Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Peter Firbas, Tomaž Amon (2021) Use of chemical, fish micronuclei, and onion chromosome damage analysis, to assess the quality of urban wastewater treatment and water of the Kamniška Bistrica river (Slovenia). *Caryologia* 74(3): 119-139. doi: 10.36253/caryologia-1177

Received: January 04, 2021

Accepted: September 24, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

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Use of chemical, fish micronuclei, and onion chromosome damage analysis, to assess the quality of urban wastewater treatment and water of the Kamniška Bistrica river (Slovenia)

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Abstract. The aim of this study was to evaluate the cytotoxic and genotoxic potential of the wastewater (WW), the effectiveness of the treatment used by the wastewater treatment plant (WWTP) with sequential batch reactors (SBR) technology, and whether its final treated effluent (FTE) can compromise the water quality of the river at the location where it is discharged. We focused our research on six examples. For analytical chemistry and *Allium* metaphase (M) test all six samples were collected. Of these, three are so-called biotechnological patterns (WW, WW after mechanical step treatment and FTE), and three are natural river environmental patterns. For the micronucleus (MN) test, fish specimens were collected from three sites in the river Kamniška Bistrica. The first two sites locations are up and down the FTE outlet. Results from these areas were compared to the third site (not polluted) reference site, the so-called natural negative control sector Drinov rob. Complementary study with analytical chemistry and biological tests shows that the treatment effect SBR in the Domžale-Kamnik central WWTP carried effectively proved to be efficient for the removal of the cytogenotoxic substances in treated effluent and consequently in aquatic environment. The upgraded and improved Domžale-Kamnik central WWTP has a very effective aerobic tertiary treatment stage. Biological rate of achievement by such SBR technology has shown excellent results. We could not find any parameters than would show the final treatment effluent (FTE) water to exceed the maximum permissible doses (MPDs).

Keywords: Chemical analysis, phytotoxicity, genotoxicity level, environmental monitoring, water quality.

INTRODUCTION

The wastewater (WW) level is increasing with intensive anthropogenic activities like industrial, agricultural and urban development (Kalia et al. 2018). WW can be characterized as complex chemical mixtures of individual organic and inorganic substances (Fijalkowski et al. 2017). All such

chemicals can be potential sources of pollution and may result in different ecotoxicological effects (Lyubenova et al. 2012), that can affect human health (Pecol 2018). However well constructed and well working wastewater treatment plant (WWTP) can solve these problems. Even better solution is if we combine this with various techniques like ecoremediations, such as constructed wetlands (Firbas and Amon 2013), processes in ecological drainage ditches (Kumwimba et al. 2017), vermicomposting process the collective action of earthworms and microbes (Bhat et al. 2018), associated with stabilization pond (Hara and Marin-Morales 2017), and the most various attractive coremediation technologies.

Just physical and chemical measurements cannot tell us the whole picture of the degree of the toxicity of the pollutant (Firbas 2015; Firbas and Amon 2017). The concentration of the toxic agent is not always proportional to the danger to human health. Environmental monitoring detected potential environmental genotoxic xenobiotics and is based on the use of vascular (higher) plants and aquatic vertebrates (Grisolia et al. 2005; Oriaku et al. 2011). Both onion plant and fish are sensitive indicators for assessment of environmental pollution (Pathiratne et al. 2015; Batista et al. 2016; Hemachandra and Pathiratne 2017).

Aquatic vertebrates accept foreign substances, e.g. xenobiotics through the epithelium, which accumulate in the body and may induce various potential toxic effects (Walker et al. 2012). These xenobiotics produce multiple consequences at organism, population, community and ecosystem level, affecting organ function, reproductive status, population size, species survival, and thus biodiversity (Bolognesi and Hayashi 2011). In order to monitor the health of aquatic organism, biomarkers have been used as effective tools in environmental risk assessment (Ghisi et al. 2016). The micronucleus (MN) assay is one of the most widely used genotoxicity biomarkers in aquatics organisms, and is used for *in situ* monitoring of water ecosystems assessing clastogenic and aneugenic events in different cell types (Al-Sabti and Metcalfe 1995; Kirsch-Volders et al. 2011). MN are cytoplasmic chromatin-containing bodies formed when centric and/or acentric chromosome fragments or whole chromosomes that are not included in the main nuclei after cell division.

Onion *A. cepa* L. is widely recognized and useful as excellent genetic model to detect environmental xenobiotics (Leme and Marin-Morales 2009; Firbas 2011; Bakare et al. 2012;Karaismailoglu 2015; Verma et al. 2016; Karaismailoglu 2017; Bonciu et al. 2018; Makar et al. 2020, Şuţan et al. 2020). This plant can absorb xenobiotics across the leaf cuticle through stomatal and root epidermis. Root growth inhibition and adverse effects on chromosomes, for example chromosome break provide on indication of likely cytotoxicity and/or genotoxicity. (Dietrich et al. 2001; Bonciu et al. 2018). The relatively large (2n = 16) and karyotype diverse chromosomes are very appropriate for the detection of morphological changes. In vivo *Allium* M test (Firbas and Amon 2013; 2014) used for study of the specific morphology (structure) exclusively of metaphase chromatids and chromosome damage. Furthermore, the chromosome and chromatid morphology is easily altered by chemical and natural compounds. By using biological (genotoxic) tests, we can ascertain those responses of the tested onion plant *A.cepa*, which result in eventual damage to its genetic material (chromosomes) regardless of the tolerance limits that can be caused by various contamination samples within an environment. In regard to the universality of the living organisms genetic codes, the research results are transferable (applicable) to human beings (Nefic et al. 2013).

Several higher plant systems were evaluated bioassays with plant root meristematic cells such: *Coriandrum satuvum* L. (Pramanik et al. 2018), *Bidens laevis* L. (Pérez et al. 2011), *Drimia polyantha* (Blatt. &McCann) (Daphedar and Taranath 2018), *Lactuca sativa* L. (Moreira et al, 2020), *Helianthus annuus* (Karaismailoglu et al. 2013), *Elodea canadensis* (Zotina et al. 2015), *Nigella sativa* (El-Ghameryand Mousa 2017) and *Trigonella foenum - graecum* L. (Mahapatra et al. 2019) possessed suitable cytological characteristics for cytotoxicity and genotoxicity testing.

The measured biological effects of some water samples appear related to the physical characteristics. Therefore, genotoxicity assays should be included, along with conventional chemical analysis, in water quality monitoring programs (Radić et al. 2010; Singh et al. 2014; Etteieb et al. 2016). So many ecotoxicological studies focus on the assessment of physical and chemical environmental parameters and biological responses of organisms (Baldantoni et al. 2018; Wijeyarante and Wadasinghe 2019). The method of combining bioassays with the analytical chemistry and monitoring as well as screening represents a powerful approach to identify organic and inorganic pollutants - the main toxic components in complex mixtures of treated wastewater (Etteiab et al., 2016; Firbas and Amon, 2017).

In this paper we studied the genotoxicity level (GL) sources (Malakahmad et al. 2017) of the river Kamniška Bistrica - both upstream and downstream of the final treated effluent (FTE) of the waste water treating plant for the cities Domžale-Kamnik. The GL levels were determined in two ways. Firstly with the fish periph-

eral blood erythrocytes MN test in the nine freshwater autochthonous salmonid and cyprinid fish species and *Allium* metaphase (M) root type cell test and secondly with the *Allium* metaphase (M) root type cell test. This combination represents a good assessment of both physical (e.g. temperature, oxygen, pH, …) and chemical (pesticides, metals, …) parameters. The work described here has been done in the period 2017-2019 and is based on the data from the renovated Domžale-Kamnik central WWTP (it was renovated in 2016). However in our article (Firbas in Amon 2017) we monitor this WWTP from 2013-2014 that is before its renovation.

MATERIALS AND METHODS

Description of the water system of the river Kamniška Bistrica

The river Kamniška Bistrica (KB) is a left tributary of the river Sava, which is a right tributary to Danube river. It is 32.8 km long and has a torrential character from its spring in the Alps to its confluence with the Sava river. The river is spread over 535 km^2 of mountain and plain area (Figure 1). Because of the construction and housing activities the river has been regulated into a moderately narrow channel running through the towns Kamnik and Domžale. In other areas the river is mainly untouched.

Some sources mention that there were probably close to 200 km of canals excavated and buried again in different times in history (Vahtar 2006). Today, there are over 40 km of active mill streams along the river Kamniška Bistrica and most of them are still in use. The flow of the river varies from 2 to 10 m^3 per second. In one year the river receives 7 million m^3 treated water from the Domžale-Kamnik central WWTP (Hvala et al 2002).

