Nutrient requirements of polar *Chlorella*-like species

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Abstract

Eukaryotic micro-algae, well adapted to extremely low and varying temperatures, varying light intensities, as well as low availability of essential macronutrients and other resources, represent ideal producers in low-temperature biotechnological processes. In order to identify the nutrient requirements of six biotechnologically perspective Arctic and Antarctic soil *Chlorella*-like strains at various temperature and light regimes, the algae were cultivated in a unit for cross gradients of temperature (-4 to 24°C) and irradiance (5 to 65 μmol m^{-2} s^{-1}), and at different nutrient treatments in each temperature-irradiance combination. The nutrient treatments included two different carbon (bicarbonate and carbonate concentrations of 1 and 5 mM) and nitrogen (nitrate concentrations of 50 amd 100 μM and ammonium concentrations 100 and 500 μM) forms at two different concentrations for each. Temperature and irradiance growth requirements were similar in the majority of strains reflecting thus comparable conditions in the original microhabitat, regardless of its geographic position. All studied strains tolerated low temperatures (1 to 5°C), but were able to grow even at temperatures above 20°C, thus, they were considered to be psychrotolerant. All experimental strains were able to grow at very low irradiances. Nutrient manipulation either did not affect the growth limits and optimum, or narrowed the growth optima; the response was strain-specific. Ammonium and nitrate additions resulted in decreased growth rates in all tested strains, with the exception of one strain in which growth stimulation was observed. The decrease in growth rate was probably due to nutrient oversaturation in the inhibited strains. Carbonate addition stimulated growth of all strains. Bicarbonate also increased the growth rate in all strains with one exception, in which bicarbonate inhibited growth, indicating thus carbon limitation during cultivation and different carbon uptake mechanisms.

Key words: Green algae, nitrogen, carbon, growth, crossed gradients of temperature and light.

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Introduction

Eukaryotic micro-algae are important primary producers in the Arctic and Antarctica (Elster 2002, Elster et Benson 2004, Friedmann et Thirstle 1993, Priscu 1998, Vincent 1988, Walton et Doake 1987) where they are exposed to various extreme environmental conditions: low and varying temperatures, varying light intensities, low availability of essential nutrients and other resources. They have evolved a complex network of adaptation reactions that compensate for the negative effect of the harsh polar environment. Due to a wide diversity of metabolic activities (Elster et Benson 2004, Elster et al. 2008, Padney et al. 2004, Rai et Gaur 2001, Shukla et Kashyap 1999, Shukla et al. 1997a, Shukla et al. 1997b, Vonshak et Torzillo 2004), they represent an important source of novel species for low-temperature biotechnology.

Chlorella-like species, their cellular constituents or products, could become such species for low-temperature biotechnology due to their growth at low temperatures (<10°C), tolerance of high temperatures (>20°C), and high photosynthesis rate across a broad temperature range (Shukla et al., submitted). They are cultured for the production and processing of desirable compounds with therapeutic potential, demonstrating that algae possess antibacterial, antifungal and anticancer activities (Becker 2004, Iwamoto 2004, Richmond 2004, Skulberg 1996, Watson 2003 etc.). However, their remarkably slow growth at low temperatures may contribute to limit the biotechnological applications of polar *Chlorella*-like strains. Thus, there is a strong pressure for optimizing growth conditions in order to achieve high growth rates and biomass yield. While temperature and light requirements, as well as temperature dependence of photosynthesis and respiration, of several polar *Chlorella*-like species are known (Shukla et al., submitted), data on their nutrient requirements, especially nitrogen and carbon, and their individual forms, are rare. Generally, polar algae should be adapted to low nutrient conditions (Dickson 2000, Elster 1996, Elster et Benson 2004, Elster et al. 1999, Elster et Svoboda 1995, Kaštovská et al. 2005, Stibal et al. 2006) and such lower nutrient demand could reduce the costs of mass cultivation. The aim of this study was to determine the requirements for nitrogen (ammonium or nitrate) and carbon (bicarbonate and carbonate) of five *Chlorella*-like species at varying temperature and light conditions. We hypothesised that nutrient additions should increase the growth rate of the experimental strains and broaden their growth optima with respect to temperature and irradiance.

