

# EXPERIMENTAL DESIGN TO OPTIMIZE BOLD'S BASAL MEDIUM TO INCREASE BIOMASS AND PIGMENT PRODUCTION IN THREE MICROALGAE SPECIES

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## INTRODUCTION

The great biotechnological potential of microalgae has aroused the interest of many industrial sectors. With biomasses rich in pigments, lipids, proteins and several other compounds, these microorganisms are a potential source of bioactive molecules for multiple applications. According to the literature, *Chromochloris zofingiensis* is an interesting source of carotenoids, specially astaxanthin<sup>1,2</sup>, *Koliella longiseta* is mainly reported as a rich source of eicosapentaenoic acid (EPA, ω-3) and arachidonic acid (ARA, ω-6)<sup>3</sup>, while *Schizomeris leibleinii* is mainly studied for wastewater treatment<sup>4,5</sup>. The imposition of abiotic stress by changing culture conditions (e.g. nutrient concentration in the culture medium, temperature, light irradiance, among others) directly influences growth and is able to modulate biomass composition. An experimental design was proposed to evaluate the effect of salinity and concentration of nitrate, ferrous sulphate and phosphate on growth and production of carotenoids and chlorophylls in these species. Higher salinity and ferrous sulphate concentration increased the final biomass concentration almost threefold in *C. zofingiensis* and twofold in *S. leibleinii* when compared to their controls. Meanwhile, *K. longiseta* was able to double its biomass concentration only by changing nitrate concentration in the medium. Regarding pigment production, higher carotenoids content was found in *C. zofingiensis* (3.16 mg·g<sup>-1</sup>) cultivated in unmodified BBM (Bold's Basal Medium) and *S. leibleinii* (3.09 mg·g<sup>-1</sup>) cultivated under nitrogen limitation and with higher salinity and ferrous sulphate concentration. Finally, higher chlorophyll content was observed in *K. longiseta* (17.77 mg·g<sup>-1</sup>) cultivated at higher ferrous sulphate concentration and *C. zofingiensis* (16.24 mg·g<sup>-1</sup>) cultivated in absence of phosphate and higher ferrous sulphate concentration.

## AIMS

- Identify and select the components of BBM that potentially affect biomass and pigments accumulation in microalgae.
- Propose an experimental design to evaluate the individual and combined effect of salinity and concentration of NaNO<sub>3</sub>, FeSO<sub>4</sub>·7H<sub>2</sub>O and potassium phosphates on growth and pigments production by *C. zofingiensis*, *K. longiseta* and *S. leibleinii*.
- Analyze the ANOVA outcomes in order to define the best culture condition for each species.

## MATERIALS AND METHODS

In order to evaluate the effect of four components of BBM<sup>6</sup> culture medium (i.e., potassium phosphates, NaNO<sub>3</sub>, NaCl and FeSO<sub>4</sub>·7H<sub>2</sub>O), a full factorial experimental planning (2<sup>n</sup>) with three central points was proposed according to the conditions described in Table 1, totaling 19 experiments (Figure 1).



Figure 1. Microalgae cultivations based on full factorial experimental planning (2<sup>n</sup>, n=4) with three central points.



Figure 2. Qualitative representation of the cultures after 9 days of cultivation.

Cultivations were carried out for 9 days at 18 ± 2 °C and 4000 lx. Cell growth was evaluated by optical density (absorbance at 750 nm) and the final biomass concentration was calculated gravimetrically.

Table 2. Experimental design by DesignExpert StatEase® software version 13.