Domžale-Kamnik central WWTP, Slovenia

Before the 2016 upgrade is central the Domžale-Kamnik central WWTP (latitude 46°07'N; longitude 14°36'E) is a conventional two-stage activated sludge plant (ASP) designed to remove organic matter from the wastewater, built in 1980 (Hvala et al. 2002). The capacity of the plant is 200,000 PE (Population Equivalent) with an average daily inflow of approximately 20.000 m^3/day. The plant influent consists of 45% municipal and 55% industrial wastewater (Hvala et al. 2002).

Upgrade in year 2016 include construction of a new aerobic biological rate of achievement of tertiary treatment by SBR technology and the construction of the input object for the reception of large quantities of waste water and appropriate mechanical pre-treatment, which will increase operational safety. After upgrading the WWTP fourth largest system for wastewater treatment in Slovenia, and ensuring the quality parameters of treated water for discharge into the watercourse, the river Kamniška Bistrica. The capacity of the upgraded

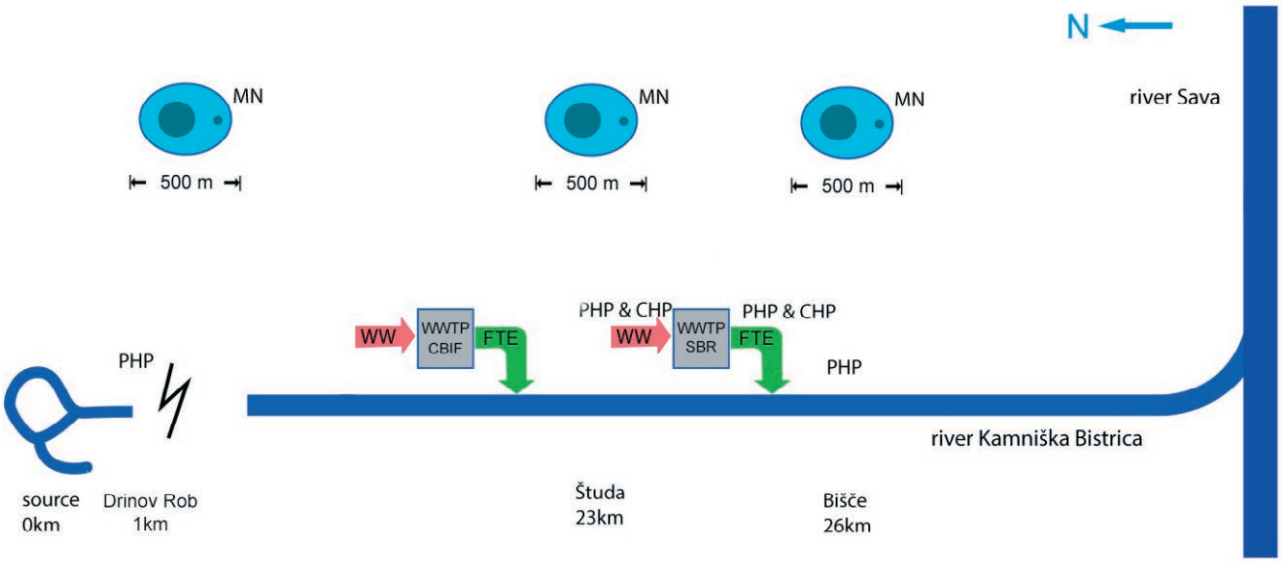

Figure 1. Study map of models WWTP including *Allium* M test and *Pisces* MN test. Samples and fishes were taken from river Kamniška Bistrica. Legends: CBIF: cart board industry factory with WWTP, WWTP: wastewater treatment plant; WW: wastewater; WWTW: wastewater treatment work; FTE: final treated effluent; PHP: physical-chemical parameter; CHP: chemical parameter.

WWTP is 149,000 PE, which means that it will accept the waste water of all residents in the reception area and other waste water (http://www.ccn-domzale.si/index.php/en/wastewater-treatment/plant-upgrade-project. Accesed 13. August 2020).

Collection of water samples

Water type definitions

Natural samples: river fresh water. The study area, which is Kamniška Bistrica river spatially lies between latitude 46°19'N and 46°04'N and longitude 14°34'E and 14°37'E (Figure 1).

Biotechnological samples: samples for all different stages of WW treatment. *Wastewater*: complex mixtures of municipal, industrial (pharmaceutical, textile, food processing, dyes-paints, timber industry, laundry textile), and rain water so called inflow. Inflow to Domžale-Kamnik central WWTP has a heterogeneous composition composed of municipal, industrial and rain water. *WW treatment after mechanical stage*: sand trapping and solid separation. *Final treated effluent*: treated water from Domžale-Kamnik central WWTP so called outflow.

Sampling locations in relation to the WWTP position

Sampling locations and biological tests according to the location of the WWTP are summarized according to Firbas and Amon (2017):

- 100 m or more upstream before the inlet into the WWTP (*Allium* M test, *Pisces* MN test),
- Influent waste water in the WWTP (*Allium* M test),
- Wastewater after solid separation (*Allium* M test),
- Effluent water from SBR (*Allium* M test),
- 100 m or more downstream after the outlet from the WWTP (*Allium* M test, *Pisces* MN test),

The detailed description of sampling locations (Figure 1)

I. Kamniška Bistrica – Drinov rob (as a negative control in the natural environmental pattern)
II. Kamniška Bistrica – village ŠTUDA (950 m upstream central WWTP – discharge point)
III. WW inflow
IV. WW after solid separation (WW treatment after mechanical stage)
V. FTE outflow from SBR
VI. Kamniška Bistrica – village BIŠČE (1750 m downstream central WWTP)

Terms of monitoringand sampling

First sampling: *Allium* M test and physical analytics 16. 02. 2017; electric fishing for *in situ* MN test 16. 02. 2017; sample WW and FTE is 24 h on average from 02/14/2017, 8:00 to 15/02/2017, 8:00.

Second sampling: *Allium* M test and physical analytics 18. 10. 2017; electric fishing for *in situ* MN test 24. 10. 2017; sample WW and FTE 24 h on average from 17/18 Oct.

Third sampling: *in vivo Allium* M test, electric fishing for *in situ* MN test and physical analytics 25. 05. 2018;sample WW and FTE is 24 h on average from24/25 May.

Fourth sampling: *in vivo Allium* M test, electric fishing *in situ* MN test and physical analysis13. 08. 2019; sample WW and FTE is 24 h on average from21/22 August.

Fifth sampling: *in vivo Allium* M test, electric fishing *in situ* MN test and physical analysis 23. 07. 2020; sample WW and FTE is 24 h on average from 22/23 July.

We sampled on three experimental sites. On each of those the samples were taken four times. We have done the measurements February 2017, in October 2017, in May 2018, and the fourth August 2019, and fifth (last) sampling was done in July 2020. Three experimental sites were chosen, namely area Študa and area Bišče, which were compared with sites Drinov rob of no sewage influence, as the control area (Figure 1).

Physical and chemical analyses

For the measuring of water physical and chemical analyses we collected six samples. Here three are natural river samples and three biotechnological samples. Samples of river water and biotechnological samples were collected from the sites, stored in bottles with thermostable boxes and transferred to the laboratory. Chemical parameters including: the metal/metalloid and organic component varied depending on the industrial profile. Physical parameters including: temperature, alkalinity (pH), electrical conductivity (EC), total suspended solid (TSS), dissolved oxygen (DO), nutrients (nitrogen, phosphor),Kjeldhal nitrogen (KN), chemical oxygen demand (COD) and biochemical oxygen demand (BOD_5)were determined in accordance with standard analytical methods (APHA, 2012).

Water physical parameters such as temperature, pH, EC, DO and TSS were measured *in situ* using HACH electrodes; TSS by (with) gravimetry; Nitrate N and nitrite N by Segmented Flow Analysis (SFA); Ammonia

by Ion selective electrode (ISE) or Spectrophotometry; and Kjeldahl N (with the composite method Digestion – Destillation – Titration). Total phosphorus, total nitrogen and COD were determined with spectrophotometry and BOD_5 with Volumetry (VOL) in incubation bottles.

Concentrations of metals (Zn, Al, Cu, Cd, Co, Cr, Ni, Ag, Pb and Fe) were analyzed using Inductively Coupled Plasma-Mass spectroscopy (ICP-MS). Benzene, toluene, ethylbenzene, xylene (BTEX) were analyzed using Gass Chromatography (DB-5 60x0.53x3 columns) – Flame Ionizator Detector(GC-FID).Absorber organic halogens (AOX) and cyanide were analyzed using Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS). Chloroalcane (C_{10}-C_{13}) were analyzed using Gass Chromatography with Electron Capture Detector (GC-ECD). Nonylphenol and nonylphenol ethoxylate were analyzed using LC-MS/MS. Di(2-ethylhexil) phthalate (DEHP) were analyzed using GC (5-MS columns 30m x 0.25mm x 0.25µm) with Electron Capture Detector (ECD). Index mineral oil were analyzed using GC with Flame Ionization Detector (FID).

