БИОТЕСТИРАНЕ НА ВОДИ ОТ РЕКА МАРИЦА – ВЪЗДЕЙСТВИЕ ВЪРХУ КУЛТУРНИ СОРТОВЕ РАСТЕНИЯ
BIOTESTING OF THE *MARITSA* RIVER WATERS – EFFECT ON CROP PLANTVARIETIES

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Abstract

The effect of heavy metals and other toxic elements on plants can be assessed by variousbiotests involving easily determinable parameters such as: length of the underground and aboveground plant parts, biomass, seed germination, photosynthetic pigments content, etc. Simultaneous use increases their sensitivity, offsets their disadvantages and allows obtaining objective results. The aim of this study was to trace the effect of water samples from various points along the *Maritsa* River (with in the regulation lines of the city of Plovdiv) on selected crops.

During the period of the survey, the *Maritsa* River waters were polluted with Cd and Pb and of satisfactory quality in relation to the presence of Cu and Zn, since their quantity was under the adopted hygiene standards for Bulgaria. Results of the experiments withcorn and sunflower seeds showed that they had a negative effect on the germination, growth and leaf pigment content ratio of the crops.

The study confirmed that the use of plants as bio-indicators is an effective method for monitoring of contaminated waters. We can point out that the use of the waters of the *Maritsa* River for irrigation poses a risk to the cultivated crops in the region of Plovdiv. As a result, it is possible to degrade the quality of the crop, which is intended for the feeding of animals and people, however without observing visible signs of metal phytotoxicity.

Key words: biotest, germination, growth, photosynthetic pigments, the *Maritsa* River.

INTRODUCTION

Use of sensitive plants in assessing the risk of environmental pollution increased in recent years. This is due both to the guiding role of plants in the formation of primary productivity of ecosystems and to the changing opinion on their sensitivity to various xenobiotics, including heavy metals, in comparison with other biological organisms (Lewis, 1995; Hock and Elstner, 2005).

Effect of heavy metals and other toxic elements to the plants can be assayed by various laboratory bioassays (Köhl and Lösch, 1999), including easily determinable parameters such as: the length of the underground (roots) and aboveground (shoots) plant parts, biomass, seed germination, concentration of photosynthetic pigments and etc. (Vangronsveld and Clijsters, 1992; An, 2004). The combined use of these parameters in the research increases their sensitivity, offset their disadvantages and allows obtaining objective results.

Most resistant to heavy metals is the process of germination of the seed, which is due to the relative impermeability of the seed coatto the metal ions. In species, in which the coat is overcome easily (eg beans, peas, etc.), seeds quickly lose their ability to germinate at high external concentrations of heavy metals (Koshkin, 2010).

Root bioassay is the most widely used method for determining the plant tolerance to heavy metals in the experimental studies. It is based on the fact that the roots are the first organs which come into contact with contaminants, and that these elements in toxic concentrations inhibit linear growth of the roots (Woolhouse, 1983; Baker and Walker, 1989). It has been shown that the length of the roots is a very sensitive indicator and that heavy metals affect both the cell division in the meristem zone andtheir extension.

Increase in the biomass of the experimental plants is also widely used as an indicator due to its integral character and easy measurement. As a rule, root growth is more sensitive to metal stress when compared with the above-ground organs, as the roots accumulate considerably more heavy metals. For this reason, their part in the total biomass of plants experienced decreases (Vassilev et al., 1993).

It is believed that the growth parameters do not always provide enough objective information on phytotoxicity of soil and therefore, it is advisable to include functional indicators in the plant bioassays (Vangronsveld and Clijsters, 1992). Photosynthetic apparatus of plants is very sensitive to heavy metals excess in the root environment. Many authors have found that heavy metals cause several ultrastructural and functional changes and disturbances in the photosynthetic process (Lidon and Henriques, 1993; Krupa et al., 1993; Vassilev, 2002; 2003). Based on comparative physiological studies on cucumber, corn, bean and salad plants, a new plant bioassay classification of phytotoxicity of heavy metals polluted soils was created (Vassilev et al., 2009).

Muhammad et al. (2009) studied the accumulation of heavy metals in different wheat varieties grown in contaminated soils with sediments. During the study, they found that the concentration of heavy metals in plants can be used as an indicator of the degree of contamination of the area, but also reveal the capabilities of the different varieties as hyperaccumulators.

