

1 **Title: Media optimisation as a first step towards the feasible production of**  
2 **biopolymers with *Botryococcus braunii*.**

3  
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14  
15 **Short title:** Optimised media for feasible production of biopolymers with *B. braunii*.

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24 **Abstract**

25 Current interest in the production of commercial biopolymers which are derived from  
26 renewable resources arises from the necessity to move from a fossil-based to a more  
27 sustainable economy. Microalgae are considered potential biopolymers producers  
28 although their use at an industrial scale is not feasible yet. In this sense, medium  
29 optimization and operational conditions can be considered the first steps leading to  
30 growth and products productivity enhancement which are a prerequisite to boost the  
31 microalgae industry. This manuscript describes media optimization for two promising  
32 strains of *Botryococcus braunii*, *B. braunii* CCALA-778 (race A) and *B. braunii* AC761  
33 (race B), which are natural producers of (exo)polysaccharides and hydrocarbons of high  
34 interest in the biopolymers industry. Media optimization was based on a three steps  
35 research process: (i) a literature survey to identify macro- and micronutrients that could  
36 be limiting *Botryococcus* growth, (ii) a 3-level factorial design experiment in microwell  
37 plates to evaluate modified media accordingly, and (iii) validation of previous results in  
38 batch cultivation in Roux flasks.

39 The results obtained prove the significant impact of culture media optimization in the  
40 growth dynamics and valuable compounds accumulation of *Botryococcus braunii*.  
41 Attention should be given to each particular *Botryococcus* race and media should be  
42 defined accordingly. But overall, the new optimised media resulted in higher  
43 productivities which might contribute to reach the sustainability in *Botryococcus*  
44 cultivation.

45

46 **Keywords:** *Botryococcus braunii*, biopolymers, culture media optimization,  
47 hydrocarbons, carbohydrates, enhanced productivities.

48

49 **1. Introduction**

50 *Botryococcus braunii* is a green colony-forming microalga which has the almost unique  
51 capacity to synthesise, accumulate and excrete large amounts of long chain hydrocarbons  
52 and interesting groups of polysaccharides, which are convertible into bio-chemicals (Díaz  
53 and Atehortúa 2014). It is known that in other microalgae, exopolysaccharides (EPSs)  
54 have shown important biological activities such as antioxidant, antitumor, and anti-  
55 inflammatory (Talero et al. 2015, Yingying et al. 2014, Zhang et al. 2019). Some of them  
56 also perform as biomaterial and biolubricant (Arad et al. 2006). On the other hand,  
57 hydrocarbons are considered a potential source of renewable and sustainable biofuels as  
58 well as bio-polymers (Kita et al. 2010). In the last two decades, these characteristics have  
59 increasingly attracted the attention of researchers in attempts to exploit *B. braunii* as  
60 renewable source of products (Li and Qin 2005).

61 According to the metabolites that *Botryococcus* can produce, it is classified into three  
62 different races: race A produces odd-chain fatty hydrocarbons (alkadiene, alkatriene);  
63 race B produces polyunsaturated and branched triterpenes called botryococcenes and  
64 methylated squalene, and race L produces tetraterpenoids hydrocarbons from isoprene,  
65 namely lycopadiene (Yoshida et al. 2012). Regarding the EPS, its composition and the  
66 amount produced by *Botryococcus* also depends on the race, ranging from 0.250 g L<sup>-1</sup>  
67 (races A and B) to 1 g L<sup>-1</sup> (race L) (Allard and Casadevall 1990). Considering the extreme  
68 variability between the different *Botryococcus* races and strains, and their slow growth,  
69 working with *B. braunii* still presents important challenges (Banerjee et al. 2002). The  
70 main reasons for their slow growth and low productivities are related to the composition  
71 of the culture media (nutrient limitation/starvation) and the morphology of the colonies,  
72 where cells are tightly connected together not allowing a fast diffusion of nutrients (Li  
73 and Qin 2005). Furthermore, different species of *Botryococcus braunii* seem to have

74 different growth requisites and necessitate a specific fine tune of the media. In fact,  
75 differences in nutrients composition and concentration result in different biomass  
76 densities and morphologies, and affect the growth rate and the accumulation of  
77 intracellular compounds (Metzger and Largeau 2005, Yang et al. 2004). Therefore, an  
78 important tool to enhance *Botryococcus* productivity is to optimise the culture media.

79 The algae selection made for this work was based on a study carried out by Gouveia et  
80 al. (2017) and in the frame of the European research project SPLASH (Sustainable  
81 Polymers from Algae Sugars and Hydrocarbons) funded by the 7<sup>th</sup> Framework  
82 Programme. In that previous work, authors concluded, from a screening of 16 strains, that  
83 2 of them - CCALA-778 (race A) and AC761 (race B) - were the most promising in terms  
84 of carbohydrates and hydrocarbons production, respectively, for their further industrial  
85 application. In this regard, the aim of the present study was to optimise growth and  
86 metabolites production in these strains of *B. braunii*. Both microalgae were cultivated in  
87 microwell plates with 46 different media, which were defined according to a literature  
88 survey on culture media composition. The performance of the best three media for each  
89 strain were then evaluated in Roux flasks to validate the results. From that experiment,  
90 the best culture medium for each microalgal strain was selected based on the improved  
91 productivities.

