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# Comparative characteristics of antioxidant capacity of some forage plants of the Baltic Sea Region (a case study of the Kaliningrad Region and Estonia)

G.N. Chupakhina[1,*], M. Shansky[2], A. Parol[2], N.Y. Chupakhina[3], P.V. Feduraev[1], L.N. Skrypnik[1] and P.V. Maslennikov[1]

[1]Immanuel Kant Baltic Federal University, Universitetskaya street 2, RU236040 Kaliningrad, Russia
[2]Estonian University of Life Sciences, Kreutzwaldi 1, EE51014 Tartu, Estonia
[3]Kaliningrad State Technical University, Sovetskiy prospect 1, RU236000 Kaliningrad, Russia
[*]Correspondence: natalie-tch@yandex.ru

**Abstract.** In this paper, we investigate changes in the antioxidant levels (anthocyanins, leucoanthocyanins, catechins) and the total water-soluble antioxidants capacity in forage plants in relation to their geography, i.e. proximity to northern or coastal areas. We demonstrate that the antioxidant content increases in unfavorable conditions, being higher in plants growing closer to the sea and in northernmost plants. Thus, since the total water-soluble antioxidants capacity is influenced by ecological factors, it may be used as one of the indicators in complex environmental assessment.

**Key words:** water-soluble antioxidants, anthocyanins, leucoanthocyanins, catechins.

## INTRODUCTION

The Baltic Sea Region countries: Poland, Lithuania, Latvia and Estonia are EU members geographically closest to Russia. Recently, they have been largely contributing to the implementation of European standards and regulations in industry and agriculture, especially in the border regions. For example, Russia is now developing its own system of field certification and precise farming, not unlike the one actively implemented by the Baltic EU member states. At the same time, the criteria for assessing quality of cultivated farmland crops are constantly being updated. The total water-soluble antioxidants capacity in agricultural crops can be used as one of such criteria (Noormets et al., 2010).

Antioxidants are sensitive components of plants, whose levels in a given plant is directly related to the state of environment (pollution of the soil and/or air with xenobiotics) and ecological conditions that determine the growth and development of plants (temperature, light, mineral nutrition) (Chupakhina, 2009). This topic is especially relevant for coastal areas, due to climatic features and specific vegetation period of plants (Chupakhina et al., 2014).

In addition, plant antioxidants are valuable biologically active compounds, with diverse biological effects. We distinguish between antioxidant enzymes and non-enzymatic (low-molecular-weight) antioxidants. The latter can be hydrophilic and hydrophobic. The hydrophilic antioxidants include ascorbic acid, reduced glutathione, and bioflavonoids. There are also fat-soluble antioxidants, such as ubiquinone, carotenoids, tocopherols. The protective effect of antioxidants is related to their ability to utilize free radicals in cells thus preventing premature aging and development of various diseases: atherosclerosis, different types of cancer, neurodegenerative and cardiovascular diseases (Maslennikov et al., 2014).

Thus, identifying plants that are high in antioxidants becomes an important task. Understanding antioxidant value of different plants is necessary not only for characterizing the quality of plant food, but also for identifying plants that can be used as nutritional supplements, functional food or genetic engineering material.

The purpose of this research was to investigate the effect of environmental factors on the total water-soluble antioxidants capacity, as well as the individual antioxidant levels of plants in meadow plant communities in the of the Baltic Sea coast regions and countries (Kaliningrad region, Russian Federation) and the Puhja municipality (Estonia).

