Impact of oil-related environmental pollutants on the ovary structure in the freshwater leech *Erpobdella johanssoni* (Johansson, 1927) (Clitellata: Hirudinea)

I. KHALED¹, H. FERJANI², A. V. SIROTKIN³, S. ALWASEL⁴, & A. H. HARRATH⁴*

¹Faculty of Science of Tunis, UR11ES12 Biologie de la Reproduction et du Développement Animal, University of Tunis El Manar, Tunisia, ²Laboratory of Research on Biologically Compatible Compounds, Dental Medicine Faculty, University of Monastir, Tunisia, ³Department Zoology and Anthropology, Constantine the Philosopher University, Slovakia, and ⁴Department of Zoology college of Sciences, King Saud University, Saudi Arabia

(Received 28 March 2017; accepted 3 May 2017)

Abstract
Toxicity of organic chemical compounds, including benzene, toluene, ethyl benzene and xylene (BTEX), is a major concern because of their induction of adverse effects in organisms, including reproductive abnormalities. In the present study, we investigated impacts of chronic exposure to BTEX at 25 µg L⁻¹ on the ovaries of the freshwater leech *Erpobdella johanssoni* at the cytological and molecular level. Based on light and transmission electron microscopy, we found that somatic cells and vitellogenic oocytes of the treated animals underwent degenerative changes, such as cytoplasmic vacuolization, mitochondrial alterations, and nuclear DNA condensation, as compared with normal. The comet test supported histological and ultrastructural results and showed that BTEX exposure induced significantly more DNA fragmentation in the ovary cells of treated leeches than in controls (*p* < 0.0001). Overall, we concluded that BTEX-induced deterioration in ovarian cells suggests the genotoxicity of BTEX on oogenesis in leech, which could impair their reproduction.

Keywords: BTEX, toxicity, reproduction, leech, ovary

Introduction
Benzene, toluene, ethyl benzene, and xylene (BTEX) are ubiquitous pollutants that were widely used in the production of many industrial products (plastics, solvent, pesticides and paints, among others) (Singh et al. 2011). Because of both occupational and non-occupational exposure, the toxicity of these hydrocarbons has proven harmful to humans and wildlife (Sirotkin & Harrath 2017). In surface water, levels were reported to be in the range of 0.1–2.1 µg L⁻¹ for benzene, 1–15 µg L⁻¹ for toluene, 0.1–1.8 µg L⁻¹ for ethylbenzene and 0.1–1.2 µg L⁻¹ for xylene. In groundwater, reported concentrations of benzene, toluene ethylbenzene and xylene were less than 0.1–1.8 µg L⁻¹, 1–100 µg L⁻¹, 0.1–1.1 µg L⁻¹ and 0.1–0.5 µg L⁻¹, respectively, whereas in drinking water, concentrations of these hydrocarbons were respectively 0.1–5 µg L⁻¹, 1–27 µg L⁻¹, 1–10 µg L⁻¹ and 0.1–12 µg L⁻¹ (ATSDR 2000, 2007a,b,c; WHO 2008).

They are environmental toxicants that exert their toxicity and disrupt cellular processes via induction of apoptosis and genotoxicity (Ross 2000; Nakai et al. 2003; Fan et al. 2009; Zhang et al. 2010; Ayan et al. 2013; Li et al. 2013; Neuparth et al. 2014). The reproductive toxicity of these compounds has recently received much attention, and many studies have been performed to address the ability of BTEX to impair reproductive functions (Chen et al. 2000; Hannigan & Bowen 2010; Sirotkin et al. 2012; Webb et al. 2014; Djemil et al. 2015). In particular, numerous studies have shown that female organisms accumulate 3.7–6.8
times more xylene than males, and ovaries are the important accumulation organ of this hydrocarbon (Suter-Eichenberger et al. 1998; Wan et al. 2007; Lin et al. 2013).