Test organisms used as bioindicators

Plant: Onion *A. cepa*, (Stuttgarter Riesen). Small onion bulbs of the same uniform size, weighing about 3 – 3.5 g, were denuded by removing the loose outer scales and scraped so that the root primordia were immersed into the different tested liquids.

Fish: Salmonids: grayling (*Thymallus thymallus*), brown trout (*Salmo trutta fario*); Cyprinids: european minnow (*Phoxinus phoxinus*), european bullhead (*Cottus gobio*), common bardel (*Barbus barbus*), mediteranean bardel (*Barbus meridionalis*), european chub (*Leuciscus cephalus*), perch (*Perca fluviatilis*), nase (*Hondrostoma nasus nasus*). Seven cyprinids and two salmonids fish species inhabiting European freshwater ecosystems, were evaluated for their as *in situ* pollution biomarkers using the micronucleus test in peripheral blood erythrocytes. As the indicators fish species were used because of their ecological significance. Fish species 150 - 200 g were collected from natural environment in the three locations.

Onion plant Allium M assay

The tests were done with the *Allium* M or *Allium* chromosome damage (CsD) test and show the degree of genotoxicity by observing the aberrations of the exclusively metaphase chromosomes of the plant *A. cepa* that are evoked by genotoxic substances in the polluted water (Firbas and Amon, 2013; 2014). Five onion bulbs are left to grow in the sample water for 72 hours. Then first the macroscopic morphological parameters are observed – the length of roots (showing the general toxicity), their shape, number, color and degree of malformation. The genotoxicity level (GL) on the microscopic observation is a general term referring to alternation to the gross structure or content of chromosome damage by exposure to toxic agents (Malakahmad et al. 2017). GL is defined by the percentage between all the metaphase cells and the cells with their chromosomes damaged. 200 random chosen metaphase cells (with well-recognized chromosomes) originate from the sample composed of ten root apex cells taken from five onion bulbs - two roots from each onion bulb (Firbas and Amon, 2013).

Chromosome preparations were set up from root meristems containing actively growing cell by the following method: developing root with bulbs were pretreated with 0.1 % aquatic solution of colchicine for 3 hours at 21 °C. After washing in distilled water for 20 min the terminal developing roots of 2 mm length were fixed for 1h in methanol:propionic acid mixture (3:1 or 1:1). Then they were macerated and stained in order to obtain a cellular suspension. This sample was stained with 0.5 % aceto-carmine for 4-5 minutes at 60 °C without hydrolysis, and squashed in aceto-carmine (Firbas and Al-Sabti, 1995). The optical microscope used in the investigation was the Olympus – BX 41 (Japan) with the photo system PM 10 SP, typical magnifications used were 400 X and anisole-immersion 1.000 X. Onion (*A. cepa*) has 16 (2n = 16) monocentric chromosomes. The possible aberrations seen at metaphase are: chromosome break, chromatide break and centromere break (Firbas and Amon 2014). The cell is called aberrant if at least one chromosome gets damaged. Sometimes 4 to 8, or even up to all 16 chromosomes in the chromosome set are damaged, with dicentric and ring chromosomes (Firbas 2015).

Electrofishing collected methods

Salmonid and cyprinid fish inhabiting European freshwater ecosystems were evaluated for their use as *in situ* pollution biomarkers using the micronucleus test in peripheral blood erythrocytes. Fish were collected with electrofishing (EF) which is a common professional survey method used to sample fish population to determine abundance, density, and species composition. When performed correctly, EF results in no permanent harm to fish, which are returned to their natural environment only two minutes after being caught.

Fish MN assay

For the MN assay, peripheral blood samples were obtained from the gill region. Blood was smeared immediately on clean grease free microscope slides, air dry for 6 hours and the fixed in absolute methanol for 18-20 minutes, the prepared slides were left to air dry at room temperature. The blood smears were stained with 5 % Giemsa in Sorenson buffer solution for 7 minutes. After washing with distilled water and left to air dry at room temperature, the slides are then ready for microscopy. Since Giemsa solution stains the nuclear material much darker than the cytoplasmic material, the MN were readily visible with anisol - 1.000 X next to the normal nuclei and a micronucleus of the erythrocyte cells. Erythrocytes, 10.000 ± 200 per specimen, were analyzed to determine the frequency of cells with one or two micronuclei and then calculated to the number of MN per 1000 erythrocytes (MN/1000 E).

Statistics calculation

Values are the mean of five replicates with standard deviation (±SD). Statistically established significant differences among the investigated samples are confirmed by the statistical calculation of paired data analysis using the two-way Fisher's exact test, which gives the *p*-value property between pairs of data calculated for a 2x2 contingency table (Agresti 1992). In the 2x2 frequency tables the statistical results as shown by the p-values sometimes do not show the significance, the addition of allium and micronucleus test clearly show the difference with other methods leads us to suggest that the picture could be more complex (Firbas and Amon 2013; 2017).

RESULTS

Chemical parameters

The chemical analysis of the FTE and maximum permissible concentrations (MPC) standard are presented in Table 1. We could not find any parameters that would show the FTE water worse in quality than the original river water. The FTE does not additionally burden the river Kamniška Bistrica.

Table 1.Chemical analysis of the final treated effluent (FTE) and maximum permissible concentrations (MPC) standard. The Domžale-Kamnik central WWTP with an upgrade of a new aerobic biological rate of achievement of tertiary treatment by SBR technology treating has been shown to be very effective.

Parameter and unit	MPC*	FTE
Zn/Zink (mg/l)	2.0	0.0414
Total cyanide (mg/l)	0.5	0.010
AOX/Absorber organic halogens (mg/l)	0.5	0.150
Chloralcanes - C_{10}-C_{13} (mg/l)	0.04	0.0035
Di(2-ethylhexil) phthalate (DEHP) (mg/l)	0.13	0.0004
Nonylphenol and nonylphenol ethoxylate (mg/l)	0.03	0.00023
Al/Aluminium (mg/l)	3.0	0.047
Cu/Cupper (mg/l)	0.5	0.010
Cd/Cadmium (mg/l)	0.025	0.001
Co/Cobalt (mg/l)	0.03	0.0010
Cr/Cromium Total (mg/l)	0.5	0.010
Ni/Nicel (mg/l)	0.5	0.010
Ag/Silver (mg/l)	0.1	0.010
Pb/Lead (mg/l)	0.5	0.010
Fe/Iron (mg/l)	2.0	0.150
Index minerals oils (mg/l)	5.0	0.100
BTX – Benzene, Tuolene, Xylene (mg/l)	0.1	0.03
Benzene (mg/l)	0.1	0.03
Toluene (mg/l)	0.1	0.03
Ethylbenzene (mg/l)	0.1	0.03
m,p- Xylene (mg/l)	0.1	0.03
o-Xylene (mg/l)	0.1	0.03

*MPC: maximum permissible concentration – Legistation (Official Leaf republic of Slovenia: 64/2012).

Physical parameters

In addition to standard parameters (COD, BOD_5, TSS) we included also the fundamental physical parameters nitrate (NO_3—N), nitrite (NO_2—N), Kjeldhal—N, ammonia (NH_4—N), phosphate (PO_4—P), electrical conductivity and pH. We measured the river Kamniška Bistrica at three locations from its location Drinov rob to the location called Bišče (Figure 1). It is this sector where the treatment water from the WWTP enters the river Kamniška Bistrica. This WWTP reduces pollutants to a level that nature can successfully process further. Detailed analysis of water physical parameters here are presented in Table 2, 3, 4, 5 and 6.

General toxicity - phytotoxicity

The results of the general toxicity are shown in combined Tables 2, 3, 4, 5, 6 and Figure 2. The location Drinov rob is taken as the negative control. General toxicity of the river samples they have more or less the same root length of the test plants ($p > 0.05$). All three of the river water samples shows longer roots and lesser general toxicity than the WW or FTE ($p < 0.05$). The FTE show longer roots and lesser general toxicity than the untreated wastewater ($p < 0.01$).

