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UNIVERSITY OF NOVA GORICA
SCHOOL OF ENVIRONMENTAL SCIENCES

INVESTIGATION OF POSSIBLE HEALTH EFFECTS RESULTING FROM THE RECONFIGURATION OF THE MRZLEK WATER SUPPLY

DISSERTATION

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Nova Gorica, 2006

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ABBREVIATIONS AND SYMBOLS

AAS	Atomic absorption spectrometry
ADI	Acceptable daily intake
ADME	Absorption, distribution, metabolism and excretion
AL	Human - hamster hybrid cell line
ALT	Alanine aminotransferase
AST	Asparate aminotransferase
ATSDR	Agency for toxic substances and disease registry
AUC	Area under the blood concentration
AU	Administrative unit
BDCM	Bromodichloromethane
BMDL	Benchmarke dose lover confidence limit
BMD	Benchmarke dose
BSO	Buthione sulfoximine
BTX	Benzene, Toluene, Xylene
BW	Body weight
CAS	Chemical abstracts service
$CHBrCl_2$	Bromodichloromethane
$CHBr_2Cl$	Dibromochloromethane
$CHCl_3$	Chloroform
CICAD	Concise international chemical assessment document
CNS	Central nervous system
DBCM	Dibromochloromethane
DBP	Disinfection by-products
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DWEL	Drinking water equivalent level
EHC	Environmental health criteria
GC-FID	Gas chromatography with flame ionization detector
GC/MSD	Gas chromatography with mass spectroscopy detector
GSH	Glutathione S-halomethane
GST	Glutamate S-transferase
HPRT	Hypoxanthine phosphoribosyltransferase
HTO	Trtiated (^3H) water
IAEA	International atomic energy agency
IARC	International agency for research on cancer
IPCS	International programme on chemical safety
IRIS	Integrated risk information system
IUPAC	International union of pure and applied chemistry
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
LMM	Linearized multistage model
LOAEL	Love-observed-adverse-effect level
MCL	Maximum contaminant level
MCLG	Maximum contaminant level goal
MN	Malignant neoplasm
MOS	Margin of exposure
mRNA	Messanger Ribonucleic acid
NCI	National cancer institute
NHATS	National Human Adipose Tissue Survey
NOAEL	No-observed-adverse-effect level
NTP	National toxicology programme
PAH	Polycyclic aromatic hydrocarbons
PBPK	Physiologically based pharmacokinetic
PCB	Polychlorinated biphenyls

PM_{10}	Particulate matter <10 μm
RfD	Reference dose
ROS	Reactive oxygen species
RSC	Relative source contribution
SIR	Standardized incidence ratio
SRI	Standardized rate of incidence
SMR	Standardized mortality rate
TDI	Tolerable daily intake
THM's	Trihalomethanes
UCL	Upper confidence limit
Uf	Uncertainty factor
US EPA	United States Environmental protection agency
VSMOW	Vienna Standard Mean Ocean Water
WHO	World Health Organization
XAD	Amberlite polymeric adsorbent

SUMMARY

It has been observed that the artificial lake water from the Solkan hydroelectric power plant mixes with the water from the natural spring Mrzlek. This mixing could bring pollutants from the river/lake into the pumped water of the Mrzlek Waterworks. The waterworks supply drinking water to more than 30,000 inhabitants.
In order to evaluate the mixing, water and sediments from the artificial lake, the raw and conditioned water from the Mrzlek Waterworks had been analysed periodically from 1985 to 2005.
The concentrations of some heavy metals, mineral oils, organic substances (particularly the THM's, which are formed during the chlorination process) had been measured by samples.
The genotoxicity of drinking water has been analysed in connection with the presence of THM's.
The incidence of seven different studied types of malignant neoplasms with the exposed and reference populations (population drinking water from the Mrzlek Waterworks and population not drinking water from the Mrzlek Waterworks) had been analysed.
On the basis of chemical analyses of some heavy metals and organic substances and their comparison with the measurements from the previous years, it may be concluded that accumulation of hazardous
substances in sediments is slight.
The constant presence of halogenated hydrocarbons in drinking (conditioned) water has also been observed. The study conducted on the mutagenic potential of drinking water from the Mrzlek Waterworks showed genotoxic effects on the test micro organisms and cells.
Regardless the results of genotoxicity tests, the standardised incidence ratios of the studied malignant neoplasms in the exposed and non-exposed population and their comparison did not show a substantial difference.
The study of the survival function from birth to onset of disease of the patients with certain types of malignant neoplasms within the two observed populations shows the probability of onset of these neoplasms at an earlier age of the exposed population.
Comparing the calculated ages at onset of particular types of neoplasms of cancer patients in the two studied populations of the Administrative unit Nova Gorica shows a longer life period -*without developing a disease* - for the group of patients in the population - not exposed population. It is not clear whether the results are just an outcome of coincidence or some rule in this study. There would be another study needed to research this question. Especially if classical risk factors,such as *smoking, alcohol and dietary habits*, which were not studied in this dissertation, may have influenced the results as confounding factors.
This research suggest the need to investigate the use of other methods for disinfection (UV light treatment, ozonization, irradiation, microfiltration…) of drinking water to determine if they have fewer potential chronic health effects than traditional chlorination disinfection.

IZVLEČEK

Opaženo je bilo, da se voda iz umetnega jezera pri hidroelektrarni Solkan meša z vodo izvira Mrzlek. To mešanje bi lahko prineslo polutante iz reke/jezera v vodo, ki jo črpa vodarna Mrzlek. Vodarna oskrbuje s pitno vodo čez 30.000 ljudi.
Da bi ocenili to mešanje, periodično so bili analizirani vzorci vode in sedimentov iz umetnega jezera ter surove in kondicionirane vode iz vodarne Mrzlek, med leti 1985 in 2005.
V vzorcih so bile merjene koncentracije nekaterih težkih kovin, mineralnih olj, organskih spojin (posebno THM, ki nastajajo med procesom kloriranja). V povezavi s prisotnostjo THM je bila testiranana tudi genotoksičnost pitne vode.
Analizirana je bila pojavnost sedmih različnih tipov malignih neoplazem v izpostavljeni in referenčni populaciji (populacija, ki pije vodo iz vodarne Mrzlek in populacija, ki ne pije vode iz vodarne Mrzlek).
Na temelju kemijskih analiz nekaterih težkih kovin in organskih spojin in njihove primerjave z meritvami iz preteklih let je mogoče zaključiti, da je akumuliranje nevarnih snovi v sedimentih jezera majhno.
Bila je opažena stalna prisotnost halogeniranih ogljikovodikov v pitni (kondicionirani) vodi.
Študija o mutagenem potencialu pitne vode iz vodarne Mrzlek je pokazala genotoksične učinke na testnih mikroorganizmih in celicah.
Standardizirano razmerje incidenc preučevanih malignih neoplazem v izpostavljeni in neizpostavljeni populaciji in njihova primerjava nista pokazala bistvene razlike, ne glede na rezultate testov genotoksičnosti.
Študija funkcije preživetja pacientov od rojstva do pojavnosti določenih tipov malignih neoplazem v obeh preučevanih populacijah kaže verjetnost pojava teh neoplazem pri nižji starosti v izpostavljeni populaciji.
Primerjava izračunanih starosti ob pojavu določenih tipov neoplazem rakavih bolnikov v obeh preučevanih populacijah v upravni enoti Nova Gorica kaže na daljšo življenjsko dobo –*brez razvoja bolezni*- za skupino pacientov v populaciji »preostanek upravne enote Nova Gorica« (neizpostavljena populacija). Vendar v tej študiji nismo ugotavljali, ali so ti izsledki posledica naključja ali kakšne zakonitosti. To bo morala opredeliti neka druga študija. Posebej če so morda “klasični” dejavniki tveganja, ki niso bili preučevani v tej študiji – *kajenje, alcohol, način prehranjevanja* – vplivali na ugotovljene rezultate, kot confounding factors.
Ta raziskava nakazuje, da je treba preučiti uporabo drugih metod (UV žarki, ozonizacija, radiacija, mikrofiltracija …) za dezinfekcijo vode, predvsem pa opredeliti, ali imajo manj potencialnih kroničnih zdravstvgenih učinkov kot tradicionalno kloriranje.

1 INTRODUCTION

1.1 Basic problem

The Mrzlek spring supplies drinking water for more than 30,000 inhabitants in the Administrative unit (AU) Nova Gorica. Because of its location – below the river bed of the Soča river, which became a 20 meter-deep lake after construction of the hydroelectric dam of the Solkan hydroelectric power plant – it is possible that the waters of the river/lake and the spring mix (Figure 1). We can reasonably expect that the river Soča may bring with it numerous pollutants, heavy metals, polyaromatic hydrocarbons (PAH), and organic substances, which in the process of chlorination may produce halogenated by-products (disinfection by-products - DBP). Some of these pollutants are known to be mutagenic and carcinogenic in humans. The effects of these potentially dangerous substances are more likely to appear in the course of lengthy exposure, as is the case with drinking water, which presents an important and constant life-long input.

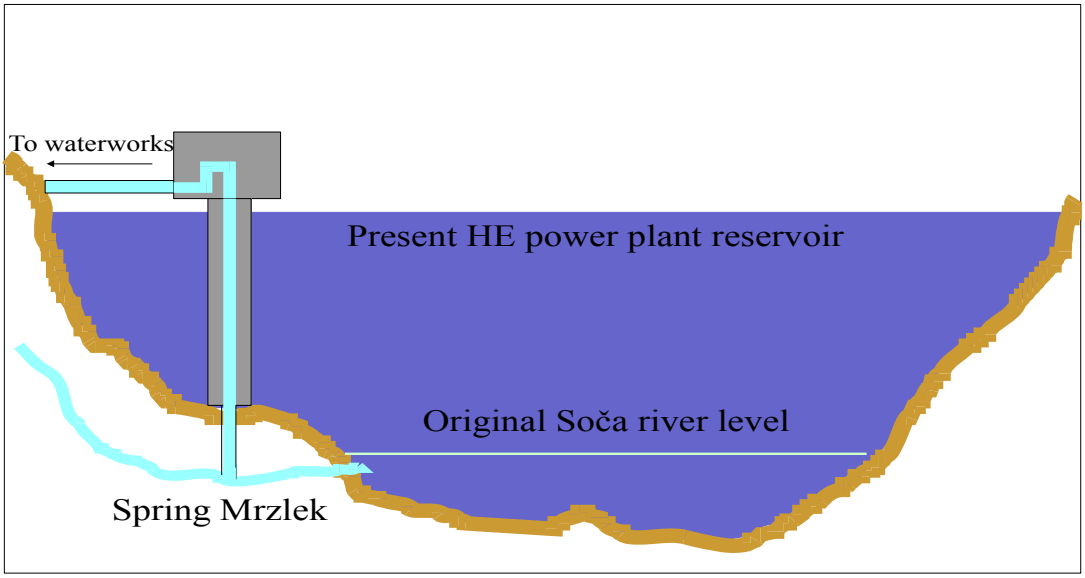


Figure 1: *Schematic diagram of the Mrzlek spring and the Mrzlek Waterworks pump station*

1.2 History of the Mrzlek Waterworks

The area of Trnovo forest and Nanos measures about 700 km^2 (3.4% of the surface of Slovenia). Even though this area is karstic in terms of geological structure, it is one of the biggest water-gathering areas in Slovenia, where 2 m^3/sec of water flow through karstic springs in dry periods and roughly 280 m^3/sec at high water levels. It is a clearly defined area from a relief point of view. It is bordered by the basin of Postojna and the deep valley of Idrijca to the northeast, the valley of Soča to the west and the edge of the Vipava valley to the south.

Mrzlek (there are actually several springs within a few hundred metres of each other) springs from the left bank of the river under the mountain Skalnica, where it reaches the surface just above the Soča water level. During strong downpours the spring is much bigger. Actually, there are two springs. An additional spring diverts the high waters at the right bank. The minimal flow of the spring is about 500 L/sec and maximum about 30 m^3/sec. The influence and correlation between Mrzlek and Soča were never studied thoroughly. They depend on the amounts of precipitation in the Mrzlek area and the Soča regime. The latter largely depends on the melting of the snow in the Julian Alps. The water temperature in Mrzlek is fairly constant, between 8 and 12°C, the ratio between Ca and Mg is between 5 and 12 in Mrzlek and between 2.5 and 4 in the Soča river (1).

A fountain was built in Gorica in the square Travnik with the financial aid of the empress Maria Theresa in 1756. The fountain got the water from the spring Jerebica above Kromberk, and can be considered a forerunner of the Mrzlek Waterworks. Wooden pipes were used in the beginning. These were replaced with lead pipes, manufactured in the Rajbelj lead mine upstream along the Soča and Koritnica. Cast iron pipes were substituted for the lead pipes in 1851. Renovation of the waterworks in 1906 allowed for a daily supply of 2,000 m^3 of water to Gorica, which was not sufficient. The Mrzlek Waterworks was built in 1935 with a capacity of 10,000 m^3 of water per day. The old Kromberk water system was joined to the Mrzlek network. The first hydrologic studies of Mrzlek were conducted after 1928, when the spring was examined regarding the amount and quality of raw water and the relation with the river Soča.

A concrete well was built on the left bank of Soča at an altitude of 50 m above sea level, to pump the strongest Mrzlek spring. Three centrifugal pumps pumped the water to the water plant at an altitude of 150 m. The water station was built with a capacity of 120 L/sec (max. 210 L/sec). It had a pool to slow down water flow, a sedimentation pool, a command room for coagulant dosage (Al-sulphate) a chlorination facility for disinfection, sand filters and an accumulation reservoir.

The foundation stone for Nova Gorica on the former Yugoslavian side was laid on September 13th 1948, and Mrzlek has been supplying the whole of Nova Gorica and a part of Gorica since then, the latter with about 5,000 m^3 of water per day. The Mrzlek Waterworks underwent a few reconstructions since then, the last in 1971 with the calculation of specific consumption of drinking water: 600 L/per capita/day and with the projection of supply – 45,000 people in the beginning of the 21st century.

Figure 2: *Solkan hydroelectric power plant*

Plans for the use of the hydro energy of the Soča existed at the time. The first investment plan for the Solkan hydroelectric power plant was made in 1966. The project of the power plant and artificial accumulation lake was completed in 1984 (Figure 2).Construction of the power plant and artificial accumulation lake made necessary a major reconstruction of the Mrzlek pumps and treatment station in the early 1980's. At that time, the treatment capacity was increased from 120 L/sec to 370 L/sec, and possible negative effects of the water from the artificial lake on the Mrzlek raw water had to be taken into account. The level of the Soča river rose 20 m, from an average level of 57 m above sea level to 77 m above sea level with the construction of the Solkan hydroelectric power plant. The Mrzlek spring was submerged in the process (Figure 3) making necessary the construction of a new water station with a capacity of 250 L/sec and a more modern design adjacent to the old one. The new station would allow treatment of polluted water from the Soča and the artificial lake. A new pumping station was built above the level of the lake and submerged pumping well. The new station next to the old waterworks contained a dynamic sedimentator, a contact reservoir (for the treatment of water with ozone), sand filters and filters with active carbon (Figure 4). The old and the new warterworks complemented each other with a common main water reservoir and common disinfection of treated drinking water with chlorine (2).

***Figure 3**: Mrzlek Waterworks pump station*

***Figure 4:** The old and the new Mrzlek Warterworks*

Investigations by experts from the Institute for Research of the Karst of Slovenian Academy of Sciences and Arts (SAZU) determined the areas of water springs on the edge of the western high karst, and the actual flow of precipitation water. Parallel measurements of precipitation, water flow and

evapotranspiration were carried out for longer than 2 years, and a water balance for the Trnovo-Banjšice plateau (western high karst) was established. The area is considered the water-gathering area of the Mrzlek spring. On the basis of isotopic and physical-chemical analysis of precipitations, spring waters and water from rivers it was discovered that most of the water flows into the Soča (Mrzlek), a smaller part into the Idrijca and the smallest fraction into the Vipava. The measurements of temperature, chemical parameters and isotopes at the Mrzlek spring and pumping station showed that the mixing of Mrzlek spring water with Soča water (artificial lake) moves deep into the spring (3).

1.3 Malignant disease - possible health effect caused by pollution of the water supply

Several sources and reports relate environmental factors to the health condition of the human population. Many malignant diseases are connected with food and water. Water is an important factor because of the constant exposure. Numerous organic and non-organic substances are known to have toxic (non-genotoxic), genotoxic, mutagenic or carcinogenic effects on human health. Three prominent institutions: International agency for research on cancer (IARC), United States Environmental protection agency (US EPA), and National Toxicology Program (NTP) classifying many:

- Agents and groups of agents,
- Mixtures,
- Exposure circumstances,

as carcinogens, probably carcinogens or possibly carcinogens to humans.
The primary question is always: does the agent induce tumours via a genotoxic mechanism? Genotoxic effects are considered to be threshold-independent. Each dose increment is believed to cause an addition to the background frequency, even at very low doses (4). Theoretically, an agent can cause carcinogenesis via tissue damage, hormonal disturbances, immune system disturbances or somatic mutations. An excess of cell multiplication over cell death is a crucial characteristic of neoplastic cell populations. The size of tissues is determined by both cell replication (rate α) and cell death (rate β) (5). The life span of normal cells in tissues and in cell-culture is approximately 40 generations (cell duplications). During this time, the accumulation of mistakes in various parts of these cells leads to their ageing and death (6,7). During carcinogenesis, these processes are disrupted and cancer cells can live longer, thus increasing the chances for further damage and changes of their DNA. Cancer cells are often described as immortal, but this naming is not completely accurate, as cancer cells die in all tumors, in quick-growing ones as well. It is therefore more suitable to speak of an extended life rather than of immortality in connection to cancer cells (8,6,9). According to these events, the genes involved in carcinogenesis can be divided in two groups. The first group are the genes affecting the length of cell life and the second those affecting the sensitivity of cells to outside stimuli. The genes of the first group code for nuclear proteins (*p53* and *myc*) (6,10,11), whereas those of the second group code for extranuclear proteins (*ras* and *erbB2*) (6,12). An exception is the mitochondrial protein *Bcl2* with an important role in the process of apoptosis, the loss of which extends the life of cells (6,13).

Certain parts of the DNA (oncogenes) can speed up the genesis and development of different types of cancer (6,8,14,15)). Certain oncogenes can trigger the genesis of cancer by themselves (HER-2/*neu*), but in most cases, at least two are needed (6).
Oncogenes work as positive regulators of cell proliferation and dominant expression is characteristic for them. Intra-chromosomal amplifications of specific loci as well as chromosomal translocations, specific for particular tumors, can be identified (16,17). The loss of function of a particular gene can also be carcinogenic (6,8,14). The results of experiments with the fusion of healthy and cancer cells have shown that the phenotype of the healthy cell dominates over the phenotype of the cancer cell. Cancer cells thus have no factors with a prominent role in normal cell functioning. The healthy cell provides these factors at fusion, after which the phenotype of the cancer cell is suppressed (6,8,15,17,18).

After lengthy breeding in cell cultures, these normal hybrids can often loose some chromosomes and acquire characteristics, typical of cancer cells and tissues. These losses are due to a later loss of some anti-oncogene at a normal chromosome. There are several genes with such inhibiting properties and many of them regulate cell proliferation and cell death. They are known as receptor genes or as the already mentioned anti-oncogenes.

That cancer genesis and development actually happen, the necessary changes must occur on both copies of the tumor-suppressor gene within the normal genome. The so-called " two-hits" model supposes that in an inheritable form of cancer, one "hit" (mutation) is inherited and present in all somatic cells, whereas the second "hit" in some of these cells is purely coincidental and can also appear early. Thus, both hits enable early cancer genesis. Examples of such tumor-suppressor genes are the *Rb1* gene, which causes retinoblastoma in children in its mutated form, and the *apc* gene, that causes colon cancer (19,20,21,22).

Finally, it must be mentioned that during carcinogenesis, changes occur in DNA fractions that do not code for proteins as well. Mutations also occur in microsatellite DNA, and these changes are present in cells of colon and endometric cancer, where they are linked to simultaneous changes in the reparative mechanisms of these cells (6,23,24).

In many tumours, the rates of both cell birth and cell death are increased over those in the tissue origin. Cell death may occur through active mechanisms:
programmed cell death – apoptosis. The term "apoptosis" was originally used to classify on morfological grounds a type of cell death characterized by condensation and fragmentation of cytoplasm and chromatin. However, other types of active cell death exist, in which cytoplasmic degradation by lysosomal, autophagic or proteasomal mechanisms may dominate. Active cell death is regulated by survival factors and death signals. Many exogenous and endogenous causes stimulate cell birth and also may inhibit cell death. Endogenous factors also exist which induce active cell death; these include transforming growth factor β1, CD95 or Fas ligand and tumour necrosis factor. Active cell death can be triggered in two principal ways:

- by toxic chemicals or injury leading to damage of DNA,
- activation or inactivation of receptors by growth-regulating signal factors (5).

Increases in cell proliferation or in cell survival induced by a chemical do not necessarily lead to cancer. Nevertheless, the widespread occurrence and relevance of active cell death in science was recognised and described more than 30 years ago (25). Cancer researchers became interested when they recognized that tumours can exhibit high rates of (active) cell death and that tumour promoters may selectively increase the survival of preneoplastic cells (25,26,27,28).

Many chemicals that induce or accelerate tumor formation affect the rates of proliferation and/or death of cells in their target tissues. Two essentialy different effects of compounds can be distinguished (29,30):

- the first is associated with the stimulation of specific tissue functions,
- the second type of effects of chemicals is associated with cell injury, cell loss, inflammatory responses and regenerative growth.

Carcinogenesis is a multi-stage process. These stages must be known for correct and timely measures to be taken:

1) Initiation is a process involving the development of the carcinogene and its subsequent binding to the DNA with an uncertain life course of affected cells due to the effects of the agent. It can be modified by active cell death in two ways: the first is elimination of cells carryng promutational lesions, which may be at risk for initiation, and the second way in which initiation can be modified is by the elimination of cells that are already initiated.
   This notion appears to contradict the "dogma" that initiation is irreversible, but, for mathematical and statistical reasons, a certain probability of extinction of initiated cells or clones must be assumed if $\beta>0$; this probability is expressed as $\beta : \alpha$ (31). Thus, with increasing rates of cell death (β), initiated clones will be extinguished with increasing probability. For $\beta>\alpha$, the (asymptotic) probability of extinction is 1 (32).
   The time required for elimination depends on the size (cell number) of the clone.

2) Promotion is a reversible process, in which clonal changes and proliferation of cells take place. Tumour promotion provides a selective growth advantage to initiated cells and enhances the rate of expansion of initiated cell clones. There are many underlying mechanisms. They can be classified into two broad categories: initiated cells may either be oversensitive to the stimulatory effects of agents, such as hormonal or hormone-like effects, or they may be less sensitive to the cytotoxic or mitoinhibitory effects of agents (29).

3) Progression is an irreversible process, where genetic changes are fixed, chromosomal abberations accumulate and benign injuries transform into maligns.

As said previously, cancer is related to DNA changes, which can be the formation of DNA adducts, oxidative damage of the DNA and failure of the DNA reparation mechanisms, or cancer is related to mutations, either by the activation of proto-oncogenes, or the inactivation of tumour-suppressor genes. This also means: “carcinogenes are mutagenes” (Bruce Ames), as most carcinogenes are also genotoxic. Mutagenesis is a process that can be caused by spontaneous modifications or due to exogenous factors following the same pattern in both cases. A mutagene (depending on absorption, distribution, metabolism) causes primary damage of a gene. The continuation develops in three directions:

a) Automatic reparation mechanisms repair the damage,
b) The cell dies,
c) The cellular damage is fixed.

DNA and chromosome damage may cause cell death, rather than mutations (4). If the fixed damage replicates, a mutation occurs first, which can die or become manifested as a mutated phenotype of somatic or germ cells.

This means that the cell and not the agent cause the mutation.

The ultimate consequences of genetic alterations in human cells are:

1) Germ cells -
- Reduced fertility,
- Reproductive failure (including fetal loss and malformation),
- Genetic (inheritable) disease;

2) Somatic cells -
- Cancer (and possibly other somatic genetic diseases).

Continued research is necessary because changing environmental factors, lifestyle, hereditary factors and coincidence and their interdependent effects influence the origin of cancer. Many authors maintains that most carcinogenic substances can be associated with the lifestyle (diet, smoking, alcohol, infections, exposure to the sun, etc**.)** (33). The most important causes of human cancer – malignant diseases are (34):

- Diet and nutrition – 35%, especially too much fat (which can include numerous dissolved substances), grilled meat (the formation of poisonous substances such as nitrosamines during the grilling process), spicy and smoked foods, a shortage of fruits and vegetables;
- Tobacco smoking – 30%, the smoke contains at least 50 carcinogenous substances;
- Chronic infections – developing countries 23%, developed countries 9%; this always means an irritation of the tissue and cells, which can be an important factor in the process of carcinogenesis. These agents provoke cancer by causing chronic inflammation and/or production of mutagenic compounds;
- Occupational exposures – 5%, among the numerous substances it is important to mention asbestos, the rubber industry, dioxines, polyaromatic hydrocarbons (PAH);
- Alcohol drinking – 3%;
- Environment pollution – 2%, pesticides, industrial contamination;
- Genetic susceptibility – up to 4%;
- Reproductive factors and hormones;
- Immunosuppression;
- Radiation;
- Medicinal drugs;
- Food contaminants – the burden of cancer attributable to food contaminations is difficult to quantify.

Cancer is one of the principal causes of death today. Close to 7 million people will die of cancer this year (2006) in the entire world – about 20% of all deaths (9 million new diagnoses every year). It is estimated that in 2020, 11 million people will die of cancer, 7 million in developed countries (35). This is a major challenge for public health.

Extreme care must be exercised regarding life-long exposure. For each hazardous substance, the Acceptable daily intake (ADI) and reference dose (RfD) must be defined or known. RfD means dose supposed to be without any harmful effect. This always demands risk assessment steps to be taken. Risk assessment steps are a series of steps, a scientific algorithm that is the basis for a final decision about the protection of public health. How is the margin of exposure or margin of safety (MOS) determined? The first step is Hazard identification – identification of the inherent toxic properties of a substance (identify critical affect). It is necessary to describe dose – response relationships and identify no-observed-adverse-effect level (NOAEL). The second step is Hazard characterisation (dose – effect/dose – response assessment or characterisation of the relationship between dose and response). The third step is Exposure assessment, which means a qualitative and quantitative assessment of exposure of a particular population. Here epidemiological data of the health state and mortality rates of the population must be used. Out of this follows the last step - risk characterisation and determination of Rfd and ADI:

RfD or ADI = NOAEL / uncertainty factor (Uf), {1}

where this factor (Uf) could be 10, 100, or 1000 (10 per variations between individuals - like kinetics, dynamics; 10 per intraspecies variations - human↔human; 10 per interspecies variations - animal↔human).

If the result (risk characterisation) > 1 measures must follow – risk reduction.

As stated above, the exposure of human population to environmental contaminants is associated with elevated risk for cancer development. Heavy metals constitute an important class of environmental carcinogens, although the mechanisms underlying their activity are unclear. For example, some compounds of cadmium (Cd), chromium (Cr), nickel (Ni) and arsenic (As) are potent mutagenes in animals and are suspected to be human carcinogens of increasing environmental and occupational concern (36). The genotoxic potential of some Cd compounds and some lead (Pb) compounds in mammalian cells is rather weak and restricted to high cytotoxic concentrations (37).

Many scientific studies on the potential dangers of various substances, chemicals and other environmental factors were published in the recent years. Assesments of the effects on the human health for the exposure to 200 chemicals (Table 1 – potential effects of some of these substances) were published in the documents of the WHO – Environmental Health Criteria Documents (38,39,40,41):

Table 1: *Potential effects of some substances and chemicals on the health of humans*

Health effect	**Endangered group**	**Main chemicals**
Cancer	Dependant of gender, elderly, young, children	Asbestos, PAH, substituted-PAH, benzene, heavy metals, radon, natural toxins, dioxin, BTX, endocrine disruptors
Cardiovascular diseases	Elderly	PM-10(respirable particles), CO, As, Pb, Cd, Co
Respiratory system diseases	Children, asthmatic patients	PM-10, SO_x, NO_x, O_3, Ni, Cr
Alergies and increased sensitivity	Children	PM-10, O_3, Ni, Cr
Reproduction	Embrio, young people	PCB, DDT, dioxines, phtalates, Pb, Hg
Nerve system disturbances	Embrio, children	Methyl-Hg, Pb, Mn, Al, organic solvents, dioxins, PCB
Osteoporosis	Elderly	Pb, Cd, Al, Se, endocrine disruptors

This information can be a solid foundation for the assesment of health effects as well as the preparation of guidelines and recommendations for sanation and prevention actions.

Exposure of human population to environmental contaminants is associated with elevated risk of cancer. Heavy metals (As, Cd, Cr-VI, Ni) constitute an important class of environmental carcinogens although the mechanisms underlying their activity are unclear (37).

The harmfulness of **arsenic** to the skin was already known in medieval times. In the past century, attention has been drawn to the incidence of skin carcinomas in zinc smelting facilities. The reason was supposedly arsenic, present in unrefined ore. Cancer of the skin was also reported in psoriatric patients, treated with arsenic compounds (42). Arsenic is ubiquitous in nature. It is found in the air, soil and water. It is most often found as arsenic trioxyde (As_2O_3). It gets into water by the washing of minerals and ore, with industrial wastewater and also with deposition from the atmosphere. In food, it is found especially in fish and meat. After ingestion, elementary arsenic is poorly absorbed through the digestive tract and is mostly excreted in an unchanged form. Soluble forms of arsenic are quickly absorbed from the digestive tract and almost completely excreted through the kidneys after methylation. Inorganic forms of arsenic can accumulate in the skin, bones or muscles. The half-life in humans is 2-40 days. Both natural forms of arsenic (three- and five-valent) are toxic. Normally, compounds of three-valent arsen or arsenite are more toxic. In the organism, arsenic is bound to sulphhydrile (-SH) groups of enzymes in the cell protoplasm, crucially affecting the cell metabolism. Skin carcinoma with multiple localisations has been described after the exposure to arsenic. Cancer of the lungs, hemangiosarcoma of the liver and fibrosis of the liver have also been described after exposure to arsenic (38). It is well established that inorganic arsenic is causally associated with lung cancer via inhalation and skin cancer via ingestion. Epidemiological evidence suggests that ingestion of inorganic arsenic may also cause other more fatal internal cancers, with the highest relative risks reported for bladder cancer (43,44). There is strong evidence of an increased risk of bladder, skin and lung cancers following consumption of water with high arsenic contamination (45).
There is very interesting to know arsenic carcinogenicity in animals. The review of arsenic carcinogenicity by IARC (46) lists for different species (mouse, rat, dog, and rabbit) given various arsenic compounds by different routes of exposure. There was no consistent demonstration of arsenic carcinogenicity in these studies.

In the literature there are two well-documented cases of severe but hidden industrial polluting by heavy metals which occured in Japan in the 1950s. In medical practice, the cases are known as syndromes, dubbed Mina-Mata and Itai-Itai; the first after the gulf of Mina-Mata (the cause was polluting of seawater by Hg) and the other by the events on the Jinzu River. The second syndrome was named after the Japanese word “itai” which means “ouch” or “painful” in English. The pain results from unusual changes in bone with multiple fractures that can be caused by the slightesr external pressure, such as coughing, and skeletal deformation takes place (47). The patients resided only in the area around the Jinzu River, the water of which is used for irrigation of rice fields. The river was polluted heavily with industrial sewage with a high concentration of cadmium (Cd). There had also been sizeable polluting of the water and underground water (thus also drinking water). Cadmium competes with calcium (Ca) in the metabolism and replaces it in the bones.

Mercury has three chemical forms: elemental mercury (Hg^0), inorganic mercury salts (Hg^+, Hg^{2+}), and organic mercury – methylmercury. Although each form of mercury can have specific toxic effects in human, each form undergoes conversion to one another. Mina-Mata disease was the first episode of epidemic intoxication occurring in an extraordinarily large area and affecting a large number of patients. The cause of disease was methylmercury formed in the marine sediments when mercury contained in wastewater and discharged from a chemical plant (acetaldehyde plant) into the sea facing Minamata. Methylmercury was concentrated in food chains and accumulated in fish. Fish was a major source of animal protein for the inhabitants in those days, almost all of them ingested methylmercury-contaminated fish (48).
Even though the degree of contamination of the environment by Hg in the area of the river Idrijca has decreased since the closure (in 1977) of the mercury mine, the consequences of centuries of mining are very high concentrations of Hg in the sediments of rivers (Soča, Idrijca) and increased concentrations of Hg in indicator organisms (shells, crabs, fish) all the way to the Gulf of Trieste (49). Consequences of physical remobilisation of abandoned mining and foundry residues are increased concentrations of heavy metals in alluvial sediments downstream and can be a substantial source of river pollution (50). A part of mine tailings and roasted ore remains of the Idrija mercury mine was deposited in the bed of the Idrijca River that transported the load downstream at high waters. It was deposited in alluvial sediments of Idrijca and Soča Rivers, and in the Gulf of Trieste, essentially contributing to mercury pollution of the environment (51). The exploitation of mercury ore in Idrija lasted from 1490 to the end of the 20th century. 12,760,700 tons of ore were excavated (52) in the 500-year period, containing 145,000 tons of mercury, but the obtained amount of mercury was actually

107,500 tons. The difference of 37,500 tons was lost (53), being mostly carried downstream to the sea by the Idrijca and Soča rivers.
The toxic effects of methylmercury are selective to the nervous system. The central nervous system and peripheral nerves are both affected. The results of the biopsy of *nervus suralis* were characterized by the general loss of myelinated fibres with a relative increase in small-size myelinated fibres (48,54).

As said previously, the genotoxic potential of **Pb and Cd** in mammalian cells is rather weak and restricted to high cytotoxic concentrations (37). They are among the most common elements in the toxicology of metals, both in acute and chronic poisonings. In the environment they appear in increased concentrations in the vicinity of metallurgic facilities in the gathering of lead and zinc. The reasons for increased levels of Pb and Cd in the organism can also be contaminated food and water. The intake of these elements in the body occurs through the respiratory and digestive systems. The typical daily intake is: 100 – 200 μg Pb/day in 10 – 35 μg Cd/day.

The presence of Pb and Cd in the sediments of the rivers Koritnica (tributary of the Soča upstream from Mrzlek) and Soča all the way to the Adriatic Sea and also in traces in the filter sand of the Mrzlek Waterworks (Tables 5 and 9) can be linked to centuries of mining in the Rajbelj lead mine in Italy (closed since 1988), as untreated waste water was diverted to the stream Roja, which is a tributary of the Koritnica, which is in turn a tributary of the Soča. Examining the results of the analyses, carried out by Institute Jožef Stefan experts in 1985 (55), it can be found that even river water of the Roja, Koritnica and Soča contains concentrations of Pb and Cd than rank these rivers in an inferior quality class according to the norms of the period (56). The concentrations of these metals in the Koritnica and Soča sediments being rather high:

- Cd: Koritnica 23.2 mg/kg; Soča 3.8 mg/kg;
- Pb: Koritnica 276 mg/kg; Soča 157 mg/kg.

These microelements can be expected in drinking water in very small concentrations, even under the level of lab detection (but in Table 3 it is notable that eg. the element Zn was identified in a substantial concentration) as most of these traces remain in the filter sand. But drinking water is still filtered through sand, in which these microelements can be traced, as the Soča permanently brings them with its flow and mixes them with the raw water from the Mrzlek spring.

While the epidemiological evidence to implicate lead as a human carcinogen is not conclusive, the findings are suggestive. Significantly inctreased rates of lung cancer, as well as cases of kidney and stomach cancer, have been documented in occupationally-exposed battery, smelter, and pigment plant workers, as well as plumbers and pipefitters exposed to lead solder fumes (46).

The genotoxicity of these heavy metals (especially Cd) in mammalian cells can be determined by mutation induction at the HPRT[1] locus and the type of induced mutations can be determined by mutation spectra analysis using mutants generated from the same population of exposed cells as those used in the studying of inhibition of DNA repair. In human-hamster hybrid cell line the mutation induction can be studied at the S1 locus of the human chromosome 11 as described by Hei *et al.* (57,58).
Cadmium and its compounds are known human carcinogens. In addition to cancer, they induce a wide variety of other adverse health effects. The mechanisms by which cadmium induces cancer are poorly understood, and this hampers accurate risk assessment upon exposure. The main difficulty is to assess the degree of involvement of cadmium and its mechanisms of action in the different stages of cancer development, namely in cancer initiation, promotion and progression. Although multiple pathways, including modulation of gene expression, signal transduction and inhibition of DNA methylation, have been proposed, two mechanisms seem to have a predominant role at the molecular level:

1. Induction of reactive oxygen species (ROS); and
2. Inhibition of DNA repair.

Increased generation of intracellular ROS is linked to mutagenesis, changed cell signaling and apoptosis, while inhibition of DNA repair processes increases genetic instability, consequently leading

[1] HPRT hypoxanthine phosphoribosyltransferase is an enzyme responsible for incorporation of thymidine in DNA. Mutation in HPRT gene makes cells resistant to base analogue hypoxantine. In the presence of the analogue only mutated cells can form colonies one reason being that cadmium-containing products are rarely re-cycled, but often dumped together with household waste.

to the increased probability for mutations. Filipič *et al.* have discussed the role of cadmium-induced ROS and interference with DNA repair in the genotoxicity and mutagenicity of this carcinogenic metal species (59).
Naturally, cadmium occurs in ore together with zinc, lead and copper. Cadmium compounds are used as stabilizers in PVC products, colour pigment, several alloys and now, most commonly, in rechargeable nickel-cadmium batteries. Metallic cadmium has mostly been used as an anti-corrosion agent. Natural sources of cadmium to the atmosphere are volcanic activity, forest fires and windblown transport of soil particles. Anthropogenic sources of cadmium to the environment are refining and use, copper and nickel smelting, fossil fuel combustion and phosphate fertilizers, which may contain high concentrations of cadmium. During the 20th century, cadmium emissions have increased dramatically,

Major occupational exposure to cadmium occurs in non-ferrous metal smelters, in the production and processing of cadmium, and in the recycling of electronic waste. Non-occupational exposure is mainly from cigarette smoke, which contains relatively high concentrations of this element. For nonsmokers who are not occupationally exposed, diet is the main route of exposure to cadmium (60). Absorption of cadmium is strongly dependent on the route of exposure (61). Only about 5% of an oral dose is absorbed by the gastrointestinal tract and > 90% of the dose is absorbed from the lung. Cadmium is absorbed through the divalent metal transporter-1 (DMT-1) and, regardless of the route, once absorbed is rapidly cleared from the blood and concentrates in several tissues. Cadmium accumulates primarily in the liver and kidney because these tissues can produce large amounts of metallothionein (MT), to which cadmium tightly binds (62). Cadmium has an extremely long biological half-life (30 years), which makes it a cumulative toxin, and to date, there is no proven treatment for chronic cadmium intoxication (61).

Cadmium has been classified as a human carcinogen by the International Agency for Research on Cancer and by the US National Toxicology Program (63,64). This classification is based on data of multiple studies that have linked occupational exposure to cadmium with pulmonary cancer in humans (65). There are also studies that indicate a role of cadmium in human renal, liver, haematopoietic system, bladder and stomach cancer in humans (65, 66,67,68).

Cadmium compounds, however, are potent clastogens. In different mammalian cells, exposure to cadmium compounds induced chromosomal aberrations, sister chromatide exchange, and DNA damage, eg, DNA single- and double-strand breaks and DNA/protein crosslinks (69,70,71,72,73,74,75). These effects are generally restricted to high, cytotoxic doses.
Puck *et al.* used human - hamster hybrid (AL^2) cell mutagenicity assay, which is highly sensitive in detecting both intragenic and multilocus mutations. AL cells contain a standard set of hamster chromosomes plus a single human chromosome 11, which encodes a series of human cell surface antigens (76). Using this test system, Filipič *et al.* found cadmium to be a potent mutagen (77).

Cadmium induced ROS formation, which is similar to hydrogen peroxide (H_2O_2) and superoxide anions, are continuously generated during oxygen metabolism. While neither molecule reacts directly with DNA, they are converted into the highly reactive hydroxyl radical via Fenton-type and Haber/Weiss-type reactions in the presence of redox-active transition metals. Radical attack on DNA can produce DNA strand breaks and altered bases, which could lead to mutations and eventually tumour development (78,79). The exact mechanism by which cadmium initiates ROS formations is not clear. Cadmium can decrease intracellular glutathione content and reduce the activities of cellular anti-oxidant enzymes, superoxide dismutase, peroxidase and catalase, which lead to the accumulation of ROS and an increase in intracellular oxidative stress in cadmium exposed cells (80,81).

