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Ditika Kopliku
Associate Professor
University of Shkodra Luigj Gurakuqi
Albania

Anila D Mesi
Associate Professor
University of Shkodra Luigj Gurakuqi
Albania

Athens Institute for Education and Research
8 Valaoritou Street, Kolonaki, 10671 Athens, Greece
Tel: + 30 210 3634210 Fax: + 30 210 3634209
Email: info@atiner.gr URL: www.atiner.gr
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Capability of *Vicia faba* L. in Evaluating Surface and Waste Waters Toxicity of Shkodra Lake, Albania

Ditika Kopliku
Associate Professor
University of Shkodra Luigj Gurakuqi
Albania

Anila D Mesi
Associate Professor
University of Shkodra Luigj Gurakuqi
Albania

Abstract

The use of bioassays to screen the mutagenic potency of complex environmental mixtures, such as surface or waste waters is getting an important part of monitoring projects. The present study was carried out to evaluate the possible toxicity of surface and waste waters collected from Shkodra Lake (a transboundary lake between Albania and Montenegro) during April and September 2013, by using in vivo Vicia faba L. assay. Physicochemical characterization of waters sampled in five stations and morpho- and cytogenetic analyzes of V. faba roots grown in these samples, including: root length, mitotic index and frequencies of micronuclei, chromosomal aberrations and types, were done.

The evaluated biological effects of some water samples appeared related to the physicochemical parameters. The results revealed seasonal fluctuation of samples toxicity, being more hazardous during September (dry season). The most polluted waters were the mouth of Moraça River (containing industrial effluents from an Aluminum plant upstream the river) and the main dump of Shkodra city (municipal wastes), inducing strong genotoxic and clastogenic effects on broad bean roots. It was noticed that mechanically pretreated waste water from Shiroka locality (municipal and touristic wastes) exerted relatively low toxic activity. Meanwhile there was observed no significant risk of anthropogenic influence in the open part of the lake.

This study highlighted the capability of V. faba assay in toxicity bio-monitoring of fresh and waste waters andthe necessity to combine physical-chemical analysis with cytogenetic approaches, for better understanding the impact of mutagens present in such waters on biota and human health.

Key words: Shkodra Lake, surface water, wastewater, *Vicia faba* assay, cytotoxicity, genotoxicity

Introduction

The assessment of water quality is considered essential for ecosystems balance and human consumption necessities. It is directly related to the application of monitoring and remediation projects aiming to minimize the risk that aquatic ecosystems undergo by hazardous substances. Distinguishing the anthropogenic inputs from the natural ones is the basis of this assessment (Wu *et al.*, 2007). Municipal, industrial and agricultural wastes can add to the environment significant amounts of mutagenic and genotoxic compounds that ultimately find the way to surface water and sediments of streams, rivers, lakes, and seas, causing long term implications on ecosystems functioning. Such contaminants may eventually pass through the food chains to humans and result in a wide range of adverse consequences including cancer induction, acceleration of ageing processes and the appearance of heritable diseases in offspring and reduction in fertility (Majer *et al.*, 2005). The contamination of water resources by genotoxins is a worldwide concern. Particularly developing countries as Albania have been suffering the impact of the increasing pollution caused by rapid urbanization and industrialization, followed by a littery discharge of different wastes without detoxification treatment in general due to high cost (Duan *et al.*, 1999; Singh *et al.*, 2014).

Shkodra region (North-West Albania) is distinguished for a unique and complicated water system, hydrologically and ecologically interdependent, which includes: Shkodra Lake (a transboundary lake with Montenegro), Buna and Drini Rivers and many streams. Shkodra Lake is the largest lake in the Balkan Peninsula, fluctuating seasonally from 370 to 600 km^2. It has some inlets, the biggest of which is Moraca River (Montenegro) providing more than 62% of the lake's water and one outlet, Buna River, which flows out to the estuary in the Adriatic Sea. The lake is situated on a karstic terrain, while many small islands and cryptodepressions (containing sublacustrine springs) are localized surrounding lake shores (Malollari *et al*., 2012). By representing one of the most diverse and interesting ecological areas of South-East Europe, it is included in the Ramsar List of Wetlands with a global importance (Ramsar, 2010). The south and south-western watersides of the lake are structured mostly by calcareous (limestone) rocks, serving for recreative and touristic activities. Meanwhile northern and north-eastern watersides are plain and siltstone, providing an extensive semi-littoral zone used as agricultural field. The ecological status of the lake is actually mesotrophic, with tendencies toward eutrophic levels during the summer period (Rakočević & Hollert, 2005; Rakaj, 2012). In spite of its importance, the lake is influenced by inflowing waters from regional rivers contaminated by the industry, municipal and agricultural activities in the area. Therefore, the lake has been the subject of various physicochemical and ecotoxicological examinations (Rastall *et al.*, 2004; Kostanjsek *et al.*, 2005; Rakočević & Perović, 2011; Kopliku *et al.*, 2011; Perovic *et al.*, 2011; Kopliku *et al.*, 2012; Malollari *et al*., 2012; Mesi & Kopliku, 2012; Neziri, 2012; Mesi & Kopliku, 2013).

