GROWTH AND METABOLISM OF *CHLORELLA VULGARIS* UNDER THE INFLUENCE OF MANGANESE AND IRON

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Abstract

The aim of this study was to investigate the effect of manganese(II) and iron(III) on the growth and basic metabolic processes of the unicellular alga *Chlorella vulgaris*. To this end, alga cultures were treated with ions of iron(III) and manganese(II) at concentrations 0.1, 0.5, 2, 5, 10, 20 and 50 mg L^{-1} and then analyzed in terms of changes in the number of cells, content of monosaccharides, proteins, chlorophyll and the activity of antioxidant enzymes: superoxide dismutase and glutathione reductase. The development of *C. vulgaris* was influenced more by manganese(II) than iron(III). The largest increase in the number of cells and concentrations of the studied biochemical parameters and antioxidant enzyme activity was induced by 20 mg L^{-1} manganese(II). The experiment also showed that an appropriate amount of iron(III) helped control the level of manganese(II) thanks to the co-precipitation of metals, and so could help in tackling the worldwide problem of eutrophication. In the case of introduction of 50 mg L^{-1} of both iron(III) and manganese(II), the number of *C. vulgaris* cells decreased, and thus the concentration of the biochemical parameters in water. The results of these experimental studies well collaborate with a study conducted earlier of surface water, which showed the existence of a strong correlation between the concentration of manganese in the water and algal biomass, whose determinant is chlorophyll *a*.

Keywords: *Chlorella vulgaris*, manganese, iron, eutrophication process

Introduction

In plants, iron is mainly associated with energy conversion required for photosynthesis and other processes. For example, iron protein compounds in chloroplasts are involved in the conversion of light into chemical energy. Iron also stimulates the formation of chlorophyll, although it is not included in its composition. It participates in a variety of redox reactions in plants that are associated with multiple metabolic processes: respiration (a component of many enzymes, e.g. cytochrome oxidase, reductase), photosynthesis, metabolism of nitrogen compounds, or takes part in the metabolism of nucleic acids. Ions of iron(II) play a very important role in biochemical processes carried out by

cyanobacteria as they activate hydrogenases and enzymes of the photoreduction and respiration systems.

Luka and Aegerter (1993) found that iron concentrations lower than 140 μg L^{-1} resulted in the slow proliferation of cyanobacteria while microcystin concentrations increased by an average of 30%; exactly the inverse relationship was observed by Utkilen and Gjølme (1995). Iron taken up by plants is mostly iron(II) in chelates. Iron is usually transported in plants in organic compounds, e.g., citric acid, or in anionic form. Environmental iron(III) is less digestible than iron(II) and therefore reduction processes on the surface of plant roots take place very slowly; reduction in further stages of metabolic processes proceed much easier. Reduction and oxidation can be carried out over a full range of pH and different aerobic conditions by autotrophic and heterotrophic micro-organisms (serving as catalysts) or without them. For example, colloidal iron(III) hydroxide precipitates even at pH>2.28 when the concentration of iron(III) in the solution is 19.4 mg L^{-1}, which corresponds to a Knop medium. When the environment becomes neutral or slightly alkaline, co-precipitation of iron and manganese occurs (Graham and Copper 1959).

Manganese is one of the trace elements necessary for the survival of plants and animals. In plants it is crucial for photosynthesis as an essential part of photosystem II (water oxidation center) which catalyzes the release of oxygen. This center is a cluster of manganese incorporated in one of the ends of the protein and is the basic unit of the enzyme catalyzing the oxygen release reaction (Fraústo de Silva and Wiliams 1991). Manganese deficiency inhibits growth and photosynthetic activity in *Chlamydomonas* (Allen et al. 2007). Manganese is a trace metal for 'special tasks', because it accumulates in enzymes such as superoxide dismutase or acid phosphatase glycoside. It also influences the reduction of nitrate ions(V) in plants, the hydrolysis of peptides, amides (peptidases) and urea (arginase). Plants absorb manganese passively and metabolically. Passive absorption concerns dissolved manganese(II); its transport in the plant occurs primarily in a Mn^{2+} form or as dissolved organic matter (Van Goor and Wiersma 1976).

Manganese deficiency in plants can be induced by the antagonistic effect of other elements, including iron. Manganese, similar to iron, undergoes multiple oxidation-reduction processes either involving or without the participation of microorganisms. For example, *Metelloglinium personatum* produces catalase that induces oxidation of manganese(II), while *Escherichia coli* produce formic acid which is able to reduce manganese(IV) oxide. Goto et al. (1999) found that algae also secrete numerous metabolites in the environment, for example sugars or amino acids, which are a source of the electrons used for the reduction of manganese(IV).

Metabolites of algae, like algae themselves, are the cause of increasing eutrophication in the world. Intensive growth of algal biomass, resulting in algal blooms, leads to the inhibition of photosynthesis, and at a later stage a significant deterioration in water aerobic conditions. This extremely important problem may be dealt with by modifying iron and manganese concentrations in the environment, among other things. Previous research has dealt with the impact

of these ions on the development of higher plants, and algae, but there is a lack of information on the impact of these metals on the basic metabolic processes of *Trebouxiophyceae* in aquatic environments especially *Chlorella vulgaris*. In recent years, there have been reports concerning only the effect of iron on the synthesis of lipids in green algae, including *C. vulgaris* (Liu et al. 2008; Ruangsomboon 2012; Ruangsomboon et al. 2013; Concas et al. 2014; JinShui et al. 2015).