Table 2. United parameters physical quantities;cytological effects of the investigated samples survey – the genotoxicity level (damage to chromosomes) and general toxicity - phytotoxicity (root length inhibition) on the test onion plant *A. cepa* and *Pisces*micronuclei (MNi)frequencies in peripheral blood erythrocytes of river fish. First sampling February 2017

Parameter	Unit	Sample sites					
		K2 – Drinov rob (Negative control)	K4 – Študa	WW – WWTP	After mechanical step	FTE – WWTP	K4 – Bišče
Physical analysis							
Water temperature	^{0}C	4.9	6.1	9.4	-	10.1	7.5
pH	-	8.2	8.0	7.6	7.7	7.6	6.7
Electrical conductivity	µS/cm	226	372	-	-	-	470
Dissolved oxygen	mg/l	11.78	10.38	-	-	-	10.98
To subside 1 h	ml/l	0	0	-	-	0	0
To subside 2 h	ml/l	0	0	14	3	0.1	0
TSS - 1µm	mg/l	2	4	236	167	13.4	3
Ammonia NH_4—N	mg/l	-	-	33	53	29	-
Kjeldahl N	mg/l	2.5	2.5	50	71	32	2.7
Nitrate NO_3—N	mg/l	0.8	1.3	2.2	-	7.4	2.0
Nitrite NO_2—N	mg/l	-	-	0.75	-	0.50	-
Total N	mg/l	-	-	34.0	-	6.3	-
Total P	mg/l	0.5	0.5	3,67	7.9	0.38	-
COD	mg/l	5.0	6.0	551	595	33	7.4
BOD_5	mg/l	3.0	3.0	347	240	5	3.0
***Allium* metaphase (M) test**							
Phytotoxicity	mm	34±2.7	33±1.5	11.0±1.2	11.0±1.1	31±1.7	34±2.4
Genotoxicity	%	2.50±0.3	4.50±0.5	34.50±2.6	21.50±1.3	11.0±0.9	4.50±0.5
Micronucleus (MN) *Pisces* test							
Leuciscus cephalus	‰	-	0.90 ±0.33				0.98 ±0.61
Thymallus thymallus	‰	-	1.29 ±0.35				0.82 ±0.34
Phoxinus phoxinus	‰	-	0.78 ±0.45				0.74 ± 0,31
Salmo trutta fario	‰	0.19 ±0.02	2.00 ±0.65				-
Cottus gobio	‰	0.44 ±0.11	4.50 ±1,50				1.36 ± 0.77

Legend. KB: Kamniška Bistricariver, K2, K4: fishing area, WW: wastewater, FTE: final treated effluent, WWTP: Central Domžale-Kamnik Wastewater Treatment Plant, %: chromosome damage (CsD) per 100 cells, GL: Genotoxicity level, ‰:micronucleiper 1000 erythrocytes.

Genotoxicity level

The results of the genotoxicity level (GL) expressed in percentage points (%), are shown in the combined Tables 2 to 6. The treated outflowing water is significantly less genotoxic than the inflowing (polluted) water. So the GL decreased from 34.5 % lowers on 11 % in the year 2017 ($p = 6.0^{-8} < 0.00001$)._The wastewater first undergoes the mechanical treatment. Afterwards the genotoxic level is significantly lower (shown by less damaged chromosomes) while the cytotoxic level remains the same (the lengths of tested *Allium* bulbs roots are not significantly changed). The Kamniška Bistrica river in K2 sector (Drinov rob) shows zero (Figure 3) or not more than one damaged chromosome in a chromosome set (Figure 4). Incoming wastewater is highly genotoxicity. Typically at least 4-10 chromosomes (Figure 5), sometimes even all chromosome get damaged (Figure 6). Also dicentric and ring chromosomes can appear. The treated wastewater from the Domžale-Kamnik central WWTP and Kamniška Bistrica river in K4 sector (Študa and Bišče) shows a much lesser degree of genotoxicity – typically two chromosomes, rarely four are damaged.

Micronucleus (MN)

The results of the MN studies are shown in Tables 2 to 5. Peripheral blood erythrocytes with MN are shown in Figure 7. Three experimental sites were chosen, namely, Drinov rob, Študa and Bišče. Sampling was carried out in February 2017, October 2017, May 2018, and

Table 3. United parameters physical quantities;cytological effects of the investigated samples survey – the genotoxicity level (damage to chromosomes) and general toxicity - phytotoxicity (root length inhibition) on the test onion plant *A. cepa* and *Pisces* micronuclei (MNi) frequencies in peripheral blood erythrocytes of river fish. Second sampling October 2017

Parameter	Unit	Sample sites					
		K2 – Drinov rob (Negative control)	K4 – Študa	WW – WWTP	After mechanical step	FTE – WWTP	K4 – Bišče
Physical analysis							
Water temperature	^{0}C	7.0	12.2	16.2	-	17.5	12.7
pH	-	8.2	8.1	7.6	7.6	7.1	7.9
Electrical conductivity	µS/cm	218	415	-	1301	-	461
Dissolved oxygen	mg/l	11.39	11.68	-	-	-	10.99
To subside 1 h	ml/l	0	0	-	-	0	0
To subside 2 h	ml/l	0	0	13	1,9	0.05	0
TSS - 1µm	mg/l	< 2	< 2	380	177	5.0	2
Ammonia NH_4—N	mg/l	0.05	< 0.015	31.90	38	0.33	< 0.015
Kjeldahl N	mg/l	-	-	24.40	55.1	2.29	-
Nitrate NO_3—N	mg/l	0.79	1.1	0.70	-	4.99	2,4
Nitrite NO_2—N	mg/l	-	-	0.32	-	0.13	-
Total N	mg/l	-	-	47	-	6.2	-
Total P	mg/l	< 0.05	< 0.05	7.00	7.5	0.28	< 0.05
COD	mg/l	< 5.0	7.3	638	599	26.1	< 5.0
BOD_5	mg/l	< 3.0	< 3.0	240	-	6	< 3.0
***Allium* metaphase (M) test**							
Phytotoxicity	mm	34.0 ±2.9	33.0 ±2.2	11.0 ±1.2	13.0 ±1.4	31.0 ±2.9	34.0 ±2.7
Genotoxicity	%	2.50 ±1.1	4.50 ±1.3	34.50 ±2.1	19.50 ±1.3	9.0 ±1.3	4.50 ±1.1
Micronucleus (MN) *Pisces* test							
Leuciscus cephalus	‰	-	4.31 ±1.55				2.98 ±0.51
Thymallus thymallus	‰	-	1.77 ±0.23				0.7 ±0,21
Chondrostoma nasus	‰	-	1.00 ±0.20				1.36 ±0.34
Salmo trutta fario	‰	0.20 ±0.1	-				-
Cottus gobio	‰	0.32 ±0.1	2.34 ±0.39				-

Legend. KB: Kamniška Bistricariver, K2, K4: fishing area, WW: wastewater, FTE: final treated effluent, WWTP: Central Domžale-Kamnik Wastewater Treatment Plant, %: chromosome damage (CsD) per 100 cells, GL: Genotoxicity level, ‰:micronuclei per 1000 erythrocytes.

August 2019. All fishes, regardless of species composition, show lower values (0.32-1.1 MN/1000 erythrocytes (E) in the Bišče sector than in the Študa sector (0.78-2.01 MN/1000 E), which is the most relevant for the species of European bullhead (*Cottus gobio*). The lowest MN abundance (0.0-0.2-0.34 MN/1000 E) is in the sector Drinov rob. The increased appearances of micronuclei in fish blood erythrocytes shows that the fish is living in water of higher genotoxic level. A significant increase in the number of MN in specimens of *Cottus gobio* at the Kamniška Bistrica river is the result of the discharge of the waste water of the Cart board industry factory (CBIF) in the sector Študa. We have monitored the frequencies of MN from 2017 to 2020 and saw that the frequency falls from year to year what points to cleaner water.

From the results of these researches we conclude that the FTE from the Domžale-Kamnik central WWTP does not adversely affect the quality watercourse of the Kamniška Bistrica river. Kamniška Bistrica is already partially contaminated above the outflow (the Študa sector) since the measured values are 2 to 5 times higher than in the sector Drinov rob.

Measurements of the genotoxicity level with *Allium* M test and physical parameters that support biological parameter implement a good basis for the risk assessment studies and Environmental Quality Standard – Ecological Status (EQS-ES) (Firbas 2015; Firbas and Amon 2017). In this article, we add the results of the *Pisces* MN tests (Table 7).

Table 4. United parameters physical quantities;cytological effects of the investigated samples survey – the genotoxicity level (damage to chromosomes) and general toxicity - phytotoxicity (root length inhibition) on the test onion plant *A. cepa* and *Pisces*micronuclei (MNi) frequencies in peripheral blood erythrocytes of river fish. Third sampling may 2018.

