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RESEARCH ARTICLE | НАУЧНАЯ СТАТЬЯ

INFLUENCE OF WIDE RANGE OF IRON AND MANGANESE CONCENTRATIONS ON THE NUMBER OF CELLS AND PIGMENT CONTENT OF *CHLORELLA VULGARIS*

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Abstract

This study investigated the effects of iron and manganese on the green microalga *Chlorella vulgaris* at different exposure times. In the experimental setup *C. vulgaris* IMBR-19 was incubated at the temperatures of +28°C, +20°C, and +10°C. The growth medium was supplemented with 1–500 μM of Fe or Mn. The number of living and dead cells and pigment amount was analyzed during the growth process. The addition of 500 μM Mn or 100-500 μM Fe resulted in the death of most microalgae. Iron had a more toxic effect than manganese in all the experiments. In the range of 75-100 μM Fe at +28°C, +20°C and 100-150 μM Fe at +10°C there was a sharp increase in the number of dead cells. Addition of 100-150 μM Mn at +10°C from 5th day of the experiment led to the adaptive growth of algae cells. With 5-300 μM of Mn at +20°C and +28°C from day 5 a similar addictive effect was observed. In a 1-week experiment with 5-300 μM Mn at +28°C, *C. vulgaris* cells contained an average of 22%, 8%, and 11% less chlorophyll *a*, chlorophyll *b*, and carotenoids than the control, respectively.

Keywords:

Chlorella, heavy metals, iron, manganese, algae pigments, temperature, toxicity

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ВЛИЯНИЕ ШИРОКОГО ДИАПАЗОНА КОНЦЕНТРАЦИЙ ЖЕЛЕЗА И МАРГАНЦА НА ЧИСЛО КЛЕТОК И СОДЕРЖАНИЕ ПИГМЕНТОВ *CHLORELLA VULGARIS*

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Аннотация

Изучено воздействие железа и марганца на зелёную микроводоросль *Chlorella vulgaris* при различной длительности эксперимента. Штамм *C. vulgaris* IMBR-19 инкубировали при температурах +28°C, +20°C и +10°C, с добавлением 1-500 мкМ тяжёлых металлов. В процессе роста анализировали количество живых и мёртвых клеток и содержание фотосинтетических пигментов. Было обнаружено, что добавление 500 мкМ Mn (II) или 100-500 мкМ Fe (III) приводило к гибели большинства клеток. Во всех экспериментах Fe (III) оказывало более токсичное воздействие, чем Mn (II). В диапазоне 75-100 мкМ Fe (III) при температуре +28°C, +20°C, а также 100-150 мкМ Fe (III) при температуре +10°C наблюдалось увеличение количества мёртвых клеток. Добавление 100-150 мкМ Mn (II) при температуре +10°C с 5-го дня эксперимента привело к адаптивному росту клеток водорослей. В диапазоне 5-300 мкМ Mn (II) при температуре +20°C и +28°C с 5-го дня наблюдался аналогичный адаптивный эффект. В недельном эксперименте с внесением 5-300 мкМ Mn (II), при температуре +28°C клетки *C. vulgaris* содержали в среднем на 22%, 8% и 11% меньше хлорофилла *a*, хлорофилла *b* и каротиноидов, чем в контроле, соответственно.

Ключевые слова:

Chlorella, тяжёлые металлы, железо, марганец, фотосинтетические пигменты, температура, токсичность

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INTRODUCTION

The term, heavy metal (HM) usually refers to metals with an atomic weight higher than Fe (55.8 g/mol) or density more than 5.0 g/cm^3. However, iron and manganese are also often considered as HM. The content of Fe and Mn in soils and aquatic ecosystems often exceeds natural levels, posing risks to both, living beings and human health [Cabala *et al.* 2011; Kacholi, Sahu 2018; Meena *et al.* 2018; Zwolak *et al.* 2019; Nájera-Martínez *et al.* 2021; Yasin *et al.* 2021]. Toxic stress associated with an excess of Fe and Mn negatively affects the metabolic pathways of algae, which leads to suppression of their growth and photosynthesis [Andresen *et al.* 2018; Al-Khiat *et al.* 2019; Rogers *et al.* 2020; Ciurli *et al.* 2021]. At the same time Mn and Fe are an essential micronutrient for many organisms including microalgae. Normal growth, development and reproduction are impossible without HM ions. Influencing enzymatic catalysis, trace elements have an effect on almost all aspects of metabolism (e.g. glycolysis, nucleotide and lipid exchange, assimilation of carbohydrates during photosynthesis, respiration and other processes) [Keren *et al.* 2002; Coelho *et al.* 2015; Fischer *et al.* 2015; Smythers *et al.* 2019; Alho *et al.* 2022].

Despite the long-term study of HMs effects on *Chlorella* enrichment cultures, there is still lack information on the effect of Mn (II) in a wide range of concentrations on the physiological and biochemical state of algae cells, in particular on photosynthesis processes. Due to its unique role in the water oxidizing activity of photosystem II, Mn is required for photosynthetic organisms. However, the mechanism of its biological action is not fully understood yet [Keren *et al.* 2002; Andresen *et al.* 2018; Alho *et al.* 2022]. In the work of Smythers *et al.* [2019] it was shown that cells in lag and early exponential phase adsorb Mn and actively increase the intracellular concentration beyond equilibrium. Algae cells reached their peak, containing 495.0 and 733.3 mM Mn for the 17.5 and 35 mM in culture, respectively, reaching a maximum of 55× the concentration of the surrounding media [Smythers *et al.* 2019]. Manganese chloride (II) at 2 μM, 10 μM and 12 μM increased the lipid content and growth parameters significantly by 14%, 16% and 15%, above the corresponding controls [Battah *et al.* 2015].

The growth of *Chlorella* sp. in the presence of limiting concentrations of Fe has been explicitly studied, nevertheless there is few information about the effect of Fe on physiological state of microalgae at different temperatures. There are also data gaps in the growth characteristics of microalgae in a wide range of Fe concentrations at different time intervals. In the work of Liu *et al.* [2008], the addition of 12 μM of Fe (III) at +20ºC in the initial media suppressed the cell growth slightly, but it stimulated lipid storage in *C. vulgaris* up to 56.6%, but within seven days cell numbers were significantly higher than that of the control. The EC50 analysis at +25ºC revealed that *Chlorella* sp. was significantly resistant to Fe compared to other microalgae. Fifty percent inhibition in growth was recorded at concentrations of 240 μM for *Chlorella* sp. [Subramaniyam *et al.* 2016]. Meanwhile in the study of Pietryczuk *et al.* [2025], Fe (II) at 2–1000 μM under +25ºC not only activated the cell division of *C. vulgaris* but also caused an increase in the concentration of chlorophylls *a* and *b* and monosaccharides in the algae. On the contrary, with 2–1000 μM of Fe (III) at +25ºC much lower concentration of that HM was needed to inhibit the growth of single-cell algae. According to literature data, excess Fe causes the degradation of chlorophylls and a decline in monosaccharides, which is probably a consequence of a reduction in the rate of photosynthesis [Cudowski, Pietryczuk 2019].