Targeted physico-chemical analysis cannot normally control and predict the mutagenic potency of complex environmental mixtures, such as surface or waste waters, especially if synergistic, additive or antagonistic effects between the components occur. They should be combined with biological assays to accomplish an integral monitoring. With bioassays, the genotoxic effect of pollutants on a test system is determined and results may be extrapolated to other organisms. Plants, which represent a crucial link in food chains, are widely utilized to evaluate the genotoxic and mutagenic effects of risk factors, due to the strongly structural preservation of their genetic material and comparative simplicity, sensitivity, low cost and good correlation to other bioassys (Majer *et al.*, 2005; Barbério, 2013). *Vicia faba* L. (2n = 12) has long been used as a model plant with multiple endpoints, providing an important method in evaluating the induced-phyto- and genotoxicity from both organic and inorganic contaminants of soil, sediment, organic material such as sewage sludge or composts and water (Ma *et al.*, 1995; Duan *et al.*, 1999; Sang & Li, 2004; El Hajjouji *et al.*, 2007; Marcato-Romain *et al.*, 2008; Amin *et al.*, 2009; Ma *et al.*, 2012). Particularly, *V. faba* root test for chromosome aberrations and micronuclei is suggested to be an effective tool for the rapid screening of hazardous chemicals due to large chromosomes (Ma *et al.*, 1995).

The chemical monitoring of Shkodra Lake, extensively performed both in Albania and Montenegro during the last decade, has been followed by relatively few biomonitoring and collaborative studies. On the other hand, to our knowledge there are no chemical analyses of liquid wastes discharged in Albanian part of the lake. The present investigation was aimed to evaluate the possible seasonal toxicity of surface and waste waters collected from the lake, by using *in vivo Vicia faba* L. assay.

Material and Methods

Samples Collection and Physicochemical Analyses

Surface and waste water samples of Shkodra Lake were collected during April and September 2013 from five stations as follows (Figure 1): Shiroka (S_1) – at the discharge point of mechanically pretreated municipal and touristic wastewaters of Shiroka and Zogaj localities (by using a sedimentation tank); Moraça mouth (S_2) – sampled after the inflow of Moraça River (mouth) to the lake, containing waste water discharged mostly from an aluminum plant (KAP) upstream of the river; middle lake (S_3); Dobër (S_4) – leaching from soil contaminated by agricultural use and several unlined open dumps; the main collector runoff of Shkodra city to the lake (S_5) – municipal waste water from sewage network. Water samples were preserved in polyethylene bottles washed with 5% hydrochloric acid and rinsed out with abundant distilled water before collection. Temperature, conductivity, oxygen concentration and pH were monitored *in situ* with a multi-parameter Hydrolab DS5 water quality monitoring system. The labeled bottles with samples were transferred to the laboratory and stored in refrigerator in 2-4°C. Chemical and toxicity analyses were performed within 48 h from collection. Total nitrogen and phosphorous were determined by using indophenols blue absorption spectrometry and molibdate blue procedures, respectively. Tap water (65 mg/L Ca, 12 mg/L Mg, 0.4-0.45 mg/L Cl and pH = 7.5±0.06) was used as negative control sample (NC) as suggested by Fiskesjö (2006).

Figure 1.*Map of Sampling Stations in Shkodra Lake. S_1-S_5 – Surface and Waste Water Sampling Points*

Vicia faba Assay

Vicia faba L. cultivar *Aguadulce* was chosen as the test plant. Healthy-looking seeds of uniform size (1.6 ± 0.1 cm) were soaked for 24 h in distilled water and then the seedlings were allowed to germinate in 18.5 cm Petri dishes, between two layers of moist cotton. The phytotoxicity test was performed second Fargašová & Lištiaková (2009) by measuring primary root length of germinated seeds exposed to lake samples. Individual sets of ten seeds were utilized for each monthly sample, rooting under controlled conditions in thermostat (dark cultivation at 24 °C and 100% humidity) for 72 h.