## RESEARCH SUBJECT AND METHOD

Our research concentrated on forage plants: timothygrass (*Phleum pratense* L.), red fescue (*Festuca rubra* L.), cock's-foot (*Dactylis glomerata* L.), white clover (*Trifolium repens* L.), red clover (*Trifolium pratense* L.), bush vetch (*Vicia sepium* L.), cow vetch (*Vicia cracca* L.), large-leaved lupine (*Lupinus polyphyllus* Lindl.); and on the plants with medicinal properties: dandelion (*Taraxacum officinale* Webb.), yarrow (*Achillea millefolium* L.), nettle (*Urtica dioica* L.) and curly dock (*Rumex crispus* L.). Plant samples from the Kaliningrad region were harvested in the Zelenogradsky urban district (near the village of Roshchino) located 2 to 4 km from the sea (coastal zone), and in the Gusevsky urban district (near the city of Gusev), located 160 to 170 km inland (continental zone). In Estonia, plant samples were collected in the Puhja municipality (aproximately 25 km from Tartu), 140 to 150 km inland.

To assess the antioxidant status of plants we determined the total water-soluble antioxidants capacity and the levels of anthocyanins, leucoanthocyanins, and catechins. The analysis was carried out on freshly harvested leaves.

Total contents of antioxidants have been estimated by an amperometric method using a TsvetYauza-01-AA (NPO Khimavtomatika Inc., Moscow, Russia) according to Yashin (Yashin, 2008). Plant extract preparation: 0.2–0.5 g of plant material was homogenized with 50 mL of eluent (solution of phosphoric acid with the molar concentration of 2.2 mM). The mixture was then filtered and used for analyse in day of preparation. Amperometric method is based on measuring an electric current in the detector cell which occurs during oxidation of the extract on the working electrode surface when certain potentials are applied. The signal was recorded as differential output curves. The areas or peak heights (of the differential curves) were calculated for the extract and for the reference substance. The average value of three to five consecutive measurements was used for the analysis. The calibration curve was made by preparing quercetin solutions at different concentrations.

The total anthocyanins content was determined spectrophotometric after their extraction from plant samples. Plant extract preparation: 0.3–0.5 g plant material was homogenized with 10 mL of extraction solvent (1% HCl water solution). The extracts were centrifuged for 30 min at 4,500 rpm. The optical density of the above supernatant was determined at 510 nm (UV-3600, Shimadzu, Japan). To correct for the content of green pigments P.V. Maslennikov suggested to consider the optical density of the extracts obtained at 657 nm. As a standard the solution of cyanidin-3-glucoside was used (Chupakhina et al., 2016).

To determine the leucoanthocyanins and catechins, the weighed portion of the plant material was homogenized in the presence of an acidified 96% ethanol (20:1). The homogenate was centrifuged at 4,500 g for 30 minutes. The content of leucoanthocyanins was measured by Butanol-HCl assay. 1 mL of the supernatant was placed into test tubes with 19 mL of a 5% solution of HCl in n-butanol. The resulting solution was vortexed. The tubes were placed in a boiling water bath for 50 minutes, after thermostating the tubes were cooled. The absorbance at 520 nm was recorded using Shimadzu UV3600 (Japan). Cyanidin-3-glucoside was used as a standard (Feduraev et al., 2011).

The vanillin method based on the reaction of vanillin with condensed tannins which lead to formation of colored complexes was used for determination of catechins. In pre-prepared tubes with 4 mL of vanillin reagent and hydrochloric acid (2.5 mL of a 5% alcohol solution of vanillin + 47.5 mL of concentrated HCl) was poured 1 mL of supernatant – starting with blank solution. The contents of each tube were mixed and transferred to the cuvettes. The absorbance was measured 5 minutes later after adding the extract to the vanillin reagent, the reference solution was used as a control. The measurements were carried out at a wavelength of 520 nm. (+)- Catechin was used as a standard (Feduraev et al., 2011).

The content of the tested antioxidants in plant samples was shown in mg $g^{-1}$ of dry weight (for the total water-soluble antioxidant capacity and catechins in curly dock), and in mg 100 g of dry weight (for anthocyanins, catechins and leucoanthocyanins).

All measurements were performed in Natural Antioxidants Laboratory of the Immanual Kant Baltic Federal University.