Parameter	Unit	Sample sites					
		K2 – Drinov rob (Negative control)	K4 – Študa	WW – WWTP	After mechanical step	FTE – WWTP	K4 – Bišče
Physical analysis							
Water temperature	^{0}C	7.8	12,1	15.7	-	17.8	14.5
pH	-	8.2	8.08	8.2	-	7.2	7.8
Electrical conductivity	µS/cm	193	287	-	-	-	338
Dissolved oxygen	mg/l	11.53	10.66	-	-	-	9.92
To subside 1 h	ml/l	0	0.1	-	-	-	< 0.1
To subside 2 h	ml/l	0	0.2	15	-	0	< 0.1
TSS - 1µm	mg/l	-	14.3	200	-	-	37.6
Ammonia NH_4—N	mg/l	-	0.04	31	39	1.34	0.016
Kjeldahl N	mg/l	(< 2.5) 0.28	(< 2.5) 0.37	-	54.2	-	(< 2.5) 0.59
Nitrate NO_3—N	mg/l	0.7	0.97	-	-	-	1.48
Nitrite NO_2—N	mg/l	-	-	-	-	-	-
Total N	mg/l	< 0.05	0.97	42	-	5.6	0,094
Total P	mg/l	-	-	6.95	7.0	0.603	-
COD	mg/l	< 5	7.6	445	467	47	10.8
BOD_5	mg/l	< 3	< 3	240	295	5.0	< 3
***Allium* metaphase (M) test**							
Phytotoxicity	mm	36 ±3.1	34 ±2.8	11.0 ±1.2	12.0 ±1.1	33 ±2.3	34 ±2.5
Genotoxicity	%	2.90 ±1.2	5.50 ±1.3	28.0 ±2.2	12.30 ±1.1	7.0 ±1.2	4.50 ±1.2
Micronucleus (MN) *Pisces* test							
Leuciscus cephalus	‰	-	-				0.81 ±0.23
Perca fluviatilis	‰	-	-				0.78 ±0.19
Barbus meridionalis	‰	-	1.78 ±0.45				1.1 ±0.11
Barbus barbus	‰	-	0.75 ±0.21				-
Salmo trutta fario	‰	0.15 ±0.10	0.82 ±0.19				-
Cottus gobio	‰	-	0.95 ±0.19				0.32 ±0.21

Legend. KB: Kamniška Bistricariver, K2, K4: fishing area, WW: wastewater, FTE: final treated effluent, WWTP: Central Domžale-Kamnik Wastewater Treatment Plant, %: chromosome damage (CsD) per 100 cells, GL: Genotoxicity level, ‰:micronuclei per 1000 erythrocytes.

DISCUSSION

The present work has been done in order to evaluate the genotoxic effects of WW and FTE on different sites in river Kamniška Bistrica using the physical and chemical analysis on *Allium* M and *Pisces* MN assays. WW treatment is an important process of considerable significance for the environment. In the eco-genotoxicology the samples are subjected to the physical and chemical analysis as well as to the genotoxical tests (Radić et al. 2010; Matsumoto et al. 2006; Grisolia et al. 2009; Okonkwo et al. 2011; Bakare et al. 2012; Polard et al. 2011; Akpoilih 2012; Firbas and Amon 2017; Francisco et al. 2019; Kaur et al. 2020). These two methods are complementary. As the water leaves the WWTP it flows into the river and again becomes the integral part of the ecosystem (Walia et al. 2013). Our results show how important is the effectiveness of this WWTP along with the continual monitoring including the study of all necessary parameters (Bolognesi and Hayashi 2011; Radić et al. 2010; Raisuddin and Jha 2004; Herrero et al. 2012; Tabres et al. 2011; Bagatini et al. 2009; Galindo and Moreira 2009).

Domžale-Kamnik central WWTP after the upgrade in 2016 represents the superb state of the technology of modern wastewater treatment in the world and high-quality clean waste water and achieves a high cleaning effect. The modernization also included additional system comprises three main process blocks: (i) a new inlet that complements the existing mechanical stage,

Table 5. United parameters physical quantities;cytological effects of the investigated samples survey – the genotoxicity level (damage to chromosomes) and general toxicity - phytotoxicity (root length inhibition) on the test onion plant *A. cepa* and *Pisces* micronuclei (MNi) frequencies in peripheral blood erythrocytes of river fish. Fourth sampling august 2019

Parameter	Unit	Samples sites					
		K2 – Drinov rob (Negative control)	K4 – Študa	WW – WWTP	After mechanical step	FTE – WWTP	K4 – Bišče
Physical analysis							
Water temperature	^{0}C	8.5	19.1	19.3/19.6*	-	21.4/21.6*	15.9
pH	-	8.2	8.2	8.0/8.2*	7.7	7.2/7.3*	7.6
Electrical conductivity	µS/cm	211	413	-	-	-	499
Dissolved oxygen	mg/l	11.85	9.15	-	-	-	8.69
To subside 1 h	ml/l	0	0	-	-	-	0
To subside 2 h	ml/l	0	0	13	0,7	< 0.05	0
TSS - 1µm	mg/l	2	4,1	290	72.5	< 2	18.3
Ammonia NH_4—N	mg/l	0.015	0.032	29.6	26.8	< 0.3	0.13
Kjeldahl N	mg/l	-	-	-	-	-	-
Nitrate NO_3—N	mg/l	0.71	1.1	-	-	-	2.7
Nitrite NO_2—N	mg/l	-	-	-	-	-	-
Total N	mg/l	-	-	6.48	-	0.84	-
Total P	mg/l	0.05	0.05	50	5.4	7.7	0,06
COD	mg/l	5	12.1	575	243	< 30	7.8
BOD_5	mg/l	-	-	280	-	< 5	-
***Allium* metaphase (M) test**							
Phytotoxicity	mm	36 ±3.1	34 ±3.0	12 ±0.9	12 ±0.7	34 ±2.5	36 ±2.7
Genotoxicity	%	3.0 ±1.3	5.50 ±1.2	29.0 ±1.9	11.0 ±1.1	6.50 ±1.3	4.50 ±1.2
Micronucleus (MN) *Pisces* test							
Leuciscus cephalus	‰	-	0.75 ±0.31				0.50 ±0.23
Thymallus thymallus	‰	-	1,21 ±0.22				1.14 ±0.19
Phoxinus phoxinus	‰	-	0.85 ±0.11				-
Barbus meridionalis	‰	-	1.06 ±0.29				-
Salmo trutta fario	‰	1.22 ±0.10	-				-
Cottus gobio	‰	0.31 ±0.17	0.96 ±0.21				1.52 ±0.27

Legend. KB: Kamniška Bistricariver, K2, K4: fishing area, WW: wastewater, FTE: final treated effluent, WWTP: Central Domžale-Kamnik Wastewater Treatment Plant, %: chromosome damage (CsD) per 100 cells, GL: Genotoxicity level, ‰:micronuclei per 1000 erythrocytes.

(ii) a new aerobic biological stage with advanced SBR technology with anaerobic selector for partial biological phosphorus removal and a new de-ionization process, (iii) the existing anaerobic biological stage with the production of biogas and cogeneration (http://www.ccn-domzale.si/index.php/en/wastewater-treatment/plant-upgrade-project).

As the environmental discharge standards are getting more advanced, the traditional (continuous flow-based) WW treatment process faces severe challenges. It has become inevitable to include tertiary treatment units for nutrient removal from WW. SBRs due to its operational flexibility and excellent process control possibility are being extensively user for the treatment of WW which nowadays is fast becoming contaminated with newer and more complex pollutants (Dutta and Sarker, 2015). Some WWTP with SBR may use additional step such as nitrogen or phosphorous removal as well as biological nutrient removal. This third and last step in the basic wastewater management system is mainly comprised of removing phosphates and nitrates (Saito et al. 2004). Nitrogen and phosphorous have become the key factors leading to eutrophication of receiving water. While achieving simultaneous nitrogen and phosphorous removal, biological methods play an important role in treating municipal and/or industrial wastewater such as SBRs (Jungles et al. 2014).

