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Description of *Neochlorella semenenkoi* gen. et. sp. nov. (Chlorophyta, Trebouxiophyceae), a Novel *Chlorella*-like Alga with High Biotechnological Potential

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Abstract: Despite many publications about *Chlorella*-like algae, their reliable and accurate identification is still difficult due to their simplicity and high phenotypic plasticity. The molecular approach has revolutionized our understanding of the diversity of 'small green balls', and a natural classification of this group is currently being developed. This work is aimed at providing a detailed study of the phylogenetic position, morphology, ultrastructure, and physiology of the biotechnologically remarkable *Chlorella*-like strain IPPAS C-1210. Based on the SSU–ITS1–5.8S–ITS2 phylogeny, genetic distances, and the presence of compensatory base changes (CBCs) in ITS1 and conserved regions of ITS2 secondary structures, we describe a new genus, *Neochlorella*, with IPPAS C-1210 as the authentic strain of the type species, *N. semenenkoi* gen. and sp. nov. In addition, we justify the reassignment of the strain *C. thermophila* ITBB HTA 1–65 into *N. thermophila* comb. nov. The distinctive ultrastructural and physiological traits of the new species are discussed.

Keywords: *Chlorella* clade; integrative approach; molecular phylogeny; morphology; new genus; physiology; ultrastructure

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1. Introduction

The genus *Chlorella* was described by M.W. Beijerinck in 1890 [1], and initially, it included unicellular coccoid green algae with cells of spherical, oval, or wide-oval shape, without bristles, less than 10 µm in size, which reproduce by autospores, and which are mainly aquatic inhabitants. In this regard, for a long time, all the so-called 'small green balls' with *Chlorella*-like morphology were attributed to this genus. Subsequently, more detailed studies of the biochemical, ultrastructural, and genetic characteristics of *Chlorella*-like strains revealed their heterogeneity, and, therefore, the genus *Chlorella* underwent several revisions. First, based on the differences in the nucleotide sequences of the 18S rRNA gene and the ITS2 spacer, Krienitz et al. [2] isolated *C. kessleri* into a separate genus *Parachlorella*. A year later, the separation of the *Chlorella* and *Parachlorella* clades was confirmed by studies of ultrastructure and synthesis of autospore cell walls [3]. Then, Luo et al. [4] summarized the available data on *Chlorella* molecular genetics, morphology, and ontogenesis and proposed a new concept of the *Chlorella* clade. According to their proposal, besides the archetype, the *Chlorella* clade included several genera with different morphologies: *Actinastrum*, *Didymogenes*, *Hegewaldia*, *Meyerella*, and *Micractinium*. Revision by Pröschold et al. [5] identified two new *Dictyosphaerium*-like genera, *Hindakia* and *Heynigia* (colonies up to 64-celled connected via mucilaginous stalks), belonging to the *Chlorella* clade.

Then, Bock et al. [6] described five species with classic *Chlorella*-like morphotype (*C. volutis*, *C. elongata*, *C. rotunda*, *C. lewinii*, and *C. singularis*), and four *Dictyosphaerium*-like species that, however, were assigned to the genus *Chlorella* (*C. pituita*, *C. pulchelloides*, *C. chlorelloides*, and *C. coloniales*). In subsequent studies, a number of new taxa belonging to the *Chlorella* clade were discovered: *C. thermophila* [7], genus *Carolibrandtia* [8,9], *Micractinium singularis*, *M. variabile*, *M. simplicissimum* [10], and *M. kostikovii* [11].

Although *Chlorella*-like algae have been studied for more than 130 years, their reliable and accurate identification is still difficult due to the simplicity and high phenotypic plasticity of their morphological properties. Though the molecular approach has revolutionized the understanding of the diversity of 'small green balls', a natural classification of this group is currently being developed. Nevertheless, many taxonomically problematic groups in the *Chlorella* clade require detailed investigation [12]. For example, besides the high diversity of the *Chlorella* clade itself, recent studies have shown that, in its present state, the genus *Chlorella* includes misidentified strains that actually belong to other independent genera [7,12–14]. Thereby, the issue of correct genera and species delimitation and identification in the *Chlorella* clade is of great importance.

The study of *Chlorella* clade representatives has not only theoretical but also practical significance. In 1919, the German cell physiologist and future Nobel laureate, O.H. Warburg, began to use a pure laboratory culture of *Chlorella* in his study of photosynthesis [15]. Since that time, *Chlorella*-like strains have been widely used as model organisms in plant physiological and biochemical studies due to their simple morphology and life cycle, fast growth in a wide range of growth conditions, and moderate nutritional requirements [16]. High biomass productivity, metabolic plasticity, and ability to produce high amounts of proteins, lipids, carotenoids, polysaccharides, and vitamins have made *Chlorella*-like strains the most cultivated eukaryotic microalgae for biofuel production and for the nutraceutical or pharmaceutical industries [17–19].

Like many strains from the *Chlorella* clade, the strain IPPAS C-1210, which is being studied here, is characterized by a high growth rate; in the exponential phase, its biomass is rich in protein and chlorophyll, while in the stationary phase, it contains mainly carbohydrates and lipids [20]. This work aimed at providing a detailed study of the phylogenetic position, morphology, ultrastructure, and physiology of this biotechnologically promising *Chlorella*-like strain, with a description of a new genus and species.

2. Materials and Methods

2.1. Isolation and Cultivation of Algal Strain

The strain IPPAS C-1210 was isolated in 2013 from the freshwater Issyk Lake in Trans-Ili Alatau, Kazakhstan (43°15′11′′ N, 77°29′05′′ E), by the enrichment culture technique and then purified to an axenic status using standard methods [21]. The strain was maintained either on agarized BG-11 medium [22] at 22 °C in a 12:12 h light/dark regime under illumination of 30 µmol photons m^{-2} s^{-1}, or on agarized BBM-3N medium [21] at 22 °C under continuous illumination of 30 µmol photons m^{-2} s^{-1}.

The strain IPPAS C-1210 was deposited at the collection of microalgae and cyanobacteria IPPAS of the K.A. Timiryazev Institute of Plant Physiology, Russian Academy of Sciences (RAS) (http://cellreg.org/Catalog/, accessed on 3 December 2022), and at the Algal Collection ACSSI of the Institute of Physicochemical and Biological Problems in Soil Science, RAS (http://acssi.org/ accessed on 3 December 2022), under number 342.

2.2. Light and Electron Microscopy

To test the phenotypic plasticity, the strain IPPAS C-1210 was grown in different media and under different cultivation conditions, as listed in Table 1: maintenance conditions (1), intensive cultivation conditions (2), and cultivation in liquid TAP medium [23] for comparison with the morphometric data obtained for *C. thermophila* ITBB HTA 1–65 by Ma et al. [7] (3).

Table 1. Cultivation conditions of the strain IPPAS C-1210 for microscopy.

#	Medium	Light/μmol Photons m^{-2} s^{-1}	Temperature	Aeration	Time of Sampling
1	solid BG-11	30, 12:12 h L/D	22 °C	none	20 days
2	liquid BG-11 +20 mM HEPES, pH 7.5	100, continuous light	32 °C	1.5–2% CO_2	3 days
3	liquid TAP	30, continuous light	32° C 22 °C	none	5 days 6 months

Morphology and life cycles were observed using a Carl Zeiss Axio Scope A1 microscope (Oberkochen, Germany) in the Collective Use Center, Institute of Physicochemical and Biological Problems in Soil Science, RAS. The results were documented using drawings and photographs obtained with a Carl Zeiss MRc 5 color digital camera (Germany). As an alternative, a Carl Zeiss Axio Imager D1 microscope with an AxioCam MRc camera (Germany) was used.

The morphology was described according to Komárek and Fott [24] and Andreyeva [25].

Transmission electron microscopy (TEM) was performed using a TEM Libra-120 (Carl Zeiss, Germany) as described previously [26]. For TEM analysis, the culture was grown as described in Table 1, line 2. The samples were taken on the third and ninth days (exponential and stationary phase, respectively).

2.3. Grazing Test

Cultures of phycophages *Paramecium caudatum*, *Daphnia pulex*, *Philodina acuticornis*, and *Brachionus rotundiformis* were used during the biotests to stimulate the development of bristles as described previously with some modifications [27]. To study the effect of the metabolites released by these predators into the culture medium, the medium with predators (500 individuals per ml) was filtered through a 0.2 μm PTFE filter. Then, 5 mL of the filtrate was added to 25 mL of IPPAS C-1210 culture grown in BG-11 medium. To study the direct effect of phycophages, 5 mL of the predator culture was added to 25 mL of IPPAS C-1210 culture grown in BG-11 medium. In the case of the test with *Brachionus rotundiformis*, the modified medium BG-11 with 25 g/L of NaCl was used [27]. The IPPAS C-1210 culture grown in standard BG-11 medium was used as a control. The density of the algal culture was at least 1.5×10^5 cells mL^{-1}. The cultures were shaken very gently by hand for 5 s twice a day. The development of bristles was examined at 24 h, 48 h, and 72 h following culturing using a light microscope. The test was performed on three replicates per exposure condition and control.

