

Presence of Dichlorodiphenyltrichloroethane (DDT) in Croatia and Evaluation of Its Genotoxicity

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1. Introduction

Pesticide is any substance or mixture of substances used for preventing, destroying or controlling any pest. According to the Food and Agriculture Organization (FAO) pest is defined as an organism that: (1) is a vector of human and animal disease; (2) can interfere with or cause harm to any step in food, wood and agricultural goods production, transport and storage; (3) can lower the quality of animal food or drugs. Extended definition of pesticide includes all substances: (1) used as plant growth regulator, defoliant and desiccant or agent for prevention of premature fruit fall; (2) applied to crops in order to protect them from deterioration during storage and transport (Food and Agriculture Organization [FAO], 2005). Every pesticide consists of active ingredient whose main function is to cause harm on the pest and inert carrier substance to improve storage, handling, application, efficiency and safety of the pesticide (World Health Organization [WHO], 1990).

For easier dealing with large numbers of pesticides, there are several classifications which divide pesticides in some classes. According to the chemical structure pesticides can be classified as: pyrethroids, carbamates, thiocarbamates, dithiocarbamates, organophosphates, organochlorides, phenoxy and benzoic acid herbicides, triazines, triazoles, ureas etc. (Kamir, 2000). Another classification is done considering the target organism, so pesticides can be classified as: insecticides, fungicides, herbicides, virucides, avicides, molluscicides, nematocides, rodenticides etc. (WHO, 1990). It is important to notice that pesticide's mode of action is not restricted just to the target organism; it also has negative effects on other organisms. That is why the World Health Organization (WHO) recommends the classification of pesticides by hazard. The base of this classification is determining the lethal dose (LD₅₀) for 50% of rat population after acute oral and dermal pesticide exposure. Depending of the LD₅₀ value, as presented in Table 1, pesticides are classified as extremely, highly, moderately or slightly hazardous (WHO, 2009a).

It can be assumed that fighting against the pests started when human civilization was introduced to agriculture which ensured enough food for further development. The first

known historical use of pesticide was observed in Mesopotamia 2500 BC. People in ancient Sumer used sulphur to treat crops thus killing pests (Miller, 2004). Around 500 BC ancient Greek historian Herodotus described the use of castor-oil plant as a mosquito repellent. People in Egypt who lived in swampy areas lit the castor-oil lamps which had an unpleasant smell. Also they fixed fishing nets around their beds before going to sleep. This information suggests that ancient Egyptians used similar techniques to deal with mosquitos as today's insecticide-treated nets (Charlwood, 2003; WHO, 2007). After long period of natural pesticides use (e.g. arsenic, mercury, nicotine sulphate, pyrethrum, etc.), in 20th century the revolution of synthesized pesticides started.

Class		LD ₅₀ for rats (mg/kg of body mass)			
		Oral		Dermal	
		Solids	Liquids	Solids	Liquids
I a	Extremely hazardous	5 or less	20 or less	10 or less	40 or less
I b	Highly hazardous	5 – 50	20 – 200	10 – 100	40 – 400
II	Moderately hazardous	50 – 500	200 – 2000	100 – 1000	400 – 4000
III	Slightly hazardous	over 500	over 2000	over 1000	over 4000

Table 1. The WHO pesticide classification based on LD₅₀ (adapted from WHO, 2005).

DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane) was one of the most used pesticides in the mid 20th century. This organochloride was synthesized in 1874 by Othmar Zeidler and its pesticides properties were discovered in 1939 by Paul Hermann Müller who was awarded Nobel Prize in Physiology or Medicine for this discovery (Agency for Toxic Substances and Disease Service [ATSDR], 2002; Turusov et al., 2002). Commercially used DDT is a mixture of several isomeric forms: *p,p'*-DDT (85%), *o,p'*-DDT (15%), *o,o'*-DDT (trace amounts) and its breakdown products DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) and DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane). The basic chemical properties of *p,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD are shown in Table 2. According to WHO, DDT is moderately hazardous pesticide with LD₅₀ of 113 mg/kg (WHO, 2009a).

In the period of almost 35 years, 2 million tons of DDT was used to control malaria and typhus, thus contaminating water, soil and air. When DDT and its metabolites enter the environment they have the potential to stay adsorbed in the sediment for more than 100 years. The best example of DDT's persistency is that it has been found in the areas 1000 km off the spraying spot and even in the Arctic animals and ice where it has never been used (ATSDR, 2002). One of the most popular acts that contributed global DDT ban in 1973, was Rachel Carson's book *Silent Spring* written in 1962 where she emphasized the problem of the DDT use (Carson, 2002). Today DDT is still legally produced (China, India, and North Korea) and used in the countries of the third world (e.g. India, Ethiopia, South Africa etc.) for malaria control (WHO, 2009b).

There are four possible ways for DDT to enter organisms: (1) ingestion, (2) inhalation, (3) dermal exposure, and (4) placental transport. Once in the body DDT is metabolized mainly in the liver and partly in the kidneys to its most familiar metabolites DDE and DDD. Detailed metabolic pathways are shown in Figure 1. Conjugated forms of 2,2-bis(4-chlorophenyl)-acetic acid (DDA) are then excreted through urine and faeces. The most dangerous property of DDT is that it possesses high bioaccumulation and biomagnification potential. As the trophic level rises, the concentration of DDT and/or its metabolites increases. All three compounds are reported to be harmful to either human or animals

(Brooks, 1986; Gold & Brunk, 1982, 1983; Morgan & Roan, 1974; Peterson & Robinson, 1964). As mentioned in the book *Silent spring*, DDT (especially metabolite DDE) has negative effects on bird's reproductive system by reducing Ca²⁺ transport which results with eggshell thinning thus increasing lethality (Carson, 2002).

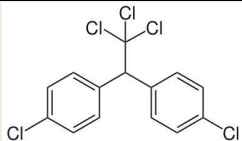
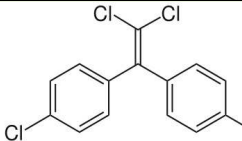
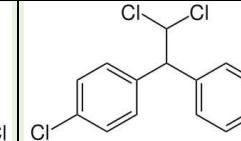
	<i>p,p'</i> -DDT	<i>p,p'</i> -DDE	<i>p,p'</i> -DDD
Chemical formula	C ₁₄ H ₉ Cl ₅	C ₁₄ H ₈ Cl ₄	C ₁₄ H ₁₀ Cl ₄
Chemical structure			
Molecular mass	354.49	318.03	320.05
Physical state	Solid ^a	Cristalline solid ^a	Solid ^a
Colour	Colourless crystals, white powder	White	Colourless crystals, white powder
Melting point	109 °C	89 °C	109 - 110 °C
Boiling point	Decomposes	336 °C	350 °C
Solubility in water	0.025 mg/L ^b	0.12 mg/L ^b	0.09 mg/L ^b
Solubility in organic solvents	Slightly soluble in ethanol, very soluble in ethyl ether and acetone	Lipids and most organic solvents	NA*

Table 2. Chemicals properties of *p,p'*-DDT, *p,p'*-DDE, and *p,p'*-DDD (adapted from ATSDR, 2002), ^a room temperature, ^b at 25°C, *NA - not available.

Not only birds are affected by this persistent pollutant, animals with higher amounts of fatty tissue have high levels of DDT and its metabolites (e.g. polar bears, orcas, belugas etc. (Crinnion, 2009; Galassi et al., 2008; Glynn et al., 2011; Okonkwo et al., 2008)). Although the acute DDT poisoning is rare, there are reports of negative effects on human health when exposed to low concentrations of DDT and its metabolites for longer periods. As shown in animals, the major damage was done on reproductive system where DDT can interfere with reproductive hormones. Also, there have been some reports regarding neurotoxicity, hepatotoxicity, immunotoxicity and genotoxicity (ATSDR, 2002). According to International Agency for Research on Cancer (IARC) DDT is classified as possible carcinogen to humans (IARC, 2009) and the increase in frequency of some cancers was detected (e.g. breast, testicular cancer etc.; Aubé et al., 2008; McGlynn et al., 2008).

