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CONTENTS

About a New Representative of the <i>Aulacoseira</i> Thwaites (<i>Bacillariophyta</i>) for the Flora of Ukraine	
<i>Genkal S.I.</i>	217
Distribution of Phytoplankton and Periphyton in the Shallow Rice-Fish Fields of Arunachal Pradesh, India	
<i>Awasthi M.</i>	223
New Localities of <i>Bambusina borrieri</i> (Ralfs) Cleve (<i>Charophyta</i>), a Species from the Red Data Book of Ukraine	
<i>Berezovska V.Yu., Burova O.V., & Raida O.V.</i>	237
Finding of a Rare Species of Diatom <i>Nanofrustulum siloi</i> (Lee, Reimer et Mcenery) Round, Hallsteinsen & Paasche, 1999 in the Periphyton of the Coastal Waters of the Black Sea	
<i>Blaginina A. & Ryabushko L.</i>	247
The Analysis of the Genetic Parameters of <i>Chlorella vulgaris</i> Beyer. Culture Growing in the Presence of Sodium Selenite, Zinc Sulfate and Chromium Chloride	
<i>Bodnar O.I., Andreev I.O., Prokopiak M.Z., Drobyk N.M., & Grubinko V.V.</i>	257
Electrophoretic Recovery of Microalgae Biomass of Straine <i>Graesiella emersonii</i> and Its Cell Analysis Post Treatment to Ensure Viable Culture Maintenance	
<i>Balachandran D. & Thomas J.</i>	269
<i>Undaria pinnatifida</i> (Harvey) Suringar and <i>Macrocystis pyrifera</i> (Linnaeus) C.Agardh from San Jorge Gulf (Argentina) as Indicators of Toxic Metals	
<i>Salomone V.N. & Riera M.</i>	281
Unattached <i>Cystoseira</i> s.l. in the Black Sea: taxonomy of <i>Gongolaria barbata</i> f. <i>repens</i> comb. nov.	
<i>Sadogurska S.S.</i>	293
Upper Pliocene Diatom Complexes and its Significance for Establishing the Lower Boundary of Quarter (South of the Far East)	
<i>Pushkar V.S.</i>	305

The Analysis of the Genetic Parameters of *Chlorella vulgaris* Beyer. Culture Growing in the Presence of Sodium Selenite, Zinc Sulfate and Chromium Chloride*

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ABSTRACT: The genetic polymorphism of *Chlorella vulgaris* under the action of trace elements selenium, zinc and chromium has been studied to optimize the method of algae cultivation and production of beneficial compounds. Metals and nonmetals, when entering the cell, have a high biochemical effect, modifying metabolic reactions, including those related to the functioning of the genetic apparatus of microalgae cells. The work uses generally accepted methods of algae cultivation, the method of DNA isolation by Rogers and Bendich (1985); molecular genetic analysis using ISSR (inter simple sequence repeats) and IRAP (inter-retransposon amplified polymorphism) markers. For all samples of *C. vulgaris*, 109 fragments were obtained, 42 of which were polymorphic (38.5%). The Jacquard genetic distances (D_j) between samples of *C. vulgaris* culture growing on nutrient media of different composition and control (culture grown under standard conditions) were: 0.232 for the action of selenite alone, 0.206 for the combined action of selenite and zinc and 0.300 for joint action of selenite and chromium. It was found that the excessive addition of trace elements to the cultivation medium causes certain modifications of the genetic apparatus of algae cells. At the same time, the detected changes in algal cells cultivated with different trace elements and their combinations are within the level of genetic polymorphism of unicellular green algae under natural growth conditions. It testifies the absence of significant genotoxic effects of trace elements and high metabolic and genetic plasticity of studied *C. vulgaris* culture.

KEY WORDS: *Chlorella vulgaris*, microelements, ISSR and IRAP markers, genetic polymorphism, Jacquard distance

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INTRODUCTION

Studies of the biotechnological potential of microalgae that are producers of commercial compounds are now aimed at elucidating the mechanisms of increasing their stress resistance, increasing productivity, intensifying metabolism and directing metabolism to activate certain biosynthetic processes and obtain useful compounds (Richmond, Hu, 2013). Along with biochemical methods of regulation, genetic methods of metabolism transformation are actively used, which consist in studying the genome of algae, expression of the corresponding genes and formation of new genetically modified strains (Alzahrani, 2013; Shrestha et al., 2013).

