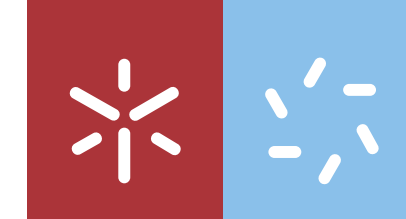


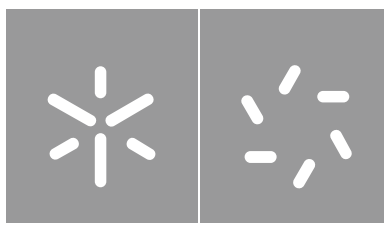


Mariana Pereira Barreiros

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electric fields on the cultivation of
microalgae**

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Escola de Ciências





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Dissertação de Mestrado

Biologia Molecular, Biotecnologia e Bioempreendedorismo em
Plantas

Trabalho efetuado sob a orientação dos

Investigador Auxiliar Ricardo Nuno Correia Pereira

Professora Auxiliar Ana Cristina Gomes Cunha

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

As microalgas, um diverso grupo de organismos presentes em ambientes marinhos e de água doce, são a fonte de uma multiplicidade de produtos de valor acrescentado, como pigmentos, antioxidantes, polissacáridos, triglicéridos, ácidos gordos, vitaminas e biomassa, que podem ser empregues comercialmente nas indústrias alimentar, nutricional, cosmética, farmacêutica e combustível. Fatores ambientais como luz, temperatura, disponibilidade de nutrientes e salinidade influenciam as microalgas, provocando alterações nos seus padrões metabólicos, composição bioquímica e produtividade em biomassa, podendo ser exploradas como estratégias de stresse para promover a acumulação de compostos de interesse.

O processamento com campos elétricos de sistemas biológicos como as microalgas tem sido alvo de exaustiva investigação. Os campos elétricos moderados (MEF), campos de baixa intensidade na gama $1-1000 \text{ V cm}^{-1}$ na forma de ondas sinusoidais/quadradas, apesar de frequentemente usados na indústria alimentar e como auxílio na extração e biotecnologia de microalgas, são ainda pouco referidos na literatura comparativamente às restantes eletrotecnologias. Estudos fundamentais sobre a sua influência no crescimento de microalgas são particularmente limitados. No presente trabalho, a influência de MEF no crescimento de *Pavlova gyrams*, uma microalga unicelular usada sobretudo na maricultura dada a sua composição bioquímica e valor nutricional, foi investigada. Usando diferentes estratégias, como a aplicação do campo em diferentes fases do crescimento, variando o tempo de aplicação e até aplicando o tratamento durante a fase escura do fotoperíodo, possíveis alterações no crescimento e composição bioquímica de *P. gyrams* como resultado do stresse causado pela aplicação do campo elétrico foram investigadas.

Apesar de não promover o crescimento, o tratamento com MEF influenciou a composição bioquímica de *P. gyrams* – aumentos máximos de 85,8 e 66,1% no conteúdo em Clorofila *a* e Carotenoides e de 4,72, 18,7 e 5,41% no conteúdo em lípidos, hidratos de carbono e proteínas resultaram de diferentes tratamentos em ensaios separados. Desta forma, o potencial da aplicação de MEF na promoção de alterações na composição bioquímica foi demonstrado.

Futuramente, para além de validar os resultados encontrados, diferentes estratégias como a combinação de estratégias elétricas com stressés conhecidos e a aplicação de eletrotecnologias na extração de compostos de interesse de *P. gyrams* poderiam ser investigadas. Mais investigação acerca da causa fundamental a nível celular das alterações encontradas é ainda necessária.

PALAVRAS-CHAVE: Campos elétricos moderados; Eletrotecnologias; Estratégias de stresse; Microalgas.

ABSTRACT

Microalgae, a largely diverse group of organisms found in marine and freshwater environments, provide a variety of added-value products, namely pigments, antioxidants, polysaccharides, triglycerides, fatty acids, vitamins and biomass, that can be harnessed for commercial use in the feed, food, nutritional, cosmetic, pharmaceutical and fuel industries. Environmental factors, such as light, temperature, nutrient availability and salinity, influence microalgae at several levels, leading to alterations in the metabolic patterns, biochemical composition and biomass productivity that can be explored as stress strategies to promote the accumulation of compounds of interest.

The influence of electric field processing on biological systems such as microalgae has been the topic of thorough investigation. Moderate electric field (MEF) application, low intensity electric fields ranging between 1-1000 V cm⁻¹ in the form of sinusoidal/square waves, although thoroughly used in the food industry and in aiding extraction and microalgae biotechnology, is still scarce in the literature when compared to other electrotechnologies, with fundamental studies on its influence on microalgae growth being particularly limited.

In the present work, the influence of MEF application on the growth of *Pavlova gyrams*, a unicellular microalgae mainly used in the feedstock market due to its biochemical composition and nutritional value, was assessed. Using different approaches, by applying the treatment in different growth stages, in varying treatment times and even during the dark phase of its photoperiod, possible alterations in *P. gyrams* growth and biochemical composition as a result of the stress caused by electric field application were investigated.

Despite not promoting growth, MEF treatment influenced the biochemical composition and pigment content of *P. gyrams* – maximum increases of 85.8 and 66.1% in the Chlorophyll *a* and in the Carotenoid content and of 4.72, 18.7 and 5.41% in the lipid, carbohydrate and protein content were found as a result of treatment in separate experiments. Hence, with this study, the potential of MEF application in the promotion of biochemical composition alterations was proven.

In the future, besides validating the results found, other approaches could be taken, for instance combining electric stress with other known stress strategies and applying electrotechnologies in the harvesting of compounds of interest from *P. gyrams*. Further investigation is also necessary in order to fully understand the fundamental causes at a cellular level of the effects found.

KEY-WORDS: Electrotechnologies; Microalgae; Moderate electric fields; Stress strategies.

TABLE OF CONTENTS

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS.....	ii
AGRADECIMENTOS.....	iii
STATEMENT OF INTEGRITY	iv
RESUMO	v
ABSTRACT	vi
LIST OF ABBREVIATIONS	x
LIST OF FIGURES	xii
LIST OF TABLES.....	xvii
LIST OF SUPPLEMENTARY MATERIAL FIGURES.....	xix
LIST OF SUPPLEMENTARY MATERIAL TABLES	xx
INTRODUCTION.....	1
1. General Introduction	1
2. Microalgae.....	2
2.1. Definition and biochemical composition	2
2.2. Microalgae culture.....	4
2.2.1. Cultivation modes and bioreactors.....	6
2.2.2. Nutritional needs	9
2.3. Microalgae species for biotechnological applications	10
2.3.1. Pavlovophyceae and the <i>Pavlova</i> genus	12
2.3.2. <i>Pavlova gyrams</i>	13
3. Culture stress strategies.....	14
3.1. Nutritional.....	15
3.1.1. Nitrogen starvation.....	15
3.1.2. Phosphorus limitation	16
3.1.3. Salinity	17
3.2. Physical	18
3.2.1. Light.....	18
3.2.2. Temperature.....	19
3.2.3. Novel strategies.....	21
4. Electric field processing	22
4.1. General overview	22
4.1.1. Electric field-based processing technologies.....	23

4.2. Current applications in microalgae biotechnology	29
5. Can MEF application lead to stress responses in microalgae?	37
MATERIALS AND METHODS	38
1. Growth conditions	38
2. Culture Conditions	38
2.1. Conductivity assessment	39
2.2. Influence of MEF application throughout growth	39
2.3. Influence of MEF application in determined time frames.....	40
2.4. Influence of MEF application during the initial growth phase for different periods of time.....	41
2.5. Influence of MEF application on the exponential growth phase for different periods of time.....	41
2.6. Influence of MEF application in the dark period as an alternative to illumination.....	41
2.7. Influence of lower MEF application on the exponential growth phase for different periods of time	42
3. Analytical Methods.....	42
3.1. Determination of the Nitrogen Consumption.....	42
3.2. Protein Quantification	43
3.3. Lipid Quantification.....	44
3.4. Pigment Quantification	44
3.5. IOMR measurement	45
3.6. Carbohydrate Quantification.....	45
3.7. Statistical analysis	46
RESULTS AND DISCUSSION	47
1. Conductivity assessment.....	47
2. Influence of MEF application throughout growth	48
3. Influence of MEF application in determined time frames.....	51
4. Influence of MEF application during the initial growth phase for different periods of time	57
5. Influence of MEF applications on the exponential growth phase for different periods of time	62
6. Influence of MEF application in the dark period as an alternative to illumination	67
7. Influence of lower MEF application on the exponential growth phase for different periods of time.....	76
8. Final remarks	81
CONCLUSIONS AND FUTURE PERSPECTIVES	86

SUPPLEMENTARY MATERIAL 88

BIBLIOGRAPHY..... 91

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AC	Alternating current
AFDW	Ash free dry weight
CaCl₂	Calcium dichloride
Chl	Chlorophyll
Chlide	Chlorophyllide
CO₂	Carbon dioxide
CO₃²⁻	Carbonate
DC	Direct current
Ddx	Diadinoxanthin
DHA	Docosahexaenoic acid
DV	Divinyl
EF	Electric field
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FP	Focused pulse
Fx	Fucoxanthin
HVED	High voltage electric discharges
KCl	Potassium chloride
LED	Light-emitting diode
MEF	Moderate electric field
MgCl₂	Magnesium chloride
MgDVP	Mg-[3,8-divinyl]-phytoporphyrin-13 ² -methylcarboxylate
MV	Monovinyl
NaCl	Sodium chloride
NH₄⁺	Ammonia
NO₃⁻	Nitrate
OD	Optical density
OH	Ohmic heating
PBR	Photobioreactors
PCA	Principal component analysis

PEF	Pulsed electric field
PO₄²⁻	Orthophosphate
POH	Pulsed ohmic heating
PUFA	Polyunsaturated fatty acid
SCP	Single-cell protein
SFA	Saturated fatty acids
sEF	Static electric field

LIST OF FIGURES

Figure 1. Microalgae cultures growth phases: (1) lag or induction phase; (2) exponential phase; (3) declining growth phase; (4) stationary phase; and (5) death phase. Source: [34].	5
Figure 2. Influence of light intensity on photoautotrophic growth of photosynthetic cells. Source: [38].	5
Figure 3. Schematic representation of nitrogen stress in microalgae cultures. Source: adapted from [4].	15
Figure 4. Variation of the conductivity (mS cm^{-1}) of the samples with (W/ Biomass) and without (W/o Biomass) biomass in a range of temperatures between 20-50 °C (n=3).	47
Figure 5. (A) Influence of the application of MEF treatment throughout growth, from day 0 to day 12, with different electric field strengths applied -0.78 V cm^{-1} (A.OH1) and 0.61 V cm^{-1} (A.OH2) – on the AFDW (g L^{-1}) of the cultures throughout time (days) alongside an untreated culture (A.Ct) (n=2). (B) Nitrate (NO_3^-) consumption (%) throughout growth. Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.	48
Figure 6. Variation of the IOMR factor of both untreated (A.Ct) and treated (A.OH1 and A.OH2) samples throughout time (days), assessed by measurement of the optical density at 254nm (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.	49
Figure 7. Variation of the lipid (A) and carbohydrate (B) content (%) of the biomass of both untreated (A.Ct) and treated (A.OH1 and A.OH2) at the end of the growth (day 12) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.	51
Figure 8. Influence of the application of different MEF treatments from day 0 to day 2 (B.OH1) and from day 4 to day 6 (B.OH2) of growth with similar electric field strengths – 0.56 V cm^{-1} (B.OH1) and 0.52 V cm^{-1} (B.OH2) – on the AFDW (g L^{-1}) of the cultures throughout time (days) in comparison with control cultures (B.Ct) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.	52
Figure 9. Variation of the IOMR factor of both untreated (B.Ct) and treated (B.OH1 – 0.56 V cm^{-1} from day 0 to day 2 and B.OH2 – 0.52 V cm^{-1} from day 4 to day 6) samples throughout time (days) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.	53

Figure 10. Variation of Chlorophyll <i>a</i> (A), Chlorophyll <i>c1+c2</i> (B) and Carotenoid (C) content (mg g ⁻¹ AFDW) throughout growth for the control and treatments B.OH1 (0.56 V cm ⁻¹ from day 0 to day 2) and B.OH2 (0.52 V cm ⁻¹ from day 4 to day 6) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average.	54
Figure 11. Variation of pigment (Chlorophyll <i>a</i> , Chlorophyll <i>c1+c2</i> and Carotenoids) content (mg g ⁻¹ AFDW) at the beginning (day 0) and at the end of the growth (day 10) for the control (B.Ct) and treatments B.OH1 (MEF application from day 0 to day 2) and B.OH2 (from day 4 to day 6) of growth with similar electric field strengths – 0.56 V cm ⁻¹ (B.OH1) and 0.52 V cm ⁻¹ (B.OH2) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average.	56
Figure 12. Variation of the lipid (A), carbohydrate (B) and protein (C) content (%) of the biomass of both untreated (B.Ct) and treated B.OH1 (0.56 V cm ⁻¹ from day 0 to day 2) and B.OH2 (0.52 V cm ⁻¹ from day 4 to day 6) at the end of the growth (day 10) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.	57
Figure 13. (A) Influence of the application of MEF treatment applied on day 0 during different periods of time – 6 h (C.OH1), 18 h (C.OH2) and 30 h (C.OH3) – and under the same electric field strength (0.15 V cm ⁻¹) on the AFDW (g L ⁻¹) of the cultures throughout time (days) in comparison with untreated control cultures (C.Ct) (n=2). (B) Nitrate (NO ₃) consumption (%) throughout growth. Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.	58
Figure 14. Variation of the IOMR factor of both untreated (C.Ct) and treated in the initial growth phase (C.OH1 – 6 h, C.OH2 – 18 h and C.OH3 – 30 h) samples throughout time (days) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.	59
Figure 15. Variation of Chlorophyll <i>a</i> (A), Chlorophyll <i>c1+c2</i> (B) and Carotenoid (C) content (mg g ⁻¹ AFDW) throughout growth for the control and treatments C.OH1 (6 h), C.OH2 (18 h) and C.OH3 (30 h) applied during the initial growth phase (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average. <i>Note:</i> samples corresponding to day 4 of growth were lost during extraction, therefore being absent from the graph presented.	60
Figure 16. Variation of pigment (Chlorophyll <i>a</i> , Chlorophyll <i>c1+c2</i> and Carotenoids) content (mg g ⁻¹ AFDW) at the beginning (day 0) and at the end of the growth (day 10) for the control (C.Ct) and treatments C.OH1 (6 h), C.OH2 (18 h) and C.OH3 (30 h) applied during the initial growth phase	

– and exposed to the same electric field strength (0.25 V cm^{-1}) ($n=2$). Error bars represent a variation coefficient resultant of the difference between sample and average..... 61

Figure 17. Variation of the lipid (A), carbohydrate (B) and protein (C) content (%) of the biomass of both untreated (C.Ct) and treated during the initial growth phase (C.OH1 – 6 h, C.OH2 – 18 h and C.OH3 – 30 h) at the end of the growth (day 10) ($n=2$). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates. 62

Figure 18. (A) Influence of the application of the electric field during the exponential growth phase from day 4 to day 6 in different periods of time – 6 h (D.OH1), 18 h (D.OH2) and 30 h (D.OH3) – on the AFDW (g L^{-1}) throughout time (days), as well as for untreated control cultures (D.Ct) ($n=2$). (B) Nitrate (NO_3^-) consumption (%) throughout growth. Error bars represent a variation coefficient resultant of the difference between sample and average. 63

Figure 19. Variation of the IOMR factor of both untreated (D.Ct) and treated during the exponential growth phase (D.OH1 – 6 h, D.OH2 – 18 h and D.OH3 – 30 h) samples throughout time (days) ($n=2$). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates. 64

Figure 20. Variation of Chlorophyll *a* (A), Chlorophyll *c1+c2* (B) and Carotenoid (C) content (mg g^{-1} AFDW) throughout growth for the control (D.Ct) and treatments D.OH1 (6 h), D.OH2 (18 h) and D.OH3 (30 h) ($n=2$) applied during the exponential growth phase. Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates ($n=2$). 65

Figure 21. Variation of pigment (Chlorophyll *a*, Chlorophyll *c1+c2* and Carotenoids) content (mg g^{-1} AFDW) at the beginning (day 0) and at the end of the growth (day 10) for the control (D.Ct), and treatments D.OH1 (6 h), D.OH2 (18 h) and D.OH3 (30 h) applied during the exponential growth phase. Error bars represent a variation coefficient resultant of the difference between sample and average. 66

Figure 22. Variation of the lipid (A), carbohydrate (B) and protein (C) content (%) of the biomass of both cultures untreated (D.Ct) and treated during the exponential growth phase – D.OH1 (6 h), D.OH2 (18 h) and D.OH3 (30 h) at the end of the growth (day 10) ($n=2$). Error bars represent a variation coefficient resultant of the difference between sample and average..... 67

Figure 23. (A) Influence of the application of MEF treatment during night time for 8 h, in alternation with culture illumination during the remaining 16 h, for different periods of time starting on day 0 of the growth experiment – 16 h (E.OH1), 32 h (E.OH2) and 48 h (E.OH3) – on the AFDW (g L^{-1}) throughout time (days), as well as for untreated control cultures (E.Ct) ($n=2$). (B) Nitrate (NO_3^-)

consumption (%) throughout growth. Error bars represent a variation coefficient resultant of the difference between sample and average.....	68
Figure 24. Variation throughout time (days) of the IOMR factor of both untreated cultures (E.Ct) and cultures treated during night time for 8 h, in alternation with culture illumination during the remaining 16 h of the day, for different periods of time (E.OH1 – 16 h, E.OH2 – 32 h and E.OH3 – 48 h) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.	70
Figure 25. Variation of Chlorophyll <i>a</i> (A), Chlorophyll <i>c1+c2</i> (B) and Carotenoid (C) content (mg g ⁻¹ AFDW) throughout growth for the control (D.Ct) and treatments E.OH1 (16 h), E.OH2 (32 h) and E.OH3 (48 h) (n=2), applied during night time in alternation with illumination during day time starting on day 0. Error bars represent a variation coefficient resultant of the difference between sample and average (n=2).	71
Figure 26. Variation of pigment (Chlorophyll <i>a</i> , Chlorophyll <i>c1+c2</i> and Carotenoids) content (mg g ⁻¹ AFDW) at the beginning (day 0) and at the end of the growth (day 14) for the control (E.Ct), and treatments E.OH1 (16 h), E.OH2 (32 h) and E.OH3 (48 h), applied at the beginning of the growth during 8 h without illumination (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average.....	72
Figure 27. Variation of the lipid (A), carbohydrate (B) and protein (C) content (%) of the biomass of both cultures untreated (E.Ct) and treated for 6 h during the dark phase throughout different periods of time starting on day 0 – E.OH1 (16 h), E.OH2 (32 h) and E.OH3 (48 h) – at the end of the growth (day 14) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average.....	74
Figure 28. (A) Influence of the application of MEF treatment during the exponential growth phase (day 6) of different duration – 6 h (F.OH1) and 30 h (F.OH2)– on the AFDW (g L ⁻¹) throughout time (days), alongside untreated control cultures (F.Ct) (n=3). (B) Nitrate (NO ₃ ⁻) consumption (%) throughout growth. Error bars represent the standard deviation of the data.....	76
Figure 29. Variation of the IOMR factor of both untreated cultures (F.Ct) and cultures treated during the exponential growth phase (day 6) of different duration – 6 h (F.OH1) and 30 h (F.OH2) – throughout time (days) (n=3). Error bars represent the standard deviation of the data.	77
Figure 30. Variation of Chlorophyll <i>a</i> (A), Chlorophyll <i>c1+c2</i> (B) and Carotenoid (C) content (mg g ⁻¹ AFDW) throughout growth of control cultures (F.Ct) and cultures treated during the exponential	

growth phase (day 6) for different periods of time – F.OH1 (6 h) and F.OH2 (30 h) (n=3). Error bars represent the standard deviation of the data. 78

Figure 31. Variation of the lipid (A), carbohydrate (B) and protein (C) content (%) of the biomass of both cultures untreated (E.Ct) and treated for 6 h during the dark phase throughout different periods of time starting on day 0 – F.OH1 (6 h) and F.OH2 (30 h)– at the end of the growth (day 14) (n=2). Error bars represent the standard deviation of the data. Statistical analysis performed using the D’Agostino & Pearson test, followed by the Kruskal-Wallis comparison test ($p < 0.05$) on Graphpad Prism 8; * stands for significantly different in comparison with the control; “ns” stands for non-significantly different in comparison with the control. 79

Figure 32. PCA plot in terms of the AFDW (g L^{-1}), pH, lipid, carbohydrate and protein content (%), Chlorophyll a, Chlorophyll c1+c2 and Carotenoid content (mg g^{-1} AFDW) and organic matter content in the supernatant of control (○, unfilled, circumference in the corresponding colour) and treated (●, ▲, ■, filled with the corresponding colour) cultures from experiments B (black icons, numbers 2-9), C (blue icons, numbers 10-17), D (green icons, 18-25), E (red icons, numbers 26-33) and F (orange icons, numbers 34-42). Analysis performed using Origin..... 84

LIST OF TABLES

Table 1. General composition of different algae (% of dry matter). <i>Source:</i> Adapted from [20].	3
Table 2. Characteristics of different types of microalgae cultivation. <i>Source:</i> Adapted from [45]..	7
Table 3. Advantages and limitations of open ponds and photobioreactors. <i>Source:</i> [16].	8
Table 4. Feasible downstream applications of various microalgae species. <i>Source:</i> Adapted from [59].	10
Table 5. Comparison between the advantages and disadvantages of ohmic heating. <i>Source:</i> [178].	22
Table 6. Novel and emergent electrotechnologies – operational characteristics and main applications in previous studies. <i>Source:</i> Adapted from [14].	28
Table 7. Electrotechnologies applied to different microalgae strains and main results achieved.	34
Table 8. Electric field parameters and treatment conditions applied to every growth experiment developed on the current work and corresponding identification code (ID code).	40
Table 9. Influence of MEF application on the growth parameters calculated for both control (A.Ct) and treated cultures (A.OH1 and A.OH2) – specific growth rate ($\mu_{\max}$, day ⁻¹) calculated for the exponential growth phase; process productivity (P_x , g.L ⁻¹ .day ⁻¹) calculated from day 0 until the first day of the stationary phase; maximum biomass achieved throughout growth ($X_{\max}$, g.L ⁻¹) (n=2; average value $\pm$ variation coefficient resulting of the difference between duplicate and average).	49
Table 10. Influence of MEF application on the growth parameters calculated for both control B.Ct and treated cultures (B.OH1 and B.OH2) – specific growth rate ($\mu_{\max}$, day ⁻¹) calculated for the exponential growth phase; process productivity (P_x , g L ⁻¹ day ⁻¹) calculated from day 0 until the first day of the stationary phase; maximum biomass achieved throughout growth ($X_{\max}$, g L ⁻¹) (n=2; average value $\pm$ variation coefficient resulting of the difference between duplicate and average).	52
Table 11. Influence of MEF application on the growth parameters calculated for both control C.Ct and treated cultures (C.OH1 and C.OH2) – specific growth rate ($\mu_{\max}$, day ⁻¹) calculated for the exponential growth phase; process productivity (P_x , g L ⁻¹ day ⁻¹) calculated from day 0 until the first day of the stationary phase; maximum biomass achieved throughout growth ($X_{\max}$, g L ⁻¹) (n=2; average value $\pm$ variation coefficient resulting of the difference between duplicate and average).	59

Table 12. Influence of MEF application on the growth parameters calculated for both control D.Ct and treated cultures (D.OH1 and D.OH2) – specific growth rate ($\mu_{\max}$, day ⁻¹) calculated for the exponential growth phase; process productivity (P_x , g L ⁻¹ day ⁻¹) calculated from day 0 until the first day of the stationary phase; maximum biomass achieved throughout growth ($X_{\max}$, g L ⁻¹) (n=2; average value $\pm$ variation coefficient resulting of the difference between duplicate and average).	64
Table 13. Influence of MEF application on the growth parameters calculated for both control E.Ct and treated cultures (E.OH1 and E.OH2) – specific growth rate ($\mu_{\max}$, day ⁻¹) calculated for the exponential growth phase; process productivity (P_x , g L ⁻¹ day ⁻¹) calculated from day 0 until the first day of the stationary phase; maximum biomass achieved throughout growth ($X_{\max}$, g L ⁻¹) (n=2; average value $\pm$ variation coefficient resulting of the difference between duplicate and average).	69
Table 14. Influence of MEF application on the growth parameters calculated for both control F.Ct and treated cultures (F.OH1 and F.OH2) – specific growth rate ($\mu_{\max}$, day ⁻¹) calculated for the exponential growth phase; process productivity (P_x , g L ⁻¹ day ⁻¹) calculated from day 0 until the first day of the stationary phase; maximum biomass achieved throughout growth ($X_{\max}$, g L ⁻¹) (n=3; average value $\pm$ standard deviation of the data). Statistical analysis performed using the D’Agostino & Pearson test, followed by the Kruskal-Wallis comparison test ($p < 0.05$) on Graphpad Prism 8; (*) stands for significantly different in comparison with the control; (ns) stands for non-significantly different in comparison with the control.	77

LIST OF SUPPLEMENTARY MATERIAL FIGURES

- Figure SM1.** Conversion factor calculated from the OD reading at 750 nm to the AFDW content (g L^{-1}) ($n=3$) – the resulting equation was used to infer on the AFDW content present in samples recovered from the experiments that followed. Error bars correspond to the standard deviation of the replicates..... 88
- Figure SM2.** Calibration curve for the determination of nitrate (NO_3^-) concentration. Standards were prepared ranging in the NO_3^- concentration range 0-0.5 mM and the resulting equation was used to infer on the concentration present on the tested samples ($n=3$). Error bars correspond to the standard deviation of the replicates. 88
- Figure SM3.** Calibration curve for the determination of protein content present in the supernatant. Standards were prepared using BSA ranging in concentration from 0 to 400 mg L^{-1} and the resulting equation was used to infer on the concentration present on the samples ($n=3$). Error bars correspond to the standard deviation of the replicates. 88
- Figure SM4.** Calibration curve for the determination of total protein content. Standards were prepared using BSA in concentrations in the range 0-0.5 mg mL^{-1} and the resulting equation was used to infer on the concentration present on the samples ($n=3$). Error bars correspond to the standard deviation of the replicates. 89
- Figure SM5.** Calibration curve for the determination of carbohydrate content. Standards were prepared using D-Glucose in the concentration range 0-500 mg L^{-1} and the resulting equation was used to infer on the concentration present on the samples ($n=3$). Error bars correspond to the standard deviation of the replicates. 89
- Figure SM6.** Differences in colour found on the 8th day of growth of the experiment where MEF treatment was applied on day 0 for different periods of time – 6 h (C.OH1), 18 h (C.OH2) and 30 h (C.OH3) – using the same electric field strength (0.15 V cm^{-1}). 89

LIST OF SUPPLEMENTARY MATERIAL TABLES

Table SM 1. Comparison between the growth parameters (specific growth rate - $\mu_{\max}$, day⁻¹; process productivity - P_x , g L⁻¹ day⁻¹; maximum biomass - $X_{\max}$, g L⁻¹), the biochemical characterization (lipid, carbohydrate and protein content) and pigment content (Chlorophyll *a*, Chlorophyll *c1+c2* and Carotenoids) at the end of the growth for each of the experiments previously addressed with the corresponding identification codes (ID code) (- stands for not analysed) ($\pm$ standard deviation of the data or variation coefficient resulting of the difference between duplicate and average). When possible, statistical analysis was performed using the D'Agostino & Pearson test, followed by the Kruskal-Wallis comparison test ($p < 0.05$) on Graphpad Prism 8; * stands for significantly different in comparison with the control; "ns" stands for non-significantly different in comparison with the control..... 90

INTRODUCTION

1. General Introduction

Microalgae are present in all existing earth ecosystems, not only aquatic but also terrestrial, amounting to a big variety of species living in a vast range of environmental conditions [1]. The vast range of resources provided by them are, nowadays, the basis of several economic activities and will offer a wide array of applications for biotechnology in the nearby future [2]. Microalgae, an essential element of marine ecosystems, are a diverse group of microscopic prokaryotic and eukaryotic photosynthetic organisms with indispensable ecological importance. Microalgae are, indeed, some of the most efficient converters of solar energy to valuable products, at the cost of natural resources [3], and are a rich source of high valuable compounds, presenting high levels of protein, carbohydrates, lipids and a diversity of pigments [4] that can be exploited for commercial use [5][6], namely in the feed, food, nutritional, cosmetic, pharmaceutical and even fuel industries [7][8].

The modern food industry has contributed to an increase in less expensive, healthier and more convenient products [9], due to the growing search of the consumer market for healthier products [10]. Microalgae can enhance the nutritional value of food and animal feed since they present a well-balanced biochemical composition, which, in turn, makes them an innovative approach for the manufacture of healthier food products [9]. Despite the efforts in linking microalgae to “healthy” food, its high production costs and the strict food safety regulations have become restricting [11], so the use of microalgae in mariculture, as food and feed additives for freshwater and marine aquatic animals, has been far more successful than its introduction to human nutrition [12].

Environmental factors, such as light, temperature, nutrient availability and salinity, impact microalgae at several levels, causing alterations even in their metabolic patterns, biochemical composition and biomass productivity [1]. These alterations can be particularly interesting, allowing the exploitation of these stress conditions as a way of obtaining highly valuable products [13].

The influence of electric field processing on biological systems, meaning techniques in which an external electric field is applied with a certain technological aim (i.e., inactivation, extraction or separation purposes), has been the topic of thorough investigation. When it comes to microalgae, techniques that encompass an efficient upstream and downstream processing have established a foothold [11]. However, for techniques as such to become successful in the areas in which they are applied, there is still a need for more fundamental research in order to fully understand its

effects and how to control them and further studies on the costs and commercial applications are still a necessity [14][15].

2. Microalgae

2.1. Definition and biochemical composition

Microalgae, ranging in size from a few micrometres to a few hundreds of micrometres, are a largely diverse group of microorganisms, comprising eukaryotic photoautotrophic protists and prokaryotic cyanobacteria. They are considered primitive plants (thallophytes), in the sense that they lack roots, stems and cells, their primary photosynthetic pigment is Chlorophyll *a* and they lack a sterile covering around the reproductive cells [16]. Microalgae may present different types of cell organization, namely unicellular, colonial and filamentous [1].

In microalgae, properties which are typical of higher plants, such as efficient oxygenic photosynthesis and simple nutritional requirements, are combined with biotechnological attributes characteristic of microbial cells, as fast growth in liquid culture and the ability to accumulate or secrete metabolites [17]. The combination of these features supports the use of microalgae in applied processes and is in the basis of microalgal biotechnology. Although the photosynthetic mechanism of microalgae is similar to that of higher plants, they usually are more efficient in the conversion of solar energy due to their simple cellular structure [11]. Besides that, since cells usually grow in aqueous suspension, their access to water, CO₂ and other nutrients is more efficient [18]. Unlike higher plants, nutrient transport in microalgae does not require a vascular system, since every single cell is photoautotrophic, absorbing nutrients directly [19].

Microalgae's diversity is also reflected in its biochemical composition, including the production of a vast array of carbohydrates, lipids, proteins, which constitute the main microalgae components [11] exploited for commercial use [5][6], namely in the feed, food, nutritional, cosmetic, pharmaceutical and even fuel industries [7]. A general overview on the main constituents of various microalgae species is compiled in **Table 1**. In addition to the diversity of biomolecules, the manipulation of their biochemical composition through the control of their growth conditions is possible due to microalgae's metabolic plasticity [11].

Table 1. General composition of different algae (% of dry matter). *Source:* Adapted from [20].

Algae	Protein	Carbohydrates	Lipids	Reference
<i>Anabaena cylindrica</i>	43-56	25-30	4-7	[20]
<i>Aphanizomenon flos-aquae</i>	62	23	3	[20]
<i>Arthrospira maxima</i>	60-71	13-16	6-7	[20]
	65	20	2	[21]
<i>Chlamydomonas reinhardtii</i>	48	17	21	[20]
<i>Chlorella pyrenoidosa</i>	57	26	2	[20]
<i>Chlorella vulgaris</i>	51-58	12-17	12-22	[20]
	47.8	8.1	13.3	[22]
<i>Dunaliella salina</i>	29.3	16.3	25.3	[23]
	25.7		18.8	[24]
<i>Euglena gracilis</i>	39-61	14-18	14-20	[20]
<i>Pavlova salina</i>	24.2	6.9	11.2	[25]
	29	7.4	12	[26]
<i>Pavlova lutheri</i>	29.7	9.1	12.3	[25]
	29	9	12	[26]
<i>Pavlova pinguis</i>	33	41	19	[27]
<i>Porphyridium cruentum</i>	28-39	40-57	9-14	[20]
	34.1	32.1	6.5	[28]
<i>Scenedesmus obliquus</i>	50-56	10-17	12-14	[20]
	52	12.5	9	[29]
<i>Spirogyra</i> sp.	6-2p	33-64	11-21	[30]
<i>Spirulina platensis</i>	61.3	15.1-15.8	7.1-8.0	[22]
	64.4	24.9	7.0	[31]
	58.2	8-14	4-9	[32]
<i>Synechococcus</i> sp.	63	15	11	[30]

In most species, starch or starch like compounds are the typical carbohydrate storage product. Many microalgae synthesise lipids as storage products, found as oil droplets in cells mostly in the form of polyunsaturated fatty acids (PUFA), such as arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). In microalgae's cellular membranes fatty acids and sterols can also be found, which are more difficult to extract than the storage lipids [11]. The average lipid content of microalgae cells varies according to species and strain and ranges between 1-40% and, under certain conditions, it can be as high as 85% of the dry weight [20].

When it comes to microalgal proteins, they are extremely diverse and are present in high concentrations in both prokaryotic, amounting to 40-60% of *Spirulina* dry weight for example, and eukaryotic cells, reaching 30-60% of *Porphyridium* dry weight, which, in turn, makes microalgae a viable alternative protein source. Contrary to carbohydrates and lipids that reach their maximum concentration in the stationary growth phase, cells become protein-rich usually when they reach the exponential growth phase [11].

The genetic and phenotypic diversity of microalgae is mirrored in its nearly ubiquitous distribution through the biosphere. They can be found in all existing Earth ecosystems, from deserts to polar seas, enduring a wide range of environmental conditions due to the different physiological systems they have developed, allowing them to adapt to several, some even extreme, conditions [11]. They thrive in varied aquatic habitats, including freshwater, brackish (<3.5% salt), marine

(3.5% salt), and hypersaline (>3.5% salt) environments with a vast range of temperatures and pH [33]. As an example, whereas green microalgae grow usually in fresh and seawater, other species grow in extremely saline conditions [34].