As mentioned above, DDT introduced revolution in control of vector-borne diseases. Today, there are reports that some *Anopheles* species are resistant to DDT (Morgan et al., 2010) which implies that new methods of fighting vectors should be introduced. According to WHO one can observe the decrease in both number of countries using pesticides to control

vectors (from 72 countries in 2006 to 61 countries in 2007) and in total amount of used DDT for vector control (from 5.1 million kg in 2006 to 3.7 million kg in 2007). Also WHO stimulates the import of new technologies to improve efficiency of insecticide-treated nets and long-lasting insecticidal nets (WHO, 2007). The importance of introducing new technologies for non harmful and efficient global pest fighting can be seen in estimation that only in United States, environmental and social costs of pesticide use are 9645 million dollars per year (Pimentel, 2005).

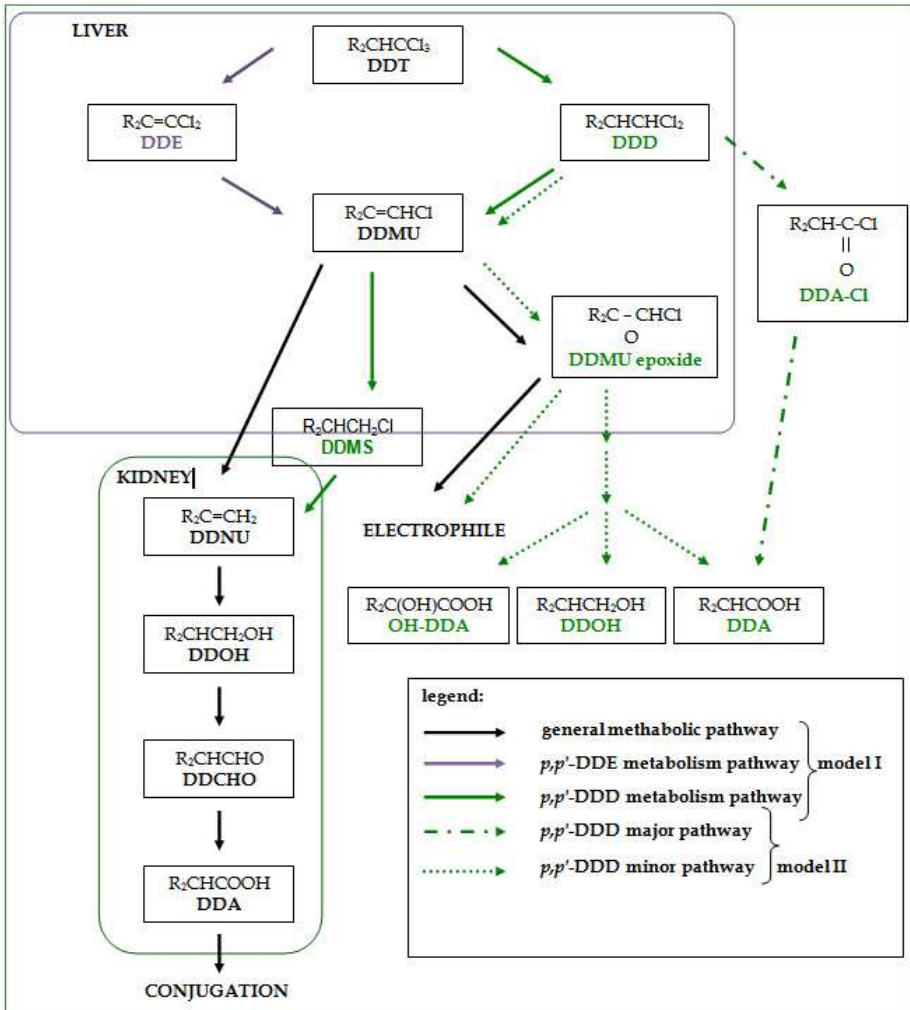


Fig. 1. Models of DDT metabolic pathways (adapted from Gold & Brunk, 1982 and Peterson & Robinson, 1964).

2. Presence of DDT in Croatia

2.1 Production and use in Croatia

Croatia, as a Mediterranean country, has been affected by malaria since the ancient times, especially in the coastal area. Only areas along the Velebit and Biokovo and the districts Hvar and Supetar have been spared from the disease. Most exposed to malaria have been the cities Nin, Benkovac, Obrovac, Skradin, Knin, Drniš, Šibenik, Imotski, Vrgorac and Metković area, the valley of the Neretva River, where malaria was called *Morbus naronianus* - Neretva disease. In 1820 French pharmacists Pierre Joseph Pelletier & Jean Biename Caventou isolated the alkaloid quinine, while doctor Lujo Adam from the island Lošinj was probably the first in the world who injected quinine sulphate into the veins of people sick from malaria. In 1902 doctor Rudolf Battara conducted the first controlled study of drug quinine prophylaxis on the overall population in Nin. The success of the study encouraged implementation of this prophylaxis in other places in Dalmatia in the upcoming years (Dugački, 2005).

DDT was first used in Croatia in 1941, when spraying was carried out against the lice of people as part of educational campaigns in the School of Public Health. The first mass use of DDT was carried out on the front of Srijem in 1945 by spraying soldiers as prevention of typhoid fever with the preparation "neocid", which was donated by the International Red Cross (Bakić, 2011). Companies Chromos from Zagreb and Zorka from Šabac, with their preparations Pantakan and Pepein, were the first producers of DDT in Croatia and the former Yugoslavia. Since 1946 DDT was produced in the form of dust, and since 1949 in the form of concentrated emulsions (Table 3). Production of pesticides in Croatia was small, but large quantities of pesticides were imported. Data on quantities of imported pesticides does not exist, except for the year 1957, when active substances for plant protection products in quantities of 1435 tons were imported. The reason for the continuing production decline of DDT was the lack of foreign exchange quotas for the purchase of organic substances necessary for the production of pesticides. Production and use of DDT in Croatia was until 1972, when its use was banned in agriculture, while in forestry DDT was still used until 1984 (Table 4; National Implementation Plan [NIP], 2004).

Year and purpose	Type of pesticide	Amount (t)	Note
1958. production	DDT	2150	1600 t sprayer concentrate
1959. plan	DDT	3000	550 t emulsifiable concentrate
1959. consumption	DDT	3122	
	DDT + Lindane	687	
1963. production	DDT	2327	
	DDT + Lindane	1854	4.6-6.6 % DDT + 0.3-0.7 lindane content of active substance in preparation

Table 3. The planned and produced quantities of DDT, according to the available data for Yugoslavia in some years (adapted from Hamel, 2003).

Year	Consumption of DDT in forestry (kg/year)	Consumption of DDT in agriculture (kg/year)
1963.	2312	-
1964.	-	1784
1965.	53428	1196
1966.	280	14051
1967.	-	16323
1968.	600	4183
1969.	-	6051
1970.	-	5450
1971.	2363	4296
1972.	4912	1078
1973.	884	0
1974.	8437	0
1975.	6907	0
1976.	8437	0
1979. – 1987.	18658	0

Table 4. Consumption of DDT in kg per year in agriculture and forestry in Republic of Croatia (adapted from NIP, 2004).

Today, list of active substances permitted for use in the Republic of Croatia is synchronized with the official list of active substances permitted in the means of the European Union. The regulations require from pesticide manufacturers to provide data on all possible risks to human health and the environment, as well as data on the effectiveness of pesticides and information on possible contamination in order to obtain licenses for the production and use.

2.2 Methodology

Residues of DDT and its metabolites were analyzed in samples of surface water, soil and food. One of the most sensitive techniques for measuring the rest of DDT and its metabolites in samples from the environment is gas chromatography with electron capture detector (GC/ECD). DDT and its metabolites were determined by applying analytical methods or modifications of the method: International Organization for Standardisation [ISO] 6468 (2002), ISO 10382 (2002), Reference Methods for Marine Pollution (United Nations Environment Programme/International Atomic Energy Agency [UNEP/IAEA], 1982) and EN 1528 1-4 (1996), according to the scheme; extraction, purification of the extracts and quantitative analysis.

