Chemistry Research and Applications

TOLUENE

Chemical Properties, Applications and Toxicology

Marco C. Palminteri
Editor

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PREFACE

Toluene, also known as methylbenzene, phenylmethane and tolulol, is a colorless and clear liquid with a distinct smell, characteristic of the aromatic hydrocarbon family of chemical compounds including benzene. In this book, the authors discuss the chemical properties, applications and toxicology characteristics of toluene. Topics include a toxicokinetic and toxicologic study of toluene and inhalation exposure levels; the immediate and persisting effect of toluene chronic exposure on hippocampal cell loss, learning and memory in adolescent and adult rats; the inhibitory, toxic and structure effects of toluene on microbial consortia involved in wastewater treatment; and the influence of toluene on postnatal neurogenesis of limbic and motor systems, metabolism and behavior of animals and correction of disturbance by antioxidants.

Chapter 1 – Toluene is one of the most important industrial chemical used as a blending component for automotive fuels, as a chemical intermediate and as a solvent primarily for paints and for inks, adhesives and pharmaceuticals.

Chemical sensibility is triggered by a large of chemicals present both indoors and outdoors including pesticides, cleaning products, perfumes, scented products and cigarette smoke. Health risk after chemical exposure depends on age, sex, genetic factors. Toluene is one of volatile organic chemicals that cause different sensitivity in individuals. It is a common contaminant of outdoor and indoor air. The Agency for Toxic Substances and Disease Registry (ASTDR 2000) reported that toluene concentrations in suburban and urban air range from 1.3 to 6.6 ppb. Indoor air concentrations are often higher than outdoor air concentrations.

Toluene is readily absorbed from the gastrointestinal and respiratory tracts, and to a lesser degree through the skin. It is distributed throughout the body with accumulation in tissues with high lipid content. It is metabolized in

the liver, primarily to hippuric acid and benzoyl glucuronide compounds that are rapidly excreted in the urine. On the other hand, although neurotoxic and immunotoxic effects of toluene have been studied extensively, the underlying mechanism remains obscure.

This chapter summarizes selected chemical and physical properties, relevant toxicokinetic and toxicologic studies of toluene and inhalation exposure levels. In view of the situation that environmental issues become more serious day by day, recent studies on practical applications of toluene removal are also reviewed.

Chapter 2 – The present study has been undertaken to determine whether toluene chronic exposure provokes immediate and/or persistent effect on the structure of hippocampus, learning and memory in adolescent and adult rats. The authors exposed male Wistar rats at ages P 28-32 (adolescents) and P 70-75 (adults) to 2000 ppm inhaled toluene for 40 days. The immediate and persisting effects of toluene misuse (immediately after the end of toluene chronic inhalation and 90-day after the end of toluene chronic inhalation, correspondingly) on (i) pyramidal cell loss in the CA1 and CA3 of the hippocampus, (ii) exploratory behavior and recognition memory in the open field and (iii) behavior in multi-branch maze were evaluated. The results reveal that toluene chronic exposure affects the structure of the hippocampus, the behavior in multi-branch maze, exploratory activity and recognition memory in the open field in adolescent and adult rats. In all cases the effect is age-dependent. In particular: in adolescent rats the more significant structural and behavioral alterations were observed immediately after toluene chronic exposure, while in adult rats the most considerable was persisting effect (90 days after withdrawal). Such data indicate that character of alterations depends upon the postnatal age of testing of the animals. The results are also additional evidence that hippocampus – the neural substrate of learning and memory, may contribute to the pathophysiology of toluene abuse in organisms of different age.

Chapter 3 – Human activities have resulted in a continuous generation of wastewater containing high concentration of organic matter and nitrogenous compounds such as ammonium, nitrate and nitrite. Some effluents such as those generated by petrochemical industry contain toluene, an aromatic volatile compound which contaminates soils, water and groundwater due to petroleum spills or to leaking storage tanks. Because of its toxicity, toluene has been classified as priority pollutant by the Environmental Protection Agency. Organic compounds and ammonium, nitrate, or nitrite can be simultaneously eliminated from municipal and industrial wastewaters using the metabolic

capacity of the nitrifying and denitrifying bacteria. Nevertheless, toluene may exert inhibiting or toxic effects on the microbial consortia. In fact, it has been reported important diminishes in the specific rates of nitrifying and denitrifying bacteria activity during toluene removal processes for wastewaters treatment. Some toxic effects have been also reported. Likewise, considering the non polar characteristic of this compound, toluene appeared to affect the exocellular substances production, sludge structure and settleability.

This chapter pretends to succinctly present the current knowledge about inhibitory and toxic effects of toluene on the biological consortia during the removal of ammonium, nitrate, nitrite and toluene from wastewaters by nitrification and denitrification. Attention is also paid on some effects of toluene on sludge settleability and exopolimeric substances content.

Chapter 4 – Alterations of the neuron quantity in the cortical and subcortical structures of the limbic and motor systems, as well as proliferative activity of granular cells in fascia dentate of hippocampus and cerebellum in albino rats at the early stages of postnatal development (P3-P21) were determined after exposure to toluene (500ppm, 1200ppm). Investigations have shown that toluene induced death of neurons in the above structures of the central nervous system (CNS) and inhibition of granular cells' proliferation and migration. Effects of toluene on patterns of migration of glial cells in the culture condition at early stages of cultivation has shown that intensity of glial cells' migration and axons growth are decreased.

Free radicals (lipoperoxide LOO•, NO) content in toluene intoxicated rats cerebral cortex were determined at P15, P30, P60 stages of development using the Electron Paramagnetic Resonance (EPR) method. The obtained data indicate the intensification of generation of oxidative stress, which promotes enhanced expression of iNOS and intensification NO synthesis in rat's cerebral cortex.

Investigations of correction of toluene-induced changes by antioxidants has shown, that Miradol decrease the number of perished neurons in the cortex and subcortex of the motor systems and impair the toluene effect on the cerebellar granule cells proliferative activity and migration properties of the glial cells and axons growth in vitro.

The peculiarities of spatial translocation and alterations of learning and memory processes have been investigated in multiway elevated maze and by the passive avoidance (PA) tests. The toluene intoxication significantly decreased manifestation of the aurioculonasocephalic (ANC) reflex and ability of finding mother's location at P7, P13. Decrease of platform finding velocity in the water corridor was found also at P15, P21. Toluene intoxication induced

the decrease of exploratory activity and motivation level, as well as the increase of emotional background, as compared to the control animals, and finally significant alterations of spatial learning and memory processes at P30 in multi-way elevated maze. Learning process was deteriorated as well in the passive avoidance (PA) test on P30. Experimental animals showed toluene intoxication-induced decrease of the memory trace consolidation, as compared to the control animals.

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Chapter 1

CHEMICAL PROPERTIES, APPLICATIONS AND TOXICOLOGY OF TOLUENE

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ABSTRACT

Toluene is one of the most important industrial chemical used as a blending component for automotive fuels, as a chemical intermediate and as a solvent primarily for paints and for inks, adhesives and pharmaceuticals.

Chemical sensibility is triggered by a large of chemicals present both indoors and outdoors including pesticides, cleaning products, perfumes, scented products and cigarette smoke. Health risk after chemical exposure depends on age, sex, genetic factors. Toluene is one of volatile organic chemicals that cause different sensitivity in individuals. It is a common contaminant of outdoor and indoor air. The Agency for Toxic Substances and Disease Registry (ASTDR 2000) reported that toluene concentrations in suburban and urban air range from 1.3 to 6.6 ppb. Indoor air concentrations are often higher than outdoor air concentrations.

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other hand, although neurotoxic and immunotoxic effects of toluene have been studied extensively, the underlying mechanism remains obscure.

This chapter summarizes selected chemical and physical properties, relevant toxicokinetic and toxicologic studies of toluene and inhalation exposure levels. In view of the situation that environmental issues become more serious day by day, recent studies on practical applications of toluene removal are also reviewed.

INTRODUCTION

Many volatile organic compounds (VOCs) are the result of industrial activity (use of fuels, solvents, production of plastics, synthetic fibers and pesticides, etc.) or of the treatment of solid and liquid waste. [1] VOCs are not only major contributors to air pollution but also as main precursors of ozone and smog formation [1–3]. In many countries, including USA, EU, Japan and Korea, stringent legislations have been put in effect to abate VOCs emission. The most common VOCs from emission sources of these types are ethane, propane, acetylene, alkanes and BTEX (benzene, toluene, ethyl-benzene and xylene) [4, 5].

VOCs emissions are of great interest because certain species of them are toxic and carcinogens; exposure to even small quantities can bring about irreparable damages to human health. Exposure to VOCs might cause toxic effects to central nervous system and internal organs, and might cause symptoms, such as the sick building syndrome (SBS) including mucous membrane irritation, headache, fatigue, respiratory tract irritation, dizziness and nausea [6-10].

Among the VOCs, toluene is one of the most widely used in many industries (paints, adhesives, glues, plastics, coatings, textiles, etc.). Toluene is a hydrophobic compound and one of the 189 hazardous air pollutants (HAPs) listed in the 1990 Clean Air Act Amendment (CAAA90) proposed by the US Environmental Protection Agency (EPA) [11]. It is rapidly absorbed from the lungs following inhalation exposure. The mechanisms of action of toluene toxicity are not well understood, and it is unclear whether toluene itself or its metabolites are responsible for its effects. The acute and chronic effects on the central nervous system are of most concern as WHO states [12]. Human studies have reported a number of symptoms of acute exposure depending on concentration levels and duration of the exposure. Symptoms progress from irritation of eyes, nose and throat, fatigue, dizziness, headache and decreased manual dexterity to narcosis as exposure levels increase. It is well established

that neurotoxicity and neurobehavioral deficits are the principal effects of long-term inhalation exposure to toluene in both humans and experimental animals. Toluene may also cause developmental decrements and congenital anomalies in humans, and these effects are supported by findings of studies on animals, for example fetal development retardation, skeletal anomalies, low birth weight and developmental neurotoxicity. The potential effects of toluene on reproduction and hormone balance in women, coupled with findings of hormone imbalances in exposed males, are also of concern. Both the human and animal data indicate that toluene is ototoxic at elevated exposures [12].

WHO (2000) has set the value of 1 mg/m^{-3} (30 min) as a recommended guideline for toluene in ambient air. Occupational exposure limits for toluene derived by ACGIH, NIOSH and OSHA are based on human studies where central nervous system toxicity and irritant effects were demonstrated at air concentrations above 40 ppm (150 mg/m^{-3}). An extensive review of toluene effects on humans and ecological receptors, toluene endpoints and limit values can be found analytically elsewhere [13].

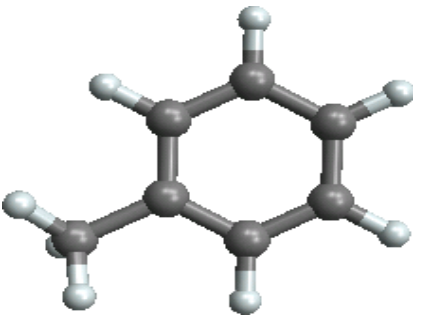
Current technical solutions for improving air quality include combinations of ionization, activated carbon adsorption, photocatalysis, biofiltration, catalytic oxidation and thermal incineration [14]. These methods can be used for the treatment of industrial emissions [15, 16], the deodorization of air emissions [17] and the treatment of indoor air [14].

1. Chemical and Physical Information Relevant to Toluene

Toluene which is also known as methylbenzene, phenylmethane and toluol is a colourless and clear liquid with a distinct smell, characteristic of the aromatic hydrocarbon family of chemical compounds including benzene. It was originally extracted from the tropical Colombian tree of Myroxylon balsamum, which has an aromatic extract known as tolu balsam. However, toluene is also a naturally occurring compound in crude though in very low levels. Some relevant physical and chemical properties of toluene are listed below [18, 19].

At room temperature, toluene is a clear-to-amber colorless liquid with a pungent, benzene-like odor. Although it is a liquid at room temperature, toluene's low vapor pressure results in extensive volatilization. It is flammable with flash point of 4.4 ˚C. Toluene is strongly reactive with a number of

chemical classes, particularly nitrogen-containing compounds, and may react with some plastics. ACGIH (2000) has a recommended an 8-hour time-weighted average (TWA) of 50 ppm (189 mg/m^3).



Structural formula: $C_6H_5CH_3$
Molecular weight: 92.14 g/mol
Density: 0.867 g/ml
Vapor pressure: 28.4 mm Hg at 25 ˚C
Water solubility: 0.59 mg/ml at 25 ˚C
Partition coefficient; Log K_{ow}: 2.72

Figure 1. Structure of Toluene.

For toluene to protect against effects on the central nervous system [20]. OSHA (1993) has promulgated an 8-hour permissible exposure limit (PEL) of 200 ppm (754 mg/m^3) [21].

Toluene is a volatile, reactive aromatic hydrocarbon that is nearly insoluble in water. It is chemically incompatible with strong oxidizing agents, sulfuric and nitric acids, nitrogen tetraoxide and chlorine. In addition, toluene reacts as a normal aromatic hydrocarbon towards electrophilic aromatic substitution. Because of the high electron density in the aromatic ring, toluene behaves as a base both in formation of charge – transfer π complexes and in the formation complexes with super acids. In this regard, toluene is intermediate between benzene and the xylenes. In the formation of π complexes with electrophiles such as silver ion, hydrogen chloride, and tetracyanoethylene, toluene differs from either benzene or the xylenes by less than a factor of two in relative basicity. The difference is small because the complex is formed almost entirely with the π electrons of the aromatic ring; the inductive effect of the methyl group provides only minor enhancement. In contrast, with HF and BF_3, which form a sigma – type complex, or in the case of reaction as with nitronium ion or chlorine where formation of sigma bonds

and complexes plays a predominant role, the methyl group participates by hyperconjegation and the relative reactivity of toluene is enhanced by several orders of magnitude compared to that of benzene. Reactivity of xylenes is enhanced again by several magnitudes over that of toluene. Thus, when only the π electrons are involved, toluene behaves much like benzene and the xylenes. When sigma bonds are involved, toluene is a much stronger base than benzene and a much weaker base than the xylenes.

Methylation of Toluene

The methylation of toluene with methanol produces xylene and water:

$$C_6H_5.CH_3 + CH_3.OH \rightarrow C_6H_4.(CH_3)_2 + H_2O$$

whereas disproportionation (DISP) takes place via reacting two toluene molecules together to produce a xylene molecule and a benzene molecule (methyl group transfer):

$$2\ C_6H_5.CH_3 \rightarrow C_6H_6 + C_6H_4.(CH_3)_2$$

The most significant side reaction taking place during both reactions is the production of trimethylbenzenes (TMBs). During toluene methylation, TMBs form via alkylating a produced xylene molecule with another methanol molecule. Here, the reaction does not encounter significant diffusion restriction, since methanol, which is relatively very small, diffuses rapidly and reacts with toluene:

$$C_6H_4.(CH_3)_2 + CH_3.OH \rightarrow C_6H_3.(CH_3)_3 + H_2O$$

Oxidation of Toluene

The qualitative toluene oxidation and pyrolysis paths already out lined have been used along with the results of recent experimental work to write the following initiation and chain propagation scheme involving toluene and its pyrolysis fragments [22-25]:

Initiation

$C_7H_8 + O_2 \rightleftarrows C_6H_5CH_2 + HO_2$
$C_7H_8 \rightleftarrows C_6H_5O_2 + H$
$C_7H_8 \rightleftarrows C_6H_5 + CH_3$

Chain Propagation

$H + C_7H_8 \rightleftarrows C_6H_6 + CH_3$
$H + C_7H_8 \rightleftarrows C_6H_5CH_2 + H_2$
$H + C_7H_8 \rightleftarrows C_6H_4CH_3 + H_2$
$CH_3 + C_7H_8 \rightleftarrows C_6H_5CH_2 + CH_4$
$CH_3 + C_7H_8 \rightleftarrows C_6H_4CH_3 + CH_4$
$C_6H_4CH_3 \rightleftarrows C_4H_3 + C_3H_4$
$C_6H_4CH_3 \rightleftarrows C_3H_3 + 2\ C_2H_2$
$C_6H_5CH_2 \rightleftarrows C_3H_3 + 2\ C_2H_2$
$CH_3 + C_6H_6 \rightleftarrows CH_4 + C_6H_5$
$C_3H_4 + M \rightleftarrows C_3H_3 + H + M$
$H + C_3H_4 \rightleftarrows C_3H_3 + H_2$
$O + C_7H_8 \rightleftarrows OHC_7H_7$
$OH + C_7H_8 \rightleftarrows C_6H_5CH_2 + H_2O$
$C_6H_5CH_2 + O \rightleftarrows C_6H_5CHO + H$
$C_6H_5CH_2 + O_2 \rightleftarrows C_7H_7O + O$

$OH + C_7H_7OH \rightleftarrows C_7H_7O + H_2O$
$C_6H_5CH_2 + OH + M \rightleftarrows C_7H_7OH + M$
$C_6H_5CH_2 + HO_2 \rightleftarrows C_7H_7O + OH$
$C_7H_7O \rightleftarrows C_6H_5CHO + H$
$C_6H_5CHO \rightleftarrows C_6H_5 + HCO$
$OHC_7H_7 + H_2 \rightleftarrows C_6H_5OH + CH_4$
$2\ C_6H_5 \rightleftarrows C_{12}H_{10}$
$C_6H_5CHO + O_2 \rightleftarrows C_6H_5CO + HO_2$
$C_6H_5CHO + OH \rightleftarrows C_6H_5CO + H_2O$
$C_6H_5CHO + HO_2 \rightleftarrows C_6H_5CO + H_2O_2$
$C_6H_5CO \rightleftarrows C_6H_5 + CO$

The species OHC_7H_7 represents a composite mixture *of* ortho, para, and meta cresols. As in the case *of* benzene oxidation, one reaction from the hydrogen-oxygen system was found to be important in the toluene oxidation mechanism, namely:

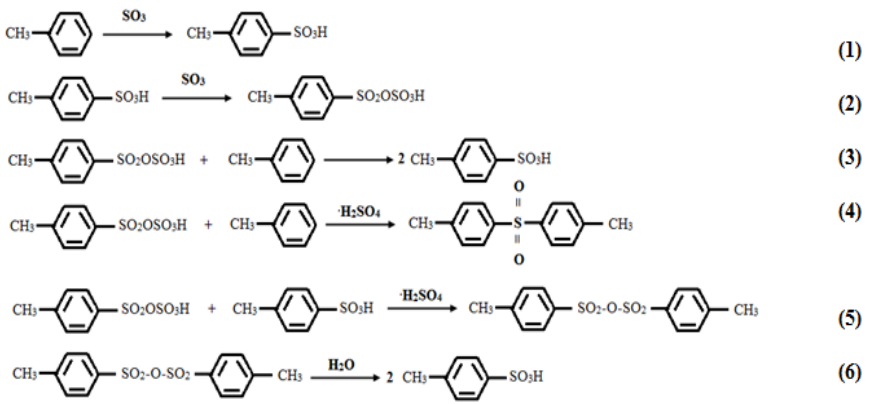
$$H + O_2 \rightleftarrows OH + O$$

This reaction is important in both initiation and chain propagation.

Sulfonation of Toluene

The solfonation of toluene with gaseous sulfur trioxide is extremely exothermic. However, it can be done safely using micro structured reactors.

The following scheme shows the reaction steps.



The stream flows through to falling-film microreactor where it reacts with the liquid toluene (reaction 1–4). As shown above sulfonic acid (reaction 1) is produced in the first reaction step. By consecutive reactions a mixed anhydride (unstable and undetectable) (reaction 2), di-tolylsulfone (reaction 3) and sulfonic acid anhydride (reaction 4) are also formed. In the residence time module the reaction of mixed anhydride with toluene to sulfonic acid (reaction 5) is completed. The last reaction step, the hydration of sulfonic acid anhydride to sulfonic acid (reaction 6), is carried out in a microstructured reactor specifically constructed for liquid phase reactions.

Transalkylation of Toluene

Toluene disproportionation, or transalkylation, catalytically converts two molecules of toluene to one molecule each of benzene and xylenes, as indicated by the reaction [26].

The transalkylation process is similar to that of toluene HDA but occurs under less severe conditions (*e.g.*, the transalkylation process operates at lower temperatures and consumes less hydrogen). Toluene is heated, combined with hydrogen, and sent to the reactor. The reacted material is moved to a separator for removal of off-gases. The product is stabilized and moved through clay towers. Benzene, toluene, and xylenes are recovered by distillation and unreacted toluene is recycled [27, 28]. Toluene disproportionation is used when the desired product is xylene. If benzene is the only product required, then HDA is a more economical and feasible process [29].

2. TOXICOKINETICS RELEVANT TO ASSESSMENTS

Toxicokinetics is the description of what rate a chemical will enter the body and what happens to it once it is in the body. Toxicokinetic studies are concerned with the change in concentration of the chemical or a metabolite with time in blood/plasma or other tissue. This information is important in relating exposure to internal dose, in determining if various biological processes, such as absorption or metabolism change as the administered dose changes, in determining if internal dose from a given exposure is different between sexes, species, old/young. Toxicokinetic data often can be extrapolated from laboratory animals to humans through mathematical modeling.

2.1. Absorption

Absorption is the process by which a chemical in the microenvironment crosses the biological barrier to enter systemic blood circulation [30]. The biological barriers relate to stratum corneum in the case of dermal exposures, alveoli in the case of pulmonary exposure, or membranes in the GI tract for oral exposure [31].

Toluene is readily absorbed from the respiratory and gastrointestinal tracts and to some degree through the skin [18]. It is estimated that 40 – 60% of inhaled toluene is absorbed [32]. Oral absorption is complete but the rate is slower than pulmonary absorption [18]. The rate of absorption of toluene through forearm skin is in the range of 14 – 23 mg cm^{-2} $hour^{-1}$ [33].

With exposure to 2,250 mg/m^3 (600 ppm) of toluene for 3.5 h a quantity corresponding to approximately 0.9% of alveolar retention has been shown to

be absorbed through the skin, as measured by the exhaled amount [34]. Exposure of skin to 1,600 mg/ml of toluene for 8 h did not result in any abnormal excretion of hippuric acid [35].

Blood levels measured in rural, urban and chemical workers not occupationally exposed to toluene were less than 3 mg/L [36]. The lowest levels were found in rural workers. Even lower blood levels have been reported by others [37]. Differences between levels measured in these studies may be based, in part, on differences in sampling and analysis procedures. Toluene was detected in most blood samples of nonoccupationally exposed individuals with a mean level of less than 1 ppb [38]. Mean blood levels in a nonoccupationally exposed population examined by Antoine et al. were 1.5 ppb [39].

Studies quantifying oral absorption of toluene are limited but have demonstrated nearly 100% absorption following a single oral exposure. In volunteers exposed to an infusion of 2 mg toluene/minute for 3 hours (~5 mg/kg) via a gastric tube, absorption of toluene, measured by monitoring exhaled air for toluene and urine for toluene metabolites, was found to be complete [40]. Turkall et al. reported that greater than 99% of a single gavage dose of radiolabeled toluene in rats was eliminated in the urine or expired air, indicating near-total absorption of the exposure dose [41].

Several studies have examined the absorption of toluene following a single inhalation exposure in humans. Benoit et al. reported an average retention of 83% in four subjects exposed to 50 ppm (189 mg/m^3) toluene for ~90 minutes [42]. Carlsson reported an average uptake (percent of inspired air) of about 55% in male subjects exposed to 300 mg/m^3 for 2 hours at rest; this value dropped to 50% during the next 2 hours of exposure at rest [43]. When the subjects exercised, the percent uptake declined with exercise time and exercise load; the absolute uptake (in mg toluene) increased with exercise time and exercise load (due to increased pulmonary ventilation). Löf et al. reported a similar absorption percentage (~50% absorbed) in groups of 10 males exposed to 3.25 $mmol/m^3$ (~300 mg/m^3) at rest for 4 hours [44]. A study by Neubert et al. found a good correlation between measured air toluene concentrations and toluene levels in the blood of rotogravure printers at the end of a 6-hour shift, though absorption itself was not quantified [45].

Toluene uptake by the inhalation route of exposure has been studied in experimental animals. The uptake ranged from 26 to 93% with a mean of 60% [46-48]. Gospe and Al-Bayati compared oral and inhalation exposures to toluene in the rat in order to determine an appropriate dosing regimen for inhalation toxicity studies [49]. Male F-344 rats were exposed to ^{14}C-toluene

by gavage or inhalation. Oral doses of 110, 336, 741, and 911 mg/kg were administered to 82 rats, and blood toluene levels were followed for six hours. For the 120 rats in the inhalation group, three-hour exposures were given at 10, 99, 549, or 1145 ppm. Blood toluene levels were measured during the uptake (exposure) phase and for a 4-hour elimination period. The data from the two exposure methods were fitted to parametric kinetic models, and the resulting curves integrated. The authors concluded that oral dosing produces blood toluene levels that are similar to those produced by inhalation; however, the shape of the time-concentration profile differed for the two methods. Inhalation curves of concentration versus time reached asymptotic levels by one to two hours. Oral blood toluene curves reached asymptotic levels from 1.6 to 6.3 hours postexposure. This suggests a slower absorption via the oral route as the concentration increased.

Table 1. Estimated toluene exposure levels in different types of exposure

Type of exposure	Observed range of concentration	Frequency of exposure	Total volume inhaled[a] or amount consumed	Inhalation or ingestion rate (mg/week)
General population				
Inhalation:				
urban areas[a]	5-145 µg/m^3	168 hours/week	140m^3	0.7-20
rural and remote areas[a]	trace-5 µg/m^3	168 hours/week	140m^3	trace-0.7
areas near user sites[b]	trace-20 µg/m^3	168 hours/week	140m^3	2800
indoor (non-industrial)[c]	trace-2.2 µg/m^3	168 hours/week	140m^3	308
Ingestion:				
drinking-water[d]	trace-10 µg/liter	2 liters/day	14 liters	0-0.3
food (fish only)[b]	0-1 mg/kg	6.5 g/day	4.5 g	0-0.045
Occupational group				
Inhalation[e]	376 mg/m^3	40 hours/week	50 m^3	18 800
Dermal[e]	0.5mg/cm^2/hour	0-30 min/week		0-1.0
Cigarette smokers				
Inhalation:				
mainstream[f]	0.2 mg/cigarette	20cigarettes/day	140 cigarettes	28
sidestream[f]	0.3 mg/cigarette			

[a] Dann et al. [54].

[b] Meek & Chan [55].

[c] Lebret et al. [56].

[d] US Environmental Protection Agency [57].

[e] Monster et al. [53].

[f] US Environmental Protection Agency [58]. Toluene content may be higher or lower depending on tobacco type.

Studies with laboratory animals [50-52] and humans [53] indicate that percutaneous absorption is slow compared to pulmonary uptake. Relative to other solvents investigated, toluene penetration of rat skin was moderate; however, rat skin may be more permeable to solvents than human skin [51]. Experimental data from volunteers simulating skin contact under occupational conditions indicate that absorption is about 0.5 mg/cm^2 per hour [53]. Absorption from the gastrointestinal tract is considered to be complete.

2.2. Distribution

Distribution, in the present context, refers to the uptake of a chemical from systemic circulation by the metabolizing, storage, and excretory organs [30]. The volume of blood, volume of tissues as well as the extent of protein binding together determine the extent of dilution or distribution of an absorbed chemical.

Toluene that is absorbed into the blood is distributed throughout the body. Human and animal inhalation studies have shown high toluene concentrations in adipose tissue, bone marrow, adrenals, kidney, liver, brain and blood [32, 33]. Ameno et al. reported that, in a 51-year-old man who died from accidental oral overdose, the highest toluene concentrations (per gram tissue) were in the liver, followed by pancreas, brain, heart, blood, fat, and cerebrospinal fluid [59]. However, Paterson and Sarvesvaran reported that a 16-year-old male, who was found dead, presumably due to inhalation overdose of toluene, had greater concentrations in the brain than the liver [60]. Takeichi et al. reported similar findings in a 20-year-old male painter who fell while working with a toluene-based paint; the greatest concentrations upon autopsy were found in the brain, followed by the liver and blood [61].

In human volunteers exposed to 80 ppm (306 mg/m^3) toluene for four, 30-minute periods at rest or in conjunction with exercise, the respective concentrations of toluene in subcutaneous fat were 0.7 and 9.9 mg/kg. Carlsson and Ljungqvist also investigated the effect of body fat on the distribution of toluene in exposed men. The estimated percentage of total toluene uptake retained in adipose tissue one hour post-exposure was 4.6% in the leanest man and 20% in the most obese. Three days after exposure, the respective fractions were estimated as 0 and 12.4%. Exercise increased the ratio of the concentration of toluene in subcutaneous fat to the concentration in arterial blood [62].

Pyykko et al. exposed groups of rats by both the oral and inhalation routes and reported greater toluene concentrations (per gram of wet tissue) in the liver than the brain by both exposure routes [63]. Following inhalation exposure during which dogs were allowed to rebreathe toluene, the liver and brain contained the highest levels (both ~190 μg/g tissue), with lesser levels in the kidneys [64]. Several studies have shown relationships between blood and tissue levels of toluene, particularly for the brain [65, 66]. Toluene is able to cross the placenta and enter the fetus [67] and can be found in breast milk [68].

2.3. Metabolism

Following inhalation or oral exposure to toluene, approximately 60 – 75% of absorbed toluene, is metabolised to benzoic acid [12, 32]. The main enzymatic pathways involved in toluene metabolism are shown in Figure 2 [69-73). The liver is expected to be the primary site of toluene metabolism. The initial step involves side chain oxidation to benzyl alcohol by cytochrome P450 enzymes. Benzyl alcohol is then further oxidised to benzoic acid by alcohol dehydrogenase and aldehyde dehydrogenase. Benzoic acid is subsequently conjugated with glycine to form hippuric acid [32]. Benzoic acid may also be conjugated with glucuronic acid to form benzoyl glucuronide in the urine. Less than 1% of absorbed toluene undergoes ring hydroxylation to form o-, and p-creosol, which are excreted in the urine as glucuronide or sulphate conjugates [32, 33]. Glutathione conjugation may also occur resulting in S-benzylglutathione and S-benzylmercapturic acid (conjugation to benzyl alcohol), or S-*p*-toluyl glutathione and S-*p*-toluylmercaptic acid (conjugation to the epoxidated ring). A detailed description of the CYP enzymes involved in the metabolism of toluene can be found in ATSDR (2000) [18].

Studies of urinary metabolites in toluene-exposed humans have identified hippuric acid as the major metabolite [40, 44, 72, 74-76, 78-85]. Minor urinary metabolites (in approximate order of decreasing abundance) include the glucuronyl conjugate of benzoic acid, the sulfate and glucuronide conjugates of *ortho*- and *para*-cresol, S-benzylmercapturic acid, and S-*p*-toluylmercapturic acid [70-72, 86].

2.4. Excretion

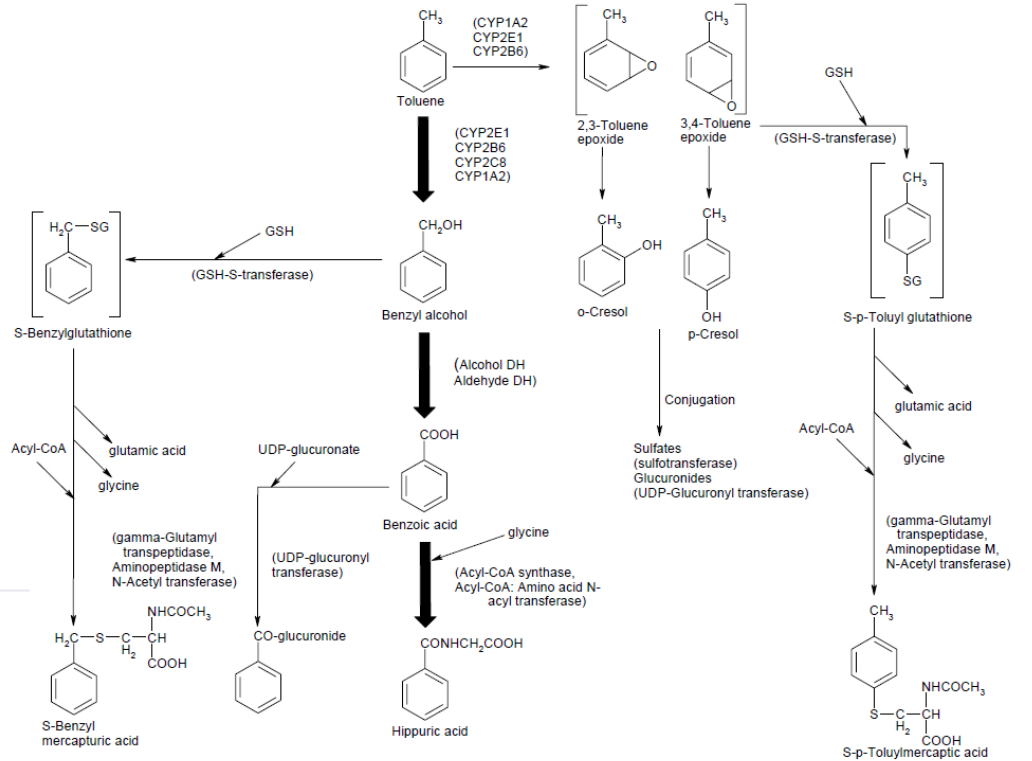
Excretion refers to the removal of the chemical and/or its metabolite from systemic circulation [30]. Studies in both humans and animals have shown that the majority of toluene in the body is eliminated in the urine, mainly as metabolites [41, 44, 83, 87, 88]. As discussed above, the primary urinary metabolite of toluene is hippuric acid, with additional metabolites (see figure metabolism) resulting from minor metabolic pathways. Elimination from the blood is rapid [43, 44, 83, 89] with three-phase elimination half times of 3, 40, and 738 minutes following a single inhalation exposure in humans [83]. A lesser, but still significant, amount of inhaled toluene is removed in the expired air [53, 90]. Elimination of toluene in the expired air is greatest at time points during or immediately after exposure and decreases rapidly there after [42]. Turkall et al. estimated that ~22% of a single oral dose is eliminated in the expired air in rats with the remainder being mainly eliminated in the urine [41].