Although there is concern regarding the influence of BTEX on reproductive processes in aquatic invertebrates, to our knowledge, only one study has reported the effects of toxic insult by BTEX on the gonads of freshwater leeches (Khaled et al. 2016); most studies have addressed effects on mammals (Zhang et al. 2010; Singh et al. 2011; Saxena & Ghosh 2012; Kumar et al. 2014), whereas those on invertebrates are lacking. In a previous study (Khaled et al. 2016), we demonstrated that considerably high-concentration exposure of BTEX (1.4 and 2.8 mg L\(^{-1}\)) for a short term (1 h) had a significant effect on male and female reproduction of the leech \textit{Limnatis nilotica}, primarily on the previtellogenic and vitellogenic oocytes. In the present study, our goal was to investigate the effects of chronic low-concentration exposure of BTEX (26 days) on the histopathology and the ultrastructure of the ovaries of the freshwater leech \textit{Erpobdella johannsoni}. Indeed, many studies have demonstrated that pollutants could have adverse effects when used at high enough concentrations but have no effects at considerably lower concentrations (Liu et al. 2010; Zheng et al. 2012; Neuparth et al. 2014). The acute toxicity of BTEX is between 1 and 10 mg L\(^{-1}\) in terms of \(LC_{50}\) or \(EC_{50}\) to aquatic organisms including fish, invertebrates and aquatic plants (OECD 2002; Zheng et al. 2012; Avramov et al. 2013). We used the species \textit{E. johanssoni} as a model because it has been regarded as a suitable organism for generating valuable information on various behavioral and physiological parameters (Petrauskiene 2004). This macrophagous predator of aquatic invertebrates has been much investigated and is considered an excellent model for the ecological assessment of invertebrate species interactions and as a bioindicator species for aquatic toxicology (Siddall 2002). Hence, investigation of the effect of this group of pollutants on the reproduction of freshwater invertebrates, in particular leeches, will be informative.

\textbf{Materials and methods}

\textit{Sample collection}

Adult leeches were carefully collected from the Tamerza waterfall (a small village and mountain oasis south of Tunisia) during the 2014 breeding season (May–June; 34°12’ 31.302°N, 7°54’56.555°E). For adaptation to the laboratory conditions, worms were kept for 1 month in an aerated glass aquarium at room temperature (20°C) and fed crushed chicken livers weekly.

\textbf{Chronic exposure of \textit{E. johanssoni} to BTEX}

Benzene, toluene, ethyl benzene and xylene with greater than 99% purity were obtained from Labo chimie PVT. Ltd. 107. To investigate the chronic toxicity of a mixture of BTEX with equal amounts of each component, a concentration of 25 µg L\(^{-1}\) was applied in triplicate to 15 leeches per replicate for 26 days. A control group containing 15 leeches was kept in uncontaminated water. The test conditions were consistent with the acclimation conditions, but aeration was removed to avoid volatilization of these pollutants. During chronic exposure, treated leeches were fed weekly and the entire exposure solution was changed daily to maintain the desired concentration of BTEX. At the end of the exposure period, all of the specimens were transferred into a pre-aerated tank and BTEX-free freshwater. The choice of this concentration was based primarily on our preliminary test results and values derived from the literature regarding chronic exposure on other aquatic invertebrates and fish (Fan et al. 2009; Li et al. 2013).

\textbf{Light microscopy}

After exposure to the pollutants at room temperature, five specimens of \textit{E. johanssoni} were dissected, and ovaries were removed and fixed in neutral buffered formalin (NBF) 4%. Then, they were embedded in paraffin wax, sectioned to 6-µm thickness, and stained with eosin and hematoxylin. Photos were taken with a camera (SONY DSC-S3000) and digitized with Adobe Photoshop 9.

\textbf{Transmission electron microscopy}

After 26 days of exposure under constant conditions, five specimens of \textit{E. johanssoni} were dissected and ovaries were removed and fixed in 3% glutaraldehyde at 4°C for 3 days. Thereafter, ovaries were rinsed with 0.1 M sodium phosphate buffer, followed by fixation with 1% osmium tetroxide (O\(_8\)O\(_4\)) for 2 h at 4°C. Then, they were dehydrated in a graded series of ethanol and finally embedded in Epon-Araldite. Semi-thin sections (0.8 µm thick) were taken and stained with toluidine blue. Ultra-thin sections were prepared using a Reichert-Jung Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined using a Joel 100SX transmission electron microscope.

