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Determination of genotoxicity in waters from the region of Tetovo

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Abstract

This research examines the genotoxicity of water samples collected from 10 locations in the region of Tetovo (Republic of North Macedonia): Dolno Sedlarce, Railway station-Tetovo, Vratnica, Lavce, Raotince, Trebos, Brvenica, Banjice, Volkovija, and Dzhepchishte. The concentrations of several heavy metals (Cr, Cu, Zn, Mn, Fe, Pb, Cd, Co, and Ni) were measured. Allium test was used to examine the genotoxicity of the water samples, with the roots being collected in three series. Mitotic index (MI), index of aberrations (IA), and the presence of various chromosomal and mitotic aberrations were determined as part of the analysis of cytogenetic parameters. The results of the chemical analysis showed that the water samples had partial contamination with heavy metals. The concentration of heavy metals was highest in the sample from the Railway station. The heavy metals with the highest measured concentrations are zinc, copper, manganese, and cadmium. MI in the second and third collection series was lowest in the bulbs placed on water samples from the Railway station. The bulbs placed in water samples from Vratnica, Lavce, Raotince, and Banjice showed an increase in MI. In the first and third series IA was highest for the bulbs placed on the water samples from Railway station. Different chromosomal and mitotic aberrations were detected including chromosomal bridges, C-mitoses, vagrants, laggards, sticky chromosomes, irregular anaphases, binucleated cells, micronuclei, elongated nuclei, fragmented nuclei, nuclear buds, and fragmented nuclei. Most of the structural chromosomal and mitotic aberrations (with frequent chromosomal bridges) were observed in the material placed on water samples from the Railway station. The results obtained from this research indicate a particular genotoxic impact of some of the waters in the region of Tetovo.

Keywords: Allium test, Tetovo region, heavy metals, chromosomal aberrations, mitotic index, genotoxicity.

Introduction

Heavy metals can cause damage to the structure of the chromatin and mitotic spindle and thus initiate various chromosomal, mitotic, and meiotic aberrations. Heavy metal ions can affect DNA and nucleoproteins directly by binding to them or indirectly creating free radicals or affecting the DNA reparation process.

Depending on the pollution level and their genetic ability to adapt, plants have developed varying degrees of sensitivity to heavy metals. Different degrees of sensitivity of the genetic material in some plant species initiated a research trend to define effective bioindicators for genotoxicity. Today there are data on various plant species that are successfully used as test organisms for genotoxicity, among which the most used are: *Zea mays* (Plewa et al. 1984; Eul et al. 2000; Grant & Owens 2006), *Arabidopsis thaliana* (Labra et al. 2003), *Pisum sativum* (Fusconi et al. 2007) different species of the genus *Tradescantia* (Kim et al. 2003), *Vicia faba* (Ünyayar et al. 2006; Shahid et al. 2011) and *Allium cepa* (Fiskesjö 1985; Rank & Nielsen 1993; Ateeq et al. 2002; Olorunfemi et al. 2012).

Allium test (A. test) was introduced as a method for detection of genotoxicity caused by chemical

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compounds (Levan 1938). Since then, this method has been modified and promoted as one of the standard methods for detecting genotoxic effects from substances of known or unknown concentration and origin. *A. cepa* is an appropriate test object for genotoxic research due to the large size of its chromosomes. Onion bulbs are easy available at low-cost (Levan 1949). The importance of the A. test has been emphasized in a study by Fiskesjö (1985), which showed that certain genotoxins causing chromosomal aberrations in *A. cepa* root meristem cells, could also cause similar effects in human cell cultures. According to Barbosa et al. (2010), A. test showed the highest sensitivity to heavy metals in water. The frequency of genotoxic effects observed in the experiment positively correlated with their measured concentrations.

Material and methods

Description of sampling locations

The area chosen for this study was the Tetovo region. The Tetovo region, located in the northwestern part of the Republic of Macedonia, is composed of the municipality of Tetovo and the neighboring municipalities. It is one of the most developed and densely populated municipalities. The Tetovo region has been facing chronic lack of water and water pollution in the last two decades. The main factors responsible for reducing water quality are the insufficiently regulated industries, agricultural practices, and inefficient waste management. There is a lack of research about the genotoxic and mutagenic effects of the waters in the Tetovo region used for drinking and agricultural practices. For better regional coverage, 10 locations were chosen as sampling locations for water samplings (Figure 1): Dolno Sedlarce, Railway station, Vratnica, Lavce, Raotince, Trebosh, Brvenica, Banjice, Volkovija, and Dzhepchishte).

- Dolno Sedlarce is a village on the south side of Tetovo, near the Tetovo-Gostivar highway, at 430 m altitude. The village is on flat terrain with dominant agricultural activity. The water samples were collected from a spring used by the local population for irrigation.
- The Railway station is near Tetovo, in vicinity of the closed metallurgical plant Silmak (former Jugohrom). Along the railway, there are several arable fields from which a large part of the agricultural products come to the markets in Tetovo. The water samples are taken from water from a canal running along the railway irrigating surrounding fields.
- Vratnica is a mountainous village located in the northern part of the Polog valley, at 730 m altitude. It is abundant with groundwater and springs, as well as dense vegetation. There are no major industrial facilities near Vratnica, except for the construction materials company IGM Vratnica. The largest industrial facility in the Polog Valley - „Silmak“ - and a potential polluter, 11 km away from

