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Redox Reactions in *Hydrocharis morsus-ranae* L. under Industrial Impacts

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Abstract. Aquatic ecosystems are very sensitive to industrial impacts, and, therefore, it is increasingly important to study the mechanisms underlying the tolerance of aquatic organisms to water pollution. Heavy metals (HMs) are among the most common and toxic pollutants of aquatic ecosystems. They have a particularly strong effect on macrophytes, which are in close contact with the aquatic environment and can accumulate metals in considerable quantities. *Hydrocharis morsus-ranae* L. is a floating macrophyte (pleistophyte) with a high capacity for accumulation of HMs. The aim of the present study was to assess the effect of industrial pollution on the redox reactions in *H. morsus-ranae* and to identify the role of low molecular weight antioxidants in adaptation of this macrophyte to unfavorable conditions. A comparative analysis of the physiological and biochemical characteristics of *H. morsus-ranae* from two (reference and impacted) water bodies was carried out. The study revealed an increased level of lipid peroxidation products in the leaves of *H. morsus-ranae* under industrial impact, which indicates oxidative stress. Nevertheless, this floating plant demonstrated fairly high resistance to adverse conditions, due to the synthesis of non-enzymatic antioxidants such as proline and soluble protein thiols. Revealing the response of macrophytes to pollution of water bodies will help predict the state of aquatic ecosystems with an increase in anthropogenic pressure.

Keywords: floating macrophyte, heavy metals, oxidative stress, low molecular weight antioxidants.

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Редокс-реакции у *Hydrocharis morsus-ranae* L. в условиях техногенной нагрузки

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Аннотация. Водные экосистемы характеризуются высокой чувствительностью к техногенным нагрузкам, поэтому все более актуальным является изучение механизмов устойчивости гидробионтов к загрязнению водных объектов. Тяжелые металлы (ТМ) относятся к наиболее распространенным и токсичным поллютантам гидроэкосистем. Особенно сильное воздействие они оказывают на макрофиты, которые контактируют с водной средой и могут накапливать металлы в значительных количествах. *Hydrocharis morsus-ranae* L. относится к плавающим макрофитам (плейстофитам), обладающим высокой аккумулятивной способностью по отношению к ТМ. Цель исследования – оценка влияния техногенного загрязнения на редокс-реакции у *H. morsus-ranae,* а также выявление роли низкомолекулярных антиоксидантов в его адаптации к неблагоприятным условиям. Проведен сравнительный анализ физиолого-биохимических характеристик *H. morsus-ranae* из двух водных объектов (фон и импакт). Исследование определило повышенный уровень содержания продуктов перекисного окисления липидов в листьях *H. morsus-ranae* в условиях техногенного воздействия, что свидетельствует об окислительном стрессе. Тем не менее этот макрофит продемонстрировал достаточно высокую устойчивость к неблагоприятным условиям, что стало возможным благодаря синтезу таких неэнзиматических антиоксидантов, как пролин и растворимые белковые тиолы. Выявление ответных реакций макрофитов на загрязнение водных объектов будет способствовать прогнозированию состояния гидроценозов при усилении антропогенного прессинга.

Ключевые слова: плавающий макрофит, тяжелые металлы, окислительный стресс, низкомолекулярные антиоксиданты.

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Introduction

Hydrocharis morsus-ranae L. (Hydrocharitaceae Juss. family) is one of the widespread floating macrophytes (pleistophytes). Macrophytes play an important role in production processes, transformation of organic compounds, and biogeochemical cycles of elements in water bodies. Aquatic ecosystems are very sensitive to industrial impacts. Therefore, it is increasingly important to study the mechanisms responsible for the tolerance of macrophytes to environmental pollution (Polechońska et al., 2017; Gałczyńska et al., 2019).

Heavy metals (HMs) are among the most common and dangerous pollutants of the hydrosphere (Kabata-Pendias, Mukherjee, 2007). As is known, with an increased HM level in the habitat, aquatic plants can accumulate HMs in rather high amounts, which are sometimes several thousand times greater than the HM content in the surface waters (Borisova et al., 2016; Gałczyńska et al., 2019).