Disagreements prevail when it comes to the taxonomic classification of microalgae. Systems of microalgae classification are based on their pigment composition, chemical nature of storage products, diversity of ultrastructural features and phylogenetic relationships [34]. Other criteria take into consideration cytological and morphological features, namely the occurrence of flagellate cells, structure of the flagella, nuclear and cell division characteristics, presence of an envelope of endoplasmic reticulum around the chloroplasts and a possible relation between the endoplasmic reticulum and the nuclear membrane [35]. The most abundant classes of microalgae are the diatoms (Bacillariophyceae), the green algae (Chlorophyceae), and the golden algae (Chrysophyceae) [34].

It is estimated that more than 50,000 species exist and around 30,000 of them have been thoroughly studied, analysed [1] and kept in collections around the world [12]. In spite of these numbers, only a limited number of species is cultivated in industrial quantities for commercial purposes [11]. The production of microalgae is expensive; however, efforts are being made towards the achievement of cost-efficient methods for mass cultivation of these organisms [17].

2.2. Microalgae culture

Microalgae growth can be divided into five different phases (**Figure 1**): the lag or induction phase (1), the exponential phase (2), the declining growth phase (3), the stationary phase (4), and the death phase (5) [1]. The lag phase is a period in which there is a physiological adaptation of the cell metabolism to the new nutrient and culture conditions. During this phase there might be a slight increase in cell density. This phase is followed by the exponential phase, in which microalgae grow as a function of time, following an exponential curve, as long as mineral substrates and light energy are saturated. In order to reduce the initial time of adaptation, the cultures can be inoculated with algae already in the exponential phase. The declining growth phase corresponds to a reduction in the cell division rate that might be due to physical or chemical limiting factors, namely nutrients, light, pH, carbon dioxide or other. During this phase, sustainable growth isn't favoured by the culture conditions, resulting in a decrease in cell density [34].

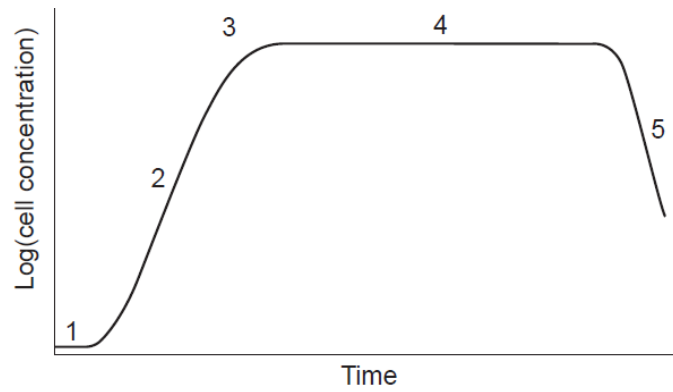


Figure 1. Microalgae cultures growth phases: (1) lag or induction phase; (2) exponential phase; (3) declining growth phase; (4) stationary phase; and (5) death phase. Source: [34].

Growth characteristics and biochemical composition of microalgae are known to be highly influenced by the cultivation conditions [4]. In fact, the cultivation of microalgae is dependent on several factors, such as light quality and quantity, nutrient supply, O_2 , CO_2 , pH, temperature and salinity [12].

Microalgae growth is affected by the type of light source, mainly because of the different coverage in wavelength range [36], and also by light intensity [37]. The influence of light intensity on the photoautotrophic growth of microalgae can be divided into distinctive phases, such as light limitation, light saturation and light inhibition, represented below in **Figure 2**.

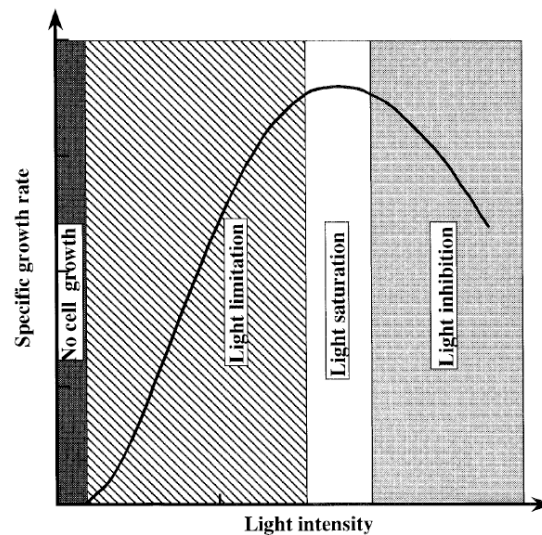


Figure 2. Influence of light intensity on photoautotrophic growth of photosynthetic cells. Source: [38].

When light intensity is below the critical intensity, as with complete darkness, cessation of cell growth, decrease in cell concentration [39] and loss of biomass [40] occur; however, at very high light intensities photoinhibition can occur [41][42]. In this way, productivity is maximum when the when light intensity saturates the photosynthetic machinery [38]. Natural light is typically used for

mass cultivation, with microalgae reaching 3-11% of light conversion efficiency; however, it is difficult to control [33]. In microalgae, as with plants, CO₂ is involved in photosynthesis and it's one of its limiting factors. A certain concentration of CO₂ is required for photosynthesis and maximum photosynthetic efficiency is usually attained when it varies between 1-5% [33].

pH is also an important environmental parameter and its optimal value for most microalgae species ranges between 7 and 9 [43]. The culture medium pH highly affects the growth of microalgae, so its maintenance in the optimal range is crucial – acidic pH values influence the cellular nutrient uptake, the production of intracellular metabolites and can lead to metal toxicity, whilst alkaline pH values decrease the affinity for CO₂ and affects cell division [44]. The feeding of CO₂ to the cultures affects its pH value [43].

Temperature is another relevant factor – microalgae are capable of tolerating temperatures up to 15 °C lower than the optimal, whilst with others changes of about 2-4 °C to the optimum temperature might lead to the loss of the culture [12]. For outdoor cultivation, temperature variations are highly dependent on the light exposure (day/night) and on seasonal changes. Adequate cultivation temperatures can promote microalgae growth, however high temperatures can lead to decreases in biomass production, mainly due to denaturation of essential proteins/enzymes as well as inhibitory effects on the cellular physiology [43].

Salinity is another important factor to consider – every microalgae has an optimum salinity range. Microalgae, bacteria and cyanobacteria are usually able to tolerate up to 1.7 M of salt concentration in marine medium [43]. Changes in salinity can lead to osmotic stress, ion (salt) stress, changes in the cellular ionic ratios due to the membrane selective ion permeability [12], production of certain metabolites to protect from salt injury and to balance the osmotic stress or to the production of specific components [43].

All these factors will influence microalgae growth and their impact can be used as the basis for different cultivation strategies, that will be discussed in a following chapter.

2.2.1. Cultivation modes and bioreactors

Microalgae cultivation conditions can be divided into four major types, namely photoautotrophic, heterotrophic, photoheterotrophic and mixotrophic [45], and shifts in metabolism in response to alterations in the environmental conditions are possible [12]. However, photoautotrophy is considered the most important and commonly used in microalgae cultivation

[11]. The following table (Table 2) presents the main differences between the different types in terms of energy and carbon source.

Table 2. Characteristics of different types of microalgae cultivation. *Source:* Adapted from [45].

Culture condition	Energy Source	Carbon Source
Photoautotrophy	Light	Inorganic carbon
Heterotrophy	Organic carbon	Organic carbon
Photoheterotrophy	Light	Organic Carbon
Mixotrophy	Light and organic carbon	Organic and Inorganic carbon

In photoautotrophic microalgae, photosynthesis is fundamental for survival [11][16]. Oxygenic photosynthesis can be divided into light and dark reactions – during the light reactions, that take place in photosynthetic membranes, light energy is transformed into chemical energy, generating NADPH₂ and adenosine triphosphate (ATP); during the dark reactions, which occur in the stroma, the NADPH₂ and ATP generated are involved in the consecutive biochemical reduction of carbon dioxide (CO₂) into carbohydrates [1]. Under these culture conditions, microalgae are usually grown in one of two systems, either open ponds or enclosed photobioreactors, being the latter more suitable for microalgae easily contaminated by other microorganisms, with the exception of some which are able to survive in extreme environments, such as high pH (e.g., *Spirulina*) and high salinity (e.g., *Dunaliella* spp.) or can grow very rapidly (e.g., *Chlorella* spp.) in open pond cultivation systems [46]. In spite of the light usage being quite efficient, microalgae phototrophic growth can be limited by light when high cell densities are reached on a large scale [47] - heterotrophic growth can be an alternative. In heterotrophic growth, microalgae use organic compounds produced by other organisms to satisfy their material and energetic needs [11], eliminating the requirement for light [48]. Microalgae are considered photoheterotrophs if they require light when using organic compounds as the carbon source [4], or chemoheterotrophs if, instead of light, they oxidize organic compounds for energy [11]. Certain microalgae are considered mixotrophic or amphitrophic, since they combine heterotrophy and autotrophy and use both organic and inorganic compounds (CO₂) for their growth. In this case, both autotrophy and heterotrophy are present simultaneously, except when in total darkness where heterotrophy dominates [11]. When compared to photoautotrophy, cells grown under these regimes are known to grow faster, resulting in higher productivities [49][50], which makes mixotrophy growth suitable for the production of compounds which are characteristic of both photosynthetic and heterotrophic metabolisms at high production rates and less dependent of light limitation [49]. However, as with heterotrophic cultivations, mixotrophic cultivations are subjected to a higher contamination risk due to the presence of organic carbon sources, becoming unfeasible in open-air reactors [11].

Bioreactors used for microalgae growth are often different in design from bioreactors used for other microorganisms, since most microalgae depend on light as an energy source [51]. In this way, in addition to the factors relevant for microalgae growth, agitation or mixing also influence biomass production [43]. Continuous mixing of a microalgae suspensions is quite important since it homogenises cell distribution, promotes light usage, facilitates the transfer of gases, eliminates thermal stratification and facilitates the even distribution of nutrients [12][52]. However, on the other hand, high liquid velocities and degrees of turbulence resultant of mechanical mixing or air bubble mixing might lead to damages in microalgae caused by shear stress [51]. In this way, microalgae cultivation systems can be classified as open systems or as controlled closed systems called photobioreactors (PBR) [53]. Open systems can be of either natural waters or artificial ponds or containers, being raceway ponds one of the most common [52], which usually consist of closed loop, oval shaped recirculation channels with mixing and circulation, required to stabilize algae growth and productivity [16]. PBR consist of an arrangement of straight glass, acrylic or plastic tubes which allow the capture of sunlight and can be aligned in different configurations [54]. There is no direct exchange of gases and contaminants between the cultivation system and the external environment, being the necessary gas exchange performed through a sterilized gas filter, avoiding contamination [43]. According to reactor geometry, closed systems can be classified into tubular, flat plate, and column PBR [55]. A comparison between advantages and limitations of open ponds and PBR is presented below in **Table 3**.

Table 3. Advantages and limitations of open ponds and photobioreactors. *Source:* [16].

Production system	Advantages	Limitations
Raceway pond	Relatively cheap Easy to clean Utilises non-agricultural land Low energy inputs Easy maintenance	Poor biomass productivity Large area of land required Limited to a few strains of algae Poor mixing, light and CO ₂ utilisation Cultures are easily contaminated
Tubular photobioreactor	Large illumination surface area Suitable for outdoor cultures Relatively cheap Good biomass productivities	Some degree of wall growth Fouling Requires large land space Gradients of pH, dissolved oxygen and CO ₂ along the tubes
Flat plate photobioreactor	High biomass productivities Easy to sterilise Low oxygen build-up Readily tempered Good light path Large illumination surface area Suitable for outdoor cultures	Difficult scale-up Difficult temperature control Small degree of hydrodynamic stress Some degree of wall growth
Column photobioreactor	Compact High mass transfer Low energy consumption Good mixing with low shear stress Easy to sterilise Reduced photoinhibition and photo-oxidation	Small illumination area Expensive compared to open ponds Shear stress Sophisticated construction

2.2.2. Nutritional needs

As with other microorganisms, microalgae require certain nutrients for their growth and metabolic activities [34]. Nutrients such as carbon (C), oxygen (O), hydrogen (H), nitrogen (N), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), sulphur (S), phosphorus (P) [33], and trace elements such as copper (Cu), manganese (Mn), selenium (Se), or zinc (Zn) need to be provided [11]. When in autotrophic growth, C, O and H are typically obtained from water and air, whilst N, P and K have to be absorbed from the culture medium [33] and their uptake is dependent on factors such as species, nutrient ratios, temperatures or pH. H and O are abundant in the medium and are never limiting to microalgae growth; C, however, is one of the major nutrients that should be supplied to cultures [34].

The C source is typically the most critical factor [43], since it is essential for algal growth and reproduction, with C representing around 50% of the cell dry weight [56]. Depending on the chosen trophic route, the culture will be supplied with either inorganic carbon, organic carbon or both, being glucose, acetic acid, and fructose some of the most commonly used C sources [11]. Since atmospheric carbon dioxide (CO_2) is not enough to satisfy the carbon necessities of autotrophic cultivation, it is quite important to supply the cultures with CO_2 and HCO_3^- . During microalgae cultivation, pH values as high as 11 are often reached due to the gradual accumulation of OH^- during active photosynthesis, leading to the necessary control of pH through the direct addition of CO_2 to the medium [1].

N is another essential element for growth, development, reproduction and other physiological activities [33], namely the production of proteins, nucleic acids [43] and chlorophyll molecules [57], accounting for 1-10% of the biomass [1]. It is mostly supplied as nitrate (NO_3^-), but ammonia (NH_4^+) or urea are frequently used as well. They are assimilated from the medium and then recycled in order to meet their ever-changing physiological needs [34]. N source and concentration are factors which influence cell composition [33] – in this way, to ensure that it doesn't become a rate-limiting factor, mediums are often formulated with an excessive amount of nutrients [1]. When convenient, altering the N concentration can also act as a strategy, leading to changes in the accumulation of PUFA, polysaccharides and pigments [11].

P is also essential for growth and for several cellular processes, namely energy transfer and biosynthesis of nucleic acids. It is preferentially supplied in the form of orthophosphate (PO_4^{3-}) and, even though algal biomass contains less than 1% P, frequently it is a growth limiting factor since it easily bounds to other ions (e.g., CO_3^{2-} and iron), leading to precipitation and becoming

unavailable for uptake [1] – this problem is usually overcome by the addition of metal chelators [11]. Microalgae are also capable of storing excess P in polyphosphate bodies, which may be used when the supply is limited. Similarly to N, P also has an influence in the composition of the produced biomass, affecting mainly the lipid content and carbohydrates [1].

2.3. Microalgae species for biotechnological applications

In the 1950s, algae were cultivated as a source of single-cell protein (SCP) but, since then, several other potential applications have arisen based on exploiting its complex biochemical pathways for the production of a large range of metabolites [58]. This class of organisms has qualities that contribute to its use for commercial and technological benefits – they are genetically diverse, comprising a wide range of physiological and biochemical characteristics; they naturally produce unusual fats, sugars and bioactive compounds; they can integrate stable isotopes into their biomass in a cost-effective way and consequently into the compounds they produce [6]. Industrially speaking, microalgae cultivation is advantageous since it is relatively easy [12] and can lead to higher growth rates and productivities when compared to forestry, agricultural crops and other aquatic plants [18], reducing the competition for arable soil with other crops [12].

Table 4. Feasible downstream applications of various microalgae species. *Source:* Adapted from [59].

Microalgae Specie	Application	Reference
<i>Chlorella</i> sp.	Health-promoting molecules	[60]
	Food additives	[61]
	Animal nutrition	[62]
	Cosmetics	[63] [64]
	Biofuels	[65]
<i>Dunaliella salina</i>	β -Carotene	[66]
	Food supplements	[62]
	Cosmetics	[62] [64]
<i>Spirulina platensis</i>	Pharmaceuticals	[67]
	Phycobiliproteins	[62]
	Human nutrition	[68]
	Cosmetics	[64]
<i>Haematococcus pluvialis</i>	Astaxanthin	[62]
	Food additives	[62]
	Pharmaceuticals	[62]
<i>Arthrospira</i>	Carotene	[62]
	Cosmetics	[69]
	Phycobiliproteins	[69]
<i>Nannochloropsis</i>	Eicosapentaenoic acid (EPA)	[70]
	Biodiesel	[18]
<i>Pavlova lutheri</i>	Aquaculture feed	[71] [72] [73] [74] [75]
	Lipids	[76]
	Potentially biodiesel	[77]
<i>Pavlova gyrams</i>	Cosmetics	[6]
	Aquaculture	[78]

Commercial microalgae growth is mainly driven by the harvesting of added-value products, namely pigments, antioxidants, polysaccharides, triglycerides, fatty acids, vitamins and biomass,

that can be used in the pharmaceutical, cosmetic, nutraceutical, food and fuel industries. They also can be useful in environmental strategies, namely in wastewater treatment and fertilization of soils, production of biofuels and phytoremediation of toxic wastes [79]. Several feasible downstream applications of various microalgae species are presented above in **Table 4**.

Microalgae can improve the nutritional content of conventional foods and lead to a positive effect in the health of humans and animals [62]. They are currently considered rich sources of protein, superior to vegetable sources, also containing several different types of sterols and innumerable antioxidant compounds, namely β -Carotene and astaxanthin, that have shown to be active against oxidative stress, responsible for an array of diseases and for ageing [12]. Dry biomass or cellular extracts attained from the genus *Chlorella*, *Dunaliella* and *Spirulina* dominate the current market [80].

β -Carotene is perhaps the most important Carotenoid available in nature [66] and *Dunaliella salina* is frequently its source for the commercial industry [6][66] – it can be used in the prevention and treatment of several ailments [81][82][83] and in the cosmetic and food industries [62][84]. Lutein, a Carotenoid obtained from *Chlorella* spp., has high nutritional value and low toxicity, being used as a colourant in the food, cosmetics, nutraceutical and pharmaceutical industries, while also being useful in the treatment of several diseases [2][79][85]. Astaxanthin is a red Ketocarotenoid with applications in the cosmetic, pharmaceutical, nutraceutical and food industries [79][84], namely in the treatment of illnesses [66][84][86], known to be accumulated by green algae *Haematococcus pluvialis*.

Long-chain PUFA, mainly EPA and DHA (ω 3 type) [72] and arachidonic acid (AA), are considered pharmacologically and therapeutically relevant and have shown to be effective in the prevention and the treatment of several illnesses [87]. These compounds are currently attained mostly from fish oils; however, due to concerns such as overexploitation of marine reserves, presence of toxic compounds in fishes, unpleasant smell and taste and oxidative instability, the finding of alternative sources is useful [10]. Thus, microalgae that naturally contain significant amounts of EPA and DHA have attracted interest as sources of such compounds to be used for the feeding of animals higher in the food chain [88]. *Nannochloropsis oculata*, *Phaeodactylum tricornutum*, and *Thalassiosira pseudonana* are capable of producing varying quantities of PUFAs [89][90]. *Pavlova lutheri* also presents high levels of PUFA, in particular EPA and DHA [91].

The use of microalgae as a food source and feed additive for freshwater and marine aquatic animals has been more successful than its use in human nutrition [12]. As mentioned before,

microalgae are capable of synthesizing large amounts of PUFA, that are poorly synthesized by several marine animals from precursor fatty acids like linoleic acid (namely, EPA and DHA), but that are essential for their normal development and, when not crucial, their insertion in the diet leads to regular growth and high survival rates for larva [92]. In this way, microalgae play an important role in mariculture, feeding larval stages of crustaceans and fish, for all stages of bivalves and feeding zooplankton, namely rotifers, copepods and brine shrimp, which in turn are fed to late larval, juvenile crustaceans and fish [72] [73]. *Isochrysis galbana* and *Tetraselmis suecica* are used in the feeding of bivalves, *Scenedesmus* spp. can be used as food for artemia and *Chlorella* spp. is adequate for feeding *Brachionus plicatilis*, but not as food for clams and oysters [92]. *Pavlova lutheri* is vastly used in the aquaculture industry, serving as live feed for marine invertebrates, namely molluscs, crustaceans and zooplankton [71][72][73][74][75]. Even though some species have to follow certain nutritional requirements, no nutritional criteria has yet been defined – however, the algae used have to be non-toxic, have an adequate size for ingestion, its cell wall must be digestible and its biochemical contents must be relevant in nutritional terms [12].

Microalgae can also be a good source of different types of renewable biofuels, namely biodiesel, methane, hydrogen, ethanol, among others [12], an application which is quite attractive considering the high costs associated with crude oil. The fact that microalgae biodiesel production won't compromise food production and other goods derived from crops [93] contributes to its potential. Other advantages are the capability of some species in increasing its lipid content, the fact that microalgae cultivation can be attained through less-costly heterotrophic growth and the possibility of genetically engineering strains to attain the desired fatty acids [79]. Algae biodiesel doesn't contain sulphur and its performance is similar to that of petroleum diesel but with reduced emissions of particulate matter, carbon monoxide, hydrocarbons and sulphur oxides [12]. *Chlorella vulgaris* and *Nannochloropsis* have shown potential for the production of biodiesel [18][65] and the possible use of *Pavlova lutheri* with this aim has also been investigated [77].

The use of microalgae, mostly *Spirullina* and *Chlorella*, in the cosmetic industry has also been reported in skin care and as sun protection and hair care products [62][64].

2.3.1. Pavlovophyceae and the *Pavlova* genus

The Pavlovophyceae class, which belongs to the Haptophyta phylum, is divided into a single sub-class, the Pavlovophycideae, one order, the Pavloales, and one family, the Pavlovaceae, which comprises four genera, namely *Pavlova*, *Diacronema*, *Exanthemachrysis* and *Rebecca*. Only

13 species have yet been characterized, which inhabit the littoral, brackish waters and, at times, freshwater environments [94]. The Pavlovophyceae are thought to represent a more primitive state, presenting characteristics related to those of the ancestral haptophyte [94].

In spite of its lower number of described species [95], Pavlovophyceae is still relevant since its members are capable of synthesising long chain PUFA, which correspond to more than half of the fatty acids present, such as DHA and EPA – PUFA in *Pavlova lutheri* comprise about 50.46% of total fatty acids [96]. Pavlovophyceae are known to produce specific sterols and conjugates, the pavlovols [97] - the bioactivities of the dihydroxy pavlovols from the genus *Pavlova* haven't been reported yet [98].

Pavlovophyceae present a relatively simple pigment signature [99]. Lenning *et al.* [99] traced a broad correlation between the morphological groupings previously distinguished within the Pavlovophyceae by Green [100], dividing them into three pigment types. The proposed pigment-based division paired together species such as *P. gyrans* and *P. pinguis*, but set apart *P. lutheri*.

Pavlova pinguis has been reported has an important species for mariculture, as feed for oyster *Crassostrea gigas* [27][101] and for scallop aquaculture [102]. However, studies have found it inadequate in feeding *Pecten maximus*, leading to poor larval development [75]. A size of 5-8 × 3-4 µm [100], a growth rate of 0.7±0.1 division day⁻¹ [75] and a 19% (in dry weight) lipid content have been described [27].

Pavlova lutheri is an economically relevant haptophyte widely used for lipid production, in aquaculture as live feed for several marine invertebrates, namely molluscs, crustaceans and zooplankton [71][73][76], since it is characterized by high levels of PUFAs, especially EPA and DHA [72], essential for marine animals [103]. Among haptophyte species, *P. lutheri* grows comparatively faster and leads to higher biomass and lipid productivities [104]. A size of 4–6 µm, a cell density of 0.4–2.5 g L⁻¹ and a 15–36% lipid content has been reported, with a doubling time of 3–4 days [93][105][106]. In most microalgal species, and particularly in *P. lutheri*, the highest proportions of EPA are obtained at low light intensities, whereas DHA levels are highest in response to high light intensities [107][108][109].

2.3.2. *Pavlova gyrans*

Pavlova gyrans, which will be used in the present work, was firstly isolated and described by Butcher [110] as the type species and inhabits brackish waters [100]. *P. gyrans* cells size ranges in 4-10 x 3-6 x 2-2.5 µm [100] and they are either elongated, ovoid or amoeboid, typically

compressed [110]. Its motile phase is dominant. A characteristic yellow-green parietal plastid with a red-orange eyespot formed by osmiophilic globules is situated close to the flagellar insertion, having parallel thylakoid lamellae [94]. It presents two unequal flagella – one forwardly directed, twice as long as the cell and with an undulatory movement, and a second one smaller in size, directed outwards with a rapid up and down movement, more difficult to detect than the first one [110]. The hairy anterior flagellum is covered with knob scales and the shorter posterior one is naked. The haptonema is frequently noticeable. An eyespot formed by osmiophilic globules can be found in the inner face close to the flagellar pit. A bulging pyrenoid is also present. The plasma membrane of the cell body is surrounded by smaller knob scales. At times, filipodia can be observed extending from the cell [94]. Propagation occurs through longitudinal division during the non-motile stage [110]. Its genome size and structure have already been studied [111].

When it comes to pigment composition, a characteristic polar Chl *c* form (Chl *cPAI*) was found in *P. gyrans* [112], alongside compounds such as MgDVP (Mg-[3,8-divinyl]-phytylporphyrin-13²-methylcarboxylate, a precursor in Chl *a* and Chl *c1+c2* synthesis) [113], Chlorophyll *c1*, Chlorophyll *c2*, Fucoxanthin (Fx), Diadinoxanthin (Ddx), Diatoxanthin (Dtx), and β,β -Carotene [99]. Its pigment composition is dominated by the Carotenoids Fx and Ddx, which together account for nearly 30% of total pigment load. When compared with other species, *P. gyrans* contains relatively large amounts of Chlorophyllide *a* (Chlide *a*), a phytol-free polar degradation product of Chl *a* [114] and three nonpolar Chl *a* derivatives (allomers) [99]. *P. gyrans* sterol composition has been studied [78][97][115][116]. The fatty acid composition of a species that closely resembles that of *P. gyrans* has also been reported [96].

Pavlova gyrans has been extensively used in the aquaculture industry for feeding bivalves, namely oysters [117], showing excellent promotion of the growth of juvenile oysters in laboratory [78]. Some microalgae are capable of producing certain compounds used to protect themselves from UV radiation and still allowing the visible radiation needed for photosynthesis to pass through – that is the case with *P. gyrans*, which produces mycosporine-like amino acids that can be harnessed to be used in the cosmetic industry [6].

3. Culture stress strategies

Environmental factors, such as light, temperature, nutrient availability and salinity impact not only the photosynthetic process and biomass productivity, but also impact the pattern, pathway and activity of the cell's metabolism and its composition [1]. If cultivated under sub- or supra-

optimal conditions, microalgae react by altering their metabolic patterns. When under extreme environmental conditions and stress, they produce several secondary metabolites, which are believed to be an attempt at retaining their growth rates or increasing the survival under these adverse circumstances, some of which can be of interest due to their possible applications as high-valuable products [118]. In this way, alterations in the conditions in which microalgae are cultured, such as nutrient deficiencies and physical changes can be used as strategies to promote the synthesis of such compounds.

3.1. Nutrimental

3.1.1. Nitrogen starvation

Nitrogen is a crucial nutrient involved in growth, development, reproduction, and other physiological activities of microalgae [33]. Nitrogen (N) starvation can occur either through N depletion or N limitation – with N depletion, microalgae are grown in a medium which lacks a source of N, whilst with N limitation the supply of N is constant but insufficient [119] – **Figure 3**.

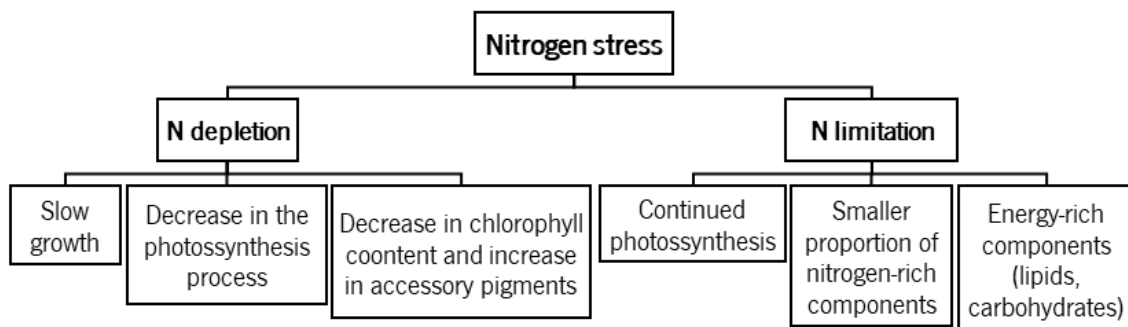


Figure 3. Schematic representation of nitrogen stress in microalgae cultures. Source: adapted from [4].

In cases where nitrogen supply is limited in comparison with other elements, the photosynthesis continues until the cell N levels reach values below a threshold [1], but the products that result from this process will have in its constitution less nitrogen-rich components [4]. Nitrogen limitation affects the photosynthesis of marine phytoplankton through a reduction of the efficiency in which energy is collected due to loss of Chlorophyll and an increase in non-photochemically active Carotenoid pigments. Besides that, the photochemical energy conversion is also directly affected due to a reduction in protein synthesis that seems to affect chloroplast proteins, and consequently the proteins of PSI and PSII reaction centres, stronger than it affects cytoplasmic proteins [120]. Ördög *et al.* [121], using freshwater microalgae *Chlorella minutissima*, found that by adding nitrogen to a nitrogen-starved inoculum, the content of Chlorophyll (a and b) increased, while Carotenoid content did not - this restoration of Chlorophyll would account for the faster growth

rates found in treatments with higher nitrogen. When nitrogen in the media was depleted, Chlorophyll was degraded, and its levels decreased, in order to reuse the nitrogen for growth.

Several studies have shown that nitrogen-limited or deprived microalgae cultures display enhanced biosynthesis and accumulation of lipids [1]. In nitrogen-depleted cells, neutral lipids in the form of triacylglycerols become the main components of lipids, which contrasts with the polar lipids of nitrogen-sufficient cells [122]. Nitrogen limitation is the most efficient and often used strategy for promoting lipid accumulation in microalgae; however, nitrogen deficiency often leads to a reduction in the microalgae growth rates, which in turn leads to negative effects on lipid productivity [43]. Widjaja *et al.* [123] found that exposing freshwater *Chlorella vulgaris* to nitrogen starvation resulted in an increase in the lipid content and that the longer the exposure to nitrogen starvation, the higher the accumulation of lipids inside the cells. In *Dunaliella salina*, data from Weldy and Huesemann [124] indicated that N-deficiency increases its lipid content, with a maximum lipid content of 44% for N-deficiency and only 38% for N-sufficient conditions. For *Neochloris oleoabundans*, the concentration of total fatty acids was positively affected by N-limitation [119]. Using *Dunaliella tertiolecta* and *Thalassiosira pseudonana*, Jiang *et al.* [125] found that decreased availability of N lowered growth rates, photosynthetic efficiency and had consequences on cell production, with the effects of N-starvation being more severe in the diatom than in the chlorophyte and with the two species presenting very different strategies to respond to nitrogen availability. Yeessang and Cheirsilp [126] tested four *Botryococcus* spp. strains and found that a combination of nitrogen deficiency, moderately high light intensity and high levels of iron lead to an increase in lipid accumulation in all four strains. Using *Chlorella pyrenoidosa*, Nigam *et al.* [127] found that the lipid content increases as nitrate concentration declines in the growth medium.

The accumulation of Carotenoids, is also a characteristic that many algae display when under nitrogen-limitation [1]. Using *Dunaliella* cells, Ben-Amotz *et al.* [128] found that nitrogen-starvation promoted the production of β -Carotene. In a study by Fábregas *et al.* [129] using *Haematococcus pluvialis*, the highest values of the Carotenoid Astaxanthin cellular content were achieved under N-deficiency for both light intensities tested, which indicates that Astaxanthin synthesis is more affected by N-deficiency than by light intensity.

3.1.2. Phosphorus limitation

Phosphorus is another essential element for microalgae growth [33] and some of the symptoms of its depletion are similar to those found in nitrogen-deficient cultures [1].

Chlorophyll *a* content has the tendency to decrease while carbohydrate levels increase in both eukaryotic and prokaryotic cells [130]. Phosphorus deficiency in *Chlorella vulgaris* caused a growth 30-40% slower than the one found in the controls [131]. There was, however, no significant alteration in total Chlorophyll *a* and *b* content. Depletion of phosphorus resulted in the accumulation of β -Carotene in *Dunaliella* [128].

In phosphorus deficiency, a drastic decrease in membrane phospholipids and its replacement with non-phosphorus glycolipids and sulfolipids occurs [132], alterations which constitute an efficient mechanism to conserve phosphorus [133]. Using *Chlorella* sp., Liang *et al.* [134] found that the lipid content increased with the decrease of the phosphorus concentration and that the phospholipid content in low phosphorus cells reduced drastically, while the glycolipid content increased from 2% to 3.35%. Khozin-Goldberg and Cohen [135], using *Monodus subterraneus* under phosphorus limitation, found that cell division and Chlorophyll synthesis were severely retarded. They found a drastic reduction in the phospholipid content (from 8.3% of total lipids to 1.4%), of phosphate-deprived cells, whilst the content of triacylglycerols increased dramatically (from 6.5% to 39.3% of total lipids). Phosphate deprivation also led to significant alterations in the fatty acid composition of *M. subterraneus*.

According to Boussiba *et al.* [136], in *Haematococcus pluvialis* cells, phosphorus depletion led to astaxanthin accumulation, but not to the extent that nitrogen deficiency did.

3.1.3. Salinity

As a response to an increase in salinity or in the osmotic pressure of the environment, low molecular weight molecules, named compatible solutes or osmolytes, are accumulated [137]. In microalgae, the polyols are the most important and the most commonly found are glycerol, mannitol, galactitol, sorbitol glycerol galactoside and trehalose [1]. In *Chlorella autotrophica*, there was an increase in proline content in response to an increase in external salinity [138]. In *Chlamydomonas* strain HS-5, increasing the NaCl concentration in the medium from 0.5 to 1.5 M led to the rapid degradation of intracellular free sugars and to the accumulation of glycerol. When *Chlorella* sp. was treated under osmotic shock, increases in the production of sugars were perceptible – after salt stress, the concentration of sucrose and trehalose went from 1.13 to 1.95 and from 0.012 to 0.019 mg g⁻¹ biomass dry weight [139].

Salt stress leads to alterations in the biomass and in the lipid, carbohydrate and Carotenoid content of microalgae.

In a study by Mohan and Devi [140], salt stress triggered significant biomass growth and lipid synthesis of microalgae, with maximum lipid synthesis being achieved for NaCl 1 g L⁻¹. In *Dunaliella* cells, the intracellular content of lipids and triacylglyceride in lipid (67% and 56%) of cells cultivated with NaCl 1.0 mol L⁻¹ were higher than the ones cultivated with NaCl 0.5 M (60% and 40%) [141]. Using *Botryococcus braunii*, Rao *et al.* [142] found that, biomass, carbohydrate and Carotenoid content varied under different levels of salinity. In *Chlamydomonas reinhardtii*, Atikij *et al.* [143] found that stress induced by NaCl led to a dramatic increase in saturated fatty acids (SFA), accounting for 70.2% of the fatty acid methyl esters (FAME) under stress, but to a decrease in PUFA composition. On the contrary, stress induced by KCl led to a slight rise in SFAs (47.05%), with the rest being PUFAs (45.77%).