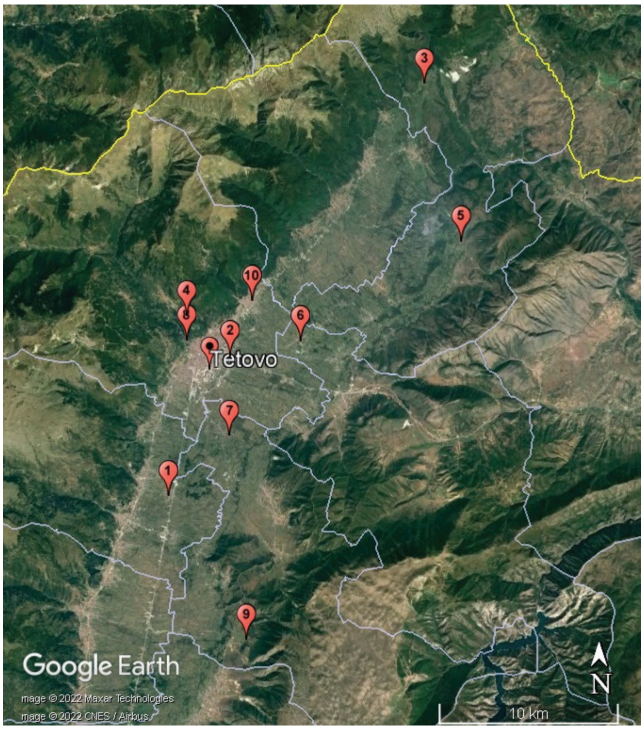

Figure 1. Locations of collection points for water samples in the Tetovo region 1) Dolno Sedlarce; 2) Tetovo railway station; 3) Vratnica; 4) Lavce; 5) Raotince; 6) Trebosh; 7) Brvenica; 8) Banjice; 9) Volkovija; 10) Dzhepciste

Vratnica. The water samples are taken from spring water used by the local population for irrigation.

- Lavce is a mountainous village located in the eastern part of the Polog valley, at 780 m altitude. It is rich in groundwater, springs, and dense vegetation. In the last ten years, a significant reduction of forest vegetation happened in the vicinity of the village due to illegal logging. There are no major industrial facilities near Lavce. Silmak, the largest industrial facility in the Polog, is located 20 km from Lavce. The water samples were collected from spring water that is used by the local population for drinking and irrigation, located 6 km from the springs that supply most of the city with water.
- Raotince is a village located in the northeastern part of the Polog valley, at 390 m altitude. The water samples were taken from the bed of the Vardar river. The Vardar river flows near the village. Only 3 km from the settlement is „Silmak"-Jegunovce and 10 km from Raotince is the Rasce spring from which most of Skopje is supplied with water.
- Trebosh is a village located northeast of Tetovo, on the left side of the Vardar river. The village is flat, with agricultural and livestock activities. There is a developed poultry and meat industry. Water samples are taken from spring water used by locals for irrigation and described as „heavy water," and it is not used for drinking. It is opaque, with a pale white color, and leaves a precipitate, especially when boiling.
- Brvenica is a plain settlement located near Tetovo, southeast of the city, at 440 m altitude. The village of Brvenica is the seat of the municipality of the same name and has developed agriculture and furniture industry. Water samples are taken from spring water used by the local population for drinking and irrigation.
- Banjice is located above Tetovo, on the slopes of Shar Mountain. Banjice is prone to floods and landslides. The water samples are from spring mineral water used for drinking by the local population.
- Volkovija is located in the far southern part of the Tetovo region, at 560 m altitude, 20 km away from Tetovo. Pastures and forests dominate the Volkovija region. The village has agricultural and livestock activities, with fishpond and poultry farms. Water samples are taken from the local well from which the village is supplied with water.
- Dzhepchishte is located north of Tetovo, not far from the city, at 4 km. The village is flat, at an altitude of 480 m. The main activity is agriculture. The village also has a developed construction industry. Water samples are taken from the local well from which the village is supplied with water.

The water samples were collected in plastic bottles and were kept in a fridge at 4°C.

Test object and experiment setup

The *A. cepa* bulbs used in this research were commercially obtained. The experiment was performed according to a modified Fiskesjo's method (Fiskesjo 1985). A total of 110 *A. cepa* bulbs were used in this experiment. The old dried roots and dry surface scales were removed from each bulb. The bulbs were placed in vials filled with water samples from the appropriate sampling locations (10 vials for each location and 10 vials for negative control). Tap water was used as a negative control. Only the top of the base of the bulbs was in contact with the water surface (Figure 2). The vials were placed at room temperature (25 °C). Three collection series were made by cutting off the roots of each of the bulbs: first series

Figure 2. Experimental setting for root system growth

(S1), second series (S2), and third series (S3). Roots were collected in intervals of 72 h.

Chemical analysis of water samples

The water samples intended for the experiment were analyzed for the presence of heavy metals (Pb, Cd, Cu, Zn, Mn, Cr, Fe, Ni, Co). The heavy metal concentration was determined by atomic absorption spectroscopy with an Agilent 240Z AA spectrometer.