\textbf{Comet assay}

Five animals from each treatment were used for the comet assay. Gonads were extracted and immersed in 100 µL of phosphate buffered saline (PBS). They were
minced with very fine scissors to obtain a cell suspen-
sion; 60 µL of the cell suspension was mixed with 60 µL
of 1.0% low-melting point agar (LMA) at 37°C and
spread on slides previously coated with a layer of 1%
(w/v) normal-melting-point agarose prepared in PBS.
After solidification at 4°C for 5–10 min, the slides were
immersed in a lysis solution (2.5 M NaCl, 100 mM
ethylenediaminetetraacetic acid (EDTA), 10 mM
Tris, and NaOH to pH 10.0) with freshly added 1%
Triton X-100 and 10% dimethyl sulfoxide (DMSO) at
4°C overnight. Slides were then randomly placed in an
electrophoresis tank containing 0.3 M NaOH and
1 mM EDTA for 20 min. Electrophoresis was con-
ducted for 20 min at 25 V and 300 mA at ambient
temperature. The slides were then neutralized using
0.4 M Tris, at pH 7.5, and immersed in absolute
ethanol for 10 min. During the assay, the slides were
stained with 40 µL ethidium bromide and viewed
under a fluorescent microscope. The experiment was
repeated 3 times for each sample. A total of 100 comets
on each slide were visually scored according to the
relative intensity of fluorescence in the tail, and placed
in one of five classes. Comet classes were given values
(0, 1, 2, 3 or 4; from undamaged, 0, to maximally
damaged, 4) as previously described by Collins et al.
(1996). The total score was calculated using the fol-
lowing equation: (percentage of cells in class 0 × 0) +
(percentage of cells in class 1 × 1) + (percentage of cells
in class 2 × 2) + (percentage of cells in class 3 × 3) +
(percentage of cells in class 4 × 4). Consequently, the
total score ranged from 0 to 400.

Statistical analysis
Statistical analyses were performed using GraphPad
prism version 5. Data were represented as mean ±
standard deviation (SD). The Mann–Whitney U test
was used to compare differences between groups.
Differences were considered statistically significant
at P < 0.01.

Results

Light microscopy
Light microscopy identified several morphological
changes that occurred in the ovary of E. johanssoni
during BTEX exposure. In fact, chronic exposure
resulted in significant effects on both nurse cells
and vitellogenic ovarian cells compared to control
cells, thereby increasing the number of degenerating
oocytes in the ovarian cord, and decreasing the num-
ber of normal oocytes (Figure 1(a) and (b)). The
morphological appearance of affected vitellogenic
oocytes was characterized by the clumping, shrink-
age, extensive alteration and vacuolization of the
cytoplasm, especially in the cortical zone (Figure 1(b)).

Transmission electron microscopy
The ultrastructure study showed obvious effects of
chronic exposure of the freshwater leech E. johans-
soni to a 25 µg L−1 concentration of BTEX. Indeed,
and in comparison to control (Figure 2(a)), many
ultrastructure alterations in somatic cells and vitello-
genic oocytes were indicated (Figure 2(b) and (c)).
The somatic cells showed a degenerating ooplasm
characterized by damaged mitochondria and devel-
oping autophagosomes, which were observed on
occasion amalgamating into a larger autophagic
vacuole (Figure 2(b)). The vitellogenic oocytes

Figure 1. Hematoxylin and eosin staining of paraffin sections of an Erpobdella johanssoni ovary. A, Histological sections of the ovarian cord from the
control group showing normal architecture: nurse cells (NC) and vitellogenic oocytes (VO). B, Ovary from treated leech revealed degeneration
shrinkage of nurse cells (NC) and vitellogenic oocytes (VO), and extensive vacuolization of their ooplasm (arrowheads). Scale bars = 20 µm.
were the most damaged cells because no glycogen granules were found in their ooplasm (Figure 2(c)). Moreover, the vitelline envelope was damaged in some zones, whereas the microvilli were damaged in other zones (Figure 2(c)).