In *Dunaliella tertiolecta*, different salt concentrations in the range 0.05–3 M of NaCl were tested and the highest Carotenoid content detected (11.73 mg L⁻¹) was achieved for a salinity of 0.5 M NaCl [144]. A study by Hashemi *et al.* [145] performed in *Dunaliella salina* showed that the higher the salt concentration, the higher the β -Carotene accumulation within the cells, reaching a maximum of 4.85 μ g of β -Carotene per mg of dry weight of microalgae at 2.5 mol L⁻¹ salinity. Kobayashi *et al.* [146] found that in *Haematococcus pluvialis* cells stressed with 0.1% NaCl, MgCl₂, KCl or CaCl₂, there was rapid cyst formation and Carotenoid synthesis in the dark.

3.2. Physical

3.2.1. Light

The influence that light exerts on biochemical composition is largely controlled by photoacclimation or photoadaptation, a process in which cells suffer alterations in composition, alongside changes in ultrastructural, biophysical and physiological properties to increase photosynthesis and growth [147]. Light quality and quantity have an influence in the growth rate and the biomass composition, with microalgal growth rates increasing with irradiance up to the level in which light-saturation occurs – increases above this level can become harmful for the photosynthetic apparatus [148].

The influence of light intensity on the growth microalgae has been investigated. For instance, the growth rate of *Dunaliella viridis* suffered a decrease to 63% when light intensity increased from 700 to 1500 μ mol m⁻² s⁻¹ [149].

In response to decreasing light intensity, the cellular content of Chl *a* and other light-harvesting pigments, namely Chl *b*, Chl *c*, phycobiliproteins and primary Carotenoids, tends to increase; however, when under high light intensity, Chl *a* and other pigments which are directly

implicated in photosynthesis decrease and photoprotective secondary Carotenoids, such as zeaxanthin, β -Carotene, astaxanthin, increase [1]. Pisal and Lele [150] exposed *Dunaliella salina* to several stress parameters, namely a combination of high irradiation and high temperature, and found that its Carotenoid content increased – the β -Carotene content per cell increased sharply with light intensity (from 1.77 ± 0.082 pg cell⁻¹ to 8.28 ± 0.61 pg cell⁻¹). In *Dunaliella salina*, exposure to high light ($1000 \mu\text{mol photon m}^{-2}\text{s}^{-1}$, as opposed to $200 \mu\text{mol photon m}^{-2}\text{s}^{-1}$) caused a decrease in expression of a β -Carotene key gene; however, a more significant decrease appeared under low light [151]. In the same study, high light caused a decrease in the Chl *a* and Chl *b* contents and an increase in Carotenoids/total Chlorophylls and Chl *a*/Chl *b* ratios. Under high light there was also an increase in the total carbohydrate, protein and lipid content. In this way, results suggest that an adaptation in gene expression, photosynthesis apparatus function, structure and biochemical cell composition occur in *Dunaliella salina* cells as a response to high light.

Light intensity influences the cellular composition of algae. Guedes *et al.* [152] studied the alterations in lipid class and fatty acid composition of *Pavlova lutheri* in response to light intensity - there were no significant alterations in the biochemical profiles in terms of total proteins and carbohydrates, however total lipid amount decreased with increasing light intensity and lipid composition suffered drastic changes with an increase in light intensity from 9 to 29 W m^{-2} . Results have shown that cultures under low light intensity possessed a higher fraction of EPA and DHA esterified in polar classes, which might be relevant for industrial applications. In a study by Pal *et al.* (2011), *Nannochloropsis* sp. accumulated the highest amount of lipids (47% of dry weight) under high light intensity ($700 \mu\text{mol m}^{-2}\text{s}^{-1}$) [153]. In *Dunaliella tertiolecta*, increasing light intensities up to saturation caused a decrease in protein content and an increase in the lipid fraction [154]. High light intensities have a tendency to promote polysaccharide production in algal cells [1]. Using cultures of *Porphyridium* sp. and *Porphyridium aerugineum*, Friedman *et al.* [155] discovered increases in polysaccharide content when light intensity increased from 75 to $300 \mu\text{mol m}^{-2}\text{s}^{-1}$. Ho *et al.* [156] applied light related strategies in order to enhance cell growth and lutein production in *Scenedesmus obliquus* – white LED lights led to a better lutein production efficiency comparatively to three other monochromatic LED lights.

3.2.2. Temperature

The influence of temperature on microalgae growth rate has been reported in several species. In *Chaetoceros pseudocurvisetus* the growth rate reached its maximum level at 25°C

[157]. Renaud *et al.* [158] found that, when operation temperature was controlled at 25-30 °C, the growth rate for *Chaetoceros* sp. was higher.

Temperature, an easily controlled parameter, is a key factor influencing biomass production, lipid accumulation and fatty acid composition [148]. The fact that temperature influences several biochemical processes, in turn, makes it relevant in terms of biochemical composition. An effect of temperature can be seen with membrane lipid composition and content – when growth temperature decreases below an optimal level, the degree of unsaturation of lipids in membrane systems usually increases [1]. Using *Scenedesmus* sp., Xin *et al.* [159] studied the influence of temperature on growth and lipid accumulation – at 25 °C, considered normal temperature for microalgae cultivation, the lipid content was around 25% (w/w); when temperature decreased to 10 and 20 °C, the lipid content was significantly higher, reaching about 31% and 35% (w/w), respectively; at 30 °C, lipid content per biomass was 22%. The content in triacylglycerols showed a positive correlation with the cultivation temperature, particularly above 20 °C. They found that the content in PUFA decreased as temperature increased - the ratio of the sum of saturated and monounsaturated fatty acids increased, and the PUFA decreased. Converti *et al.* [160] found that increasing the temperature from 20 to 25 °C led to an almost doubling of lipid content in *Nannochloropsis oculata* from 7.90 to 14.92%, while in *Chlorella vulgaris* increasing the temperature from 25 to 30 °C decreased the lipid content from 14.71 to 5.90%. Han *et al.* [161] studied the influence of a high temperature of 40 °C as a stimulus for lipid accumulation in *Scenedesmus quadricauda* in different time periods – biomass concentration showed an increase after the first day of treatment, but then decreased with time of exposure; lipid content increased significantly to 33.5% after 1 day of being under stress, reaching its maximum and decreasing afterwards; carbohydrate content increased slowly and then remained almost constant; protein content gradually increased with time of exposure.

Temperature also seems to influence Carotenoid content. For instance, according to Tjahjono *et al.* [162], when temperature increased from 20 to 30 °C, a threefold increase in Astaxanthin formation was reported in *Haematococcus* cells. Liu and Lee [163] found a similar response in *Chlorococcum* sp., where total Carotenoid content nearly doubled from 4.4 mg g⁻¹ to 8.4 mg g⁻¹ dry weight with an increase in temperature from 20 °C to 35 °C. Carotenoid composition was also altered – a threefold increase in astaxanthin content was reported at 35 °C comparatively to 25 °C, while β -Carotene and Canthaxanthin levels decreased.

3.2.3. Novel strategies

Despite microalgae's potential, there is still a long way to go between the technology available currently and the one necessary to fulfil the world's demands [164]. Strategies where multiple products can be obtained from a single source might be of value, following the premise of a biorefinery – this approach might, in turn, make the production of microalgae strains at an industrial scale feasible [16][165], surpassing obstacles like total production and processing costs, largely caused by energy consumption, and the initial investment and operational costs [166], since the total ending value surpasses the total cost for the algae production, making it worthwhile [167]. Hence, in order to completely take advantage of the biomass produced by microalgae, mild extraction techniques are needed; however, techniques conventionally used for extraction and separation, such as bead milling, homogenization, high pressure, heating, osmotic shock and the use of chemicals, tend to focus on attaining a specific product, whilst damaging the remaining fractions [165]. In this way, new approaches and technological solutions must be developed.

Techniques such as ultrasound, where a sound with a pitch above human hearing is used, have been applied for several purposes [168], for instance, in the food industry, for processing preservation and extraction [169] and in microalgae biotechnology, in the extraction of lipids from *Chlorella vulgaris* [170]. Ultrasounds act mostly through the formation of cavitation bubbles/cavities which grow and implosively collapse in liquids, releasing significant amounts of localized energy [171][172]. Considered an environmentally friendly technology, it is highly efficient in terms of productivity, yield and selectivity, product quality is enhanced, it is economically viable and processing time is reduced when compared to other conventional methods [169][173].

Pulsed light is a non-thermal method used in food preservation and in the decontamination of surfaces/package materials, in which an intense broad spectrum of pulses rich in UV-C light are applied in order to rapidly inactivate microorganisms [174][175]. In this way, the need for chemical disinfectants and preservatives is reduced [176]. Through non-thermal processing, temperature is kept at lower values than with thermal treatments and minimal or no changes are caused to vitamins, essential nutrients and flavours present in food [177], contributing to the quality of the final product.

The branch of electrotechnologies, meaning the application of an electric current to biomaterials with a technological intent, is also gathering more and more attention [14] due its promising applications.

4. Electric field processing

4.1. General overview

Applying an electric field to a biological or bio-based system leads to dissipation of heat, since the system acts as a semi-conductor [14], through the Joule effect, a process which is commonly known as ohmic heating (OH). An ohmic heater or a joule heater is an electrical heating device in which a liquid's electrical resistance is used for the generation of heat [178], which is formed in the fluid as a consequence of the passage of an alternating electric current (I) through a conductive material with a resistance (R), resulting in energy generation and in an increase in temperature [179]. This heating is instigated by friction that occurs through molecular agitation caused by the movement of charged molecules within the material [180]. Electrical conductivity is the most relevant parameter influencing the heating rate and the ohmic heating process and can be measured by the "quantity of electricity transferred across a unit area, per unit potential gradient and per unit time" and is reported for numerous products [181]. In ohmic heating, the product itself acts as the conductive medium and solid electrodes are used, which are in close contact with the treated product and are fed by an electric power supply [181]. Ohmic heaters aid in solving problems often associated with commonly used heating techniques for liquids, which rely on the transfer of heat from a hot surface, such as the requirement of a temperature gradient, the fouling of surfaces that results from the heating process and the difficult heating of very viscous fluids or ones with particulates [178]. A summarized comparison between the advantages and disadvantages of ohmic heating is present below, in **Table 5**.

Table 5. Comparison between the advantages and disadvantages of ohmic heating. *Source:* [178].

Advantages	Disadvantages	Suggestions for Improvement
Required temperature achieved rapidly	Lack of generalized information	Develop predictive, determinable and reliable models of ohmic heating patterns
Rapid uniform heating of liquids with faster heating rates	Requested adjustment according to the conductivity of the dairy liquid	Further research should be conducted to develop a reliable Feedback control to adjust the supply power according to the conductivity change of the dairy liquid
Reduced problems of surface fouling	Narrow frequency band	Developing real-time temperature monitoring techniques for locating cold-spots and overheated regions during ohmic heating
No residual heat transfer after shut off of the current	Difficult to monitor and control	Developing of adequate safety and quality-assurance protocols in order to commercialization ohmic heating technology
Low maintenance costs and high energy conversion efficiencies	Complex coupling between temperature and electrical filed distribution	
Instant shutdown of the system		
Reduced maintenance costs due to the lack of moving parts		
A quiet environmentally friendly system		
Reducing the risk of fouling on heat transfer surface		

Ohmic heating displays several potential applications, such as blanching, evaporation, dehydration, fermentation and extraction. This technology is particularly appropriate for viscous products and foods that contain particulates, since the heat is created in the interior of the food product [182]. In this way, it has been applied in the pasteurization of milk [183], in the cooking of processed meats [184], to fruit purees [185] and to fresh fruits and different cuts of fresh meat [186]. It has also been used in seawater desalination, since the heat generated can be used as an alternative heating method to the use of steam boilers [187]. Industrially speaking, ohmic heating can be of use for waste treatment, namely in sterilization of animal wastes [188], sewage sludge [189], heating of clay slip and other slurries and compost leachate [178]. This process can also be applied for microbial control, providing a rapid and even heating which leads to less thermal damage in comparison to conventional methods [186] – past studies have suggested that the microorganism inactivation during ohmic heating was mainly due to thermal effects [190][191]; however, research states that mild non-thermal damage to the cells might occur due to the exposure to an electric field [192][193]. Several studies have reported the use of ohmic heating in the inactivation of microorganisms, such as *Geobacillus stearothermophilus* spores [194], *Bacillus subtilis* spores [192] and *Alicyclobacillus acidoterrestris* spores in orange juice [195].

Another effect of the application of the application of electric fields is electroporation. When biological membranes are exposed to an external electric field, a transmembrane potential is formed. When this potential overcomes a threshold value (0.2 – 1 V) [14], electric conductivity and permeability suffer a sudden and large increase, known as electro-permeabilization, that can be reversible or irreversible [196] and cause an increase in the flux of compounds through the membrane, cell damage and destruction [197]. The application of an electric field can also lead to charge-related events, since almost every natural molecule presents an intrinsic electric charge [14]. When these molecules are exposed to a continuous or non-uniform electric field, electrophoresis or dielectrophoresis might occur, respectively, which presents the possibility of using these phenomena in focusing, trapping or fractioning biological material [198]. Secondary events, namely electrochemical reactions, shock waves and light emission can also be resultant of electric field application [197].

4.1.1. Electric field-based processing technologies

Currently, electrotechnologies can be classified in terms of type of electric flow (direct or alternating), application in pulses or not, electric field strength (voltage applied by the section

length), extension of heat deposition, along with others [14]. The operational characteristics and applications in previous studies of these novel electrotechnologies are summarized in **Table 6**.

4.1.1.1. Pulsed electrotechnologies

Pulsed electrotechnologies are based on the application of electric pulses, usually of high intensity, during short periods of time [199]. Techniques such as pulsed electric fields (PEF), pulsed ohmic heating (POH) and high voltage electrical discharges (HVED) are representative, presenting distinct influence on the elevation of temperature, mechanical damage and other features of the materials exposed to treatment [200].

Pulsed Electric Field (PEF) processing is a non-thermal technique which consists of the application of high voltage pulses, very short in duration, delivered to a target which is arranged between two electrodes confining the treatment gap of the chamber [201][202]. When exposed to PEF, biomaterials suffer the loss of their membrane barrier functions, since the application of an electric field causes the formation of pores inside the membrane and lead to increasing permeability [203], through an event called electroporation or electroporomeabilization [199]. Electric field strength is quite a relevant parameter – with its increase, electroporation can shift from the enlargement of already existing pores to the generation of new ones, with a temporary or permanent in character, and can even influence intracellular structures and cause cell lysis (electroplasmolysis) [14]. Besides electric field strength, parameters such as pulse form, pulse duration and frequency, treatment time, specific energy input are also relevant [204]. Exposure of food to high-intensity electric pulses can also result in resistance heating [182], electrolysis [205] and disruption of cell membranes [206], which, in turn, contribute to inactivate microorganisms [207]. In spite of being usually referred to as a non-thermal technique, there is indeed some heat deposition present, which leads to an increase in temperature, typically from a few degrees to dozens – however, if needed, temperature can be kept below the necessary to incite inactivation of enzymes and microorganisms [180][14]. Pulsed electric field technology has been of use in the inactivation of microorganisms, such as *Escherichia coli* [205], *Salmonella senftenberg* [208], in the electroporation of cell membranes [209], in the softening of tissues [210][211][212] and in assisting the extraction of high-added value compounds, for instance from fruit peels [213][214]. The implementation of this technique is still hindered by the extensive cost of pulse generators [215] and the lack of understanding when it comes to factors such as processing conditions, types of microorganisms and properties of the treatment medium, equipment design and characteristics of the treatment target [216].

Recently, Pulsed Ohmic Heating (POH), a technique that conjugates the thermal effects to the application of electric pulses, has garnered attention, in particular when it comes to extraction processes [14]. Enhancements in extraction resultant of the combination of the electrical and thermal treatments have been reported [242][243][246][247].

High Voltage Electrical Discharges (HVED) are characterized by the application of a direct high voltage and high current pulse to a liquid medium, which leads to a sudden release of energy and to the generation of a plasma channel and also to secondary events such as shock waves, cavitation, emission of light and formation of radicals [14][200]. The high electric field strength can often cause electroporation; however, cell structure damage and destruction are mainly due to the mechanical stress caused by shock waves [180]. This technique is often used in the chemical removal of organic impurities from water and in conjunction with PEF for the extraction of bioactive compounds from several food matrices. In comparison with conventional techniques, HVED allows greater mass transfer, leads to a higher extraction rate, reduces the processing time, decreases the processing temperature, diminishes the use of solvents, lessens the degradation of compounds which are thermosensitive, improves the purification level of extracts and leads to less energy costs and to a smaller environmental impact [215].

4.1.1.2. Non-pulsed eletrotechnologies

The non-pulsed eletrotechnologies consist of techniques in which the electric current flows unidirectionally (Direct Current) or at times reverses its direction (Alternating Current) without there being interruptions for substantial time periods [14].

Direct Current (DC) consists of a unidirectional electric charge flow throughout two electrodes, one negatively charged (cathode) and one positively charged (anode), which leads to the electrophoretic movement of charged particles in the direction of the electrode with opposite polarity [180]. As the electrodes polarize, electrochemical reactions, such as the electro-oxidation of the electrodes and water electrolysis, can occur and result in corrosion of the electrodes and bubbling [219]. Depending on the amperage, the treatment time, the type of cell and the features of the medium, different outcomes can occur from the application of a DC to living cells, such as inhibition or stimulation of the proliferation of cells [220]. The DC induced electrophoretic movement appears to result in a slight separation of cells, possibly allowing the separation and concentration of cells in a culture medium [221]. The strength of the electric field applied is an important parameter in this process, influencing the rate at which the particles move and, if

sufficiently high, leading to permeabilization [180]. Factors as the high energy required, the excessive heat generated and the scarce durability of the electrodes impair the application of DC in the electrokinetic separation processes [220]. DC appears to have antimicrobial potential, which is possibly due to the adverse electrochemical reactions that occur as a result of the application, in particular near the electrodes [222][223].

Alternating Current (AC) is characterised by an electric waveform whose voltage causes the periodical alternation of its polarity [180]. Its applications tend to be associated with Moderate Electric Fields (MEF), a technique in which a low intensity electric field, usually ranging between 1 and 1000 V cm⁻¹, lower than that of PEF, with or without heating, in the form of a sinusoidal or square wave [224][225]. MEF processing, which relies on either the non-thermal effects of electricity or on the combination of the electrical and thermal events [224] is quite relevant, since it is less expensive than PEF processing, whilst still attaining several of the same effects [207]. Despite its main application in the food industry being, for the past decade, the sterilization of food mixtures [224], depending on the relative electrical and thermal exposure of a treatment, several other results from their application have been described [226]. Non-thermal inactivation of *Escherichia coli* under MEF treatment at temperatures below 25 °C has been described and further analysis revealed alterations on the cell membrane, which might be the cause of cell death [207]. Inactivation of *Bacillus subtilis* spores by ohmic heating in comparison to conventional heating has been reported, with the ohmic treatment resulting in a greater rate of spore death; it was concluded that the spore inactivation during this treatment was primarily caused by the thermal effect, but that the electric current was additionally causing spore death as well [192]. The influence of OH on the growth kinetics of *Lactobacillus acidophilus* has also been studied, demonstrating its potential application to fermented foods [227][228]. The application of MEF treatments has also resulted in the permeabilization of eukaryotic cells [224]. In mint tea leaves, the application of MEF treatment enhanced the extraction from fresh cellular material in comparison to conventional heating alone – this improvement is attributed to a possible breakdown of the cell membrane induced by the field; it was also found that with decreasing frequencies, the permeabilization and molecular transport suffer an increase; besides that, treated samples showed chloroplast compression [226]. Improvements on the drying of vegetable tissues as a result of moderate electric field treatment is applied has also been reported [229]. The application of MEF treatments in the blanching process, a step involved in the preparation of vegetables before its preservation, has been reported due to the rapid heating rate it provides [230]. The application of moderate

electrothermal treatment to fruit juice has been described, with lower frequencies leading to an increase in moisture diffusion, which in turn resulted in higher juice yields; possible savings on energy were also reported [231]. The effect of ohmic heating on the peeling of vegetables, namely tomato, has also been reported and led to promising results [232]. Moderate electric field treatment is capable of effectively disintegrating sugar beet tissue – at the given conditions, the disintegration is attributed to an electroporation phenomenon [233] – which can contribute to the extraction of cellular constituents, as with the extraction of betanin from sugar beets, that might be enhanced by MEF treatment [224]. The application of MEF on the extraction of food constituents, namely oils, triglycerides, polysaccharides and hydrocolloids, and other bioactive compounds, such as pigments and phenolic compounds, has been thoroughly compiled by Gavahian *et al.* [234]. For instance, the application of a MEF treatment led to an improvement in the colour and amount of β -Carotene and lycopene extracted from *Momordica cochinchinensis*, otherwise known as gac fruit, in comparison to conventional heating – after the extraction with ohmic heating, it was also noted that there was more cell wall rupture than with the conventional treatment [235].

Table 6. Novel and emergent electrotechnologies – operational characteristics and main applications in previous studies. *Source:* Adapted from [14].

Electric Field	Technological Common Names	Electric Field (kV cm ⁻¹)	Frequency (Hz)	Waveform	Operational temperature	Treatment time (s)	Main Applications
Pulsed	Pulsed electric Fields (PEF)	1 to 50	1 to 2000	Unipolar or bipolar square or exponential wave (from 4 to 20 μ S)	Typically, from room temperature to 60°C	1×10^{-6} to 2.4×10^{-3}	Non-thermal inactivation of microorganisms Electroporation of cell membranes Tissue softening Assisted extraction of compounds
	Pulsed Ohmic Heating (POH)	< 1	50 to 25000	Bipolar square pulses (delay > 15 μ s)	No theoretical upper limit	No time restrictions	High temperature and short time (HTST) pasteurization and sterilization protocols Thermal and electrical inactivation of microorganism and enzymes Assisted HTST extraction of molecules Avoidance of electrochemical reactions Thermal extraction of compounds
	High Voltage Electric Discharges (HVED)						Extraction of compounds Chemical removal of organic impurities from water
Non-Pulsed	Ohmic Heating (OH)	< 1	50 to 25000	Alternating sinusoidal or square	No theoretical upper limit	No time restrictions	Continuous or batch thermal processing High temperature short-time pasteurization and sterilization of materials Thermal extraction of bio compounds Microbial inactivation
	Moderate Electric Fields (MEF)	< 1	50 to 25000	Alternating sinusoidal or square	Typically performed at sub-lethal temperature	No time restrictions	Non-thermal inactivation of microbial cells Effects on functional properties of globular proteins Controlling activity of some enzymes and bacteria (e.g., reducing lag phase of fermentation) Assisted extraction of thermo-labile bio compounds Extraction of food products

4.2. Current applications in microalgae biotechnology

The application of electric field technologies to microalgae has been reported for several species (Table 7). Their simplicity in structure, high growth rates, benefits to the environment and the presence of certain metabolites, that can be harnessed and used in several commercial products, justify why they have garnered such interest [236]. The application of electrotechnologies to microalgae processing involve both upstream, such as the use of electroporation in genetic transformation, the capability of controlling contaminants and the modulation of growth kinetics, and downstream processes as with harvesting and extraction [180].

The application of electrotechnologies in the genetic transformation of microalgae strains has been reported [237] – for instance, in *Dunaliella salina* cells electroporation allowed the efficient introduction of foreign DNA and its retaining for a considerable period of time, enabling the study of gene expression and function in the cells [238].

When it comes to growth modulation, may that be through growth arrest or increases in cell growth or in the biomass yield, several studies using PEF treatment have been published. Qin *et al.* [239] studied the effect of PEF treatment on the inactivation of *Spirulina* sp. – the application of 100 and 500 high-field impulses of 33.3 kV cm^{-1} caused growth arrest; if magnitude increases to 66.7 kV cm^{-1} , growth stops with only 50 impulses. No structural damage was found by visual inspection. A study using *Arthrospira platensis* showed that the repetitive application of a nano second PEF (nsPEF) treatment to highly proliferating cells led to a significant increase in cell growth [240], an effect which was most pronounced after five days – cell structure analysis confirms that the treatment doesn't cause morphological alterations to the plasma membrane. Gusbeth *et al.* [241] reported a boost in growth resulting of nsPEF treatment – in *Chlorella vulgaris* a 30 min non-lethal treatment applied during the exponential growth phase led to a 10-20% increase in biomass yield, an effect that was significant after 5 days. Using phototrophic *Chlorella vulgaris*, Haberkorn *et al.* [242] also found a significant increase of up to 18% by applying three 100 ns PEF treatments during the early exponential growth phase – the treatment showed limited influence in the carbon and pigment contents; however, protein content suffered a decrease of about 6%.

The application of PEF in extraction is thoroughly described in the literature. On the matter of protein extraction, Buchmann *et al.* [243] developed a cyclic protein extraction process using PEF treatment on *Chlorella vulgaris*, in which the rate protein extraction increased with the increase in electric field strength up to nearly 97% of free protein; nevertheless, growth was negatively influenced by PEF treatment. In order to extract non-degraded proteins from *Haematococcus*

pluvialis living cells, Gateau *et al.* [244] employed PEF treatment and reached 46% of the total protein amount that could be attained through total destructive extraction; in spite of that, increasing electric field strengths did not lead to improvements in the extraction yield. In *Chlorella vulgaris*, the use of PEF treatment led to the extraction of up to half of the proteins existing in the cell [245] – biomass concentration and temperature throughout incubation showed to highly impact the efficiency of extraction, with the efficiency decreasing as the biomass concentration increases. It was shown that, when exposed to PEF treatment, the cells suffer programmed cell death. Akaberi *et al.* [246] studied the influence of PEF pre-treatment on the enzymatic protein hydrolysis of *Scenedesmus almeriensis* – both of the treatments applied (PEF and high-pressure homogenization) revealed a final degree of hydrolysis of about 50% and resulted in incomplete protein hydrolysis; nonetheless, since PEF is capable of preserving cell shape and the separability of the biomass, it is appointed as an alternative downstream method. In spite of that, the application of PEF treatment in the extraction of proteins and carbohydrates from microalgae cells led, at times, to unsatisfactory results, since the electroporation effect might not have been sufficient to liberate a considerable amount of the compounds of interest [14]. Even when applied in conjunction with temperature, PEF treatments were incapable of disintegrating the resistant cells in order to cause the release of protein and carbohydrates in comparison with other more effective extraction methods [247][248]. For instance, using *Chlorella vulgaris* and *Neochloris oleoabundans*, Lam *et al.* [247] concluded that the electroporation effect resultant of PEF treatment wasn't enough to cause the considerable release of intracellular proteins – even using harsh conditions led to the release of only 13% of proteins, in comparison with the 45-50% protein release caused by bead milling. In a study by Postma *et al.* [248] using *Chlorella vulgaris*, the application of PEF treatment with temperatures from 25 to 55 °C, in spite of increasing the conductivity in 75% due to membrane permeabilization, only led to the release of 25–39% of carbohydrates and 3–5% of proteins, values which weren't up to par when compared with bead milling (40–45% protein, 48–58% carbohydrates). Pataro *et al.* [249] reported that, in spite of the application a PEF treatment to *Chlorella vulgaris* enabling liberation of substantial amounts of ionic solutes, carbohydrates and phenolic compounds, the degree of membrane permeabilization achieved wasn't sufficiently effective to allow the release of high quantities of large molecules like proteins, which had an extraction yield lower than 8%.

PEF treatment has also been applied in order to improve the extraction of pigments from microalgae. Luengo *et al.* [250] reported the use of PEF as a pre-treatment on *Chlorella vulgaris*

prior to the extraction of Carotenoids and Chlorophylls. The results revealed that after PEF treatment there was reversible and irreversible electroporation, with treatments ranging from 20 to 25 kV cm⁻¹ leading to irreversible electroporation of the majority of the *C. vulgaris* population even when shorter pulses were applied. PEF treatments higher than 15 kV cm⁻¹ and longer than 15 µs significantly improved the yield of extraction of intracellular compounds. Carotenoid and Chlorophyll *a* and *b* extraction improved 0.5, 0.7 and 0.8 times, respectively, with a 20 kV cm⁻¹ 75 µs treatment; however, when the Carotenoid extraction from PEF-treated cells was performed after a 1 h incubation, the extraction yield also improved in 1.2, 1.6 and 2.1-fold for Carotenoids and Chl *a* and *b*, respectively. Käferböck *et al.* [251] managed to improve the extraction of C-phycocyanin from *Arthrospira maxima* by 90% in comparison to bead milling through a combination of freeze-thawing and PEF treatment and also found that the phycocyanin and bulk protein fractions achieved through extraction were purer. A PEF pre-treatment applied to stressed *Haematococcus pluvialis* biomass and a subsequent 6 h incubation in growth medium led to the extraction of 96% of total Carotenoids, which was superior in comparison to the achieved with the other methods tested (80%) [252]. Parniakov *et al.* [253] found that the application of PEF in assisting the extraction of compounds of interest from *Nannochloropsis* spp. through the use of a binary mixture of organic solvents and water led to an improvement in the extraction efficiency using DMSO and EtOH aqueous solutions. The two-stage procedure, with assistance of PEF, allowed for a better and faster extraction of green pigments, an effect that was attributed to the electroporation of membranes induced by PEF, which allowed the removal of the water-soluble content and, in this way, eased the pigment extraction. When DMSO was used as a solvent, PEF assisting showed a significant influence on the extraction of proteins, phenolic compounds and on the antioxidant capacity. Using EtOH, PEF application improved the extraction of proteins and phenolic compounds.

The use of PEF treatment in order to enhance lipid recover has also been reported. The application of PEF through the form of a Focused Pulse (FP) technology in *Scenedesmus* led to a 3.1-fold increase in the crude lipids and FAME yield of extraction with different solvents [254], while also leading to a 12-fold reduction in the usage of toxic solvents necessary to extract comparable yields and reducing the energy required for mixing. Sheng *et al.* [255] tested different methods of lipid extraction from *Synechocystis*, two of them being PEF treatment with and without temperature control. PEF treatment without a control of temperature led to destruction of the cell envelope and plasma membrane, while also disarranging the intracellular thylakoid membranes. It was revealed by soluble chemical oxygen demand (SCOD) measurement that heat improves the liberation of cell

material, since PEF without temperature control led to a larger increase in SCOD than the same treatment under controlled temperature (4.9% and only 1.4%, respectively). Lipid analysis revealed an increase of 9% in total extracted lipids (as FAME) under PEF with temperature control. PEF treatment has also been used as an intensification pre-treatment for a greener extraction of lipids, leading to an enhancement in lipid recovery from *Ankistrodesmus falcatus* [256]. The use of a less energy consuming PEF treatment in combination with typical solvents led to the lysis of 90% of the cells and to a considerable improvement in the rate of lipid recovery, with the second being caused by the electrical treatment and not by thermal effects. In *Auxenochlorella protothecoides*, Eing *et al.* [257] found that the application of PEF treatment to concentrated microalgae suspensions can lead to improvements in the extraction of water-soluble compounds – the treatment was capable of releasing 15% of the initial biomass content of the suspension into the extracellular medium but no lipids were found, due to the presence of the cell wall that is permeable to water-soluble molecules smaller in size and ions, but not to lipid droplets. As a result, PEF was applied in combination with solvent extraction – using freeze-dried or wet microalgae slurry, both previously PEF treated biomass, there were 9- and 3-times higher lipid yields, respectively, in comparison with untreated samples. The study supports the theory that for there to be an optimal access of the solvent to the interior of the cell and thus an effective lipid recovery, complete irreversible membrane permeabilization is necessary.

PEF exposure has also proved to be useful in the extraction of water-soluble compounds. That is the case with a study by Goettel *et al.* [258], where PEF treatment was applied to *Auxenochlorella protothecoides* and, independently of the parameters applied, were able to cause cell disintegration and consequent release of intracellular soluble components – the efficiency of cell disruption increased with increasing specific treatment energies. In spite of the algae containing about 20% of lipids, the exposure to PEF was only able to spontaneously release soluble components, which displays the possibility of selective extraction. Using *Auxenochlorella protothecoides*, Silve *et al.* [259] also found improvements in the extraction of water-soluble compounds like ions, carbohydrates and even lipids, if followed by solvent extraction, through PEF application when followed by a 20 h incubation at 25 °C, and in addition enabling the reduction of the specific energy required for treatment. In *Nannochloropsis* sp., Grimi *et al.* [260] and Parniakov *et al.* [261] demonstrated that electrically based techniques such as PEF and HVED enabled the selective extraction of ionic compounds soluble in water, microelements, low molecular weight organic

compounds such as amino-acids and of water-soluble proteins; however, they were inefficient in the extraction of pigments, for which techniques such as sonication were required.

HVED has been used as a pre-treatment in a multi-step aqueous and non-aqueous extraction procedure that aimed towards the extraction of bio-molecules from *Phaeodactylum tricornutum* [262] – it was shown that HVED enabled the selective extraction of ionic products soluble in water in the first and second steps and was more efficient in the non-aqueous extraction of pigments and lipids in the third and fourth steps; nevertheless, HVED wasn't capable of reaching the amount of extracted compounds that was attained with high pressure homogenization. Zhang *et al.* [263] studied the influence of several physical treatments, namely HVED and PEF, on the extraction of carbohydrates, proteins and Chlorophyll *a* from three different microalgae species (*Nannochloropsis* spp., *P. tricornutum* and *P. kessleri*). It's worth noting that HVED treatment led to highest extraction yield of carbohydrates for the three species studied, while PEF treatment led to the smallest extraction yield, only superior to untreated samples.

The application of MEF treatment to microalgae has been far less described in comparison with PEF; however, its effect in growth modulation, as an enhancer in cellular compounds labelling and as a pre-treatment or an auxiliary in pigment extraction has been reported. Su *et al.* [264] were capable of using AC to improve the labelling of cellular lipids of *Chlorella vulgaris* and *Spirulina* sp. with fluorescent probes. The fluorescence intensity of *Spirulina* sp. AC treated cells doubled and its labelling was faster. In *Chlorella vulgaris*, fluorescence decay suffered a decrease from more than 50% after 10 min for untreated samples to only 1.22% per minute for AC treated samples. Jaeschke *et al.* [265] studied the combination of MEF treatment with ethanol for the extraction of Carotenoids and lipids from *Heterochlorella luteoviridis*, an approach that proved to be interesting since it resulted in high extraction yields, whilst using a less harmful and non-toxic solvent. Carotenoid extraction was influenced by MEF treatment and ethanol concentration, with the combined application yielding up to 73% of Carotenoid extraction; however, lipid extraction showed to be only affected by ethanol concentration. The application of MEF as a pre-treatment on Carotenoid extraction also from *Heterochlorella luteoviridis* at different temperatures has been described [266]. The MEF pre-treatment intensified extraction in comparison with control experiments, with 40 and 50 °C leading to up to 86% of extraction of total Carotenoids, which supports the use of MEF as a pre-treatment for extraction at lower temperatures. The promotion in extraction by MEF was attributed to probable cell electroporation.