It is known that algae contain a significant number of groups of active organic components of different chemical structure and different properties. They include polysaccharides (alginates, laminarin, agar), pigments (chlorophylls, carotenoids, lutein), lipids (saturated and polyunsaturated fatty acids), peptides and proteins, as well as vitamins and minerals. Many of these compounds are characterized by a wide range of potential therapeutic and prophylactic effects (antioxidant, anticancer, antiviral, antibacterial, antiallergic, antidiabetic, anti-inflammatory, etc.) on animals and humans (Liu, Hu, 2013; Richmond, Hu, 2013). Therefore, a relevant and promising way to treat and prevent many diseases may be the use of natural dietary supplements from algae (Skrivan et al., 2010; Liu, Hu, 2013). Due to their high adsorption properties, algae are able to absorb and accumulate metals and non-metals against the concentration gradient and include them in intracellular organic molecules (Richmond, Hu, 2013). Given this, algae complexes of selenium (a component of the antioxidant system) and biologically active metals zinc (one of the most important regulatory trace elements in living organisms) and chromium (glucose tolerance factor) are of interest (Skrivan et al., 2010; Yoshida et al., 2011; Vincent, 2013).

Chlorella, due to its rapid growth and reproduction and unpretentiousness to growing conditions, is a classic object for various laboratory studies and raw material for useful products in aquaculture (Afkar et al., 2010; Liu, Hu, 2013; Richmond, Hu, 2013). In contrast to morphological, biochemical and physiological features, which largely depend on the age of the culture and the conditions of cultivation, and do not always have a clear species affiliation, genetic features are usually more stable.

Today, molecular methods are widely used to identify organisms and reveal their phylogenetic relationships, including unicellular algae (Roshani et al., 2012; Wongsawad et al., 2015).

We have previously found that the use of sodium selenite alone and in combination with zinc sulfate or chromium chloride for biotechnological cultivation of *C. vulgaris* to obtain beneficial compounds enriched in trace elements is optimal given the modeling of

metabolic status of algae and activation of lipid metabolism (Lukashiv et al., 2017; Bodnar et al., 2018). At the same time, it remains unknown how cultivation under such conditions affects the genetic characteristics of algal cells.

Alzahrani (2013) showed that along with physiological and biochemical changes in the adaptation of *C. vulgaris* culture to elevated concentrations of copper, there are changes in the genetic characteristics of the culture. The differences, determined by ISSR-PCR (method of molecular genetic analysis based on polymerase chain reaction PCR), between the culture of wild algae and selected for resistance to copper culture exceeded 60%.

Therefore, a comprehensive approach to studying the effects of metals and nonmetals as effective regulators of metabolism in algae (which is the use of physiological, biochemical and molecular methods) may help to determine the level of genetic changes in the culture growing in the medium with excess content of trace elements, and establish their optimal concentrations. This will allow more efficient selection of resistant to potentially toxic chemical elements of algal strains (Alzahrani, 2013; Kebeish et al., 2014).

One of the most effective tools for the study of genetic polymorphism, due to its simplicity, high sensitivity and speed, are methods of molecular genetic analysis based on PCR (Malyshev, Kartel, 1997; Mostafa et al., 2011), in particular using multilocus ISSR (inter simple sequence repeats) markers (Mostafa et al., 2011; Alzahrani, 2013) and IRAP (inter-retransposon amplified polymorphism) markers (Malyshev, Kartel, 1997).

MATERIALS AND METHODS

The object of the laboratory study was algologically pure culture of green alga *Chlorella vulgaris* HPDP-119 from the collection of the Institute of Hydrobiology of NAS of Ukraine. It was grown on Fitzgerald medium in the modification of Zender and Gorham No 11 at 22–25 °C and light intensity of 2500 lx for 16 h (Topachevskiy, 1975).

Aqueous solutions of Na_2SeO_3 based on the number of Se(IV) ions – 10.0 mg/dm³ separately and together with $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ based on Zn^{2+} – 5.0 mg/dm³ or with $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ based on the content of Cr^{3+} – 5.0 mg/dm³ were added to the culture medium. The control was a culture grown in a standard medium without the addition of sodium selenite and salts of chromium and zinc. Cells for DNA isolation were taken on the 7th day of cultivation.