C. vulgaris (*Trebouxiophyceae*) is a crucial component of natural phytocenoses and it is a model experimental object in plant biochemistry because it can be cultivated on a simple mineral medium and is characterized by rapid cell division. Moreover, perception of the signalling molecule and biochemical response take place within the same cell.

Experimental procedures

Plant Material and Growth Conditions

C. vulgaris culture was sourced from the Department of Plant Physiology collection at the University of Bialystok (Poland). Microalgae were cultivated in stable conditions; humidity 45±5% and temperature 25±1 ºC. Illumination was supplied over a 16 hour photoperiod (8 hours dark period) by a bank of fluorescent lights, yielding a photon flux density of 50 µmol $m^{-2} s^{-1}$ of photosynthetically active radiation (PAR) at the surface of the tubes. PAR was measured with an FF-01 phytophotometer (SOMOPAN, Poland). Permanent synchronous growth was established according to the method by Pirson and Lorenzen (1966). The culture medium used was Knop medium with the following components: 500 mg KNO_3, 500 mg $Ca(NO_3)_2 \cdot 4H_2O$, 200 mg KH_2PO_4, 150 mg $MgSO_4 \cdot 7H_2O$, 10 mg $FeCl_3 \cdot 6H_2O$, 3 mg H_3BO_3, 2 mg $MnCl_2 \cdot 4H_2O$, 0.3 mg NH_4VO_3, 0.2 mg $ZnSO_4 \cdot 7H_2O$, 0.1 mg $(NH_4)_6Mo_7O_{24} \cdot 7H_2O$ per liter of distilled water (Bajguz and Asami 2004). The culture medium was sterilized by autoclaving at 125 ºC for 25 minutes. Besides this, glassware and bacteriological stoppers were sterilized in a thermal chamber at 105 ºC for 4 hours. The pH of the medium was adjusted to 6.8 with 1 mol L^{-1} NaOH (Pietryczuk et al. 2014). The pH of the culture medium was based on literature data on the optimal pH for the growth of *C. vulgaris*. Algae were cultured in Erlenmeyer flasks containing 250 mL of appropriate medium and shaken at 150 rpm in a rotary shaker. The culture from which the inoculum was taken was in a logarithmic growth phase. The initial cell density was about $12 \cdot 10^4$ cells per ml in all experiments.

In the present work, the effects of Mn(II) and Fe(III) concentrations applied at 0.1, 0.5, 2, 5, 10, 20 and 50 mg L^{-1} were analyzed. For this purpose, appropriate amounts of $FeCl_3$ and $MnCl_2$ were added to the Erlenmeyer flasks with Knop medium to obtain the desired concentrations of the tested ions. The experimental and control cultures were performed on Knop medium deficient in manganese(II) and/or iron(III). Control cultures contained either no manganese(II) or no iron(III). Cultures of the activity of manganese(II) were examined in the medium free of manganese(II), and in the medium without

manganese(II) and iron(III). Similarly, algae cells treated with various amounts of exogenous iron(III) were cultured in a medium devoid of iron(III) and in a medium containing neither iron (III) nor manganese(II). Cell number and proteins, monosaccharides, photosynthetic pigment level as well as antioxidant enzyme activity in response to manganese(II), iron(III) were analyzed. The cell number and biochemical parameters were determined at 24, 72 and 120 hours of cultivation. Cultures were conducted in three replicates.

Determination of cell number

The number of algae cells was determined by direct counting in a Bűrker chamber.

Determination of proteins

Protein concentrations were determined by spectrophotometric method according to Lowry et al. (1951). Folin's reagent was used in determinations. Bovine serum albumin of a known concentration was used as a standard. The absorbance of the extract was measured on a Beckman DU-650 spectrophotometer.

Determination of monosaccharides

The concentration of monosaccharides was determined by spectrophotometric method according to Somogyi (1954). Sugars were extracted in ethanol for 24 hours. Arsenic-molybdenum reagent was used for the assay. Pure glucose standard of a known concentration was used as a standard. The absorbance of the extract was measured on a Beckman DU-650 spectrophotometer.

Determination of photosynthetic pigments

The concentration of chlorophyll was determined by spectrophotometric method according to Wellburn (1994). Chlorophylls were extracted in 99.9% methanol at 70 °C for 30 minutes. The absorbance of the extract was measured on a Beckman DU-650 spectrophotometer. Chlorophyll concentrations were calculated using the equation proposed by Wellburn (1994).