2.4. DNA Isolation and Sequencing

Total genomic DNA was isolated from algal cells using the GenEluteTM PlantGenomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. Amplification was carried out with the GenAmp 2720 machine (Life Technologies, Grand Island, NY, USA) using Hot Start Taq polymerase (Syntol, Moscow, Russia). The primers and conditions for the SSU and ITS1-5.8S-ITS2 amplification are listed in Table 2. All primers were synthesized by Evrogen (Evrogen JSC, Moscow, Russia). Amplified partial SSU and ITS1-5.8S-ITS2 fragments were separated by agarose gel electrophoresis; DNA was stained with ethidium bromide, visualized, and excised under weak ultraviolet light. DNA was purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania) and sequenced by Sanger sequencing (Evrogen JSC, Moscow, Russia) using the same primers as used for the initial PCR. The obtained sequences were assembled using the SeqMan Pro module of the Lasergene v. 12.3.1 software package (DNAStar Inc., Madison, WI, USA) and deposited in GenBank under accession numbers MT897850 (SSU) and MT890143 (ITS1-5.8S-ITS2).

Table 2. Primers and PCR conditions for the SSU and ITS1-5.8S-ITS2 regions.

Amplified Sequence	Primer	Sequence (5′–3′)	PCR Conditions	Reference
SSU ~2200 bp	EukA F	AACCTGGTTGATCCTGCCAGT	95 °C 10 min; 35 cycles (95 °C 30 s, 62 °C 30 s, and 72 °C 2 min); and 72 °C 6 min	[28]
	18L R	CACCTACGGAAACCTTGTTACGACTT		[29]
ITS1-5.8S-ITS2 785 bp	ITS5 F	GGAAGTAAAAGTCGTAACAAGG	95 °C 10 min; 35 cycles (95 °C 30 s, 55 °C 30 s, and 72 °C 1 min); and 72 °C 6 min	[30]
	ITS4 R	TCCTCCGCTTATTGATATGC		

2.5. Phylogenetic Analysis

Phylogenetic analysis was performed on a concatenated dataset of the SSU and ITS1-5.8S-ITS2 sequences. All of the sequences were searched using the BLASTn algorithm in GenBank (https://blast.ncbi.nlm.nih.gov, accessed on 10 September 2021). The sequences were selected based on the criteria of highest identity (≥95%), read quality (without degenerate and unknown nucleotides), read length (≥2300 bp), and, mainly, belonging to the type species and collection of authentic strains. A data set of 103 sequences with 2611 aligned base positions was used for the phylogenetic analyses; introns were excluded. *Dictyosphaerium ehrenbergianum*, *Parachlorella beijerinckii*, and *P. kessleri* were chosen as an outgroup. The taxon names are listed according to the international electronic database AlgaeBase [31]. Multiple alignment was performed in BioEdit 7.2.5 using the ClustalW algorithm [32]. Based on the AIC in jModelTest [33], the GTR + I + G nucleotide substitution model was selected as the optimal model for Maximum Likelihood (ML) and Bayesian Inference (BI). ML was performed using PhyML [34] with 1000 bootstrap replicates. BI was performed using BEAST v. 1.8.4 [35] with 1,000,000,000 generations of Markov chain Monte Carlo iterations, and the parameters were saved every 100,000th tree, while discarding the first 25% as burn-in. The calculation of genetic distances was performed in the MEGA 6.0 program [36]. To compare the tree topology, we used data from articles [2,5–9,37–41]. The folding of ITS1 and ITS2 was performed using the RNAfold web server (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi, accessed on 20 November 2021) in accordance with the principle of minimum energy. The correctness of the predicted secondary structure of the ITS1 and ITS2 regions was verified [42–45]. The comparison of the secondary structure of the spacers between strains and the search for conservative motifs and compensatory base changes (CBCs) were carried out in the 4SALE program [46,47]. For species delimitation, we used the search of CBCs in the ITS2 secondary structure. According to the classical approach, the presence of even one CBC in the conservative regions of ITS2 (5 bp of helix I, 10 bp of helix II, and whole helix III) in two microalgae correlates with their belonging to different species [43,44]. We also took into account the recommendations of Hoshina et al. [8,38] and Chae et al. [10] that, within the framework of the *Chlorella* clade, CBCs in non-conservative regions of ITS1 or ITS2, as well as stable differences in their secondary structures, may also indicate belonging to different species. The secondary structures of the spacers were visualized in the PseudoViewer3 program. To analyze the level of genetic differences, the nucleotide sequences of ITS2 were aligned, taking into account the secondary structure in the 4SALE program. Then, the genetic distances were calculated in the MEGA 6.0 program (using the Kimura 2-parameter model). The results were interpreted based on the works by Hoshina et al. [14,38,48].

2.6. Physiological Tests

Several physiological tests were performed to determine the optimal growth conditions and stress tolerance limits of the strain IPPAS C-1210. We tested temperature, pH, and osmotic effects on the growth of IPPAS C-1210, as well as the ability of this strain to use different sources of carbon and nitrogen.

For the physiological experiments, the strain was pre-grown for 7–14 days in 300 mL Erlenmeyer flasks with 150 mL of BG-11 medium buffered with 20 mM HEPES (pH 7.5) or BBM-3N medium. The cultures were grown at room temperature on an orbital shaker with an average illumination of 50 µmol photons m^{-2} s^{-1} from a warm white light.

The effect of temperature on microalgal growth was investigated using the Laboratory System for Intensive Cultivation described in Gabrielyan et al. [49]. The culture grown for four days at a temperature of 32 ± 1 °C was used as an inoculum. Cultivation was carried out in a glass vessel with 200 mL of buffered BG-11 medium under an average illumination of 100 or 500 µmol photons m^{-2} s^{-1} and aeration with sterile air containing 1.5–2% CO_2 at four different temperatures, 24 ± 1 °C, 30 ± 1 °C, 36 ± 1 °C, and 41 ± 1 °C, for 7–9 days. Each temperature variant was grown in triplicate. Culture growth was followed by optical density measurements at 750 nm (OD_{750}), and the initial OD_{750} was in the range of 0.08–0.09.

The following physiological experiments were carried out in a growth chamber MLR-351 (SANYO, Japan) at 32 ± 1 °C under continuous illumination of 50 µmol photons m^{-2} s^{-1}. The temperature of 32 °C was chosen based on data from a study of the temperature effect on the growth of the strain IPPAS C-1210 (see Section 3.4.).

Evaluation of the effect of pH on growth was carried out in 75 mL Erlenmeyer flasks with 25 mL of BBM-3N medium with pH values adjusted to 4–11 (with step 1) using either NaOH or HCl, with each pH variant in triplicate. The cultures were incubated for eight days. Culture growth was estimated based on changes in OD_{750} (ΔOD_{750}), and the initial OD_{750} was 0.01–0.02. The experiment was repeated twice independently.

The tolerance of the strain IPPAS C-1210 to the combined effect of NaCl and $NaHCO_3$ was investigated based on the method described in Mikhodyuk et al. [50] with modifications. The BBM-3N medium was used with a cross gradient of $NaHCO_3$ and NaCl concentrations (0–2 M; steps 0.2 M and 0.4 M, respectively). Cultivation was held in 10 mL serum vials filled with 10 mL of the corresponding media and closed with cotton plugs. Culture growth was estimated after seven days of cultivation based on ΔOD_{750}, and the initial OD_{750} was 0.2. The experiment was repeated twice independently.

The ability of the strain to use different carbon and nitrogen sources for growth was observed using BG-11 medium with modifications. The tested compounds and their concentrations were chosen based on Shihira and Krauss [51].

In the first experimental series, the strain was cultivated in BG-11 media buffered with 20 mM HEPES (pH 7.5) with different nitrogen sources: $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, and $CO(NH_2)_2$ (urea); in all variants, the nitrogen concentration was adjusted to 17.6 mM. Cultivation was caried out in 75 mL Erlenmeyer flasks with 25 mL of the corresponding medium. The culture grown in standard BG-11 with 20 mM HEPES (pH 7.5) was used as an inoculum after centrifugation and washing with the corresponding growth medium. Growth was estimated after 12 days of cultivation based on changes in OD_{750}, and the initial OD_{750} was 0.03. Each medium variant was grown in triplicate.

In the second series of experiments, the strain IPPAS C-1210 was grown on plates with modified BG-11 media buffered with 20 mM HEPES (pH 7.5) and containing different nitrogen and carbon sources. As nitrogen sources, 17.6 mM sodium nitrate, 0.01% yeast extract, 0.01% casamino acids, and 0.01% tryptone were used. As carbon sources, atmospheric carbon dioxide, 0.1% glucose, 0.1% mannose, 0.1% lactose, 0.1% galactose, 0.1% glycerol, 0.001 M acetate, and 0.01 M acetate were used. For the experiment, the inoculum grown in standard liquid BG-11 medium with 20 mM HEPES (pH 7.5) was concentrated by centrifugation, and then a series of 5 tenfold dilutions with the same medium was prepared. Three drops of 10 µL of each dilution were plated on nutrient agar plates, with two plates per medium variation. One Petri dish was wrapped in aluminum foil (dark condition, heterotrophic growth) and another Petri dish was held under light (light condition, autotrophic or mixotrophic growth). The variants grown on standard BG-11 medium with 20 mM HEPES (pH 7.5) were used as a control. After 14 days of incubation, when colonies in the highest dilutions were clearly seen, the growth was estimated based on the number and size of the colonies.

2.7. Statistics

The significance of the effects from the temperature, pH, and nitrogen source experiments on the growth of IPPAS C-1210 was analyzed using a one-way ANOVA with a Tukey's post hoc test (https://astatsa.com/OneWay_Anova_with_TukeyHSD/, accessed on 3 December 2022). The data were considered significantly different at $p < 0.05$.

