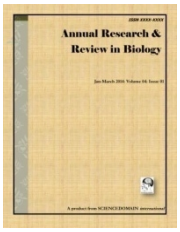

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New Data on the *Ceratophyllum demersum* L. as an Environmental Pollution Bioindicator

N. V. Ilyashenko[1*], M. B. Petrova[1], N. V. Pavlova[1], E. A. Kharitonova[1] and L. A. Kurbatova[1]

[1]Department of Biology, Tver State Medical Academy, Sovetskaya Str., 4, 170100 Tver, Russia.

Authors' contributions

This work was carried out in collaboration between all authors. Author NVI designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MBP, LAK and NVP managed the analyses of the study. Author EAK managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To perform an experimental analyses of the chemical composition and anatomic structure of polluted higher aquatic plants with the aid of combined physical methods of characterization by infrared spectroscopy, scanning electron microscopy and X-ray microanalysis to validate their use for environmental pollution bioindication.
Methodology: We used Fourier transform spectroscopy (FTIR), scanning electron microscopy (SEM) and X-ray microanalysis for the detection of anthropogenic pollution in nature and in the model experiments on the chemical composition and anatomic structure of bioindicator plants (hydrophytes) *Ceratophyllum demersum* L.
Results: A correlation between the changes existing in the IR spectrum of the plant samples and anthropogenic pollution of the plant inhabitation is established. Deformation and epidermis cell disruption were revealed in the samples from polluted sites and under the influence of salts of heavy metals (Hg_2SO_4, $NiSO_4$) and ammonium salts ($(NH_4)_2SO_4$, NH_4NO_3).
Conclusion: By the use of combined physical methods it was proved that higher aquatic plants have a capability to respond actively on the water chemical composition changes by the increase of absorption bands intensity related to contaminants.

**Corresponding author: Email: nadya_bioecology@mail.ru;*

Keywords: Fourier IR spectroscopy, scanning electron microscopy, X-ray microanalysis, Ceratophyllum demersum L., model experiment, anthropogenic pollution.

1. INTRODUCTION

A great role in bioindication is played by a number of higher equatic plants capable of accumulation of polluting substances in quantities exceeding the background values thus providing their use in systems of monitoring and environment control and making them ideal test objects for the determination of anthropogenic chemical loading of the water body in both native and modelling conditions [1].

A particular place among these plants are resided by dark green hornwort (*Ceratophyllum demersum* L.) and *Lemnamínor* L., which, in addition to its bioindicative properties, is also a source of valuable fodder vegetable substances [1-5].

Thus, *C. demersum* is an indicator of water organic pollution, acidification and contamination with heavy metals [1].At the same, *L. mínor* has the ability to indicator to the organic pollution and eutrophication (nitrogen, phosphorus) [1, 2].

In this view the interest increases in the realization of adequate biomonitoring and comprehesive analysis of the ecologically stressed zones effect on the vegetable making use of the modern exact analytical methods [5-7].

IR spectroscopic data on the chemical composition changes in bioindicator plants may be informative for the estimation of hydrosphere pollution in industrial regions. The exact identification of the types of compounds formed in the plant as a result of accumulation of various pollutants enables the use of Fourier transform IR spectroscopy (FTIR) for biomonitoring of acid pollutions (sulphur and nitrogen dioxides), petroleum products and also organic compounds [2, 7-11].

As far as bioindicator plants are subject to change at chemical and anatomic levels due to the action of anthropogenic environment pollution, these transformations may be effectively monitored with the aid of FTIR spectroscopy, scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) [12].

Combination of SEM and EDX makes it possible to determine the element composition in a volume of ~ 1 cubic micrometer by means of registration the X-ray characteristic irradiation occurring during the primary electron interaction with the sample surface. EDX is an analytical technique used for the chemical characterization of a sample [12].

The aim of this study was to perform an experimental analyses of the chemical composition and anatomic structure of polluted higher aquatic plants with the aid of combined physical methods to validate their use for environmental pollution bioindication.

2. MATERIALS AND METHODS

2.1 Plant Collection

C. demersum is a submerged perennial macrophyte which will normally grow with the base of its stem buried in sandy or silty substrates. It does not form roots (Fig. 1) [1,9].