The range of growth temperature within the genus *Chlorella* sp. is variable. Significant fluctuations are observed among different strains of one species. Common representatives of *C. vulgaris* are characterized by optimum +25°C – +28°C and maintain its viability at +30°C – +35°C, in some cases *Chlorella* sp. might be grown at +50°C (*C. pyrenoidosa*) [Serra-Maia *et al.* 2016; Ahmad *et al.* 2020; Denïz

2020]. Low temperature limit is also strain and species-specific. Some strains of *Chlorella* sp. have optimum at +14°C and might be also grown at +4°C [Wang *et al.* 2024]. Temperature directly affects the sensitivity rate of green algae to HMs depending on the HM type, algae strain and temperature range [Yuqin *et al.* 2021; Wang *et al.* 2022].

Thus, our study aimed to establish the effect of wide range of concentrations of Fe (III) and Mn (II) on the number of living and dead cells and pigment content in *Chlorella vulgaris* at 10–28°C, thereby expanding the available data on the combined effect of HMs and temperature.

MATERIALS AND METHODS

Microalgae strain. Freshwater axenic strain of green microalgae *Chlorella vulgaris* IMBR-19, without bacterial contamination, maintained in sterile conditions, obtained from the A.O. Kovalevsky Institute of Biology of the Southern Seas (Russian Academy of Sciences) was utilized in current study.

Experimental design and growth performance. Experiments were performed in controlled laboratory conditions. The algae were incubated in a growth chamber (accuracy of temperature maintenance ±0.1°C) in conical flasks at +28°C – line 1 (L1) and +10°C – line 2 (L2) in an enrichment culture mode using liquid medium BG-11 [Rogers *et al.* 2020]. All the experiments were done in 24-well plates at the temperatures of +28°C, +20°C (with L1) and +10°C (L2) under a light intensity of 6500 lux with 12h:12h light:dark photoperiod on a light panel with evenly arranged LEDs and frosted glass, which gave a uniform, instrumentally measured luminous flux (fig. 1). The empirically established optimal growth temperature for this strain was +28°C, +10°C is the lower limit at which a significant cell growth was visible during the one week of cultivation, +20°C is the midpoint. The initial pH of the culture medium was 7.0. The sowing aliquot of *Chlorella* contained 1.5-1.6 $\times 10^6$ cells/ml in the well with final volume 2.3 ml.

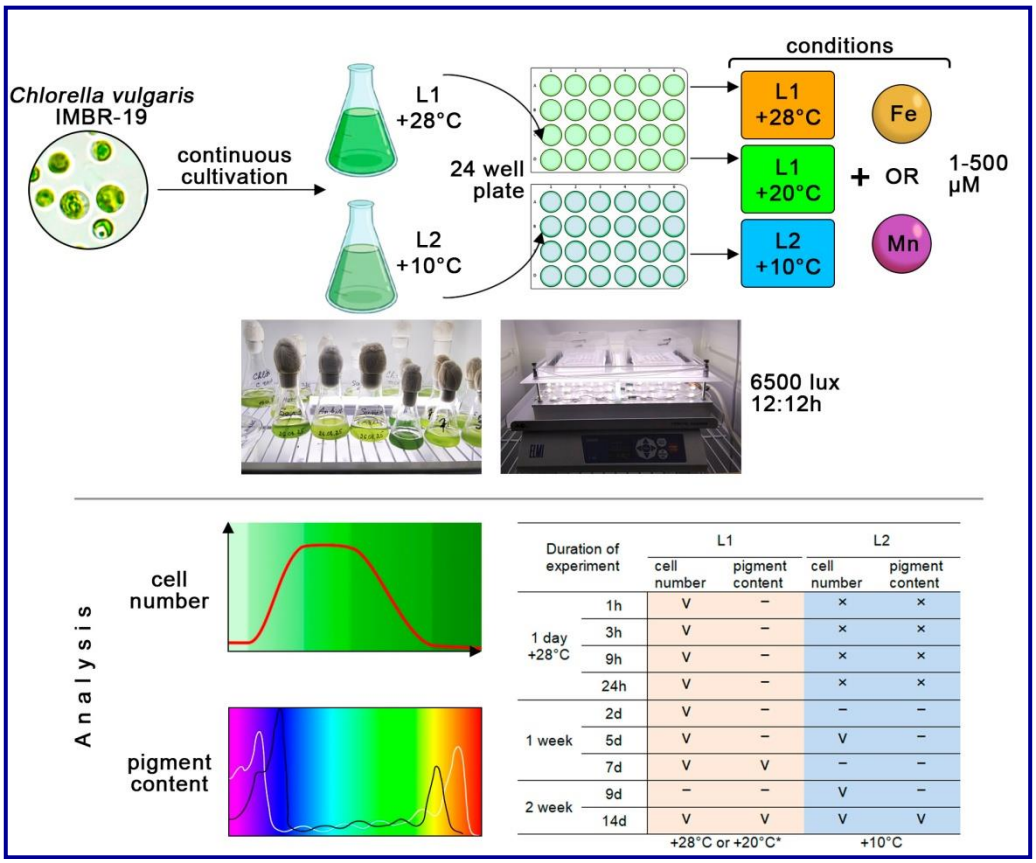
In trials, $MnCl_2 \times 4H_2O$ and $FeCl_3 \times 6H_2O$ of analytical grade were added to the final concentration of 1; 5; 10; 25; 50; 75; 100; 150; 200; 300 and 500 μM of respective metal beyond the 3 and 2 μM of Fe (III) and Mn (II) in the base medium respectively (fig. 1).

Cell count. The number of *Chlorella vulgaris* cells (viable and/or dead) was determined by adding the methylene blue dye with direct counting in the Goryaev chamber using standard light microscopy methods and ZEISS Axiostar Plus microscope (Germany), with further conversion of the results in units of culture density (cells/ml) [Dvoretsky *et al.* 2017]. The suspension was stirred before counting and 100 μl of it was mixed with 2 μl of 1% dye in 96-well immunological plates (Khimmed, Russia) then incubated for 3 minutes. The percentage of dead cells was assessed using the formula: dead cells (%) = dead cells / total number of cells×100. Experiments were conducted in three biological replicates. Determination of cell number was carried out during the first day (after 1, 3, 9, 24 hours for L1 at +28°C), on the 2nd, 5th, 7th, 14th day for L1 at +20°C and +28°C, and on the 5th, 9th, 14th day for L2.

Photosynthetic pigment assay. For photosynthetic pigment assay, 3 ml samples of cell suspension (from identical variant wells) were harvested on the 7th (L1) and 14th (L1 and L2) day by centrifugation at 10,000 rpm for 10 min. Supernatant was discarded, and 3 ml of pre-cooled acetone (80%) was mixed with the cell pellet. Then the mixture was incubated in darkness at 4°C for 12 hours or longer, until the color of the pellet became white. The absorbance of the supernatant was measured at 470, 663, 645 and 750 nm on Cary 100 (Agilent Technologies, USA). The pigment concentration (μg/ml) (chlorophylls *a*, *b* and carotenoids) in algae cells was calculated with the formulas of Lichtenthaler [1987]. The pigment index was evaluated as the ratio of acetone extracts of total chlorophyll to carotenoids ($\sum C_{chl}/C_{car}$) and chlorophyll *a* and *b* ($C_{chl\ a}/C_{chl\ b}$).

Figure 1. Schematic illustration of the study. "v" – collected samples; "×" - no experimentation; "–" - no data (sample collection did not take place); "*" - at +20°C, the L1 trials were conducted only in a two-week variant

Рисунок 1. Схема исследования. "v" – отобраны образцы; "×" - экспериментов не проводилось; "–" - нет данных (отбор образцов не проводился); "*" - при температуре +20°C обработка L1 проводилась только в двухнедельном варианте



Statistical analysis. Statistical analysis was done with Microsof Excel 2010 and Statistica 10.0 software tool packs. All results are considered statistically significant at $p < 0.05$ and expressed as mean values of three replicates with standard deviation. The correlation was analyzed using a Pearson test (two-tailed). The significance of the various parameters was tested using one-way analysis of variance.