The role of ROS in $CdCl_2$ mediated cytotoxicity and mutagenicity in AL mutagenicity test system
Cadmium induced mutagenicity was studied using two complementary approaches. In the first approach, AL cells were exposed to $CdCl_2$ in the presence of free radical scavenger and in the second approach, cells were depleted of intracellular glutathione before exposure to $CdCl_2$. Concurrent treatment with free radical scavenger (0.5% DMSO) reduced the mutagenic potential of $CdCl_2$ by 2.5-fold, but had no effect on the cytotoxicity of $CdCl_2$ (77). This finding, that cadmium-induced mutations were suppressed by the hydroxyl radical scavenger DMSO, is consistent with observations

[2] The AL human-hamster hybrid cell line contains a standard set of CHO-K1 chromosomes and a single copy of human chromosome 11 (72). Chromosome 11 contains the *CD59* gene that encodes the CD59 cell surface antigen that renders AL cells sensitive to killing by specific monoclonal antibody E7.1 in the presence of rabbit serum complement. In the presence of antibody and rabbit serum only mutated cells can grow

that anti-oxidant enzymes, such as superoxide dismutase and catalase, as well as d-mannitol and trolox, suppressed cadmium-induced DNA single-strand breaks (72,75), chromosomal aberrations (69), and gene mutations in *hprt locus* (82).

In the second approach, cultures were pre-treated with the thiol-depleting drug buthione sulfoximine (BSO), a competitive inhibitor of g-glutamyl cysteine synthetase, to reduce intracellular glutathione before treatment with $CdCl_2$. Glutathione and other low molecular weight thiols, such as cysteine and cysteamine, are considered to have significant free radical scavenging abilities that contribute to the maintenance of cell integrity (83). Although a decrease in cellular glutathione by itself may not result in cell death, its depletion has been shown to enhance the cytotoxicity and mutagenicity of various agents, including ionising radiation, heavy metals, some chemotherapeutic drugs and asbestos (83,84,85). Pre-treatment of AL cells with BSO increased both the cytotoxicity and mutagenicity of $CdCl_2$.

Current evidence indicates that under specific experimental conditions, cadmium has substantial mutagenic potency, however the relevance of this finding for cadmium carcinogenesis has to be elucidated. In a simplified model, the mechanism of cadmium mutagenesis is depicted by a sequence of events where cadmium produces intracellular ROS, which can directly generate DNA damage (shown below in Figure 5; to sum up ref. 59):

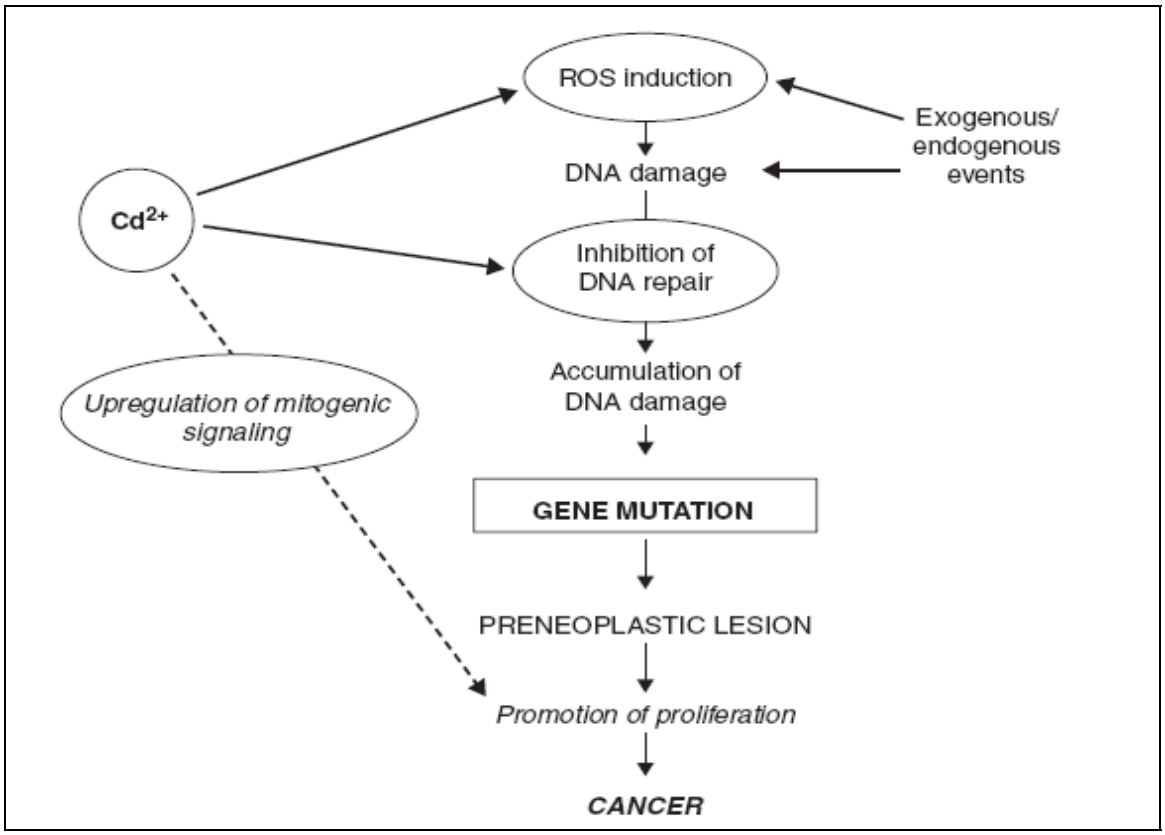


Figure 5: *sequence of events to produces intracellular ROS (59)*

Concurrent cadmium mediated inhibition of DNA repair leads to the accumulation of DNA damage generated by ROS as well as DNA damage induced by spontaneous events, and exogenous DNA damaging agents, which in turn increase the mutation rate. This model is supported by evidence from the epidemiological study of workers co-exposed to cadmium, lead and cobalt, where increased levels of DNA single-strand breaks in blood cells correlated to cadmium exposure and cadmium levels in blood (86). In addition, this study showed that the capacity of blood cells to repair 8-OHdG adducts decreased with increasing cadmium exposure and inversely correlated to the level of DNA strand breaks.

Mutagenicity assays have been used to study the mutagenic potential of drinking water. The epidemiological investigation of the relation between exposure to chlorinated drinking water and cancer occurrence is problematic because any increase in relative risk over that in people drinking unchlorinated water is likely to be small and therefore difficult to detect in epidemiological studies. It is particularly important to obtain valid assessment of disease status, of confounding factors and of the level of exposure to chlorinated water. Nevertheless, many of epidemiological studies have shown that a correlation between genotoxicity of drinking water and increased cancer risk, exist (87,88,89). Morris *et al.* (90) measured (by meta-analysis) of exposure to chlorination by-products in the drinking water and cancer. The data support a significant association between bladder cancer and exposure to DBP in drinking water. The results from Dunnick and Melnic study (91) suggest that organic by-products of chlorination are compounds of greatest concern in assessment of the carcinogenic potential of chlorinated drinking water. In the workshop report (92) many of experts agree with the opinion that the sites of cancers most frequently associated with chlorinated water are bladder, colon and rectum. Morales Suarez-Varela *et al*. (93) found an association between stomach and bladder cancer mortality with the type of water source in Valencia Province. Koivusalo and Jaakkola (94) found statistically significant exposure-response association between exposure and incidence of bladder, kidney, and stomach cancer. In an ordinary municipality using chlorinated surface water, this exposure would indicate relative risk of 1.2 for bladder cancer and of 1.2 to 1.4 for kidney cancer compared with municipalities where nonmutagenic drinking water was consumed. In another case-control study (95) in Ontario, Canada King and Marrett found the relationship between bladder cancer and exposure to chlorination by-products in public water supplies. Odds ratios (OR) adjusted for potential confounders were used to estimate relative risk. Those exposed to chlorinated surface water for 35 or more years had an increased risk of bladder cancer compared with those exposed for less than 10 years. These results indicate that the risk of bladder cancer increases with both duration and concentration of exposure to chlorination by-products. In the similar case-control study McGeehin *et al*. (96) found similar results. The OR for bladder cancer increased for longer duration of exposure compared with no exposure.

Many of these studies have reported the mutagenicity of tap water, river water, lake water and ground water using the Ames assay (97,98,99,100) or other short-term epidemiological - biological tests. Halogen substituted methanes are mutagenic in bacteria and carcinogenic in rodents (101,102,103,104). In addition the presence of trihalomethanes (THM's) in drinking water has been related to the incidence of cancer in humans (105). The source of halogen substituted methanes (THM's) in drinking water from Mrzlek is the pollution of water resources due to an extensive use of these substances in agriculture and industry, where they are used as pesticides, solvents, propellants in aerosols, coolants, anesthetics, etc. (106).

Water pollution by organic pollutants is one of the most critical problems concerning drinking water bodies. Many ecological studies correlated age and gender adjusted regional human mortality rates with water treatment practices (107). These studies suggested an association between chlorination and the development of cancer of digestive or urinary malignoma neoplasms, particularly of the colon, rectum and urinary bladder. Because of the correlation at a community level (as opposed to an individual level), and the inability to control for most prominent confounders, these studies could only suggest associations and were unable to provide definitive evidence for an association. The presence of genotoxic substances presents a serious problem since large populations are exposed. Additionally, single and combined biological effects for most pollutants are not known. A possible way of approaching the problem of risk assessment is to use biological tests that have a non-specific response to the pool of pollutants present, but are reliable in the proving of these pollutants.

This thesis deals with the quality of drinking water from the Mrzlek spring and its possible pollution, as well as with the effects on the incidence rate of certain malignant diseases of the population drinking this water. On the basis of the chemical analyses of water and sediments from the artificial lake at the Solkan hydroelectric power plant, of raw and conditioned water from the Mrzlek Waterworks, genotoxicity assays and toxicity dossier of some substancies and on the basis of the epidemiological studies of the incidence rate of the exposed population in comparison with the reference population, it will be possible to confirm or reject the points of the null hypothesis. These are as follows:

1. Soča river and the artificial lake at the Solkan hydroelectric power plant do not bring pollutants: heavy metals, mineral oils and other organic pollutants, which could have negative effects on the quality of the raw water from the Mrzlek spring;

2. The drinking water from the Mrzlek Waterworks does not contain any supposed mutagenic and/or carcinogenic potential;

3. These are no negative health indicators (an increased incidence of malignant diseases) linked with the supposed mutagenic/carcinogenic potential of the water from the Mrzlek Waterworks within population drinking this water;

4. Two communities within AU Nova Gorica divided on the basis of using different sources of drinking water have the same probability to develop a digestive or urinary malignant neoplasms regarding age as long as the other social ecomomic characteristics are not a confounding factor.

These points of the null hypothesis have been verified during a longer period of time, on the basis of the results of laboratory analyses, demographic data and the Cancer Registry data of the last two decades. Considering the known effects of particular pollutants – especially THM's on human health, it will be possible to process statistical and epidemiological data concerning the incidence rate of certain malignant neoplasms among the population drinking the water from the Mrzlek spring.

1.4 Study arrangement

1.4.1 Chemical part

Chemical substances as environmental pollutants are mostly determined as:

- Industrial chemicals,
- Pollutants caused by environmental damage,
- Pharmaceuticals,
- Food additives,
- Plant protection products,
- Biocides.

To determine the presence of dangerous pollutants in drinking water, it was necessary to establish, based on a thoroughly planned research protocol:

a) Key places for water and sediment sampling,
b) Chemical parameters which could be analyzed in the laboratory using tested and standardized methods for laboratory analysis.

In the course of this study, Mladen Franko from the Polytechinc Nova Gorica contributed significantly to the evaluation of the scientific protocol.
Chemical analyses of the investigated substances in the water and sediments were carried out by Mirjam Hojak and Ljuba Maver from the laboratory of the Institute of Public Health of Nova Gorica. Isotope analyses (oxygen and tritium) from the water samples were carried out by Sonja Lojen from the Jožef Stefan Institute.

1.4.2 Toxicological part

To study the supposed genotoxic/mutagenic pollutants from drinking water, especially halogenated methanes, it was necessary to execute:

- Extraction of halogenated methanes by stripping,
- Genotoxicity assays with standardized methods.

Based on the obtained results and related with health risk assessment, it was necessary to prepare a toxicological dossier for each of the three most problematic THM's: chloroform, bromdichloromethane (BDCM), dibromochloromethane (DBCM).

The extraction of halogenated methanes from the analysed water samples by stripping was carried out by Emil Žerjal from the Institute of Public Health of Maribor and Polonca Trebše from the Polytechnic Nova Gorica.
Genotoxicity analyses were carried out by Bojana Žegura, according to the advice and under the guidance of Metka Filipič from the National Institute of Biology.
Tanja Fatur from the Institute of Public Health of Slovenia contributed with advice to the writing of toxicologic studies for each particular substance – trichalomethanes.

1.4.3 Epidemiological part

Epidemiological data are reliable indicators of environmental health effects. The focus is, of course to discover possible negative health indicators (an increased incidence of certain malignant diseases) of the population, drinking water from Mrzlek Waterworks, compared to the reference population (the part of the population of the AU Nova Gorica, which does not depend on the Mrzlek Waterworks).
The question is whether it is possible to correlate any of these malignant diseases with the alleged effects caused by the substances present in this water:

a) In this study the simplest and most widely used incidence dynamic response model for specifying non-proportionality in hazards over time and a non-proportionality among two communities is used. Having in mind the hypothesis that changing frailty of the individual human being caused by the toxicity component of water might be reflected in the observed incidence rate. This observation considers data for a period of 18 years, from 1985 to 2002. Within this period, it should be presented that complicated dynamics are not results of only random variation of the cancer incidence but be interferred by the underlying causes such as physiological changes over time. This model serves as a framework for incorporation different analytical scenarios.
b) It is necessary to compare two comparable and independent populations by age – standardized method as a support for the basic thesis on the possibility to develop a digestive or urinary malignoma neoplasm due to the presence of specific pollutants in drinking water.

Even though the latency period for the development of malignant diseases can normally be 20 years or more, the research was focused on the last 20 years. It is reasonable to suppose that, even though DBP were probably formed earlier during the process of chlorination at the Mrzlek Waterworks, the situation regarding the possibilities of introduction and quantities of mutagenic/carcinogenic pollutants changed significyntly for the worse after the construction of the Solkan hydroelectric power plant, as this created possibilities for constant mixing of lake water with the water of the Mrzlek spring.

Expert mathematician and statistician Miljana Vegnuti, currently working at the Golnik Clinic, took part in the selection of statistical methods and analyses, statistical calculations and the representation of the results in this study.

1.5 Further steps based on the results of the study

- Is an evaluation of drinking water quality standards necessary?

Giving consideration to the results obtained, the regulations defining the quality of drinking water may be discussed. Regulations and norms are developed and modified over a longer period of time. They defined the maximum contaminant level (MCL) for several substances. For those substances that are possibly mutagenic and/or carcinogenic the agencies recommend or set goals – maximum contaminant level goals (MCLG). The goals for carcinogens should be zero (0), especially if the results will be the identification of genotoxic substances.

2 MATERIALS AND METHODS

2.1 Chemical analysis

Nine sampling points were selected in the artificial lake at the Solkan hydroelectric power plant for collecting water and sediment samples. The water samples were collected from cross-sections of the lake (the left bank, the bottom in the middle, the right bank) at three locations: 1. At the Solkan power plant hydroelectric dam; 2. At the Mrzlek water pump station; 3. At Prelesje village. Samples from the tenth point on the Soča in Deskle served as controls. In order to obtain water samples from the Mrzlek spring, the sampling site at the water pump in the Mrzlek Waterworks was selected. The sampling site for drinking (conditioned – tap water) water was in the laboratory of the Polytechnic.

Thanks to the kindness of the management of the Public Health Institute of Nova Gorica and Goriški Vodovodi enterprise, we were able to examine the results of many years of regular monitoring of drinking water from numerous smaller waterworks systems, which are used by the part of the population of the AU Nova Gorica, which does not depend on the Mrzlek Waterworks. It was found that besides occasional increased oxygen demand due to turbidity, occasional microbiological unsuitability and traces of nitrogen compounds, the presence of mineral oils or heavy metals was never detected. Neither were THMs detected, as these waterworks systems are chlorinated only occasionally. That is why we did not sample these waterworks systems and also why the results of this regular monitoring are not shown explicitly in Chapter 3 - Results.

The results for the determinations of some heavy metals (Cr, As, Cd, Pb, Zn, Hg) and mineral oils from the years 1985, 86, 87, 88 and 89 were found and studied in the archive of the Institute of Public Health Nova Gorica (108) (Table 56 - Annex II.).

In 1997, two samplings of waters and sediments from the lake, of raw and conditioned water, and of the sand from the filters in the Mrzlek Waterworks were conducted. In all the samples the concentrations of some heavy metals (Cr, As, Pb, Zn, Hg), mineral oils (Table 57 – Annex II.) and organic compounds - THM's were measured (Table 4 – Chapter 3.).

Chemical analyses of waters and sediments were carried out with the use of the following methods:

a) For heavy metals atomic absorption spectrometry (AAS) with the graphite furnace (Cr, As, Cd, Pb, Zn), and cold vapor method (Hg);
b) For mineral oils the method gas chromatography with flame ionization detector (GC-FID using column temperature program from 55° to 265°C);
c) The presence of THM's was established with the use of the method gas chromatography in combination with mass spectroscopy (Head space- GC/MSD).

On the basis of the results obtained in 1997, the number of sampling sites for the extraction of water and sediment samples from the Soča river were reduced in 1998. This was done assuming there were no crucial differences in the quality of the water and sediments along the river profile. Subsequently, samples were collected only at the Mrzlek water pump station and at the controlling point in Deskle. The sampling of raw and conditioned water, and of the sand from the filters at the Mrzlek Waterworks remained unchanged (Tables 5, 6 and 10 – Chapter 3).

In 1999, another series of sampling and analyses was carried out. The locations of sampling sites remained unchanged. In the samples of sediments and sands the concentrations of some heavy metals (Cr, As, Cd, Pb, Zn, Hg,) and mineral oils were measured (Table 9 – Chapter 3).

Special attention was given to assessing the presence of genotoxic volatile halogen substituted methanes (THM's) in drinking water (Tables 11, 15, 17 and 20 – Chapter 3).

Some samples of radioactive isotope ^{3}H and ratio of $^{18}O/^{16}O$ isotops (Tables 7 and 8 – Chapter 3) were taken and analyzed to assess the possible mutual effect between the rainwater from the catchment area of the Mrzlek spring, water from the river Soča and raw and conditioned water from the Mrzlek Waterworks.

The ratio of stable oxygen isotopes $^{18}O/^{16}O$ in water samples was determined with mass spectroscopy. The method is based on the exchange of oxygen isotopes between water and CO_2 (109). The isotopic composition/ratio between the part of the heavier and the lighter oxygen isotope is expressed by the value "δ", which is a relative difference in isotopic composition of the studied sample (vz) according to the standard (st) and is measured in ‰:

$$\delta^{18}O = \frac{R_{vz} - R_{st}}{R_{st}} x1000 \qquad \{2\}$$

Where R is the ratio between isotopes $^{18}O/^{16}O$ in the sample or standard, when marked vz or st respectively. The International Standards for Isotopic Measurements are set by the International Atomic Energy Agency (IAEA) in Vienna. The default value for oxygen is the standard of mean ocean water – Vienna Standard Mean Ocean Water (VSMOW) (110) at the depth of one meter at the temperature of 298 K. The defined ratio of oxygen isotope ratio in Rst is 0,002005.
Tritium (^3H) is found in the normal water cycle. It comes from the air into the groundwater with rainwater. In that way it is posible to study how fast is groundwater regenerated. The major source of natural tritium is the atmosphere, were it is formed mainly from the interaction of cosmic-ray neutrons with nitrogen and oxygen. One source of natural tritium is $^{14}N + {}^1n \rightarrow {}^{12}C + {}^3H$. Since the early fifties, nuclear explosions added artificial tritium to the atmosphere. As expected, after atomic weapon tests ceased, tritium level decreased substantially over the past four decades. About 99% of the ^3H inventory is converted to tritiated water (HTO) and participates in the natural water cycle. Tritium enters food crops in the form of HTO and is partly incorporated into organic matter. In general the annual absorbed doses obtained in that way are of the order of 10^{-8} Gy in all tissues (111).
To determine the concentration of Tritium (3H) a 300 cm3 water sample was transferred into a pyrex glass bottle and distilled to remove volatile and organic impurities and β particles (112). 300 mL of the distilled sample was added to an electrolysis cell for the enrichment of tritium according to the IAEA procedure (113). The water samples, obtained from precipitation, the river or the spring were mixed with a liquid scintillator ULTIMA GOLD LLT after electrolysis, and the 3H activity of the mixture was measured by liquid scintillation counting.

As mentioned previously, after the construction of the Solkan hydroelectric power plant dam, the waters of Mrzlek spring and the Soča river have been mixing.

2.2 Genotoxicity analysis and toxicological dossier

To establish the genotoxic potential of drinking water and to evaluate the genotoxic potential of samples, halogenated methanes from Mrzlek chlorinated drinking water (1999) were extracted by stripping (Table 11). Chloroform, bromodichloromethane (BDCM) and dibromochloromethane (DBCM) were detected by GC/MSD in the concentrate. Genotoxicity of concentrated THM's was assayed with the SOS/*umu* test and the Ames test. The test organism for SOS/*umu* response test (114,115) was bacterium *S. typhimurium* TA1535/pSK1002. The samples were tested at five different concentrations in three parallel repetitions, and in three experiments, carried out at different times. The testing was conducted with and without exogenous metabolic activation with the S9 mix (Table 12 and Figure 10 – Chapter 3). The samples were also assayed using the standard bacterial test of reverse mutations (the Ames test) (116,117,118) with bacteria *Salmonella typhimurium* TA98 and TA100 in two separated experiments. They were tested at four different concentrations with and without metabolic activation (S9 mix) (Tables 13 and 14 and Figure 11 – Chapter 3).

Next, the water samples were tested for the potential genotoxicity of substances in the non-purgeable fraction (1999). The sources of these samples were: 1) the Soča river (because of it's potential influence on the Mrzlek spring), 2) raw – non-chlorinated water from the Mrzlek Waterworks, and 3) drinking – chlorinated water from the Mrzlek Waterworks. The potentially genotoxic substances were isolated with solid phase adsorption (C 18 – paraphine reverse phase) and elution with methylene chloride. The extracts were tested with the SOS/*umu* test with two bacterial strains of *Salmonella typhimurium:* TA1535/pSK 1002 and NM2009. Strain NM2009 has an increased O-acetyltransferase

activity and is more sensitive to the activity to nitropolyaromatic hydrocarbons. The extracted non-purgeable substances were not genotoxic (the results are not shown).

Comet assays (119,120,121.) were made (2003) on four samples in two separated experiments (Tables 15 and 16 and Figures 12 and 13 – Chapter 3). The samples tested were:
1) Chlorinated tap water – sample (a) from Pohorje (Slovenska Bistrica), 2) Mrzlek chlorinated tap water – sample (b) from Polytechnic N.G., 3) sample (c) of chlorinated water prepared *in vitro* – Polytechnic laboratory (it was supposed to mimic sample "a" regarding THM's concentration) and
4) sample (d) of chlorinated water prepared *in vitro* – Polytechnic laboratory (it was supposed to mimic sample "b" regarding THM's concentration). HepG2 cells were treated with samples containing 10 vol% of trihalomethanes in William's medium E for 4 hours. Cells were washed with PBS, trypsinized, centrifuged at 1000 rpm for 10 minutes and the cell pellets frozen at -80°C. In each experiment the vehicle control - 1% Dimethyl sulphoxide (DMSO) and the negative control (non-treated cells) were included in order to exclude possible effects of the solvent. In all the experiments the results of trihalomethane treated cells are compared to the vehicle control. One-way analysis of variance (ANOVA, Kruskal-Wallis) was used to analyse the differences between treatments within each experiment. Dunnett's test was used for multiple comparison versus the vehicle control; $p<0,05$ was considered as statistically significant (more about that – Discussion part 4.2).

In the year 2005, special attention was given to assessing the presence of potential genotoxic substances in the non-chlorinated, raw water from Pohorje (Slovenska Bistrica) and in the non-chlorinated, raw water from Mrzlek. The samples were assayed with the standard bacterial test of reverse mutations (the Ames test) with bacteria *Salmonella typhimurium strains* TA98 and TA100 in two separated experiments, without and with metabolic activation (S9 mix) (Tables 17, 18 a and b, 19 a and b and Figures 14, 15, 16 and 17 – Chapter 3). The results were negative (were not genotoxic).
THM's were constantly present in the treated water of the Mrzlek water supply (Table 20 – Chapter 3). Continual monitoring was begun in 1987.

To determine with more certainty if the THM's are really causes of the appearance of some malignant diseases in the population constantly drinking the water from Mrzlek the base of information from the toxicologic study of THM's was extrapolated. Toxicologic files for chloroform, BDCM and DBCM, the three most common chlorinated substances found in the Mrzlek drinking water were made for this purpose (in Chapter 3 – Results Section 3.2 and Chapter 4 - Disussion part 4.2). The file for each particular substance was made according to the steps and instructions for toxicologic studies from the documentats of WHO and International programme on chemical safety (IPCS) (122,123,124):

1. Physical and chemical properties,
2. Kinetic and metabolism data,
3. Toxicity data and toxicity evaluation,
4. Exposure,
5. Toxicity, hazard and risk estimation,
6. Risk evaluation.

2.3 Population data – statistical analysis

2.3.1 Demographic characteristics

The age distribution characteristics of population of the AU Nova Gorica did not change during the observation period. Overall changes of age group sizes during the period from 1985 to 2002 do not exceed 0.6%. The stability is observed in both parts of AU Nova Gorica. The two distributions presented for the last year of observation (Table 2) and cumulative distribution for the average period's age groups seem quite similar (Figure 6), except the loss of ages 50 to 60 years found at "the rest of AU Nova Gorica" (not exposed population).

The age group from 50 to 60 in the "not exposed population" is relatively smaller regarding the distribution of age groups in the "exposed population". The biggest difference is observed in the class from 50 to 54.

Table 2: *The age groups distribution of population of AU Nova Gorica, population drinking water from Mrzlek (Mrzlek community a - exposed populationa) and the rest of AU Nova Goricab (not exposed populationb) in 2002*

AGE GROUPS	AU NOVA GORICA	%	EXPOSED TO MRZLEK	%	NOT EXPOSED TO MRZLEK	%
0 - 4	2503	*4.2*	1291	*4.1*	1212	*4.3*
5 - 9	2746	*4.6*	1371	*4.4*	1375	*4.9*
10 - 14	3262	*5.5*	1605	*5.1*	1657	*5.9*
15 - 19	4037	*6.8*	1832	*5.9*	2205	*7.8*
20 - 24	4664	*7.8*	2447	*7.8*	2217	*7.8*
25 - 29	4285	*7.2*	2496	*8.0*	1789	*6.3*
30 - 34	4378	*7.4*	2303	*7.4*	2075	*7.3*
35 - 39	4336	*7.3*	2462	*7.9*	1874	*6.6*
40 - 44	4750	*8.0*	2451	*7.9*	2299	*8.1*
45 - 49	5162	*8.7*	2822	*9.0*	2340	*8.3*
50 - 54	3551	*6.0*	1455	*4.7*	2096	*7.4*
55 - 59	3370	*5.7*	1598	*5.1*	1772	*6.3*
60 - 64	3302	*5.6*	2002	*6.4*	1300	*4.6*
65 - 69	3031	*5.1*	1584	*5.1*	1447	*5.1*
70 - 74	2618	*4.4*	1455	*4.7*	1163	*4.1*
75 - 79	2001	*3.4*	1181	*3.8*	820	*2.9*
80 -	1466	*2.5*	860	*2.8*	606	*2.1*
TOTAL	59462	*100*	31215	*100*	28247	*100*

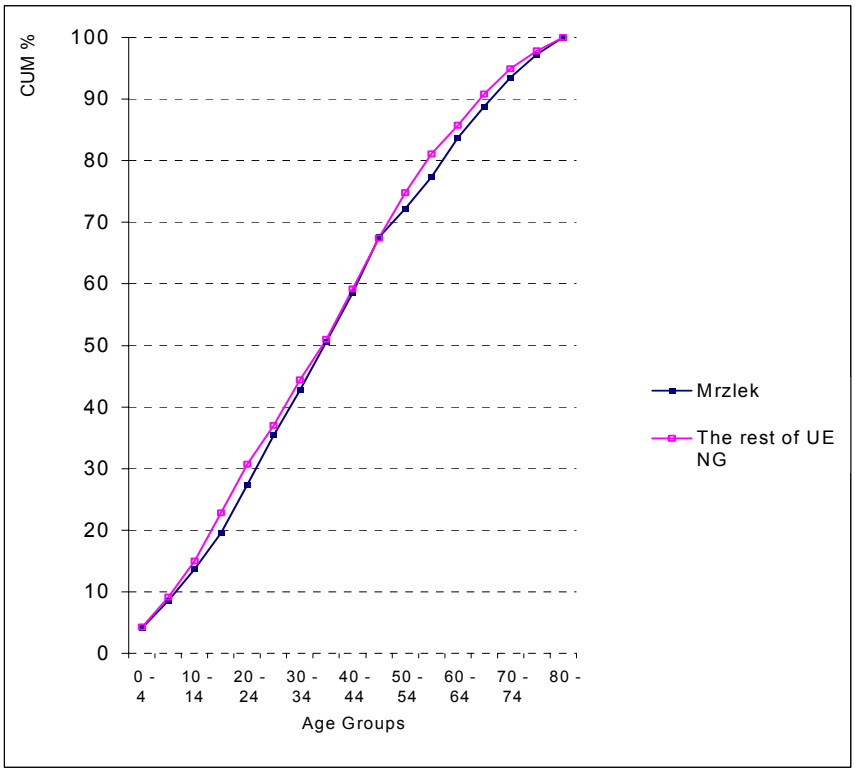


Figure 6: *The average number of age groups in the exposed populationa compared to the not exposed population in the period 1985-2002 showed as cumulative distribution over the 5-years age groups*

The differences between the cumulative distributions of the populations (the exposed population and the not exposed population) observed in the graph, arise at intersections of younger and older age groups – in the age interval between 45 and 50 (125).

2.3.2 Statistical techniques

In the study of incidence rates of some malignant neoplasms of the observed population compared to the reference population, according to the International statistical classification of diseases and related health problems – 10th (126), different statistical-mathemathical calculations and models were used for the work on seven different malignant neoplasms:

2.3.2.1 Average annual crude rate of incidence

Data on incidence are presented as rates. For a specific tumour and population, a crude rate is calculated simply by dividing the number of new cancers observed during a given time period by the corresponding number of people in the population at risk. The result are expressed as an annual rate per 100,000 persons at risk.

2.3.2.2 Age standardized rate of incidence– direct method

An age-standardized rate of incidence is a summary measure of a rate that a population would have if it had a standard age structure. Standardization is necessary when comparing several populations that differ with respect to age because age has such a powerful influence on the risk of cancer. We used Slovenian standard population. The result are expressed per 100,000.

2.3.2.3 Direct Standardized Incidence Ratios

The standardized incidence ratio (SIR) is defined as a weighted average of the age-specific rates, where the weights are represented by the standard, AU Nova Gorica, distribution for age. Practically, each age-specific rate is weighted with the standard in the same age group, simply multiplying the age specific rate by this weight (118,127).

$$\textbf{SIR(D)} = [\text{Sum}_{\text{age groups}} (M_{ar} P_{as})]/P_s \times 1000 \quad \{3\}$$

M_{ar} is the age-specific morbidity rate for the community,
P_{as} is the number of people in the age group in the standard population,
P_s is the total standard population.

2.3.2.4 Indirect Standardized Incidence Ratios

This standardized incidence ratio uses the indirect method of adjustment to compare the cancer experience of both AU Nova Gorica communities (“Mrzlek community” and “the rest of AU Nova Gorica”) with Slovenian data on incidence (Slovenian standard population) by the calculation:

Standardized Incidence Ratio (SIR) = (Observed events / Expected Events) (128) {4}

For obtaining the exact confidence limits the following method SIRL and SIRU for the true SIR was used. Assuming D is Poisson distributed with mean m = E(D), confidence limits for m are obtained using the relationship between the Poisson distribution and the chi-square distribution. Then these limits are divided by the total number of expected events, E*, to obtain the limits:

$$SIRL = (\chi^2_2 D, \alpha/2)/ 2E^* \quad \text{and} \quad SIRU = (\chi^2_2 (D+1), 1- \alpha/2/ 2E^* \quad \{5\}$$

where χ^2_2D,α is the 100a percentile of the chi-square distribution with *v* degrees of freedom (129,130).

2.3.2.5 Survival function

Survival times in this study are data that measure the time from birth to the developement of malignant neoplasms at seven locations (stomach - C16, colon - C18, rectosigmoid junction - C19, rectum - C20, liver and intraheptic bile ducts - C22, kidney, exept renal pelvis - C64 and bladder - C67) in the two population of AU Nova Gorica established according to the drinking water resources. These times are subject to random variations. The distribution of survival times is described by three functions: the survival function, the probability density functions and the hazard function. These three functions are mathematically equivalent, if one of them is given, the other two can be derived (117,131).

Survival function employed in the further analysis of incidence of new malignoma cases is denoted by *S(t)*, which is defined as the probability that an individual survives longer than *t.* This function is also known as the cumulative survival rate. The graph of *S(t)* presents the survival curve and is used to find the median and other percentiles of survival time and to compare two survival distributions, one for "Mrzlek community" and one for "the rest of AU Nova Gorica" community.

The Kaplan-Meier procedure was employed as a method of estimating time-to-event models in the form of non-censored cases, since the number of incidence dates represent the realizations in the defined time period and their survival times are exact and known.

The model is based on estimating conditional probabilities at each time point – age of an individual when a cancer disease occurs and taking the product limit of those probabilities to estimate the survival rate at each age. The assumption was that within both compared samples, the exact survival times are independent and identically distributed. Each sample of subjects is a random sample from the population of AU Nova Gorica, so that they are independent of each other, both within each sample and among samples. To compare both samples Log-rank (Mantel-Cox) and Gehan-Breslow test were used, which are both nonparametric.

The log-rank test compares the equality of two distributions one at each observed event time, and calculating a statistic based on the observed and expected values for contingency tables. Gehan-Breslow tests is weighted variants of the log-rank test, weighted by the number of cases at risk at each time point.

Tests of goodness of fit, independence and association between two variables were made by chi-square tables.

The data on patient health status are a complex assessment of the burden of cancer in the observed population. They reflect the success of all programs of oncologic protection, from mass screening and early discovering, to treatment, rehabilitation and long-term monitoring of patient health state.Several factors can influence on that, linked either to patients themselves (age, gender, physical capabilities and other diseases) or to cancer: location and distribution of the disease at diagnosis, histologic type, manner of treatment (132).

Regardles of this, the main goal of this study is to find out whether there are differences in the incidence rates in two parts of the AU Nova Gorica, and whether the possible difference in survival function of the two populations is linked to the incidence of cancer.

3 RESULTS

3.1 Laboratory study - tables and figures

Table 3: *THM's in Mrzlek drinking water (µg/L) – results from 1995-1997*

Time of sampling	Feb. 95	Jan. 96	Jul. 96	Apr. 97	Aug. 97
THM's	8.0	10.8	13.6	6.9	5.9

It was always possible to detect the presence of these volatile organic substances in the regular monitoring of THM's, executed by the Institute of Public Health Nova Gorica.

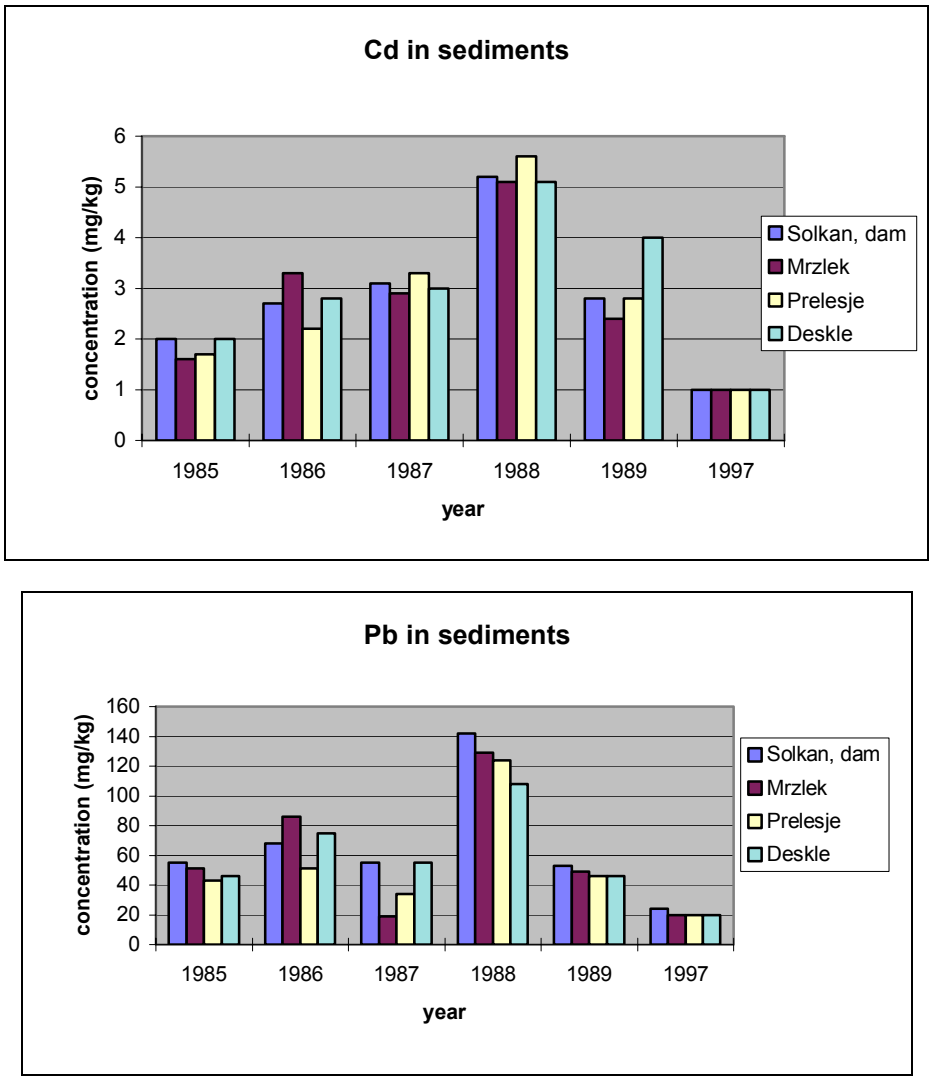


Figure 7: *Concentrations of Cd and Pb in sediments from river Soča*

A fall of the concentration of heavy metals in the sediments is notable after 1988, which coincides with the closure of the lead mine upstream in the catchment area of the river Soča.

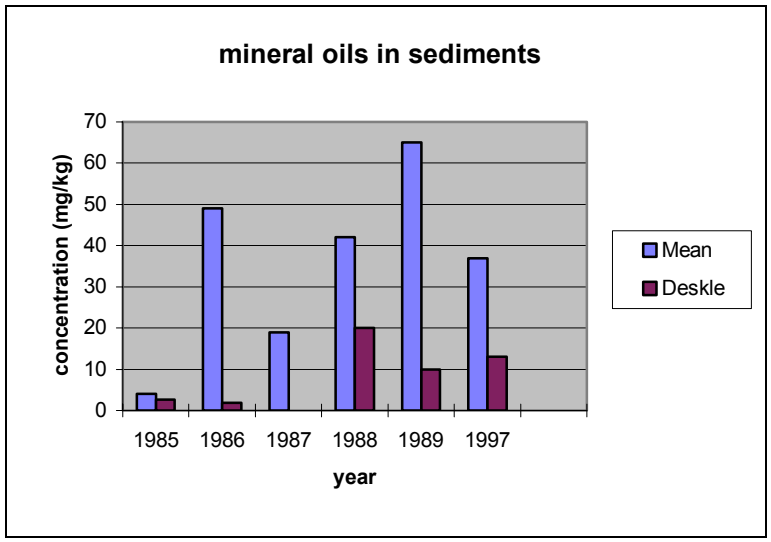


Figure 8: *Mean values of concentrations of mineral oils in sediments from river Soča*

Mineral oils are constantly present in the Soča sediments.

