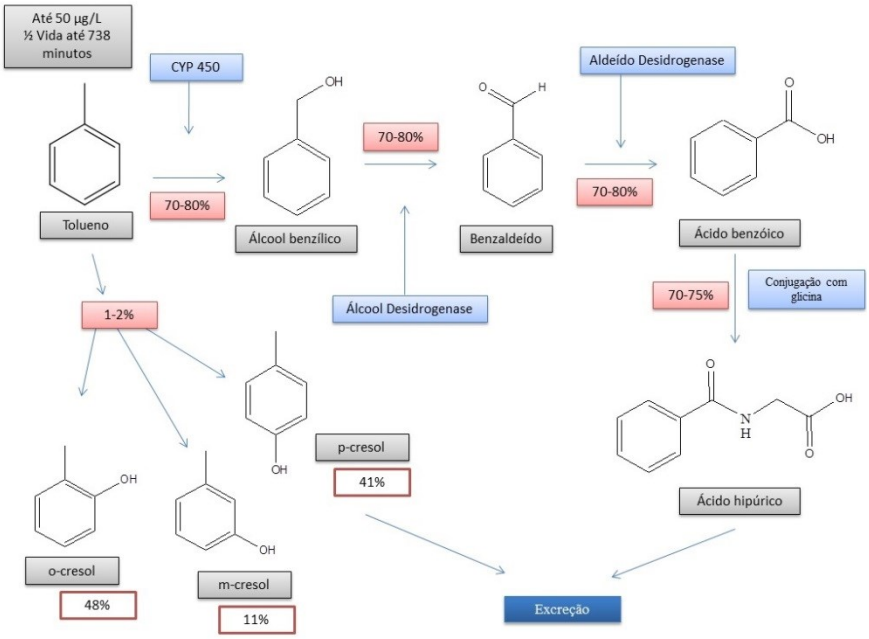
Apesar da existência de regulamentações, podemos observar que ainda assim existem trabalhadores sendo expostos a níveis superiores aos aceitáveis. Conforme um estudo de Jahangiri et al., (2014), ao analisar a presença de hidrocarbonetos presentes no ar do local de trabalho, uma empresa de pulverização de tintas automotivas, perceberam que os equipamentos de proteção individual utilizados pelos trabalhadores não estavam adequados, pois se observou que benzeno ultrapassava o filtro dos respiradores; bem como os índices de benzeno, tolueno e xileno presentes no local do trabalho, estavam significativamente acima do permitido pelo órgão regulamentar, a ACGIH.

Dentre as misturas de solventes orgânicos que são utilizados em processos industriais destaca-se a gasolina, majoritariamente composta de solventes orgânicos voláteis como benzeno, xileno e tolueno. Visto que postos de gasolina estão localizados em ambientes abertos, este seria um grande colaborador para poluição do ar externo por compostos orgânicos voláteis e um potencial risco contra a saúde dos trabalhadores e da população. O tolueno, constituinte da gasolina, é um potencial contribuidor para os problemas de saúde pública e ocupacional, sendo este solvente o nosso alvo de estudo nesse trabalho (WATKINS; KLAASSEN, 2012).

## 3.2 Tolueno

O tolueno ou metilbenzeno (Fig. 1) é um solvente orgânico com significativa lipossolubilidade e volatilidade, estando presente em tintas, colas, adesivo e, principalmente, na gasolina. É absorvido pela via pulmonar, sendo que 40% são absorvidos rapidamente pelos pulmões após a exposição, atingindo um equilíbrio em alguns minutos, se mantendo constante até o fim da exposição. Além disso, a realização de esforços físicos, aliados a uma temperatura elevada no local de exposição causam aumento significativo na taxa de absorção (WATKINS; KLAASSEN, 2012).

**Figura 1.** Estrutura química do Tolueno e sua biotransformação.




**Fonte**: Software ChemDraw Ultra 8.0; ACGIH (2014) e Environmental Protection Agency (EPA, 2005).

Sua distribuição ocorre rapidamente, iniciando pelos tecidos vascularizados, seguindo para os tecidos moles e, por último, sua distribuição mais lenta para tecido adiposo e medula óssea. Algumas características físico-químicas do tolueno são apresentadas na tabela 1, sendo que devido às suas características lipofílicas, consegue ultrapassar as membranas biológicas, inclusive a barreira hemato-encefalica, exercendo efeitos no Sistema Nervoso Central (SNC). Grande parte do tolueno absorvido sofre biotransformação hepática (ANDERSSON et al., 1983; KENYON et al., 2008).

A principal rota de entrada do tolueno é pela via pulmonar, assim sua acumulação ocorre no trato respiratório, no entanto a interação com outros agentes podem modificar seu comportamento (ROBERTS, S. M. et al., 2018). Variações no ambiente e fatores fisiológicos de cada indivíduo podem modular a taxa de absorção do solvente, parâmetros estes comprovados pela utilização de modelos *in vivo* e modelagem farmacocinética matemática (MARCHAND et al., 2015; TARDIF et al., 1995).

O SNC é o alvo principal da ação do tolueno, sendo a área mais afetada o cerebelo. Morata et al., (1997), observou que trabalhadores de impressora em rotogravura apresentavam perda auditiva significativa, comprovada por exames de audiometria, e que este efeito seria devido às exposições aos ruídos e ao tolueno. Corroborando a isso, estudos *in vitro* e *in vivo* demonstraram que o tolueno causa perda auditiva por induzir disfunção coclear, pela diminuição das células ciliadas externas (LIU; RAO; FECHTER, 1997).

Exposições crônicas ao tolueno geram também hepatotoxicidade, conforme constatou um estudo de Carvalho et al., (2006), que avaliou trabalhadores de uma refinaria de petróleo. Os autores observaram através de exames de alanina aminotransferase (ALT) e gama-glutamiltransferase (GGT), que o grupo de trabalhadores da refinaria apresentava uma prevalência maior de alterações hepáticas em relação a um grupo de pessoas externas. Já quando utilizado ratos Wistar, Tas et al., (2011), demonstram que a exposição inalatória ao tolueno aumenta significativamente os níveis de ALT, AST e Malondealdeído em amostras de fígado e sangue dos ratos, ratificando o efeito hepatotóxico do tolueno.

A exposição ao tolueno também é responsável por gerar danos renais. No trabalho de Ana-Lilia et al., (2006), foi encontrado que os níveis urinários de o-cresol estavam bastante elevados em trabalhadores, bem como a atividade da enzima N-acetil-beta-D-glucosaminidase (NAG), uma enzima utilizada como marcador da função renal. Ahmadizadeh et al., (2014), utilizando ratos expostos ao tolueno, demonstraram que tolueno gerou lesão renal bem como elevação dos niveis de nitrogênio uréico e creatinina e diminuição dos niveis GSH. Com base

nisso, os autores apontam o tolueno como responsável por induzir lesão em células tubulares proximais, e que o possível mecanismo de ação possa ser devido a um estresse oxidativo, uma vez que observaram diminuição dos niveis de GSH no rim.

Como já discutido, o tolueno possui ação ao nível de SNC, promovendo alterações neurocomportamentais e alterando os niveis de alguns neurotransmissores (OGA; CAMARGO; BATISTUTO, 2008). Tais relatos são sustentados com efeitos em curto prazo. Porém os mecanismos pelos quais o tolueno atua foram pouco investigados em modelos *in vivo*. Tal estudo se faz necessário para que possa elucidar as vias de neurotoxicidade mediadas pela exposição ao tolueno, principalmente com exposições crônicas, bem como direcionar estratégias para combater os efeitos tóxicos.

**Tabela 1.** Características e parâmetros físico-químicos do tolueno

| Propriedade/Característica | Tolueno |
|---|---|
| **IUPAC** | Metilbenzeno |
| **Número CAS** | 108-88-3 |
| **Fórmula molecular** | $C_6H_5CH_3$ |
| **Coeficiente de Partição n-octanol/água** | Log kow – 2,73 |
| **Aspecto** | Límpido e incolor |
| **Solubilidade** | Solúvel em solventes orgânicos, em água 587 mg/L |
| **Densidade** | 0,871 g/mL |
| **Ponto de ebulição** | 110,6 ºC |
| **Massa molar** | 92,14 g/mol |
| **Coeficiente de Partição - Partículas suspensas no ar** | 1591.8 |
| **Pressão de vapor** | 3769.3 Pa |
| **Distribuição pelo ar** | 97,7% |
| **½ vida no ar** | 57,1 horas |

**Fonte**: FISPQ - Ficha de Informações de Segurança de Produtos Químicos/Petrobras.

## 3.3 Outros alvos de ação do tolueno

O tolueno é conhecido por seus efeitos no cérebro, sendo estes semelhantes aos do etanol, promovendo efeito estimulatório na fase inicial de exposição, seguido por efeitos depressores intensos com o passar do tempo. Por conta dessas características, muitos jovens abusam do uso do tolueno buscando efeitos estimulantes, tornando isso um grande problema de saúde pública, visto que é comprovado que exposições em longo prazo, geram efeitos neurotóxicos graves (FLANAGAN et al., 1990). Esse abuso constante pode ser explicado pelo aumento de neurotransmissores em algumas áreas do cérebro. Alguns pesquisadores demonstraram com experimentos *in vitro* que o tolueno estimula diretamente os neurônios dopaminérgicos na área tegmental ventral, assim aumentando a liberação de dopamina no núcleo accumbens cerebral e promovendo a sensação de recompensa e bem estar aos usuários (RIEGEL et al., 2007). Além disso, já foi observado que usuários crônicos de tolueno apresentavam atrofia difusa cerebral, cerebelar e do tronco encefálico, bem como alterações nas substâncias branca e cinza no cérebro (ROSENBERG et al., 1988).

Com base em estudos anteriores, pesquisadores investigaram os efeitos da exposição ao tolueno em culturas primárias de neurônios do hipocampo de rato e observaram que o tolueno causou aumento na sinalização em neurônios glutamatérgicos via receptor NMDA e diminuição na atividade de neurônios GABA (BALE, A. S. et al., 2005). Mais recentemente, foi publicado um trabalho no qual se evidenciou que a exposição crônica com 10.000 ppm de tolueno em ratos gerou déficits comportamentais que foram acompanhados por alterações na expressão da subunidade dos receptores NMDA e GABA (FURLONG et al., 2016). Outro alvo de ação descrito na literatura seria o sistema colinérgico. Assim utilizando oócitos cultivados de *Xenopus* que expressam receptores nicotínicos se avaliou o possível efeito por conta da exposição ao tolueno, no qual se comprovou que o tolueno promoveu a inibição dos receptores, o que poderia explicar os efeitos neurocomportamentais que a exposição ao tolueno promove (BALE, AMBUJA S.; SMOTHERS; WOODWARD, 2002).