Report by the Basin Directorate – Plovdiv (Section 2 of the Management Plan for the river basin in the East Region 2010-2015, Volume 1 – Maritsa) pointed heavy metals as amain contaminants for the status of surface waters. Typical elements in Maritsa River are zinc, copper, manganese, and as priority shall indicate cadmium, lead and nickel. Contamination by these heavy metals is found in much of the watershed of the Maritsa River, particularly pronounced at the confluence of Topolnitsa River. Their distribution suggests accumulation in the food chain and possible negative environmental consequences.

Aim of this study is to trace the effect of water samples from various points along the course of Maritsa River (in regulation lines of Plovdiv city) on some eco-physiological parameters of selected crops.

MATHERIALS AND METHODS

Selection of water sampling points. Along the course of Maritsa River in Plovdiv city were selected five sampling points: Point 1 - western periphery of the city of Plovdiv, ring road; Point 2 - Sugar Factory, after discharge of wastewater; Point 3 –central city part, under the bridge Gerdzhika; Point 4 - eastern periphery of the city of Plovdiv, ring road; Point 5 - eastern periphery of the city of Plovdiv, after the outflow of water from wastewater treatment plants (Fig. 1).

Sixty liters of water have been taken from the middle of river flowinany point. Temperature, pH and conductivity of the water were measured immediately after sampling. Then, the content of lead, copper, zinc and cadmium was measured using express tests and portable device for field studies (pHotoFlex - WTW, Germany).

Water samples were transported to the Laboratory of Ecology, Faculty of Biology, University of Plovdiv “Paisii Hilendarski”, in order to set a bioassay with seeds of selected crops.

Biotest – experimental setup and methodology. We used seeds of twoeconomically important crops that have been grown in agricultural areas around the Maritsa River - *Zea mays* L. (Maize, variety Laureate - FAO 350) and *Helianthus annuus* L. (Sunflower, variety Heliasol).

Fifty seeds of maize and sunflower, respectively, were sown in containers with perlite at a depth of 2 cm. In each container were added 100 ml of the sampled water from a studied point. Control containers with seeds and dechlorinated water also were prepared. All containers were placed in chamber, at room temperature, period 12/12 day/night, for 14 days. Periodically they were irrigated with the same water. Before reporting, the plant material was fixed with 60% ethanol (Velcheva et al., 2013, with modification). Each experiment was conducted in three replications.

For each test variant were measured the following parameters:

- Germination = (number of sprouted seeds/ total number of seeds) × 100%
- Average length of shoots, cm
- Average length of roots, cm
- Shoot-to-root ratio
- Biomass of shoots, g fresh weight
- Biomass of roots, g fresh weight
- Concentration of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids in leaves, mg/g fresh weight
- Ratio chlorophyll *a*/chlorophyll *b* and ratio total chlorophyll/carotenoids.

Fig. 1. *Map of Plovdiv city and location of sampling points*

Length of the shoots and roots of experimental plants was recorded using graph paper. Biomass was measured with analytical balance (Model H440-21N, Kern & Sohn Gmb, Germany).

Extraction of the photosynthetic pigments was carried out according to the methodology of Schlyk (1965) using 90% acetone. Absorption was read on a spectrophotometer at a wavelength of 662 nm for chlorophyll *a*, 644 nm for chlorophyll *b* and 440,5 nm for carotenoids. All analyzes were performed in triplicate.

Concentrations of the pigments was calculated according to the following formulas:

$$C_a = 9{,}784 \times E_{662} - 0{,}990 \times E_{644}$$
$$C_b = 21{,}426 \times E_{644} - 4{,}650 \times E_{662}$$
$$C_{a+b} = 5{,}134 \times E_{662} + 20{,}436 \times E_{644}$$
$$C_{car} = 4{,}695 \times E_{440,5} - 0{,}268 \times C_{a+b},$$

where C_a – concentration of chlorophyll *a*, mgl^{-1},

C_b – concentration of chlorophyll *b*, mgl^{-1},
C_{a+b} – concentration of total chlorophyll, mgl^{-1},
C_{car} - concentration of carotenoids, mgl^{-1}.