Table 7. Electrotechnologies applied to different microalgae strains and main results achieved.

	Conditions Applied	Microalgae Strain	Main Results	Reference
PEF	PEF treatment with an exponential decay waveform with 45 kV peak voltage, 360 ns 1/e pulse duration.	<i>Ankistrodesmus falcatus</i>	a) PEF treatment caused lysis in 90% of the cells. b) PEF increased the rate of lipid recovery using ethyl acetate as a solvent.	[256]
	15, 20 and 25 kV cm ⁻¹ PEF assisted extraction, square bipolar pulses with 4–32 µs of width and a pulse frequency of up to 300 Hz.	<i>Arthrospira maxima</i>	a) Phycocyanin content in PEF treated (25 kV cm ⁻¹) samples more than double. b) PEF combined with freeze-thawing increased phycocyanin extraction efficiency to 90%.	[251]
	Nano second PEF treatments of 10 kV cm ⁻¹ with constant pulse duration and electric field.	<i>Arthrospira platensis</i>	a) nsPEF treatment increased cell proliferation after successive treatments applied in the exponential growth phase - the effect was most visible 5 days after treatment, accompanied by pigment increase.	[240]
	PEF treatment with a field amplitude of at least 35 kV cm ⁻¹ and pulse duration of 1 µs.	<i>Auxenochlorella protothecoides</i>	a) PEF treatment can improve the extraction of water-soluble cell components. b) PEF combined with solvent extraction increased the lipid recovery yield from both freeze-dried and wet slurry. c) Amount of extracted lipids increased at a specific energy of 100 kJ kg ⁻¹ and higher.	[257]
	Square pulsed PEF treatment with with 1 µs of duration. Different specific treatment energies (52–211 kJ kg ⁻¹ suspension) and electric field strengths (23–43 kV cm ⁻¹) were tested.	<i>Auxenochlorella protothecoides</i>	a) PEF treatment induced cell disintegration led to release of soluble intracellular matter. b) Disintegration efficiency increased with increasing specific treatment energies. Field strength had hardly any influence.	[258]
	PEF treatment with 1 µs rectangular pulses at 3 Hz and 40 kV cm ⁻¹ . Temperature varied between 25 °C - 35.8 °C (±) 3.19.	<i>Auxenochlorella protothecoides</i> <i>Chlorella vulgaris</i>	a) PEF treatment didn't immediately affect the mechanical stability of the cells. b) Both treated and control samples displayed a similar percentage of intact cells after each pass.	[267]
	Continuous PEF treatment of 4 MV m ⁻¹ with a fixed pulse duration of 1 µs and frequency of 0.1 Hz and 3 Hz.	<i>Auxenochlorella protothecoides</i>	a) Extraction efficiency of water-soluble components improved strongly when an incubation (20 h at 25 °C) was done after PEF treatment – there's a reduction in the PEF-treatment specific energy without impairing the high extraction yields.	[259]
	PEF treatment of 40 kV cm ⁻¹ by rectangular-shaped pulses at frequency 0.2 Hz and pulse duration of 100 and 1000 ns.	<i>Auxenochlorella protothecoides</i>	a) <i>A. protothecoides</i> showed more sensitivity to long pulses, particularly at low specific treatment energies. b) For identical treatment energies, organelles exposed to shorter treatment times showed lower damage than the ones exposed to longer treatment experiments. c) PEF treatment significantly influences photosystem II.	[268]
	PEF treatment with a pulse duration between 10-200 µs, with electric field intensities of 5 or 25 kV cm ⁻¹ .	<i>Auxenochlorella protothecoides</i> <i>Chlorella vulgaris</i>	a) There is microalgae precipitation on the treatment electrodes used when PEF treatment is applied (mainly at the anode). b) Application of shorter pulses seems to reduce unwanted precipitation.	[269]
	30 min PEF treatment at 40 kV cm ⁻¹ . Pulses of 25 ns.	<i>Chlamydomonas reinhardtii</i> <i>Chlorella vulgaris</i>	a) 40 kV cm ⁻¹ 25 ns pulses didn't affect algae growth. b) 30 min PEF treatment applied to <i>C. vulgaris</i> in the exponential phase led to an increase of about 10-20% in biomass yield.	[241]
	Protein extraction using PEF treatment up to 20 kV cm ⁻¹ with a pulse width of 50 µs.	<i>Chlorella vulgaris</i>	a) Increasing EF strength led to increases in protein extraction (up to 96.6% of free protein). b) After PEF, increasing extraction rates were accompanied by a negative influence on the growth – however, a 29.1% protein extraction rate was achieved without significantly affecting growth after 168h.	[243]
	Continuous nano second PEF (nsPF) treatments of 10-25 kV cm ⁻¹ with up to a pulse width of 100 ns were applied 1-3 times with an interval of 3 h between them.	<i>Chlorella vulgaris</i>	a) Three 100 ns, 5 Hz, 10 kV cm ⁻¹ PEF treatments in the early exponential growth phase increased biomass yields in 17.53%. b) Carbon and pigment contents weren't affected by treatment, while protein content decreased.	[242]

Continuous PEF using 1–40 square wave monopolar pulses with 0.05–5 ms of duration at an electric field strength of 7.5–30 kV cm ⁻¹ .	<i>Chlorella vulgaris</i> <i>Neochloris oleoabundans</i>	a) PEF treatment both species led to the release of ions, demonstrating cell permeabilization. b) Electroporation was not efficient enough to cause the substantial release of intracellular proteins. c) When using a severe energy input, only 13% of proteins were liberated from the cells.	[247]
50 square waveform pulses of 3 µs at 10, 15, 20, and 25 kV cm ⁻¹ . Frequency of pulse delivery was 0.5 Hz.	<i>Chlorella vulgaris</i>	a) 20-25 kV cm ⁻¹ PEF caused irreversible electroporation of the majority of the population. b) Extraction performed right after 20 kV cm ⁻¹ 75 µs PEF led to increases in Carotenoid and Chlorophylls <i>a</i> and <i>b</i> extraction yields of 0.5, 0.7, and 0.8 times. c) Extraction 1h after 75 µs 20 kV cm ⁻¹ PEF increased Carotenoid and Chlorophyll <i>a</i> and <i>b</i> extraction yield in 1.2, 1.6, and 2.1 times.	[250]
PEF treatment by 5 µs square pulses with an electric field strength of 27 or 35 kV cm ⁻¹ .	<i>Chlorella Vulgaris</i>	a) >20 kV cm ⁻¹ PEF, capable of irreversibly permeabilizing the cells, substantially released small ionic solutes, carbohydrates and phenolic compounds. b) Degree of cell membrane permeabilization achieved didn't release high amounts of large molecules (i.e., proteins). c) Increases in energy input seem to be more effective in contributing to intracellular compound leakage than increases in electric field strength.	[249]
PEF-temperature treatment (average EF strength of 17.1 kV cm ⁻¹ ; 25-65 °C), monopolar square wave electric pulses of 5 µs at 50-200 Hz.	<i>Chlorella vulgaris</i>	a) PEF combined with 25-55 °C increased conductivity in 75% as result of cell membrane permeabilization. b) 25–39% of carbohydrates and 3–5% of proteins were released and a synergistic effect at 55 °C was found for carbohydrates.	[248]
PEF treatment of 40 kV cm ⁻¹ and a duration of 1 µs, resulting in a specific treatment energy of 150 J g ⁻¹ .	<i>Chlorella vulgaris</i>	a) PEF and a 24 h incubation step were capable of extracting up to half of the proteins present in the cell. b) PEF treated cells went into programmed cell death.	[245]
PEF electroporation with exponential electric pulses of 1000 V cm ⁻¹ .	<i>Dunaliella salina</i>	a) Efficient introduction of foreign DNA and its retaining for a considerable period of time (at least 72h), enabling the study of gene expression and function in the cells.	[238]
PEF treatment with 5 or 9 pairs of pulses at 9.62 Hz and duration of 2 ms. 6 different field strengths (0, 0.5, 1, 2, 3 or 6 kV cm ⁻¹) tested.	<i>Haematococcus pluvialis</i>	a) 1 kV cm ⁻¹ PEF treatment led to the extraction of 10.2 µg protein per mL of culture (46% of total protein that could be attained by complete extraction). b) Extraction yield didn't increase with the field strength and did not depend on pulse frequency.	[244]
PEF treatment with 10 pulses of 5 ms at 1 kV·cm ⁻¹ (50 kJ kg ⁻¹).	<i>Haematococcus pluvialis</i>	a) PEF pre-treatment and subsequent incubation in growth medium increased astaxanthin ethanol extraction yield to 96% of the total content.	[252]
PEF treatment of 20 kV cm ⁻¹ , 1–4 ms, 13.3–53.1 k kg ⁻¹ .	<i>Nannochloropsis</i> sp.	a) PEF and HVED selectively extracted water-soluble ionic components and microelements, small molecular weight organic compounds and water-soluble proteins (5.2% water-soluble proteins extraction degree).	[260]
PEF treatment at varying frequencies (1, 2, 3, 4 and 5 Hz) and duty cycles (5, 7, 9, 11, and 13%).	<i>Nannochloropsis</i> sp.	a) Optimal treatment variables for extraction were 1 Hz frequency and 7% duty cycle.	[270]
PEF treatment of 20 kV cm ⁻¹ , comprised of variable successive pulses (1-600 pulses) of variable duration (0.01–6 ms).	<i>Nannochloropsis</i> spp.	a) PEF treatment enabled selective extraction of ionic components and water-soluble proteins, but not of pigments. b) PEF treatment led to extracts with better purity.	[261]
PEF treatment by 400 pulses distanced by 2 s of 20 kV cm ⁻¹ .	<i>Nannochloropsis</i> spp.	a) Two-stage PEF-assisted procedure achieved high levels of extraction of non-degraded proteins at the first stage and improved pigment extraction in the second stage.	[253]
1 µs PEF treatment of 40 kV cm ⁻¹ and 150 k kg ⁻¹ or 75 kJ kg ⁻¹ .	<i>Scenedesmus almeriensis</i>	a) PEF treatment at both energy inputs led to the same final degree of protein hydrolysis as high-pressure homogenization (50%±2%).	[246]
FP treatment of 30.6 KWh m ⁻³ (1-pass treatment).	<i>Scenedesmus</i> spp.	a) FP treatment led to a 3.1x increase in FAME recovery using hexane in comparison to the control, increasing the FAME-to-crude-lipid ratio for every solvent tested as well, while not affecting the FAME profile.	[254]
PEF treatment with a variable number of pulses (50, 100, 500, and 1000) using 33.3 and 66.7 kV cm ⁻¹ .	<i>Spirulina</i> sp.	a) 100-500 33.3 kV cm ⁻¹ pulses stop microalgae growth. b) 66.7 kV cm ⁻¹ stops growth with 50 pulses.	[239]

	PEF treatment with (36 kWh m ⁻³ , final temperature 36 °C) and without temperature control (36 kWh m ⁻³ , final temperature 46 °C).	<i>Synechocystis</i> sp.	a) PEF with temperature control led to the smallest increase in total lipid extraction (9%). b) Temperature-controlled PEF is well suited for large-scale cell disruption, improving lipid recovery and retaining most lipids in the suspended phase.	[255]
HVED	HVED pre-treatment of 40 kV cm ⁻¹ , 1–8 ms.	<i>Phaeodactylum tricornutum</i>	a) HVED selectively extracted water-soluble ionic products. b) In non-aqueous extractions, pigment and lipid extraction assisted by HVED was the most efficient.	[262]
	20 kV cm ⁻¹ PEF treatment. 40 kV cm ⁻¹ HVED treatment. Application of 600 successive pulses at 0.5 Hz and with a duration of 0.01-6 ms.	<i>Nannochloropsis</i> sp <i>P. tricornutum</i> <i>P. kessleri</i>	a) Carbohydrate extraction efficiency: PEF < ultrasonication < HVED b) Protein and pigment extraction efficiency: PEF < HVED < ultrasonication.	[263]
	Application of a uniform constant electric field of 2.77 kV cm ⁻¹ (Moderate sEF) for 0, 10, 30, 50, and 70 min to cultures containing 0.25, 0.4 and 1.0 g CDW L ⁻¹ .	<i>Chlorella vulgaris</i>	a) Highest growth stimulation (51%) was achieved after a 50 min treatment to 0.4 g L ⁻¹ microalgal suspension. b) Chlorophyll content increased in 26% in suspensions of 0.25 g L ⁻¹ after 30 min of treatment; in 0.4 and 1 g L ⁻¹ suspensions the increase was of about 59% and 89% after 50 and 70 min of treatment. c) Extended EF exposure increased peroxide accumulation in the medium, masking the stimulatory effect of the treatment. d) Chlorophyll content increased with the growth rate; total Carotenoids levels were only insignificantly altered.	[271]
MEF	AC, 500–2000 V cm ⁻¹ , 10 MHz sine wave.	<i>Chlorella vulgaris</i> <i>Spirulina</i> sp.	a) 2000 V cm ⁻¹ caused a reduction of 1.22% per minute on the fluorescence decay. b) EF application allowed rapid labelling of cells and increased fluorescence intensity to 270%.	[264]
	MEF assisted extraction 0–40 V cm ⁻¹ , 60 Hz, ≤35 °C, 60 Hz of frequency.	<i>Heterochlorella luteoviridis</i>	a) MEF application, in conjunction with the use of methanol as a solvent, led to a 73% of Carotenoid extraction.	[265]
	10 min MEF pre-treatment (0–180 V) at different temperatures (30, 40, 50 and 60 °C).	<i>Heterochlorella luteoviridis</i>	a) MEF pre-treatment led to higher Carotenoid concentration at 30, 40 and 50 °C. b) Highest extraction yield (86% of total Carotenoid content) was achieved for 40 and 50 °C with MEF pre-treatment.	[266]

5. Can MEF application lead to stress responses in microalgae?

In comparison with other systematically applied electrotechnologies, the application of MEF on matters other than the food industry and in aiding extraction or microalgal biotechnology is still scarce in terms of literature. The application of MEF on the growth of microalgae was approached by Nezammahalleh *et al.* (2016) using *Chlorella vulgaris* and a stimulatory effect on algal growth was found, reaching a maximum increase of 51% (50 min treatment to 0.4 g L⁻¹ algal suspension) - the growth rate increased with treatment time, which was attributed to membrane permeability increase, and subsequently diminished due to the accumulation of peroxide in the growth medium. Total cellular Chlorophyll content increased to a maximum and subsequently declined after exposure to treatment – a maximum Chlorophyll content was reached for suspensions of 0.4 g L⁻¹ and 1.0 g L⁻¹, where increases of 59% and 89% were observed after 50 and 70 min of treatment, respectively [271]. The content in total Carotenoids didn't suffer significant changes after exposure to treatment. With increasing concentration of the algal suspension (from 0.25 g L⁻¹ to 1.0 g L⁻¹) there was a decrease of up to 55 and 50% in total Chlorophylls and Carotenoids. No significant variations were reported for the carbohydrate, lipid, and protein contents after EF treatment. In this study, a uniform constant moderate sEF of 2.77 kV cm⁻¹ was applied, which is superior to the one typically described for MEF. In this sense, the influence of low to moderate electric fields is still undisclosed. No other studies focusing on the application of MEF on the growth and alterations on the biochemical composition of microalgae were found.

In this way, the present study intended to evaluate the effect of MEF treatment on the growth of microalgae *Pavlova gyrams*, whilst trying to uncover possible alterations on the final microalgae biochemical composition and pigment accumulation as a result of the stress applied. Different approaches, that will be thoroughly described in the following chapter, were taken in order to explore the application of MEF on the growth of *P. gyrams*.

It has been suggested that the application of electric fields affects biological systems at both a cell level and as a culture – biochemical processes such as the separation of photochemical charges in the photosystems, modulation of the signalling ion-ligand interaction, regulation of the activity of macromolecules through conformational alterations [272]. Hence, in this study for each of the approaches taken in the MEF application, the growth was followed through variations in the optical density of the cultures and through variations in the biochemical composition.

MATERIALS AND METHODS

1. Growth conditions

Non-axenic *Pavlova gyrams* RCC1553 used in the present work was provided by the Roscoff, France collection. Stock cultures were maintained in f/2 medium with a salinity of 30 g L⁻¹ and kept under constant illumination of 100 μ mol of photons in 2-L glass balloons. Cultures were aerated and 0.7 % v/v CO₂ was sparged for 24 h, in order to maintain the pH in the range 7.7-8.3.

2. Culture Conditions

All the following experiments were carried in 1-L bubble column reactors laterally illuminated with 200 μ mol of photons, aerated with 800 L min⁻¹ of air. Non-axenic cultures were grown under the conditions described previously.

In the reactors in which the electrical treatment was applied, two aluminium electrodes with 1.5 cm of width were inserted and positioned at 2.5 cm apart. In the control reactors, in order to mimic the shadow caused by the electrodes in the culture throughout growth, two aluminium foil strips with 1.5 cm of width were symmetrically placed. In every experiment, as a way to control the temperature of the system and to prevent it from surpassing 28 °C, a temperature probe was inserted in one of the reactors.

The experiments developed in the current work were of exploratory nature. Firstly, MEF treatment was applied constantly throughout growth in order to gather a general understanding of the effects on growth. Then, considering the results found for constant application, the treatment was applied in shorter time frames during both the initial and the exponential growth phase (from day 0 to day 2 and from day 4 to day 6). Afterwards, the duration of the treatment was once again reduced, this time to 6 h, 18 h and 30 h, and cultures were exposed to it during the initial growth phase and, in a separate experiment, during the exponential growth phase in order to explore microalgae's composition in the different growth stages. Next, taking into consideration the thorough investigation found in the literature on the effects of photoperiod alterations on microalgae growth and biochemical composition, the photoperiod was reduced to 16 h of light and 8 h of dark and MEF treatment was applied during the dark phase.

To follow microalgae growth, optical density (OD) was measured spectrophotometrically at 750 nm using a microplate reader Bio-Tek Synergy HT. Microalgae growth was also followed by gravimetric determination of the ash free dry weight (AFDW) (g L⁻¹). The gravimetric analysis was conducted after microalgae harvesting using a vacuum filtration system. Glass microfiber filters

with a pore size of 0.7 μm (Whatman GF/A) were used to collect the AFDW. Based on the gravimetrical analysis, a conversion factor from the OD at 750 nm to the AFDW content (g L^{-1}) was determined (**Figure SM1**). OD measurements were conducted in triplicates with using the microplate reader Bio-Tek Synergy HT. The AFDW present in each sample recovered from the experiments that followed was calculated using the following equation (**Equation 1**):

$$\text{AFDW (g L}^{-1}\text{)} = 0.8293 \times \text{OD}_{750\text{nm}} + 0.1054 \quad (1)$$

Throughout the growth, pH and salinity variations were also measured using the Hanna Instruments HI 2210 pH Meter and the Hanna HI96822 Seawater Refractometer, respectively.

At the end of each growth experiment, the culture remaining in the reactors was centrifuged at $1792 \times g$ (Centurion, model CR7000R, UK) in order to recover the biomass and stored at $-20\text{ }^{\circ}\text{C}$ until it was lyophilized.

Maximum specific growth rate ($\mu_{\text{máx}}$, day^{-1}) (**Equation 2**) and maximum biomass productivity (P_X , $\text{g L}^{-1} \text{ day}^{-1}$) (**Equation 3**) were calculated in order to serve as a comparison parameter between the different experimentations using the following equations.

$$\mu_{\text{máx}} = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (2)$$

$$P_X = \frac{X_2 - X_1}{t_2 - t_1} \quad (3)$$

in which X_1 and X_2 stand for the AFDW (g L^{-1}) at a certain time 1 (t_1) and time 2 (t_2), respectively, belonging to the experimental growth phase.

2.1. Conductivity assessment

In order to infer on the conductivity of the cultures used in the experiments, the variation of the conductivity (mS cm^{-1}) in a range of temperatures between 20 and $50\text{ }^{\circ}\text{C}$ was assessed, in order to better understand the range in which the following experiments should be developed. Culture samples (30 g L^{-1} of salinity) with and without biomass (supernatant previously obtained by centrifugation) were exposed to ohmic heating ($2\text{ to }5\text{ V cm}^{-1}$) and the conductivity data obtained was plotted as a function of the temperature.

2.2. Influence of MEF application throughout growth

Non-axenic *P. gyrans* was cultivated under the conditions previously described. The experiment was carried out in three different conditions in duplicate – a control (A.Ct) without electrical treatment, and two different treatments (A.OH1 and A.OH2), where the cultures were

electrically treated with 0.78 and 0.61 V cm⁻¹, respectively, electric field conditions which are described below in **Table 8**. The electrical treatment was applied throughout growth. The salinity of both the control (A.Ct) and the electrically treated cultures (A.OH1 and A.OH2) was adjusted to 30 g L⁻¹.

Samples of about 10 mL were taken every 2 days. Its absorbance was measured spectrophotometrically at 750 nm using a microplate reader Bio-Tek Synergy HT. Biochemical analysis of the samples recovered was carried out as described in the following chapter.

Table 8. Electric field parameters and treatment conditions applied to every growth experiment developed on the current work and corresponding identification code (ID code).

Application		Amperage (A)	Voltage (V)	Electric Power (W)	Electric Field Strength (V cm ⁻¹)	ID Code
Phase	Time					
		No electrical treatment applied				A.Ct
Permanently applied throughout growth		0.85	1.96	1.66	0.78	A.OH1
		0.39	1.53	0.60	0.61	A.OH2
		No electrical treatment applied				B.Ct
Initial growth phase	Days 0-2 (48 h)	0.59	1.39	0.82	0.56	B.OH1
	Days 4-6 (48 h)	0.32	1.30	0.42	0.52	B.OH2
		No electrical treatment applied				C.Ct
Initial growth phase	6 h	2.18	0.34	0.74	0.14	C.OH1
	18 h	2.08	0.40	0.83	0.16	C.OH2
	30 h	2.19	0.39	0.84	0.16	C.OH3
		No electrical treatment applied				D.Ct
Exponential growth phase (day 4)	6 h	0.63	1.49	0.94	0.60	D.OH1
	18 h	0.63	1.34	0.84	0.54	D.OH2
	30 h	0.65	1.48	0.96	0.60	D.OH3
		No electrical treatment applied				E.Ct
First 16 h	6 h	0.21	0.98	0.21	0.39	E.OH1
First 32 h		0.21	0.92	0.19	0.37	E.OH2
First 48 h		0.20	0.92	0.18	0.37	E.OH3
		No electrical treatment applied				F.Ct
Exponential growth phase (Day 6)	6 h	0.27±0.009	1.38±0.134	0.37±0.025	0.55	F.OH1
	30 h	0.29±0.017	1.30±0.073	0.38±0.034	0.52	F.OH2

2.3. Influence of MEF application in determined time frames

P. gyrans was cultivated under the conditions previously described and the experiment was carried out in three different conditions in duplicate – a control (B.Ct), grown under the circumstances described and without any electrical treatment, and two different treatments (B.OH1 and B.OH2), where the cultures were subjected to electrical treatment with 0.56 and 0.52 V cm⁻¹, respectively – further electrical parameters were previously designated in **Table 8**. However, instead of being applied throughout growth as before, the electrical treatment was only applied in

certain time intervals - from day zero to day two and from day four to day six of growth for both treatment A (A) and treatment B (B), respectively. The salinity of both the control (B.Ct) and the electrically treated cultures (B.OH1 and B.OH2) was adjusted to 30 g L⁻¹.

Sampling and OD reading were done as described in 2.2. for the previous experiment.

2.4. Influence of MEF application during the initial growth phase for different periods of time

As with before, *P. gyrans* was cultivated under the conditions previously described. The experiment was carried out in four different conditions in duplicate – a control (C.Ct), grown under the circumstances described under no electrical treatment, and three different treatments (C.OH1, C.OH2 and C.OH3), where the cultures were exposed to an electrical field of 0.14, 0.16 and 0.16 V cm⁻¹, respectively, with the further electrical parameters presented before in **Table 8**. In this experiment the time frame used in the previous experiment for treatment B.OH1 were chosen (initial phase of growth, from day 0 to day 2) and the electrical treatment was applied in variable times – for 6-, 18- and 30 h starting on day zero in treatments C.OH1, C.OH2 and C.OH3, respectively. The salinity of the electrically treated cultures was adjusted to 30 g L⁻¹, as with the control cultures.

Sampling and OD reading were done as described in 2.2.

2.5. Influence of MEF application on the exponential growth phase for different periods of time

Following the details used in the previous experiment, *P. gyrans* was cultivated under the conditions previously described. The experiment was carried out in four different conditions in duplicate – a control (D.Ct), grown under the circumstances described, and three different treatments (D.OH1, D.OH2 and D.OH3), where the cultures were treated with an electric field of 0.60, 0.54 and 0.60 V cm⁻¹, respectively, parameters which are further presented in **Table 8**. Here the electrical conditions were kept similar between treatments and they were applied in the exponential phase of growth (day 4 to day 6) in variable times – for 6-, 18- and 30 h starting day four in treatment D.OH1, D.OH2 and D.OH3, respectively. The salinity of the electrically treated cultures was adjusted to 30 g L⁻¹, as with the control cultures.

Sampling and OD reading were done as described in 2.2.

2.6. Influence of MEF application in the dark period as an alternative to illumination

In this experiment, *P. gyrans* was cultivated under the conditions previously described. Four different conditions were used in duplicate – a control (E.Ct), grown under the circumstances

described initially, and three different treatments (E.OH1, E.OH2 and E.OH3), in which cultures were exposed to an electric field treatment of 0.39, 0.37 and 0.37 V cm⁻¹, according to the parameters described earlier in **Table 8**. The scheme and electrical conditions of the present experiment were similar to the ones applied before. However, in this trial, periods of illumination during the day (16 h) were followed by periods of darkness during the night (8 h) in which the cultures were electrically treated (6 h). These periods of electrical treatment whilst in darkness were of variable time – 16-, 32- and 48 h for the treatments E.OH1, E.OH2 and E.OH3. The salinity of the electrically treated cultures was adjusted to 30 g L⁻¹, as with the control cultures.

Sampling and OD reading were done as described in 2.2.

2.7. Influence of lower MEF application on the exponential growth phase for different periods of time

After analysing the results found for the experiment where the influence of MEF application on the exponential growth phase for different periods of time was assessed, which were seemingly promising in terms of pigment content, and the apparent relation between the electric field conditions applied and the results obtained, in the last experiment slightly lower voltages were applied to the cultures in an attempt to promote increasing lipid and carbohydrate contents.

P. gyrams was cultivated under the conditions previously described. The experiment was carried out in three different conditions in triplicate – a control (F.Ct), grown under the circumstances described, and two different treatments (F.OH1 and F.OH2), where the cultures were exposed to an electric field treatment of 0.55 and 0.52 V cm⁻¹, with the electric field parameters previously presented in **Table 8**. The electrical treatment was applied in the exponential growth phase, starting in the sixth day of growth, in variable times – for 6- and 30 h in treatment A and B, respectively. The salinity of the electrically treated cultures was adjusted to 30 g L⁻¹, as with the control cultures.

Sampling and OD reading were done as described in 2.2.

3. Analytical Methods

3.1. Determination of the Nitrogen Consumption

Nitrate consumption over time was also used to follow microalgae growth. Nitrate (NO₃) determination was attained using the Ultraviolet Spectrophotometric Screening Method described by the American Public Health Association [273]. After centrifugation (7840 xg) of the samples recovered throughout growth, 0.125 mL of the supernatants were treated by adding 0.3 mL of HCl

1 M and mixing thoroughly. A standard curve was achieved by preparing NO₃⁻ calibration standards in the range 0 to 7 mg NO₃⁻-N L⁻¹ by diluting to 50 mL the following volumes of intermediate nitrate solution (0, 1.00, 2.00, 7.00 ... 30.0 mL) in triplicates. The NO₃⁻ standards were treated in the same way as the samples. The absorbance of the standards and the samples was read using a microplate reader Bio-Tek Synergy HT against distilled water set at zero absorbance at a wavelength of 220 nm to obtain the NO₃⁻ reading and a wavelength of 275 nm to determine the interference of dissolved organic matter. For both samples and standards, the absorbance reading at 275 nm was subtracted twice from the reading at 220 nm to obtain the absorbance due to NO₃⁻ (corrected absorbance). To attain a standard curve, absorbance due to NO₃⁻ was plotted against the NO₃⁻-N concentration of the standards (**Figure SM2**), resulting in the following equation (**Equation 4**).

$$[\text{NO}_3^-] \text{ (mM)} = (\text{Corrected OD}_{220\text{nm}} - 0.0093)/1.8226 \quad (4)$$

Using corrected sample absorbances, sample concentrations were obtained directly from the standard curve.

3.2. Protein Quantification

Supernatant protein was quantified using the Bradford assay [274], as described by Parniakov *et al.* [261]. Supernatant samples were obtained by centrifugation at 2822 xg for 10 min at room temperature. 0.2 ml of the extract (diluted if necessary) and 1.8 ml of three-fold diluted Bradford Reagent prepared were mixed. The tubes with the mixture were vortexed for 10 s and the samples were kept at room temperature for 5 min. Then, the absorbance of each sample was measured at the wavelength of 595 nm using the microplate reader Bio-Tek Synergy HT. Bovine Serum Albumin (BSA) (Sigma Aldrich) was used in the calibration, varying the concentration of BSA between 0 and 400 mg L⁻¹ (**Figure SM3**), proceeding in the same way as described for the measurement of the samples, resulting in the following equation (**Equation 5**).

$$[\text{BSA}] \text{ (mg L}^{-1}\text{)} = (\text{OD}_{595\text{nm}} - 0.0438)/0.0015 \quad (5)$$

Using corrected sample absorbances, sample protein concentrations were obtained directly from the standard curve.

In order to quantify total protein levels, the Bradford method [274] was also used after a hydrolytic treatment in 0.1 N NaOH at 80 °C in a water bath for 1 h [275] was applied to 15 mg of lyophilized biomass. Samples attained were centrifuged at 8000 xg [276] and 0.1 mL of sample were added to 0.9 mL of Alfa Aesar Bradford Dye Reagent, Ready-to-Use soln., followed by

vortexing. After an incubation period of 15-30 minutes, the absorbance of the solution was read at the wavelength of 595 nm using the microplate reader Bio-Tek Synergy HT. Again, BSA solutions at several concentrations (0-0.5 mg mL⁻¹) (**Figure SM4**) were used for calibration, proceeding as above, resulting in the following equation (**Equation 6**).

$$[\text{BSA}] \text{ (mg mL}^{-1}\text{)} = (\text{OD}_{595\text{nm}} + 3\text{E-}05)/0.4632 \quad (6)$$

Using corrected sample absorbances, sample protein concentrations were obtained directly from the standard curve.

3.3. Lipid Quantification

Lipid extraction was performed using the method described by Bligh and Dyer (1959) with minor alterations [277].

For each sample, 50 mg of microalgae powder was added to 1 mL of a solvent mixture of chloroform/methanol (2:1, v/v) and the mixture homogenized by vortexing 2 min, followed by incubation on a heating block at 30 °C for 30 min. The mixture was then centrifuged at 493 *xg* for 10 min and the organic phase was collected to a pre-weighed glass tube. The biomass residue was re-extracted seven more times with 1 mL of solvent or until the supernatant has no pigmentation. The organic phase was dried under a stream of nitrogen gas, the tube was weighed and the initial extract weight was recorded. To remove non-lipid contaminants, the initial extract was re-dissolved in 2 mL of chloroform and 1 mL of methanol, vortexed and for complete extract solubilization sonicated for 10 min. 0.75 mL of water was added to the organic phase followed by vortexing for 2 min. In order to promote phase separation, the mixture was centrifuged for 10 min at 492 *xg* and the organic (lower) phase was collected to a new pre-weighed tube and the aqueous phase was re-extracted with 2 mL of chloroform. The combined organic phases were dried under a stream of nitrogen gas and weighed. The dry weight of the residue obtained was subtracted from the initial weight. Total lipid extracts were transferred to dark vials previously dried and weighed and stored at -20 °C until analysis.

3.4. Pigment Quantification

Pigments were quantified according to Henriques *et al.* [278] with slight modifications, using the equations described by Jeffrey [279] for Chlorophyll *a* and *c1+c2* (**Equations 7 and 8**) and by Strickland and Parsons [280] for Carotenoids (**Equation 9**).

In order to follow pigment variation throughout growth, 5 mL of culture were collected and centrifuged at 1968 *xg* for 10 minutes and the pellet was frozen until further analysis. The pellet

was resuspended in 3 mL of acetone 90% and vortexed for 2 min at 40 Hz using the VELP Scientifica Zx³ vortex. In order to promote extraction, the samples were put in an ultrasonic water bath (VWR Ultrasonic Cleaner USC-TH) for 10 minutes – during ultrasonication, samples were kept in ice to prevent temperature increase. The samples were then centrifuged at 1109 $\times g$ for 10 min in a Hettich EBA 20 centrifuge. This extraction process was repeated until the supernatant was clear. The absorbance of the supernatants was read at the wavelengths used in the equations below using the Jasco V-560 UV/Vis Spectrophotometer.

$$\text{Chlorophyll } a = 11.47 E_{664} - 0.40 E_{630} \quad (7) \quad [279]$$

$$\text{Chlorophylls } c1+c2 = 24.36 E_{630} - 3.73 E_{664} \quad (8)$$

$$\text{Carotenoids} = 10.0 E_{480} \quad (9) \quad [280]$$

3.5. IOMR measurement

After treatment, 500- μ L samples were centrifuged at 11290 $\times g$ for 10 min. The supernatant was collected and its absorbance was measured at a wavelength of 254 nm in the SynergyTM HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., USA). This procedure, described by Geada *et al.* [281], allowed to detect the intracellular organic matter release (IOMR) from the cells at different conditions. The extracellular organic matter present in the original sample (control) was also determined by applying the same method. To simplify the interpretation of results, an IOMR factor was calculated for each sample tested according to the following equation (**Equation 10**):

$$\text{IOMR factor} = \frac{\text{Abs}_t - \text{Abs}_{t0}}{\text{Abs}_{t0}} \quad (10)$$

where Abs_t refers to the absorbance measured at 254 nm after treatment for a certain period of time (t, min) and Abs_{t0} is the absorbance measured at 254 nm of the original sample (before treatment).

3.6. Carbohydrate Quantification

In order to quantify the carbohydrates at the end of each growth trial, the anthrone method, which was initially described by Dreywood [282], was used with the modifications described by Chen and Vaidyanathan [283] after hot acid hydrolysis.

For each sample tested, to 10 mg of lyophilized biomass 5 mL of H₂SO₄ 1 M were added and the tubes were vortexed for 2 min and then boiled in a water bath at 100 °C for 3 hours [102]. 0.2 mL of the hydrolysed sample were mixed with 0.4 mL of a 75% H₂SO₄ solution (previously kept on ice) and the tubes were vortexed. 0.8 mL of an anthrone solution (2 g L⁻¹ in 75% H₂SO₄) were added to the mixture and the tubes were then placed in a water bath at 100 °C for 15 min. The tubes cooled down to room temperature and the samples' absorbance was read at 578 nm [283].