RESULTS

Standard growth curves. According to empirically determined growth curves (fig. 2) for L1 a rapid growth rate was recorded during the first 5 days. After 2 days, the algae cell count increased by 11 times (to 11.0-19.7×10^6 cells/ml), and on the fifth day, L1 cell number expanded by another 1.5-2.0 times (up to 30.3 and 29.7×10^6 cells/ml) at +20°C and +28°C, respectively. From the 5th day the culture entered into a stationary growth phase. Cell number of L2 was increasing gradually and only by the 9th day reached 16.0×10^6 cells/ml (fig. 2). On the 9th day, L2 entered into a stationary growth phase. According to available data, *Chlorella* sp. achieves the best growth rates in "standard conditions" after 7-10 days [Blair *et al.* 2014; Safi *et al.* 2014].

One day experiment. Fe (III) concentrations ranged from 1 to 25 µM within 24 h did not affect apparently the number of algae cells in comparison to the control values. At 50-100 µM of Fe (III) the cell population was slightly decreased, at 150 µM – by 8-17% less than the control within 1-24 h, at 300 and 500 µM – it reduced by almost half in relation to the control variants (fig. 3). The proportion of dead cells in test suspensions that were below 50 µM of Fe (III) was identical to that of controls. At 100-150 µM it reached 5-8% of the total number after 9 h and 17% after 24 hours (at 100 µM).

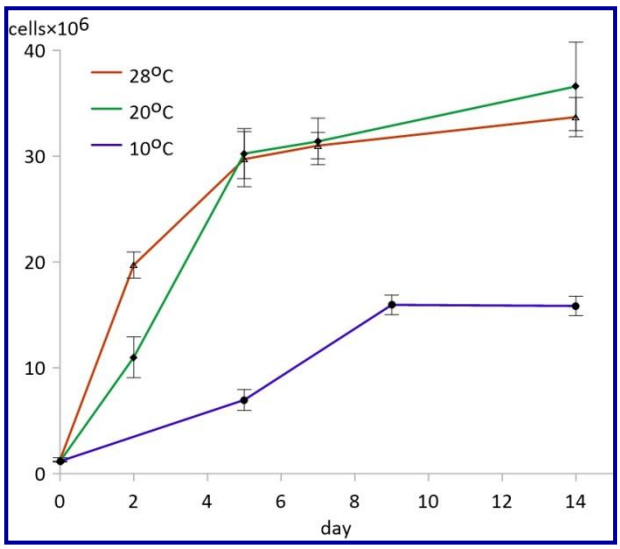


Figure 2. Standard curve of *Chlorella* growth at +28°C, +20°C (L1), and +10°C (L2)

Рисунок 2. Стандартная кривая роста хлореллы при температуре +28°C, +20°C (L1) и +10°C (L2)

Microalgae exposed to 50-150 μM of Mn (II) during the first 1-3 h showed no significant differences in the cell number between the control and test samples (fig. 4). Faint increase in dead cell proportion occurred in variants supplemented with 150 μM of Mn (II) meanwhile at 500 μM it was 6 times higher than control one (fig. 4). Due to low growth rate at +10°C (fig. 2) there were no experiments with L2 during the first day and up to a week. Also in the initial period,the differences between +20°C and +28°C were small, for this reason only data for L1 at +28°C is given hereafter for trials with duration less than a week.

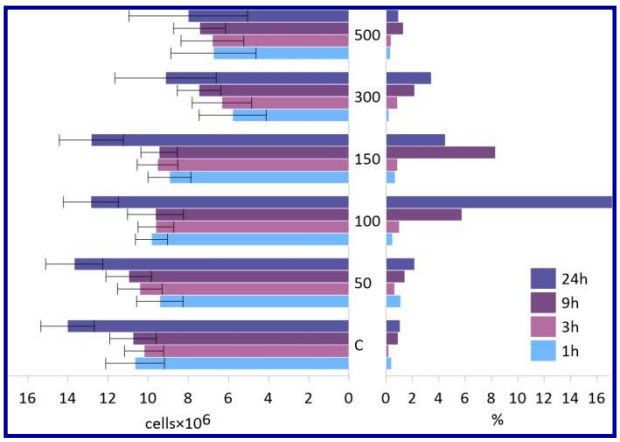


Figure 3. The effect of 1-500 μM of Fe(III) on the cell number (left) and dead cell proportion (%, right) of L1+28°C in the stationary growth phase in a daily experiment (concentrations below 50 μM are omitted), "C"-control

Рисунок 3. Влияние 1-500 мкМ Fe(III) на количество клеток (слева) и долю мертвых клеток (%, справа) при температуре L1+28°C в стационарной фазе роста в суточном эксперименте (концентрации ниже 50 мкм не учитываются), "С" -контроль

One week experiment, Fe(III). Evaluation of the effect of Fe (III) on the number of *C. vulgaris* showed no changes after 2 and 5 days with the addition of 1 μM of Fe (III). At 5-75 μM, a reduction in cell number was observed in accordance with Fe (III) concentration rise (correlation coefficients: −0.93, −0.88 and −0.99; $p \leqslant 0.01$ for 2, 5 and 7th day). Meanwhile, the number of dead cells was higher than the control starting with 5 μM. At 100 μM of Fe (III), the number of cells after 2 and 5 days was 2.2% and 4.8% of the respective control. In the case of 150 μM and higher concentrations, there was no cell growth. On day 7, the effect of Fe (III) could be detected even at 1 μM. At 100 μM, the cell number was just 24% of the respective control. At higher concentrations, cell growth was nearly absent, and the proportion of dead cells reached 95-100% (fig. 5).

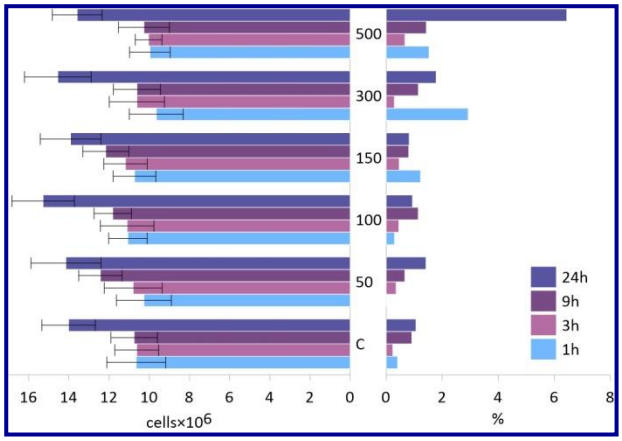


Figure 4. The effect of 1-500 μM of Mn(II) on the cell number (left) and dead cell proportion (%, right) of L1+28°C in the stationary growth phase in a daily experiment (concentrations below 50 μM are omitted), "C"-control

Рисунок 4. Влияние 1-500 мкМ Mn(II) на количество клеток (слева) и долю мертвых клеток (%, справа) при температуре L1+28°C в стационарной фазе роста в суточном эксперименте (концентрации ниже 50 мкм не учитываются), "С" -контроль