Com o desenvolvimento de novos trabalhos, alguns autores investigaram os efeitos do tolueno frente à reprodução e ao desenvolvimento, como a exposição de ratos machos e fêmeas a 2.000 ppm de tolueno por duas gerações, sendo expostos inclusive durante o período de acasalamento e gravidez. Observaram que o tolueno não induziu alterações em comportamento ou reprodução, porém, inibiu o crescimento nas progênies de ambos os sexos nas duas gerações investigadas (ROBERTS, L. G.; BEVANS; SCHREINER, 2003). Já usando concentrações maiores, 8.000 e 12.000 ppm de exposição em ratas grávidas e

analisando sua prole, outros pesquisadores obtiveram como resultados que a exposição ao tolueno provocou uma diminuição significativa do ganho de peso materno e na prole, bem como o aumento do número de malformações e mortes pós-natal precoce (BOWEN, SCOTT E.; HANNIGAN, 2013). Também já foi evidenciado que metilhidroquinona e metilbenzoquinona, produtos de biotransformação do tolueno, promovem fragmentação do DNA e que tais resultados sustentam a hipótese de que o tolueno promove carcinogênese e disfunção reprodutiva (MURATA et al., 1999).

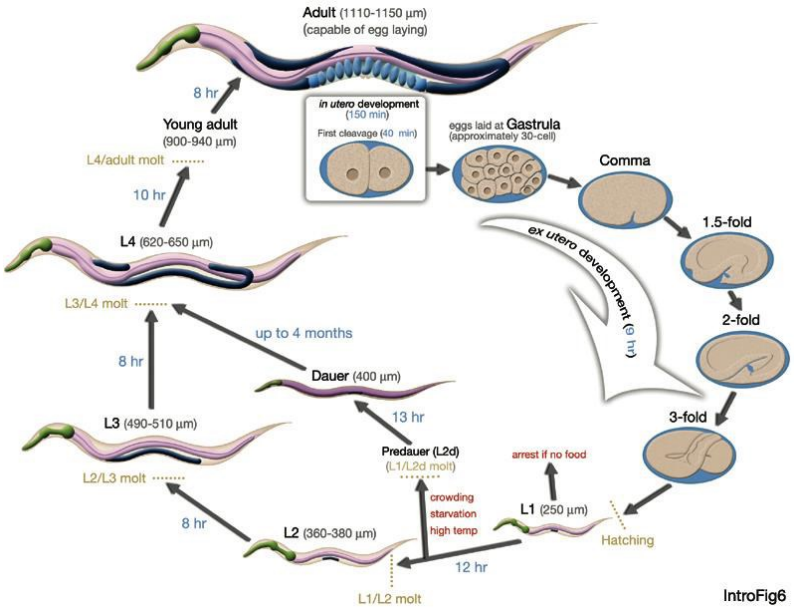
Dentre os solventes já citados, sabe-se que o benzeno é classificado como uma substância do grupo I, considerado carcinogênico pelo IARC (*international agency for research on cancer*). Já o xileno e o tolueno não são classificados desta forma, porém algumas pesquisas foram publicadas na literatura contrariando tal classificação. Em um trabalho utilizando fragmentos de DNA humano, foi descoberto que os produtos de biotransformação do tolueno promoveram dano ao DNA, ainda que com menor potência comparada aos metabólitos do benzeno (MURATA et al., 1999). Adicionalmente, uma pesquisa com ratos expostos a 1.500 ppm de tolueno por via inalatória  demonstrou que a exposição gerou um aumento dos níveis 8-hidroxi-2'-desoxiguanosina (8-OH-dG)em alguns tecidos dos ratos como pulmões, fígado, rins e na espermatogonia. Logo, pelo é possível sugerir que o tolueno causa danos ao DNA (TOKUNAGA et al., 2003).

Corroborando com esse contexto, Cassini et al., (2011), investigaram possíveis efeitos genotóxicos em trabalhadores expostos a tintas, focando em biomarcadores para tolueno. Foi demonstrado que o grupo exposto ao tolueno apresentou maiores níveis de ácido hipúrico e diminuição no índice de divisão nuclear em relação a um grupo controle não exposto. Também evidenciaram pelo ensaio cometa um índice de dano ao DNA muito maior no grupo exposto em relação ao grupo controle. O efeito genotóxico do tolueno também foi investigado em um modelo alternativo, a mosca da fruta *Drosophila melanogaster.*  Foi observado que houve um aumento nos marcadores apoptóticos e genotóxicos de maneira significativa e dose-dependente nas moscas expostas a benzeno, tolueno e xileno (SINGH et al., 2011).

## 3.4 O Modelo Alternativo *Caenorhabditis elegans*

O *Caenorhabditis elegans* (Fig. 2) é um nematóide de vida livre, que vem sendo amplamente usado como um modelo alternativo para os mais diversos tipos de pesquisa. Dentre as vantagens de sua utilização estão o ciclo de vida curto, seu tamanho pequeno, o que facilita sua manutenção e seu processo de reprodução, no qual um hermafrodita pode gerar até 300 descendentes (NAYAK; GOREE; SCHEDL, 2005).

**Figura 2.** Ciclo de vida do *C. elegans*.



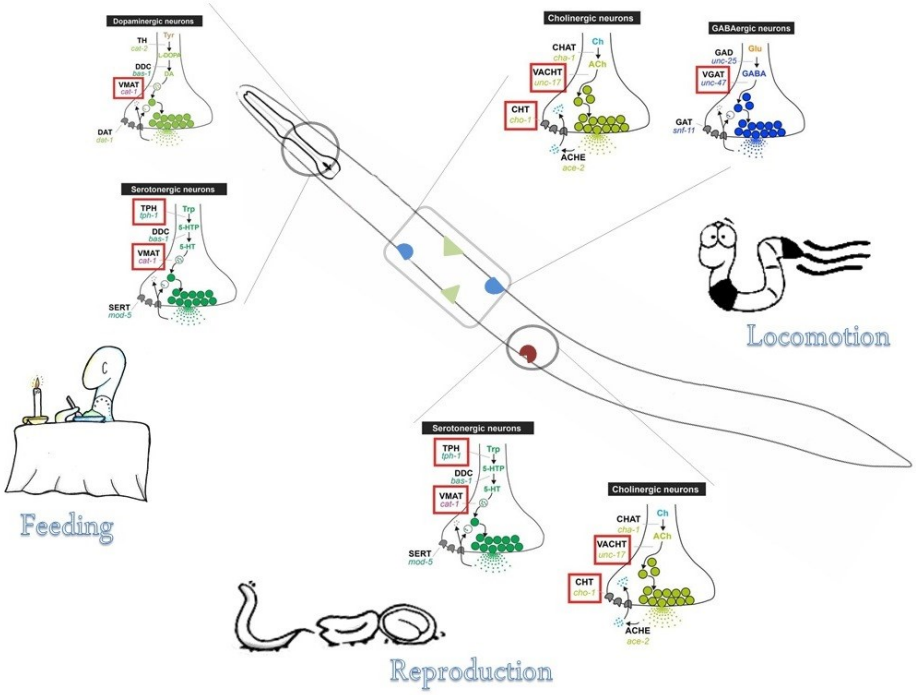

**Fonte**: Wormatlas (ALTUN; HALL, 2005).

Outra grande vantagem de sua utilização é que existem inúmeras possibilidades relacionadas à genética, já que cerca de 60-70% dos genes presentes em *C. elegans* possuem homólogos em mamíferos (KALETTA; HENGARTNER, 2006). Devido a sua característica de corpo transparente, permite com que pesquisadores possam, com apenas o uso de um microscópio, realizar estudos sobre desenvolvimento celular com maior facilidade, com a vantagem de utilizar um modelo *in vivo* sem necessidade de dissecações. Outra possibilidade é que as proteínas fluorescentes podem ser utilizadas para estudar esses processos de desenvolvimento, marcar neurônios ou produtos com GFP (Green fluorescent protein), bem como caracterizar interações de proteínas *in vivo* (CHALFIE et al., 1994).

Os nematóides hermafroditas adultos possuem 302 neurônios, sendo dentre estes neurônios do tipo dopaminérgicos, colinérgicos, serotoninérgicos e GABAérgicos (Fig. 3). Com a tecnologia da geração de transgênicos expressando GFP em proteínas de interesse, é

possível marcar essas células, permitindo a identificação de mutantes defeituosos em vários processos celulares, através de medidas da intensidade da fluorescência (HUSSON; GOTTSCHALK; LEIFER, 2013). Além disso, muitos comportamentos podem predizer o funcionamento normal da atividade de neurônios, como comportamentos de quimiotaxia, estímulos ao toque e locomoção (BARGMANN, 2006; GOODMAN, 2006).

**Figura 3.** Distribuição de alguns neurônios e sua função em *Caenorhabditis elegans.*




**Fonte**: Adaptado pelo autor de Wormatlas (ALTUN; HALL, 2005) e (SERRANO-SAIZ et al., 2017).

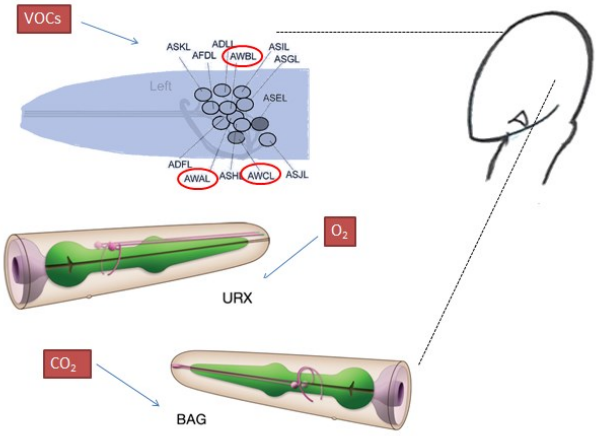
Outra vantagem de sua utilização é que seu comportamento reprodutivo possui ampla descrição na literatura e muitos parâmetros já estão bem caracterizados no modelo (SCHAFER, 2005; SOMMER, 2005). Como já dito anteriormente, há a possibilidade do uso da fluorescência para estudos nas células germinativas em *C.elegans,* o que facilita ainda mais os estudos em biologia celular e do desenvolvimento, visto que há a possibilidade da utilização de tecnologia de modificação genética para a expressão de proteínas fluorescentes em sua linha germinal, que aliado a sua estrutura corporal transparente nos permite em tempo real avaliar alguma alteração com o animal ainda vivo (GREEN et al., 2008; HUBBARD; GREENSTEIN, 2005; KIMBLE; CRITTENDEN, 2005; STROME, 2005).

Com base nesses dados, se torna necessário que haja maiores informações quanto aos efeitos do tolueno sobre reprodução. Cabe apontar que trabalhos avaliando tais efeitos do tolueno em *Caenorhabditis elegans* são muito pouco explorados atualmente. Considerando que o modelo possui um sistema reprodutivo bem definido, isso o torna uma ferramenta interessante para que os mecanismos dos efeitos do tolueno sejam elucidados.