A blank sample, made of 0.2 mL sample mixed with 1.2 mL of the 75% H₂OSO₄ solution, without the anthrone reagent, was used to correct the absorbance of the samples tested. So as to estimate the carbohydrates present in the samples tested, a standard curve was traced using D-glucose in concentrations varying between 0 and 250 mg mL⁻¹ (**Figure SM5**), proceeding for the preparation of the glucose samples in the same way as earlier described, resulting in the equation that follows (**Equation 11**):

$$[\text{D-Glucose}] \text{ (mg mL}^{-1}\text{)} = (\text{OD}_{578\text{nm}} - 0.0109)/0.0018 \quad (11)$$

Using corrected sample absorbances, sample protein concentrations were obtained directly from the standard curve.

3.7. Statistical analysis

Experimental results were given as the average values of two independent experiments and the associated error was represented by a variation coefficient corresponding to the difference between sample and average. On the last growth experiment performed, however, the results represent the average of three independent experiments and the associated error corresponds to the standard deviation.

The D'Agostino & Pearson normality test, followed by the Kruskal-Wallis nonparametric comparison test, were performed using GraphPad Prism version 8.0.2 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com". *p* values ≤ 0.05 were considered to be statistically different.

Biochemical composition – i.e., percentage of lipids, proteins and carbohydrates, pigments concentration (including Chlorophyll and Carotenoids concentration – collected from 42 microalgae growth reactors at the end of the growth stager, were analysed through Principal Component Analysis (PCA) using PAST – Paleontological Statistics Software Package for Education and Data Analysis, version 3.26 [284]. Prior to PCA analysis data was scaled and centred. Ellipse was established within highlighted group with 95% of confidence interval.

RESULTS AND DISCUSSION

1. Conductivity assessment

Prior to any experiment, the conductivity of the *Pavlova gyrams* cultures (with a salinity of 30 g L⁻¹) was assessed using samples with and without biomass (W/ Biomass and W/o Biomass, respectively) in a range of temperatures between 20 and 50 °C (**Figure 4**) in order to understand the range in which the following experiments should be developed.

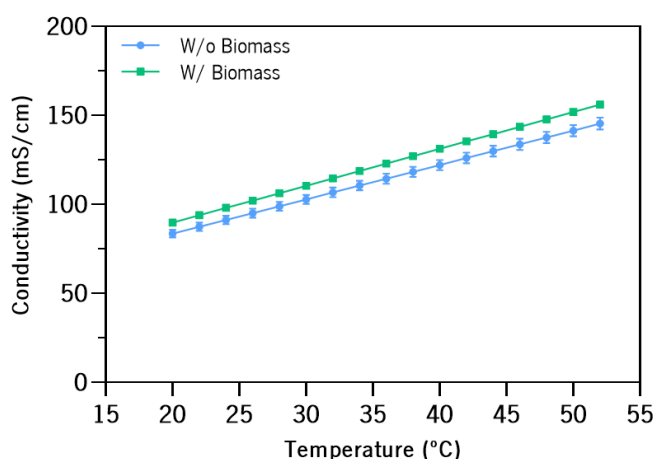


Figure 4. Variation of the conductivity (mS cm⁻¹) of the samples with (W/ Biomass) and without (W/o Biomass) biomass in a range of temperatures between 20-50 °C (n=3).

This preliminary experiment allowed to conclude that the presence of microalgae cells turned the medium more conductive. Every living cell similar in structure presents a cytoplasm enclosed by a membrane and, in the case of plants, for instance, they are further surrounded by a cell wall. Different cellular structures display determined electrical properties – as an example, the cell's cytoplasm is quite complex, containing large quantities of salts, proteins and nucleic acids and several smaller membranous structures that can influence the dielectric properties [285]. Biological tissues present characteristics of insulators and conductors, due to the presence of dipoles and charges that can move in a constrained manner – as both positive and negative ions move oppositely, internal charges can separate within the tissue causing effective internal polarization [286].

In the temperature range tested, the increase of conductivity was linear with increasing temperature, independently of the presence of microalgae biomass. A tissue's electrical properties are dependent on the temperature, with the mobility of the ions transporting the current increasing with temperature, while the extracellular fluid's viscosity increases [286]. In this way, due to the quick heating of the cultures (i.e., about 6 °C min⁻¹, results not shown), in the following experiments

low electric fields were applied in order to not over-heat the cultures and to prevent any cell damage. Temperature of growth trials was always maintained below 28 °C during experiments.

2. Influence of MEF application throughout growth

Firstly, the effect of the constant application of MEF on the growth *P. gyrans* was evaluated by following the AFDW (g L⁻¹) content of the cultures and the nitrate consumption (%) (Figure 5) throughout time – two different electric field strengths were tested: 0.78 V cm⁻¹ (A.OH1) and 0.61 V cm⁻¹ (A.OH2), alongside an untreated control culture (A.Ct).

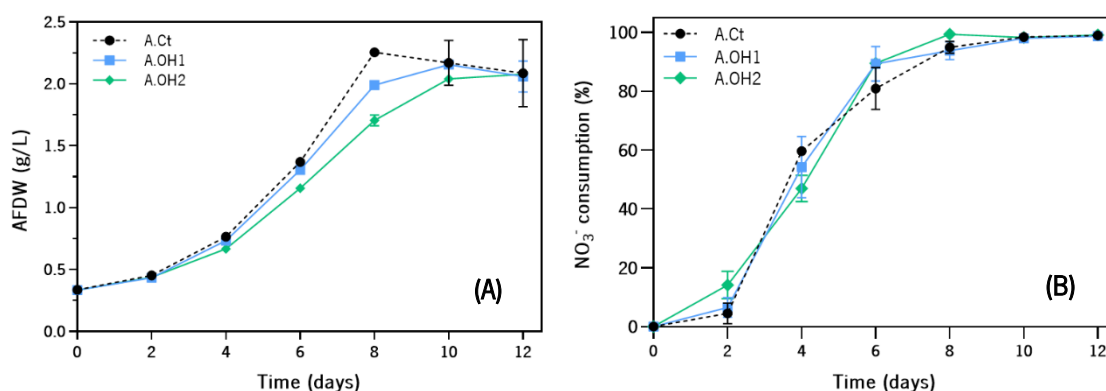


Figure 5. (A) Influence of the application of MEF treatment throughout growth, from day 0 to day 12, with different electric field strengths applied –0.78 V cm⁻¹ (A.OH1) and 0.61 V cm⁻¹ (A.OH2) – on the AFDW (g L⁻¹) of the cultures throughout time (days) alongside an untreated culture (A.Ct) (n=2). (B) Nitrate (NO₃⁻) consumption (%) throughout growth. Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.

The measurement of the AFDW (g L⁻¹) revealed differences between the treated cultures (A.OH1 and A.OH2) and the untreated ones (control A.Ct) (Figure 5A) – the AFDW of the control cultures is superior to that of the treated ones throughout growth, with treatment A.OH1 presenting a superior value to A.OH2, a pattern that was maintained until the end of the growth. These differences start to become noticeable after day 2; however, they become more obvious after day 6, when a clear separation between treatments is visible. A maximum dry weight of 2.25 g L⁻¹ was achieved for the control samples on day 8. The analysis of the nitrate consumption throughout growth (Figure 5B), however, doesn't mirror the noticeable differences that the AFDW content revealed, with the consumption profile being quite similar between treated and untreated cultures throughout growth.

The calculation of the specific growth rates (μ , day⁻¹) for the exponential growth phase, the process productivity (P_x , g L⁻¹ day⁻¹) and the maximum biomass achieved throughout (X_{max} , g L⁻¹) (Table 9) mirror the alterations in the dry weight, with the control (A.Ct) presenting higher values

for all three parameters, followed by treatment A.OH1 and then by treatment A.OH2. In this way, MEF treatment negatively influenced the growth of treated cultures, but it did not lead to growth arrest. This influence varied according to the electric field intensity applied – the higher intensity of electric field applied (in this case, A.OH1), the lesser the reduction in the dry weight and, consequently, in the calculated parameters.

Table 9. Influence of MEF application on the growth parameters calculated for both control (A.Ct) and treated cultures (A.OH1 and A.OH2) – specific growth rate (μ_{max} , day⁻¹) calculated for the exponential growth phase; process productivity (P_x , g.L⁻¹.day⁻¹) calculated from day 0 until the first day of the stationary phase; maximum biomass achieved throughout growth (X_{max} , g.L⁻¹) (n=2; average value $\pm$ variation coefficient resulting of the difference between duplicate and average).

ID Code	μ_{max} (day ⁻¹)	P_x (g L ⁻¹ day ⁻¹)	X_{max} (g L ⁻¹)
A.Ct	0.271 $\pm$ 0.005	0.238 $\pm$ 0.002	2.255 $\pm$ 0.013
A.OH1	0.249 $\pm$ 0.004	0.187 $\pm$ 0.001	2.155 $\pm$ 0.003
A.OH2	0.235 $\pm$ 0.001	0.181 $\pm$ 0.001	2.079 $\pm$ 0.010

In order to better understand the alterations found in the growth parameters analysed, the organic matter content in the supernatant of untreated and treated cells was analysed through the measurement of absorption at 254 nm, being presented in **Figure 6** as an IOMR factor. Between days 0 and 4 there was no clear separation between the IOMR factors of treated and untreated samples; however, after day 4 a clear separation was observed that was maintained until the end of the growth. Control samples achieved higher IOMR factor values, as it happened with the dry weight. In this way, the application of treatment did not seem to contribute to the appearance of organic matter in the supernatant of the cultures.

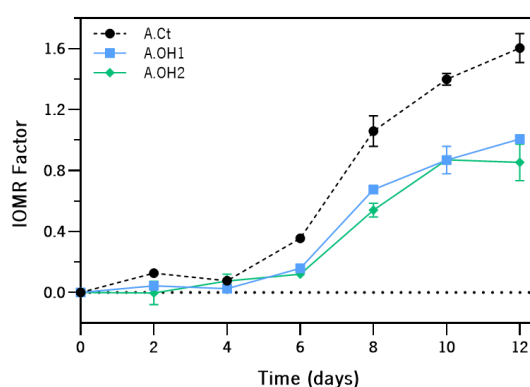


Figure 6. Variation of the IOMR factor of both untreated (A.Ct) and treated (A.OH1 and A.OH2) samples throughout time (days), assessed by measurement of the optical density at 254nm (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.

During growth, microalgae are known to release organic metabolites either naturally [287][288][289] or when cell lysis happens [290]. In fact, the production of extracellular organic

matter containing components such as carbohydrates, amino acids, proteins, lipids and organic acids has been reported [291] and the influence of certain environmental stresses on the production of such compounds has been studied. For instance, in *Dunaliella salina* the production of extracellular polymeric substances increased from 56 to 944 mg L⁻¹ when salt concentration increased from 0.5 to 5 M NaCl [292]. Similarly, high salinity also leads to increases in the extracellular polymeric substances of *Botryococcus braunii* [293]. Such an effect might be occurring as a result of electrical stress – consequently, the liberation of intracellular compounds to the extracellular medium or, in turn, their confinement inside the cell, can be influencing the organic matter content in the supernatant. On the other hand, depending on the growth phase, microalgae secrete different compounds into the extracellular space, which might also explain the variations found in the organic matter content in the supernatant throughout microalgae growth. *Ochromonas danica* releases a combination of free fatty acids into the medium; however, this fatty acid secretion might be restricted to the early and middle exponential growth phase [294]. Polyunsaturated aldehydes, which are released in response to cell damage, can also be liberated into the surrounding environment from intact cells in the late stationary growth phase of microalgae [295].

It is known that electroporation occurs as a result of electric field treatment, altering the membrane permeability [196] and promoting a flux of compounds through the membrane [197], with the exact cellular mechanisms implicated not yet being fully understood [296]. In this way, it would be expected that treated cultures would present higher organic matter content in the supernatant; however, the opposite was found here.

Additionally, MEF treatment might be reducing contamination in the cultures and, thus, leading to the reduction in organic matter in the supernatant found here – ohmic heating has been reported to lead to the inactivation of microorganisms such as *Escherichia coli*, *Bacillus subtilis* and *Lactobacillus acidophilus* [192][207][227][228]. In previous studies, microorganism inactivation was attributed to a lethal thermal effect and non-thermal lethality to *B. subtilis* spores caused by electric exposure, with treatment injuring spores and turning them vulnerable to ohmic heating [192]; in *E. coli* cell death was non-thermal, with alterations in the cell membrane being pointed out as a possible cause of cell death [207]; ohmic heating was also described as a sub-lethal strategy to maintain temperature throughout fermentation, altering the growth and the metabolic activity of *L. acidophilus* [227].

The analysis of the lipid and carbohydrate content from biomass recovered at the end of the growth revealed apparent differences between treated and untreated samples (**Figure 7**).

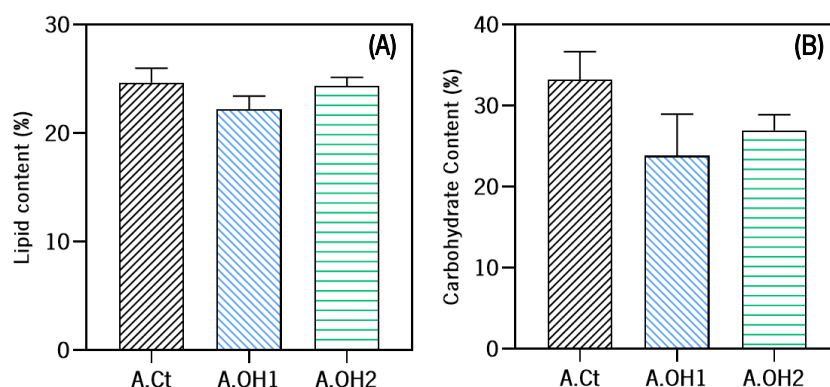


Figure 7. Variation of the lipid (A) and carbohydrate (B) content (%) of the biomass of both untreated (A.Ct) and treated (A.OH1 and A.OH2) at the end of the growth (day 12) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.

When it comes to the lipid content (**Figure 7A**), the application of the treatment doesn't appear to remarkably influence the lipid accumulation, causing a slight decrease of 2.4% and 0.3% for samples A.OH1 and A.OH2, respectively, in comparison with the control (A.Ct). The carbohydrate content (**Figure 7B**) was more clearly negatively influenced by the treatment, regardless of the conditions applied – there was a decrease of 9.4% and 6.3% for cultures A.OH1 and A.OH2, respectively. Hence, the application of MEF treatment in these conditions seems to negatively influence the content of lipids and carbohydrates at the end of the growth experiment, with higher electric field strengths leading to higher decreases in the contents of these compounds. No further analysis of the protein and pigment content was done to the samples recovered in this experiment.

3. Influence of MEF application in determined time frames

After analysis of the results previously obtained for the permanent treatment throughout growth, MEF was applied to *P. gyrans* but only in specific time frames, from day 0 to day 2 (B.OH1) and from day 4 to day 6 (B.OH2) – similar electric field strengths were applied: 0.56 V cm⁻¹ (B.OH1), 0.52 V cm⁻¹ (B.OH2). This strategy allowed to evaluate if MEF applied in different growth stages can result in different outcomes.

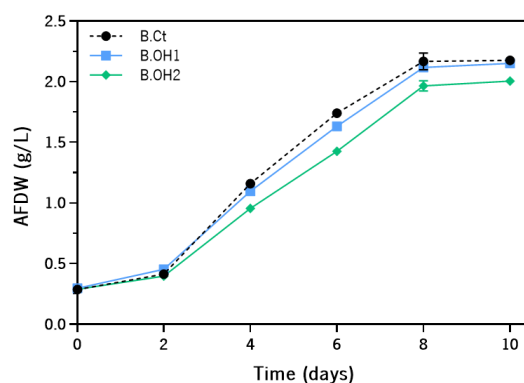


Figure 8. Influence of the application of different MEF treatments from day 0 to day 2 (B.OH1) and from day 4 to day 6 (B.OH2) of growth with similar electric field strengths – 0.56 V cm^{-1} (B.OH1) and 0.52 V cm^{-1} (B.OH2) – on the AFDW (g L^{-1}) of the cultures throughout time (days) in comparison with control cultures (B.Ct) ($n=2$). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.

The application of the MEF treatment didn't seem to drastically affect the dry weight profile (**Figure 8**); nevertheless, the control cultures (B.Ct) always display a superior level of dry weight, followed by treatments B.OH1 and B.OH2. A maximum value of dry weight of 2.18 g L^{-1} was achieved for the control samples after 10 days. Taking a closer look at the graph, and considering that culture B.OH1 was treated from day 0 to day 2, after day 2 its dry weight value seemed to become similar to the one corresponding to the control, while culture B.OH2 also remained close but at a lower dry weight value. Seeing as culture B.OH2 was treated from day 4 to day 6, the treatment didn't seem to cause a drastic effect on its dry weight value.

The calculation of the specific growth rate for the exponential phase, the process productivity and the maximum biomass achieved are presented in **Table 10** – there are differences between control (B.Ct) and treated cultures (B.OH1 and B.OH2), with the control cultures presenting higher values for all three parameters in comparison with the treated cultures. In this way, MEF treatment under the conditions tested didn't cause growth arrest, but appears to influence the growth parameters analysed, as observed previously.

Table 10. Influence of MEF application on the growth parameters calculated for both control B.Ct and treated cultures (B.OH1 and B.OH2) – specific growth rate (μ_{max} , day^{-1}) calculated for the exponential growth phase; process productivity (P_x , $\text{g L}^{-1} \text{ day}^{-1}$) calculated from day 0 until the first day of the stationary phase; maximum biomass achieved throughout growth (X_{max} , g L^{-1}) ($n=2$; average value $\pm$ variation coefficient resulting of the difference between duplicate and average).

ID Code	μ_{max} (day^{-1})	P_x ($\text{g L}^{-1} \text{ day}^{-1}$)	X_{max} (g L^{-1})
B.Ct	0.360 ± 0.006	0.253 ± 0.001	2.177 ± 0.018
B.OH1	0.321 ± 0.009	0.246 ± 0.001	2.150 ± 0.002
B.OH2	0.319 ± 0.007	0.240 ± 0.003	2.004 ± 0.015

The analysis of the organic matter present in the supernatant of the samples tested, represented as the IOMR factor (**Figure 9**), showed differences between control and treated samples.

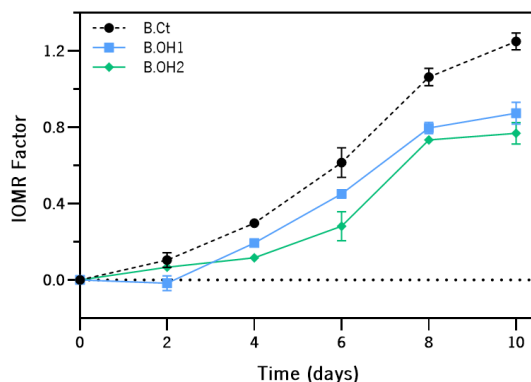


Figure 9. Variation of the IOMR factor of both untreated (B.Ct) and treated (B.OH1 – 0.56 V cm^{-1} from day 0 to day 2 and B.OH2 – 0.52 V cm^{-1} from day 4 to day 6) samples throughout time (days) ($n=2$). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.

All throughout growth there seems to be a disparity between the organic matter content in the supernatant of control and treated cultures – the control (B.Ct) presents higher values throughout growth, culture B.OH1 remains at an intermediate value and culture B.OH2 presents the lower IOMR values. In this way, the application of the electric field seems to be leading to a decrease in the organic matter present in the supernatant of the cultures, as it was previously seen. It's noticeable that in culture B.OH1, during the period of time when the treatment was applied (day 0 to day 2), its IOMR factor is the lowest in comparison with the other two; the same happens with culture B.OH2, which presents the lowest value during the time it's being treated (day 4 to day 6). This decrease in the organic matter content can be an evidence of contaminant reduction as a result of electric field application or of an alteration in the release of intracellular metabolites as a consequence of electrical stress, as previously addressed.

The influence of the treatments on the cultures was also followed by analysing the pigment content of the cultures throughout growth (**Figure 10**).

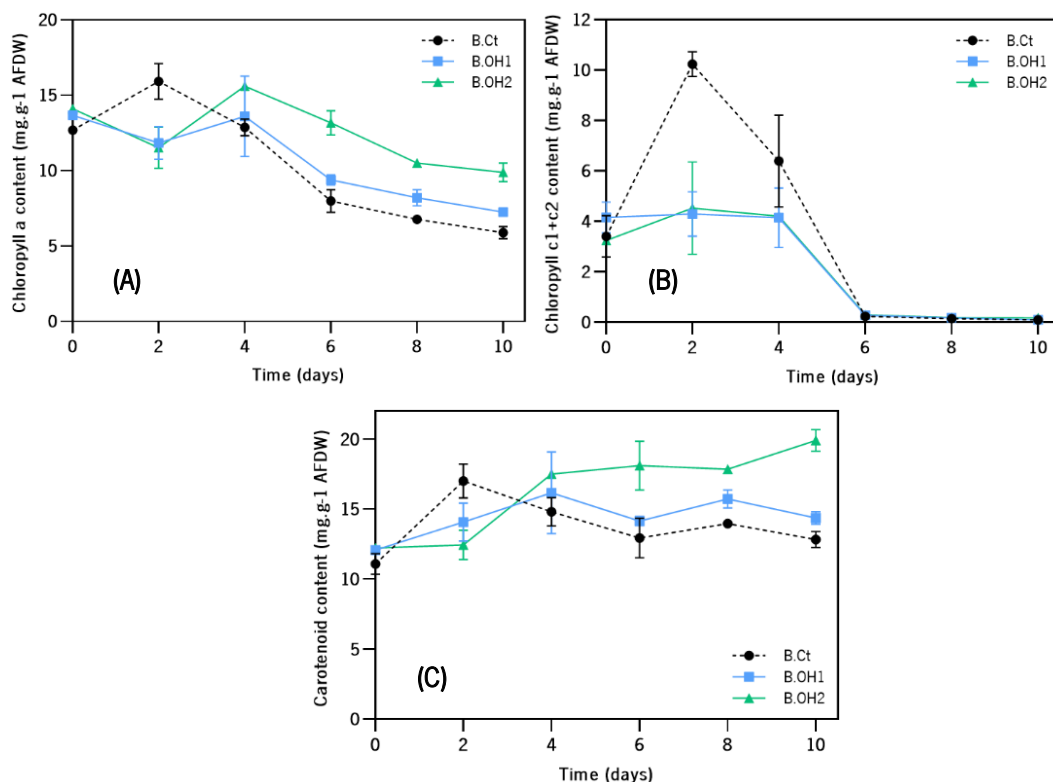


Figure 10. Variation of Chlorophyll *a* (A), Chlorophyll *c1+c2* (B) and Carotenoid (C) content (mg g⁻¹ AFDW) throughout growth for the control and treatments B.OH1 (0.56 V cm⁻¹ from day 0 to day 2) and B.OH2 (0.52 V cm⁻¹ from day 4 to day 6) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average.

The application of MEF treatment seems to influence the pattern of Chlorophyll *a* accumulation throughout time (**Figure 10A**) in comparison with the control culture (B.Ct). In culture B.Ct there is an increase in the content from day 0 to day 2 (3.24 mg.g⁻¹ AFDW), reaching the maximum value achieved throughout growth (15.9 mg.g⁻¹ AFDW), followed by a constant decrease until the end of the growth. As previously addressed, N is an essential nutrient for microalgae growth [33] that influences several cellular processes, namely the production of chlorophyll molecules [57]. In previous experiments, reductions in the Chlorophyll content were found as a result of nitrogen deficiency or starvation, which is possibly related to the insufficiency of N atoms that constitute the Chlorophyll structure [297]. In *Chlorella vulgaris*, the Chl *a* content decreased with the consumption of external KNO₃ as cell growth continued [298]. The total Chlorophyll concentration of N-starved *Chlorella fusca* declined throughout growth [299]. The Chlorophyll *a* content of *Scenedesmus subspicatus* also decreased with the reduction N concentration (from 19.6 to 0.8 mg mL⁻¹) [300]. In *Scenedesmus dimorphus*, N starvation also caused a reduction in the yield and productivity of Chlorophyll *a* and *b* [301]. N-restriction in *Chlamydomonas reinhardtii* caused a reduction of around 64% in Chlorophyll content on the first day of culture and of about 78% on the seventh day [302]. In the current experiment, it could be suggested that as N present

in the medium is being consumed, the Chlorophyll content decreases; however, in order to prove this assumption, the measurement of N consumption would be necessary. In cultures B.OH1, where the treatment was applied in the first two days of growth, a decrease in Chlorophyll *a* content can be seen (1.83 mg g⁻¹ AFDW), followed by a slight increase of the same value, and then the pattern becomes similar to the one displayed by B.Ct cultures, yet always attaining slightly higher content levels. The Chlorophyll *a* content in cultures B.OH2 presents a similar behaviour throughout growth – there was an initial decrease in content (2.59 mg g⁻¹ AFDW, respectively), followed by an increase of about 4.08 mg g⁻¹ AFDW, higher than the one presented by cultures B.OH1, and a subsequent decrease until growth ends, reaching higher values at the end of the growth than the ones cultures B.Ct and B.OH1 display. A similar behaviour has been described for the Chlorophyll content of *Chlorella vulgaris* – it increases until a maximum is attained (day 4) and then content declines as a result of EF exposure [271].

As it happened with the Chlorophyll *a* content, the Chlorophyll *c1+c2* content also suffered alterations under MEF treatment (**Figure 10B**). For B.Ct cultures, there was an initial increase in content, reaching a maximum of 6.83 mg g⁻¹ AFDW, followed by a constant decrease until the end of the growth. For cultures B.OH1 and B.OH2 there is no initial increase in content, staying more or less constant until day 4, after which there is a continuous decrease.

The Carotenoid content (**Figure 10C**), however, follows a different pattern throughout growth. For B.Ct cultures, there was an initial increase of about 5.92 mg.g⁻¹ AFDW and a subsequent decrease. For cultures B.OH1 the initial Carotenoid content increase (2.01 mg g⁻¹ AFDW) was not as high as the one found for B.Ct, yet, after that initial increase, its pattern of accumulation seems to become similar to the one shown by control cultures, but always at a slightly higher content level. In regards to cultures B.OH2, the pattern is quite different – after day 2, the Carotenoid content increased and stayed more or less constant until the end of the growth, reaching the maximum content of 19.9 mg g⁻¹ AFDW on day 10. The increase in the Carotenoid content of cultures B.OH2 after day 4 can be caused by MEF treatment; however, it might also be caused by the slight increase in temperature resultant of the treatment (from room temperature to a maximum of 28 °C) that, coincidentally, was applied from day 4 to day 6 – in *Haematococcus* cells and in *Chlorococcum* sp., increases in temperature from 20 to 30 °C and from 20 to 35 °C, respectively, led to significant increases in the Carotenoid content and to alterations in composition [162][163].

In order to better compare the variation that occurred, the pigment content (mg g^{-1} AFDW) at the beginning (day 0) and at the end of the growth experiment (day 10) for the control and each of the treated cultures was compared (Figure 11).

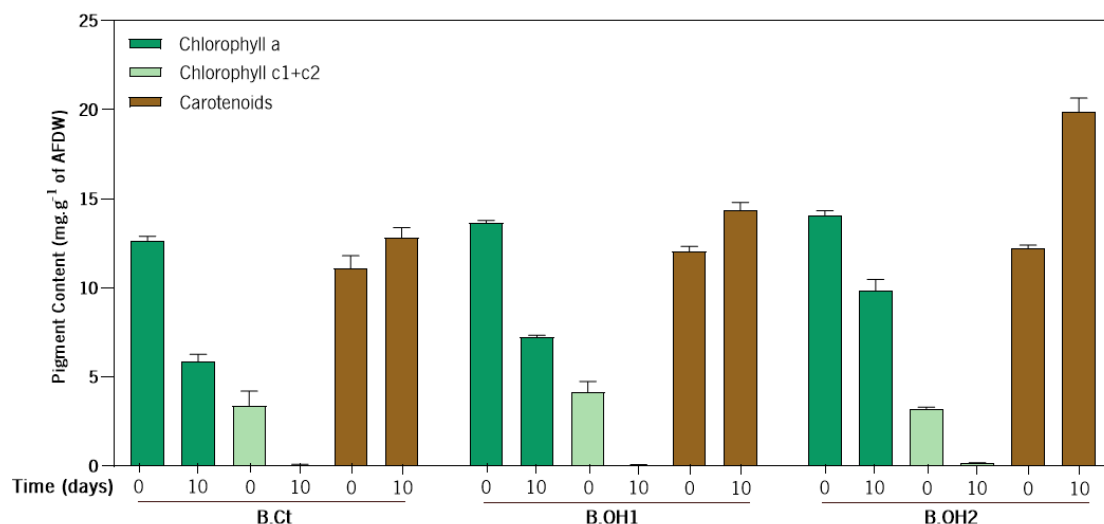


Figure 11. Variation of pigment (Chlorophyll *a*, Chlorophyll *c1+c2* and Carotenoids) content (mg g^{-1} AFDW) at the beginning (day 0) and at the end of the growth (day 10) for the control (B.Ct) and treatments B.OH1 (MEF application from day 0 to day 2) and B.OH2 (from day 4 to day 6) of growth with similar electric field strengths – 0.56 V cm^{-1} (B.OH1) and 0.52 V cm^{-1} (B.OH2) ($n=2$).

Error bars represent a variation coefficient resultant of the difference between sample and average.

From the beginning until the end of the growth, there was always a decrease in Chlorophyll *a* content for both treated (B.OH1 and B.OH2) and untreated (B.Ct) cultures; however, that decrease was higher for control cultures and B.OH1 treated cultures (6.80 and 6.41 mg g^{-1} AFDW, respectively) than for cultures B.OH2 (4.23 mg g^{-1} AFDW, respectively). In this way, the Chlorophyll *a* content at the end of the growth for cultures B.OH1 and B.OH2 represent an increase of 23.3% and 67.9% in comparison to B.Ct cultures. The Chlorophyll *c1+c2* content also suffered a decrease from beginning to end for both control and treated cultures B.OH1 and B.OH2 – 3.32 , 4.06 and 3.07 mg g^{-1} AFDW, respectively – with the decrease being more noticeable for cultures B.OH1. Contrarily, the Carotenoid content always increased from day 0 to day 10. For control cultures, that increase was of 1.75 mg g^{-1} AFDW, while for cultures B.OH1 and B.OH2 it's of 2.29 and 7.67 mg g^{-1} AFDW, respectively, which corresponds to an increase of 12.0% and 55.1%. In this way, it can be concluded that MEF treatment under the conditions applied seems to cause apparent alterations to the profile of accumulation of pigments in *P. gyrans*.

The biochemical analysis of the biomass – regarding lipids, carbohydrates and proteins – attained at the end of the growth experiments for both untreated (B.Ct) and treated (B.OH1 and B.OH2) cultures is present in Figure 12.

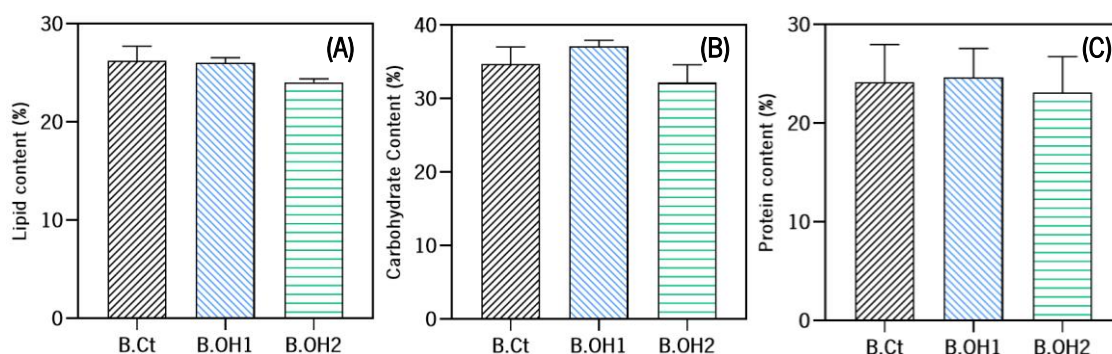


Figure 12. Variation of the lipid (A), carbohydrate (B) and protein (C) content (%) of the biomass of both untreated (B.Ct) and treated B.OH1 (0.56 V cm⁻¹ from day 0 to day 2) and B.OH2 (0.52 V cm⁻¹ from day 4 to day 6) at the end of the growth (day 10) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.

No noticeable alterations were found in the biochemical analysis. Concerning the lipid content (**Figure 12A**), the application of treatment B.OH1 didn't seem to influence the content at the end of the growth since the value attained (26.0%) is quite close to the one achieved for the B.Ct cultures (26.2%); nevertheless, treatment B.OH2 led to a slight decrease in lipid content, reaching content levels of 24%. The carbohydrate content at the end of the growth (**Figure 12B**), however, seems to be influenced by both treatments – treatment B.OH1 leads to 37.1% and B.OH2 to 32.2% of carbohydrates, in comparison to B.Ct where the carbohydrate content was of 34.7%. In this way, there was a slight increase in content when treatment B.OH1 is applied. The protein content at day 10 (**Figure 12C**) for B.Ct cultures was of 24.1% and for cultures B.OH1 is 24.6%, quite similar values, while for treatment B.OH2 there seems to be a reduction in content to 23.1%.

4. Influence of MEF application during the initial growth phase for different periods of time

Knowing from previous results that MEF application at the initial growth phase brought different outcomes regarding pigment composition, another strategy was evaluated. The effect of MEF treatment on the initial growth phase of *P. gyrans* (day 0) but for different periods of time – treatment C.OH1 (6 h), treatment C.OH2 (18 h) and treatment C.OH3 (30 h) – and exposed to the same electric field strength (an average of 0.15 V cm⁻¹) was then assessed, alongside untreated control cultures (C.Ct) (**Figure 13**).