The high ability of *H. morsus-ranae* to accumulate such metals as Mn, Fe, Co, Ni, Zn, and Cu has already been noted (Brekhovskikh et al., 2009; Polechońska, Samecka-Cymerman, 2016). Polechońska and Dambiec (2014) reported that the contents of Mn, Fe, and Cu in the leaves of *H. morsus-ranae* exceeded the average levels observed for other floating macrophytes, even in cases of low metal content in water bodies. At the same time, the average concentrations of Zn, Mn, and Fe exceeded physiological values and corresponded to the level of toxicity (Polechońska, Dambiec, 2014).

The study of the HM accumulation by *H. morsus-ranae* during the growing season showed that the maximal contents of most HMs in plants and the highest bioconcentration factors were noted in June. It has been suggested that the increased content of metals in the floating macrophyte at the beginning of the growing season may be associated with the contact of hibernating buds with contaminated sediments during the winter (Polechońska et al., 2017).

The HM bioaccumulation in *H. morsus-ranae* shoots depends on the type of industrial activities. The highest levels of Cd and Co were found near plants producing organic compounds and the automotive industries; elevated concentrations of Cr, Cu, and Pb were noted near thermal power plant and former ferrochrome industry. At the same time, the concentrations of alkali metals, Co, and Fe in *H. morsus-ranae* were higher than in other aquatic plants regardless of their amounts in the environment (Polechońska, Samecka-Cymerman, 2016).

HM concentrations in the *H. morsus-ranae* leaves were ranked as follows: Mn > Fe > Zn > > Cu > Hg. Based on the bioaccumulation and translocation factors, it was concluded that *H. morsus-ranae* is an accumulator of Co, Cr, Cu, Fe, K, Mn, Ni, Pb, and Zn (Polechońska, Samecka-Cymerman, 2016). Moreover, the roots accumulate more HMs than the leaves (Gałczyńska et al., 2019). Significant positive correlations were also found between the contents of Zn, Fe, and Hg in *H. morsus-ranae* and in water, which indicates the possibility of using this floating macrophyte in the biomonitoring of water pollution (Brekhovskikh et al., 2009).

Thus, a lot of data have been reported on the high tolerance of *H. morsus-ranae* to environmental pollution with HMs and its accumulative abilities. However, there is only fragmentary information on the adaptive physiological and biochemical mechanisms contributing to the growth and vitality of this floating macrophyte even under considerable industrial impacts.

The aim of the study was to assess the effect of industrial pollution on redox reactions in *H. morsus-ranae* and identify the role of low molecular weight antioxidants in adaptation of this species to unfavorable conditions.

Materials and methods

H. morsus-ranae is a cosmopolitan perennial plant with numerous roots hanging down into the water column. The leaf blade is rounded; the aerenchyma is not developed. The plant has a high growth rate and vegetative reproduction, prefers water bodies with an average trophic status, grows best in low-flow habitats such as swamps, river backwaters, lakes, and reservoirs (Polechońska, Dambiec, 2014; Polechońska, Samecka-Cymerman, 2016).

Sampling of surface waters, sediments, and plant material was carried out in the Chelyabinsk Region (South Ural, Russia) in July 2018–2019. Samples were collected from two sites: the reference site (Site 1) and the impacted site (Site 2). The coastal water of the Irtyash Lake (55°52'22'' N 60°42'16'' E) was used as the reference site (Table 1). This reservoir lake is located in the Kaslinsky district of the Chelyabinsk Region. According to its primary productivity and nutrient content, the Irtyash Lake is a mesotrophic water body.