92

## 93 **2. Material and Methods**

### 94 **2.1. Microalgal strains and culture conditions**

95 *Botryococcus braunii* CCALA-778 (race A) and *B. braunii* AC761 (race B) were  
96 obtained from the Culture Collection of Autotrophic Organisms, Trebon, Czech Republic  
97 and the Laboratoire de Chimie Bioorganique et Organique Physique, Ecole Nationale  
98 Supérieure de Chimie de Paris, France, respectively. In order to maintain viable cultures,

99 both strains were cultivated in modified Chu 13 medium (Gouveia et al. 2017) in 1L Roux  
100 flasks inside a culture room at 25 °C. Continuous fluorescent illumination was provided  
101 at an incident light intensity of 100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (Photosynthetic Active  
102 Radiation; PAR), and the cultures were bubbled with air containing 2.5% (v/v) CO<sub>2</sub> as  
103 the only carbon source.

104

## 105 **2.2. Maximum photosynthetic efficiency.**

106 Maximal photosynthetic efficiency of Photosystem II (PSII), also named as maximum  
107 Quantum yield ( $Q_y = F_v/F_m$ ), was used as an indirect indicator of cells viability (Maxwell  
108 and Johnson 2000). Chlorophyll fluorescence ( $F_v/F_m$ ) was determined using the pulse  
109 amplitude modulation (PAM) technique by an AquaPEN AP-100 device (Photon System  
110 Instruments, Czech Republic) according to Cuaresma et al. (2012). 2 mL aliquots of  
111 *B. braunii* cultures were dark-adapted during 15 min ( $F_0$ ) prior to the application of a  
112 saturating pulse of actinic light ( $F_m$ ). From these values the maximum quantum yield was  
113 calculated according to Cosgrove and Borowitzka (2011) (equation 1).

$$114 \quad \frac{F_v}{F_m} = \frac{(F_m - F_0)}{F_m}$$

115

## 116 **2.3. Biomass dry weight determination.**

117 For dry weight determinations, 2 to 5 mL aliquots of cell cultures (depending on cell  
118 density) were filtered through pre-weighed 2.7  $\mu\text{m}$  glass microfiber filters (Filter-Lab  
119 MFV4, Filtros Anovia, Barcelona, Spain) using a vacuum pump (Gast, DOA-P504-BN,  
120 United States) to separate the cells from the medium. The filters containing the algae  
121 were dried at 100 °C for 24 h in an oven (Mettler, Germany) and after that, were placed  
122 1 h into a desiccator to cool down. Dry weight was calculated as the difference between

123 the filter weight before and after the filtration step (Sartorius Balance, CP225D,  
124 Germany).

125

#### 126 **2.4. Productivity calculation**

127 Biomass volumetric productivities ( $P_v$ ) were calculated as the increase in dry weight  
128 ( $\Delta C_x$ ) in a given culture volume and for a specific period of time ( $\Delta t$ ) in the linear phase  
129 of the microalgal growth (equation 2).

$$130 \quad P_v = \frac{\Delta C_{x(t, t-1)}}{\Delta t_{(t, t-1)}}$$

131 Similarly, the specific productivity of each relevant metabolite (carbohydrates and  
132 hydrocarbons) produced by both microalgal strains was calculated as the increase in the  
133 concentration of this product in a given culture volume, over a given period of time.

134

#### 135 **2.5. Extraction of carbohydrates.**

136 Carbohydrates content in *B. braunii* CCALA-778 was analysed according to a  
137 colorimetric method based on Dubois principle (Dubois et al. 1956). 3-10 mg of freeze-  
138 dried biomass were used. Acid hydrolysis was carried out by addition of HCl 2.5 M and  
139 3 hours of incubation at 100 °C in a waterbath. After that, samples were neutralized with  
140 NaOH 2.5 M and incubated in a water bath at 35 °C with phenol and H<sub>2</sub>SO<sub>4</sub>, resulting in  
141 an orange-yellow coloured complex compound which could be measured  
142 spectrophotometrically at 483 nm (Thermo Fisher Scientific, Evolution 201, United  
143 States).

144

#### 145 **2.6. Extraction of hydrocarbons**

146 Hydrocarbons in *B. braunii* AC761 were extracted using organic solvents  
147 (methanol:dichloromethane) according to the methodology describes by Folch et al.

148 (1957), and Bligh and Dyer (1959). It was necessary 1mL of sample with a minimum  
149 biomass concentration of 0.5 g L<sup>-1</sup>. Samples with the solvents were incubated in an  
150 incanter (Rotator SB3, Stuart, UK) for 4 hours. After that time, it was added NaCl in order  
151 to obtain two phases. The phase which contained the hydrocarbons was dried under  
152 nitrogen gas, re-suspended in 1mL of hexane and later identified and quantified by gas  
153 chromatography (Agilent 7890A, FID detector, United States). Squalene (SUPELCO  
154 442785, C30 analytical standard, USA) was used as standard for the quantification.