Os vermes fazem trocas gasosas, mas não possuem um sistema respiratório. Estes animais apresentam neurônios específicos, URX e BAG (Fig. 4), que sinalizam esse processo, que ocorre via difusão por poros presentes em sua cutícula (ATKINSON, 1980). Outra característica dos vermes é o reconhecimento de diversas classes de compostos voláteis via neurônios da classe amphid, AWA; AWB e AWC, podendo ou não modular comportamentos distintos (BARGMANN; HARTWIEG; HORVITZ, 1993). Por exemplo, usando *Caenorhabditis elegans* como modelo de estudo, alguns pesquisadores investigaram os efeitos da exposição aérea ao tolueno e etanol. Foi evidenciado que apesar da semelhança entre seus efeitos apontados na literatura, diferentes genes, são responsáveis por induzir alterações comportamentais, *slo-1* e *rab-3* necessários para efeitos do etanol, já alterações pelo tolueno são independentes destes genes, logo a exposição a eles gerou efeitos distintos na locomoção (DAVIES et al., 2012). Entretanto, nenhum outro estudo para investigar estes achados mais a fundo foram realizados.

Diante das características apresentadas, o modelo escolhido para realização do trabalho encaixa-se perfeitamente ao que planejamos estudar e adequado para desenvolver todas as investigações acerca dos efeitos que o tolueno possa vir a demonstrar.

**Figura 4.** Neurônios envolvidos no reconhecimento de gases em *Caenorhabditis elegans.*




**Fonte**: Adaptado pelo autor de Wormatlas (ALTUN; HALL, 2005)

# 4 Manuscrito

## Airborne Toluene exposure promotes germline apoptosis and neurobehavioral alterations by neuronal damage pathway in *Caenorhabditis elegans*

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## Abstract

Toluene is one of the organic solvents present in gasoline, has a high degree of volatility, and it is absorbed mainly by the pulmonary route and easily reaches the central nervous system promoting toxic action. The toxicity of toluene has been described but not well stablished. The present work aimed to evaluate airborne exposure to toluene in the *in vivo* model *Caenorhabditis elegans*, in order to validate it for this type of exposure and elucidate possible mechanisms of toxicity of this solvent. Worms at the first or fourth larval stages were exposed to toluene for 48 or 24 hours, respectively, in a laboratory-developed vapor chamber at concentrations of 450; 850; 1,250 and 1,800 ppm. We observed an increase in worm's mortality and a significant developmental delay in a concentration-dependent manner. An increase in the apoptotic events in germline cells was evidenced which corroborated with the reduction of the reproductive rate. In addition, toluene promoted significant behavioral alteration in swimming movements and in radial locomotion which was associated to changes in the fluorescence intensity and morphology of GABAergic and cholinergic neurons. We can conclude that toluene presented a significant toxicity in *C. elegans* through the induction of apoptosis and neuronal damage.


Keywords: Toluene, reprotoxicity, locomotion, volatile, gasoline.

## Introduction

Organic solvents refer to a group of chemical substances widely used in industrials processes, which main characteristics are the high volatility and lipossolubility, since the beginning of their use and also nowadays these solvents have been implicated in various health problems of occupational origin (Carlini et al., 1988; Thetkathuek et al., 2015). Currently, the industries are the main contributors to atmospheric pollution; however, gasoline components, their production and combustion, generate thousands of aromatic volatile molecules that can have a strong impact to human health, with carcinogenic potential (Montero-Montoya et al., 2018).

The gasoline has many solvents in its composition, being mainly aromatics hydrocarbons, of which we highlight toluene. This is an organic solvent present in many industrial and commercial products such as gasoline and paint thinners. Toluene has great volatility and therefore its main route of absorption is through the respiratory system, but remarkably its lipossolubility provides cutaneous absorption (Kang et al., 2005). Due to this lipophilic nature, toluene can easily cross the biologic membranes, including the blood-brain barrier, exerting negative impact in the central nervous system (SNC) (Eisenberg, 2003).

Neurotoxicity of toluene is well-known effect reported in scientific field. Chronic exposures are responsible for generating dysfunction or atrophy in cerebellum and hippocampus (Deleu and Hanssens, 2000; Morata et al., 1993; Morata et al., 1997). Acute exposures to toluene are responsible by promoting behavioral alterations as lethargy, sleep, memory loss and locomotor defects in mammals and invertebrates (Braunscheidel et al., 2017; Davies et al., 2012; Thetkathuek et al., 2015), but how and which neurons are affected still remains little explored.

In addition, toluene has also been shown to alter reproduction and development in animals. Female rats and their descendants exposed to toluene have shown to developmental delay (Gospe et al., 1994). Exposure to toluene has been revealed to promote a significant decrease of maternal and offspring body weight, as well as an increase in the number of malformations and early postnatal deaths (Bowen and Hannigan, 2013). Toluene metabolites have been suggested to promote DNA damage, indicating carcinogenic promotion and reproductive dysfunction (Murata et al., 1999).

Unlike benzene, which is classified as a genotoxic and carcinogenic substance by IARC (*international agency for research on cancer*), toluene has not been considered carcinogenic. However, there are *ex vivo* (Cassini et al., 2011), *in vitro* (Murata et al., 1999)

and *in vivo* (Tokunaga et al., 2003) studies contradicting such classification, and demonstrating that toluene presents genotoxic potential.

For these reasons, it is necessary to deeply evaluate and evidence the effects of toluene to understand its mechanisms of action. However, the use of a proper route of exposure is a constant limitation of these studies. Therefore, a useful experimental model that follows the 3R policy in toxicology research and that also applies the proper airborne exposure is invaluable. Among the alternative/complementary models is the nematode *Caenorhabditis elegans,* which has been used due the high genetic homology with mammals, besides the conservation of metabolic and neurotransmission pathways (Kaletta and Hengartner, 2006). In addition, this worm has a short life cycle (~21 days) which speeds up the long-term effect studies; has easy culture and maintenance protocols; body transparency that allows the *in vivo* observation of cellular proteins by tagging them with fluorescent proteins; and well established reproductive and nervous systems (Riddle et al., 1997). Remarkably, the *C. elegans* nervous system comprises 302 neurons that can be marked, for instance, with a Green Fluorescence Protein (GFP) and observed by fluorescence microscopy allowing the observation of neuronal degeneration processes induced by toxicants as pesticides, mycotoxins and metals (González-Hunt et al., 2014; McCarthy et al., 2004; Moyson et al., 2018; Rappold et al., 2011; Wang and Xing, 2008; White et al., 1986; Xing et al., 2009; Xu et al., 2017; Zhou et al., 2013). Notably, worms have neurons that signal levels of internal and environmental $CO_2$ (BAG and AFD neurons) (Bretscher et al., 2011) and others that are sensitive to the presence of $O_2$ (URX, AQR and PQR neurons) (Carrillo et al., 2013), which are responsible for signaling the need for gas exchange. Nematodes do not have a respiratory system; they perform their gas exchange with the environment via diffusion through the tissues or through pores present in their cuticle (Atkinson, 1980), and some studies have shown that worms can respond to volatile organic compounds (VOCs) (Bargmann et al., 1993; Sengupta et al., 1993).

In view of the above considerations, we evaluated the effects of airborne exposure to toluene in the *C. elegans* model and correlate them to findings in other experimental models, thus validating this nematode to this end. We also investigated the neurotoxic effects of toluene and a possible reprotoxicity, as this has not been evidenced *in vivo* yet.

## Materials and Methods

## Reagents and strains

Toluene in its liquid form was purchased from Sigma-Aldrich (St. Louis, MO, USA), with degree of purity of 99%. The strains used in this work were wildtype N2 Bristol, MD701 (bcIs39 [lin-7p::ced-1::GFP + lin-15(+)]), EG1285 (oxIs12 [*unc-47p*::GFP + *lin-15*(+)]) and LX929 (vsIs48 [*unc-17*::GFP]), which were obtained from the Caenorhabditis Genetics Center (CGC University of Minnesota, MN, USA).

## Worm's maintenance and synchronization

All strains were maintained in the petri dishes with nematode growth medium (NGM) seeded with *Escherichia coli* (*E. coli*) OP50 at 20 ºC in an incubator. Worms had their larval stage synchronized from adult pregnant hermaphrodite nematodes, which were treated with lysis solution (1mL of 10M NaOH (0,25%) + 4mL of sodium hypochlorite (1%) + 5mL of $H_2O$) (Stiernagle, 2006). The released eggs were washed with M9 buffer (22.04 mM $KH_2PO_4$, 42.26 mM $Na_2HPO_4$, 85.56 mM NaCl, 1 mM $MgSO_4$, sterilized by autoclaving) and left in this buffer overnight at 20 ºC. After 14 hours, the eggs hatched and released the first larval worms (L1), which were used for exposure or were grown to L4 stage. All the steps were performed at a controlled temperature of 20–22 ºC.

## Validation of the concentrations of toluene in the chamber

For analyzes a passive sampler SKC VOC 575-002 series was used. The sampler was placed inside the chamber and left in contact with toluene for a period of 48 hours in a room with temperature control at 20-21ºC, after this period the sampler was stored at <4ºC, according to its manual. Followed analysis, desorption of the toluene from the sampler was performed using 2.5 mL of dichloromethane and mixed for 30 min in a shaker. The desorbed toluene was transferred to a vial, and 1 µL was injected into a Gas Chromatography with flame ionization detector (GC-FID; Varian, Middleburg, The Netherlands). The chromatography conditions were: OV-1 column (30 m x 0.32 mm x 1 µm), carrier gas (helium) flow rate of 4 mL $min^{-1}$, using temperature gradient (35 °C for 7 min, an increase of 10 °C per min up to 90 °C, which was maintained for 4 min, and after that increase of 30 °C per min up to 150 °C, and it was maintained until the end of the analysis which was 15 min). The retention time was 11.8 min. The limit of quantification was 0.19 µg $mL^{-1}$ (Figure S2).

## Exposure

2,000 worms at the first larval stage (L1) or larval stage four (L4) were transferred to NGM plates with *E.coli* OP50 in duplicates. These plates were placed inside of a glass vapor chamber developed by our group based on a previous study (Davies et al., 2012), which was placed into a fume hood chamber with air flow. Inside the airborne chamber, toluene was pipetted to a filter paper at different volumes to obtain different concentrations. To calculate the final concentrations, we have used a Gas Chromatography with flame ionization detector (GC-FID; Varian, Middleburg, The Netherlands) (Nelson, 1971). The chamber was sealed and a fan placed within was switched on to evaporate toluene. Oxygen levels were measured with **KXL-803 Oxygen meter equipment**, to verify that hypoxia was not an interfering variable. The worms were chronically or sub-chronically submitted to these different concentrations for 48 hours (from L1 stage) or 24 hours (from L4 stage), respectively, in a temperature-controlled environment at 22ºC (Figure 1). We will not describe here the developed airborne chamber in details as it has been submitted to registry as a utility model.