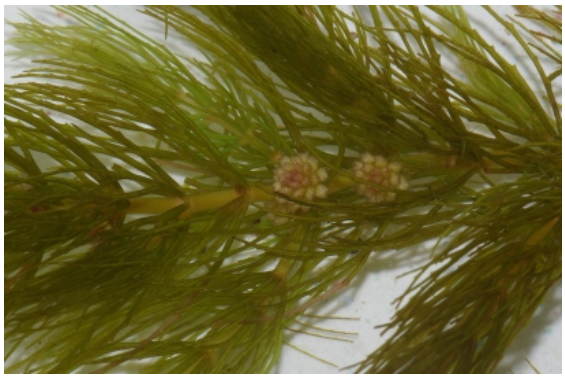


Fig. 1. Photo of investigated *C. demersum* sample

C. demersum can be used as a measure of lake pollution, as it can contain trace metals such as cadmium and lead in plant tissue [1, 2]. It can also be successfully used for heavy metal removal under dilute metal concentration. *C. demersum* is recommended for use in plantings for remediation of a dump site in Europe [1, 2, 9].

Ceratophyllum demersum L. occurs in Tver region in stagnant reservoirs with slowly flowing water, ponds, peaceful river backwater, cutoff meanders, and is able to grow both in clear and contaminated inhabitants [1, 2, 4]. Collection of the plants and water intake for chemical analysis were performed in water bodies of the city of Tver and Tver region classified by the factor of proximity to the sources of contamination in two groups – control and polluted (Table 1).

Table 1. Sites of *C. demersum* collection

o	Water body	Ecological status / Source of pollution [13, 14]		Main pollutants [13, 14]
I	Mezha river, Big Fyodorov village, Nelidov district of Tver region	SPNT* (control area), Central-Forest National Natural Biosphere Park		-
II	Coolant lake Udomlya, Udomlya, Tver region	Ecologically contaminated sites	Kalinin nuclear power station	Petroleum products, small radiation doses
III	Cunette, Redkino township, Konakov district, Tver region		Public corporation «Redkino pilot plant»	Petroleum products, inorganic anions, ammonia, aromatic compounds, anionic surfactants

** SPNT – Special Protected Natural Territory*

2.2 Analysis of the Water

In parallel the chemical analysis of the water from the sites of *C. demersum* growth was made to ensure the proper interpretation of the element composition and IR spectra of the aquatic plants from industrial regions. Chemical analysis was performed with the aid of a spectrofluorimeter "Fluorat-02-Panorama" and capillar electrophoresis system «Kapel-105» (Lumex). Determination of the contents of inorganic anions, surfactants, petroleum products,

phenols in water was made in accordance with standard methods described in [14-18]. We replicated the experiment three times and the average values are presented in this paper.

2.3 Sample Preparation

The IR spectra of the samples under study were recorded in the range of 400–4000 cm^{-1} by a standard method with potassium bromide [19] making use of the Fourier transform spectrometer «Equinox 55» (Bruker).

Scanning electron microscope studies were made in high vacuum regime with JEOL 6610LV SEM (Japan), EDX was performed with the INCA Energy system (OXFORD INSTRUMENTS, UK). The morphology of the epidermal surfaces was examined at the magnifications of ×500, ×600 and ×1000. Samples of the plants for SEM and EDX studies were dried at 30–40 ^{o}C and fixed by a graphite adhesive tape [6,12].

2.4 Model Experiment

The modelling of the effects of a number of pollutants (heavy metal salts (Hg_2SO_4, $NiSO_4$) and ammonium salts (($(NH_4)_2SO_4$, NH_4NO_3)) on *C. demersum* were performed in artificial conditions. For the model experiment a choice was made of the dark-green hornwort from a natural population of hydrophyte plants of control area (sample I) (Table 1). As it was already shown in a natural experiment these objects are highly sensitive to small concentrations of metal ions and other pollutants [1, 6, 9].

Under the laboratory conditions the plants were inserted at room temperature into vessels with the solutions of heavy metal salts (Hg_2SO_4, $NiSO_4$) and ammonium salts (($(NH_4)_2SO_4$, NH_4NO_3)with the concentrations of 0,02% for *C. demersum*. Preliminary the salt concentration was selected such that the plants under study demonstrated the properties of test organisms. The control group of hydrophytes was kept for 3 weeks in glass vessels filled with water without additions of model pollutants.