One-way analysis (ANOVA) was performed using the SigmaPlot 12.3 (Systat Software GmbH, Erkrath, Germany). Before ANOVA data were checked for normality and the homogeneity of variance. To identify the difference between species in different region the 1-factorial ANOVA was conducted for each zone separately. And then the reliability of differences in means of antioxidants content between different regions for each plant species was calculated. Difference among means were determined by Tukey's test at a significance level of $p < 0.05$. The results were reported as mean ± standard deviation (SD).

## RESULTS AND DISCUSSION

The total water-soluble antioxidants capacity in forage plants of meadow plant communities in the Kaliningrad Region, located at different distances (2–4 km and 160–170 km) from the sea are shown in Fig. 1.

The total water-soluble antioxidant capacity was significantly higher in cock's-foot, timothy-grass, red clover and large-leaved lupine growing in close proximity to the Baltic coast. We found no major differences in water-soluble antioxidant capacity in white clover and cow vetch. Red clover ranked the highest in total water-soluble antioxidant capacity: with more than 2.5 mg g it had up to 5 times the capacity of other plant species.

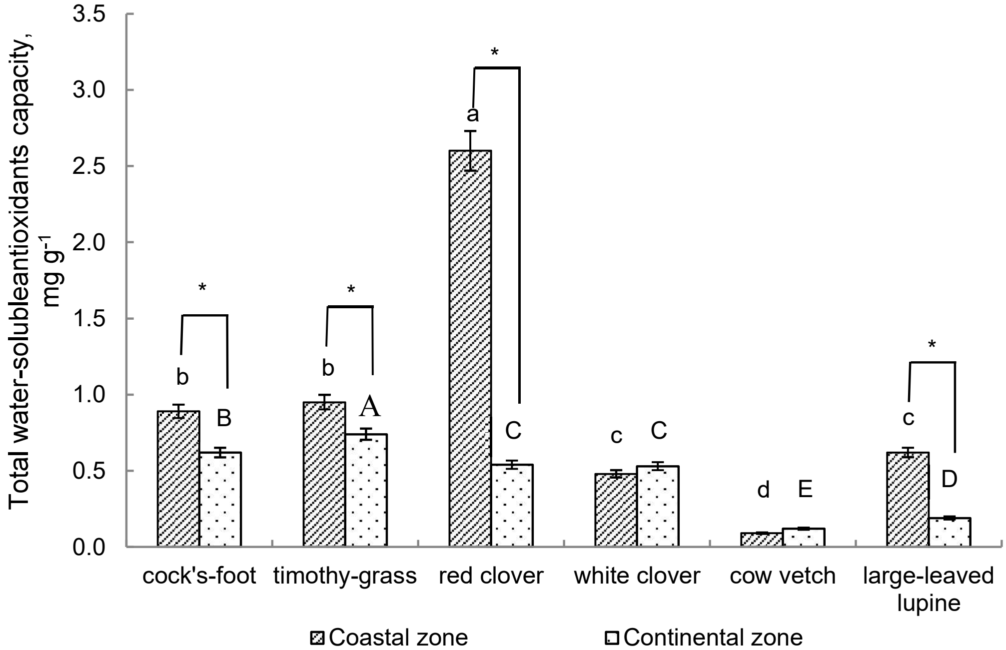


**Figure 1.** Total water-soluble antioxidant capacity in plants of the Kaliningrad region at different sites. Different lower case letters indicate significant differences among plant species in coastal zone, upper case letters indicate significant differences among plant species in continental zone ($p < 0.05$), asterisk * indicate significant differences among coastal and continental zone ($p < 0.05$) based on post hoc Tukey's tests.

Anthocyanins were higher in timothy-grass, cock's-foot and curly dock from meadow plant communities of the coastal part of the region compared to those growing in the continental zone (Fig. 2). In general, we found the greatest amount of anthocyaninsin the leaves of curly dock in coastal zone (more than 5 mg per 100 g).