Material and methods

Six soil *Chlorella*-like strains originating from various polar regions and habitats (Table 1) were obtained from the Culture Collection of Algae at the Laboratory of Algology of the Institute of Botany, Třeboň, Czech Republic, and the Institute of Soil Biology, České Budějovice, Czech Republic. All strains were pre-cultivated at a temperature of 8°C and an irradiance of 80 μmol m^{-2} s^{-1} of photosynthetically active radiation.

In order to test the effect of nutrient additions across gradients of temperature and irradiance, the microplate method of

Species	Strain No.	Alternative strain No.	Locality	Microenvironment	Isolated by	Culture collection
Bracteacoccus sp.	N2	1999/2	Arctic, Ny-Ålesund	Deglaciated soil	Řeháková	CCALA
Pseudomuriella sp.	N7	1998/7	Arctic, Ny-Ålesund	Deglaciated soil	Elster	CCALA
Chlorella vulgaris sp.	L5	1994/5	Arctic, Ellesmere Island	Deglaciated soil	Lukešová	ISB
Chlorella minutissima	L15	1999/15	Antarctic, Anchorage Island	Bare ground	Lukešová	ISB
Chlorella homosphaera	L24	1997/24, SPH 15	Antarctic, King George Island	Deglaciated soil	Lukešová	ISB
Chlorella minutissima	L32	1996/109, EG-1	Antarctic, King George Island	Deglaciated soil	Lukešová	ISB

Table 1. List of used strains. Culture collection abbreviations: CCALA – Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň, Czech Republic; ISB – Institute of Soil Biology, Biological Centre AS CR, České Budějovice, Czech Republic.

cross gradients cultivation was used (Kvíderová et Lukavský 2005). Temperature ranged from -4°C to 24°C and irradiance from 5 to 65 μmol m^{-2} s^{-1} corresponding to mean conditions on the surface or slightly below the soil surface (Kaštovská et al. 2005, Tscherko et al. 2003). At each pre-defined microplate position, temperature was measured by a digital thermometer (Omega, USA) and irradiance by a PU-550 digital luxmeter (Metra Blansko, Czech Republic) with modified PAR sensor.

Abbreviation	Treatment
C	Control (Z medium)
+ 200 A	Z medium + 200 μM NH_4Cl
+ 500 A	Z medium + 500 μM NH_4Cl
+ 50 N	Z medium + 50 μM KNO_3
+ 100 N	Z medium + 100 μM KNO_3
+ 1 BC	Z medium + 1 mM $NaHCO_3$
+ 5 BC	Z medium + 5 mM $NaHCO_3$
+ 1 C	Z medium + 1 mM Na_2CO_3
+ 5 C	Z medium + 5 mM Na_2CO_3

Table 2. Nutrient treatment abbreviations.

A homogeneous algal suspension in mineral nutrient medium Z (Staub 1961), with a volume of 0.2 ml and initial cell density of 10^5 cells ml^{-1}, was inoculated in wells of 36 serological plates used for 36 different combinations of temperature and light. There were six replicates (in six wells of one column) for each treatment in every plate. Treatments consisted of a positive control (Z-medium), different nutrient additions (200 and 500 μM NH_4Cl, 50 and 100 μM KNO_3, 1 and 5 mM $NaHCO_3$, 1 and 5 mM Na_2CO_3, treatment abbreviations are summarized in Table 2), and a negative control (distilled water). Only one strain was tested in one cultivation experiment. The stability of gradients was checked regularly by periodic temperature and irradiance measurements between individual strain cultivation experiment.

Cultivation occurred in the unit for crossed gradients of temperature and light (Labio, Czech Republic; see Kvíderová et Lukavský 2001). To prevent carbon limitation during the cultivation, the air was permanently pumped under a Perspex cover. In order to synchronise the algal populations, the plates were incubated in dark for one-day. Absorbance (light scattering) at 750 nm, $A_{750,}$ was measured on every second day using an iEMS Plate Reader (LabSystems, Ltd., Finland). The experiments lasted 8 days until a steady-state of the algal culture was reached. The measured A_{750} values were converted to number of cells, N (cells ml^{-1}) and dry weight, DW (mg ml^{-1}) according to individual conversion curves and equations (see Kvíderová et Lukavský 2003) for conversion equation estimations). Growth rate μ (d^{-1}) was calculated as the slope of a linear regression of the dependence of N vs. time

(Kvíderová et Henley 2005). The optimum and growth limits were estimated from contour graphs where the data were smoothed by the least square method.