Results were converted in mg g^{-1} as follows:

$$C_a' = (C_a \times V \times R) * g^{-1}$$
$$C_b' = (C_b \times V \times R) * g^{-1}$$
$$C_{a+b}' = (C_{a+b} \times V \times R) * g^{-1}$$
$$C_{car}' = (C_{car} \times V \times R) * g^{-1}$$

where C_a – concentration of chlorophyll *a*, mgl^{-1},

C_b – concentration of chlorophyll *b*, mgl^{-1},
C_{a+b} – concentration of total chlorophyll, mgl^{-1},
C_{car} – concentration of carotenoids, mgl^{-1},
V – volume of extract, l,
R – dilution,
g – fresh weight of the plant material, g.

Raw data from all analyses were processed using statistical software package Statistica 7.0. for Windows (Stat Soft Inc., 2006).

RESULTS AND DISCUSSION

Characteristics of water samples

Data on physico-chemical parameters of the tested water samples and control (dechlorinated) water are presented in Table 1. Differences in the measured pH, temperature and conductivity of all water samples turned out to be significant, although a slight trend towards alkalinity of pH towards the exit of the city was noticed.

We found that the content of Cd and Pb in the studied water samples from Maritsa River was higher while the content of Cu and Zn was lower than the maximum permitted concentrations according to the Regulation Norm № 18 on the quality of water for irrigation of crops (MOEW, 2009).

As regards cadmium, copper and lead, it was seen a growing trend towards the river through the city, as highest levels for cadmium were measured in the water samples from Point 2 and Point 5, for copper - from Point 3 and Point 4 and for lead - from Point 5. Zinc trend was decreasing and the highest value was measured in the east periphery of Plovdiv city (Point 1 and Point 2).

Biotest – morphometrical and physiological parameters

Results of the effect of water samples from the Maritsa River on germination, length and biomass of aboveground and underground plant partsof studied crops are presented in Table 2. Data on the content of photosynthetic pigments in leaves are presented in Table 3.

• Maize

Germination of seeds of maize exposed to the water samples from Point 2 and Point 3 was lower compared to control whilewe recorded a stimulatory effect of the water from the remaining three points (Fig. 2 a). Our results are confirmed by a number of authors as Wierzbicka and Obidzinska (1998), An

Table 1. Physico-chemical parameters of water samples in the beginning of the experiment

Water sample	Temperature	pH	Conductivity	Cd, mg l^{-1}	Cu, mg l^{-1}	Pb, mg l^{-1}	Zn, mg l^{-1}
Control	12°C	7,34	382 µS	<0,005	0,01	<0,01	0,07
Point 1	8,7°C	7,37	329 µS	0,029	0,03	0,24	1,18
Point 2	8,8°C	7,53	316 µS	0,038	0,02	0,25	1,47
Point 3	9,3°C	7,61	308 µS	0,021	0,08	0,27	0,53
Point 4	9,1°C	7,65	323 µS	0,025	0,07	0,29	0,71
Point 5	8,8°C	7,86	322 µS	0,044	0,05	0,35	0,68

Table 2. Germination, length and biomass of roots and shoots of studied crops after 14 day period exposition to the water from Maritsa River

Water sample	Crop plant	Germination	Germination to the control	Shoot length, cm	Root length, cm	Shoot-to-root ratio	Shoot biomass, g	Root biomass, g
Control	***Maize***	42%	-	12,22	11,71	1,04	13,4	23,9
	Sunflower	19%	-	12,34	6,76	1,83	10,4	5,6
Point 1	***Maize***	51%	121%	11,91	14,38	0,83	13,5	22,5
	Sunflower	7%	37%	11,6	5,2	2,23	3,2	1,2
Point 2	***Maize***	36%	86%	7,25	11,14	0,65	8,7	13,2
	Sunflower	6%	32%	13,5	8,25	1,64	1,8	6,5
Point 3	***Maize***	32%	76%	8,3	13,11	0,63	10,5	17,1
	Sunflower	17%	89%	13,18	9,06	1,45	17,2	14,0
Point 4	***Maize***	50%	119%	12,52	14,93	0,84	18,1	29,0
	Sunflower	12%	63%	14,69	9,03	1,63	6,8	2,2
Point 5	***Maize***	49%	117%	11,77	15,63	0,75	18,9	42,2
	Sunflower	10%	53%	12,19	7,76	1,57	8,2	2,0

Table 3. Content of photosynthetic pigments in the leaves of studied crops after 14 day period exposition to the water from Maritsa