3. Results

3.1. Morphology and Ultrastructure

Morphological observations by light microscopy revealed that the strain IPPAS C-1210 had a *Chlorella*-like morphotype (Figure 1). The cells were solitary, planktonic, and without bristles. Mucilage was absent. The vegetative cells were spherical to ellipsoidal, and 3.5−6.5 µm in diameter in all tested variants. Chloroplasts were single, parietal, and cup-shaped, with a spherical pyrenoid covered by a segmented starch sheath. Old cells accumulated oil droplets (Figure 1d). Reproduction was by 2−8 autospores, and sporangium size was 5.5–8 µm in diameter (Figure 1e–g). Autospores were equal in size (1.5–2.0 × 1.5–2.5 µm) and exhibited liberation by rupture of the sporangium cell wall (Figure 1g). Zoospores and sexual reproduction were not observed.

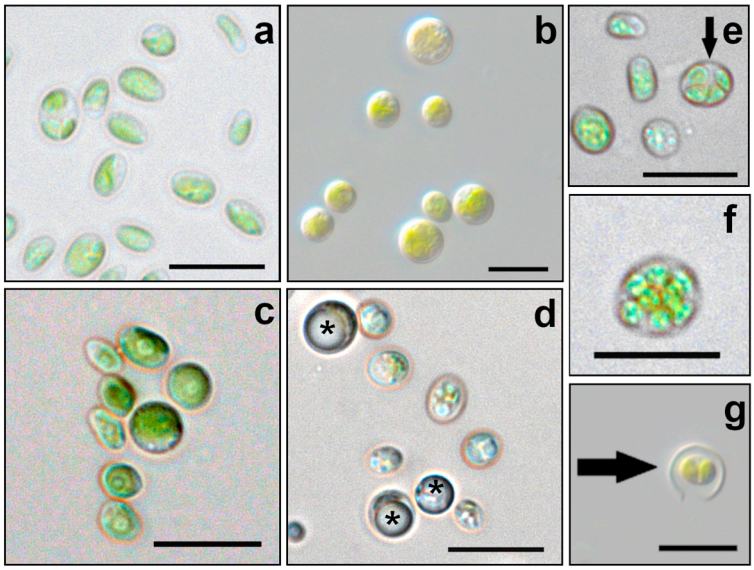

Figure 1. Cell morphology of *Neochlorella semenenkoi* IPPAS C-1210. Intensively growing three-day-old culture in liquid BG-11 medium (**a**); twenty-day-old culture grown on BG-11 plates (**b**); five-day-old (**c**) and six-month-old (**d**) cultures grown in liquid TAP medium; and cells with four (**e**), eight (**f**) and two autospores (**g**). The arrows indicate parent cell envelopes, and the asterisks indicate lipid droplets. Scale bars: 10 µm.

Upon TEM observation, a double thylakoid that dissected the pyrenoid matrix and starch sheath was observed (Figure 2a,b,d). Starch grains were scattered among the thylakoids in the chloroplast (Figure 2a–d); in mature cells, numerous lipid bodies were located in the cytoplasm close to the plasma membrane (Figure 2d,f). A single nucleus was peripherally positioned (Figure 2a,b). Young cells had thin single-layered microfibrillar cell walls with an average thickness of 20–40 nm (Figure 2a). The cell walls of cells in the stationary phase of growth were significantly thicker (100–200 nm) and had a non-homogeneous ultrastructure (Figure 2d–f). Autospore cell walls were clearly seen in the autosporangia as thin electron-dense layers (Figure 2c).

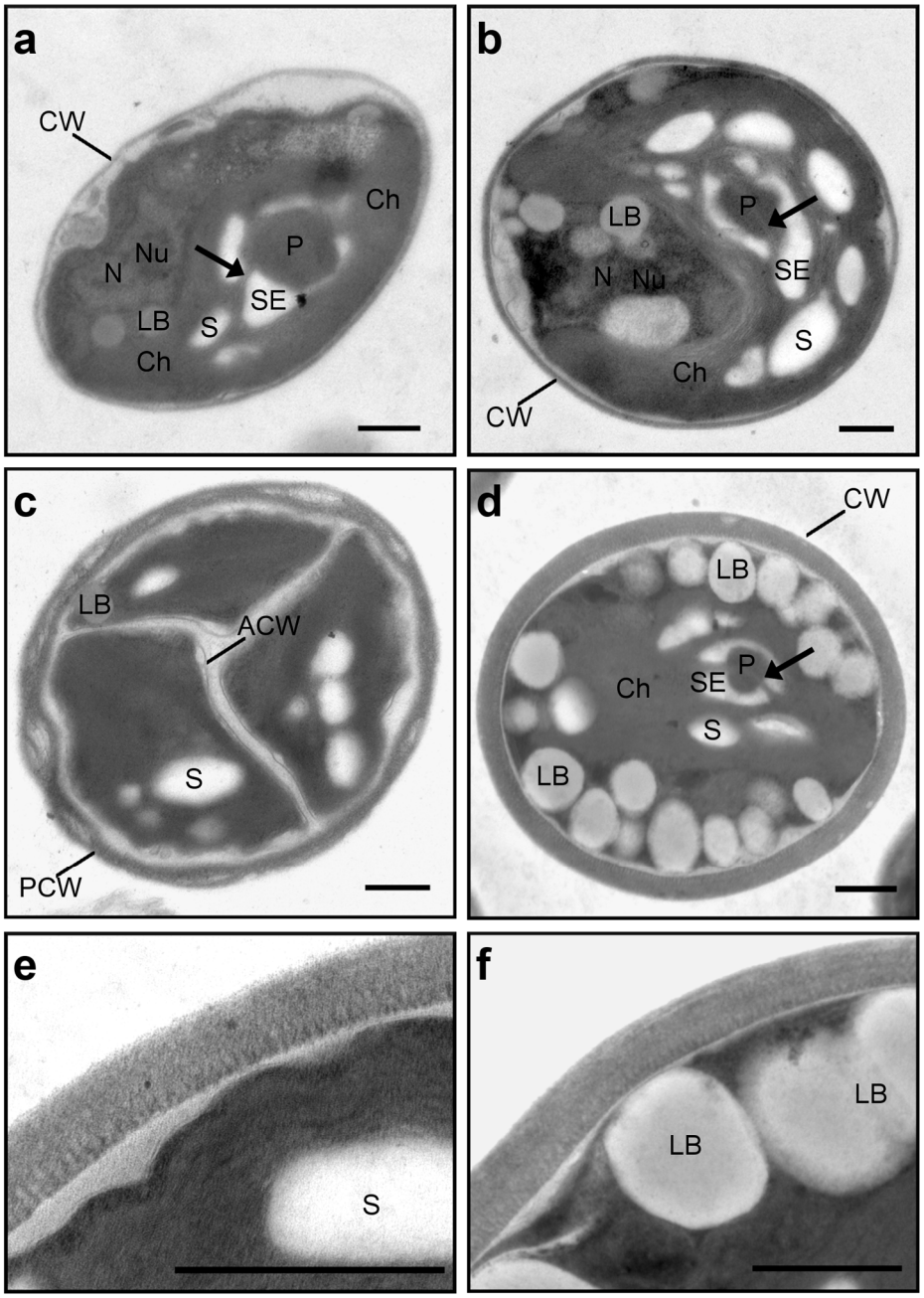

Figure 2. Cell ultrastructure of *Neochlorella semenenkoi* IPPAS C-1210 in exponential (**a**–**c**) and stationary (**d**–**f**) growth phases. Young ellipsoid cell with thin cell wall (**a**); mature spherical cell (**b**); parent cell with autospores (**c**); cell in stationary growth phase with thickened cell wall (**d**); and thickened cell walls of old cells (**e**,**f**). Ch: chloroplast; CW: cell wall; ACW: autospore cell wall; LB: lipid body; PCW: parent cell wall; N: nucleus; Nu: nucleolus; P: pyrenoid; S: starch grain; SE: starch envelope. The arrows point to the thylakoid pair penetrating the pyrenoids. Scale bars: 0.5 μm.

The grazing test had no effect on the IPPAS C-1210 morphology: the strain did not produce bristles either under direct pressure from phycophages or in contact with their metabolites (Figure S1).

3.2. Phylogenetic Analysis

Based on the 18S–ITS1–5.8S–ITS2 phylogeny, the studied strain IPPAS C-1210 clustered with the strain ITBB HTA 1–65 (PP—1.00, BP—100%) (Figure 3). The latter strain (hereafter, ITBB HTA 1–65) is the authentic strain of *C. thermophila* [7]. The genetic distance between

the IPPAS C-1210 and ITBB HTA 1–65 was 3.9%. A related phylogenetic lineage was formed by the strain CCAP 211/120 (PP—1.00, BP—86%), which is the authentic strain of *C. volutis* [6]. The genetic differences between this strain and the cluster containing IPPAS C-1210 and ITBB HTA 1–65 ranged from 3.6% to 4.4%. This group occupied a unique phylogenetic position within the *Chlorella* clade and did not cluster with *C. vulgaris*, which is the type species of the genus *Chlorella*, or with the type species of any other genera. In contrast to the ITBB HTA 1–65 and *C. volutis* CCAP211/120, the SSU rDNA of IPPAS C-1210 possessed an intron of 440 bp.