155

### 156 **2.7. Quantification of hydrocarbons by gas chromatography (GC-FID).**

157 For the quantification of hydrocarbons, a gas chromatographer was used (Agilent 7890A,  
158 FID detector, United States). Squalene was used as analytical standard for hydrocarbons  
159 quantification (SUPELCO 442785, C30 analytical standard, USA). It included an  
160 automatic sampler (7683 B) and an FID detector, with a fused silica capillary column  
161 (Restek Rxi-5ms) 30 m long, 0.25 mm wide and 0.25 µm internal diameter. The carrier  
162 gas was He, and the flame was obtained with a mixture of H<sub>2</sub>: Air (40:400 mL·min<sup>-1</sup>). In  
163 order to quantify the hydrocarbons which were present in each sample, a calibration curve  
164 with a squalene pattern was previously made at concentrations ranging between 50 and  
165 850 ppm. Concentration of hydrocarbons was calculated according to the area of the  
166 chromatogram peaks and the equation obtained during the calibration process.

167

### 168 **2.8. Experimental approach**

169 Media optimization started with a literature survey, in which different media used for  
170 *B. braunii* cultivation were identified. It allowed to recognize critical micro- and  
171 macronutrients which might need to be optimised. An experimental design was defined  
172 based on the results from the media analysis and the corresponding experiments were

173 carried out in micro-well plates. 46 new media (Online Resource 1) were evaluated in  
174 order to identify potential media which might allow better productivities. Finally, the best  
175 media were validated in a new experiment in Roux flasks and optimised media could be  
176 selected for each *Botryococcus* strain.

177

#### 178 *2.8.1. Literature survey and experimental design*

179 As a first approach, a literature survey was carried out with the purpose of identifying the  
180 culture media in which *Botryococcus* strains are normally cultivated. The reference media  
181 used in this work (modified Chu 13) (Gouveia et al. 2017) was then compared to the  
182 identified media in terms of molar composition, as well as to other standard culture media  
183 commonly used to grow other microalgal species. The comparison might allow to identify  
184 possible nutrient limitations in the reference media to be optimised. In total, the  
185 composition of 38 culture media was analysed and the relative weight of each nutrient  
186 was assessed in terms of frequency of occurrence and average concentration. Comparison  
187 of reference medium nutrients with the calculated average concentrations allowed the  
188 identification of macro- and micronutrients to be optimised. In order to simplify the  
189 comparison, the media were classified in 2 groups: 1) culture media commonly used to  
190 grow microalgae, and 2) culture media commonly used to grow *Botryococcus* strains. For  
191 each group, a Box Plot analysis was done in order to visually summarize, compare and  
192 identify outlier data during the comparison of the molar composition of the media.

193 The 5 chemical elements which resulted in the highest deviations to the average  
194 concentration values obtained for other media -which already proved to allow high  
195 biomass productivities- were selected to be included in the experimental design. A 3-level  
196 factorial experimental design (Design Expert software) was used to define 46 different

197 combinations of the selected nutrients at different concentrations to be experimentally  
198 tested in well-plates.

199

#### 200 2.8.2. Media optimisation in well-plates

201 The optimisation experiment was carried out axenically in 24-well plates incubated at  
202 25 °C in a CO<sub>2</sub> enriched environment, with **continuous** incident light of 100 μmol photons  
203 m<sup>-2</sup> s<sup>-1</sup> and mechanical agitation by rotation in an Orbital Shaker (OL30-HE, OVAN,  
204 Spain). Prior to the real experiment, both *B. braunii* strains were pre-adapted to the new  
205 media and to the experimental conditions by incubation in the well-plates for one week  
206 at an initial optical density (O.D.<sub>750nm</sub>) of 0.1 and 0.2 for *B. braunii* CCALA-778 and  
207 AC761 respectively. After that time, biomass from each media was independently  
208 harvested, centrifuged and used to inoculate new plates for the real experiment. In both  
209 stages the different algae strains were inoculated at a low cell density with the aim to  
210 avoid photo-limitation in the plates. In order to avoid contamination, all the material used  
211 was autoclaved, media were sterilised by sterile filtration (0.2 μm pore size syringe filter,  
212 Labware, USA) and manipulation was done inside a laminar flow cabinet (Telstar PV-  
213 100, Terrassa, Spain).

214 Each plate row contained a different media, inoculated in 5 replicates plus a media blank.  
215 Moreover, in order to confirm the conditions inside the plates were reproducible, each  
216 plate contained the reference media in a different position.

217 Growth was daily monitored during one week with a plate reader (Elx 800, Biotek, Bad  
218 Friedrichshall, Germany). Optical density at 680 and 750 nm was measured and values  
219 for the different media were compared with the reference medium. After one week all the  
220 biomass was harvested and pH, photosynthetic efficiency, dry weight, carbohydrates

221 and/or hydrocarbons content were analysed. The three media with the best growth and  
222 product productivity were pre-selected for each *Botryococcus* strains.

223

224

### 225 2.8.3. Validation of Well-plates results in Roux flasks

226 The performance of the best 3 media from the well-plates experiment was validated at a  
227 larger volume. For that, Roux bottles were selected as cultivation flasks because of their  
228 volume (800 mL) and their flat shape. Compared to Erlenmeyer flasks, the shorter light  
229 path allows a better light integration. Performance of pre-selected media was compared  
230 to reference medium (Gouveia et al. 2017) in terms of growth and product accumulation.  
231 Prior to the real validation, biomass was acclimated to the different media and the  
232 cultivation conditions for 3 weeks (until stationary phase was reached). After that period,  
233 biomass was partially harvested, centrifuged and used to inoculate the new flasks in  
234 **experimental duplicates** with fresh media at an initial optical density of 0.1 (OD at 750  
235 nm). Daily analysis included optical density, pH and photosynthetic efficiency (measured  
236 as maximal efficiency of Photosystem II). Dry weight and product (carbohydrates and  
237 hydrocarbons) content were punctually analysed along the growth curve which allowed  
238 getting insight into product accumulation.