DNA was isolated according to a standard protocol from algal biomass dried at 37 °C (Rogers, Bendich, 1985). As a result of preliminary screening of eight ISSR primers and four IRAP primers, we selected seven and one primers, respectively, which provided the synthesis of clear reproducible amplicons (primer sequences are given in Table 1).

Amplification was performed in a thermal cycler Tertsik MS2 (Biotechnology, Russia). The reaction mixture contained: 20 ng DNA (IRAP analysis), 30 ng DNA (ISSR

analysis), 0.2 mm dNTP (Fermentas, Lithuania), 1.25 U Taq polymerase (Amplisens, Russia), 0.5 µm primer, 1 × PCR buffer on (NH₄)₂SO₄ with 2.5 mM MgCl₂ (Fermentas, Lithuania). 15 µL of mineral oil was layered on top.

TABLE 1: Nucleotide sequences of ISSR and IRAP primers

No	Type of primer	Primer's name	Nucleotide sequence, (5' – 3')
1	ISSR	UBC#03	5' ACA CAC ACA CAC ACA CTT 3'
2		UBC#04	5' ACA CAC ACA CAC ACA CAG 3'
3		UBC#05	5' ACA CAC ACA CAC ACA CTG 3'
4		UBC#23	5' ACA CAC ACA CAC ACA CTA 3'
5		UBC#807	5' AGA GAG AGA GAG AGA GT 3'
6		UBC#836	5' AGA GAG AGA GAG AGA GYA 3'
7		UBC#840	5' GAG AGA GAG AGA GAG AYT 3'
8		UBC#811	5' GAG AGA GAG AGA GAG AC 3'
9	IRAP	642	5' TTTGAAAACCTGGCGGCAACG 3'
10		866	5' ACCAGCCCGGGCCGTCGACC 3'
11		1651	5' TGACCAAGGGCGCGTATCGTG 3'
12		1681	5' ATACCTCGGAGGCGCTGCACCTG 3'

Note. Primers that provided the synthesis of clear reproduction sequences and were used for genetic analysis are highlighted in bold; Y = C/T.

A standard DNA-free reaction mixture was used as a negative control. The following modes were set for PCR: ISSR-PCR: 94 °C – 2 min, 35 cycles (94 °C – 30 s, 53 °C – 30 s, 72 °C – 1.5 min), 72 °C – 2, 5 min; IRAP-PCR: 94 °C – 2 min, 35 cycles (94 °C – 30 s; 58 °C – 30 s; 72 °C – 1.5 min), 72 °C – 2.5 min. The amplification products were separated by electrophoresis in a 1.3% agarose gel with the addition of 0.5 µg/mL ethidium bromide in 1 × SB-buffer (5 mM Na₂B₄O₇, pH 8.5) for 5–6 h at an electric field strength of 4–5 V/cm. To determine the length of the fragments we used a molecular weight marker (100 bp + 1.5 Kb + 3 Kb DNA Ladder), which contained DNA fragments of the following sizes: 100; 200; 300; 400; 500; 600; 700; 800; 900; 1000; 1500; 3000 BC (Ltd.-SibEnzym-M, Moscow).

Processing of electrophoregrams was performed using the program TotalLab TL120 (Nonlinear Dynamics). FAMD 1.25 (Schluter, Harris, 2006) and GenAlEx 6.5 (Peakall,

Smouse, 2006) were used for statistical evaluation of PCR analysis data. Parameters such as the proportion of polymorphic amplicons and Jacquard genetic distances (D_J) were used to assess the level of genetic differences. The obtained experimental data were processed using the program Statistica 6.0.

In experimental studies, reagents from Sigma, Lachema, Reanal, Khimreaktiv (Ch.D.A.), Amplisens, Fermentas, and OOO-SibEnzym-M were used.

RESULTS AND DISCUSSION

The ISSR and IRAP primers used provided fragment amplification in the range of 180–2600 bp and 190–3400 bp accordingly. The Figure shows electrophoregrams that show the polymorphism of the PCR profiles of samples of *C. vulgaris* cultures growing on media of different composition.