The plant samples for the analysis were selected weekly for a period of three weeks, washed and dried at t = 30-40^{o}C. Model samples were studied by the methods of IR spectroscopy, scanning electron microscopy and EDX at magnifications of 300, 500 and 1000 [6, 12, 19].

3. RESULTS AND DISCUSSION

3.1 Chemical Analysis of the Water in Reservoirs under Study

Results of the water analysis of the sites specified in Table 1 confirmed the existence of pollutants listed in Table 2, Fig. 2.

Main water indices (Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, phenols, anionic surfactants) in samples II, III are much higher as compared with the control (I).

In the water sample III the content of phenols and inorganic anions is by a factor of several tens larger than in sample I thus indicating discharges of chemical pollutants from the Redkino pilot plant (Table 2, Fig. 2).

Table 2. Chemical analysis of the water samples from the reservoirs under study

Index	Content, mg/l			EU Indicator index of water [20]
	I (control)	II	III	
Cl^-	1.2	6.2	64.0	250
NO_2^-	<0.2	0.9	5.0	0.5
SO_4^{2-}	2.8	7.4	42.3	250
NO_3^-	<0.2	1.2	<0.2	50
PO_4^{3-}	<0.2	<0.2	<0.2	0.5
Petroleum products	0.2	-	0.2	0.1
Phenols (total)	0.001	-	0.4	0
Anionic surfactants	<0.1	-	0.1	0.01

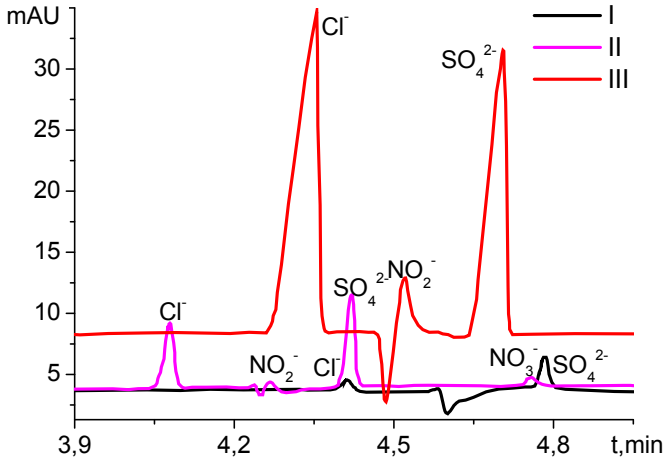


Fig. 2. Electrophoregrams of water samples from the reservoirs under study: I (control), II (lake Udomlya), III (Cunette, Redkino township), AU - absorbance unit

It should be noted that in a number of cases the data given in Table 2 for the concentration of pollutants in water bodies are higher than water quality parameters accepted by the EU countries [20].

The existence of various pollutants in the water reservoirs should be taken into account because aquatic plants are able to accumulate the contaminants (Table 2) [1, 2, 7, 11].

3.2 IR Spectral Analysis of *C. demersum* Samples

The IR spectra of *C. demersum* samples collected from different reservoirs are presented in Fig. 3.