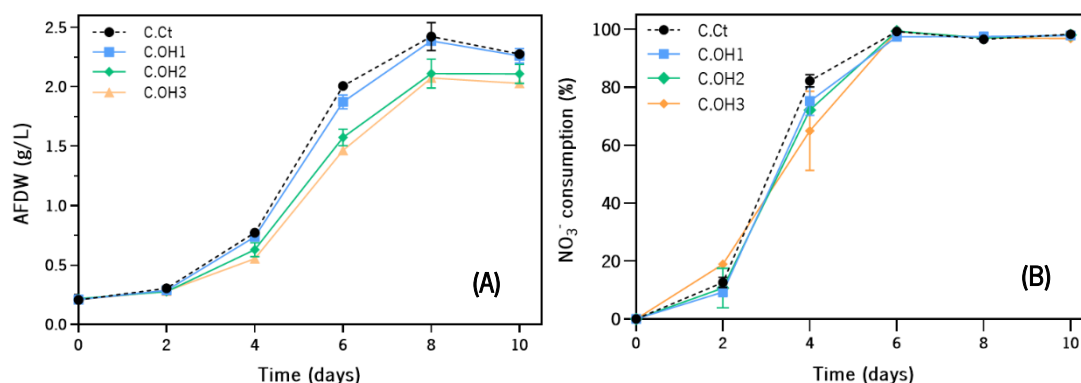


Figure 13. (A) Influence of the application of MEF treatment applied on day 0 during different periods of time – 6 h (C.OH1), 18 h (C.OH2) and 30 h (C.OH3) – and under the same electric field strength (0.15 V cm^{-1}) on the AFDW (g L^{-1}) of the cultures throughout time (days) in comparison with untreated control cultures (C.Ct) ($n=2$). (B) Nitrate (NO_3^-) consumption (%) throughout growth. Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.

The application of MEF treatment seems to influence the AFDW content of the cultures after day 2 of growth (**Figure 13A**), since there were visible differences in content between untreated and treated samples; however, independently of MEF treatment, growth didn't suffer arrest. While C.Ct and C.OH1 cultures seem to stay at close superior values throughout growth, cultures C.OH2 and C.OH3 maintained lower similar values until the last day. A maximum dry weight content was reached for the C.Ct cultures on the 8th day (2.42 g L^{-1}). In this way, it is important to note that longer treatment times (18 h and 30 h – C.OH2 and C.OH3, respectively) seem to lead to lower AFDW contents, meaning that growth might be more affected than when shorter treatments are applied (6 h – C.OH1). These differences in the growth as a result of treatment can also be seen in the nitrate consumption (**Figure 13B**) – between days 2 and 6, treated cultures appear to be consuming less nitrate than control cultures.

The noticeable differences in the AFDW content were consistent with the specific growth rate and process productivity calculated and with the maximum biomass achieved (**Table 11**) – C.Ct cultures present the highest values of all three parameters; however, they were close to the ones displayed by cultures C.OH1, whilst cultures C.OH2 and C.OH3 presented lower values.

In the present study, a 0.15 V cm^{-1} MEF treatment was applied to cultures with an average of 0.21 g L^{-1} of dry weight. In a study using *Chlorella vulgaris* at 0.25 g L^{-1} , where a MEF treatment of 2.77 kV cm^{-1} was applied for shorter periods of time (10, 30, 50 and 70 min), EF treatment promoted growth – maximum growth was achieved for 10-30 min treatments, corresponding to a growth rate 25% superior to that of untreated cultures [271]. However, in the present study, the application of MEF treatment caused a slight decrease in the specific growth rate, which is most noticeable for the longer treatment times (18 h and 30h for C.OH2 and C.OH3, respectively).

Table 11. Influence of MEF application on the growth parameters calculated for both control C.Ct and treated cultures (C.OH1 and C.OH2) – specific growth rate (μ_{max} , day⁻¹) calculated for the exponential growth phase; process productivity (P_x , g L⁻¹ day⁻¹) calculated from day 0 until the first day of the stationary phase; maximum biomass achieved throughout growth (X_{max} , g L⁻¹) (n=2; average value $\pm$ variation coefficient resulting of the difference between duplicate and average).

ID Code	μ_{max} (day ⁻¹)	P_x (g L ⁻¹ day ⁻¹)	X_{max} (g L ⁻¹)
C.Ct	0.472 $\pm$ 0.005	0.307 $\pm$ 0.010	2.422 $\pm$ 0.118
C.OH1	0.469 $\pm$ 0.005	0.305 $\pm$ 0.006	2.388 $\pm$ 0.017
C.OH2	0.436 $\pm$ 0.018	0.284 $\pm$ 0.003	2.112 $\pm$ 0.123
C.OH2	0.413 $\pm$ 0.013	0.287 $\pm$ 0.002	2.074 $\pm$ 0.035

The analysis of the organic matter present in the supernatant, presented below as an IOMR factor, revealed differences between control and treated samples (Figure 14).

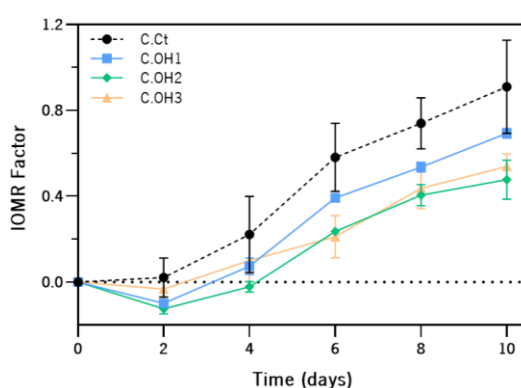


Figure 14. Variation of the IOMR factor of both untreated (C.Ct) and treated in the initial growth phase (C.OH1 – 6 h, C.OH2 – 18 h and C.OH3 – 30 h) samples throughout time (days) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.

In spite of the overall behaviour of treated and untreated cultures being quite similar, with an initial decrease in organic matter content followed by increases until the end of the growth, control cultures always displayed higher content in the supernatant than treated ones. The application of MEF treatment appears to reduce this content – longer treatment times (18 h and 30 h) seem to lead to a more noticeable reduction in the organic matter present in the supernatant than shorter treatment times (6 h), thus confirming previous results. As previously mentioned, this decrease in the organic matter content can be credited to a reduction in the presence of contaminants caused by electric treatment. Besides that, there might also be alterations in the liberation of intracellular compounds as a result of MEF treatment which can, in turn, be influencing the organic matter content present in the supernatant.

The variation in pigment content (Chlorophyll *a*, Chlorophyll *c1+c2* and Carotenoids) throughout growth as a result of MEF treatment is presented in Figure 15.

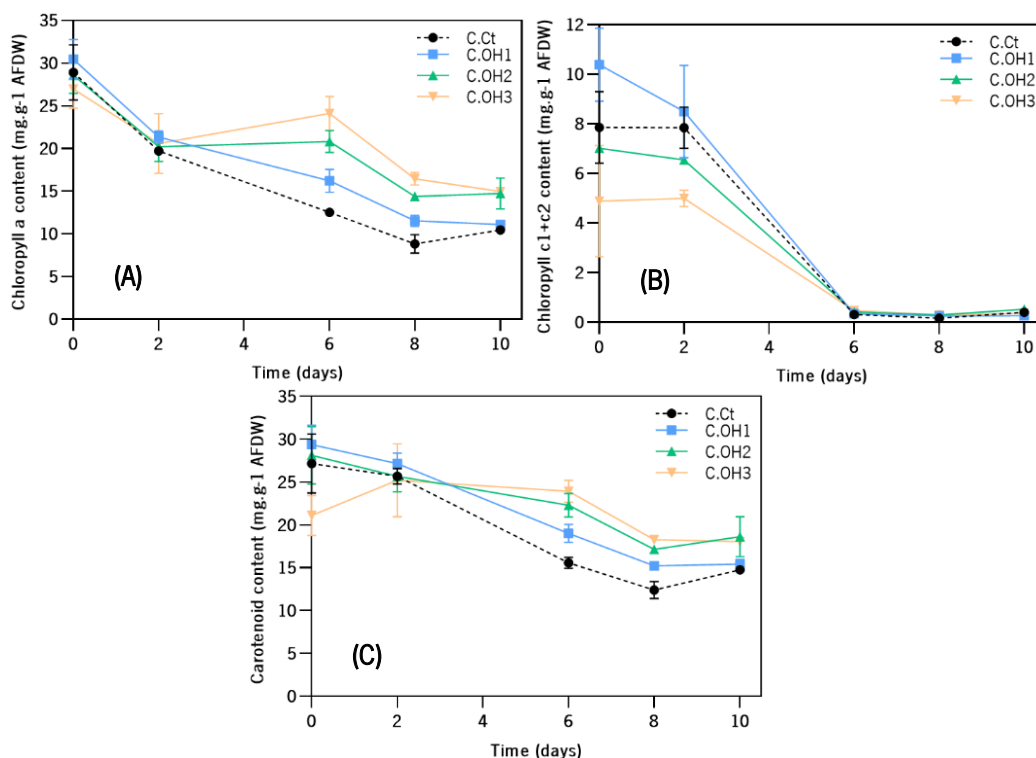


Figure 15. Variation of Chlorophyll *a* (A), Chlorophyll *c1+c2* (B) and Carotenoid (C) content (mg g⁻¹ AFDW) throughout growth for the control and treatments C.OH1 (6 h), C.OH2 (18 h) and C.OH3 (30 h) applied during the initial growth phase (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average. *Note:* samples corresponding to day 4 of growth were lost during extraction, therefore being absent from the graph presented.

Chlorophyll *a* content (**Figure 15A**) seems to decrease in the first two days of growth for both treated and untreated conditions; however, while control C.Ct and treatment C.OH1 cultures seemed to maintain that decreasing tendency, which has been previously associated with N consumption, cultures C.OH2 and C.OH3 increase after day 2 - reaching a Chlorophyll content of 20.8 and 24.1 mg g⁻¹ AFDW on day 6, the maximum value achieved, apart from the initial amount. After that increase, cultures C.OH2 and C.OH3 seem to follow the pattern described for C.Ct and C.OH1 cultures, but always at superior content levels. The Chlorophyll *c1+c2* content (**Figure 15B**) for both treated and untreated cultures decreases from day 0 to day 6, after which it seemed to stabilize at residual levels. The Carotenoid content (**Figure 15C**) displayed a different behaviour than the previously addressed pigments – there seems to be a constant decrease in content, a decrease that was most noticeable in the C.Ct samples, followed by treatment C.OH1 samples; however, no notorious alterations in its content were found as a result of MEF treatment, which is in accordance with what was previously reported for *C. vulgaris* and *C. kessleri* exposed to electric and magnetic field treatment [271][303][304].

Noticeable colour differences were found in the culture samples recovered on the 8th day of growth (**Figure SM6**) – treated cultures (C.OH1, C.OH2 and C.OH3) presented a more intense colour than control cultures (C.Ct), with colour intensity increasing with treatment time, a variation which is in accordance with the pattern found for Chlorophyll *a* content on that same day.

As a way to better compare the content levels at the beginning and at the end of each of the growth conditions tested, the variation in content from day 0 to day 10 for both control and treated cultures is displayed in **Figure 16**.

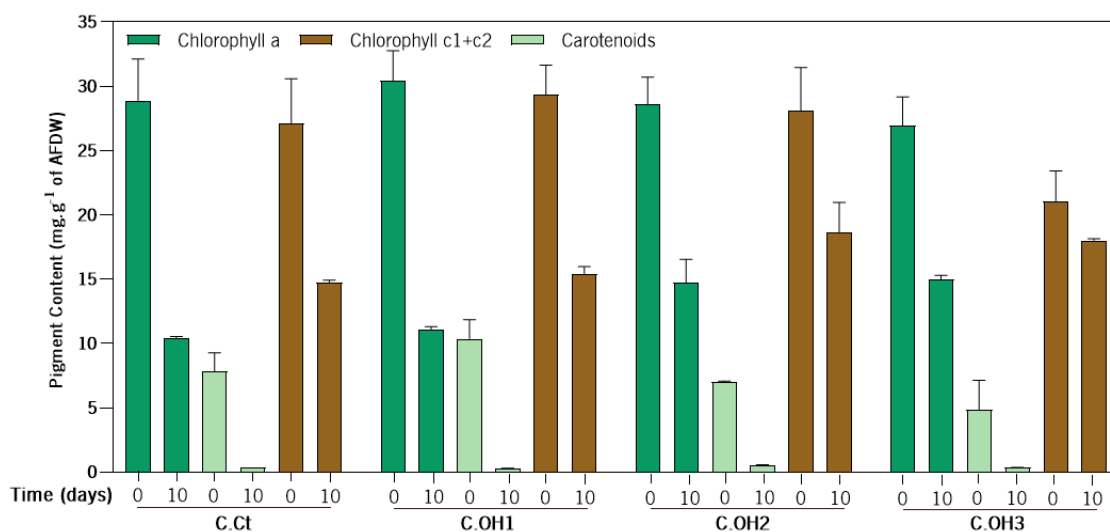


Figure 16. Variation of pigment (Chlorophyll *a*, Chlorophyll *c1+c2* and Carotenoids) content (mg g⁻¹ AFDW) at the beginning (day 0) and at the end of the growth (day 10) for the control (C.Ct) and treatments C.OH1 (6 h), C.OH2 (18 h) and C.OH3 (30 h) applied during the initial growth phase – and exposed to the same electric field strength (0.25 V cm⁻¹) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average.

As seen before with **Figure 15A**, the Chlorophyll *a* content always decreased from day 0 until day 10, however, it was most visible in control C.Ct and treatment C.OH1 samples, where there were reductions of 18.4 and 19.4 mg g⁻¹ AFDW, respectively. For treatments C.OH2 and C.OH3 there was still a decrease in content of 13.9 and 12.0 mg g⁻¹ AFDW, which was smaller than the ones suffered by C.Ct and C.OH1. Chlorophyll *c1+c2* also decreases in value from the start until the end of the growth for control and treated samples, with the most noticeable one being that of treatment C.OH1, where the content decreases 10.1 mg g⁻¹ AFDW. The Carotenoid content also suffered a reduction throughout time, with the most drastic one being that of treatment C.OH1 (14.0 mg g⁻¹ AFDW).

Biochemical analysis of the biomass recovered at the end of the growth allowed the understanding of the influence of MEF treatment under the conditions tested on the lipid, carbohydrate and protein content of treated samples (**Figure 17**).

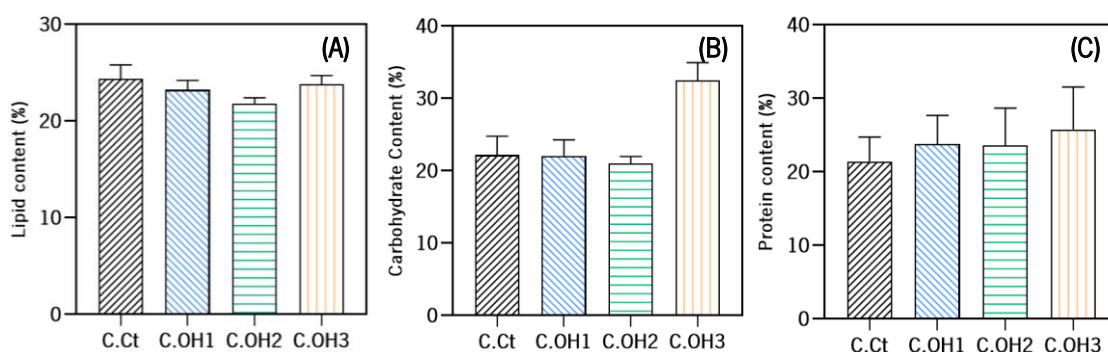


Figure 17. Variation of the lipid (A), carbohydrate (B) and protein (C) content (%) of the biomass of both untreated (C.Ct) and treated during the initial growth phase (C.OH1 – 6 h, C.OH2 – 18 h and C.OH3 – 30 h) at the end of the growth (day 10) (n=2).

Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.

The analysis of the lipid content at the end of the growth (**Figure 17A**) showed differences between treated and untreated cultures – in comparison with the lipid content of the control C.Ct cultures (24.4%), treated cultures C.OH1, C.OH2 and C.OH3 presented a lower value (23.2%, 21.6% and 23.8%, respectively), with treatment C.OH2 leading to the most noticeable reduction. The carbohydrate content (**Figure 17B**) seemed to also be influenced by treatment, not so much for cultures C.OH1 and C.OH2 (22.0% and 21.0%) in comparison with the control (22.2%), but mostly in the case of cultures C.OH3, where the carbohydrate content increased to 32.5%. The application of MEF treatment also seems to lead to alterations in the protein content (**Figure 17C**), with all treated cultures C.OH1, C.OH2 and C.OH3 (23.7%, 23.6% and 25.7%) presenting higher content levels than the control cultures (21.4%), with treatment C.OH3, once again, showing the most noticeable difference. No alterations in these biochemical components were found when *Chlorella vulgaris* at 0.25 g L⁻¹ was exposed to a MEF treatment of 2.77 kV cm⁻¹ [271].

5. Influence of MEF applications on the exponential growth phase for different periods of time

The effect of MEF treatment (an average of 0.58 V cm⁻¹) when applied to the exponential growth phase of *P. gyrans* was then studied, by applying a similar electrical treatment to the previous one, in different time periods for 6 h (D.OH1), 18 h (D.OH2) and 30 h (D.OH3), but starting on the fourth day of growth. The influence of treatment on the growth parameters followed is portrayed in **Figure 18**.

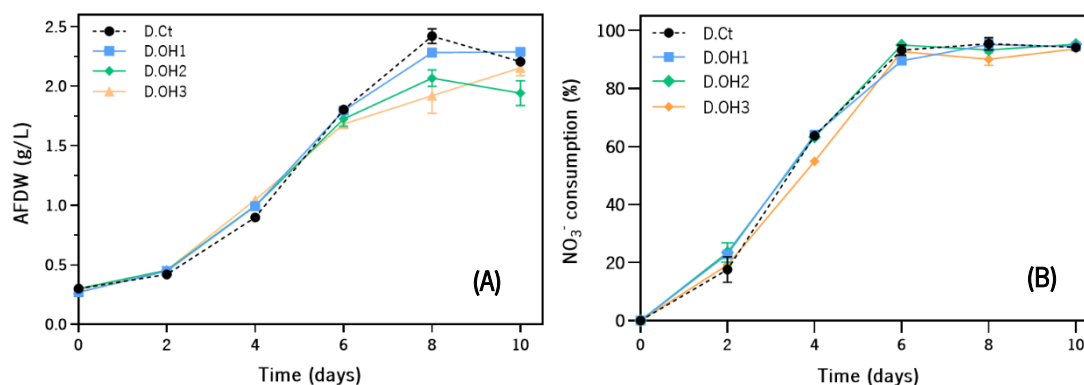


Figure 18. (A) Influence of the application of the electric field during the exponential growth phase from day 4 to day 6 in different periods of time – 6 h (D.OH1), 18 h (D.OH2) and 30 h (D.OH3) – on the AFDW (g L^{-1}) throughout time (days), as well as for untreated control cultures (D.Ct) ($n=2$). (B) Nitrate (NO_3^-) consumption (%) throughout growth. Error bars represent a variation coefficient resultant of the difference between sample and average.

According to **Figure 18A**, and in spite of the treatment being applied on the fourth day of growth, differences only became noticeable after the sixth day – until that point, both untreated (D.Ct) and treated cultures (D.OH1, D.OH3 and D.OH4) presented a similar growth pattern; yet, from that point on, there appeared to be a slight separation between D.Ct and D.OH1, that remained closer and at a higher AFDW content, while treated cultures D.OH2 and D.OH3 presented a lower content. A maximum AFDW content was achieved for the D.Ct cultures on the 8th day of growth (2.42 g L^{-1}). On day 8 there appears to be a tendential separation between the different treatments – as previously stated, the control presents the highest AFDW content, followed by the 6 h treatment (D.OH1), then by the 18 h treatment (D.OH2) and lastly the 30 h treatment (D.OH3) – seemingly showing that with the increase of treatment time, the influence of MEF on the growth was more noticeable, never leading, however, to growth arrest. No noticeable changes were found in the nitrate consumption profile (**Figure 18B**) to corroborate the differences found in growth after the 6th day; nevertheless, it's important to mention that after that day the amount of nitrate available for consumption was relatively low, therefore minimal alterations in pattern might not have been detected.

These differences between control and treated cultures and between the treatments themselves are also reflected on the parameters present in **Table 12**, with the control presenting the highest value for all three. Here, a MEF treatment of around 0.58 V cm^{-1} was applied to *P. gyrans* cultures with an average dry weight of 0.98 g L^{-1} . In a study by Nezammahalleh *et al.* [271] where an electric treatment of 2.77 kV cm^{-1} was applied to 1 g L^{-1} *C. vulgaris* cultures for periods ranging from 10 to 70 min, a promotion in growth was found for all treatments tested, with the most notorious stimulation in growth being attained for the longer treatments (50 and 70 min),

leading to a peak specific growth rate 20% higher than that of the control. Unlike what was found in that study, here decreases in the specific growth were found as a result of MEF treatment – with increasing treatment times leading to more noticeable decreases in the specific growth rate.

Table 12. Influence of MEF application on the growth parameters calculated for both control D.Ct and treated cultures (D.OH1 and D.OH2) – specific growth rate (μ_{max} , day⁻¹) calculated for the exponential growth phase; process productivity (P_x , g L⁻¹ day⁻¹) calculated from day 0 until the first day of the stationary phase; maximum biomass achieved throughout growth (X_{max} , g L⁻¹) (n=2; average value $\pm$ variation coefficient resulting of the difference between duplicate and average).

ID Code	μ_{max} (day ⁻¹)	P_x (g L ⁻¹ day ⁻¹)	X_{max} (g L ⁻¹)
D.Ct	0.365 $\pm$ 0.001	0.261 $\pm$ 0.002	2.421 $\pm$ 0.061
D.OH1	0.347 $\pm$ 0.001	0.267 $\pm$ 0.003	2.290 $\pm$ 0.038
D.OH2	0.334 $\pm$ 0.008	0.241 $\pm$ 0.003	2.068 $\pm$ 0.069
D.OH3	0.327 $\pm$ 0.005	0.235 $\pm$ 0.009	2.155 $\pm$ 0.067

The presence of organic matter on the supernatant of the cultures was followed throughout growth and is represented below as an IOMR factor (**Figure 19**).

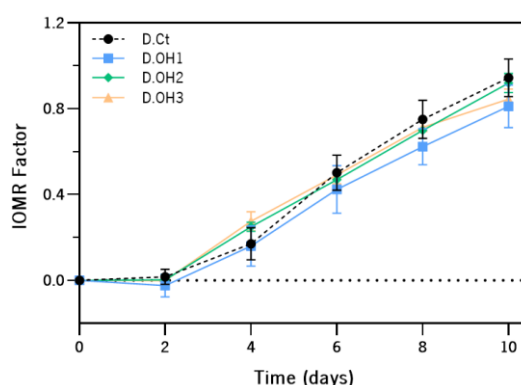


Figure 19. Variation of the IOMR factor of both untreated (D.Ct) and treated during the exponential growth phase (D.OH1 – 6 h, D.OH2 – 18 h and D.OH3 – 30 h) samples throughout time (days) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.

Unlike what was found in the previous experiments, no significant differences were detected in the IOMR factor of control D.Ct and treated cultures (D.OH1, D.OH2 and D.OH3). However, after day 6 all three treated cultures appeared to have a slightly lower organic matter content in the supernatant, with treatment D.OH1 leading to the lowest IOMR factor.

The influence of MEF treatment on the pigment content (Chlorophyll *a*, Chlorophyll *c1+c2* and Carotenoids) was followed throughout growth and is displayed below, in **Figure 20**.

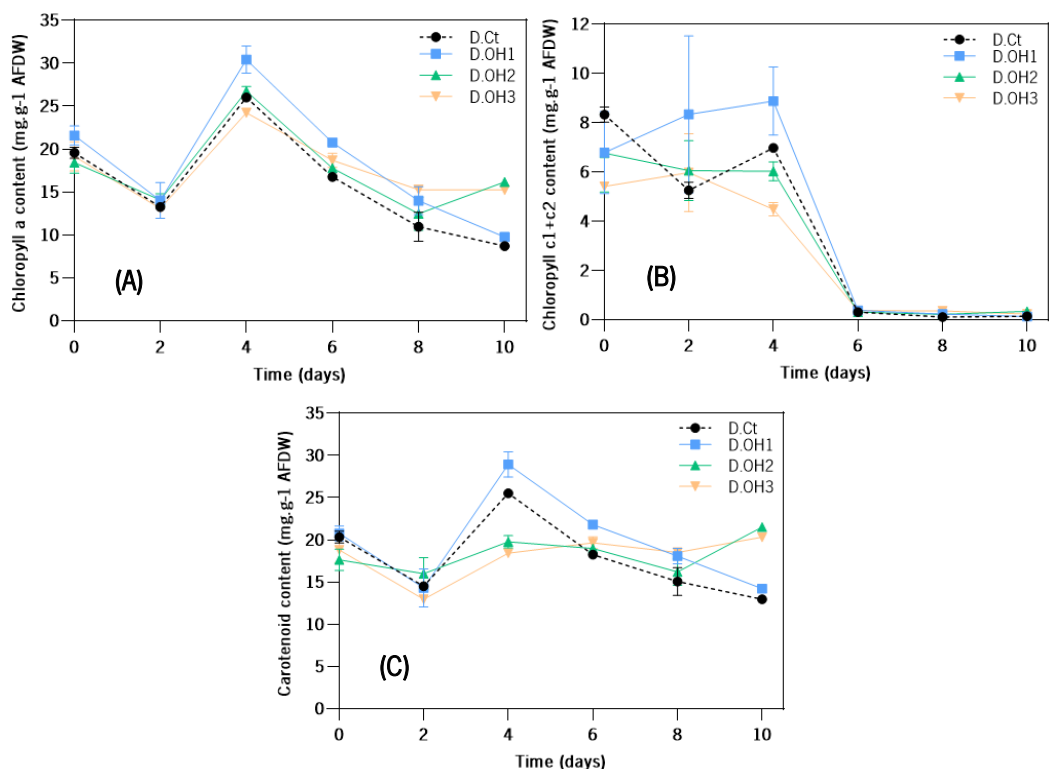


Figure 20. Variation of Chlorophyll *a* (A), Chlorophyll *c1+c2* (B) and Carotenoid (C) content (mg g⁻¹ AFDW) throughout growth for the control (D.Ct) and treatments D.OH1 (6 h), D.OH2 (18 h) and D.OH3 (30 h) (n=2) applied during the exponential growth phase. Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates (n=2).

The pattern followed by the Chlorophyll *a* content throughout growth (**Figure 20A**) didn't appear to suffer significant alterations when comparing control D.Ct and treated cultures D.OH1, D.OH2 and D.OH3 in the first two days. Nevertheless, after that, D.OH1 6 h treated cultures appear to suffer a higher increase in content up to 30.4 mg g⁻¹ AFDW, which was also the maximum content achieved throughout growth for any of the cultures. Chlorophyll *c1+c2* content (**Figure 20B**) also showed higher levels for cultures treated for 6 h (D.OH2) until day 4, after which the content of untreated D.Ct cultures and all treated cultures remained at residual levels until the end of the growth. As described by Nezammahalleh *et al.* [271] for 1 g L⁻¹ *C. vulgaris*, here the Chlorophyll content also increased until a maximum is reached (day 4), followed by a decline when exposed to EF treatment. The Carotenoid content (**Figure 20C**) displayed a similar behaviour throughout growth as the one described for Chlorophyll *a* content – after day 2, cultures D.OH1 showed an increase in content up to 28.9 mg g⁻¹ AFDW; after that point, the Carotenoid content of all cultures (untreated – D.Ct and treated - D.OH1, D.OH2, D.OH3) suffered a decrease until the end of the growth. However at end of the growth period (day 10) MEF treated samples D.OH2 and D.OH3 presented higher concentration of Carotenoids when compared with D.Ct samples. The consistent declines in the Chlorophyll *a* and Carotenoid contents observed in the control and D.OH1 cultures

after day 4, can be a result of the increasing algal concentration in suspensions of these cultures (from 0.90 g L⁻¹ AFDW on day 4 to 2.42 g L⁻¹ on day 8 for D.Ct and from 0.99 g L⁻¹ AFDW on day 4 to 2.28 g L⁻¹ AFDW for D.OH1), which can cause shading of light [271].

In order to better understand the differences that occurred from the beginning until the end of the growth, the pigment content on day 0 and on day 10 for both control (D.Ct) and treated cultures (D.OH1, D.OH2 and D.OH3) was presented separately (Figure 21).

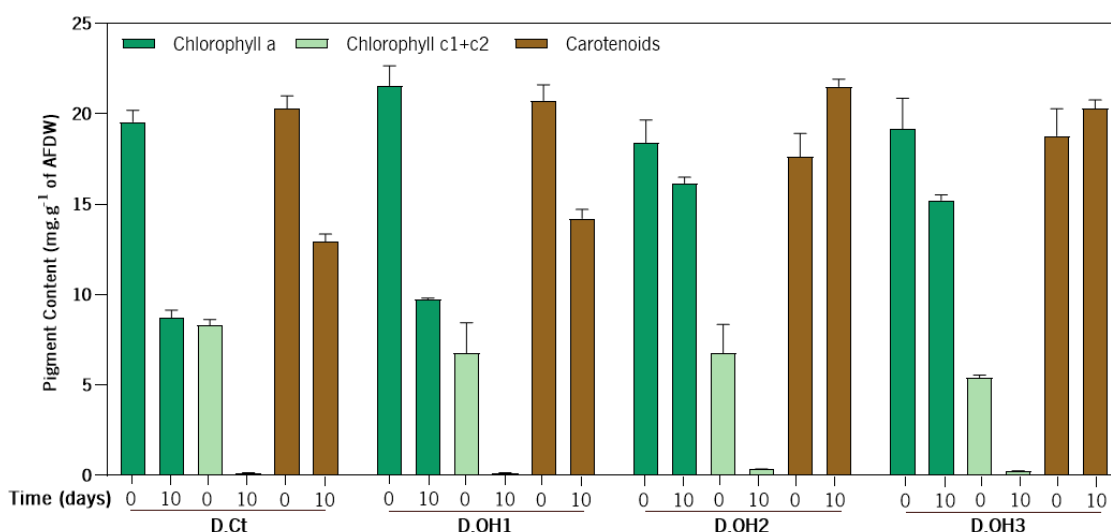


Figure 21. Variation of pigment (Chlorophyll *a*, Chlorophyll *c1+c2* and Carotenoids) content (mg g⁻¹ AFDW) at the beginning (day 0) and at the end of the growth (day 10) for the control (D.Ct), and treatments D.OH1 (6 h), D.OH2 (18 h) and D.OH3 (30 h) applied during the exponential growth phase. Error bars represent a variation coefficient resultant of the difference between sample and average.

In the D.Ct samples and in the ones treated for 6 h (D.OH1) it appears to be a drastic decrease of 10.9 and 11.8 mg g⁻¹ AFDW in the Chlorophyll *a* content from day 0 to day 10, while in the samples treated longer (18- and 30 h, D.OH2 and D.OH3, respectively) that decrease was less noticeable (2.25 and 3.93 mg g⁻¹ AFDW, respectively). When comparing the values of treated cultures at the end of the growth period to the ones shown by the control cultures, increases in Chlorophyll *a* content of 11.9%, 85.9% and 74.9% for treatments D.OH1, D.OH2 and D.OH3, respectively, were registered. No relevant differences were found in the Chlorophyll *c1+c2* content for either control or treated samples. A relevant finding, however, was that in all the treated cultures the Carotenoid content at the end of the growth, when compared to the control (D.Ct), increased in about 9.80%, 66.1% and 56.9% for the 6-, 18- and 30 h treatments, respectively, unlike what was described in previous studies for *C. vulgaris* and *C. kessleri* [271][303][304], where no significant alterations in the Carotenoid content as a result of electric or magnetic field treatment were reported. In this way, it can be concluded that the application of MEF treatment under the

conditions tested appears to influence both the pattern of Chlorophyll *a* and Carotenoid accumulation throughout growth, as well as the levels of both pigments at the end of the growth.

The biochemical analysis of the biomass recovered at the end of the growth period (**Figure 22**) revealed differences between treated and untreated cultures.

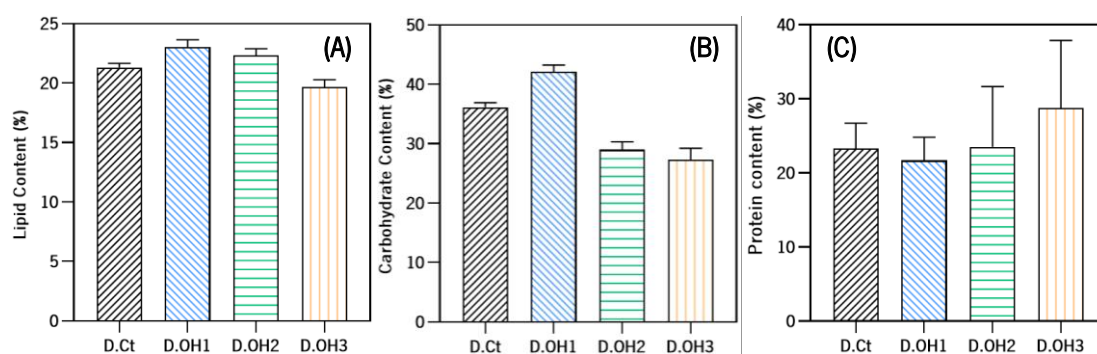


Figure 22. Variation of the lipid (A), carbohydrate (B) and protein (C) content (%) of the biomass of both cultures untreated (D.Ct) and treated during the exponential growth phase – D.OH1 (6 h), D.OH2 (18 h) and D.OH3 (30 h) at the end of the growth (day 10) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average.

In terms of the lipid content (**Figure 22A**), treatment for 6 h (D.OH1) and 18 h (D.OH3) led to higher values (23.0% and 22.3%, respectively), whilst 30 h of treatment led to a slight decrease in content (19.7%) when compared to the control (21.3%). The carbohydrate content (**Figure 22B**) also increased to 42.12% with the shorter treatment (D.OH1) in comparison with the control samples (32.2%); however, longer treatments led to decreases in the content (29.0% and 21.3% for D.OH2 and D.OH3). Protein content (**Figure 22C**) was affected differently, with the shorter treatment (D.OH1) causing a slight decrease in content to 21.7% when compared to the control D.Ct (23.3%), the 18 h treatment (D.OH2) causing no alteration (23.5%) and longer 30 h treatment (D.OH3) causing a slight increase to 28.7%. Unlike the results found in the present work, Nezammahalleh *et al.* [271] found no significant variation in the lipid, carbohydrate and protein content of 1 g L^{-1} *C. vulgaris* when a 2.77 kV cm^{-1} was applied.

6. Influence of MEF application in the dark period as an alternative to illumination

The possibility of applying MEF treatment to cultures while in the darkness instead of during the photoperiod was explored in this experiment – for 16 hours the cultures were artificially illuminated and during the remaining 8 hours of the day an electric field of 0.38 V cm^{-1} was applied. This treatment scheme was applied for 16 h, 32 h and 48 h starting on day 0 of the growth experiment, corresponding to treatments E.OH1, E.OH2 and E.OH3, respectively. The influence of the treatment on the growth parameters of the cultures is presented in **Figure 23**.