Statistical analyses were performed using Statistica 10.0 (StatSoft, U.S.A.) and the Principal Component Analysis (PCA) using Canoco (CPRO-SLO, The Netherlands). The results were significant for $p \leq 0.05$. Before evaluation, the raw data were subjected to K-criterion for outlying values for six samples at p-level = 0.05; any outlying values were excluded from further evaluation.

Results

All studied environmental variables (temperature, irradiance and nutrients) and their combinations significantly influenced the growth rates of all strains (ANOVA; only $p \leq 0.05$ considered). The PCA distinguished a similar ecophysiological response for strains N7, L15, L32 and L5, regardless of their geographic origin, and different requirements for L5 and L24 (Fig. 1). The PCA also found negative effects of nitrogen addition in both forms (ammonium and nitrate) and positive effects of carbon (bicarbonate and carbonate) treatments in all strains. Nutrient manipulation either did not affect the optima ranges and growth limits of the individual strains, or resulted in narrower optima ranges. However, the response to individual treatments seems to be strain specific (Table 3). As revealed by the PCA, the ammonium nitrogen addition decreased the growth rate of all strains in all cases, with the exception of strain N2 (multiple-ANOVA, n=216, $p<0.001$ in all cases; Fig. 2a). Nitrate nitrogen addition also reduced the growth rate of all strains with the exception of strain N2 (multiple-ANOVA, n = 216 p = 0.039 for strain N7 and $p < 0.001$ for the remaining strains; Fig. 2). This could indicate possible nitrogen saturation or even over-saturation, and/or toxic ammonium effects on strains N7, L5, L15, L24 and L32. On the other hand, strain N2 seems to be nitrogen limited and could probably utilize both nitrogen forms.

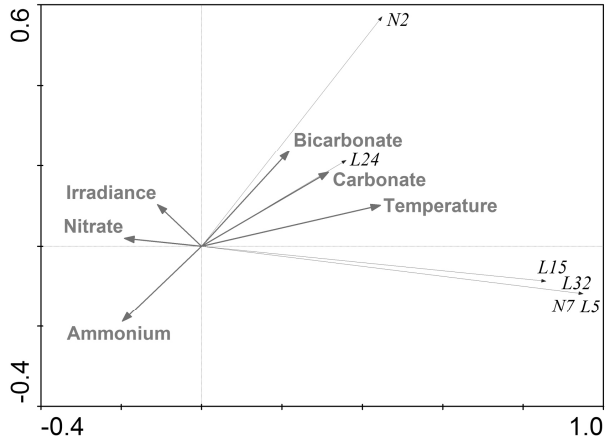

Fig. 1. The PCA analysis diagram. The first axis explains 85.2%, the second axis 10.1% and the third axis 4.6% of total variance.

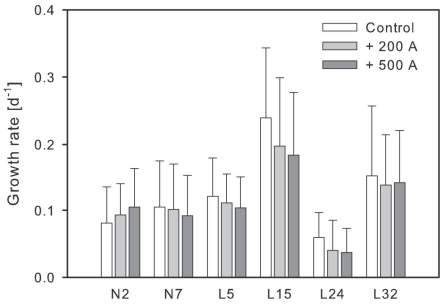

Fig. 2. The effect of ammonium treatments on growth rate of individual strains.

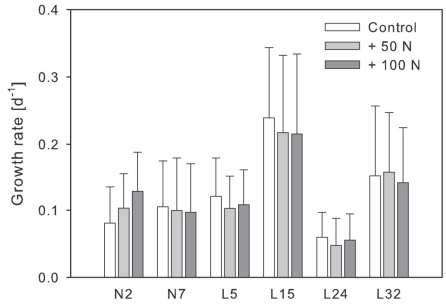

Fig. 3. The effect of nitrate treatments on growth rate of individual strains.