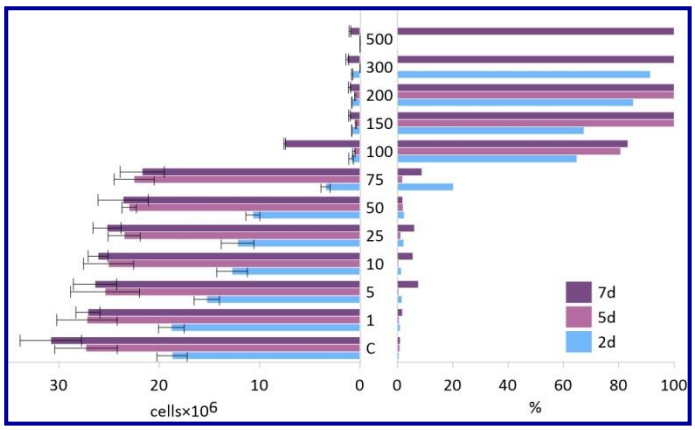


Figure 5. The effect of 1-500 μM of Fe(III) on the growth (left) and dead cell proportion (%, right) of L1+28°C in a 7-day experiment, "C"-control

Рисунок 5. Влияние 1-500 мкМ Fe(III) на рост (слева) и долю мертвых клеток (%, справа) при температуре L1+28°C в 7-дневном эксперименте, "C"-контроль

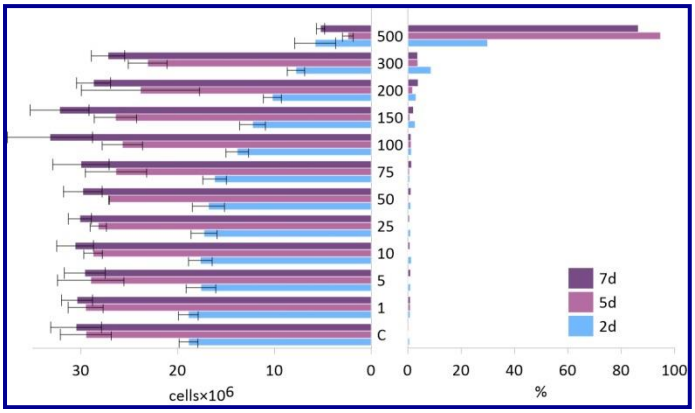


Figure 6. The effect of 1-500 μM of Mn(II) on the growth (left) and dead cell proportion (%, right) of L1+28°C in a 7-day experiment, "C"-control

Рисунок 6. Влияние 1-500 мкм Mn(II) на рост (слева) и долю мертвых клеток (%, справа) при температуре L1+28°C в 7-дневном эксперименте, "C"-контроль

One week experiment, Mn(II). At a concentration of 1 μM Mn (II), there were no significant differences in cell number and dead cell proportion compared to the control throughout the experiment (fig. 6). On day 2, there was a noticeable decline in cell number with increasing Mn (II) content from 5 to 500 μM. The cell numbers were 2.5 and 3.2 times lower than the control in variants with 300 and 500 μM, respectively. Dead cell percentage was significantly different from the control (0.6% from total cell number) at 150 μM and higher, reaching 8.6% and 30% at 300 and 500 μM, respectively. After 5 days, a similar decrease in cell numbers was observed in the range of 50-300 μM Mn (II), with only 2.4×10^6 cells/ml (8% of the control) remaining at 500 μM, where dead cell proportion was 95%. By day 7, a significant decrease in *Chlorella* population was seen at 200 μM and higher (94% and 17% from the control variant for 200 and 500 μM respectively). Dead cell number was substantial only at 500 μM (accounting for 86% of the total cell number).

Two week experiment, Fe(III). A 2-week experiment was conducted to study the impact of Fe (III) and Mn (II) on *Chlorella* at lower temperatures due to slow growth of L2 culture (fig. 2). Results showed that after 2 days at +28°C, the number of L1 cells increased by 1.5—2.0 times greater than at +20°C (fig. 7) with 5-75 μM of Fe (III), while the percentage of dead cells ranged from 1.5 to 20.0% (at +20°C and +28°C respectively). Starting with 100 μM of Fe (III), growth was suppressed and dead cells rose to 66-100%.

After 5 days, in controls, culture density was $27\text{-}29\times10^6$ cells/ml with a slight decrease at 5-75 μM of Fe (III). At +20°C cell numbers were 5-10% higher than at +28°C. With higher Fe (III) concentrations (100-500 μM), the number of vital L1 cells in most cases did not exceed 0.5×10^6 cells/ml and dead cells increased to 80-100%. Almost complete suppression of culture growth was observed from 100 μM. On day 5, L2 culture at +10°C had 3-4 times lower cell numbers than L1 and the active

culture growth continued even at 100 μM of Fe (III), while the percentage of dead cells climbed up with increasing of Fe (III) concentration (fig. 7). At 150-500 μM in L2, the *Chlorella* cell density was low (0.8–1.2 $\times 10^6$ cells/ml) compared to values at 5-100 (5.6–6.7$\times 10^6$ cells/ml).

After 7 days in L1 with 5 and 75 μM Fe (III), the number of *Chlorella* cells at +28°C and +20°C was 22-26$\times 10^6$ cells/ml, with the proportion of dead cells three times higher at +28°C than at +20°C. At 100 μM and higher, over 80% of cells were non-living in both variants. On day 7, L2 cell number remained consistent with day 5 values, prompting sampling on day 9, where the control had 15$\times 10^6$ cells/ml with only 2.5% dead cells. The apparent growth of culture was also observed in the range of 5-100 μM of Fe (III), while the proportion of dead cells was up to 3.4%, increasing significantly at 150-500 μM to 75% or more. No living cells were observed in L1 at 150 μM in both temperature variants.

By day 14, cell density of L1 was 2 or more times higher than L2 (both, in control and experiment) in the range up to 100 μM of Fe (III). From day 7 to 14 in L1 at 5 and 75 μM of Fe (III) cell amount increased by no more than 20% (fig. 7). The number of cells on 14th day with 100 μM at +28°C was 14 times greater than at +20°C. No growth was observed in L1 at 150-500 μM Fe (III). In L2, growth occurred at 150 μM of Fe (III) with 56% dead cells. At 300–500 μM and above no living cells were present.

Two week experiment, Mn(II). After 2 days of experiment in the control and at 5-300 μM of Mn (II), *Chlorella*'s cell number was nearly double at +28°C compared to +20°C. The lowest cell counts were observed with 500 μM of Mn (II) (5.8$\times 10^6$ cells/ml at +28°C and 2.7$\times 10^6$ cells/ml at +20°C) (fig. 8). The values of dead cell proportion in the range of 5-300 μM were larger in L1 at with the highest percentage at 500 μM (30% at +28°C and 83% at +20°C).

On day 5, the *Chlorella*'s population in L1 at 5-200 μM Mn (II) was 25-30 $\times 10^6$ cells/ml (at +20°C and +28°C), sharply reducing at higher concentrations (300 and 500 μM). The proportion of non-living cells increased with rise of metal concentration from 5 to 300 μM, reaching 95% and 89% at +28°C and +20°C, respectively. In the experiment with L2 the number of algae cells reached 7.6$\times 10^6$ cells/ml, following a standard growth curve. Manganese (II) concentrations from 5 to 500 μM suppressed culture growth by 15–78% (correlation coefficient: 0.62; $p \leqslant 0.05$) with the amount of dead cells increased with a growth of metal concentration in the medium reaching 55% at 500 μM (fig. 8).