TABLE 2: Characteristics of PCR products of *Chlorella vulgaris* in accordance with our data from ISSR and IRAP markers

Primer	Number of amplicons	Number of polymorphic amplicons
ISSR-03	15	7
ISSR-04	13	1
ISSR-05	10	1
ISSR-23	17	7
ISSR-807	13	1
ISSR-836	11	7
ISSR-840	21	17
IRAP-642	8	1
Totally	109	42

When determining the genetic polymorphism in *C. vulgaris* using ISSR primers, we obtained PCR products in the range of 200–2600 bp which is similar with those of *C. vulgaris* and *C. pyrenoidosa* amplified using the same type of primers (Shen, 2008). The total number of fragments for all samples synthesized using 1 IRAP and 7 ISSR markers (Table 2) was 109, of which 42 were polymorphic (38.5%), and primer UBC # 840 (ISSR) showed the highest level of polymorphism (81%), and UBC # 04 (ISSR) – the lowest (7.7%). Shen (2008) showed that the proportion of polymorphic amplicons in four clones of *C. vulgaris* under standard cultivation conditions was 39.6%, which corresponds to our data on the effects of selenium alone and together with zinc or chromium.

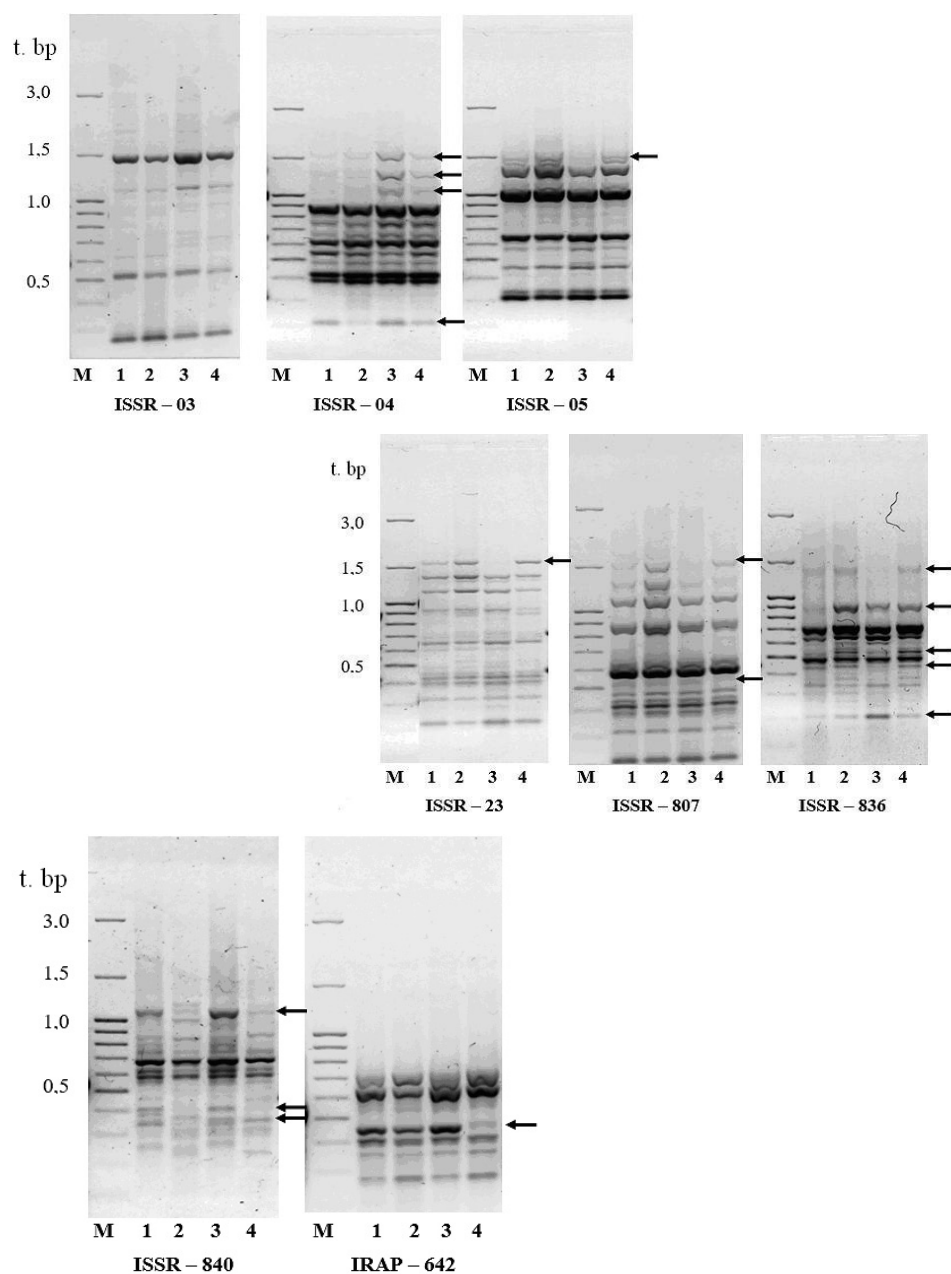


FIGURE: Polymorphism of the spectra of PCR products of samples of *Chlorella vulgaris* cultures growing on media of different composition: 1 – control; 2 – algae grown with the addition of Se(IV) 10.0 mg/dm³; 3 – Se(IV) 10.0 mg/dm³ and Zn(II) 5.0 mg/dm³, 4 – Se(IV) 10.0 mg/dm³ and Cr(III) 5.0 mg/dm³; M is a marker of DNA molecular weight

We determined the values of Jacquard genetic distances (D_j) between samples of culture of *C. vulgaris*, growing on nutrient media of different composition (with the addition of selenium, zinc and chromium), and control. D_j values calculated on the basis of PCR analysis showed that the largest differences from the control (0.300) had a sample of *C. vulgaris* culture grown on medium containing sodium selenite and chromium chloride, and the smallest value (0.206) had a sample of *C. vulgaris* culture grown in the presence of sodium selenite and zinc sulfate (Table 3).

TABLE 3: Jacquard genetic distances, calculated on the basis of ISSR- and IRAP-PCR data

Nutrient medium	1	2	3	4
Control	–	–	–	–
Se(IV)	0.232	–	–	–
Se(IV) + Zn(II)	0.206	0.216	–	–
Se(IV) + Cr(III)	0.300	0.148	0.298	–

Legend: 1 – control; 2 – algae cultivated with Se(IV) 10.0 mg/dm³; 3 – with Se(IV) 10.0 and Zn(II) 5.0 mg/dm³; 4 – with Se(IV) 10.0 and Cr(III) 5.0 mg/dm³.