The Egoza River backwater (55°44'03'' N 60°30'57'' E) near the town of Kyshtym was used as an impacted site (Table 1). There are several industrial facilities in Kyshtym, and due to their activities, various pollutants, including HMs, enter the river. In addition, the increased contents of many HMs are associated with the presence of serpentinite rocks, which contain

Table 1. The pH value, specific electrical conductivity, and total index of toxic load in the study sites

Parameter	Site 1	Site 2
pH value of water	6.9 ± 0.1	6.8 ± 0.2
Specific electrical conductivity of water, µS/cm	392.7 ± 33.2	551.3 ± 35.3*
Si (water)	1.0	3.4
Si (sediments)	1.0	2.0

Data presented as Mean ± SE; asterisk (*) indicates significant differences between the study sites at $p < 0.05$.

high concentrations of Ni, Fe, Cr, and Co (Tripti et al., 2021).

The pH and specific electrical conductivity of the water were measured using a pH meter/conductometer (Hanna Instruments, Germany).

To determine the HM content, water and sediment samples were taken at four points of each site and mixed to prepare a composite sample. The content of metals was determined in three replicates by inductively coupled plasma atomic emission spectrometry (iCAP 6500 Duo, Thermo Fisher, U.S.A.) after wet digesting with 70 % HNO_3. As an integrated indicator of water and sediment pollution, the total index of toxic load (*Si*) was used, which was calculated using the formula (Bezel et al., 1998):

$$Si = (1/n)\sum_{i=1}^{n}\left(\frac{C_i}{C_{\text{reference}}}\right) \quad (1)$$

where, C_i is the concentration of metal in water/sediments of the impacted site; $C_{\text{reference}}$ is the concentration of metal in the water/sediments of the reference site; n is the number of metals studied.

Physiological and biochemical characteristics such as the rates of lipid peroxidation, content of carotenoids, free proline, phenolic compounds, soluble thiols and proteins were measured in the leaves of *H. morsus-ranae* spectrophotometrically on a PD-303UV (APEL, Japan), according to standard methods.

The rate of lipid peroxidation was determined by the reaction of malonic dialdehyde (MDA) with thiobarbituric acid (TBA). Absorbance was measured at 532 and 600 nm (Heath, Packer, 1968). The contents of photosynthetic pigments (chlorophylls and carotenoids) were measured at 470, 647, and 663 nm after extraction in 80 % acetone. The carotenoid content was calculated according to Lichtenthaler (1987). Free proline content was determined after extraction in hot water (95 °C) and boiling in a water bath for 20 min (Kalinkina et al., 1990). For staining, a mixture of ninhydrin reagent with glacial acetic acid (1:1) was used; absorbance was measured at 520 nm. The total content of soluble phenolic compounds was determined at 760 nm using the Folin-Chiocalteu reagent after preliminary extraction with 70 % ethanol solution for 24 hours. Gallic acid was used as a standard (Singleton et al., 1999). The extraction and determination of protein and non-protein thiols were performed as described by Borisova et al. (2016). The total content of soluble thiols was determined after reaction with Elman's reagent (5.5'-dithiobis (2-nitrobenzoic) acid) at 412 nm. The content of protein thiols was calculated by subtracting the amount of non-protein thiols previously obtained by precipitation of proteins with 50 % trichloroacetic acid from the total soluble fraction. Reduced glutathione was used as a standard (Borisova et al., 2016). The content of soluble protein was determined at a wavelength of 595 nm according to Bradford (1976). Bovine serum albumin was used as a standard. Determination of physiological and biochemical parameters was carried out in 4 biological and 3 analytical replicates (n = 12). All parameters were measured on fresh plant material and then calculated as per one g of dry weight (DW).

The significance of differences was assessed using the nonparametric Mann–Whitney test at a significance level of $p < 0.05$. The tables and figures show the mean values (Mean) and their standard errors (SE); asterisks indicate significant differences between the study sites.

Results and discussion

The study sites did not differ significantly in water pH, while the specific electrical conductivity of the surface waters in the impacted site (the Egoza River) was 1.4 times higher than in the reference one (the Irtyash Lake, Table 1).

The total index of toxic load at the impacted site (Site 2) was calculated from the contents of four metals (Fe, Zn, Mn, and Ni). The toxic load for water was 1.7 times higher than for sediments. The average value of this index for surface waters and sediments was 2.7 (Table 1).