Cytogenetic methods

The top meristem tissue from the roots (1-2 mm) exposed to the collected water samples was used for cytogenetic analysis. The analysis was performed for all three series of collections. The material was processed according to Tjio & Levan (1950), as well as the standard "squash" method after Battaglia (1955). Chromosome staining was done with hematoxylin after Gomori, according to Konstantinov et al. (1985). Ten slides were analyzed for each of the series (30 slides per water sampling collection), with 1000 analyzed cells per slide. For each slide, a mitotic index (MI) was calculated as a ratio between the cells in mitosis and the total number of counted cells, multiplied by 100, based on the average of 1000 analyzed cells per slide. Also, for each slide, the index of aberrations (IA) was calculated as a ratio between the aberrations per 100 cells and the total number of counted cells, multiplied by 100, based on the average of 1000 analyzed cells per slide. The aberration index shows the frequency of chromosomal and mitotic aberrations.

Statistical methods

The results were statistically processed with STATSOFT STATISTICA 8 software for Windows. After checking the normality with the Kolmogorov-Smirnov test and the homogeneity of the data, it was necessary to transform the data because there was no normal distribution. Two-way ANOVA and post-hoc Tukey tests were performed to compare different categories of cytogenetic parameters between the different locations and series of collections. The results are presented as mean values followed by the standard deviation.

The relationship between chemical analysis and cytogenetic findings through linear correlation was also investigated. The numerical values of the measured ion concentrations were correlated with the numerical values of the cytogenetic parameters. To quantify the strength and direction of the relationship between each of the examined variables, the correlation coefficient (r) was calculated. The bond strength and direction were determined by a scale of six different degrees for the values of r (Lamorte 2021). The values were considered statistically significant for $p < 0.05$.

Results

Chemical analysis of water samples

The concentrations of measured heavy metal ions are shown in Table 2. They are compared to the allowed thresholds from the Regulation for Water Classification (Government of Republic of Macedonia, 1999), which classifies the waters in Macedonia in five classes, according to the degree of cleanness, the use and the effects on the living organisms (Table 1).

The highest concentrations of heavy metals were measured in the water samples from the Railway station (Table 2), where the concentration of Zn was the highest. High concentrations of Zn were also registered in the water samples from Dzhepchishte and Vratnica, while the lowest concentrations of Zn were measured in the Banjice samples. High concentration of Cu was detected in the water samples from Volkovija and the lowest concentration of Cu was measured in samples taken from Brvenica. The concentration of Pb was high

Table 1. Classes of waters according to the "Regulation for Water Classification" (1999) of Republic of North Macedonia

Class	Description
Class I	Oligotrophic, clean waters, which in their natural state can be used for drinking and for the needs of the food industry (these waters do not contain inorganic pollutants, but they could contain small, occasional, anthropogenic pollution with organic pollutants).
Class II	Low pollution waters which in their natural state can be used for hygiene.
Class III	Moderately eutrophic waters which in their natural state can be used for irrigation.
Class IV	Strongly polluted, eutrophic waters which can be used for other needs, only after previous treatments.
Class V	Heavy polluted, hypertrophic waters, which in their natural state cannot be used for whatever use; the concentration of compounds present in the water can have heavy toxic effect for the organisms.

Table 2. Concentrations of heavy metals measured in water samples (μg/l) from 10 different locations

Sampling location		Cu	Pb	Cd	Co	Ni	Cr	Mn	Fe	Zn
1.	D. Sedlarce	16.4	2.3	0.4	<0.1	0.1	0.3	0.4	0.9	<0.1
2.	Railway station	262**	39.4*	3.8	0.1*	6.9	26.6	2.9	238*	8.4
3.	Vratnica	120**	2.2	1.4	<0.1	<0.1	0.7	0.2	1.7	0.3
4.	Lavce	9.1	2.8	1.3	<0.1	0.1	3.6	0.1	1.1	<0.1
5.	Raotince	10.1	2.5	1.1	<0.1	0.1	0.8	0.2	19.3	0.3
6.	Trebosh	13.2	3.3	2.5	<0.1	<0.1	0.1	0.1	0.6	<0.1
7.	Brvenica	12.4	1.5	0.5	<0.1	<0.1	0.4	0.3	1.1	<0.1
8.	Banjice	6.8	2.2	0.9	<0.1	0.2	1.0	0.2	16.2	0.3
9.	Volkovija	38.1	5.7	1.3	<0.1	<0.1	0.3	<0.1	1	<0.1
10.	Dzhepchishte	128*	1.9	1.4	0.1	<0.1	0.5	0.2	2.3	0.1

* Concentrations belonging to Class III and IV

** Concentrations belonging to Class V

in the water samples from Trebosh, and the lowest in the samples taken from D. Sedlarce. Cd concentration was high in the water samples from Dzhepchishte, and low concentrations were measured in Lavce, Raotince, Trebosh, Brvenica, Banjice and Volkovija. Co concentrations were lowest in the water samples from Trebosh. In the water samples from Volkovija there was no Co detected. High concentration of Ni was measured in the samples from Lavce and its lowest concentrations were measured in the water samples collected from Trebosh. Cr concentrations were high in D. Sedlarce, with the lowest concentrations detected in Volkovija. Mn concentrations were the high in Raotince and Banjice. Lowest concentrations of Mn were measured in the water samples from Trebosh. Fe concentrations were high in Vratnica and Raotince, and the lowest concentrations were measured in the water samples from Lavce, Trebosh and Brvenica.