When culturing *C. vulgaris* on medium with sodium selenite D_j was 0.232 comparing to the control. With the addition of sodium selenite and zinc sulfate, this index decreased by 12% (Table 3). Therefore, it can be assumed that zinc modulates and controls the accumulation of genetic changes in the culture of *C. vulgaris*, because their number is lower than in the sample, which was cultured only with sodium selenite.

Thus, it was found that the cultivation of *C. vulgaris* in the presence of selenium, zinc or chromium is accompanied by changes in the genetic characteristics of algal culture. These changes could be explained by the mutagenic effect of these elements in the concentrations used. However, given the short duration of the culture period, during which only about three cell divisions could occur, and the available literature, this assumption seems unlikely.

Sun et al. (2014) showed that the lowest of the studied concentrations of Se (≤ 75 mg/dm³) have both a positive effect on the growth of *C. vulgaris* and a clear antioxidant effect, inhibiting lipid peroxidation and the formation of reactive oxygen species during cultivation for 144 h (6 days). Under these conditions, a significant acceleration of cell growth and an increase in the content of organic Se in algae was also

observed. Inhibition of algal growth was observed only when the concentration of Se in the medium was above 100 mg/dm³ (Sun et al., 2014).

The biological activity of selenium compounds in cells is directly or indirectly related to proteins: selenocysteine, selenium methionine, glutathione peroxidase, thioredoxin reductase, etc. (Yoshida et al., 2011). The appropriate amount of selenium in these compounds ensures the normal course of protection of DNA and chromosomes from breakage, rupture, deletions and the formation of adducts. Selenium compounds can also modulate DNA methylation or inhibit the deacetylation of histone proteins (Ferguson et al., 2012). Additional protective function of selenium relative to the genetic apparatus of cells through selenium methionine-induced DNA repair and increased activity of reparative enzymes: DNA glycosylases (primarily p53, BRCA1 and Gadd45), which repair damaged DNA (Fischer et al., 2006; Bera et al., 2013).