The maximum difference between sites in the contents of metals was found for Fe (5.6 times), Zn (2.4 times), Mn (2.2 times), Ni (3.0 times), and Co (4.0 times) in water, and for Ni, Pb, and Zn in sediments (4.2, 2.7, and 1.6 times, respectively) (Table 2).

Most of the concentrations of metals in water exceeded the maximum permissible concentrations (MPC) for fishery water bodies (Water quality standards …, 2016). The highest excess of MPC was noted for mercury in the water of Site 2 (66 times), while for zinc, iron, and manganese it was 5 times higher on average. Among the metals studied, the most toxic to plant organisms are Hg, Co, Ni, Cu, and Pb (Kabata-Pendias, Mukherjee, 2007). Their contents in Site 2 were higher both in water and in sediments. The exception was Pb, whose concentration in the water of the impacted site was slightly lower than in the reference one (Table 2).

As is well known, an excess of HMs in the habitat causes inhibition of photosynthesis in plants, impaired transport of assimilates and mineral nutrition, growth inhibition, and changes in the water and hormonal status. Increased concentrations of HMs (especially of such redox-active ones as copper, iron, and manganese) can promote the generation of reactive oxygen species (ROS) (Blokhina et al., 2003; Gill, Tuteja, 2010). Lipid peroxidation, whose rates are assessed by the MDA content, is a reaction indicating damage to cell membranes (Blokhina et al., 2003; Sharova, 2016).

An assessment of the lipid peroxidation rate in the leaves of *H. morsus-ranae* showed that the MDA content in plants in Site 2 was 1.2 times higher than in Site 1 (Fig. 1a). An increase in the MDA content in the plants under anthropogenic load indicates the development of oxidative stress, despite the fact that most metals accumulate in the *H. morsus-ranae* roots.

The concentration of ROS formed in the cell is maintained at a sufficiently low level by a multicomponent antioxidant defense system, the state of which largely determines plant resistance to stress (Blokhina et al., 2003; Sharova, 2016). ROS and antioxidants interacting with them are presently considered as redox-active molecules, which are the main participants in redox processes (Pradedova et al., 2017). As is well known, ROS

Table 2. The contents of heavy metals in water and sediments of the study water bodies

Metal	Metal content in water, µg/L		Metal content in sediments, mg/kg	
	Site 1	Site 2	Site 1	Site 2
Fe	94.27 ± 8.87	527.90 ± 39.81*	26505.48 ± 1429.05	28602.91 ± 1293.90
Zn	25.42 ± 2.76	60.06 ± 1.36*	72.15 ± 2.51	115.19 ± 9.75*
Mn	19.27 ± 0.77	43.40 ± 1.50*	614.02 ± 3.06	768.17 ± 14.47*
Ni	9.16 ± 0.55	27.12 ± 2.08*	32.40 ± 1.05	136.90 ± 7.85*
Cu	19.53 ± 1.75	23.45 ± 0.19	60.06 ± 1.25	76.92 ± 1.35*
Co	0.50 ± 0.01	2.06 ± 0.16*	8.33 ± 1.65	10.94 ± 0.68
Hg	0.40 ± 0.01	0.66 ± 0.09*	13.44 ± 0.64	14.38 ± 0.33
Pb	5.41 ± 0.16	3.04 ± 0.40*	45.34 ± 1.26	120.46 ± 8.48*

Data presented as Mean ± SE; asterisk (*) indicates significant differences between the study sites at $p < 0.05$.