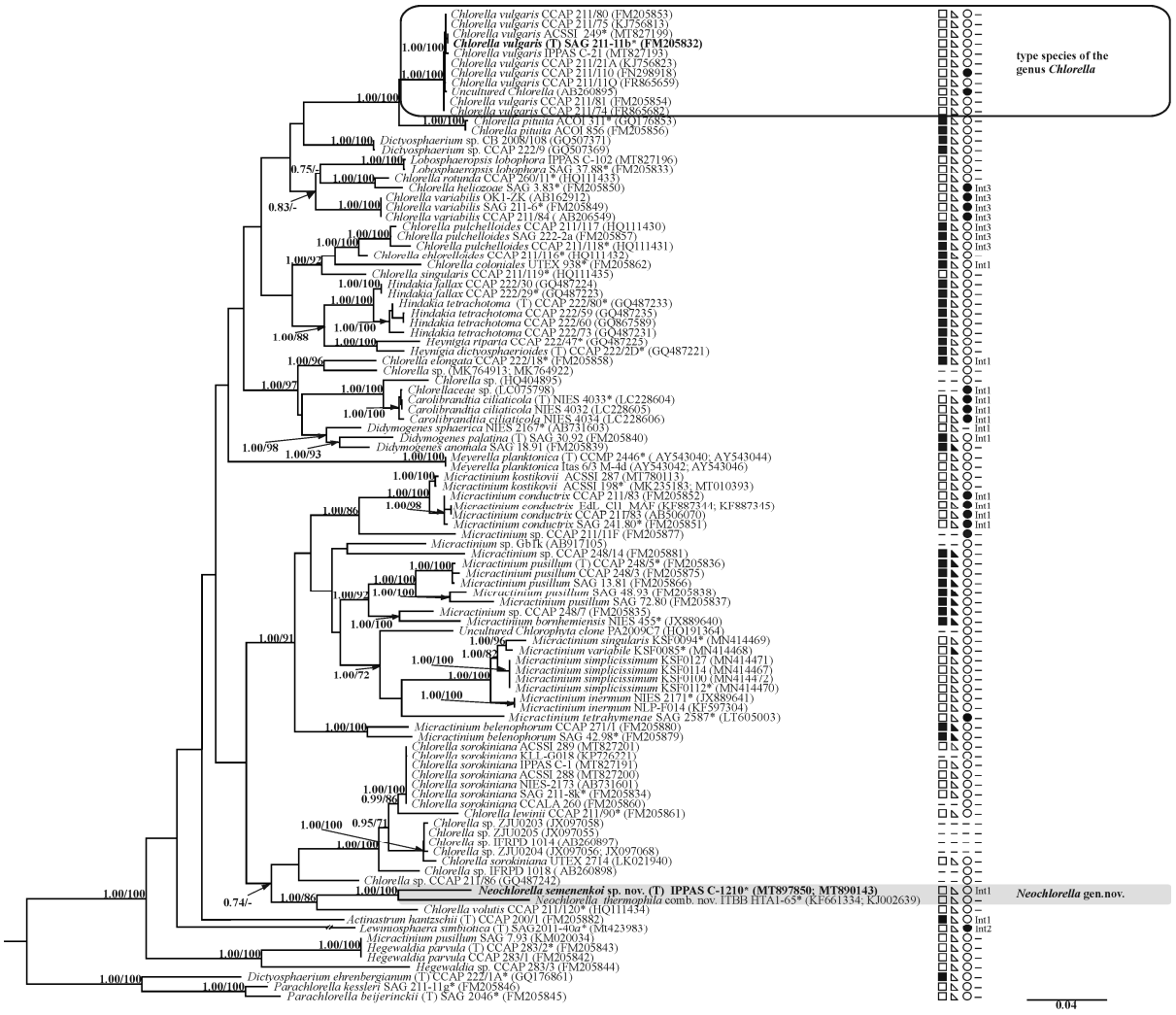

Figure 3. A Bayesian phylogenetic tree of the *Chlorella* clade (Trebouxiophyceae, Chlorophyta) based on the comparison of the nucleotide sequences of the SSU-ITS1-5.8S-ITS2 region (2611 bp). The support values are given for Bayesian Inference and Maximum Likelihood (PP/BP). The cut-off values for BI and ML are 0.7 and 70%, respectively. The model of nucleotide substitutions: GTR + I + G. The newly sequenced strain IPPAS C-1210 is highlighted in bold. Members of the genus *Neochlorella* gen. nov. are highlighted by a gray box. The strains of *C. vulgaris*, which is the type species of the genus *Chlorella*, are enclosed by a rectangle. The authentic strain *C. vulgaris* SAG 211-11b is in bold. Designations: *—authentic strain; T—type species; black square—forms colonies; white square—single cells; white triangle—does not produce bristles; black triangle—produces bristles; white circle—a free-living organism; black circle—an endosymbiotic organism; "−"—no information; Int—presence of introns, and the number of introns is indicated next to it; "–"—no intron. Scale bar: 0.04 substitutions/site.

3.3. ITS1 and ITS2 Secondary Structures

The secondary structures of the ITS1 regions of the strains IPPAS C-1210, ITBB HTA 1–65, and *C. volutis* CCAP211/120 are shown in Figure 4. The length of the ITS1 of IPPAS C-1210 was 278 bp, and it was 280 bp for ITBB HTA 1–65 and 260 bp for *C. volutis* CCAP 211/120. The ITS1 secondary structures of these strains corresponded to the generalized description of the model, which was suggested for eukaryotic organisms by A. Coleman [44] and included four unbranched helices. The helices I−III were located next to each other. The short helix IV was separated from others by unpaired nucleotides. Following the helix IV, a single-stranded A-rich region was adjacent to the 5.8S rRNA gene. The strains IPPAS C-1210 and ITBB HTA 1–65 had no CBCs in ITS1. Compared to *C. volutis* CCAP 211/120, the strains IPPAS C-1210 and ITBB HTA 1–65 exhibited three CBCs in ITS1: 13th bp (G–C → U–A) and 16th bp (C−G → G−C) in the helix II and, 1st bp (U−A → G−C) in the helix IV. The CBC (U–A → G–C) at the base of the helix IV distinguished the strains IPPAS C-1210 and ITBB HTA 1–65 from all other members of the *Chlorella* clade.

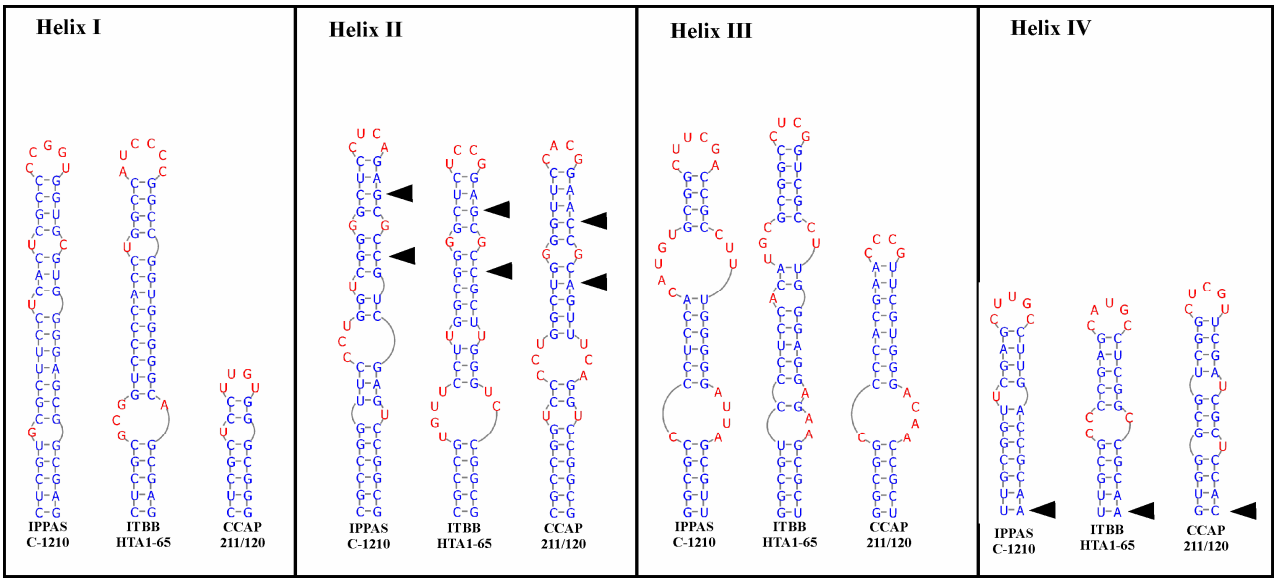

Figure 4. ITS1 secondary structure of *Neochlorella semenenkoi* IPPAS C-1210, *N. thermophila* ITBB HTA 1–65, and *C. volutis* CCAP 211/120. Black arrow—CBC.

The length of the ITS2 regions of the strains IPPAS C-1210 and ITBB HTA 1–65 was 261 bp and 266 bp, respectively. The ITS2 length of the strain *C. volutis* CCAP 211/120 was shorter (249 bp). The ITS2 secondary structure of these strains under consideration had common features specific for green microalgae (Chlorophyta): four unbranched helices, a pyrimidine–pyrimidine mismatch in the helix II, and the conservative motif GGUAGG on the 5′-side of helix III [42,43]. One CBC was found in the conserved region of the ITS2 helix I between the strain IPPAS C-1210 and the strain ITBB HTA 1–65 (3rd bp: G−C → U−A) (Figure 5). This CBC can be considered the molecular signature of the strain ITBB HTA 1–65 in the entire *Chlorella* clade.

CBC in the 7th bp (A-U-G-C) of the ITS2 helix III was one more molecular signature of the strains IPPAS C-1210 and ITBB HTA 1–65 that distinguished them from other species of the *Chlorella* clade. Another CBC between the strains IPPAS C-1210, ITBB HTA 1–65, and *C. volutis* CCAP 211/120 was found in the conserved region of the helix III (20th bp: G−C → A−U).