Run	Factor 1 A: NaNO <sub>3</sub> (g·L <sup>-1</sup> )	Factor 2 B: NaCl (g·L <sup>-1</sup> )	Factor 3 C: K <sub>2</sub> HPO <sub>4</sub> /KH <sub>2</sub> PO <sub>4</sub> (g·L <sup>-1</sup> )	Factor 4 D: FeSO <sub>4</sub> ·7H <sub>2</sub> O (g·L <sup>-1</sup> )
1	0.250	0.025	0.161	0.025
2	0.000	0.025	0.000	0.005
3	0.000	0.025	0.161	0.025
4	0.125	5.858	0.080	0.015
5	0.125	5.858	0.080	0.015
6	0.000	11.69	0.000	0.005
7	0.000	0.025	0.000	0.025
8	0.250	11.69	0.000	0.025
9	0.250	11.69	0.000	0.005
10	0.250	0.025	0.161	0.005
11	0.000	11.69	0.161	0.025
12	0.250	11.69	0.161	0.025
13	0.125	5.858	0.080	0.015
14	0.000	11.69	0.161	0.025
15	0.000	0.025	0.161	0.005
16	0.250	0.025	0.000	0.005
17	0.000	11.69	0.000	0.025
18	0.250	11.69	0.161	0.005
19	0.250	0.025	0.000	0.025

Note: Run 10 (green box) represents the control condition (unmodified BBM). While runs 4, 5 and 13 (dark red box) are the three central points.

Table 1. Definition of the conditions for the experimental design.

Factors	Stress condition*	References	Proposed range (g·L <sup>-1</sup> )
[PO <sub>4</sub> <sup>3-</sup> ]	↓	[7]	0-0.161
NaNO <sub>3</sub>	↓	[8]	0-0.25
NaCl	↑	[9,10]	0.025-11.69
FeSO <sub>4</sub> ·7H <sub>2</sub> O	↑	[11]	0.005-0.025

\* Stress conditions claimed to increase biomass or pigments production by *C. zofingiensis*.

The experimental planning was analyzed by analysis of variance (ANOVA) using DesignExpert StatEase® software version 13 with 95% of confidence, being statistically significant when p<0.05.

Pigments extractions were performed according to Fernandes and Cordeiro<sup>12</sup> with some modifications (instead of ultrasonating the samples for 90 min, they were ultrasonicated for 30 min three times).

Total of carotenoids (Car<sub>T,E</sub>) and chlorophylls (Chl<sub>T</sub>) were calculated through Eq. (1) and (2)<sup>13</sup>, respectively.

$$Car_{T,E} (mg \cdot L^{-1}) = \frac{(1000 \cdot A_{430} - 1.90 \cdot Chl_{T,E} - 63.14 \cdot Chl_{D})}{214} \quad (1)$$

$$Chl_T (mg \cdot L^{-1}) = (11.24 \cdot A_{661.6} - 2.04 \cdot A_{644.8}) + (20.13 \cdot A_{644.8} - 4.19 \cdot A_{661.6}) \quad (2)$$