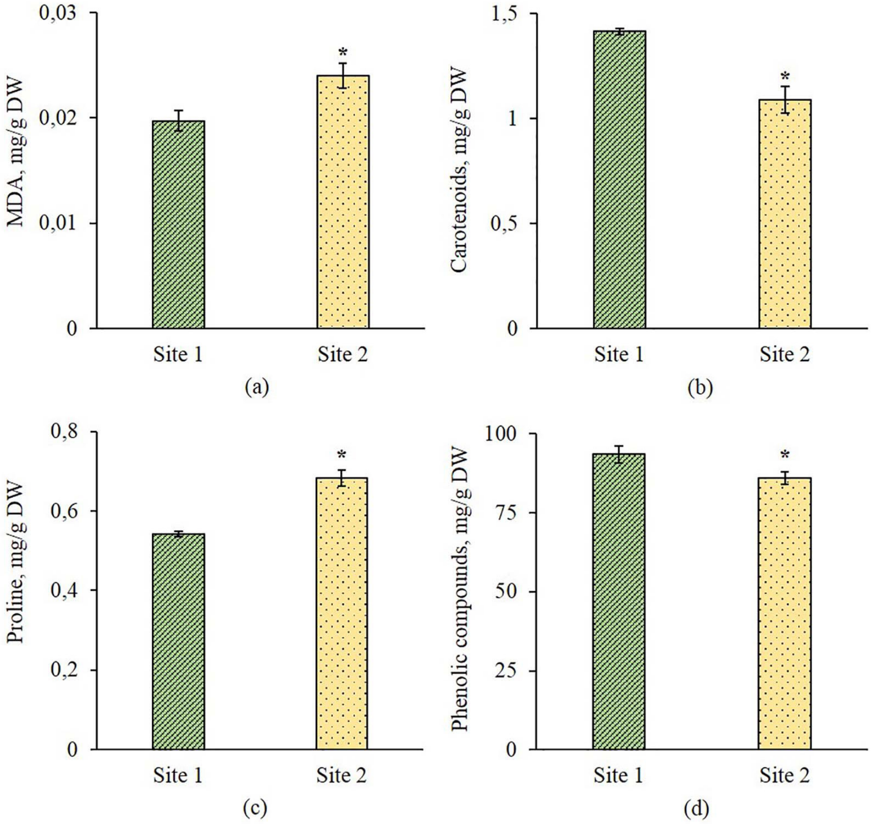


Fig. 1. Contents of malondialdehyde (a), carotenoids (b), free proline (c), and soluble phenolic compounds (d) in the leaves of *H. morsus-ranae*. Data presented as Mean ± SE; asterisk (*) indicates significant differences between the study sites at $p < 0.05$

in plants are involved in the regulation of many vital processes. At the same time, antioxidants play a leading role in the understanding of the physiological functions of ROS (Sharova, 2016; Pradedova et al., 2017).

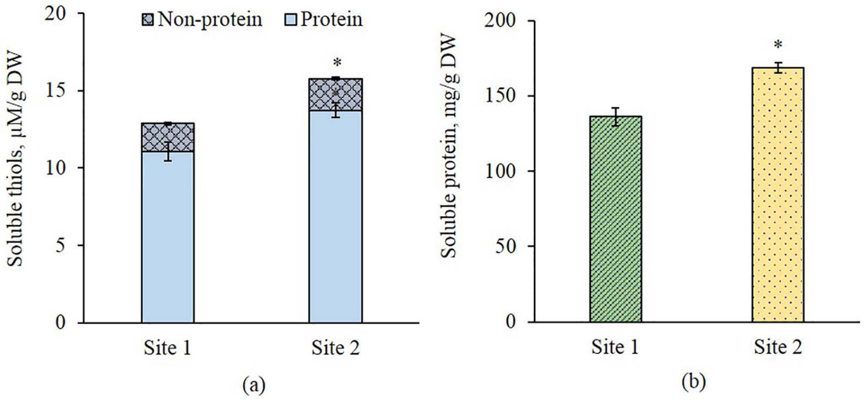
Carotenoids are the multifunctional compounds of plants. Not only do they take part in the absorption of light energy and the protection of green pigments from photodegradation, but they also have antioxidant activity (Strzalka et al., 2003). The content of carotenoids in *H. morsus-ranae* in Site 2 was significantly (1.4 times) lower than in Site 1 (Fig. 1b). A decrease in the carotenoid content in a habitat contaminated with metals was also noted in our previous study of the helophyte *Typha latifolia* L. (Maleva et al., 2019). Carotenoid molecules have double bonds, and they are oxidized when interacting with ROS (Strzalka et al., 2003). Thus, a decrease in the content of carotenoids in plants from contaminated habitats is apparently a consequence of their oxidative degradation.