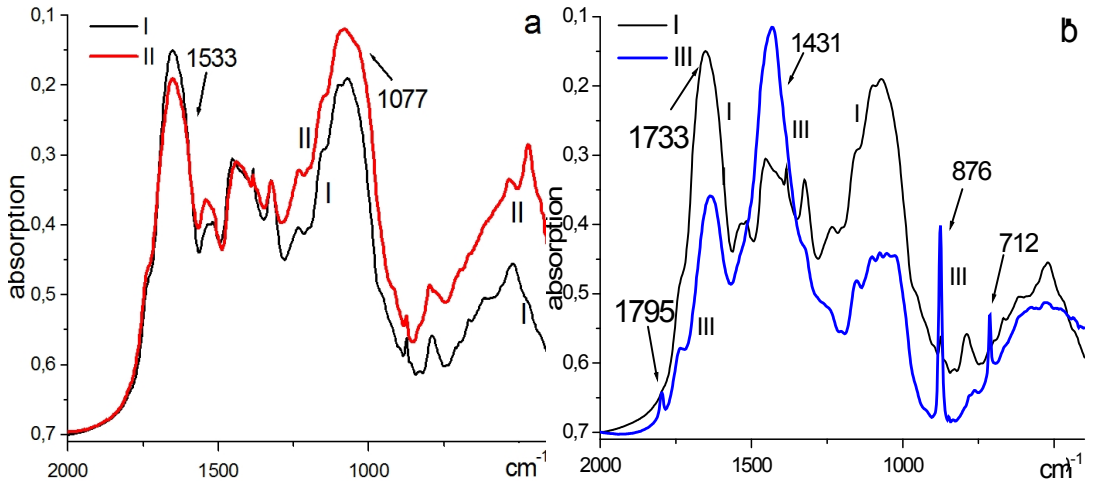


Fig. 3. IR spectra of *C. demersum* samples:
I (control), II (lake Udomlya), III (Cunette, Redkino township)

The IR spectral analysis shows that all samples under study have absorption bands corresponding to the main chemical components of the plant: carbohydrates ~56 % (of the absolute dry weight), proteins ~18 %, fats ~1 % [1]. The existence of carbohydrates in the plant is testified by absorption bands due to stretching vibration of CH_2 - groups at a frequency of ~2925 cm^{-1} and OH - groups at ~3400 cm^{-1} [5, 21]. The existence of proteins is evidenced by the absorption bands at ~1640 (Amid I), ~1535 (Amid II), and ~1235 cm^{-1} (Amid III) [7, 22]. The presence of fat may be judged by the existence of absorption bands at ~1735 ($\nu_{C=O}$), ~1446 (δ_{CH2-}) [2, 5, 7, 11, 19].

Spectra of plant samples from contaminated sites (Fig. 3) demonstrate essential changes at the following frequencies: ~2514 cm^{-1} (III), due to stretching vibration of S-H groups; ~1794 cm^{-1} because of stretching vibration of C=O groups (III); ~876 cm^{-1} owing to stretching symmetrical vibration of the S-O-C groups (III); and ~712 cm^{-1} by virtue of stretching vibration of C-S-C groups (III) [11, 19].

The absorption band at ~ 1431 cm^{-1}, attributed to $\nu_{as}(SO_2)$, δ(N-H), observed in all spectra of samples from contaminated sites, but it is mostly intensive in the spectrum of sample III on account of high concentration of sulphur-bearing anions absorbed from the water by the plant (Fig. 3).

Comparison of the IR spectra of *C. demersum* samples taken from control and contaminated reservoirs demonstrates considerable changes in the chemical composition of the plants. It is important that most significant changes of the absorption bands intensity correspond to the samples collected in the regions of industrial contamination. In samples collected from the reservoirs not subjected to direct contamination the band intensity conforms to the control values [7, 8, 11].

3.3 Examination of Hydrophytic Plant Samples with the Aid of EDX and SEM

Electron images and X-ray spectra of *C. demersum* samples studied with the aid of SEM and EDX are presented in Fig. 4.