## RESULTS AND DISCUSSIONS

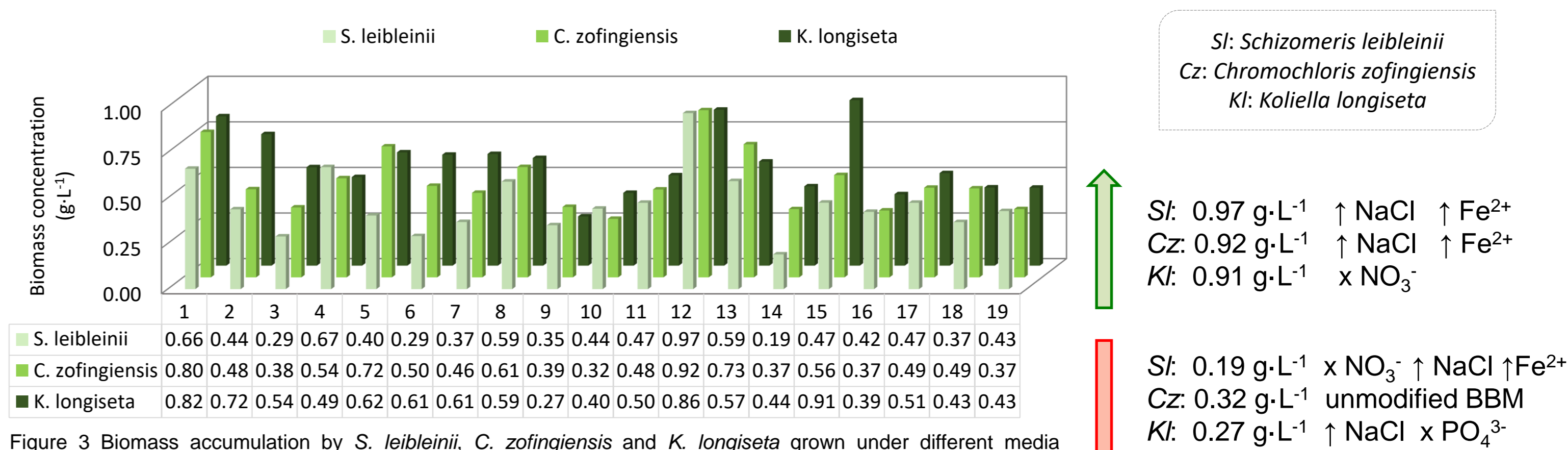


Figure 3. Biomass accumulation by *S. leibleinii*, *C. zofingiensis* and *K. longiseta* grown under different media compositions.

✓ Higher salinity and ferrous sulphate concentration seems to increase biomass accumulation in *S. leibleinii* and *C. zofingiensis*. Surprisingly, *K. longiseta* achieved a higher biomass concentration under nitrogen starvation.

✗ Nitrogen starvation seems to drastically impair biomass accumulation in *S. leibleinii*. Whereas *K. longiseta* was more affected by higher salinity combined with potassium phosphate starvation. Finally, all conditions tested improved biomass production in *C. zofingiensis* when compared to the control.