Water sample	Crop plant	Chl *a,* mg g^{-1}	Chl *b,* mg g^{-1}	Total chlorophyll, mg g^{-1}	Carotenoids, mg g^{-1}	Ratio chlorophyll *a/b*	Ratio chlorophyll/ carotenoids
Control	*Maize*	21,51	13,67	35,18	6,44	1,57	5,46
	Sunflower	8,11	4,42	12,53	2,92	1,84	4,29
Point 1	*Maize*	17,81	9,64	27,45	5,53	1,85	4,97
	Sunflower	8,84	4,85	13,69	4,22	1,82	3,25
Point 2	*Maize*	17,73	10,07	27,81	5,56	1,76	5,00
	Sunflower	7,64	3,68	11,32	2,70	2,07	4,19
Point 3	*Maize*	13,50	6,75	20,24	4,31	2,00	4,70
	Sunflower	8,85	4,70	13,55	3,23	1,88	4,19
Point 4	*Maize*	17,54	8,72	26,26	5,20	2,01	5,05
	Sunflower	8,58	3,90	12,49	3,05	2,20	4,10
Point 5	*Maize*	16,25	8,45	24,70	4,84	1,92	5,10
	Sunflower	9,22	5,61	14,84	3,58	1,64	4,14

(2004), Mahmood et al. (2005) and Bashmakov et al. (2007) which have indicated the relative stability of the maize seed to the effects of heavy metals, as well as their good germination in the presence of low concentrations of copper.

As regards the shoot length, the impact of all water samples was inhibitory (Fig. 2 c), while in the root length inhibition was recorded only in the water from Point 2 (Fig. 2 d). This result led to the decrease of the shoot-to-root ratio in comparison with the control (Fig. 2 b). Similar results were reported by Ghani (2010b) who found that in the presence of high concentrations of pollutants maize shoots are characterized by difficulty increase, while underground parts remain relatively unaffected.

Reduction of biomass was found under the influence of water from Point 1, 2 and 3 (Fig. 2 e, f). In Point 2 and Point 3 could be assumed that this is a consequence of reduced germination and growth suppression. In Point 1, however, an explanation for this result could be sought in the opinions of other authors like Ghani (2010), which establishes a significant decrease of the growth and protein content in maize plants and their biomass under the influence of manganese, chromium, cadmium, lead, mercury and zinc, as well as combinations of these elements.

We found a statistically significant reduction of thecontent ofphotosynthetic pigments (Table 3). According Lagriffoul et al. (1998) the reduction of the amount of chlorophyll and carotenoids in the leaves of maize plants is one of the first visible biomarkers for phytotoxic effects caused by heavy metals. Values of the ratio chlorophyll a/b and ratio total chlorophyll/carotenoids were lower in all test containers (Fig. 2 g, h) in comparison with the control, which is a sign of increased synthesis of antioxidants as a result of induced stress.

• ***Sunflower***

In the biotests with sunflower seeds we found an inhibition of germination in all experimental variants, the most pronounced was the effect of the water samples from Point 1 and Point 2 (Fig. 3 a). Sunflower seeds have been shown to be relatively resistant to the effects of a range of heavy metals and toxic elements in many studies, but have been reported and similar to our results. Ahmad et al. (2009) tested the germination of seeds of different sunflower varieties with increasing concentrations of nickel from 0 to 60 mg l^{-1}. Its study revealed that at doses up to 20 mg l^{-1}, this element had positiveeffect on the process of germination and above 40 mg l^{-1} - negative. Fozia et al. (2008) found an inhibition of germination in three varieties of sunflower crops after adding chromium in the substrate at concentrations above 20 mg kg^{-1}.

Despite the reported negative impact on germination, we have found inhibition of growth only in the sunflower plants irrigated with water from Point 1 (Fig. 3 c, d), wherein the shoot-to-root ratio was maximal (Fig. 3 b). Biomass synthesis was reduced in all test variants with the exception of water samples from Point 3 (Fig. 3 e, f). Similar negative effect was reported by Gopal and Khurana