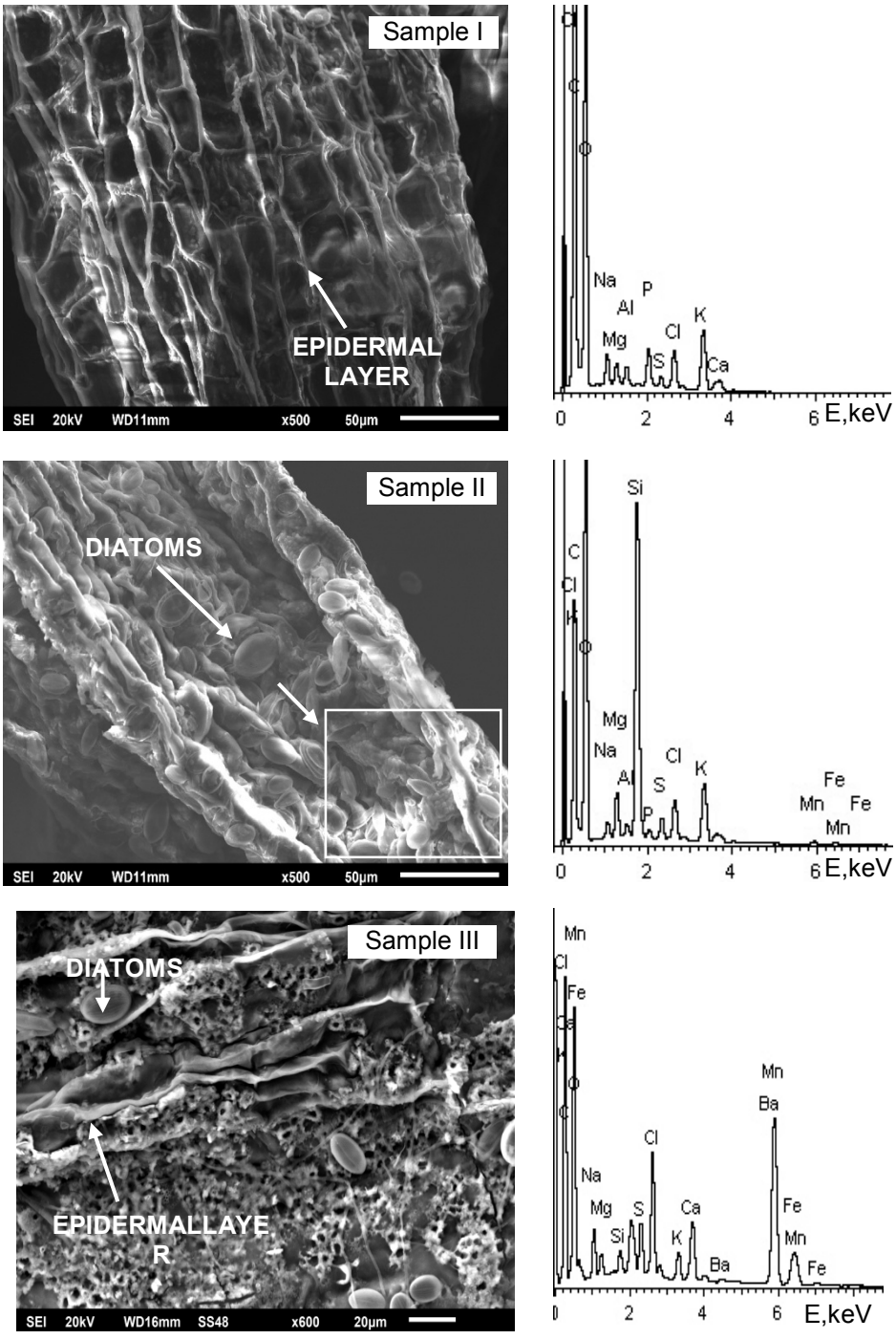


Fig. 4. SEM images and energy X-ray spectra of *C. Demersum* leaf samples I, II and III at magnification ×500, ×600

It is seen that in samples II and III collected in polluted sites there are deformation and destruction of epidermis resulting in violation of the epidermal cell layer integrity.

In sample II (Udomlyalake) the attention is attracted by the high density of diatoms (Diatomea sp.) at the epidermal layer of the hydrophytic plant under study. Such kind of hornwort leaves encrustation by diatoms (fouling) is to all appearance stipulated by the favourable conditions for their vital activity due to heightened thermal regime of the Udomlyalake of the Kalinin nuclear plant [14].

The elemental composition and quantitative chemical analysis of *C. demersum* plants was performed with the aid of EDX. Given below (Table 3) are the data for elemental composition and occurence of various chemical elements in the tissues of *C. demersum* from some water bodies of Tver region.

Table 3. Elemental chemical composition of *C. demersum*

Sample	Chemical element	C	O	Na	Mg	Al	P	S	Cl	K	Ca	Si	Mn	Fe	Ba	Total
I	Wt%	56.35	40.90	0.60	0.27	0.18	0.41	0.10	0.40	0.67	0.12	–	–	–	–	100
II		48.34	45.31	0.34	0.68	0.11	0.12	0.23	0.50	0.75	–	3.48	0.07	0.06	–	100
III		35.78	30.48	1.72	0.53	–	–	1.15	3.27	0.80	1.90	0.41	13.40	2.09	0.42	100

The EDX data reflect the overall chemical composition of *C. demersum* [1]. However in samples II and III the manganese, iron, sulphur, barium and chlorine content is higher than in the control sample, which is in accordance with the corresponding chemical analysis data of the water given in Table 2, Fig. 2. In addition, high content of silicon in sample II should be mentioned. Apparently the silica-containing frustules of diatoms observed on the hornwort foliage serve as a source of silicon [1, 9].