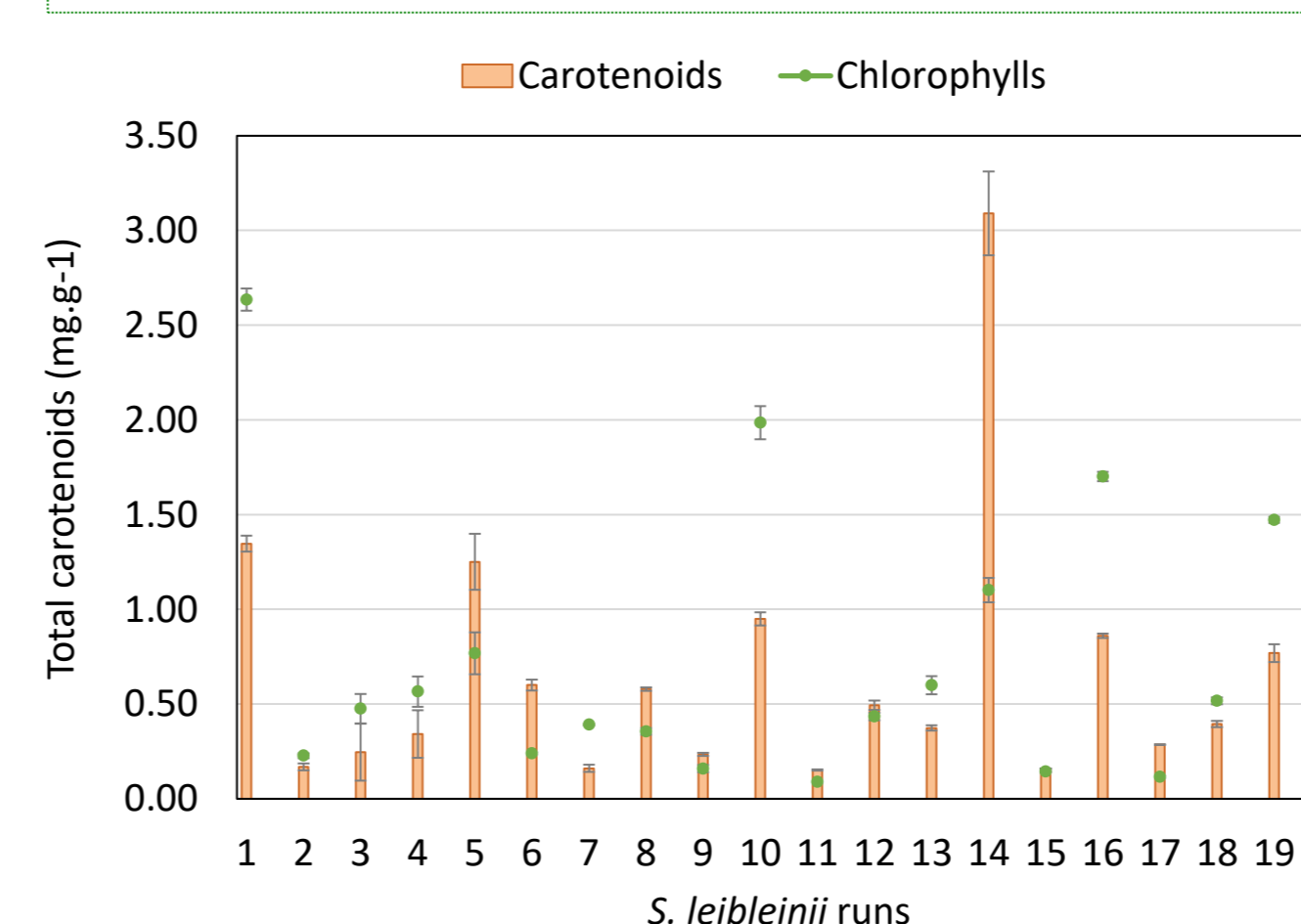


Figure 4. Carotenoids and chlorophylls accumulation in *S. leibleinii* cultivated under different media compositions.