The character of WWs effluents varies greatly, dependent on the nature of the specific industry involved, both in terms of the likely BOD_5 loading of

Table 6. United parameters physical quantities; cytological effects of the investigated samples survey – the genotoxicity level (damage to chromosomes) and general toxicity - phytotoxicity (root length inhibition) on the test onion plant *A. cepa* and *Pisces* micronuclei (MNi) frequencies in peripheral blood erythrocytes of river fish. Fifth sampling July 2020

Parameter	Unit	Sample sites					
		K2 – Drinov rob (Negative control)	K4 – Študa	WW – WWTP	After mechanical step	FTE – WWTP	K4 – Bišče
Physical analysis							
Water temperature	^{0}C	8,3	15.0				15.0
pH	-	7,9	8,2	7,5	7,7	7,8	7,7
Electrical conductivity	µS/cm	210	362	812	908	871	438
Dissolved oxygen	mg/l	11,02	9,3				9,07
To subside 1 h	ml/l	0	0	16	1,2	0	0
To subside 2 h	ml/l	0	0	15	1,5	0	0
TSS - 1µm^2	mg/l						
Ammonia NH_4—N	mg/l	0,1	0,06	19,7	10,6	0,3	0,06
Kjeldahl N	mg/l	<2,5	2,5	37,9	30,1	2,5	2,5
Nitrate NO_3—N	mg/l	0,58	0,94	0,36	0,34	6,06	1,8
Nitrite NO_2—N	mg/l						
Total N	mg/l						
Total P	mg/l	0,026	0,075	6,6	3,9	0,97	0,12
COD	mg/l	<5	11,1	534	125	22,2	9,1
BOD_5	mg/l	<3	<3	255	85	4,5	<3
***Allium* metaphase (M) test**							
Phytotoxicity	mm	39	34	12	13	35	38
Genotoxicity	%	2,5	6,50	30,0	10,5	6,50	4,50
Micronucleus (MN) *Pisces* test							
Cottus gobio	‰	-	0.9±0.33				0,7±0.24
Leuciscus cephalus	‰	-	0,7±0.22				-
Phoxinus phoxinus	‰	-	-				0,5±0.13
Barbus meridionalis	‰	-	1,1±0.35				-
Thymallus thymallus	‰	-	0,5±0.12				-
Salmo trutta fario	‰	0,3±0.11	-				-

Legend. KB: Kamniška Bistricariver, K2, K4: fishing area, WW: wastewater, FTE: final treated effluent, WWTP: Central Domžale-Kamnik Wastewater Treatment Plant, %: chromosome damage (CsD) per 100 cells, GL: Genotoxicity level, ‰:micronuclei per 1000 erythrocytes.

any organic components and the type of additional contaminants which may also be present. Accordingly, the chemical industry may offer WWs with high COD and rich various toxic compounds is another high BOD_5 that effluent contains (Evans and Furlong 2011). Parameters of the COD, BOD_5 and TSS properties are the key parameters for the standardized monitoring of the cleaning process in the WWTP and at the same time they show the how much the environmental picture gets modified after mixing with the effluent of the WWTP. The correlation between COD, BOD_5 and TSS properties is linearly proportional to the results obtained from the genotoxicity tests (Firbas and Amon 2013).

Test systems need to be developed on the basis of criteria that allow a realistic assessment of GL and are of major ecological importance in environmental screening and monitoring at the cell, organism, population and ecosystem levels. Currently, clastogenic and/or aneugenic bio-marker so called MN and CsD are most frequently and trustworthy for genotoxicity testing in aquatic environments. Many toxic and potentially toxic chemical substances, some of natural origin and others due to human activities, are released into the environment daily (Obiakor et al. 2012). It has been shown that the chemical analysis alone is not enough to assure that the effluent water is really clean. To protect human and ecosystem health, it is necessary to perform also the biological analysis and to develop sensitive assays and to identify responsive cells and species and their life stages (Raisuddin and Jha 2004).

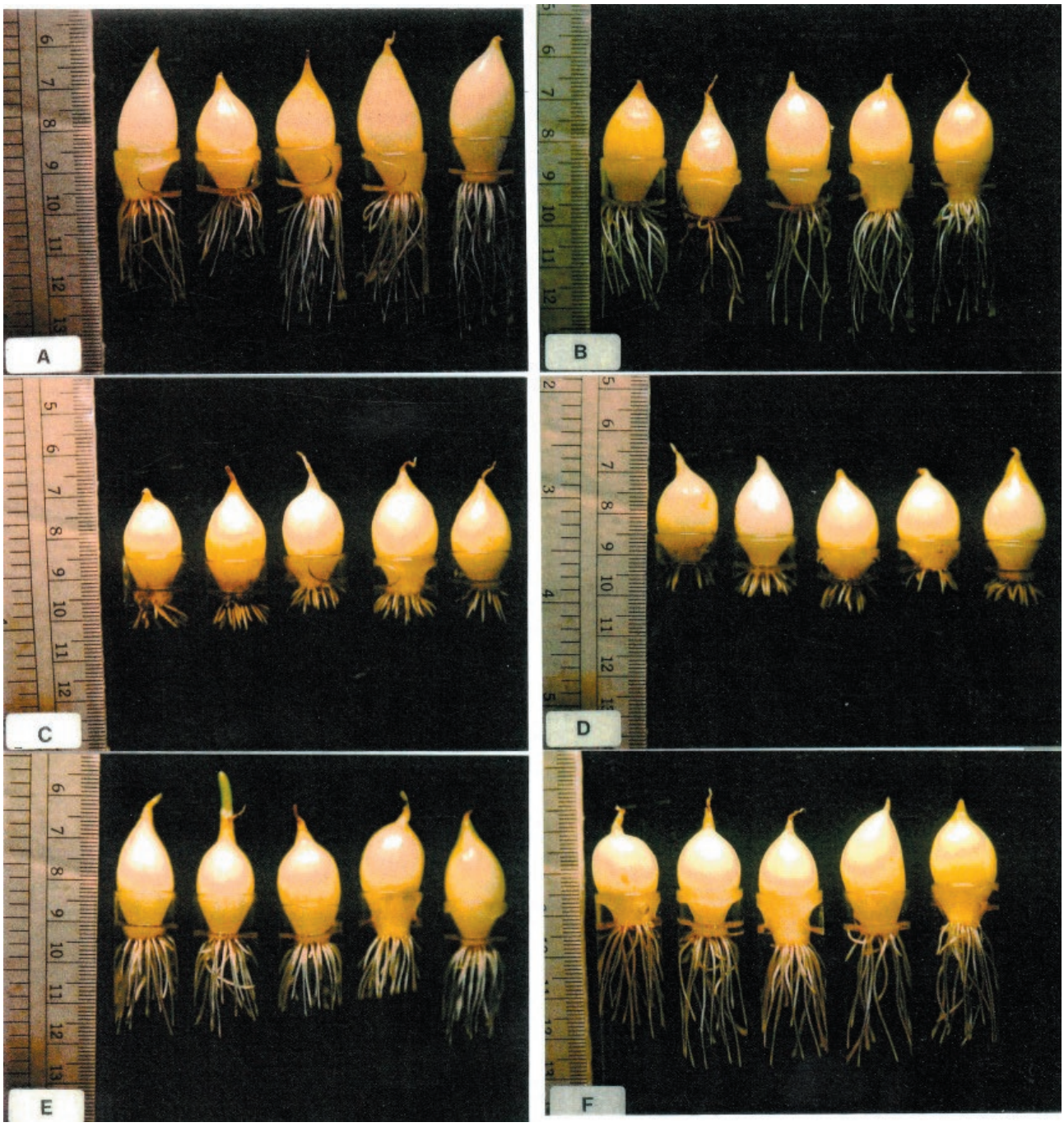

Figure 2. Examples of series of onions cultivated 72 h in different biotechnological and environmental river samples. A) Drinov rob, B) Študa, C) wastewater, D) WW after mechanical step, E) final treated effluent, F) Bišče.

The *in situ* quantification of MN fish erythrocytes has shown to be an adequate bio-marker in the evaluation of aquatic ecosystems quality (Al-Sabti and Metcalfe 1995; Minissi et al. 1996). Given the nucleated nature of erythrocytes in fish the MN test has gained high relevance in bio-monitoring of aquatic environments, also including assessment of water quality (Palacio-Betancur et al. 2009). Aquatic vertebrates and invertebrates are directly exposed to many pollutants dissolved or suspended in the surface water (Bolognesi and Hayashi 2011; Smital and Kurelec 1997; Bolognesi and Fenech 2012; Beršiene et al. 2012; Walker et al. 2012). Salmonids

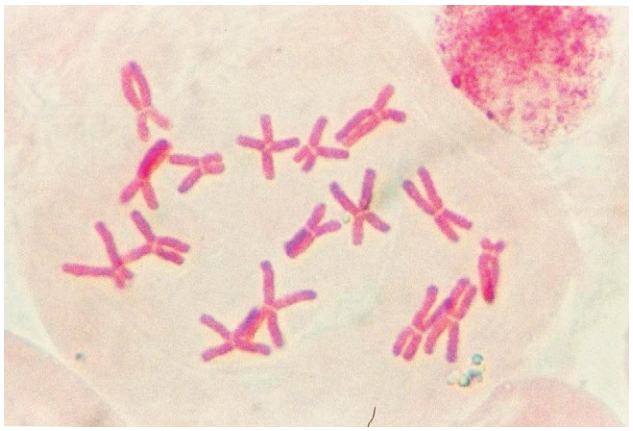

Figure 3. Diploid monocentric metaphase chromosome from the root meristem cells of the onion (*Allium cepa* L.), containing 2n of 16, with basic chromosome number x=8 (2n=16).