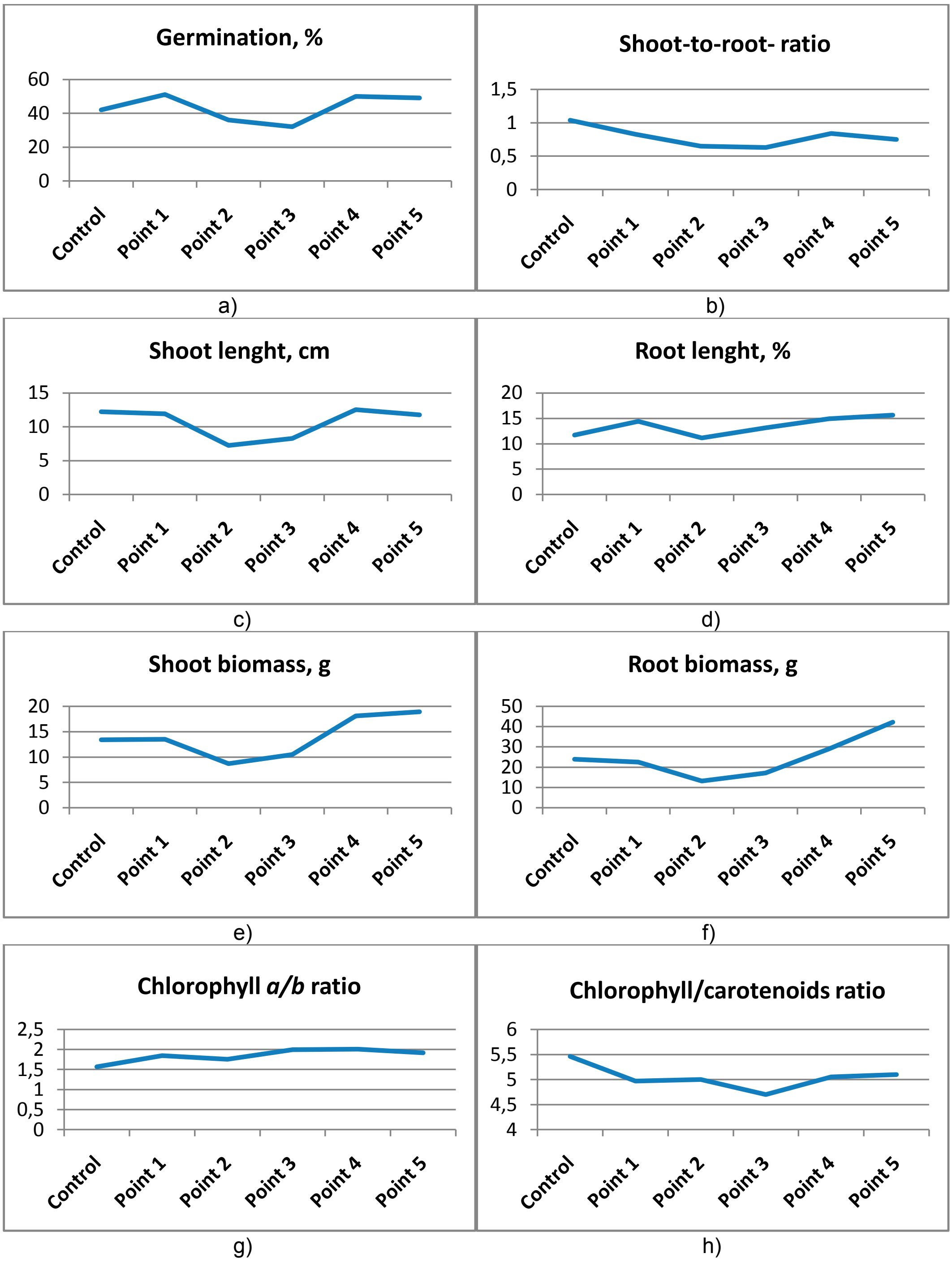

Fig. 2. *Results of biotest with maize seeds*

(2011) and Ullah et al. (2011) which experiments revealed with a reduction of 94% (chromium), 90% (nickel), 81% (cobalt), 69% (Cd), and 20% (Pb) of the biomass in comparison with the control.

Excluding watersample from Point 2, all other water samples proved a stimulating effect on the synthesis of chlorophylls and carotenoids (Table 3). Raise in the level of photosynthetic pigments in sunflower plants was also observed by Singh et al. (2004). In the course of their experiments with industrial waste water, after 30 days period of exposure the content of chlorophyll was higher, however, with time extension effect has become unfavorable and led to a reduction of its quantity relative to the control. This fact could explain the observed decrease of the ratio chlorophyll/carotenoids in all test variants (Fig. 3 g, h) in our biotests.