3.4 Examination of Hydrophytic Plant Samples under the Conditions of Model Experiment

In the model experiment on the effect of heavy metal salts (Hg_2SO_4, $NiSO_4$) and ammonium salts ($(NH_4)_2SO_4$, NH_4NO_3) on the natural population of hydrophyte plants (*C. demersum*) from the background zone the changes in the samples on both chemical and morpho-anatomical levels were detected.

The modification of characteristic absorption bands of IR spectra containing sulphonate groups was observed in the plant samples under the action of sulphate-containing salts (Fig. 5). The exposure of hornwort samples to ammonium salts results in a difference between the absorption band intensity of nitrogroups1384 $см^{-1}$ ($\nu_{s\text{-}O\text{-}NO2}$), which increases with the increase of the exposition time [7,9,11,19].

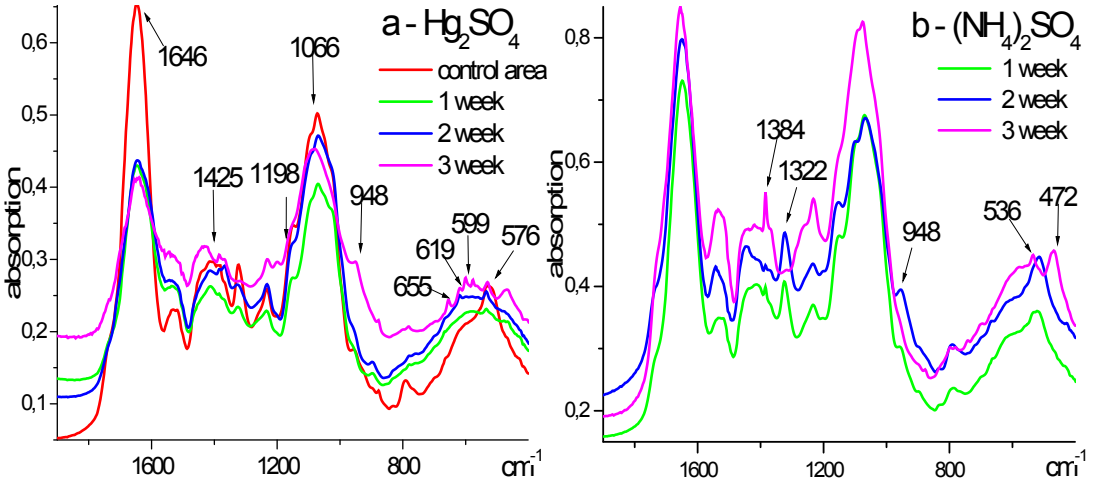
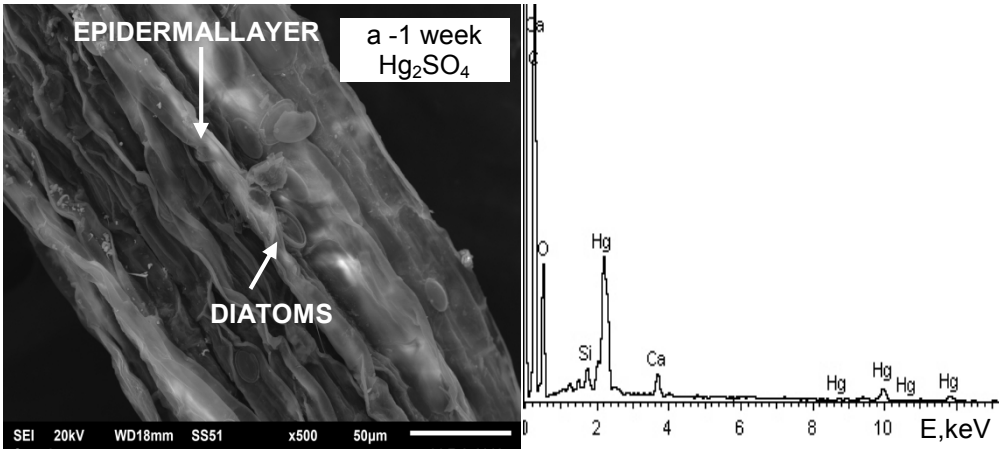