The results of cytogenetical analyses are presented in Table 3.

Table 3. Results of cytogenetical analyses in A. cepa bulbs exposed to negative control (tap water) and different water samples (test liquids from different sampling sites). Mitotic index (MI) and Index of aberrations (IA) are presented as mean values, followed by standard deviation in brackets.

		MI			IA		
No.	Collections	S1	S2	S3	S1	S2	S3
	Negative control	36 (5)	38 (10)	37 (8)	6 (7)	5 (11)	4 (8)
1.	Dolno Sedlarce	40 (5)	44 (6)	46 (9)	19 (23)	10 (15)	21 (21)
2.	Railway station	35 (2)	34 (4)	33 (3)	69 (74)	29 (58)	30 (33)
3.	Vratnica	46 (8)	64 (14)	47 (7)	42 (53)	58 (93)	12 (15)
4.	Lavce	38 (14)	44 (17)	65 (19)	4 (5)	10 (15)	13 (20)
5.	Raotnice	54 (18)	51 (18)	62 (14)	12 (12)	6 (9)	6 (9)
6.	Trebosh	34 (8)	48 (9)	46 (9)	8 (14)	21 (26)	13 (14)
7.	Brvenica	46 (8)	43 (7)	46 (4)	15 (13)	12 (18)	14 (17)
8.	Banjice	58 (20)	55 (10)	40 (8)	23 (26)	28 (27)	11 (20)
9.	Volkovija	43 (10)	45 (6)	41 (10)	3 (6)	2 (4)	5 (12)
10.	Dzhepchishte	49 (7)	41 (7)	45 (19)	9 (13)	6 (11)	5 (8)

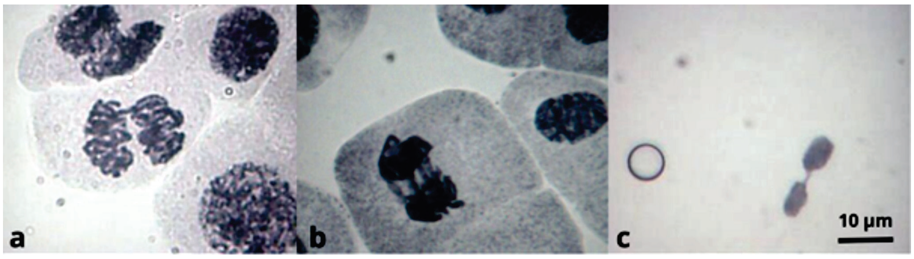

Fig. 3. Types of chromosomal bridges: a) single chromosomal bridge; b) multiple chromosomal bridge; c) interphase bridge

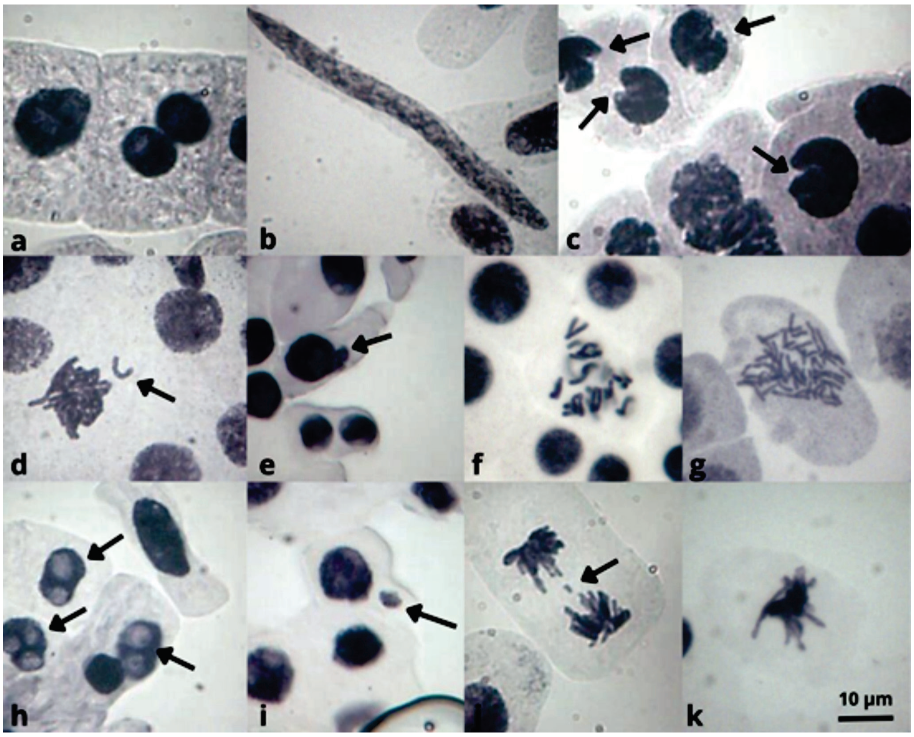

Fig. 4. Chromosomal and mitotic aberrations in root tips of *A. cepa* bulbs exposed to different water samples: a) binucleated cells; b) elongated nuclei; c) fragmented nuclei; d) vagrants; e) nuclear buds; f) irregular anaphases; g) C-mitosis; h) "vacuolated" nuclei; i) micronuclei, j) laggards; k) sticky chromosomes

In the first collection series, the highest MI was registered in Banjice, followed by Raotince. The lowest MI was registered in Trebosh. In the second collection, there was a significant increase in MI in Vratnica, followed by Banjice. The lowest MI was registered in the materials placed on water samples from Railway station. In the third collection, MI was the highest in Lavce, followed by Raotince. The lowest MI was detected in the materials placed on water samples from Railway station.