After 7 days, growth of *Chlorella* L1 at +28°C and +20°C followed standard curve. When exposed to Mn (II), growth was similar to the control except at 500 μM where it was significantly suppressed to 17% of the control. In L1 at +20°C with Mn (II), culture density was under the control values. The proportion of dead cells in L1 (at +28°C and +20°C) increased slightly with Mn (II) concentration up to 300 μM, then sharply rose to 90-100% at 500 μM (fig. 8). On day 9, the cell density of *Chlorella* L2 in the control reached 16.6$\times 10^6$ cells/ml (stationary growth phase). With the addition of 5-200 μM Mn (II), the cell number decreased slightly, but was approximately 2-times lower than in L1 on 7th day. At 300 and 500 μM, it was 45% and 32% of the control values, respectively. The proportion of dead cells did not exceed 4% at metal concentrations up to 200 μM, At 500 μM rose sharply to 68%. This index of dead cells was significantly lower than in L1.

Figure 7. The effect of 5-500 μM of Fe(III) on the total cell number and ratio of living and dead cells of L1 (+28°C, +20°C) and L2 (+10°C) in a 14-day experiment, "C"-control

Рисунок 7. Влияние 5-500 мкМ Fe(III) на общее количество клеток и соотношение живых и мертвых клеток L1 (+28°C, +20°C) и L2 (+10°C) в 14-дневном эксперименте, "C"-контроль

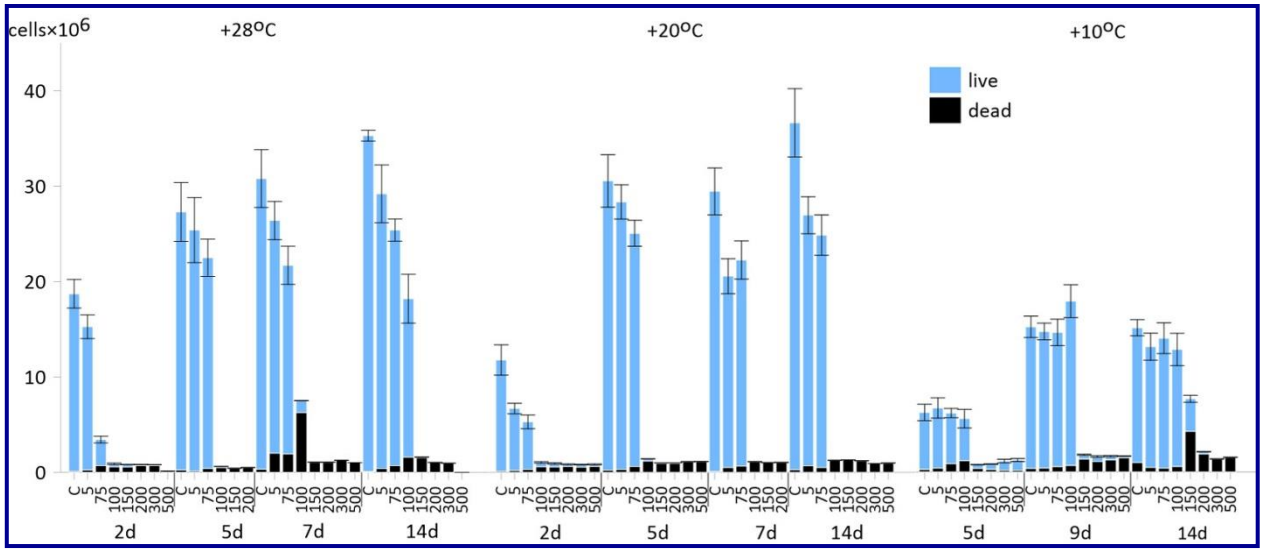
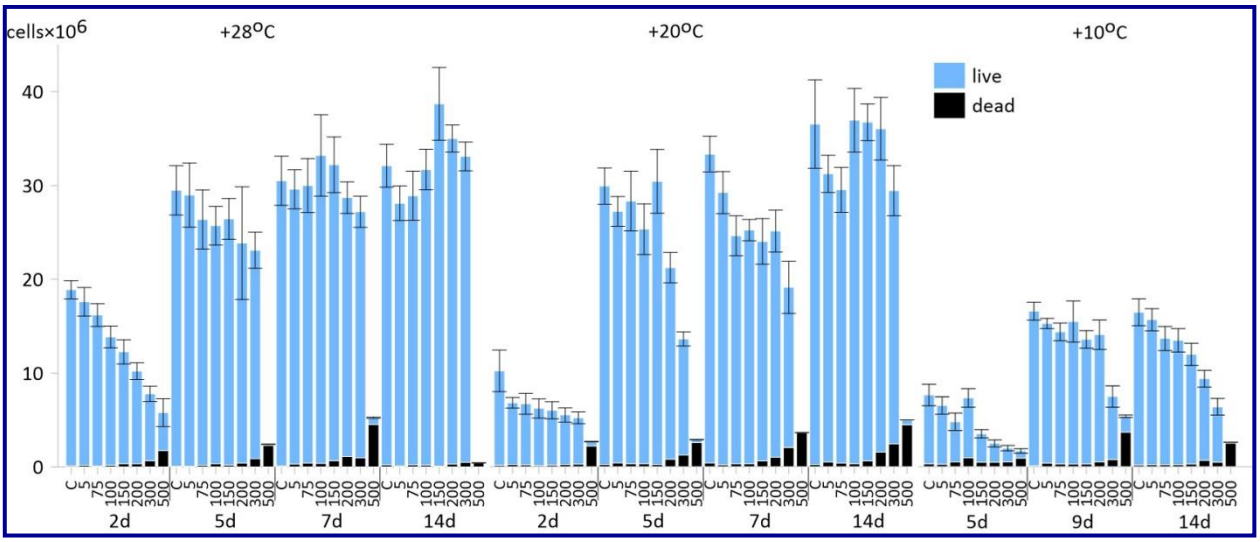


Figure 8. The effect of 5-500 μM of Mn(II) on the total cell number and ratio of living and dead cells of L1 (+28°C, +20°C) and L2 (+10°C) in a 14-day experiment, "C"-control

Рисунок 8. Влияние 5-500 мкМ Mn(II) на общее количество клеток и соотношение живых и мертвых клеток L1 (+28°C, +20°C) и L2 (+10°C) в 14-дневном эксперименте, "C"-контроль



By day 14, the number of microalgae in the control was higher at +20°C. In the presence of 150-200 μM Mn (II) at +28°C, it surpassed the respective control, while at +20°C matched it. The proportion of dead cells in L1 experiments was significantly higher at +20°C than at +28°C. In L2, after two weeks, the culture reached 16.5×10^6 cells/ml in the control, which is 2-2.5 times lower than L1. The cell number in experiments with 5-300 μM Mn (II) gradually decreased from 15.7 to 6.4 $\times10^6$ cells/ml. The proportion of dead cells at concentrations up to 100 μM in L2 did not exceed 2%, increasing to 7.7% at range 150–300 μM. In L1 at +20°C and +28°C, the mortality rates were 1.6-8.3% and 0.2-1.5% in the same range, respectively. The critical concentration was 500 μM Mn (II) in all cases, leading to 90-100% dead cells.