There are several studies of toluene elimination in humans. A positive correlation between the concentration of toluene in alveolar air and in blood both during and after exposure has been demonstrated [43, 91, 92]. Nomiyama and Nomiyama reported that the excretion of hippuric acid in humans exposed to 107 ppm toluene in air reached a maximum rate of approximately 190 mg/hour within two hours of the start of exposure. These investigators also estimated elimination half-life for toluene-derived hippuric acid to be 117 and 74 minutes in males and females, respectively [93].

For humans, the elimination of hippuric acid is not considered as reliably associated with toluene exposure as it was formerly considered to be. The variation among individuals within the human population is too large for this association to be considered suitable for biological monitoring. One possible explanation for the variability is the differing levels of benzoic acid within the human diet [83, 94].

2.5. Physiologically-Based Pharmacokinetic (PBPK) Models

PBPK models are now commonly used in drug development and regulatory toxicology to predict the kinetics and metabolism of substances in the body, with a focus on the effective dose at the expected target site [95-98].



* Proposed enzymes are noted in parentheses.
Sources: Angerer et al., 1998; ATSDR, 2000; IARC, 1999; Nakajima and Wang, 1994; Nakajima et al., 1997; Tassaneeyakul et al.,
** CoA = coenzyme A; CYP = cytochrome P-450; DH = dehydrogenase; GSH = glutathione; UDP = uridine 5'-diphosphate.

Figure 2. Proposed pathways for toluene metabolism.

The physiological basis of PBPK models makes them especially suited to explore, understand and predict the determinants of inter- or intra-individual variability in pharmacokinetics. Those translate into variability of target doses and can have direct consequences for therapeutic safety and the likelihood of toxicity, especially for compounds with narrow therapeutic windows or a steep dose–response for toxicity. Therefore, simulation of inter-individual variability has become an integral part of the assessment of pharmacokinetics in humans [99,100].

PBPK models are available that describe the kinetics of toluene after inhalation exposure: two for humans [101-103] and three for rats [104-107]. These models are modifications of the standard four-compartment PBPK model developed for styrene [108] in which

- Absorption into the lung blood is assumed dependent on the inhaled concentration of toxicant, the concentration of toxicant in alveolar air, blood flow to the lung, blood/air partition coefficient, and alveolar ventilation rates
- Exchange of toxicant between arterial blood and tissue compartments is flow-limited
- Changes in the amount of toxicant in three nonmetabolizing tissue compartments (adipose tissue, slowly perfused tissues, and rapidly perfused tissues) are described by mass transfer differential equations with tissue volume, blood flow through the tissue (i.e., tissue perfusion rate), arterial blood toxicant concentration, and tissue/blood partition coefficients as explanatory variables
- Changes in toxicant amount in the liver (the fourth compartment) are described by similar differential equations that additionally include a Michaelis-Menten term for overall rates of toxicant metabolism

The five-compartment human model for toluene developed by Pierce et al. includes an additional equation describing mass balance across the lung that has a single Michaelis-Menten metabolic term to represent total toluene metabolism [102]. A five-compartment rat PBPK model developed by DeJongh and Blaauboer is similar in design to the Tardif et al. rat PBPK model except that it contains an additional nonmetabolizing compartment representing the brain [104, 106]. The above models have all been partially- or fully-validated using *in vivo* pharmacokinetic data in the appropriate species. Van Asperen et al. utilized a five-compartment model that also included a pulmonary blood-alveolar air gas exchange compartment to study the impact

of the exposure scenario (constant vs. fluctuating) on the behavior and toxicokinetics of the rat [107]. This analysis utilized high exposure concentrations (2700-8000 ppm toluene) for short periods of time and found the difference in toxicokinetics after constant or fluctuating exposure at high dose levels to be small but that fluctuating exposure patterns may produce different toxic effects than continuous exposures, even when the external exposure conditions have the same time-weighted average.

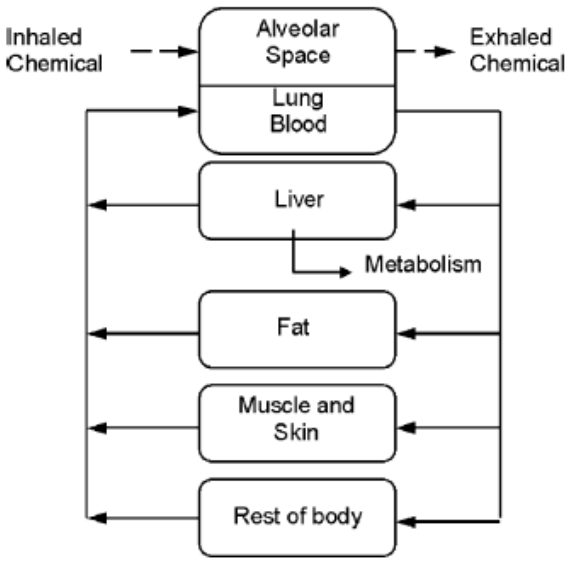


Figure 3. Conceptual representation of a PBPK model.

3. TOXICITY OF TOLUENE

The health risks of inhalation exposure to volatile organic compounds are usually estimated based on 8 h time-weighted average occupational exposure limits (TLV-TWA). These limits are set using the total external dose (expressed as concentration×time) as the dose metric for risk assessment. Animal experimental or human studies to determine the acute effects on which exposure limits are based typically use constant concentration exposure scenarios. However, recent insights recognise that the toxicity of inhaled solvents may not only be related to the total external dose but also to the pattern of exposure. For example, exposure to regularly occurring peak

concentrations of organic solvents has been speculated to play an important role in the development of chronic toxic encephalopathy (CTE) [109-111]. Some recent rat data indicate that also acute effects of industrial solvents are differentially affected following fluctuating exposure including peak concentrations or following exposure to a constant concentration [107, 112].

Toluene, a volatile organic solvent, is widely used in industry and a number of commercial products such as cosmetics, inks, adhesive, paints and glues [113]. As a widely used chemical of commercial importance as well as a drug of human abuse, there is much general information in the literature about toxic effects of toluene exposure. Inhalation is the typical route of human exposure to toluene, although it can be absorbed through the skin and gastrointestinal tract. The occupational exposure limit for toluene is 50 ppm in Japan [114], although the updated threshold limit value is 20ppm in the United States [115]. Toluene readily crosses the blood-brain-barrier after inhalation and produces effects similar to that of sedative-hypnotics, such as alcohol and benzodiazepines [116]. Studies on the health effects of toluene exposure have revealed that toluene mainly affects the central nervous system, causing an increased tendency to sleep, frequent headaches, eye irritation and memory impairment in humans [117]; dizziness, depression, and fatigue in paint workers [118, 119]; and cerebellar dysfunction and cerebral and hippocampal atrophy as well as a loss of brain volume in chronic toluene abusers [120-122]. In addition, the inhalation of low concentrations of toluene induces a persistent deficit in spatial learning and memory in humans [123-125] and in animals [126, 127]. Toluene-induced impairments of memory function are associated with occupational exposure levels [128, 129]. The abuse of toluene by pregnant woman can lead to a form of embryopathy known as “fetal solvent syndrome,” in which growth retardation and microencephaly are the major effects in newborns, accompanied by a typical facial appearance with deep-set eyes, low-set ears, a flat nasal bridge, micrognathia and cognitive deficits [130, 131].

3.1. Health Effects in Animals

The acute toxicity of toluene either inhaled or ingested, is relatively low [132]. Estimates for median lethal doses (LD50, LC50) for acute toluene exposures have been reviewed [9, 113, 133, 134]. The median lethal dose of toluene was estimated in rats, mice, guinea pigs, and rabbits for various routes of exposure [135]. In rats, the oral LD50s range from 2,600 to 7,530 mg/kg.

Lethal doses following dermal administration are 1.5 to 5-fold higher than those for ingestion. The range of LC50 values for inhalation is 20,299 to 153,200 mg/m^3 (5300 to 40,000 ppm) depending on the duration of exposure and the species [134].

Narcosis, instability, incoordination, depression, restlessness, tremors, and prostration were consistently observed in rats and mice exposed to high doses of toluene via inhalation [137, 138]. Various related manifestations have occurred over a wide range of exposures, including limb paralysis and contractions, headshakes, and loss of the righting reflex [137-140].

Studies in the rat and mouse have detected effects of toluene inhalation on brain constituents and morphological and biochemical parameters. High levels approaching 2000 ppm have caused ataxia, prostration and tremors in rats exposed for 7 days [141]. At the completion of this 26-week study, there were no treatment-related effects. Regional loss of cells in the hippocampus coupled with abnormal electrical activity in this region was observed when rats were exposed to a toluene concentration of 500 ppm for 8–16 hours/day for up to 5 weeks [142]; it was concluded that toluene causes irreversible effects in this area of the brain.

Inhaled toluene in rats has been detected in all brain regions, with the highest concentration in the brainstem, an area also found to be involved in neurological sequelae in toluene abusers [143]. Uptake was correlated with lipid content in each brain region. Enzyme activities and receptor binding was most affected in the brainstem of rats exposed subchronically and chronically [144].

Currently, many researchers are involved in the study of neuroimmune interactions after environmental chemical exposure. Twelve years ago, Kipnis et al. demonstrated that cop-1 (a synthetic antigen that apparently acts as a weak agonist for a wide range of self-reactive T cells) vaccination in healthy animals resulted in an increase in the accumulation of T cells in the CNS and the local population of neurotrophic factors [145]. In addition, the same research group has reported that a deficiency in peripheral T cells leads to cognitive and behavioral impairments and that these impairments are restored by the passive transfer of mature T cells [146]. CNS-specific T cells are necessary for spatial learning and memory and for the expression of neurotrophic factors in the dentate gyrus, suggesting that a common immune-associated mechanism underlies different aspects of hippocampal plasticity and cell renewal in the adult mouse brain [147]. Using wild-type and athymic nude mice, our laboratory showed for the first time that a specific link exists between the presence of T cells in the hippocampus and the up-regulation of

memory function related gene expressions in the hippocampus of wild-type mice exposed to low levels of toluene [148].

Neurotrophins are a group of structurally related polypeptides that support the survival, differentiation, and maintenance of neuronal populations expressing appropriate high-affinity neurotrophin receptors. Neurons in the hippocampus are maintained by neurotrophins such as nerve growth factor (NGF), BDNF, and the tyrosine kinase (Trk) family of neurotrophin receptors. Neurotrophins and their related receptors have been identified as targets for neurotoxicants and are known to play a role in bidirectional signaling between cells of the immune and nervous systems. We suggest that toluene exposure affects the function of the hippocampus by modulating neurotrophin-related genes and signaling pathways and that allergic stimulation as an immune stressor may influence the threshold for sensitivity. Currently, we are investigating inter species variations in sensitivity to the expression of toluene-mediated neurotrophins and related receptors in the hippocampi of three strains (C_3H/HeN, BALB/c, C57BL/10) of mice and examining the combined effect of toluene exposure and allergic challenge on neurogenesis-related markers in a sensitive mouse strain. We have found that low levels of toluene exposure can up-regulate the expression of neurotrophins and their related receptors in the mouse hippocampus in a strain-dependent manner and that allergic stimulation lowers the threshold for sensitivity to toluene in C_3H/HeN mice, the most sensitive strain [149].

Rats exposed to toluene (1500 ppm for 4 h per day for 7 days) showed increased adrenal gland weight, the size of adrenocortical cells, immuno reactivity of corticotrophin-releasing factor (CRF) in the paraventricular nucleus and plasma adrenocorticotropic hormone (ACTH) [150]. These findings suggest that toluene exposure might induce adrenocortical hypertrophy via HPA axis.

The rats exposed to organic solvents showed significant changes in catecholamine levels in the hypothalamus, the site of higher centers of the autonomic nervous system [151, 152].

A variety of tests have been employed to test learned behaviour in rats [153]. Acute exposure of rats to toluene concentrations of up to 1000 ppm have not been shown to impair avoidance performance [154]. However, effects were found at repeated exposure to levels as low as 900 ppm [155]. Long-lasting impairment of operant behaviour was observed in rats exposed to 1000 ppm for 21 hours/day, 5 days/week, for 4 weeks [156]. Impairment of spatial learning and memory accompanied by a persistent increase in dopamine-mediated locomotor activity was observed in rats exposed to 80 ppm for 6

hours/day, 5 days/week for 4 weeks [126]. Although spontaneous locomotor activity was not affected, apomorphine-induced locomotion and activity was increased by toluene exposure in this protocol [157]. Exposure of rats to 1000 ppm, 21 hours/day for up to 11 weeks caused disturbances in vestibulo-oculomotor function [158].

A variety of studies suggest that toluene is only minimally toxic to the liver and kidney. Exposure of rats to toluene at concentrations of 30 and 300 ppm for 6 hours/day, 5 days/week for four weeks produced only minimal histological changes and had no effect on AST, liver weight, or mixed function oxidases [159]; serum alkaline phosphatase was elevated at 300 ppm. There were no histopathological changes in the kidney.

Information gained from animal studies supports the observations in humans that toluene can be ototoxic. Ototoxicity has been observed in rats when toluene was injected subcutaneously [161], via gavage dosing [161] or by inhalation [162]. Li et al. demonstrated that toluene exposure canaggravate auditory degeneration in genetically predisposed mice [163].

3.2. Health Effects in Humans

The health risk after environmental chemical exposure depends on age, sex, genetics, socioeconomic status, nutritional status and environmental factors [164]. Individuals with different genetic backgrounds have different sensitivities to toxic chemical exposure, and some people are more susceptible to chemical exposure than others because of their genetic makeup. The adverse health effects of chemicals depend on the chemicals' toxicities and how people are exposed to the chemicals, as well as individual susceptibility.

Differences in sensitivities to chemical exposure may be due to environmental, genetic and physiological factors; however, no data exists concerning whether some people are more sensitive to toluene exposure as a result of their genetic background.

Toxicity studies in humans have primarily involved evaluation of individuals exposed to toluene via inhalation in experimental or occupational settings or as a result of intentional abuse. Only those studies involving primarily toluene are described.

An increasing trend in the prevalences of allergic disease, such as asthma, rhinitis and eczema, has been reported in many industrialized countries. Outdoor and indoor air pollutants are environmental risk factors that have been shown to contribute to the development of respiratory infection and allergy.

Rapidly increasing evidence has suggested that environmental agents may cause adverse health effects by disrupting the homeostatic mechanisms of nervous and immune systems. Under physiologic conditions, the local production of neurotrophin is low in both human and animal studies [165].

Echeverria et al., studied forty-two college students (21 female and 21 male) who were exposed to 0, 74 ppm (279 mg/m^3), or 151 ppm (569 mg/m^3) toluene for 7 hours over 3 days. This exposure sequence was repeated for a total of 42 exposures over a 3- month period. The odour of toluene was masked. A battery of performance tests was administered to each participant prior to starting the exposures and again at 4 and 7 hours during the exposure; the initial test served as a control for those tests performed during the exposure. A 5-10% decrement in performance was considered significant if consistent with a linear trend. Test results for visual perception differed from control values for both exposure levels. Results of a manual dexterity test differed from control values at the higher but not the lower exposure level. Psychomotor test results were unaffected by toluene exposure. Subjective symptomatology increased with exposure with increasing numbers of complaints of eye irritation, headache, and somnolence. A NOAEL of 74 ppm (279 mg/m^3) is indicated for these results. The duration-adjusted value is 122 mg/m^3 for these acute effects [166].

An extensive database on human exposure to toluene indicates that dysfunction of the CNS is of primary concern. Deficits in neurobehavioural functioning have been viewed as precursors of more serious indications of CNS toxicity [167, 168]. Their measurement is generally held to be more reliable than subjective symptoms, which may involve situational factors unrelated to a cause–effect relationship with exposure. Potential confounders such as age, alcohol, drugs and education need to be assessed to ensure correlations of toxicity with exposure.

Toluene abuse typically involves acute or chronic exposure to unknown, high levels. Cognitive dysfunction may be the most disabling and frequent feature of chronic toluene abuse [169]. Gradual resolution of some effects was found upon discontinuance of toluene abuse, although persistent neurological impairment was foreseen some individuals. The types of effects seen in abuse cases include: cerebral, cerebellar and brainstem atrophy, ataxia, muscular incoordination, neuronal degeneration and personality disorders [170]. Magnetic resonance imaging or computerized tomography have been useful tools for examining affected areas of the brains of toluene abusers [171].

Evidence of abnormal brainstem auditory evoked potential, considered to be an early indicator of CNS injury, and has also been found in chronic toluene abusers [172]. Slow-wave abnormalities upon electroencephalography have been demonstrated in some case reports [173]. Workers exposed to high concentrations of toluene for extended periods of time have been reported to exhibit signs of mild chronic toxic encephalopathy [173].

Abnormalities in visual evoked potential in printers exposed to a mean toluene concentration of 2000 mg/m^3 or 530 ppm (measured over a ten-year period) were suggested as evidence of subclinical CNS dysfunction [174]. Toluene exposure of workers was also suggested as a cause of altered visual evoked potential, although an exposure–response relationship was not demonstrated [175].

The developing human brain is inherently much more susceptible to injury caused by environmental toxic chemicals than the adult brain. Currently, the potential health effects of chemicals in children have become a public issue, particularly with regard to neurodevelopmental disorders resulting from exposure to environmental toxicants during brain development. Prenatal toluene exposure reportedly leads to significant embryopathy, known as fetal solvent syndrome, in which women who abuse solvents during pregnancy are prone to bearing infants with congenital defects such as developmental delays, microcephaly, and cognitive deficits [130, 131]. However, the exact mechanism underlying toluene embryopathy remains unclear. Neonatal toluene exposure during postnatal days 4–7 induces long-term but reversible changes in the function and composition of NMDA receptors and may contribute to the cerebellar dysfunction observed in fetal solvent syndrome [176]. In addition, young rats show long-term behavioral deficits after prenatal toluene exposure on gestational day (GD) 8 through GD20 [177]. In addition, neuronal proliferation, differentiation, migration, synaptogenesis, apoptosis, gliogenesis and myelination begin at an early stage of embryogenesis and extend well into postnatal life. Toluene may induce alterations in neurogenesis and neuronal migration [178] and may disrupt synaptogenesis in hippocampal neurons [179]. Inflammatory signals reportedly induce neurotrophin expression in human microglia cells through the transcription factor NF-κB pathway [180]. Proper communication between neurons, immune cells and neurotrophins is potentially responsible for regulating and controlling neuroimmune crosstalk. Therefore, developmental exposure to low-level toluene might disturb neuroimmune interactions and persist in later life.

Although brain is a primary target for toluene exposure, toluene also causes autonomic and peripheral nervous dysfunction [181]. In human studies,

a considerable percentage of workers exposed to mixed organic solvents, mainly toluene, have shown symptoms related to the autonomic nervous system such as palpitation, nausea, bound bowels, or irritation [182, 183]. Electrocardiographic findings in workers exposed to mixed organic solvents including toluene show that R–R intervals was significantly reduced especially in respiratory sinus arrhythmia which reflects parasympathetic component [181, 184].

F. Pariselli et al. studied on biological effects of low constant measurable concentrations of benzene and toluene and their mixture, representing the key contaminants in indoor non-occupational environments [186], on human tumour lung epithelial cells (A549). The relevant indoor air mixture of the two VOCs, toluene and benzene, increased cytotoxicity without any modification of the glutathione redox status. The analysis of the exposed cells by the comet assay showed the most interesting effects. DNA repair occurring after toluene exposure alone was suppressed in the presence of benzene. This could be due to an intracellular interaction between both chemicals [185].

Although toluene is defined as a class 3 substance (not classifiable as carcinogenic to humans) [74], some studies have shown evidence for toluene genotoxic effects in exposed subjects [187-189]. Biological monitoring, using different exposure, effect, or susceptibility biomarkers, has a fundamental role in occupational risk assessment [190]. Several biomarkers are available to assess internal exposure to toluene; however, with the reduction of toluene content in some preparations coupled with better industrial hygiene conditions, more specific markers are required for biological monitoring of low-level exposure to toluene [191]. In addition to biomarkers of exposure, biomarkers of the effects caused by toluene, such as genotoxicity biomarkers, are needed to assess exposures to mutagens or genotoxic carcinogens [190].

Angela M. Moro et al. evaluated genetic damage caused by low-level exposure to toluene [192]. They biomonitored painters from an industry in Rio Grande do Sul, Brazil. Painters enrolled in this study had a mean age of 28.9, ranging from 18 to 50 years old. In the control group the mean age was 29, ranging from 19 to 55 years old. The workers assessed were occupationally exposed to toluene in an average time of 46.15 ± 9.94 months. Urinary hippuric acid levels were not statistically different between the groups ($p > 0.05$). Although HA concentrations were higher in painters, they were still below the biological exposure index (1.60 g g^{-1} creatinine), according to ACGIH (American Conference of Governmental Industrial Hygienists). Painters and controls showed no significant difference in relation to HA levels, indicating the low specificity of this biomarker as a monitor to toluene

exposure. Concerns about the value of hippuric acid as an exposure biomarker were raised in recent years, since occupational exposures to toluene have been gradually decreasing, and also because hippuric acid can be found in the urine of non-exposed subjects. The value of hippuric acid as a marker of occupational toluene exposure is further challenged by the presence of benzoate in some soft drinks added as a preservative, because benzoate is metabolized and excreted as hippuric acid in the urine [193, 194]. painters presented significantly higher DNA damage indices in comparison to the control group. These findings are consistent with previous studies [188, 189, 193], which also showed cytogenetic damage under low level exposure conditions. With regard to MN frequency in buccal cells, no significant difference was observed between painters and control subjects. This can be explained by the low levels of toluene found in paints and by the short-term (less than 4 year) exposures of the painters, which were insufficient to induce detectable damage. A recent report found a significant association between the time spent at work and MN frequency in buccal cell [195], thus highlighting the influence of exposure time on DNA damage. According to Heuser et al. [189], differences in the findings of the comet and MN assays could be associated with the kinds of exposure and/or damage caused. Our results showed that genotoxic effects of toluene exposure could be detected by the comet assay, but did not affect MN frequency. The micronucleus assay is used for assessing DNA damage at the chromosomal level, and differs from the comet assay, which can detect repairable damage [196]. Despite low levels of toluene exposure, painters showed elevated oxidative damage. Our previous study has shown that workers exposed to paints also showed changes in lipid peroxidation and endogenous antioxidants [197]. Elevated MDA levels observed in painters could be linked to toxic effects of toluene by the formation of free radicals and reactive oxygen species (ROS) during its biotransformation, causing damage to biological membranes [198]. The ROS production during toluene biotransformation could also be responsible for protein oxidation. Our results showed increased PCO levels in painters, indicating oxidative damage in proteins, reflecting the cellular damage induced by multiple forms of ROS [199-200]. In addition to the formation of protein carbonyls, another proteins oxidation was observed, with albumin as the main target. Elevated production of ROS during toluene biotransformation could transiently modify the N-terminal region of albumin and result in increased IMA levels [201, 202].

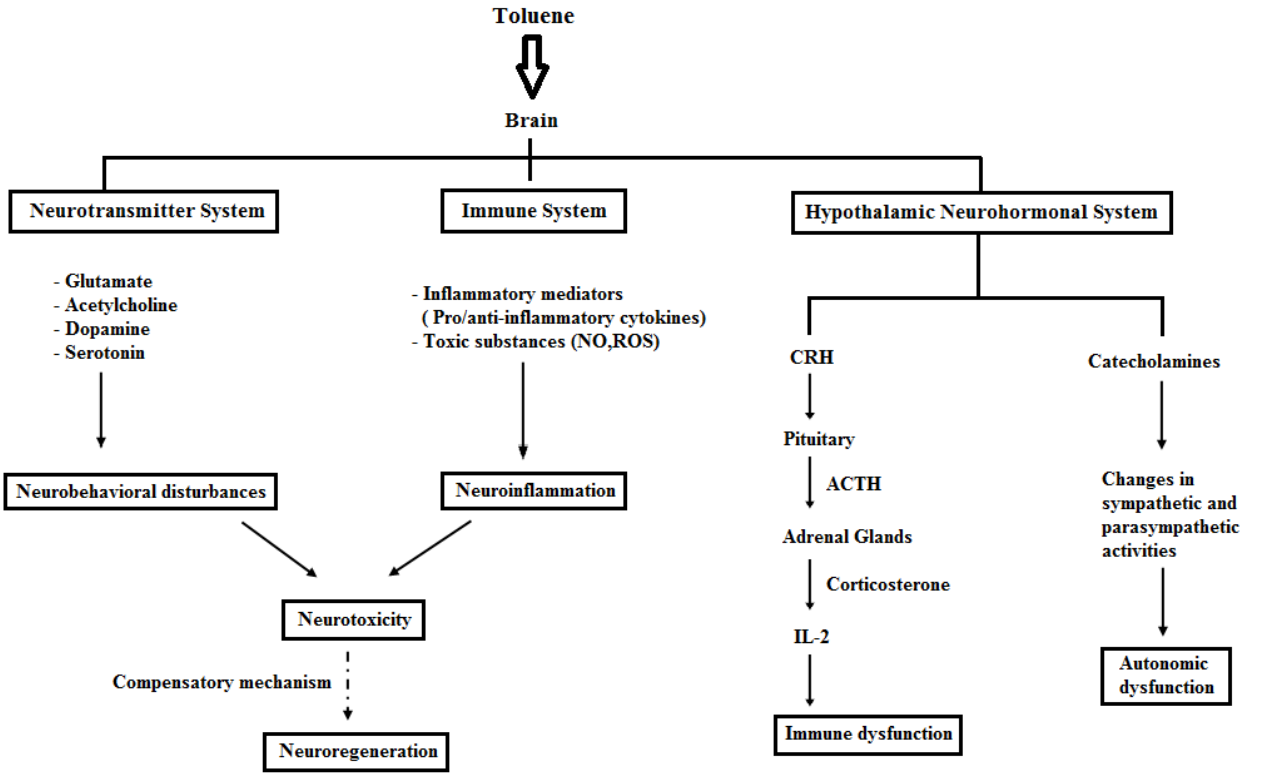


Figure 4. Potential mechanisms involving toluene sensitivity in the brain. (NO: nitric oxide; ROS: reactive oxygen species; CRF: corticotrophin releasing factor; ACTH: adrenocorticotropic hormone).

There are many sources of DNA damage, but damage caused by free radicals and ROS may often be significant [203]. The linear correlation found between DNA DI and the biomarker of lipid peroxidation (MDA) is consistent with genotoxic effects related to oxidative stress. Furthermore, linear correlations were also observed between DNA DI and biomarkers of toluene exposure; higher levels of blood toluene and urinary HA were observed in subjects with higher DNA DI. Formation of ROS during toluene biotransformation could be involved in the genotoxic effects. The oxidative alterations present in lipids and protein give a measurement of exposure-induced oxidative stress that results in inflammation and damage to macromolecules, including DNA, proteins and lipids [204, 205]. Although the comet assay is not able to discriminate the etiology of DNA damage, the genotoxic effects observed in painters may be due to production of free radicals during toluene biotransformation. The increase in DNA DI was also associated with increased age and exposure time.

4. Application for Elimination of Toluene

Volatile organic compounds (VOCs) are important group of air pollutants that cause great harm to environment and human health [206, 207]. VOCs include any organic compound present in the atmosphere. Vehicle emissions, power generation and solvents emissions are considered to be the major sources of VOCs [208]. Among them, BTX (i.e. benzene, toluene and xylene) are of particular concern due to their high toxicity for human beings [209-211]. Therefore, the development of effective and reliable methods for complete elimination of VOCs is of great importance considering their harmful effects on human health and the environment.

Several countries and organizations are now regulating the VOC levels of industrial emissions with the goal of improving air quality. Perhaps being stricter of all, California has been regulating the VOC content even in consumer products for several years now. Such VOC containing products are subject to Environmental Protection Agency (EPA) laws. For instance, Wood floor Varnish (finishes) would have a maximum allowable limit of 350 g/l. Environmental legislation has imposed increasingly stringent targets for permitted levels of VOCs emission. For instance, the maximum VOC emission amount by 2020 in the EU member countries should be reduced by nearly half as compared to the base year 2000 according to the Goteborg protocol [212].

The choice of VOCs control technology depends on the actual operating conditions and the physical and chemical properties of organic compounds [213]. The most common used methods for VOC removal include adsorption, catalytic oxidation, biofiltration and photocatalysis.

4.1. Adsorption

Adsorption is a reliable alternative to eliminate organic compounds from industrial waste gases because of the flexibility of the system, low energy and cheap operation costs, which is favored by the majority of researchers [214-216]. Activated carbons are generally used in many adsorption processes because of their higher adsorption capacity and low cost. However, their efficiency is limited by their sensibility at high temperatures and their difficult regeneration [217]. Therefore, the most research is focused to select the adsorbent with a good stability and regeneration performance recently. New porous materials such as zeolites [218-220] and metal oxides [221] are proposed to be used as adsorbents for VOCs removal.

To well understand the nature of the adsorption process, the influence of pore size, surface properties, pore structure and morphology of the adsorbents on VOCs adsorption has been reported [222-224]. However, most research is focused on investigating the influences of one or two kinds of similar adsorbent on the VOCs adsorption. In addition, few authors have reported the specific impact of pore structure of different kinds of materials in the adsorption/desorption process. Kosuge et al. have investigated the porous properties of various adsorbents and VOCs adsorption/desorption, just focusing on the pore structure and morphology of mesoporous silica [225]. The interaction features of zeolites with adsorbent molecular are very important to understand the nature of the adsorption/desorption process.

Zhang et al. used to four different types of adsorbents, SBA-15, MCM-41, NaY and SiO_2, were used to study the dynamic adsorption/desorption of toluene. To further investigate the influence of pore structure on its adsorption performance, two SBA-15 samples with different microspores were also selected. It is shown that there existed a strong relationship between porous structure and the adsorption and desorption performance for toluene. Microporous material NaY showed the largest toluene adsorption amount, but its complete desorption temperature was much higher than other materials. Mesoporous MCM-41 and amorphous SiO_2 showed relatively lower adsorption capacity for toluene. SBA-15 materials with “bimodal pore system”

showed a moderate adsorption capacity and desorption performance, and SBA-15 with more microporous volumes showed a better adsorption performance. The pore structure of absorbents was found to play a significant role in the adsorption properties of VOCs. The size of microporous pores, which was similar to the dynamic size of toluene, was beneficial for the adsorption of toluene, but the space resistance was larger and showed a negative effect on the desorption process. Mesoporous pores showed a positive effect on the diffusion of toluene, while the adsorption capacity of VOCs was not ideal. Thus, the significant dynamic adsorption and desorption performance should be the synergistic effect of mesopores and the large number of microspores in silica walls [226].

4.2. Catalytic Oxidation

The removal of VOCs by means of catalytic combustion is believed to be one of the most effective and environmental-friendly routes [227]. The key issue for such an approach is the availability of an efficient catalyst. In the past years, various kinds of catalysts such as perovskite-type oxides [228], supported noble metals [229, 230] and transition metal oxides [231] have been developed for VOC catalytic oxidation. However, the good performance for metal oxides is only observed at relatively high temperatures (>600 ˚C), and the inevitable outcomes are high-energy consumption and fast deactivation of catalysts [227]. In contrast, noble-metal-based catalysts show excellent low temperature activity and stability for the VOC oxidation. Especially, Pd-loaded catalysts were widely studied due to its high activity, thermal stability and low cost compared to other noble metals [232].