The highest IA in the first collection series was registered in Railway station, followed by Vratnica. The lowest MI was registered in Volkovija, followed by Lavce. The increase of IA in Railway station was followed by an increase in the frequency of chromosomal bridges. In the second collection series, IA was the highest in Vratnica, and the lowest was in Volkovija. In the third collection, IA was the highest in the Railway station. The lowest IA was recorded in Volkovija and Dzhepchishte.

The following chromosomal and mitotic aberrations were identified: chromosomal bridges (Figure 3), C-mitosis (Figure 4g), vagrants (Figure 4d), laggards (Figure 4j), sticky chromosomes (Figure 4k), irregular anaphases (Figure 4f), binucleated cells (Figure 4a), micronuclei (Figure 4i), elongated nuclei (Figure 4b), fragmented nuclei (Figure 4c), nuclear buds (Figure 4e), and "vacuolated" nuclei (Figure 4h).

The research identified three different types of chromosomal bridges: single chromosomal bridges (Figure 3a), multiple chromosomal bridges (Figure 3b), and interphase bridges (Figure 3c). The nuclei in the detected binucleated cells had standard shape, size, and symmetry. "Vacuolated" nuclei had single or multiple gaps with different sizes and shapes in

Table 4. Correlation coefficient (r)* for each of the heavy metals with the cytogenetic parameters ($p<0.05$)

	Zn	Cu	Pb	Cd	Co	Ni	Cr	Mn	Fe
MI	-0.18	-0.25	-0.14	-0.21	-0.25	-0.23	-0.24	-0.23	-0.24
IA	0.28	0.26	0.23	0.29	0.27	0.27	0.28	0.27	0.28
Chromosome bridges, %	0.20	0.25	0.18	0.23	0.25	0.25	0.26	0.25	0.25
Binucleated cells, %	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Micronucleus, %	Ns	Ns	Ns	Ns	0.11	0.11	0.13	0.11	0.11
C-mitosis, %	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Vagrants and laggards, %	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Sticky chromosomes, %	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Elongated nuclei, %	0.20	0.15	0.14	0.18	0.16	0.16	0.15	0.15	0.16
Fragmented nuclei, %	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Nuclear buds, %	Ns	0.12	Ns	0.12	0.12	0.12	0.13	0.12	0.12
„vacuolated" nuclei, %	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Irregular anaphases, %	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns

*r values: -0.8 – -1 (strong negative correlation); -0.4– -0.8 (medium negative correlation); 0 – -0.4 (weak negative correlation); 0 – 0.4 (weak positive correlation); 0.4– 0.8 (medium positive correlation); 0.8 – 1 (strong positive correlation); Ns-statistically insignificant correlations

their structure. Part of the ”vacuolated” nuclei had deformations in their shapes.Some of the elongated cells had chromatin gaps. These nuclei have certain deformations in the shape of chromatin extensions like buds or micronuclei positioned close to the nucleus connected with a short chromatin bridge. The detected laggards were in the shape of single or multiple chromosomes or chromosomal segments, which fall behind the other chromosomes in anaphase and are not attached to neither of the poles. In some cases, single or multiple laggards have been identified in combination with polyploidy, bridges, and vagrants. The identified micronuclei in this research have been of different sizes and numbers (1 or 2), while in some of the nuclei, there has been visible damage in the nuclei chromatin.

The correlation coefficients between the cytogenetic parameters and the heavy metals are presented in Table 4.

The concentrations of all heavy metals correlate negatively with the MI. The concentrations of heavy metals positively correlate ($p<0.05$) with the frequency of chromosomal bridges and elongated nuclei. Cu, Cd, Co, Ni, Cr, Mn, and Fe concentrations positively correlate with the nuclear buds frequency. All heavy metals positively correlate with the IA (Table 4).

Discussion

Mitotic index

Within the analyzed slides from the first collection series, it was noticed that there are relatively similar MI between the samples and the control (Table 3). The possible reason for this trend may be the short period of exposure of the bulbs to the active substances in the analyzed water samples and the delayed activity of the heavy metals. In the roots from the bulbs grown in water samples from Banjice, a significantly higher mitotic index was observed in the first collection series in relation to the control, without significant differences in terms of the measured heavy metals in the water. This phenomenon may be due to the fact that the Banjice spring is known as a source of mineral water. There can be a presence of certain micronutrients that did not fall within the scope of this study and may have a stimulating effect on mitosis. According to the Center for Public Health Tetovo (CPHT 2021), the Banjice spring is rich in magnesium, calcium and potassium, which in specific concentrations can act as mitogen (Phillips 1968; Becerra & Lopez-Saez 1978).