2.2.1 Sample extraction and cleanup

Water samples, two to four litres, were extracted with methylene chloride (HPLC grade for spectroscopy, Merck, Darmstadt, Germany) as solvent using an Ultra Turrax system. Extracts were dried over granular anhydrous sodium sulphate and concentrated using rotary evaporator (ISO 6468, 2002). The soil sample was sieved (<2 mm) and stored at room temperature for two days before the experiments. 10 g soil sample (dry matter) was extracted in an ultrasonic bath (ISO 10382, 2002) and the extract was dried by passing them through anhydrous sodium sulphate. Determination of DDT in food samples was based on

method EN 1528 1-4 (1996). Approximately 10-20 g of food were homogenized and extracted with hexane, the extract was evaporated in a rotary evaporator and in nitrogen stream to ensure dryness. Contents of fat were determined weighing a dry sample. Milk fat portions were extracted from each individual sample of milk, cheese, cream and yoghurt according to the method by Sannino et al. (1996). Butter does not normally require extraction procedures. An automated gel permeation chromatographic (GPC) procedure was used to determine DDT residues in fatty foods. About three grams of the fat or less was dissolved into methylene chloride and cleaned up by GPC with a Biobeads SX3 column (OI Analytical, College Station, TX, USA) and a methylene chloride as eluant. About 10 g of fish tissue was weighed, homogenized with anhydrous sodium sulphate and extracted with pesticide grade hexane (Merck, UNEP/IAEA, 1982). The extract was condensed in a rotary flask vaporator to a specific aliquot (5 ml). The aliquot was then subjected to acid treatment by adding concentrated sulphuric acid (Merck). All samples were cleaned up with florisil, commercially available cartridges, 3-6 mL, 500-1000 mg (Kodba & Voncina, 2007). Extracts were concentrated using evaporator in nitrogen stream. An aliquot of each extract was transferred to vials for the quantitative analysis.

2.2.2 Gas chromatograph analysis

The samples were analyzed by gas chromatograph (GC) Shimadzu (Models GC 2010 and GC 17A series, Tokyo, Japan) equipped with autosampler and electron capture detector (ECD) on two fused silica capillary columns of different polarity. Nitrogen was used as the carrier gas with a flow rate of about 1.4 mL/min and as the makeup gas. The injection was set at splitless mode. The injection port and detector temperature were 250 and 300 °C, respectively. The compounds *p,p'*-DDE, *p,p'*-DDD, *o,p'*-DDT and *p,p'*-DDT were identified by comparing peak retention times between samples and known standards. The standard samples of the 18 pesticides were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany, and AccuStandard Inc., New Haven, CT, USA) with the purities of 97-99%. Calibration standard curves were created and DDT residues were quantitatively determined by comparison of the retention times and peak areas of the sample chromatogram with those of standard solutions run under the same operating conditions. Peak confirmation was done by running the samples and the standard on another column and comparing. The concentrations of DDT residues in each sample were reported as ng/L or µg/kg. Limit of quantification for every matrix is presented in Table 5. The laboratory has participated (annually) in intercalibration study proficiency test.

Limit of quantification					
		<i>p,p'</i> -DDE	<i>p,p'</i> -DDD	<i>o,p'</i> -DDT	<i>p,p'</i> -DDT
Water	µg/L	0.0005	0.001	0.001	0.001
Soil (dry mater)	µg/g	0.002	0.003	0.004	0.003
Milk (liquid) and milk products (milk fat)	µg/g	0.0001	0.0004	0.0004	0.0004
Fish and fish products (wet weight)	µg/g	0.0005	0.001	0.0006	0.001
Food (fat)	µg/g	0.001	0.002	0.004	0.002

Table 5. Limit of quantification for *p,p'*-DDE, *p,p'*-DDD, *o,p'*-DDT and *p,p'*-DDT.

2.3 Results

During his lifetime a human being has been exposed to DDT and other pesticides through food, water and the environment. A level of these exposures can be established by analysing of samples from the environment. All food samples come from the market in Croatia while samples of surface water and soil were from different areas of Croatia. Food, particularly dairy products, meat and fish, has been identified as the primary immediate intake route of DDT and other organochlorine pesticides for the population (Johansen et al., 2004).

Samples of surface water of the rivers Sava, Krka, Mrežnica, Kupa, Zrmanja and Cetina were analyzed. The concentration of total DDT in surface waters in Croatia for the period 2009-2010 amounted from below detectable limit to 0.021 µg/L, with median value 0.0016 µg/L (Table 6). The average concentrations of total DDT in surface waters were 0.0026 µg/L. These results comply with those reported by Drevenkar et al. (1994), Drevenkar & Fingler (2000), and Fingler et al. (1992) in their papers. DDT may reach surface waters primarily by runoff, atmospheric transport and drift, or by direct application (e.g. to control mosquito-borne malaria). DDT is practically insoluble in water; but some DDT may be adsorbed onto the small amount of particulate matter present in water (ATSDR, 2002).

The concentration of DDT and metabolites in analyzed samples of soil were below 0.5 µg/g of dry matter (legally prescribed limit values). These analyses were conducted on the samples collected over the last two years and only from a few locations and therefore cannot be found sufficient to make any kind of general conclusions about the current situation. There is no systematic monitoring of DDT in soil in Croatia, resulting with little relevant data. Studies such as Picer et al. (2004) are rare examples of organized research in this area in Croatia, conducted as a part of post war damages in areas where there were concerns that soil was contaminated.

Data on levels and distribution of DDT and other persistent organic pollutant (POPs) in surface waters and soils in Croatia is insufficient, despite these hydrophobic substances being extremely important for assessing environmental contamination. DDT compound in sample of fish tissue were present in very low concentrations, although it is well known that DDT bioaccumulates in marine species. The mean value of DDT in fish tissue, analyzed in year 2007, was 3.8 µg/kg wet weights, with median value of 4.7 µg/kg. Similar results are reported by Krautchaker & Reiner (2001), and Bošnjir et al. (2007). The five dairy products have been examined for the rest of DDT: milk, butter, cheese, cream and yoghurt. The mean values of the residual concentrations of total DDT in the examined dairy products were 29.6 µg/kg fat, respectively, with median 25.2 µg/kg (Table 6). The presence of DDT in milk and milk products has also been reported by Krautchaker & Reiner (2001) and Bošnjir et al. (2010) as well as in other countries (Nevein et al., 2009; Dawood et al., 2004; Bulut et al., 2010). These studies have found that DDT complex were the most frequent contaminants in dairy products. Heck et al. (2006) concluded there is no difference in DDT in raw and pasteurized milk. The concentration of DDT in meat products sampled at food markets was 1.2-740.0 µg/kg fat, with median value 16.2 µg/kg fat (Table 6). These results should be taken with caution since the origin of meat is unknown; whether the meat is from domestic production or imported from other countries of the world. Meat is imported primarily from developing countries where there is limited or no control over the use and/or control of pesticide residues in foods. These results comply with those reported by Krautchaker & Reiner (2001), Covaci et al. (2004), and Tompić et al. (2011).

	Value range	Mean	Median
Surface and ground water (ng/L)	0.6 – 20.5	2.6	1.6
Soil ($\mu\text{g/g}$; dry matter)	0 – 0.005	0.002	0.002
Fish and fish products ($\mu\text{g/kg}$; wet weight)	0.2 – 31.0	3.8	4.7
Meat and meat products ($\mu\text{g/kg}$; fat)	1.2 – 74.0	51.7	16.2
Milk and milk products ($\mu\text{g/kg}$; milk fat)	11.3 – 79.9	29.6	25.2

Table 6. Concentrations of DDT compounds in different samples.

Lamb meat results (Table 7) demand special attention, since the concentrations of *p,p'*-DDE were generally high. Similar results were found by Tompić et al. (2011) in samples of lamb imported from Bulgaria. *p,p'*-DDE are found in every examined sample of lamb meat, which indicates the need for continuous monitoring of concentrations of this metabolites in samples of lamb meat.

	<i>p,p'</i> -DDE ($\mu\text{g/kg}$)	<i>p,p'</i> -DDD (ng/g)	<i>p,p'</i> -DDT (ng/g)	Total DDT (ng/g)	Total DDT ($\mu\text{g/kg}$)
Lamb meat	739.0	-	0.4	74.3	743.0
Lamb meat	173.0	-	0.5	17.8	178.0
Lamb meat	609.0	0.6	-	1.5	615.0
Lamb meat	964.0	6.1	1.2	103.7	1037.0
Lamb meat	354.0	1.4	0.5	37.3	373.0

Table 7. Concentrations of DDT compounds in samples of lamb meat.

Generally, it was observed that the total DDT or its metabolites residues were bellow acceptable and legally prescribed boundaries. These results highlight the need for regular analyzing of a larger number of samples from the environment to DDT residues and other chemicals of POPs, especially in imported food.

