

Xylene: weight of evidence approach case study to determine the need for an extended one generation reproductive study with a developmental neurotoxicity animal cohort



L. G. Higgins ¹, F. Faulhammer ², M. Rooseboom ³, N. Aygun Kocabas ⁴, J. H. Arts ⁵, A. Cordova ⁶, E. Freeman ⁶, M. Nahar ⁶, E. Richmond ⁷, S.Schneider ², and K. Morris-Schaffer ⁶

¹Penman Consulting Ltd, Wantage, United Kingdom; ²BASF, Ludwigshafen, Germany; ³Shell Global Solutions International, The Hague, Netherlands; ⁴TotalEnergies Refining & Chemicals, Seneffe, Belgium; ⁵Nouryon, Deventer, Netherlands; ⁶Exponent, Menlo Park, CA; and ⁷Exponent, Harrogate, United Kingdom.
Conflict of Interest: The authors declare that there is no real or perceived conflict of interest. The authors are employed by entities that manufacture or distribute the material that is subject to this presentation or have received services fees from those entities.

Introduction

Xylene, an aromatic hydrocarbon organic solvent, is currently undergoing substance evaluation under Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). Xylene substances (**Table 1**) recently received testing decisions on one-generation reproductive toxicity (EOGRT) studies with additional developmental neurotoxicity (DNT) cohorts for each of the three isomers based on DNT concerns in published literature¹⁻⁴.

Table 1. Chemical ID of Xylene Isoforms, Xylene, and Xylene Reaction Mass (RM) Substances

Xylene Isomers								
Substance	ortho (o)-xylene		meta (m)-xylene		para (p)-xylene			
CAS #	95-47-6		108-38-3		106-42-3			
EC #	202-422-2		203-576-3		203-396-5			
	Typical conc. ^a	Conc. Range ^a	Typical conc.	Conc. range	Typical conc.	Conc. range		
	98%	>98%	98%	>98%	95%	>90-100 %		
Structure								
Xylene and Xylene Reaction Mass (RM) Substances								
Substance	Xylene		RM EB ^b and Xylene		RM EB ^b and m-Xylene		RM EB ^b , m-Xylene and p-Xylene	
CAS #	1330-20-7		-		-		-	
EC #	215-535-7		905-588-0		905-570-2		905-562-9	
Constituent	Typical conc.	Conc. range	Typical conc.	Conc. range	Typical conc.	Conc. range	Typical conc.	Conc. range
o-Xylene	20%	20 - 30%	>10%	5 - 60%	5%	<10%	2.5%	<10%
m-Xylene	50%	40 - 60%	>10%	10 - 60%	>10%	10 - 79.9%	>10%	10 - 65%
p-Xylene	20%	15 - 30%	>10%	5 - 60%	5%	<10%	>10%	7 - 40%
EB ^b	-	>3 - <10%	>10%	10 - 75%	>10%	10 - 60%	>10%	10 - 60%

^a All typical concentrations and concentration ranges are (w/w); ^b EB – ethylbenzene.

As a legacy industrial chemical with a significant amount of existing animal and human data, the xylene substances present a unique opportunity for a weight of evidence WoE review to investigate whether additional animal toxicology testing is truly necessary to meet the specified regulatory data requirements or if it would be contradictory to the ECHA commitment to the 3R principles.

Methods

Historical DNT studies were published before implementation of current testing guidelines and requirements. These published literature, including those cited by ECHA (1, 2, and 3) to justify the inclusion of a DNT cohort in an EOGRTS, were re-evaluated using current state-of-science perspectives to gauge whether additional animal testing on xylene substances are warranted.

To address the need for further vertebrate testing, a comprehensive WOE review of published data on xylene was performed including the following:

- Pharmacokinetics,
- Central nervous system (CNS) narcotic effects,
- Narcotic mode of action (MOA),
- Strength of DNT signal.

The Lower Olefins Aromatics (LOA) REACH Consortium also provided new unpublished study data from multiple updated guideline studies (OECD TG 408 and OECD TG 422) on xylene (CAS: 1330-20-7), xylene reaction mass substances, and xylene isomers to be included in the review.