Pigment content. Pigment content on day 7 in the culture L1 at +28°C, it was shown that the Chl *a* and Chl *b* content in the control reached values 1.71 and 0.49 mg/l respectively. The highest concentration of chlorophylls was recorded in the variant with addition of 50 μM of Fe (III) (table 1). At the same time, significant correlations between chlorophylls content and concentration of Fe (III) were noted (correlation coefficients: −0.70 and −0.65; $p \leqslant 0.01$). In tests with Mn (II), Chl *a* and *b* values were lower than in the control (−0.44 and −0.64; $p \leqslant 0.05$). The Chl *a* and *b* ratio in both, control and

experimental variants was in the range of 2.98–3.97 (3.22 ± 0.07 in average), that indicated high photosynthetic activity of *Chlorella* L1 at +28°C (table 1). Normally, this ratio corresponds to 2.2–3.0. Carotenoid content did not differ significantly from the control. The total ratio of chlorophylls to carotenoids was highest at 50 µM of Fe (III) and Mn (II). No significant relationships were found between carotenoid content and HMs concentration.

Table 1. The effect of Fe(III) and Mn(II) on concentration of pigments and their ratio in *Chlorella* cells within 7 days (L1, +28°C)

Таблица 1. Влияние Fe(III) и Mn(II) на концентрацию пигментов и их соотношение в клетках хлореллы через 7 дней (L1, +28°C)

Metal concentration, µM	Pigment concentration, mg/l			$C_{chl\ a}$/ $C_{chl\ b}$	$\sum C_{chl}$/ C_{car}
	Chlorophyll *a* ($C_{chl\ a}$)	Chlorophyll *b* ($C_{chl\ b}$)	Carotenoids (C_{car})		
Fe (III)					
Control	1.71 ± 0.17	0.49 ± 0.10	0.70 ± 0.13	3.49	2.81
5	1.51 ± 0.30	0.51 ± 0.13	0.69 ± 0.16	2.96	2.93
50	1.91 ± 0.27	0.67 ± 0.19	0.63 ± 0.14	2.85	4.09
75	1.35 ± 0.31	0.34 ± 0.14	0.78 ± 0.20	3.97	2.17
100	0.54 ± 0.20	0.19 ± 0.08	0.25 ± 0.11	2.84	2.92
Mn (II)					
Control	1.75 ± 0.06	0.53 ± 0.04	0.84 ± 0.14	3.30	2.72
5	1.28 ± 0.27	0.43 ± 0.09	0.87 ± 0.16	2.98	1.96
50	1.50 ± 0.33	0.46 ± 0.11	0.51 ± 0.21	3.26	3.84
75	1.29 ± 0.24	0.41 ± 0.08	0.84 ± 0.17	3.14	2.02
100	1.30 ± 0.14	0.42 ± 0.10	0.82 ± 0.20	3.09	2.09
150	1.51 ± 0.18	0.44 ± 0.09	0.85 ± 0.24	3.41	2.28
200	1.41 ± 0.15	0.40 ± 0.08	0.76 ± 0.26	3.52	2.38
300	1.23 ± 0.13	0.41 ± 0.12	0.60 ± 0.11	3.07	2.72

Within 14 days, pigment content was measured in cultures L1 (+28°C and +20°C) and L2 (+10°C). Significant differences in algal pigment values at different temperatures were observed (*t*-test). Despite an increase in algae cell number at +28°C in both the control and experiments with HMs, pigment concentrations in the control decreased significantly in L1 on the 14th day compared to the 7th day (by 5.4 times for Chl *a*, 3.7 times for Chl *b* and 1.2 times for carotenoids) (table 2). The low chlorophyll values in L1 at +28°C were attributed to resource depletion in a limited cultivation volume and aging of the algae culture. A disruption in *Chlorella*'s photosynthetic activity was also indicated by the pigment index ($\sum C_{chl}$/ C_{car} < 1 in control, at 5-75 µM Fe (III) and 5-100 µM Mn (II)) (table 2). Overall, at low pigment concentrations in L1 at +28°C, an increase in Chl *a* and Chl *b* was observed at 75-200 µM Mn (II) or 100 µM Fe (III), correlating with an increase in cell density (fig. 7, 8).

Positive relationships were found between Chl *a* (0.76, 0.92) and Chl *b* (0.84, 0.92) content and the Mn (II) and Fe (III) concentrations in the medium at +28°C ($p \geq 0.05$). At +20°C in L1 with Fe (III), the synthesis of chlorophylls *a* and *b* was at its highest at 75 µM, while with Mn (II) – at 100 µM (table 2). At the same time, these parameters did not show significant correlations with metal concentrations. The ratio of total chlorophyll to carotenoids rose up with higher HMs concentrations in the medium, but the Chl *a*/ Chl *b* proportion decreased.

At +10°C (L2) Chl *a* and *b* values were higher in the control than in trials, but lower than at +20°C (L1). Negative correlations were found between Chl *a* and HMs concentration (–0.84 and –0.73 for Mn (II) and Fe (III)). For Chl *b*: –0.83 and –0.62 respectively. Carotenoid levels remained

Table 2. The effect of Mn(II) and Fe(III) on pigment concentration (mg/l) and their ratio in Chlorella cells within 14 days (L1 +28°C, +20°C; L2 +10°C)

Таблица 2. Влияние Mn(II) и Fe(III) на концентрацию пигмента (мг/л) и их соотношение в клетках хлореллы через14 дней (L1 +28°C, +20°C; L2 +10°C)

Metal conc., μM	Chlorophyll *a* C_{chla}			Chlorophyll *b* C_{chlb}			Carotenoids C_{car}			$\sum C_{chl}/ C_{car}$		
Mn(II)	+28°C	+20°C	+10°C	+28°C	+20°C	+10°C	+28°C	+20°C	+10°C	+28°C	+20°C	+10°C
Control	0.31 ± 0.03	3.11 ± 0.20	2.34 ± 0.40	0.12 ± 0.01	0.63 ± 0.07	0.41 ± 0.05	0.65 ± 0.03	1.66 ± 0.09	1.16 ± 0.07	0.66	2.25	2.37
5	0.24 ± 0.05	3.06 ± 0.61	2.15 ± 0.28	0.12 ± 0.03	0.64 ± 0.06	0.37 ± 0.07	0.65 ± 0.07	1.49 ± 0.06	1.09 ± 0.14	0.55	2.48	2.31
75	0.36 ± 0.05	3.24 ± 0.90	2.02 ± 0.19	0.18 ± 0.06	0.71 ± 0.09	0.40 ± 0.09	0.63 ± 0.04	1.46 ± 0.05	0.99 ± 0.15	0.86	2.71	2.44
100	0.48 ± 0.09	4.29 ± 0.48	2.29 ± 0.18	0.13 ± 0.05	1.03 ± 0.12	0.40 ± 0.07	0.78 ± 0.10	2.07 ± 0.12	1.14 ± 0.11	0.78	2.57	2.36
150	0.72 ± 0.11	3.14 ± 0.60	2.06 ± 0.32	0.23 ± 0.05	0.75 ± 0.09	0.39 ± 0.11	0.86 ± 0.22	1.34 ± 0.15	0.93 ± 0.09	1.11	2.90	2.63
200	0.81 ± 0.38	2.90 ± 0.61	1.75 ± 0.44	0.29 ± 0.07	0.68 ± 0.13	0.32 ± 0.13	0.86 ± 0.19	1.11 ± 0.09	0.77 ± 0.14	1.28	3.23	2.69
300	0.58 ± 0.27	2.32 ± 0.57	1.13 ± 0.36	0.30 ± 0.08	0.66 ± 0.11	0.19 ± 0.09	0.63 ± 0.13	0.75 ± 0.10	0.54 ± 0.13	1.40	3.97	2.44
Fe(III)	+28°C	+20°C	+10°C	+28°C	+20°C	+10°C	+28°C	+20°C	+10°C	+28°C	+20°C	+10°C
Control	0.33 ± 0.04	2.83 ± 0.51	2.40 ± 0.23	0.16 ± 0.06	0.69 ± 0.14	0.41 ± 0.09	0.76 ± 0.06	1.56 ± 0.07	1.16 ± 0.04	0.65	2.26	2.42
5	0.25 ± 0.04	2.53 ± 0.43	2.28 ± 0.17	0.12 ± 0.04	0.64 ± 0.11	0.30 ± 0.05	0.64 ± 0.09	1.28 ± 0.05	1.05 ± 0.05	0.58	2.48	2.46
75	0.35 ± 0.19	2.96 ± 0.67	2.23 ± 0.29	0.21 ± 0.09	0.72 ± 0.22	0.38 ± 0.11	0.64 ± 0.12	1.42 ± 0.08	0.94 ± 0.08	0.88	2.59	2.78
100	0.50 ± 0.23	“–“	2.39 ± 0.52	0.23 ± 0.10	“–“	0.43 ± 0.01	0.57 ± 0.14	“–“	1.05 ± 0.06	1.28	“–“	2.69
150	“–“	“–“	1.02 ± 0.48	“–“	“–“	0.13 ± 0.06	“–“	“–“	0.36 ± 0.07	“–“	“–“	3.19