3. Cytogenetic methods for detection of pesticide genotoxicity

Pesticide exposure is ubiquitous, due not only to agricultural pesticide use and contamination of foods, but also to the extensive use of these products in and around residences. Because of their biological activity, the use of pesticides may cause undesired effects to human health. Pesticides tend to be very reactive compounds that can form covalent bonds with various nucleophilic centers of cellular biomolecules, including DNA (Crosby, 1982). For instance, the induction of DNA damage can potentially lead to adverse reproductive outcomes, the induction of cancer and many other chronic diseases (Ribas et al., 1996; Lander et al., 2000; Meinert et al., 2000; Ji et al., 2001). A great variety of tests and test systems based on microbes, plant and animals have been developed in order to asses the genotoxic effects of xenobiotic agents, including pesticides. Biomonitoring studies on human populations exposed to pesticides are employing circulating lymphocytes as biomarkers of exposure (and perhaps of effect). Those studies have essentially focused on cytogenetic end-points such as chromosomal aberrations (CA), sister-chromatid exchanges (SCE) and micronuclei (MN) frequency. Genetic damage at the chromosomal level entails an alternation in either chromosome number or chromosome structure, and such alternations can be measured as CA or MN frequency. The SCE analysis was also adopted as an indicator of genotoxicity, although the exact mechanism that leads to an increased exchange

of segments between sister chromatids is not known in detail at present (Palani-Kumur & Panneerselvam, 2008). Recent studies revealed the nucleotide pool imbalance can have severe consequences on DNA metabolism and it is critical in SCE formation. The modulation of SCE by DNA precursors raises the possibility that DNA changes are responsible for the induction of SCE and mutations in mammalian cells (Popescu, 1999; Ashman & Davidson, 1981). While increased levels of CA have been associated with increased cancer risk (Hagmar et al, 1994, 1998), a similar conclusion has not been reached for SCE or MN. However, high levels of SCE and MN frequency have been observed in persons at higher cancer risk due to occupational or environmental exposure to a wide variety of carcinogens (Fučić et al, 2000; Vaglenov et al, 1999; Fenech et al, 1997). Evidence of CA increases, mainly as structural chromosomal aberrations in occupationally exposed populations. The sensitivity of SCE is lower than that of the CA test in detecting genotoxic effects related to pesticide exposure and fewer data are therefore, available for MN than for the other cytogenetic endpoints (Bolognesi, 2003). Exposure to potential mutagens or carcinogens can provide an early detection system for the initiation of cell dysregulation. Biomarkers of effect are generally pre-clinical indicators of abnormalities and the most frequently used in genotoxicity assessment are comet assay and cytokinesis-block micronucleus test that are being proposed as a useful biomarkers for early effects. The cytogenetic endpoints can give indication of genetic damage; hence they are used as effective biomarkers of exposure *in vivo* and *in vitro*. In recent years, the comet (single-cell gel) assay has been established as a useful technique for studying DNA damage and repair (Tice, 1995). The comet assay combines the single-cell approach typical of cytogenetic assays with the simplicity of biochemical techniques for detecting DNA single strand breaks. The advantages of the comet assay include its simple and rapid performance, its sensitivity for detecting DNA damage, and the use of extremely small cell samples (Hartmann et al., 1998). The advantage of micronucleus assay is its simplicity and speed over the assay of chromosomal aberration. Both techniques have become an important tool for genotoxicity testing because of their simplicity of scoring and wide applicability in different cell types. These techniques became the methods of choice for studies of environmental and occupational exposure to air pollutants, metals, radiation, pesticides, and other xenobiotics.

3.1 Comet assay

The comet assay, also known as the single-cell gel electrophoresis assay (SCGE), is a method for detecting DNA strand breakage (single-strand DNA breaks, alkali-labile sites, double-strand DNA breaks, incomplete repair sites, and inter-strand cross-links) in virtually any nucleated cell (Collins et al., 2004, 2008; Shaposhnikov et al., 2008).

First quantification of DNA damage in individual cells was done by Rydberg & Johanson (1978). After gamma-irradiation they embedded cells in agarose on microscopic slides and lysed under mild alkali conditions. Upon neutralization, the cells were stained with acridin orange and the extent of DNA damage was measured by the ratio of green (indicating double-stranded DNA) to red (indicating single-strand DNA) fluorescence. To enhance the sensitivity for the DNA damage detection, Östling & Johanson (1984) proposed that strand breaks would enable DNA loops to stretch out upon electrophoresis, so the microelectrophoretic procedure under pH of 9.5 was developed. As reported by Singh et al. (1988) this pH of 9.5 is below the limit for DNA unwinding, and was notified to detect only double strand breaks (DSB), with more strongly alkaline conditions (pH 10 or above) needed for unwinding and detection of single strand breaks (SSB). It has been shown that

neutral or mildly alkaline comet assay has the same limit of detection of DNA damage (SSB) as the alkaline comet assay, although the use of neutral pH does effect the comet image obtained (Collins, 2004). The comet tails are less pronounced at neutral pH, and this can be an advantage when a less sensitive method is needed, for example when investigating cells that have large amount of background, or induced damage is high (Angelis et al., 1999).

Alkaline version of the comet assay was presented by Singh et al. (1988) in which DNA is allowed to unwind at $\text{pH} > 13$. In their paper DNA damage was measured as the migrating distance of DNA from the nucleoid. In 1990, Olive et al. (1990) also under alkaline conditions developed the concept of the tail "moment", a combination of tail length and DNA content, as a measure of DNA damage. Also, in 1990, the name "Comet assay" was introduced and the application of the first image analysis program was described (Olive, 1989; Olive et al., 1990; Sviežená et al., 2004). Image analysis has become essential for objective measurement of low-dose effects, or for distinguishing small differences among sub-populations of cells. Strong alkaline conditions enabled clearer images, and besides SSBs other types of DNA damage could be detected, such as alkaline labile sites (Tice et al., 2000). Olive et al. (1990) revealed that employing milder alkaline ($\text{pH} 12.3$) conditions prevents conversion of alkaline labile sites into breaks. Under the $\text{pH} > 13$ alkali labile sites are formed into SSBs, thus revealing otherwise hidden damage. Therefore, by modifying the pH of lysis and/or electrophoresis over the range of 9.5–13.5, one can apply a comet assay of different sensitivity, but of similar limits of detection (Collins et al., 1997; Angelis et al., 1999; Wong et al., 2005).

In its basic form, comet assay gives limited information on the type of DNA damage being measured. Single strand breaks detected by standard alkaline method are not the most interesting of lesions, because they are quickly repaired, and are not regarded as a significant lethal or mutagenic lesion. Many genotoxic agents do not induce strand breaks directly. They may create apurinic/pyrimidinic (AP) sites, which are alkali labile and are probably converted to breaks while DNA is in the electrophoresis solution at high pH. Furthermore, it is not possible to determine whether the high level of breaks in the comet assay is the indicator of high damage or efficient repair, due to temporary presence of breaks in the lesions repair via base excision or nucleotide excision (Collins et al., 1997).

More recently, the assay was modified further to enable the detection of specific kinds of DNA damage by combining the assay with the use of a purified DNA repair enzymes, which recognize the lesions along the DNA and convert them into the breaks expressed as an increase in comet DNA migration. Briefly, the DNA in the gel, following lysis, is digested with a lesion-specific repair endonuclease, which introduces breaks at sites of damage. In principle, any lesion for which a repair endonuclease exists can be detected in this way. To date, endonucleases most commonly used in the modified comet assay are the bacterial enzymes which recognize different types of oxidative damage. The first enzyme to be used was endonuclease III, a glycosylase which recognizes a variety of oxidized pyrimidines in DNA and removes them, leaving an AP site (Doetsch et al., 1987). Formamidopyrimidine DNA glycosylase (FPG) has the ability to convert altered purines, including 8-oxoguanine, into DNA breaks (Collins, 2007; Dušinská & Collins, 1996). When using these enzymes to measure oxidative DNA damage the usual practice is to incubate a slide with buffer alone in parallel with the enzyme slide. Slide with buffer would be a valid control slide, due to small increase in strand breaks on incubation without enzyme (Collins, 2009; Gajski et al., 2008). Recently, a mammalian analogue of FPG, 8-oxoguanidine DNA glycosylase, or OGG1, has been applied in the comet assay (Smith et al., 2006). OGG1 is the major base extension repair

enzyme that initiates the repair of oxidative base lesion. It is a bifunctional DNA glycosylase possessing both DNA glycosylase and AP lyase activities (Boiteux & Radicella, 2000). In human it is named hOGG1. hOGG1 recognizes both 8-oxoguanine (8-oxodG) and 8-oxoadenine (8-oxodA) and removes these oxidized bases from double-stranded DNA, initiating the base lesion repair process (Smith et al., 2006). 8-hydroxy-2-deoxyguanosine (8-OHdG) lesion causes G→T and A→C transversions (Moriya, 1993) that have been reported as the sites of spontaneous oncogene expression and ultimately cancer manifestation (Valko et al., 2004; Bartsch, 1996; Shinmura & Yokota, 2001). Deletion of the hOGG1 gene was shown to be associated with accumulation of 8-OHdG lesion and increase in mutational risk (Hansen & Kelley, 2000; El-Zein et al., 2010).