The MI within the second collection series (Table 3) is the highest in the analyzed bulbs grown on water samples from Vratnica, significantly higher than the control. At the same time, in the water samples from Vratnica, a relatively higher concentration of zinc than allowed was detected (120.2 µg/l). Zinc is an essential nutrient in specific amounts (Steinkellner et al. 1998). According to Reis et al. (2018), zinc concentrations lower than 2000 µg/l are non-toxic to plant cells and have a mild mitogenic effect, while concentrations higher than 2000 µg/l can significantly reduce the MI and increase the number of dividing cells with aberrations. Zinc increases the activity of mitogen-activating protein kinases (MAPK) in the rice root system (Lin et al. 2005). It is also an essential element in the construction of “zinc finger” proteins (Ciftci-Yilmaz & Mittler 2008), especially in the enzymes and factors involved in transcription (Broadley et al. 2007) and cell division (Zhao & Wu 2017). Zinc also participates in the synthesis of tryptophan (Brown et al. 1993) and thus in the synthesis of auxins (Brennan 2005) which stimulate the cell division of the top root meristem cells (Perrot-Rechenmann 2010). Such an increase in the MI was not observed in Dzhepchishte, although the zinc concentrations are similar to those in Vratnica. This indicates the possible presence of more complex interactions between zinc, other ions, and meristem cells. Namely, certain free radicals that can originate from various stressors can effectively prevent the activation of zinc-induced MAPK (Lin et al. 2005).

Within the third collection series, MI was significantly higher in the analyzed roots from Lavce and Raotince samples, without significant differences in the concentrations of the measured heavy metals (Table 3). This may indicate the possible presence of other, non-measured mitogen compounds in the water samples. In this collection series, MI was lowest in the examined material from bulbs grown on water samples from the Railway station. Given the high concentrations of some of the measured ions in the water samples (Zn, Cu, Cd, Mn), together with the negative correlations between MI and heavy metals, there is a possibility for a suppressive effect of some of the heavy metals on the division of meristem cells. Several studies showed that the increase in zinc concentrations was directly proportional to the inhibition of mitosis in the top meristem cells (Świeboda 1976; Jain et al. 2010; Raskar & Laware 2014; Reis et al. 2018). These findings are also confirmed with A. test (Kocik et al. 1982). According to Powell et al. (1986), the treatment of *Festuca rubra* L. with Zn at concentrations of 100 and 200µg/l causes a slowing of the cell cycle of 40 % and 132 % respectively, and the slowing is most evident in the G_1 phase. According to Qin et al. (2015), concentrations of copper accumulated in the roots in *A. cepa* samples treated with 2.0 or 8.0 µm copper salts after 12 and 36 hours increased by 38.7 % and 218.5 %, respectively, more than in controls. Increased concentrations of copper affected depolymerization and microtubule organization, and decreased the α-tubulin content, thus decreasing MI. Cadmium also effects the microtubular cytoskeleton of the top meristem cells of *Allium sativum* L. (Xu et al. 2009). It affects the mechanisms of control of cytoskeleton organization, as well as the processes of tubulin assembly and disassembly, stimulating the formation of abnormal discontinuous and fragmented microtubular rays. According to the authors, increased concentration of Cd reduces the MI. Decreased MI was observed in *A. cepa* bulb roots treated with 0.03 % manganese salt solutions for 72 hours (Doroftei et al. 2010). Manganese also caused a decrease of MI in *Zea mays* meristem cells (Ertürk et al. 2021).

Index of aberrations

The roots from the bulbs placed on water samples from the Railway station had the highest IA in the first collection series (Table 3), significantly higher in relation to the control. Given the high concentration of the heavy metals in the water samples from this location, there is likely a possibility of genotoxic influence. The lowest IA was observed in samples from Volkovija and Lavce, where there are lower concentrations of measured ions.

The highest IA in the second collection series was observed in the slides from the bulbs' roots placed on water samples from Vratnica. The IA in these samples was also significantly higher than in control. This could be explained by the high MI and high frequency of divisions in these root tips, which may increase the possibility of a higher incidence of errors in mitosis. The increase of IA at Vratnica is mostly due to the presence of elongated nuclei. Such trends suggest the possibility of a mitogenic activity, with the simultaneous presence of a factor that prevents the cell from finishing the G1/S phase (Yasuhara & Kitamoto 2014; Mercado & Caleño 2020). In the water samples from Vratnica, a relatively higher concentration of zinc than allowed was detected (120 µg/l). Analyzes of different stages of cell division, affected by relatively high doses of zinc (130 µg/l), revealed chromosomal aberrations in 66.66 % of cells in metaphase and 57.77 % of cells in anaphase, compared with 3.38 % and 3.63 % of cells with two-phase aberrations in controls treated with 0.065 µg/l (Jain et al. 2010). Copper also causes different types of high-frequency aberrations (Qin et al. 2015). Also, with an increasing Cd concentration the frequency of irregular divisions also increases (Xu et al. 2009).

Within the third collection series, IA is highest in the bulbs' roots placed in water samples from the Railway station, which is correlated to the higher concentrations of heavy metals in the samples taken from the same location.

Types of detected chromosomal and mitotic aberrations

The frequency of chromosomal bridges within this study is positively correlated with all heavy metals (Table 4). Heavy metals often cause chromosomal bridges in the chromosomes of plant meristem cells. According to Abubacker & Sathya (2017), chromium is the one that most often causes chromosomal bridges, followed by lead. Although these two heavy metals in the water samples from the Railway station have concentrations within the allowed standards, their concentrations are still several times higher than at other sites. The presence of an increased number of chromosomal bridges in bulbs treated with known concentrations of chromium, lead and cadmium have been confirmed by Glińska et al. 2007. Some studies have shown that a higher cadmium concentration increases the number of chromosomal bridges (Zhang & Yang 1994; Liu et al. 2003; Pizzaia et al. 2019). Zinc and manganese can also cause chromosomal bridges in higher concentrations (Doroftei et al. 2010; Mumthas et al. 2010; Shaymurat et al. 2012). Chromosomal bridges can also be caused by copper solutions. A. test is suspective to the presence of copper ions (Jiang et al. 2001; Souguir et al. 2008; Yildiz et al. 2009; Qin et al. 2015).