Determination of superoxide dismutase (SOD) activity

For the extraction of superoxide dismutase, a fresh sample of *C. vulgaris* was passed through a paper filter under pressure and was homogenized using liquid nitrogen and subsequently a lysis buffer containing 0.1 mol L^{-1} phosphate buffer

(pH=7.8), 3 mmol L^{-1} $MgSO_4$, 1 mmol L^{-1} dithiotreitol (DTT) and 3 mmol L^{-1} EDTA. The homogenate was centrifuged at 12000 G for 10 minutes and the resulting supernatant was used to determine enzyme activity. SOD (EC 1.15.1.1) activity was measured based on inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to Beauchamp and Fridovich (1971). The reaction mixture consisted of 2.2 mL of 0.1 mol L^{-1} phosphate buffer (pH=7.8), 0.25 mL of 0.156 mmol L^{-1} riboflavin, 1.1 mL of 0.156 mmol L^{-1} methionine, 0.25 mL of 0.756 mmol L^{-1} NBT and 50 μL of enzymatic extract. Samples were incubated for 20 minutes at room temperature. The absorbance of the extracts was measured spectrophotometrically at 560 nm. One unit of SOD activity was defined as the enzyme concentration required to inhibit the reduction of NBT by 50%.

Determination of glutathione reductase (GR) activity

For the extraction of glutathione reductase, a fresh weight of *C. vulgaris* was passed through filter paper under pressure and homogenized using liquid nitrogen and subsequently a lysis buffer containing 0.02 mol L^{-1} phosphate buffer (pH=7.0). The homogenate was centrifuged at 12000 G for 10 minutes and the resulting supernatant was used to determine enzyme activity. The entire extraction procedure was carried out at 4 °C. Glutathione reductase (EC 1.6.4.2) activity was determined according to Foyer and Halliwel (1976). The reaction mixture consisted of 0.25 mol L^{-1} KCl in 1 mL of 0.02 mol L^{-1} phosphate buffer (pH=7.0), 0.25 mL of 7 mmol L^{-1} glutathione disulfide and 0.1 mL of ezymatic extract. The reaction was initiated by adding 0.5 mL of 0.7 mol L^{-1} NADPH. NADPH concentration was defined as the optical density at 340 nm, and activity was calculated using the extinction coefficient e=62.2 mmol L^{-1} cm^{-1} for NADPH. One unit of glutathione reductase was defined as the amount of enzymes needed to break down 1 μmol L^{-1} of NADPH per 1 mg soluble protein per minute.

Replication and statistical analysis

Each treatment consisted of 3 replicates and each experiment was carried out on at least two different occasions (N=6). The results were subjected to statistical analysis in SPSS 19. Kruskal-Wallis tests were used to estimate the difference between means. The standard deviations from the means of all tested parameters were not greater than 5%.

Results

Variant B – where iron and manganese were absent in the Knop medium and different amounts of iron were then added to the experimental cultures – was characterized by very low changes in all biochemical parameters (fig. 1B-6B) and number of cells (fig. 7).

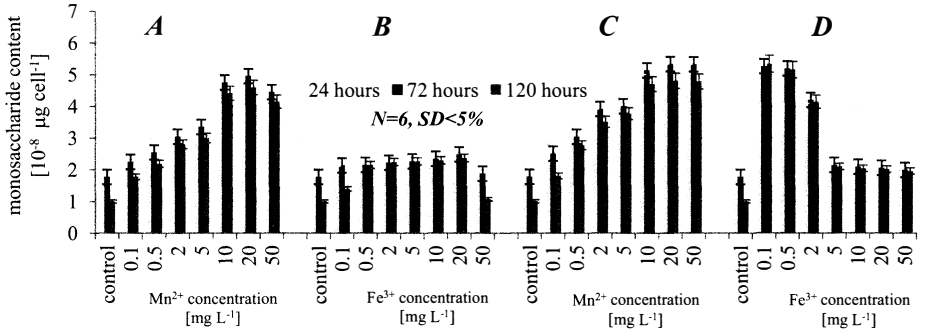

Figure 1. Change in monosaccharide concentrations in time induced by various concentrations of an introduced analyte, in a situation when ***A*** – iron and manganese were absent in the medium and manganese(II) was introduced; ***B*** – iron and manganese were absent in the medium and iron was introduced (III); ***C*** – manganese was absent in the medium and manganese(II) was introduced; ***D*** – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

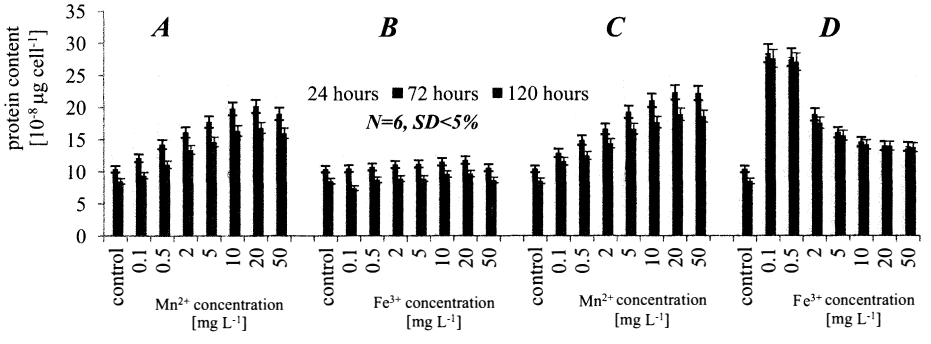

Figure 2. Change in protein concentrations in time induced by various concentrations of an introduced analyte, in a situation when ***A*** – iron and manganese were absent in the medium and manganese(II) was introduced; ***B*** – iron and manganese were absent in the medium and iron was introduced (III); ***C*** – manganese was absent in the medium and manganese(II) was introduced; ***D*** – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