Table 4: *Concentrations of heavy metals, mineral oils and THM's (µg/L) in water from the Soča, and in conditioned and raw water from Mrzlek (1997)*

Parameter	DAM		PRELESJE		DESKLE		MRZLEK CONDIC.		MRZLEK RAW	
(µg/L)	Mar.	Nov.	Mar.	Nov.	Mar.	Nov.	Mar.	Nov.	Mar.	Nov.
Cr	< 50	< 50	< 50	< 50	< 50	< 50	< 50	< 50	< 50	< 50
As	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
Pb	< 5	< 5	< 5	< 5	9	< 5	< 5	< 5	< 5	7
Zn	50	< 20	30	< 20	70	< 20	30	< 20	20	< 20
Hg	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Mineral oils	7	< 5	5	< 5	19	< 5	130	< 5	7	7
Chloroform	1.1	0.7	0.8	0.6	0.8	0.7	1.3	0.5	4.3	2.7
BDCM	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.7	0.6
DBCM	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.2	< 0.1

The concentrations of some heavy metals in water samples are normally below the limit of laboratory detection, but the constant presence of mineral oils and THM's in water samples from the Mrzlek Waterworks is a cause of concern. Results, which are very similair to those in Table 4, can be found in occasional measurements, which were carried out in the years before and after the measurements in Table 4.The results are in the archiveof the Institute of Public Health of Nova Gorica.

Table 5: *Heavy metals in the sand from the filters in the Mrzlek Waterworks*

Mg/kg dry substance	March 1997		July 1998	
	OW	NW	OW	NW
Cr	4.2	5.1	4.0	3.8
As	0.6	0.6	0.7	0.5
Pb	< 2.5	< 2.5	< 2.5	5
Zn	6	7	4	5
Hg	0.05	0.09	<0.05	0.06

OW The old waterworks
NW The new waterworks

Heavy metals can be traced in the filter sand of the Mrzlek Waterworks, regardless of the changing of the sand in the filters. The particulate matter in the raw water from the spring that mixes with the river water also brings heavy metals.

Table 6: *Heavy metals and mineral oils in sediments – river Soča*

Mg/kg dry substance	April 1998		July 1998		October 1998	
	Deskle	Mrzlek	Deskle	Mrzlek	Deskle	Mrzlek
Cr	32	35	43	32	*	62
As	2.2	4.8	5.8	6.5	*	8.3
Pb	20	< 16	19	< 25	*	31
Zn	140	98	142	90	*	365
Hg	2.6	11	27	25	*	25
Min. oils	*	*	48	36	*	22

* The sample was not suitable for analyse
The concentrations of heavy metals, especially mercury (centuries of mining in Idrija) and arsenic are a cause for concern. Mineral oils are constantly present.

Table 7: *Mean values of ^{18}O in water from the Soča, in conditioned and raw water from Mrzlek, and in rainwater from the catchment area of the Mrzlek spring*

Water from…	Jan. 1998	Apr. 1998	Avg. 1998	Avg. 1999
River Soča	7.8	8.17	8.71	8.44
Condicioned water Mrzlek	7.76	7.97	8.29	8.46
Raw water Mrzlek	8.47	8.45	8.42	8.35
Rainwater (Banjšice)	6.44	6.80	6.75	6.95

Comment on Table 7.:
Isotopic composition of oxygen in water from the Soča, in conditioned and raw water from Mrzlek, and in rainwater from the catchment are of the Mrzlek spring, expressed as a relative deviation (δ) of the $^{18}O/^{16}O$ ratio from the reference $^{18}O/^{16}O$ ratio established by the VSMOW standard, expressed in ‰Δ (3).
In the researches betwen 1993 and 1996 (3) the mean values of ^{18}O were:
1. Soča river 8,46 ± 0,30 ‰
2. Mrzlek – raw water 8,24 ± 0,29 ‰
3. Banjšice - rainwater 7,76 ± 0,17 ‰
Our results (presented in Table 7) do not differ significantly from the reference quoted.

Table 8: *Activity of ^3H (in Bq/m^3) in water from the Soča, in conditioned and raw water from Mrzlek and in rainwater from the catchment area of the Mrzlek spring*

Water from…	Oct. 1997	July 1998	Oct. 1998	Apr. 1999
River Soča	995	1025	1350+/-380	910+/-160
Conditioned water Mrzlek	950	850	*	*
Raw water Mrzlek	*	1175	1510+/-380	1285+/-170
Rainwater (Banjšice)	*	920+/-175	1260+/-330	750+/-180

* The sample was not suitable for analyse

The isotope analyses of the samples of the rainwater from the catchment area of the Mrzlek spring, water from the river Soča and raw and conditioned water from the Mrzlek spring
are important. In the case of ^3H (910 – 1350 Bq/m^3 for the Soča; 1175 – 1510 Bq/m^3 for the Mrzlek raw water), the measurements have shown higher activities for Mrzlek. These are also higher than the activities of ^3H in rainwater (750 – 1260 Bq/m^3).

The results of the isotopic analyses of water from the Soča and Mrzlek, and of rainwater are similar to those from the period 1993-1996 (3). With parallel measurements of precipitations, of the rates of flow of water, and of evapotranspiration, the water balance of the Trnovsko-Banjška planota Plateau (the drainage area of the Mrzlek spring) was established, showing the major drainage of water into the Soča (the Mrzlek spring), less drainage into the Idrijca river and the least into the Vipava river. The measurements of temperature, chemical parameters and isotopes (3) in water of the Soča, of the Mrzlek spring and in the waterworks, proved the mixing of the spring water with the Soča moves deeply into the spring, especially during dry spells in the hinterland.

Table 9: *Concentrations of heavy metals and mineral oils in sediment samples from the Soča and in sand samples from the filters in the Mrzlek Waterworks, March 1999*

Element	Sediments - Soča		Sand from the filters - Mrzlek	
(mg/kg dry substance)	Deskle	Mrzlek	Old waterworks	New waterworks
Cr	43 ± 4	42 ± 4	3.4 ± 0.3	5.3 ± 0.5
As	6.3 ± 0.6	5.8 ± 0.6	0.66 ± 0.07	0.62 ± 0.07
Cd	0.3 ± 0.03	< 0.3	< 0.3	0.3 ± 0.03
Pb	29 ± 3	23.5 ± 3	4.3 ± 0.4	3.9 ± 0.04
Zn	95 ± 9	84 ± 9	6.3 ± 0.6	12 ± 1
Hg	32 ± 3	8 ± 1	<0.03	0.05 ± 0.01
Mineral oils	8.8 ± 0.8	49 ± 5	*	*

* The sample was not suitable for analyse

There is a constant presence of heavy metals and mineral oils in the sediments from the Soča and in the sands from the filters.

Table 10: *THM's – conditioned water from Mrzlek*

(μg/L)	April 1998	July1998	October 1998
Chloroform	2.3	1.9	4.9
BDCM	0.9	1.7	1.7
DBCM	0.4	0.2	< 0.2
Bromoform	< 0.2	< 0.2	< 0.2

It is always possible to detect trace THM's in the conditioned water from Mrzlek regardless of the minimal amounts of chlorine used for desinfection.

The volatile halogenated methanes were isolated from the water samples according to a “special laboratory method” (shown in Figure 9). THM’s were extracted by stripping (purging) from the 54 L of the chlorinated drinking water sample with 20mL/min helium (>99.999% pure) and collecting the stripped volatile fraction in 10 mL DMSO (Figure 9 – stripping scheme) and thus prepared for laboratory analysis which will be discussed in the Chapter 4 – Discussion part 4.2.

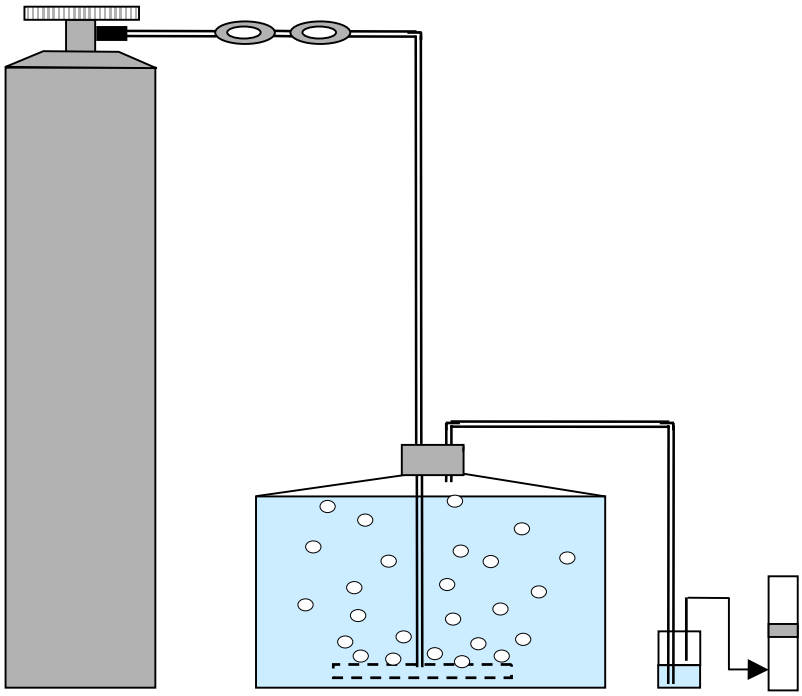


Figure 9: *Scheme of device to extracted THM's by stripping (purging)*

Table 11: *Concentration of THM’s in a sample of drinking water from Mrzlek (1999)*

THM	Before the extration (μg/L)	At the end of extraction (μg/L)	Extracted THM for testing genotoxicity (μg/L DMSO)
Chloroform	6.9	2.0	44.1
BDCM	2.0	0.8	10.8
DBCM	1.0	0.5	4.5

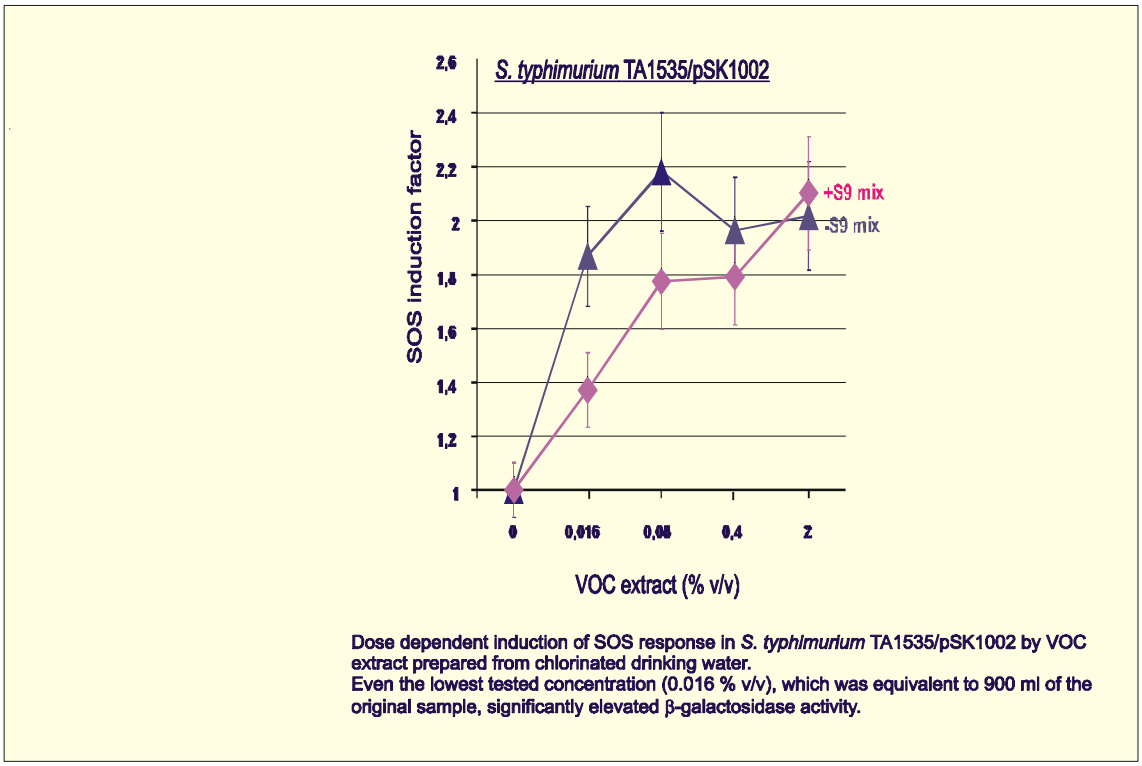
Table 11 shows the concentrations of THM's in the sample before and after the isolation of THM's by stripping and the final calculated concentration in the sample for the testing of genotoxicity. The concentration of isolated THM's in the samples for the testing of genotoxicity (which had final volumes of 6 mL) was calculated from the differences in the measured concentrations of THM's in the sample (54 L) before and after extraction. The differences of pre- and post-extraction concentrations were multiplied with the volume of the sample {x 54 (L)} from which the THM's were stripped and divided with the final volume of the sample (÷ 6 mL) for the testing of genotoxicity.

Table 12: *Induction of SOS response in test microorganisms*

	β-galactosidase	- S9		β-galactosidase	+ S9	
Conc. % v/v	**(Enz. unit)**	**±SD**	**SOSIF[a]**	**(Enz. unit)**	**±SD**	**SOSIF[a]**
0	19.81	2.4	**1.00**	42.93	8.0	**1.00**
0.016	31.11	0.5	**1.87 ***	58.90	11.3	**1.37**
0.08	36.31	2.6	**2.18 ***	76.17	18.0	**1.77**
0.4	32.69	1.0	**1.96****	77.01	17.9	**1.79 ***
2	33.60	4.6	**2.02****	90.24	5.5	**2.10****
10	36.69	2.5	**2.20****	123.21	24.0	**2.87****
PC[b]	40.15	3.9	**2.41****	90.44	10.8	**2.11****

* p 0.05
** p 0.005
SOSIF[a] (induction factor) means the ratio between enzyme units of β-galactosidase activity in the tested sample / enzyme units of β-galactosidase activity of the control.
PC[b] (positive control): - S9: MNNG (10 μM); + S9: BaP (10 μg/mL).

The sample for the testing of genotoxicity induced an SOS response without metabolic activation and with metabolic activation. The induction of the SOS response was caused by the lowest tested concentration (0.016% v/v) which is equivalent to 900 mL of the original sample.



Dose dependent induction of SOS response in *S. typhimurium* TA1535/pSK1002 by VOC extract prepared from chlorinated drinking water.
Even the lowest tested concentration (0.016 % v/v), which was equivalent to 900 ml of the original sample, significantly elevated β-galactosidase activity.

Figure 10: *Dose dependent induction of SOS response in S. Typhimurium TA1535/pSK1002*

Table 13: *Induction of reverse mutations after treating S. typhimurium TA98 with a sample of THM's isolated from drinking water from Mrzlek*

		- S9			+ S9	
Conc. µl/pl	**Rev./plate**	**SD**	**MI[a](IR)**	**Rev./plate**	**SD**	**MI[a](IR)**
0	66.7	2.5	**1.00**	91.7	3.2	**1,00**
0.5	nt			83.7	5.1	**0.91**
2	89.0		**1.34**	82.7	4.2	**0.90**
20	106.7	5.7	**1.60****	85.3	4.2	**0.93**
100	90.7	3.1	**1.36****	94.0	2.5	**1.02**
AFB1				441.3	20.1	**4.81****

* p 0.05
** p 0.005
MI[a] (mutation index; induction ratio) is defined as the ratio between the number of revertants grown in the presence of the sample and the number of spontaneous revertants.
AFB1 is aflatoxin B1 used it as positive control.

Table 14: *Induction of reverse mutations after treating S. typhimurium TA100 with a sample of THM's isolated from drinking water from Mrzlek*

		- S9			+ S9	
Conc. µl/pl	**Rev./plate**	**SD**	**MI[a](IR)**	**Rev./plate**	**SD**	**MI[a](IR)**
0	102.3	1.5	**1.00**	125.0	3.6	**1.00**
0.5	112.3	3.2	**1.10***	118.0	2.6	**0.94**
2	109.3	6.8	**1.07**	124.7	7.0	**0.99**
20	116.0	5.6	**1.13***	124.0	9.6	**0.99**
100	145.3	6.0	**1.42****	145.7	5.5	**1.65***
AFB1				330.0	11.1	**2.64****

* p 0.05
** p 0.005
MI[a] (mutation index; induction ratio) is defined as the ratio between the number of revertants grown in the presence of the sample and the number of spontaneous revertants.
AFB1 is aflatoxin B1 used it as positive control.

The sample for the testing of genotoxicity was tested with a standard bacterial test of return mutations (Ames test) with the use of *Salmonella typhimurium* TA98 (Table 13) and *Salmonella typhimurium* TA100 (Table 14). The sample was mutagenically active without an exogenous metabolic activation, but the sample was not active in the presence of the S9 mix (metabolic activation).

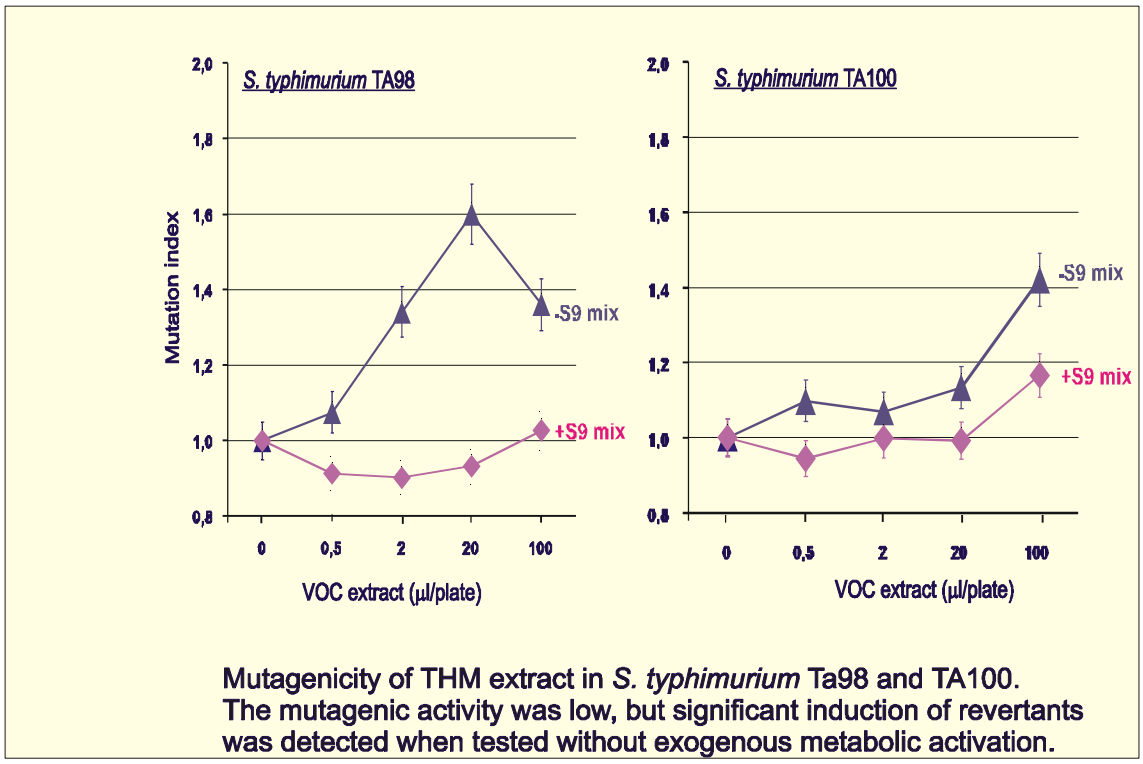


Figure 11: *Mutagenicity of THM's extract in S. typhimurium TA98 and TA100*

Table 15: *THM's - results 2003*

a) Chlorinated tap (drinking) water – sample from Pohorje (Slovenska Bistrica)

Tap water Pohorje (1)	Before isolation (µg/L)	After isolation (µg/L) (7 days)	In the asmple for genotoxicity testing (µg/L DMSO)
Chloroform	29.3	17.6	105.3
BDCM	1.32	0.43	8.01

b) Mrzlek chlorinated tap (drinking) water – sample from Polytechnic N.G.

Tap water Polytechnic (2)	Before isolation (µg/L)	After isolation (µg/L) (5 days)	In the asmple for genotoxicity testing (µg/L DMSO)
Chloroform	12.7	7.7	45
BDCM	1.12	n.d.	9

c) Sample of chlorinated water prepared *in vitro* – Polytechnic lab.

Lab. sample N° (3)	In "vitro" prepared the sample for genotoxicity testing (µg/L DMSO)
Chloroform	129.6
BDCM	30.6

d) Sample of chlorinated water prepared *in vitro* – Polytechnic lab.

Lab. sample N° (4)	In "vitro" prepared the sample for genotoxicity testing (µg/L DMSO)
Chloroform	46.8
BDCM	10.2

Table 15 shows the concentrations of THM's in the samples (a- Pohorje tap water; b- Mrzlek tap water) before and after the isolation of THM's by stripping and the final calculated concentration in the sample for the testing of genotoxicity. Samples c) and d) were prepared *in vitro* in Polytechnic lab (they were supposed to mimic samples "a" and "b").
The samples above were tested in Comet assay and they showed positiv results (Table 16 and Figures 12 and 13).

Table 16: *Comet assay - results 2003*

Mean values % DNA in the tail:

	A	B	aver.
0	12.91	12.53	12.72
0 (DMSO)	10.28	11.77	11.03
1	24.88	22.31	23.59
2	32.42	23.25	27.83
3	26.34	24.19	25.26
4	25.17	22.72	23.95

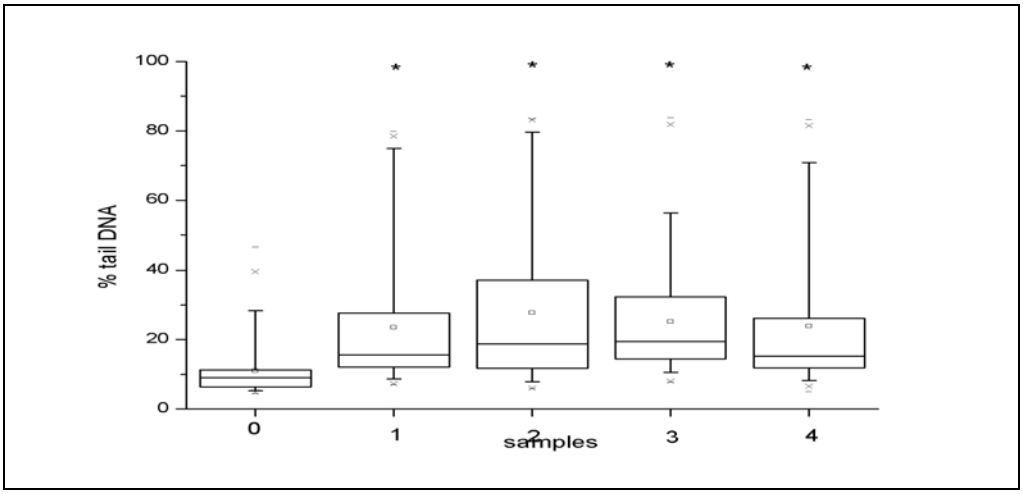


Figure 12: *Comet assay (2003)*

Comment on Figure 12*:* The level of THM's-induced DNA strand breaks in HepG2 cells

The cells were treated with 10 vol% trihalomethane samples (1-4) for 4 hours. The levels of DNA strand breaks are expressed as percent of tail DNA. Fifty cells were analyzed per experimental point in each of two independent experiments. Data are presented as quantile box plots. The edges of the box represent the 25th and 75th percentiles, the median is a solid line through the box, mean values are represented as square (□), and the error bars represent the 95% confidence intervals. (*) Denotes a significant difference between sample treated groups and non-treated group (Kruskal–Wallis test, $P < 0.05$).

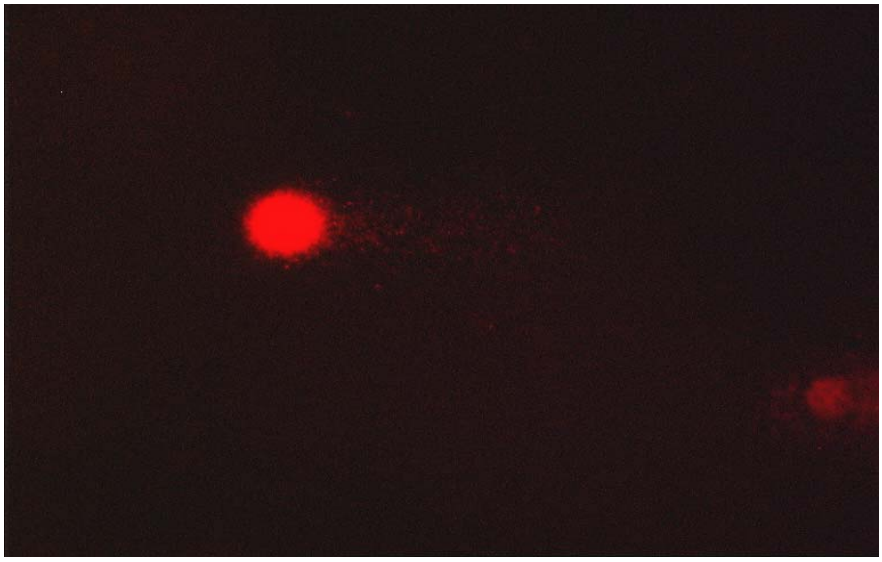


Figure 13: *Comet tail (2003)*

Table 17: *THM's results 2005*

Raw water Pohorje (Slo. Bistrica)	Before isolation (μg/L)	After isolation (μg/L) (7 days)	In the asmple for genotoxicity testing (μg/L DMSO)
Chloroform	1.03	n.d.	9.3
BDCM	n.d.	n.d.	n.d.

Raw water Mrzlek	Before isolation (μg/L)	After isolation (μg/L) (5 days)	In the asmple for genotoxicity testing (μg/L DMSO)
Chloroform	n.d.	n.d.	n.d.
BDCM	n.d.	n.d.	n.d.

Table 17 shows that there are no THM's present in raw water (before chlorination) from the Mrzlek Waterworks, sampled in 2005, while the raw water (before chlorination) from Pohorje contains a minimal concentration of chloroform of unknown origin. Raw water from Pohorje is interesting, as it contains considerable natural concentrations of humic and fulvic substances (acids), which are identified as THM's precursors.

Table 18: *Results Ames test, strain TA98*

a)

Strain		Without activation			With activation		
	Conc. µl/pl	Average n° colonies/pl.	± STD	MI (IR)	Average n° colonies/pl.	± STD	MI (IR)
TA98	C	28.3	5.1	1.00	36.7	0.6	1.00
Raw w. Pohorje DMSO	PC	96.0	4.2	3.39	132.5	13.4	3.61
	1.25	27.7	5.5	0.98	38.0	7.9	1.04
	2.5	29.3	7.8	1.04	36.0	9.5	0.98
	5	30.7	9.3	1.08	31.3	3.5	0.85
	10	31.67	1.53	1.12	31.3	7.5	0.85
TA98	C	28.3	5.1	1.00	36.7	0.6	1.00
Raw w. Mrzlek DMSO	PC	96.0	4.2	3.39	132.5	13.4	3.61
	1.25	31.3	1.5	1.11	29.7	2.9	0.81
	2.5	26.0	5.0	0.92	33.0	1.7	0.90
	5	31.7	4.2	1.12	30.3	3.8	0.83
	10	26.7	6.0	0.94	39.7	1.5	1.08

MI (mutation index; induction ratio) is defined as the ratio between the number of revertants grown in the presence of the sample and the number of spontaneous revertants.
C - control.
PC - positive control

b)

R.w.Poh.	Without S9	STD	With S9	STD	**R.w.Mrz**.	Without S9	STD	With S9	STD
PC	96.00	4.24	132.50	13.44	PC	96.00	4.24	132.50	13.44
0	28.33	5.13	36.67	0.58	0	28.33	5.13	36.67	0.58
1.25	27.67	5.51	38.00	7.94	1.25	31.33	1.53	29.67	2.89
2.5	29.33	7.77	36.00	9.54	2.5	26.00	5.00	33.00	1.73
5	30.67	9.29	31.33	3.51	5	31.67	4.16	30.33	3.79
10	31.67	1.53	31.33	7.51	10	26.67	6.03	39.67	1.53

The results of the Ames test using the strain *Salmonella typhimurium* TA98, with and without activation were negative (Table 18 a and b; Figures 14 and 15).

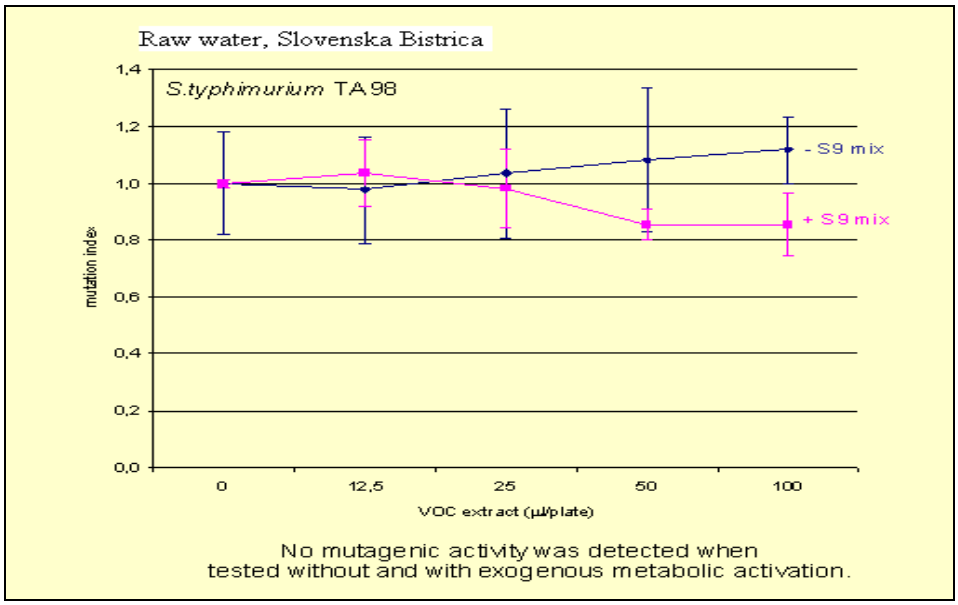


Figure 14: *R.w.Poh., results Ames test, strain TA98*

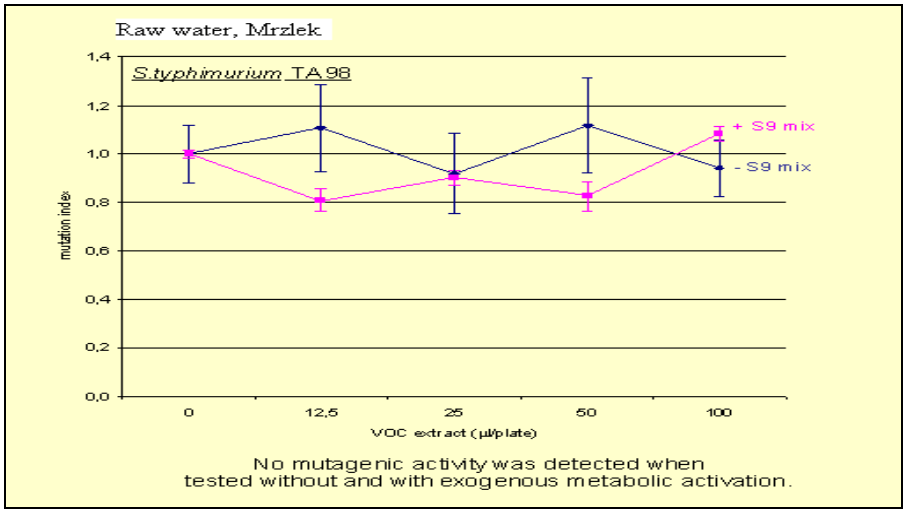


Figure 15: *R.w.Mrz., results Ames test, strain TA98*

Table 19: *Results Ames test, strain TA100*

a)

Strain		Without activation			With activation		
	Conc. µl/pl	Average n° colonies/pl.	± STD	MI (IR)	Average n° colonies/pl.	± STD	MI (IR)
TA100	C	108.0	9.5	1.00	107.3	13.3	1.00
Raw w. PohorjeDMSO	PC	418.7	59.9	3.88	842.7	84.0	7.85
	1.25	109.0	7.5	1.01	111.7	9.3	1.04
	2.5	92.3	9.5	0.85	102.7	2.1	0.96
	5	110.3	26.1	1.02	101.0	11.1	0.94
	10	106.00	9.54	0.98	94.7	2.5	0.88
TA100	C	108.0	9.5	1.00	107.3	13.3	1.00
Raw w. Mrzlek DMSO	PC	418.7	59.9	3.88	842.7	84.0	7.85
	1.25	88.7	10.2	0.82	119.7	6.4	1.11
	2.5	101.7	4.9	0.94	122.0	15.1	1.14
	5	96.7	3.2	0.90	106.7	5.1	0.99
	10	84.0	6.0	0.78	124.0	20.8	1.16

MI (mutation index; induction ratio) is defined as the ratio between the number of revertants grown in the presence of the sample and the number of spontaneous revertants.
C - control.
PC - positive control

b)

R.w.Poh.	Without S9	STD	With S9	STD	**R.w.Mrz**.	Without S9	STD	With S9	STD
PC	418.67	59.91	842.67	84.03	PC	418.67	59.91	842.67	84.03
0	108.00	9.54	107.33	13.32	0	108.00	9.54	107.33	13.32
1.25	109.00	7.55	111.67	9.29	1.25	88.67	10.21	119.67	6.43
2.5	92.33	9.50	102.67	2.08	2.5	101.67	4.93	122.00	15.13
5	110.33	26.08	101.00	11.14	5	96.67	3.21	106.67	5.13
10	106.00	9.54	94.67	2.52	10	84.00	6.00	124.00	20.81

The results of the Ames test using the strain *Salmonella typhimurium* TA100 with and without activation were negative (Table 19 a and b; Figures 16 and 17).

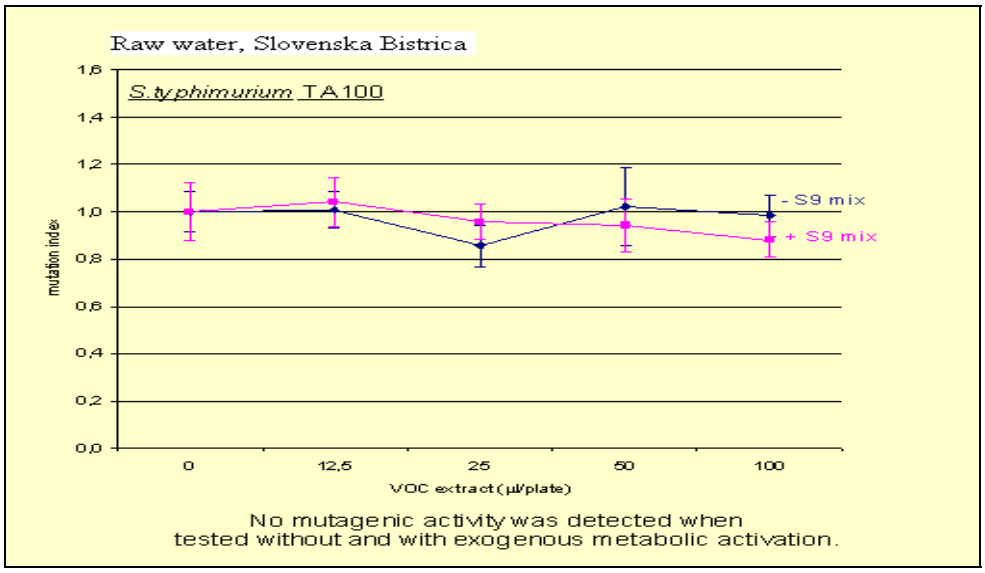


Figure 16: *R.w.Poh., results Ames test, strain TA100*

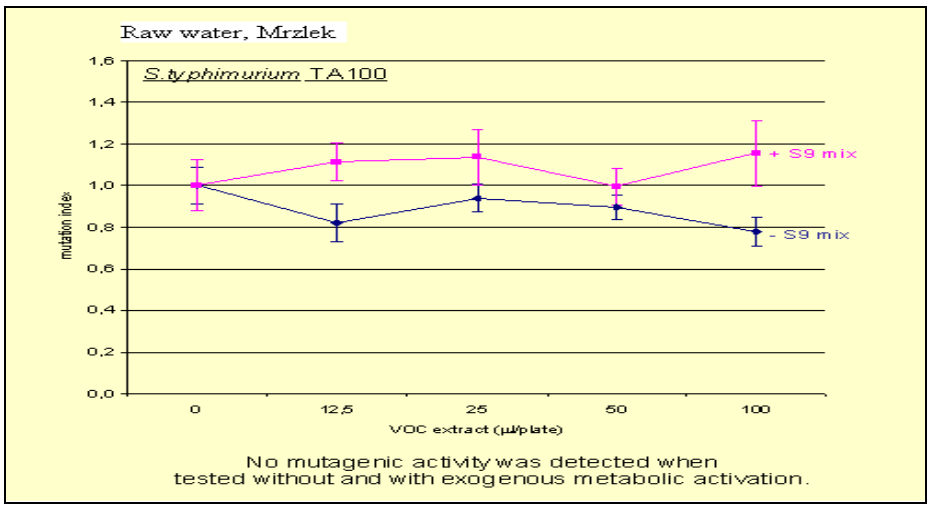


Figure 17: *R.w.Mrz., results Ames test, strain TA100*

Table 20: *Sum of THM's (μg/L) in drinking water from the Mrzlek Waterworks**

Time of sampling	Sum of THM's (μg/L)		Time of sampling	Sum of THM's (μg/L)
1987	8.0		1998	6.6
1988	2.0			6.8
1989	7.0		1999	2.3
	10.0			4.3
1990	10.0		2000	4.3
1991	2.0			8.3
1992	3.0		2001	4.9
	6.0			9.1
1994	4.5		2002	4.9
1995	8.6			7.1
1996	10.0		2003	4.6
	13.6			5.5
1997	6.9		2004	7.4
	5.9			6.5

*With the kind permission of Goriški vodovodi

Regular monitoring of THM's in the Mrzlek conditioned water started in 1987 by "Goriški vodovodi." THM's are constantly present in drinking water. As the Mrzlek water has been chlorinated constantly and continuously since the beginning of the operating of the waterworks (in 1935), it can be assumed that THM's were always present in drinking water from Mrzlek.

3.2 THM's - toxicological study (dossier)

THM's are formed in drinking water by a reaction between the disinfectant chlorine, organic matter and bromide. In this doctoral thesis, the toxicity of **chloroform, bromodichloromethane and dibromochloromethane** exposure and risk assessment for human health are discussed in detail.

3.2.1 Chloroform ($CHCl_3$)

Chloroform is a chemical that has been extensively used as an anaesthetic, in dentifrices, in liniments and in untitussives (THM, WHO)(133). Anthropogenic sources of chloroform are direct processing of the substance (production, storage, transit or use), formation during paper bleaching, in pulps and paper mills, formation during municipal wastewater treatment and formation as a by-product in the production of chlorofluoromethane. The most important natural source of chloroform are marine microalgae.
General population may be exposed to chloroform present in chlorinated drinking water, ambient air and some foods (International agency for research on cancer - IARC, 1999)(134).

3.2.1.1 Physical and chemical properties of chloroform

Toxicologically relevant physical and chemical properties of $CHCl_3$ are shown in Table 21.

Table 21: *Summary of physical and chemical properties of chloroform (135,136).*

PROPERTY	INFORMATION
Chemical name (IUPAC)	Trichloromethane
Synonyms	Chloromethane, methane trichloride, trichloroform, methyl trichloride, formyl trichloride
CAS No.	67-66-3
Chemical formula	$CHCl_3$
Molar mass	119.4 g/mol
Physical state (22 ºC)	Liquid
Colour	Colourless
Odour	Pleasant etheric
Solubility in water at 25ºC	7.2-9.3 g/L
Density at 25ºC	1.48 g/cm^3
Log P_{ow}	1.98
Vapour pressure at 20ºC	160 mm Hg

3.2.1.2 Kinetic and metabolism data

Many studies were performed on metabolism of chloroform in different species; humans, rats, mice and monkeys.

Absorption and excretion: In **humans** that were given a single oral dose of 0.5 g of chloroform 50-52 % were absorbed and exhaled almost completely in the form of carbon dioxide. The peak concentration in blood was detected at 1.5 hours, and the half-life of chloroform was determined to be 13 minutes. After a single inhalation exposure to 5 mg of [^{38}Cl]chloroform, 80 % were absorbed (Concise international chemical assessment document - CICAD, Morgan *et al.*, 1970)(135).
Studies of absorption and excretion of chloroform in animals are gathered in Table 22.