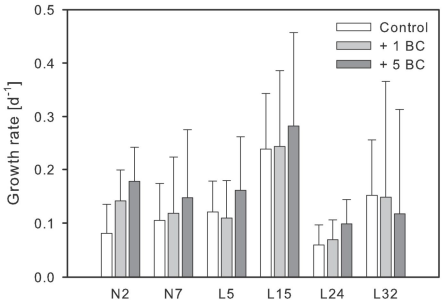

Fig. 4. The effect of bicarbonate treatments on growth rate of individual strains.

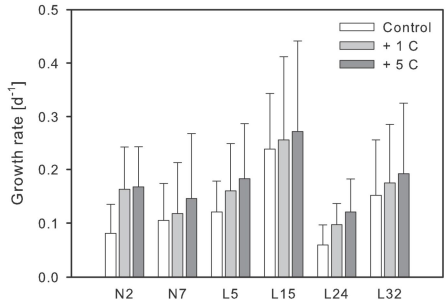

Fig. 5. The effect of carbonate treatments on growth rate of individual strains.

A different response of the experimental strains was observed in the bicarbonate treatment. While the growth rate was significantly higher in strains N2, N7, L5, L15 and L24 after its addition, a slight growth inhibition was detected in L32 (multiple-ANOVA, $n=216$, $p<0.001$ in all cases; Fig. 3). Contrary to the heterogeneous response to ammonium, nitrate and bicarbonate, the effect of the carbonate treatment was uniform in all studied strains, since its addition resulted in increased growth rates (multiple-ANOVA, $n=216$, $p<0.001$ in all cases; Fig. 4). This could indicate carbon limitation during cultivation and possible different carbon uptake mechanisms, especially in strain L32, which may not be able to utilize bicarbonate.