Cyto- and genotoxicity test was done second El Hajjouji *et al*. (2007) with few modifications. After 3 days, the 2-3 cm long primary roots of untreated seedlings were suspended in aerated tap water tanks for 4 days and then their tips cut off in order to let the secondary roots grow. Exposure time was 30 h for the NC and 6 h for the treated groups followed by a 24 h recovery period. The newly emerged roots, being 1-2 cm in length, were used for microscopic analyzes. Root tips (10 mm) randomly chosen from each treatment set, were placed on slides and the terminal root tips (1-2 mm) were cut off and used for further preparation. Five slides were prepared per each treatment in accordance with the standard procedure for Feulgen staining of squashed material. Cytogenetic endpoints in root meristem, such as: mitotic index (MI), frequencies of interphase cells with micronuclei (MNC) and abnormal mitotic cells (FAC) with chromosome aberrations (CA), were observed and evaluated. By scanning slides, data for MI and FAC were collected on number of dividing cells out of 1000 cells, while at list 1500 interphase cells were counted for the formation of micronuclei. All values were scored as percent ratio.

Statistical Analysis

All experiments were set up in a completely randomized design and the results were expressed as the mean of three replicates per sample ± standard deviation (SD). Analysis of Variance (One-way ANOVA) and post-hoc Student Newman-Keuls (SNK) tests were used to test for significant values of evaluated parameters. Differences against corresponding NC were assumed statistically significant at $P<0.05$ and $P<0.001$.

Results

Physicochemical Analyzes

Data from the physicochemical analyzes of surface and waste lake water samples are summarized in table 1. The highest conductivity values were recorded in $S_5$and S_2 samples and generally during September. The detected dissolved O_2, total N and total P values of both S_5 seasonal samples were noticeably higher compared to others, especially middle lake (S_3). Additionally it was noted a high value of P content at Moraça sample (S_2). The physicochemical characterization of S_1, S_3 and S_4 samples revealed in general low seasonal variation, except for total N, which reached really higher values in September. The temperature spectrum varied 16.8 (S_1) – 18.3°C (S_4) and 21.3 (S_2) – 24.9°C (S_4) during April and September, respectively. The pH rates of the water samples were slightly alkaline and differed between 7.6 and 8.4.

Table 1. *Seasonal Physicochemical Analyses of Shkodra Lake Surface and Waste Waters*

Parameter	**Month**	**Lake water samples**				
		S_1	**S_2**	**S_3**	**S_4**	**S_5**
Conductivity (µS/cm)	April	207	228	219	211	249
	September	239	273	226	234	281
Temperature (°C)	April	16.8	18.2	17.7	18.3	17.4
	September	24.1	21.3	23.7	24.9	24.5
Dissolved O_2 (mg/L)	April	8.58	8.52	8.64	8.57	8.69
	September	8.66	8.63	8.71	8.92	9.10
Total N (mg/L)	April	0.27	0.31	0.011	0.38	0.53
	September	0.61	0.49	0.55	0.67	0.88
Total P (mg/L)	April	0.003	0.007	0.001	0.002	0.036
	September	0.002	0.024	0.002	0.005	0.044

Root Lengh and Cytogenetic Analyzes of V. faba Seedlings

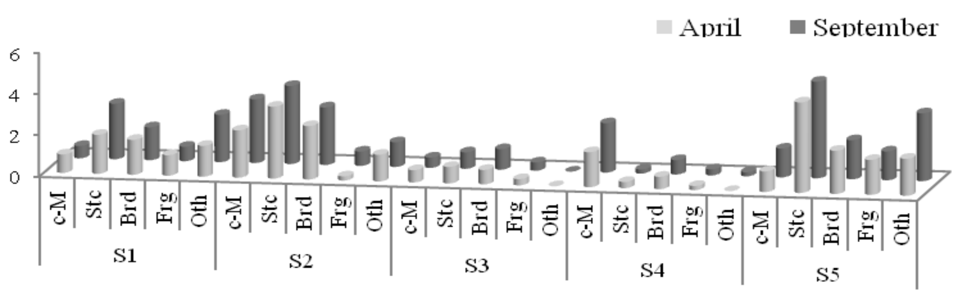
The results concerning length and cyto-genetic analyzes of *Vicia faba* seedling roots treated with lake and tap water samples are presented in table 2 and figure 2. Primary roots exposed to tap water (NC) for 72 h had a mean length of 2.96 cm. The two months bio-monitoring showed significant root length reduction of 21-29.9% (April) and 26-36.8% (September) through lake samples, compared to NC (P<0.05 using ANOVA test). The highest decrease of root length was induced by both seasonal Moraça samples (S_2), being significantly different from each other (p<0.05 with SNK test), as well. Significant inhibition of mitotic index (MI) in secondary root meristem of broad bean was detected across months only in following samples: S_2 (28-34%), S_5 (23-26%) and S_1 (20-24%) compared to the corresponding NC of 10.98% (P<0.05). In general, the reduction of mitotic activity resulted more severe during September.