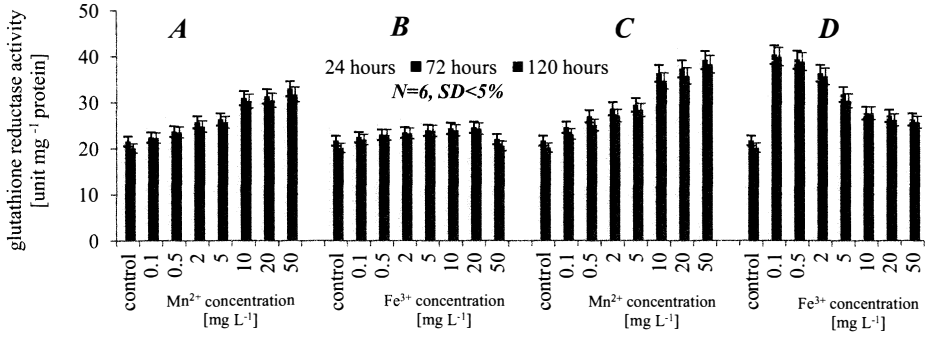

Figure 3. Change in GR activity in time induced by various concentrations of an introduced analyte, in a situation when ***A*** – iron and manganese were absent in the medium and manganese(II) was introduced; ***B*** – iron and manganese were absent in the medium and iron was introduced (III); ***C*** – manganese was absent in the medium and manganese(II) was introduced; ***D*** – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

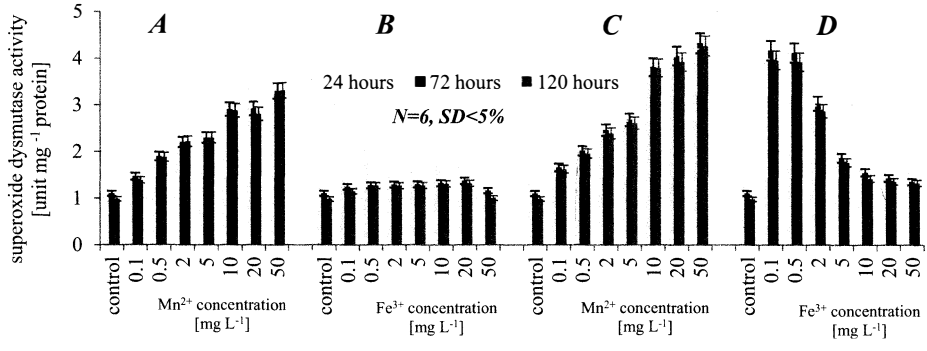

Figure 4. Change in SOD activity in time induced by various concentrations of an introduced analyte, in a situation when ***A*** – iron and manganese were absent in the medium and manganese(II) was introduced; ***B*** – iron and manganese were absent in the medium and iron was introduced (III); ***C*** – manganese was absent in the medium and manganese(II) was introduced; ***D*** – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

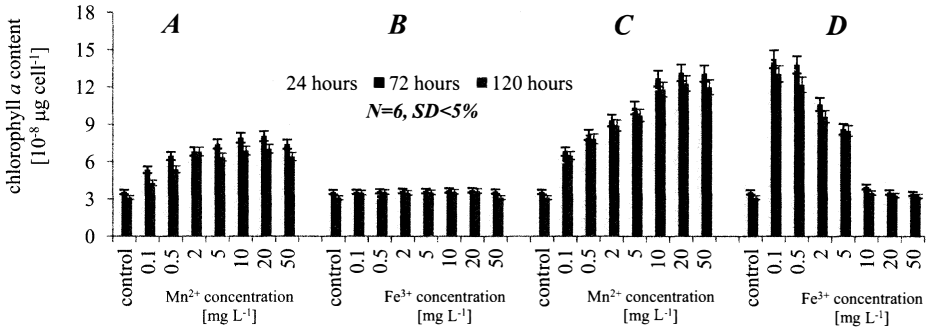

Figure 5. Change in chlorophyll *a* concentrations in time induced by various concentrations of an introduced analyte, in a situation when ***A*** – iron and manganese were absent in the medium and manganese(II) was introduced; ***B*** – iron and manganese were absent in the medium and iron was introduced (III); ***C*** – manganese was absent in the medium and manganese(II) was introduced; ***D*** – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

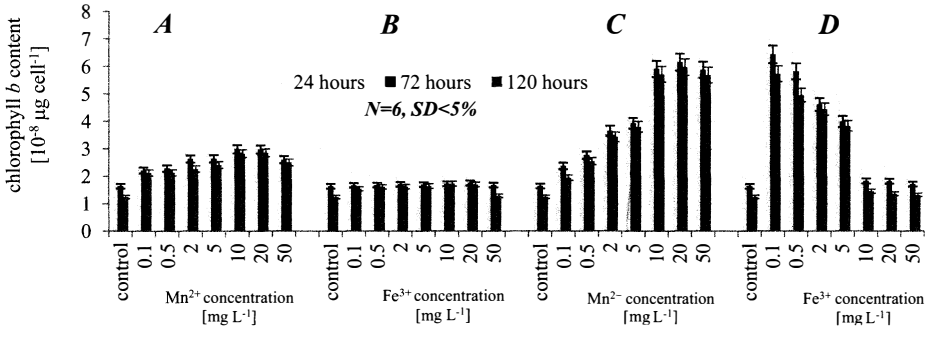

Figure 6. Change in chlorophyll *b* concentrations in time induced by various concentrations of an introduced analyte, in a situation when ***A*** – iron and manganese were absent in the medium and manganese(II) was introduced; ***B*** – iron and manganese were absent in the medium and iron was introduced (III); ***C*** – manganese was absent in the medium and manganese(II) was introduced; ***D*** – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