239

## 240 2.9. Statistical Analysis

241 Unless otherwise indicated, the presented data are the average value of **four replicates (2**  
242 **experimental replicates + 2 analytical replicates)** and they are expressed as means with  $\pm$   
243 standard deviation (SD). Mean values are presented in the corresponding figures with  
244 error bars as standard deviation. The differences among reference medium and the new  
245 culture media were evaluated using one-way analysis of variance (ANOVA). If ANOVA

246 results were significant, comparisons among means were followed by a post-hoc using  
247 Fisher's Least Significant Difference (LSD) multiple comparison tests, with a confidence  
248 level of 0.05. The mean variability was indicated by the standard deviation and was  
249 analyzed statistically using the R project software version 3.2.3 (R Development Core  
250 Team, 2015). Boxplot were plotted to check outliers and to show differences among each  
251 new culture medium and the reference one.

252

### 253 **3. Results**

#### 254 **3.1. Culture media Box Plot analysis and experimental design**

255 In total 26 different culture media were identified during the literature research, most of  
256 them as a modification of the medium originally proposed by Largeau in 1980 (Ambati  
257 et al. 2010, Cheng et al. 2013, Dayananda et al. 2005, 2007, Fernandes et al. 1991,  
258 Furuhashi et al. 2013, Ge et al. 2011, Kawachi et al. 2012, Kojima and Zhang 1999,  
259 Largeau et al. 1980, Lee et al., 1998, Li and Qing 2005, Liu et al. 2013, Lupi et al. 1994,  
260 Qin 2005, Rao et al. 2007, 2012; Ruangsomboon 2012, Sakamoto et al. 2012, Shimamura  
261 et al. 2012, Song et al. 2012, Tanoi et al. 2011, Weiss et al. 2012, Yang et al. 2004,  
262 Yoshimura et al. 2013). The molar composition of these media was compared to other  
263 culture media commonly used to grow green algae: BBM, BG-11, F2, K9, M8 and  
264 Sueoka, among others (Bischoff and Bold 1963, Stanier et al. 1971, Guillard 1975, Ruiz-  
265 Domínguez et al. 2014, Cuaresma et al. 2009, Sueoka et al. 1967).

266 As it can be seen in Figure 1a and 1b, the main macronutrients which clearly differed  
267 between culture media commonly used to grow microalgae and those commonly used to  
268 grow *Botryococcus* were, potassium, sodium, nitrogen and phosphorus. In general,  
269 *Botryococcus* media showed much lower concentrations of such macronutrients.  
270 Regarding the micronutrients, main differences were found in the content of boron,

271 EDTA, iron, molybdenum, selenium and zinc (Figures 1c, 1d). At the same time, some  
272 of the media used to cultivate *Botryococcus* presented other trace elements which were  
273 never present in the other media group -coloured in red in the box chart-, as aluminium,  
274 bromine, chromium, iodine and selenium among others. As well as with macronutrients,  
275 most of the trace elements were present in minor quantities in the culture media used for  
276 *Botryococcus*.

277 According to the biological importance of elements such as nitrogen, phosphorus, iron  
278 and molybdenum, and considering the deviations between the two groups of media, it  
279 was decided to optimise the concentration of such elements in the reference culture  
280 medium selected for *Botryococcus* in this work (modified Chu 13) (Gouveia et al. 2017).  
281 Apart from that, reference medium also contained selenium (Se). Selenium is relevant to  
282 the enzyme-dependent antioxidant cell response in humans. Nevertheless, excess  
283 selenium can be toxic to humans (Brown and Arthur 2001) and, therefore, it becomes a  
284 problem when medium/large volumes of culture media need to be handled. Thus, it was  
285 decided to, at least, reduce its concentration relative to the reference medium. Table I  
286 shows the averaged values found for the different element concentrations in the different  
287 media groups. Based on the media composition analysis, a range of concentration was  
288 defined for each nutrient and 46 different media compositions were prepared by means  
289 of a 3-level factorial design (Online Resource 1). According to it, concentration of  
290 nitrogen, phosphorus and molybdenum was increased, iron was evaluated at an  
291 intermediate concentration and selenium was reduced or not added.

**Comentado [MCF1]:** Eli, esta parte tiene que ir en discusión, ya que es la justificación de por qué seleccionamos en base a las diferencias observadas y comentadas.

### 293 3.2. Pre-selection of potential media in well plates.

294 The 46 new media were evaluated in 24-well plates, as described in Materials and  
295 Methods section. Final product productivity of carbohydrates and hydrocarbons allowed

296 the pre-selection of several media to be further tested. The absence of selenium was also  
297 considered as an important criterium for the final selection.

298 In general, *B. braunii* CCALA-778 showed a faster growth during the experimental time,  
299 resulting in a major biomass density at the end of the cultivation in the well plates. It also  
300 resulted in higher biomass productivities compared to *B. braunii* AC761 (data not shown).

301 The analysis of the biomass and product (carbohydrates and hydrocarbons) productivity  
302 allowed to compare all the new media with their corresponding reference cultures.