DNT

Five published studies (1, 2, 3, 14, 15) were extensively reviewed for DNT. Severe methodological deficiencies including experimenter bias confounding scientific comparisons between control and treated groups, poor study design including insufficient number of animals tested and lack of positive controls, and results lacking in detail including no reporting of individual animal data are common throughout the publications.

The lack of positive controls in these studies make it difficult to determine whether the laboratories had any valid methods for detecting any biological relevant changes in behavior. Additionally, study results cannot be interpreted fully with the lack of individual animal data.

In one study (2) rats were housed with and without enrichment materials and it was acknowledged that there were no statistically significant learning findings in rats housed with enrichment. Use of enriched housing is a required condition for animal welfare in current guideline studies; therefore, this study outcome has more relevance than the spurious findings exhibited by the non-enriched group.

Only one of the reviewed studies used a purified form of xylene (14), and although underpowered and lacking a positive control group, the dosages applied were high and the lack of any evident neurobehavioral signal change suggests that xylene does not target the developing CNS in the offspring. Furthermore, the presence of a significant amount of ethylbenzene (1,2,3) prevents results from being considered as directly comparable to more purified forms of xylene isomers and from identifying the effects of xylene in isolation.

Considering all lines of evidence in the literature, there was no convincing data found which indicates the xylene evokes developmental neurotoxicity.

CNS Narcotic Effects

The key feature common to all narcotics is that their associated neurological symptoms and behavioral states are transient and fully reversible. Xylene is well-established and recognized as a narcotic with multiple animal and human studies demonstrating transient, reversible signs of intoxication, with no evidence of a permanent or deleterious effect on the CNS. A review of narcotic organic solvents (7) concluded that a link between narcosis and DNT cannot be assumed.

Humans

- Multiple clinical studies have demonstrated transient narcotic effects with no evidence of irreversible deleterious CNS effects.

Animals

- Nine unpublished new studies (2 x OECD 408, 7 x OECD 422) indicate no treatment-related CNS adverse findings up to 1000 mg/kg bw/day, indicating an absence of transient or permanent injury to the CNS.
- Only one of three published (non-guideline) studies (8, 9, and 10) indicated a persistent neurobehavioral effect which was determined to be consistent with an adapted learned fear response to the stressful odors which is elicited in rats by xylene.

Narcotic MoA

Aromatic hydrocarbon organic solvents that demonstrate narcotic effects, such as xylene, are theorized to have a MOA based on nonspecific perturbation of nervous cell membrane phospholipids, which occurs by penetrating the cell membrane and increasing its fluidity, such that membrane-bound proteins and their functions are also generally affected.

- Membrane fluidity was demonstrated in pig microsomes (11) and mouse synaptosomes (12).
- A linear correlation between membrane fluidity and the octanol/water coefficients (12).
- Decreased activity of the membrane-bound ATPase (12 and 13) and acetylcholinesterase (13).

Available *in vivo* neuronal receptor binding assays do not demonstrate clear, consistent or preferential receptor phenotype which aligns with nonspecific behavioral phenotypes observed in repeated exposure studies; however, the odor of xylene would elicit a fear or stress response, and stress alone has been shown to readily alter receptor binding in these studies.

This evidence supports that xylene exerts its narcotic effects on the CNS through lipophilic uptake into the neural cell membrane, resulting in reversible fluidity perturbation of the phospholipid bilayer and subsequently, in nonspecific transient narcotic effects.

Pharmakokinetics

Xylene is well-absorbed *via* inhalation and oral routes of exposure in humans and animals and is readily metabolized in the liver to methyl hippuric acid (MHA) which is primarily excreted through urine. Xylene isomers are rapidly absorbed and distributed to areas of high levels of phospholipids i.e. body fat, bone marrow and white matter of the brain (**Figure 1**), before being rapidly metabolized to MHA and distributed to the blood, liver, lung, kidney and adrenal medulla.