**BTEX-induced DNA fragmentation in ovaries of E. johannsoni**

Results of the visual scoring of total DNA damage in ovaries of the leech *E. johannsoni* after 26 days of treatment with BTEX are illustrated in Figure 3. We observed an increase in DNA fragmentation caused by BTEX treatment for 26 days in ovary cells \( (p < 0.0001) \). In fact, DNA damage was higher by approximately 7-fold than in the control group.

**Discussion**

BTEX has been identified as potential groundwater pollutants, which cause deleterious effects on organisms, including humans (Chen et al. 2008; Li et al. 2013; Lim et al. 2014; Liu et al. 2014). Our histological findings demonstrated obvious alterations in the progression of oogenesis in *E. johannsoni* with BTEX treatment of 25 µg L\(^{-1}\) for 26 days. Our findings agreed with previous studies that also investigated the effect of BTEX on the sensitivity of freshwater animals (Dórea et al. 2007; Avramov et al. 2013). However, the level of sensitivity of organisms to pollutants seems to vary among species, during exposure, and by concentration and life stage. Reproductive toxicity in leeches has seldom been addressed. In fact, it was shown that cadmium...
exposure induced a detrimental effect on the structure and development of reproductive tissue in the freshwater predatory leech *Nephelepsis obscura* (Westcott 1997). Davies and Gates (1991) also found a decrease in reproductive potential in *Nephelepsis obscura* exposed to cadmium with reduced production of ova and spermatozoa as well as reduced masses of the ovisacs, epididymys and testisacs. Rao et al. (1983) observed that exposure to reserpine and chlorpromazine induces a decrease in ovary and testis indices in the leech *Poecilobdella viridis*. It has also been noted that pesticides such as mercuric chloride and copper sulfate decreased testis and ovarian indices and inhibited testis and ovarian maturation in the leech *Poecilobdella viridis* (Kulkarni & Deshpande 2007). Kiceniuk and Khan (1983), and Khan and Kiceniuk (1989), reported that water-soluble fractions (WSF) of a crude oil affected the reproduction process, egg production and hatching of egg, and survival in the marine leech *Johanssonia arctica*.

Our results show that the majority of somatic cells and vitellogenic oocytes were affected by BTEX treatment. This result is especially important because the toxic effect of BTEX may disrupt the later phases of vitellogenesis because of the accumulation of reserve material (mainly proteins, lipids and saccharides) for use during the later stage of embryonic development (Świątek 2005; Ben Ahmed et al. 2010, 2013). It is known that BTEX are highly lipid-soluble toxicants, and they can be easily accumulated in high levels in the ovaries. It has been shown that exposure to benzene, toluene and xylene induced pathological alterations in the earthworm *Eudrilus eugeniae* (Eseigbe et al. 2013), whereas exposure to benzene only induced severe histopathological injuries in rat ovaries (Singh & Rana 2010). Volatile organic compounds (VOCs), especially BTEX, lead to potential damage to the reproductive and developmental health of women, resulting in preterm birth, infertility, birth defects and spontaneous abortion in women exposed to toluene and benzene in their work environment (Lin et al. 2013; Webb et al. 2014). Exposure to toluene and formaldehyde led to several injuries in the ovaries of adult female mice, causing disruption in the follicular growth process and alteration of their histological structure (Kareem et al. 2014). Chen et al. (2000) reported that many solvents including toluene and benzene damage ovaries by inducing disruption in the follicular growth process, whereas Sirotkin et al. (2012) demonstrated that exposure to BTEX induced apoptosis of porcine and bovine ovarian cells.