We also looked at catechins content in plants of meadow plant communities at different distances from the Baltic Sea. It was established that the most coastal zone samples had higher catechins values (Fig. 3). However, timothy-grass plants from continental zone were characterized by higher content of catechins. It is necessary to note there was no significant difference between catechins contents in curly dock of costal and continental zones. Curly dock had the highest accumulated catechins content - up to 400 mg 100 g, almost 50 times higher than in the other plants.

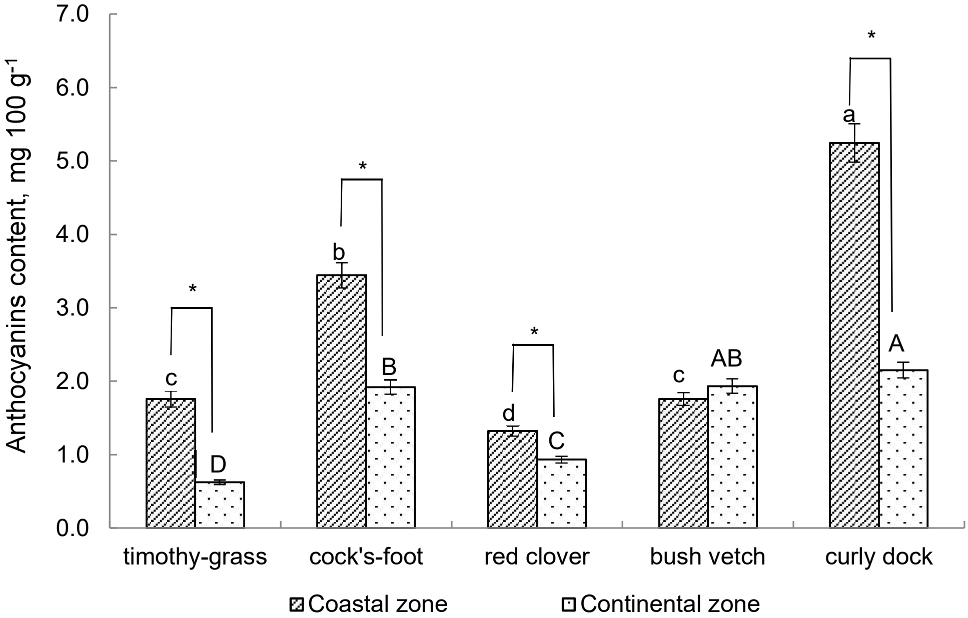


**Figure 2.** Anthocyanins content in plants of the Kaliningrad region at different sites. Different lower case letters indicate significant differences among plant species in coastal zone, upper case letters indicate significant differences among plant species in continental zone ($p < 0.05$), asterisk * indicate significant differences among coastal and continental zone ($p < 0.05$) based on post hoc Tukey's tests.