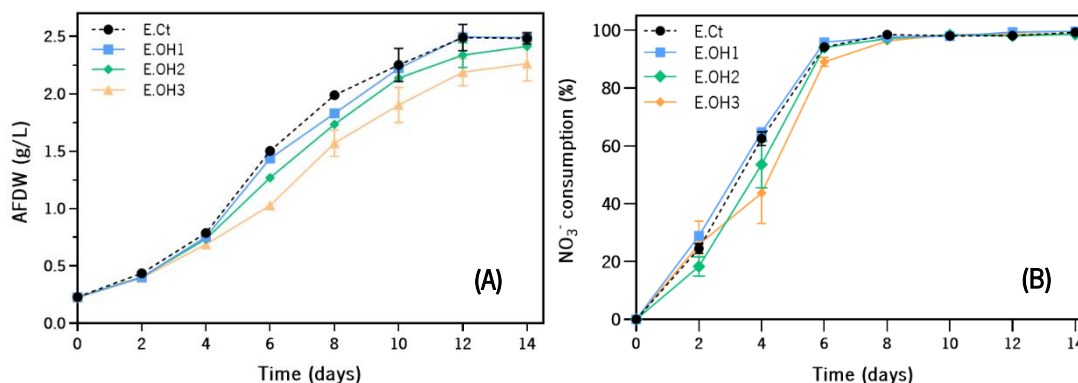


Figure 23. (A) Influence of the application of MEF treatment during night time for 8 h, in alternation with culture illumination during the remaining 16 h, for different periods of time starting on day 0 of the growth experiment – 16 h (E.OH1), 32 h (E.OH2) and 48 h (E.OH3) – on the AFDW (g L^{-1}) throughout time (days), as well as for untreated control cultures (E.Ct) ($n=2$). (B) Nitrate (NO_3^-) consumption (%) throughout growth. Error bars represent a variation coefficient resultant of the difference between sample and average.

Firstly, it's worth noting that, unlike the previous experiments where growth took 10 days until completion, in this one both untreated (E.Ct) and treated (E.OH1 and E.OH2) cultures took an additional 4 days as a result of the 8 h alteration in its photoperiod, not due to MEF application. Treatment application seems to influence the AFDW content of cultures throughout growth (**Figure 23A**) after the fourth day of growth – when the treatment scheme was applied for a shorter period of time (16 h, E.OH1), the dry weight of the cultures was maintained quite close to that of the control cultures (E.Ct); the application of the treatment scheme for 32 h (E.OH2) and 48 h (E.OH3) seemed to negatively influence the dry weight content of the cultures, with the longer treatment leading to a more noticeable decrease throughout growth. However, none of the treatments applied led to growth arrest, only to slight decreases in AFDW content. A maximum AFDW content was achieved for cultures E.Ct and E.OH1 (2.49 g L^{-1}) on the day 12 of growth.

The nitrate consumption profile (**Figure 23B**) shows a slight consumption delay in cultures E.OH2 between day 0 and day 6 and E.OH3 between day 2 and 8, after which nitrogen was apparently all consumed. When compared to previous experiments, where nutrient depletion happened after day 6 (with the exception of cultures A.Ct, A.OH1 and A.OH2), here the complete nutrient consumption by all cultures suffered a delay of 2 days (day 8) as a result of the photoperiod alteration. A longer delay (3.5 days) was described by Kato *et al.* [305] for *Chlamydomonas* sp. under light/dark conditions.

The dry weight content, the specific growth rate and process productivity (**Table 13**) calculated for cultures E.Ct and E.OH1 were quite similar; however, the parameters calculated for

cultures E.OH2 and E.OH3 showed decreases in comparison with the control, reflecting the differences found in the AFDW content.

Table 13. Influence of MEF application on the growth parameters calculated for both control E.Ct and treated cultures (E.OH1 and E.OH2) – specific growth rate (μ_{max} , day⁻¹) calculated for the exponential growth phase; process productivity (P_x , g L⁻¹ day⁻¹) calculated from day 0 until the first day of the stationary phase; maximum biomass achieved throughout growth (X_{max} , g L⁻¹) (n=2; average value $\pm$ variation coefficient resulting of the difference between duplicate and average).

ID Code	μ_{max} (day ⁻¹)	P_x (g L ⁻¹ day ⁻¹)	X_{max} (g L ⁻¹)
E.Ct	0.312 $\pm$ 0.003	0.199 $\pm$ 0.003	2.489 $\pm$ 0.114
E.OH1	0.308 $\pm$ 0.001	0.200 $\pm$ 0.004	2.494 $\pm$ 0.003
E.OH2	0.288 $\pm$ 0.003	0.194 $\pm$ 0.002	2.413 $\pm$ 0.010
E.OH3	0.257 $\pm$ 0.005	0.199 $\pm$ 0.001	2.262 $\pm$ 0.152

Shah *et al.* [93] also reported decreases in the dry weight content of *P. lutheri* cultures when the photoperiod was reduced from 24 h to 12 h; however, little differences were found in the specific growth rates calculated, even when cultures were grown under total darkness unlike what was found in the current experiment. Bashir *et al.* [104], however, reported that no alterations were found in the growth of phototrophic *P. lutheri* cultures as a result of alterations in the photoperiod (0, 6, 12, 18, and 24 h were investigated); nevertheless, in mixotrophic *P. lutheri* cultures maximum growth was found for 18 h of photoperiod, whilst superior ones led to slight reductions in cell density. Alterations in the illumination regime under which microalgae species other than *Pavlova* sp. were grown led to different responses. In *Nannochloropsis* sp., decreasing the photoperiod from 24 h to 18 h and 12 h led to decreases in cell density [306]; reductions in the biomass concentration (g L⁻¹) were also found for the same microalgae species when the photoperiod was altered from 24 h to 18 h, 12 h and 6 h [307]. However, in *Chlorella vulgaris*, the exposure to a photoperiod of 16 h led to the maximum growth rate and the maximum biomass content found in the study [308]. In the present experiment, a decrease in the specific growth rate of control cultures (0.312 day⁻¹) was found as a result of an 16 h photoperiod, when compared to the value found for previous experiments with 24 h illumination (an average of 0.358 day⁻¹). In addition, within this experiment, a reduction in the specific growth rate was also found as a consequence of MEF treatment.

When the illumination of different green algae was altered from constant 24 h illumination to a 12 h light : 12 h dark cycle, the growth of the cultures varied – the growth of *Botryococcus braunii* and *Scenedesmus obliquus* was more efficient under constant illumination, with the photoperiod of 12 h leading to considerable decreases in biomass productivity [309]. In the current

study, a similar result was found, with the productivity of the cultures under a 16 h illumination regime (considering E.Ct, 0.312 day^{-1}) being lower than the ones achieved in the previously addressed experiments under constant illumination (for instance 0.360 day^{-1} for cultures B.Ct and 0.365 day^{-1} for cultures D.Ct).

The presence of organic matter in the supernatant was followed throughout the growth of untreated and treated cultures by the calculation of an IOMR factor (Figure 24).

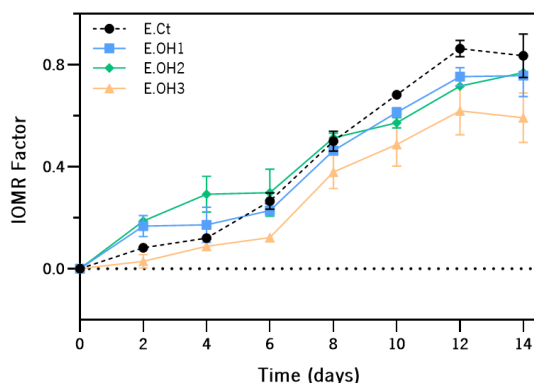
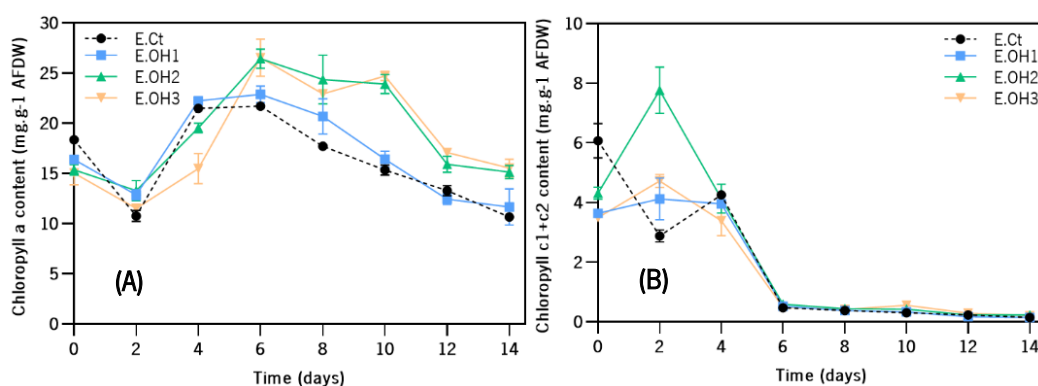


Figure 24. Variation throughout time (days) of the IOMR factor of both untreated cultures (E.Ct) and cultures treated during night time for 8 h, in alternation with culture illumination during the remaining 16 h of the day, for different periods of time (E.OH1 – 16 h, E.OH2 – 32 h and D.OH3 – 48 h) ($n=2$). Error bars represent a variation coefficient resultant of the difference between sample and average of the duplicates.

The organic matter content in the supernatant increased throughout growth for all the cultures; however, a few differences can be found between the different cultures – treated cultures E.OH3 always presented the lowest content levels; the organic matter content in the supernatant of cultures E.OH1 and E.OH2 is superior to that of the control from day 0 to day 6, but after day 8 the content in the supernatant of control cultures surpasses all others.

Analysis of the pigment accumulation profile (Figure 25) reveals noticeable fluctuations in content throughout time for untreated and treated cultures.



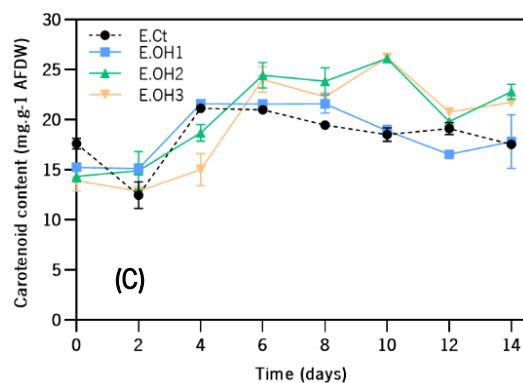


Figure 25. Variation of Chlorophyll *a* (A), Chlorophyll *c1+c2* (B) and Carotenoid (C) content (mg g⁻¹ AFDW) throughout growth for the control (D.Ct) and treatments E.OH1 (16 h), E.OH2 (32 h) and E.OH3 (48 h) (n=2), applied during night time in alternation with illumination during day time starting on day 0. Error bars represent a variation coefficient resultant of the difference between sample and average (n=2).

Alterations can be observed in the Chlorophyll *a* content throughout time (**Figure 25A**) – control E.Ct and treated cultures displayed a similar pattern in the beginning, with a decrease in content from day 0 to day 2, followed by an increase until day 4; however, after day 4 the Chlorophyll *a* content of cultures E.Ct and E.OH1 stayed constant until day 6 and afterwards decreased until the end of the growth. For cultures E.OH2 and E.OH3 after day 4 there was an increase in content, reaching at day 6 the maximum content of 26.4 and 26.5 mg g⁻¹ AFDW, respectively, followed by fluctuations until day 10 after which the Chlorophyll *a* content decreased until the end of the growth, always presenting higher levels than E.Ct and E.OH1. Chlorophyll *c1+c2* content (**Figure 25B**) also appeared to be influenced by treatment – while control cultures E.Ct suffered an initial decrease in content, followed by an increase and subsequent decrease until the content stabilizes at residual levels. For treated cultures there was an initial increase in content, followed by constant decreases until it stabilizes at the same residual levels. This increase in content was most noticeable for cultures E.OH2, reaching a maximum of 7.76 mg g⁻¹ AFDW on day 2. Carotenoid content throughout time (**Figure 25C**) also suffered alterations as a result of treatment, following a tendency similar to the one followed for the Chlorophyll *a* content. In cultures E.Ct and E.OH1, after a few less noticeable variations in the first two days of growth, the Carotenoid content increased until day 4, after which it seems to more or less stabilize until the end of the growth. As for cultures E.OH2 and E.OH3, after the initial increase from day 0 to day 4, the cultures suffered further increases in content, reaching higher Carotenoid contents than cultures E.Ct and E.OH1 and maintaining those higher levels until the end of the growth. Similar to what occurred to Chlorophyll *a* content, the Carotenoid content plateaued between day 6 and 10, but the maximum Carotenoid content was achieved on day 10 for cultures E.OH2 and E.OH3 (26.1 and 26.2 mg g⁻¹

AFDW, respectively); however, unlike Chlorophyll *a*, the subsequent decline in the Carotenoid content was almost negligible. It is also relevant to point out that while treatment was applied for 16 h, 32 h and 48 h after day 0, the most noticeable alterations in Chlorophyll *a* and Carotenoid content seem to appear only after day 4, when treatment had already ceased.

Other relevant conclusions can be taken when looking at the variation in pigment content from the start (day 0) until the end of the growth (day 14) – **Figure 26**.

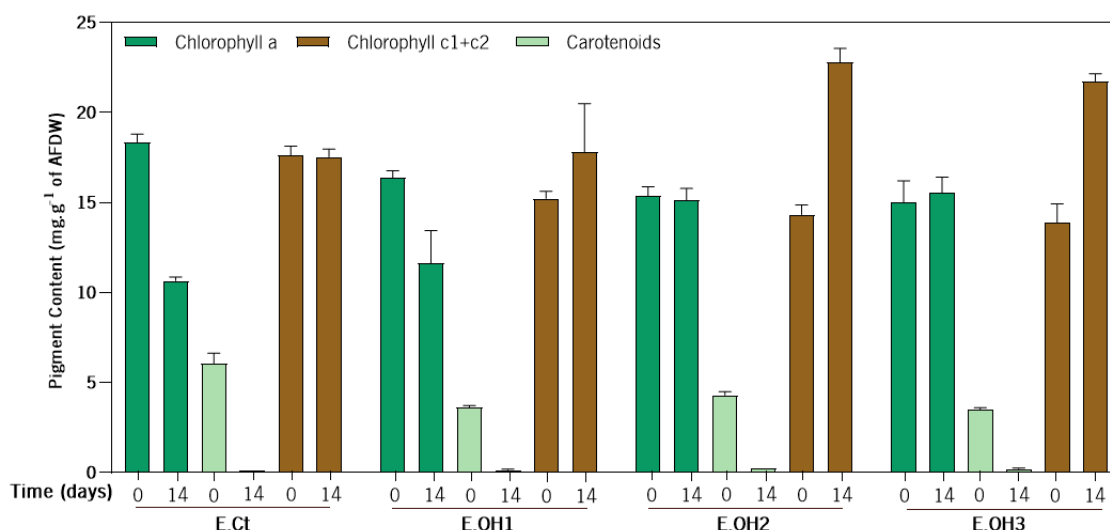


Figure 26. Variation of pigment (Chlorophyll *a*, Chlorophyll *c1+c2* and Carotenoids) content (mg g⁻¹ AFDW) at the beginning (day 0) and at the end of the growth (day 14) for the control (E.Ct), and treatments E.OH1 (16 h), E.OH2 (32 h) and E.OH3 (48 h), applied at the beginning of the growth during 8 h without illumination (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average.

While for control cultures E.Ct there was a considerable decrease of 7.71 mg g⁻¹ AFDW in Chlorophyll *a* content from day 0 to day 14, as seen with the previous experiments, for cultures E.OH1 that decrease was less noticeable (-4.73 mg g⁻¹ AFDW) and for cultures E.OH2 and E.OH3 the variations were negligible (-0.23 and +0.50 mg g⁻¹ AFDW, respectively). Up to this point, and in spite of treatments at times reducing the decrease in Chlorophyll *a* content from the beginning until the end of the growth, no treated or control culture showed close content values to the ones found at the end of the growth of cultures E.OH2 and E.OH3. Chlorophyll *c1+c2* content from start to the end of the growth period doesn't appear to be influenced by treatment, since its behaviour is quite similar in both control and treated cultures, but it is important to highlight that the final values are almost null. The Carotenoid content, however, showed quite relevant differences between control and treated cultures – for control cultures E.Ct, the Carotenoid content suffered a negligible decrease of 0.06 mg g⁻¹ AFDW from day 0 to day 14; yet, for treated cultures E.OH1, but specially

E.OH2 and E.OH3 there was an increase in content from day 0 to day 14, representing an increment of 1.56%, 29.9% and 23.9% when compared to the control.

It is known that varying the light intensity and the light : dark cycles under which microalgae are grown leads to significant changes in the pigment content [310]. In *Nannochloropsis* sp. reducing the photoperiod from 24 h to 18 h led to decreases of about 7.23 and 2.44 $\mu\text{g mL}^{-1}$ in the Chlorophyll *a* and Carotenoid content [307]. Cheirsilp and Torpee (2012), using *Chlorella* sp. and *Nannochloropsis* sp., found increases in the Chlorophyll content with increasing light intensities [311]. A similar effect was found in *Ankistrodesmus falcatus* as a result of increasing light intensities; in addition, photoperiods of 12 h and 18 h led to increases in the Chlorophyll *a* and Carotenoid content in comparison with 24 h illumination [312] – in that study, it was proposed that these alterations in pigment content are an adaptation mechanism driven by the exposure to high light. Similar results were found in the current study.

If a comparison is to be drawn between the Chlorophyll *a* content achieved at the end of the growth for cultures E.Ct (10.67 mg of Chlorophyll *a* per g of AFDW), grown under 16 h illumination, and the previous control cultures (an average of 8.35 mg of Chlorophyll *a* per g of AFDW), grown under continuous illumination (24 h), increases in content can also be found. The content in Chlorophyll *c1+c2* at the end of the growth of control cultures E.Ct grown under a light regime of 16 h (0.145 mg of Chlorophyll *c1+c2* per g of AFDW) was lower than the one found for control cultures in previous experiments (an average of 0.214 mg of Chlorophyll *c1+c2* per g of AFDW). The Carotenoid content of cultures E.Ct (17.55 mg of Carotenoids per g of AFDW) also appeared to increase in comparison with the one achieved for the control cultures in previous experiments (an average of 13.5 mg of Carotenoids per g of AFDW). Carotenoids in microalgae, accessory pigments involved in photosynthesis, are also known to function as structural components in the light harvesting complexes and to have a photoprotective role [313], so it would be expected for their content to increase with irradiance [105]. In the current experiment, growth under a photoperiod of 16 h appears to increase the Carotenoid content at the end of the growth (17.55 mg of Carotenoids per g of AFDW) in comparison with the content achieved under 24 h of illumination in previous experiments (an average of 14.62 mg of Carotenoids per g of AFDW). This increase in Carotenoid content at the end of the growth is further promoted by MEF treatment - 17.82, 22.80 and 21.75 mg of Carotenoids per g of AFDW (in cultures E.OH1, E.OH2 and E.OH3, respectively). In this way, these variations in pigment content appear to be caused by a synergistic

combination of the alteration in the photoperiod and of MEF treatment – nevertheless, further experiments would be required to support this claim.

In regard to the analysis of the biochemical composition of the biomass recovered at the end of the growth (**Figure 27**), some alterations in lipid, carbohydrate and protein content were found.

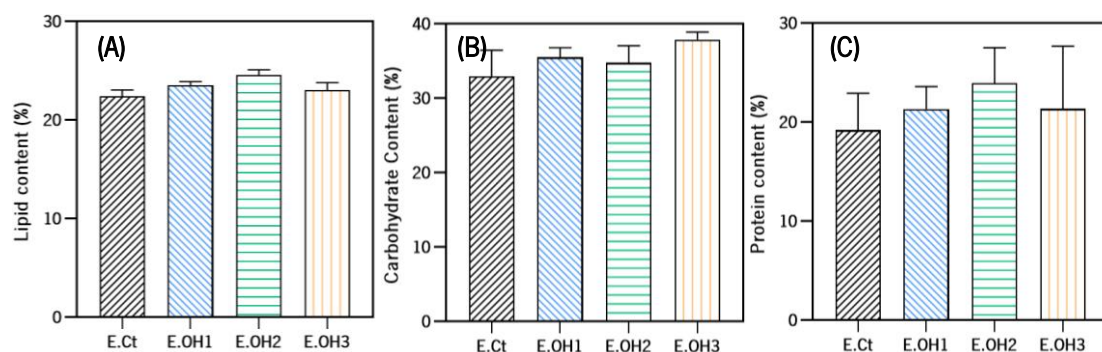


Figure 27. Variation of the lipid (A), carbohydrate (B) and protein (C) content (%) of the biomass of both cultures untreated (E.Ct) and treated for 6 h during the dark phase throughout different periods of time starting on day 0 – E.OH1 (16 h), E.OH2 (32 h) and E.OH3 (48 h) – at the end of the growth (day 14) (n=2). Error bars represent a variation coefficient resultant of the difference between sample and average.

As previously stated, the quality and quantity of light to which microalgae are exposed heavily influences both the growth rate and the composition of the biomass recovered [148].

The lipid content (**Figure 27A**) of cultures treated for 16 h, 32 h and 48 h (E.OH1, E.OH2 and E.OH3) only slightly increased (1.13%, 2.17% and 0.62%, respectively) in comparison with control cultures E.Ct. An increase of 5% in the lipid content of *P. lutheri* was reported by Shah *et al.* [93] when the photoperiod was reduced from 24 h to 12 h. In *Nannochloropsis* sp., maximum lipid content was achieved under a photoperiod of 18 h, an increase of about 3.35% in comparison with 24 h of constant illumination [306]. *Chlamydomonas* sp. grown under light/dark conditions presented a lower maximum lipid productivity in comparison with continuous illumination [305]. In the current experiment, besides the exposure to electrical treatment in the treated cultures, the photoperiod of all cultures was reduced from the 24 h of constant illumination used in the previous experiments to only 16 h of light. In this way, if a comparison was to be established not between the electrically treated cultures, but between control cultures E.Ct (22.4%), not electrically treated and grown with only 16 h of illumination, and the control cultures of previous experiments (an average of 24.1%), no significant increases in lipid content were found; in fact there was a slight decrease of about 1.70% in content. Perhaps reductions in the illumination period larger than the 6 h tested here would further promote lipid accumulation.

As aforementioned, a significant amount of studies using different microalgae species have reported alterations in the lipid content of cultures as a result of alterations in light intensity [314] [315][316][298][317]. In fact, Guedes *et al.* [152], described decreases in the total lipid content as well as drastic changes in lipid composition in *P. lutheri* under increasing light intensities. In *Chlorella vulgaris* changes in the light regimes also led to variations in the fatty acid composition [308]. Further studies on whether those alterations in the lipid content and composition would also occur if, besides varying light intensity, there was also electric field exposure would be relevant to investigate.

An increase was found in the carbohydrate content (**Figure 27B**) of treated cultures E.OH1, E.OH2 and E.OH3 (35.50%, 34.76% and 37.87%) in comparison with control E.Ct (32.94%). In *Thalassiosira pseudonana*, decreasing the photoperiod from 24 h to 12 h during the logarithmic phase led to a decrease in the carbohydrate content (from 19% to 6% of dry weight), while alterations in the photoperiod during the stationary phase caused a more noticeable decrease from 36% to 13% of the dry weight [314]. In *Ankistrodesmus falcatus*, the carbohydrate content increased with a regime of 6 h of light and 18 h of darkness (7.77%) in comparison with the one reached under constant illumination (5.91%) [312]. In the current experiment, when comparing, for instance, the carbohydrate content of the control E.Ct (32.94%) with the average carbohydrate content of the previous experiments (31.55%), a slight increase can still be found, regardless of it not being as noticeable as the one reported by Brown *et al.* [314], as a result of solely the alteration in the illumination regime. The cultures treated in the current experiment (E.OH1, E.OH2 and E.OH3), in addition to the stress caused by the alteration of the photoperiod, were also exposed to electrical treatment and yet show a higher carbohydrate content than the control – MEF treatment appears to be further promoting carbohydrate accumulation.

Similarly, the protein content (**Figure 27C**) seems to increase as a result of MEF treatment in comparison with the control – 19.18% for E.Ct, 21.32% for E.OH1, 23.94% for E.OH2 and 21.33% for E.OH3. In the present study, unlike what happened with the carbohydrate content, protein content attained for control E.Ct cultures as a result of the alterations in the illumination regime (19.18%) is somewhat inferior to the one achieved for control cultures under constant illumination in previous experiments (an average of 22.94%). This decrease is in accordance with what was found for *Ankistrodesmus falcatus*, in which protein content decreased from 38.6% to 37.9% when instead of 24 h illumination a photoperiod of 18 h was used [312]. Contrarily, in a study using *Chlorella vulgaris* and *Scenedesmus obliquus* exposed to alterations in the photoperiod,

higher protein contents were found for both species under a photoperiod of 14 h in comparison with continuous illumination [318].

Taking all this into account, the application of the combined treatment regime described appears to cause increases in the lipid, carbohydrate and protein content of all treated cultures, yet the magnitude of the increment varied according to treatment time – 32 h of treatment (E.OH2) led to the highest lipid and protein content attained, while the maximum carbohydrate content was achieved for 48 h (E.OH3) of treatment.

7. Influence of lower MEF application on the exponential growth phase for different periods of time

Considering the alterations found in the pigment content as a result of MEF application during the exponential growth phase (on day 4) for different time periods (6 h , 18 h and 30 h in treatments D.OH1, D.OH2 and D.OH3, respectively), in the current experiment a lower in amperage MEF treatment (0.54 V cm^{-1}) was also applied to *P. gyrans* during the exponential growth phase (1.6 g L^{-1}), but this time starting on day 6 and applied for 6 h (F.OH1) and 30 h (F.OH2). The influence of treatment on the growth parameters is displayed in **Figure 28**.

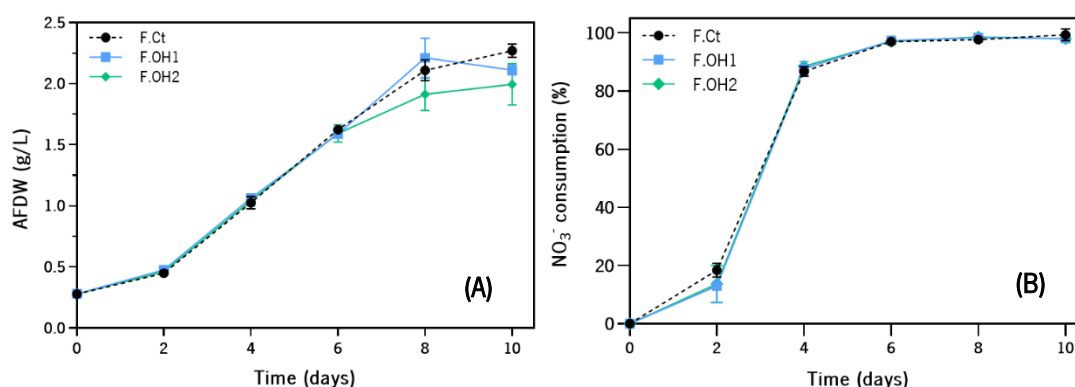


Figure 28. (A) Influence of the application of MEF treatment during the exponential growth phase (day 6) of different duration – 6 h (F.OH1) and 30 h (F.OH2)– on the AFDW (g L^{-1}) throughout time (days), alongside untreated control cultures (F.Ct) ($n=3$). (B) Nitrate (NO_3^-) consumption (%) throughout growth. Error bars represent the standard deviation of the data.

According to the variation of the AFDW of the cultures presented on **Figure 28A**, the application of MEF treatment on the 6th day of growth appears to lead to alterations – the dry weight of treated cultures F.OH1 and F.OH2 is negatively influenced by the treatment, since it leads to a reduction in content after application. Longer treatment time (30 h) further decreases the AFDW when compared to the shorter treatment applied (6 h). No alterations were visible in the nitrate consumption profile (**Figure 28B**).

Since the treatment was applied on day 6, the comparison between the specific growth rates (Table 14) calculated for the exponential growth phase (day 2 to day 6) doesn't contribute with any relevant information – up until the day 6, the triplicates of the different conditions tested (F.Ct, F.OH1 and F.OH3) were grown under the same conditions. The calculation of the process productivity reflects the slight increase in AFDW found for F.OH1 cultures on day 8.

Table 14. Influence of MEF application on the growth parameters calculated for both control F.Ct and treated cultures (F.OH1 and F.OH2) – specific growth rate (μ_{max} , day⁻¹) calculated for the exponential growth phase; process productivity (P_x , g L⁻¹ day⁻¹) calculated from day 0 until the first day of the stationary phase; maximum biomass achieved throughout growth (X_{max} , g L⁻¹) (n=3; average value $\pm$ standard deviation of the data). Statistical analysis performed using the D'Agostino & Pearson test, followed by the Kruskal-Wallis comparison test ($p < 0.05$) on Graphpad Prism 8; (*) stands for significantly different in comparison with the control; (ns) stands for non-significantly different in comparison with the control.

ID Code	μ_{max} (day ⁻¹)	P_x (g L ⁻¹ day ⁻¹)	X_{max} (g L ⁻¹)
F.Ct	0.324 $\pm$ 0.004	0.253 $\pm$ 0.006	2.270 $\pm$ 0.056
F.OH1	0.301 $\pm$ 0.001 (*)	0.258 $\pm$ 0.009 (ns)	2.212 $\pm$ 0.160 (ns)
F.OH2	0.310 $\pm$ 0.008 (ns)	0.240 $\pm$ 0.009 (ns)	1.995 $\pm$ 0.170 (ns)

The analysis of the organic matter present in the supernatant of the cultures throughout time (Figure 29) shows the influence of the treatment – after treatment application on day 6, there was an increase in content with increasing treatment time, with control cultures F.Ct displaying the lowest levels, followed by the 6 h treatment F.OH1 and then by the 30 h treatment F.OH2, that presented the highest level of organic matter in the supernatant.

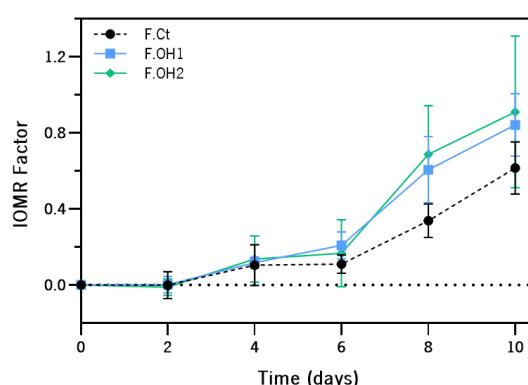


Figure 29. Variation of the IOMR factor of both untreated cultures (F.Ct) and cultures treated during the exponential growth phase (day 6) of different duration – 6 h (F.OH1) and 30 h (F.OH2) – throughout time (days) (n=3). Error bars represent the standard deviation of the data.

Unlike with previous experiments, here MEF treatment did not lead to a reduction in the organic matter present in the supernatant; in fact, after its application on day 6 that content increased. In this way, a reduction in contamination caused by electric treatment cannot be proposed here. However, it can be argued that, since in the current experiment MEF was applied

later on in the growth of non-axenic *P. gyrans* (day 6), the electric treatment applied might not have been as effective as before in controlling contamination. Besides that, MEF application in this later growth phase might have influenced the membrane permeability in a different manner, leading to more substantial leakage of intracellular compounds, in addition to the release that naturally occurs [287][288][289]. Studies suggest that, depending on the species, growth phase influences the cellular lipid yield and composition, which may influence the synthesis of membrane lipids [319]. In fact, the lipid classes of *Pavlova lutheri* vary throughout growth – during the lag phase, hydrocarbons are abundant (48%) and mostly non-polar; in the exponential phase, there is an overall decrease in total lipids; during the stationary phase, triglycerides accumulate, becoming the main lipid reserve (40%) [320]. In this way, the influence of MEF application on the cell membrane might be different in different growth phases. Nevertheless, additional investigation on the topic is necessary in order to draw any further conclusions.

The pigment content (Chlorophyll *a*, Chlorophyll *c1+c2* and Carotenoids) of both untreated and treated cultures was analysed throughout growth (Figure 30).

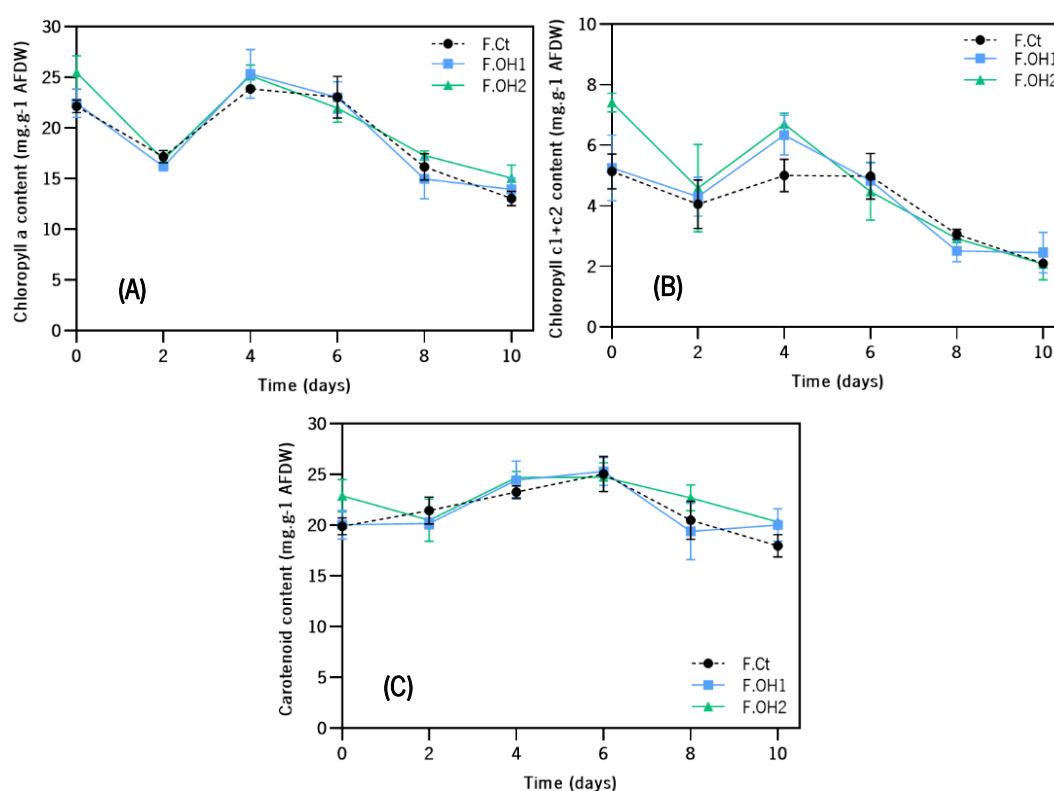


Figure 30. Variation of Chlorophyll *a* (A), Chlorophyll *c1+c2* (B) and Carotenoid (C) content (mg g⁻¹ AFDW) throughout growth of control cultures (F.Ct) and cultures treated during the exponential growth phase (day 6) for different periods of time – F.OH1 (6 h) and F.OH2 (30 h) (n=3). Error bars represent the standard deviation of the data.