Table 2. *Seasonal Phyto- and Genotoxic Effects of Shkodra Lake Surface and Waste Waters on Roots of Viciafaba Seedlings*

Samples	Month	MRL (cm)	MI (%)	MNC (%)	FAC (%)
NC		2.96 (0.56)	10.98 (1.14)	0.21 (0.08)	1.9 (0.4)
S_1	April	2.40 (0.41)	8.78 (0.62)*	0.64 (0.11)*	7.4 (0.6)**
	September	2.19 (0.27)*	8.34 (0.97)*	0.72 (0.07)*	7.9 (0.2)**
S_2	April	2.02 (0.32)*/a	7.91 (0.44)*/a	1.47 (0.12)**	11.6(0.8)**/a
	September	1.87 (0.19)*	7.25 (0.28)*	1.31 (0.28)**	9.8 (1.2)**
S_3	April	2.72 (0.47)	9.11 (0.72)	0.28 (0.04)	2.4 (0.1)
	September	2.61 (0.22)	9.87 (0.51)	0.36 (0.09)	2.7 (0.4)
S_4	April	2.34 (0.29)*	8.89 (0.37)	0.33 (0.03)	2.8 (0.2)
	September	2.46 (0.64)	8.56 (0.83)*	0.38 (0.06)	3.8 (0.7)*
S_5	April	2.21 (0.13)*	8.42 (0.22)*	1.09(0.16)**/a	11.0(1.3)**/a
	September	2.08 (0.25)*	8.13 (0.49)*	1.28±0.09**	12.7 (2.4)**

Within each column means labeled with asterisks and letters are significantly different respectively from negative control according to One-Way ANOVA test (* P<0.05; ** P<0.001) and between monitoring periods in SNK test (a p<0.05). NC-negative control; S_1-S_5 – lake surface and waste water samples; MRL-mean root length; MI-mitotic index; FAC-frequency of aberrant cells with chromosome aberrations; MNC- frequency of micronucleated interphase cells; standard deviations in parentheses

Figure 2. *Seasonal Spectrum of Chromosomal Aberrations in V. faba Root Tip Meristem exposed to Shkodra Lake Water Samples*



S_1-S_5 – lake surface and waste water samples; c-M- c-mitosis; Stc-stickiness; Brd-bridges; Frg-fragments; Oth-others: laggard, vagrant and ring chromosomes, multipolar anaphases

The frequency of abnormal dividing cells with chromosomal aberrations (FAC) of faba secondary root meristem exposed to S_1, S_2, S_4 (only in September) and S_5 samples resulted significantly different from respective tap water of 1.9% ($P<0.05$ and 0.001) and generally greater during September. Scored FAC values through monitoring periods were: 5.8-6.7 (S_5), 5.1-6.1 (S_2), 3.9-4.2 (S_1) and 2 (S_4) folds greater than NC. Even though S_3 samples (middle lake) caused slightly increased FAC values (2.4-2.7%), none were statistically significant compared to NC. The spectrum of observed chromosome aberrations (CA) was more or less the same through seasonal samples, but it notably varied between lake samples. The most frequent CA types included: stickiness, bridges involving one or more chromosomes, c-mitosis and fragments (Fig. 2). Sticky chromosomes resulted predominantly induced by S_5 (40-43% of respective FAC), S_2 (33-35%) and S_1 (25-34%) samples, which contain municipal and industrial wastes. Bridges (mainly in anaphase) as well were produced in high frequency by these lake samples (15-27%). Compared to other samples, S_4 (containing mostly agricultural wastewater) caused significant mitotic damage on *V. faba* root-tip cells, mainly in the form of c-mitosis (61-63%). Fragments as the least frequent CA type were considerably observed only in seedlings group treated with S_5 samples (11-15%). Meanwhile other CA types as: laggard, vagrant and ring chromosomes and multipolar anaphases were detected in lower frequency. It was noticed a relatively high rate of micronuclei in interphase cells of plant sets treated with lake samples (Tab. 2), especially S_2, S_5 and S_1, significantly exciding as high as: 6.2-7 (S_2), 5.2-6.1 (S_5) and 3-3.4 (S_1) folds the corresponding NC of 0.21% ($P<0.05$ and 0.001), respectively.