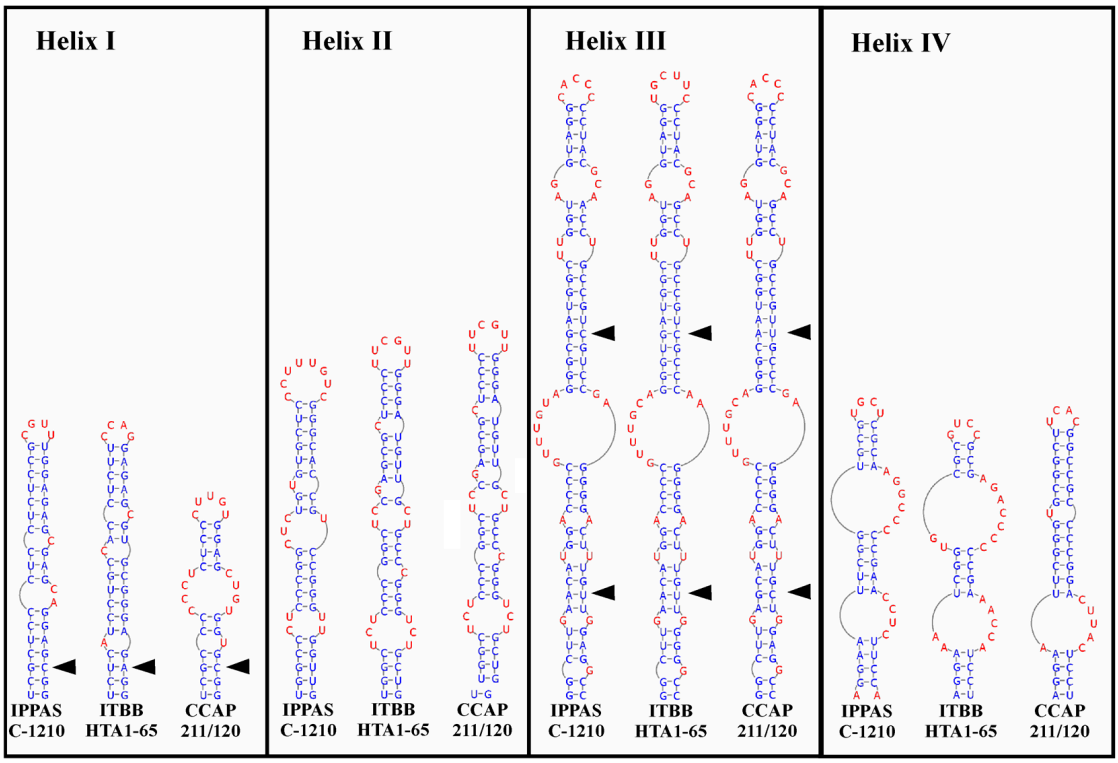

Figure 5. ITS2 secondary structure of *Neochlorella semenenkoi*. IPPAS C-1210, *N. thermophila* ITBB HTA 1–65, and *C. volutis* CCAP 211/120. Black arrow—CBC.

3.4. Physiological Tests

Temperature and light intensity effects. The strain IPPAS C-1210 grew well at temperatures ranging from 24 to 36 °C, with the highest growth rate (μ_{max} = 3.024 day^{-1}, doubling time of 5.5 h) at 30 °C and irradiation of 500 µmol photons m^{-2} s^{-1} (Figure 6). At 24 °C, the cultures grew at a similar rate as at 30 °C at lower irradiance of 100 µmol photons m^{-2} s^{-1}, but the growth was notably slower at higher irradiance of 500 µmol photons m^{-2} s^{-1}. At 36 °C, the growth significantly slowed down after four days, especially at higher irradiance. At 41 °C, retardation of growth was observed after two days at lower irradiation and after one day at higher irradiation. Microscopic observation on the fourth day of incubation at 41 °C revealed that all cells were bleached, but the cultures were able to resume their growth after being transferred to 30 °C.

Since the optimal growth temperature for the IPPAS C-1210 strain was found to be 30 °C, the following physiological tests were performed in a growth chamber with a temperature of 32 °C, which was the closest to the optimal temperature among available variants.

The effect of pH. The highest biomass increment was observed in the media with an initial alkaline pH of 8–11 (Figures 7 and S2). At pH < 6, growth was very weak, although after eight days of incubation, the average pH values in these variants increased by 0.3–0.6 units. The final pH values in the variants with an initial pH of 8–11 were in the range of 8–9, which was defined as optimal.

NaCl and $NaHCO_3$ tolerance. The experiments with the combined effect of $NaHCO_3$ and NaCl revealed that the optimal growth of the strain IPPAS C-1210 was at 0.2 M $NaHCO_3$ and in the absence of NaCl (Figures 8 and S3). The strain was able to maintain growth with $NaHCO_3$ up to 1.2 M in the medium and NaCl up to 2 M. Tolerance to NaCl decreased as $NaHCO_3$ concentration increased, and vice versa.

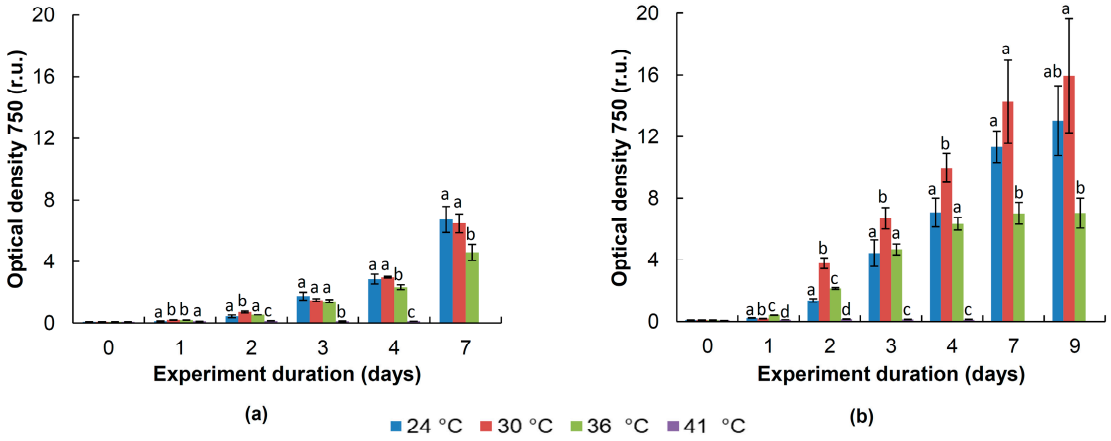

Figure 6. Growth curves of *Neochlorella semenenkoi* IPPAS C-1210 at different temperatures under 100 (**a**) and 500 (**b**) µmol photons m^{-2} s^{-1}. The error bars show standard deviations of the mean (n = 3). The temperature variants with the same letter are not significantly different (one-way ANOVA analysis, post hoc Tukey HSD test; $p < 0.05$).

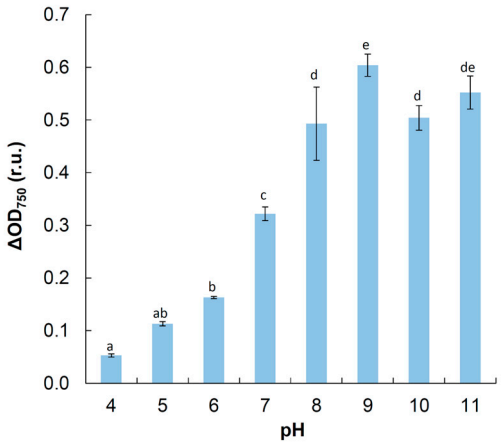

Figure 7. Growth of *Neochlorella semenenkoi* IPPAS C-1210 at different initial pH values. The error bars show standard deviations of the mean (n = 3). The variants with the same letter are not significantly different (one-way ANOVA analysis, post hoc Tukey HSD test; $p < 0.05$). One representative experiment out of two is shown.

Carbon and nitrogen sources. In the first series of the experiments with different nitrogen sources, the highest biomass yield was in the BG-11 medium with urea (Figure 9). In the variants with nitrate and ammonium, the biomass yield was approximately two times lower.

The tests on agar plates with different nitrogen and carbon sources revealed that the addition of 0.1% glucose under the mixotrophic and heterotrophic conditions improved growth most significantly (Figure 10). The addition of 0.1% galactose and 0.01 M or 0.001 M acetate also improved growth, especially under the dark conditions. Mannose and lactose had no effect on growth, and glycerol inhibited it. All tested organic sources of nitrogen (0.01% yeast extract, 0.01% casamino acids, and 0.01% tryptone) improved growth under the light conditions and could be used as a carbon source under the heterotrophic conditions, with tryptone and yeast extract being more effective.

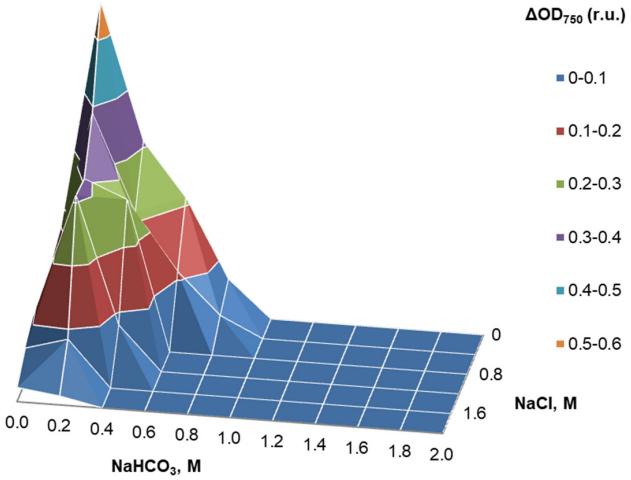

Figure 8. Growth of *Neochlorella semenenkoi* IPPAS C-1210 in the concentration matrix of $NaHCO_3$ and NaCl. One representative experiment out of two is shown.