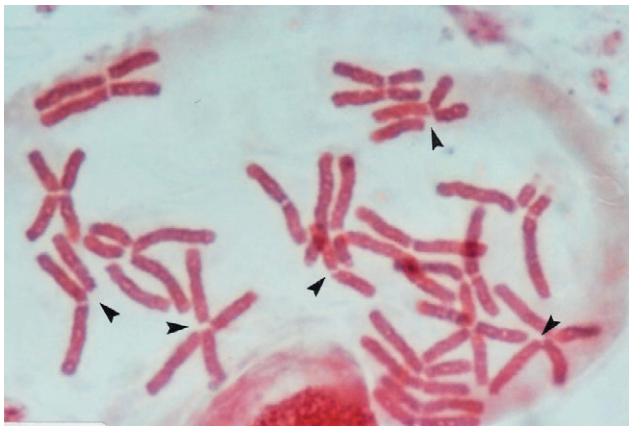

Figure 5. Different number chromosome damage in metaphase cells obtained from the meristem root-type cells of onion (*A. cepa* L.).

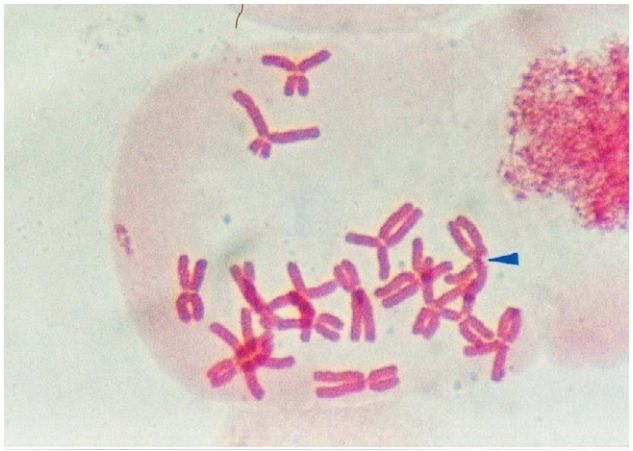

Figure 4. One chromosome is damaged.

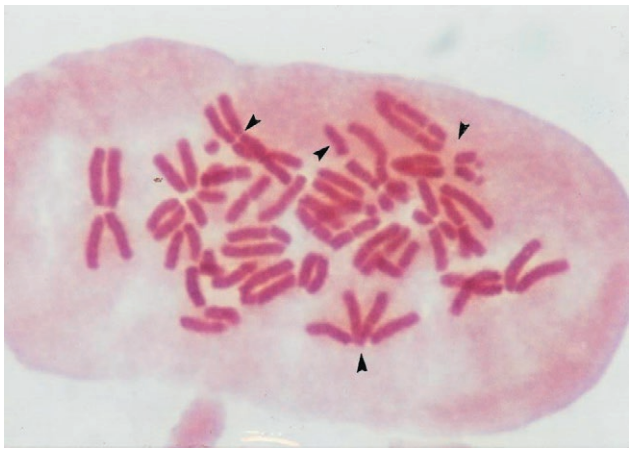

Figure 6. Whole chromosome set is damaged.

T. thymallus, *S. trutta fario*, and cyprinids*P. phoxinus*, *C. gobio*, *B. barbus*, *B. meridionalis*, *L. cephalus*, *P. fluviatilis*, *H. nasus nasus* that are inhabiting European freshwater ecosystem were evaluated for their use as *in situ* using the micronucleus test (Rodriguez-Cea et al. 2003; Minissi et al. 1996). *In situ* surveys of wild freshwater ecosystems with different levels of pollution showed that cyprinids fish in moderately pollution sites do not present higher micronuclei averages than those caught in clean rivers system, whereas micronuclei are induced by *Thymallus thymallus*, *Salmo trutta fario* and *Cottus gobio* inhabiting moderately polluted sites. Our results demonstrated the suitability of *Cottus gobio* for *in situ* monitoring of freshwater ecosystems using the *Pisces* MN test.

Some researchers have reported the sensitivity of this species, including in the detection of genotoxicity effects: Frequency of micronucleated erythrocytes (E) in blood of *Phoxinus phoxinus* in the laboratory conditions and in environment is 0.3-0.7 MN/1000 E (Bolognesi and Hayashi M 2011; Ayllon and Garcia-Vaszquez 2000). In blood of *Leuciscus cephalus* that lives in partially contaminated river waters one finds levels of micronucleated erytrocytes 0.7-2.9 MN/1000 E (Piccoli et al. 2010). The low to high frequency (0.5-5 MN /1000 E) MN in the erythrocytes (E) is known for *Chondrostoma nasus i*n an uncontaminated environment 0.5 MN/1000 E and 4 MN/1000 E in a contaminated environment (Koca and Koca 2008). The *Barbus barbus* also has 0.5 MN/1000 E in uncontaminated environment and 3 MN/1000 E in a contaminated environment (Boettcher et al 2010). The frequency of MN in the erythrocytes of *Salmo truta fario* specimens was increased after exposure to a con-

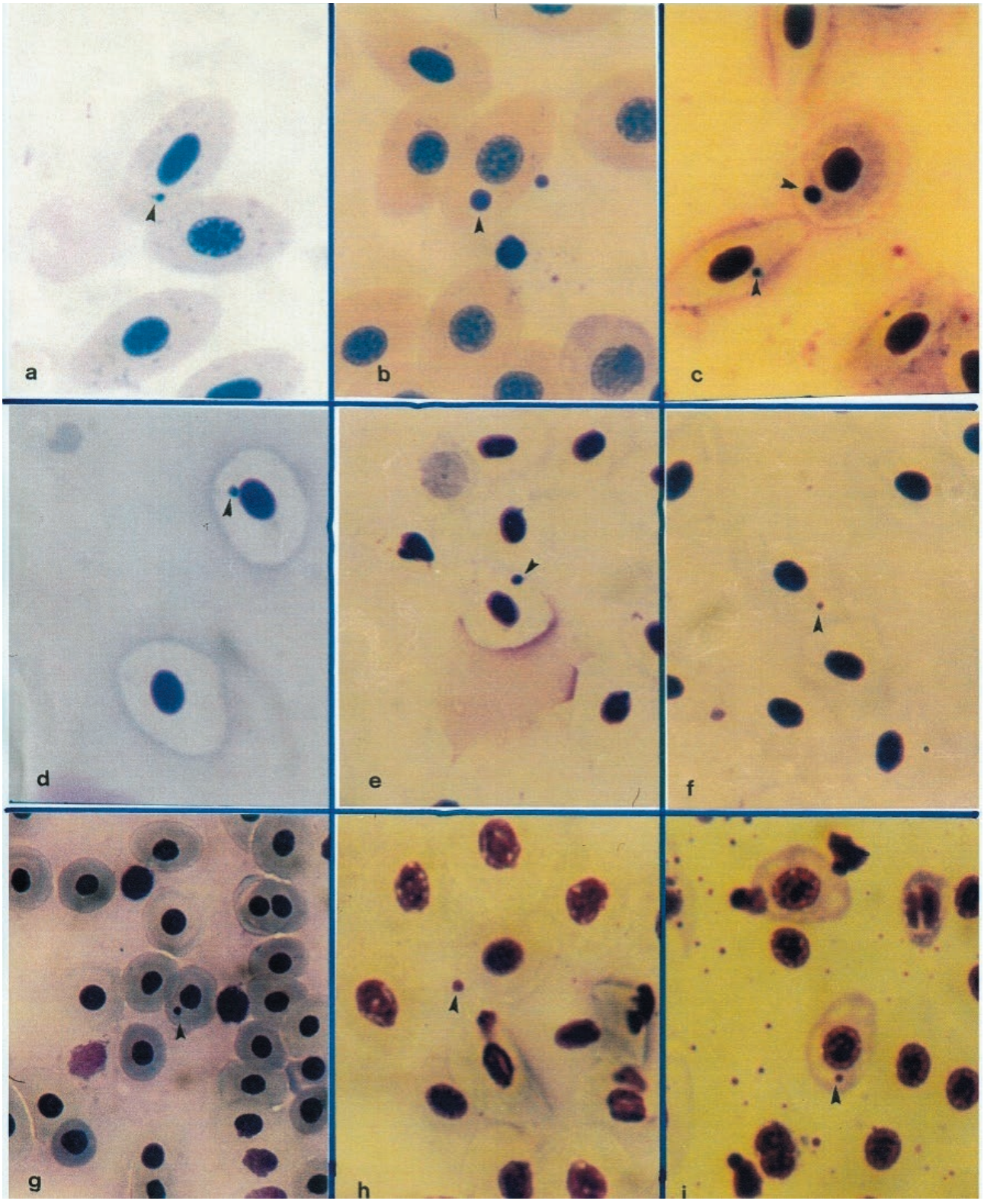

Figure 7. Normal mononucleated erythrocytes, and micronucleated erythrocytes in peripheral blood.a) *Salmo trutta fario,* b) *Thymallus thymallus*, c) *Barbus barbus*, d) *Barbus meridionalis*, e) *Phoxinus phoxinus*, f) *Leuciscus cephalus*, g) *Cottus gobio*, h) *Hondrostoma nasus*, i) *Perca fluviatilis*.