Fig. 5. IR spectra of *C. demersum* samples in the model experiment: a – Hg_2SO_4 (1-3 weeks), control area, b – $(NH_4)_2SO_4$ (1-3 weeks)

The IR spectral analysis of hydrophytes has shown that the main changes in the IR spectra of the samples related with the effect of heavy metal salts Hg_2SO_4 , $NiSO_4$, manifest themselves in the region of low-frequency oscillations whose intensity also increased in proportion to the time of the experiment (Fig. 5) [7,11,19].

During the study of the hornwort samples by the SEM and EDX methods the destruction – changes in the epidermal layer becoming apparent in the thickening of the cell walls in the epithelium, evidently due to the salts used in the model experiment, was observed in addition to the fouling of the plants with diatom weeds (Fig. 6)[6,21,22].



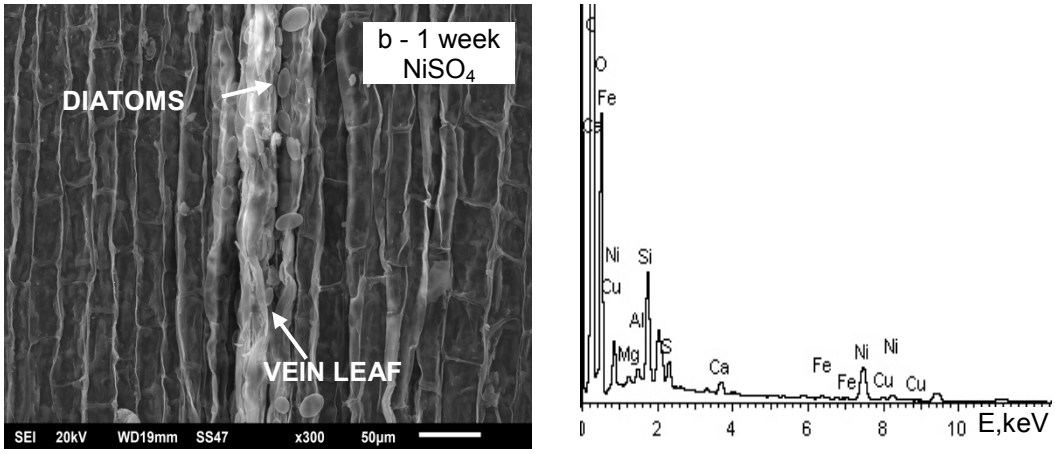


Fig. 6. SEM images and energy X-ray spectra of *C. demersum* leaf samples in the model experiment: a - 1 week, 0,02% Hg_2SO_4 (×500), b - 1 week, 0,02% $NiSO_4$ (×300)

In addition, crystals and concretions of metal and ammonium salts were observed at the epithelial surface of hydrophyte plants [2, 22].

4. CONCLUSION

The results of the study evidenced that the FTIR spectroscopy may be recommended for the effective application in biomonitoring of contaminated water bodies. An increase of the metal (Fe, Mn, Ba) and also S, Cl and Si concentration in the lamina of *C. demersum,* sprouting in a cunette (sample III) was demonstrated; furthermore formation of concretions and diatom weeds on the surface of the damaged epidermal layer was observed as a possible source of increasing silicon concentration.

Model experimentation devoted to the study of the effect of metal and ammonium salts (Hg_2SO_4, $NiSO_4$, $(NH_4)_2SO_4$, NH_4NO_3) on the samples of hydrophyte plants under study revealed similar changes in the chemical composition of *C. demersum*.

Thereby the comparative study of *C. demersum* hydrophytes has shown that higher aquatic plants possess high selective abilities in the accumulation of not only macro-, but also microelements and heavy metal salts.

The described physical methods of analysis may be effectively employed in biomonitoring of the ambient environment.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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