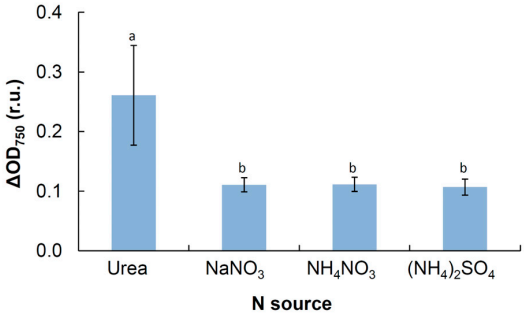

Figure 9. Growth of *Neochlorella semenenkoi* IPPAS C-1210 in modified BG-11 media with 20mM HEPES (pH 7.5) and different N sources. The error bars show standard deviations of the mean (n = 3). The variants with the same letter are not significantly different (one-way ANOVA analysis, post hoc Tukey HSD test; $p < 0.05$).

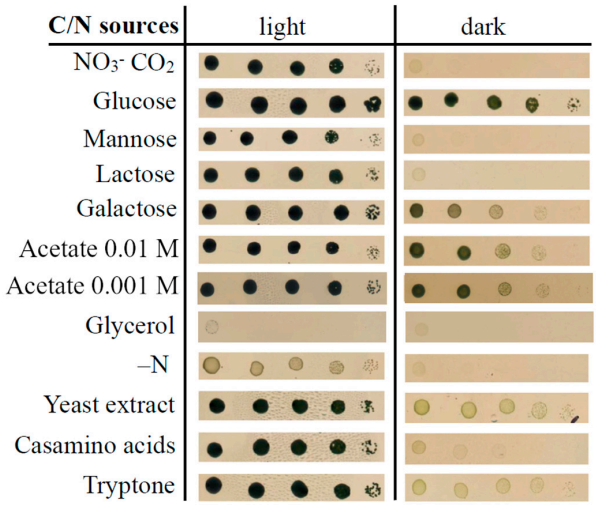

Figure 10. Growth of *Neochlorella semenenkoi* IPPAS C-1210 on modified BG-11 agar media with different C and N sources. A series of 5 tenfold dilutions were spotted on the modified BG-11 media with 20 mM HEPES (pH 7.5) and containing different nitrogen sources (nitrate, 0.01% yeast extract, 0.01% casamino acids, and 0.01% tryptone) and carbon sources (atmospheric carbon dioxide, 0.1% glucose, 0.1% mannose, 0.1% lactose, 0.1% galactose, 0.1% glycerol, 0.001 M acetate, and 0.01 M acetate). The variants grown on standard BG-11 medium with 20 mM HEPES (pH 7.5) were used as a control. One of three repetitions is shown.

3.5. Formal Description

Neochlorella Krivina, Temraleeva, Bobrovnikova et Sinetova gen. nov.

Description: Cells are solitary, planktonic, spherical or ellipsoidal shape, without bristles. Mucilage is absent. Chloroplast is single, parietal, and cup-shaped, with a spherical pyrenoid covered by two starch grains. Asexual reproduction occurs by equal autospores. Zoospores and sexual reproduction were not observed. Autospores are released through disruption of the parent cell wall. The genus differs from other genera of the family by the SSU and ITS rRNA gene sequences.

Distribution: Among the representatives of this genus, there are freshwater and aeroterrestrial organisms.

Type species: *Neochlorella semenenkoi* Krivina, Temraleeva, Bobrovnikova et Sinetova sp. nov.

Etymology: From Greek 'neo-'= new and the previously described genus *Chlorella*, *Neoclorella* can be translated as "new *Chlorella*". The name reflects that it is a new genus of *Chlorella*-like algae.

Holotype: The cryopreserved culture of the authentic strain *Neochlorella semenenkoi* IPPAS C-1210.

Holotype locality: The holotype culture was obtained from the freshwater lake Issyk, Kazakhstan (43°15′11.16″ N, 77°29′4.92″ E).

Holotype deposit: Material from the authentic strain *Neochlorella semenenkoi* IPPAS C-1210 is stored at the IPPAS collection of microalgae and cyanobacteria, Moscow, Russian Federation (metabolically inactive cryopreserved culture).

Isotypes: The authentic strain IPPAS C-1210 was deposited in the Algal Collection of Soil Science Institute (ACSSI) under the designation ACSSI 342.

Neochlorella semenenkoi Krivina, Temraleeva, Bobrovnikova et Sinetova sp. nov.

LM and TEM observation (Figure 1): Cells are solitary, planktonic, and spherical shaped, without bristles, and 3.5−6.3 µm in diameter. Mucilage is absent. Chloroplast is single, parietal, and cup-shaped, with a spherical pyrenoid, and is covered by two starch halves. Asexual reproduction occurs by 2−8 equal autospores. In the process of development, they change their shape from initially spherical and ellipsoid shaped to spherical shaped. Zoospores and sexual reproduction were not observed.

Holotype: Material from the authentic strain IPPAS C-1210 is stored in the IPPAS collection of microalgae and cyanobacteria, Moscow, Russian Federation (metabolically inactive cryopreserved culture).

Isotype: The authentic strain IPPAS C-1210 was deposited at the Algal Collection of Soil Science Institute (ACSSI) under the designation ACSSI 342.

Etymology: The species is named in honor of Prof. Victor Efimovich Semenenko, a founder of microalgal biotechnology in Russia, in recognition of the many contributions he made to our knowledge about the physiology of *Chlorella*-like algae.

Authentic strain: IPPAS C-1210.

GenBank accession number: MT897850; MT890143.

Neochlorella thermophila (Ma, S., Han, B., Huss, V.A.R., Hu, X., Sun, X. and Zhang, J.) Krivina, Temraleeva, Bobrovnikova et Sinetova, comb. nov.

Synonym: *C. thermophila* Ma, S., Han, B., Huss, V.A.R., Hu, X., Sun, X. and Zhang, J. [7].

LM and TEM observation: Cells are solitary, planktonic, spherical, or ellipsoidal shaped, without bristles, and 1.5−2.5 µm in diameter. Mucilage is absent. Cell walls are smooth and double-layered. Chloroplast is single, parietal, and cup-shaped, with a spherical pyrenoid covered by two starch grains. Asexual reproduction occurs by 2−4 equal autospores. Zoospores and sexual reproduction were not observed [7].

Holotype: Deposited as strain HTA 1–65 in cryopreserved and active forms in the Microorganism Collection Center of the Institute of Tropical Bioscience and Biotechnology (ITBB), CATAS, Hainan, China [7].

Type location: Rooftops, Haikou, Hainan Province, China [7].

Etymology: The species is named after its thermo-tolerance [7].

Authentic strain: ITBB HTA 1–65 [7].

GenBank accession number: KF661334; KJ002639 [7].

4. Discussion

Identification of asexual small coccoid green algae is very difficult due to the simplicity and scarcity of morphological characteristics. One of the most striking examples of this is the genus *Chlorella*. At present, the name 'Chlorella' indicates the morphotype of the organism rather than its taxonomic status. Such a morphotype is a result of convergent evolution and occurs among various microalgae [52–55]. In this regard, the most effective way to determine the true taxonomic status is a combined use of morphological, ecophysiological, and molecular phylogenetic methods [8,14,41,56]. Based on such an integrative approach, we describe a new genus, *Neochlorella*, with the IPPAS C-1210 as the authentic strain of the type species, *N. semenenkoi* gen. and sp. nov. In addition, we justify the reassignment of the strain *C. thermophila* ITBB HTA 1–65 into *N. thermophila* comb. nov.

4.1. Morphology and Ultrastructure

The light and TEM observations of *N. semenenkoi* IPPAS C-1210 showed the typical *Chlorella*-like morphology, but correct species affiliation based on morphology and ultrastructure alone was not possible (Table 3). At the same time, our strain had a number of differences from neighboring phylogenetic lineages [6,7]. Thus, in contrast to *C. volutis* CCAP 211/120, with adult cells having only a spherical shape, the adult cells of *N. semenenkoi* IPPAS C-1210 and *N. thermophila* ITBB HTA 1–65 are both spherical and ellipsoidal. The cells of *N. semenenkoi* IPPAS C-1210 are larger in size (3.5−6.3 µm) than the cells of *N. thermophila* ITBB HTA 1–65 (1.5−2.5 µm), but they are smaller than the cells of *C. volutis* CCAP 211/120 (5.0−6.5 µm). The chloroplasts of *N. semenenkoi* IPPAS C-1210 and *N. thermophila* ITBB HTA 1–65 are predominantly cup-shaped, unlike those of the strain *C. volutis* CCAP 211/120, which may be saucer-shaped. All of them have one pyrenoid with a segmented starch sheath. In the stationary phase, the cells of *N. semenenkoi* IPPAS C-1210 accumulate large lipid droplets (Figures 1d and 2d,f), which makes them different from the cells of *N. thermophila* ITBB HTA 1–65 with only a few small oil bodies [7].

Table 3. Comparative characteristics of some *Chlorella* clade representatives.