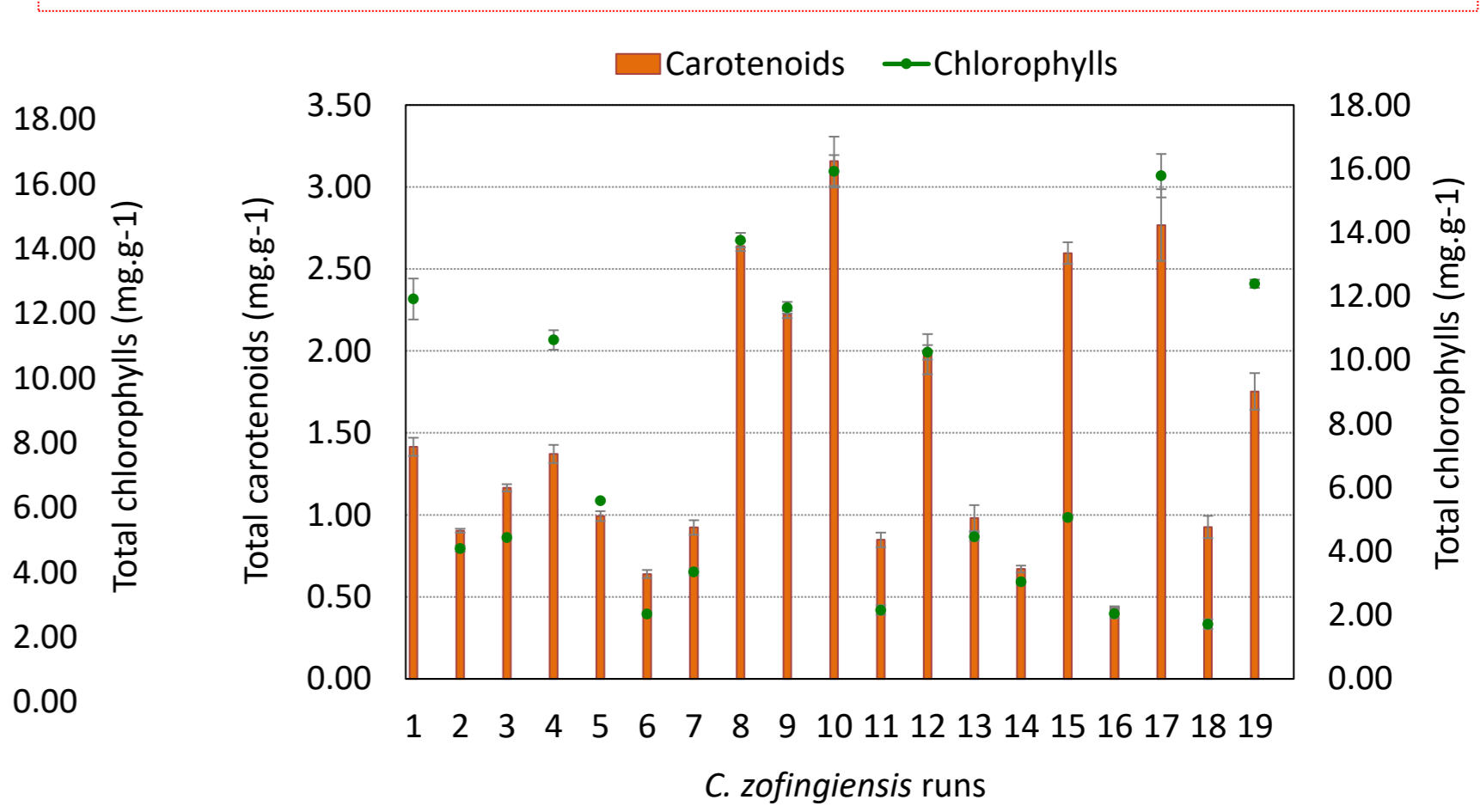


Figure 5. Carotenoids and chlorophylls accumulation in *C. zofingiensis* cultivated under different media compositions.