303 Figure 2 shows the volumetric biomass productivity data of *B. braunii* CCALA-778 and  
304 AC761 respectively. In order to avoid the influence of the experimental cultivation  
305 conditions, the results were grouped according to the three independent experiments in  
306 which the different culture media were evaluated. Each experiment had its own reference  
307 cultures.

308 Despite of the dispersion of the data for some of the new culture media, related to the  
309 difficulties to perform accurate sampling in the well plates with a colony forming  
310 microalgae, a clear trend could be observed for each experiment. As it can be seen in  
311 Figure 2, many of the new media showed better productivities compared to their  
312 corresponding reference media (78% for *B. braunii* CCALA-778 and 46% for *B. braunii*  
313 AC761), which might indicate that the higher availability of nutrients as nitrogen, but also  
314 iron and molybdenum -which are essential in nitrogen assimilation-, could partially  
315 enhance the *B. braunii* biomass productivities (Raven 1998). However, in order to assess

**Comentado [MCF2]:** Esto también sería discusión

316 the potential of *B. braunii* as metabolite producer, the carbohydrates and hydrocarbons  
317 productivities were also calculated (Figure 3), improvements being only observed for  
318 some of the new culture media.

319 For *B. braunii* CCALA-778, 3 culture media were selected to be further validated in a  
320 new experiment in Roux flasks among the best media that showed either higher or non-

321 significant difference in carbohydrates productivity compared to the reference medium.  
322 M15 was selected as the best medium from experiment 1 although it contained selenium.  
323 From experiment 2, it was decided to select M21 because it did not contain selenium.  
324 And finally, from experiment 3, M44 was selected due to the higher photosynthetic  
325 efficiency (data not shown) and better biomass productivity compared to the reference  
326 medium.

327 For *B. braunii* AC761, many of the new media resulted in improved hydrocarbons  
328 productivities. From all media, 4 were selected to be further validated in a new experiment  
329 in Roux flasks. The final selection was M11, M22, M28 and M35. From the experiment 1,  
330 M11 was selected due to the lack of Se and its higher biomass productivity. From  
331 experiment 2, M28 and M22 were selected as the best two media. M28 due to the absence  
332 of selenium and M22 as example of a media containing an intermediate amount of Se  
333 (25  $\mu$ M). Finally, from experiment 3 it was decided to select M35 due to the fact that it  
334 was the only medium in this experiment that presented both, higher biomass and  
335 hydrocarbons productivities respect to the reference medium and, besides, it did not  
336 contain Se.

337

### 338 **3.3. Validation of pre-selected media in Roux-flasks.**

339 Previously selected media were decided to be evaluated in Roux flasks in order to obtain  
340 more information about the growth kinetics and product accumulation. Prior to the  
341 experiment, all the cultures were adapted to the new experimental conditions (media and  
342 experimental set-up) through an adaptation period of 12 days. Once the cultures were  
343 considered to be adapted the experiment started.

344

#### 345 3.3.1. *B. braunii* CCALA -778

346 From the preselected media, M44 showed to be a promising media for the cultivation of  
347 *B. braunii* CCALA-778. However, one of the objectives of media optimisation was to  
348 avoid the presence of selenium and M44 only reduced the Se content to 25 µM. For that  
349 reason, it was also evaluated M44 without selenium (new media called M44m). At the  
350 same time, the preselected media M21 showed a very low N/P ratio (1.2) and in order to  
351 see if productivity could be further enhanced, it was decided to also evaluate M21 with  
352 an increased nitrogen content (22mM) (new media called M21m). Results in Figure 4  
353 show that M44m clearly resulted in higher biomass and carbohydrates productivities  
354 compared to the other media, showing an increase of 13.5% in biomass and 50.5% in  
355 carbohydrates respect to the reference medium. Furthermore, the obtained values were  
356 even higher than in other studies previously published with different *B. braunii* race A  
357 strains (Blifernez-Klassen et al. 2018, Díaz and Atehortúa 2014).

358

### 359 3.3.2. *B. braunii* AC761

360 In the case of *B. braunii* AC761, the pre-adaptation phase was carried out during 10 days  
361 and after that, cultures were re-inoculated. In terms of biomass productivity, all the new  
362 media presented improved values. The maximum rate was obtained with M22, being 20%  
363 higher respect to the reference medium (Figure 5). However, none of the pre-selected  
364 media for this microalga resulted in better hydrocarbons productivities than the reference,  
365 registering values between 12.7% (M22) and 25.5% (M28) lower.

366 Considering that among the new media M22 presented the best results but it contained  
367 selenium, M35 could be proposed as a good medium to cultivate *B. braunii* AC761.  
368 However, further optimisation might be needed in order to improve the production of  
369 hydrocarbons with such strain.

**Comentado [MCF3]:** Esto también sería discusión. Y aquí añadiría una frase al final donde diría que analizar la productividad máxima no fue el objetivo de este trabajo, si no más bien comparar medios para optimizar y que la productividad tiene que ser evaluada en sistemas más controlados y a mayor escala.

370 The final productivity results obtained from the cultivation of *B. braunii* CCALA-778  
371 and AC761 in Roux flasks are presented in summary in Online Resource 2.