There is a significant positive correlation between C-mitosis and MI (Table 4), which is to be expected given that in the presence of agents that can damage the mitotic spindle, as the frequency of divisions increases, so does the number of mitotic aberrations and aberrant divisions. Particular influence on the MI and C-mitoses in the root meristem cells has been observed in plants treated with Zn salts (Świeboda 1976).

Vagrants are chromosomes that move unsynchronized (forward or sideways) from their chromosome set, leading to unequal separation of chromosomes in newly formed cells (Sabeen et al. 2020). On the other hand, laggards occur when chromosomes do not connect at all or fail to remain connected to the microtubules of the mitotic spindle and remain positioned around the middle of the metaphase plate (Khanna & Sharma 2013). The increased number of chromosome vagrants may indicate potential negative influences on the process of spindle formation (El-Ghamery et al. 2003; Rank 2003). Mitotic spindle dysfunctions may be associated with disturbances in calcium ion homeostasis in cells, which may affect microtubule polymerization (Zhang et al. 1992). Other possible causes of spindle dysfunction may be changes in cytoskeletal proteins initiated by ion interactions with heavy metals or cytoskeleton reorganization due to changes in cell charges (Hedberg et al. 1991; Dovgalyuk et al. 2003; Yemets et al. 2021). In our study, the frequency of vagrants and laggards correlated positively with binucleated cells, elongated nuclei, fragmented nuclei, and "vacuolated" nuclei (Table 4). Binucleated cells may be the product of aberrations in the spindle formation in early anaphase or failed cytokinesis after telophase (Bonea et al. 2018). Some of the elongated nuclei may be forms of polyploid nuclei that result from endomitosis or endoreplication. Such phenomena, if induced, are associated with complete dysfunction of the spindle, which may be caused by increased concentrations of heavy metals in the meristem cells (Morimura et al. 1978). The appearance of fragmented and "vacuolated" nuclei, in turn, is essentially the result of reduction of the chromatin inside of the interphase nucleus, due to chromatin deficiency. This condition could result from the loss of chromosomes through the formation of vagrants and laggards in the anaphases that preceded the formation of such nuclei. Such a process would explain the positive correlation between vagrants and laggards on the one hand and fragmented and "vacuolated" nuclei on the other. The common etiology of vagrants, laggards and binucleated cells could be a possible reason for their positive correlation. However, at the time of writing, to the best of our knowledge, no data were found in the available literature that would confirm or otherwise explain the reasons for the association between the appearance of vagrants and laggards on the one side and binucleated cells, elongated nuclei, fragmented nuclei and "vacuolated" nuclei on the other. Regarding correlations with other factors, the frequency of vagrants and laggards does not correlate with any of the heavy metals.

Binucleated cells may indicate spindle cell division disorders in meristem cells, which would also mean the presence of an agent or group of agents with a degrading effect on this cellular component (Mueller et al. 1999; Zalacain et al. 2005). However, binucleated cells can also occur in plants as part of the polyploid cell formation process (Stebbins 1980; Weiss-Schneeweiss et al. 2013; Wendel 2015). In our research, binucleated cells are only positively correlated with vagrants (Table 4), which would confirm the association of the two aberrations through defects in the mitotic spindle.

The micronucleus is an indicator of malformations in the chromatin structure and may indicate certain genotoxic effects. Micronucleus can be formed by various mechanisms: from dislocated chromosomes during the previous metaphase, from vagrants or laggards occurring in previous bipolar or multipolar anaphases, and from chromosomal fragments torn from chromosome bridges in anaphase (Huang et al. 2011). Micronuclei showed a significant positive correlation with cobalt, nickel, manganese, chromium and iron (Table 4). These results confirm the findings of some studies that have detected the influence of heavy metals on the formation of micronuclei (De Marco et al. 1988; Steinkellner et al. 1998; Majer et al. 2002). Compared to other aberrations, the frequency of micronuclei showed a positive correlation only with the elongated nuclei. This correlation may be due to the role of the lamina in maintaining the stability of the core. Namely, lamina consists of connecting protein filaments that form a network of polymers in the form of a coating around the periphery of the nucleus. The binding of specific reactive molecules to these proteins can alter the stability and shape of the nucleus which in turn can lead to the formation of micronuclei and extremely elongated nuclei (Butin-Israeli et al. 2015; Stephens et al. 2017; Stephens et al. 2018).

Elongated nuclei usually occur as a result of aberrations in the chromatin structure and nucleoproteins, primarily lamins, which are proteins responsible for maintaining the structure of the nucleus. Various heavy metals can cause elongated nuclei (Doroftei et al. 2010; Papp et al. 2011; Zanin et al. 2017). A positive correlation was also found between the frequency of the elongated nuclei and the frequency of the micronucleus and the vagrants, indicating a certain correlation of all three aberrations through the separation processes of the chromatin material within or outside of the nucleus. In our research, no statistically significant increases in this type of aberrations were observed in relation to the control, however, evidently higher frequencies are visible within the first and second collection of water samples from the Railway station and Vratnica, where there have also been registered higher concentrations of zinc, copper, cadmium, and manganese.