## Lethality assay

A concentration-response curve was performed at concentrations of 450; 850; 1,250 and 1,800 ppm of toluene in L1 worms. After 48 hours of exposure, the plates were removed from the chamber and the live worms were scored, in order to calculate the $LC_{50}$. To assay L4 worms, they were exposed to two concentrations, 1,250 and 1,800 ppm for 24 hours (to avoid progeny). The independent experiments were done in duplicates and repeated at least for four times.

## Worms Body length

At 48 hours of exposure, ten worms from each group were separated randomly to be photographed. The worms were transferred to a glass slide and paralyzed with levamisole solution (57.84 mM) to obtain the images. The pictures were processed in Image J program, in which we performed a quantification of the worm's body length from the head to tail. The independent experiments were done in duplicates and repeated at least for four times.

## Apoptosis assay

To this assay, we have used the strain MD701 [lin-7p::ced-1::GFP + lin-15(+)] that possess the CED-1 marked with green fluorescent protein (GFP). CED-1 is a caspase protein present in the apoptosis pathway and in the worms is expressed in gonadal arms of the

germline cells that suffer apoptotic events. Worms at L1 stage were transferred to plates with *E.coli* OP50 for 48 hours until reaching the L4 stage. Then, these nematodes were exposed for 24 hours to toluene at all concentrations. Ten worms of each treatment group and control group were separated to be photographed in a fluorescent microscope EVOS FLoid Cell Imaging Station (Thermo Fisher Scientific) to obtain representative images. We then scored the number of apoptotic cells in each group (Chalfie et al., 1994; Gartner et al., 2008). The experiment was done in L4, for better observation of germline development in this larval stage. The independent experiments were done in duplicates and repeated at least three times.

## Reproduction Evaluation

To verify the worm's reproduction, we evaluated egg laying, brood size and the rate of viable eggs. After 48 hours of exposure, one individual worm from each treatment (in triplicates), were transferred to petri plates with *E.coli* OP50 and during fertile period (four to five days), the number of eggs laid was counted. In the next day the number of hatched larvae was scored. Based on these counting, we calculated the viability percentage of the eggs from exposed worms (Figure 1). As a control MD701 worms were analyzed in parallel with N2. The independent experiments were done in triplicates and repeated at least three times.

## Swimming Assay

We scored the number of swimming movements in the worms according to Ghosh and Emmons, (2008) with some adaptations. Worms exposed or not to toluene from L1 stage were transferred to a twelve-well plate containing 200 µL of M9 and were observed in a stereomicroscope Olympus SZ2-LGB model. The number of body bends was counted for one minute and six worms per group were observed. The independent experiments were repeated at least four times.

## Radial locomotion

This assay was performed according to Han et al., (2016), with some alterations. Six worms of each group were transferred to a NGM plate of 5.5 cm of diameter, without bacteria, in which the center of plate was marked with a circle. Using a worm pick we transferred worms one at a time, positioning nematodes at the center of the plate. After five minutes we measured the crawling distance from the center with the aid of a stereomicroscope Olympus SZ2-LGB. The independent experiments were repeated at least for four times.

## Locomotion speed and swimming distance assay

Animals at the first larval stage (L1) exposed to 48 hours or worms at the fourth larval stage (L4) exposed for 24 hours with toluene were transferred to a six-well plate, adapted by our laboratory, containing 30 µL of M9, and a 30 seconds recording (30 frame per second) was captured in a stereo microscope Olympus SZ2-LGB using a Fujifilm FinePix JX500 camera. Tracker video analysis and modeling tool software was then used to track the speed of each worm. An average speed and locomotion distance for each group of 6 animals was calculated. At least three independent experiments were performed.

## Neuronal assessment

Worms at L1 stage were transferred to plates NGM with *E.coli* OP50 for 48 hours to develop and reach L4 stage. Then, these nematodes were exposed for 24 hours to toluene and, at the end of the exposure, 10 worms of each group were photographed in a fluorescence microscope EVOS FLoid Cell Imaging Station (Thermo Fisher Scientific). Representative images were analyzed in the software Image J to quantify the fluorescence intensity of these worms. The data was expressed in total sum of pixels. Alterations in transgenic strains LX929 and EG1285 were evaluated according to the criteria used in previous studies (Negga et al., 2011; Negga et al., 2012; Zlotkowski et al., 2013). The independent experiments were repeated at least three times.

## Pharyngeal Pumping

The pharyngeal pumping rate is defined as the number of contraction and relaxation of the pharynx. Ten worms at L1 or L4 stages were treated with the higher concentrations of toluene as described previously. The pharyngeal pumping rate of worms was count for 30s using a stereo microscope Olympus SZ2-LGB model. This assay was performed to eliminate the hypothesis that toluene promotes alterations in the pharynx leading to food deprivation and then interfering in our results. The independent experiments were repeated at least three times.

## *E.coli* growth assay in the presence of toluene

We performed an assay to detect whether toluene affected the growth of *E.coli* OP50, and causes a consequent food deprivation to worms chronically exposed to toluene. We used acrylic cuvettes in which 1 mL of Luria-Bertani (LB) medium was added to 50 µL of bacterial

suspension and then exposed for 48 hours to toluene by using the same airborne chamber developed in our laboratory. After exposure, these cuvettes were read at 600 nm to evaluate their changes in optic density (OD) in a SpectraMax M5 Series Multi-Mode Microplate Reader. The independent experiments were done in duplicates and repeated at least three times.

### Statistical analysis

Statistical analysis was generated with GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). The data were expressing as media ± standard error of media (SEM). Differences between the treated groups and control groups were determinate using parametric analyses of variance of one or two pathway (ANOVA), followed by Tukey's *post-hoc* test, and non-parametric analyses were realized with method of Kruskal-Wallis, followed by Dunn's *post hoc* test. A value of $p < 0.05$ was considered significative.

## Results

## Airborne toluene exposure lethality

The results demonstrate that toluene caused a high toxicity to the worms in a concentration-dependent manner when worms were exposed at both larval stages, L1 and L4. As depicted in Figure 2, all concentrations caused a significant reduction in the worm's survival, being the concentration of 1,800 ppm the most lethal. We verified that L4 worms presented a slightly greater resistance if compared with L1s exposed at the same concentrations. $LC_{50}$ for L1 exposure was 1,340 ppm. For L4, as we did not perform a concentration-response curve, we could not calculate $LC_{50}$. The survival test in L4 was performed to confirm toluene toxicity, as L1 exposed worm's demonstrated developmental delay (Figure 3) and therefore neuronal evaluation would not be accurate.

## Pro-apoptotic effect of toluene in germline cells

MD701 exposed to 450 and 850 ppm toluene depicted a greater number of apoptotic corpses in comparison to control group (Figure 4). Curiously, at high concentrations, 1,250 and 1,800 ppm, worms presented a smaller number of apoptotic processes when comparing with other concentrations of toluene.

## Reprotoxicity following toluene exposure

Based on previous results, in which was demonstrated that toluene exposure increased the number of apoptotic corpses in germline cells, we sought to evaluate the impact this effect in the parameter of reproduction. The results demonstrate that all tested concentrations of toluene reduced the number of eggs laid as well as the hatched larvae in wild type worms (Figure 5a); however, in strain MD701, only the number of hatched larvae was reduced in a significant manner (Figure 5a). Analyzing the viability results (Figure 5b), all concentrations promoted a greater number of unviable eggs in exposed N2 worms, whereas MD701 mutants depicted a reduction in viable eggs only when exposed at 850 ppm

## Toluene induces neurobehavioral alterations

Next, we performed two locomotor behaviors assay. First, we scored the number of swimming movements of the worms in liquid medium, and it was observed that worms from all the groups presented a significant reduction in their swimming movements, when exposed from L1 or L4 stages (Figure 6a). Following, we observed a reduction in the treated worm's capacity to move from the center to the plate's edges in the radial locomotion assay for both exposure protocols (Figure 6b). We also verified that worms exposed at L1 or L4 stages to toluene presented a significant reduction in their mean velocity (Figure 7a and c) and swimming distance (Figure 7b and d). Corroborating with the changes in the number of movements and reduced distance, we have also observed that the pattern of the sinusoidal locomotion was altered, as demonstrated in Supplementary Figure 1.

## GABAergic damage induced by toluene is more pronounced than cholinergic

Based on neurobehavioral results, we decided to elucidate which neurons would be affected by toluene exposure that could explain the behavioral alterations. In Figure 8, we demonstrate that L4 worms exposed to 1,800 ppm for 24 hours present significant fluorescence reduction of GABAergic neurons. Furthermore, we have observed alterations in neuronal morphology and degeneration of some ventral neurons (Figure 8). Analyzing cholinergic neurons, we have also verified reduction in the neurons fluorescence from nematodes exposed to 1,800 ppm. Notably, morphologic alterations such as Y ramifications in motor neuron commissures were observed (Figure 9), but total absence of neurons, as evidenced in GABAergic neurons, were not observed. L4 worms were used in this assay to avoid developmental delay bias.

## Feeding endpoints by toluene exposure

To exclude the possibility that toluene would alter the *C. elegans* feeding and promote starvation, thus causing all the observed effects, we evaluated whether toluene exposure could alter pharyngeal pumping. In Suplementary Figure 1 we demonstrate that exposure to toluene did not affect this behavior in a significant manner in relation to control group. A second hypothesis was that toluene could be bactericide or bacteriostatic, thus changing the worms feeding choice. We have observed that vapor presence of toluene at high concentrations did not promote alteration in bacterial growth (Figure S2).

## Discussion

Currently we have been exposed to multiple toxics agents in the air, including the organic solvents present in gas stations, for instance. Notably, the workers are occupationally exposed and studies highlight the toxic effects of these aromatic compounds, from neurologic symptomatology to genetic alterations (Rekhadevi et al., 2010; Thetkathuek et al., 2015). Our study is contributing to the literature by demonstrating the multiple targets of action of airborne toluene exposure. First, the cholinergic and GABAergic neuronal damage evidenced *in vivo* through behavioral and imaging assays. Second, the reduction of the reproductive rate due to apoptosis in germline cells, a compelling result since its action on reproduction is controversial using other models. Since the apoptotic effect in the *C.elegans* germline is a phenomenon strictly related to DNA damage (Lans and Vermeulen, 2015), our findings could be a warning to the population and regulatory agencies, since at present this solvent is not classified as carcinogenic agent by IARC.

The results obtained in our study demonstrate that the chronic airborne exposure to toluene generates a significative decrease in worm's survival, with an $LC_{50}$ of 1,340 ppm. There are a few data regarding lethal doses of toluene for animals and humans. A study with *Metamysidopsis elongata atlantica* determined a $LC_{50}$ of 235 µg/mL (Vieira, 2004); the rat $LC_{50}$ was estimated at 26,700 ppm following 1 hour of exposure in a closed environment (Pryor et al., 1984), whereas the U.S.EPA, (2005) reported that the oral toluene $DL_{50}$ is approximately 5,000 mg/kg for rats, while that the $LC_{50}$ for inhalation exposure is 4,000 ppm.