At the same time, the optimal amounts of this trace element for the life of a particular organism depend primarily on its genotype and biological characteristics (Ferguson et al., 2012). More detailed studies of the mechanisms of selenium's influence on DNA repair processes will help to explain the contradictory data available in the literature regarding its minimum amounts for maximum protection and stability of the genome in all organisms (Bera et al., 2013).

Maeda et al. (1990) showed that the concentration of zinc in the nutrient medium up to 20 mg/dm³ had no effect on the functioning of *C. vulgaris* cells, while the concentration of 10 mg/dm³ led to better and faster growth compared to the control. In another study, the growth of *C. pyrenoidosa* culture at Zn²⁺ concentrations of 5–10 mg/dm³ did not change in comparison with the control (Zhou et al., 2012).

Zinc is necessary for the functioning of many proteins, containing the so-called zinc fingers – areas of the protein that are stabilized by one or two zinc ions and have a characteristic spatial structure. Such regions are part of the protein domains responsible for the binding of DNA, RNA, other proteins and small molecules. Zinc-containing proteins are very common in eukaryotic cells and perform a variety of functions: DNA recognition, RNA packaging, activation of transcription, regulation of apoptosis, formation of the spatial structure of the protein, and lipid binding (Laity et al., 2001). In the study of transcription factors in microalgae, representatives of *Haptophyta*, *Bacillariophyta*, *Heterokontophyta*, *Chlorophyta* and *Rhodophyta* (Thiriet-Rupert et al., 2016), it was found that on average, up to 10% of them belong to the class of proteins with zinc fingers. The active participation of this group of proteins in cell responses to biotic and abiotic stressors has also been shown (Deng et al., 2012; Peng et al., 2012).

The problem of the biological activity of chromium still remains controversial and primarily depends on the species and genotype of the organism. Chromium has been found

to be toxic to many lower plant species, including algae (Cervantes et al., 2001; Fang et al., 2014). In high concentrations, this metal has a carcinogenic effect with a significant violation of metabolism and a complex mechanism of mutagenesis. Cr(III) has been shown to be less dangerous than Cr(VI) (Fang et al., 2014).

Thus, the concentrations of selenium and zinc used in our work do not significantly affect the increase in algal biomass, and therefore do not have a significant genotoxic effect. Only chromium at such concentrations, which is able to partially inhibit the growth of *C. vulgaris* (Meisch, Schmitt-Beckmann, 1979; Qian et al., 2013), could potentially have a mutagenic effect, but with longer cultivation.

Therefore, there must be other reasons for the changes we found in the genetic characteristics of the culture of *C. vulgaris*. The most likely cause may be the initial genetic heterogeneity of the culture used. Shen (2008) using ISSR-PCR compared individual clones of two species of algae and showed that under natural conditions of their growth, the genetic distances (D_j) between four clones of *C. vulgaris* ranged 0.218–0.321, and between three strains of *C. pyrenoidosa* they varied from 0.190 to 0.275 (Shen, 2008). Therefore, we can assume that the genetic changes we have identified are the result of selective selection of a certain part of the genetically heterogeneous population of *C. vulgaris*, most adapted to the changed culture conditions (Hovde et al., 2018). The result was changes in the genetic structure of the cell population, which were reflected in the appearance of the genetic differences we found between culture variants obtained on different cultural media. However, to test this assumption, as well as to determine the genotoxic effects of the elements used in the work, it is necessary to conduct additional experiments on cultures of algae obtained by pre-cloning.

CONCLUSIONS

Cultivation of *Chlorella vulgaris* in the presence of sodium selenite separately and together with zinc sulfate and chromium chloride changes the genetic characteristics of the culture, according to the results of ISSR and IRAP-PCR. The genetic distances (D_j) between the obtained culture variants (micropopulations) of *Chlorella* vary in the range of 0.206–0.300. Additional introduction of salts of selenium, zinc and chromium into the culture medium of *C. vulgaris* in the studied concentrations testified to the absence of their significant genotoxicity on algae. Therefore, we assume that these compounds can be used in the biotechnological process to obtain algal biomass enriched in trace elements.

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