	Temperature [°C]			Irradiance [µmol m^{-2} s^{-1}]		
	Lower limit	Optimum	Upper limit	Lower limit	Optimum	Upper limit
N2						
C	1 - 4.5	10.1 - 20.5	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 200 A	**4.5**	**10.1 - 18.4**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 500 A	**4.5**	**10.1 - 18.4**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 50 N	**4.5**	**10.1 - 18.4**	>20.5	<12.3	<12.3 - >50.5	>50.5
+100 N	**4.5**	**10.1 - 18.4**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 1 BC	**1**	**10.1 - 18.4**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 5 BC	**1**	**10.1 - 18.4**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 1 C	**4.5**	**10.1 - 18.4**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 5 C	**4.5**	**10.1 - 14.3**	>20.5	<12.3	<12.3 - >50.5	>50.5
N7						
C	1 - 4.5	10.1 - >20.5	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 200 A	**1**	10.1 - >20.5	>20.5	<12.3	**<12.3 - 27.8**	**50.5**
+ 500 A	**1**	10.1 - >20.5	>20.5	<12.3	**<12.3 - 27.8**	**50.5**
+ 50 N	**1**	10.1 - >20.5	>20.5	<12.3	**<12.3 - 27.8**	**50.5**
+100 N	**1**	10.1 - >20.5	>20.5	<12.3	**<12.3 - 27.8**	**50.5**
+ 1 BC	**1**	10.1 - >20.5	>20.5	<12.3	**<12.3 - 35.7**	>50.5
+ 5 BC	**1**	10.1 - >20.5	>20.5	<12.3	**<12.3 - 35.7**	>50.5
+ 1 C	**4.5**	10.1 - >20.5	>20.5	<12.3	**<12.3 - 35.7**	>50.5
+ 5 C	**4.5**	10.1 - >20.5	>20.5	<12.3	**<12.3 - 35.7**	>50.5
L5						
C	1	4.5 - >20.5	>20.5	<12.3	<12.3 - 35.7	50.5
+ 200 A	1	4.5 - >20.5	>20.5	<12.3	<12.3 - 35.7	50.5
+ 500 A	1	4.5 - >20.5	>20.5	<12.3	<12.3 - 35.7	50.5
+ 50 N	1	4.5 - >20.5	>20.5	<12.3	**<12.3 - 15.9**	50.5
+100 N	1	4.5 - >20.5	>20.5	<12.3	**<12.3 - 15.9**	50.5
+ 1 BC	1	**10.1 - 18.4**	>20.5	<12.3	**<12.3 - 15.9**	50.5
+ 5 BC	1	**10.1 - 18.4**	>20.5	<12.3	**<12.3 - 15.9**	50.5
+ 1 C	1	**10.1 - >20.5**	>20.5	<12.3	**<12.3 - 15.9**	**27.8**
+ 5 C	1	**14.3 - >20.5**	>20.5	<12.3	**<12.3 - 21.5**	**27.8**
L15						
C	1	4.5 - >20.5	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 200 A	1	4.5 - >20.5	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 500 A	1	**4.5 - 20.5**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 50 N	1	4.5 - >20.5	>20.5	<12.3	<12.3 - >50.5	>50.5
+100 N	1	4.5 - >20.5	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 1 BC	1	**10.1 - >20.5**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 5 BC	1	**10.1 - >20.5**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 1 C	1	**10.1 - >20.5**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 5 C	1	**10.1 - >20.5**	>20.5	<12.3	<12.3 - >50.5	>50.5
L24						
C	1 -10.1	10.1 - >20.5	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 200 A	**10.1**	**14.3 - 20.5**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 500 A	**4.5**	**10.1 - 20.5**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 50 N	**4.5**	10.1 - >20.5	>20.5	<12.3	<12.3 - >50.5	>50.5
+100 N	**4.5**	10.1 - >20.5	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 1 BC	**1**	**18.4 - >20.5**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 5 BC	**1 - 4.5**	**18.4 - >20.5**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 1 C	**4.5**	**>20.5**	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 5 C	**4.5**	**>20.5**	>20.5	<12.3	<12.3 - >50.5	>50.5
L32						
C	1 - 4.5	10.1 - >20.5	>20.5	<12.3	<12.3 - >50.5	>50.5
+ 200 A	**1**	**14.3 - >20.5**	>20.5	<12.3	**<12.3 - 35.7**	**50.5**
+ 500 A	**1**	**14.3 - >20.5**	>20.5	<12.3	**<12.3 - 35.7**	**50.5**
+ 50 N	**1**	**14.3 - >20.5**	>20.5	<12.3	**<12.3 - 35.7**	**50.5**
+100 N	**1**	**14.3 - >20.5**	>20.5	<12.3	**<12.3 - 35.7**	**50.5**
+ 1 BC	1 - 4.5	**14.3 - 18.4**	**20.5**	<12.3	<12.3 - >50.5	>50.5
+ 5 BC	1 - 4.5	**14.3 - 18.4**	**20.5**	<12.3	<12.3 - >50.5	>50.5
+ 1 C	**1**	14.3 - >20.5	>20.5	<12.3	**<12.3 - 27.8**	**35.7**
+ 5 C	**1**	14.3 - >20.5	>20.5	<12.3	**<12.3 - 27.8**	**50.5**

Table 3. The ecophysiological characteristics of the experimental strains at different nutrient treatments. For treatment abbrevivatons, see Table 2)

Discussion

All tested strains tolerated low temperatures (1 to 5°C). According to their temperature growth optima and limits, all strains could be regarded as psychrotolerant (Morita 1975). All strains were able to grow at very low irradiances, however, tolerance to higher irradiances (above 55 μmol m^{-2} s^{-1}) as well as the lowest irradiance causing photoinhibition remains to be determined. The broad temperature tolerance and low light tolerance could reflect adaptation/ acclimatization (*sensu* Elster 1999) to conditions of polar soil environments (Kaštovská et al. 2005, Tscherko et al. 2003). Similarity and/or difference of ecophysiological requirements indicates a key role of the original microenvironment in defining the growth limits and optimum ranges regardless of the geographic position of the original locality, as was already observed in *Stichococcus* strains (Kvíderová et Lukavský 2005).