Our work concentrations, in principle, do not mimic real exposures that the workers of gas stations are exposed since they work in an open environment and constant gas changes occur. However, we do not rule out that concentrations similar to ours can be observed in

other places, for instance, in workplace incidents (Longley et al., 1967). However, the chosen concentrations are smaller in relation those used in other models of the literature (Batis et al., 2010; Bowen et al., 2010; Bruckner and Peterson, 1981). Another aspect that should be emphasized is that the concentrations must be adjusted according to the model to be used, since there are anatomical differences to toxicological evaluations (de Lima et al., 2017; Hodson, 1985; Schneider et al., 2013).

In our work we demonstrated that the exposure to toluene also promotes a significant delay of nematodes development. This corroborates with some studies in the literature that report that some toxic agents can generate DNA damage, and consequently promote apoptosis in the germline, or even inhibit the cell cycle, thus significantly altering the worms development (Feng et al., 2016; Shin et al., 2019). Evaluating the inhalation exposure in rats, Ono et al observed that the exposure to 2,000 ppm of toluene in males and females generate significative toxic effects in the fertility and reproduction (Ono et al., 1996), while other authors observed that exposure of pregnant rats to 1,200 ppm resulted in reduced corporal weight of mother and their descendants (Thiel and Chahoud, 1997).

Furthermore, it has been reported that exposure to toluene (8,000 to 16,000 ppm) significantly reduced the growth of the offspring, including decrease in the placenta weight and fetal weight (Bowen et al., 2009). So far no putative mechanism has been reported to explain how toluene promotes these impairments. Corroborating with these results, we reported that toluene significantly reduced the eggs viability, therefore modifying the reproductive rate of the exposed animals. Considering the reprotoxicity caused by toluene, we investigated whether these effects were due to apoptosis induced by the solvent. It is known that apoptotic processes in the germline cells can occur due to the activation of a pathway composed by pro-apoptotic proteins denominated caspases. Some works have demonstrated that this process can be activated by multiple types of stress, in both pathways. One path is dependent of the activation of CEP-1/EGL-1, homologous of the protein p53 and another path independent of CEP-1, which both activate the cellular apoptotic machinery CED-9, CED-4 e CED-3 (Lettre and Hengartner, 2006). Any damage to DNA is a recognized activator of the cascade, being dependent of CEP-1, therefore genotoxic agents can induce apoptosis of germline cells (Gartner et al., 2008).

Indeed, DNA damage induced by gamma radiation exposure generates an increase in the number of apoptotic events in worms germline cells (Gartner et al., 2000). Notably, a recent study defined that deaths in the germline can be used as a reliable parameter for DNA damages (Lans and Vermeulen, 2015). Our results have shown that toluene increased the

number of apoptotic corpses in the germline, and this induced a decrease in reproductive rate of the worms. This pro-apoptotic effect caused by toluene exposure has been reported in *ex vivo* assays (Al-Ghamdi et al., 2004; Sarma et al., 2011). Based on that, we suggest that DNA damage is the putative mechanism of action behind of the increased number of apoptotic events in the germline cells, thus reducing reproductive rate. Another hypothesis could be a direct activation of some protein that can trigger the apoptotic processes pathway.

Of note, we observed that just the lower tested concentrations, 450 and 850 ppm, triggered this increase of apoptotic corpses, whereas at 1,250 and 1,800 ppm we could not detect a significative increase. Such a result, can be explained by fact that the mutant strain CED-1::GFP labels only early apoptotic corpses, and in conditions that a greater number of apoptosis events occur it may become hard to detect these cells as they must be already dead (Gartner et al., 2008; Schumacher et al., 2001; Schumacher et al., 2005). Another explanation can be that at high concentrations toluene stimulates cellular death by necrosis pathway and not by apoptosis pathway.

Another way to activate the apoptosis pathway is by starvation stress, being this pathway independent of CEP-1/EGL-1 (Salinas et al., 2006). More recently, it was discovery that the starvation stress promotes accumulation of LIN-35 protein, and this is responsible by down-regulation of CED-9, culminating with an increase of apoptotic responses (Lascarez-Lagunas et al., 2014). Therefore, starvation and hypoxia could have been involved in all the observed results and not toluene. Worms are aerobic, and live in environments with $O_2$ levels between 12-21%, and hypoxia effects in *C.elegans* occur when oxygen levels are below 4% (Gray et al., 2004). In addition, hypoxia has been implicated in damage to the nervous system of worms exposed as embryos (Pocock and Hobert, 2008) or as adult (Samokhvalov et al., 2008). Nevertheless, we observed that toluene did not change any parameter related to feeding, as *E.coli* growth was normal under the same exposure conditions and worms pharyngeal pumping were not affected by the solvent. In addition, $O_2$ levels inside the chamber were 20.6%. Therefore, we discarded the starvation and oxygen deprivation hypothesis and confirmed that the effects observed were indeed caused by toluene.

The nervous system is the main target of toluene intoxications, as evidenced that long term exposures generate serious neurotoxic effects (Flanagan et al., 1990). *In vitro* assays demonstrated that toluene directly stimulates the dopaminergic neurons in the tegmental ventral area, thus increasing dopamine release in some brain regions (Riegel et al., 2007). In another work, authors have demonstrated that individuals chronically exposed to toluene presented diffuse brain and cerebellar atrophy, as well alterations in white substance and grey

in the brain (Rosenberg et al., 1988). A subchronic exposure in rats has caused a significative neurobehavioral alteration associated to a neurochemical dysfunction as changes in dopamine and serotonin levels were found (Berenguer et al., 2003).

In the present study, toluene airborne exposure promoted a significant impairment in locomotor movements. In agreement to the fact that locomotion movements on solid medium and swimming in liquid media are specifically controlled by neuronal stimulus of the GABA and cholinergic neurons (Hobert, 2005), we have observed a decrease in the neuronal fluorescence of animals exposed to 1,800 ppm, which may indicate neuronal damage. Damage to GABA neurons due to toluene exposures has not yet been reported, only some evidences that toluene reduced the neuronal signaling by altering the expression of GABA receptor subunits (Bale et al., 2005; Furlong et al., 2016).

Our results also indicate that toluene promotes alterations in the cholinergic neuronal system, by causing alteration in the neuronal morphology such as aberrant branches in the commissures responsible for the transmission of the cholinergic stimulus. Furthermore, we have visualized ruptures in the neuronal networks in those worms exposed to 1,800 ppm. Likewise the GABAergic system, the toluene- induced cholinergic damage has been barely described in the literature (Bale et al., 2002). Based on our findings which toluene exposure induced the formation of apoptotic corpses in germline cells, we can hypothesize that this process may be occurring in these neurons, therefore affecting worm's physiological functions.

Altogether, our findings corroborate with the few data in the literature that establish toluene as a neurotoxic agent (Abbate et al., 1993; Benignus et al., 2007; Eisenberg, 2003; Thetkathuek et al., 2015). A possible damage or dysfunction in the worms neuronal system as indicated by the behavioral alterations in association with the reduced fluorescence intensity and morphological alterations, corroborates with previous findings (Berenguer et al., 2003; Thetkathuek et al., 2015), thus validating our animal model. Additionally, we have validated the vapor chamber developed by our group, reinforcing the application of this uncommon exposure via in *C. elegans* to study many other volatile molecules.

## Conclusion

Our work demonstrates that *C. elegans* model is adequate for exposures to volatile compounds, and that the developed vapor chamber is effective to expose nematodes using airborne pathway. With these tools at hand, we could characterize toluene effects that had not been reported in the literature using *in vivo* models, such as the reproductive defect due to apoptosis and neuronal damage to GABAergic and Cholinergic neurons.

The results are still to be further explored, mainly in relation to the mechanism by which toluene induces these phenomena. A possible mitochondrial dysfunction must be studied by robust techniques, and also gene expression related to the neurotransmitters systems affected must be sought. In addition, the nature of the degeneration (reversible or irreversible) will be further assessed.

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# Figures

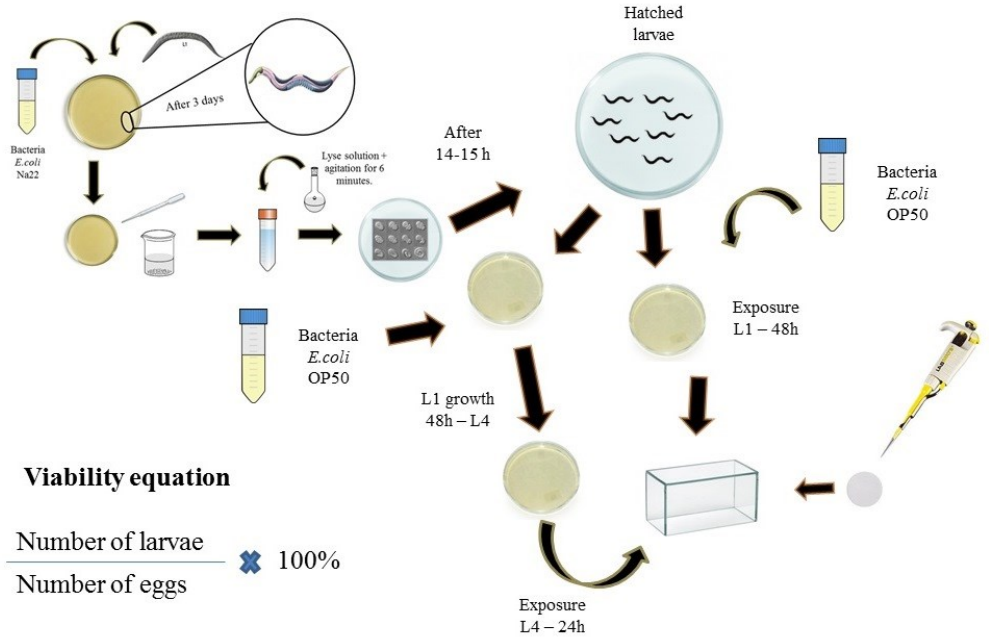


**Figure 2** Representative scheme of the experimental design of airborne exposure to toluene, reproduction viability equation.