Discussion

In the current study the *V. faba* assay was applied to assess the possible toxicity of surface and waste waters collected from Shkodra Lake through root growth inhibition (phytotoxicity) and cytogenetic damage (cyto- and genotoxicity) induced. Related to root growth as the combination of cell division and elongation, significant reduction of length is a true indicator of rhizotoxicity, being a general phenomenon caused by most pollutants (Fiskesjö, 2006). Additionally, the mitotic index is considered to reliably identify the presence of cytotoxic pollutants in the environment (Grover & Kaur, 1999). MI reduction of 50% is considered a threeshold value for sublethal and lethal effects on test organism (Sharma, 1983). Data from literature have shown that the decreased mitotic activity in various test organisms treated with wastewaters might be due to the presence of trace metals and pesticides (Ivanova *et al.*, 2008; Amin *et al.*, 2009). In this investigation, the reduction of both root length and mitotic index could be positively correlated, showing that inhibition of root growth may be a consequence of mito-depressive effect of tested lake samples. On the basis of these phytotoxicity parameters the samples could be arranged in the rank order of root growth inhibition as follows: $S_2 > S_5 > S_1 > S_4 > S_3$.

Root meristematic tissue is presumed as a suitable tool for screening mutagenic potency of chemical pollutants present in water and the suppression of its mitotic activity is usually accompanied by an increase of chromosome aberrations (Power & Boumphrey, 2004). Additionally the inducement of micronucleated interphase cells is another biomarker of genetic damage accumulated during cell cycle, truly indicating mutagenic effect (Ma *et al*., 1995). Micronuclei are cytoplasmic chromatin-containing bodies, resulting from chromosome breaks or lagging that require a passage through mitosis to be recognizable. By the cytogenetic analyses of *V. faba* root meristem, it was revealed the presence of genotoxins in the tested water samples. The highest genotoxic effect was induced by: S_5, taken closed to the discharge point of the main sewage collector of Shkodra city to the lake and S_2, sampled after the inflow of Moraça River (mouth) to the lake, which contain mostly industrial effluents discharged from the Aluminium plant upstream to the river. The increase of abnormal cells with chromosome aberrations and micronuclei as high as 12.7 and 1.28%, respectively, induced by S_5 could be due to the synergistic and/or additive effects of genotoxic compounds found in sewage sludge, high heavy metal content, household detergents and chlorophenols (Ivanova *et al*., 2008). On the other hand unknown complex chemicals generated from industrial activity may attribute to the high cyto- and genotoxicity of Moraça sample. Unlike S_5, this sample could induce higher rate of MNC compared to corresponding FAC. Despite the mechanical pretreatment of municipal and touristic wastewater of Shiroka and Zogaj villages, located in the west coastline of the lake, the detected genotoxic effects of S_1 sample may be due to the mutagenicity of certain residual organic substances. Meanwhile rural runoff in S_4 sample, which susceptibly contains certain agriculturally originated organic substances (being toxic even at a low concentration), may be the reason of the relatively high genotoxicity induced. According to Rakočević & Hollert (2005), Shkodra Lake is on betamesosaprobic level of saprobity, which means that it is moderately polluted with organic compounds.

The most observed chromosome aberration types were due to chromatin dysfunction (stickiness, bridges and fragments) or spindle failure and damage (c-mitosis, laggards and multipolarity). Compared to other CA types, appreciable rates of sticky chromosomes and bridges, scored in wastewater-containing lake samples indicated their aneugenic action on the genetic material of *V. faba* roots. Stickiness is considered a common sign of highly toxic and irreversible effects on chromosomes. It is caused probably through immediate reaction of chemicals with DNA during its inhibition period, inducing DNA-DNA or DNA-protein cross-linking or through altering the physicochemical properties of nucleic acids and/or nucleoproteins, consequently leading to high percentage of bridges in anaphase and telophase and thereby to abnormal cytokinesis (Amin *et al*., 2009). Concerning c-mitosis, it has been suggested that pollutants as metals inhibit the activity of mitotic key enzymes by disturbing the cytoplasmic Ca^{2+} homeostasis (Liu *et al.*, 1995).