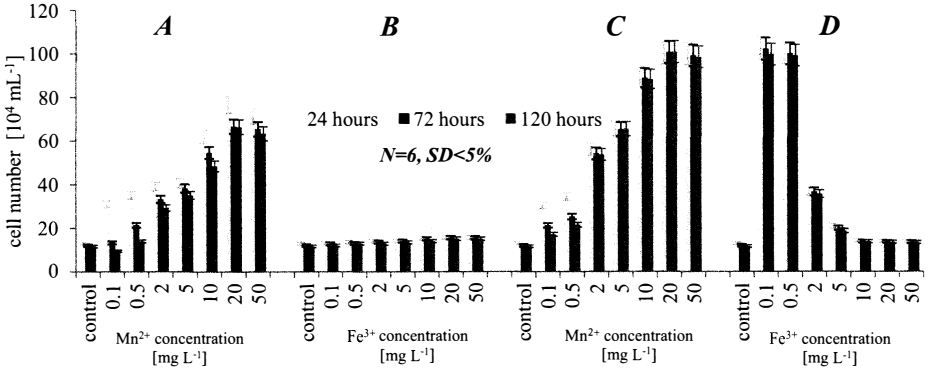

Figure 7. Change in the number of *chlorella vulgaris* cells in time induced by an introduced analyte, in a situation when ***A*** – iron and manganese were absent in the medium and manganese(II) was introduced; ***B*** – iron and manganese were absent in the medium and iron was introduced (III); ***C*** – manganese was absent in the medium and manganese(II) was introduced; ***D*** – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

Introducing iron to the medium at 0.1-20 mg L^{-1} resulted in a very slight increase in the concentrations of all the biochemical parameters in *C. vulgaris* cells, but the increase was not statistically significant relative to the control. When the algae were treated with 50 mg Fe L^{-1} all parameters increased slightly in the cells, including the number of cells (fig. 7) relative to the 20 mg Fe L^{-1}. The maximum values of concentrations of all the biochemical parameters examined were observed when iron was introduced to the medium at 20 mg Fe L^{-1}, regardless of the age of the culture (fig. 1B-6B). These regularities also applied to the activity of the studied enzymes, glutathione reductase and superoxide dismutase.

Variant C, in which only manganese was absent in the Knop medium and varying amounts of manganese were added to the solution, showed the highest variation in concentrations of all parameters and activity of the enzymes in the cells of *C. vulgaris* (fig. 1C-6C), and in the number of cells (fig. 7C). Similar to variant B, the introduction of manganese into the solution at 0.1-20 mg L^{-1} caused an increase in all parameters and concentrations of enzyme activity in the algae cells, and was statistically significant ($p<0.005$) compared to the control. The addition of 50 mg Mn L^{-1} to the solution caused, as in variant B, concentration decreases (not significant) in proteins and in chlorophylls a and b compared to when iron was introduced to the medium at 20 mg L^{-1} (fig. 2C, 5C, 6C). The solution of 50 mg Mn L^{-1} resulted in a further increase in the activity of glutathione reductase and superoxide dismutase (fig. 3C-4C), while monosaccharide levels in the algae cells did not differ from the culture grown with 20 mg Mn L^{-1} (fig. 1C). The average increase in concentration of monosaccharides when the solution of 50 mg Mn L^{-1} was introduced was more than 3-fold higher relative to the control (fig. 1C), while proteins increased more than 2 times (fig. 2C) and chlorophyll *a* and *b* more than 3.5 times (fig. 5C-6C). The activity of

glutathione reductase increased more than 2 times compared to the control (fig. 3C), while the activity of superoxide dismutase increased more than 3.5 times (fig. 4C).

In variant A, with a Knop medium deficient in both iron and manganese and where manganese was introduced to the medium in different amounts, concentrations of all biochemical parameters and enzyme activity (fig. 1A-6A) as well as cell number (fig. 7A) showed greater differences than in variant B and lower differences than in variant C. As in the case of variant B, there was an increase in the activity of glutathione reductase and superoxide dismutase over the whole range of concentrations of manganese introduced to the solution (fig. 3A-4A). The average increase in glutathione reductase activity was 1.5 times higher relative to the control (fig. 3A), and superoxide dismutase was almost 3 times higher (fig. 4A). Introduction of 50 mg Mn L^{-1} decreased ($p<0.01$) the levels of monosaccharides, proteins, chlorophylls a and b in *C. vulgaris* cells compared to the culture treated with 20 mgMn·L^{-1} (fig. 1A, 2A, 5A, 6A) and also cell number (fig. 7A). The average concentration of chlorophyll *a* in the cells in variant A was 30% lower than in variant C (fig. 5A, 5C), and in the case of chlorophyll *b* the difference was greater – 40% (fig. 6A, 6C).