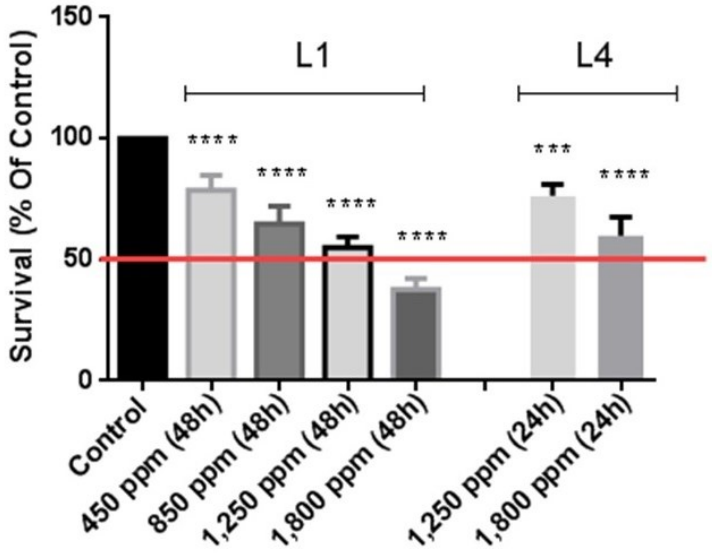


**Figure 1** Toluene concentration-response curve after airbone exposure in larval stage one (48h) and larval stage four (24h). Red line represents 50% of worm's survival. Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. Symbols show significant differences compared to the control group: *** $p$ <0.001 and **** $p$ <0.0001. $LC_{50}$ = 1,340 ppm in larval stage one.

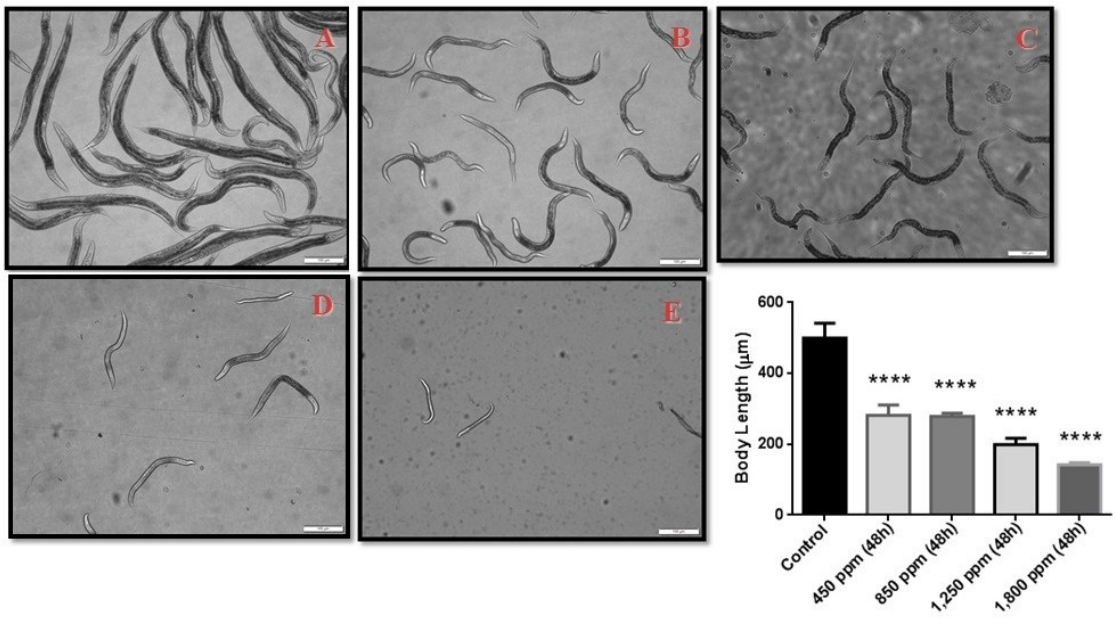


**Figure 3** Representative images of worms exposed to toluene at different concentrations. A) control group, B) 450 ppm, C) 850 ppm, D) 1,250 ppm and E) 1,800 ppm; alongside a graphic representation of the quantification of worms size. Statistical significance was determined using One-way ANOVA test, followed by Tukey's post hoc test. Asterisks indicate significant differences compared to the control group: **** p <0.0001.

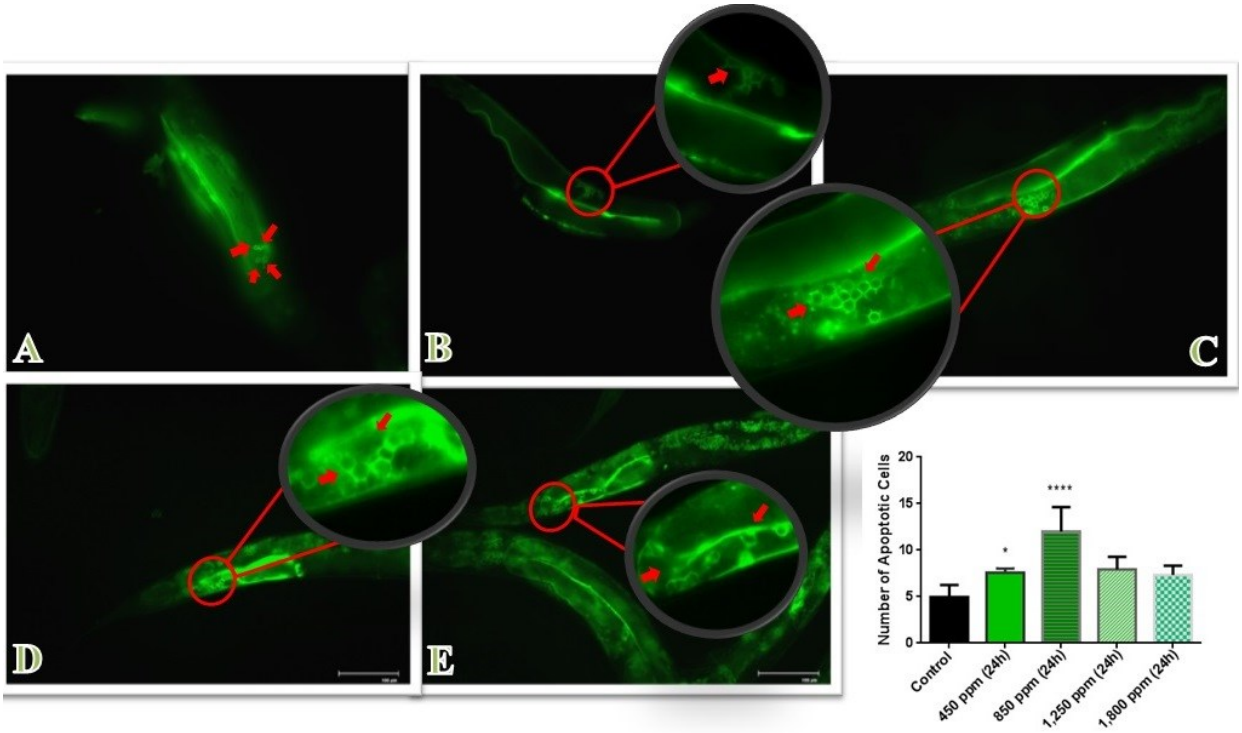


**Figure 4** Representative fluorescence microscopy images of the MD701 bcIs39 strain [lim-7p :: ced-1 :: GFP + lin-15 (+)] after 24 hours of airborne exposure to toluene. For the analysis, 10 worms per group were used. A) control group, B) 450 ppm, C) 850, D) 1,250 ppm and E) 1,800 ppm; alongside a graphical representation of the quantification of apoptotic cells. Statistical significance was determined using One-way ANOVA test, followed by Tukey's post hoc test. Asterisks indicate significant differences compared to the control group: * $p < 0.05$ and **** $p$ <0.0001.

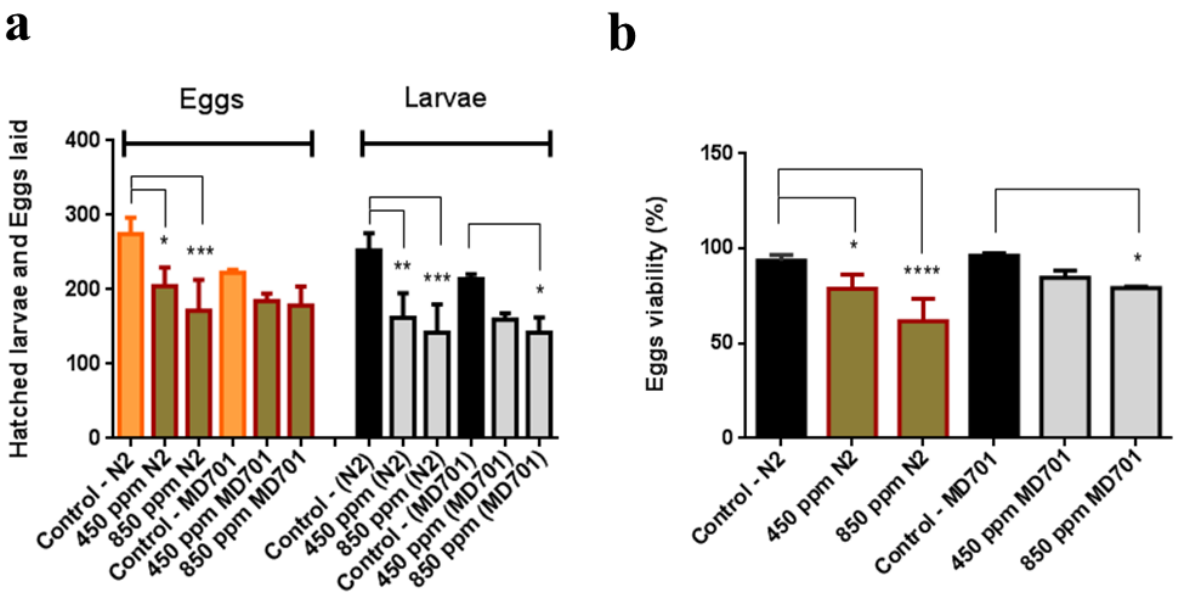


**Figure 5** Reproduction assessment after 48h to exposure to toluene. A) number of eggs laid and hatched larvae; B) rate of eggs viability. Statistical significance was determined using two-way ANOVA test, followed by Tukey's post hoc test. Symbols show significant differences compared to the control group: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ and ****,$p < 0.0001$.