“–“ missing of pigments

consistent up to 150 μM of Mn (II) and 100 μM of Fe (III). The pigment index varied irregularly from 2.1 to 3.2 in experiments with HMs and showed no clear dependency on metal concentration. Thus, temperature has a significant impact on *Chlorella*'s cell number and pigment content when exposed to HMs. As follows from our experiments, on the one hand at +10°C L2 *Chlorella*'s cell number was lower than in L1, also Chl *a* and *b* synthesis significantly suppressed, which shows that the culture is probably experiencing cold stress.

DISCUSSION

According to published researches there is a significant variation in concentrations of Mn (II) and Fe (III) which might have an either stimulating or inhibitory effect on *Chlorella*, thus it does not allow us to make an exact conclusion on the effect of one or another metal concentration on the growth and physiological state of that algae [Keren *et al.* 2002; Mousavi *et al.* 2011; Liu *et al.* 2018; Smythers *et al.* 2019]. However, the effect of a wide range of Fe (III) concentrations (1.8-900 μM) and an increase in the number of cells at a concentration of 360 μM was shown in the work of Cudowski, Pietryczuk [2019]. At +28°C, using a seeding dose of 2×10^6 cells/ml and cultivating for 18 days with low Fe (III) concentrations (0.2 – 1.8 μM), previous research has shown that the most significant impact on the growth of *Chlorella* culture occurred with Fe (III) concentrations between 0.36-0.72 μM. This resulted in a 50% growth enhancement on the 12th day of cultivation compared to the control [Al-Khiat *et al.* 2019]. Similar data on the positive effect of 12 μM of Fe (III) for *Chlorella* on the 13th-15th days were given in studies of Liu *et al.* [2008].

In our case, in 1-2–week experiments, at +20°C – +28°C, we observed a notable decrease in cell number with an increase in Fe (III) concentration up to 75 μM. Beyond this threshold, the number of dead cells sharply increased at 100 μM and higher. Conversely, an increase in Mn (II) concentration resulted in a more gradual emergence of negative effects, nearly completely suppressing the growth of microalgae only at 500 μM. Interestingly, at 100-150 μM Mn (II), the cell count surpassed the control values. At +10°C *Chlorella* culture remained viable even at 100 μM of Fe (III) or 300-500 μM of Mn (II). Additionally, it is worth noting that the concentration of chlorophylls was significantly higher than in control at +28°C within 7 days under the influence of 50 μM of Fe (III) and within 14 days at +20°C under the 100 μM of Mn (II). Earlier studies indicated that the concentrations of Fe (III) or Mn (II) that has a positive or negative effect on *Chlorella* widely varied. In particular, the cultivation of *Chlorella* sp. and *Scenedesmus obliquus* showed that microalgae grew well at low Fe (III) levels (4.0 – 6.3 μM), likewise in our study [Liu *et al.* 2008; Iriani *et al.* 2011; Abd El Baky *et al.* 2012]. While a significant decrease in the cell concentration of those algae was observed during the alteration from 6 μM to 83 μM of Fe (III), after 8-10 days of cultivation. The maximum abundance values (about 10^7 cells/ml) were noted on day 12 at 6 μM [Iriani *et al.* 2011]. We also emphasized the devastating effects of Fe (III) during the transition from 50 to 100 μM. But, according to other sources, the EC50 for *Chlorella* sp. was 240 μM of Fe (III) at +25°C within 4 days and even 900 μM of Fe (III) did not lead to its complete death [Subramaniyam *et al.* 2016]. In another study, it was demonstrated that Fe concentrations exceeding 200 μM led to just a reduction in the growth intensity of the culture at +25°C over a period of 25 days [Estevez *et al.* 2001]. Additionally, in Pietryczuk *et al.* [2025] Fe (II) was used by algae more effectively than Fe (III). The study showed that the biggest increase in the number of cells, studied biochemical parameters and antioxidant enzyme activity took place under the influence of 89 μM Fe (II) (highest concentration) within 120 hours [Pietryczuk *et al.* 2025].

According to literature data, Mn (II) can stimulate growth even in relatively high concentrations (more than 100 μM) at the beginning of cultivation. And the negative effect can be apparent with gradual increase in the concentration of Mn (II), in some studies – up to 1000 μM [Battah *et al.* 2015; Cudowski, Pietryczuk 2019; Smythers *et al.* 2023; Pietryczuk *et al.* 2025]. This is generally consistent with our data, where we observed slow suppression of microalgae growth to a concentration of 300 μM Mn (II). However surprisingly, as noted by Alho *et al.* [2022], Mn (II) is toxic to freshwater microalgae Chlorophyceae, *Raphidocelis subcapitata* (median inhibitory concentration after 72 h was 4 μM).

Temperature has a direct effect on the growth of microalgae. As it rises by 10°C, the number of microalgae doubles until the optimal growth temperature is reached [Han *et al.* 2016]. According to Dai *et al.* [2022], most industrial microalgal species have an optimal growth temperature ranging from +15 to +25°C, and the maximal growth temperature is usually between +25°C and +35°C. Optimal growth temperatures vary greatly for different strains of *C. vulgaris*, so the choice of cultivation conditions should be strain-specific. Also some studies have found that at higher temperatures the algal cell size reduces [Padfield *et al.* 2016; Ahmad *et al.* 2020]. The optimal temperature for the strain IMBR-19 studied in the current work was +28°C. During the cultivation of two lines of this strain, it was shown that lower temperatures (L2, +10°C) facilitated viability of microalgae exposed to HMs impact. Moreover we noticed that at optimal temperature cells tended to be smaller. However, when the strain was subjected to both lower and higher temperatures, the cell size increased, and the time intervals between cell divisions also appeared to lengthen.