372

#### 373 **4. Discussion**

374 *B. braunii* CCALA-778 and AC761 selection was based on a previous study carried out  
375 by Gouveia et al. 2017. According to the authors, *B. braunii* CCALA-778 showed  
376 potential for industrial applications as carbohydrate producer since it generates large  
377 quantities of fucose and galactose which are considered of high industrial value whilst  
378 not hydrocarbon production was detected. In *B. braunii* AC761 only low quantity of  
379 galactose was measured, however, it was able to produce an important amount of  
380 hydrocarbons, hence being considered a promising hydrocarbon producer. Apart from  
381 these differences, there is evidence that a trade-off between the synthesis of carbohydrates  
382 and lipids exists in microalgae that makes one of them to predominate over the other. And  
383 in some cases, a decrease in one of these compounds is obtained while increasing the  
384 other (Suastes-Rivas et al. 2020). This assumption led us to optimise only the  
385 biomolecules synthesized in greater quantity in each case and with industrial application.  
386 The culture media optimisation started with the selection of the elements to be assessed,  
387 which was ultimately based on their biological importance. Nitrogen and phosphorus are  
388 essential elements for all life forms. After carbon, nitrogen is considered the most  
389 important nutrient for biomass development and it has an important role in regulating  
390 photosynthesis (Smith 1979). The nitrogen content of the biomass can range from 1% to  
391 more than 10% and nitrogen limitation can be also used as a tool to promote the  
392 accumulation of N-lacking compounds such as polysaccharides and acylglycerides  
393 containing polyunsaturated fatty acids (Becker 1994). Phosphorus is an essential  
394 component of nucleic acids and many intermediary metabolites, such as sugar phosphates

395 and adenosine phosphates, which are crucial molecules in the metabolism of all life forms  
396 (Correll 1998). Iron is one of the most important elements required by most microalgae;  
397 for instance, iron mediates catalysis of nucleic acid processing enzymes and is also part  
398 of iron–sulfur clusters found in a variety of metalloproteins involved in biochemical redox  
399 reactions. Specifically, in microalgae cells iron is also involved in photosystem II  
400 electron-transfer reactions, nitrogen assimilatory reduction reactions and chlorophyll  
401 synthesis (Hutchins and Bruland 1998). Consequently, deficient or excessive iron can  
402 generally reduce the photosynthetic efficiency of microalgae (Geider and La Roche  
403 1994). And finally, molybdenum is a very scarce element playing key roles in the  
404 metabolism of nitrogen and sulphur, in hormone biosynthesis and acts as enzyme-  
405 cofactor (Llamas et al. 2011, Tejada-Jiménez et al. 2011). Taking into account all of the  
406 above, the range of concentrations to be studied of each of these elements was carefully  
407 chosen. Selenium was also included in the optimisation since although trace amounts are  
408 necessary for cellular function in many organisms, it presents toxic effects in large  
409 amounts. Several microalgae, such as haptophyte, show Se essential requirements and  
410 incorporate Se into metalloproteins through a well-known Se-assimilation metabolic  
411 pathway (Araie and Shiraiwa 2009, Gojkovic et al. 2014). Nevertheless, the role of Se in  
412 *Botryococcus* metabolism is not clear. Only few authors mention its role as growth  
413 enhancer for other microalgae, but even in these studies the Se concentrations used are  
414 much lower than the one in our reference medium (Araie and Shiraiwa 2009, Yoshimura  
415 et al. 2013). Other studies with selenium focus on its bioaccumulation and  
416 biotransformation to Se-aminoacids, taking advantage of this detoxification mechanism  
417 with the aim to produce Se-enriched microalgae biomass with value in the  
418 food/feed/health-care industry (Gojkovic et al. 2015, Kouba et al. 2014). Altogether, the  
419 results obtained in this research work indicated that selenium might not be essential for

420 *Botryococcus* growth. On the contrary, the rest of the studied elements seemed to play an  
421 important role. In the case of the well-plate experiments, higher biomass productivities  
422 were observed in the majority of the new media compared to the reference, whereas  
423 metabolites productivities were only improved for some of them. These results are  
424 consistent since biomass production and secondary metabolites biosynthesis compete for  
425 the metabolic destination of photosynthetically assimilated inorganic carbon. And as  
426 accumulation and/or excretion of several biomolecules has been proposed as a stress  
427 response of living cells (Cheng et al. 2013), the higher availability of nutrients in most of  
428 the new media might result in lower carbohydrates and hydrocarbons content. The best  
429 culture media, in terms of carbohydrate and hydrocarbon production that did not  
430 compromise biomass growth of both strains, were selected to be validated in higher  
431 volume in Roux flasks. For *B. braunii* AC761 those media were M11, M22, M28 and  
432 M35. In the case of *B. braunii* CCALA-778 the chosen media were M15, M21 and M44.  
433 A modified version of M21, with a higher nitrogen concentration (M21m), and M44  
434 (M44m), without selenium, were also evaluated. Focusing on the nutrients' composition,  
435 it can be observed that the N/P ratio for M44m (37:1) was the highest, followed by M21m  
436 (7:1) and M15 (4:1). This was consistent with a study carried out by Choi and Lee (2015),  
437 in which a continuous increase in biomass was obtained as the N/P ratio increases up to  
438 a maximum value from which it begins to decrease. According to Redfield (1934), M21m  
439 and M15 present nitrogen limitation ( $N/P < 16$ ) while M44m is phosphorus-limited  
440 ( $N/P > 16$ ). This shows that nitrogen limitation has a stronger impact on biomass  
441 productivity and growth compared to phosphorus deprivation state, which has more  
442 influence on carbohydrates production (Hopkins and Hüner 2009, Markou et al. 2012b).  
443 Regarding the strain AC761, the lower hydrocarbon productivities obtained with the new  
444 media respect to the reference media could be justified since although, in this case, all the