From an ultrastructural perspective, oogenesis in the normal ovary of *E. johanssoni* starts with the oogonia that undergoes a marked increase in volume because of the accumulation of reserve material to form previtellogenic oocytes and huge vitellogenic oocytes (Ben Ahmed et al. 2013). The ooplasm formed during vitellogenesis is filled with cytoplasmic constituents primarily composed of cell organelles, lipid droplets and yolk. At a later stage, the vitellogenic oocyte has an ooplasm filled with glycogen granules and lipid droplets, and is limited by a dense layer of microvilli and the vitelline envelope (Ben Ahmed et al. 2013). However, in the ovary of treated leeches, the majority of somatic cells and vitellogenic oocytes underwent degenerative changes, such as cytoplasmic vacuolization and nuclear condensation, resulting in these oocytes not completing oogenesis and degenerating instead. Using histological outcomes combined with ultrastructure results, we can conclude that BTEX induced deterioration of somatic cells and vitellogenic oocytes of treated *E. johanssoni*. To determine whether BTEX induced DNA fragmentation in ovarian cells of treated *E. johanssoni*, we used the alkaline comet assay, which is a sensitive, rapid and simple method that has been widely used for detection of DNA damage (Chen et al. 2008; Azqueta et al. 2016). The comet assay outcomes were clearly associated with the histopathological and ultrastructural results because they demonstrated the ability of BTEX to induce DNA damage in leech ovaries. The latter result is in agreement with that found during the investigation of aluminum pollution and the level of DNA damage in hemocytes of the medicinal leech (Mihaljević et al. 2009). It has also been shown that the gasoline water-soluble fraction (GWSF) induced genotoxic and mutagenic toxicity in the bivalve mollusk *Corbicula fluminea* (Fedato et al. 2010) and in the freshwater amphipod *Quadrevirgulus affluti* (Lacaze et al. 2011; Weber et al. 2013). Siu et al. (2004) observed that benzo [a] pyrene leads to genotoxic damage in hemocytes of the green mussel *Perna viridis*.

Several previous studies focused on the ability of a wide variety of environmental toxicants to trigger cell death, including cadmium, chromium, methyl mercury, organotin compounds and volatile organic compounds (Robertson & Orrenius 2000; Mathur et al. 2011; Fouad & Jresat 2015; Zhuang et al. 2016). Moreover, most of the mitochondria in the ovary of treated animals lost their integrity as compared to those in control animals. The affected mitochondrial integrity increased the permeability of the outer mitochondrial membrane, allowing the release of cytochrome, thereby inducing the formation of apoptosomes and the subsequent activation of the caspase-dependent mitochondrial pathway (Chang
Influence of pollutants on leech ovaries

This work was supported by the King Saud University [RGP-164].

References

Ateeq B, Farah MA, Ahmad W. 2006. Evidence of apoptotic effects of 2, 4-D and butachlor on walking catfish, *Clarias batrachus*, by transmission electron microscopy and DNA degradation studies. Life Sciences 78:977–986. DOI:10.1016/j.lfs.2005.06.008.

ATSDR. 2000. Toxicological profile for toluene. USA: US Department of Health and Human Services, Agency for Toxic Substances and Disease Registry. 2007a. Toxicological profile for benzene. USA: US Department of Health and Human Services, Agency for Toxic Substances and Disease Registry.

ATSDR. 2007b. Draft toxicological profile for ethylbenzene. USA: US Department of Health and Human Services, Agency for Toxic Substances and Disease Registry. 2007c. Toxicological profile for 2,4-D. USA: US Department of Health and Human Services, Agency for Toxic Substances and Disease Registry.

Avramov M, Schmidt SI, Griebler C. 2013. A new bioassay for the ecotoxicological testing of VOCs on groundwater invertebrates and the effects of toluene on *Niphargus incisus*. Aquatic Toxicology 130–131:1–8. DOI:10.1016/j.aquatox.2012.12.023.

Ayan M, Tas U, Sogut E, Kuloglu T, Cayli S, Kocaman N, Karaca ZI, Sahin M. 2013. The apoptotic effect of a high dose of toluene on liver tissue during the acute phase: An experimental study. Toxicology and Industrial Health 29:728–736. DOI:10.1177/0748233712442731.

Azqueta A, Costa-Amaral IC, Collins AR. 2016. High-throughput measurement of DNA breaks and oxidised bases with the comet assay. In: Dhawan A, Anderson D, editors. The comet assay. UK: Royal Society of Chemistry. pp. 65–92.

Ben Ahmed R, Fuchs AZ, Tekaya S, Harrath AH, Świątek P. 2010. Ovary cords organization in *Himantoderma Johnsonia*, 1816 and *Linnatiunolotus* (Savigny, 1822) (Clitellata, Hirudinea). Journal of Comparative Zoology 249:201–207.