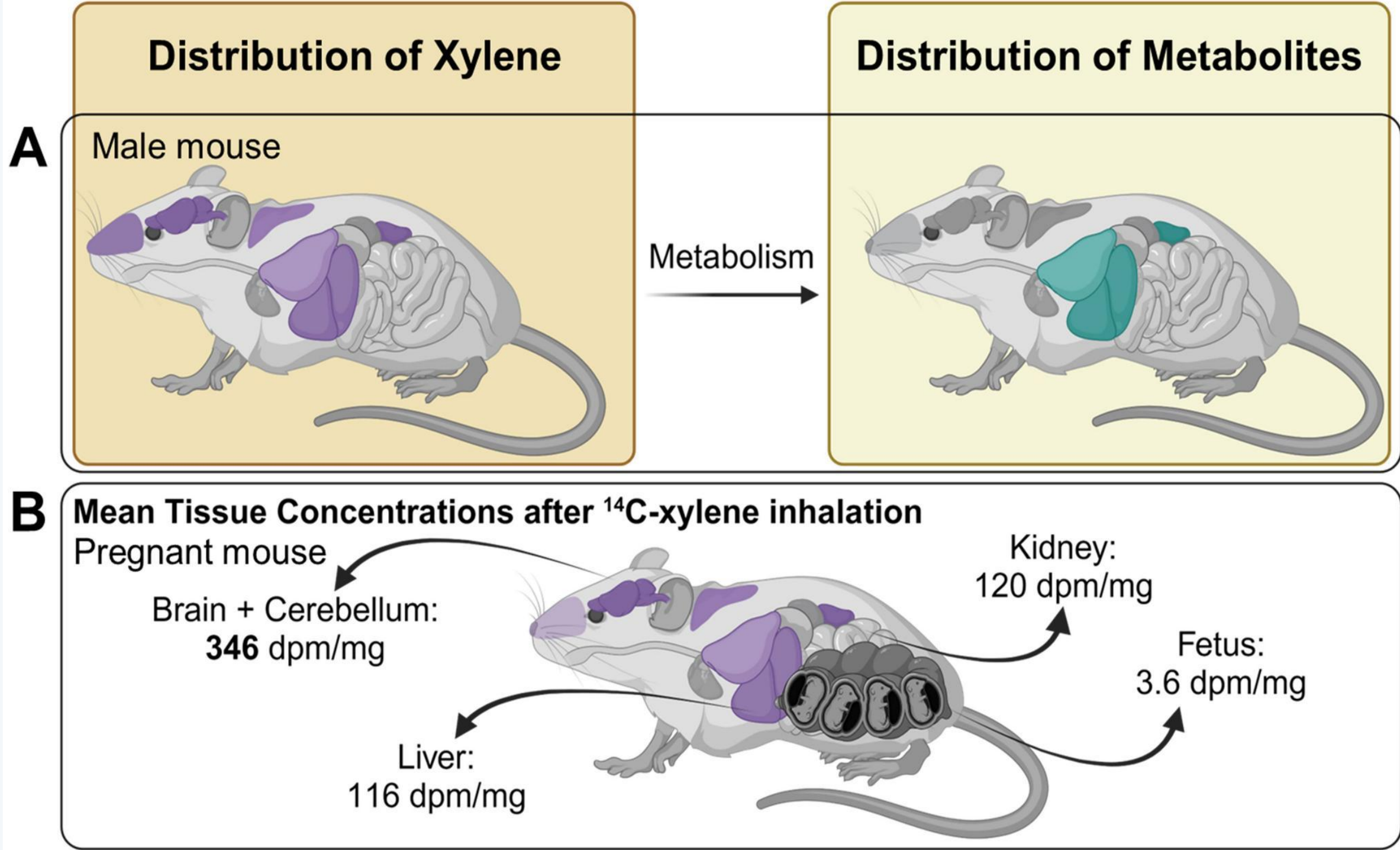


Figure 1. Images are adapted from autoradiogram images from (A) Bergman and Appelgren (4) and (B) Ghantous and Daneilsson (5). Purple indicates presence of xylene and teal indicates presence of xylene metabolites, as detected by whole body autoradiography.