445 new culture media and the reference one presented a N/P ratio which shows a nitrogen-  
446 limited state, the higher concentrations of nitrogen and phosphorus in such media respect  
447 to the one used as reference must be the cause of their higher biomass production. It has  
448 been described that high nitrate or high phosphate concentration may interfere with  
449 hydrocarbon production (Casadevall et al. 1985, Cheng et al. 2013). In addition to find  
450 the right proportion among these nutrients, adding the appropriate quantities also plays  
451 an important role (Nieuwerburgh et al. 2004). Our results are in accordance with a study  
452 carried out by Ruangsomboon (2012) in which the author found that for *B. braunii*  
453 KMITL-2 increasing the phosphorus concentration led to an increase in biomass but a  
454 decrease in lipid content. Regarding the micronutrients influence, the effect of iron  
455 evaluated in another *B. braunii* race B strain BOT-22 resulted in very slow growth when  
456 the alga was cultivated in low-iron medium compared with that of the iron-rich culture  
457 (Tanoi et al. 2014). Similarly, the higher concentration of iron and/or molybdenum in the  
458 new media used in our study may have influenced the increase in biomass production at  
459 the expense of hydrocarbon accumulation.

460 Comparing the effect of iron and molybdenum on biomass and metabolite yields of  
461 *B. braunii* CCALA-778 and AC761, differences are visible between both strains.  
462 Although the results obtained cannot establish a specific pattern, it is concluded  
463 that the influence of trace elements on growth and production of molecules of  
464 interest varies for each microalga. This is not surprising since in a study carried  
465 out with a strain of *B. braunii* lipids accumulated when high levels of iron were  
466 combined with a nitrogen-limited state and moderate light intensity, whereas in  
467 another work with a different strain, the authors concluded that nickel and  
468 manganese were more important than molybdenum and iron in regulating algal  
469 hydrocarbon production (Song et al. 2012, Yeesang and Cheirsilp 2011). The

470 effect of micronutrients, such as iron or molybdenum, on biomass composition of  
471 eukaryotic microalgae is quite complex as these elements influence many  
472 metabolic functions.

473 The difference in the behavior of both strains, depending on the nutrients rate and  
474 concentration, can be supported by other studies in which microalgal preference for  
475 optimal N/P ratios varies among species since it mainly depends on the physiological  
476 requirement for nutrients (Lagus et al. 2004, Mills and Arrigo 2010). Therefore, whereas  
477 more work should be done in optimising the medium to increase the hydrocarbons yield  
478 in *B. braunii* AC761, a new and improved culture medium for *B. braunii* CCALA-778  
479 was obtained which might contribute to reinforce the potential of that strain for the  
480 production of carbohydrates. Future research should be done to validate these promising  
481 results at large-scale.

482

## 483 5. Conclusions

484 Culture media optimisation allowed to improve growth and products productivity of two  
485 *B. braunii*'s strains (CCALA-778 and AC761) of high interest in the biopolymers  
486 industry. The results indicated that for *B. braunii* CCALA-778 (carbohydrates producer),  
487 a nitrogen amount 5.5-fold higher than the reference medium, while maintaining the same  
488 phosphorus concentration, significantly improved biomass and carbohydrates  
489 productivities. In the case of *B. braunii* AC761 (hydrocarbons producer), the results  
490 obtained did not show a significant improvement in the productivities so the selection  
491 was based on the lack of selenium. The absence of selenium in the media did not affect  
492 biomass and metabolites (carbohydrates and hydrocarbons) productivity which is an  
493 important achievement in terms of safety and environmental protection, especially at  
494 large scale cultivation.

**Comentado [MCF4]:** Eli, acabo de darme cuenta que en algunos sitios usamos optimization con ese y en otros con z. Revisa que usemos siempre lo mismo, en cualquier forma de esta palabra. Si haces una búsqueda en el documento con "optim" te las va a ir indicando para que las cambies. 😊

495 Overall, the results showed that for each algal strain, growth and metabolite production  
496 were affected by different N:P ratios. Therefore, attention should be given to each  
497 particular *Botryococcus* strain in order to optimise its culture media. However, media  
498 optimisation resulted in higher productivities which might contribute to reach the  
499 sustainability in *Botryococcus* cultivation.