For evaluation of DNA specific damage the comet assay has also been coupled with the method of fluorescent in situ hybridization (Comet-FISH). Since its initial development, Comet-FISH has been used to handle a number of quite different questiones. First, it was used to identify chromosome-specific areas on electrostretched DNA fibres and to determine their special distribution (Santos et al., 1997). Further applications were then to detect region-specific repair activities (Horvathova et al., 2004; McKenna et al., 2003; Mellon et al., 1986), genotoxic effects in total DNA and in telomeres (Arutyunyan et al., 2005), or in tumor relevant genes, like TP53 (Schaeferhenrich et al., 2003). Also specific chromosomal alternations (Harreus et al., 2004) were studied as were genetic instabilities (Tirukalikundram et al., 2005). Comet-FISH has also been used to discriminate between DNA double-and single-strand breaks (Fernandez et al., 2001). Whereas results from the Comet assay alone reflect only the level of overall DNA damage, the combination with the FISH-technique allows the assignment of the probed sequences to the damaged or undamaged part of the comet (tail or head, respectively). If two fluorescence signals are obtained with a probe for a particular gene in the head of a comet, this indicates that the gene is in an undamaged region of DNA, whereas the appearance of a spot or several spots in the tail of a comet indicates that a break or breaks has/have occurred in the proximity of the probed gene.

3.2 Micronucleus assay

Human exposure to environmental mutagens can be monitored using cytokinesis-block micronucleus (CBMN) assay (Natarajan et al., 1996) which is an efficient biomarker for diagnosing genetic damage and/or genome instability at the chromosome/molecular level in animal and/or human cells. It provides a comprehensive measure of chromosome breakage, chromosome loss, chromosome rearrangements, non-disjunction, gene amplification, necrosis and apoptosis (Fenech, 2000, 2006; Kirsch-Volders et al., 2000).

In the classical cytogenetic techniques, chromosomes are studied directly by observing and counting aberrations in methaphases (Natarajan & Obe, 1982). The complexity and laboriousness of enumerating aberrations in methaphase and the confounding effect of artefactual loss of chromosomes from methaphase preparations has stimulated the development of a simpler system of measuring chromosome damage. More than a century ago micronuclei were described in the cytoplasm of erythrocytes and were called "fragment of nuclear material" by Howell or "corpuscules intraglobulaires" in the terminology of Jolly in the late 1800s and early 1900s. To the hematologists these structures are known as "Howell-Jolly bodies". Similar structures were described in mouse and rat embryos and in *Vicia faba* (Thoday, 1951) and called "fragment nuclei" or "micronuclei". In the early 1970s the term micronucleus test was suggested for the first time by Boller & Schmidt (1970) and

Heddle (1973) who showed that this assay provided a simple method to detect the genotoxic potential of mutagens after *in vivo* exposure of animals using dividing cell population such as bone marrow erythrocytes. A few years later it was shown by Countryman & Heddle (1976) that peripheral blood lymphocytes could also be used for the *in vivo* micronucleus approach and they recommended using micronuclei as a biomarker in testing schemes. As only dividing cells could express micronuclei, for the *in vitro* micronucleus studies it was necessary to establish cell proliferation and micronucleus induction at the same time. The decisive breakthrough of micronuclei as assay for *in vitro* genotoxicity testing came with work of Fenech & Morley (1986) that developed the CBMN assay. In the CBMN assay, cells that have completed nuclear division are blocked from performing cytokinesis using cytochalasin-B and are consequently readily identified by their binucleated appearance (Fenech & Morley, 1985, 1986). Whereas micronuclei originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division, their occurrence is proven in binucleated cells. As a consequence, the CBMN assay has been shown to be more accurate and more sensitive than the conventional methods that do not distinguish between dividing and nondividing cells (Fenech & Morley, 1986; Fenech, 1991; Kirsch-Volders & Fenech, 2001).

Baseline or spontaneous micronucleus frequencies in culture human lymphocytes provide an indicator of accumulated genetic damage occurred during the lifespan of circulating lymphocytes. The half-life and mean lifespan of T-lymphocytes has been estimated to be three to four years, respectively (Natarajan & Obe, 1982; Buckton et al., 1967). The observed genetic instability may also reflect accumulated mutations in the stem cell lineage from which the mature lymphocytes originate. The type of mutations that could contribute to spontaneous micronuclei include: 1) mutations to kinetochore proteins, centromeres, and spindle apparatus that could lead to unequal chromosome distribution or whole chromosome loss at anaphase, and 2) unrepaired DNA strand breaks induced endogenously or as a result of environmental mutagens, which may result in acentric chromosome fragments. Studies using kinetochore antibodies to identify whole chromosomes suggest that approximately 50% of spontaneously occurring micronuclei are the consequence of whole chromosome loss and the rest are presumably derived from acentric chromosome fragments (Thompson & Perry, 1988; Fenech & Morley, 1989; Eastmond & Tucker, 1989). The spontaneous micronucleus frequency refers to the incidence of micronucleus observed in the absence of the environmental risk or exposure that is being assessed. The spontaneous micronucleus frequency of a population has to be established to determine acceptable normal values as well as providing baseline data for those situations when spontaneous micronucleus frequencies for individuals is not known before exposure. Micronuclei harbouring whole chromosomes are primarily formed from failure of the mitotic spindle, kinetochore, or other parts of the mitotic apparatus or by damage to chromosomal sub-structures, alterations in cellular physiology, and mechanical disruption. An increased number of micronucleated cells is a biomarker of genotoxic effects and can reflect exposure to agents with clastogenic modes of action (chromosome breaking; DNA as target) or aneugenic ones (aneuploidogenic; effect on chromosome number; mostly non-DNA target) (Albertini, 2000). The advantage of the CBMN assay is its ability to detect both clastogenic and aneugenic events, leading to structural and numerical chromosomal aberrations, respectively (Kirsch-Volders et al., 2002; Mateuca et al., 2006). Micronuclei observed in cultured lymphocytes are believed to arise primarily *in vitro* from: 1) chromatid-type chromosomal aberrations formed during DNA replication on a damaged template, 2)

chromosome-type aberrations initiated before the mitosis and duplicated at replication, or 3) disturbances of mitotic apparatus leading to chromosome lagging. Micronuclei arising *in vivo*, inducible by both clastogenic and aneugenic mechanisms, can be scored in exfoliated epithelial cells (Salama et al., 1999) sampled, e.g., from buccal or nasal mucosa or urine, or in peripheral blood mononuclear cells e.g., isolated lymphocytes (Surrallés et al., 1996; Albertini, 2000). The CBMN assay is the preferred method for measuring micronuclei (MNi) in cultured human and/or mammalian cells because scoring is specifically restricted to once-divided cells. For scoring MNi uniform criteria should be used. Only MNi not exceeding 1/3 of the main nucleus diameter, clearly separable from the main nucleus and with distinct borders and of the same color as the nucleus, should be scored. In practice, 1000–2000 cells are often scored per subject in lymphocyte studies utilising the CBMN technique, while more cells (3000–5000 per subject) are evaluated in epithelial cells due to the lower baseline MNi frequency. In the CBMN method, only binucleate cells should be analysed for MNi; further divisions of a binucleate cell, usually resulting in cells with 3–4 nuclei, are highly irregular and show high MNi rates (Fenech et al., 2003).