Ben Ahmed R, Tekaya S, Malota K, Świątek P. 2013. An ultrastructural study of the ovary cord organization and oogenesis in *Bipodella johannsoni* (Annelida, Clitellata: Hirudinida). Micron 44:275–286. DOI:10.1016/j.micron.2012.07.005.

Chang HY, Yang X. 2000. Proteases for cell suicide: Functions and regulation of caspases. Microbiology and Molecular Biology Reviews 64:821–846. DOI:10.1128/MMBR.64.4.821–846.2000.

Chen CS, Hseu YC, Liang SH, Kuo J-Y, Chen SC. 2008. Assessment of genotoxicity of methyl-tert-buty1 ether, benzene, toluene, ethylbenzene, and xylene to human lymphocytes using comet assay. Journal of Hazardous Materials 153:351–356. DOI:10.1016/j.jhazmat.2007.08.053.

Chen H, Song L, Wang X, Wang S. 2000. Effect of exposure to low concentration of benzene and its analogues on luteal function of female workers. Wei Sheng Yan Jiu= Journal of Hygiene Research 29:351–353.

Collins AR, Dusinská M, Gedík CM, Stětina R. 1996. Oxidative damage to DNA: Do we have a reliable biomarker? Environmental Health Perspectives 104:465–469. DOI:10.1289/ehp.96104s3465.

Davies RW, Gates TE. 1991. The effects of different oxygen regimes on the feeding and vertical distribution of *Nephelopsis*
Dörera HS, Bispo JR, Aragão KA, Cunha BB, Navickiene S, Alves JP, Romão PL, Garcia CA. 2007. Analysis of BTEX, PAHs and metals in the oilfield produced water in the State of Sergipe, Brazil. Microchemical Journal 85:234–238. DOI:10.1016/j.micpro.2006.06.002.

Eseigbe FJ, Doherty VF, Sogbanmu TO, Otitololu AA. 2013. Histopathology alterations and lipid peroxidation as biomarkers of hydrocarbon-induced stress in earthworm, Eudriluseugeniae. Environmental Monitoring and Assessment 185:2189–2196. DOI:10.1007/s11031-012-0736-z.

Fan YW, Zhou QX, Wang YY, Zhu S. 2009. Toxic effects of BTEX in water on Daphnia magna and Limnodrilus hoffmeisteri and safety assessment of the aquatic environment. Journal of Environmental Sciences 29:1485–1490.

Fedato RP, Simonato JD, Martinez CBR, Soares AC. 2010. Reproductive toxicity and teratology of abused toluene. Systems Biology in Reproductive Medicine 56:70–80. DOI:10.1016/j.sibrm.2010.05.012.

Fouad AA, Jresat I. 2015. Thymoquinone therapy abrogates toxic effect of cadmium on rat testes. Andrologia 47:417–426. DOI:10.1111/and.2015.47.issue-4.

Hannigan JH, Bowen SE. 2010. Reproductive toxicity and histopathology alterations of toxic pollutants and their associated health risks in the library of Jawaharlal Nehru University, New Delhi. Environmental Science and Pollution Research/Genetic Toxicology and Environmental Mutagenesis 70:700–85. DOI:10.1016/j.mrgento.2010.05.012.

Khaled I, Ferjani H, Ben Ahmed R, Harrath AH. 2016. Effects of oil-related environmental pollutants on gonads of the freshwater leech Limnatis nilotica (Annelida, Hirudinea). Invertebrate Reproduction & Development 60:263–270. DOI:10.1080/07924259.2016.1208118.

Khan RA, Kiceniuk JW. 1989. Sublethal effects of crude oil on a cold-water marine leech, Johansonia arctica, following chronic exposure. The Bulletin of Environmental Contamination and Toxicology 43:590–596. DOI:10.1007/BF01701940.

Kiceniuk JW, Khan RA. 1983. Toxicology of chronic crude oil xposure: Sublethal effects on aquatic organisms. In: Nrgiu JO, editor. Aquatic toxicology. New York: John Wiley & Sons. pp. 425–443.