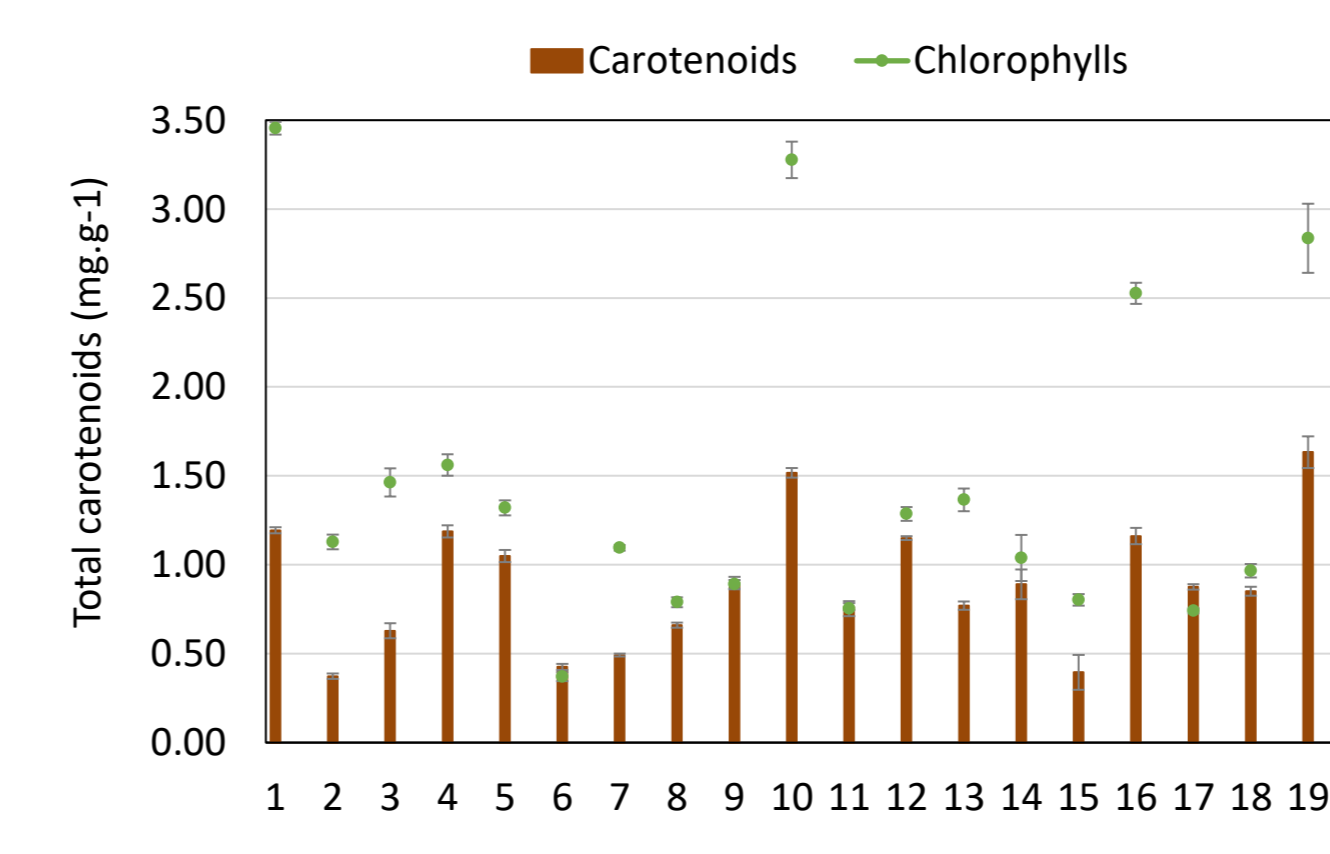
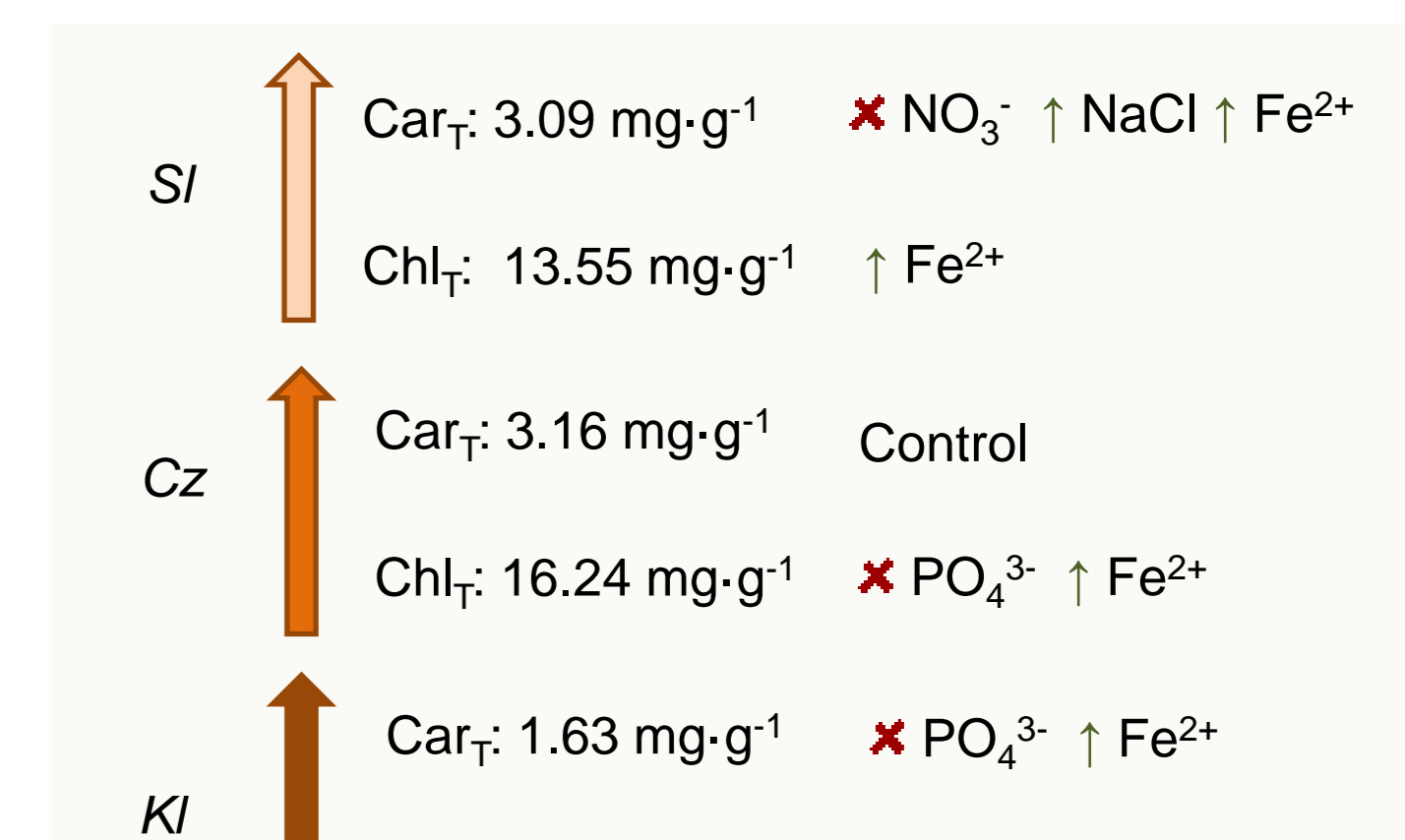