All the biochemical parameters in variant D, where iron was completely absent in the Knop medium and iron was added in various concentrations, behaved completely differently than the other variants. Introduction of 0.1 mg Fe L^{-1} to the solutions resulted in a sharp increase in all the biochemical parameters in the algae and the number of cells, while the introduction of larger amounts of iron to the culture medium resulted in a decrease in the concentrations of all the biochemical parameters in *C. vulgaris* (fig. 1D-6D) and in cell number (fig. 7D). In the case of monosaccharides, chlorophylls *a* and *b*, and superoxide dismutase, 5 mg Fe L^{-1} resulted in a sharp decline in the concentrations of the indicators and activity of enzymes (fig. 1D, 4D-6D). For all the biochemical parameters there was a statistically significant difference ($p<0.001$) between the control and experimental cultures treated with iron at 0.1-2.0 mg Fe L^{-1}. In the case of the protein concentration and activity of the antioxidant enzymes tested, a statistically significant difference ($p<0.05$) was demonstrated between the control and the various cultures compared to the corresponding iron introduced into the solution at 5-50 mg L^{-1}, and in the case of both monosaccharides and the statistical significance of chlorophylls, a difference did not exist.

The described changes in the concentrations of biochemical parameters, the activities of enzymes, and the numbers of cells, were identical regardless of the age of cultures.

Discussion

There is a good number of literature data concerning the effect of manganese(II) and iron(III) on the growth of higher plants and basic metabolic processes such as photosynthesis, respiration, or antioxidant defense. It is also known that these elements are involved in the regulation of the growth and

metabolism of lower plants, especially algae (Pramod et al. 2014). However, there is a lack of detailed information on their impact on changes in the content of basic metabolites in the *Trebouxiophyceae* cells. Therefore, understanding the biochemical mechanisms behind the action of iron and manganese in the algae seems to be extremely important for the control of phytoplankton growth in water bodies; modifying the concentrations of these elements in the aquatic environment may have a beneficial effect. Previous studies of surface water showed that there is a strong relationship between the concentration of manganese in the water and algal biomass (Górniak and Cudowski 2006; Cudowski and Górniak 2008; Cudowski 2015). Intensive growth of algal biomass, resulting in their `blooms`, leads to inhibition of photosynthesis and in a further step to a significant deterioration of water oxidation, which is the first stage of eutrophication. This process has become a global problem, as it is recorded in lakes, seas, and rivers around the world (Imai et al. 2006; Selaman et al. 2008).

In our study, manganese(II) and iron(III) induced the growth of *C. vulgaris* cell numbers compared with the control culture. Manganese(II) is a stimulator of the growth of algae (Rousch and Sommerfeld 1999, Chen et al. 2010), and similar to other reports, in our study resulted in a much larger increase in the number of *C. vulgaris* cells than under the influence of iron(III). Treatment of seedlings of *Vicia faba L.* with manganese(II) at a concentration of 4 g L^{-1} by about 9% compared to control, while iron(III) at a concentration of 4 g L^{-1} caused an increase in angular length of 4% (Abd El-Razek et al. 2013). Also in the sunflower there was more growth, increased biomass and leaf surface as compared to the control plants after treatment with manganese(II) (Jabeen and Ahmad 2011). In contrast, the addition of exogenous manganese(II) and iron(III) to the culture medium, at a concentration of 4 g L^{-1} resulted in the largest increase in the length of the stem relative to control, by as much as 12% (Abd El-Razek et al. 2013), which confirms the results of our study on a single-celled algae. The greatest increase in the number of cells was observed in *C. vulgaris* growing on medium containing iron(III), into which the various concentrations of manganese(II) were introduced. In a reverse situation, when the culture was growing on a medium with manganese(II) and iron(III) added in the concentration range of 0.1-50 mg L^{-1}, a gradual decrease was observed in the number of cells up to the level of control, which is probably associated with the co-precipitation of iron(III) and manganese(II) in the form of iron-manganese concretions, which make manganese(II) unavailable to plants. Silveira et al. (2007) noted that the concentration of manganese in the dry weight of stem and root of rice fell below control when the plants were treated with 500 mg L^{-1} iron(III). The available literature data shows that iron(III) is also a stimulator of plant (Silveira et al. 2007) and algae (Chen et al. 2010) growth. However, compared to manganese(II), iron stimulates growth in the number of algal cells to a much lesser extent than manganese. In addition, treating *C. vulgaris* with iron(III) at a concentration of 50 mg L^{-1} caused a decrease in the number of algal cells. Silveira et al. (2007) observed that Fe(III) at a concentration of 500 mg L^{-1} induced a slight decrease in the dry weight of rice stems with respect to rice treated with 6.5 mg L^{-1}. It seems, therefore, that a much lower concentration of iron is needed to

inhibit the growth of single-celled algae, which is also confirmed by our results on changes in the content of other biochemical parameters under the influence of various concentrations of iron(III).