Table 22: *Summary of absorption, metabolism and excretion studies of chloroform*

ROUTE OF EXPOSURE	SPECIES	DOSE	RESULTS	REFER-ENCE
Oral	Rats Mice Monkeys	60 mg/kg bw (body weight)	In 48 hours 90 % of chloroform were exhaled in all species. Mice: 85 % exhaled as CO_2 and 5 % as $CHCl_3$. 2-3% excreted in urine/faeces. Rats: 18 % exhaled as CO_2 and 79 % as $CHCl_3$. 8% excreted in urine/faeces. Monkeys: 67 % exhaled as CO_2 and 2 % as $CHCl_3$. 2-3% excreted in urine/faeces.	CICAD
Inhalation	Mice Rats	0, 49, 440 and 1790 mg/m^3, 6 hrs 0, 460, 1740 and 5100 mg/m^3, 6 hrs	At low doses metabolism was extensive in both species. Exhaled and excreted amount [mg equivalents/kg bw]: • In mice: 7.22 CO_2, 0.03 $CHCl_3$, 0.95 in urine and 0.05 in faeces, • In rats: 31.84 CO_2, 0.76 $CHCl_3$, 3.34 in urine and 0.04 in faeces. At about 1750 mg/m^3 partial saturation of metabolism was observed. Exhaled and excreted amount [mg equivalents/kg bw]: • In mice: 217.85 CO_2, 23.03 $CHCl_3$, 21.24 in urine and 3.48 in faeces, • In rats: 54.85 CO_2, 16.15 $CHCl_3$, 6.53 in urine and 0.81 in faeces.	CICAD

Chloroform is readily absorbed through the skin of humans and animals. Significant absorption of chloroform during showering has been demonstrated. It seems that hydration accelerates the absorption of chloroform through the skin (THM, WHO, Jo *et al.*, 1990)(133).

Distribution: Mice were exposed to 280 mg/kg bw (body weight) of [^{14}C]chloroform by inhalation. Autoradiography was carried out immediately after the exposure and 2 hours after that. High concentration were found in fat, blood, liver, kidneys, spinal cord, nerves, meninges and cerebrall cortex. Non-volatile radioactivity was bound in the bronchi, nasal mucosa, liver, kidneys, salivary glands and duodenal contents. High levels of extractable or volatile radioactivity were determined in testes, perputial gland and epididymis. Transplancental transfer of chloroform was demonstrated in rats, mice, guinea pigs and humans (CICAD, THM WHO)(133,135).

Metabolism: Oxidative and reductive pathways of chloroform metabolism were identified *in vitro* and *in vivo*. However, in intact organisms mostly oxidative pathway takes place due to the oxygen tension. By oxidative dechlorination of chloroform, a reactive metabolite phosgene (CCl_2O) is formed. Phosgene binds covalently to tissue proteins, polar heads of phospholipids, and it can react with water to produce carbon dioxide and hydrogen chloride (hydrochloric acid). Phosgene binds with glutathione.
The major enzyme involved in chloroform metabolism is cytochrom P450 2E1 or CYP2E1 (ethanol-inducible mono-oxygenase). This was demonstrated by using CYP2E1 inducers and inhibitors in laboratory animals, CYP2E1 deficient mice and anti-CYP2E1 monoclonal protein. Regions of observed liver lesions in mice and rats correlate well with hepatic distribution of CYP2E1 and glutathione. CYP2B1 may also have a minor role in metabolism of chloroform since after induction of rats with phenobarbitone (CYP2B1 inducer) chloroform hepatotoxicity was potentiated compared to rats where no induction with phenobarbitone took place.
Phosgene covalently binds to liver macromolecules and damages the tissue. A probable mechanism that takes part in the detoxification of chloroform is binding with glutathione which prevents covalent binding in liver cells. After glutathione depletion continued chloroform exposures resulted in covalent binding and lipid peroxidation in the liver.
The highest metabolic activity was observed in liver, nose and kidney after oral exposure of rats to [^{14}C]chloroform (CICAD, Smith *et al.,* 1979)(135).
Physiologically based pharmacokinetic (PBPK) models were used to determine the metabolism of chloroform in humans, rats and dogs. The Canadian model is specific for human metabolism. This model is based on human metabolic parameters of liver and kidneys that were collected after metabolism of two known CYP2E1substrates by microsomal fractions of 18 humans, data on ventilation rate, blood flow and cardiac output (CICAD, Corely *et al*., 1990)(135). Using this model, it was calculated that the risk for cancer would increase for 5 % in with lifelong daily ingestion drinking water with 37 mg chloroform/L or lifelong daily inhalation of air with 9.8 mg chloroform/m^3 (95th percentile value: 12 mg/L and 3.4 mg/m^3). This value was used in the benchmark dose approach of calculation of tolerable daily intake (TDI).

3.2.1.3 Toxicity data and toxicity evaluation

Acute toxicity
Chloroform is moderately toxic to rats, with LD_{50} ranging from 0.45 to 2.0 g/kg bw. In mice LD_{50} values range from 36-1366 mg/kg bw. Clinical signs after acute oral exposure were narcosis and anaesthesia in rodents, and changes in liver and kidneys. After acute inhalation of chloroform major clinical signs were depression of the central nervous system (CNS) and kidney damage. Kidney damage was also observed after acute dermal exposure to chloroform.

Studies performed on acute toxicity of chloroform are summarised in Table 23.

Table 23: *Summary of acute toxicity studies on chloroform (137)*

SPECIES	EXPOSURE	DOSE	RESULTS	REFERENCE
Rat (F344 and Osborne-Mendel)	Oral gavage (or gastric intubation)	0, 10 and 90 mg/kg bw	Proliferation of renal cells, at 90 mg/kg bw lesions and epithelial cell proliferation in the nasal passage.	CICAD
Rat (male F344)	Oral gavage	0, 30 and 60 mg/kg bw	Values determined for serum enzyme changes indicating liver damage. NOAEL= 30 mg/kg bw LOAEL= 60 mg/kg bw	CICAD
Rat (Wistar)	Oral gavage	0, 67, 135 and 338 mg/kg bw	Dose-dependent ↑ of necrotic hepatocytes in centrilobullar region and ↑ alanine aminotransferase (ALT) level.	CICAD
Rats	Oral gavage	250 mg/kg bw	Liver and kidney changes.	CICAD
Mouse (B6C3F1, male)	Oral gavage	150 mg/kg bw	Cell proliferation in liver and kidneys, severe necrosis in the kidneys.	CICAD
Mouse	Oral gavage	240 mg/kg bw	Hepatic necrosis.	CICAD
Rat	Inhalation	No data.	LC_{50}= 9.2 g/m^3.	CICAD
Rat (F344)	Inhalation	0, 5 and 10 g/m^3 for 6 hrs	No mortalities at 5 g/m^3, at 10 g/m^3 17/20 animals died. Clinical signs: depression of the central nervous system.	CICAD
Rat	Inhalation	2,1 g/m^3 for 4 hrs	Significant subnarcotic effects.	CICAD
Mouse (OF1, females)	Iinhalation	No data.	LC_{50}= 2.1 g/m^3.	CICAD
Mouse (BDF1)	Inhalation	Females: 0, 2.5 and 40 g/m^3, 6 hrs. Males: 0, 59 and 120 mg/m^3, 6 hrs.	Females: no deaths occurred at 2.5 g/m^3, animals died at 40 g/m^3 (centrilobullar liver necrosis). Males: 1/10 died at 59 mg/m^3 and 8/10 mg/m^3 (cause of death: necrosis of proximal tubules of the kidneys). Males were much more susceptible than females.	CICAD
Rabbit	Dermal	1 g/kg bw, 24 hrs, covered application	Kidney tubule degeneration, no gross changes in the liver.	CICAD

According to the results of acute toxicity studies and European Directive 67/548/EEC on classification and labelling of dangerous substances chloroform should be classified as harmful if inhaled or swallowed (Xn, R20, R22).

Toxicity after repeated exposure

Results of the most relevant repeated exposure toxicity studies are summarised in Table 24.

Table 24: *Summary of repeated exposure toxicity studies of chloroform (136,137)*

SPECIES	EXPOSURE			RESULTS	NOEL	REFER-ENCES
	route	dose	duration			
Rat (F344)	Oral	0, 34 and 100 mg/kg bw/day	21 days (3weeks)	Lesions and changes in olfactory epithelium, changes in the nasal passages were seen at 34 mg/kg bw/day after 4-5 days of administration. After 3 weeks these changes were seen at 100 mg/kg bw/day, but not at 34.	NOAEL< 34 mg/kg bw/day	CICAD
Mouse (B6C3F1)	Oral	0, 32, 64, 97, 145, 290 and 436 mg/kg bw/day with drinking water	90 days	436 mg/kg bw/day: 160-250 % ↑ in liver fat and atrophy of the spleen was observed. Histological examination: ↑ in centrilobullar fat at 290 and 436 mg/kg bw/day.	NOAEL= 145 mg/kg bw/day, LOAEL= 290 mg/kg bw/day	IRIS (Jorgenson and Rushbrook , 1980)

Continued on next page

Continued from previous page: ***Table 24:*** *Summary of repeated exposure toxicity studies of chloroform (136,137)*

Mouse (CD-1)	Oral	0, 50, 125 and 250 mg/kg bw/day in drinking water	90 days	250 mg/kg bw/day: ↑ liver weight, ↓ hepatic microsome activity, ↓ humoral immunity and ↓ cellular immunity (♀ only). 50 and 125 mg/kg bw/day: ↓ bw gain, ↓ humoral immunity.	LOAEL= 50 mg/kg bw/day	THM WHO; Munson *et al.*, 1982.
Rat (F344), Mouse (B6C3F1 and BDF1)	Inhal-ation	2-300 ppm (10-1460 mg/m^3), 6 hours/day, 5 to 7 days /week	90 days	↑ histopathological lesions of mice and rats: liver lesions (vacuolated hepatocytes, necrotic foci, inflammation), renal lesions (vacoulation, basophilic appearance, tubule cell necrosis, enlarged cell nuclei), nasal lesions (atrophy of olfactory epithelium). Liver and kidney lesions were observed at 30 ppm or higher doses, while the effect on nasal epithelium was determined at 2 ppm.	Rat: no NOAEL determined, LOAEL=2 ppm B6C3F1 mice: NOAEL = 10 ppm BDF1 mice: NOAEL= 5 ppm.	IRIS (Larson *et al.*, 1996, Templin *et al.*, 1996a, Templin *et al.*, 1996b).
Rat (F344)	Inhal-ation	0, 9.8 and 50 g/m^3, 6 hrs /day.	4 and 7 days	After 4 days of exposure to 9.8: cell proliferation was observed in the ethmoid region of the nose. After 4 days only mild lesions were seen after exposure to 50 g/m^3, but after 7 days: lesions in nasal turbinates, including cell proliferation in central, proximal and distal regions of first endoturbinate and histological changes in the central turbinate bone.	Not determined.	IRIS (NCI, 1976)
Rat (Osborne-Mendel)	Oral	0, 90 and 180 mg/kg bw/day in corn oil, 5 days/week,	78 weeks	At 180 mg/kg bw/day the incidence of kidney epithelial tumors increased for 24 %.	Not determined.	IRIS (NCI, 1976)
Mouse (B6C3F1)	Oral	0, 60, 130 and 270 mg/kg bw/day in corn oil and 2 % emulgophor	90 days	Measurements of serum enzyme levels, tissue triglyceride levels and histological examination of the livers have shown that hepatotoxic effects were more pronounced after administration of chloroform in corn oil compared to water.	NOAEL (water)= 270 mg/kg bw/day NOAEL(corn oil) =130 mg/kg bw/day	IRIS (Bull *et al.*, 1987)

Toxicological effects of chloroform were more pronounced after administration in corn oil compared to the water. The authors of one study (Bull *et al.*, 1987)(137) suggest that the reason might be the reaction of chloroform with the corn oil or altered absorption kinetics in the presence of corn oil. Another probable explanation would be that by single oral gavage the dose of chloroform in much higher compared to the low doses of chloroform distributed over the longer period when it is administered in the drinking water (Integrated risk information system - IRISb)(136).

Genotoxicity/mutagenicity

Many *in vitro* and *in vivo* genotoxicity studies have been performed with chloroform. But the results of these studies must be interpreted with caution:

(1) chloroform is very volatile and actual concentration of chloroform in the test vessel might be lower than expected,
(2) metabolites of the chloroform (phosgene) mainly react with DNA, so appropriate active P450 enzyme based metabolic system should be used,
(3) metabolites are highly reactive, therefore tests using exogenous metabolic systems give unreliable results,
(4) in tests where ethanol is used as a solvent results may be confounded, because in the reaction between phosgene formed from the chloroform and ethanol ethyl or diethyl carbonate is produced. The ethyl or diethyl carbonate is an alkylating agent,

(5) in tests performed with high toxic doses of chloroform positive results might be a consequence of overall cytotoxicity.

The results of genotoxicity studies are presented in Table 25.

Table 25: *Summary of genotoxicity/mutagenicity studies of chloroform (138,139.)*

***IN VITRO* STUDIES**	**ENDPOINT**	**TEST SYSTEM/EXPOSURE**	**RESULTS**	**REFERENCES**
	Reverse mutations	*Salmonella typhimurium* TA 98, TA100, TA1535, TA1537	Positive result in 1 study (- activation) – no strain specified, Negative result all strains 2 studies (+/- activation system).	THM WHO Roldan-Arjona and Pueyo, 1993, Le Curieux *et al.*, 1995.
	Mammalian cell forward mutation	Chinese hamster cells	Negative.	IARC, 1987.
	Sister chromatid exchange	Human lymphocytes	Negative.	IARC, 1987.
		Chinese hamster cells	Negative.	IARC, 1987.
	Chromosomal aberration	Human lymphocytes	Negative.	IARC, 1987.
	Unscheduled DNA synthesis	Human lymphocytes	Negative.	IARC, 1987.
	DNA damage, mutation, mitotic recombination, aneuploidiy and gene conversion	*Saccharomyces cerevisiea*	DNA damage. Positive. Negative for other endpoints. Positive results when endogenous levels of cytochrome P450 were increased.	IARC, 1987.
***IN VIVO* STUDIES**	DNA binding	Rats and mice, liver and kidney cells at 742, 119 and 48 mg/kg bw. At 2.9 mg/kg bw: After pre-treatment with phenobarbitone: no increase in DNA binding.	Negative. Positive.	(IRIS, EPA) Diaz Gomez and Castro, 1980, Pereira *et al*., 1982, Reitz *et al*., 1982, Colacci *et al*., 1991.
	Micronuclei formation	Mouse (B6C3F1), 371 and 800 mg/kg bw. Rats and mice- several studies. Rats and mice: 400-600 mg/kg bw.	Negative. Negative. Positive.	THM WHO, Shelby and Witt, 1995. IRIS EPA Topham, 1980. Robbiano *et al*., 1998.
	Chromosomal aberrations	Mouse (B6C3F1).	Positive.	THM WHO, Shelby and Witt, 1995
	Sister chromatid exchange	Mouse. 50 and 200 mg/kg bw.	Positive.	Morimoto and Koizumi, 1983.

In *in vitro* genotoxiciy test systems the results of chloroform toxicity were mostly negative. But the results of *in vivo* studies were both negative and positive. Genotoxic effects *in vivo* were observed mostly at doses that were also cytotoxic for the animals. These data indicate that chloroform is not genotoxic.

Carcinogenicity

Some of the relevant studies on carcinogenicity of chloroform are presented in Table 26.

Table 26: *Summary of long-term toxicity and carcinogenicity studies with chloroform (135,140)*

SPECIES	EXPOSURE			RESULTS	NOEL	REFER-ENCES
	Route	Dose	Duration			
Mouse (B6C3F1)	Oral	0, 138 and 277, mg/kg, males, 0, 238 and 477, mg/kg, females.	92 weeks.	477 mg/kg bw/day: ↓ survival. ↑ incidence of hepatocellular carcinomas in all treted groups. 138 mg/kg bw/day ♂: nodular hyperplasia of the liver was observed.	LOAEL = 138 mg/kg bw/day	CICAD
Dog (beagle)	Oral	0, 15 and 30 mg/kg bw/day, 6 days/week in toothpaste base	7.5 years, followed by 20-24 weeks of recovery	15 mg/kg bw/day: 30-50 % ↑ of ALT . 30 mg/kg bw/day: ALT level was doubled. This could be the result of minimal liver damage. After microscopic examination ↑ in fatty cysts (aggregations of vacuolated histiocytes) was observed in the liver of treated dogs (control 1/27, low-dose 9/15, high-dose 13/15).	LOAEL = 15 mg/kg bw/day	IRIS (Hey-wood, 1979)
Rat (Osborne-Mendel ♂) and mouse (B6C3F1♀)	Oral	0, 200, 400, 900 and 1800 ppm/day in drinking water	6 months	Rats: no treatment related findings. Mice: ↑ mortality in first 3 weeks, reduced water consumption and consequently lassitude and lack of vigor. ↑ in liver fat after 3 months at 65 mg/kg bw/day and at 130 and 236 mg/kg bw/day after 6 months.	Rat: NOAEL = 160 mg/kg bw/day Mouse: NOAEL= 34 mg/kg bw/day	IRIS (Jorgen-son *et al*., 1982)
Rat (Osborne-Mendel males) and mouse (B6C3F1 females)	Oral	0, 200, 400, 900 and 1800 ppm/day in drinking water	104 weeks	Rats: ↑ incidence of kidney tumors at 160 mg/kg bw/day. At 81 and 160 mg/kg bw/day: ↑ of renal tubular injury (basophilia, cytoplasmic vacuolation, hyperplasia of proximal tubules, single-cell necrosis, karyomegaly). Mice: No ↑ of hepatocellular carcinoma.	Rat: NOAEL = 38 mg/kg bw/day (400 ppm), Mouse: NOAEL= 236 mg/kg bw/day	IRIS (Jorgen-son *et al*., 1985)
Rat (F344 males)	Oral	0, 900 and 1800 ppm in drinking water	100 weeks	At 1800 ppm: ↑ of hepatocellular proliferative lesions, ↑ multiplicy of adenomas and carcinomas in the liver. The only histopathological lesion in the liver observed was the midzonal vacuolization, due to the fat accumulation.	NOAEL = 45 mg/kg bw/day	IRIS (De-Angelo, 1995).
Rat (F344), mouse (BDF1)	Inha-lation	Rats: 0, 10, 30 and 90 ppm. Mice: 0, 5, 30 and 90 ppm. 5 days/week	104 weeks	Rats and mice: necrosis and metaplasia of olfactory epithelium and gobbler cell hyperplasia of the respiratory epithelium. At the lowest dose: ossification of the nasal turbinate and the nasal septum. At 30 and 90 ppm: ↑ renal cell adenoma and renal cell carcinoma was observed in the male mice.	NOAEL not determined.	IRIS (Nagano *et al*., 1998).

Chloroform induced kidney tumours, hepatocellular carcinomas and adenomas in rats and liver tumours and renal cell carcinoma and renal cell adenomas in mice. The most sensitive species in long-term study (7.5 years) was the dog and NOAEL 15 mg/kg bw/day was determined.

Further studies were performed to assess the initiating and promoting potential of carcinogenic activity of chloroform if administered in the drinking water. In several studies, administration of chloroform in drinking water did not initiate tumor formation. Chloroform did not act as a co-carcinogen, nor did it promote the development of liver tumor. One oral study was performed to determine initiation potential of chloroform, but after a single dose of 180 mg/kg/bw, no such effect was observed. In one study liver tumor formation was induced by diethyl nitrosamine, after that the animals were treated with the single

dose of 180 mg chloroform/kg/bw for 2 months. In this study no promoting potential of chloroform was observed. In the study performed by Demi and Oesterle (1985)(135), oral administration of chloroform enhanced the incidence of liver preneoplastic tumors in rats treated with a single dose of N-nitroso diethylamine (CICAD).
A possible explanation of carcinogenic activity of chloroform is the alteration of gene expression in the cells. H-ras, *myc* and *fos* (mRNA), methylation of ras oncogenes and p53 gene were considered as the genes that could be affected. After exposure to chloroform (0.5-2% v/v), hypermethylation of p53 gene observed in rat liver epithelial cells. Hypermethylation could be the result of protein kinase C stimulation and this might represent the alternative mode of action in chloroform carcinogenicity (CICAD).

Human data: Investigations have attempted to assess the correlation of the exposure to chloroform from extensively chlorinated drinking water with cancer incidence (IARC, 1987). In areas with chlorinated drinking water, increased community-based rates on cancer mortalities have been reported. Correlations were found between mortality due not only to bladder cancer, but also to rectum/large intestine, brain, kidney cancer and lymphoma (IARC, 1987). A mortality study of anaesthesiologists who worked at the time chloroform was used provided no significant information.

Two cohort studies of cancer and drinking-water quality were carried out in the United States. The first one showed increased mortality from cancers of the liver and breast, while the second one indicated increased risk of colon cancer, lung and skin melanoma associated with chloroform concentrations in drinking-water (IARC, 1999). Eight case–control studies have been reported on bladder cancer in relation to chlorinated drinking-water. Significant increasing trends in the risk for bladder cancer were seen in two studies. One showed increasing risk with years of exposure to chlorinated water and the other increasing risk with lifetime intake of THM's (from drinking-water), but only in men and not in women (1999). Exposure to chloroform in the workplace was addressed in two case–control studies, both of which had limited statistical power. The study showed associations with cancers of the prostate and lung, but no association was seen with bladder cancer (IARC, 1999).

However it is not possible to evaluate any effect of chloroform from these studies. While chloroform is the most ubiquitous product of water chlorination, other disinfection by-products and impurities that may harm the health are present in such water too. Therefore it is difficult to isolate the role of chloroform in various site cancer formation from other possible causes. In majority of the studies, other sources of chloroform exposure were emphasized, but drinking water was ignored.

Chloroform was first evaluated by IARC in 1979. There was sufficient evidence that chloroform is carcinogenic to animals (IARC, 1979)(141). In the second evaluation chloroform was classified as Group 2B – possibly carcinogenic to humans (IARC, 1987)(138).

Reproductive toxicity
Summary of reproductive toxicity studies of chloroform is presented in Table 27.

Table 27: *Summary of reproductive toxicity of chloroform (136)*

STUDY TYPE	ANIMAL SPECIES	EXPOSURE, DOSE LEVEL AND DURATION	RESULTS	NOEL	REFER-ENCES
Teratology study (1)	Rat (Sprague-Dawley)	Oral; 0, 79 126, 300, 316 and 516 mg/kg bw/day. Days 6-15 of gestation.	Mothers: at all doses alopecia, rough hair and eczema. ↓ bw gain and ↓ food consumption at 126 mg/kg bw/day and higher doses. 316 and 516 mg/kg bw/day nephrosis, hepatitis and maternal death. Foetus: Fetotoxic effects at 316 and 516 mg/kg bw/day.	Maternal: 79 mg/kg bw /day, Offspring: 300 mg/kg bw/day	IRIS (Thompson , 1974)
Teratology study (2)	Rat (Sprague-Dawley)	Oral; 0, 20, 50 and 126 mg/kg bw/day. Days 6-15 of gestation.	Mothers: ↓ bw gain and mild fatty changes in the liver at 50 and 126 mg/kg bw/day. Foetus: ↑ frequency of bilateral extra lumbar ribs and ↓ bw at 126 mg/kg bw/day.	Maternal: 20 mg/kg bw /day, Offspring: 50 mg/kg bw/day	IRIS

Continued on next page

Continued from previous page: ***Table 27:*** *Summary of reproductive toxicity of chloroform (136)*

Range finding study.	Rabbit (Dutch-belted)	Oral; 0, 25, 63, 100, 159, 251 and 398 mg/kg bw/day in corn oil. Days 6-18 of gestation.	Mother: ↓ survival at 100 mg/kg bw/day and higher doses. At 63 mg/kg bw/day: anorexia, weight loss, diarrhea, abortion and 1 death. Animals exposed to 25 mg/kg bw/day: mild diarrhea and intermittent anorexia. Foetus: no data.	No NOAEL determined.	IRIS
Teratology study (3)	Rabbit (Dutch-belted)	Oral; 0, 20, 35 and 50 mg/kg bw/. Days 6-18 of gestation.	Mother: ↓ bw gain and 4/15 death at 50 mg/kg bw/day. Foetus: ↓ bw at 20 and 50 mg/kg bw/day. Data at 20 mg/kg bw/day were considered not an evidence of teratogenic effect.	Maternal: 35 mg/kg bw/day. Foetus: 35 mg/kg bw/day.	IRIS
Twogenera-tion reproduction study	Mouse (CD-1)	Oral; 0, 6.6, 16 and 41 mg/kg bw/day in corn oil. 7 days/week, 18 weeks.	F_0 and F_1 generation: no effect on fertility index, nr. of litters per pair, litter size, proportion of live pups, sex ratio, pup weight at birth. F_1 females: ↑ liver weight and liver lesions (degeneration of centrilobular hepatocytes) at 41 mg/kg bw/day. F_1 males: ↑ epididymal weight. Sperm count, motility and percent of abnormal sperms were not altered.	Not determined (no histological examination at low and mid dose).	IRIS (NTP, 1988)
Teratology study (4)	Rat	Oral; 0, 100, 200 and 400 mg/kg bw/day. Days 6-15 of gestation.	Mothers: At all doses: ↓ bw gain, ↓ hemoglobin levels, ↓ hematocrit level, ↑ liver size. At high dose: ↑ of inorganic phosphorus, ↑ cholesterol levels, ↑ kidney weight, ↓ RBC count. Foetus: at 400 mg/kg bw/day ↓ bw. At 200 and 400 mg/kg bw/day: ↑ sternebrea aberrations.	Maternal: <100 mg/kg bw/day, Foetal: 100 mg/kg bw/day	IRIS (Ruddick, 1983)
Embryoto-xicity study (1)	Rat (Wistar)	Inhalation; 0, 30, 100 and 300 ppm (0, 146, 488 and 1464 mg/m^3); 7 hours/day. Days 7-16 of gestation.	Mothers: dose dependent ↓ in food consumption and bw gain. ↑ dead foetuses at all doses. Foetuses: at 300 ppm: ↓ bw, ↓ crown-rump lenght.	Maternal: < 146 mg/m^3, time-weighted concentration 46,1 mg/m^3. Foetal: < 146 mg/m^3, time-weighted concentration 46,1 mg/m^3.	IRIS (Baeder, 1988)
Embryoto-xicity study (2)	Rat (Wistar)	Inhalation; 0, 3, 10 and 30 ppm (0, 15, 52.2 and 147 mg/m^3); 7 hours/day. Days 7-16 of gestation.	Mothers: ↓ food consumption and bw gain at 10 and 30 ppm. ↑ dead foetuses at all doses. Foetuses: at 30 ppm: ↓ bw (<3 g), ↓ crown-rump length, ↑ incidence of foetuses with slight or no ossification of individual skull bones.	Maternal: < 3 ppm (15 mg/m^3). Foetal: 10 mg/m^3 (52.2 mg/m^3).	IRIS (Baeder, 1991)
Embryo- and fetotoxicity (3)	Rat (Sprague-Dawley)	Inhalation; 0, 30, 100 and 300 ppm (0, 146, 464 or 1420 mg/m^3). 7 hours/day. Days 6-15 of gestation.	Mothers: at all doses: ↓ food consumption and bw gain. At 300 ppm: ↓ absolute liver weight, 61 % of implantations were resorbed. Foetuses: at 300 ppm ↓ bw and crown-rump length. At 100 ppm: litter with acaudia or imperforate anus, ↑ frequency of missing ribs and subcutaneous oedema. At 30 ppm: ↑ incidence of wavy ribs.	Maternal: < 30 ppm. Foetal: 30 ppm.	IRIS (Schwetz, 1974)

Only one two-generation study was performed on rats. In this study chloroform did not affect reproduction potential of the animals: fertility index, number of litters per pair, litter size, proportion of

live pups, gender ratio and pup weight at birth. However, the liver weight of F_0 and F_1 females was increased, and there was also an increase in the epididymal weight in F1 males.
Oral teratology studies on rats have shown that chloroform was fetotoxic only at toxic doses for the mother. In the rabbit teratology study the effect of chloroform on the mother and the foetus was observed at the same dose, but lower bw of the foetus is probably the result of lowered bw of the mother.
Effects on foetuses were observed also in inhalation embryotoxicity studies and again at concentrations the same or higher to concentrations that induced parental toxicity. From these data we can conclude that chloroform is toxic for reproduction and development but only at the doses that are generally toxic for parent animals.

Human data: Some epidemiological studies were performed to investigate the association between exposure to chloroform and other disinfection byproducts in chlorinated water and the occurance of adverse reproductive consequences. In one case, there was a significant relationship between chloroform levels and decreased intrauterine growth (IRIS, Kramer *et al.*, 1992)(142). In two other studies, low birth weight, oral cleft defect, cardiac defects and retarded fetal growth were observed (IRIS: Bove *et al*., 1995, Gallagher *et al*., 1998)(143,144). These studies are very useful in evaluating the repro- and fetotoxic effects of chloroform, however they are not adequate to establish a causal link between chloroform exposure in the drinking water and occurrence of adverse reproductive effects. In chlorinated drinking water also other harmful disinfection products might be present as well as other contaminants (pesticides, heavy metals,..) so these effects are not necessarily due to chloroform exposure from the drinking water.

3.2.1.4 Exposure

In this case, the focus is on the estimation of exposure of adult person to chloroform from chlorinated drinking water.
It is difficult to determine the exact amount of chloroform in the drinking water in Slovenia, because the analytical methods used in the monitoring of drinking water measure only the total amount of THM's.

The values determined for total THM's in the drinking water are 30 μg/L or less. As the worst case, the total quantity of THM's in the drinking water was considered to be chloroform.

Scenario:
Daily intake of water: 2 L,
Chloroform concentration: 30 μg/L,
Average weight of an adult person: 60 kg,

Estimated chloroform exposure = (30 μg/L 2L/day)/60 kg = 1 μg/kg bw/day.* {6}

WHO prepared (WHO, 2005)(133) the estimation of total daily intake of chloroform. Exposure of general population to chloroform was estimated to be 2-3 μg/kg bw/day (if the tap water contains relatively high concentrations of chloroform it may reach 10 μg/kg bw/day):

- indoor air: 0.3 – 1.1 μg/kg bw/day,
- intake during showering (10 minutes with warm water) – dermal and inhalation: 0.5 μg/kg bw/day,
- ingestion of chlorinated drinking water: 0.7 μg/kg bw/day,
- food: 1 μg/kg bw/day.

3.2.1.5 Toxicity, hazard and risk estimation

Chloroform is readily absorbed after oral, dermal or inhalation exposure. It is widely distributed through the body and excreted mostly through the exhaled air. It does not accumulate in the body. Chloroform

is mainly metabolised in the liver, with CYP2E1 being the major enzyme involved in chloroform metabolism.
In acute and repeat-dose toxicity studies critical targets of chloroform were liver, kidneys and olfactory nasal epithelium. Observed lesions included cellular degradation, vacuolisation, necrosis and change in weight of a target organ. Nasal effects were the result of absorbed chloroform, rather than of direct contact because the effect was seen after oral or inhalation exposure.
Chloroform was reprotoxic in teratogenic at the same or higher concentrations that induced toxic effects on the parents. Repro- and fetoxicity are therefore secondary effect of parental toxicity.
Chloroform increased the formation of liver and kidney tumors in several species by various routes of exposure. Carcinogenic effects are the result of non-genotoxic mechanisms. One model of tumour induction by chloroform involves: metabolism of chloroform in target organs, induction of sustained cytotoxicity by metabolites and subsequent persistent regenerative cell proliferation (Environment Canada, Health Canada, 2001)(145).

Setting the reference dose:
To determine the oral reference dose for chloroform the traditional NOAEL/LOAEL and benchmark dose approach were used (IRIS).

NOAEL/LOAEL approach: To determine TDI of chloroform (the dose that does not adversely affect health of the exposed people) reliable long-term exposure studies, were included. Also included were the studies of reproductive and developmental toxicity. In various long-term studies, liver toxicity was the most sensitive endpoint. As an appropriate study to determine the TDI, the 7.5 years long study on dogs was chosen (Heywood, 1985). In dogs, aggregations of vacuolated histiocytes were observed in the liver, the critical target organ. This study is also suitable because the animals were exposed to chloroform in the drinking water, which is the main route of exposure of humans. The study performed by Heywood (1985) only identified the LOAEL (lowest observed adverse effect level) 15 mg/kg bw/day, because this was the lowest concentration used. TDI was derived:

15 mg/kg bw/day =	LOAEL defined in 7.5 years study on dog,
6 days/7 days =	adjustment to account for exposure 6 days/week,
1000 =	assessment factor; 10 for using the LOAEL instead of NOAEL, 10 for interspecies variations and 10 for intraspecies variation,

TDI= (15 mg/kg bw/day (6 days/7 days))/ 1000 = 0.013 mg/kg bw/day* {7}

Benchmark dose approach: The benchmark dose (BMD) approach utilizes mathematical models to characterise the dose response curve for the given endpoint (10 % increase in toxic effects on liver). BMD based on the incidence of fatty cysts in dogs (Heywood, 1985) was 1.7 mg/kg bw/day and lower confidence limit of the BMD (BMDL) was calculated to be 1.2 mg/kg bw/day. This value was adjusted because the dogs were exposed 6 days/week:

*BMDL = 1.2 mg/kg bw/day *(6 days/7 days) = 1 mg/kg bw/day* {8}

TDI = (1 mg/kg bw/day)/ 100 = 0.01 mg/kg bw/day {9}

Coincidently the values calculated using both approaches were the same.

Risk assessment for adult person:
TDI: 0.013 mg/kg bw/day,
Estimated exposure from drinking water: 0.001 mg/kg bw/day,

Risk assessment = (0.001 mg/kg bw/day)/(0.013 mg/kg bw/day) = 7.7% {10}

The dose of chloroform that people are exposed to from drinking water is 7.7 % of TDI (if chloroform was the only THM's present in the water).

In Slovenia it is determined by the rule for drinking water that the concentration of total THM's should not exceed 100 μg/L water (U.l. RS 19/04)(146). Again, in the worst case if all of the THM's in water was chloroform, the estimated exposure of an adult to chloroform would be 3 μg/kg bw/day and this would account for 26 **%** of TDI.

WHO estimated daily exposure to chloroform on 3 μg/kg bw/day what would make 23 % of TDI (in the worst case 10 μg/kg bw/day is 77 % of TDI).

3.2.1.6 Risk evaluation

At exposure lower than the reference value, there should be no non-cancer or cancer adverse effects on health of the consumers.
Estimated exposure of adult person to chloroform by drinking chlorinated water was calculated to be 7.7 % of TDI in the scenario where people are exposed to chloroform only by drinking contaminated water and 23 % in case that total THM's concentration in drinking water is 100 μg/L and the only THM present in the water is chloroform. There is no risk for human health after ingestion of drinking water containing such amount of chloroform.
If the WHO estimation of total chloroform daily intake 2-3 μg/kg bw/day is suitable for Slovenia (to our knowledge in Slovenia no such estimation was performed) human health is also not at risk, because this exposure represents only 23 % of TDI.

3.2.2 Bromodichloromethane ($CHBrCl_2$)

BDCM is used in the synthesis of other chemicals or as a solvent (THM; WHO)(133).
BDCM is found in chlorinated drinking water as a consequence of the reaction between chlorine, used as a water disinfectant, and natural organic substances in the presence of bromide (124). Bromide exists widely in natural water, of which the concentration is much higher in river and ground water in coastal areas because of seawater invasion. BDCM is one of the major components of the organohalides produced by marine algae (IARC, 1991)(147). Sea water normally contains about 65 mg/L Br and about 19,000 mg/L Cl. The soil contains <0,001 – 0,01 mg/L Br and 7 – 50 mg/L Cl. Surface flowing water contains about 0,005 – 140 mg/L Br and 5 – 35 mg/L Cl (148). Sometimes, it is possible to expect up to 2000 mg/L Br in natural water, according to the geological structure of the soil (149).
The major route of human exposure to BDCM is by drinking chlorinated water.

3.2.2.1 Physical and chemical properties of BDCM

Toxicologically relevant physical and chemical properties of BDCM are summarised in Table 28.

Table 28: *Summary of physical and chemical properties of BDCM* (150)

PROPERTY	INFORMATION
Chemical name (IUPAC)	Bromodichloromethane
Synonyms	Dichlorobromomethane, monobromodichloromethane
CAS No.	75-27-4
Chemical formula	$CHBrCl_2$
Molar mass	163.83 g/mol
Physical state	Lliquid
Colour	Colorless
Odour	Sweet
Solubility in water at 20 ºC	4.5 g/L
Log P_{ow}	2.1
Vapour pressure at 20 ºC	50 mm Hg
Melting point	-57.1 ºC
Boiling point	90 ºC
Density at 20 ºC	1.98 g/cm^3

3.2.2.2 Kinetic and metabolism data

Studies on absorption, distribution, metabolism and excretion of BDCM were performed on rats and mice. Mink *et al*. (1983 in Environmental health criteria - EHC)(151) compared pharmacokinetics between rats and mice exposed to radioactively labelled BDCM. B6C3F1 mice were exposed to 150 mg/kg bw in corn oil by gavage. 93 % of radioactivity was recovered in 8 h: 81 % were expired in air as CO_2 and 7.2 % as parent compound. 2.2% were excreted in urine and 3.2 % was detected in organs. Elimination half-life of BDCM in mice was 2.5 hours. Sprague-Dawley rats were exposed to 100 mg/kg bw in corn oil by gavage and only 63 % of radioactivity were recovered after 8 hours. Only 14 % was detected in form of CO_2 in expired air, the highest residual radioactivity was observed in liver, kidneys and lungs. The elimination half-life of BDCM in rats was 1.5 hours. Results of this study strongly suggest that absorption, metabolism and excretion of BDCM is much faster in mice compared to rats and this might also be a reason for rats being more susceptible than mice in the following toxicological studies.

Lilly *et al*. (1994 EHC)(152) studied absorption, distribution, metabolism and excretion (ADME) in F344 after receiving 50 or 100 mg/kg bw of BDCM in corn oil or aqueous emulsion. The animals were administered the corn oil solution by gavage while they drank the aqueous emulsion. After exposure to BDCM in water the peak level in blood was determined at 6 minutes after exposure with the maximum concentration of 16 and 26 mg/L. When animals were given BDCM in corn oil the peak concentration 5 and 9 mg/L was reached after 15 or 30 minutes. Elimination half-life of BDCM in the blood was 1 h for lower and 1.5 h per higher dose. After administration in aqueous emulsion the amount of unmetabolised BDCM in expired air was higher (8.9 % and 13.2 %) compared to administration in corn oil (5.3 % and 5.8 %).

Mathews *et al*. (1990, EHC) has shown that there is no effect of saturation of metabolism and excretion in F344 rats. The rats were exposed to 0, 1, 10, 32 and 100 mg/kg bw/day for 10 days. 24 hours after exposure more than 90 % of radioactivity was recovered in non-fecal excreta samples and tissues what indicates that BDCM is rapidly absorbed and metabolised in rats. Repeated doses had no effect on tissues distribution of BDCM and significant bioaccumulation was not observed (0.9-1.1 % total retention of the label) (BDCM was administered by gavage).

Metabolism: BDCM is probably metabolised the same way as chloroform to phosgene and/or CO_2. In metabolism of BDCM CYP2E1 and CYP2B1/2 are involved (EHC, Thorton-Manning *et al*., 1994)(153), as well as a theta-class glutamate S-transferase (GST).

Human data: There are only a few data regarding ADME in humans. A BDCM concentration of 14 ng/mL blood was found in a blood sample from one resident living near the waste site in New York (Toxnet: Barkley *et al*., 1980)(154). BDCM was not detected in human fat studied in the National Human Adipose Tissue Survey (NHATS) (IRIS).

3.2.2.3 Toxicity data and toxicity evaluation

Oral toxicity of BDCM was assessed in many studies on experimental animals. Some of the relevant studies and studies used to determine the reference dose of BDCM as well as studies crucial for classification of BDCM are presented in this chapter. To our knowledge no studies on toxicity of BCDM after inhalation or dermal exposure were performed.

Acute toxicity
Studies performed on acute toxicity of BDCM are summarised in Table 29.