Among other CA types it was noted the relatively high percentage of chromosome laggards, induced by a weak c-mitotic effect, which fitted well with the markedly increased MNC frequency in interphase cells treated with S_2, S_5 and S_1 wastewater-loaded samples.

As represented in table 2, it was detected a certain correlation between cyto- and genotoxic endpoints of *V. faba* root meristem treated with the lake surface and waste waters. Tap water and middle lake samples, which did not induce any apparent citotoxic effects, did not produce any significant genotoxic effects, too. Contrariwise, S_1, S_2 and S_5 samples that induced strong inhibition of mitotic activity indicated simultaneously distinct genotoxicity. However, the recorded cytotoxicity rate of S_5sample (closed to the main sewage collector run-off to the lake) was not strictly correlated to the corresponding genotoxicity. This collector discharge considerable daily quantities of N and P salts, which might be responsible for the temporarily mito-stimulatory effect observed in seedlings set exposed to this sample. Despite the reported low genotoxic potential of municipal wastewaters (White and Rasmussen, 1998; Mesi & Kopliku, 2012), such wastewaters (mostly domestic) can achieve loading values that are several orders of magnitude greater than wastes from industries (Radić *et al*., 2010). This fact explains the higher genotoxic activity of S_5 compared to S_2 (industrial effluents).

The data of chemical analyzes and the corresponding toxic potential of lake samples generally correlated with (Tab. 1 & 2). S_5 and S_2 samples that had the highest levels of dissolved salts (determined by conductivity) resulted the most cyto- and genotoxic ones. Dissolved O_2 values of the municipal and agricultural wastewater samples (S_5, S_1 and S_4) were relatively higher compared to other water samples, most likely due to the discharge of organic matter. Additionally, the results revealed seasonal fluctuation of chemical proprieties and toxicity inducement of samples, which resulted more hazardous during September (dry season). This may be related to the dilution factor of abundant precipitation added to the lake during the rainy season (April). Ma *et al.* (1985), using *Tradescantia* MNC test, assumed that in general genotoxicity is decreased in lake water during rainy season. On the other hand we suggest that more pronounced genotoxic effects scored in seedlings set treated with S_2 April sample could be due to the large amount of industrial effluents accumulated down streaming Moraça River during the rainy season.

The unique features and wide range of endemic and rare or endangered species resulted in the classification of Shkodra Lake as a wetland site of international significance. In spite of its importance, the lake is influenced by contaminated inflowing waters due to different human activities in the surrounding area (Rastall *et al.*, 2004; Kostanjsek *et al.*, 2005). The main pollution sources in Montenegrin part of the lake include: the Aluminium plant (KAP) upstream on the Morača River; steel plant in Nikšić, untreated or inadequately treated wastewater, urban municipal (solid) waste, mineral waste oils and agricultural runoff in the Zeta plain (Rakočević & Perović, 2011). In Albanian part, more than 50-70% of the urban waste is deposited to the lake, while in general no wastewater treatment is conducted. The lake waters are also polluted by different unlined open dumps. Agricultural waste disposals in the east shore are another source of contamination for the lake. Most of the field is private property with farming activity. That's why actually it is difficult to control the agrochemicals usage, which has led to high amount of nutrients and organic compounds at this side of the lake. Moreover Drini River waters bring to the lake microelements from the chromium, copper, and iron/nickel mines (Kopliku *et al.*, 2012; Malollari et al., 2012; Mesi & Kopliku, 2012; Neziri, 2012; Mesi & Kopliku, 2013).

The current analyses of *Vicia faba* seedling roots in terms of growth inhibition, reduction of mitotic activity and induced-mutagenicity and genotoxicity revealed the presence of genotoxins mostly in the tested Shkodra Lake waters containing discharged wastes. Their identification and availability should be determined by chemical analyses in order to enable discovering the origin and to minimize the risk that this ecosystem undergoes by such hazards.

Conclusions

In conclusion the results of morpho- and cytogenetic analyses of *Vicia faba* seedling roots performed in this study revealed the presence of genotoxins mostly in the tested Shkodra Lake waters containing discharged wastes. Additionally, seasonal fluctuation of clima conditions (temperature, precipitations, etc.) can influence the availability of such pollutants and consequently their toxic impact. In order to protect the lake from further contamination, the treatment and recycling of wastewaters discharged into the lake is needed. This study also highlighted the capability of *V. faba* assay in toxicity bio-monitoring of fresh and waste waters and the necessity to combine physical-chemical analysis with cytogenetic approaches, for better understanding the impact of mutagens on biota and human population health.

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