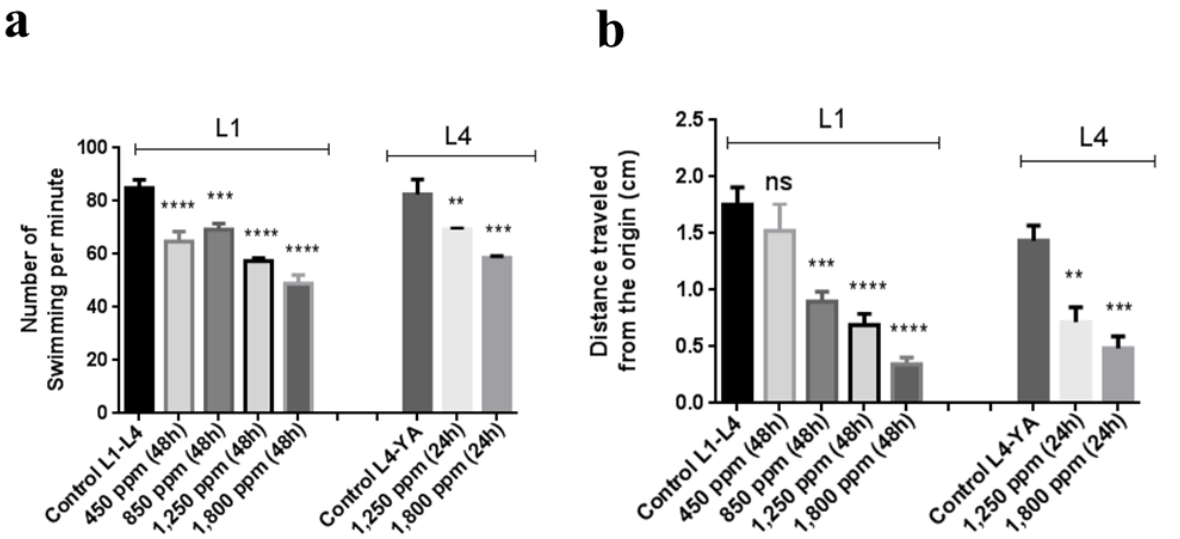


**Figure 6** Behavioral tests performed after 24 (from L4 to young adult-YA) or 48 hours (from L1) airborne exposure to toluene A) number of swimming movements; B) radial locomotion. Statistical significance was determined using one-way ANOVA, followed by Tukey's post hoc test. Symbols depict significant differences compared to the control group: ** $p < 0.01$; *** $p < 0.001$ and ****,$p < 0.0001$.

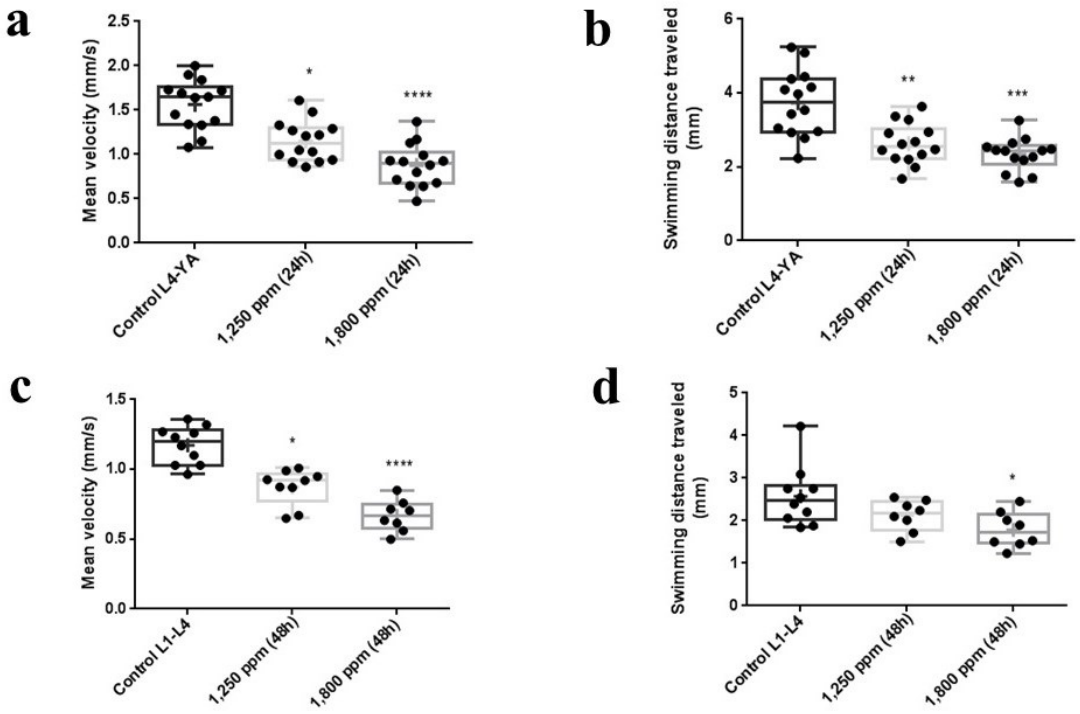


**Figure 7** Locomotion assay of worms exposed to toluene after 24 (from L4 to young adult-YA) or 48 hours (from L1) airborne exposure to toluene. Average speeds of worms in 10 s period following A) 24 h and C) 48h airborne exposure to toluene; average of swimming distance traveled B) 24 h and D) 48h airborne exposure to toluene. Significance was determined using Kruskal-Wallis test, followed by Dunn's *post hoc* test. Symbols show significant differences compared to the control group: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$.

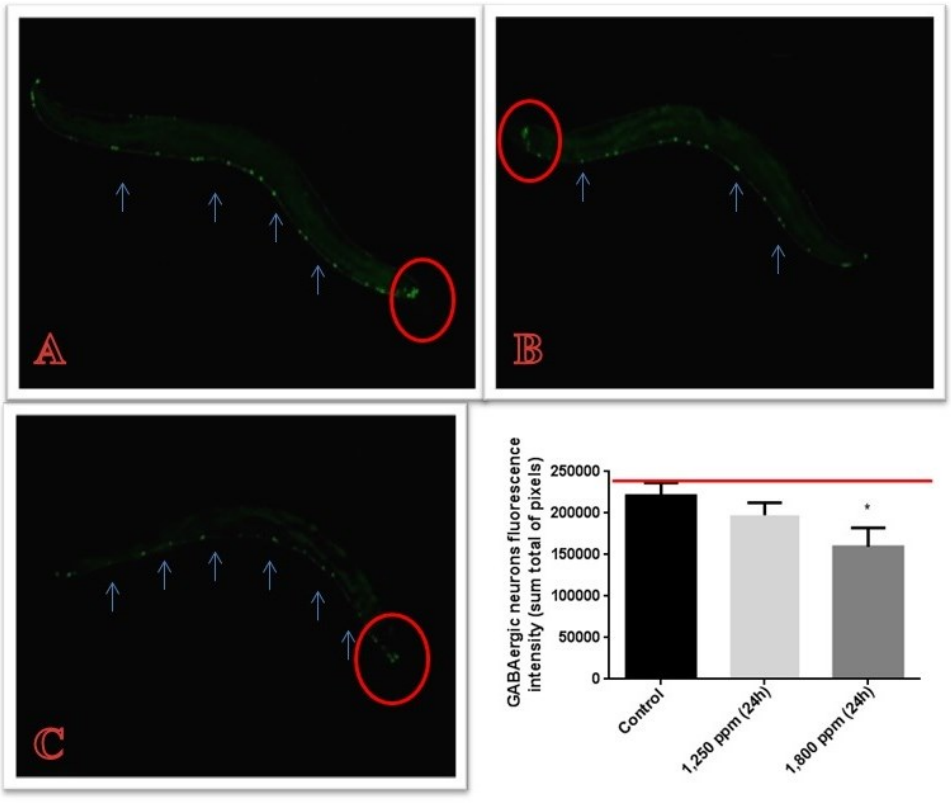

**Figure 8** Representative fluorescence microscopy images of the EG1285 oxIs12 strain [unc-47p :: GFP + lin-15 (+)] and neuronal fluorescence quantification following 24 hours of airborne exposure to toluene. Statistical significance was determined using one-way ANOVA, followed by Tukey's post hoc test. Symbols show significant differences compared to the control group: * p <0.05.

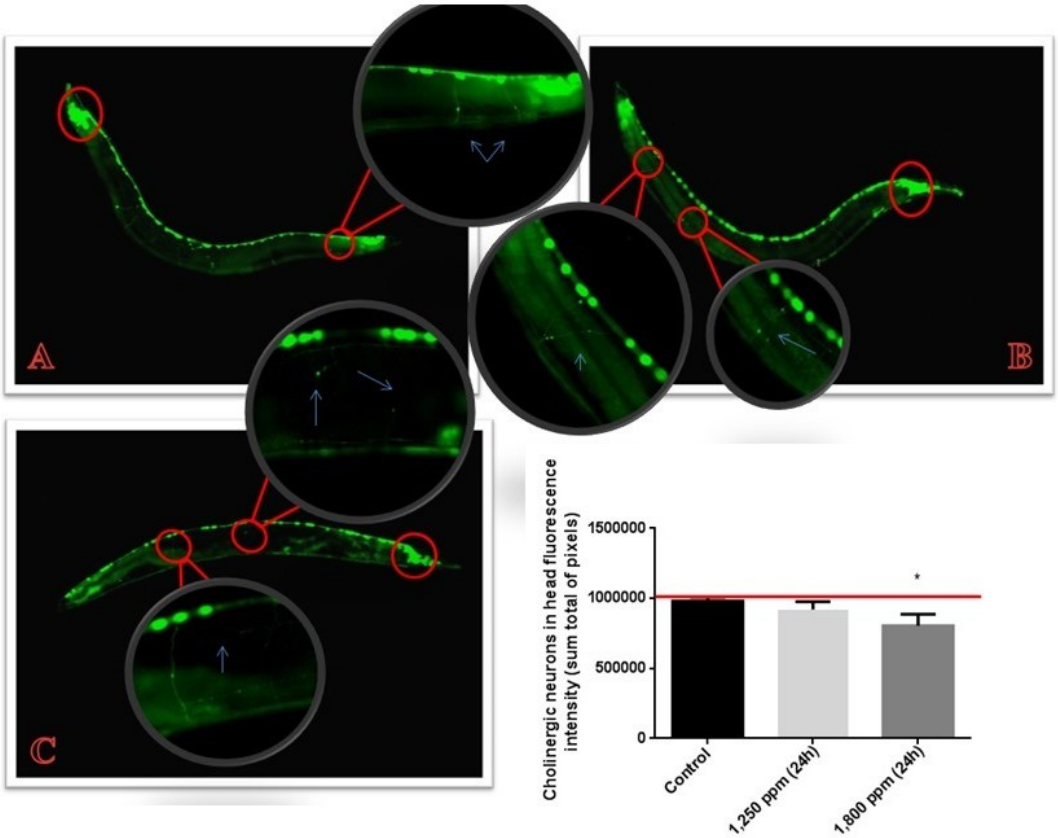


**Figure 9** Representative fluorescence images of the strain LX929 (vsIs48 [unc-17::GFP]) and neuronal fluorescence quantification after 24 hours of airborne exposure to toluene. Statistical significance was determined using one-way ANOVA, followed by Tukey's post hoc test. Asterisk indicates significant difference compared to control group: * p <0.05.

## 5. Conclusão

Este trabalho demonstrou, em um modelo *in vivo*, os efeitos tóxicos que a exposição pelo ar ao tolueno promove, sendo analisados parâmetros como letalidade, desenvolvimento, reprodução, comportamento e avaliação neuronal. Pode-se demonstrar que a exposição ao tolueno causou efeito tóxico ao *C. elegans* em todos os parâmetros avaliados. Além disso, foi possível validar o modelo experimental para estudos futuros com exposições a compostos voláteis.