Characteristics	*N. semenenkoi* IPPAS C-1210	*N. thermophila* ITBB HTA 1–65	*C. volutis* CCAP 211/120	*C. sorokiniana* SAG 211-8k	*C. lewinii* CCAP 211/90	*C. vulgaris* SAG 211-11b
Cells	solitary					
Bristle	no					
Mucilage	no					
Adult cell shape	spherical or ellipsoidal	spherical or ellipsoidal	spherical	ellipsoidal or spherical	oval and egg shaped	always spherical
Young cell shape	spherical to ellipsoidal	spherical to ellipsoidal	spherical to slightly oval	ellipsoidal	oval	spherical
Cell size (µm)	3.5−6.3	1.5−2.5	5.0−6.5	4.5–5.5 × 3.5–5.4	4.0–6.0	2–6
Chloroplast	single, parietal, and cup-shaped	single, parietal, and cup-shaped	single, parietal, and cup- or saucer-shaped	single, shallow, and cup-shaped	single, parietal, and cup-, girdle- or saucer-shaped	single and deep cup-shaped
Pyrenoid	single, spherical, and 0.4−0.8 µm in diameter	single, spherical, and 0.4−0.6 µm in diameter	single, ellipsoid to spherical	single	single andbroadly ellipsoidal to spherical	single
Starch envelope	two starch halves					
Cell wall	20–40 nm single-layer in young cells, and 100–200 nm non-homogenous in mature cells	~60–80 nm and double-layered	ND	22 nm and single-layered in young cells; 60 nm and multilayered in older cells	ND	180–200 nm
Main storage products	Lipids and starch	Starch	ND	Starch and lipids	ND	Lipids and starch

Table 3. *Cont.*

Characteristics	*N. semenenkoi* IPPAS C-1210	*N. thermophila* ITBB HTA 1–65	*C. volutis* CCAP 211/120	*C. sorokiniana* SAG 211-8k	*C. lewinii* CCAP 211/90	*C. vulgaris* SAG 211-11b
Starch envelope	two starch halves					
Reproduction	by 2–8 autospores	by 2–4 (sometimes more, needs clarification) autospores	ND	by 2–8 (16) autospores	ND	by 2–16 autospores
Intron in the SSU	440 bp	no	no	no	no	no
Type location	freshwater reservoir	rooftops	freshwater reservoir	freshwater reservoir	soil in pond	freshwater reservoir
NaCl optimum/limits	0 M/2M	ND	ND	ND/1–3% (0.2–0.6 M)	ND	ND/3–4% (=0.6–0.7 M)
Temperature optimum/limits	30 °C/36 °C (41 °C–43 h)	33 °C/42 °C (45–3h)	ND	38–39 °C/42 °C	ND	20–25 °C/28–30 °C
pH optimum/lower, upper limits	8–9/4, 11	ND	ND	ND/3.5–5	ND	5–8/4, 9.5
Organic C sources						
acetate	+	+?	ND	–	ND	+
glucose	+	N/D		+		+
galactose	+	N/D		+		N/D
mannose	–	N/D		–		N/D
glycerol	–	N/D		N/D		+ (under light)
N sources						
nitrate	+	N/D	+	+	+	+
ammonium	+	+	N/D	+	N/D	N/D
urea	+	N/D	N/D	N/D	N/D	N/D
tryptone/peptone	+	N/D	N/D	N/D	N/D	+
yeast extract	+	N/D	N/D	–	N/D	N/DN/D
casamino acids	+	N/D	N/D	+	N/D	
References	Present study	[7]	[6]	[3,51,57–60]	[6]	[1,59–65]

ND = no data.

Reproduction of all these strains is by autospores. *N. semenenkoi* IPPAS C-1210 autosporangia form 2–8 autospores, while *N. thermophila* ITBB HTA 1–65 has been reported to produce 2–4 autospores per autosporangium [7]. Nevertheless, according to the available illustrative material (Figure 1B in Ma et al. [7]), *N. thermophila* autosporangia also may contain more than four autospores (at least five are clearly seen).

Cells of *N. semenenkoi* IPPAS C-1210 have a thick (100–200 nm) non-homogenous cell wall in the stationary phase (Figure 2d–f). It may be different from cells of the sister species *N. thermophila* ITBB HTA 1–65, which have cell walls that are significantly thinner (60–80 nm, as can be estimated from Figure 1C,D in Ma et al. [7]). To confirm the difference, it is necessary to study the cell wall ultrastructure of *N. thermophila* in cells at different growth stage. Unfortunately, no data are found on the cell wall ultrastructure of the authentic strains of *C. volutis* and *C. lewinii*. The cell wall ultrastructure of the *C. sorokiniana* type strain SAG 211-8k (=IAM C-212 = UTEX 1230) was studied in detail by Yamamoto et al. [3], Němcová and Kalina [60] and Rosen et al. [66]. Cell walls of both young and adult cells of *C. sorokiniana* are also much thinner than those of *N. semenenkoi* IPPAS C-1210 (22 and 60 nm, respectively [3], and Figures 3 and 4 in Němcová and Kalina [60]). The images of cell walls of the authentic strain of *C. vulgaris* SAG 211-11b (=H1955) were found only in the work by Němcová and Kalina [60]. Mature cells of *C. vulgaris* have a thick cell wall (estimated from Figures 1 and 2 in Němcová and Kalina [60] as 180–200 nm), but its ultrastructure is indistinguishable on the available images. Cell wall ultrastructure has been repeatedly suggested as a distinctive trait for *Chlorella*-like algae [3,16,60,67,68]. New studies based on type strains are needed to clarify the role of cell wall ultrastructure and composition as a taxonomic marker.

4.2. Phylogenetic Analysis

The results of phylogenetic analysis are generally consistent with previous studies [7,12–14]. All taxonomically recognized, non-monotypic genera (*Hindakia*, *Heynigia*, *Didymogenes*, and *Micractinium*) are well-supported clusters. The phylogenetic tree confirms

that the boundaries of the genus *Chlorella* are currently incorrectly defined and need to be revised. In particular, the species *C. pulchelloides*, *C. chlorelloides*, *C. singularis*, *C. colonialialis*, *C. elongata*, *C. lewinii*, *C. sorokiniana*, and *C. volutis* are misidentified and actually belong to other independent genera that need to be studied, described, and validated.

The expansion of the phylogenetic dataset makes it possible to identify groups in the *Chlorella* clade with strong statistical support. According to the results obtained, *N. semenenkoi* IPPAS C-1210 and *N. thermophila* ITBB HTA 1–65, together with *C. volutis* CCAP 211/120, form a cluster with a unique phylogenetic position within the *Chlorella* clade. Unfortunately, for the entire *Chlorella* clade, it is impossible to clearly and unambiguously determine the interspecific and intergeneric level of genetic differences. For example, in the genus *Micractinium*, the interspecific level of genetic differences varies from 0.3 to 4.3%, and in the genus *Hegewaldia*, it is 3.9%. At the same time, the genetic distance between the genera *Heynigia* and *Hindakia* is 2.2–2.5%, and between *Heynigia* and *Didymogenes*, it is 2.2–2.9%. Nevertheless, it can be confidently stated that the genetic distance between *N. semenenkoi* IPPAS C-1210 and *N. thermophila* ITBB HTA 1–65 (3.9%) corresponds at least to the interspecific level. The *Neochlorella* clade is separated from the authentic strain *C. vulgaris* SAG 211-11b by a genetic distance of 5.2–5.4% and from the authentic strains of type species of other genera by genetic distances of $\geq$4.4%, which definitely correspond to the intergeneric level.

N. semenenkoi IPPAS C-1210 possesses a 440-nucleotide insertion in the 18S rRNA gene as mentioned above, but this intron is not present in any of its closely related strains (Figure 3). The use of the main characteristics of intron (composition and position in the SSU) as a tool for distinguishing algal species is quite effective, including the differentiation of morphologically cryptic species [13,38,56,69,70]. It should be noted, however, that the presence or absence of an intron, as well as differences in its structure or length, can be observed between different populations of the same species in some cases. For example, when studying the representatives of *Chlorella variabilis*, Hoshina et al. [14] found differences in the length and structure of an intron between strains of different populations. Nevertheless, in the present case, the presence of an intron in the 18S rRNA gene can be considered an additional confirmation of the difference between *N. semenenkoi* and its sister species, *N. thermophila*.

4.3. ITS1 and ITS2 Secondary Structures

Currently, the analysis of the secondary structures of ITS1 and ITS2 is widely used to distinguish the species of green algae [42,44,71]. *N. semenenkoi* IPPAS C-1210 shows differences in one CBC in the conserved regions of ITS2 compared to *N. thermophila* ITBB HTA 1–65. In comparison to *C. volutis* CCAP 211/120, both *Neochlorella* species have three CBCs in the ITS1 secondary structures and two CBCs in the conserved regions of ITS2. Another additional CBC was found in the conserved regions of ITS2 between *N. thermophila* ITBB HTA 1–65 and *C. volutis* CCAP 211/120. Since even one CBC is a rare evolutionary event within the *Chlorella* clade [10,13,38], the results of the analysis of the internally transcribed spacers undoubtedly indicate the independent species status of each of these strains. In addition, unique CBCs in ITS1 (1st bp, helix IV) and ITS2 (7th bp, helix III) were found in all *Neochlorella* species, which distinguish them from all other *Chlorella* clade genera. In our opinion, they can be considered molecular signatures of the genus *Neochlorella*.

Hoshina et al. [14,38] found that when analyzing ITS2, it is important to take into account not only CBCs and secondary structure features but also genetic distances. For two organisms to be compared, the difference between the ITS2 sequences is usually either less than 2% or more than 10%. In the first case, the organisms are representatives of the same species, and in the second case, they belong to different species [14,38]. The genetic difference between *N. semenenkoi* IPPAS C-1210 and *N. thermophila* ITBB HTA 1–65 is 17.6%, and between *N. semenenkoi* IPPAS C-1210 and *C. volutis* CCAP 211/120, it is 20.4%. Such values unambiguously correspond to the interspecific level within the *Chlorella* clade.