The discovery of kinetochore-specific antibodies in the serum of scleroderma CREST (*Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly and Telangiectasia*) patients (Moroy et al., 1980) has made it possible to determine the contents of micronuclei. Kinetochore immunofluorescence has been rapidly developed for the *in situ* detection of aneuploidy and chromosome breakage in human micronuclei (Degrassi & Tanzarella, 1988; Hennig et al., 1988; Eastmond & Tucker, 1989). Also, the FISH technique using chromosome-specific DNA probes has improved the detection and evaluation of structural chromosomal aberrations. The combination of the micronucleus assay with FISH using a DNA probe specific to the centromeric regions, or with antibodies that specifically stain kinetochore proteins, provides the methodology to distinguish between micronuclei containing either one or several whole chromosomes, which are positively labeled (centromere positive micronucleus), or acentric chromosome fragments, which are unlabeled due to the absence of centromere (centromere negative micronucleus) (Natarajan et al., 1996; Mateuca et al., 2006; Benameur et al., 2011). Except for cytogenetic damage measured by the number and distribution of micronuclei, according to the new criteria for micronuclei scoring, the CBMN assay also detects the nucleoplasmic bridges (NPBs), as well as nuclear buds (NBUDs). Current evidence suggests that NPBs derive from dicentric chromosomes which the centromeres have been pulled to the opposite poles of the cell during the anaphase stage, and are therefore indicative of the DNA mis-repair, chromosome rearrangement or telomere end-fusion. According to the new criteria applicable to the CBMN assay, NBUDs arise from the elimination of the amplified DNA and possibly from the elimination of the DNA-repair complexes, which therefore, may be considered a marker of gene amplification and altered gene dosage (Fenech, 2006; Fenech & Crott, 2002; Fenech et al., 2011; Garaj-Vrhovac et al., 2008; Lindberg et al., 2006; Thomas et al., 2003).

The significance of the CBMN assay lies in the fact that every cell in the system studied is scored cytologically for its viability status (necrosis, apoptosis), its mitotic status (mononucleated, metaphase, anaphase, binucleated, multinucleated) and its chromosomal instability or damage status (presence of MNi, NPBs, NBUDs and number of centromere probe signals amongst nuclei of binucleated cell if such molecular tools are used in combination with the assay). In this respect, the micronucleus assay has evolved into a comprehensive method employed in measuring chromosomal instability of the phenotype and altered cell viability and represents an effective tool to be used in research of cellular

and nuclear dysfunctions caused by *in vitro* or *in vivo* exposure to toxic substances (Fenech, 2006; Garaj- Vrhovac et al., 2008; Thomas & Fenech, 2011).

4. Genotoxicity of *p,p'*-DDT in human peripheral blood lymphocytes

4.1 Methodology

4.1.1 Chemicals

Chromosome kit P was from Euroclone, Milan, Italy; RPMI 1640, was from Invitrogen, Carlsbad, CA, USA; cytochalasin B, histopaque-1119, ethidium bromide, low melting point (LMP) and normal melting point (NMP) agaroses were from Sigma, St Louis, MO, USA; heparinised vacutainer tubes from Becton Dickinson, Franklin Lakes, NJ, USA; acridine orange from Heidelberg, Germany; Giemsa from Merk, Darmstadt, Germany; All other reagents used were laboratory-grade chemicals from Kemika, Zagreb, Croatia. DDT was administered as *p,p'*-DDT (Sulpeco, Bellefonte, PA, USA) in final concentration of 0.025 mg/L at different time points.

4.1.2 Blood sampling and treatment

Whole blood samples were taken from a healthy female donor who had not been exposed to ionizing radiation, vaccinated or treated with drugs for a year before blood sampling. Whole venous blood was collected under sterile conditions in heparinised vacutainer tubes containing lithium heparin as anticoagulant. The comet assay and the micronucleus assay were conducted on whole blood cultivated at 37 °C in an atmosphere with 5% CO₂ (Heraeus Heraecell 240 incubator, Langenselbold, Germany). The whole blood was treated with 0.025 mg/L *p,p'*-DDT for 1, 2, 4, 8, 24 and 48 h for the cytotoxicity assay and alkaline comet assay and 24 and 48 h for the CBMN assay. In each experiment, a non treated control was included.

4.1.3 Cell viability (cytotoxicity) assay

The indices of cell viability and necrosis were established by differential staining of human peripheral blood lymphocytes (HPBLs) with acridine orange and ethidium bromide using fluorescence microscopy (Duke & Cohen, 1992). Lymphocytes were isolated using a modified Ficoll-Histopaque centrifugation method (Singh, 2000). The slides were prepared using 200 µL of HPBLs and 2 µL of stain (acridine orange and ethidium bromide). The suspension mixed with dye was covered with a cover slip and analyzed under the epifluorescence microscope (Olympus AX 70, Tokyo, Japan) using a 60× objective and fluorescence filters of 515–560 nm. A total of 100 cells per repetition were examined. The nuclei of vital cells emitted a green fluorescence and necrotic cells emitted red fluorescence.

4.1.4 The alkaline comet (SCGE) assay

The alkaline comet assay was carried out as described by Singh et al. (1988). Briefly, after the exposure to *p,p'*-DDT, 5 µL of whole blood was mixed with 100 µL of 0.5% LMP agarose and added to fully frosted slides pre-coated with 0.6% NMP agarose. After solidifying, the slides were covered with 0.5% LMP agarose, and the cells were lysed (2.5 M NaCl, 100 mM EDTANa₂, 10mM Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethyl sulfoxide, pH 10) overnight at 4°C. After the lysis the slides were placed into alkaline solution (300 mM NaOH, 1 mM EDTANa₂, pH 13) for 20 min at 4°C to allow DNA unwinding and subsequently electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized in

0.4 M Tris buffer (pH 7.5) for 5 minutes 3 times, stained with EtBr (20 $\mu\text{g}/\text{mL}$) and analyzed at 250 \times magnification using an epifluorescence microscope (Zeiss, Göttingen, Germany) connected through camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., Haverhill, Suffolk, UK). The tail length parameter was used to measure the level of DNA damage and a total of 100 randomly captured nuclei were examined from each slide. Tail length (i.e. the length of DNA migration) is related directly to the DNA fragment size and is presented in micrometers (μm). It was calculated from the centre of the nucleus.

4.1.5 Cytokinesis-blocked micronucleus (CBMN) assay

The micronucleus assay was performed in agreement with guidelines by Fenech & Morley, (1985). After the exposure to *p,p'*-DDT the whole blood (500 μL) was incubated in a Euroclone medium at 37 °C in an atmosphere of 5% CO_2 in air. Cytochalasin-B was added at a final concentration of 3 $\mu\text{g}/\text{mL}$ 44 h after the culture was started. The cultures were harvested at 72 h. The lymphocytes were fixed in methanol-acetic acid solution (3:1), air-dried and stained with 5% Giemsa solution. All slides were randomised and coded prior to analysis. Binuclear lymphocytes were analyzed under a light microscope (Olympus CX41, Tokyo, Japan) at 400 \times magnification. Micronuclei, nucleoplasmic bridges and nuclear buds were counted in 1000 binucleated cells and were scored according to the HUMN project criteria published by Fenech et al. (2003).

4.1.6 Statistics

For the results of the comet assay measured after treatment with *p,p'*-DDT statistical evaluation was performed using Statistica 5.0 package (StaSoft, Tulsa, OK, USA). Multiple comparisons between groups were done by means of ANOVA on log-transformed data. Post hoc analyses of differences were done by using the Scheffé test. Differences in the frequency of micronuclei, nucleoplasmic bridges, and nuclear buds were assessed using the chi-square test. $P < 0.05$ was considered significant.

4.2 Results

4.2.1 Vital staining using ethidium bromide and acridine orange

The viability of HPBLs exposed to aqueous *p,p'*-DDT (0.025 mg/L) for different lengths of time as determined by acridine orange and ethidium bromide staining, using fluorescence microscopy was not significantly affected (data not shown). Changes were determined according to the different staining of the nucleus (Figure 2).

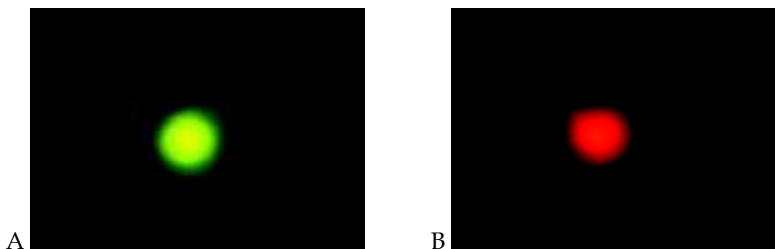


Fig. 2. Cell viability microphotographs represent viable lymphocyte from the un-exposed sample (A; green), and dead lymphocyte (B; red) from sample treated with aqueous solution of *p,p'*-DDT.