Table 29: *Summary of acute toxicity studies (155).*

STUDY TYPE	SPECIES	RESULTS	REFERENCES
Acute oral toxicity (1)	Rat	LD_{50} = 510 mg/kg bw ♀ LD_{50} = 430 mg/kg bw ♂	ATSDR BCDM; Aida *et al*., 1987.
Acute oral toxicity (2)	Rat: Sprague-Dawley	LD_{50} = 969 mg/kg bw ♀ LD_{50} = 916 mg/kg bw ♂	ATSDR BCDM; Chu *et al*., 1980.
Acute oral toxicity (3)	Mouse: ICR	LD_{50} = 450 mg/kg bw ♂ LD_{50} = 900 mg/kg bw ♀	ATSDR BCDM; Bowman *et al*., 1978

According to the results of acute toxicity studies BDCM is toxic by the oral route, and in accordance with European directive 67/548 EEC on classification and labelling of dangerous substances, BDCM should be classified as harmful by ingestion (Xn, R22). In acutely exposed animals ataxia, disturbed coordination, sedation, lethary and anaesthesia were observed (500 mg/kg bw). After single exposure also hepatic and renal toxicity was observed.
Additional study was performed in F344 rats after acute exposure to BDCM in drinking water (EHC, Lilly *et al*., 1994, 1997a)(156). The maximal liver toxicity was observed after dosing with 164-492 mg/kg bw as observed by the increased level of serum, ALT, AST (aspartate aminotransferase), sorbitol dehydrogenasis, LDH (lactate dehydrogenasis) and histopathological observations of centrilobular vacuolar degeneration and hepatocellular necrosis. Significant abatement of hepatic toxicity was noted 48-hours post-dosing. The acute oral NOAEL and LOAEL in this study were 41 and 82 mg/kg bw. Kidney damage was detected after single oral dose of BDCM in corn oil or aqueous solution (200-492 mg/kg bw). Indicators of renal damage were: increased kidney weight, urinary N-acetyl-β-glucosaminidase, AST, ALT, LDH and protein, serum urea and creatinine, renal tubule degeneration and necrosis (EHC, Lilly, 1994, 1997a)(156). Nephrotoxicity was observed already at 200 mg/kg bw. BDCM is equipotent acute hepatotoxicynt as chloroform and even more acutely toxic for kidneys compared to chloroform (Lilly *et al*., 1997a).

Toxicity after repeated exposure
Results of repeated exposure toxicity studies of BDCM are summarised in Table 30.

Table 30: *Summary of repeated exposure toxicity studies of BDCM (157).*

SPECIES	EXPOSURE			RESULTS	NOEL	REFER-ENCES
	Route	Dose	Duration			
Rat (F344) 10/gender/ dose	Oral (corn oil)	0, 19, 38, 75, 150 or 300 mg/kg bw/day, 5 days/week	13 weeks	Rats; 300 mg/kg bw/day: 50 % of ♂ and 20 % of ♀ died. Hepatic centrilobullar degeneration, mild bile duct hyperplasia and athrophy of the thymus, spleen and lymph nodes in both genders. Renal tubular degeneration and necrosis in ♂.	Rat: 75 mg/kg bw/day	EHC (NTP, 1987)
Mouse (B6C3F1)		0, 50, 100, 200 and 400 mg/kg bw/day, 5 days/week		Mice: 100 mg/kg bw/day: degeneration and necrosis of the kidney. 200 and 400 mg/kg bw/day: centrilobullar degeneration of the liver.	Mouse: 50 mg/kg bw/day	

Continued on next page

Continued from previous page: ***Table 30:*** *Summary of repeated exposure toxicity studies of BDCM (157).*

Mouse (CD-1)	Oral (aqueous gavage)	0, 50, 125 and 250 mg/kg bw/day	14 days	250 mg/kg bw/day: nephrotoxicity (↑ blood urea nitrogen). 125 and 250 mg/kg bw/day: effect on humoral immune system (↓ antibody forming cells and haemaglutination titres), ↑ liver weight.	50 mg/kg bw/day	EHC (Munson *et al*., 1982)
Mouse (CD-1)	Oral (corn oil)	0, 37, 74 and 147 mg/kg bw/day	14 days	74 and 147 mg/kg bw/day: renal damage. 147 mg/kg bw/day: liver toxicity	37 mg/kg bw/day	EHC (Condie *et al*., 1983).

In repeated exposure studies main toxic effects of BDCM were observed on liver and kidneys which are the main active sites of BDCM metabolism. Covalent adducts formed by dihalomethyl or dihalocarbonyl free radicals and cellular proteins or lipids probably cause cell damage by impairing the function of these macromolecules (EHC, Gao *et al*., 1996). BDCM produced significantly higher amounts of these adducts in hepatic or renal microsomes compared to chloroform (EHC, Gao and Pegram, 1992). Another mechanism proposed to be involved in tissue damage induced by BDCM is induction of lipid peroxidation by free radical metabolites (EHC, de Groot and Nool, 1989).
Minor effect was observed on immune system.

Genotoxicity/mutagenicity
Results on genotoxicity studies of BDCM are gathered in Table 31. All studies are described in Agency for toxic substances and disease registry (ATSDR) document on BDCM.

Table 31: *Summary of genotoxicity/mutagenicity studies of BDCM (155)*

	ENDPOINT	**TEST SYSTEM/EXPOSURE**	**RESULTS**	**REFER-ENCES**
***IN VITRO* STUDIES**	Reverse mutations	*Salmonella typhimurium* TA 98, TA100, TA1535, TA1537	Positive results: TA 100 in 2 studies (-activation system) TA1537(-activation system), Negative results: all strains 1 study (+/- activation system).	Simmon *et al*., 1972, Varma *et al*., 1988, Ishidate *et al*., 1982, Mortelmans *et al*., 1986.
	Mitotic recombination	*Saccharomyces cerevisiae* D3	Negative(+/- activationsystem)	Simmon and Kauhanen, 1978.
	Mammalian cell forward mutation	Mouse L5178Y lymphoma cells/TK locus	Positive (+ activation) and negative (- activation).	NTP, 1987.
	Sister chromatid exchange	Human lymphocytes	Positive results: 2 studies (- activation).	Morimoto and Koizumi, 1983, Sobti 1984.
		Rat liver cells	Positive (- activation).	Sobti, 1984.
		Chinese hamster ovary cells	Negative (+/- activation).	NTP, 1987.
	Chromosomal aberration	Chinese hamster fibroblasts	Positive (+ activation).	Ishidate *et al*., 1982.
		Chinese hamster ovary cells	Negative (+/- activation).	NTP, 1987.
***IN VIVO* STUDIES**	Sister chromatid exchange	Mouse (CR/SJ), bone marrow cells	Positive.	Morimoto and Koizumi, 1983.
	Micronuclei formation	Rat and mouse, bone marrow cells	Negative	Ishidate *et al*., 1982, Hayashi *et al*., 1988.
	Chromosomal aberrations	Rat (Long-Evans), bone marrow cells, oral or intraperitoneal exposure (16.4 mg/kg bw)	Positive.	Fujie et al., 1990.
	DNA strand breaks	Rat (F344, kidney cells), gavage (246 mg/kg bw, 7 days)	Negative.	Potter *et al*., 1996.
	Unscheduled DNA synthesis	Rat (liver of males), 134 or 450 mg/kg bw/day intubation of aqueous solution, single dose.	Negative.	Stocker *et al*., 1997.

BDCM was mutagenic in most of the *in vitro* systems used, but the responses with and without metabolic activation system are less consistent.
In *in vivo* studies BDCM exerted some genotoxic potential indicated by increased sister chromatid exchange in mice and chromosomal aberrations in rats. However, BDCM did not induce micronuclei formation, DNA strand breaks or unscheduled DNA synthesis in experimental animals.
In metabolism of BDCM, highly reactive metabolite phosgene was formed. Phosgene can react with glutathione and although the reaction is protective for renal and hepatic toxicity, direct conjugation with brominated THM and GSH may lead to genotoxicity. Currently it is not known if this reaction also takes place in humans.

Carcinogenicity
Carcinogenicity studies of BDCM are summarised in Table 32.

Table 32: *Summary of long-term toxicity and carcinogenicity studies with BDCM (150)*

SPECIES	DOSE	DURATION	CRITICAL EFFECTS	NOAEL/ LOAEL	REFER-ENCES
Rat (F344/N) 50/gender/ dose Mouse (B6C3F1) 50/gender/ dose	0, 25, 50 and 100 mg/kg bw/day by gavage in corn oil	102 weeks	↓ bw 100 mg/kg bw/day: ↑ in adenomas and adenocarcinomas of the kidney. Males: cytomegalia and tubular cell hyperplasia of the kidney, fatty metamorphosis of the liver. Dose dependent ↑ adenocarcinomas and adenomatous polyps in the large intestine. Females: Eosinophillic cytoplasmic changes, focal cellular change, fatty metamorphosis of the liver and kidney tubular cell hyperplasia. ↑ of large intestine adenocarcinomas and adenomatous polyps.	LOAEL: 25 mg/kg bw/day, adjusted to 5 days treatment/ 7 days: 17.9 mg/kg bw/day	(EPA IRISb) NTP, 1986.
	Males: 0, 25 and 50 mg/kg bw/day, Females: 0, 75 and 150 mg/kg bw/day, by gavage in corn oil.	102 weeks	↓ bw Males: renal cytomegaly, fatty metamorphosis of the liver, thyroid follicular cell hyperplasia, tubular cell adenomas in kidney. ↑ of tubular cell adenomas and adenocarcinomas at 50 mg/kg bw/day. Females: follicular cell hyperplasia of the thyroid gland, hepatocellular adenomas (at 75 and 150 mg/kg bw/day). ↑ of hepatocellular adenomas at 75 and 150 mg/kg bw/day and ↑ of hepatocellular carcinomas at 150 mg/kg bw/day.		
Rats (Wistar) 40/gender/ dose	Males: 0, 6, 26 and 128 mg/kg bw/day, Females: 0, 8, 32 and 168 mg/kg bw/day. In diet.	2 years	↑ liver weight at all doses, hepatic fatty degeneration and granuloma. ↑ kidney weight and cholangiofibrosis in the high dose group.	LOAEL= 6 mg/kg bw/day	EPA IRISb.
Rat (Wistar)	1.2 ml of BDCM in drinking water (males 200 mg/kg bw/day and females 150 mg/kg bw/day)	140 – 180 weeks.	↓ in bw (35-40 %). Females: ↑ hepatic neoplastic nodules. Adenomas and adenocarcinoma were observed in treated animals (2 ♂ and 1 ♀) but not in controls.	Not determined.	Tumasonis *et al*., 1985. EPA IRIS b.
Mouse (CBA×C57 Bl/6) 50/gender/ dose	0.0076, 0,76 and 76 mg/kg bw/day) in drinking water	104 weeks	No statistically significant increase in the incidence of tumor formation in mice.	Not determined.	Veronin *et al,* 1987, EPA IRIS b.

BDCM was carcinogenic in rats and mice. It induced kidney adenomas and adenocarcinomas in rats and mice, large intestine adenomas and adenomatous polyps in rats and hepatocellular carcinomas in mice.
Mechanisms involved in carcinogenicity of BDCM could be genotoxicity of BDCM and formation of covalent adducts, formation of free radicals induced in BDCM metabolism (dihalomethyl free radical, and formation of products of conjugation of GSH with dihalomethane). Additional *in vivo* studies are required to confirm the mechanisms underlying BDCM carcinogenicity.

There is sufficient evidence that BDCM is carcinogenic to experimental animals (positive results in two species), but inadequate evidence for the carcinogenicity of BDCM in humans. Therefore, it was classified by IARC as possibly carcinogenic to humans – Group 2B (IARC, 1991b)(147).

Reproductive toxicity
Summary of reproductive toxicity studies of BDCM is presented in Table 33.

Table 33: *Summary of reproductive toxicity of BDCM (158,159,160,161)*

STUDY TYPE	ANIMAL SPECIES	DOSE LEVEL	RESULTS	NOAEL	REFER-ENCES
Two generation, reproduction study	Mouse (ICR)	0, 17, 171 and 685 mg/kg bw/day in drinking water	685 mg/kg bw/day: ↓ fertility and ↓ gestation index in F_1 generation. ↓ fertility in F_2 generation. 171 and 685 mg/kg bw/day: ↓ litter size, ↓ viability, ↓ lactation index and ↓ postnatal bw. No dominant lethal or teratogenic effects were observed.	Parental: 17 mg/kg bw/day, Reproductive: 17 mg/kg bw/day, Offspring: 17 mg/kg bw/day	EHC Borzellec a and Carchma n, 1982.
Teratology study (1)	Rat (Sprague-Dawley)	0, 50, 100 and 200 mg/kg bw/day on gestational days 6-15.	Mother: at 200 mg/kg bw/day ↓ bw gain, ↑liver and kidney weight. Offspring: sternebral anomalies.	Parental: 100 mg/kg bw/day, Developmental: 200 mg/kg bw/day, Offspring: 200 mg/kg bw/day	EHC. Ruddick *et al*., 1983.
Teratology study (2)	Rat (F344)	0, 25, 50 and 75 mg/kg bw/day in corn oil or aqueous vechicle.	Full litter resorptions: - corn oil: 8 % at 50 mg/kg bw/day and 83 % at 75 mg/kg bw/day, - water: 17 % at 50 mg/kg bw/day and 21 % at 75 mg/kg bw/day, Mother: toxic effect were observed at 50 and 75 mg/kg bw/day in previous studies.	Maternal: 25 mg/kg bw/day, Foetal: 25 mg/kg bw/day	EHC, Narotsky *et al.,* 1997.
Fertility effect	Rat (F344)	0, 22 and 39 mg/kg bw/day for 52 weeks in drinking water	39 mg/kg bw/day: ↓ sperm motility: ↓ mean straight line, ↓ average path and curvilinear velocity of sperm from cauda epididymis.	22 mg/kg bw/day	EHC; Klinefel-ter *et al*., 1995.

The multigeneration study was performed on mice. Fetotoxic effect was observed at the dose that decreased mother's body weight gain by 40 %. In one of the teratology studies, the effect on the offspring was observed at the levels that were toxic for the mother. These data suggest that BDCM is not toxic for reproduction, neither does it effect development of the pups, but it clearly reduced the fertility (indicated by decreased sperm motility).

Human data: In one epidemiological study, an association between increased risk of spontaneous abortion and BDCM was noted. This is an informative datum, but based on this study, no direct correlation between BDCM exposure and adverse effect on reproduction can be drawn. BDCM is only one of the by-products of water chlorination that can harmfully affect reproduction (Kramer *et al.,* 1992)(142).

A BDCM dose-dependent reducion in the secretion of bioactive and immunoreactive chorionic gonadotropin from human placental trophoblasts in culture was also observed (Chen *et al*., 2003)(162). This indicates that placental trophoblats are target cells for BDCM and reduction of chorionic gonadotropin could have adverse effects on the pregnancy since this hormone is necessary for maintaining pregnancy.

Neurotoxicity

As described above, acute exposure of animals to BDCM induced ataxia, disturbed coordination, sedation, lethary and anaesthesia (500 mg/kg bw). Some repeated exposure studies were performed to determine neurotoxicity of BDCM. When ICR mice were dosed 1.2 or 11.6 mg/kg bw/day by aqueous gavage for 90 subsequent days, no effect was observed in various behavioural tests. After dose 100 mg/kg bw/day for 30 days, BDCM did not effect passive avoidance learning. Animals exposed to 100 or 400 mg/kg bw/day for 60 days, exhibited decreased response rates in an operant behaviour test. The effect was expressed at the beginning of the treatment and did not change during the course of the study.

3.2.2.4 Exposure

For nonoccupationally exposed people the major route of exposure to BDCM is consumption of chlorinated drinking water, beverages and food products. Contamination is a result of chlorination of drinking water and its further use in preparation of food. Exposure can also occur through inhalation of ambient air and through dermal exposure in chlorinated swimming pool water.

The exact amount of BDCM in the drinking water in Slovenia can not be determined, since in the monitoring of drinking water only the total amount of trichalomethanes (THM's) is measured. The values determined in the drinking water are 30 μg/L or less. As the worst case we considered all of the THM amount in the drinking water to be BDCM.

Scenario:
Daily intake of water: 2 L,
BDCM concentration: 30 μg/L,
Average weight of an adult person: 60 kg,

Estimated BDCM exposure = (30 μg/L 2L/day)/60 kg = 0.001 mg/kg bw/day* {11}

3.2.2.5 Toxicity, hazard and risk estimation

Target organs of BDCM toxicity: central nervous system, the liver, the kidney and the intestine, have been identified in oral exposure studies on experimental animals. Primary target tissues are the active sites of metabolism of BDCM. Reactive metabolites produced in this reaction then bind to macromolecules and elicit cytoxic and genotoxic responses. The most concerning effect of BDCM was carcinogenicity. Neoplasms were observed in liver, kidneys and intestine. In addition, BDCM gave positive results in some *in vitro* genotoxicity assays and it also increased sister chromatid exchange in bone marrow cells of mice exposed *in vivo*. BDCM is the most potent rodent carcinogen of THM's. In epidemiological study the exposure to BDCM was reported to be associated with colorectal cancer in humans. This was not the result of underlying epi-genetic or cytotoxic mechanism.
In reproduction toxicity studies in rat and mice BDCM affected reproduction and development of foetuses but only at doses toxic for the mother.
Several studies indicate that there is a difference in susceptibility to BDCM between species and between genders. For example, no intestinal tumors were observed in mice after exposure to BDCM, while male and female rats developed this kind of tumors. The basis of this difference might be different disposition and metabolism of BDCM between genders and species. Therefore it is difficult to extrapolate toxicology data to humans. But until further data are published on mechanistic and toxicokinetic differences, it must be assumed that effects similar to those observed in animals will occur in humans after exposure to comparable doses of BDCM.

Mechanism proposed to be involved in cytotoxic and genotoxic effects of BDCM are the formation of dihalomethyl free radicals and subsequently the formation of covalent adducts with cell proteins and lipids. Another important mechanism in this view is lipid peroxidation and direct conjugation of dihalomethanes with GHS and the product of this reaction then directly reacts with guanine.

Setting the reference dose:
Calculation of TDI is usually based on NOAEL value for the most sensitive species from chronic toxicity/carcinogenicity studies. For calculation of TDI the LOAEL from the carcinogenicity study on rats should be used (LOAEL = 6 mg/kg bw/day). For further calculation we have included assessment factor 1000:

6 mg/kg bw/day =	LOAEL defined in 2 years study on rats,
1000 =	assessment factor; 10 for using the LOAEL instead of NOAEL, 10 for interspecies variations and 10 for intraspecies variation,

TDI = 6 mg/kg bw/day/ 1000 = 0.006 mg/kg bw/day {12}

However, since the data on genotoxicity of BDCM *in vivo* are not clear, it is difficult to set a TDI, because in case of genotoxic agents there is no threshold for the effect.

Risk estimation for adult person:
In this study we have focused on the risk estimation for adult people exposed to BDCM through the drinking water alone.

TDI: 0.006 mg/kg bw/day,
Estimated exposure from drinking water: 0.001 mg/kg bw/day,

Risk assessment = (0.001 mg/kg bw/day)/(0.006 mg/kg bw/day) = 0.166 {13}

The dose of BDCM that people are exposed to from drinking water is 16.6 % of TDI (if BDCM was the only THM present in the water).

In Slovenia it is determined by the rule for drinking water that the concentration of total THM's should not exceed 100 μg/L water (U.l. RS 19/04)(146). Again, in the worst case if all of the THM in water was BDCM, the estimated exposure of an adult to BDCM would be 0.003 mg/kg bw/day and this would account for 55 **%** of TDI.

3.2.2.6 Risk evaluation

Human health risk evaluation
At exposure lower than the reference value there should be no non-cancer or cancer adverse effects on health of the consumers.
Estimated exposure of adult person to BDCM by drinking chlorinated water was calculated to be 16.6 % of TDI in the scenario where people are exposed to BDCM only by drinking contaminated water and 55 % in case that total THM's concentration in drinking water is 100 μg/L and the only THM present in the water is BDCM. There is no risk for human health after ingestion of drinking water containing such amount of BDCM.

3.2.3 Dibromochloromethane ($CHBr_2Cl$)

Dibromochloromethane (DBCM) is used industrially as a chemical intermediate in the manufacture of refridgerants, pesticides, propellants and other organic chemicals (THM; WHO)(133). It is formed in drinking water as a result of a reaction between chlorine and organic substances in the presence of bromide. It is a major component of organohalide emissions from marine algae (IARC, 1991)(163).

People are mostly exposed to DBCM via chlorinated drinking water.

3.2.3.1 Physical and chemical properties of DBCM

The toxicologically relevant physical and chemical properties of DBCM are summarised in Table 34.

Table 34: *Summary of physical and chemical properties of DBCM (164)*

PROPERTY	**INFORMATION**
Chemical name (IUPAC)	Dibromochloromethane
Synonyms	Chlorodibromomethane
CAS No.	124-48-1
Chemical formula	$CHBr_2Cl$
Molar mass	208.28 g/mol
Physical state	Liquid
Colour	Colorless to pale yellow
Odour	Sweet, similar to chloroform
Solubility in water at 20 ºC	2.7 g/L
Log P_{ow}	2.16
Vapour pressure at 25 ºC	76 mm Hg
Melting point	- 20 ºC
Boiling point	120 ºC
Density at 20 ºC	2.451 g/cm^3

3.2.3.2 Kinetic and metabolism data

Absorption: There were no studies performed on inhalation exposure to DBCM. Based on physical-chemical properties and analogy with chloroform, it is expected that DBCM would be well absorbed in the lungs. There are some data on human inhalation exposure and absorption. DBCM level was measured in five swimmers swimming in chlorinated water. The concentration of DBCM in the water was 0.8 μg/L, concentration in the air before and after swimming were 5.2 μg/m^3 and 11.4 μg/m^3 respectively.
Estimated DBCM uptake during the one hour of sitting near the pool ranged from 1.5–2.0 μg/hour and uptake during one hour of swimming in the water ranged from 14–22 μg/hour (ATSRD DBCM, Aggazzotti *et al*., 1998)(165).
One study was performed on oral absorption of DBCM in rats after single gavage dose (ATSDR, da Silva *et al*., 1999)(166). DBCM was rapidly absorbed with the peak concentration in plasma 1 hour after exposure.
The relationship between the dose of DBCM (52 and 104 mg/kg bw) and area under the blood concentration versus time curves (AUCs) was nonlinear. For DBCM 52 and 104 mg/kg bw the AUCs were 31.2 and 85.6 μM/hour. These results suggest that the metabolism of DBCM can be saturated at higher doses. When DBCM was administered together with chloroform (25 mmol/kg of each THM's) the AUC's were much higher compared to AUC's for DBCM alone.
The results on another study of DBCM absorption showed that if DBCM was administered in the mixture with other THM's (100 mg/kg bw for rats and 150 mg/kg bw for mice) 90 % of it was absorbed in gastrointestinal tract (radioactivity was found in urine, expired air and internal organs) (DBCM; ATSDR, 1997). However the absorption of halocarbon in gastrointestinal tract might be slower when the substance is given in oil compared to the substance given in the water (ATSDR; DBCM, Withey *et al*., 1983)(167).

Distribution: In rats 8 hours after a single dose of ^{14}C-labeled DBCM, measurable amounts of radioactively were detected in the brain, kidney, liver, lungs, muscles, pancreas, stomach, thymus and urinary bladder (ATSDR DBCM, Mink *et al*., 1986)(151). Similar results were obtained in mice, but in mice a significant amount of DBCM was found in the blood. In vitro data indicate that haemoglobin is the major target of DBCM in the blood (ATSDR DBCM; Beliveau and Krishnan, 2000b)(168).

Metabolism: DBCM is metabolised in the same way as the other THM's. In the first step, DBCM is oxidised by cytochrome P450 in the liver to trihalomethanol that is further decomposed to dihalocarbonyl. Dihalocarbonyl is a highly reactive metabolite, and it either forms covalent adducts, reacts with glutathione (oxidized glutathione and CO are formed) or it may be hydrolysed to yield CO_2. The conversion to CO_2 is the major pathway (Mink *et al*., 1986).
During oxidation of dibromochloromethane by cytochrome P450 the trihalomethyl free radicals can be formed. Trihalomethyl free radicals are highly reactive and cause lipid peroxidation in cells. The formation of trihalomethyl free radicals can be an important mechanism of DBCM toxicity and carcinogenicity.
The major enzyme involved in metabolism of DBCM is CYP2E1. This was determined by 1) decreased production of bromide and carboxyhemoglobin in rats that were pre-exposed to diethydithicarbamate (inhibitor of CYP2E1) and 2) increased production of bromide in rats pretreated with isoniazid (inducer of CYP2E1). The pre-treatment of rats with phenobarbital (inducer of CYP2B1) also increased bromide concentration in plasma and indicates that CYP2B1 is another enzyme involved in DBCM metabolism (ATSDR DBCM, Pankow *et al*., 1997)(164). In extrahepatic tissues glutathione-S transferase might an important component in toxic mechanisms of DBCM (ATSDR DBCM, Ross and Pegram, 2004)(169).

Excretion: When ^{14}C-labeled DBCM was administered in the mixture with other THM's (100 mg/kg bw for rats and 150 mg/kg bw for mice), most of DBCM was excreted during the first xx hours by exhaled air in the form of CO_2 (Mink *et al*., 1986)(151) for mice, but not for rats. Data are presented in Table 35.

Table 35: *Excretion of DBCM from rats and mice.*

	EXHALED AIR				URINE	ELIMINATION HALF-LIFE
	% of radioactivity	Form	% of radioactivity	Form	% of radioactivity	
Rats	18.2	CO_2	48.1	DBCM	5	1.2 hours
Mice	71.58	CO_2	12.31	DBCM	5	2.5 hours

Faster metabolism and excretion of DBCM in mice is probably the reason why mice are less susceptible to DBCM than rats.

Regarding the data on metabolism, distribution and excretion of DBCM, it is unlikely to accumulate in organs or tissues.

3.2.3.3 Toxicity data and toxicity evaluation

Many studies were performed on oral toxicity of DBCM in experimental animals. Some of the relevant studies and studies used to determine the reference dose of DBCM as well as studies crucial for classification of DBCM are presented in this section. To our knowledge no studies on inhalation or dermal exposure were performed with DBCM.

Acute toxicity
Some studies performed on acute toxicity of DBCM are summarised in Table 36.

Table 36: *Summary of acute toxicity studies (164).*

STUDY TYPE	SPECIES	RESULTS	REFERENCES
Acute oral toxicity (1)	Rat: Sprague-Dawley	LD_{50} = 848 mg/kg bw ♀ LD_{50} = 1186 mg/kg bw ♂	Chu *et al*., 1982a
Acute oral toxicity (2)	Mouse: ICR	LD_{50} = 800 mg/kg bw ♂ LD_{50} = 1200 mg/kg bw ♀ At 500 mg/kg bw: sedation, anaesthesia.	Bowman *et al.,* 1978
Acute oral toxicity (3)	Hamster: Golden Syrian	LD_{50}= 145 mg/kg bw ♂	Korz and Gatterman, 1997

According to the results of acute toxicity studies and European Directive 67/548/EEC on classification and labelling of dangerous substances DBCM should be classified as harmful if swallowed (Xn, R22). Regarding tha data obtained in the study on hamsters it should be classified as toxic if swallowed, but there were no experimental details in the report of this study, so the relevance of this study is questionable (ATSDR). Neurotoxic effects were observed after acute exposure of mice to DBCM.

Toxicity after repeated exposure
Results of repeated exposure toxicity studies of DBCM are summarised in Table 37.

Table 37: *Summary of repeated exposure toxicity studies (170,171).*

SPECIES	DURATION	NOAEL	LOAEL/ EFFECTS	REFERENCES
Rat (F-344)	14 days	125 mg/kg bw/day	250 mg/kg bw/day ↓ bw gain, mottled liver, darkened renal medullae. At 500 mg/kg bw/day: lethargy and ataxia.	ATSDR. NTP, 1985.
Rat (F-344)	7 days	160 mg/kg bw/day	310 mg/kg bw/day ↓ serum testosterone, ↓ bw.	
Mouse (CD-1)	14 days	50 mg/kg bw/day	125 mg/kg bw/day ↑ absolute liver weight, ↓serum glucose, ↑SGPT and SGOT, impaired humoral immunity.	ATSDR. Munson *et al*., 1982.
Rat (Wistar)	30 days	18.3 mg/kg bw/day	56.2 mg/kg bw/day: hepatocellular vacoulation. 100 mg/kg bw/day: hepatocellular necrosis.	ATSDR, Aida *et al.*, 1992.
Rat (F-344)	13 weeks (5 days/week)	30 mg/kg bw/day	60 mg/kg bw/day hepatocellular vacuolation, toxic nephropathy, ↓ bw gain, salivary gland hyperplasia.	ATSDR, NTP, 1985.
Mouse (B6C3F1)	13 weeks (5 days/week)	125 mg/kg bw/day	250 mg/kg bw/day hepatocellular vacuolisation, toxic nephropathy	ATSDR, NTP, 1985.
Rat (Sprague-Dawley)	90 days	50 mg/kg bw/day	100 mg/kg bw/day ↓ thymus weigth, ↓ absolute brain weight.	ATSDR. Daniel *et al.*, 1990.

Target organs of DBCM were: liver (vacoulation, hepatocellular ballooning and proliferation), kidneys (darkened medullae, mesangial hyperplasia) and immune system (impaired humoral immunity). Some neurotoxic effects were observed (lethargia, ataxia, sedation) and also the effect on endocrine system (decreased testosterone level).

Genotoxicity/mutagenicity
Results on genotoxicity studies of DBCM are collected in Table 38.

Table 38: *Summary of genotoxicity/mutagenicity studies (164,171)*

	ENDPOINT	TEST SYSTEM/EXPOSURE	RESULTS	REFER-ENCES
***IN VITRO* SUDIES**	Reverse mutations	*Salmonella typhimurium* TA 98, TA100, TA1535, TA1537	Negative results: TA100 3 studies (+/- activation system), Positive results: 1 study (+ activation system, positive in TA 1535 and 1537 – activation system).	Simmon *et al*., 1977, LeCurieux *et al*., 1995, Kubo *et al*., 2002, NTP 1985.
	Mammalian cell forward mutation	Mouse L5178Y lymphoma cells	Positive (- activation).	McGregor *et al*., 1991.
	Sister chromatid exchange	Human lymphocytes	Positive results: 2 studies (- activation).	Morimoto and Koizumi, 1983, Sobti 1984.
		Rat liver cells	Positive (- activation).	Sobti, 1984.
	Chromosomal aberration	Mouse L5178Y lymphoma cells	Positive (+ activation).	Sofuni *et al.*, 1996.
		Chinese hamster lung cells	Positive (- activation).	Matsuoka *et al*., 1996.
	DNA single strand breaks	Primary rat hepatocytes	Negative (+/- activation).	Getter *et al.*, 2003a.
		Human lymphoblastic leukaemia cells	Positive (+/- activation).	Getter *et al.*, 2003a.
***IN VIVO* STUDIES**	Micronucleus in bone marrow	Mouse, intraperitoneal,	Negative.	Hayashi *et al*., 1988.
	Chromosomal aberrations	Rat, bone marrow cells, intraperitoneal (20.8 mg/kg bw, single dose).	Positive.	Fujie *et al*., 1990.
		Rat, bone marrow cells, gavage (10.5-1041 mg/kg bw/day, 5 days)	Weakly positive.	Fujie *et al*., 1990.
	DNA strand breaks	Rat (kidney cells), gavage (125 mg/kg bw, single dose)	Negative.	Potter *et al.*, 1996.
		Rat (liver, kidney and duodenum cells), 125 mg/kg bw/day in drinking water, 2 weeks	Negative.	Getter *et al*., 2003a.
	Sister chromatid exchange	Mouse (bone marrow cells), oral (25-200 mg/kg bw/day, 4 days)	Positive.	Morimoto and Koizumi, 1983.

In vitro studies of genotoxicity of DBCM gave positive results in 1 of 4 studies on reverse mutations in bacteria. Positive results were obtained also in mammalian cells (forward mutation, sister chromatid exchange, chromosomal aberrations and 1/2 DNA strand breaks formation). These data suggest that DBCM is genotoxic *in vitro*.
In *in vivo* studies DBCM induced sister chromatid exchange and chromosomal aberrations, while it did not induce micronuclei formation in bone marrow cells or DNA strand breaks in several organs if exposed animals. These data suggest that DBCM is a weak mutagen *in vivo*.

Carcinogenicity
Human data**:** there are some studies that indicate that there is a correlation between human exposure to DBCM and increased risk of bladder, rectal or colon cancer in humans. But these studies do not specify if the effects observed are due to the DBCM or one/more by-products present in the chlorinated drinking water.

Animal data**:** Carcinogenicity studies of DBCM are summarised in Table 39.

Table 39: *Summary of long-term toxicity and carcinogenicity studies with DBCM (171)*

SPECIES	DURATION/DOSE	NOAEL	LOAEL/ EFFECTS	REFERENCES
Mouse (B6C3F1)	105 weeks/ 0, 50 and 100 mg/kg bw/day, 5 days/week oral gavage	< 50 mg/kg bw/day	50 mg/kg bw/day Hepatocellular adenoma/carcinoma, fatty metamorphosis of the liver, nephrosis, thyroid follicular cell hyperplasia, ↓ bw.	ATSDR. NTP, 1985.
Rat (F-344)	105 weeks/ 0,40 and 80 mg/kg bw/day, 5 days/week oral gavage	< 40 mg/kg bw/day	Liver fatty changes, ground glass cytoplasmic changes, ♀: ↑ kidney nephrosis.	IRIS EPA. NTP, 1985.
Mouse (CBA×C57B1/6)	Lifetime exposure/ 0, 0.008, 0.76 and 76 mg/kg bw/day, in drinking water	Not given.	No increase in tumour incidence.	IRIS DBCM; Veronin *et al*., 1987.

DBCM induced dose-depenedent increase in hepatic adenoma and carcinoma in one study on mice. In rats it did not induce the formation of malignant tumours.

IARC (1991a, 1999)(163) has decided that DBCM *is not classifiable* as to its carcinogenicity to humans (Group 3), since there is inadequate evidence for the carcinogenicity in humans and limited evidence (carcinogenic effect detected only in 1 species) for the carcinogenicity in experimental animals.

Immunotoxicity studies

In one study conducted on Immunotoxic effects of DBCM. CD-1, mice were orally exposed to DBCM for 14 days. After the treatment impaired humoral immunity was observed in females exposed to 125 mg/kg bw/day. NOAEL value 50 mg/kg bw/day was set in this study (ATSDR, Munson *et al*., 1982)(155).

Reproductive toxicity

Human data**:** There were some studies performed on humans trying to identify the connection between DBCM exposure and adverse effects on reproduction and foetal development.

In the first study population case-controle study (ATSDR, Kramer *et al*., 1992)(164) pregnant women were exposed to DBCM from drinking water. The risk of premature delivery was not increased at DBCM concentrations of 4 μg/L and higher. No assocaiation was found between ≥ 4 μg/L DBCM in tap water and increased risk of low birth weight or intrauterine growth retardation. The results of the second study were similar (ATSDR, Waller *et al*., 1998)(172). The risk of spontaneous abortion was not significantly associated with DBCM levels.

The percentage of pregnancies ending with spontaneous abortions were 9.7, 9.6 and 10.4 % for DBCM levels of 0.1-30 and ≥ 31 μg/L, respectively.

The third study examined the possible association between THM in drinking water and menstrual cycle lenght. The menstrual cycle was followed by concentration of hormones in the urine of women (403 participants) for 5.6 menstrual cycles. In women exposed to concentrations ≥ 20 μg/L the menstrual cycle length was reduced by 1.21 day and folicular phase length by 1.1 day. No associations between lutheal phase length, menses length or cycle variability were found. The interpretation of this study is difficult due to co-exposure to other THM in the drinking water.

Animal studies:

Summaries of reproduction toxicity studies are gathered in Table 40.

Table 40: *Summary of reproduction toxicity studies of DBCM (158,159)*

SPECIES	DURATION/DOSE	NOAEL REPRODUCTION/ DEVELOPMENT	LOAEL/ EFFECTS	REFERENCES
Rat (Sprague-Dawley) and mouse (B6C3F1)	13 weeks/ Up to 250 mg/kg bw/day, 5 days/week	> 250 mg/kg bw/day	No effect on reproduction.	ATSDR. Daniel, 1990, NTP, 1985.
Rat	2 years/ 0, 80 or 100 mg/kg bw/day, 5 days/week	> 100 mg/kg bw/day	No effect on reproduction.	ATSDR, NTP, 1985.
Mouse (ICR)	Twogeneration study/ 0, 17, 171 and 685 mg/kg bw/day	Maternal NOAEL = 17 mg/kg bw/day Foetal NOAEL = 17 mg/kg bw/day	685 mg/kg bw/day: ↓ fertility, ↓ litter size, ↓ gestational survival, ↓ postnatal survival and postnatal body weight. ↓ bw gain, ↓ survival, ↑ and discoloured liver. No foetal skeletal, visceral or soft-tissue abnormalities. 171 mg/kg bw/day: ↓ bw gain, ↑ in occurrence of gross liver pathology.	ATSDR. Borzelleca and Charchman, 1982.
Rat	35 days/40.3 mg/kg bw/day.	>40.3 mg/kg bw/day	No alterations in reproduction or fertility indices.	ATSDR, NTP, 1996.
Rat (Sprague-Dawley)	Days 6-15 of gestation, up to 200 mg/kg bw/day	>200 mg/kg bw/day	No alteration in no. of resorption sites, litter size, foetal bw, fetal gross malformations, no skeletal or visceral abnormalities.	ATSDR,. Ruddick *et al*., 1983.

Results of reproductive toxicity studies show that DBCM does not affect the reproduction or development of experimental animals at doses that do not cause obvious maternal toxicity. Based on presented data DBCM is not toxic for reproduction and foetal development.

3.2.3.4 Exposure

The general population may be exposed to DBCM via inhalation of ambient air, ingestion of drinking water containing this disinfection by-product and dermal contact with this compound or other products containing DBCM.
The focus of this study was the risk assessment for the people who were exposed to DBCM in the drinking water.
Due to analytical methods currently used to determine the amount of THM's in the drinking water in Slovenia the exact amount of DBCM is not determined, because only total THM's concentration in drinking water is assessed. The values determined in the drinking water are 30 μg/L or less. As the worst case scenario, it was assumed all of THM in the drinking water was DBCM.

Daily intake of water: 2 L,
DBCM concentration: 30 μg/L,
Average weight of an adult person: 60 kg,

Estimated DBCM intake = (30 μg/L 2L/day)/60 kg = 0,001 mg/kg bw/day* {14}

3.2.3.5 Toxicity, hazard and risk estimation

Target organs of DBCM toxicity liver, kidneys and CNS have been identified in toxicity studies. These targets are active sites of DBCM metabolism. DBCM is readily absorbed and excreted. It is metabolised predominantly by CYP2E1 or CYP2B1.
DBCM was genotoxic *in vitro*, but also *in vivo* it induced chromosomal aberrations in rats and sister chromatid exchange in mice. DBCM induced hepatocellular adenomas and carcinomas in mice, but no neoplasms were observed in rats after exposure to DBCM. Therefore DBCM was classified by IARC as Group 3. Reproduction and foetal development were affected only at doses toxic for the parents.

Setting the reference dose:
For the calculation of TDI, the NOAEL value for the most sensitive species from chronic toxicity/carcinogenicity studies is used. In case of DBCM in carcinogenicity studies the lowest NOAEL value was 50 mg/kg bw/day. But in the 90-days study on rats (NTP, 1987) the NOAEL of 30 mg/kg bw/day was determined. And since the NOAEL was lower in the study of shorter duration, this value was used to calculate TDI.

30 mg/kg bw/day =	NOAEL defined in 90-days study on rat,
5 days/7 days =	adjustment to account for exposure 5 days/week,
1000 =	assessment factor; 10 for shorter duration of study, 10 for interspecies variations and 10 for intraspecies variation,

TDI= (30 mg/kg bw/day (5 days/7 days))/ 1000 = 0.021 mg/kg bw/day* {15}

Risk estimation for adult person:
The focus of this study was to estimate the risk for adult people exposed to DBCM through the drinking water alone.

TDI: 0.021 mg/kg bw/day,
Estimated exposure from drinking water: 0.001 mg/kg bw/day,

Risk assessment = (0.001 mg/kg bw/day)/(0.021 mg/kg bw/day) = 0.047 {16}

The dose of DBCM that people are exposed to from drinking water is 5 % of TDI, if DBCM was the only THM present in the water.

In Slovenia it is determined by the rule for drinking water that the concentration of total THM's should not exceed 100 μg/L water (U.I. RS 19/04)(146). Again, in the worst case if all of the THM in water was DBCM, the estimated exposure of an adult to DBCM would be 0.003 mg/kg bw/day and this would account for 16 % of TDI.

3.2.3.6 Risk evaluation

At exposure lower than the reference value there should be no adverse effects on health of the consumers.
Estimated exposure of adult person to DBCM by drinking chlorinated water was calculated to be 5 % of TDI in the scenario where people are exposed to DBCM only by drinking contaminated water, and 16 % in the case where the total THM's concentration in drinking water is 100 μg/L and the only THM present in the water is DBCM. There is no risk for human health after ingestion of drinking water containing such amount of DBCM.