Knałkievicz T. 2014. Planarians as invertebrate bioindicators in freshwater environmental quality: The biomarkers approach. Ecotoxicology and Environmental Contamination 9:1–12. DOI:10.5132/ecc.

Kulkarni GK, Deshpande GV. 2007. Effects of mercuric chloride and copper sulphate on gonadal indices and gametogenesis of a freshwater leech, Pseudeobdella viridis (Blanchard). Aquaculture 93:94–102.

Kumar A, Singh BP, Pania M, Singh D, Kumar K, Jain VK. 2014. Assessment of indoor air concentrations of VOCs and their associated health risks in the library of Jawaharlal Nehru University, New Delhi. Environmental Science and Pollution Research 21:2240–2248. DOI:10.1007/s11356-013-2150-7.

La Caze E, Devaux A, Mons R, Bony S, Garric J, Geffard A, Geffard O. 2011. DNA damage in caged Gammarus fossarum amphipods: A tool for freshwater genotoxicity assessment. Environmental Pollution 159:1682–1691. DOI:10.1016/j.envpol.2011.02.038.

Li X, Zhou Q, Luo Y, Yang G, Zhou T. 2013. Joint action and lethal levels of toluene, ethylbenzene, and xylene on midge (Chironomus plumosus) larvae. Environmental Science and Pollution Research 20:957–966. DOI:10.1007/s11356-012-1264-7.

Lim SK, Shin HS, Yoon KS, Kwak SJ, Um YM, Hyeon JH, Kwak HM, Kim JY, Kim TH, Kim YJ, Roh TH, Lim DS, Shin MK, Choi SM, Kim HS, Lee B-M. 2014. Risk assessment of volatile organic compounds benzene, toluene, ethylbenzene, and xylene (BTEX) in consumer products. Journal of Toxicology and Environmental Health, Part A 77:1502–1521. DOI:10.1080/15287394.2014.959505.

Lin C-C, Huang C-N, Wang J-D, Hwang Y-H, Shie R-H, Chang Y-Y, Weng S-P, Chen P-C. 2013. Exposure to multiple low-level chemicals in relation to reproductive hormones in premenopausal women involved in liquid crystal display manufacture. International Journal of Environmental Research and Public Health 10:1406–1417. DOI:10.3390/ijerph10041406.

Liu FF, Escher BI, Were S, Duffy L, Ng JC. 2014. Mixture effects of benzene, toluene, ethylbenzene, and xylenes (BTEX) on lung carcinoma cells via a hanging drop air exposure system. Chemical Research in Toxicology 27:952–959. DOI:10.1021/tx5005552.

Liu Y, Zhou Q, Xie X, Lin D, Dong L. 2010. Oxidative stress and DNA damage in the earthworm Eisenia fetida induced by toluene, ethylbenzene and xylene. Ecotoxicology 19:1551–1559. DOI:10.1007/s10646-010-0540-x.

Mathur PP, Huang L, Kashou A, Vairinthanathan S, Agarwal A. 2011. Environmental toxicants and testicular apoptosis. Open Reproductive Science Journal 3:114–124. DOI:10.2174/1874255601003101114.

Mihaljević Z, Ternjej I, Stanković I, Kerovec M, Kopjar N. 2009. Application of the comet assay and detection of DNA damage in haemocytes of medicinal leech affected by aluminium pollution: A case study. Environmental Pollution 157:1565–1572. DOI:10.1016/j.envpol.2009.01.002.

Nakai N, Murata M, Nagahama M, Hirase T, Kawanishi S. 2003. Oxidative DNA damage induced by toluene is involved in its male reproductive toxicity. Free Radical Research 37:69–76. DOI:10.1080/107157602100033103.

Novoarth T, Capela R, Pereira SPP, Moreira SM, Santos MM, Reis-Henriques MA. 2014. Toxicity effects of hazardous and noxious substances (HNS) to marine organisms: Acute and chronic toxicity of p-xylene to the amphipod Gammarus locusta. Journal of Toxicology and Environmental Health, Part A 77:1210–1221. DOI:10.1080/15287394.2014.921867.

OECD (Organisation for Economic Cooperation and Development). 2002. Ethylbenzene. USA: UNEP Publication. pp. 177.