It is found that the support nature plays an important role in the improvement of catalyst efficiency by providing large pores and surface areas to disperse the active particles, particularly in oxidation reactions [233]. Moreover, researchers proposed that the acid-base property of support had significant influences on the Pd dispersion and oxidation state [234]. Currently, different types of supports were applied for VOC catalytic oxidation (e.g., Al_2O_3, TiO_2, ZrO_2, CeO_2, SiO_2, C and porous materials) [235-241]. However, Pd atoms as well as other metal nanoparticles are often unstable (sintering) on the metal oxide supports at elevated reaction temperatures and their activities rapidly decay [242].

Noble metal catalysts are known to be very active in nonhalogenated VOC removal. Supported Pt and Pd catalysts have been most widely studied for this

purpose. In general, noble metal catalysts had a greater activity than other metal catalysts, but their manufacturing cost was high and can be easily deactivated by either sintering or poisoning by chlorine, sulfur or metals in the gas stream. For these reasons, there are strong interests to develop alternative efficient oxide based catalysts for VOC oxidation, especially utilizing natural abundant cheap resources. The advantages of developing catalysts based on transition metal oxides were associated with their lower cost, as well as the possibly higher thermal stability, resistance to humidity, increased specific surface area and greater resistance to common poisons [243–247]. The catalytic combustion of toluene has been studied with catalysts including zeolite-based metal oxides [248, 249].

Zeolites were renowned as promising supports to stabilize transition metals with great potential as oxidation catalysts [250]. The availability of zeolites with several porous structures, different composition and hydrophobicity degree, as well as the possibility to control the acidic properties and location of exchanged cations have contributed to the increased usage of zeolites [251]. HY and HZSM- 5 (HMFI) zeolites exchanged with copper (1–5 wt.%) and cesium (5–10 wt.%) have been studied as catalysts for the combustion of toluene [248]. Zeolites, i.e. clinoptilolite, have been claimed as promising supports to stabilize and provide high surface area support to transition metals [249].

The availability of zeolites with myriad of pore structures, composition and degree of hydrophobicity as well as the possibility to control the acidic properties have contributed to the increased usages of zeolites [251].

We investigated catalytic combustion of toluene over Fe, Co, and Mn transition metal oxides catalysts supported on clinoptilolite (CLT). Increase in activity was paralleled by increasing metal oxide content for all three-catalyst types up to 9.5% by weight. It has been found that manganese oxide exhibited higher catalytic activity for the low-temperature catalytic combustion of toluene than other two oxides. 9.5MnO_2/NaCLT catalyst showed the catalytic conversion of toluene to 93% at a temperature of 350 ˚C. However, due to the interaction between the exchanged ion and the zeolite, increasing combustion temperature above 350 ˚C led to major decrease in activity. Addition of metal oxide into clinoptilolite resulted in a decrease in combustion temperature and an increase in toluene conversion [252].

In 2012, we study combustion of toluene (1000 ppm) over MnO_2 modified with different supports. α-Al_2O_3 and γ-Al_2O_3 obtained from Boehmite, γ-Al_2O_3 (commercial), SiO_2, TiO_2 and ZrO_2 were used as commercial support materials. In view of potential interest of this process, the influence of support

material on the catalytic performance was discussed. The deposition of 9.5MnO_2 was performed by impregnation over support. Amongst the MnO_2-based catalysts supported on α-Al_2O_3 (from Boehmite), γ-Al_2O_3 (from Bohmite), γ-Al_2O_3, SiO_2, TiO_2, and ZrO_2, (Mn/α-Al_2O_3) presented better catalytic performance for the combustion of toluene. Mn/α-Al_2O_3 catalyst with 9.5%Mn loading had a high activity and toluene conversion of 90% which was obtained at a low temperature of 289 ˚C. Considering all the characterization and reaction data reported in this study, it is concluded that support surfaces affected surface active MnO_2 species. The lattice oxygen and surface oxygen species are effective for catalytic combustion of toluene. α-Al_2O_3 (from Boehmite) type support not only facilitated the combustion and lowered light-off temperature too. Manganese oxides impregnated α-Al_2O_3 support showed unusually high catalytic combustion activity for the commercial catalysts reported in open literature [253].

4.3. Biofiltration

Among the various technologies for VOC treatment, biological methods (conventional biofilters and biotrickling filters) have proven their potential to remove VOCs at low-moderate concentrations of contaminants (lower than 5–6 g m^{-3}) and high flows (from 1000 to 10,000 Nm^3 h^{-1}) [255-257]. Moreover, biofiltration is an environmentally friendly and cost-effective alternative because pollutants are finally converted into innocuous compounds such as CO_2, H_2O, and biomass at room temperature [258].

These methods can be used for the treatment of industrial emissions [15, 16], the deodorization of air emissions [17] and the treatment of indoor air [14].

Many reports have noted the inhibiting effects or enhancing response of biofilters when feeding separate substrates or their mixtures [259-261]. García-Peña et al. found that benzene degradation was negatively affected by both toluene and ethylbenzene and that the toluene degradation rate was hindered by the presence of benzene [261]. Nikolova and Nenov found that ethylbenzene was easily degraded by different fungal strains in mixtures [260]. Nevertheless, only a handful of researchers have evaluated the performance of biofiltration in the removal of ethylbenzene as a sole contaminant in gas streams [262, 263].

One key factor in biofilter performance is temperature. Most biofilter studies have been operated at room temperature of 20–35 ˚C, typical of

mesophilic microorganisms [264]. However, many industrial streams emitting from the processes such as pulp and paper manufacturing, coating process (i.e. spry paint) and waste treatment (i.e. dry activated sludge cake) frequently demonstrate high temperatures (40–70 ˚C), which is higher than the temperature ranges corresponding to the optimum activity of mesophilic microorganisms [265, 266]. One option for the treatment of high temperature gases is cooling these exhausts below 40 ˚C prior to biological reactors, which is quite expensive [267]. Another alternative would be the use of thermophilic microorganisms. The application of these microorganisms active at temperatures over 40 ˚C would demonstrate great cost savings and extend the feasibility of biofilters [268].

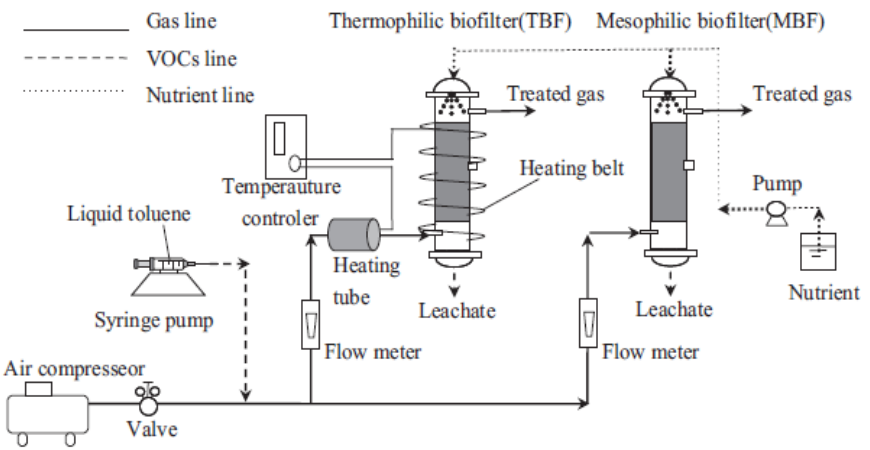


Figure 5. Experimental setup for the thermophilic biofilter (TBF) and the control mesophilic biofilter (MBF).

Only a few studies are available on the thermophilic treatment of waste gases. Lu et al. [269] observed a decrease in performance when switching from mesophilic to thermophilic conditions. However, several other biofiltration studies demonstrated higher removal rates in thermophilic biofilters compared with mesophilic ones. For instance, a higher removal rate for ethylacetate was achieved in a biofilter at 45–50 ˚C compared to a mesophilic one [256]. Kong et al. reported effective removal of a-pinene and methanol at biotrickling filter temperatures of 60 and 70 ˚C, respectively [270]. The activity of microorganisms in thermophilic biofilters, in terms of its growth and pollutant utilization rate, is usually expected to be higher than the

operation under mesophilic conditions. A similar result has been recently observed in biotrickling filters treating H_2S-containing malodorous gas, reaching higher removal efficiency of 95% at 60 ˚C [271].

Most of previous experiments focused on the pollutants' removal and the biomass accumulation process in the thermophilic biofilter for a long-term operation were paid little attention. Wang et al. investigated the long-term performance and the biomass accumulation process of two biofilters under various inlet toluene concentrations and gas flow rates. Furthermore, a carbon balance was also made to show the removal fate of toluene inside the biofilters. Finally, the dominant microorganisms from both TBF and MBF were isolated and identified.

The thermophilic biofilter (TBF) achieved high performance (the removal efficiency was over 90%) to remove high temperature gaseous toluene (55 ˚C) under low inlet loading conditions (less than 100 g m^{-3} h^{-1}). In the long-term operation, the biomass in the TBF showed a slow accumulation process and presented a lower pressure drop of filter bed than that of the mesophilic biofilter (MBF), which suggested a more stable potential operation capability. The leachate from both biofilters was also analyzed. In addition, the TBF presented a neutral pH and higher TOC values in leachate than those from MBF. The results of three-dimensional fluorescence spectra suggested that the products of toluene biodegradation included some organic acids. The carbon mass balance analysis showed that a relative higher fraction of the removed toluene was converted to biomass in the MBF, which led to rapid biomass accumulation process.

Gallastegui et al. investigated the long-exposure performance of two laboratory-scale biofilters to treat toluene and ethylbenzene as separate substrates [272]. This model of laboratory-scale biofilter used organic wastes for obtaining the packing material and the acclimated inoculum. Two biofilters filled with an organic waste material for treating ethylbenzene and toluene were operated under similar conditions for 415 and 472 days, respectively, and the influ-ence of several relevant operating parameters was reported over time. As expected during Stages I and II, the higher EBRT in both cases (230 s for ethylbenzene and 229 s for toluene) rendered a linear response between the IL and the concentra- tion of removed carbon ($C_{REMOVED}$). However, when EBRT was decreased to 100 s for ethylbenzene and 89 s for toluene, the average RE was as low as 72.7 % for the former and 61.0 % for the latter. As far as the degradation profile along the biofilter is concerned, as inlet concentration was increased, the mass transfer capacity and the reaction rate of the upper section of the bed were exceeded and the contaminants moved into the downstream

section. The MC of the packing material proved to be a crucial factor for bioreactor efficiency. A sudden decrease in the performance of both biofilters occurred when the MC value was higher than 37% for ethylbenzene and 30% for toluene. Thus, the recommended MC for this organic material ranged between 15% and 30%, which is a very low value in comparison with other materials. The bioreactors' high RE when operated at low MC was attributed to the synergism effect between a prevailing fungi colony and bacteria attached to the fungal hyphae. Nevertheless, the individual contributions of fungi and bacteria to CO_2 production (and consequently to EC) in the two biofilters could not be established.

4.4. Photocatalysis

Most research on VOCs has focused mainly on the toxicity of VOCs sources or the degradation conditions rather than the by-products generated during the treatment procedures [273].

Recently, photocatalytic oxidation (PCO) has become an attractive technology for VOCs abatement compared with adsorption, biofiltration or thermal treatment [274,275]. It is noteworthy that, although many works have accomplished the degradation of VOCs by PCO, only a few investigations have focused on evaluating the detoxification of organic pollutants in the environment.

In particular, titanium dioxide (TiO_2) has proved to be a promising candidate for photoassisted pollutant degradation due to its nontoxicity, high oxidative capacity, low cost and good stability against photocorrosion [276, 277]. TiO_2 nanotubes as one of one-dimensional nanostructure materials [278, 279] can provide high surface area associated with size in nanometer scale, which is very beneficial for the utilization of TiO_2. Recently, there has been a growing interest in the synthesis of TiO_2 nanotubes by template method [280] due to the simplicity of materials preparation and handling, as well as better controllability over the synthesis process in comparison with other methods.

One drawback remains that TiO_2 has high recombination rate of electron–hole pairs formed in photocatalytic processes. In order to overcome this limitation, many modification methods have been conceived to increase the hole-electron separation efficiency, such as noble metal doping [280, 281], coupling with other semiconductors [282], and dye sensitization [283]. In addition to these methods, coupling TiO_2 with SiO_2 [284, 285], or embedding the element of Si into TiO_2 matrix [286], can lead to novel structures, which

have been demonstrated with excellent photocatalytic activity. Iwamoto et al. prepared silicon-modified titania photocatalyst via a glycothermal method [287]. They found that the incorporation of silicon atoms into TiO_2 matrix increased thermal stability and decreased the crystallite size of the silicon-modified titania. Park and Jung [288] reported the effect of Si-doping on photocatalytic behavior of TiO_2, and suggested the enhanced photoactivity was attributed to the increase of surface area and crystallinity through embedding amorphous silica into titania.

The methods for TiO_2–SiO_2 composite reported so far are mainly focusing on the preparation of TiO_2–SiO_2 particles with irregular morphologies or thin films, such as mixed particles of both preformed oxides [289], silica supports covered with titania films [290], mesoporous silica with attached TiO_2 [291], composites by flame co-hydrolysis of $TiCl_4$ and $SiCl_4$ vapors [292], or hydrothermal co-gelation from the corresponding titania and silica precursors [293]. In comparison with random-shaped nanoparticles, TiO_2–SiO_2 nanotubes could possess the advantages of flexibility, higher chemical stability, electrochemical activity and three-dimensional mesoscopic structures with mechanical integrity, which are desirable for the adsorbing and photocatalytic degradation of gaseous pollutants.

Zou et al. reported a facile sol–gel technique to prepare TiO_2–SiO_2 composite nanotubes by employing ZnO nanowires as template [294]. This approach has the advantages of reproducibility, flexibility and high microscopic structural homogeneity. The composite nanotubes were applied in photocatalytic degradation of gaseous toluene and their performance was compared with commercial TiO_2 (P25, Degussa Co.). Compared with the pure TiO_2 nanotubes, the UV–vis absorption edge of TiO_2–SiO_2 composite nanotubes showed a small blue shift due to the formation of Ti–O–Si group. The photocatalytic activity of the composite nanotubes in degrading gaseous toluene was significantly higher than that of P25 due to the large surface hydroxylation of the surface of the composites, which demonstrates they have potential application in photocatalytic removal of indoor VOCs.

On the other hand, many studies have been devoted to the improvement of photocatalytic activity of TiO_2 by depositing noble metals [295, 296]. Ag is considered as the relatively cheap noble metal and Ag loaded TiO_2 can enhance the photocatalytic activity of TiO_2 effectively according to the previous reports [297].

Commonly, the Ag-doping TiO_2 nanotube powder was fabricated by hydrothermal or template-based synthesis method with chemical reduction [298], deposition precipitation and photodeposition [299].

Li et al. reported a facile method to synthesize TiO_2 nanotube powder by using rapid breakdown anodization, which can produce massive powder consisting of a large number of TiO_2 nanotubes [300]. Then Ag was loaded in nanoparticulate form via a simple wetness impregnation method. Their photocatalytic activities were investigated by the degradation of toluene. As a comparison, commercial TiO_2 (P25, Degussa Co.) and Ag-doping P25 were also employed as the photocatalyst. The photocatalytic oxidation of toluene over different samples under UV light irradiation is investigated. The initial concentration of 120 ppm for toluene was used for reaction. The conversion of toluene was about 79 % for P25 after 6 h reaction, and pure TiO_2 nanotubes and 1% Ag-doping TiO_2 nanotubes presented much higher photodegradation efficiency (90% and 98%, respectively) for toluene under the same conditions. Meanwhile, it was also found that the degradation efficiency of toluene slightly increased with the increasing of calcination temperature for pure TiO_2 nanotubes. Especially, the conversion of toluene over 1% Ag doping TiO_2 nanotubes had achieved 98% after 4 h reaction and the produced CO_2 was about 300 ppm, which could be attributed to the following reasons: (1) The doping Ag species can promote the anatase to rutile phase transformation at lower calcination temperature. This mixture phase would be beneficial for photocatalytic activity [301]. (2) According to the SPV analysis, the existing impurity band can reduce the recombination of photoinduced electron–hole, which can transfer electrons more efficiently to the oxygen adsorbed on the surface of TiO_2 nanotubes. In addition, (3) Ag-doping TiO_2 nanotubes showed porous and higher specific surface area. These advantages of the Ag-doping TiO_2 nanotubes remarkably improve their photocatalytic performance. Meanwhile it was provided that the photocatalytic reaction followed a pseudo-first-order reaction, the rate constant of toluene decomposition over 1% Ag-doping TiO_2 nanotubes, which were estimated to be about 0.76 h^{-1}, were faster than the other samples. Moreover, under visible light irradiation, the photocatalytic activity of 1% Ag-doping TiO_2 nanotubes was also obviously higher than the other samples.

CONCLUSION

In the work environment, toluene comes into contact with the skin and the respiratory tract primarily in the form of vapors or liquid. Only very small quantities of toluene vapors are absorbed through the skin. Liquid toluene can be absorbed through the skin, from which further distribution is difficult. The

risk of intoxication as a result of skin exposure will, in the case of normal occupational use (cleaning included), be extremely small because toluene quickly diffuses out again after skin contact ceases. Inhalation of toluene vapors is therefore the only real intoxication hazard in the work environment.

Toluene is readily absorbed from the respiratory and gastrointestinal tracts and to some degree through the skin [18]. It is estimated that 40 – 60% of inhaled toluene is absorbed. Oral absorption is complete but the rate is slower than pulmonary absorption. The amount absorbed increases as the work load (2-3 times), fat deposits, or inhaled air concentration increases. The absorption may reasonably be described by mathematical models. Absorbed toluene is distributed through the blood to tissues and organs. With the exception of fatty tissue, the concentration in the organs will be one to three times higher than that in the blood.

Fifteen to twenty percent of inhaled toluene is exhaled unchanged. Besides, small quantities are excreted unchanged in the urine. The rest of absorbed toluene is converted to benzoic acid. Most of this (about 80%) is conjugated with glycine and excreted as hippuric acid in the urine. The remaining 20% of benzoic acid is conjugated with glucuronic acid and excreted in the urine as glucuronide. A very small part of the toluene may possibly be converted to phenolic derivatives and excreted as glucuronides and sulfates in the urine. Other products and excretions are of no importance. Women eliminate. Less toluene through the lungs, while their initial rate of excretion of hippuric acid is higher than that observed in men.

Studies on the health effects of toluene exposure have revealed that toluene mainly affects the central nervous system, causing an increased tendency to sleep, frequent headaches, eye irritation and memory impairment in humans; dizziness, depression, and fatigue in paint workers; and cerebellar dysfunction and cerebral and hippocampal atrophy as well as a loss of brain volume in chronic toluene abusers. In addition, the inhalation of low concentrations of toluene induces a persistent deficit in spatial learning and memory in humans and in animals.

Studies in the rat and mouse have detected effects of toluene inhalation on brain constituents and morphological and biochemical parameters. High levels approaching 2000 ppm have caused ataxia, prostration and tremors in rats exposed for 7 days. Inhaled toluene in rats has been detected in all brain regions, with the highest concentration in the brainstem, an area also found to be involved in neurological sequelae in toluene abusers. Uptake was correlated with lipid content in each brain region. Enzyme activities and receptor binding

was most affected in the brainstem of rats exposed subchronically and chronically.

A variety of studies suggest that toluene is only minimally toxic to the liver and kidney. Exposure of rats to toluene at concentrations of 30 and 300 ppm for 6 hours/day, 5 days/week for four weeks produced only minimal histological changes and had no effect on AST, liver weight, or mixed function oxidases, serum alkaline phosphatase was elevated at 300 ppm. There were no histopathological changes in the kidney.

The health risk after environmental chemical exposure depends on age, sex, genetics, socioeconomic status, nutritional status and environmental factors [164]. Individuals with different genetic backgrounds have different sensitivities to toxic chemical exposure, and some people are more susceptible to chemical exposure than others because of their genetic makeup. The adverse health effects of chemicals depend on the chemicals' toxicities and how people are exposed to the chemicals, as well as individual susceptibility.

An extensive database on human exposure to toluene indicates that dysfunction of the CNS is of primary concern. Deficits in neurobehavioural functioning have been viewed as precursors of more serious indications of CNS toxicity. Their measurement is generally held to be more reliable than subjective symptoms, which may involve situational factors unrelated to a cause–effect relationship with exposure. Potential confounders such as age, alcohol, drugs and education need to be assessed to ensure correlations of toxicity with exposure.

Toluene can reach the hippocampus after acute and chronic exposure, affecting the synaptic environment including pre- and post-synaptic neurons, microglia, astrocytes and immune cells.

There are still many uncertain factors because the basis of human diagnosis is not very specific and because it is very seldom possible to determine exposure levels in a satisfactory way.

Volatile organic compounds (VOCs) are important group of air pollutants that cause great harm to environment and human health. VOCs include any organic compound present in the atmosphere. Vehicle emissions, power generation and solvents emissions are considered to be the major sources of VOCs.

The choice of VOCs control technology depends on the actual operating conditions and the physical and chemical properties of organic compounds. The most common used methods for VOC removal include adsorption, catalytic oxidation, biofiltration and photocatalysis.

Although numerous research and development have proposed, further technological breakthroughs are required for practical applications of toluene removal.

In summary, we can expect that the continuous improvements of the material property and the reactor design would create a large number of effective environmental purification systems. These systems are necessary for a healthy and comfortable living environment.

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Chapter 2

IMMEDIATE AND PERSISTING EFFECT OF TOLUENE CHRONIC EXPOSURE ON HIPPOCAMPAL CELL LOSS, LEARNING AND MEMORY IN ADOLESCENT AND ADULT RATS

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ABSTRACT

The present study has been undertaken to determine whether toluene chronic exposure provokes immediate and/or persistent effect on the structure of hippocampus, learning and memory in adolescent and adult rats. We exposed male Wistar rats at ages P 28-32 (adolescents) and P 70-75 (adults) to 2000 ppm inhaled toluene for 40 days. The immediate and persisting effects of toluene misuse (immediately after the end of toluene chronic inhalation and 90-day after the end of toluene chronic inhalation, correspondingly) on (i) pyramidal cell loss in the CA1 and CA3 of the hippocampus, (ii) exploratory behavior and recognition memory in the open field and (iii) behavior in multi-branch maze were evaluated. The

results reveal that toluene chronic exposure affects the structure of the hippocampus, the behavior in multi-branch maze, exploratory activity and recognition memory in the open field in adolescent and adult rats. In all cases the effect is age-dependent. In particular: in adolescent rats the more significant structural and behavioral alterations were observed immediately after toluene chronic exposure, while in adult rats the most considerable was persisting effect (90 days after withdrawal). Such data indicate that character of alterations depends upon the postnatal age of testing of the animals. The results are also additional evidence that hippocampus – the neural substrate of learning and memory, may contribute to the pathophysiology of toluene abuse in organisms of different age.

1. INTRODUCTION

Toluene and toluene-containing volatile substances are the most widely abused solvents with demonstrative addictive potential in humans. Experimentation with these substances is especially common during adolescence, since they are widely used, easily accessible, legal and inexpensive. Besides, because toluene is broadly used as an industrial solvent in manufacturing of chemical pharmaceuticals and multiple widely-spread household and commercial products, such as gasoline, glue, rubber, cement or paints, it has the highest potential for abuse for adult co-workers also. For the same reasons potentially millions persons are exposed to occupational levels of toluene at their home [1-3]. Therefore, the abuse of toluene-containing solvents is an important health and social problem around the world. The understanding of the chronic effects of toluene has evolved over the last five decades. Numerous clinical and experimental studies have demonstrated that the exposure to toluene vapor leads to diverse consequences at the level ranging from the cell to the whole organism. As the primary target of toluene chronic exposure the central nervous system has been identified and various, sometimes long-lasting neurological and neurobehavioral impairments as well as diffuse changes in brain matter in the organisms of different age were described [4-8]. It became evident that toluene shares common cellular mechanisms and has similar actions to other drugs of abuse, namely, activates the mesolimbic dopaminergic reward system, the major substrate of addiction and related structures [9-12]. But, compared with other abusive substances, relatively few studies have assessed outcomes of chronic misuse of toluene-containing inhalants in the organisms of different age. Most studies concerning

the neurological and neurobehavioral consequences of toluene chronic exposure have used adult animals while the data obtained on younger animals are comparatively rare. Especially limited work has been conducted investigating the neurobiological impact of toluene misuse during adolescence, despite the facts, that just the adolescents represent the most numerous group of toluene abusers and a large number of adults who use this substance started as teenagers [5, 12, 13]. However it is well-known that since specific physiology of the brain in different aging groups, adolescents and adults react differentially to substances of abuse [14-18]. Thus, the age-differences of behavioral, biochemical and structural alterations (such as development of sensitization to the locomotor stimulant effect, risk-taking or exploratory activities, reorganizations of neurons from brain areas involved in addiction) in the case of other drugs of abuse (such as amphetamine, cocaine, methylphenidate or inhalants) have been reported before [14-17]. In a few number of studies such difference was revealed in the case of toluene misuse also [5, 13, 19]. Based on these data it was suggested that studying a unique profile of various alterations in organisms of different age may provide valuable information regarding the mechanism of action of toluene.

Another area of uncertainty in the toluene studies is related with clarification of long-term outcome in toluene abusers. The biggest part of existing clinical and experimental research concerning toluene misuse has mostly focused on its acute effect with limited work examining delayed outcome in toluene abusers who abstain from inhalant abuse. Consequently, there are insufficient data concerning reversibility/on-reversibility of toluene-produced alterations once the toxic effect of toluene is no longer present. However to fully understand the nature of toluene addiction, studies need to be done where both, acute and persisting effect of toluene chronic misuse in organisms of different age will be compared. Clarification of possible difference between immediate and persisting responses of adults and adolescents could help to develop appropriate age-specific treatment for toluene abusers.

Significant number of clinical and experimental studies indicates that as a result of toluene chronic exposure evident alterations in learning and memory in organisms of different age take place [4, 5, 18, 19]. But because of differences in age, species, length of exposure, dose and rate of administration, it is not always possible to conclude whether adolescent experience results in changes in learning and memory comparable to that seen in adults. The special examination of the age-dependent difference in responsivity to toluene was done only in a few numbers of studies [19, 21]. Clearly, additional

investigations need to be done where the unique responses to toluene during various periods of development will be particularly elucidated.

The present research is designed to evaluate the immediate and persisting consequences of toluene chronic exposure on hippocampal structure and learning and memory in adolescent and adult rats. Specifically, in the first series of experiment we studied the effects of toluene misuse on the pyramidal cell loss in the hippocampus – the major substrate for learning and memory. The second series of experiments was designed to investigate the recognition memory - ability to acquire and use spatial or non-spatial information - and exploratory behavior in the open field in chronic toluene-exposed animals. In the third series of study we clarified the immediate and persisting effect of toluene chronic exposure on the behavior in the multi-branch maze, with the purpose to reveal whether experimental conditions affect learning process in rats of different age. All these questions never reported before.

2. EXPERIMENTAL DESIGN

In all series of experiments male Wistar rats (Department of Animal Care, I. Beritashvili Center of Experimental Biomedicine) of two age groups 28-30 days (d) (weighting 100-120 g) as adolescent [22-24] and 90-100 d (weighting 200-220 g) as adult were used. During experimental period the rats were housed under controlled environment (temperature 20-22 C^0, humidity – 55-60%, light on 07.30 – 19.30). Standard food pellets and tap water were available. Experimental protocol was approved by Animal Studies Committee of I. Beritashvili Center of Experimental Biomedicine.

2.1. Experimental and Control Animals: Exposure to Toluene Vapor and Clean Air

Each rat was placed separately in the glass cylindrical chamber (45 cm height x 35 cm diameter) and was exposed to toluene vapor at the concentration 2000 ppm or clean air for 4 min/day, during 40 days.

2.2. Groups of Animals

After 40 d of toluene exposure rats of each age group were divided into 2 subgroups. For the purpose to clarify immediate effect of toluene misuse on hippocampal structure, learning process in the multi-branch maze, exploratory activity and recognition memory in the open field, the studies have been undertaken on the next day after the end of toluene exposure (animals of first subgroup), while for to clarify persisting effect, the rats of second subgroup were studied 90 days after the end of toluene inhalation. Correspondingly, control rats were evaluated on the next day and 90 d after the clean air exposure. As a consequence the following animal groups were studied:

Adolescents:

- Control animals: immediate and persisting effects;
- Experimental animals: immediate and persisting effects.

Adults:

- Control animals: immediate and persisting effects;
- Experimental animals: immediate and persisting effects.

3. Effect of Toluene Exposure on Hippocampal Structure

Hippocampus, along with amygdale, is considered to be critical for cue-elicited drug-seeking taking [25-27]. Thus, hippocampal pathways have been shown to be highly implicated in drug-seeking that is elicited by contextual stimuli [20, 27, 28]; the hippocampus participates in strengthening connections in the areas that are involved in addiction, suggesting that drug-induced changes in the hippocampal function could produce long-lasting functional changes in these areas [28-30]; manipulation of hippocampal cells alters dopamine levels and firing rates of dopaminergic cells in the areas of mesolimbic system - the neural substrate that contribute to the pathophysiology of drug abuse [31, 32]. These properties make the hippocampus as an intriguing area in regard to addiction.

It is well-established that detailed neuroanatomical analysis can provide insight into alterations provoked by addictive drugs in the morphology of mesolimbic reward pathway and corresponding structures, including the hippocampus. Significant structural disorders of this region have been

described as a result of chronic misuse of various addictive drugs such as nicotine, alcohol, cocaine, methamphetamine and others [26, 33]. Such alterations, considering as associated with modifications in hippocampal learning-related cell signaling present documentation of the importance of learning, memory and synaptic plasticity in addiction of above-mentioned drugs [34, 35].

Recently in the hippocampus some molecular and biochemical alterations as a result of toluene chronic exposure were described [1, 13]. These data point out the involvement of this region in the toluene addiction. However it remains uncertain whether such exposure alters the structure of the hippocampus in animals of different age or, in the case of such alteration, is the structure of the hippocampus of adults and adolescents differentially susceptible to this exposure. For the reason to clarify this issue, the immediate and persisting toluene effect on pyramidal cell loss in the CA1 and CA3 of the hippocampus in adolescent and adult rats was evaluated.

Figure 1. Section of hippocampal subregion, demonstrating one of the levels used for cell counting: control rat brain. Magnification 3.6; scale = 1.4.

3.1. Perfusion and Material Processing

Under pentobarbital injection (100mg/kg), experimental and control animals underwent transcardiac perfusion with heparinized 0.9% NaCl, followed by 500 ml of 4% paraphormaldehyde in 0.1 M phosphate buffer (PB), pH-7.4 at a perfusion pressure 120 mm Hg. The brains were removed from skull, hippocampi were isolated, placed in 30% sucrose in 0.1 M PB until equilibrating, then were blocked, frozen and sectioned in the coronal plane (30 μm) with freezing microtome. Consecutive coronal sections were collected between - 2.28 mm and - 3.48 mm from bregma [36]. Sections were kept in serial order, placed in 0.1 M PB, and every 3rd section was stained for Thionin in order to identify the spatial distribution of pyramidal cell loss in the CA1 and CA3. Using anatomical land marks totally 6-10 sections/animal of similar hippocampal levels within and between experimental groups were selected (Figure 1).

3.2. Cell Counting

For cell counting a systematic random sampling was used and the neurons with distinct nucleus and nucleolus were counted with 2-dimensional counting grid (250 μm x 250 μm) in the CA1 and 3 fields in the CA3 in 5 non-overlapping fields at the magnification 400x in the sections of each condition. Totally 30 fields per animal were analyzed and average of cell central profiles per field was used to assess anatomical damage of CA1 and CA3 in abovementioned groups of animals. The sections were analyzed with a microscope Leica MM AF.

3.3. Statistical Analysis

To determine whether toluene chronic exposure provokes immediate or persisting effect on number of pyramidal cells of the hippocampus, the one-way ANOVA of quantitative data was performed separately in adolescent and adult rats. The results were presented as mean ± standard error (SE). A p-value less than 0.05 were considered as statistically significant. In the case of significant effect planned comparisons were carried out using t –tests**.**

3.4. Results

3.4.1. CA1 Area

3.4.1.1. Adolescent Rats

One-way ANOVA revealed effect of experimental conditions (immediate and persisting outcomes of toluene exposure) on the number of pyramidal cells in the CA1 of adolescent rat [F (3,12) = 17.97, $p < 0.001$] (Table 1). The significant pyramidal cell loss was observed immediately (29%, $p < 0,01$) and 90 d after the end of toluene chronic exposure (39%, $p < 0.01$) vs. control. No significant difference was observed between control and these experimental animal groups ($p > 0.05$) (Table 2; Figure 2a and b).