Xylene crosses into the blood of the fetus at a lower concentration than in maternal blood (6) and accumulation in fetal tissues is limited (**Figure 1B**), which suggests maternal metabolism markedly decreases fetal uptake.

Xylene and its metabolites are excreted in the urine and/or via exhalation within 24 hours.

Discussion and Conclusion

Based on this comprehensive review of published data on xylene pharmacokinetics, CNS narcotic effects (human and animal), narcotic MOA and lack of a robust DNT signal with xylene, it is concluded that there is no trigger for an additional DNT cohort (2A and 2B) in an EOGRT study to be conducted for xylene.

Furthermore, due to the study design of the additional DNT cohort in an EOGRT study, transient CNS effects cannot be differentiated thus will produce unreliable data in contrast to studying DNT effects in, for example, a developmental neurotoxicity study such as an OECD 426 testing guideline.

1. Hass U, Jakobsen BM. 1993. Prenatal toxicity of xylene inhalation in the rat: a teratogenicity and postnatal study. *Pharmacol Toxicol.* 73(1):20–23;
2. Hass U, Lund SP, Simonsen L, Fries AS. 1995. Effects of prenatal exposure to xylene on postnatal development and behavior in rats. *Neurotoxicol Teratol.* 17(3):341–349;
3. Hass U, Lund SP, Simonsen L. 1997. Long-lasting neurobehavioral effects of prenatal exposure to xylene in rats. *Neurotoxicology.* 18(2):547–551;
4. Bergman K, Appelgren LE. 1983. Application and results of whole-body autoradiography in distribution studies of organic solvents. *Crit Rev Toxicol.* 12(1):59–118;
5. Ghantous H, Danielsson BR. 1986. Placental transfer and distribution of toluene, xylene and benzene, and their metabolites during gestation in mice. *Biol Res Pregnancy Perinatol.* 7(3):98–105;
6. Ungváry G, Tátrai E, Huddák A, Barcza G, Lőrincz M. 1980. Studies on the embryotoxic effects of ortho-, meta- and para-xylene. *Toxicology.* 18(1): 61–74;
7. De Groot DMG. 2017. Scientific review on the link between the narcotic effects of solvents and (developmental) neurotoxicity. Final report. Prepared for ECHA;
8. Korsak Z, Sokal JA, Gorny R. 1992. Toxic effects of combined exposure to toluene and m-xylene in animals. III. Subchronic inhalation study. *Int J Occup Med Environ Health.* 5(1):27–33;
9. Gralewicz S, Wiaderna D. 2001. Behavioral effects following subacute inhalation exposure to m-xylene or

trimethylbenzene in the rat: a comparative study. *Neurotoxicology.* 22(1):79–89;
10. Gralewicz St, Wiaderna DO, Tomas TA. 1995. Development of spontaneous, age-related nonconvulsive seizure electrocortical activity and radial-maze learning after exposure to m-xylene in rats. *Int J Occup Med Environ Health.* 8(4):347–360;
11. Engelke M, Bergmann U, Diehl HA. 1993. Fluidity of the microsomal membrane and cytochrome P450 reduction kinetics of pig liver microsomes as a consequence of organic solvent impact. *Xenobiotica.* 23(1):71–78;
12. Tani H, Huang J, Ohyashiki T, Hashimoto K. 1994. Physical-chemical-activity relationship of organic solvents: effects on Na⁺-K⁺-ATPase activity and membrane fluidity in mouse synaptosomes. *Neurotoxicol Teratol.* 16(6):575–582;
13. Korpela M. 1989. Inhibition of synaptosome membrane-bound integral enzymes by organic solvents. *Scand J Work Environ Health.* 15(1):64–68;
14. Rosen MB, Crofton KM, Chernoff N. 1986. Postnatal evaluation of prenatal exposure to p-xylene in the rat. *Toxicol Lett.* 34(2–3):223–229;
15. Mirkova E, Hinkova L, Vassileva L, Bogdanova N. 1979. Xylene neurotoxicity in pregnant rats and fetuses. *Activ Nerv Sup (Praha).* 21:265