Our results show that manganese(II) and iron(III) not only activate cellular division in *C. vulgaris*, but also increase the content of chlorophyll *a* and *b*, and monosaccharides. Literature data show that manganese(II) is a trace element essential in the biosynthesis of chlorophylls, and its deficiency causes a disturbance of photosynthesis, contributing to the disintegration of photosynthetic pigments in terrestrial plants, such as *Cassia grandis* (Li et al. 2010), *Pisum sativum* (Gangwar et al. 2010) and also aquatic plants such as *Salvinia minima* and *Spirodela polyrhiza* (Lizieri et al. 2011), *Lemna gibba* (Doganlar et al. 2012), which is confirmed by our results. In the cells of algae *C. vulgaris*, manganese(II) at a concentration of 50 mg L^{-1} caused a decrease in chlorophyll *a* and *b* and monosaccharides. This is probably due to the fact that high concentrations of manganese displace cellular magnesium, essential for the biosynthesis of chlorophyll (due to competitive reaction between the metals). Furthermore, manganese may induce iron deficiency in plants (Hauck and Spribille 2002). It can be assumed that manganese(II) at a concentration of 50 mg L^{-1} will result in a gradual displacement of magnesium from cells of *C. vulgaris* and induce iron deficiency causing a decrease in the concentration of chlorophylls. However, this decrease is small, and the content of chlorophylls does not fall below the control, suggesting that a much higher concentration of manganese is required to cause the complete inhibition of photosynthesis and biosynthesis of photosynthetic pigments in *C. vulgaris.* For comparison, in the cells of *V. faba* a decrease in chlorophyll content was reported in cells treated with a much lower manganese concentration (4.4 mg Mn L^{-1}).

A manganese(II)-induced increase in chlorophyll *a* and *b*, on average by 40% compared to control, has been reported in studies on *V. faba*. Furthermore, that study revealed an iron-induced increase in the content of chlorophyll in cells, but only by 18-20% relative to the control culture. A similar increase in concentration of photosynthetic pigments (by about 18%) occurred after treatment of plants with manganese(II) and iron(III) simultaneously (Abd El-Razek et al. 2013). Our research showed that the content of chlorophylls *a* and *b* in algae cells increased nearly 3 times when iron(III)-containing medium was added to different concentrations of manganese(II). In contrast, manganese(II) added to the culture medium devoid of iron(III) resulted in only a 2-fold increase in the concentrations of these pigments. This indicates that iron is an essential micronutrient for *C. vulgaris*, required for the proper synthesis and function of chlorophylls in its cells. Moreover, in higher plants iron causes a 2-3-fold increase in the content of photosynthetic pigments, for example in *Citrullus lanatus*, and its deficiency induces a significant decrease in the concentration of chlorophylls, carotenoids, proteins, and the rate of net photosynthesis, primarily through disrupting the electron transport in the PSII system (Abadia et al. 1999). In turn, manganese deficiency inhibits the growth and photosynthetic activity in *Chlamydomonas* (Allen et al. 2007). It has been shown that manganese induces PSII in *Euglena gracilis* (Ferroni et al. 2004), *Amphidinium sp.* (Chunhui et al. 2011).

The crucial role of manganese and iron in photosynthesis is demonstrated in our study where algae cells treated with exogenous manganese(II) and iron(III) had an increased concentration of monosaccharides. *C. vulgaris* cells growing on an iron(III)-containing medium into which manganese(II) was added, had a much higher increased content of sugars in relation to the control than the cells treated with manganese(II) and growing on a medium without iron(III). In addition, *C. vulgaris* cells experienced a slight decline in photosynthetic pigments and monosaccharides when treated with iron (III) at a concentration of 50 mg L^{-1}. According to literature data, excess iron causes the degradation of chlorophylls and a decline in monosaccharides, which is probably a consequence of a reduction in the rate of net photosynthesis. Xing et al. (2010) shows that even at 10 mg L^{-1} iron causes a significant decrease in the content of chlorophylls (by 20% compared to control) and in monosaccharides (down to 0) in the cells of *S. polyrhiza*. Such a drastic decline in these biochemical parameters was not recorded in our study, even when the algae cells treated with exogenous iron grew on the medium without manganese(II).

Manganese is an essential element for the proper course of redox processes in plant cells, such as electron transport in photosynthesis. It is also involved in the biosynthesis of chlorophyll – the presence of which is necessary for the proper functioning of the PSII photosystem (Mousavi et al. 2011, Nusrat and Rafiq 2011). Increased photosynthesis efficiency may result in an increase in the concentration of monosaccharides in plant cells. The literature on higher plants confirms that manganese causes an increase in the content of reducing sugars, as observed, for example, in *V. faba* (Abd El-Razek et al. 2013). An increase in monosaccharide content compared to control was also observed in algal cells treated with iron(III) concentrations in the range of 0.1-50 mg L^{-1}, although small compared to the effect of manganese(II). This is consistent with literature data on higher plants (Abd El-Razek et al. 2013) and is probably due to the fact that iron is one of the factors which regulate the activity of enzymes involved in the metabolism of sugars. It is known that during the first 48 hours iron(III) reduces the activity of one of the Krebs cycle enzymes – malate dehydrogenase, which inhibits the breakdown of glucose in cells (Russo et al. 2010). In addition, in the first day of culture, iron(III) stimulates the activity of phosphoenolpyruvate carboxylase (PEPCase) activity in *Zea mays* (Russo et al. 2010). An increase in the activity of this enzyme can lead to increased levels of sugars in the cell, which could also have occurred in *C. vulgaris*, in which the first day of culture was marked by the highest levels of monosaccharides in cells treated with various concentrations of iron(III).