Petrauskienė L. 2004. The medicinal leech as a convenient tool for water toxicity assessment. Environmental Toxicology 19:336–341. DOI:10.1002/tox.20039.

Rao AB, Anand CSK, Kulkarni GK. 1983. Effect of tranquilisers on the reproduction of a freshwater leech, Pseudeobdella viridis (Blanchard). Proceedings: Animal Sciences 92:323–331.

Robertson JD, Orrenius S. 2000. Molecular mechanisms of apoptosis induced by cytotoxic chemicals. Critical Reviews in Toxicology 30:609–627. DOI:10.1080/1040840009851122.

Ross D. 2000. The role of metabolism and specific metabolites in benzene-induced toxicity: Evidence and issues. Journal of Toxicology and Environmental Health, Part A 61:357–372. DOI:10.1080/00984100050166361.
Wan YI, Wei Q, Hu J, Jin X, Zhang Z, Zhen H, Liu J. 2007. Levels, tissue distribution, and age-related accumulation of synthetic musk fragrances in Chinese sturgeon (Acipenser sinensis): Comparison to organochlorines. Environmental Science & Technology 41:424–430. DOI: 10.1021/es061771r.

Weaver CV, Liu S-P. 2008. Differentially expressed pro- and anti-apoptogenic genes in response to benzene exposure: Immunohistochemical localization of p53, Bag, Bad, Bax, Bel-2 and Bel-w in lung epithelia. Experimental and Toxicologic Pathology 59:265–272. DOI: 10.1016/j.etp.2007.02.012.

Weaver CV, Liu S-P, Lu J-F, Li B-S. 2007. The effects of benzene exposure on apoptosis in epithelial lung cells: Localization by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) and the immunocytochemical localization of apoptosis-related gene products. Cell Biology and Toxicology 23:201–220. DOI: 10.1007/s10565-006-0165-2.

Webb E, Bushkin-Bedient S, Cheng A, Kassotsis CD, Balise V, Nagel SC. 2014. Developmental and reproductive effects of chemicals associated with unconventional oil and natural gas operations. Reviews on Environmental Health 29:307–318. DOI: 10.1515/reveh-2014-0057.

Weber L, Carvalho L, Sá N, Silva V, Beraldini N, Souza V, Conceição M. 2013. Genotoxic effects of the water-soluble fraction of heavy oil in the brackish/freshwater amphipod Quadrirvisio afflitzi (Gammaridea) as assessed using the comet assay. Ecotoxicology 22:642–665. DOI: 10.1007/s10646-013-1055-z.

Westcott FM. 1997. The effects of low-level chronic cadmium exposure on a freshwater leech. Thesis submitted to the faculty of graduate studies in partial fulfilment of the requirements for the degree of master of science. University of Calgary. pp. 101–112.

WHO. 2008. Guidelines for drinking water quality. Third Edition incorporating the first and second addenda. Switzerland, Geneva: World Health Organization.

Wu J, Jing L, Yuan H, Peng S-Q. 2011. T-2 toxin induces apoptosis in ovarian granulosa cells of rats through reactive oxygen species-mediated mitochondrial pathway. Toxicology Letters 202:168–177. DOI: 10.1016/j.toxlet.2011.01.029.

Zhang M, Wang Y, Wang Q, Yang J, Yang D, Liu J, Li J. 2010. Involvement of mitochondria-mediated apoptosis in ethylbenzene-induced renal toxicity in rat. Toxicological Sciences 115:295–303. DOI: 10.1093/toxsci/kfp046.

Zheng S, Zhou Q, Gao J, Xiong H, Chen C. 2012. Behavioral alteration and DNA damage of freshwater snail Bellamya aeruginosa stressed by ethylbenzene and its tissue residue. Ecotoxicology and Environmental Safety 81:43–53. DOI: 10.1016/j.ecoenv.2012.04.016.

Zhuang Y, Liu P, Wang L, Luo J, Zhang C, Guo X, Cao H. 2016. Mitochondrial oxidative stress-induced hepatocyte apoptosis reflects increased molybdenum intake in caprine. Biological Trace Element Research 170:106–114. DOI: 10.1007/s12011-015-0450-0.