The Chlorophyll *a* content (**Figure 30A**) didn't appear to suffer alterations throughout time as a result of treatment, with control F.Ct and treated F.OH1 and F.OH2 cultures presenting similar behaviours through growth – there is an initial decrease in content, followed by an increase that reaches the maximum content achieved throughout growth (25.3 mg g⁻¹ AFDW for cultures F.OH3), after which there are continuous decreases in content until the end of the growth. The Chlorophyll *c1+c2* content (**Figure 30B**) was also not apparently influenced by MEF treatment on day 6 – for untreated and treated cultures there was an initial decrease in content, followed by a slight increase (up to 6.71 mg g⁻¹ AFDW for cultures F.OH3) and afterwards there were decreases in content until the end of the growth. This decrease in Chlorophyll content after day 6 has also been present in the previous experiments described (with the exception of cultures E.OH2 and E.OH3, in which the decrease is more noticeable after day 8), which corresponds to the day after which nitrogen appears to be consumed in totality. It is in fact reported that N-starvation leads to decreases in the Chlorophyll content [321][322][323]. The Carotenoid content of the cultures throughout growth (**Figure 30C**) isn't altered as a consequence of MEF treatment – the pattern found for control cultures E.Ct is similar to the one found for treated cultures E.OH1 and E.OH2.

In spite of the pigment content not showing variations as noticeable as the ones found in previous experiments, the analysis of the biochemical composition of the biomass recovered at the end of the growth (**Figure 31**) shows differences as a result of treatment.

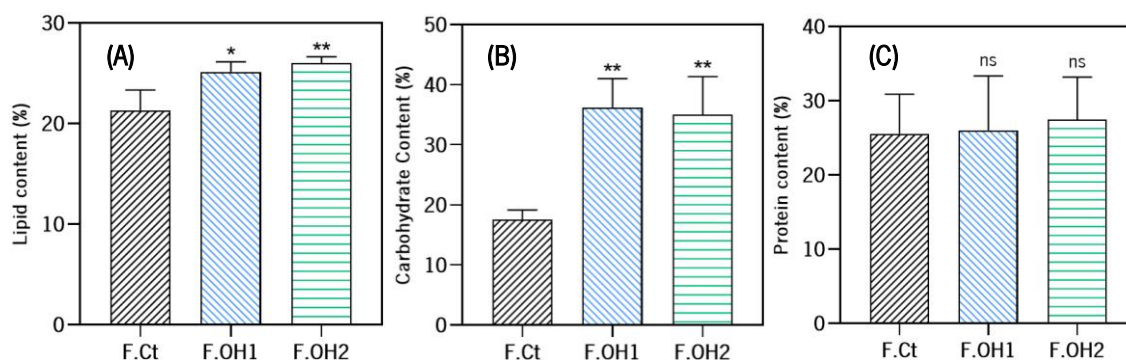


Figure 31. Variation of the lipid (A), carbohydrate (B) and protein (C) content (%) of the biomass of both cultures untreated (E.Ct) and treated for 6 h during the dark phase throughout different periods of time starting on day 0 – F.OH1 (6 h) and F.OH2 (30 h) – at the end of the growth (day 14) (n=2). Error bars represent the standard deviation of the data. Statistical analysis performed using the D'Agostino & Pearson test, followed by the Kruskal-Wallis comparison test ($p < 0.05$) on Graphpad Prism 8; * stands for significantly different in comparison with the control; "ns" stands for non-significantly different in comparison with the control.

In regard to the lipid content (**Figure 31A**), the application of MEF treatment caused significant increases of 3.85% and 4.72% in the content of both F.OH1 and F.OH2, respectively, in comparison to the control F.Ct and the lipid content significantly increased with increasing

treatment times. In the literature it is thoroughly reported that, when grown under altered/stressful conditions, microalgae suffer changes in their lipid content and composition – in order to deal with the stress that is limiting growth, whether that may be light intensity, temperature, salinity, nutrient, pH and UV-radiation, microalgae increase lipid and/or starch accumulation as a strategy to combat it [324]. For instance, it is described that in *P. lutheri* exposed to 8 days of UV radiation treatment there was a significant increase in cell lipid concentration [325]. The increases in lipid content found in the present study couldn't be attributed to nitrogen limitation, under which several microalgae accumulate lipids [326][327][328], since, as previously addressed, the nitrate consumption profile of control F.Ct and treated F.OH1 and F.OH2 (**Figure 28B**) was quite similar. It could also be argued that the significant increase in the lipid content might be due to the slight increase in temperature (from room temperature to 28 °C) resultant of the electric field application, since variations in the lipid content of *P. lutheri* as a consequence of alterations in the growth temperature have been reported [105][329]. However, in the previous experiments described, where the same temperature controlling system was used, the lipid content at times seemingly decreased as a result of electrical treatment.

The carbohydrate content (**Figure 31B**) also suffered significant increases with MEF treatment when compared to that of the control cultures F.Ct – increases of 18.65% and 17.46% for treatments F.OH1 and F.OH2, respectively. Nutrient limitation, such as nitrate and phosphorus, has been used as a strategy to increase carbohydrate content in microalgae [328][330][331]; however, as aforementioned for the lipid content, nitrate consumption in the cultures that presented higher carbohydrate content (F.OH1 and F.OH2) was quite similar to the one displayed by the control cultures F.Ct – in the current study, nitrate limitation didn't appear to be promoting carbohydrate accumulation.

The protein content (**Figure 31C**) wasn't significantly affected by MEF application.

In this way, despite not significantly affecting the pigment and protein content of the cultures, the application of MEF treatment under these conditions led to significant increases in both the lipid and carbohydrate contents of the cultures at the end of the growth when exposed to 6 h (F.OH1) and 30 h (F.OH2) of treatment.

8. Final remarks

In order to better compare the results found for the different conditions tested, the growth parameters calculated, biochemical characterization and pigment content of both control and treated cultures of all the experiments developed were summarized in **Table SM1**.

The lipid content achieved in all the experiments (an average of 23.4% for control cultures) is in accordance with what is described in the literature. The lipid content of *Pavlova lutheri* varied between 4.61% and 47.2% depending on the growth and stress conditions applied and on the method used for extraction [12][76][93][104][332][320], a range at times higher than the one found in the present study. *Pavlova pinguis*, nevertheless, has a lower described lipid content of about 7.6% [333]. *Pavlova salina* is reported to present a 35.5% lipid content [12]. The highest lipid content achieved as a result of electrical treated was found in cultures B.OH2 (26.0%), treated with 0.56 V cm⁻¹ from day 0 to day 2, and F.OH2 (26.0%), treated with 0.54 V cm⁻¹ for 30h on day 6. However, the lipid content of cultures B.OH2 represents a slight decrease from the one achieved for the control D.Ct of that experiment (26.24%), while the one reached in cultures F.OH2 represents a significant increase from the content of control cultures F.Ct (21.29%).

The carbohydrate content achieved in the present study (an average of 29.5% for control cultures) is superior to the one described for *P. lutheri* (9%) and *P. salina* (6.9–7.4%) [27][25]. However, it's important to note that certain pigment-based characterizations consider *P. gyraus* more closely related to *P. pinguis* [99], that reportedly has a superior carbohydrate content (41%) [27]. The highest carbohydrate content achieved in the previously described experiments as a result of MEF treatment was found for cultures D.OH1 (42.1%) as a result of a 0.58 V cm⁻¹ 6 h treatment, which was superior to the content found for control cultures D.Ct in the same experiment (36.2%).

Regarding the protein content, in the literature the content described for *P. lutheri* (29.0–29.7%), *P. salina* (24.2–29.0%) and *P. pinguis* (33.0%) [27][25] are quite similar but slightly superior to the one found in the current experiment for control cultures (an average of 22.7%). In the experiments aforementioned, maximum protein content was achieved for cultures D.OH3 (28.7%), treated with 0.58 V cm⁻¹ for 30 h starting on the 4th day of growth, which represented an increase in comparison with the control cultures D.Ct (23.3%). Cultures F.OH2 also presented a high protein content of 27.46%, representing a non-significant increase in comparison with control cultures F.Ct (25.5%) as a result of 30 h of 0.54 V cm⁻¹ treatment on day 6.

The methods used in the biochemical characterization of the samples only allowed quantification of the classes as a total – a finer analysis of these samples would be of value, since it would indicate what specific classes of compounds are responsible for the increases in total content. The analysis of the lipid classes that are contributing to the alterations in the total content would be of particular relevance. Members of the Pavlovophyceae family, to which *P. gyrans* belongs to, are capable of synthesising valuable long chain PUFA, such as DHA and EPA acids [72], a vital constituent of the diet of marine animals [103] – in this way, if the increases in lipid content found in the current experiments were due to increases in lipids containing PUFA, additional strategies could be considered in order to further promote its increase and its following extraction.

In the present study, Chlorophyll *a* content reached about 37.8% of total pigment content for control cultures at the end of the growth. The Chlorophyll *c1+c2* content was, on average, 1.60% of total pigment content at the end of the growth for control cultures, lower in content than the other pigments quantified, a result that was expected since Chlorophyll *c1+c2* is typically present in quite low quantities. According to the literature, Carotenoids account for the biggest class in the pigment composition of *P. gyrans*, with Fx and Ddx accounting for almost 30% of the total pigment load [99]. In the experiments developed, Carotenoids also dominated the total pigment content, accounting for an average of 59.7% of total pigments quantified for control cultures at the end of the growth.

Maximum Chlorophyll *a* content at the end of the growth was achieved for cultures D.OH2 (16.2 mg g⁻¹ AFDW) as a result of 0.58 V cm⁻¹ treatment for 16 h on day 4, which corresponds to almost double the content for control cultures D.Ct (8.71 mg g⁻¹ AFDW). Cultures E.OH2 and E.OH3 also display an interesting Chlorophyll *a* content at the end of the growth (15.1 and 15.5 mg g⁻¹ AFDW, respectively), which likewise represent an increase in comparison with control cultures E.Ct (11.7 mg g⁻¹ AFDW) as a result of 0.38 V cm⁻¹ MEF treatment applied in conjunction with a reduction to 16 h of light in the photoperiod for 32 h and 48 h, respectively. The Chlorophyll *c1+c2* content at the end of the growth reached maximum value in cultures F.OH1 (2.45 mg g⁻¹ AFDW), an increase comparatively to control cultures F.Ct (2.09 mg g⁻¹ AFDW) as a result of 0.54 V cm⁻¹ MEF treatment for 8 h. Carotenoid content was maximum for cultures E.OH2 and E.OH3 (22.8 and 21.8 mg g⁻¹ AFDW, respectively) as a result of 0.38 V cm⁻¹ MEF treatment applied in conjunction with a reduction to 16 h of light in the photoperiod for 32 h and 48 h, respectively. Further analysis

of the final pigment content would also be of value, since it would help comprehend to what classes the variations found can be credited to.

The organic matter content in the supernatant seems to always increase from the beginning until the end of the growth. However, there appears to always be a reduction in cultures where an electrical treatment was applied (with the exception of experiments E and F), with control cultures displaying a higher IOMR factor. These variations found can be attributed to alterations in the release of intracellular compounds as a result of exposure to electrical stress, as it has been reported for the release of extracellular polymeric substances from *Dunaliella salina* [292] and *Botryococcus braunii* [293] when exposed to salt stress. Besides that, the decrease found in the organic matter content of treated cultures can be due to a reduction in contamination as result of electrical treatment [192][207][227][228] or of those variations in the release of intracellular compounds from the microalgae cells that might, in turn, be influencing the microorganisms that grow mostly at the expense of these extracellular products [334]. The decrease in content can also be explained by possible precipitation or degradation of the organic matter - when under certain conditions, events such as sorption, colloid formation, precipitation or degradation can occur to dissolved organic matter [335], with biodegradation and photodegradation being the main processes influencing dissolved organic matter [336][337][338].

In order to screen for potential relationships between the various parameters studied and to better understand the impact of the different electrical treatments on the parameters tested, a Principal Component Analysis (PCA) was carried out (**Figure 32**) using the AFDW (g L^{-1}), pH, lipid, carbohydrate and protein content (%), Chlorophyll *a*, Chlorophyll *c1+c2* and Carotenoid content (mg g^{-1} AFDW) and organic matter content in the supernatant from the cultures used in the experiments from B to F. PCA enables the reduction of the dimensionality of the data, whilst keeping the majority of the data set variation [339], meaning that the most important information from the data table is extracted and is represented as a new set of orthogonal variables – the principal components [340], that are a result of linear combinations of the original variables and through which the data variation is maximal [341]. By plotting the data, it is possible to visually infer on the similarity or disparity amongst the samples and to understand if the samples can be separated into groups [341].

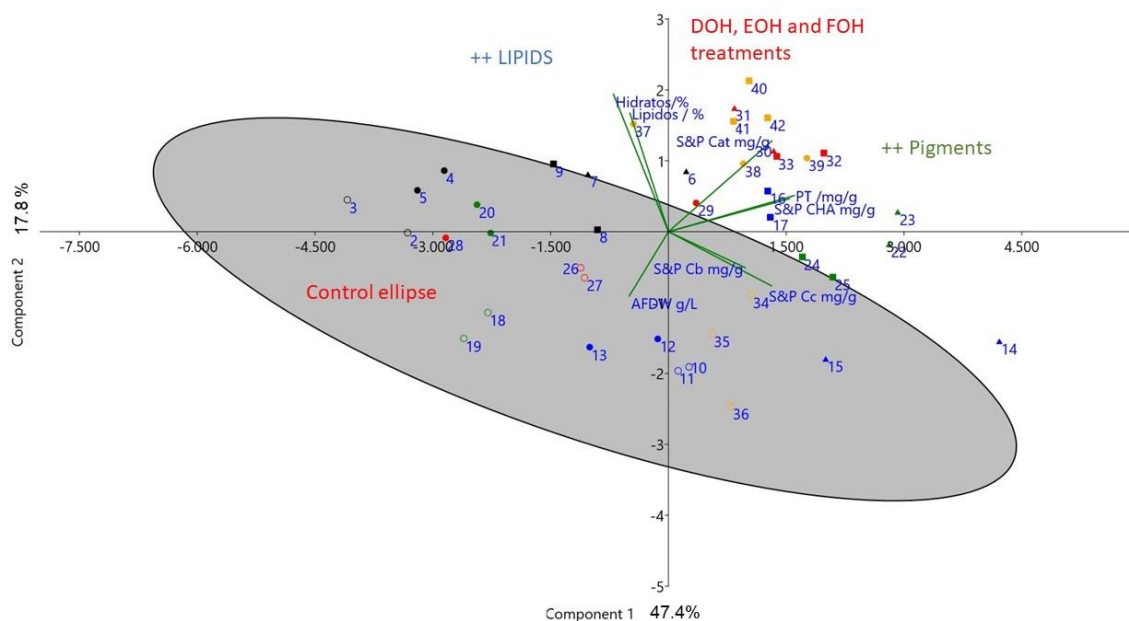


Figure 32. PCA plot in terms of the AFDW (g L^{-1}), pH, lipid, carbohydrate and protein content (%), Chlorophyll *a*, Chlorophyll *c1+c2* and Carotenoid content (mg g^{-1} AFDW) and organic matter content in the supernatant of control ($\circ$, unfilled, circumference in the corresponding colour) and treated ($\bullet$, $\blacktriangle$, $\blacksquare$, filled with the corresponding colour) cultures from experiments B (black icons, numbers 2-9), C (blue icons, numbers 10-17), D (green icons, 18-25), E (red icons, numbers 26-33) and F (orange icons, numbers 34-42). Analysis performed using Origin.

According to PCA, the first component (horizontal axis) and second component (vertical axis) correspond to approximately 50% and 20% of the variability in data, thus being acceptable to reduce data into a bivariate dimension. The top left quadrant encompasses the samples which displayed higher lipid and carbohydrate content, such as the treated samples from experiment B (B.OH1 and B.OH2); while in the bottom left quadrant are the samples which showed higher AFDW (g L^{-1}) at the end of the growth, for instance the control samples from experiments C (C.Ct), D (D.Ct) and E (E.Ct). On the top right quadrant are the samples that showed higher Chlorophyll *a* and Carotenoid content and also higher protein content – only treated cultures are displayed in this quadrant, with the treated cultures from experiments E and F being examples. On the bottom right quadrant, the samples with higher Chlorophyll *c1+c2* levels are displayed, i.e. the control from experiment F (F.Ct) and a few treated cultures. It is important to highlight that even when control samples (independently of the growth strategy) are included in an ellipse with 95% of confidence interval (represented in grey colour), MEF treated samples can be distinguished, meaning that inherent differences to each growth can be surpassed by the influence of the electric field. There appears to be a connection between the variations in pigment content, biochemical composition of the samples and growth parameters: cultures which displayed higher Chlorophyll *a*, Carotenoids and protein content, displayed lower carbohydrate levels, and vice-versa; cultures in which the

pigment content was superior, the growth was impaired, showing a lower AFDW and the other way around. These relationships can serve as a guiding method for choosing the approach to be followed, depending on the compound of interest – for instance, if the goal was to promote pigment accumulation, the experiments which resulted in the culture samples displayed in the top right quadrant should be chosen.

As previously addressed, not many studies in which the influence of electric field application on growth was investigated were found in the literature concerning microalgae biotechnology. There are, however, a few studies approaching static electric field (sEF) application and its effect on plant growth that can serve as a basis of comparison to the present study, in spite of not using similar EF strengths, growth conditions or experimental setup.

Studies on sEF application have shown varying consequences on the germination rate, growth and yield of plants [342]. In some, beneficial results were found with the exposure to sEF ($0.25\text{--}400\text{ kV m}^{-1}$) promoting germination, plant growth or its yield, for instance in barley [343], wax beans [344], potato seeds [345] and pea seeds [346]; nevertheless, in others EF treatment ($15\text{--}200\text{ kV m}^{-1}$) applied to orchard grass led to a deacceleration of growth [347], at times, to damage in the leaf tips [343][347] and to decrease in the crop yield of sorghum grains [348].

The influence of EF application on the metabolic activity of plants has also been studied – increases in the respiratory activity of several plant materials when exposed to sEF of $5\text{--}6\text{ kV m}^{-1}$ has been described [349]; a delay in biochemical reactions has been reported for tomato fruits exposed to $100\text{--}300\text{ kV m}^{-1}$ [350][351]. In *Taxus chinensis*, exposure to PEF (50 Hz , 10 V m^{-1}) appears to promote its secondary metabolism – a significant increase of 30% in the intracellular accumulation of Taxuyunnanine C was found when cells in the early exponential growth phase were treated for 30 min; results showed that PEF triggered a defence response and might have changed the dielectric properties of the cell or of its membrane [352]. It can be questioned whether such events would also happen to the microalgae used in the present work, in spite of the lower EF intensities used in this study - further fundamental investigation on the influence of EF treatment on the metabolic activity of *P. gyrans* would be required.

CONCLUSIONS AND FUTURE PERSPECTIVES

With the present work, the premise of evaluating the influence of MEF application on the growth of *P. gyrans* was achieved. In spite of not promoting growth, MEF treatment led to valuable alterations on the biochemical composition and pigment content that, if explored, could surpass the increase in the yield of added-value compounds only by marginal impacts on biomass productivity. A maximum increase of 85.8% was achieved for Chlorophyll *a* content and of 66.1% for Carotenoid content as a result of treatment. In terms of the biochemical composition, maximum increases of 4.72%, 18.7% and 5.41% were found for the lipid, carbohydrate and protein content, respectively. A finer analysis of the pigment content and biochemical composition is required in order to better understand what alterations are occurring in terms of compound classes present as a result of electrical treatment.

In the future, besides validating the results found here and completing the missing analyses, several other approaches could be explored. Further investigation on the combined effects of the factors photoperiod and light intensity in combination with MEF treatment could be developed, since with the current work only a very limited part of this approach was studied. The evaluation of the inactivation of microorganisms by the electric field, which is described in the literature as a consequence of EF application, would be quite interesting, since a reduction in competitive microbial contaminants would be useful. Since the current work was an attempt to understand what happened directly to the cultures when a treatment was applied, it would also be of value to understand what is fundamentally causing the alterations and what cellular mechanisms are being affected – possible shifts in metabolic pathways in which enzymes are being influenced; alterations in the photosynthetic machinery and activity that are leading to changes in the pigment accumulation pattern. Looking at EF application from a different perspective, while with the present work the main focus was the influence of treatment on growth, the evaluation of its effect on the harvesting of *P. gyrans* would also be valuable, since it is known to contain compounds of interest. The possibility of combining MEF treatment with other stress strategies described in the literature (salinity, medium supplementation, nutrient limitation) could also be explored. In future works, a finer control of the electrical and growth conditions would be required – here, variations were found between the control cultures of the different experiments which could be attributed to slight variations in the salinity of the cultures, influencing conductivity, or to an inadequate system assembly, which may have resulted in alterations in the spacing between the electrodes, thus influencing the EF applied.

The lack of studies on the short and long-term application of MEF treatment to microalgae that motivated the explorative approach taken in the present work was, at times, an obstacle, since no term of comparison and further discussion could be traced between the results found here and the ones described in the literature.

In this way, the present study demonstrated the potential of MEF application to microalgae as an innovative strategy to promote growth alterations and to influence the pigment accumulation patterns and the biochemical composition of the biomass achieved.

SUPPLEMENTARY MATERIAL

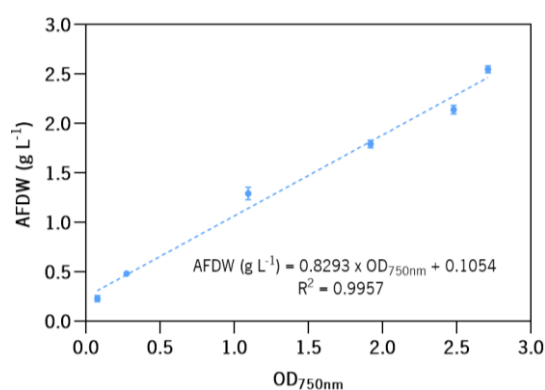


Figure SM1. Conversion factor calculated from the OD reading at 750 nm to the AFDW content (g L⁻¹) (n=3) – the resulting equation was used to infer on the AFDW content present in samples recovered from the experiments that followed. Error bars correspond to the standard deviation of the replicates.

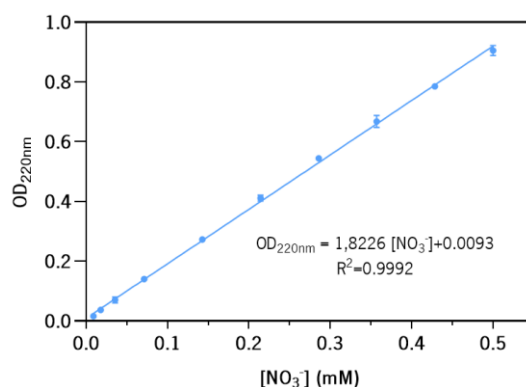


Figure SM2. Calibration curve for the determination of nitrate (NO₃⁻) concentration. Standards were prepared ranging in the NO₃⁻ concentration range 0-0.5 mM and the resulting equation was used to infer on the concentration present on the tested samples (n=3). Error bars correspond to the standard deviation of the replicates.

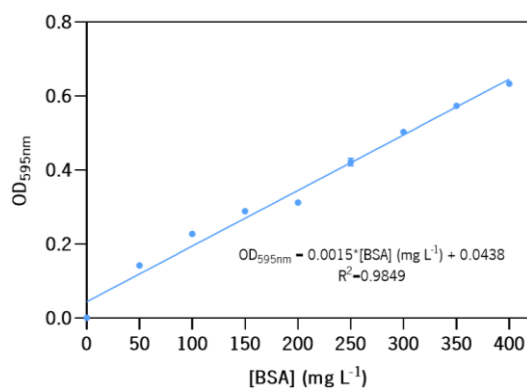


Figure SM3. Calibration curve for the determination of protein content present in the supernatant. Standards were prepared using BSA ranging in concentration from 0 to 400 mg L⁻¹ and the resulting equation was used to infer on the concentration present on the samples (n=3). Error bars correspond to the standard deviation of the replicates.

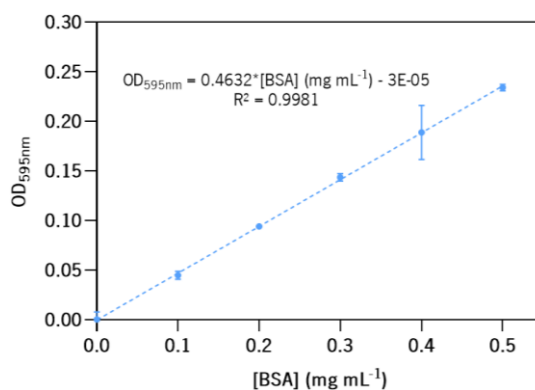


Figure SM4. Calibration curve for the determination of total protein content. Standards were prepared using BSA in concentrations in the range 0-0.5 mg mL⁻¹ and the resulting equation was used to infer on the concentration present on the samples (n=3). Error bars correspond to the standard deviation of the replicates.

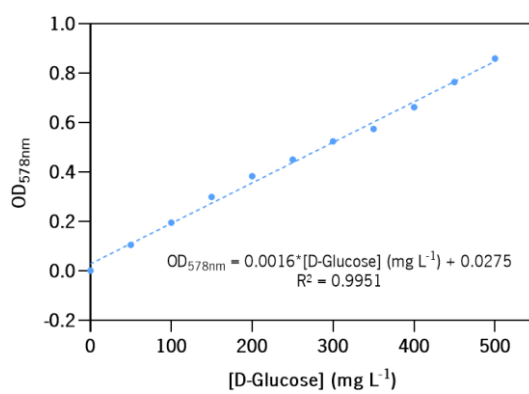


Figure SM5. Calibration curve for the determination of carbohydrate content. Standards were prepared using D-Glucose in the concentration range 0-500 mg L⁻¹ and the resulting equation was used to infer on the concentration present on the samples (n=3). Error bars correspond to the standard deviation of the replicates.

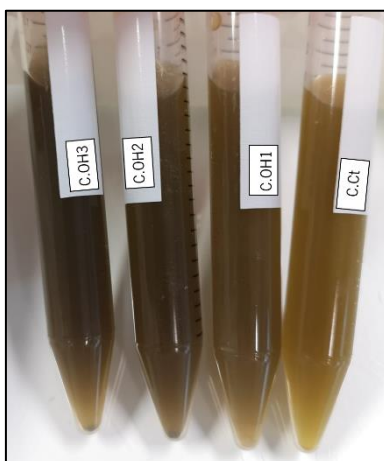


Figure SM6. Differences in colour found on the 8th day of growth of the experiment where MEF treatment was applied on day 0 for different periods of time – 6 h (C.OH1), 18 h (C.OH2) and 30 h (C.OH3) – using the same electric field strength (0.15 V cm⁻¹).

Table SM 1. Comparison between the growth parameters (specific growth rate - μ_{max} , day⁻¹; process productivity - P_x , g L⁻¹ day⁻¹; maximum biomass - X_{max} , g L⁻¹), the biochemical characterization (lipid, carbohydrate and protein content) and pigment content (Chlorophyll *a*, Chlorophyll *c1+c2* and Carotenoids) at the end of the growth for each of the experiments previously addressed with the corresponding identification codes (ID code) (- stands for not analysed) ($\pm$ standard deviation of the data or variation coefficient resulting of the difference between duplicate and average). When possible, statistical analysis was performed using the D'Agostino & Pearson test, followed by the Kruskal-Wallis comparison test ($p < 0.05$) on Graphpad Prism 8; * stands for significantly different in comparison with the control; "ns" stands for non-significantly different in comparison with the control.

ID code	Growth parameters ($\pm$ variation coefficient or standard deviation)			Biochemical characterization (% , $\pm$ standard deviation)			Pigment content (mg g ⁻¹ AFDW, $\pm$ variation coefficient or standard deviation)		
	μ_{max} (day ⁻¹)	P_x (g L ⁻¹ day ⁻¹)	X_{max} (g L ⁻¹)	Lipid content	Carbohydrate content	Protein content	Chlorophyll <i>a</i>	Chlorophyll <i>c1+c2</i>	Carotenoids
A.Ct	0.271 $\pm$ 0.005	0.238 $\pm$ 0.002	2.255 $\pm$ 0.013	24.65 $\pm$ 1.341	33.20 $\pm$ 3.465	-	-	-	-
A.OH1	0.249 $\pm$ 0.004	0.187 $\pm$ 0.001	2.155 $\pm$ 0.003	22.20 $\pm$ 1.209	23.83 $\pm$ 5.112	-	-	-	-
A.OH2	0.235 $\pm$ 0.001	0.181 $\pm$ 0.001	2.079 $\pm$ 0.010	24.35 $\pm$ 0.784	26.94 $\pm$ 1.940	-	-	-	-
B.Ct	0.360 $\pm$ 0.006	0.253 $\pm$ 0.001	2.177 $\pm$ 0.018	26.24 $\pm$ 1.475	34.69 $\pm$ 2.320	24.13 $\pm$ 3.831	5.877 $\pm$ 0.409	0.092 $\pm$ 0.025	12.83 $\pm$ 0.569
B.OH1	0.321 $\pm$ 0.009	0.246 $\pm$ 0.001	2.150 $\pm$ 0.002	26.02 $\pm$ 0.511	37.07 $\pm$ 0.836	24.62 $\pm$ 2.949	7.245 $\pm$ 0.116	0.099 $\pm$ 0.015	14.37 $\pm$ 0.442
B.OH2	0.319 $\pm$ 0.007	0.240 $\pm$ 0.003	2.004 $\pm$ 0.015	24.03 $\pm$ 0.365	32.16 $\pm$ 2.437	23.08 $\pm$ 3.663	9.867 $\pm$ 0.629	0.171 $\pm$ 0.030	19.90 $\pm$ 0.019
C.Ct	0.472 $\pm$ 0.005	0.307 $\pm$ 0.010	2.422 $\pm$ 0.118	24.37 $\pm$ 1.433	22.16 $\pm$ 2.582	21.37 $\pm$ 3.358	10.45 $\pm$ 0.098	0.402 $\pm$ 0.001	14.76 $\pm$ 0.167
C.OH1	0.469 $\pm$ 0.005	0.305 $\pm$ 0.006	2.388 $\pm$ 0.017	23.21 $\pm$ 0.978	21.98 $\pm$ 2.272	23.74 $\pm$ 3.919	11.07 $\pm$ 0.248	0.269 $\pm$ 0.032	15.42 $\pm$ 0.559
C.OH2	0.436 $\pm$ 0.018	0.284 $\pm$ 0.003	2.112 $\pm$ 0.123	21.75 $\pm$ 0.625	20.97 $\pm$ 0.968	23.58 $\pm$ 5.083	14.72 $\pm$ 1.816	0.523 $\pm$ 0.049	18.63 $\pm$ 2.336
C.OH3	0.413 $\pm$ 0.013	0.287 $\pm$ 0.002	2.074 $\pm$ 0.035	23.79 $\pm$ 0.894	32.47 $\pm$ 2.441	25.74 $\pm$ 5.764	14.99 $\pm$ 0.344	0.366 $\pm$ 0.011	18.03 $\pm$ 0.140
D.Ct	0.365 $\pm$ 0.001	0.261 $\pm$ 0.002	2.421 $\pm$ 0.061	21.30 $\pm$ 0.362	36.15 $\pm$ 0.763	23.33 $\pm$ 3.402	8.709 $\pm$ 0.439	0.148 $\pm$ 0.008	12.95 $\pm$ 0.402
D.OH1	0.347 $\pm$ 0.001	0.267 $\pm$ 0.003	2.290 $\pm$ 0.038	23.02 $\pm$ 0.610	42.12 $\pm$ 0.124	21.68 $\pm$ 3.149	9.745 $\pm$ 0.071	0.131 $\pm$ 0.020	14.22 $\pm$ 0.498
D.OH2	0.334 $\pm$ 0.008	0.241 $\pm$ 0.003	2.068 $\pm$ 0.069	22.32 $\pm$ 0.574	28.96 $\pm$ 1.351	23.52 $\pm$ 8.132	16.18 $\pm$ 0.323	0.349 $\pm$ 0.016	21.52 $\pm$ 0.411
D.OH3	0.327 $\pm$ 0.005	0.235 $\pm$ 0.009	2.155 $\pm$ 0.067	19.67 $\pm$ 0.602	27.27 $\pm$ 1.946	28.74 $\pm$ 9.128	15.23 $\pm$ 0.302	0.237 $\pm$ 0.020	20.32 $\pm$ 0.459
E.Ct	0.312 $\pm$ 0.003	0.199 $\pm$ 0.003	2.489 $\pm$ 0.114	22.39 $\pm$ 0.648	32.94 $\pm$ 3.505	19.18 $\pm$ 3.741	10.67 $\pm$ 0.207	0.145 $\pm$ 0.001	17.55 $\pm$ 0.417
E.OH1	0.308 $\pm$ 0.001	0.200 $\pm$ 0.004	2.494 $\pm$ 0.003	23.52 $\pm$ 0.371	35.50 $\pm$ 1.272	21.32 $\pm$ 2.274	11.65 $\pm$ 1.806	0.147 $\pm$ 0.041	17.82 $\pm$ 2.688
E.OH2	0.288 $\pm$ 0.003	0.194 $\pm$ 0.002	2.413 $\pm$ 0.010	24.56 $\pm$ 0.513	34.76 $\pm$ 2.301	23.94 $\pm$ 3.571	15.14 $\pm$ 0.648	0.226 $\pm$ 0.001	22.80 $\pm$ 0.769
E.OH3	0.257 $\pm$ 0.005	0.199 $\pm$ 0.001	2.262 $\pm$ 0.152	23.01 $\pm$ 0.785	37.87 $\pm$ 1.027	21.33 $\pm$ 6.326	15.54 $\pm$ 0.882	0.202 $\pm$ 0.066	21.75 $\pm$ 0.410
F.Ct	0.324 $\pm$ 0.004 •	0.253 $\pm$ 0.006 •	2.270 $\pm$ 0.056 •	21.29 $\pm$ 2.042 •	17.55 $\pm$ 1.580 •	25.54 $\pm$ 5.326 •	13.03 $\pm$ 0.716 •	2.091 $\pm$ 0.100 •	17.95 $\pm$ 1.100 •
F.OH1	0.301 $\pm$ 0.001 • (*)	0.258 $\pm$ 0.009 • (ns)	2.212 $\pm$ 0.160 • (ns)	25.14 $\pm$ 0.992 • (*)	36.21 $\pm$ 4.813 • (**)	26.03 $\pm$ 7.309 • (ns)	13.95 $\pm$ 1.143 • (ns)	2.452 $\pm$ 0.668 • (ns)	20.00 $\pm$ 1.616 • (ns)
F.OH2	0.310 $\pm$ 0.008 • (ns)	0.240 $\pm$ 0.009 • (ns)	1.995 $\pm$ 0.170 • (ns)	26.00 $\pm$ 0.643 • (**)	35.01 $\pm$ 6.360 • (**)	27.46 $\pm$ 5.716 • (ns)	15.07 $\pm$ 1.281 • (ns)	2.079 $\pm$ 0.530 • (ns)	20.30 $\pm$ 0.412 • (ns)

• $\pm$ standard deviation

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