In the present study manganese(II) and to a lesser extent iron(III) stimulated the overall growth in protein content in *C. vulgaris*. A significant increase in the concentration of these metabolites in *L. gibba* cells induced by 0.25-16 mg L^{-1} manganese(II) was also observed by Doganlar et al. (2012). Literature data show that iron(III) is a factor that induces an increase in the accumulation of proteins in plant cells. An increase in this parameter induced by biochemical iron(III) was also observed in *S. polyrhiza*. Higher concentrations of iron(III) (higher than 10 mg L^{-1}) caused a gradual decline in these metabolites in the cells of *S. polyrhiza*

(Xing et al. 2010) and *Brassica napus* (Pourgholam et al. 2013). An increase in protein content in algal cells treated with exogenous manganese(II) may be associated with stimulation of RNA polymerase activity (Mousavi et al. 2011), or an induced increase in the concentration of simple sugars, essential components of DNA and RNA. Furthermore, the addition to the culture medium of manganese at 50 mg L^{-1} did not result in a statistically significant decrease in protein in algae as compared with other biochemical parameters, which may have been due to the synthesis of stress proteins in response to excess manganese in cells.

It is known that iron(III) (Sajedi et al. 2011, Jucoski et al. 2013) and manganese(II) (Li et al. 2010, Arya and Roy 2011, Sajedi et al. 2011) at physiological concentrations and higher enhance the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX). Moreover, both elements respond to oxidative stress by increasing the activities of the antioxidant enzyme superoxide dismutase (SOD) in *Thalassiosira pseudonana* and *T. oceanica* (Peers and Price 2004). In this study cells of *C. vulgaris* treated with manganese(II) in a range of 0.1-50 mg L^{-1} had a significantly increased activity of antioxidant enzymes, in particular superoxide dismutase (EC 1.15.1.1), compared to control. However, in algal cells treated with various concentrations of iron(III) an increase in the activity of superoxide dismutase and glutathione reductase (EC 1.6.4.2) was not statistically significant and was maintained at a control level. The largest increase in the activity of both enzymes was observed under the influence of manganese at 50 mg L^{-1} (II), which indicates that this concentration may already be toxic and induce oxidative stress in *C. vulgaris.* This is supported by the observation that manganese(II) in the aforementioned concentration caused a decrease of the content of chlorophylls, monosaccharides, and algae cell number. It seems that the increase in the number of algae cells and sugars under physiological concentrations of manganese(II) (0.1-20 mg L^{-1}) is indirectly caused by an increased activity of SOD which activates cell division and inhibits the catabolism of glucose via the inhibition of Krebs cycle enzymes (Morgan et al. 2008). Furthermore, stimulation of the activity of SOD by manganese(II) may be positively correlated with an increase in GR activity. It is known that manganese (Arya and Roy 2011) and iron (Abadia and Zaharieva 2003) significantly increase the amount of reduced glutathione. Therefore, it cannot be excluded that a similar relationship exists in *C. vulgaris.* Reduced glutathione is a cell division-inducing factor via stimulation of the transition of cells from G1 to S phase (Kawano 2003) and protects plants against chlorosis (Ramirez et al. 2013), which may explain the persistence of the control level of chlorophyll under all iron(III) concentrations or an increase above control when the cells were treated with manganese(II) over the range of concentrations used in *C. vulgaris* in this study.

The study confirmed the important role of the concentration of manganese(II) in the growth of algae in water bodies (Górniak and Cudowski 2006, Cudowski and Górniak 2008, Grabowska 2012), because the higher the concentration of manganese in the solution the stronger the growth of algae. All the results indicate that the introduction of an appropriate amount of iron(III) helps control the level of manganese(II) by the co-precipitation of the metals. In this way it

may help control eutrophication, observed in lakes, seas, and rivers around the world (Selaman et al. 2008).

Conclusion

The obtained results clearly indicate that both manganese(II) and iron(III) induce significant changes at a cellular level not only in higher plants but also in unicellular algae. The conducted research shows that the largest increase of concentrations of biochemical parameters and enzyme activity took place in a situation where manganese was removed from the medium and then was re-introduced in different portions to the solution. In the situation in which iron was completely removed from the medium and then re-introduced in various amounts, co-precipitation of iron and manganese occurred, since iron in the acidic medium precipitates in the form of colloidal $Fe(OH)_3$. As a result of the aforementioned reactions, both metals were not available, hence the observed decrease in the concentrations of studied biochemical parameters and enzyme activity. The experiment also showed that an appropriate amount of iron(III) helped control the level of manganese(II) thanks to the co-precipitation of metals, and so could help in tackling the worldwide problem of eutrophication. Our results also showed a greater impact of manganese(II) than iron(III) on the growth of *C. vulgaris*. When both metals were completely removed from the medium, the introduction of manganese(II) resulted in a rapid increase of concentrations of biochemical parameters and enzyme activity, while when iron(III) was added, the increase was low.

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