4.4. Physiology

The representatives of the cluster under study have different habitat preferences (Table 3). *N. semenenkoi* IPPAS C-1210 and *C. volutis* CCAP 211/120 are free-living inhabitants of freshwater reservoirs, as are most members of the *Chlorella* clade [6,13]. In contrast, rooftops as a typical habitat are indicated for the strain *N. thermophila* ITBB HTA 1–65 [7]. Therefore, it can be assumed that it has an aerophytic life strategy, but this needs to be clarified.

N. semenenkoi IPPAS C-1210 is moderately thermotolerant, as evidenced by its ability to grow at 36 ± 1 °C and maintain viability after one to two days of incubation at 41 ± 1 °C. The best growth was observed at 30 ± 1 °C and a high irradiance of 500 µmol of photons m^{-2} s^{-1}.

The optimum pH was determined to be in the range of 8–9, but the strain grows normally at higher pH values, acidifying the medium. *N. semenenkoi* IPPAS C-1210 grows well in media with 0–0.4 M of NaCl or 0–0.2 M of $NaHCO_3$ and could withstand higher concentrations of NaCl up to 2 M and $NaHCO_3$ up to 1.2 M. It can be concluded that *N. semenenkoi* IPPAS C-1210 is halotolerant and is capable of using bicarbonate as a carbon source.

It has also been shown that *N. semenenkoi* IPPAS C-1210 is able to use nitrate, ammonium salts, and urea as nitrogen sources; yeast extract, casamino acids, and tryptone as nitrogen and carbon sources; and glucose, galactose, and acetate as carbon sources for mixotrophic and heterotrophic growth.

Thus, *N. semenenkoi* IPPAS C-1210 is characterized by thermotolerance, halotolerance, alkaliphily, and ability to use a wide range of carbon and nitrogen sources. These properties make the strain especially attractive for biotechnological applications. For example, the cultivation of microalgae in closed photobioreactors requires temperature control to avoid overheating, which is very expensive in the case of large-scale cultivation, but using thermotolerant strains helps to reduce temperature control costs [72]. Robust species that are able to grow in a wide range of temperatures are also suitable for outdoor cultivation with significant daily temperature fluctuations [73]. Halotolerant strains may be grown without using fresh water, and seawater or brackish water can be used instead. Alkaliphily is a beneficial trait in biotechnology because intensively growing microalgae raise the pH of their media, and a high pH will not inhibit the growth of an alkaliphilic strain. In addition, many contaminants do not survive at high pH. The ability to use bicarbonate as a carbon source helps solve the problem of carbon supply in large-scale cultivation [74]. The ability of the strain to use a wide range of carbon and nitrogen sources allows its growth in heterotrophic and mixotrophic cultures with a high cell density. In addition, such strain can be used for wastewater remediation [75].

The physiological characteristics of other authentic strains of the species assigned to the genus *Chlorella* have been studied to a lesser extent (Table 3). In terms of the resistance of algae to biotic and abiotic stresses, one of the most studied is resistance to high temperatures. Currently, among the representatives of the *Chlorella* clade, for which the reaction to exposure to high temperatures has been studied, *C. sorokiniana* 7-11-05 (=SAG 211-8k) has the highest thermal stability, growing at temperatures up to 42 °C [51,57]. The strain *N. thermophila* ITBB HTA 1–65 is considered the second most thermally stable since it has an optimal growth temperature of 33°C and could grow at 42 °C, although at a relatively low rate. In addition, this strain could tolerate heat up to 45 °C for at least three hours a day [7]. The studied strain IPPAS C-1210 also demonstrates thermotolerant properties, which, however, are somewhat lower than those of *C. sorokiniana* and *N. thermophila*. For comparison, *C. vulgaris* CCAP 211/11B (=SAG 211-11b) could grow at temperatures no higher than 30 °C [61,65]. Thus, it is likely that thermotolerance is characteristic for the genus *Neochlorella*.

Other notable physiological features of *N. semenenkoi* are its halotolerance (up to 2 M NaCl) and alkaliphily (optimal pH 8–9, upper limit pH 11). In comparison, the type species of the genus *Chlorella*, *C. vulgaris* SAG 211-11b, can tolerate only 3–4% of NaCl (0.6–0.7 M) [59,76], and its optimal pH is in the range of 5–8, with an upper limit pH of 9.5 [63]. The more closely related *C. sorokiniana* SAG 211-8k is also less halotolerant, surviving only in 1–3% of NaCl (0.2–0.6 M) [59], but we were not able to find information

about its upper pH limit. No available information was found on the pH and salt effects on the growth of other closely related *Chlorella*-like strains, including the sister species *N. thermophila* ITBB HTA 1–65.

The ability to use different nitrogen and carbon sources was suggested as a taxonomic marker for species delimitation by Shihira and Krauss [51]. The ability to grow mixotrophically and heterotrophically using various organic substrates is common to many strains of the *Chlorella* clade [51,64]. Acetate can be used as a carbon source for heterotrophic and mixotrophic growth by *N. semenenkoi* IPPAS C-1210 and *C. vulgaris* UTEX 259 (=SAG 211-11b) [64]. Ma et al. [7] grew *N. thermophila* ITBB HTA 1–65 in TAP medium containing acetate. Thus, we can assume the ability of *N. thermophila* to use acetate as a carbon source, but this has yet to be proven. *C. sorokiniana* 7-11-05 (=SAG 211-8k) is not able to use acetate for its growth in darkness [51]. Glucose can be used as a carbon source for heterotrophic and mixotrophic growth by *N. semenenkoi* IPPAS C-1210, *C. vulgaris* SAG 211-11b [63,64], and *C. sorokiniana* 7-11-05 [51]. Galactose can be used by *N. semenenkoi* IPPAS C-1210 and *C. sorokiniana* 7-11-05 [51]. Mannose inhibits the growth of *C. sorokiniana* 7-11-05 [51] and has no effect on *N. semenenkoi* IPPAS C-1210. Glycerol inhibits the growth of *N. semenenkoi* IPPAS C-1210, while *C. vulgaris* UTEX 259 uses it as a carbon source in light [64]. No information on the heterotrophic growth of *C. volutis* CCAP 211/120 and *C. lewinii* CCAP 211/90 is available. In addition, it is very important to check the ability of *N. thermophila* ITBB HTA 1–65 to grow autotrophically.

N. semenenkoi and all related strains under study are able to use nitrate as a nitrogen source; the only exception is *N. thermophila* ITBB HTA 1–65 because it was grown in TAP media with ammonium as a nitrogen source, and its ability to use nitrate has not yet been studied. Ammonia is a suitable nitrogen source for *N. semenenkoi* IPPAS C-1210, *N. thermophila* ITBB HTA 1–65 [7], and *C. sorokiniana* 7-11-05 [51], and no information about other strains is available. Furthermore, *C. sorokiniana* 7-11-05 can use casamino acids [51], *C. vulgaris* SAG 211-11b can use triptone and peptone [1], while *N. semenenkoi* IPPAS C-1210 can use all of them. It remains to be investigated whether other relative strains can utilize organic nitrogen. The ability to utilize different carbon and nitrogen sources seems to be a possible taxonomic marker, being easy for testing and useful for biotechnological purposes.

5. Conclusions

The results of this study have again demonstrated the need for a thorough revision of the algal strains with *Chlorella*-like morphology that are mistakenly assigned to the genus *Chlorella*. The morphological and ultrastructural studies showed that the studied strain IPPAS C-1210 has a typical *Chlorella*-like morphology and ultrastructure. It differs from closely related strains only by cell size and thickness of cell wall.

The phylogenetic analysis based on 18S–ITS1–5.8S–ITS2 sequences revealed that the studied strain IPPAS C-1210 groups in a highly supported cluster with the authentic strain of *C. thermophila* ITBB HTA 1–65. A related phylogenetic lineage is formed by *C. volutis* CCAP 211/120. This cluster occupies a unique phylogenetic position within the *Chlorella* clade and is clearly separated from the cluster containing *C. vulgaris*, the type species of the genus *Chlorella*, as well as from other genera of the *Chlorella* clade. Based on genetic distances and CBCs in ITS1 and ITS2 secondary structures, we describe a new Trebouxiophycean genus, *Neochlorella* gen. nov. Within this genus, two new species are described: *N. semenenkoi* sp. nov. (autentic strain IPPAS C-1210) and *N. thermophila* comb. nov. (authentic strain ITBB HTA 1–65). A more complete study of the related strain *C. volutis* CCAP 211/120 is required to clarify its taxonomic identity.

Our study shows that the newly described *N. semenenkoi* IPPAS C-1210 is characterized by thermotolerance, halotolerance, alkaliphily, and ability to use a wide range of carbon and nitrogen sources. These properties make the strain especially attractive for biotechnological applications.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d15040513/s1. Figure S1: Cell morphology of *Neochlorella semenenkoi* IPPAS C-1210 after the grazing test: a 3-day-old culture grown in BG-11 with the phycophagus *Brachionus rotundiformis*; Figure S2: Growth of *Neochlorella semenenkoi* IPPAS C-1210 at different pH. The error bars show standard deviations of the mean (n = 3). The variants with the same letter are not significantly different (one-way ANOVA analysis, post hoc Tukey HSD test; $p < 0.05$). The second experiment out of two is shown; Figure S3: Growth of *Neochlorella semenenkoi* IPPAS C-1210 in the concentration matrix $NaHCO_3$ and NaCl. The second experiment out of two is shown.

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