Table 1. Immediate and persisting effect of toluene chronic exposure on the number of pyramidal cells in the hippocampal CA1 and CA3 areas in adult and adolescent rats. Summary of one-way ANOVA results: *F*-variance ratio from one-way ANOVA, *P* - probability

Age	Adolescent		Adult	
	$F_{(3, 12)}$	p	$F_{(3, 12)}$	p
CA1 Area Pyramidal layer	17.97	0.000	27.03	0.000
CA3 Area Pyramidal layer	5.39	0.014	3.74	0.042

Table 2. Immediate and persisting effect of toluene chronic exposure on the number of pyramidal cells in the hippocampal CA1 area in the adolescent and adult rats. Summary of two sample *t*-test. Data are given as mean ± SE; † - indicates vs. control group, ‡ - indicates vs. immediate effect group, ¥ - indicates vs. control of immediate effect group

Hippocampus, CA1 Area								
Age	Adolescent				Adult			
Effect	Immediate		Persisting		Immediate		Persisting	
Groups	Control	Exp	Control	Exp	Control	Exp	Control	Exp
Mean	51.73±3.3	36.92±2.0	50.03±2.5	30.90±1.2	76.08±2.3	60.45±0.87	75.50±3.1	45.45±3.9
p-value		0.019†	0.699 ¥	0.002† 0.064‡		0.008†	0, 888¥	0.002 † 0.034

3.4.1.2. Adult Rats

As in the hippocampus of adolescent rats, in adult rats one-way ANOVA revealed significant effect of experimental conditions on the number of pyramidal cells [$F(3,12) = 27.03$, $p < 0.001$] (Table 1). Like in the adolescents, in adult animals significant cell loss was observed both, immediately (21%, $p < 0.01$) and 90 d after cessation of toluene chronic exposure (40%, $p < 0.01$). The cell number was the same in the both control groups ($p > 0.05$) and significantly differs between animals investigated immediately after the end of toluene exposure and 90 days after (25%, $p < 0.05$) (Table 2).

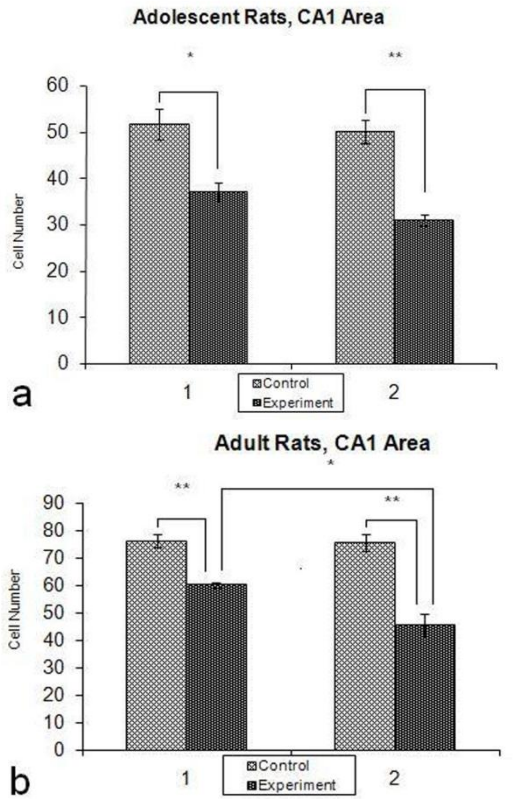

Figure 2 (a) Measurement of pyramidal cell number (n) in the CA1 area of adolescent rats after toluene chronic exposure: immediate and persisting effects. (b) Measurement of pyramidal cell number in the CA1 area of adult rats after toluene chronic exposure: immediate and persisting effects. 1 – Immediate effect, 2 – persisting effect; data are given as mean ± SE. *$p \leq 0.05$; **$p \leq 0.01$.

Thus: In the CA1 of adolescent and adult rats both, immediate and persisting effects of toluene chronic exposure were observed. In adolescent rats both effects were almost the same, while in adult rats the more significant was persisting effect (Figure 2a and b).

3.4.2. CA3 Area

3.4.2.1. Adolescent Rats

One-way ANOVA revealed effect of experimental conditions on the number of pyramidal cells [$F(3,12) = 5.39$, $p < 0.01$] (Table 1). Like the CA1, in the CA3 significant loss of pyramidal cells was observed immediately (24%, $p < 0.05$) and 90 d after cessation (33%, $p < 0.05$) of toluene exposure. Both effects were almost the same (no difference between aging groups was observed) (Table 3; Figure 3a,b, 4a).

Table 3. Immediate and persisting effect of toluene chronic exposure on the number of pyramidal cells in the hippocampal CA3 area in the adolescent and adult rats. Summary of two sample *t*-test. Data are given as mean ± SE; † - indicates vs. control group, ‡ - indicates vs. immediate effect group, ¥ - indicates vs. control of immediate effect group

Hippocampus, CA3 Area								
Age	Adolescent				Adult			
Effect	Immediate		Persisting		Immediate		Persisting	
Groups	Control	Exp	Control	Exp	Control	Exp	Control	Exp
Mean	32.75±2.3	25.07±1.4	34.92±2.5	23.30±0.68	45.22±3.3	41.11±2.2	44.93±3.3	33.82±1.8
P-value		0.037†	0.640¥	0.023† 0.327‡		0.350†	0.951¥	0.043 † 0.053‡

3.4.2.2. Adult Rats

According to one-way ANOVA [$F(3,12) = 3.74$, $p < 0.05$] (Table 1), in adult animals the outcome of experimental conditions on the principal cell loss is less expressed. Statistically significant effect is observed only after 90 d of toluene exposure (25%, $p < 0.05$) vs. control. The significant difference is also revealed between both experimental groups [immediate effect vs. persisting effect (18%, $p \leq 0.05$)] (Table 3).

Thus: In the CA3 of adolescent rats both, immediate and persisting effects of toluene chronic exposure were present. No significant difference between these effects was detected. In adult rats only persistent effect (90 d after withdrawal) was observed (Figure 4b).

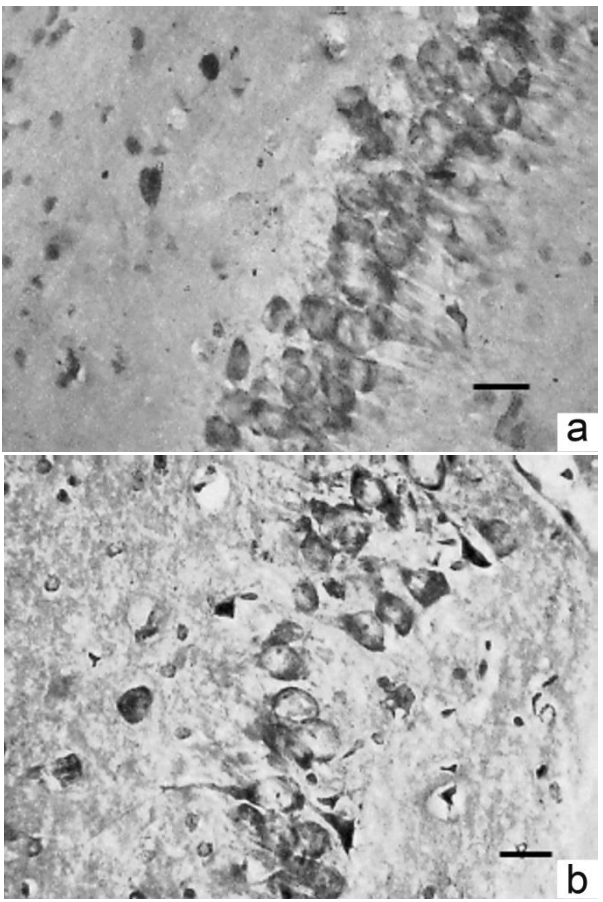

Figure 3. Persisting effect of toluene chronic exposure (90 days after withdrawal). (a) Representative photomicrograph, demonstrating the CA3 area of control adolescent rat. Magnification 200x; scale = 25 μm**.** (b) Representative photomicrograph demonstrating depletion of pyramidal cell layer in the hippocampal CA3 area of adolescent rat. Magnification 200x; scale = 25 μm.

3.5. Summary

Histological studies show that toluene chronic exposure provokes pyramidal cell loss in the CA1 and CA3 of the hippocampus in adolescent and adult rats. Among these areas the most significant effect is observed in the CA1. The character of alterations depends on the age of animal. In both areas of adolescents immediate and persisting effects were present; both effects were

almost the same. Therefore, in adolescent rats the alterations do not progress during 90 d period of withdrawal. On the contrary, in adult rats more prominent was persisting effect. Therefore, in adult animals the alterations progress during 90 d period of withdrawal.

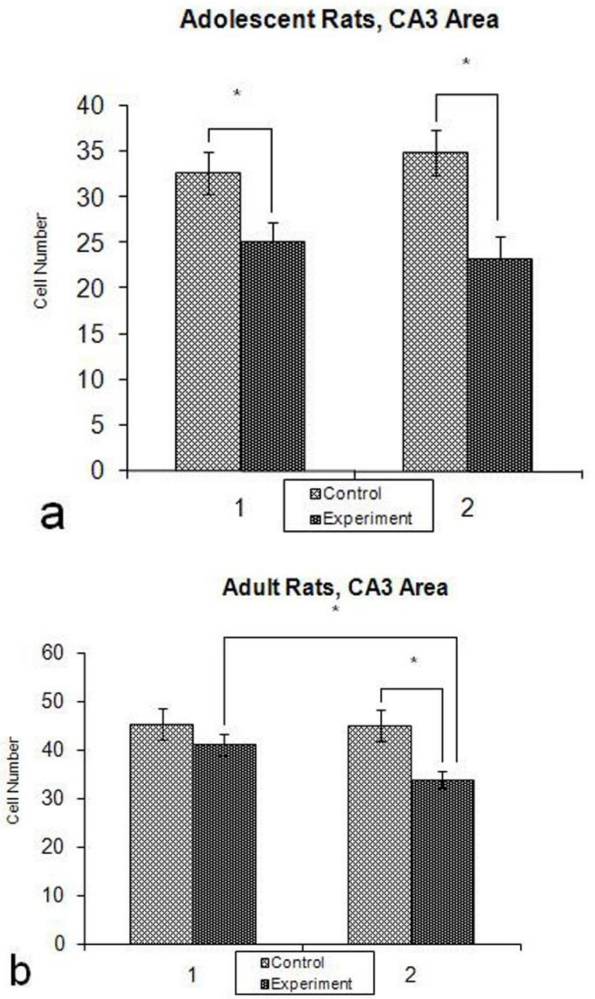

Figure 4. (a) Measurement of pyramidal cell number (n) in the CA3 area of adolescent rats after toluene chronic exposure: immediate and persisting effects; (b) Measurement of pyramidal cell number in the CA3 area of adult rats after toluene chronic exposure: immediate and persisting effects. 1 – Immediate effect, 2 – persisting effect; data are given as mean ± SE. *$p \leq 0.05$.

4. Effect of Toluene Chronic Exposure on Exploratory Behavior and Recognition Memory

The purpose of these series of experiments was to investigate the ability to acquire and use spatial or non-spatial information as well as to habituate exploratory activity over time in chronic toluene-exposed rats in the open field.

Reaction to novelty is a behavior frequently observed in many mammalian species when they are confronted with a change in their physical environment. For example, a new object added to a series of familiar objects is selectively explored in preference to the familiar objects [37, 38]. Furthermore, mammals are also able to detect a specific change of location of one or more familiar objects that have been set up in an open field. Previous studies have shown that normal animals, such as gerbils, hamsters, and rats, usually react to that change by renewed exploration of the entire apparatus and/or by selective reinvestigation of the displaced object [39, 40]. If the modification does not induce an increase of the exploratory activity, it may be concluded that the feature of the situation that has been affected by the modification has not been encoded in the representation of the animal, or that the change has not been noticed by animal. Therefore, this method may be appropriate for assessing possible differences between normal and toluene-exposed animals in terms of their ability to acquire and use spatial (or non-spatial) information as well as to habituate exploratory activity over time. An increased exploration of the displaced and substituted objects being usually interpreted as an index of the ability of animals to detect and react to the spatial and non-spatial changes [39-41]. Thus, it allows the comparison of the effects of chronic toluene exposure on two different behavioral responses.

4.1. Methods

4.1.1. Behavioral Apparatus

An open-field square arena enclosed by walls (65 x 65 x 75 cm) made from wood and illuminated by a 60 W light bulb mounted 1 m above the area was used for the behavioral test. The floor of the arena was divided into 16 equal squares by white lines. The walls inside the arena were surrounded with a white cloth to a height of 1.5 m (for the purpose to prevent the rat from looking out into the room and thereby, to maximize attention to the object of

stimuli); therefore no external stimuli could be seen during the experiment. An overhead camera and a video recorder were used to monitor and record the animal's behavior for subsequent analysis. The objects to be distinguished were made of glass, plastic, or metal and existed in duplicate. The weight of the objects ensured that they could not be moved by the rats. As far as could be ascertained, the objects had no natural significance for the rats and they had never been associated with a reinforcer. In order to exclude the odor traces, after each session, each object was substituted with a new one of same color, shape and size. After each test, the open-field was cleaned with a solution of 20% ethanol and then dried with a cloth.

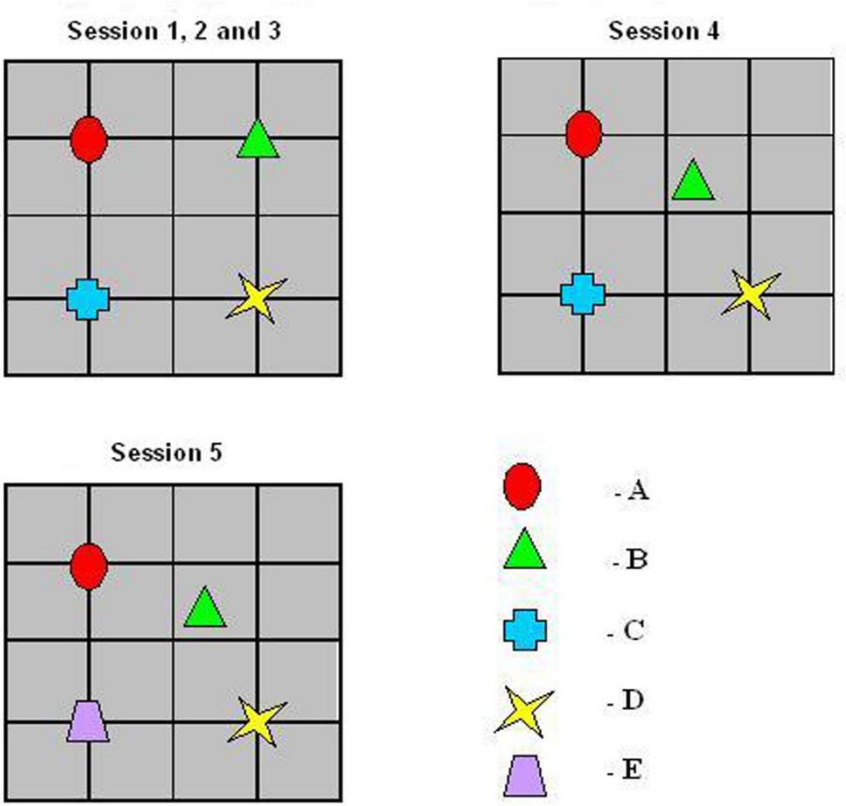

Figure 5. A schematic illustration of the configuration of objects in the open field.

4.1.2. Behavioral Procedure

Rats (n = 8 for each experimental and control group) were individually given five 3-min sessions, each of which was separated by 24-hour delay,

during which subjects were returned to their home cage. In all sessions animals were gently placed in the center of the open field and allowed to explore the open-field environment. During Session 1 four different (by color, shape and size) objects (A, B, C, D) were simultaneously presented in the open field (Figure 5).

The rat was placed into the open field to familiarize it with the apparatus and to record the baseline level of locomotor activity and object exploration. The same configuration of the objects was presented during Session 2 and Session 3. For Session 4, the spatial location of the object (B) was modified and a response to spatial change was assessed by comparing the time in contact with the objects belonging to each category (displaced and non-displaced). In Session 5, one of the familiar non-displaced object (C) was substituted with a new one (object E) at the same location. Response to the new object was assessed by comparing the time in contact with the objects belonging to each category (novel and familiar).

4.1.3. Behavioral Measures

Locomotor activity was assessed by counting the number of grid crossed by each animal while moving in the open field across the five sessions. The decrease in the number of crossings between Session 1 and Session 3 was taken to be a measure of habituation to the environment.

The amount of time spent by each animal for the object exploration was recorded. Exploration was considered to be directing the nose at a distance <2 cm to the object and/or touching it. The habituation index was calculated by comparing the time spent for exploring the objects when they were first introduced in Session 1 with the time spent in re-exploring the same objects in Session 3. To measure the amount of habituation, we calculated a difference score by subtracting the time spent for exploring four objects in Session 3 from the time spent for the same objects during Session 1. The larger the habituation index, the greater the habituation.

The rats' responses to the spatial change (in Session 4) and object novelty (in Session 5) were evaluated as discrimination indexes (DIs) that takes into account individual differences in the total amount of exploration [42, 43]. Response to the spatial change was assessed by comparing the time in contact with the displaced object during Session 4 and the mean time spent in contact with the remaining (three) non-displaced objects. The following equation was used for displacement discrimination index, DI_D: $DI_D = t_D/tN_D + t_D$, where t_D = exploration time of the displaced objects and tN_D = mean exploration time of the non-displaced objects. The object novelty discrimination index, DI_N was

calculated as: $DI_N = t_N/t_F + t_N$, where t_N = exploration time of the novel object and t_F = mean exploration time of the familiar objects.

4.1.4. Statistical Analysis

A one-way ANOVA was used to compare immediate and persisting effects of toluene chronic exposure on the locomotor activity and object exploration, also on the habituation and displacement or object novelty discrimination indexes. Between group comparisons was performed by two-sample *t*-test. In Sessions 4 and 5, the DIs (displacement discrimination index and object novelty discrimination index) were also compared with chance performance (DI = 0.5) by a one-sample *t*-test. Differences were considered significant when $p < 0.05$.

4.2. Results

4.2.1. Adolescent Rats

4.2.1.1. Locomotor Activity and Habituation to the Environment

Figure 6a illustrates the immediate and persisting effects of toluene exposure on the locomotor activity during five sessions of adolescent rats. The different control groups were combined into one control group, because there was a not significant difference among the groups ($p > 0.10$). ANOVA revealed specific effect of experimental conditions on locomotor activity [$F(2,117)=6.43$; $p < 0.01$]. Specifically, significant difference was observed between control rats and the rats studied immediately after the end of toluene exposure ($p < 0.01$). Locomotor activity differs significantly between control animals and the animals investigated after 90 d of toluene exposure ($p = 0.01$). One-way ANOVA for the habituation index also showed significant effect of toluene exposure [$F(2,21) = 29.70$; $p < 0.001$]. This effect was expressed only between the control rats and the rats that performed the open field test 90 d after the end of toluene exposure ($p < 0.001$). The habituation index did not differ significantly between the control rats and the rats that were tested immediately after the end of toluene exposure ($p = 0.1$). The habituation index significantly differs between animals of both experimental groups ($p < 0.001$) (Figure 6b). The results of the rat locomotor activity are presented in Tables 4 and 5.

Table 4. Immediate and persisting effect of toluene chronic exposure on the exploratory activity, habituation (HI) and recognition memory (defined by discrimination indexes - DI_D, DI_N) of adolescent and adult rats. Summary of one-way ANOVA results: *F*-variance ratio from one–way ANOVA, *P*-probability

	Adolescent		Adult	
	F (2,117)	P	F (2,117)	P
Locomotor Activity	6.43	0.002	1.58	0.211
Object Exploration Time	24.68	0.000	25.92	0.000
	F (2,21)	P	F (2,21)	P
HI of Locomotor Activity	29.70	0.000	31.36	0.000
HI of Object Exploration	14.50	0.000	24.68	0.000
DID	17.29	0.000	25.82	0.000
DIN	10.26	0.001	15.44	0.000

4.2.1.2. Object Exploration and Habituation to the Objects

Figure 6c shows immediate and persisting effects of toluene exposure on the object exploration in adolescent rats. The ANOVA revealed significant effect of treatment on the object exploration [$F(2,21)=14.50$; $p < 0.001$]. The habituation index to the object significantly differs between the control rats and the rats that were tested in the open field immediately after the end of toluene exposure ($p < 0.01$), also between control rats and the rats that were tested 90 d after the end of toluene exposure ($p < 0.001$). The habituation index did not differ significantly between experimental groups ($p > 0.05$) (Figure

6d). The results of the object exploration are presented in Tables 4 and 5.

4.2.1.3. Detection of Spatial Novelty

Figure 7a shows the difference between the responses to spatial change (defined by DI) at Session 4 by control and toluene-treated rats. One-way ANOVA for the displacement discrimination index revealed significant effect of experimental conditions [$F(2,21) = 17.29$; $p < 0.001$]. Significant difference was observed between control rats and rats investigated immediately after the end of toluene exposure ($p < 0.0001$). The displacement discrimination index did not differ significantly between control animals and the animals investigated 90 days after exposure ($p = 1.000$). The results of the spatial novelty detection are presented in Tables 4 and 5.

Table 5. Immediate and persisting effect of toluene chronic exposure on the exploratory activity, habituation (HI) and recognition memory (defined by discrimination indexes - DI_D, DI_N) of adolescent and adult rats. Summary of two sample *t*-test. Data are given as mean value ±SE (Standard Error); † indicates vs. control, ‡ indicates vs. immediate effect

Age	Adolescent			Adult		
Groups	Control	Immediate	Persistent	Control	Immediate	Persistent
Locomotor Activity	162.8±10	122.4±4.8	200.1±5.6	199±10	187±5.3	173.8±12
P-value		0.007†	0.010† 0.000‡		0.294†	0.113† 0.317‡
Object Exploration	68.38±3.4	24.88±1.9	57.06±2.4	56.9±4	39.8±3.0	30,75±1.4
P-value		0.000†	0.018† 0.000‡		0.050†	0.000† 0.022‡
HI of Locomotor Activity	50.25±3.1	56.50±1.4	19.3±5.3	34.5±3.0	3.90±3.4	5.0±2.9
P-value		0.104†	0.000† 0.000‡		0.000†	0.000† 0.808‡
HI of object Exploration	18.50±1.5	11.69±1.0	7.63±1.7	5.65±1.1	6.81±1.1	0.60±0.56
P-value		0.003†	0.000† 0.067‡		0.465†	0.002† 0.000‡
DI_D	0.74±0.03	0.48±0.023	0.74±0.049	0.75±0.017	0.6±0.025	0.51±0.022
P-value		0.000†	1.000† 0.001‡		0.000†	0.000† 0.019‡
DI_N	0.76±0.016	0.65±0.13	0.75±0.025	0.64±0.016	0.54±0.013	0.51±0.022
P-value		0.000†	0.746† 0.006‡		0.000†	0.000† 0.257‡

The mean discrimination ratios in control rats ($DI_D = 0.74 \pm 0.03$) and rats investigated 90 days after the end of toluene exposure ($DI_D = 0.74 \pm 0.05$) were significantly higher from chance performance ($t = 7.95$, $P < 0.0001$; $t = 4.90$, $P < 0.01$, respectively). The mean discrimination ratio (DI_D) in rats investigated immediately after the end of toluene exposure ($t = 0.77$, $p > 0.05$) did not differ significantly from chance level ($p > 0.05$**).** The results of one-sample *t*-test are presented in Table 6.

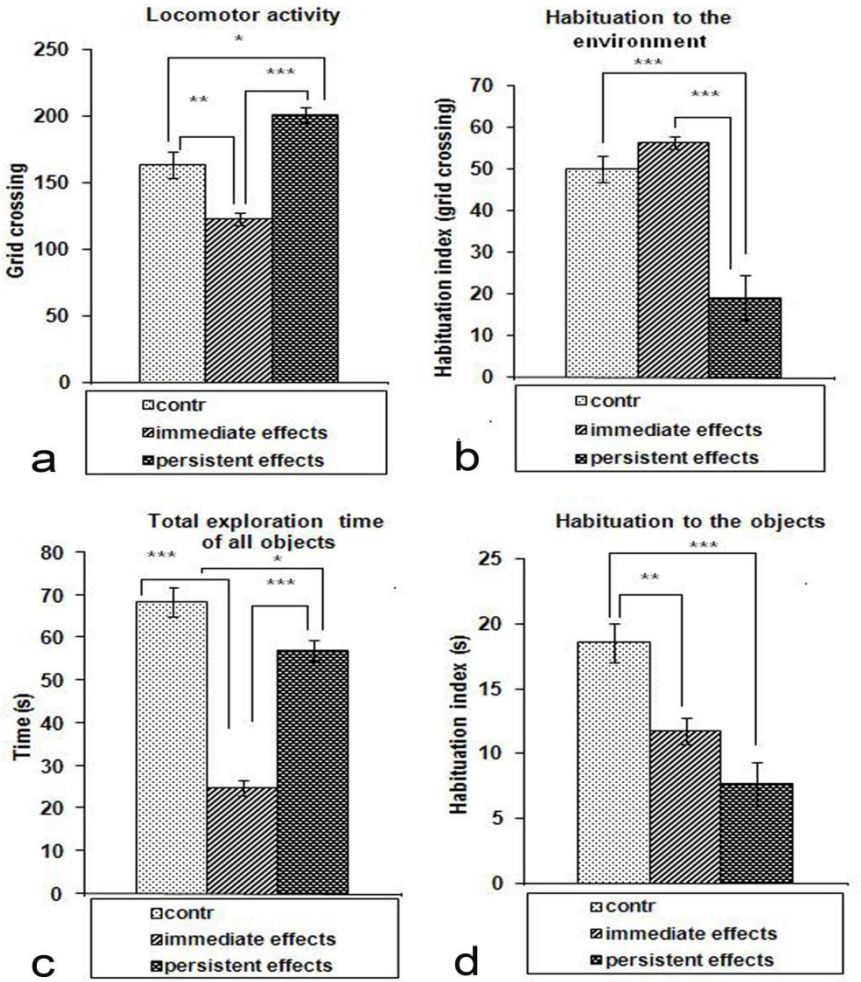

Figure 6. Immediate and persisting effect of toluene chronic exposure on the exploratory behavior in the open field: adolescent rats. (a) Effect of toluene chronic exposure on the locomotor activity (assessed by counting the number of grid crossed during five sessions) and (b) on the habituation to the environment (measured by the decrease in the amount of locomotor activity between Sessions 1 and 3); (c) Effect of toluene chronic exposure on the object exploration (the amount of time spent by animals for the object exploration during five sessions) and (d) on the habituation to the objects (measured by the decrease in the amount of exploration of the same objects between Sessions 1 and 3. Data are given as mean ±SE. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Table 6. Immediate and persistent effect of toluene chronic exposure on the discrimination indexes of displacement (DI_D) and object novelty (DI_N) of adolescent and adult rats. Summary of one sample *t*-test of DI_D and DI_N comparison with chance performance (DI = 0.5)

Adolescent		Mean	StDev	SE Mean	T-value	P-value
DI_D	Control	0.740	0.0854	0.0302	7.95	0.000
	Immediate	0.483	0.0645	0.0228	- 0.77	0.468
	Persistent	0.740	0.1387	0.0490	4.90	0.002
DI_N	Control	0.7600	0.0450	0.0159	16.33	0.000
	Immediate	0.6500	0.0378	0.0134	11.22	0.000
	Persistent	0.7500	0.0721	0.0255	9.81	0.000
Adult						
DI_D	Control	0.7500	0.0481	0.0170	14.70	0.000
	Immediate	0.6000	0.0707	0.0250	4.00	0.005
	Persistent	0.5100	0.0632	0.0224	0.45	0.668
DI_N	Control	0.6400	0.0466	0.0165	8.50	0.000
	Immediate	0.5400	0.0355	0.0125	3.19	0.015
	Persistent	0.5100	0.0614	0.0217	0.46	0.659

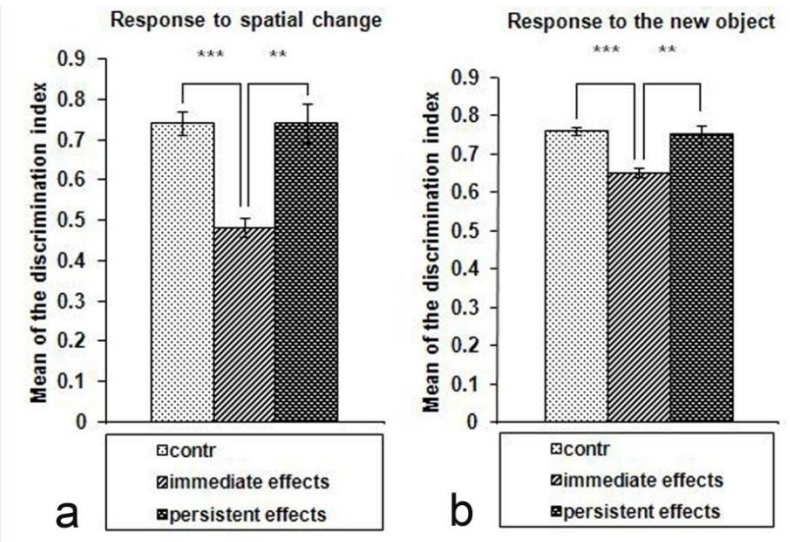

Figure 7. Immediate and persisting effect of toluene chronic exposure on the behavioral response to the spatial change (a) and object change: adolescent rats. The histograms represent difference between spatial change scores (a) or object change scores (b) defined by discrimination indexes (DIs). Data are given as mean ±SE. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

4.2.1.4. Detection of Object Change

One-way ANOVA for the object novelty discrimination index showed significant effect of experimental conditions [$F(2,21) = 10.26$; $p = 0.001$] in adolescent rats. Significant difference was observed between control animals and the animals investigated immediately after the end of toluene exposure ($p < 0.001$). The displacement discrimination index did not differ significantly between control animals and the animals investigated 90 days after exposure ($p > 0.05$). Significant difference was observed between experimental groups ($p < 0.01$) (Figure 7b). The results of the object novelty detection are presented in Tables 4 and 5.

The mean discrimination ratios in control rats ($DI_D = 0.76 \pm 0.02$) and rats investigated immediately ($DI_D = 0.65 \pm 0.013$) or 90 days after the end of toluene exposure ($DI_D = 0.75 \pm 0.025$) were significantly higher from chance performance *(*$t = 16.33$*;* $t = 11.22$*;* $t = 9.81$ respectively*;* $p < 0.001$*).* The results of one-sample *t*-test are presented in Table 6.

4.2.2. Adult Rats

4.2.2.1. Locomotor Activity and Habituation to the Environment

Figure 8a shows immediate and persisting effects of toluene exposure on the locomotor activity (measured by grid crossings) during five sessions in adult rats. The different control groups were combined into one control group, because there was a not significant difference among the groups ($p > 0.1$). According to one-way ANOVA in adult animals the outcome of experimental conditions on the locomotor activity is less expressed [$F(2,117) = 1.58$; $p > 0.05$]. The locomotor activity didn't differ significantly between groups (Figure 8a). The control rats generally decreased their level of activity across sessions, demonstrating habituation to the environment as they were exposed to the same or similar environment repeatedly. One-way ANOVA for the habituation index showed significant effect of experimental conditions [$F(2.21 = 31.36$; $p < 0.001$]. Significant difference was observed between the control rats and the rats that were tested in the open field immediately or 90 d after the end of toluene exposure ($p < 0.001$). Significant difference was not observed between experimental groups ($p > 0.05$) (Figure 8b). The results of the rat locomotor activity are presented in Tables 4 and 5.