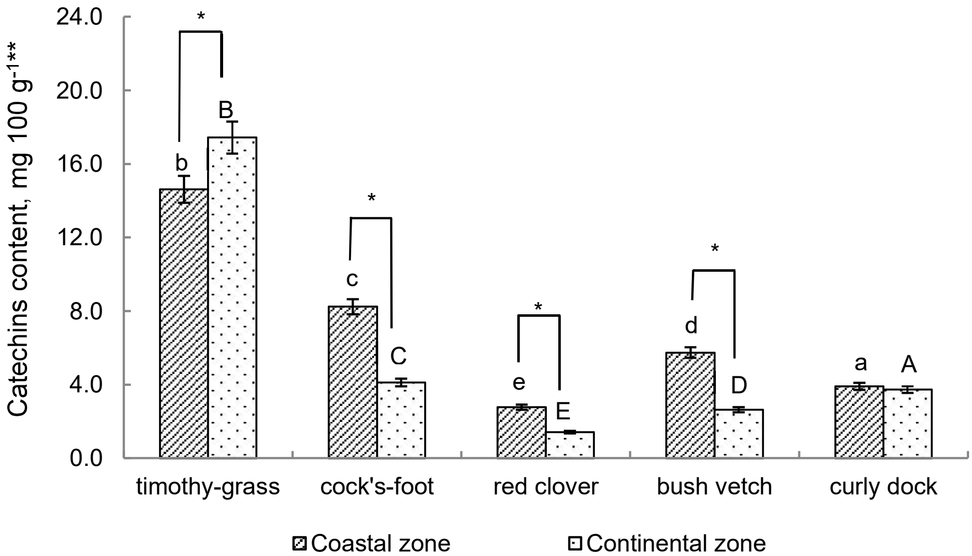


**Figure 3.** Catechins content in plants of the Kaliningrad region at different sites (** – catechins content in curly dock are shown in mg $g^{-1}$). Different lower case letters indicate significant differences among plant species in coastal zone, upper case letters indicate significant differences among plant species in continental zone ($p < 0.05$), asterisk * indicate significant differences among coastal and continental zone ($p < 0.05$) based on post hoc Tukey's tests.

A comparative study of leucoanthocyanins showed that their levels were higher in curly dock, cock's-foot, timothy-grass and bush vetch harvested in the coastal area (Fig. 4). Curly dock showed the highest content of these compounds, more than 35 mg 100 $g^{-1}$.

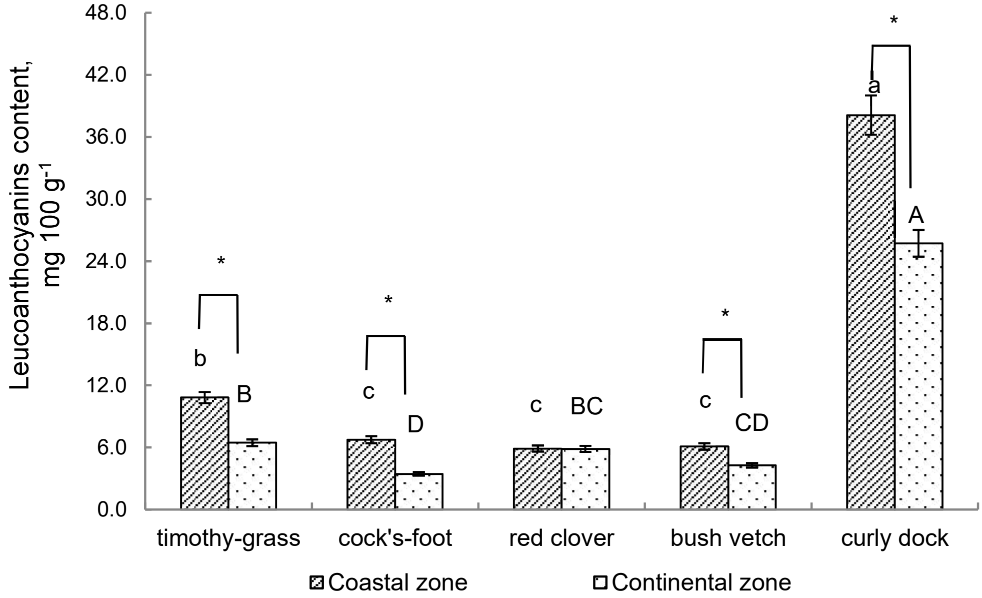


**Figure 4.** Leucoanthocyanins content in plants of the Kaliningrad region at different sites. Different lower case letters indicate significant differences among plant species in coastal zone, upper case letters indicate significant differences among plant species in continental zone ($p < 0.05$), asterisk * indicate significant differences among coastal and continental zone ($p < 0.05$) based on post hoc Tukey's tests.

The results of the studies of total water-soluble antioxidants capacity in fodder plants of meadow plant communities in the territory of the Puhja municipality, Estonia, as well as a comparison of the collected data with the results for the samples of the same plant species harvested near the city of Gusev in the Kaliningrad region, also located more than 150 km inland are shown in Fig. 5.