Figure 6. Carotenoids and chlorophylls accumulation in *K. longiseta* cultivated under different media compositions.



ANOVA outcomes:

- Nitrate alone and through the combined effect with other factors seems to be an important parameter that significantly affects the variables studied.
- None of the factors had significant influence on biomass accumulation in *S. leibleinii* and *C. zofingiensis*.

Table 3. Significant model terms according to ANOVA (p<0.05).

Factor	<i>S. leibleinii</i>	<i>C. zofingiensis</i>	<i>K. longiseta</i>
Car <sub>T</sub>	AB	A	A, AB
Chl <sub>T</sub>	A, B, C, D, AB, CD, ABCD	A	A, B, C, D, AB
Biomass	None	None	AB, AC, AD, D

A: NO<sub>3</sub><sup>-</sup>; B: NaCl; C: PO<sub>4</sub><sup>3-</sup>; D: Fe<sup>2+</sup>; Car<sub>T</sub>: total carotenoids; Chl<sub>T</sub>: total chlorophylls.

Table 4. Numerical optimization by analysis with DesignExpert StatEase® software version 13.

Species	Optimized concentrations				Predicted responses		
	NaNO <sub>3</sub> (g·L <sup>-1</sup> )	NaCl (g·L <sup>-1</sup> )	K <sub>2</sub> HPO <sub>4</sub> /KH <sub>2</sub> PO <sub>4</sub> (g·L <sup>-1</sup> )	FeSO <sub>4</sub> ·7H <sub>2</sub> O (g·L <sup>-1</sup> )	Biomass concentration (g·L <sup>-1</sup> )	Car <sub>T</sub> (mg·g <sup>-1</sup> )	Chl <sub>T</sub> (mg·g <sup>-1</sup> )
<i>S. leibleinii</i>	0.250	5.840	0.161	0.025	0.83	0.92	7.82
<i>C. zofingiensis</i>	0.250	11.69	0.157	0.025	0.94	1.90	10.10
<i>K. Longiseta</i>	0.250	0.025	0.161	0.025	0.82	1.21	17.73
BBM standard	0.250	0.025	0.161	0.005	-	-	-

## CONCLUSIONS AND PERSPECTIVES

- Nitrogen concentration seems to have a great influence on pigment accumulation, by itself and through combined effect. However, to optimize the results simultaneously, it is suggested to keep its concentration as in unmodified BBM, almost the same for potassium phosphates.
- Increasing the ferrous sulphate concentration seems to be a good strategy to optimize growth conditions for all species, however, salinity works quite differently for each of them.
- Carotenoid accumulation is drastically reduced when biomass accumulation is enhanced, especially in *S. leibleinii* and *C. zofingiensis*.
- Analyze the composition of the biomasses in terms of carbohydrate, carotenoid, chlorophyll and lipid profiles.
- In addition to nutrients, investigate the effect of light irradiation at different wavelengths.

## ACKNOWLEDGMENTS



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