3.3 Statistical study – results

3.3.1 The incidence of seven types of malignant neoplasms in "Mrzlek community" (exposed population) compared to "the rest of AU Nova Gorica" (not exposed population)

Table 41: *The number of new cancer cases and mean age at onset of disease in the period from 1985 to 2002*

	EXPOSED TO MRZLEK		NOT EXPOSED TO MRZLEK		**Total**	
	N	Mean	N	Mean	N	Mean
C16 MN Stomach	113	*66.6*	104	*69.6*	217	*68.0*
C18 MN Colon	153	*66.8*	114	*70.1*	267	*68.2*
C19 MN Rectosigmoid junction	42	*68.1*	27	*68.8*	69	*68.4*
C20 MN Rectum	93	*67.0*	86	*70.6*	179	*68.7*
C22 MN Liver	37	*66.9*	27	*68.2*	64	*67.5*
C64 MN Kidney	44	*63.6*	43	*64.1*	87	*63.9*
C67 MN Bladder	79	*69. 7*	70	*71.6*	149	*70.6*
Total	561	*67.1*	471	*69.5*	1032	*68.2*

MN - malignant neoplasm

The calculation of ages at onset of the particular types of neoplasms in two communities of AU Nova Gorica gives a longer life pderiod -*without developing a disease*- with population of **"**the rest of AU Nova Gorica**"** (not exposed population) in general, which probably means the onset of the disease later in life.

While the age distribution of two samples in comparison is essential, and does not differ because the source of variation between the age-groups is not significant(F= 0.86, p=0.36), the descriptive characteristics concerned with the incidence of neoplasms show differentiation between two locations of AU Nova Gorica. The heterogeneity between the mean age at diagnosis for the specific causes of neoplasm is an important observation.

In Table 41, the distributions of seven malignant neoplasms are listed by numbers of cases and their mean age at onset. As the mean age at onset in general differs by 2.4 years, the cancer of rectum is a source of the greatest differences between the two parts of AU Nova Gorica (3.6 years), while the differences of stomach cancer and colon cancer are 3.0 and 3.3 years respectively, with better results for the not exposed population.

3.3.2 The incidence of malignant neoplasms within "Mrzlek community" (exposed population) and within "the rest of AU Nova Gorica" (not exposed population)

3.3.2.1 Average annual crude rate of incidence

To study the burden of cancer more in detail, we calculated the average annual crude rate of incidence per 100,000 inhabitants for seven types of cancer within the two populations of AU Nova Gorica in the period from 1985 to 2002. The findings are shown in Figures 18 to 24 below.

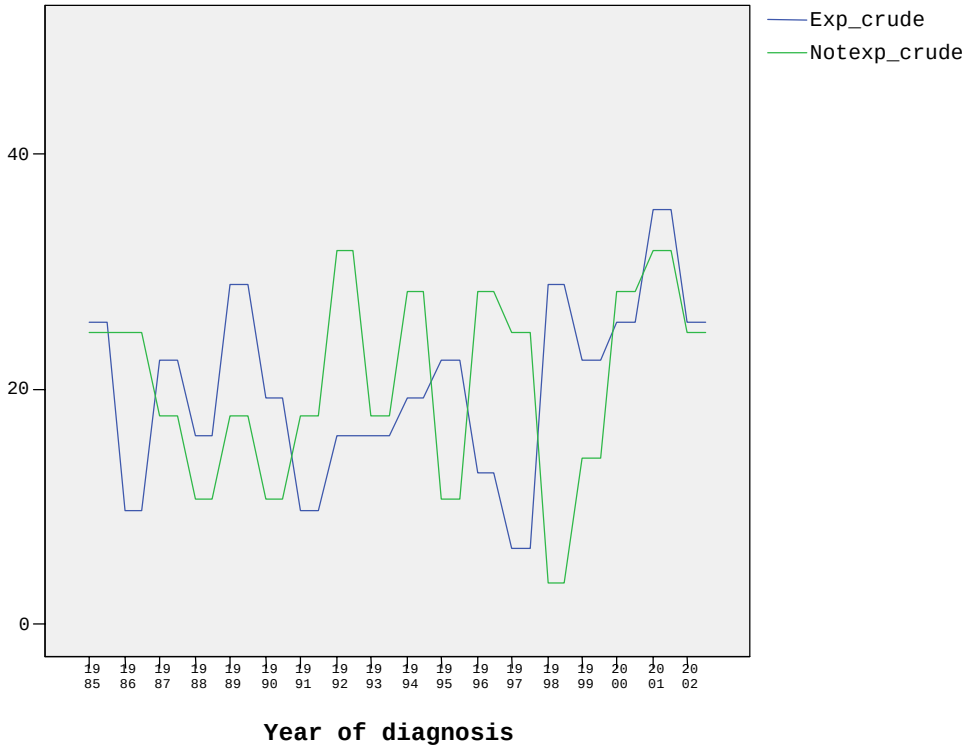


Figure 18: *Malignant neoplasms of stomach; Exp_crude = Mrzlek community crude rate/100.000, Notexp_crude = the rest of AU Nova Gorica crude rate/100.000*

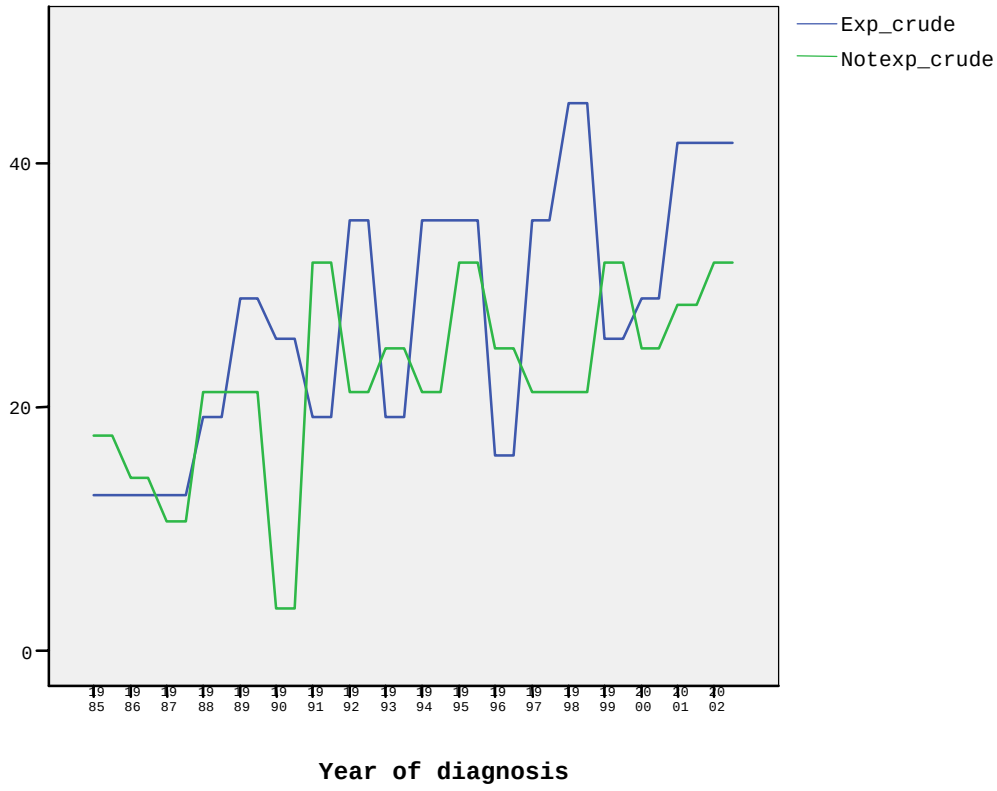


Figure 19: *Malignant neoplasms of colon; Exp_crude = Mrzlek community crude rate/100.000, Notexp_crude = the rest of AU Nova Gorica crude rate/100.000*

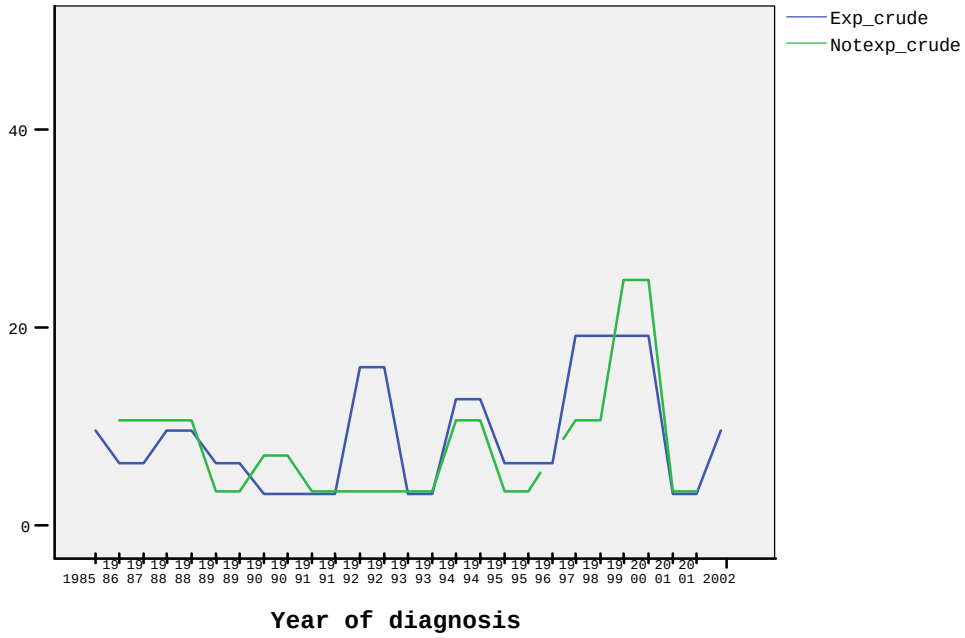


Figure 20: *Malignant neoplasms of rectosigmoid junction; Exp_crude = Mrzlek community crude rate/100.000, Notexp_crude = the rest of AU Nova Gorica crude rate/100.000*

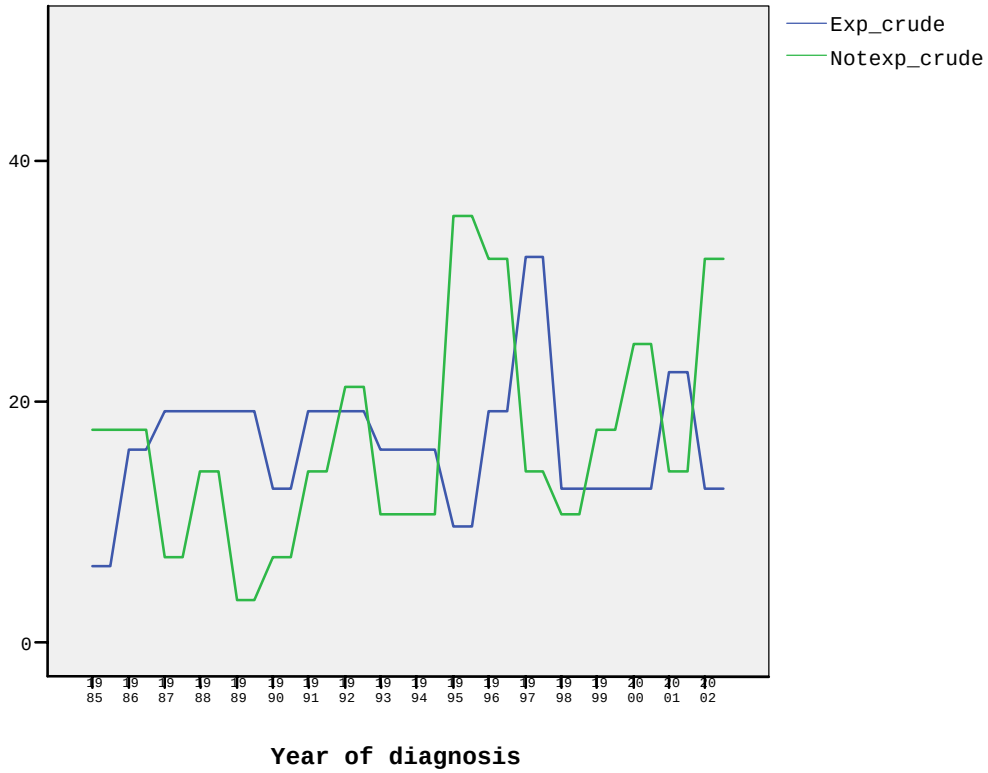


Figure 21: *Malignant neoplasms of rectum; Exp_crude = Mrzlek community crude rate/100.000, Notexp_crude = the rest of AU Nova Gorica crude rate/100.000*

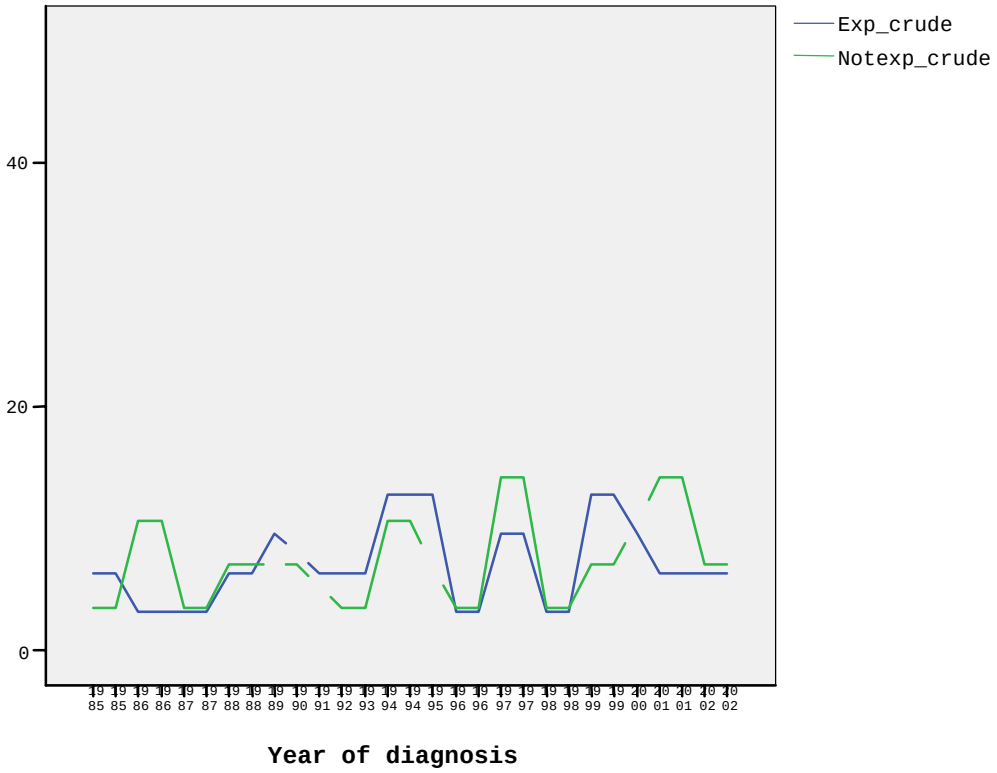


Figure 22: *Malignant neoplasms of liver and intrahepatic bile ducts; Exp_crude = Mrzlek community crude rate/100.000, Notexp_crude = the rest of AU Nova Gorica crude rate/100.000*

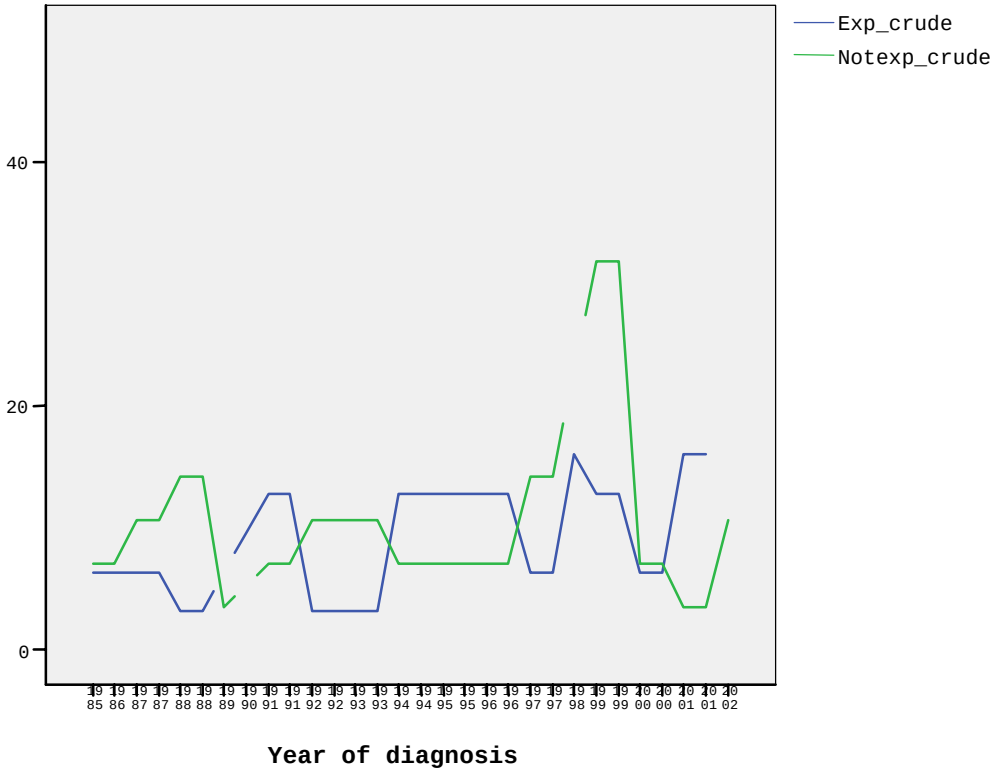


Figure 23: *Malignant neoplasms of kidneys*; *Exp_crude = Mrzlek community crude rate/100.000, Notexp_crude = the rest of AU Nova Gorica crude rate/100.000*

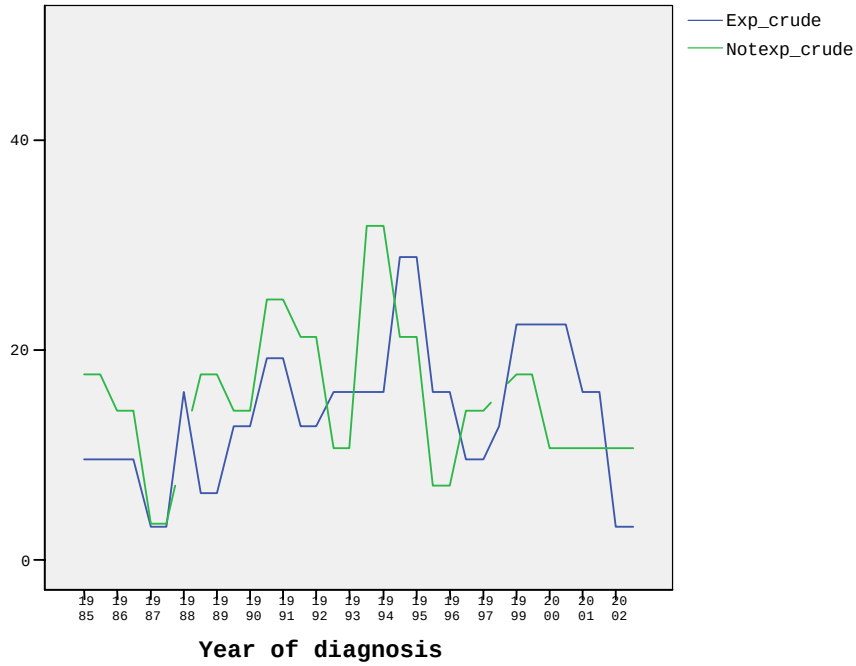


Figure 24: *Malignant neoplasms of urinary bladder; Exp_crude = Mrzlek community crude rate/100.000, Notexp_crude = the rest of AU Nova Gorica crude rate/100.000*

3.3.2.2 Age standardized rate of incidence – direct method

In subsequent studying of the burden of cancer within the two populations of the AU Nova Gorica, we calculated the age standardized rate of incidence for the period from 1985 to 2002 according to the direct method per 100,000 inhabitants for seven types of cancer. The findings are shown in Figures 25 to 31 below.

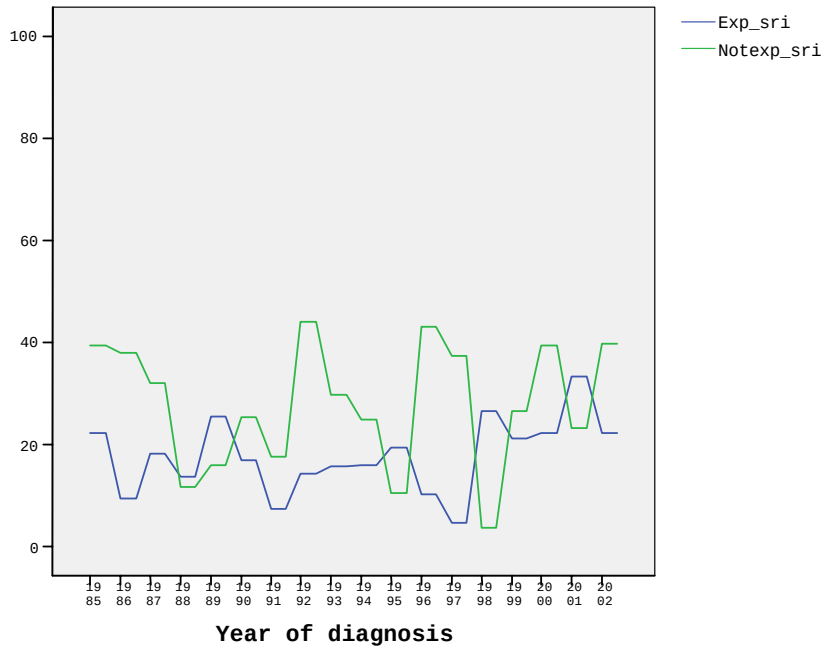


Figure 25: *Malignant neoplasms of stomach; Exp_sri = Mrzlek community SRI direkt/100.000, Notexp_sri = the rest of AU Nova Gorica SRI direkt/100.000*

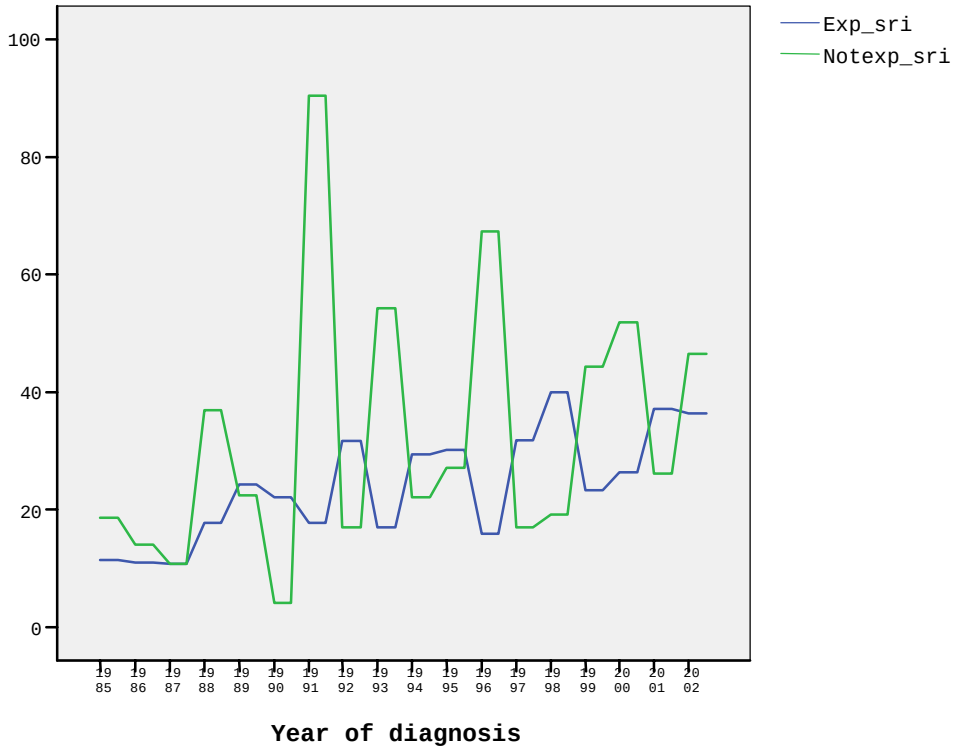


Figure 26: *Malignant neoplasms of colon; Exp_sri = Mrzlek community SRI direkt/100.000, Notexp_sri = the rest of AU Nova Gorica SRI direkt/100.000*

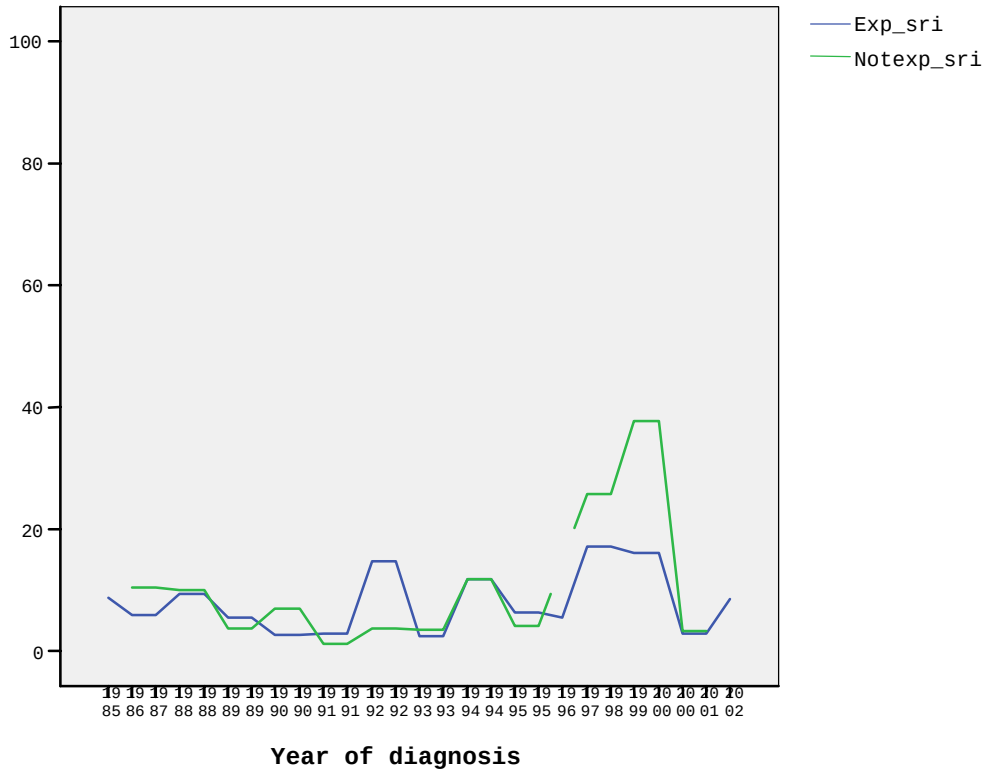


Figure 27: *Malignant neoplasms of rectosigmoid junction; Exp_sri = Mrzlek community SRI direkt/100.000, Notexp_sri = the rest of AU Nova Gorica SRI direkt/100.000*

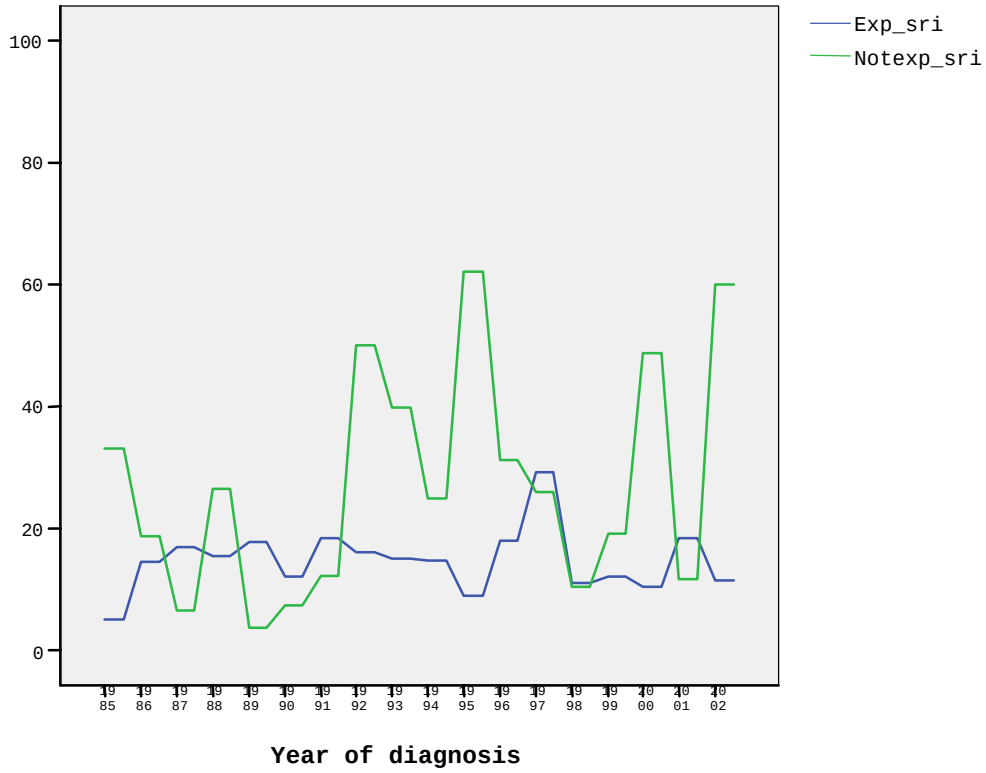


Figure 28: *Malignant neoplasms of rectum; Exp_sri = Mrzlek community SRI direkt/100.000, Notexp_sri = the rest of AU Nova Gorica SRI direkt/100.000*

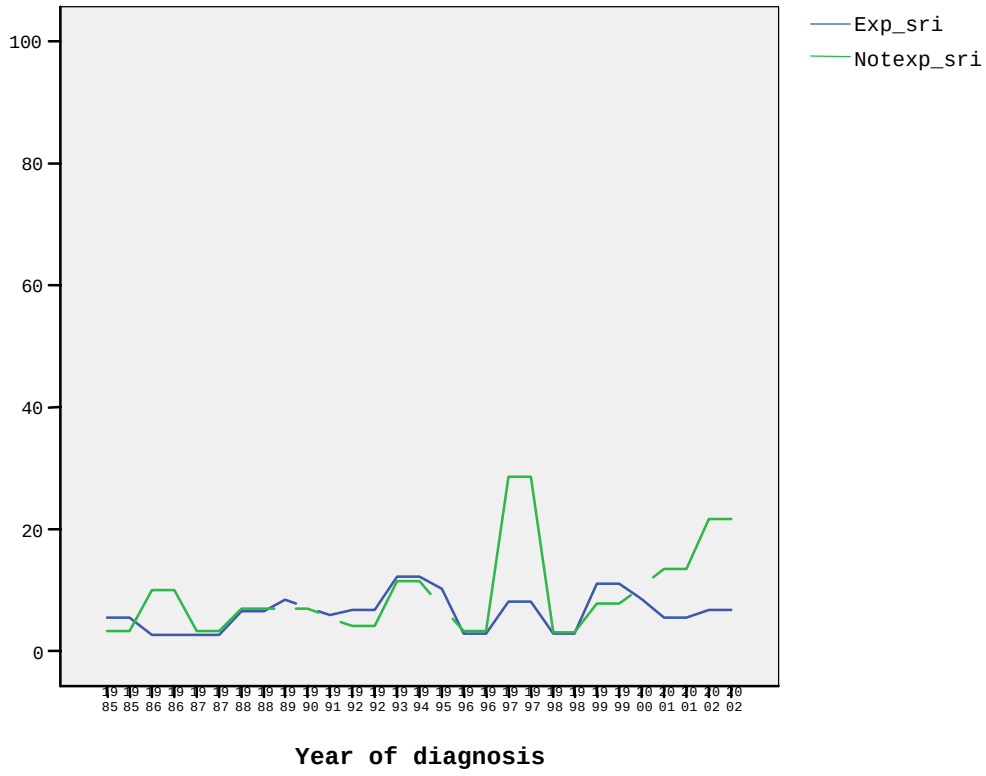


Figure 29: *Malignant neoplasms of liver and intrahepatic bile ducts; Exp_sri = Mrzlek community SRI direkt/100.000, Notexp_sri = the rest of AU Nova Gorica SRI direkt/100.000*

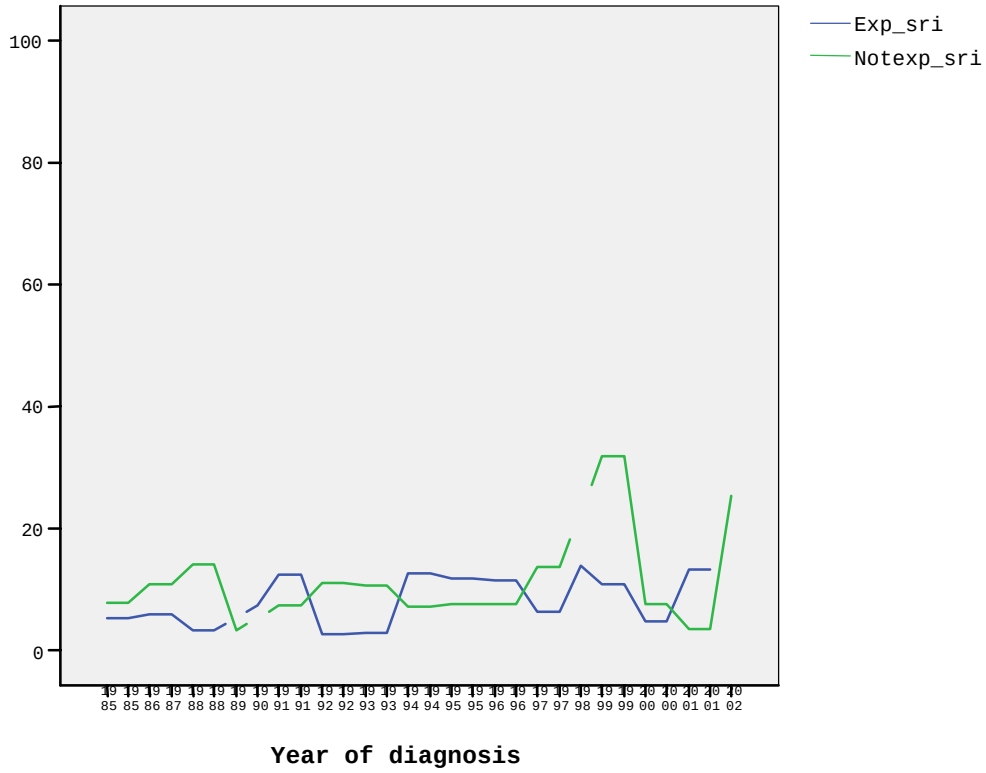


Figure 30: *Malignant neoplasms of kidneys; Exp_sri = Mrzlek community SRI direkt/100.000, Notexp_sri = the rest of AU Nova Gorica SRI direkt/100.000*

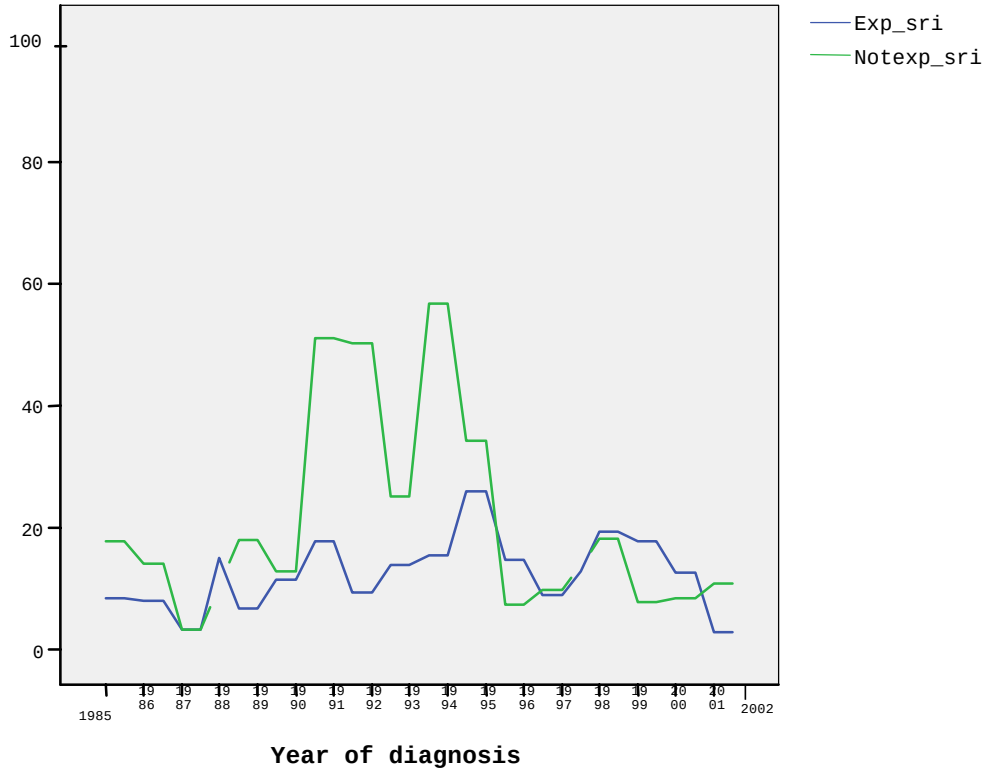


Figure 31: *Malignant neoplasms of urinary bladder; Exp_sri = Mrzlek community SRI direkt/100.000, Notexp_sri = the rest of AU Nova Gorica SRI direkt/100.000*

The crude rate of incidence and the age standardized rate of incidence – direct method which were calculated per 100,000 inhabitants and shown in Figures 18 to 31 show markedly random events and are not under the influence of some rule (do not show specific pattern). Sometimes there are more events while sometimes there are less. It shows a random number of cancers in a longer period. Substantial differences between the incidence of cases of seven studied types of cancer of the exposed and non-exposed (reference) population can not be observed.

Both direct and indirect standardized incidence ratios are used in order to overcome the problems of masking differences in crude rate in particular age groups (Section 3.3.2.3 and 3.3.2.4)

3.3.2.3 Direct Standardized Incidence Ratios

Directly standardized rates give an indication of the number of events that would occur in a standard population, if the population had the same age-specific rates of the local area. We know the age structure and the age distributions of the observed and reference population and we also know the age at incidence. We can thus compare them directly.

The standardised incidence rates for two communities separately, and for AU Nova Gorica as total, show the occurencies of different types of malignoma. The values indicate the rates per 100.000 inhabitants respectively to some malignant neoplasms of digestive organs and malignant neoplasms of urinary organs from 1985 to 2002. The overview of total rates basically presents higher incidence rates for "the rest of AU Nova Gorica". The rate of malignant neoplasm of rectosigmid junction is higher for "Mrzlek community" only.
These standardized incidence ratios are tabulated below (Table 42).

Table 42: *The standardized incidence ratios (direct standardization, SIR$_{direct}$) and Crude incidence rates - in "Mrzlek community" (exposed population) and in "the rest of AU Nova Gorica" (not exposed population) from 1985 to 2002*

	EXPOSED TO MRZLEK		NOT EXPOSED		AU NG	
	Per 100.000		Per 100.000		Per 100.000	
	SIR*direct*	Crude	SIR*direct*	Crude	SIR*direct*	Crude
C16 MN Stomach	17.8	20.1	27.9	20.5	22.9	20.3
C18 MN Colon	24.2	27.2	32.8	22.4	28.5	24.8
C19 MN Rectosigmoid junction	6.8	7.5	6.8	5.3	6.8	6.4
C20 MN Rectum	14.8	16.6	27.4	16.9	21.1	16.7
C22 MN Liver	6.0	6.6	6.9	5.3	6.5	5.9
C64 MN Kidney	7.0	7.8	9.4	8.5	8.2	8.1
C67 MN Bladder	12.5	14.1	19.2	13.8	15.9	13.9

3.3.2.4 Indirect Standardized Incidence Ratios

Indirect standardization compares actual numbers of newly registered cases of specific malignoma neoplasms to expected numbers, adjusting for age and gender. The expected number of incidence is taken from the number of Slovenian reference population. This means that two population samples, »Mrzlek community« and »The rest of AU Nova Gorica«, were compared to the standard Slovene population.

The SIR of the reference population showed in the Table 43, a value of lower than 1 means that fewer new cases of malignomas than expected occurred in the local population after adjusting for differences in age and gender; more than 1 means that there have been more cases than expected**.** These standardized incidence ratios for the exposed and control populations are tabulated below (Table 43).