The data presented in Fig. 5 shows that plants growing in Estonia generally display higher total water-soluble antioxidants capacity. Reliably higher values were established for all plant species, except dandelion and nettle, where the difference in the antioxidant content between the plants of the Kaliningrad region and Estonia was statistically inconsistent. The same species of plants were characterized by a generally lower content of water-soluble antioxidants, compared to other plant species both in Estonia and in the Kaliningrad region.

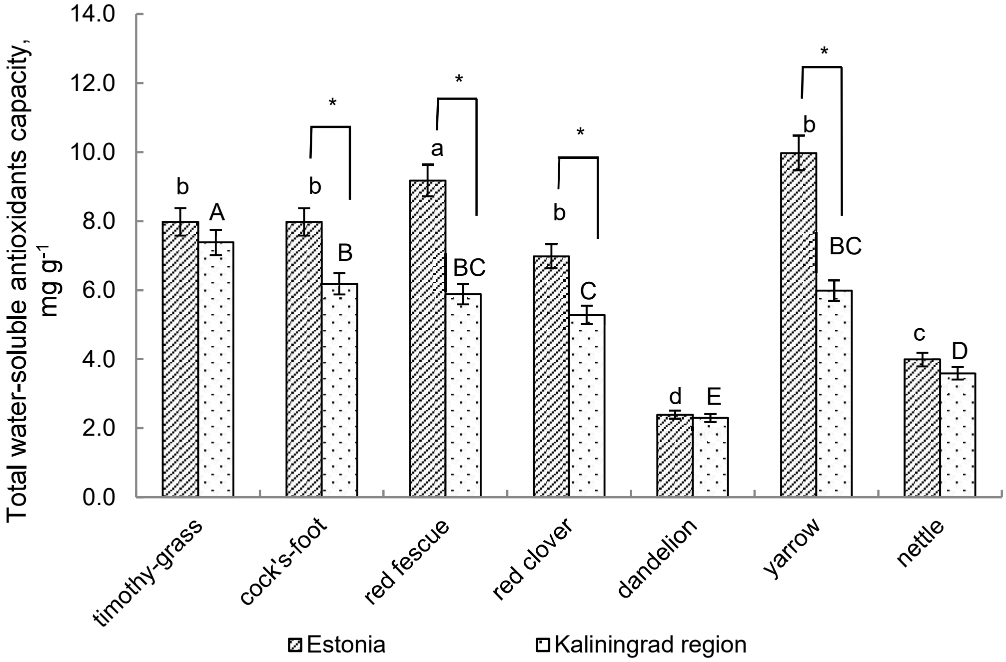


**Figure 5.** Comparative characteristics of the total water-soluble antioxidants capacity in the meadow plant species of the Puhja municipality (Estonia) and the vicinity of Gusev (Kaliningrad region). Different lower case letters indicate significant differences among plant species in Estonia, upper case letters indicate significant differences among plant species in Kaliningrad region ($p < 0.05$), asterisk * indicate significant differences among Estonia and Kaliningrad region ($p < 0.05$) based on post hoc Tukey's tests.

A study of the accumulation of antioxidants in plants of the Kaliningrad region at different sites showed that almost all plant species of the coastal zone (cock's-foot, timothy-grass, red clover, large-leaved lupine) had higher antioxidant content. In these plants, the total water-soluble antioxidants capacity (Fig. 1) was higher (or significantly, up to 5 times higher, as in the case of red clover), and the level of individual antioxidants – anthocyanins (Fig. 2), catechins (Fig. 3), leucoanthocyanins (Fig. 4) – was also higher. The climate features are illustrated by the Table 1. The Baltic Sea creates special microclimatic conditions in this part of the region: the wind is constant and is generally stronger, the summer temperatures are relatively low and the humidity is high (Dedkov & Fedorov, 2015). Thus, increased levels of antioxidants in the coastal plants may probably be explained by the antioxidants' protective functions. It is known that under the influence of stress created, for example, by low temperatures, the content of ascorbic acid (Golovina et al., 2008), anthocyanins (Chon et al., 2012), polyphenols (Ramakrishna & Ravishankar, 2011) increases in plants. The results obtained in this study are consistent with the findings of a previous study of the total water-soluble antioxidants capacity in forage plants at different sites of the Kaliningrad region, which also revealed a direct correlation between the distance from the coast and the level of antioxidants in plants (Chupakhina et al., 2013). The protective function of antioxidants can explain a higher level of water-soluble antioxidants in plants growing in Estonia (near Tartu), located further north from the Kaliningrad region (58° 22'00"N and 54° 35'00" N, respectively).