4.2.2 Induction of DNA strand breaks

The whole blood was exposed to aqueous solution of *p,p'*-DDT (0.025 mg/L) and the DNA damage in HPBLs was determined with the alkaline comet assay. Figure 3 represents different levels of DNA fragmentation between non-exposed sample and sample exposed to aqueous solution of *p,p'*-DDT. Statistically significant ($P<0.05$) increase in the amount of DNA strand breaks was observed after all exposure times to *p,p'*-DDT (Figure 4).

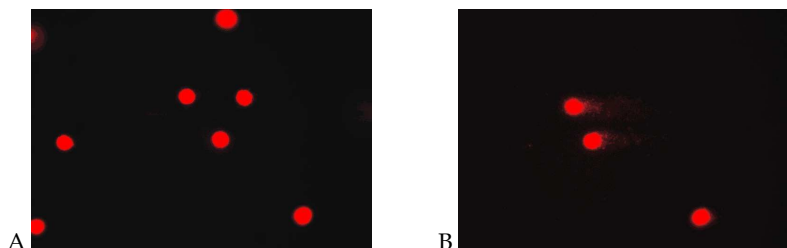


Fig. 3. Comet assay microphotographs represent undamaged lymphocytes from the unexposed sample (A). Image (B) represents damaged lymphocytes that have comet appearance after the treatment with aqueous solution of *p,p'*-DDT.

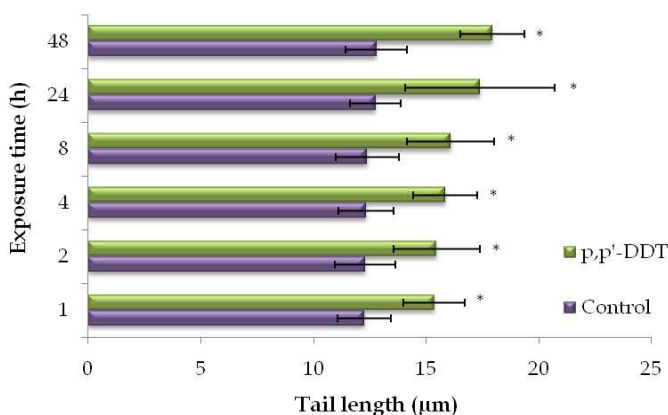


Fig. 4. Tail length (length of DNA migration) as comet assay parameter in human peripheral blood lymphocytes after exposure to low concentration of aqueous *p,p'*-DDT. * Statistically significant compared to corresponding control ($P<0.05$).

4.2.3 Induction of micronuclei, nucleoplasmic bridges and nuclear buds

The genotoxic activity of *p,p'*-DDT (0.025 mg/L) was further evaluated using the CBMN assay. Figure 5 represents binucleated lymphocytes from the non-exposed sample and samples exposed to aqueous solution of *p,p'*-DDT. Following *p,p'*-DDT treatment for 24 and 48 h, increase in the frequency of MNi was detected for both exposure times (Table 8). Additionally, statistically significant induction ($P<0.05$) of NBP and NBUDs was also observed following *p,p'*-DDT treatment for 24 and 48 h (Table 9).

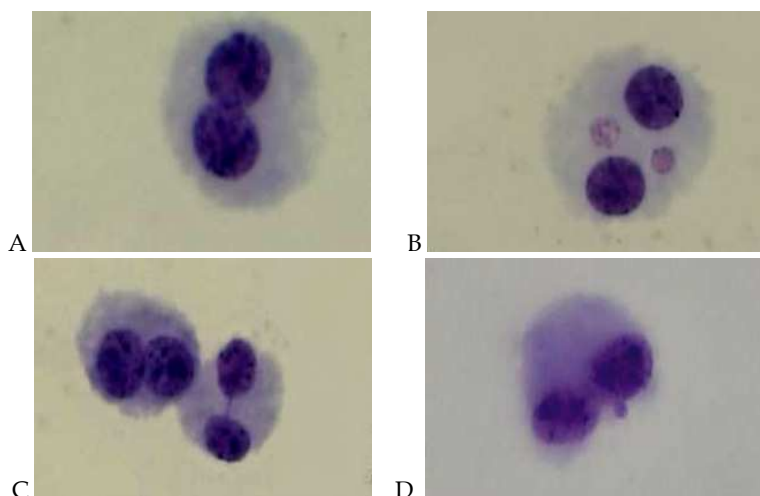


Fig. 5. Cytokinesis-block micronucleus assay microphotographs represent binucleated lymphocyte from the un-exposed sample (A). Image (B) represents binucleated lymphocyte with two micronuclei, image (C) binucleated lymphocyte with nucleoplasmic bridge (right), and image (D) binucleated lymphocyte with nuclear bud after the treatment with aqueous solution of *p,p'*-DDT.

Exposure time (h)	Sample	1 MN	2 MNi	3 MNi	Total no. of MNi
24	Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	<i>p,p'</i> -DDT	28.50±13.44*	1.50±2.12	0.50±0.71	33.00±19.80*
48	Control	6.00±1.41	0.00±0.00	0.00±0.00	6.00±1.41
	<i>p,p'</i> -DDT	30.50±9.19*	1.50±2.12	0.50±0.71	35.00±15.56*

Table 8. Incidence of micronuclei (MNi) as cytokinesis-block micronucleus assay parameter in human peripheral blood lymphocytes after exposure to low concentration of aqueous *p,p'*-DDT, * Statistically significant compared to corresponding control ($P<0.05$).

Exposure time (h)	Sample	Total no. of NPBs	Total no. of NBUDs
24	Control	0.00±0.00	0.00±0.00
	<i>p,p'</i> -DDT	10.50±0.71*	10.50±4.95*
48	Control	0.00±0.00	2.50±0.71
	<i>p,p'</i> -DDT	14.00±11.31*	20.50±3.54*

Table 9. Incidence of nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) as cytokinesis-block micronucleus assay parameters in human peripheral blood lymphocytes after exposure to low concentration of aqueous *p,p'*-DDT, * Statistically significant compared to corresponding control ($P<0.05$).

5. Discussion

Due to uncontrolled use for several decades, many pesticides, among them DDT, probably the best known and the most useful insecticide in the world has damaged wild life and might have adverse effects on human health. Because of its current use in countries of the Third World, DDT still enters environment and in that way it can still represent health risk for human population, even in countries that have banned its use almost 40 years ago (ATSDR, 2002; Crinnion, 2009; Ecobichon, 2000, 2001; Eskenazi et al., 2009; Gajski et al., 2007; Turusov et al., 2002).

Large number of epidemiological studies regarding health risk of DDT confirmed that it represents major threat for wild life and human health (Aronson et al., 2010; Demers et al., 2000; Donato & Zani, 2010; Ecobichon, 1995; Martin et al., 2002; van Wendel de Joode et al., 2001; Wojtowicz et al., 2004, 2007; Woolcoot et al., 2001). Although tested in several genotoxicity studies on bacterial and animal models (Amer et al., 1996; Binneli et al., 2007, 2008a, 2008b; Canales-Aquirre, et al., 2011; Donnato et al., 1997a, 1997b; Galindo Reyes et al., 2002; Gauthier et al., 1999; Uppala et al., 2005) there is still necessity for conducting cytogenetic research regarding its genotoxicity employing sensitive methods to reveal the exact mechanisms of action of this chemical.

Combining different cytogenetic methods may play an important role in assessing genotoxic damage from different environmental chemical or physical agents. With these methods it is possible to evaluate the level of primary DNA damage or the dynamics of its repair even after short-term exposure to these agents (Garcia-Sagredo, 2008; Gajski & Garaj-Vrhovac, 2008). The comet assay is a sensitive method for measuring and analyzing DNA damage at the single cell level, and can be used both in *in vivo* and *in vitro* (Collins, 2004; Collins et al., 2008; Dušinská & Collins, 2008). The comet assay detects single and double stranded breaks at the level of DNA molecule, sites of incomplete repair, alkali labile sites, and DNA-DNA and DNA-protein cross-links (Piperakis, 2009). Furthermore, micronucleus assay can indicate cellular and nuclear dysfunction caused by *in vitro* or *in vivo* exposure to toxic substances. It is a reliable method for measuring chromosomal instability and altered cellular viability (Fenech, 2009; Fenech et al., 2003). It includes micronuclei, which are biomarkers of chromosome breakage and whole chromosome loss, nucleoplasmic bridges, which are biomarkers of DNA misrepair and telomere end-fusions, and nuclear buds, which are biomarkers of elimination of amplified DNA and DNA repair complexes (Fenech, 2007; Garaj-Vrhovac et al., 2008).