Table 43: *The incidence ratios by indirect age standardization*

	EXPOSED TO MRZLEK		NOT EXPOSED TO MRZLEK	
	SIR	95 % CI	SIR	95 % CI
C16 MN Stomach	0.76	0.62-0.90	0.87	0.70-1.04
C18 MN Colon	0.72	0.61-0.84	0.66	0.54-0.79
C19 MN Rectosigmoid junction	1.24	0.87-1.62	1.01	0.63-1.39
C20 MN Rectum	0.67	0.53-0.81	0.76	0.60-0.92
C22 MN Liver	0.79	0.54-1.05	0.72	0.45-0.99
C64 MN Kidney	2.55	1.80-3.30	3.09	2.17-4.02
C67 MN Bladder	1.19	0.93-1.45	1.33	1.02-1.64

Confidence intervals are used to give a range of values within which there is a degree of certainly that the values are correct, and to assess if values are significantly different from those of the reference population. The range of variation of “Mrzlek community” SIR are within the 95% confidence intervals of “the rest of AU Nova Gorica” and we can be 95% certain that the real value will fall somewhere between the values of the two confidence limits 95 times out of 100. According to the previous statement the indirect standardized ratios show that there are no significant differences in the number of newly registered malignoma cases between two populations.
Table 43 shows the SIR’s with it’s confidence intervals in the exposed and not exposed area for all malignant neoplasms locations compared in the analysis. The SIR’s in the exposed population are not signifcantly diferent from the not exposed SIR’s, as the values of the not exposed population SIR’s are included througout in the confidence intervals of exposed population SIR’s.

3.3.2.5 Trends

The standardized incidence ratio trends for the exposed and control populations are summarized in Figure 32.

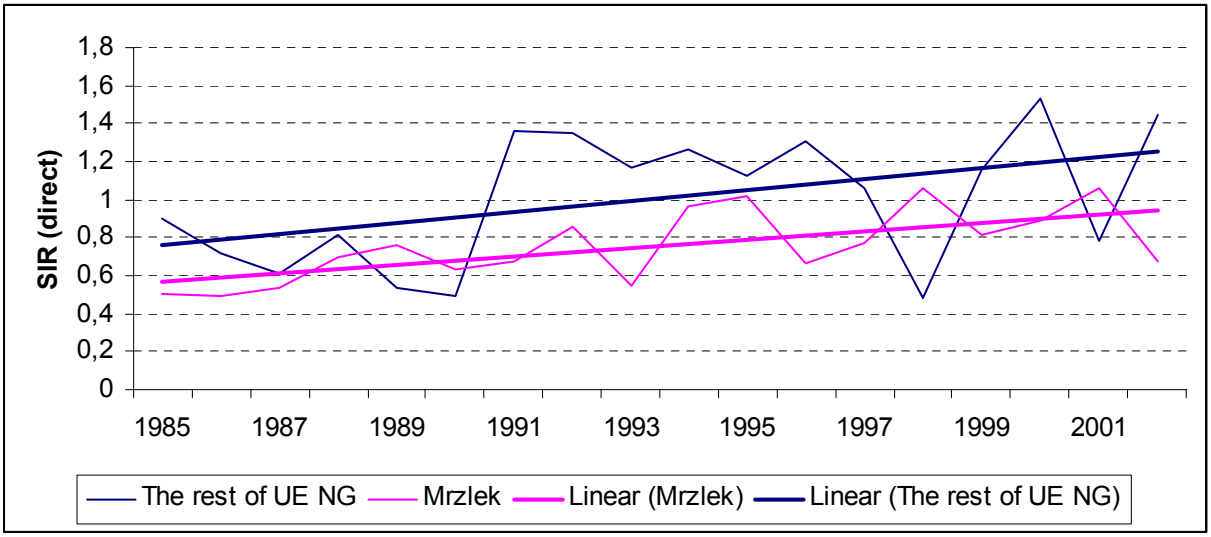


Figure 32: *The trend of SIR from 1985 to 2002, “Mrzlek community” and “the rest of AU Nova Gorica”*

The particular malignant neoplasms incidence rates as neoplasm of stomach, colon, rectosigmoid junction, rectum, liver, kidney and bladder provides information about the number of observations, which *per se* establishes the frequency of disease. Especially the standardised incidence rates for these malignant neoplasm do not show stability and are slightly moving upward within this period for both communities particularly for malignant neoplasm of colon (Figure 32). The trend of SIR data on 7 malignant neoplasms from 1985 to 2002 moves upward with both observed community, “Mrzlek community” and “the rest of AU Nova Gorica”.

3.3.3 Survival function on incidence of 7 types of malignant neoplasms with "Mrzlek community" and with "the rest of AU Nova Gorica"

Survival function after Kaplan - Meier is highly useful to determine the time of an event as a nonparametric method, such as the time of development of a malignant disease according to the age of the patients in an observed population. Unlike with incidence, which shows what and to what extent happened in a year, this method will be used to observe the age of the patients at the onset of the disease.

3.3.3.1 The length of malignant neoplasms free time at "Mrzlek community" and with "the rest of AU Nova Gorica"

Survival time is defined as the time to the occurrence of a development of disease registered by cancer diagnosis. In this case survival time is cancer-free time, which is subject to random variations. Application of survival in this study is targeted to an objective demonstration of possible differences in life period before diagnosis of the patients of the two populations. The examination of the reasons of any possible differences would require either a different approach to the study or a new study altogether.

Survival times for the exposed population is summarized below in Table 44.

Table 44: *Estimate of means and medians for survival time for "Mrzlek community"*

	Mean				Median			
	Esti-mate	Std. Error	95% Confidence Interval		Esti-mate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
C16 MN Stomach	75.6	1.2	73.2	77.9	77	1.4	74.2	79.8
C18 MN Colon	74.0	1.0	72.1	75.8	74	1.2	71.7	76.3
C19 MN Rectosigmoid junction	72.9	1.5	69.9	75.9	74	1.9	70.3	77.7
C20 MN Rectum	74.8	1.1	72.7	76.9	76	1.3	73.4	78.6
C22 MN Liver	72.5	1.7	69.1	75.9	74	2.4	69.4	78.6
C64 MN Kidney	71.4	1.7	68.0	74.8	73	2.2	68.7	77.3
C67 MN Bladder	78.8	1.7	75.5	82.1	78	1.9	74.3	81.7
Overall	78.8	1.7	75.5	82.1	78	1.9	74.3	81.7

The mean is used to describe the central tendency of the distribution, but in survival distributions the median is more appropriate because of small number of individuals with exceptionally long or short cancer-free times would cause the mean survival time to be disproportional.

Survival times for the control population is tabulated below (Table 45).

Table 45: *Estimate of means and medians for survival time for "the rest of AU Nova Gorica"*

	Mean				Median			
	Esti-mate	Std. Error	95% Confidence Interval		Esti-mate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
C16 MN Stomach	76.8	1.1	74.7	78.9	78	1.4	75.2	80.8
C18 MN Colon	77.2	0.9	75.4	79.0	79	1.1	76.8	81.2
C19 MN Rectosigmoid junction	76.5	1.6	73.4	79.5	76	1.8	72.4	79.6
C20 MN Rectum	76.2	1.0	74.3	78.1	77	1.5	74.1	79.9
C22 MN Liver	75.9	1.8	72.4	79.3	77	1.2	74.7	79.3
C64 MN Kidney	71.5	1.6	68.3	74.7	72	1.7	68.6	75.4
C67 MN Bladder	79.5	1.5	76.5	82.4	79	1.3	76.5	81.5
Overall	79.5	1.5	76.5	82.4	79	1.3	76.5	81.5

Median survival is the time at which the cumulative survival function is equal to *0.5*. Overal survival time is 78 years for “Mrzlek community” (exposed population) and survival time is 79 years for “the rest of AU Nova Gorica” (not exposed population).

Below, survival functions and *Chi-square* tests for all seven types of observed malignant tumors are shown.

1. C16 - MALIGNANT NEOPLASM OF STOMACH

Within the observed period there were registered 113 malignant neoplasms with the “Mrzlek community” and 104 with “the rest of AU Nova Gorica”. The median survival time is 77 years for “Mrzlek community” and 78 years for “the rest of AU Nova Gorica”.

The survival function for the exposed and control population are shown in Figure 33.

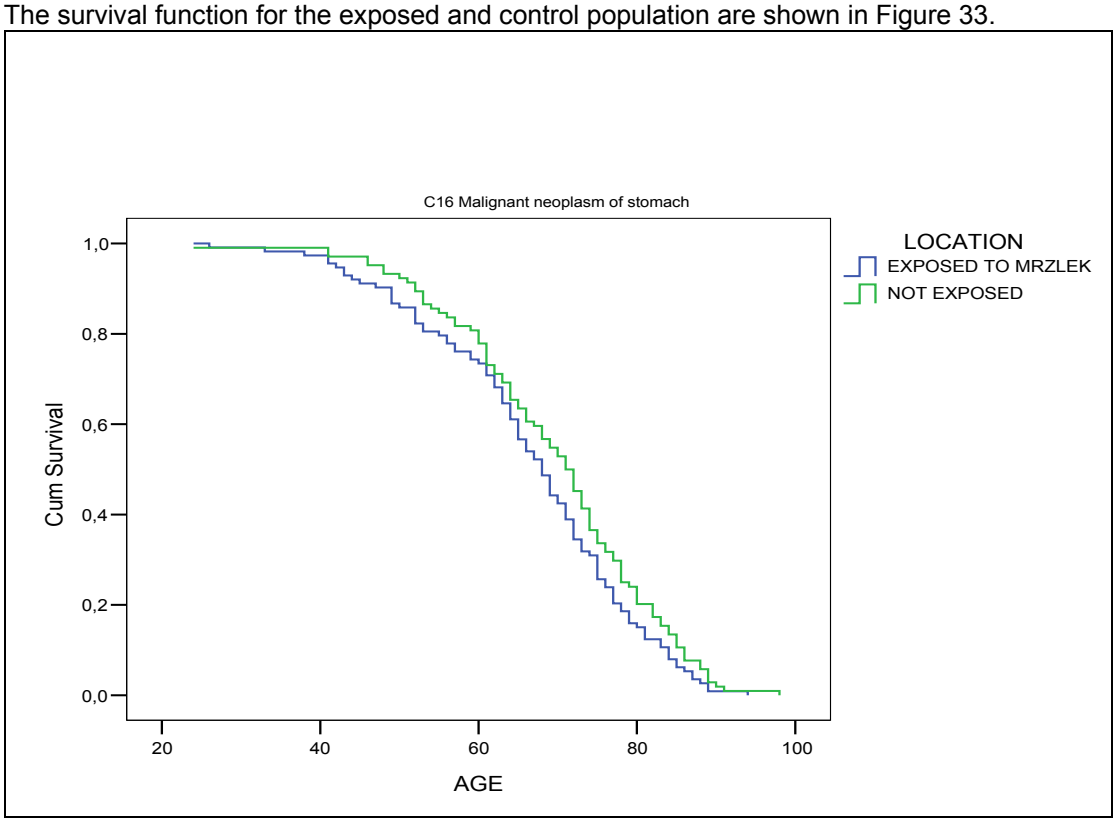


Figure 33: *Survival function for malignant neoplasm of stomach - C16*

The survival curve shows that the people from “the rest of AU Nova Gorica” had better survival experience than people from “Mrzlek community” regarding age of onset of the malignoma of stomach.

Table 46.: *C16 Chi-square*

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	2.801	1	.094
Breslow (Generalized Wilcoxon)	2.420	1	.120

Using Log - Rank, the difference in survival distribution of the human ages at the onset of the malignoma of stomach with two areas of AU Nova Gorica is found to be .09 and is insignificant at the level of 5% probability.

Figure 33 and Table 46 show the cumulative proportion of cases surviving up to the respective interval. The difference in cumulative proportions, which are computed by multiplying out the probabilities across all the previous intervals, do not provide enough evidence to reject the null hypothesis that two the survival distributions are equal. This means that the error probability (the mistake made by rejecting the null hypothesis is greater than the mistake made by accepting it) of the rejecting the null hypothesis is larger than of the accepting the statement of no differences. The survivorship represents the probability of onset the stomach cancer at younger age at "Mrzlek community" than at "the rest of AU Nova Gorica" (not exposed population).

2. C18 - MALIGNANT NEOPLASM OF COLON

The cancer of colon is one of the most frequent cancers occurring in the group of digestive organs. In 19 years, there were 153 cases of colon cancer diagnosed in the population from "Mrzlek community" and 114 cases in the rest of AU Nova Gorica population. The median survival time is 74 years for "Mrzlek community" and 5 years more for the rest of AU Nova Gorica (79 years).
The survival function for the exposed and control population are shown in Figure 34.

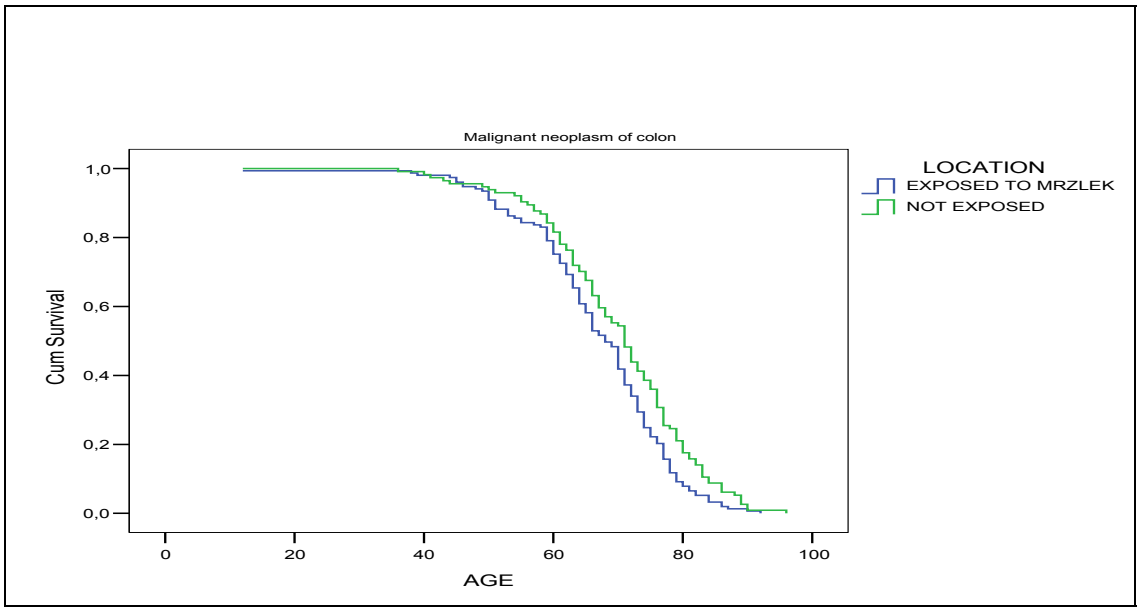


Figure 34: *Survival function for malignant neoplasm of colon - C18*

Table 47: *C18 Chi-square*

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	6.798	1	.009
Breslow (Generalized Wilcoxon)	4.923	1	.027

Since the probabilities of survival that disease has not yet occurred is much higher for "the rest of AU Nova Gorica", the "Mrzlek community" has lower cancer-free life period. Both Log - rank test and Breslow, which takes into consideration the number of cases as a weight, are significant (Table 47). It is very likely that the onset of colon cancer happens sooner in the life with the inhabitants of "Mrzlek community" than with the inhabitants of "the rest of AU Nova Gorica".

3. C19 – MALIGNANT NEOPLASM OF RECTOSIGMOID JUNCTION

The diagnosis of cancer of rectosigmoid junction was identified 42 times in “Mrzlek community” and 27 times at “the rest of AU Nova Gorica”. The median cancer-free time is 74 years for “Mrzlek community” and 76 years for “the rest of AU Nova Ggorica”. The survival function by its shape represents two sections of changing cumulative probability, the first decay of “Mrzlek community” survival happens up to 60 years in comparison to the people living in “the rest of AU Nova Gorica”. The second section represents those cases getting the disease at very old age, and there are more such cases in the not exposed population. The survivorships without cancer of rectosigmoid junction in those communities is reflected by the median estimate of two years gap between “Mrzlek community” and the other community, which begins at the 'younger' ages but there are more cases of disease also at the very old age. The differences between communities are not significant.

This is shown by the survival function in Figure 35.

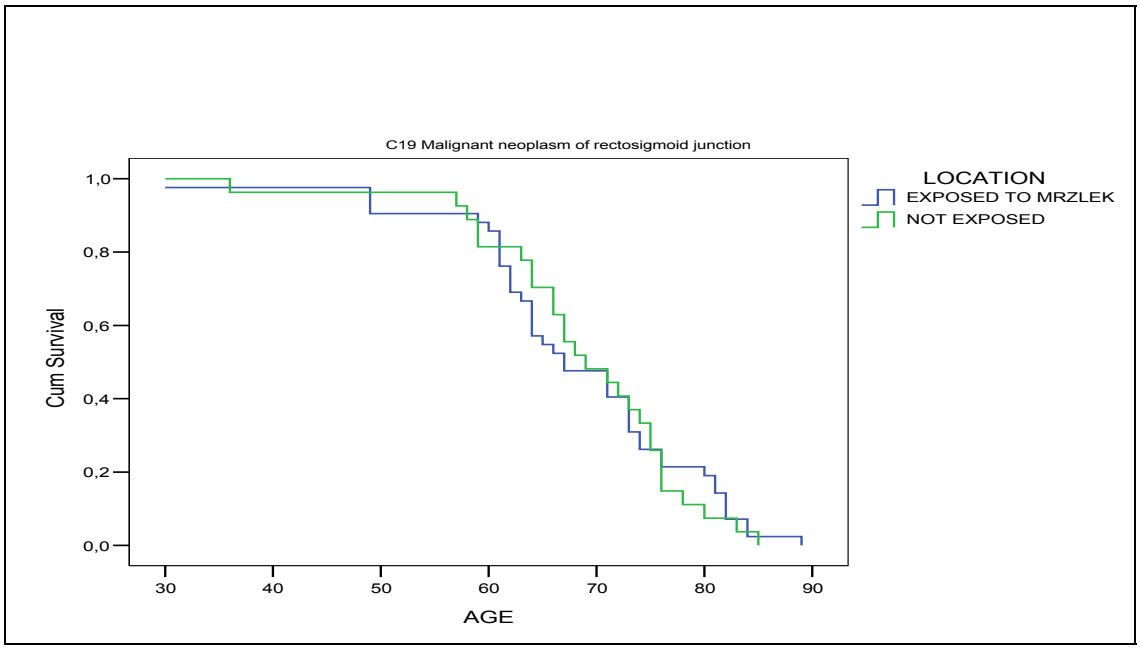


Figure 35: *Survival function for malignant neoplasm of rectosigmoid junction - C19*

Table 48.: *C19 Chi-square*

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.033	1	.855
Breslow (Generalized Wilcoxon)	.148	1	.700

The vector of trend weights is -1. 1. There are no characteristic differences between the ages of both populations at the onset of the disease.

4. C20 – MALIGNANT NEOPLASM OF RECTUM

There were 93 cases of malignant neoplasm of rectum in the “Mrzlek community” inhabitants. In “the rest of AU Nova Gorica”, 86 cases of cancer of rectum were diagnosed.

The survival function for malignant neoplasm of the rectum is shown in Figure 36.

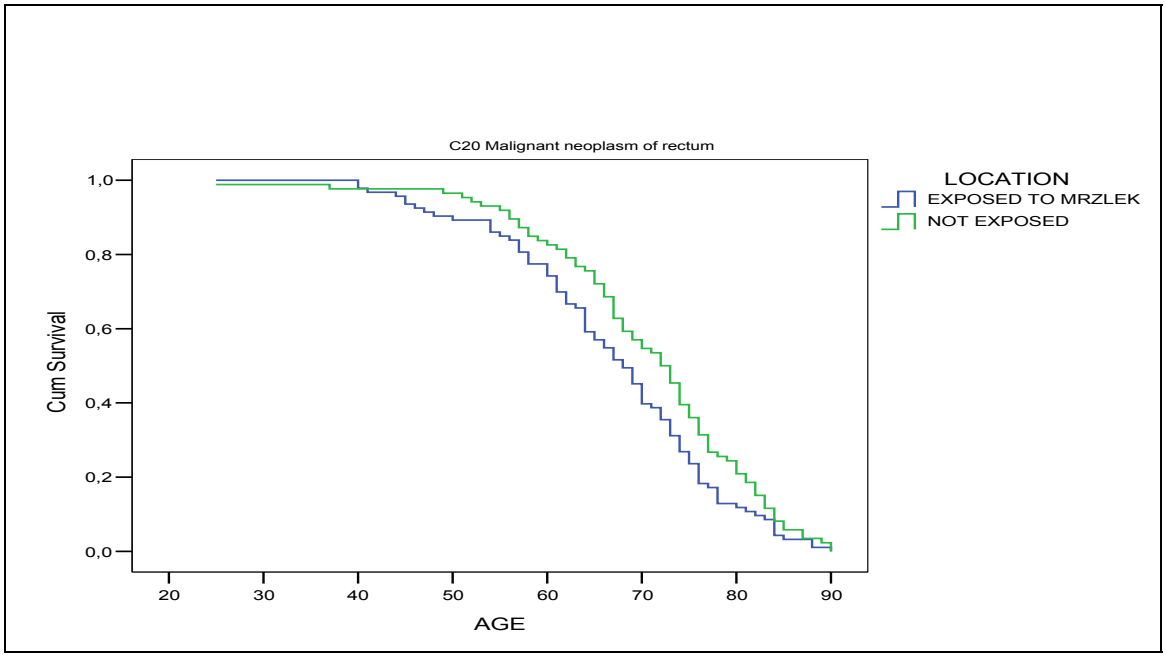


Figure 36: *Survival function for malignant neoplasm of rectun - C20*

Table 49.: *C20 Chi-square*

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	3.888	1	.049
Breslow (Generalized Wilcoxon)	4.953	1	.026

The vector of trend weights is -1. 1. The cummulative cancer-free probability, which begin to decline with “Mrzlek community” at the age of 40 is distributed evenly and constantly with a lower cancer-free probability for “Mrzlek community”. The differences in survival are significant both at Log Rank test as well as its weighted variant Breslow (Table 49).

5. C22 – MALIGNANT NEOPLASM OF LIVER

Malignant neoplasm of liver was registered 37 times among members of the “Mrzlek community” and 27 times for “the rest of AU Nova Gorica”. With regard to the rarity of disease, its characteristic is also to develop very early in life. The lower probability of cancer free period is observed in the “Mrzlek community” among the younger population (up to 65 years) compared to “the rest of AU Nova Gorica”. In general the differences in survivorship are not significant.

The survival functions are shown in Figure 37.

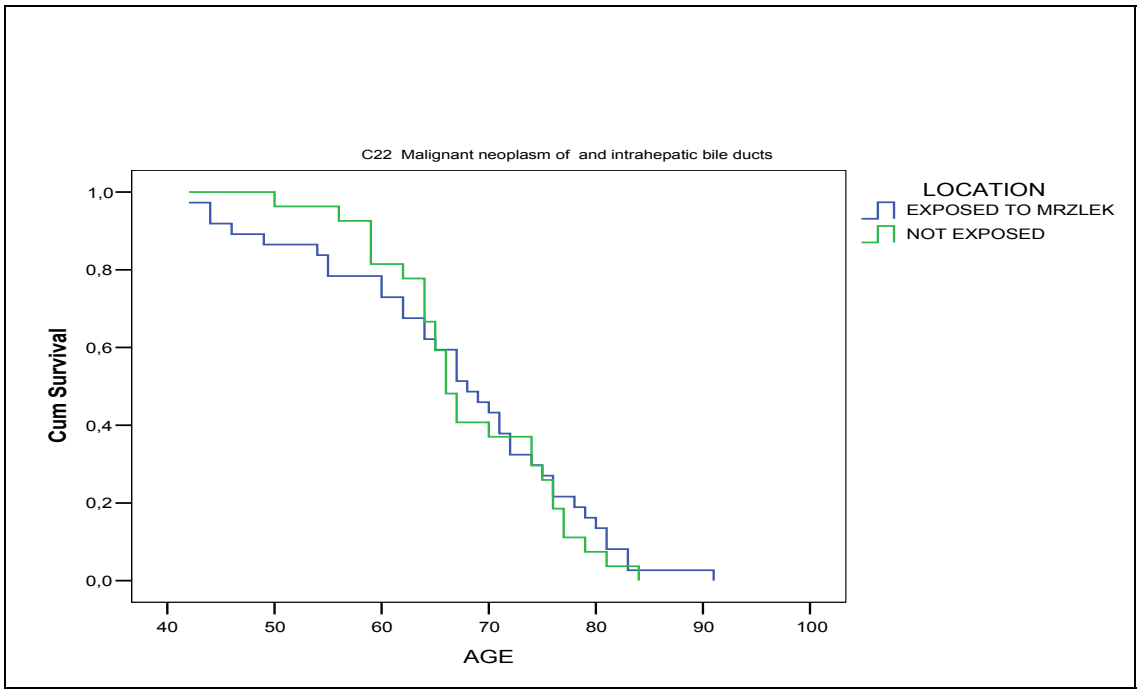


Figure 37: *Survival function for malignant neoplasm of the liver - C22*

Table 50: *C22 Chi-square*

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.159	1	.690
Breslow (Generalized Wilcoxon)	.007	1	.935

The vector of trend weights is -1. 1. There are no characteristic differences between the ages of both populations at the onset of the disease.

6. C64 – MALIGNANT NEOPLASM OF KIDNEY, EXCEPT RENAL PELVIS

44 cases of malignant neoplasm of kidney were observed among members of the “Mrzlek community”, and 43 cases for “the rest of AU Nova Gorica”. The median survival time was 73 years for “Mrzlek community” and 72 years for “the rest of AU Nova Gorica”. Regarding the Figure 37, it can be seen that there are two characteristics with phenomena of developing a neoplasm of kidney. The first of these kinds of malignoma should take place very early in life as the probability of no-disease is even not equal 1. With “the rest of AU Nova Gorica”, the onset occurrs at the years of 30. With the “Mrzlek community”, the probability of cancer-free time is lower for a certain period between 45 and 75 years, approximately. On the count of the second wave of probable development of a disease, which is more common with the people at “Mrzlek community”, there is final surplus of lifetime is in favour of “Mrzlek community”. The comparison of survivorship shows no significant differences among communities. The survival functions and chi-square values are given in Figure 38 and Table 51, respectively.
The early onset of kidney neoplasm in the observed populations is perhaps connected with occurences of the hereditary Wilms tumour, a rare type of kidney cancer. This would required extra research in another study.

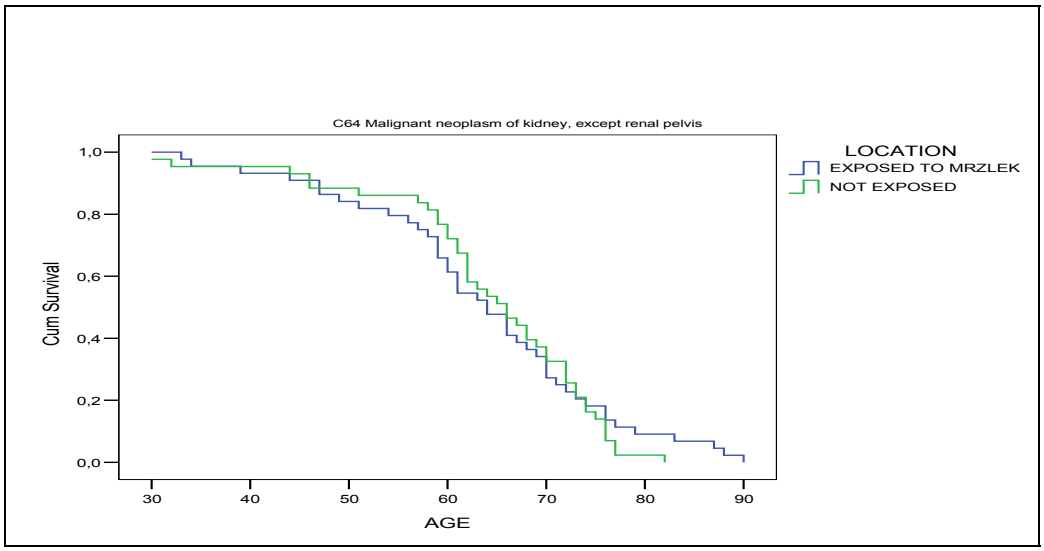


Figure 38: *Srvival function for malignant neoplasm of the kidney - C64*

Table 51.: *C64 Chi-square*

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.145	1	.704
Breslow (Generalized Wilcoxon)	.268	1	.605

The vector of trend weights is -1. 1. There are no characteristic differences between the ages of both populations at the onset of the disease.

7. C67 – MALIGNANT NEOPLASM OF BLADDER

The median survival time is 78 years for “Mrzlek community” and 79 years for “the rest of AU Nova Gorica”, with the 79 and 70 registered cases, respectively. The survival functions and chi-square values are given in Figure 39 and Table 52, respectively.

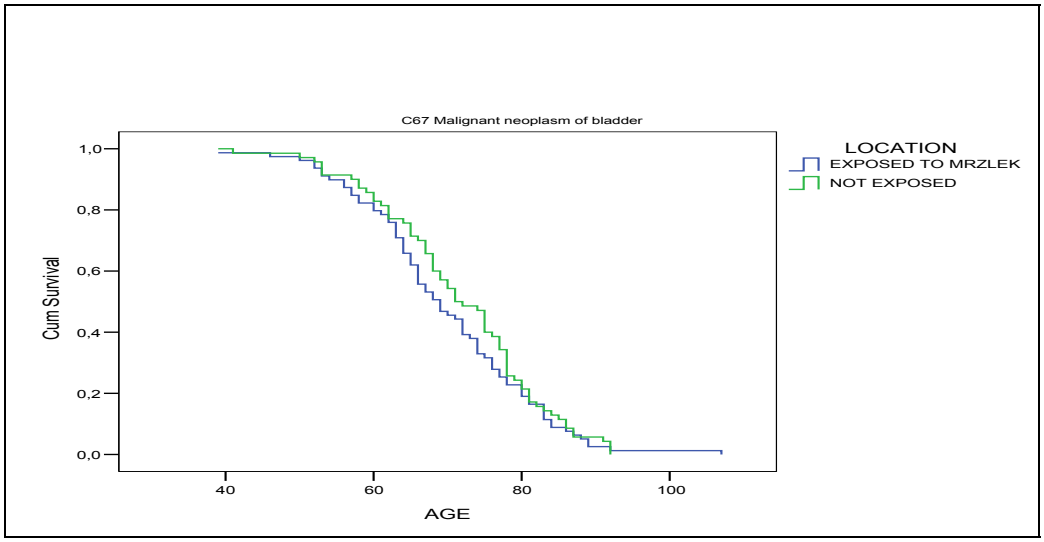


Figure 39: *Survival function for malignant neoplasm of the bladder - C67*

Table 52: *C67 Chi-square*

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.826	1	.363
Breslow (Generalized Wilcoxon)	1.523	1	.217

The vector of trend weights is -1. 1. The difference in median survival between “Mrzlek community” and with “the rest of AU Nova Gorica” is one year, and the probability to live cancer-free life is lower constantly for the “Mrzlek community” except at the end of possible life span. The differences between communities are not significant.

4 DISCUSSION

On the basis of the studies conducted for the purpose of the doctoral thesis, based on:

4.1 Chemical analysis of water and sediments from the artificial lake at the Solkan hydroelectric power plant, of raw and drinking (conditioned) water and sand from the filters in the Mrzlek Waterworks;

4.2 The toxicological studies (part I, part II);

4.3 The epidemiological - statistical studies of the potentially exposed population ingesting water from the Mrzlek Waterworks;

it has been established:

- whether there are hazardous pollutants in the water and sediments of the lake at the Solkan hydroelectric power plant accumulating in quantities which could endanger the quality of water from the Mrzlek Waterworks;
- whether the lake water has an important influence on the quality of water from the Mrzlek Waterworks;
- whether there are substances in drinking water which could be carcinogenic in humans;
- whether there are any negative health indicators in the population drinking water from the Mrzlek Waterworks that are connected with the supposedly mutagenic and/or carcinogenic potential of the water;

and after that it will be possible to confirm or reject the points of the null hypothesis.
In the end:

4.4 An outcome from this research could be the evaluation of drinking water quality standards.

4.1 Characterization of drinking water from the Mrzlek Waterworks

As said previously, the Mrzlek spring supplies drinking water for more than 30,000 inhabitants in the AU Nova Gorica. The population of AU Nova Gorica, which count about 60,000 inhabitants, was devide in two parts:
a) population drinking water from Mrzlek (Mrzlek community[a] - exposed population[a]),
b) and the rest of AU Nova Gorica[b] (not exposed population[b]).
Since special attention was given in this doctoral thesis to the assessment of the presence of genotoxic volatile halogen substituted methanes (THM's) in drinking water, paralel data for the drinking water used by the control population is not listed. This is due to the fact that the control population drinks water from numerous small water systems, which are generally poorly maintained and are not chlorinated at all (therefore, there are no THM's present in this water, as they appear during chlorination). Generally, water from these systems is chemically in accord with the requirements of the drinking water quality edict, but it can sometimes be microbiologically unsuitable (173). Data of the monitoring of this water is held in the documentation of the Institute of Public Health Nova Gorica (174) and Goriški Vodovodi enterprise.

In the course of several years' studies, the concentrations of some heavy metals (Cr, As, Cd, Pb, Zn and Hg) and mineral oils in sediments and in the sand from filters have been measured, as well as concentrations of heavy metals, mineral oils, organic compounds (THM's) in the lake water and in raw and drinking water from the Mrzlek water supply.

In the case of sediments, it can be concluded on the basis of analyses and comparison with the measurements made in previous years that the accumulation of sediments in the artificial lake at the Solkan hydroelectric power plant is small. The concentrations of Cd and Pb are decreasing, especially after the year 1988. This coincides with the closing down of the lead mine Rajbelj in Italy, which presented an important source of pollution upstream of the rivers Koritnica and Soča in Slovenia. The mineral oils in sediments of the lake are also constantly present, although the concentrations of them are higher than the concentrations at the controlling point (the Soča at Deskle, before the artificial lake), which indicates the possibility of accumulation of organic pollutants in the lake.

Concentrations of pollutants in none of the analysed substances were higher than the allowed concentrations in water (175,176). These poisonous and carcinogenic substances (As, Hg, Cd, Pb) were found in the river/lake sediments and in the filter sand of the Mrzlek Waterworks, while they were under the laboratory detction treshold in drinking water. Regardless of that, the concentrations of some heavy metals in water samples are normally below the limit of laboratory detection, but the traces of mineral oils and constant presence of THM's in water samples is a cause of concern.
The widespread use of chemicals and the often uncontrolled and rough polluting of the general environment is cause for concern. The pollution of the general environment has a great influence on public health (177). Unfortunately, due to insufficient knowledge of certain parameters, (scientifically) agreed acceptable risk must be substituted for the NOAEL, or the so-called Pre-cautional Principle, which are more strict than would possibly be necessary in the setting of norms, must be employed.

The environmental isotopes oxygen – 18 (^{18}O) and tritium (^{3}H) are suitable for tracing the origin of the water in the hydrological cycle because they are constituens of the water molecule. These isotope analyses of the samples of the rainwater from the catchment area of the Mrzlek spring, water from the river Soča and conditioned and raw water from the Mrzlek Waterworks are important to assess the possible mutual effect between water from different sources. Based on the oxygen – 18 (^{18}O) and tritium (^{3}H) analysis it can be concluded that the Mrzlek groundwater is several months old (3). The ^{3}H values of samples from the Soča river correspond with the actual ^{3}H content of precipitation, thus may indicate a relatively short mean residence time of the spring water in the underground. A fairly large range in the isotope signal was measured in samples from Mrzlek spring, however, the large variability can in this case be attributed to a stronger influence of the Soča river.

The measurements of isotopes in water of the Soča and in the Mrzlek spring, proved the mixing of the spring water with the river moves deeply into the spring, especially during dry spells in the hinterland.

As said previously, many of those substances were found in the artificial lake and the Mrzlek Waterworks, so the first point of the null hypothesis is:

1. “Soča river and the artificial lake at the Solkan hydroelectric power plant do not bring pollutants: heavy metals, mineral oils and other organic pollutants, which could have negative effects on the quality of the raw water from the Mrzlek spring”

Thus not in accordance with the obtained results, and we can reject this point of the null hypothesis.

4.2 Risk assessment of DBP carcinogenic potential

4.2.1 (part I.)

Chlorine in one form or another is by far the most commonly used chemical for the disinfection of water supplies. It is also active for other purposes associated with water treatment and supply, such as prevention of algal, bacterial and general slime growths in treatment plants and pipeworks, control of tastes and odours, and removal of iron, manganese and colour (178). One of the first reported uses of chlorination for the disinfection of water supplies was in 1897, when bleach solution was used to disinfect a water main in Maidstone, Kent, UK, following an outbreak of typhoid. Regular use in water treatment began around the beginning of the twentieth century. In the UK, the first known regular use was in Lincoln after a typhoid epidemic. In 1908 in Chicago, IL, USA, G.A. Johnson instituted chlorination by adding “chloride of lime” to contaminated river water.

The addition of chlorine to waters containing dissolved organic compounds results in complex reactions that lead to chlorination by-products. There are several of them:

a) Trihalomethanes,
b) Halogenated acetic acids,
c) Halogenated acetonitriles,
d) Chlorinated ketones,
e) Halogenated phenoles,
f) Other halogenated hydrocarbons,
g) Chlorinated furanones,
h) Miscellaneous chlorination products.

The results are quite similar in this studied project. The constant presence of halogenated hydrocarbons is observable in conditioned (chlorinated) drinking water from the Mrzlek Waterworks. Among the substances that were searched for and measured in drinking water, the trihalomethanes were identified as the most critical. The sum of trihalomethanes was, according to the measurements presented in Table 19, close to the value 15µg/L. The preliminary research of mutagenic and genotoxic potential of drinking water from the Mrzlek Waterworks showed genotoxic effects on test micro organisms from the trihalomethanes present in one liter of drinking water. The results are shown in Chapter 3 (Results) in Tables (3, 4, 10, 11, 12, 13, 14, 15, 16, 17, 18 a&b, 19 a&b and 20) and in Figures (10, 11, 12, 13, 14, 15, 16 and 17). The comment on sampling and methods is given in Chapter 2 (Materials and Methods). Even though THM's concentrations were always within the regulated norms, several sources as well as the toxicologic dossier suggest, that THM's can help to increase cancer morbidity in a population drinking water that contains THM's. The development of cancer diseases can be linked to a genotoxic potential shown by THM's. The genotoxicity of isolated THM's was assayed with the SOS/umu test (114,115), the Ames test (116) and the Comet assay (119,120,121).

The presence of mutagenic chemicals in concentrated extracts of drinking water is inferred from the positive results obtained in bacterial mutagenicity assays such as the *Salmonella*/microsome mutagenicity assay (116,179,180). Organic compounds present at low concentrations in drinking water must be extracted and concentrated prior to assays for mutagenicity. No single technique is capable of extracting all organic material from water. The most widely used technique involves adsorption on XAD macroreticular resin.
In this study the THM's from the water samples were concentrated for the genotoxicity tests (Tables 11, 15 in 17 in Chapter 3, Results - Section 3.1) by "special laboratory method" (Figure 9). That was as follow**:**
from the barrel containing 54 L of water we isolated volatile halogenated methanes by stripping them using helium of purity 99.999%. Helium (flow rate of 20 mL/min) was introduced to the glass container filled with sample of water up to the top through the metal frit at the bottom of the container. Helium, containing volatile halogenated compounds, was trapped into the vessel, containing 10 mL of DMSO. The purging lasted up to 7 days.

The concentration of isolated THM's in the water sample for the genotoxicity testing was calculated from the concentrations, measured before and after stripping, replicated with the volume of 54 L, from which were isolated, and divided with the final volume of the sample (6 mL).
The decrease of THM's concentrations was followed periodically using method, developed at HP (Application Note 228-379).
Calibration solutions of the four target solutes were prepared in pentane. Water samples were taken from the barrel container periodically. Then, l-lM aliquits were pipetted into 2 mL automatic sampler vials. The compounds were extracted by vortex mixing for 1 minute at medium speed. The two solvent layers were allowed to sit for 1 minute to separate. Then the sample vial was transferred to autosampler for analysis.

The SOS/*umu* test is based on the detection of expression of the *umu* gene. The *umu* gene is included in the system for the repair of DNA damage (the so-called SOS reparation system). That is why it is activated and expressed when DNA is damaged. The test micro-organism is the bacteria *Salmonella typhimurium* strain TA1535/pSK1002. It has the *umu* gene implanted in the plasmid, which is linked to the gene *lacZ*, which codes for the synthesis of β-galactosidase. When there is an increased expression of the *umu* gene due to DNA damage, there is also a simultaneous increase in the *lacZ* gene expression and thus an increase in β-galactosidase synthesis that is measured

colourimetrically with the help of the suitable substrate. β-galactosidase activity is the measure for induction of the SOS repairing response due to DNA damage. As stated, the samples with isolated THM's (isolated from the sample of conditioned water from the Mrzlek Waterworks) were tested with five different concentrations in three parallel repeats, and at least in three separate experiments. The testing was executed with and without exogenous metabolic activation with the S9 mix. The S9 mix is a microsome fraction of rat liver, induced with Arochlore 1254 and includes a cytochrome P450-linked monooxygenase enzyme system. As it can be seen in Table 11 and Figure 9, the sample induced an SOS response in the test without metabolic activation and with metabolic activation, taking into account that the definition of SOSIF[a] (induction factor) means the ratio (÷) between enzyme units of β-galactosidase activity in the tested sample / enzyme units of β-galactosidase activity of the control.
The lowest test concentration (0.016 % v/v) that is the equivalent of 900 ml of the original sample caused the induction of the SOS response in the test without metabolic activation. This can mean that people who drink this water in the average daily amount of 2-3 L are potentially exposed to a health risk due to the genotoxicity of THM's that are constantly present in this drinking water.