**Table 1.** Climatic parameters in different zones (coastal and continental) of the Kaliningrad Region and Estonia for the summer period (April – October)

| | Zone | Average temperature, °C | Precipitation, mm | Average air humidity, % | Average wind speed, m $s^{-1}$ |
|---|---|---|---|---|---|
| Kaliningrad | Coastal | 18.7 | 525 | 76 | 5–6 |
| | Continental | 21.4 | 475 | 70 | 3.5–4 |
| Estonia* | Coastal, Vilsandi | 11.9 | 358 | 81 | 5.6 |
| | Continental Tartu-Tõravere | 11.8 | 457 | 75 | 2.9 |

Source of data: *The characteristics of Estonian climate are based on the database of the Estonian Weather service (Riigi Ilmateenistus, 2018) of the Kaliningrad region the climate characteristics are based on the information from the Hydrometeorology and Environmental Monitoring Center of Kaliningrad (Maslennikov et al., 2018).

Moreover, the results obtained during the study give the opportunity to consider the level of antioxidants as an indicator of the state of the environment. For this purpose, either total water-soluble antioxidant capacity or concentrations of individual antioxidants could be used. The greatest change under the influence of environmental factors is established for the content of anthocyanin pigments. It must be noted, however, that such change is not specific to this cause. A number of studies have shown an increase in the number of anthocyanins stimulated by a deficiency of mineral components in the soil (Henry et al., 2012; Msilini et al., 2014), by various pollutants (Chupakhina & Maslennikov, 2004; Maslennikov et al., 2013), high light intensity (Zhang et al., 2010), or by UV irradiation (Gao & Yang, 2016). Therefore, an integral indicator (total water-soluble antioxidant capacity) is better suited for judging the state of the environment.

Among the studied so far plants, legumes were the most sensitive to environmental changes. The pool of water-soluble antioxidants in legumes was less stable than in grasses (Poaceae), where the level of antioxidants was less dependent on whether the plant community was coastal or not. Since accumulation of antioxidants is species-specific (Maslennikov et al., 2014), comparative studies of plants in different climatic and/or environmental conditions should focus on a single species to eliminate inter-species variations of indicator values. For the region in this study the species of choice can be red clover.

## CONCLUSION

We have demonstrated that the antioxidant status of plants species dominant in the meadow plant communities of the Kaliningrad region is, among other factors, determined by the proximity to the sea: the level of antioxidants was higher in plants growing closer to the Baltic Sea that in those growing further inland. Geographical zoning, and, more specifically, latitude, also plays a role: in more northern locations the antioxidants capacity of plants was higher than that in the southern ones. These conditions affect the content of anthocyanins, leucoanthocyanins, catechins, and the total water-soluble antioxidants capacity of plants. The latter can be used as a marker in assessing the state of the environment. Legumes are well-suited to serve as indicator species, since their pool of water-soluble antioxidants is not as stable as that of grasses (Poaceae). For the studied region, we recommend using red clover as an indicator species.

Red clover is native to Europe, Western Asia and North Africa and is naturalised in many other parts of the world (Edwards et al., 2015) that can also be explained by an active system of antioxidant protection (Kaurinovic et al., 2012), which makes it possible to adapt the physiology of this plant to various environmental conditions.

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