The causes of fragmented nuclei can be multifactorial. According to some studies, the formation of fragmented nuclei may be the result of: senescence of cells (Farage-Barhom et al. 2011), aborted apoptosis (Tubio & Estivill 2011), telomere dysfunction (Tubio & Estivill 2011) or premature chromosome condensation and asynchronous mitosis (Pantelias et al. 2019). The increased frequency of fragmented nuclei in meristem cells may indicate some genotoxic effect on the structure and consistency of chromatin material within the nucleus (Fusconi et al. 2006; Yıldız et al. 2009). The frequency of fragmented nuclei within our research did not show a significant increase at any of the collection s, in any series. A significant positive correlation was shown between fragmented nuclei only with the vagrants. This correlation has been observed in other studies (Mustafa & Suna Arikan 2008; Kumari et al. 2011; Renjana et al. 2013).

The nuclear buds frequency may indicate the presence of substances with a direct genotoxic effect on the chromatin. Such nuclei may be precursors to micronucleus formation (Huang et al. 2011). Our research found that bud nuclei frequency did not show a significant increase at any of the collection s, at any series. The only minor increase in terms of control was registered in the material from the bulbs placed in samples from the Railway station. Nuclear buds showed a significant correlation with only the fused chromosomes. It is indicative that bud nuclei did not show a positive correlation with the elongated nuclei and micronuclei, although this was expected due to similar formation mechanisms associated with the lamina dysfunction (Butin-Israeli et al. 2015; Stephens et al. 2017; Stephens et al. 2018). However, Stephens et al. (2018) found that chromatin and lamins have different roles providing the structure, shape and stability of the nucleus. Lamins stabilizie the nucleus from the inside and are essential for a more robust stress response mechanism that targets the entire nucleus, while chromatin is more important for a shorter response to stresses on the nucleus. In this case, the indications from our research suggest that the micronuclei and elongated nuclei may be the result of the former type of mechanism and the nuclear buds of the latter. However, more research is needed to define the whole core dynamics of the occurrence of these aberrations in more details.

The "vacuolated" nuclei may result from a reduction in chromatin material within the interphase and prophase nuclei. Some authors have suggested that a possible cause of such aberrations is DNA deficiency in the nucleus (Croker 1953; El-Ghamery et al. 2003; Youssef & Elamawi 2020). According to El-Ghamery et al. (2003), certain chemical changes in the structure of nucleoproteins or DNA can lead to a change of these molecules, with chromatin losing its ability to bind with the dyes or granulating more densely, and such a change in certain regions results in paler colored formations similar to vacuoles. These changes may also be the result of genotoxic effects from heavy metals. This change can occur if nucleoproteins bind to heavy metal ions, altering in that way the surface protein charge, hydrophobicity, internal stability, and other physical properties (Cabaleiro-Lago et al. 2010), thereby inhibiting their normal function. The most sensitive target for such binding are the histones, which control the structure and organization of chromatin in the nucleus (Klosterman et al. 2003; Zhang 2003). According to some studies, zinc and copper ions in particular, have the ability to cause such nuclei in meristem cells (El-Ghamery et al. 2003; Nagaonkar et al. 2015; Youssef and Elamawi 2020).The frequency of "vacuolated" nuclei within our research did not show significant deviations at any of the sites. The only slight increase compared to the control was observed in Dolno Sedlarce. The "vacuolated" nuclei did not correlate with any heavy metals. The "vacuolated" nuclei correlated positively only with the vagrants.

Conclusions

The water samples from the ten locations in the Tetovo region have partial contamination with heavy metals. The highest concentrations were measured in the samples from the Railway station, located near a large industrial and transport center. In general, zinc, copper, manganese and cadmium ions were present in higher concentrations in all 10 locations. MI in the first collection series was highest in the material from the bulbs placed on water samples from Banjice and lowest in Trebosh. However, relatively similar MI was observed between the samples and the control. In the second series, MI was significantly higher in the material from the bulbs placed in water samples from Vratnica, while the lowest was in samples from the Railway station. Within the third collection series, MI was highest in the water samples from Lavce, with a significant increase in Raotince, and the lowest in the water samples from the Railway station. IA in the first collection series was highest in the material from the bulbs placed on water samples from the Railway station, in the second collection series in the material from the water samples from Vratnica, and in the third series again the water samples from the Railway station. The following chromosomal and mitotic aberrations were detected: chromosomal bridges, C-mitoses, vagrants and laggards, sticky chromosomes, irregular anaphases, binucleated cells, micronuclei, elongated nuclei, "vacuolated" nuclei, and fragmented nuclei.

Heavy metals have a negative correlation with the MI and positive correlation with the IA, indicating their potential mitosuppressive and genotoxic influence. A particularly significant increase is seen in chromosomal bridges and elongated nuclei.

These findings will contribute to a better understanding of the water pollution in the Tetovo region and the risks to the genetic material of organisms exposed to such waters. Through appropriate standardization and implementation of the A. test, and with the support of the relevant institutions in Macedonia, a sustainable system for biomonitoring of the genotoxic effects of waters could be built in the future.

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