Considering the lack of data on the effect of DDT on the cellular genome, and taking into account its usage in some countries of the Third World and its environmental persistence, the aim of this study was to evaluate the genotoxic potential of a low concentration of aqueous *p,p'*-DDT upon *in vitro* exposure of HPBLs of different duration, by using alkaline comet assay and CBMN assay. Our results showed that exposure of HPBLs to aqueous *p,p'*-DDT increased DNA damage in time dependent fashion as measured by the comet assay. In addition, CBMN assay parameters revealed a wider scale of chromosomal alterations after *p,p'*-DDT treatment.

Cytogenetic studies of DDT are mainly based on *in vitro* research on the animal models and its genotoxicity was evaluated in a variety of test systems. Results obtained by studying cytogenetic effects of DDT on DNA of shrimp larvae (*Litopenaeus stylirostris*) indicated that DDT causes DNA adducts and/or breaks (Galindo Reyes et al., 2002). DDT and its

metabolites DDE and DDD showed a clear genotoxic effect on haemocytes of zebra mussel (*Dreissena polymorpha*) specimens in different concentrations that have been found in several aquatic ecosystems worldwide, with a greater genotoxic potential of the DDE in respect to the other two chemicals (Binneli et al., 2008a, 2008b). DDT has also the ability to induce chromosomal aberrations in mouse spleen indicating its genotoxicity (Amer et al., 1996). In addition, DDT was genotoxic towards lymphocytes and mammary epithelial cells of female rats showing an increase in lipid peroxidation, the outcome of the growth level of free oxygen radicals, which lead to an oxidative stress (Canales-Aquirre et al., 2011). DDT also induces cellular and chromosomal alterations in the rat mammary gland, which is consistent with the hypothesis that it can induce early events in mammary carcinogenesis (Uppala et al., 2005). Additionally, beluga whales (*Delphinapterus leucas*) inhabiting the St. Lawrence estuary are highly contaminated with environmental pollutants including DDT which can induce significant increases of micronucleated cells in skin fibroblasts of an Arctic beluga whale (Gauthier et al., 1999).

Regarding human test system, the cytogenetic effect of DDT was investigated both *in vitro* and *in vivo*. *In vitro*, certain DDT concentrations have the effects on human leukocyte functions (Lee et al., 1979), are causing chromosomal aberrations (Lessa et al., 1976), DNA strand breaks (Yáñez et al., 2004), and apoptosis induction which is preceded by an increase in the levels of reactive oxygen species (Pérez-Maldonado et al., 2004, 2005). *In vivo*, DDT is able to induce chromatid lesions (Rabello et al., 1975), increase in chromosomal aberrations and sister chromatid exchanges (Rupa et al., 1989, 1991), DNA strand breaks (Yáñez et al., 2004; Pérez-Maldonado et al., 2006), apoptosis (Pérez-Maldonado et al., 2004) as well as cell cycle delay and decrease in mitotic index (Rupa et al., 1991).

Before 1973 when it was banned, DDT entered the air, water and soil during its production and use as an insecticide. DDT is present at many waste sites and from these sites it might continue to contaminate the environment. DDT still enters the environment because of its current use in other areas of the world. DDT may be released into atmosphere in countries where it is still manufactured and used; it can also enter the air by evaporation from contaminated water and soil and then it can be deposited on land or surface water. This cycle of evaporation and deposition may be repeated many times and as a result, DDT can be carried long distances in the atmosphere (ATSDR, 2002; Crinnion, 2009; Donato & Zani, 2010; Gajski et al., 2007; Torres-Sánchez & López-Carrillo 2007). These chemicals have been found in bogs, snow and animals even in the Arctic and Antarctic regions, far from where they were ever used. DDT can last in the soil for a very long time, potentially for hundreds of years. Most DDT breaks down slowly into DDE and DDD, generally by the action of microorganisms and can be deposited in other places like in the surface layers of soil; it may get into rivers and lakes in runoff or get into groundwater. In surface waters, DDT will bind to particles in the water, settle and be deposited in the sediment. DDT is then taken up by small organisms and fish in the water. It accumulates to high levels in adipose tissue of fish and marine mammals, reaching levels many thousands of times higher than in water. DDT can also be absorbed by some plants and by the animals that can directly impact human population and like that represent a major health threat (ATSDR, 2002; Beard, 2006; Gajski et al., 2007; Gauthier et al., 1999).

All of these findings suggests that DDT is still present not only in poorly developed countries of the Third World but it can still be found in other countries that have banned its use almost 40 years ago due to its stability and long persistence in the environment.

6. Conclusion

Significant levels of DDT and its metabolites can still be found in biological samples of serum, adipose tissue and maternal milk of populations that are not occupationally exposed. People are usually exposed to DDT through food, inhalation or dermal contact. Also, there are evidences on damages to the health, specially related to the reproductive area, and more recently damages at cellular level, as well as, alteration in the psychomotor development of children exposed in uterus. Although there are studies dealing with adverse effects of pesticide exposure there is still great need for elucidating the exact mechanism and health consequences related to DDT exposure and its metabolites. Our data in conjunction with other available data regarding pesticide genotoxicity have identified that DDT induces DNA strand breaks in human peripheral blood lymphocytes *in vitro* as well *in vivo*. In our study, this effect was noted even after the treatment with very low concentration of aqueous DDT. These results also confirms previous findings that DDT induces alterations of the ultrastructure of cells and DNA damage by causing single strand breakage and adducts in DNA molecule. Present study also confirms that combinations of sensitive techniques like alkaline comet assay and cytokinesys-block micronucleus assay are useful for the assessment of cellular and DNA alterations after exposure to mutagens and carcinogens from the environment. Results obtained in this research indicate the need for further environmental and food monitoring, and cytogenetic research using sensitive methods in detection of primary genome damage after exposure to DDT to establish the impact of such chemicals on human genome and health.

7. Acknowledgment

The study is a part of a research project supported by the Ministry of Science, Education and Sports of the Republic of Croatia (Grant no. 022-0222148-2125).

8. References

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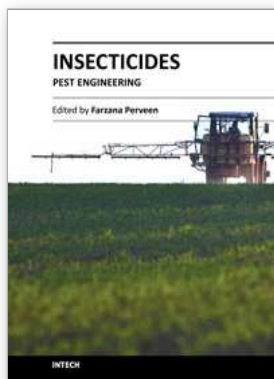
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Insecticides - Pest Engineering

Edited by Dr. Farzana Perveen

ISBN 978-953-307-895-3

Hard cover, 538 pages

Publisher InTech

Published online 15, February, 2012

Published in print edition February, 2012

This book is compiled of 24 Chapters divided into 4 Sections. Section A focuses on toxicity of organic and inorganic insecticides, organophosphorus insecticides, toxicity of fenitrothion and permethrin, and dichlorodiphenyltrichloroethane (DDT). Section B is dedicated to vector control using insecticides, biological control of mosquito larvae by *Bacillus thuringiensis*, metabolism of pyrethroids by mosquito cytochrome P40 susceptibility status of *Aedes aegypti*, etc. Section C describes bioactive natural products from sapindaceae, management of potato pests, flower thrips, mango mealy bug, pear psylla, grapes pests, small fruit production, boll weevil and tsetse fly using insecticides. Section D provides information on insecticide resistance in natural population of malaria vector, role of *Anopheles gambiae* P450 cytochrome, genetic toxicological profile of carbofuran and pirimicarp carbamic insecticides, etc. The subject matter in this book should attract the reader's concern to support rational decisions regarding the use of pesticides.

How to reference

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Goran Gajski, Marko Gerić, Sanda Ravlić, Željka Capuder and Vera Garaj-Vrhovac (2012). Presence of Dichlorodiphenyltrichloroethane (DDT) in Croatia and Evaluation of Its Genotoxicity, *Insecticides - Pest Engineering*, Dr. Farzana Perveen (Ed.), ISBN: 978-953-307-895-3, InTech, Available from: <http://www.intechopen.com/books/insecticides-pest-engineering/presence-of-dichlorodiphenyltrichloroethane-ddt-in-croatia-and-evaluation-of-its-genotoxicity>

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