Usually researchers examine the influence of HMs at a constant temperature, and there are few articles that show combined effects of HMs and temperature. *Chlorella* culture is rarely cultivated for more than 7 days at temperatures exceeding 20°C. In a one-week experiment of Wang *et al.* [2022], on the effects of Pb (II) and Cr (VI) within the range 15°C – 30°C, the increase in cell number and pigment content was proportional to rise of cultivation temperature. Also researchers showed that *Chlorella* which was growing at higher temperatures under normal conditions on days 11-12 either reached a plateau or partially decreased in number [Wang *et al.* 2024]. Similarly, Iriani *et al.* [2011] noted a sharp increase in cell number of *Chlorella* sp. between days 8-12, followed by a noticeable decrease from day 14. In another study, regardless of the amount of HM added at 25°C, the *Chlorella* culture exhibited robust growth for up to 8 days, and then began to die [Ajayan *et al.* 2018]. In a study by Kyratzopoulou *et al.* [2025] when exposed to different concentrations of some HMs, the maximum cell number was obtained on day 5, followed by a decline.

High levels of HMs in the environment can disrupt the photosynthetic process in algae by affecting key parameters of the photosynthetic apparatus. Chlorophyll (Chl) levels are crucial for algal growth and serve as an indicator of photosynthetic activity. Since Chl *a* is associated with the reaction centers of photosystems, then the higher its content, the more active photosynthesis proceeds [Kozlov 2001]. At the same time, a decrease in the content of Chl *a* in *Chlorella* cells is a frequent sign of heavy metal toxicity [Tremper *et al.* 2004]. Carotenoids are less susceptible to the negative effects of HMs and serve not only as additional pigments but also shielding chlorophylls free radicals oxidation caused by HMs [Demmig-Adams 1990]. The ratio of total chlorophyll content to carotenoid (pigment index $\sum C_{Chl}/C_{Car}$) reflects the level of metabolic activity and the functional state of cells.

Previous studies have shown that *Chlorella*'s chlorophyll content decreased by more than three times in four days when grown with 90 – 900 μM of Fe (III) [Subramaniyam *et al.* 2016]. Also, Fe (III) concentrations exceeding 200 μM led to decreased growth intensity of the culture at +25°C over 25 days-long study. On the contrary, the base Fe (III) concentration (90 μM) in the medium had no effect

on carotenoid production. We also found no significant correlations between Fe (III) concentration and carotenoid levels in current study. The optical density of the culture and the chlorophyll content reached their maximums at 100 – 200 μM of Fe (III) [Estevez *et al.* 2001]. According to Cheng *et al.* [2022] the medium with high Fe concentration (17 μM) achieved higher OD faster and chlorophyll accumulation was higher. In our investigation, highest concentration of chlorophyll was found under 50 μM of Fe (III) at +28°C. But, Fe (III) concentrations more than 100–150 μM suppressed culture growth, making it almost impossible to reliably measure the amount of pigments. At +20°C (L1) on day 14, chlorophyll synthesis was highest at 75 μM Fe (III).

Unlike other metals found in the photosynthetic electron transport chain the Mn excess enhances photosynthetic activity rather than diminish it [Smythers *et al.* 2023]. Contrary to our data, some sources suggest that even small concentrations of Mn (II) can have negative effects on the growth and pigment content of microalgae. The presence of this metal diminished algae growth and the average fluorescence of Chl *a* at the highest concentrations of this HM (3.6–14.6 μM). The authors report that Mn (II) concentrations from 1.5 to 14.6 μM at +25°C notably reduced the number of microalgae (at 14.6 μM by 82%) and the content of Chl *a*. Manganese (II) at 900 μM also caused a disruption of photosynthetic activity (decrease in the content of Chl *a* and *b*) in cells of *C. vulgaris* and *S. quadricauda* [Smythers *et al.* 2023]. According to Cudowski, Pietryczuk [2019] in the cells of *C. vulgaris* Mn (II) at a concentration of 1000 μM resulted in a gradual displacement of magnesium from algae cells and induced Fe deficiency causing a decrease in the concentration of chlorophylls. However, this decrease was small, and the content of chlorophylls did not fall below the control, suggesting that a much higher concentration of Mn (II) is required to cause the complete inhibition of photosynthesis and biosynthesis of photosynthetic pigments in *C. vulgaris* [Cudowski, Pietryczuk 2019]. In our study, concentrations up to 300 μM, showed no considerable negative changes in pigment content, compared to the control on the 7th day at +28°C, with a moderate decrease in chlorophylls. Carotenoids remained stable up to 150 μM. By the 14th day, a general decrease in pigment levels was observed at +28°C (unrelated to the effects of Mn), while at +10°C and +20°C, by contrast there was a noticeable increase in pigments at 100 μM.

When considering other microalgae as test objects, it is evident that the extent of HMs impact is strongly dependent on the species-specific sensitivity. For instance, *S. armatus* exposed to 340 μM of Mn (II) exhibited a slight increase in cell number relative to the control during the first three days. However, from the fourth day onward, its population was significantly reduced compared to control levels [El-Enany, Issa 2001]. *Gonyostomum semen* (Raphidophyceae) required higher (>4 μm) Fe(III) concentrations – up to 11 μM, to support growth in the culture medium [Münzner *et al.* 2021]. Subramaniyam *et al.* [2016] reported that the microalgae *Chlamydomonas* sp. and *Chlorococcum* sp. exhibited lower tolerance to Fe(III) relative to *Chlorella* sp., with EC50 values determined at 236, 230, and 200 μM for *Chlorella* sp., *Chlamydomonas* sp. and *Chlorococcum* sp. respectively. A decrease in chlorophyll concentration occurred at 90 μM of Fe(III) and above [Subramaniyam *et al.* 2016]. In contrast, such strains as *C. fusca, Ankistodesmus braunii, S. obliquus, C. saccharophila* and *Leptolyngbya* sp. were able to maintain normal growth pattern up to 900 μM of Fe (II), while the concentration beyond it inhibited the growth [Zada *et al.* 2021].

CONCLUSIONS

The current study results showed that high levels of HMs had a toxic effect (death of most algae cells) at different cultivation temperatures and experiment lengths. We found that Fe (III) had a more

toxic effect than Mn (II) when cultivating *Chlorella* at different temperatures. Iron (III) at 150 μM and Mn (II) at 300 μM became critical for *Chlorella*.

Noticeable adaptive reaction of algae was observed at +20°C and +28°C, but the tolerance to various HMs concentrations was higher at +10°C. With an extension of incubation time the adaptive capabilities of algae to high concentrations of HMs were observed.

Our research showed that culture growth was significantly slowed at low temperature, but gradual inclusion of HMs in the metabolism of algae under cold conditions had a lesser impact on *Chlorella* due to its slow growth, allowing it to tolerate higher concentrations of HMs (e.g. in L2 case compared to L1). Therefore, for industrial-scale applications in water treatment for Fe(III) removal the use of *Chlorella* is not recommended at a concentration of 75 μM or higher when the temperature is ⩾20°C. Similarly, at temperatures 10–20°C, concentrations exceeding 100 μM are not advisable. Regarding Mn(II), it is important to note that concentrations above 300 μM are unsuitable under both temperature conditions.

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CONFLICTS OF INTERESTS

The authors declare that they have no conflicts of interest related to this study.

AUTHOR CONTRIBUTIONS STATEMENT

All authors have made substantial contributions to this work. Material preparation, data collection, and analysis were performed by Dmitry Y. Sharavin and Polina G. Belyaeva. The first draft of the manuscript was written by Dmitry Y. Sharavin. All authors read and approved the final manuscript.

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