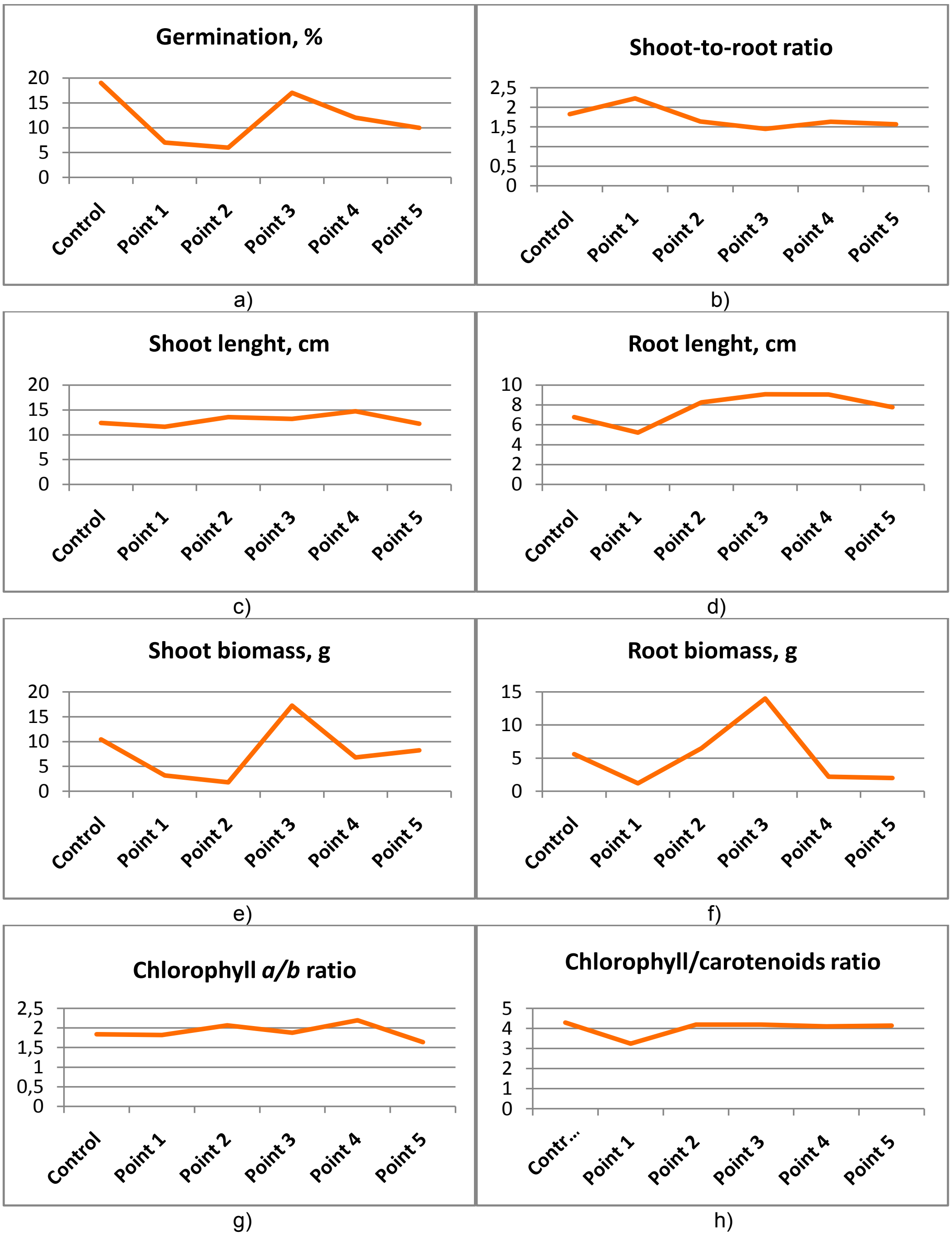

Fig. 3. *Results of biotest with sunflower seeds*

CONCLUSIONS

1. In the period of the survey, the Maritsa River waters were polluted with Cd and Pb. Studied water samples were of satisfactory quality in relation to the presence of Cu and Zn, since their quantity was under the adopted hygiene standards for Bulgaria.

2. Results of the experiments with corn and sunflowerseeds showed that they have a negative effect on the germination, growth and pigment content ratioof the crops.

3. This study confirmed that the use of plants as bio-indicators is an effective method for monitoring of contaminated waters. We can point out that the use of waters of the Maritsa River for irrigation poses a risk to the cultivated crops in the region of Plovdiv.

4. As a result, it is possible to degrade the quality of the crop, which is intended for the feeding of animals and people, without also observed visible signs of metal phytotoxicity (chlorosis, necrosis, etc.).

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