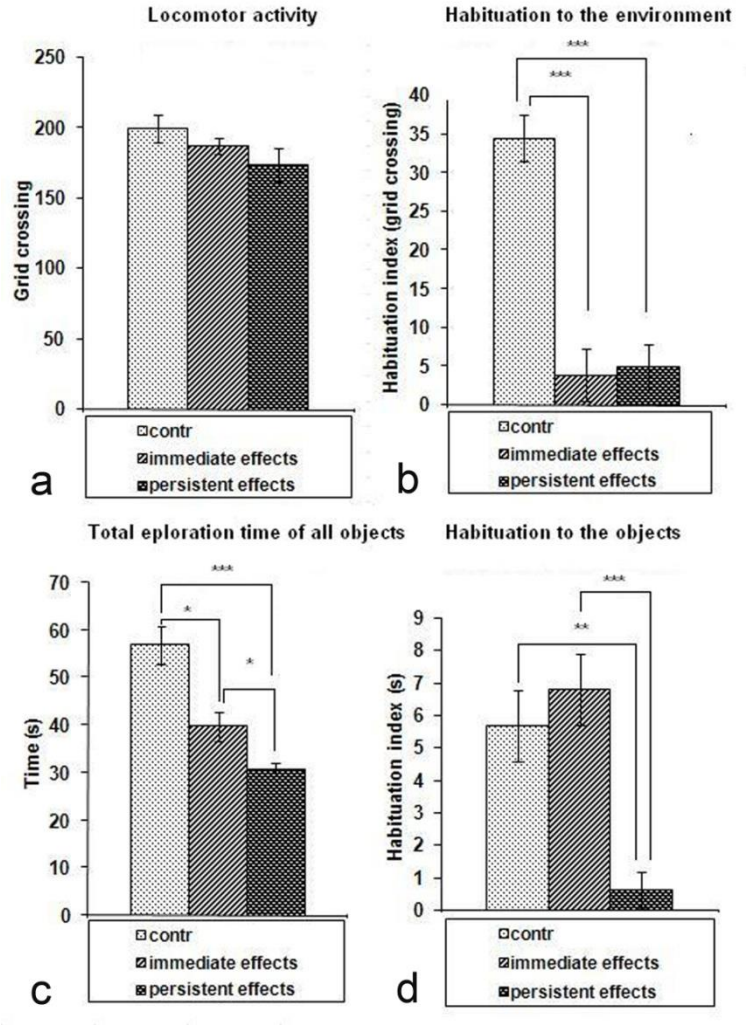

Figure 8. Immediate and persisting effect of toluene chronic exposure on the exploratory behavior in the open field: adult rats. (a) Effect of toluene chronic exposure on the the locomotor activity (assessed by counting the number of grid crossed during five sessions) and (b) on the habituation to the environment (measured by the decrease in the amount of locomotor activity between Sessions 1 and 3); (c) Effect of toluene chronic exposure on the object exploration (the amount of time spent by animals for the object exploration during five sessions) and (d) on the habituation to the objects (measured by the decrease in the amount of exploration of the same objects between Sessions 1 and 3. Data are given as mean ±SE. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

4.2.2.2. Object Exploration and Habituation to the Objects

Although the activity level measured by grid crossing reflects general habituation of the rats to the environment, it does not show that the habituation specifically occurred to the objects in the environment. Figure 8c demonstrates the total time of object exploration during five sessions. ANOVA revealed significant effect of treatment on the object exploration [$F(2,117 = 25.92; p < 0.001$]. The total time of object exploration in the rats that were tested in the open field immediately after the end of toluene exposure was lower than that in the control animals ($p = 0.05$). More significant alterations in object exploration were observed 90 d after the end of toluene exposure ($p < 0.001$). More significant alterations in object exploration were observed 90 d after the end of toluene exposure. One-way ANOVA for the habituation index showed significant effect of toluene exposure [$F(2,21) = 24.68; p < 0.001$]. Significant difference was observed between control rats and rats studied immediately or 90 days after the end of toluene exposure ($p < 0.001$). Significant difference was not observed between experimental groups ($p > 0.05$) (Figure 8d). The results of the object exploration are presented in Tables 4 and 5.

4.2.2.3. Detection of Spatial Novelty

In Session 4 one of the objects (object B) was removed from its original location and was placed in different location in the open field. One-way ANOVA for the displacement discrimination index showed significant effect of experimental conditions [$F(2,21) = 25.82; p < 0.001$]. Significant difference was observed between control animals and the animals investigated immediately after the end of toluene exposure ($p < 0.0001$) or 90 days after exposure ($p < 0.001$) (Figure 9a). The displacement discrimination indexes significantly differ between experimental groups ($p < 0.05$). The results of the spatial novelty detection are presented in Tables 4 and 5.

The mean discrimination ratio in control rats ($DI_D = 0.75 \pm 0.017$) was significantly higher from chance performance ($t = 14.70$, $p < 0.001$). In rats investigated immediately after the end of toluene exposure the mean discrimination ratio ($DI_D = 0.60 \pm 0.025$) significantly differs from chance level ($t = 4.00$, $p < 0.05$) but in rats investigated 90 days after the end of toluene exposure the mean discrimination ratio ($DI_D = 0.51 \pm 0.022$) did not differ significantly from chance level ($t = 0.45$, $p > 0.05$). The results of one-sample *t*-test are presented in Table 6.

4.2.2.4. Detection of Object Change

In Session 5 one of the familiar non-displaced object (C) was substituted with a new one (object E) at the same location. The results indicate that the control rats clearly react to the object novelty by exploring the new object more than familiar ones. One-way ANOVA for the object novelty discrimination index showed significant effect of experimental conditions [$F(2,21) = 15.44$; $p < 0.001$]. Significant difference was observed between control animals and the animals investigated immediately after the end of toluene exposure ($p < 0.001$) or 90 days after exposure ($p < 0.001$). The object novelty discrimination index did not differ significantly between experimental groups ($p > 0.05$) (Figure 9b). The results of the object novelty detection are presented in Tables 4 and 5.

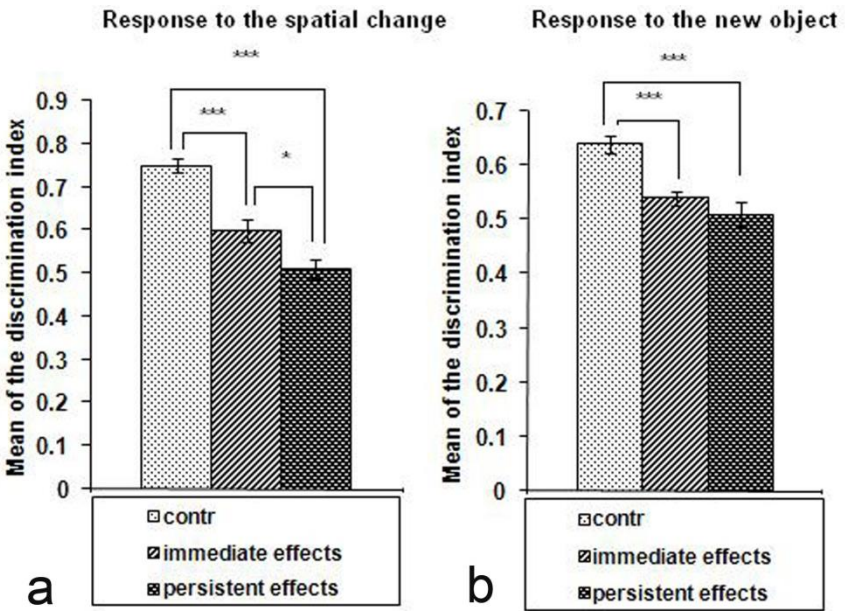

Figure 9. Immediate and persisting effect of toluene chronic exposure on the behavioral response to the spatial change (A) and object change: adult rats. The histograms represent difference between spatial change scores (A) or object change scores (B) defined by discrimination indexes (DIs). Data are given as mean ±SE. *$p \leq 0.05$; ***$p \leq 0.001$.

The mean discrimination ratio in control rats ($DI_N = 0.64 \pm 0.017$) was significantly higher from chance performance ($t = 8.50$, $p < 0.001$). In rats investigated immediately after the end of toluene exposure discrimination ratio

($DI_N = 0.54 \pm 0.013$) significantly differs from chance level ($t = 3.19$, $p < 0.05$) and in rats investigated 90 days after the end of toluene exposure ($DI_N = 0.51 \pm 0.022$) did not differ significantly from chance level ($t = 0.44$, $p > 0.05$). The results of one-sample *t*-test are presented in Table 6.

4.3. Summary

The behavioral results demonstrate that toluene chronic exposure provokes alterations in exploratory behavior in the open field and recognition memory in adolescent and adult rats. Like in histological studies, character of these alterations depends on the age of animal. In adolescent rats in the majority of cases the immediate effect was the most expressed; alterations did not progress during 90 d period abstinence. The exception was the habituation index (object observation), which was significantly higher after abstinence period than immediately after the end of toluene exposure. On the other hand: in adult rats the most expressed was persistent effect: in these animals alterations progress during 90 d withdrawal.

5. Effect Of toluene Chronic Exposure on Behavior of Rats in Multi-Branch Maze

The multiple T-maze studies were used to determine whether experimental conditions affect learning in animals, usually in rodents. Performance in this labyrinth is easy to evaluate because each intersection is usually identical and has a clear right or wrong answer.

5.1. Behavioral Procedure and Measures

The maze used in this study was composed from the nest-box, firing pad and multiple T-junctions with several “blind-alleys” – deadlocks (Figure 10). The maze was located in the room without external cues (such as window, clock on the wall, head lamp etc.). During the experiment, each rat was transported to the maze 60 min prior to the start and was placed separately at the nest-box for acquired tolerance with maze. At the beginning of experiment each rat was put up on the firing pad from which it begins to explore the maze.

No reward was done during all experiment. As a final destination the return of rat in the pad and then at the nest-box was considered. Each rat performed the maze test 5 times at day, during 10 d, at the same time: from 10.00 until 12.00. The activity of rats in maze was assessed by: (i) the number of errors made by rats – entrance in deadlocks – while searching for optimal way to nest-box and (ii) exploration period – time, which the rat spent into the maze. For each group five animals were used.

5.2. Statistical Analysis

To determine whether toluene chronic exposure provokes immediate or persisting effect on the behavior of rats in maze (evaluating the number of errors and the time needed for passing the entire maze) the one-way ANOVA of quantitative data was performed separately in adolescent and adult rats. The results were presented as mean ± standard error (SE). A p-value less than 0.05 were considered as statistically significant. In the case of significant effect planned comparisons were carried out using t -tests.

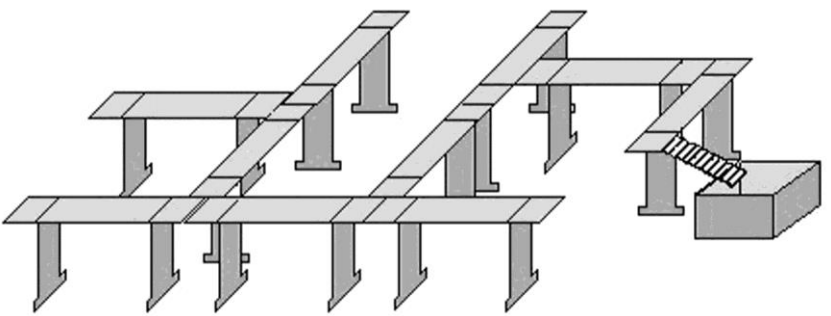

Figure 10. Schematic design of multi-branch maze.

5.3. Results

5.3.1. Adolescent Rats

The one-way ANOVA results demonstrate statistically significant influence of experiment conditions on the number of errors and time during maze test performance [correspondingly $F(2,29) = 26.65$, $p < 0.001$; $F(2,29) = 3.26$, $p < 0.05$]. According to the two-sample t-test the rats that performed the

maze test immediately after the end of toluene exposure, spend significantly more time in the maze (89.90 ± 6.8 sec vs. 67.5 ± 0.013; $p < 0.05$) (Figure 11) and make significantly more mistakes (entrance in "blind-alley") than control peers (numbers of errors – 2.455 ± 0.057 vs. 1.13 ± 0.14; $p < 0.001$) (Figure 12).

Table 7. Immediate and persisting effect on the behavior of adolescent and adult rats in maze (evaluating the number of errors and the time needed for passing the entire maze). Summary of one-way ANOVA results. *F*- Variance ratio from one-way ANOVA, *p* - probability

	Adolescent		**Adult**	
	$F_{(2,29)}$	*p*	$F_{(2,29)}$	*p*
Time	3.26	0.047	3.22	0.049
Number of Errors	26.65	0.000	11.17	0.000

Table 8. Immediate and persisting effect on the behavior of adolescent and adult rats in maze (evaluating the number of errors and the time needed for passing the entire maze). Summary of two sample *t*-test.results

	Adolescent			Adult		
	Control	Immediate	Persistent	Control	Immediate	Persistent
Time	67.5±0.013	89.90±6.8	75.50±7.5	78.4±8.3	84.8±5.7	105.0±8.8
P-value		0.013†	0.364† 0.174‡		0.536†	0.042† 0.073‡
Number of errors	1.13±0.14	2.455±0.057	1.325±0.19	0.465±0.11	1.05±0.17	1.68±0.24
P-value		0.000†	0.415† 0.000‡		0.010†	0.001† 0.050‡

After 90 days of toluene exposure rats that passed the maze needed almost the same time (75.50 ± 7,5 sec vs. 67.5 ± 0.013 sec; $p > 0.05$) (Fig,11) and make the same number of errors (entrance in "blind-alley") as their control counterparts (1.325 ± 0.19 vs. 1.13 ± 0.14; $p > 0.05$) (Figure 12). Comparison between persistent and immediate effect revealed significant difference in number of errors made by adolescents during maze test performance ($p < 0.001$).

Thus: according maze test, in adolescent rats toluene chronic exposure provokes significant alterations only immediately after the end of toluene chronic exposure; after 90 d withdrawal the behavior of rats in maze was almost the same as observed in control animals.

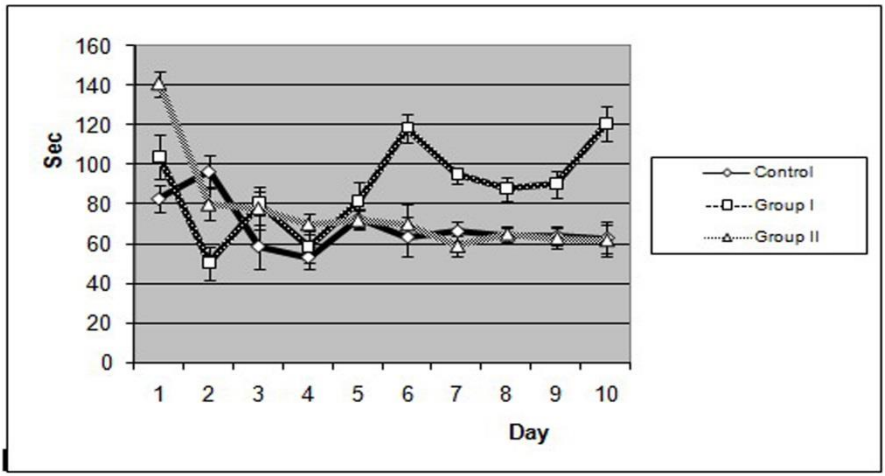

Figure 11. Duration of multi-branch maze test by control and experimental adolescent rats. Group I – animals started maze test directly after the end of toluene exposure. Group II – animals started maze test 90 days after the end of toluene exposure.

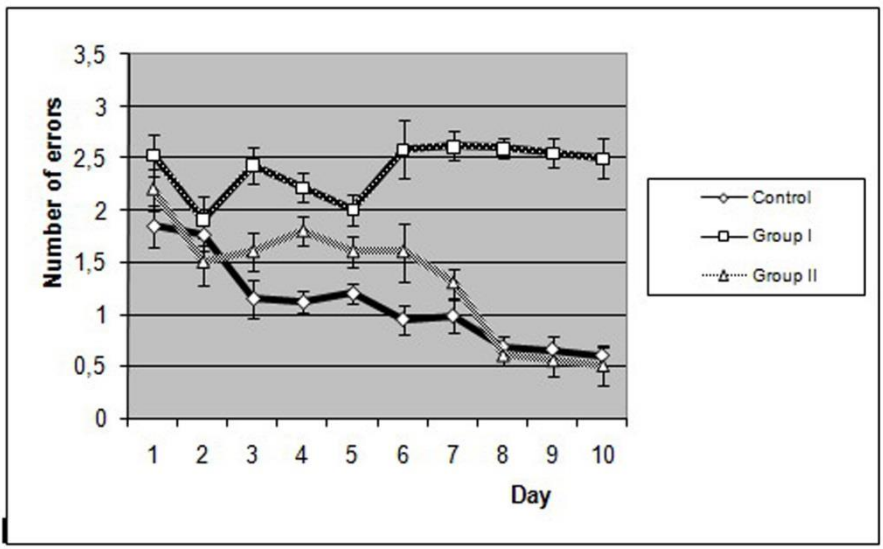

Figure 12. Measurement of number of errors (entering to "blind-alleys") made by control and experimental adolescent rats in multi-branch maze. Group I – animals started maze test directly after the end of toluene exposure. Group II – rats started maze test 90 days after the end of toluene exposure.

5.3.2. Adult Rats

The one-way ANOVA results demonstrate statistically significant influence of experimental conditions on the number of errors and time of maze test performance [correspondingly $F(2,29) = 11.17$, $p < 0.001$; $F(2,29) = 3.22$, $p < 0.05$] in adult rats too. According to the two-sample *t*-test the rats that performed the maze test immediately after the end of toluene exposure, spent in maze almost the same time, as their control counterparts (84.8 ± 5.7 sec vs. 78.4 ± 8.3 sec; $p > 0.05$) (Figure 13). However, during maze passing they make significantly more mistakes (entry in "blind-alleys") than control peers (1.05 ± 0.17 vs. 0. 465 ± 0.11; $p \leq 0.01$) (Figure 14).

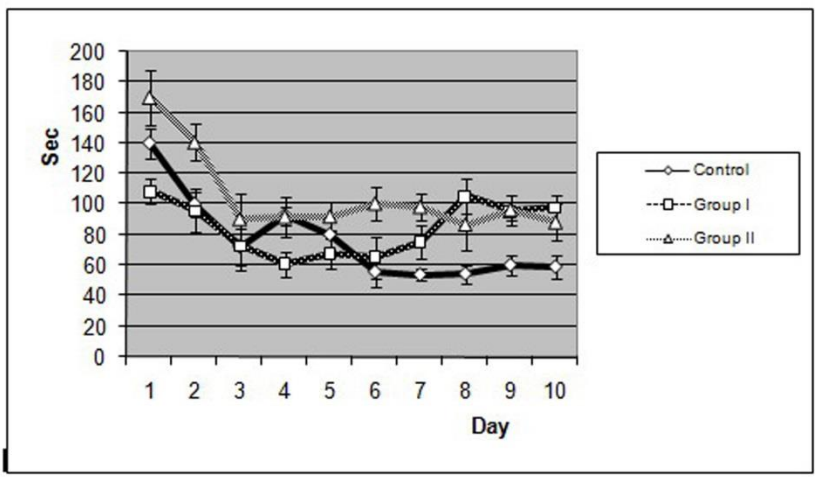

Figure 13. Duration of multi-branch maze test by control and experimental adult rats. Group I – animals started maze test directly after the end of toluene exposure. Group II – animals started maze test 90 days after the end of toluene exposure.

Unlike these animals, rats that passed the maze 90 d after the end of toluene chronic exposure, spent in the maze significantly more time than control rats (105.0 ± 8.8 sec vs. 78.4 ± 8.3 sec; $p < 0.05$) (Figure 13). They also made significantly more mistakes (entry in "blind alley") than their control peers (1.68 ± 0.24 vs. 0.46.5 ± 0.11; $p \leq 0.001$) or rats that performed maze test immediately after the end of toluene exposure (1.68 ± 0.24 vs. 1.05 ± 0.17; $p \leq 0.05$) (Figure 14).

Thus, in adult rats, toluene chronic exposure provokes both, immediate and persisting effect on behavior in maze. The most significant was persisting effect – observed 90 d after withdrawal.

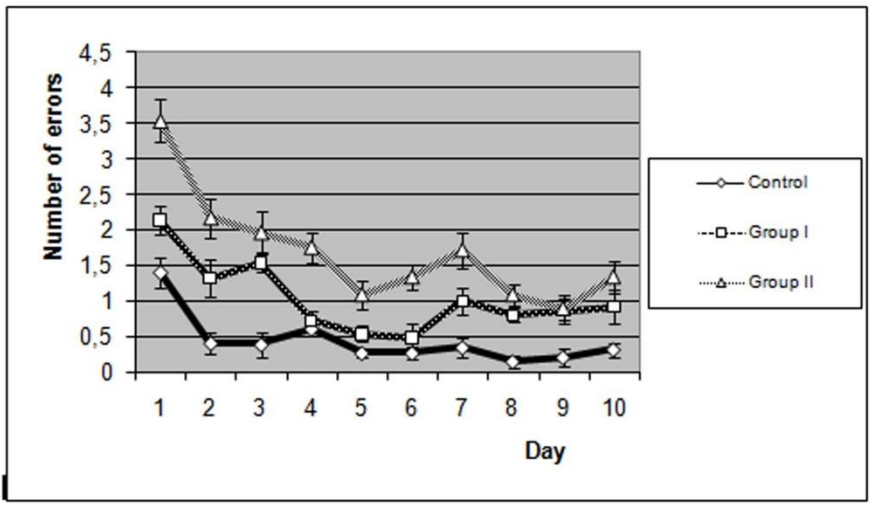

Figure 14. Measurement of number of errors (entering to "blind-alleys") made by control and experimental adult rats in multi-branch maze. Group I – animals started maze test directly after the end of toluene exposure. Group II – rats started maze test 90 days after the end of toluene exposure.

5.4. Summary

The results demonstrate that toluene chronic exposure provokes alterations in learning process of adolescent and adult rats. These alterations depend from the age of animals: in adolescent rats the more significant was immediate effect: the alterations did not progress significantly during period of withdrawal. On the contrary, in adult rats the most significant was persisting effect: 90 d after withdrawal.

7. DISCUSSION

In the present research we studied immediate and persisting effect of toluene chronic exposure on learning process (assessed in multi-branch maze), exploratory activity in the open field, recognition memory and hippocampal structure in adolescent and adult rats. There were the following major findings in the study: (1) toluene chronic exposure alters learning, memory and hippocampal structure in adolescent and adult rats; (2) the level of behavioral

and structural alterations depends upon the postnatal age of testing animals. In particular: in adolescent rats the most significant behavioral and structural alterations were observed by the day following toluene chronic exposure. These alterations do not progress significantly during abstinence period: some altered parameters were almost the same as observed the day following immediately after toluene misuse and others were very close to observed in control animals. Therefore, in adolescent rats the most expressed was immediate effect of toluene misuse. Contrary to it: in adult rats most alterations significantly progress during 90 d period of abstinence. So, in these animals more substantial was persistent effect of toluene chronic exposure. The discussion of these findings is done below.

The ability of addictive substances to alter hippocampus and other neural substrates of learning and memory would explain the capacity of these substances to produce long-lasting and maladaptive behavioral and cellular changes [14, 15, 28, 34, 44, 45]. Like other addictive drugs toluene-containing volatiles have been known to produce alterations in learning, memory and corresponding substrates. However while the neurotoxic subsequences of toluene misuse have been studied extensively, the changes provoked by this inhalant in learning, memory and related brain areas were described only in a few number of studies [46-49]. The least numerous are data concerning toluene effect on learning, memory and corresponding brain structures in adolescents.

Numerous clinical and experimental data strongly indicate that all alterations provoked by toluene chronic exposure are dose-dependent. The dose used in our study is 2 000 ppm. The Occupational Safety and Health Administration considers toluene level of 2 000 ppm as dangerous for health and life. Clinically, this dose is comparable to the inhaled exposure which produces euphoria in humans. Thus, euphoria usually appears at levels near 800 -1 500 ppm, but some people may be more or less sensitive to the effect of inhaled toluene. Because euphoria is the desired effect, individuals with toluene abuse deliberately expose themselves to toluene levels of at least 800 – 1 500 and higher. For instance, the person who has used toluene by sniffing glue can achieve levels of exposure estimated at 500 – 1 200 ppm. Several authors consider the 2 000 ppm as low level toluene inhalation (comparably to 5 000 – 12 000 ppm – dose, usually used by chronic abusers). But this “low” dose is known to alter the brain neurotransmitter levels (for example, to gradually decrease acetylcholine and increase GABA) [10, 11, 32, 50] and some types of behavior (for example, diminishes avoidant behavior) [40].

The results of present study indicate that 40 d exposure to 2 000 ppm of inhaled toluene in adolescent and adult rats to simulate human toluene abuse, in addition to abovementioned biochemical and behavioral changes provokes alterations in the exploratory behavior and recognition memory in the open field, behavior in multi-branch maze and the structure of the hippocampus, one of the key brain regions responsible in learning and memory. The majority of alterations were observed both, immediately after the last day of exposure (immediate effect of toluene chronic inhalation) and after 90 day's abstinence (persisting effect of toluene chronic inhalation), herewith the age-dependent difference was revealed. The age-dependent difference of several behavioral and neurochemical consequences in the case of toluene misuse have been reported in a few numbers of recent studies. Thus, less sensitization and attenuation of neurochemical responses in adolescents in comparing with adults as a result of toluene chronic exposure was revealed. Also, differential effect of inhaled toluene on locomotor activity in animals of different age was described [4, 13, 19]. Following these authors we show (for our knowledge for first time) the age-dependent difference of toluene effect on learning process in maze, several types of memory and hippocampal structure. Taking together, these data indicate that as a result of toluene chronic misuse the level of behavioral and structural alterations depends upon the postnatal age of testing animals.

Several factors may play a role in these differences. At this step only some assumptions are possible. Thus, such distinction, at least partly, could be related with the age-difference in toluene-pharmacokinetics, which is also shown. Hence, levels of hepatic enzymes, responsible for toluene metabolism to benzyl alcohol and *o* - and *p* - cresol vary across age in drug-naïve rats, suggesting that younger rats might metabolize toluene more quickly than adults [51-53]. However some facts (for example: adolescent and adult animals are differentially sensitive to the acute effect of some CNS depressants that have other pharmacokinetic mechanism than toluene) argue against such position [4, 5]. Therefore, additional multidirectional investigations are necessary to evaluate the causes and mechanisms that may play the role in these differences. Besides difference in pharmacokinetics, this phenomenon is most likely related to multiple factors one of which could be the unique developmental profile of the adolescent brain (in rat: 2 weeks period P28-P42) [22, 24]. The adolescence, as developmental transition is characterized by a high level of neuroplasticity, set of neurobehavioral alterations, biochemical rearrangements, neuronal pruning and brain re-organization including developmental changes in receptors and

neurotransmitter systems. The difference in the effects of toluene in adolescents as compared to adults could be to some extent related with toluene interaction with multiple neurotransmitter systems. First at all, it could be interaction with dopamine system and GABA(A) or NMDA receptors, specific alterations of which are considered to be one of the common mechanisms of action for abused inhalants [54]. Hippocampus is one of the brain structures that still undergo multilateral developmental changes during adolescence. Significant alterations in adolescent hippocampus take place in dopamine system and GABA(A) and NMDA receptors [54]. Observed in our study age-dependent difference of structural alterations in the hippocampus could be, at least partly, related with this fact.

The identification of toluene induced cell loss pathway (apoptosis, necrosis) in adolescents and adults and the age dependent differences in corresponding mechanisms should also help to understand different effect of toluene chronic inhalation on the hippocampus structure in the animals of various ages. The potential of toluene to induce apoptosis and/or necrosis is demonstrated by several authors; the character of cell death depends on brain region, cell type, developmental stage of animal, duration of exposure and dosage [54-58]. For example, the expression of several peptides that participate in apoptosis and necrosis differs in adult and adolescent brain. Some of such peptides, for instance, the p75NTR, the member of the TNF receptor superfamily, are shown to participate in toluene-induced cell death [59]. It is very likely that unique character of hippocampal cell death in adolescents and adults as a result of toluene misuse is related to the difference in expression of the p75NTR and other peptides participating in the toluene-provoked apoptosis and necrosis. Therefore, comparative studies in several directions, such as identification of cell death pathway, clarification of factors responsible for cell death and determination of the level of their expression in adolescent and adult animals in concrete conditions of toluene intoxication, are considered as future direction of our research.

Another finding of histological research observed in both aging groups, is the greater vulnerability of the CA1 to toluene chronic exposure in comparing with the CA3. Among hippocampal regions, the CA1 is known to be the most susceptible to multiple experimental treatments and disorders. Several structural and molecular peculiarities of this area (for example, the presence of comparatively large number of electrical synapses along with chemical forms or specific assortment of $Ca2^{+}$ - binding proteins) can only partly explain such vulnerability. However to fully understand the nature of toluene addiction the contributing factor/s of the CA1 area vulnerability should be also determined.

In the present, histological part of research we are focused on pyramidal cells of the hippocampus. Earlier we investigated the effect of toluene chronic inhalation on hippocampal radial and oriental interneurons (unpublished data). The comparison of our data strongly indicates that as a result of toluene misuse in both aging groups the pyramidal cell loss is more substantial than the loss of interneurons. Thus, we suggest that in the hippocampus of rats of different age the special population of hippocampal neurons – principal cells – is more vulnerable to toluene chronic exposure. What peculiarities of hippocampal pyramidal neurons provoke such susceptibility is the subject of future investigations. In summary: present histological results are additional evidence that hippocampus, the neural substrate for learning and memory, may contribute to pathophysiology of addiction and toluene abuse in particular.

Our data point out that as a result of toluene misuse, structural disorganization of the hippocampus is associated with alterations in learning and memory. The most interesting fact is the certain coincidence of structural and behavioral alterations. Thus, our data indicate that the alterations in learning (assessed in multi-branch maze) are reflected on the structure of hippocampus, one of the brain regions responsible for the learning processes. Moreover, our results give the possibility to assume the involvement of the hippocampus in object and spatial recognition memory. There are various opinions about the role of the hippocampus in different types of memory. Thus, it is well established that dysfunction of the hippocampus causes spatial learning deficits in the tasks such as the Morris water maze [60]. However, the role of the hippocampus in recognition tasks has been controversial: some investigations show impaired recognition performance [61-64] and some does not [65-67].

Recognition memory involves making judgments about whether a stimulus has been encountered before. However, it can be argued that this type of memory is not a unitary process, as distinct types of information are used to form judgments of prior occurrence, including the relative familiarity of an object or location or when or where an object was previously encountered. Several behavioral studies show that under conditions in which recognition memory has a spatial or temporal component, the hippocampus appears to be critical [68, 69]. But a number of experiments show that hippocampal or fornix lesions produce no effect in object recognition [70-72]. In these experiments, the sample phase involves the presentation of two identical objects in the sample phase and replaced one of these objects with an object never previously encountered by the subject. Unlikely this experimental design, in our research the sample phase (sessions 1, 2, 3) involves the presentation of

four different objects (e.g., objects A, B, C, D) and, following the retention delay, one of the objects (e.g., object C) is replaced with the novel object E. Thus, the presence of four different objects in the sample phase might create additional contextual cues or object–object associations that are themselves vulnerable to hippocampal damage. Indeed, current studies have suggested that the hippocampus can play a role in object recognition if such cues are present [73, 74]. Recently it was shown that even single toluene injection impairs hippocampus-dependent non-spatial memory retention in adult mice; such impairment is accompanied by selective modulation of NMDA receptor subunit expression [75]. Our data indicate that alterations in recognition memory in adult rats take place not only after the end of toluene misuse, but also after 90 day's period of abstinence. In another study it was described that adolescent toluene exposure leads to cognitive impairment, including the impairment in recognition of novel objects – at adulthood [76]. Present results are opposite with these data. But the design of these two studies differs. Thus, our experiments were performed on rats while first results were obtained on mice. The age of animals also differs. The rats used in our study were younger, while mice used by Lin et al. [76] were more aged and more close to adulthood. Therefore, the plasticity of the central nervous system in these animals is lesser than of adolescent rats used in our study. This and some abovementioned factors could explain the difference in the results described in these two studies.

Several autopsy data show relatively specific damage to myelin with axonal sparing [77, 78]. Based on such studies it was suggested that brain damage induced by toluene chronic exposure may be considered as potentially reversible with abstinence or even amenable to future treatments designed to restore the myelin using the existing scaffolding of preserved axons. Furthermore, clinical experience suggests that toluene abusers who abstain from toluene misuse may show partial recovery from once the toxic effect of toluene is no longer persists [77-79]. Our data do not give us the possibility for such assumption. However describing the age-difference of toluene response, we show that in adolescent rats, the alterations provoked by toluene abuse in learning process, different forms of memory and hippocampal structure do not progress significantly during period of abstinence: the long-term outcome was almost the same as observed immediately after the end of toluene misuse while some parameters were even normalized. Taking into consideration the high plasticity of developmental brain, it is possible to predispose that in the adolescents the long-term abstinence could be associated with certain reversibility of pathological alterations.

Our data underline the necessity for understanding the consequences of toluene misuse on the developing and developed brain.