Proline is a proteinogenic heterocyclic amino acid, which plays an important role in the plant cells. It is involved not only in osmoregulation, but also in the stabilization of proteins, membranes, and subcellular structures. Proline is also able to chelate HMs, maintain cellular redox potential, and participate in ROS neutralization (Hare, Cress, 1997; Sharova, 2016). The free proline content in *H. morsus-ranae* from Site 2 increased by 26 % compared to Site 1 (Fig. 1c), which indicates its active role in the adaptation of the macrophyte to the environmental pollution.

Many phenolic compounds are known to have antioxidant properties. Due to OH groups, phenols can participate in the detoxification of ROS (Sharova, 2016). Phenolic substances readily interact with reactive oxygen species. Initially, they are oxidized to phenoxyl radicals, the further oxidation of which leads to the formation of quinones. They can also chelate HMs and stabilize membranes, which limits the diffusion of free radicals and reduces the rate of lipid peroxidation (Michalak, 2006). As a rule, under stress, the synthesis of phenolic compounds is enhanced (Pourcel et al., 2007; Sharova, 2016). However, the present study demonstrated that the content of soluble phenolic components in the leaves of *H. morsus-ranae* in Site 2 was 17 % lower than in Site 1 (Fig. 1d). It can be assumed that, as a result of the high level of toxic load, the rate of destruction of phenols was higher compared to its synthesis reactions. Interestingly, our previous study of the helophyte plant *Typha latifolia* L. revealed an opposite trend for phenolic compounds (Maleva et al., 2019). That species demonstrated higher tolerance to industrial pollution than *H. morsus-ranae*. At the same time, they share similar trends in changes of the contents of other non-enzymatic antioxidants, which indicates a major role of these secondary metabolites in the formation of tolerance to HMs.

Compounds containing SH-groups (thiols), which can be divided into protein and non-protein ones, play an important part in the antioxidant protection of plants. Thiols can both bind HMs and act as antioxidants, participating in the neutralization of ROS formed during oxidative stress (Cobbett, 2000; Sharova, 2016). A previous study demonstrated that the contents of soluble thiols in different species of aquatic plants correlated with the accumulation of HMs (Borisova et al., 2016). The present study showed that the content of soluble thiols in the contaminated site (Site 2) was significantly (1.2 times) higher than in the reference site (Fig. 2a). Moreover, protein thiols prevailed over non-protein ones: their content was about 86 % of the total amount of soluble thiols.

Many proteins are involved in the antioxidant defense system of plants. They are capable of both directly chelating HMs and acting as enzymes, catalyzing the reactions of ROS neutralization (Kulaeva, Tsyganov, 2011). Determination of the total content of soluble protein revealed an increase in its amount in plants from Site 2 compared to the reference ones (by an average of 24 %, Fig. 2b).



(a) (b)

Fig. 2. Contents of soluble thiols (a) and protein (b) in the leaves of *H. morsus-ranae*. Data presented as Mean ± SE; asterisk (*) indicates significant differences between the study sites at $p < 0.05$.

Thus, the study of the *H. morsus-ranae* redox reactions to environmental pollution showed that the development of oxidative stress was accompanied by the accumulation of proline, soluble thiols, and proteins in cells, suggesting activation of their synthesis under anthropogenic load.

Conclusion

Comparative analysis of the redox reactions of the floating macrophyte *H. morsus-ranae* from natural habitats with different levels of industrial impact revealed some adaptive features of the plant. The increased level of lipid peroxidation products in the leaves of *H. morsus-ranae* from the impacted site indicates oxidative stress. At the same time, the macrophyte demonstrated a fairly high resistance to the pollution of the habitat by heavy metals, which was probably due to the increased synthesis of such non-enzymatic antioxidants as proline and soluble protein thiols.

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