The sample was also tested with a standard bacterial test of return mutations (Ames test) with the use of strains *Salmonella typhimurium* TA98 and TA100 in four different concentrations with and without metabolic activation (S9 mix). As evident from Tables 13 and 14 and Figure 11, the sample showed mutagenous activity in testing without exogenous metabolic activation, while it was not active in the test in the presence of the S9 mix, with MI[a] (mutation indeks; induction ratio) defined as the ratio between the number of revertants (mutants) grown in the presence of the tested sample and the number of spontaneous revertants (mutants).

Attention was directed to possible non-purgeable substances, supposedly carried with the Soča, which mixes with the water from the Mrzlek spring. The water samples were tested for the potential genotoxicity of substances in the non-purgeable fraction (1999): 1) the Soča river (because of it's potential influence on the Mrzlek spring), 2) raw – non-chlorinated water from the Mrzlek Waterworks, and 3) drinking – chlorinated water from the Mrzlek Waterworks. The potentially genotoxic substances were isolated with solid phase adsorption (C 18 – paraphine reverse phase) and elution with methylene chloride. The extracts were tested with the SOS/*umu* test with two bacterial strains of *Salmonella typhimurium:* TA1535/pSK 1002 and NM2009. Strain NM2009 has an increased O-acetyltransferase activity and is more sensitive to the activity to nitropolyaromatic hydrocarbons. The extracted non-purgeable substances were not genotoxic.

The testing of the drinking water samples was also made with the Comet assay. In the comet assay a single cell suspension is embedded in agarose on a microscope slide, lyzed by detergents and high salt concentrations at pH 10 and then electrophoresed for a short time under alkaline conditions. Lysis removes the cell contents except the nuclear material so that nucleoids with highly super-coiled DNA remain. When placed in alkali DNA starts to unwind from sites of strand breaks and the cells with increased DNA damage display increased migration of DNA from nucleoids towards anode under the electrical current, giving the appearance of a "comet tail" (Figure 13). The nucleoids are stained with fluorescent DNA binding dye, visualised by fluorescence microscopy and the DNA damage is quantified by image analysis or visual scoring (119). The DNA damage detected by the alkaline comet assay are frank SSB and DSB, and transiently present SSB and ALS which occur as intermediates during base and nuclear excision repair. The high level of breaks detected with the comet assay indicates either high DNA damage or efficient repair of DNA damage (120). At pH conditions of 12.6 and higher ALS are quickly transformed to SSB and because almost all genotoxic agents induce orders of magnitude more SSB and ALS than DSB, the alkaline (pH>13) version is recommended as the optimal for identifying the agents with genotoxic activity (121).
The major applications of the comet assay are in genetic toxicology for identifying genotoxic agents, in DNA repair studies, eco-toxicology and environmental biomonitoring, nutrition toxicology, clinical applications as well as for biomonitoring in human population studies, Cell-cycle analysis and free-radical biology.
The samples tested in two independent experiments were carried out for each treatment scheme. HepG2 cells were grown in William's medium containing 15% foetal bovine serum,
2 mM L-glutamine and 100 U/mL penicillin/streptomycin at 37°C in 5% CO_2. The cells were used at passages between 7 and 9. In each experiment the vehicle control (1% DMSO) and the negative control (non-treated cells) were included in order to exclude possible effects of the solvent (DMSO). In all the experiments the results of trihalomethane treated cells are compared to the vehicle control.

In addition HepG2 cells were treated with samples containing 10 Vol% of trihalomethanes in William's medium E for 4 hours. Cells were washed with PBS, trypsinized, centrifuged at 1000 rpm for 10 minutes, and the cells pellets frozen at -80°C.
The assay was performed as described by Sing *et al* (181). Thirty micro-litres of cell suspension (<400,000 cells/mL) was mixed with 70 mL of 1% LPM agarose and added to fullyfrosted slides that had been covered with a layer of 1% NMP agarose. Subsequently the slides were lysed for 1h at 4°C, rinsed with distilled water, placed in the electrophoresis solution for 20 minutes to allow DNA unwinding, and electrophoresed for 20 minutes at 25 V and 300 mA. Finally the slides were neutralised, stained with ethidium bromide and analysed using a fluorescence microscope. The % of tail DNA was used to measure DNA strand breaks.
One-way analysis of variance was used to analyze the differences between treatments within each experiment. Dunnett's test was used for multiple comparisons versus the vehicle control. Results were positive; $p<0,05$ was considered as statistically significant. The samples showed genotoxic potential.

Special attention was given to the analysis of unchlorinated – raw water. It is expected that THM's will not be identified in unchlorinated water. This was the reason for collecting samples of raw water and analysing them from the waterworks at Mrzlek and in Slovenska Bistrica, which gets its water from sources in the Pohorje. It is known that natural water from Pohorje contains substantial concentrations of humic and fulvic substances (acids), which are identified as THM's precursors, due to the geologic qualities of the Pohorje region. We also attempted to concentrate THM's, if these substances are perhaps present in raw water samples, including the pre-chlorination phase. The result of the concentration was unsuccessful for the Mrzlek Waterworks and minimal concentrations of chloroform of unknown origins were detected in the Pohorje water. The samples were then tested with the Ames test. The results were negative (Tables 17, 18 a&b, 19 a&b; Figures 14,15,16 and 17) which suggests that the chlorination process has a crucial role in the formation of highly-volatile substances – THM's, that raw unchlorinated water does not show a genotoxic potential, whereas chlorinated (conditioned) water shows genotoxic potential in all samples and in all the executed tests.

After the statements above, the second point of the null hypothesis is:

2. "Drinking water from the Mrzlek Waterworks does not contain any supposed mutagenic and/or carcinogenic potential"

Thus not in accordance with the obtained results, and we can reject this point of the null hypothesis.

4.2.2 (part II.)

The toxicity identification of THM's is presented based on the toxicological study (Section 3.2):

- THM's are primarily metabolised to CO_2 and/or CO.
- The rate of metabolism of THM's is DBCM>BDCM>CHLOROFORM (THM; WHO). PBPK (Physiologically based pharmacokinetic) has shown that after exposure to mixture of chloroform and DBCM or chloroform and BDCM There are higher amounts of unmetabolised chloroform in blood compared to exposure to chloroform alone (THM, WHO, da Silva, 2000).
- Acute exposure of animals to chloroform affected CNS, liver and kidneys. Observed neurotoxic effects were depression of CNS, piloerection, ataxia, sedation and prostration. Critical effects on target organs were cellular degeneration, damage and/or necrosis.
- After repeated exposure the major target organ of THM's toxicity was the liver. However, the histopatholigical effects were mild and reversible. Some effects were determined also on the immune system, but they were not dose-dependent.
- There were some epidemiological studies performed trying to establish the correlation between reproductive toxicity and exposure to THM's in drinking water but the data were not sufficient to determine the quantitative assessment of reproduction and developmental risk. However some reproduction and fetotoxic effects were recognised for chloroform and BDCM in animal studies and in the case of BDCM also the reduction of male fertility.
- Both BDCM and DBCM induced sister chromatid exchange in human lymphocytes *in vitro* and micronuclei formation in mouse bone marrow cells *in vivo*. Results of genotoxicity studies of chloroform are predominantly negative, while there is some evidence for genotoxicity of

brominated THM's. This is probably a consequence of metabolic activation of brominated THM's with glutathione S-transferase theta.

- Based on animal carcinogenicity data and on epidemiological studies on humans chloroform and BDCM were classified by IARC as possibly carcinogenic for humans and DBCM as not classifiable as to its carcinogenicity for humans. The tumours induced by chloroform and BDCM were hepatomas and kidney tumours and intestinal tumours for BDCM. There were some indication of increase in bladder, colon, rectal, brain and pancreatic cancer, but no data
- are strong enough to draw the correlation between THM's exposure and cancer incidence.

Regarding the toxicological properties of THM's and the Directive 67/548/EEC on classification and labeling of dangerous substances chloroform, bromodichloromethane and dibromochloromethane should be classified as shown in Table 53 below:

Table 53: *classification of some THM's*

a) CHLOROFORM	
Carcinogen category 3, R40	Limited evidence of a carcinogenic effect
Xn, R20	Harmful if swallowed.
R48/20/22	Danger of serious damage to health by prolonged exposure through inhalation and if swallowed.
Xi, R38	Irritating to skin.
b) BDCM	
Carcinogen category 3, R40	Limited evidence of a carcinogenic effect
Xn, R20	Harmful if swallowed.
R48/22	Danger of serious damage to health by prolonged exposure if swallowed.
R46	May cause mutation.
c) DBCM	
Xn, R20	Harmful if swallowed.

In the **risk assessment** performed in Section 3.2 it was shown that the concentration of CHLOROFORM, BDCM and DBCM found in drinking water in Slovenia does not present a risk for the people drinking this water. But there are still some uncertainties that were not taken into account in this study:

- In performed risk assessment only exposure through the drinking water was taken into account. However, people who live in areas with chlorinated drinking water use it also for cooking, brushing teeth, showering, bathing or swimming. These chloroform polluted indoor air and ingestion of food, are additional sources of exposure for general population. In case of showering, bathing or swimming people are exposed to THM's through dermal absorption, inhalation and also ingestion. There were no data performed on dermal absorption of chloroform, BDCM and DBCM. In addition, no studies were performed on inhalation exposure to BDCM and DBCM. There is a need of additional studies on dermal and inhalation toxicity, dermal absorption, and ADME data after dermal or inhalation exposure to THM's to estimate the exposure of general public to certain THM more accurately.
- The absorption, distribution, metabolism and excretion of certain metabolites could be different if the people are exposed to combination of these compounds compared to most toxicity studies that were performed after exposure to a single compound at a time.
- It is questionable if at the TDI there is no risk for the health of the consumer, since the genotoxic activity of BDCM *in vivo* was not clearly excluded.
- The data on combined toxicity of various THM's are scarce. The effect of the combination of various THM's simultaneously could be additive, synergistic or not altered.

WHO has set the guideline values for CHLOROFORM in drinking water at 300 μg/L, BDCM at 60 μg/L and for DBCM at 100 μg/L (WHO, 2005).
Although in case of mutagenic carcinogens (proposed mechanism for BDCM carcinogenicity) the threshold for the effect cannot be determined and it is not excluded that at this concentration people are not at higher risk for malignant tumour development.
To conclude, much is known about the toxicity of specific THM's and the risk for human health from the oral exposure in the drinking water can be made. However, it would healthier to use modern methods for disinfection of drinking water and not chlorination. But at this time, the immediate remedy

is to employ the lowest amount of chlorine to disinfect the water and try and keep the concentrations of THM's in the drinking water as low as practically achievable, but the disinfection of drinking water must never be compromised.

4.3 Epidemiological - statistical studies of the potentially exposed population

Even though the concentrations of some heavy metals, mineral oils and THM's (which are regarded as a burden in the drinking water from the Mrzlek Waterworks in this thesis) in the analysed samples are not critical, the different laboratorial-epidemiologic short-term tests performed on these samples all showed a genotoxic potential in these samples. The typical positive Comet test of 2003 is especially important, while the repeat of the Ames test of 2005 (analysis of unchlorinated water samples**)** showed negative results of genotoxicity, which is a good control test of the previous positive tests.

In general, all of the epidemiological studies were handicapped by the extreme difficulty of identifying a very small effect in a population. Many of them were seriously limited by the absence of data on past exposures, which are the only ones that are directly relevant to cancer that has already been diagnosed. Similarly, many of the studies were deficient in identifying populations that were stable in the areas where the water quality was studied. The methodological complexities inherent in epidemiological studies of human populations exposed to multiple contaminants at low concentrations in drinking water make it virtually impossible to establish a casual link between pollutants (e.g. THM's) and an increase in cancer of the bladder or of any other site. In addition there are difficulties in controlling for a multitude of factors that are known to affect cancer incidence: cigarette smoking, diet, occupation, use of alcohol, drugs, socio-economic status, and nonaqueous sources of pollutants.

This dissertation is a longitudinal study with a control population, as exact data from the Cancer Registry of Slovenia which follows these information longitudinally as well as cohortly were obtained and processed. Population parameters, namely age and gender were controlled explicitly, supposing that social and economic characteristics of both populations are distributed randomly and in such a way that they do not differ. This supposition is based on the appertainance to similar geographic environments, similar genetic forms, social and economic order and standards of living (there is no proof that one population is more poor or less educated than the other, for example). The control of age and gender was carried out by means of direct and indirect standardization and with the use of other statistical techniques taking into account the differences of age and gender. Generally, differences in the incidence of cancers are undoubtedly linked to the age and gender of the population.

A comment of the results of Chapter 3, Section 3.3: the age distribution characteristics of population of the AU Nova Gorica did't change during the observation period. The stability is observed in both parts of AU Nova Gorica (“Mrzlek community” – exposed population and “the rest of AU Nova Gorica”). In this study, different statistical calculations were used for analysed patterns of incidence of seven different malignant neoplasms. The particular malignant neoplasms incidence rates as neoplasm of stomach, colon, rectosigmoid junction, rectum, liver, kidney and bladder provides information about the number of observations, which *per se* establishes the frequency of disease. Both direct and indirect standardized incidence ratios (SIR's) are used in order to overcome the problems of a crude rate masking differences in particular age groups. The SIR’s with it’s confidence intervals in the exposed and not exposed area for all malignant neoplasms locations compared in the analysis. The SIR’s in the exposed population are not signifcantly diferent from the not exposed SIR’s, as the values of the not exposed population SIR’s are included througout in the confidence intervals of exposed population SIR’s.

After the statement above, the third point of the null hypothesis are:

3. “These are no negative health indicators (an increased incidence of malignant diseases) linked with the supposed mutagenic/carcinogenic potential of the water from the Mrzlek Waterworks within population drinking this water ”

Thus that is accordance with the obtained results, and we can accepted this point of the null hypothesis.

The other view takes into consideration the age of the population at the onset of a disease. In the context of this dissertation, the term survival time means **the time from birth to the development** of seven locations of malignoma neoplasms: stomach (C16), colon (C18), rectosigmoid junction (C19), rectum (C20), liver and intraheptic bile ducts (C22), kidney, except renal pelvis (C64) and bladder (C67) in two population of AU Nova Ggorica. Table 41 (calculation of age at onset of particular types of neoplasms in two communities) show that **"**the rest of AU Nova Gorica**"** (not exposed population) has a greater life period -*without developing a disease* - in general, which probably means the onset of the disease later in life. As the mean age at onset in general differs by 2.4 years, the cancer of rectum is a source of the greatest differences between the two parts of AU Nova Gorica (3.6 years), while the differences of stomach cancer and colon cancer are 3.0 and 3.3 years respectively, with more promising results for the not exposed population.

As said above survival time is cancer-free time, which is subject to random variations. The survival curve for malignant neoplasm of stomach – C16 (Figure 33) shows that the people from "the rest of AU Nova Gorica" had a greater age of onset of the malignomas than people from "Mrzlek community" regarding age of onset of the malignoma of stomach. The survivorship represents the probability of onset of stomach cancer at a younger age in "Mrzlek community" than in "the rest of AU Nova Gorica" (not exposed population).
The median survival time for malignant neoplasm of colon – C18 (Table 44) is 74 years for "Mrzlek community" and 5 years more for "the rest of AU Nova Gorica" (79 years, Table 45). It is very likely that the onset of colon cancer happens sooner in life with the inhabitants of the "Mrzlek community" than with the inhabitants of "the rest of AU Nova Gorica" (Figure 34).
The cumulative cancer-free probability (Figure 36, malignant neoplasm of rectum – C20), which begin to decline with "Mrzlek community" at the age of 40 is distributed evenly and constantly with a lower cancer-free probability for "Mrzlek community".
The difference in median survival (Figure 39, malignant neoplasm of the bladder – C67) between "Mrzlek community" and with "the rest of AU Nova Gorica" is one year, and the probability to live cancer-free life is lower constantly for the "Mrzlek community".

At long term, from an epidemiologic point of view, the incidence of cancer (an otherwise rare disease) in an observed population does not tell much, and in our exposed population the "Mrzlek community" the absolute incidence is even slightly lower. With the length of life, the risk for cancer disease increases. Therefore, our conclusion is that these cancers appear in younger age groups in the "Mrzlek community" than in "the rest of the AU Nova Gorica" (not exposed). Even though in this study, we did not examine whether these findings are a result of a coincidence or some rule. Some other study will have to establish this. Especially if classical risk factors,such as *smoking, alcohol and dietary habits*, which were not studied in this dissertation, may have influenced the results as confounding factors, although this is unlikely. As said, the study deals with two very similair segments of the same population, which have the same historical, cultural, geographic, social-economic, health and other characteristics, which determine a population.

After the statement above, the fourth point of the null hypothesis are:

4. "Two communities within AU Nova Gorica divided by the usage of different sources of drinking water have the same probability to develop a digestive or urinary malignoma neoplasm regarding age as long as other social ecomomic characteristics are not a factor".

Thus not in accordance with the obtained results, and we can reject this point of the null hypothesis.

4.4 Is an evaluation of drinking water quality standards necessary?

The maintenance of an adequate supply of unpolluted water is a requirement for both human health and good environmental quality. Our demands upon the planet's water are great, and in some regions dangerously so. Water is used for drinking, irrigation and industry and is returned as industrial discharge, agricultural run-off and microbiologically contaminated, treated or untreated sewage. The most critical characteristic of water for human health is its microbiology. Microbiological contamination of water is controlled by disinfection methods based on oxidants, like chlorine. In consequence, drinking water may contain a variety of potentially carcinogenic agents, including chlorination by-

products and arsenic. It is desirable to reduce such contamination without reducing the rigour of disinfection procedures (182) There are substantial and irrefutable benefits of disinfection of water supplies by chlorination. Any major change to these programs would need to be evaluated fully as to its costs and benefits in regards not only to the need of maintaining microbiological safety but also to the possible long-term adverse effects of alternatives to chlorination. Nonetheless, it is now known that the interaction of chlorine with naturally occurring humic and fulvic acids in water supplies results in the formation of by-products such as trihalomethanes and others, some of which are either known or suspected carcinogens.

The International Agency for Research on Cancer (IARC) concluded (147):

a) that there was inadequate information from epidemiologic studies and experiments in animals to assess the risk for development of cancer in humans from chlorinated drinking water alone,
b) the chlorination of drinking water remains essential in prevention of disease.

United States Environmetal Protection Agency (US EPA) is considering alternative ways to disinfect water supplies, including the use of chloramine, which, compared with chlorine, lowers the formation of trihalomethanes by 90% (183). The maximum permissible contaminant level for total trihalomethanes in drinking water in the U.S. is 100 μg/L. The same norm is valid in Slovenia (173).

The regulations and drinking water standards are formed and modified over a longer period of time. They define the MCL for many substances. These are the maximum permissible levels of contaminants in water that enter the distribution system of a public water system. Agencies recommend or set the goals (MCLG) for substances that can be mutagenic and/or carcinogenic. The MCLG's are maximum levels of contaminants in drinking water for lifetime consumption, which are non enforceable health goals and are strictly health based, and do not include a technical feasibility or economic evaluation. The goals for carcinogenic substances are zero (184). In Slovenia the MCLG have not yet been defined. In US National Primary Drinking Water Standards (1977), recommendation for trihalomethanes is zero.

How could we establish drinking water regulations and quality standards? The primary question is always: does the agent cause toxic or genotoxic effects? Depending on that, estimating noncarcinogenic toxicity and estimating cancer risk is necessary. Two approaches are used for risk assessment of chemicals in drinking water: one for noncarcinogens and one for carcinogens (184). For noncarcinogenes, data from chronic studies and an uncertainty factor (Uf) approach are used on a threshold phenomenon for the critical health endpoint of concern. For carcinogens assumed not to have a threshold in the absence of convincing data, a commonly used approach is mathematical modeling for quantitative risk assessment. Various models are used for carcinogenic risk assessment, a common one being the linearized multistage model (LMM) (however, these models lack scientific validity).

The scientific process for risk assessment used for the setting of permissible levels for chemicals in drinking water are shown in Figures 40 and 41 below (to sum up ref. 185):

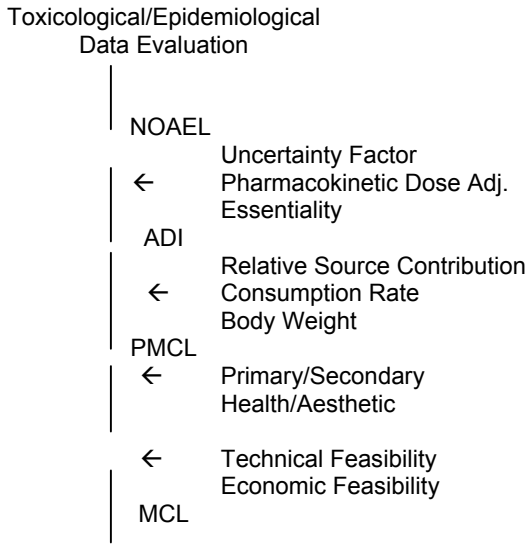


Figure 40: *Development of water standards for noncarcinogens (185)*

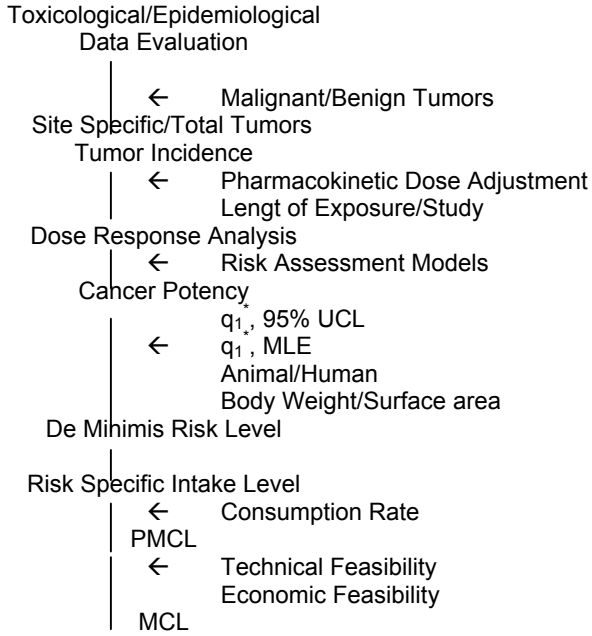


Figure 41: *Development of water standards for carcinogens(185)*

For noncarcinogens, the first step in the risk assessment process, hazard identification, would allow an identification of one or more health effect endpoints of concern. A NOAEL or LOAEL (lovest-observable-adverse-effect level) is then derived for the critical endpoint as a reference exposure value for comparison to an individual's total exposure from all sources to the chemical. The reference value sets a limit which should not be exceeded by an individual for the purpose of health protection. Application of the Uf and relative source contribution (RSC) would provide a calculation of the permissible level in water as follows (184):

$$\frac{(\text{NOAEL/LOAEL, mg/kg-d})\ (70\text{-kg})\ (\text{RSC})}{(\text{Uf})\ (\text{W, L/d})} \qquad \{17\}$$

RSC is usually assumed to be 20% or 0.2, if adequate data on other sources of human exposure such as diet are absent but can reasonably be believed to contribute to major exposure. The RSC can be 100% if water is believed to be the likely sole source. W is the daily water consumption rate of 2 L/d for drinking water only for an adult.

Using an another equation would be established drinking-water-equivalent level (DWEL) as follows (186):

$$DWEL = \frac{RfD * Body\ weight\ (bw)}{Drinking\ water\ (DW)\ L/d} \quad \{18\}$$

After that, MCLG maybe calculated:

MCLG = DWEL x % DW contribution {19}

For carcinogens, risk assessment methodology has undergone considerable debate regarding issues such as the use of data from combined tumor types and the use of which ratio for animals-to-humans body surface conversion. The strength of carcinogenic activity of a chemical, expressed as carcinogenic potency, is first determined. The human carcinogenic potency for a chemical can be estimated directly from human epidemiological data or for experimental animal data. However, human data is rarely available or adequate for cancer risk assessment. Therefore, animal data is often used to derive the human potency estimate, using a matematical model (e.g.linearised multistage model) fitted to the animal tumor incidence data from carcinogenicity studies in rodents. This default approach uses the 95% upper-bound estimate (95% Upper Confidence Limit, UCL) of the low-dose slope obtained from animal dose-response data, assuming linearity at very low doses. In fitting the linearised multistage model to the animal cancer data, experimental data on dosing regimen are converted to lifetime average daily doses. When available and appropriate, other tissue dosimetry, which include pharmacokinetic behaviour are also considered. In cases of a shorter than lifetime study duration, the potency values are corrected for inter current mortality by the factor $(L/Le)^3$ where Le is the duration of the experiment and L is the life span of the animal, usually 102 weeks is used for rats and mice. To extrapolate animal potency data to humans, a conversion based on the surface area or body weight ratio is used as follows (186):

$$q_{1}^{*}(H) = q_{1}^{*}(A)(W_h/W_a)^{1/3} \quad \{20\}$$

where q_1^* (H) is the human potency in $(mg/kg\text{-}d)^{-1}$, q_1^* (A) is the animal potency in $(mg/kg\text{-}d)^{-1}$, H_h is the standard adult human body weight in kg (70 kg), and W_a is the animal (rat or mouse) weight in kg. Using the potency determined from above, the permissible level in drinking water for carcinogen can be derived as follows (184):

$$\frac{(Risk)(70kg)(RSC)}{\{q_1^*(H),(mg/kg-d)^{-1}(W,L/d)} \quad \{21\}$$

Risk is the theoretical de minimus (significant) individual excess lifetime cancer risk of 10^{-6}; 70 kg is adult body weight; RSC is the relative source contribution often, but not always, assumed to be 1.0; q_1^*(H) is the human potency in $mg/kg\text{-}d^{-1}$, and W is the water consumption rate in liters per day or liter equivalents per day if additional exposure routes other than drinking water are considered.

Three prominent institions: IARC, US EPA, and NTP use classical classification (tabulated below in table 54) to presented “weight of evidence criteria for classifying carcinogens” (184):

- IARC: **1, 2A, 2B, 3** and 4;
- US EPA: **A, B_1, B_2, C, D** and **E**;
- NTP: **a** and **b**.

Table 54: *Weight-of-evidence criteria for classifying carcinogens*

Institution	Category	Criterion
International Agency for Research on Cancer	1	Carcinogenic to humans (sufficient epidemiologic evidence)
	2A	Probably carcinogenic to humans (at least limited evidence of carcinogenicity to humans)
	2B	Possibly carcinogenic to humans (no evidence of carcinogenicity to humans)
	3	Not clasifiable as to carcinogenicity to humans (sufficent evidence of carcinogenicity in experimental animals)
	4	Probably not carcinogenic to humans
U.S. Environmetal Protection Agency	A	Human carcinogen (sufficent evidence of carcinogenicity from epidemiological studies)
	B_1	Probably human carcinogen (limited evidence of carcinogenicity to humans)
	B_2	Probably human carcinogen (suficent evidence from animal studies and inadequate evidence or no data on carcinogenicity to humans)
	C	Possibly human carcinogen (limited evidence from animal studies; no data for humans)
	D	Not classifiable because of inadequate evidence
	E	No evidence of carcinogenicity in at least two animal tests in different species or in both animal and epidemiologic studies
National Toxicology Program	a	Known to be carcinogenic (evidence from studies on humans)
	b	Reasonably anticipated to be a carcinogen (limited evidence of carcinogenicity in humans or sufficient evidence in animals

Neverthelass MCLG may be based on carcinogenicity divided in three categories shown in Table 55 (186):

Table 55: *Relationship between MCLG and evidence of carcinogenicity*

Category	Evidence of Carcinogenicity Via Ingestion	Setting MCLG
I	Strong	Set a zero
II	Limited or equivocal	Calculate based on RfD plus added safety margin or set within cancer risk range of 10^{-5} to 10^{-6}
III	Inadequate or none	Calculate RfD

As said previously, the THM's (e.g. Chloroform and Bromodichloromethane) were constantly present in the treated water of the Mrzlek water supply. These substances may be clasified in category 2B – possible human carcinogens. Two options are considered to establish MCLG for those substances:

1) MCLG based on RfD + Uf (1 – 10),
2) MCLG based on lifetime risk from 10^{-5} to 10^{-6}.

After that linearised multistage model (LMM) data is used to calculate a human carcinogenic potency factor for each of the substances.

People have come to fear that chlorine in their drinking water may cause cancer. During disinfection with chlorine several disinfection by-products (DBP) are formed in drinking water. Our study undoubtedly shows a genotoxic potential of chlorinated water from the Mrzlek Waterworks. Based on epidemiologic studies, it is possible to speak of a specific health effect with high probability in the population drinking this water, due to early morbidity of the population due to the development of particular types of cancer.

Basic characteristic of genotoxic/carcinogens is stohastic mechanism that means – no threshold dose, just a chance of mutation of genes. As mentioned previously, genetic change is generally considered to be a stochastic event, increasing with dose even at very low levels. Furthermore, the possible consequences of genetic change are serious, e.g. cancer or inheritable genetic disease. Therefore, any increase of genotoxic exposure to humans is regarded as unacceptable.

Nevertheless, one of the most vexing issues in the effort to provide a safe water supply is that of risk tradeoffs. Efforts to reduce one risk in drinking water may introduce a different risk to the population using the water supply. As said previously, people have come to fear that chlorine in their drinking water may cause cancer. But chlorine is used to combat serious waterborne microbial diseases. If we stopped adding chlorine to drinking water in an effort to reduce the risk of long term cancer, would we thereby increase the risk of waterborne microbial disease? Would we just trade one form of risk for another?

5 CONCLUSIONS

- The measurements of temperature, chemical parameters and isotopes at the Mrzlek spring and pumping station showed that the mixing of Mrzlek spring water with Soča water (artificial lake) moves deep into the spring.

- Some heavy metals, especially mercury (centuries of mining in Idrija), lead, cadmium, and arsenic (centuries of mining in Rabelj) and mineral oils are constantly present in sediments – river Soča and in the sands from the Mrzlek Waterworks filters, while they were under the laboratory detction treshold in drinking water.

- THM's were constantly present in the treated water of the Mrzlek water supply even though THM's concentrations were always within the regulated norms.

- The study conducted on the mutagenic potential of drinking water from the Mrzlek Waterworks showed genotoxic effects on the test micro organisms and cells:
    1. The sample for the testing of genotoxicity induced an SOS response.
    2. The sample for the testing of genotoxicity with a return mutations (Ames test).
    3. The samples were tested in Comet assay.

- In samples where there are no THM's present in raw water, the results of the Ames test with and without activation were negative.

- The extracted non-purgeable substances from the Mrzlek Waterworks were not genotoxic.

- The direct and indirect standardized ratios show that there are no significant differences in the number of newly registered malignoma cases between two populations.

- The calculation of ages at onset of the particular types of neoplasms in two communities of AU Nova Gorica give a longer life period -*without developing a disease* - with the population of **"**the rest of AU Nova Gorica**"** (not exposed population) in general.

- As the mean age at onset in general differs by 2.4 years, the cancer of rectum is a source of the greatest differences between the two parts of AU Nova Gorica (3.6 years), while the differences of stomach cancer, colon and bladder cancer are 3.0, 3.3 and 1.9 years respectively, with better results for the not exposed population.

- The survival curves show that the people from "the rest of AU Nova Gorica" had a greater age of onset of the malignomas than people from "Mrzlek community" regarding age of onset of the malignomas of stomach, colon, rectum and bladder. The survival functions represent the probability of onset for this type of cancer at younger age at "Mrzlek community" than at "the rest of AU Nova Gorica" (not exposed population). The cummulative cancer-free probability, which begins to decline with "Mrzlek community" at the younger age is distributed evenly and constantly with a lower cancer-free probability for "Mrzlek community". The probability to live cancer-free life is lower constantly for the "Mrzlek community".

- The study design and the limited assessment of only one risk factor (drinking water) in this population do not allow for any conclusions about the cause of the health effects observed in this study. Nonetheless, this research does indicate that there is an association between chlorinated drinking water and a limited expressions of health status in the study population of Nova Gorica.

- A critical characteristic of water for human health is its microbiology. Microbiological contamination of water is controlled by disinfection methods based on oxidants, like chlorine. In consequence, drinking water may contain a variety of potentially carcinogenic agents, including chlorination by-products (DBP).

- This research suggest the need to investigate the use of other methods for disinfection (UV light treatment, ozonization, irradiation, microfiltration…) of drinking water to determine if they have fewer potential chronic health effects than traditional chlorination disinfection.

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ANNEX A

International presentations:

1. Vudrag M, Hojak M, Maver L, Franko M 1997. Solkan Hydroelectric Power Plan and the Drinking Water Quality in Nova Gorica. International Symposium an Environmental Epidemiology in Central and Eastern European Countries – Critical Issues for Improving Health, Smolenice, Slovakia. Sept.1997.
2. Vudrag M. 1998. Hidroelektrarna Solkan in kakovost pitne vode v Novi Gorici. 2.Slovenski kongres preventivne medicine, 6–10 junij 1998.
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4. Filipič M, Žerjal E and Vudrag M 1999. Halogenated methanes related genotoxicity of drinking water sample. 29th Anual Meeting of the European Environmental Mutagen Society. Copenhagen, Denmark, 4 – 9 julij 1999.
5. Jug T, Vudrag M, Franko M 1999. Recent Measurement of Water Quality in Spring Mrzlek, Groundwater Pollution in Karst – Preserving Water Quality in Karst Systems. Ljubljana, Slovenija, 4–6 sept. 1999.
6. Filipič M, Fatur T, Vudrag M 2006. Molecular mechanisms of cadmium induced Mutagenicity. V Human & Experimental Toxicology 25: 67 – 77, 2006. www.hetjournal.com.

ANNEX B: Laboratory results

B.1 Some laboratory results – early period

Table 56: *Concentrations of heavy metals and mineral oils (µg/g) in sediments – older results (49)*

Para-meter	Samplingsite	Year				
		1985	1986	1987	1988	1989
Cr	1.	21	9	4.6	31	11.7
90 (23)*;	2.	14	8	1.6	20	< 10
	3.	24	12	5	25	16.8
25 (24)*	4.	29	17	4.9	36	25.3
	5.	0.68	12	8.6	23	19.1
	6.	20	31	6.6	28	16.9
	7.	18	16	7.5	25	17.4
	8.	16	12	1.9	28	17.4
	9.	26	23	13	30	13.1
	10.	13	-	2.7	23	10
As	1.	16	< 20	1.6	< 10	< 10
4 (23)*;	2.	33	43	1.6	< 10	< 10
	3.	13	-	0.23	< 10	< 10
- (24)*	4.	19	52	2.1	< 10	< 10
	5.	< 1	120	2.1	< 10	< 10
	6.	4.8	93	2.4	< 10	< 10
	7.	19	56	0.37	< 10	< 10
	8.	11	100	0.3	< 10	< 10
	9.	1.3	71	3.2	< 10	< 10
	10.	34	< 20	0.55	< 10	< 10
Cd	1.	1.3	2.9	2.8	5.9	2.7
0,3 (23)*;	2.	2	2	3.3	5.4	2.8
	3.	1.8	1.8	4.2	5.6	2.9
0,002- 2 (24)*	4.	2	3.8	4.2	4.9	1.7
	5.	1	3.1	4.7	5.4	2.7
	6.	1.9	3.1	3.7	5.1	2.8
	7.	2	1.6	3.2	5.4	2.7
	8.	2	3	1.8	4.9	2.8
	9.	2.1	3.6	4.2	5.4	3
	10.	2	2.8	3	5.1	4
Pb	1.	34	67	55	145	43
20 (23)*;	2.	45	41	13	98	41
	3.	49	45	33	129	55
100 (24)*	4.	95	120	32	126	43
	5.	10	61	39	129	58
	6.	49	78	16	132	46
	7.	46	44	44	136	51
	8.	58	70	33	142	56
	9.	60	89	89	148	52
	10.	46	75	55	108	46

Continued on next page

Continued from previous page: ***Table 56:*** *Concentrations of heavy metals and mineral oils (µg/g) in sediments – older results*

Zn	1.	91	62	100	300	144
95 (23)*;	2.	68	24	25	80	142
	3.	130	49	20	167	200
120 (24)*	4.	170	100	120	149	134
	5.	17	70	150	117	239
	6.	140	155	200	170	118
	7.	96	102	110	165	181
	8.	400	65	68	220	236
	9.	220	220	125	210	180
	10.	17	40	27	82	91
Hg	1.	0.44	14	0.55	-	16.1
0,4 (23)*;	2.	0.15	3.9	0.76	-	2.4
	3.	0.033	2.6	0.57	-	3.8
0,01 – 1 (24)*	4.	0.023	0.43	0.27	-	< 0.5
	5.	1.2	6	0.19	-	6.1
	6.	< 0.01	9.7	0.46	-	3.9
	7.	1.1	3.1	1.9	-	3.1
	8.	0.23	14	0.013	-	4.9
	9.	1,3	8.9	0.46	-	6
	10.	0.032	3	1.4	-	1.3
MO	1.	5	-	5	138	20
-	2.	3.9	-	7	16	27
	3.	5.6	16	62	17	87
	4.	6.3	95	9	13	54
	5.	2.7	35	12	13	133
	6.	3.8	45	20	113	49
	7.	4.4	50	19	46	61
	8.	3.1	75	25	12	51
	9.	1.6	26	14	10	103
	10.	2.6	1.9	-	20	10

*The results are compared with some general values for heavy metals in volcanic rock (vs. sediments) according to some references (187,188).

Legend:

1. Prelesje – left bank
2. Prelesje – the bottom
3. Prelesje – right bank
4. Mrzlek – left bank
5. Mrzlek – the bottom
6. Mrzlek – right bank
7. the dam – left bank
8. the dam – the bottom
9. the dam – right bank
10. Deskle – the bottom

MO – mineral oil

B.2 Some laboratory results – later period

Table 57: *Concentrations of heavy metals and mineral oils (µg/g) in sediments*

Parameter	Sampling site	Mar. 97	Nov. 97
Cr	1.	12	16
90 (23)*; 25 (24)*	2.	9	13
	3.	18	11
	4.	15	15
	5.	12	27
	6.	16	16
	7.	16	21
	8.	26	29
	9.	18	16
	10.	10	-
As	1.	3.6	3.8
4 (23)*; - (24)*	2.	2.5	3.9
	3.	4.8	3.7
	4.	3.8	4.3
	5.	3.9	5.5
	6.	4.0	5.2
	7.	3.5	8.0
	8.	6.2	6.5
	9.	4.0	6.4
	10.	3.2	-
Pb	1.	< 20	21
20 (23)*; 100 (24)*	2.	< 20	15
	3.	22	19
	4.	< 20	14
	5.	< 20	30
	6.	< 20	19
	7.	< 20	23
	8.	28	28
	9.	20	17
	10.	< 20	-
Zn	1.	52	79
95 (23)*; 120 (24)*	2.	37	84
	3.	102	47
	4.	95	97
	5.	59	238
	6.	115	83
	7.	78	110
	8.	120	113
	9.	156	82
	10.	32	-

Continued on next page

Continued from previous page: ***Table 57:*** *Concentrations of heavy metals and mineral oils (µg/g) in sediments*

Parameter	Sampling site	Mar. 97	Nov. 97
Hg	1.	13	10
0,4 (23)*;	2.	11	13
0,01 – 1 (24)*	3.	24	14
	4.	22	21
	5.	12	7
	6.	18	18
	7.	19	9
	8.	2,9	7
	9.	26	19
	10.	2	-
MO	1.	16	25
	2.	5	24
	3.	120	14
	4.	16	14
	5.	24	49
	6.	50	30
	7.	17	20
	8.	18	23
	9.	30	24
	10.	13	-

*The results are compared with some general values for heavy metals in volcanic rock (vs. sediments) according to some references (187,188).

Legend:

1. Prelesje – left bank
2. Prelesje – the bottom
3. Prelesje – right bank
4. Mrzlek – left bank
5. Mrzlek – the bottom
6. Mrzlek – right bank
7. the dam – left bank
8. the dam – the bottom
9. the dam – right bank
10. Deskle – the bottom

MO – mineral oil

Certain heavy metals can be detected in the sediment from the river Soča. Also due to the century-long mining of mercury and lead upstream in the area of the river Soča.