CONCLUSION

The results of the present histological and behavioral studies indicate that toluene chronic exposure provokes some disorders in the structure of the rat hippocampus and learning and memory. The level of these disorders depends on the age of animals. On the bases of our data it is possible to suggest that adolescent rats may show partial recovery from once the toluene toxic effect no longer persists.

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Chapter 3

INHIBITORY, TOXIC AND STRUCTURE EFFECTS OF TOLUENE ON MICROBIAL CONSORTIA INVOLVED IN WASTEWATER TREATMENT

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ABSTRACT

Human activities have resulted in a continuous generation of wastewater containing high concentration of organic matter and nitrogenous compounds such as ammonium, nitrate and nitrite. Some effluents such as those generated by petrochemical industry contain toluene, an aromatic volatile compound which contaminates soils, water and groundwater due to petroleum spills or to leaking storage tanks. Because of its toxicity, toluene has been classified as priority pollutant by the Environmental Protection Agency. Organic compounds and ammonium, nitrate, or nitrite can be simultaneously eliminated from municipal and industrial wastewaters using the metabolic capacity of the nitrifying and denitrifying bacteria. Nevertheless, toluene may exert inhibiting or toxic effects on the microbial consortia. In fact, it has been reported important diminishes in the specific rates of nitrifying and

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denitrifying bacteria activity during toluene removal processes for wastewaters treatment. Some toxic effects have been also reported. Likewise, considering the non polar characteristic of this compound, toluene appeared to affect the exocellular substances production, sludge structure and settleability.

This chapter pretends to succinctly present the current knowledge about inhibitory and toxic effects of toluene on the biological consortia during the removal of ammonium, nitrate, nitrite and toluene from wastewaters by nitrification and denitrification. Attention is also paid on some effects of toluene on sludge settleability and exopolimeric substances content.

INTRODUCTION

Human activities have resulted in the increase of carbon and nitrogen content in wastewater and groundwater affecting the environment [1]. One of the most important sources of carbon pollution is the petrochemical industry. Monoaromatic hydrocarbons such as toluene, benzene and isomers of xylene (also known as BTX compounds) are extracted and produced from crude oil [2]. Toluene is also produced by catalytic reforming (dehydrogenating process of the aliphatic compounds) and by alkylation and transalkylation processes [3]. A crescent toluene production has been observed all over the world. For instance, in 2000, the USA toluene production was close to 6.5 million of tons. Toluene is widely used for producing paints, rubber, adhesives, varnishes, agrochemicals, polymers and gasoline among many other products. In fact, gasoline is rich in toluene as almost 16% of gasoline content corresponds to this compound [4]. Accidental spills, evaporation of industrial sources, leakage from storage tanks in gas stations, industrial discharges and car combustion have resulted in environmental pollution with toluene. The great mobility abilities and toxicity of this compound are of major concern for environment and human health [5]. Toluene is a relatively water-soluble compound and has a high vapor pressure. Some of the physical and chemical properties of toluene are illustrated in Table 1. In this sense, toluene presence in soil, air, surface and groundwater has been increasingly reported [6, 7].

Concentrations ranging from 23 to 81 mg/l of toluene have been reported in groundwater [8]. Considering the non polar characteristic of this compound, there is an important bioaccumulation of toluene in the lipid fraction of cell membrane. The narcotic and neurotoxic properties of toluene are the major health hazards in humans due to its tendency to accumulate in adipose tissue.

Toluene is highly lipophilic affecting the central nervous system [9]. Acute intoxication from inhalation is characterized by euphoria, hallucinations, dizziness, confusion, headache, ataxia, stupor, and coma. Exposition over long periods of time may produce neuropsychosis, cerebral degeneration with ataxia, peripheral neuropathies, cognitive ability problems, ototoxicity and deafness [10]. According to Mexican legislation (NOM-127-SSA1-1994) [11], the maximum level established in potable water for toluene is 0.3 mg/l, whereas in the United States of America, the maximum level is 1 mg toluene/l [12]. All of these toluene characteristics clearly illustrate why this compound represents an environmental and health challenge.

Table 1. Some physical-chemical properties of toluene

Chemical structure	Molecular weight (g/mol)	Density (g/ml)	Water solubility (g/l) at 25ºC	Vapor pressure (mm Hg)	Partition coefficient octanol/water (log K_{ow})	Henry´s law constant at 25ºC (kPa m^3/mol)	Polarity
	92	0.87	0.47	28	2.69	0.67	Non polar

On the other hand, the increase of nitrogen compounds such as nitrate, nitrite and ammonium in superficial and groundwater has caused several environmental effects such as eutrophication, toxicity to aquatic organisms, and loss of biodiversity [13]; and human h ealth damages as methahemoglobinemia [14]; formation of nitrosoamines which are potentially carcinogenic compounds [15] and gastric cancer [16]. Nitrate is one of the most common contaminants present in aquifers all over the world [17]. It has been estimated that in the United States of America, the content of nitrate is higher than the maximum levels (10 mg NO_3^--N/l) established by the United States Environmental Protection Agency [18], in close to 22% of the aquifers [16], whereas in Europe, almost 33% of the aquifers exceeds the maximum levels established by Europe Environment Agency (11 mg NO_3^--N/l) [16]. In the north of Gulf of Mexico, an important hypoxic zone has been detected where oxygen concentration is lower than 2 mg/l due to high nitrate discharges and eutrophication [19, 20]. Nitrate concentrations between 7 and 156 mg/l have been determined in aquifers of middle and south of Mexico [21]. These nitrate concentrations are also higher than the maximum levels (15 and 40 mg

total nitrogen/l) established by Secretary of Environmental and Natural Resources (SEMARNAT) (NOM-003-ECOL-1997) [11]. Therefore, it is clear the need of applying effective wastewater treatments for reducing nitrogen contamination.

There are different alternatives for carbon and nitrogen compounds removal from contaminated water, such as physical-chemical and biological processes. Gravity separation, volatilization, adsorption, dialysis, inverse osmosis, ultrasonic radiation or chemical reactions as phentom are some of the most used physical-chemical processes [22]. Nevertheless, most of these processes are not cost-effective, are poorly efficient when highly contaminated streams are treated and generally give undesirable residues [23]. On the other hand, microbiological treatment processes may be economically feasible, result in highly nitrogen and carbon consumption efficiencies and conversion to innocuous products such as N_2, CO_2 and H_2O, respectively.

Nitrogen compounds can be biologically removed from water by the coupling of nitrification and denitrification processes [24]. Nitrification is the oxidation of ammonium to nitrate and denitrification is the reduction of nitrate to molecular nitrogen. Nitrifying and denitrifying microorganisms are widely spread in nature (soils, rivers, lakes, oceans, sea sludge, etc.) and play a key role in the biological removal of nitrogen in both nature and wastewater treatment systems. Nitrifying bacteria use ammonium and nitrite as energy sources, carbon dioxide as a carbon source, and molecular oxygen as the final electron acceptor [25]. The nitrifying process is composed of two consecutive steps: 1) the oxidation of ammonium to nitrite that is mainly carried out by ammonium-oxidizing bacteria (AOB) and 2) the oxidation of nitrite to nitrate that is catalyzed by nitrite-oxidizing bacteria (NOB). Nitrifying bacteria are sensitive to variations of the environmental conditions and their growth is slow and scarce, even in optimal conditions [26]. Thus, nitrification is commonly the rate limiting step of the overall nitrogen removal. Denitrifying microorganisms obtain energy from the reduction of nitrate to molecular nitrogen. Denitrification requires the concomitant oxidation of an electron donor in order to reduce nitrate to N_2. Organic sources are used in organotrophic denitrification whereas inorganic compounds are used for lithotrophic denitrification. The activity of denitrifying microorganisms can be affected by several environmental factors, such as oxygen tension, presence of nitrogen oxides, pH, temperature, carbon/nitrogen (C/N) ratio and the type of electron source [24]. Sewage treatment plants are usual receptors of xenobiotic

compounds that have to be treated with municipal wastewaters before being discharged to the water environment. The presence of aromatic contaminants in effluents, such as BTX compounds, may inhibit irreversibly sensitive biological processes in wastewater treatment, such as nitrification and denitrification. BTX are often present in municipal treatment plants [27]. According to investigations of the content of BTX in the sewage collected from several municipal wastewater treatment plants, the substance which had always been present in sewage, usually in the highest concentration from 14 to 1156 mg/l, was toluene [28]. Organic solvents with a partition coefficient in *n*-octanol and water (log K_{ow}) below 4.0, as toluene (log K_{ow} = 2.69), are extremely toxic for microorganisms because they accumulate in the cytoplasmic membrane of bacteria and disrupt the cell membrane structure. This damage is often followed by cell lysis [29]. Recalcitrant aromatic compounds, such as toluene, can also provoke inhibitory effects on microbial metabolism, decreasing the specific rates of the process. In the literature, there is significant research on the biodegradation of toluene under aerobic and anaerobic conditions [4]. For instance, rapid toluene consumption has been reported under sulfate [30], hem [31] and methanogenic [32] conditions. Most of these studies are focused on the disappearance of the aromatic compound; however, there is scarce information describing inhibition and toxic effects of toluene on microbial consortia. Considering that biological wastewater treatment by nitrification and denitrification is widely used and that toluene can be present in treatment plants, it is of interest to investigate more on the inhibitory and toxic effects of toluene on microbial nitrifying and denitrifying consortia involved in wastewater treatment. Understanding of the inhibitory and toxic effects of toluene on microbial sludge is not only important for achieving deeper knowledge of the whole wastewater treatment process, but also for predicting the possible impact of this contaminant once released into the environment (e.g. surface water, groundwater, etc.).

Biological treatment of wastewater can be successfully carried out by using upflow anaerobic sludge blanket (UASB) reactors, sequencing batch reactors (SBR) or completely mixed reactors. All of them depend on the formation of flocs and granules with good settlement ability. Thus, the efficiency of the wastewater treatment depends on the stability of the sludge structure. Granules and biofilms formation and their stability have been related with extracellular polymeric substances (EPS) among other factors [33, 34]. The EPS include chemical substances where 70-80% of the organic carbon

present in their structures could be attributed to proteins and carbohydrates [35, 36]. Exopolymeric carbohydrates and proteins have been considered to maintain the structure stability of sludge granules and biofilms [37]. In some cases, during wastewater treatment poor sludge settleability can be observed. This problem can be observed as instability of sludge structure resulting in sludge flotation, or in the worst situation in sludge bulking [38], which implies serious problems in solid-liquid separation [39, 40]. Finally, the lost of sludge and therefore of the biological process may occur in few hours. This lack of settlement ability has been related to several factors, where extracellular polymeric substances and type of carbon sources play an important role [41, 42]. Several studies have reported that toluene provokes damage in cellular structure exerting denaturing of proteins and lipids and producing lost of ions or metabolites [43, 44]. Moreover, toluene removal procedures may result in high biomass production. This situation affects the sludge structure as its accumulation could also provoke bulking. The presence of aromatic compounds such as toluene in wastewater could enhance operational troubles in the biological reactors operation. Information about the effect of toluene on sludge settleability is still scarce.

This chapter pretends to succinctly present the current knowledge about inhibitory, toxic and structure effects of toluene on the biological consortia during the removal of ammonium, nitrate, nitrite and toluene from wastewaters by nitrification and denitrification. The chapter is divided into three parts. In the first part, a brief description of microbiology and physiology of nitrification and denitrification is made. In the second part, inhibitory and toxic effects of toluene on the nitrifying and denitrifying processes are described. Finally, attention is paid on some effects of toluene on sludge settleability and extracellular polymeric substances content.

1. Nitrification and Denitrification Processes Used for Wastewater Treatment

1.1. Nitrification

Nitrification has been extensively investigated as a very useful process in the first step of nitrogen removal in biological wastewater treatment. Nitrification is an aerobic respiratory process where nitrifying bacteria use

ammonium and nitrite as energy sources, carbon dioxide as a carbon source, and molecular oxygen as the final electron acceptor [25]. The oxidation of ammonia and nitrite by nitrifying processes generates nitrate for the denitrifying process where nitrate is converted to molecular nitrogen.

Nitrification is carried out by two groups of Gram negative chemolithoautotrophic bacteria that belong to the Proteobacteria group: the ammonium- and nitrite-oxidizing bacteria. The ammonium-oxidizing bacteria (AOB) are species of the following genera: *Nitrosomonas*, *Nitrosospira*, *Nitrosolobus*, *Nitrosococcus*, and *Nitrosovibrio*, being *Nitrosomonas* and *Nitrosomonas europaea* the genus and species better studied, respectively. Ammonia can also be transformed by a number of heterotrophic fungi and bacteria but it is generally accepted that chemolithotrophs are the primary nitrifiers in many systems [45]. Recently, it has been reported that ammonium-oxidizing archaea, such as *Candidatus* "*Nitrosopumilus maritimus*" and *Candidatus* "*Cenarchaeum symbiosum*", are also believed to be involved in ammonium oxidation [46]. The genera of nitrite-oxidizing bacteria (NOB) are: *Nitrobacter*, *Nitrococcus*, *Nitrospina*, and *Nitrospira*. Most of the physiological and biochemical investigations have been carried out with members of the genus *Nitrobacter*. Some strains of *Nitrobacter* have been shown to be able to grow mixotrophically but their growth is very slow [47].

The nitrifying process is composed of two consecutive steps: 1) the oxidation of ammonium to nitrite that is mainly carried out by AOB and 2) the oxidation of nitrite to nitrate that is catalyzed by NOB (Table 2). As indicated by the free energy change (ΔG°') values, the oxidation of hydroxylamine (NH_2OH) to nitrite is the main step where the AOB obtain energy. The ΔG°' is lower for nitrite oxidation than for ammonium oxidation and the consequence is a lower growth yield for NOB than for AOB. Due to the low energy availability for cellular biosynthesis in the respiratory process, growth of nitrifying bacteria is scarce, even in optimal conditions.

Table 2. Reactions and ΔG°' values of ammonium and nitrite oxidizing processes in nitrification [48]

Reactions	Equations	ΔG°' (kJ/reaction)
Ammonium oxidation	$NH_4^+ + 0.5O_2 \rightarrow NH_2OH + H^+$	-8
	$NH_2OH + O_2 \rightarrow NO_2^- + H^+ + H_2O$	-267
Global reaction	$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$	-275
Nitrite oxidation	$NO_2^- + 0.5O_2 \rightarrow NO_3^-$	-74

In the ammonium oxidizing process, the first reaction is catalyzed by an ammonia monooxygenase (AMO) and the second one by the complex enzyme system hydroxylamine oxidoreductase (HAO). AMO is located in the cytoplasmic membrane while HAO in the periplasmic space [49]. It is generally accepted that ammonia (NH_3) rather than ammonium (NH_4^+) is the real substrate for the enzyme AMO. The AMO has shown to be able to co-oxidize numerous organic compounds, including recalcitrant aliphatic, aromatic, and halogenated molecules [50-53]. Nevertheless, this ability would result in inhibitory effects of the organic substances on the AMO enzyme, due to competition for its active site. The second stage in nitrification is the oxidation of nitrite to nitrate and is catalyzed by the nitrite oxidoreductase enzyme (NOR). NOR is located in the cytoplasmic membrane and is composed of cytochromes *a* and *c*, a quinone and a deshydrogenase dependent on NADH [54, 55].

Nitrification is affected by environmental factors such as temperature, pH, substrates concentrations (O_2, NH_4^+, and NO_2^-), and the presence of organic matter [26]. Such parameters can cause an effect on both the anabolic (cellular biosynthesis) and catabolic (respiration) processes. The sensitivity of nitrifying bacteria to the toxic or inhibitory effects of organic compounds is well-documented, and it is known that the stability of nitrifying systems in wastewater treatment can be altered by the presence of organic matter [56]. Most of the studies on effects of organic compounds on nitrification have used axenic cultures or consortia such as activated sludge as inocula. It has been shown that the effects mainly depend on the type and chemical structure of the organic pollutant as well as its concentration and hydrophobicity but also the type of culture (axenic or consortium) and the origin of the sludge [57, 58].

1.2. Denitrification

Denitrification is a biological process that microorganisms use for obtaining energy from the reduction of nitrate to molecular nitrogen and requires the concomitant oxidation of an electron donor. Organic sources are used in organotrophic denitrification whereas inorganic compounds are used for lithotrophic denitrification. Soluble and recalcitrant compounds such as methanol, glycerol, benzoic acid, acetate, glucose, lactate, *p*-xylene, benzene, toluene, phenol and *p*-cresol have been successfully removed by organotrophic denitrification [41, 59, 60-65]. Regarding lithotrophic denitrification, hydrogen (H_2), elemental sulfur (S^0), sulfide (S^{2-}), thiosulfate ($S_2O_3^{2-}$), sulfite

(SO_3^{2-}) and reduced iron (Fe°) among others can be used as electron donors [66].

Different taxonomic groups are involved in denitrification including Gram negative and positive bacteria. Their remarkable characteristic is their facultative respiration. Some genera as *Rhodobacter* are phototrophic, whereas many others can use organic sources as *Bacillus* [67], *Paracoccus* [68], *quaspirillum* [69], *Halomonas* [70] or sulfur compounds, as *Beggiatoa* [71], *Thiobacillus* [72], *Paracoccus* [73] and *Pseudomonas* [74], among others.

The denitrification process could be described as a modular organization in which every biochemical reaction is catalyzed by specific reductase enzymes [24]. The reactions take place when environmental conditions become anaerobic [75]. Four enzymatic reactions take place in the cell where reduced ubiquinone (UQH_2), ubiquinone (UQ); reduced cytochrome(c^{2+}); oxidized cytochrome (c^{3+}) have been identified [76]:

(i) Nitrate is reduced to nitrite by nitrate reductase *(Nar).* The reaction can take place in the cell membrane and periplasmic space:
$NO_3^- + UQH_2 \rightarrow NO_2^- + UQ + H_2O$

(ii) A subsequent reduction of nitrite to nitric oxide is carried out by one of two nitrite reductase (*Nir*) located at the periplasmic space:
a) $NO_2^- + Cu^{1+} + 2H^+ \rightarrow NO + H_2O + Cu^{2+}$ or
b) $NO_2^- + c^{2+} + 2H^+ \rightarrow NO + H_2O + c^{3+}$

(iii) Afterwards, in the cell membrane, nitric oxide is reduced to nitrous oxide by the enzyme nitric oxide reductase (*Nor)*:
$2NO + 2c^{2+} + 2H^+ \rightarrow N_2O + H_2O + 2c^{3+}$

(iv) Finally, nitrous oxide is reduced to N_2 by the enzyme nitrous oxide reductase (*Nos)* which is located at the periplasmic space:
$N_2O + 2c^{2+} + 2H^+ \rightarrow N_2 + H_2O + 2c^{3+}$

Several environmental factors, such as oxygen tension, the presence of nitrogen oxides, pH, temperature, carbon/nitrogen (C/N) ratio and the type of electron source, affect denitrification. Among these factors, the type of electron source determines the denitrifying performance as this variable has an important effect on thermodynamic and kinetic. $\Delta G^{o\prime}$ values of denitrification with several electron sources have been compared [24]. It was concluded that in any case the processes are clearly exergonic and the magnitude of $\Delta G^{o\prime}$ is depending on the type of electron source. High $\Delta G^{o\prime}$ value is associated with the use of toluene as electron donor; thus, important biomass production might also be expected. Regarding to kinetic terms, differences in specific

consumption rate (q_s) of denitrifying process are expected to occur with distinct electron donors as q_s values depend on the concentration and type of substrate. In fact, each electron donor has a particular affinity constant (Ks) value. Therefore, when organic compounds with high Ks values are used, such as aromatic compounds, their consumption rates may represent the limiting step of denitrifying process.

2. Inhibitory and Toxic Effects of Toluene on Nitrification and Denitrification Processes

Taking into account the physicochemical properties of toluene, it is expected to observe some level of inhibition or toxic effects on the microbial processes. In fact, toluene may be a highly toxic compound and concentrations as low as 0.1% (v/v) would be sufficient to kill most microorganisms [77]. The inhibitory and toxic effects of toluene on the microbial metabolism could be observed at several levels, affecting microbial growth (anabolism), and/or the energy production processes (catabolism). The metabolism of microbial cultures can be stated by the following equation [24]:

$$q_s = \frac{\mu}{Y_{x/Scx}} + \frac{q_p}{Y_{p/Scp}}$$

Where q_s is the specific consumption rate of substrate; μ is the specific growth rate; q_p is the specific formation rate of product; $Y_{x/Scx}$ and $Y_{p/Scp}$ are the yields for biomass and products respect to the consumed substrate, respectively. The equation summarizes the metabolism of many cell cultures; where $\mu/Y_{X/Scx}$ expresses the biosynthetic process and $q_p/Y_{P/Scp}$ the respiratory process. Generally, in biological wastewater treatment, it is desirable to obtain dissimilative processes where μ will be very small for obtaining negligible biomass production and high substrate conversions (e.g. to molecular nitrogen, nitrate, and bicarbonate, depending on the biological process applied). In this sense, inhibitory effects of toluene could be related to a decrease of specific consumption or production rates. Obtaining predictions about the inhibitory effects on these parameters is highly recommended for practical purposes.

2.1. Inhibitory and Toxic Effects of Toluene on Nitrifying Sludge

There are numerous studies in the literature on the inhibitory effects of organic compounds on nitrification. Some works with axenic cultures of *Nitrosomonas* sp. or *Nitrobacter* sp. have been focused on the growth inhibition of bacteria [78, 79], others on the activity of the AMO [53]. There is also extensive information on the effects of different organic substances on the nitrifying activity of microbial consortia [57, 80, 81]. Nitrification has been studied in the presence of inhibitory aromatic compounds such as phenolic compounds (phenol, cresols, *p*-hydroxybenzaldehyde, 2-chlorophenol) [82-85]. However, less attention has been paid to the negative influence of BTX compounds on nitrifying microorganisms and further work is required for understanding how BTX compounds can affect the performance of nitrifying treatment systems.

The effects of toluene on nitrifying activity have been evaluated with axenic cultures, consortia, and soil microbial populations (Table 3).

Data found in the literature are very heterogeneous. The reasons for these differences may depend on differences in methodology (inoculum size, use of axenic cultures or consortia, floc or biofilm, time of incubations, among others) or physiological state of the microorganisms (origin and history of the inoculum) but also on the response variables used for evaluating the inhibitory effects. For instance, Fuller and Scow [90] defined the IC_{50} as the toluene concentration causing 50% of decrease in the specific rate of nitrite production by ammonia oxidation while Blum and Speece [89] determined the IC_{50} value as the toluene concentration causing 50% of decrease in the ammonium consumption efficiency. It is important to note that very few studies evaluated the complete nitrifying respiratory process (oxidation of ammonium to nitrate) using the necessary response variables as a whole: ammonium consumption efficiency, nitrite and nitrate production yields and specific rates of ammonium consumption and nitrate production. This information can be used for characterizing the ammonium and nitrite oxidizing processes in nitrifying sludge in the presence of toluene and for understanding better the possible inhibition mechanisms. On the other hand, difference between inhibition and toxic effect is not always clearly defined in the literature. In some studies, the decrease in the ammonium consumption efficiency is used as the criterion for inhibition or toxicity on the ammonium oxidizing process. However, various authors agree with the fact that inhibitory effects should be related to a decrease in specific rates of the microbial process. Toxicity is generally associated with the loss of cellular viability but there are few studies where

viable cells are counted. On the other hand, several investigators have carried out experiments designed to test the reversibility of toluene effects on nitrifying sludge activity and then associated the results with toxicity.

In spite of the discrepancy between the different studies reported in the literature, it can be seen in Table 3 that toluene generally caused a negative impact on nitrification, diminishing the ammonium consumption efficiency, the nitrate or nitrite production yields, or the volumetric or specific rates of nitrification processes. Various studies have been conducted with axenic cultures of *Nitrosomonas europaea.* Firstly, Keener and Arp [52] examined the inhibition of ammonia oxidation by *N. europaea* with various aromatic compounds. The inhibition was determined as a reduction in the NO_2^- production. They showed that a toluene concentration of 13.8 mg/l inhibited the activity nearly 80%. When cells were exposed for 5 h to toluene at 27.6 mg/l and then washed, ammonia oxidation was fully recoverable. In the study of Radniecki et al. [86], experiments were also performed to determine if inhibition was reversible. After three hours of *N. europaea* cells exposure to 3.7 mg/l of toluene, they were harvested, washed, and placed into fresh media. Toluene inhibition was found to be completely reversible. Thus, the authors mentioned that the inhibition observed during exposure to toluene was apparently nontoxic. Lauchnor et al. [88] reported that the addition of 8.9 mg/l of toluene resulted in 53% decrease in NH_3 oxidation rate by exposed *N. europaea* biofilms. Additionally, the recovery of NO_2^- production rate was monitored after toluene addition was terminated. Biofilms inhibited by toluene returned to 100% of previous NO_2^- production rates within 24 h of stopping toluene addition, indicating that toluene inhibition in biofilms was reversible, which is consistent with previously reported results with suspended cells [86]. In studies with *N. europaea* cells, results are in relation to the ammonium-oxidizing process and less information is available on the effects of toluene on the nitrite-oxidizing process.

Fuller and Scow [90] in their study on the effects of toluene on the activity of soil microbial populations, observed that the ammonia oxidation showed greater sensitivity to toluene than the nitrite oxidation. Assays designed to test the reversibility of effects on nitrification caused by incubation with toluene, indicated that ammonia and nitrite oxidation increased slightly but never returned to the levels observed in the control soil. According to their data of the most probable number of ammonia oxidizers, the slow recovery of ammonia oxidizing activity in soil cannot be attributed completely to reductions in population size. The authors proposed that toluene impact may also be the result of effects on membrane permeability or metabolism.

Table 3. Effect of toluene on microbial nitrification

Inoculum	Toluene concn. (mg/l)	Effects	Reference
Nitrosomonas europaea	0.9-27.6	Decrease in final NO_2^- production	[52] [86]
Nitrosomonas europaea	1.8	Decrease in NO_2^- production rate	[87]
Nitrosomonas europaea	1.3-5.9	Decrease in NO_2^- production rate IC_{50} = 3.4 mg/l	[88]
Nitrosomonas europaea	1.8-11	Decrease in NO_2^- production rate Biofilm: IC_{50} = 9.2 mg/l Suspended cells: IC_{50} = 1.8 mg/l	
Nitrifying enrichment cultures	-	AOA: IC_{50} = 84 mg/l	[89]
Nitrifying consortium	5.5-55	Decrease in ammonium consumption efficiency, q_{AOA} and q_{NOA}	[58]
Soil microbial populations	20-200	Decrease in q_{AOA} and q_{NOA} AOA: IC_{50} = 1 mg/l; K_i = 9.2 µg/L	[90]
Activated sludge	-	IC_{50} = 20 mg/l	[91]
Activated sludge	50-300	Decrease in ammonium consumption efficiency	[92]
Activated sludge	0.25-1.25	Decrease in ammonium consumption efficiency	[27]

AOA: Ammonia oxidation activity; NOA: Nitrite oxidation activity.
q_{AOA} and q_{NOA}: specific rates of AOA and NOA.
IC_{50}: toluene concentration causing 50% inhibition in rate, efficiency or nitrite and nitrate yield.

In the study of Zepeda et al. [58], the effect of different initial concentrations of toluene on a nitrifying consortium produced in steady-state nitrification was evaluated in batch reactors by means of ammonium consumption efficiency, nitrate yield and nitrification specific rates. The authors observed that at 10 mg C/l of toluene, there was no significant effect on nitrification efficiency and the ammonium removal efficiency was of

100%. Between 20 and 50 mg C/L of toluene, the nitrifying process was affected as the efficiency dropped to 20%. In all cases, the consumed NH_4^+-N was totally oxidized to NO_3^--N. The nitrifying yield was close to 1.0 and nitrite concentration was negligible, indicating that the process was mainly dissimilative. These results suggested that wastewaters containing up to 10 mg C/l of toluene would not affect the efficiency of ammonium conversion to nitrate in a treatment system. However, toluene (5-20 mg C/l) induced a significant decrease in the values for specific rates of NH_4^+ consumption (77-96%) and NO_3^- production (46-92%), affecting mainly the ammonium-oxidizing pathway (Table 4). These results indicated that, in the presence of toluene, the nitrification process was inhibited but the nitrifying metabolic pathway was only altered at the specific rate level as nitrate was still the main end product. The same authors evaluated the toxic influence of the aromatic compound on the sludge previously exposed to toluene for 16 h. They reported that the sludge exposed to 10 mg C/l of toluene recovered completely its nitrifying activity, showing high values for efficiency and nitrate yield. However, from 20 to 50 mg C/l of toluene, there was no recovery of the nitrifying activity as the ammonium consumption efficiency was null suggesting a toxic effect.

Table 4. Nitrification specific rates in the nitrifying cultures in the absence and presence of toluene (modified from Zepeda et al. [58])

Initial toluene concn (mg C/l)	Specific rates (g N/g microbial protein-N.h)	
	NH_4^+-N consumption	NO_3^--N production
0	1.389 ± 0.079	0.577 ± 0.030
5	0.317 ± 0.012 (− 77%)[a]	0.310 ± 0.007 (− 46%)
10	0.225 ± 0.006 (− 84%)	0.221 ± 0.004 (− 62%)
20	0.054 ± 0.004 (− 96%)	0.047 ± 0.007 (− 92%)

[a]Percentages of decrease for nitrification specific rates were calculated by using the values obtained in the control culture as references.

Various hypotheses have been proposed to explain the negative effects of organic matter on nitrification. According to Radniecki et al. [86], aromatic compounds as toluene can potentially inhibit nitrification activity in multiple ways; including acting as a cometabolic energy drain, forming cytotoxic daughter products, or disrupting vital cell processes via solvent effects on the biological membranes [52, 93]. In axenic cultures of *N. europaea*, it has been observed that the AMO had a broad substrate range for catalytic oxidation and

the inhibitory effects of many organic substances, including aromatic compounds, would be due to competition for the active site [50-52, 94, 95]. Keener and Arp [52] reported that *N. europaea* cells were able to oxidize toluene to benzyl alcohol and benzaldehyde. These transformations were initiated only by cells with active AMO and they were prevented by treatment of the cells with C_2H_2 to inactivate AMO. According to these results, the decrease in nitrification activity is believed to be caused by competition between toluene and ammonia for the active site on AMO, the enzyme responsible for ammonia oxidation. Radniecki et al. [86] also showed that the inhibition was directed at the AMO enzyme as no inhibition was noticed on the hydroxylamine oxidoreductase enzyme. It was also found that benzyl alcohol and benzaldehyde, the products of toluene oxidation by *N. europaea* cells, did not cause inhibition on ammonia oxidation [52, 86, 88]. As regards the effects of solvents as toluene on membrane, the information in the literature is wide. Many cyclic hydrocarbons are toxic and/or inhibitory to microorganisms because of their hydrophobic character and their disturbing effects on biological membranes [43, 96]. The toxicity of aromatic compounds generally correlates with their hydrophobicity, which is described by their partition coefficients in a mixture of *n*-octanol and water. Compounds with a log K_{ow} between 1 and 4, such as toluene (log K_{ow} = 2.69), may be cytotoxic due to their solubility in biological membranes [93]. Toluene exposure has been shown to increase cell membrane permeability and alter metabolic functions [43]. Exposure to 1% toluene has been shown to remove considerable amounts of protein, phospholipid, and polysaccharides from cells, particularly from the cytoplasmic membrane [97]. Bacteria respond to aromatic compounds by either metabolizing the compound, removing the aromatic hydrocarbon from the cell via efflux pumps or rigidifying the outer-membrane by increasing the membrane's protein content and/or shifting the cis/trans ratio of their ester-linked fatty acids [29, 98]. However, in the case of *N. europaea*, Radniecki et al. [87] did not observe a strong correlation between log K_{ow} and $IC_{50.}$ The investigators suggested that other factors besides membrane disruption are involved, such as the inhibition of AMO or the interaction of the aromatic hydrocarbons with other cellular constituents. Aromatic hydrocarbons have been shown to alter bacterial outer-membranes and genes associated with lipid biosynthesis, membrane proteins, and fatty acid metabolism were found to be up-regulated in response to benzene inhibition [86]. These authors investigated the transcriptomic responses of *N. europaea* during the cometabolism of toluene to benzyl alcohol and benzaldehyde. They observed that there were no significant up- or down-

regulation of genes in *N. europaea* cells exposed to 1.8 mg/l of toluene, which caused 50% inhibition of ammonia oxidation rate. However, exposing *N. europaea* to 3.1 mg/l of benzene, which caused a similar degree of inhibition, resulted in the up-regulation of seven genes, including genes as NE 1545 and NE 1546, that appear to be involved with fatty-acid metabolism, lipid biosynthesis, and membrane protein synthesis. Additionally, transmission electron microscopy images revealed a shift in outer membrane structure and thickness in *N. europaea* cells exposed to benzene but no changes in the membrane were observed in cells exposed to toluene. Studies have also shown decreases in cell size of bacteria exposed to various aromatic hydrocarbons [99, 100]. However, Radniecki et al. [87] showed that aromatic hydrocarbons that contain no polar group substitutions such as toluene caused only a 5% decrease in cell volume of *N. europaea*.