Além da validação do modelo, também foi possível caracterizar os efeitos neurotóxicos do tolueno. Outro aspecto inovador foi o desenvolvimento da metodologia de exposição por via aérea nos nematoides, visto que muitos dos trabalhos publicados utilizam uma exposição oral ao tolueno. Levando em consideração que a população alvo se expõe ao solvente principalmente pela via inalatória, nosso método de exposição é mais adequado.

Foi visto que a exposição ao tolueno promoveu reprotoxicidade, por gerar um aumento nos eventos apoptóticos nas células da linhagem germinativa dos vermes, efeito este ainda não muito caracterizado como mecanismo para este solvente. Também foi possível apontar possíveis alvos da neurotoxicidade induzida pelo tolueno, uma vez que observamos alterações nos neurônios GABA e colinérgicos dos vermes, e por conta de tal alteração, foram evidenciados defeitos comportamentais na locomoção dos nematóides.

## 6. Perspectivas

Nossos resultados ainda devem ser mais aprofundados, principalmente em relação aos eventos mecanísticos envolvidos na degeneração dos neurônios estudados. Buscaremos avaliar se processos apoptóticos também estão ocorrendo nos neurônios e se estes efeitos são de natureza reversível ou irreversível.

Pretendemos descobrir mais sobre a toxicocinética do tolueno nos vermes, e posteriormente, com base em nossas hipóteses, avaliar uma possível bioacumulação neuronal do tolueno e seu efeito a nível mitocondrial. Tentaremos investigar se o tolueno poderia estar promovendo uma neurodegeneração via disfunção mitocondrial por danos na membrana ou por danos ao DNA mitocondrial. Visando uma perspectiva de mimetizar exposições que ocorrem em humanos, também iremos avaliar o grupo BTEX em conjunto, visto que no ambiente não estaríamos sendo expostos a um ou outro composto isoladamente, mas sim a um cenário mais complexo de compostos voláteis.

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# Supplementary material

## Airborne Toluene exposure promotes germline apoptosis and neurobehavioral alterations by neuronal damage pathway in *Caenorhabditis elegans.*

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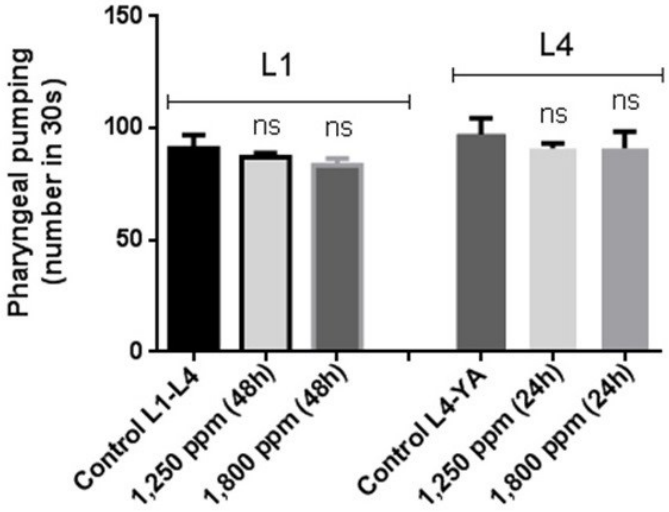
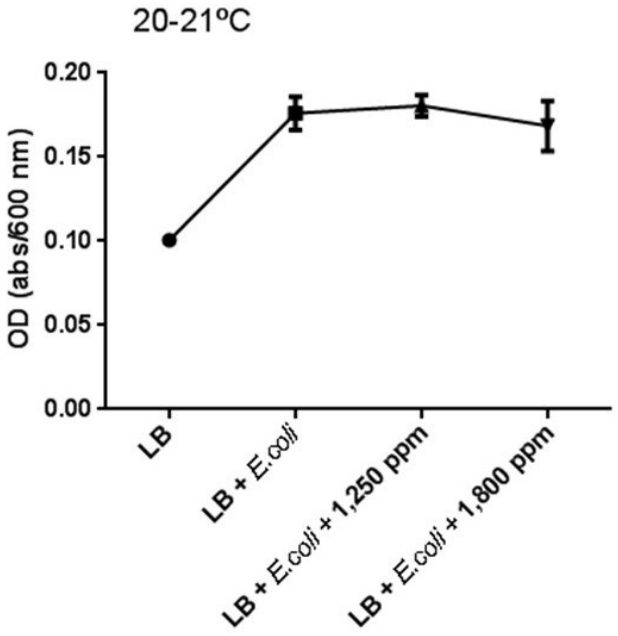
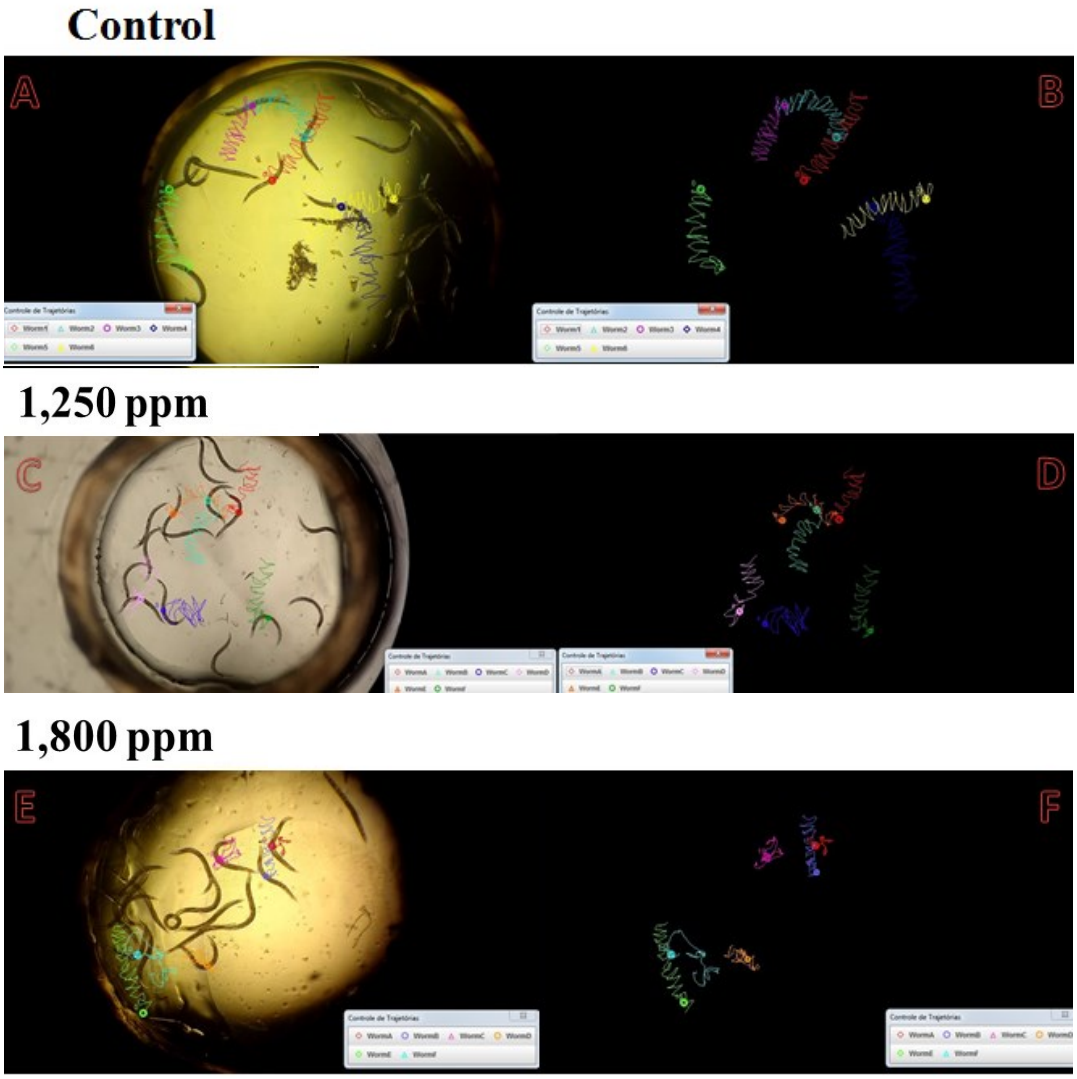


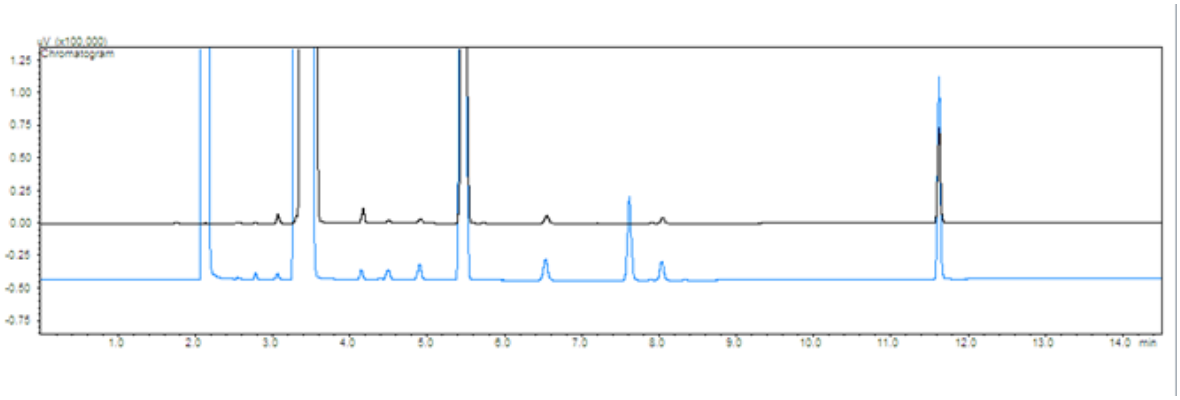

**Suplementary Figure 1.** Number of pharyngeal pumping in the worms, in larval stage one and four, after airborne exposure to toluene. Significance was determinate using One-way ANOVA, followed by Tukey's *post hoc*. No significant differences were observed between groups.




**Suplementary Figure 2.** Graphic of *E.coli* OP50 growth, after airborne exposure to toluene for 48 hours. Significance was determinate using One-way ANOVA, followed by Tukey's *post hoc*. No significant differences were observed between groups.




**Suplementary Figure 3** Microscopic images of the worm's mass center and trashes in liquid medium. Figure A and B) control group, C and D) 1.250 ppm and E and F) 1,800 ppm. A, C and E, representative images and B, D and F, isolated trajectories.




**Suplementary Figure 4** Chromatogram of the process of validating the presence of toluene in the vapor chamber. Chromatogram of toluene standard (blue line) and chromatogram of the sampler (black line). Toluene retention time: 11.8 min.