Table 7.Correlation between chromosome damage (CsD) of the *Allium* M test and *Pisces* MN test of the genotoxicitylevel (GL) with parallel physical parameters (BOD_5, NO_3 – N). The relationship is directly related to the Environmental Quality Standard-Ecological Status (EQS-ES) and environmental risk assessment (ERA).

MN *Pisces* MN test (υ MN/1000 E)	CsD *Allium* M test υ CsD /100 C	Level to endanger (Risk assessment)	Environmental samples	EQS-ES*	BOD_5 (O_2 mg/l)	NO_3 – N (mg/l)
-	< 2	Natural mutagenicity test organisms	High quality drinking water		> 0.5	> 1.5
0.09/0.20	2 -5	NO RISK	Spring (drinking) water I. Quality class rivers and lakes	WERY GOOD	1.6 – 2.4	3.2 – 7.0
0.30/0.50/1.26	4 – 10	LOW LOW/MIDLE	I.- II. Quality class rivers and lakes	GOOD	2.0 – 5.4	6.5 – 9.5
1.21/2.01/5.50	9 – 21	MIDLLE MIDLE/HIGH	II. Quality class rivers and lakes,	MODERATION	< 8.5	>9.6
-	22 – 39	HIGH	Wastewater (municipal waste) Treated wastewater, FTE	WEACLY	15 – 20	9 – 12
-	40 – 55	CRITICAL	Wastewater (industrial, leachate, intensity chemical)	BADLY	100 – 500	> 20

centration 25 ppm EMS (Ethyl methanesulfonate) under laboratory condition to 1,8-2,7 MN/1000 E and concentration 780 pg/ml PCB (Polychlorinated biphenyl) induced 1,5-1,7 MN/1000 E (Belpaeme et al. 1996).

The relevance of this MN test is also confirmed by the work by De Flora et al. (1993), Schultz et al. (1993), Al-Sabti and Metcalf (1995), Marlasca et al. (1998), Ayllonand Garcia-Vazquez(2000), Ayllón et al. (2000), Ayllón et al. (2001), Raisuddin and Jha (2004), Bagdonas and Vosyliene (2006), Kim and Hyun (2006), Baršieneet al. (2006), Ali et al. (2008), Palacio-Betancur et al. (2009),Boettcher et al. (2010), Bolognesi and Hayashi (2011) and Llorente et al. (2012).

The frequency of occurrences of CsD in root cells (CsD/200 cells) in the plant of common onion (*Allium cepa* L.) in uncontaminated laboratory and natural conditions is 2.0-2.5 damaged chromosome cells/200 cells (%) and is evaluated as a negative control (Firbas and Amon 2014).

Very little of *Allium* test studies are focused on clastogenic and/or aneugenic effects, thus, chromosome damage and chromosome number changes in the chromosome set. The use of colchicine during chromosome preparation destroys microtubules, but influencing the chromosome movement, increased frequency of metaphase with arranged condensed chromosomes and reduced transition from metaphase to anaphase, allowing a better observations of the exclusively metaphase chromosome (Ray et al. 2013; Firbas and Amon 2014; Kundu and Ray 2017). However this research strategy is provided by the *Allium* metaphase (M) test. Chromosome preparation is a key and crucial step in all cytogenetic techniques (Kirov et al. 2014), and cytogenetic assay are classical method to detect chromosome damage (aberration, anomalies). The metaphase chromosome anomalies as detected in the *Allium* M test procedure are not excluded to occur also in the human chromosomes when exposed to similar pollutants. Plant cytogenetic, using *Allium* M test, identifies same chromosome damages as they are identified in human cytogenetic such as: chromosome and chromatid damages, dicentric chromosomes, aneuploidy, euploidy and translocation (Stimpson et al. 2013; Firbas and Amon 2014; Firbas 2015; Polsikovsky et al. 2018). Mentioned chromosome aberrations cause clinical defects on human body (Schauer 1981; Pardee et al. 2007; Gibbs 2008; Duesberg 2005; Duesberg 2007; Gardner 2009). The study of DNA damage at the chromosome level is an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis (Fenech 2000), the genotoxic disease syndrome (Kurelec 1993) and evaluated genotoxic effect in autoimmune diseases by the micronucleus test assay (Torres-Bugarín et al. 2015).

The *Allium* M test—*Pisces* MN test means that these two independent testing system technologies show the same results in our research (Firbas and Amon 2017). *Allium* M test—*Pisces* MN test is a reliable, preferred and accurate method for the monitoring of the WWTP and water quality in aquatic ecosystem. Both tests help us to monitor the chromosome damages caused by the water pollution. The test system *Allium* M test—*Pisces* MN test has two restrictions: (i) MN can produce also a whole and undamaged chromosome and (ii) in the *Allium* M test cytostatic colchicine can mask the occurrence of

C-mitosis. In both cases here we talk about the malfunction of the mitotic process, however, this is not a chromosomal or chromatid lesion.

For testing the genotoxicity of the collected water samples, two assays were used: chromosome aberration assay in metaphase mitotic cell (Kumar and Panneerselvam 2007; Panneerselvam et al. 2012; Ragunathan and Panneerselvam 2007) and MN assay in interphase cell (Bolognesi et al. 2006). The CsD and MN studies have shown to be highly reliable and preferred in genotoxicity testing. The aim of this study was to determine of river water quality by conducting an experiment involving biomonitoring of water constituents of genotoxicity in fish and onion inhabiting these sites. In summary, WW treatment process in one of the most important environmental conservation processes that should be encouraged worldwide.

CONCLUSION AS AN ENVIRONMENTAL ESSAY

How healthy is a water (Firbas 2016) and/or living environment is definitely an opinion-forming issue. Its message is an integral part of biological science, thus opening up a world which enables us to determine the quality of any living environment by using current biological observation. This concerns the different lengths of a tested onion plant's roots, and any injuries to the chromosomes within their cells and micronucleus (MN) in blood erythrocytes indigenous fish in relation to their environment.

Physical and chemical analysis alone do not provide any reliable answer to the question of how healthy the water is. However complementary research in association with biological and chemical studies are needed in order to obtain a fully comprehensive picture, because it is difficult to identify a wide variety of effects (chemical pollution) within the environment. The biological method reveals an integrated impact on the growth and development of living cells or organisms, and detects the presence of harmful substances within the limits and capabilities of analytical methods. By using biological (genotoxicity) tests, we see that the outcomes of both plant and animal testing show damage of their genetic material (chromosomes) regardless of the tolerance limits that can be caused by various contamination sample concentrations within an environment. In regard to the universality of the living organisms "genetic codes", the research results are transferable (applicable) to human beings. It is time for us to act in a responsible way, thus ensuring a healthy environment which based on high quality drinking water.

The different feedback from the tested plant's root growth is a general quality indicator of an environmental sample. Their straight growth is an indicator of how adequate is the environment they are growing in. Looking at the cellular level of the tested plant's root-tip growth, especially when monitoring the cells and determining the ratio between the undamaged and damaged chromosomes, and the presence or absence of micronuclei in the blood of erythrocytes gives us a very detailed picture of the environmental living quality, respectively answering the question as to how healthy the living environment is.

Co-dependence of pollution within an environment is substantial evidence that some genotoxic stuff causes chromosomal damage, known as genotoxicity, and displays carcinogenic properties, as well as hormone disrupting chemicals (HDC) features. Risk assessment is insufficient just to find out how threatened we are due to different kinds of pollution within the environment, namely, it is very important to also discover how serious it is (how does it create adverse effects in biological systems). The evidence is indisputable that there are no safe doses, and that maximum permissible doses (MPDs) are, by agreement, subordinate to the practical applications. There are biological tests in response to these challenges, showing the synergistic and cumulative effects of harmful pollution, mechanisms of transmission, and the transformation of harmful pollution within biological systems.

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