In microbial consortia, the competition between heterotrophs and autotrophs for ammonia and oxygen is another hypothesis commonly mentioned for explaining the nitrification inhibition by organic matter [101]. Zepeda et al. [58] also observed that the inhibitory effects of toluene on nitrification seemed to be related to its persistence in the nitrifying cultures. At low concentrations, the chemical structure of the BTX compounds appeared to be the predominant factor whereas at higher concentrations, their hydrophobicity played an important role. The presence of different functional groups as well as their nature can influence the metabolism and toxicity of aromatic compounds [102]. The absence of functional groups may confer to benzene higher stability and persistence to biotransformation while the presence of methyl groups may facilitate the mechanisms of biotransformation for toluene and *m*-xylene. According to the results of Zepeda et al. [103], the inhibitory effect of BTX compounds on the nitrifying process seemed to be higher when they were present in mixtures than individually. As observed by Zepeda et al. [58, 103] in the case of BTX compounds, in spite of the inhibitory effects of organic compounds on nitrification, in some cases and under controlled experimental conditions, nitrification processes could successfully proceed [104, 105]. It is clear that further research is needed to identify and understand better how toluene affects nitrification processes, including studies with axenic cultures and nitrifying consortia, in batch cultures as in biological reactors. Such information will contribute to a better control of the nitrifying activity of microbial consortia used in wastewater treatment.

2.2. Inhibitory and Toxic Effects of Toluene on Denitrifying Sludge

For kinetically purposes and considering that denitrification is a respiratory process where reduction of nitrate is simultaneously linked to electron donor oxidation, the specific substrate consumption rate (q_S) can be expressed for nitrate or electron donor. Likewise, specific production rate can be described in terms of N_2 or HCO_3^- generation. Low microbial growth rates are desired to obtain, as a result of dissimilative process during biological denitrification. Therefore, as it has been previously pointed out, the inhibitory effect of toluene on denitrification process will be discussed either in terms of decrease in substrate consumption rate (toluene q_T, or nitrate consumption, q_{NO3}) or in production rate (N_2 or HCO_3^- generation). Nevertheless, many research studies have been only focused in the substrate consumption efficiency values.

There are studies in the literature indicating the feasibility in using toluene as carbon source for sustaining growth of denitrifying bacteria [106] and anoxygenic photothrops as *Blastochloris sulfoviridis* [107]. In fact, toluene is considered as the easiest of the BTX compounds for being assimilated as a carbon source or oxidized as electron donor. Attempts for toluene removal under anoxic conditions using different electron acceptors such as nitrate [4, 60, 61], sulfate [108] and CO_2 [109] have been conducted. Thermodynamic data for several respiratory processes with toluene as electron source are illustrated in Table 5. The denitrification process is exergonic and appears to be thermodynamically favored when compared with anaerobic processes such as methanogenesis or sulfate reduction.

Some kinetic studies based on pure cultures of the denitrifying strain T1 have shown maximum growth rates (μ_{max}) values ranging from 0.10 – 0.13 h^{-1} [110] and 0.14 h^{-1} [111] when this microorganism was growing at 92 mg/l of toluene. A similar μ_{max} value (0.10 h^{-1}) was reported by Jørgensen et al. [112] when using a mixed culture coming from a sewage treatment plant which was grown at 10.8 mg toluene/l. Likewise, Kim et al. [113] reported a μ_{max} of 0.283 h^{-1} in a sand-column experiments inoculated with *Thauera aromatica* T1 at 13 mg toluene/l. It is important to note that all these studies were conducted at low toluene concentrations, suggesting that a minimal growth inhibition was observed. Studies on the denitrifying "*Aromatoleum aromaticum"* strain EbN1 have shown its metabolic ability to remove toluene with high efficiency in aquifer microcosms [114]. The inhibitory effect of toluene on growth rate has been assessed on this strain by Trautwein et al. [115]. The authors reported a

growth rate of 0.012 h^{-1} at a toluene concentration of 6.4 mg/l. Growth rate was significantly reduced as toluene concentration was increased, indicating a semi-inhibitory concentration (about 50% growth inhibition) at 68.1 mg/l of toluene, where growth rate was 0.003 h^{-1}. Microbial growth was completely inhibited at 79.1 mg/l of toluene. Cultures of strain EbN1 adapted to anaerobic growth with succinate achieved growth rates of 0.053 h^{-1}. The specific growth rate decreased to 0.014 h^{-1} when these cultures were suddenly exposed to 110.4 mg/l of toluene, whereas complete growth inhibition was observed upon shock with 276 mg/l toluene. According to their results, Trautwein et al. [115] have indicated that strain EbN1 is highly solvent tolerant. The authors proposed that considering that petroleum-contaminated groundwater may contain toluene at a concentration of 1 mg/l [116]; this strain should be able to survive and proliferate in these contaminated environments and could be effectively used for biological treatment.

Table 5. ΔGº' values of several respiratory processes with toluene as electron source

Respiratory process	Equation	ΔGº' (kJ/reaction)
Denitrification	$C_7H_8 + 7.2NO_3^- + 0.2H^+ \rightarrow 3.6N_2 + 7HCO_3^- + 0.6H_2O$	-3524
Aerobic respiration	$C_7H_8 + 9\ O_2 \rightarrow 7\ CO_2 + 4\ H_2O$	-3831
Methanogenesis	$C_7H_8 + 7.5\ H_2O \rightarrow 4.5\ CH_4 + 2.5\ HCO_3^- + 2.5\ H^+$	- 131
Sulfate reduction	$C_7H_8 + 4.5\ SO_4^{2-} + 3H_2O \rightarrow 7\ HCO_3^- + 2.5\ H^+ + 4.5\ HS^-$	- 205
Hem reduction	$C_7H_8 + 94\ Fe(OH)_3 \rightarrow 7\ FeCO_3 + 29\ Fe_3O_4 + 145\ H_2O$	- 3398

There is some information on the effects of toluene on the denitrifying respiratory process of microbial consortia. Specific toluene consumption rate values reported in literature are generally low indicating the inhibitory effect of toluene on denitrification (Table 6). However, it is difficult to make comparisons among these data considering that operational and environmental conditions, as well as biomass concentration used are different.

Table 6. Some specific consumption rates of toluene at different denitrifying culture conditions

Initial concentration (mg toluene/l)	Inoculum	Toluene consumption rate	Reference
92	Pure culture Strain T1	0.32 mg toluene/mg protein h	[110]
92	Pure culture Strain T1	30 µmol NO_3-N/mg protein h	[111]
1.5	Mixed culture	78 µg toluene/l d	[109]
16	Enriched mixed culture	0.28 g toluene/g cells d	[117]
11	Mixed culture exposed to alkylbenzenes	0.71 mg toluene/mg protein h	[112]
55	*Azoarcus tolulyticus*	0.89 mg toluene/mg biomass d	[118]
17-93	Denitrifying consortium	0.005 - 0.025 mg toluene-C /mg VSS d	[60]
50	Enriched mixed culture	1.67 mg/l d	[62]
20	Denitrifying consortium	0.007 mg toluene-C /mg VSS d	[65]

The inhibitory effect of toluene has been evidenced in batch assays with a denitrifying consortium fed with similar concentrations of acetate or toluene (as mg C/l) as electron source by Peña-Calva et al. [60]. The authors reported an acetate consumption rate (q_A) of 0.36 ± 0.01 mg acetate-C/mg VSS d and a nitrate consumption rate (q_{NO3}) of 0.29 ± 0.01 mg nitrate-N/mg VSS d. These authors determined that the specific toluene consumption rate was 17 times lower than the acetate consumption rate. Similarly, the nitrate consumption rate in the presence of toluene was 16 times lower than that obtained when acetate was the electron donor. As a consequence, the metabolic mineralization of toluene diminished resulting in lower specific production rates of HCO_3^- and N_2. Results obtained by the same authors indicated that specific consumption rates of toluene (q_T, mg of toluene-C/mg of VSS d) increased to 4.5 times as the initial toluene concentration increased from 15 to 70 mg C/l (Figure 1). A slight inhibitory effect was observed at a toluene concentration of 85 mg/L, as q_T decreased by 2.5%. However, a significant inhibition was determined at 100 mg of toluene-C/l as q_T was reduced in 21%. A similar behavior in nitrate consumption rate was observed. Inhibition of

denitrification has been also reported by Elmen et al. [118] in axenic cultures of *Azoarcus tolulyticus*, where specific toluene consumption rate decreased in 25% at a toluene concentration of 102 mg/l. These results suggested that no substrate inhibition would be detected in wastewaters containing less than 85 mg C/l of toluene, as the removal rate of the treatment system will not be affected. Nevertheless, generalizations about this situation must be avoided as previous physiological state of sludge, biomass concentration and the rest of culture conditions might affect kinetic data and performance of the respiratory process.

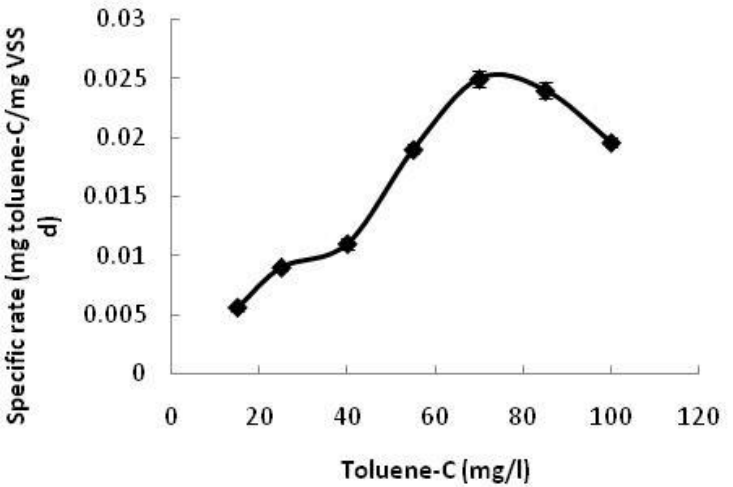

Figure 1. Specific toluene consumption rates at different initial toluene concentrations in a denitrifying process in batch culture (modified from Peña-Calva et al. [60]).

Toluene toxicity has been generally correlated with its hydrophobicity as toluene is preferentially dissolved in biological membranes [93]. Consequently, membrane fluidity increases, which leads to a loss of ions, ATP and other cellular metabolites. This damage might be followed by cell lysis [29]. Likewise, dissipation of the proton motive force and denaturation of membrane proteins result in energetic and metabolic problems in solvent-exposed cells [43]. In this regard, Trautwein et al. [115] have reported changes in abundances of denitrifying enzymes (nitrite reductase and nitrous oxide reductase) in the strain EbN1 exposed to a semi-inhibitory concentration of toluene. Therefore, the decrease in both growth rate and specific rate of toluene consumption was probably owing to the denaturing effect of toluene on the cytoplasmic membrane.

Table 7. Kinetic data of toluene consumption in single assays and in BTX mixtures under denitrifying conditions (modified from Texier et al. [48])

Initial toluene concentration in single and in mixture with BTX or acetate (mg/l)	Inoculum	Toluene removal rate	Reference
44 mg toluene/l	Denitrifying consortium	0.011 mg toluene-C /mg VSS d	[60]
90 BTX/43 toluene	Denitrifying consortium	0.021 mg toluene-C/mg SSV d	[119]
50 mg toluene/l	Enriched mixed culture	1.67 mg/ld	[62]
300 BTX/50 toluene	Enriched mixed culture	1.29 mg toluene/ld	[62]
22 mg toluene/l	Denitrifying consortium	0.007 mg toluene-C /mg VSS d	[65]
65 acetate/22 toluene	Denitrifying consortium	0.014 mg toluene-C /mg SSV d	[65]

The inhibitory effect of toluene on denitrification might be diminished or potentiated by the presence of other aromatic compounds such as benzene and the isomers of xylenes. Several assays with mixtures of BTX have indicated substrate interactions among these compounds. Differences in toluene consumption rate values have been observed when mixtures of BTX were assayed and compared with the values obtained in assays with the single toluene compound (Table 7). A diminishment of the inhibitory effect of toluene was observed, as enhancement in toluene consumption rate between two times was determined in mixtures of toluene, benzene and *m*-xylene [119], suggesting a positive interaction in hydrocarbons consumption when they are in mixture. In contrast, Dou et al. [62] reported a higher inhibitory effect when mixtures of BTX containing toluene concentrations higher than 5 mg/l were assayed; diminishing the toluene consumption rate when compared with the q_T obtained in single toluene tests. Assays with toluene added with an easily consumable substrate such as acetate have been explored as another possibility for diminishing the inhibitory effect of toluene on microbial consortia with no previous contact to BTX [65]. The authors reported a significant decrease in toluene inhibition at the acetate/toluene ratio of 65/22 (mg/l) where the specific consumption rate of toluene was twice compared to that obtained at 22 mg toluene/l alone (Table 7). These results indicated that toluene inhibition could be diminished when the acetate concentration is clearly higher than

toluene. All these results indicate that the use of some BTX mixtures or addition of easily consumable substrates might be helpful for diminishing the inhibitory effects of toluene on bacterial denitrifying metabolism.

3. Effects of Toluene on Structure and Settleability of Microbial Consortia

Most of the microorganisms involved in biological wastewater treatment are forming microbial aggregates such as sludge flocs, sludge granules and biofilms. These microbial aggregates are keeping together in a three-dimensional gel matrix mainly composed by extracellular polymeric substances (EPS) [34, 36]. EPS are a complex high-molecular-weight mixture of polymers secreted by microorganisms or produced by cellular lysis and hydrolysis of macromolecules which are present both outside of cells and in the interior of microbial aggregates. EPS have a significant influence on physicochemical properties of microbial aggregates such as structure, stability and settling properties, as EPS bind with cells through different interactions as London forces, electrostatic interactions or hydrogen bonds [120], in order to form a structure which maintains and accelerates the formation of microbial aggregates [35, 121]. The content and composition of EPS extracted from different microbial aggregates are reported to be heterogeneous [122]. Carbohydrates and proteins are usually found to be the major components of EPS; however, humic substances, lipids, nucleic acids and some inorganic components have also been found in sludge in biological wastewater treatment systems [123, 124]. In spite of exopolymeric carbohydrates and proteins have been respectively considered to be the backbone in the sludge structure and provide a certain level of protection against diffusion process of toxic compounds to cytoplasm (e.g. enzymes and biocides) and prevent washing out of exoenzymes [125], many studies have shown that a high concentration of EPS results in a decrease in the settleability of microbial aggregates [126]. Particularly, high protein contents in EPS would have more significant effects on the sludge settleability. The sludge volume index (SVI) is generally used to characterize the sludge settleability [127, 128]. SVI values lower than 100 ml/g indicates good settleability, whereas sludge settleability and structural stability of sludge decrease as SVI increases. In fact, bad settlement abilities of sludge have been related to SVI values higher than 150 ml/g and high exopolymeric protein content [41, 42, 129].

The type of electron donor or substrate has a substantial effect on the metabolism of microbial communities involved in wastewater treatment. Therefore, production of EPS would also be influenced by these parameters. In fact, it has been reported that sludge fed with glucose had more EPS production than that fed with acetate [41, 130]. Other reports suggest that microorganisms would excrete more EPS under unfavorable conditions or when they are in presence of toxic or biocide compounds. Furthermore, under toxic conditions, the increase of the protein content far exceeded that of other components in EPS [34]. This has also been reported by Hernández et al. [131] and Beristain-Montiel et al. [129] with anaerobic sludge exposed to toluene and 2-chlorophenol, respectively. Thus, the presence of aromatic compounds such as toluene in wastewater could enhance the EPS production and consequently, result in operational troubles during the biological wastewater treatment.

Sequencing batch reactors (SBR) have been successfully applied for treating effluents containing toxic or recalcitrant substances under both aerated and anoxic conditions [104, 129]. Nevertheless, changes in sludge settleability during SBR operation may become a serious trouble. The fact that toluene might provoke damage in cellular structure, the denaturation of proteins and lipids and the lost of ions or metabolites [43, 44] could also affect the stability of microbial aggregates and finally their settleability. Important biomass production has been reported in aerobic systems treating toluene [132, 133]. Likewise, it has been reported that sludge accumulation along cycles in SBR systems may also result in operational malfunction [134]. Nevertheless, information about the effect of toluene on sludge settleability is still scarce. Hernández et al. [131] have evaluated in a denitrifying SBR fed with 70 mg toluene-C/l or 70 mg acetate-C/l, the behavior of sludge settleability and the effect of volatile suspended solids (VSS) content on the sludge settlement abilities. The authors reported an efficient process as toluene or acetate were completely eliminated and mineralized to CO_2 whereas nitrate was reduced to N_2. When the SBR was fed with acetate, the exopolymeric protein (EP) content was 26.5 ± 10.3 mg/l while exopolymeric carbohydrate (EC) was 6.3 ± 2 mg/l. When toluene was fed in the SBR, EP content was 1.75 times higher than the value determined with acetate. Similarly, EC content with toluene was 3.4 times higher than the EC content obtained with acetate. Therefore, the change in electron source from acetate to toluene resulted in an increase in the exopolymeric content of the sludge. Nevertheless, in both cases, the sludge presented good settlement abilities as SVI values remained between 14 ± 3 and 40 ± 3 ml/g. When the VSS concentration was increased from 2 to 4 and 8 g

VSS/l (at a constant concentration of 70 mg toluene-C/l), the EP and EC contents were twofold higher than that observed with 2 g VSS/l. In fact, in the assay with 8 g VSS/l, the EP content was very close to 100 mg EP/l. Therefore, the increase in biomass concentration had an important effect on the production of exopolymeric substances, particularly in EP. Concomitant to the increase in EP, a diminishing in sludge settleability was observed. Similar results have been reported by Cuervo-López et al. [41] where sludge settlement ability decreased when EP concentration was higher than 100 mg/l. These results suggest that concentrations up to 70 mg toluene-C/l could be effectively eliminated in a SBR system without influencing sludge settleability; however, sludge concentration should be maintained between 2 and 4 g VSS/l in order to avoid operational troubles. Nevertheless, it would be of practical application to have more information about sludge stability at higher concentrations of toluene as the diminishing in sludge settlement ability was accompanied with an increase in the EP content of the sludge. Considering the importance of adequate wastewater treatment operation based on the sludge settlement properties, further research on this topic is needed.

CONCLUSION

Toluene is often present in sewage and its impact on the performance of the biological treatment processes, such as nitrification and denitrification, should be more investigated. The information presented in this chapter indicates the potential for toluene to adversely influence the biological nitrogen cycle by affecting both nitrifying and denitrifying processes. Nitrification, as a lithoautotrophic process, appeared to be highly sensitive to toluene, whereas the denitrification process seemed to be an option for using toluene as energy and carbon source. The main effect of toluene on both processes is at a kinetic level, decreasing significantly the metabolic rates. However, in both cases, under controlled experimental conditions and low toluene concentrations, microbial consortia can be able to maintain their nitrifying and denitrifying activity as well as their settlement ability, and be used in wastewater treatment. Some strategies for diminishing the inhibitory impact of toluene on the microbial consortia activity such as the use of BTX mixtures or the addition of easily consumable substrates have been proposed. However, further investigation should be conducted, taking into account physiological, microbiological, kinetic, sludge settleability and engineering aspects, among others.

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Chapter 4

INFLUENCE OF TOLUENE ON POSTNATAL NEUROGENESIS OF LIMBIC AND MOTOR SYSTEMS, METABOLISM AND BEHAVIOR OF ANIMALS AND CORRECTION OF DISTURBANCE BY ANTIOXIDANTS

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ABSTRACT

Alterations of the neuron quantity in the cortical and subcortical structures of the limbic and motor systems, as well as proliferative activity of granular cells in fascia dentate of hippocampus and cerebellum in albino rats at the early stages of postnatal development (P3-P21) were determined after exposure to toluene (500ppm, 1200ppm). Investigations have shown that toluene induced death of neurons in the above structures of the central nervous system (CNS) and inhibition of granular cells' proliferation and migration. Effects of toluene on patterns of migration of glial cells in the culture condition at early stages of cultivation has shown that intensity of glial cells' migration and axons growth are decreased.

Free radicals (lipoperoxide LOO•, NO) content in toluene intoxicated rats cerebral cortex were determined at P15, P30, P60 stages of development using the Electron Paramagnetic Resonance (EPR) method. The obtained data indicate the intensification of generation of oxidative stress, which promotes enhanced expression of iNOS and intensification NO synthesis in rat's cerebral cortex.

Investigations of correction of toluene-induced changes by antioxidants has shown, that Miradol decrease the number of perished neurons in the cortex and subcortex of the motor systems and impair the toluene effect on the cerebellar granule cells proliferative activity and migration properties of the glial cells and axons growth in vitro.

The peculiarities of spatial translocation and alterations of learning and memory processes have been investigated in multiway elevated maze and by the passive avoidance (PA) tests. The toluene intoxication significantly decreased manifestation of the aurioculonasocephalic (ANC) reflex and ability of finding mother's location at P7, P13. Decrease of platform finding velocity in the water corridor was found also at P15, P21. Toluene intoxication induced the decrease of exploratory activity and motivation level, as well as the increase of emotional background, as compared to the control animals, and finally significant alterations of spatial learning and memory processes at P30 in multi-way elevated maze. Learning process was deteriorated as well in the passive avoidance (PA) test on P30. Experimental animals showed toluene intoxication-induced decrease of the memory trace consolidation, as compared to the control animals.

INTRODUCTION

Effect of different toxic substances (exhaust gases, toxins formed after processing of petroleum products) on living organisms due to contamination of the environment is one of the most important environmental problems. Among the xenobiotics exerting the damaging effects it toluene, being one of the major products of petroleum refining and being included in the toxicological screening system of the environment, should be noted. Drug addicts often use organic solvents, such as a hallucinogenic substance by with of inhalation to achieve euphoric state through which it has a significant effect on the nervous system. The deliberate inhalation is a problem for public health worldwide.

The main component of solvents is toluene, known as a neurotoxic agent. Toluene is commonly characterized as a lipophilic substance that affects biological membranes, changing the stability of proteins, lipids, and chromatin [1, 2]. Violation of lipid-protein interactions is assumed as the mechanism of inhibition [3]. In the offspring of pregnant women consuming toluene fetal toluene syndrome FSS (Fetal Solvent Syndrome) was detected, causing delay of fetal growth, reduction of the size and weight of the brain, decrease the number of nerve cells and the amount of DNA in their nuclei, death of the developing cells by apoptosis [4, 5, 6, 7].

Consumption of toluene was most frequently observed among children and adolescents [8, 9] leading to disruption of the structure and function of the CNS [10]. Low cost and easy availability of organ solvents leads to an increase in the number of young drug addicts in many countries. In the early period of postnatal development neural structures are characterized by a special sensitivity to external toxic factors [11]. Among chronic users of toluene one can observed disturbance of the processes of learning and memory, as well as various neurological disorders, autism, cerebral palsy etc. [12, 13, 14]. Several authors have shown that toluene affects the processes of proliferation, disturbs formation of the brain structures and further on leads to a change in the future of behavioral parameters and memory deficit [6, 15]. Studies with computer tomography and magnetic resonance imaging have demonstrated atrophy of certain areas of the brain [16, 17, 18], as well as violations in the white matter [19]. After chronic intoxication with toluene reduction of pyramidal neurons in the CA2-CA3 fields of the hippocampus was associated with induction of free radicals [20, 21].

The CNS in the early stages of development, in contrast to adults is more sensitive to the effects of damaging factors since in the development process are particularly sensitive periods for the brain. There is no possibility of

recovery of violations in this period, which consequently causes further dysfunction of the brain [22, 23]. In the early period of development following exposure to toluene of newborn rats a decrease in the volume of hilus and granule cell layer in the dentate gyrus is revealed [20, 24]. A well-pronounced degeneration of granule cells and an increase in argyrophylic cells in the granule layer are observed [25]. Prenatal toluene intoxication in the sensorimotor cortex of rats in the early stages of postnatal development can affect the proliferation and migration of precursor cells [26, 27]. As a result of destructive processes in the motor system, there is atrophy of neurons [18], as well as reduction the number of neurons in the olfactory bulb at pre-and postnatal toxicity [28, 29]. Cytotoxic effect of toluene leads to the degeneration of neurons, their death by necrosis or apoptosis, and structural changes in primary cultures of astrocytes and neurons [30, 4].

Proceeding from the foregoing, special interest acquires to attempt to ameliorate the destructive processes caused by toluene with antioxidants possessing neuroprotective properties. In recent years pharmacologic agents and plant antioxidants are widely used [31-35].

Thus, analysis of the data reported in the literature concerning the morphological and functional abnormalities observed in the CNS following toluene intoxication showed that the above problems require consideration of the issues that underlie the formation of substance abuse. In this regard, the use of preventive agents will help ameliorate the pathological processes, caused by toluene.

The aim of this study is to identify violations of neurogenesis as a result of destructive processes caused by the toluene inhalation in rats and their possible correction of with an antioxidant.

The present study addresses the following questions: the structural, metabolic and functional abnormalities in the cortical and subcortical structures of the limbic and motor systems in the early stages of postnatal development of white rats after toluene intoxication. In particular, investigated were the changes in the number of nerve cells in the aforementioned structures, the proliferative activity of the dentate gyrus granule cells in the hippocampus and cerebellum, the processes of migration and the intensity of glioblasts migration and growth of axons in cultures of the cerebral cortex, the intensity of the dynamics of free radicals in the cerebral cortex and changes in learning and memory processes of animals being under the influence of toluene within 21 days of postnatal development.

Experiments were carried out on mongrel albino rats within early stages of their postnatal development (P3-P21). Group I - the intact (control) animals ,

group II - experimental animals which inhaled 1200 ppm toluene, group III - experimental animals were subjected to 500 ppm, IV - experimental animals of III groups received Miradol with maternal milk (female rats during lactation were received Miradol 1.4g daily).

Toluene was inhaled by the rats at 500ppm and 1200 ppm, for 10-12 min per day, within 21 days (5 days per week). Total numbers of nerve cells in the cortical and subcortical structures of limbic system were counted at 3, 7, 15, 21 days of postnatal development (P3, P7, P15, P21).

In order to eliminate the effect of hypoxia, the animals were placed in desiccator without toluene vapors for 10-12 min. It was shown that all parameters (growth rate, weight, time insight, the rhythm of breathing, motor activity, etc.) did not differed from respective values in control group.

To collect the material the animals were sacrificed under ether anesthesia in accordance with the Ethical Guidelines for Investigators of Experimental Animals.

The following methods were used:

1. To determine number of the neurons in the cortical and subcortical structures of the limbic (cingulate gyrus, entorhynal cortex, hippocampus, supraoptic, paraventricular and ventro-medial nuclei of hypothalamus,lateral and medial nuclei of septum) and motor (sensorimotor cortex, ventrolateral thalamic nucleus, nucleus caudatus, globus pallidus,) systems, as well as in cerebellar on the early stadies of postnatal development (P3, P7, P15, P21). Following brain fixation with Carnoy's fluid, paraffin sections (7μm) were stained with cresyl-violet. The number of neurons in the cortical and subcortical limbic and motor systems by the light microscope (Amplival Zeiss, Germany) was determined with an aid of the eyepiece micrometer grid, at magnification of 10x40 on the surface area amounting 0.025 mm^2.

   A stereotaxic atlas of rat brain coordinate [36] was used to identify localization of the limbic and motor systems structures.
2. In order to study a proliferation activity, the mitotic index of the granule cells in the hippocampal dentate fascia and in cerebellum, at P3, P7, P15 was assessed on the cresyl-violet-stained and 7μm thick slices, with the light microscope (Amplival, Zeiss, Germany), with an aid of the eyepiece micrometer grid, at magnification of 10x40.
3. With a view of revealing the effect of toluene and Miradol on migrating ability of glial cells and intensity of axonal growth in the

cortical explants from 1-2 day old newborn rats we used cultivation in Maximov's chamber on collagen in the nutrition medium, composed of modified medium of Eagle (DMEM, Sigma), Bovine serum F-1051 (Sigma). The cultures were explored at every 24h, 48h and 72h in the phase and interference contrast by Nomarsky. After fixation in Carnoy's fluid cultures were stained with cresyl-violet. Three series cultures was observed: I - the cultures with intact nutrition medium; II – nutrient medium contained toluene (100mM); III - nutrient medium containing toluene(100mM) and Miradol (10^{-5} M) with a view of revealing the preventive effect of Miradol on migrating ability of glial cells.

4. Free radicals in the cerebral cortex and liver were determined in rats at P15, P30 and P60 (after toluene intoxication during P3-P21) using the method of Electron Paramagnetic Resonance (EPR). For the detection of lipoperoxid free radical (LOO·) and free nitric oxide (NO) EPR signal the spin traps, PBN (α-phenyl-N-tert-butil-nitron) (Sigma) and DETS (Sodium Diethil-ditiocarbamate) (Sigma) were used. Brain and liver tissues were placed in a plastic tubes (with thickness 20-25mm, length 150 mm) and then contained in liquid nitrogen. Registration of the EPR spectra was performed on the radiospectrometer RE-1307 (Russia) at a liquid nitrogen temperature (-196°C), using the quartz Dewar (Wilmad).
5. Peculiarities of spatial behavior at early stages of postnatal development (P3, P7, P15, P21), as well as disorders in learning processes and memory in P30 rats after intoxication with 1200ppm toluene have been investigated. Following experiments were carried out:
    1) Peculiarities of spatial ambulation: a) "dummy mother" test – prior to opening ayes in P7 and P13 rats. Animals were removed from the nest and began to move toward a warm container, covered with cloth smelling of mother rat. The distance to the dummy for P7 and P13 animals corresponded to 8 and 12 cm. A pattern of movement and the time necessary to reach the dummy have been investigated. b)"swimming test"- after opening the ayes in P15 and P21 rats. We studied peculiarities of swimming in a water corridor (60×10 cm for P15 and 100×10 cm for P21) with a visible platform at the end (five tests per day). The time necessary to reach the platform was measured.

2) A capacity for consolidation of the memory traces after the toluene intoxication have been investigated by passive avoidance (PA) test, using standard chamber with two compartments. The floor in the dark compartment was electrified, and the animal entering this compartment received pain stimulation of the limbs (1.5 mA during 2 sec). The level of preservation of PA reaction was checked 24 and 48 h later. If the animals after re-testing 24 h later did not enter the dark section during 5 min. we believed that the PA reaction was preserved. The number of animals of groups I and II that successfully performed the task was taken into account; we also measured the latency time before of the first entry into the dark compartment.
3) Spatial learning and formation of memory were estimated in the elevated-type multi-way maze [37]. The maze included 10 platforms (40×10 cm) fixed at a height of 25 cm. The motivation for movement along the maze under test conditions was the animal's wish to escape ethologically negative conditions by passing into a box-nest fixed at the end of the maze. Experience in the use of such maze test allowed us to conclude that the corresponding experimental conditions create a sufficient level of motivation for training even without food reward. Experiments were carried out during 7 days (five trials each day); at the beginning of the experiment, the animal was placed on the starting pad; the rat began to move, entered different parts of the maze, and opened up, by trial-and-error, the optimum trajectory of the movement to the nest-box. On the first day, the experimenter helped the animal (by gentle shakings) to find the optimum trajectory. The studied parameters were recorded from the second testing day. We calculated the number of errors (deviations from the optimum trajectory) and measured the total time of crossing the maze. Analysis of the obtained numerical data allowed us to estimate the dynamics and result of the learning process. The error-free passing of the labyrinth during 10-15 sec and the achievement of automatism in behavior were considered a criterion of completion of the learning process.

Experiments were carried out on two groups of rats, I – control group (intact animals); II – experimental group (animals subjected daily to toluene intoxication during P3-P21). Each group included at least 9 animals.

The obtained numerical data were processed statistically using the Fisher−Student *t*-test.

The determination of the number of the neurons in the cortical and subcortical structures of the limbic and motor systems on early stages of postnatal development (P3, P7, P15, P21) after intoxication by toluene (500ppm and 1200ppm) has shown that toluene induces death of the neurons in the above structures of the CNS. After toluene exposure the pyramidal neurons of entorhinal cortex of limbic cortex was more sensitive, than the neurons in the cingulate gyrus. The Number of the perished pyramidal neurons in the entorhinal cortex ranged within 32-38%, while in the cingulate gyrus this number corresponded to 20-30% (Table 1). These results are consistent with the data, according to which toluene particularly damages development and arborization of the pyramidal neurons dendrites and causes a significant damage to the cytoarchitectonic of the entorhinal cortex [38, 15].