The long-term cultivation in the culture collection conditions may also contribute to the similarity of strain growth requirements, however this acclimation (sensu Elster (1999), *i.e.* adaptation to cultute conditions) does not occur in all strains, i.e. some *Stichoccoccus* strains kept their original strain-specific growth requirements even after one year in the culture collection (Kvíderová et Lukavský 2005). If the culture collection conditions (15°C, 45 μmol m^{-2} s^{-1}) are similar to the growth optimum, there is no selection pressure to shift the optimum range to different temperature or irradiances. For example, strain *Stichococcus bacillaris* Hindák 1984/15 isolated from a temperate lake, kept its optimum temperature in range of 19 to 24°C despite of 20 years in the culture collection (Kvíderová et Lukavský 2005).

Freshwater terrestrial and hydro-terrestrial ecosystems in the polar regions, especially newly deglaciated ones, are usually oligotrophic and nitrogen limited (Dickson 2000, Elster 1996, Elster et Benson 2004, Elster et al. 1999, Elster et Svoboda 1995, Kaštovská et al. 2005, Stibal et al. 2006), so nitrogen addition could increase the growth rate and primary productivity, as has been observed in *in situ* manipulation experiments (Elster et Svoboda 1995, Chapin et al. 1994, Chapin et Shaver 1985), and broaden the growth optima ranges. Contrary to this hypothesis, none or opposite effects were observed in our experiments, with the exception of strain *Bracteacoccus* sp. N2. The response of individual strains could reflect adaptation to nutrient availability in the original microhabitats; unfortunately these data are not available for further analysis. The inhibitory effects of ammonium and nitrogen in strains *Pseudmuriella* sp. N7, *Chlorella vulgaris* L5, *Chlorella minutissima* L15, *Chlorella homospaera* L24 and *Chlorella minutissima* L32 could be caused by nutrient oversaturation. Such nutrient oversaturation was already recorded in bacteria (Ishida et Kadota 1981), or algae (Kvíderová et Elster, submitted) originating from extreme oligotrophic environments; however, these studies are rare. The different taxonomical position of the experimental strains probably could not explain the different nitrogen requirements since both the nitrogen-limited strain *Bracteacoccus* sp. N2 and nitrogen-inhibited strain *Pseudomuriella* sp. N7 are within the class Chlorophyceae (Fučíková et al. 2011), while the other strains belong to Trebouxiophyceace (Lepka 2007).

Contrary to the nitrogen treatments, which rather inhibited growth, at least one of the carbon forms (bicarbonate or carbonate) stimulated the growth rates of all strains, indicating thus possible carbon limitation, even if CO_2 was supplied to the cultivation space during the experiment. Since all strains, with the exception of

Chlorella minutissima L32, had increased growth rates in both the bicarbonate and carbonate treatments, these algae probably could utilize both carbon forms (Colman et al. 2002, Espie et Colman 2005, Raven 1997, Raven 2003, Raven et al. 2005). The opposite response of the strain *Chlorella minutissima* L32 to the bicarbonate and carbonate treatments was probably caused by having a different carbon uptake mechanism than in the rest of the strains. There are several carbon uptake and concentrating mechanisms (Colman et al. 2002, Espie et Colman 2005, Raven 1997, Raven 2003, Raven et al. 2005), so the preferred carbon utilization mechanism could be relevant to the original chemical microenvironment. However, the data concerning the chemistry of the original microenvironment are missing. Since the pH of the Z medium (Staub 1961) varied between 7 and 7.3, the dominant form of carbon should be bicarbonate (Falkowski et Raven 2007), however the photo-synthetic activity could significantly increase the medium pH to 9 to 10 (Shiraiwa et al. 1993), affecting thus the available carbon forms and carbon uptake mechanism. The precise mechanism of carbon uptake in the strain *Chlorella minutissima* L32 remains to be investigated.

In general, five experimental strains, *Pseudmuriella* sp. N7, *Chlorella vulgaris* L5, *Chlorella minutissima* L15, *Chlorella homospaera* L24 and *Chlorella minutissima* L32, have similar features (low and high temperature tolerance, ability to grow even at very low irradiance, and low nutrient requirements), making them perspective species for low temperature biotechnology applications. The carbon limitation observed in our experiments and common in mass algal cultivation (Brune et Novak 1981) could be eliminated by using a suitable gas mixing system. These strains will be further screened for production of biotechnologically interesting compounds, such as fatty acids, etc.

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