In subcortical structures, at all stages of development, the reduction number of neurons was also identified. Particularly pronounced reduction of neurons was found at P15 and P21 among the pyramidal neurons of the Ammony horn by 42% and 52% and as well as in the ventromedial and supraoptic nuclei of the hypothalamus at P15 and P7 respectively. Reduction in number of neurons in other subcortical nuclei was also observed, however, to a lesser extent (Table 1).

Inhalation of 500ppm toluene, as well as in the limbic cortex revealed a decrease in the number of pyramidal neurons in the motor cortex, subcortical nuclei and cerebellum, Determination of density of the pyramidal neurons in the motor cortex of animals showed that in the development process, due to the growth of the neuropil, the pyramidal neurons at P7, P15, P21, compared to control animals, decreased by 25%, 8% and 17% respectively (Table 2). In the cerebellum, the number of Purkinje cells on the same stage of development decreased by 29%, 46% and 11% respectively. In subcortical nuclei especially pronounced changes were observed in the ventromedial nucleus - the number of cells decreased by 47%, 26% and 43% (Table 2). In accordance with our earlier evidence the number of Purkinje cells in the cerebellum decreased by 36 and 44% after one-and two-month intoxication, respectively [27]. Long-term inhalation of toluene induces destructive changes including Purkinje cell atrophy [39, 40].

Table 1. Number neurons in cortical and subcortical structures of the rat's limbic system during toluene intoxication

Age / Structures	3 day		7 day		15 day		21 day	
	Control	Toluene	Control	Toluene	Control	Toluene	Control	Toluene
Entorhinal cortex	26,6 ± 0,7	18,2 ± 0,7	28,4 ± 0,4	17,6 ± 0,5	22,4 ± 0,5	13,9 ± 0,3	20,9 ± 0,4	13,5 ± 0,4*
Cingulate gyrus	40,0 ± 0.6	31.7 ± 0,8*	37,3 ± 0,6	29,4 ± 0,4*	26,6 ± 0.4	22.3 ± 0,4*	21.7 ± 0,5	15.1 ± 0,4*
Ammonis horn	450,2 ± 6.5	350,1± 5.8 *	441,5 ± 3.5	353,4 ± 2,8 *	466,7 ± 3.1	271,0 ± 3.3 *	470,3 ± 8,9	227,1±3.4*
Supraoptic nucleus of hypothalamus	143,9 ± 0,5	112,6 ± 2,4 *	141,4 ± 1,8	80,8 ± 1,4 *	129,8 ± 0,7	101,6 ± 1,2 *	125,6 ± 1,1	113,8 ± 2,2
Paraventricular nucleus of hypothalamus	408,8 ± 4,4	304,2 ± 3,3 *	359,4 ± 1,5	274,6 ± 2,3 *	181,7 ± 0,7	120,5 ± 0,9 *	170,9 ± 3,2	127,1±2,1*
Ventromedial nucleus of hypothalamus	203,7 ±0,9	186,1± 0,8*	236,9 ± 1,3	177,3 ± 1,3*	188,9 ±1,4	109,0 ± 1,8*	155,3 ± 1,1	104,1±1,2*
Lateral nucleus of septum	129,8 ± 2,7	98,4 ± 2,0 *	120,6 ± 1,0	94,2 ± 2,0 *	114,0± 2,1	88,7 ± 1,4 *	110,4 ± 2,0	92,0 ±1,5 *
Medial nucleus of septum	118,6 ± 2,8	91,1 ± 1,8 *	117,5 ± 1,6	93,4 ± 1,8 *	110,1± 1,8	84,3 ± 1,1 *	100,8 ± 1,3	68,6 ± 1,0*

* Significant change between Control and Toluene ($P < 0.01$).

Table 2. Number of neurons in cortical and subcortical structures of the motor system during toluene and Miradol consumption

Age / Structures	3 day			7 day			15 day			21 day		
	I group	II group	III group	I group	II group	III group	I group	II group	III group	I group	II group	III group
Motor cortex	87±1.1	74±2.6*	68±1.1**	79±1.3	59±1.6*	64±1.1**	39±1.1	36±1.2*	36±0.3	23±0.5	19±0.2*	22±0.5**
Ventrolateral nucleus of thalamus	64±2.1	39±1.5*	64±1.2**	91±2.5	48±1.9*	73±1.9**	85±2.0	63±2.7*	73±1.6**	91±3.4	52±2.2*	67±1.5**
Nucleus caudatus	620±2.1	435±4*	505±1.4**	445±4.3	375±5.3*	392±2.7**	312±8.0	272±5.8*	279±1.7**	193±2.3	158±2.2*	156±1.4**
Globus pallidus	258±5.7	202±2.9*	209±2.2**	199±3.0	176±7.2*	181±2.0**	151±3.3	113±1.9*	118±1.3**	129±0.6	70±2.7*	77±1.1**
Purkinje cells	20±0.6	11±1.4*	17±0.2**	17±0.5	12±0.5*	20±0.6**	13±0.6	7±0.2*	11±0.7**	9±1.2	8±1.1*	11±0.3**

Comment: I group – control animals, II experimental group – intoxication by toluene, III experimental group – received toluene and Miradol.

* Significant change between Control and Toluene ($P < 0.01$).

** Significant change between Toluene and Toluene + Miradol ($P < 0.01$).

The early stages of postnatal development are highly sensitive to the impact of various xenobiotics [41]. Neurogenesis of the brain involves the stratification of the cortex, formation of subcortical structures and granular cell layer of the cerebellum.

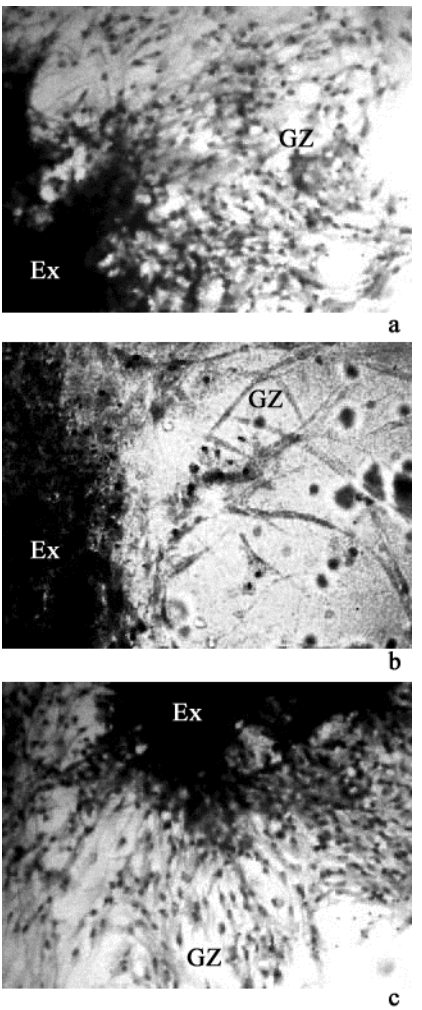
The continued proliferation of granule cells and neuronal migration occur simultaneously with synaptogenesis, apoptosis and development of neuropil. The consumption of toluene during lactation disturbs the course of processes of proliferation and migration underlying neurogenesis.

After toluene exposure a decline of mitotic activity of granule cells in fascia dentate and cerebellum at P3 P7 and P15 (Table 3) was revealed early postnatal development of the CNS is considered as one of the critical periods of brain development [23]. Reduction of neurons is particularly expressed in the hippocampus and cerebellum, formation of which occurs within the first month after birth, when there is active proliferation of granule cells and the formation of structures [20, 25, 42]. A decrease in the number of neurons in cortical and subcortical structures of the limbic and motor systems, as well as in the cerebellum in the early stages of postnatal development of albino rats after intoxication with toluene causes death of neurons, leading to a violation of the stratification of the cortex, subcortical nuclei formation, the integrity of afferent and efferent pathways including intrahippocampal and cerebellar connections, which can have an impact on behavioral activity. Perish of projection neurons and interneurons in the cortical and subcortical structures of the brain destroy nerve pathways, which consequently affects the motor activity and declarative memory of animals [43, 44].

Table 3. Mitotic index of granule cells of dentate fascia and cerebellum during toluene and Miradol consumption

	Dentate fascia	Cerebellum
3 day		
Control	9.0 ± 0.5	3.7±0.2
Toluene	6.1 ± 0.4*	2.8±0.4*
Toluene+Miradol	4.1 ± 0.3**	3.4±0.2
7 day		
Control	6.2 ± 0.7	9±0.8
Toluene	4.0 ± 0.4 *	2.3±0.1*
Toluene+Miradol	5.2 ± 1.0	5.6±0.1**
15 day		
Control	1.4 ± 0.3	5.1±0.2
Toluene	1.2 ± 0.1 *	3.0 ± 0.1*
Toluene+Miradol	1.0 ± 0.2	1.7 ± 0.1 **

Designations are the same as in the Table 2.



a – Inhibition of glial cell migration from explant after introduction in nutrition medium of toluene.
b – Intensive migration of glial cells from explant in the growth zone of intact explants.
c – Glial cell migration from explant after introduction in nutrition medium of toluene and Miradol.
Ex – Explant.
GZ – Growth zone.
Magnification: oc x 10, obj x 20, cresyl-violet-stained.

Figure 1. The brain cortical area of one-day old newborn rats in vitro (72h cultivation).

For the prevention of toxic effects of toluene a variety of antioxidants, possessing neuroprotective, anti-inflammatory and antioxidant properties [31, 32, 35] is often used. A number of investigators demonstrated that melatonin significantly reduces the toluene-induced reduction of dendritic branching in the cerebral cortex [45, 46]. The early studies performed by us showed that adding LB-plaferon to diet of 2-3 months old white rats reduced the cytotoxic effect of prolonged toluene exposure, manifested in death of neurons of cortical and subcortical structures of motor system [27].

We used Miradol to reduce toluene-induced damages. Miradol contains epofen, a new generation antioxidant, with prominent ability of detoxication, blocking formation of free radicals, promoting optimal oxygen consumption by cells and tissues, increasing resistance to oxygen deficiency. Miradol also contains amino acids, macro-and micronutrients, polysaccharides, vitamins: B, PP, H and vitamin E, which in turn increase the preventive effect of Miradol.

A simultaneous exposure to toluene and Miradol of the developing brain reduced the number of perishing neurons in the cortical and subcortical structures of the motor system and the cerebellum. Partial improvement of proliferative activity of granule cells of dentate gyrus and cerebellum was observed at P3, P7 and P15, but the more pronounced features of prevention by Miradol were noted at P7 and P15 (Table 3).

In order to identify the characters of the influence of toluene on the migration of glial cells and on axonal growth, we used an experimental model of nerve tissue culture. In vitro toluene exposure on the migration of glial cells in the cultures of cerebral cortex showed that addition of toluene to the nutrient medium of cultures had an inhibitory effect on the development of the growth zone. Disruption of intercellular contacts, a significant reduction in the number of glial cells deported from explants were observable. The growth zone was mainly represented by cells such as fibroblast and by individual glial cells (Figure 1a), in contrast to the intact cultures where there was active migration of the glial cells in the growth zone (Figure 1b).

Inhibition of glial cells' migration ability and the intensity of axonal growth, after toluene intoxication in culture conditions are consistent with the data of researchers who discovered reduction of neuronal activity in vitro, after toluene exposure [47]. Toxic effects of toluene on primary cultures of hippocampal neurons in rats were manifested in swelling of neuronal cell bodies, the condensation of heterochromatin in nuclei, degeneration of organelles, decrease in the number of cells and apoptosis, which may be due to the ability of toluene to strengthen the flow of Ca^{2+} ions. It is also described the reduction of specific markers of synapses, which causes disruption of

synapse formation [48, 49, 50]. To prevent the observed changes caused by supplementation of toluene in culture medium, simultaneously with it, in the culture medium was administered Miradol. In these series of cultures growth of neuritis was weak there was mainly observed active eviction of glial cells in growth zone of explants (Figure 1c).

The observed by us active migration of glial cells from the explants after the addition to the nutrient medium of Miradol, together with toluene may be due not only the effect of an antioxidant – epofen, but also the influence of vitamin E, which is also part of Miradol. In studies conducted by us earlier, it was shown that the addition of vitamin E to the nutrient medium of cultures of the cerebral cortex of newborn rats caused a decrease in the intensity of oxidative stress and consequently the degree of damaged cells induced by the addition of the nutrient medium cultures of H_2O_2 [51]. Analysis of the data makes it possible to conclude that the cortical and subcortical motor system in the early stages of development antioxidant effects of Miradol attenuates the cytotoxic effect of toluene, and in vitro preventive effect of Miradol is to inhibit the destructive effects and normalization of the culture conditions.

The particular sensitivity of the nervous system to toluene is caused by big amount of fatty acids in the membranes of nerve cells. Toluene, or methylbenzene, is a lipid-soluble aromatic hydrocarbon. Because of its high lipid solubility, toluene accumulates in lipid-rich tissues such as brain. Due to the lipophilic properties of toluene, it induces alterations in membrane lipid bilayer. Studies of the effects of toluene on membrane fluidity demonstrated toluene dose-dependent influences on the synaptosomal membrane fluidity [52]. It was suggested that these effects developed due to disruption of lipid–protein interactions [3]. Chronic exposure to toluene in animals causes increased levels of lipid peroxidation in the membranes of the cerebral cortex, hippocampus and cerebellum [53]. The free radical effects correlated with oxidative damage of DNA, increase in an apoptotic marker (caspase 3) in the cortex and cerebellum, indicating that apoptosis may play a role in toluene neurotoxicity [54, 55].

Normal brain consumes a large quantity of oxygen, naturally forms oxidants for auto-oxidation of some neurotransmitters, and is relatively poor in antioxidant capacity, which makes it particularly vulnerable to oxidative damage [56]. Redox system plays an important role in regulation of the functioning of the nervous system, including development processes, control of cellular metabolism and the initiation of a cascade of neurotoxic events. The redox-active molecules include reactive oxygen species (ROS) and lipoperoxids, as well as nitric oxide (NO). The impact of free radicals causes

damage of cell membranes, disruption of stability of proteins and chromatin, as well as respiratory chain in mitochondria, which leads to cell death by necrosis or apoptosis and therefore formation of dystrophic processes in the brain structures [21, 20, 5, 46]. An increase in the formation of free radicals has been revealed in hippocampus, cerebral cortex and cerebellum of rats, exposed to toluene intoxication [31].

The membrane lipids are the main target for free radicals, formed during toluene metabolism and play a major role by which toluene induces brain damage. NOS has three major isoforms: neuronal, endothelial (constitutional cNOS isoforms) and inducible (iNOS) [57]. Isoforms of NOS-synthase in the brain are presented in the form of a constitutional (cNOS) and inducible (iNOS) isoforms, and are included in a number of physiological and pathophysiological functions.

NO plays the role of intra-and extracellular mediator in the nervous system, having many diverse functions [57]. Under normal conditions, NO involves in the functioning of the central nervous system by the implementation of the interneuronal connections (as a neurotransmitter), and synaptic transmission, establishment of interneuronal synaptic interactions during development of the nervous system (immune mediator) [58, 59] NO is synthesized by neuronal NO-synthase (nNOS) in response to the increased flow of calcium ions into excitable neurons and its spread to neighboring cells causes the formation of cGMF, which is able to adjust the conductivity of the membrane ion channels and thereby alter neuronal electrogenesis. Level of expression of iNOS in intact neurons is very low, but increases dramatically during various pathological conditions like ischemia [60], trauma [61], Parkinson's disease [62], Alzheimer’s disease [63], as under the influence of various toxic factors [64, 65]. The large amount of NO produced by iNOS has been closely correlated with the pathophysiology in a variety of diseases and inflammation [66]. One of the mechanisms by which NO affects cellular metabolism - interaction with superoxide radicals with the formation of highly reactive, toxic oxidizing agent, peroxynitrite, which is included in the pathogenesis of various diseases by nitration of proteins and interaction with membrane lipids, involves in the pathogenesis of various diseases.

Formation of iNOS, after intoxication with toluene causes damage to cell membranes, a violation of the stability of proteins and chromatin, as well as the respiratory chain in mitochondria, which promotes the cell death by necrosis and leads to the formation of dystrophic processes in the brain structures [28, 8, 55].

As follows from the results of our study, after the intoxication with toluene at P15, P30 and P60 stages in the rat brain cortex and liver an increase of intensity of the spin-trapped signal of lipoperoxide ($LOO^{\cdot}$) was revealed that indicates on the intensification of generation of reactive oxygen species (ROS), development of oxidative stress and lipid peroxidation processes in the explored tissues. The intensification of oxidative stress under the influence of chronic intoxication with toluene started especially rapidly in the animals liver, which was revealed by the particularly higher intensity of EPR signal of lipoperoxides; in the liver signal intensity of $LOO^{\cdot}$ was reached a maximum at P15, whereas in the cerebral cortex its intensity reached a maximum value only after one month of intoxication (Table 4). ROS generation during toluene catabolism may be promoted by cytochrome P450 and aldehydedehydrogenase oxidation [28].

In rats subjected to intoxication with toluene oxidative stress is able to promotes enhanced expression of iNOS, which is manifested in the increase of free NO in the EPR spectrum the the tissues. In the cerebral cortex intensity of spin-labeled free NO EPR signal increased at the later stages of development (P30 - P60) (Table 4), which may be due to the increased expression of iNOS in oxidative stress conditions. Sharp intensification of oxidative stress in liver at an early stage of rat's intoxication (P15) contributes to transformation of NO to peroxynitrite, which manifests itself in a slight decrease of EPR signal intensity of spin-trapped free NO.

Table 4. Intensity of signals of free nitrogen oxide (NO) and lipoperoxides ($LOO^{\cdot}$) in the cerebral cortex and liver of rats during toluene intoxication

	NO		$LOO^{\cdot}$	
K P15	Cerebral Cortex	Liver	Cerebral Cortex	Liver
Control	0,7±0,02	1,2±0,03	-	-
Toluene	0,9±0,04*	1,0±0,05*	0,2±0,03	0,8±0,04*
P30				
Control	0,9±0,01	1,0±0,04	-	-
Toluene	1,0±0,03*	1,0±0,02	0,5±0,04*	0,8±0,06*
P60				
Control	0,8±0,02	1,0±0,03	-	-
Toluene	1,2±0,05*	1,0±0,04	0,5±0,03*	0,8±0,05*

Designations are the same as in the Table 1.

Our results coincide with the existing data and confirm the opinion of the special sensitivity of the process of neurogenesis to the effects of toluene in the early postnatal period [13, 3, 20].

The results of comparison of the body masses of the animals at P15 and P21 intoxicated with toluene significantly lagged behind the control animals. A decrease in growth rate and body weight, degree of body hair, they began to see clearly a day later. Intoxication, used in our experiments, readily promotes a decrease in the body mass via the effect of toluene on the olfactory system and its central structures, which results in limitation of food consumption by the animals.

It is known that young rats prior to opening of the eyes, when sensing specific thermal and olfactory stimuli, can crawl, predominantly by rotary motion of the body, and this ability begins to be manifested on the 4th to 5th postnatal days. Clearly pronounced manifestations of the auriculonasocephalic reflex (ANC) are typical of the neonatal period. The young rat, when removed from the nest, reproduces smelling movements, twists its head around, and opens and closes its mouth ("looking" for its mother). Studying the peculiarities of spatial movements in the animal groups I and II, we found that, both on P7 and P13, control animals of group I, using mostly rotatory movements, reached the dummy of the mother more rapidly than the experimental rats ($P < 0.01$). Despite the fact that the distance for the movement increased from 8 to 12 cm, the reaching time measured on day P13 significantly decreased in both groups (Figure 2). In the first trials, animals of group I demonstrated intense search activity; these rats also showed effect of learning (especially on P13). As compared with the group I, most of group II characterized a weakly expressed ANC reflex and demonstrated some typical behavioral manifestations, such as quivering stopping in their tracks, tremor, and return to the start position; motions by means of rotary motion were observed rarely. Blind young rats were able to rapidly find the mother within the first days after birth. Intact offspring subjected to no chemical or any other external intervention until day P10 are characterized by intense rotary movements of the body. These movements have an obvious adaptive importance because they help young rats to remain near the nest. In our experiments, the investigated young rats subjected to the action of toluene demonstrated practically no such motor strategy for solution of this spatial task.

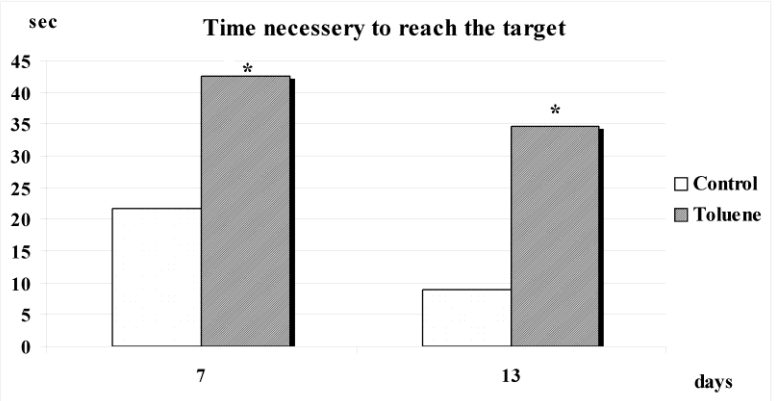


Figure 2.

Results of testing of the examined animals (P15 and P21) in the water corridor showed that rats after intoxication with toluene, for reaching the platform, spent on average for longer time than intact animals ($P<0.01$) (Figure 3). Experimental group of animals demonstrated a delay on the start, floating in one and the same place, and returned back to the start despite the fact, that the platform was readily visible. At the same time, in these animals impairment of the goal realization was not associated with deterioration of their ability to swim. Unlike them, animals of the control group improved the time value of the goal attainment from trial to trial; they had a pronounced effect of learning (particularly at P21).

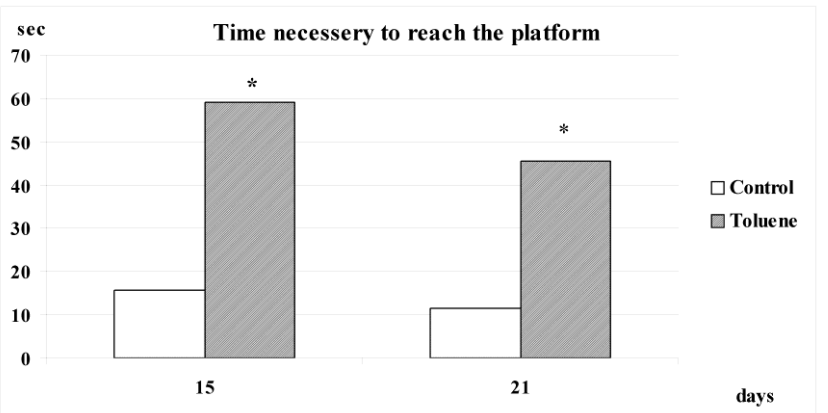


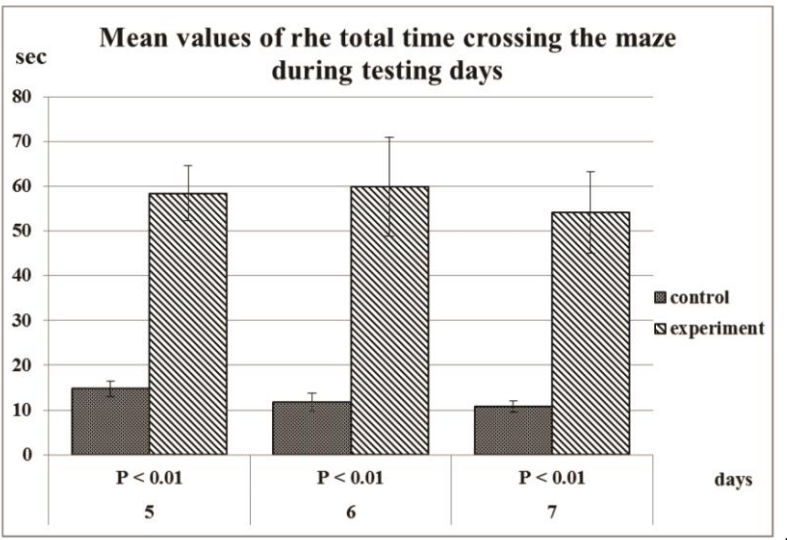
Figure 3.

The results obtained to examine the characters of the spatial movements of animals of two groups indicate that the effect of toluene significantly impairs the ANC reflex and respectively, the ability to find the nest. At the same time, toluene causes significantly decreases of platform finding velocity in the water corridor in experimental P15 and P21 animals, which points at decreased motivation level and deterioration of the learning processes in absence of motor deficit..

The results obtained with the use of the PA test showed that under conditions of re-testing after 24 h, only one rat of the control group (n = 14) entered the dark compartment of the test chamber on the 285 sec. of the experiment, while seven rats of the experimental group (n =11) entered the dark compartment, where they received pain stimulation the day before. Therefore, the index of learning for PA in animal groups I and II corresponded to 93% and 36% and mean values of the latency time during re-testing differed significantly ($P<0.01$) and were 298.9±1.1 sec and 208.7±3.45 sec. respectively. No one of the control group entered the dark compartment, while 9 animals of the second group entered the dark chamber. Re-testing carried out in 48 h once more confirmed that control animals were much successful in the PA test. Delays of attempts of entry into the dark compartment in these two animal groups differed significantly ($P < 0.01$), and these values for groups I and II were on average 300±0 and 160±37.1 sec. We noted that intoxicated animals are less active compared with the control ones. Increasing of anxiety was observed, in a light section they mostly moved on the periphery, along the wall and spent more time in a dark section, than control animals. In animals of II group exploratory motivation was decreased and stereotyped grooming reactions were noted. Thus, results obtained with the use of the PA test on P30 showed that the ability of experimental animals to consolidate memory traces was disturbed significantly.

Monitoring of the processes of spatial learning of animals of two groups in the elevated maze showed that animals of group I (30 day-old intact rats, n=9), when placed in the maze for the first time, needed the help of the experimenter only in two trials of the first testing day. Later on, they practically independently opened up the new environment and demonstrated intense research activity. The rather low level of emotional background in these animals was confirmed by the fact that, in initial trials of the first day, acts of defecation (boluses) were only single. On the 4th day, four rats of this group completely opened up spatial information. Other rats made only few errors and significantly shortened the passage time along the maze. On the 5th day, all 9 rats of this group unmistakably identified the shortest way to the target and

spent, on average, 14.7±1.5 sec for the movement. Moreover, at the end of the experiment, the majority of the animals in some trials could pass the maze during 4-6sec.



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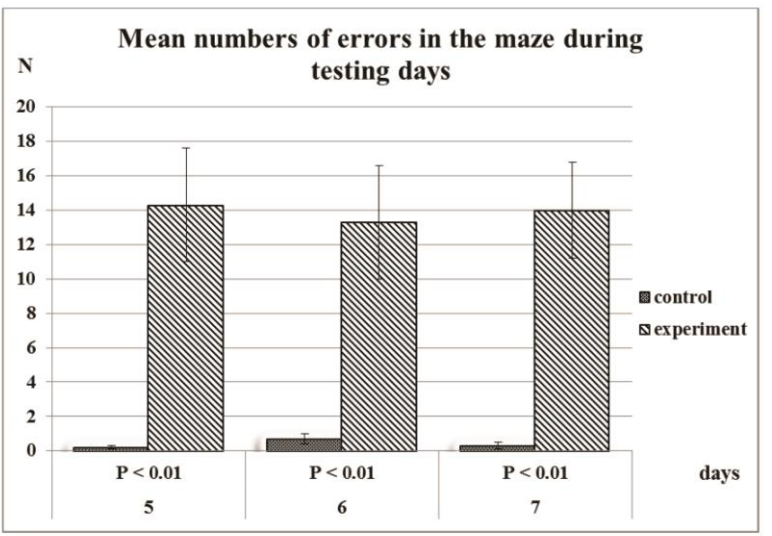

b

Figure 4.

Animals of group II (experimental P30 rats, n=9) in contrast to the animals of group I were characterized by high emotional background, evidenced by accompanied vegetative components (frequent defecation and urination) in the first 3 days, as well as restriction of movement - in the process of animals' movement in the maze, as it were "dragging" the hind paws. On the first day of the experiment, not a single rat was able to learn the entrance to the nest, even from the last platform. On II, III, IV days, they were characterized by perpetual motion, return to the starting pad, often of the site, crossbar adjacent to the nest, froze or stopped and made a lengthy grooming at the crossroads of the maze crossbars and mean number of errors were 34.9, 28 and 17.3 respectively. Determining the time of passage of the maze showed that the majority of animals on II-IV days could not come down to the nest within 3 min. (therefore obtained results are presented on Figure 4 from V testing day). On V testing day in spite of the fact that in all trials experimental animals committed errors and often return an starting pad, they significantly decreased the number of errors and were able to reached the finish in less than 3 min. Learning effect was manifested in the fact, that they from the middle part of the maze significantly increased the of the rate of run to the finish. However, despite the visible improvement spatial learning processes on V and VII testing days results obtained from experimental animals differed quite significantly ($P<0.001$) from the control group in both studied parameters (number of errors and timed crossing the maze) (Figure 4a,b).

As we see above, toluene intoxication significantly decreased a capacity for consolidation of the memory traces in PA test, and ability of spatial learning in the elevated maze in P30 animals. Our earlier studies allowed as to conclude that P30- adolescent intact rats make errors twice more rarely and reach the specified training criterion earlier than adult P60 animals. A relatively low emotional background and high research activity observed within the initial stages of the experiment accelerated the process of spatial maze learning of P30 rats [67]. It is known that hippocampal structures are especially related to the processes of learning and consolidation of memory traces. The data obtained over the last 10 years confirm that, in the course of performance of the labyrinth tests by animals, granule cells of the dentate gyrus play a specific role in the formation of spatial memory [68, 69]. It was also found that the glutamatergic system of the hippocampus is involved in the processes of spatial learning and memory and those NMDA receptors play a crucial role in acquisition of the corresponding information and its storage. On the 30th day of postnatal life of rats, the hippocampus is a quite finally organized structure where 85% of granular cells have been formed. It was also

found that the number of NMDA receptors in the hippocampus of these animals changes during ontogenesis. This index is rather limited up to P7, then rapidly increases, is stabilized, and reaches the level typical of adult rats on P28 [70]**.** It is known that the hippocampus is the primary target for the neurotoxic effects of toluene, which is accomplished through the glutamatergic neurotransmitter systems [71, 72]. Disturbance of the hippocampus and its connections causes a delay in maze learning, performance of which depends on the working and spatial memory [72].

According to our results, toluene intoxication in rats decreases the number of pyramidal neurons in the hippocampus and also inhibition of proliferative activity resulting in dramatic decreases of the number of granular cells in the dentate fascia on the early stages of postnatal development. Thus, naturally, should induce impairment of both intrahippocampal connections and connections between the hippocampus and other structures of the limbic system, i.e., of the communications responsible for realization of learning and memory processes.

CONCLUSION

Our data showed that toluene intoxication (500rrm and 1200rrm) on the early stages of postnatal ontogenesis of albino rats, causes a decrease in the number of neurons in cortical and subcortical structures of the limbic and motor system in result of delay of proliferation and cells death.

After intoxication with toluene the cerebral cortex and liver of rats at P15, P30 and P60 revealed increased signal intensity of spin-labeled lipoperoxids ($LOO^{\cdot}$). These data indicate an intensification of oxidative stress and lipid peroxidation in these tissues. Increased oxidative stress contributes to the expression of iNOS, which is manifested in an increase of free NO in the EPR spectrum of the cerebral cortex.

Alteration the number of nerve cells delays the stratification of the cortex and formation of the subcortical structures, leading to disruption of afferent and efferent connections. Reduction of the number of pyramidal neurons in Ammony horn may cause disruption of the intrahippocampal links and connections between the hippocampus and other limbic system structures.

The results obtained to examine the characters of the spatial movements of animals of two groups indicate that the effect of toluene significantly impairs the ANC reflex and respectively, the ability to find the nest. At the same time, toluene causes significantly decreases of platform finding velocity in the water

corridor in experimental P15 and P21 animals, which points at decreased motivation level and deterioration of the learning processes in absence of motor deficit. As we see above, toluene intoxication significantly decreased a capacity for consolidation of the memory traces in PA test, and ability of spatial learning in the elevated maze in P30 animals.

The use of Miradol, in order to reduce the damaging effects of toluene showed a reduction in the number of perishing neurons in the cortical and subcortical motor system and cerebellum, partially restoring the proliferative activity of granule cells in the hippocampus and cerebellum, and migration ability of glial cells in vitro.

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