500

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505

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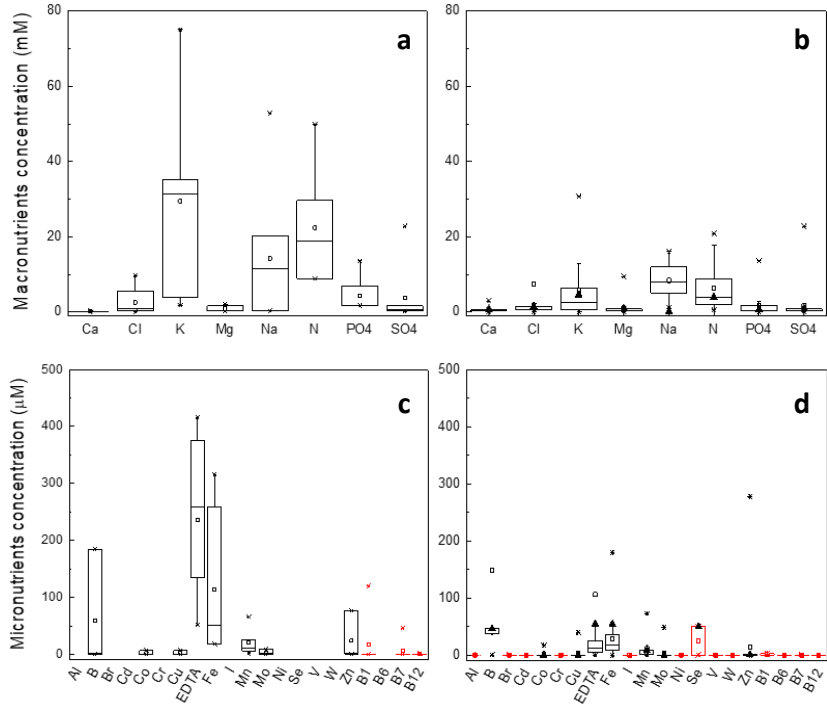
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**Caption to Figures**

**Fig. 1** Box chart representing the macronutrients (a, b) and micronutrients (c, d) composition of the culture media commonly used to grow algae (a, c) and the media used to grow *Botryococcus* strains (b, d). Values corresponding to the reference medium used in this work (modified Chu 13) are presented with black triangles in the right plots. Elements which were scarcely present in the different media are coloured in red. Box represents 25-75% percentiles, range bar represents 1 and 99% percentiles, (\*) beyond these bars represent outliers and (□) mean values

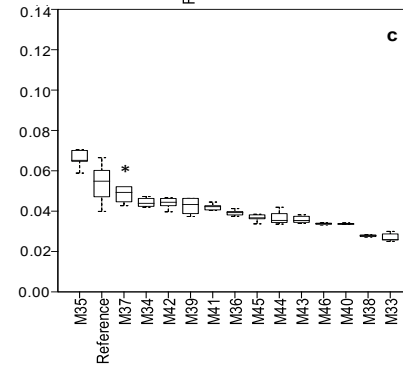
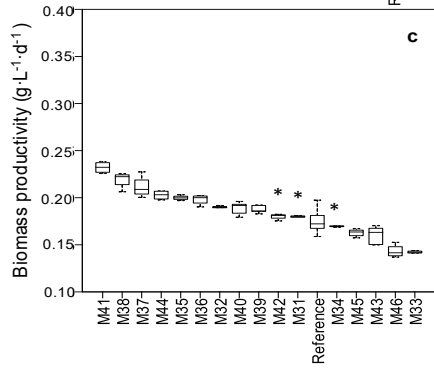
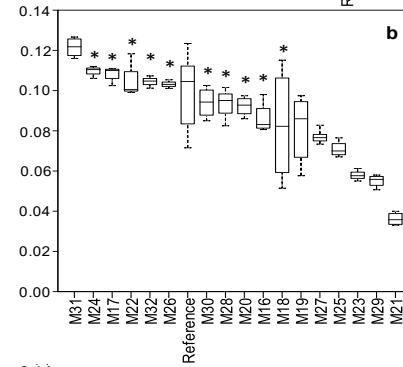
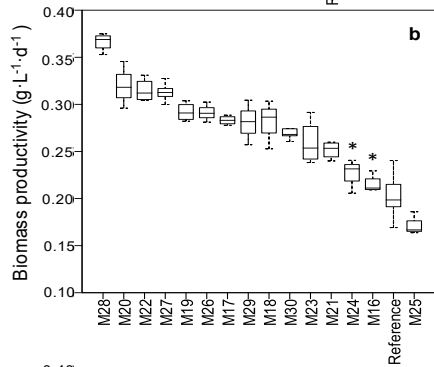
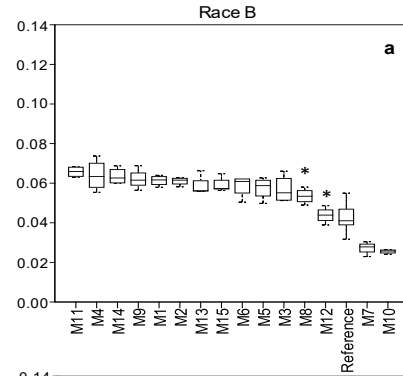
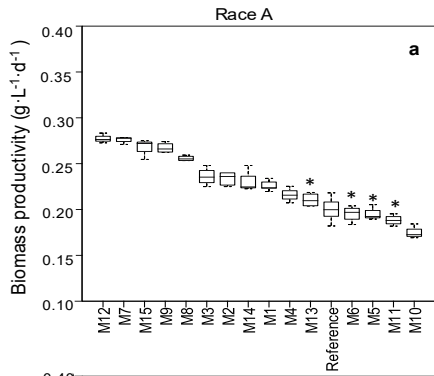


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729 **Fig. 2** Box plots of biomass productivity data, expressed as grams of biomass produced  
730 per liter of culture broth and per day ( $\text{g L}^{-1} \text{d}^{-1}$ ) of each culture media in experiment 1 (a),  
731 experiment 2 (b) and experiment 3 (c), for strains CCALA-778 (left column) and AC761  
732 (right column), after 116 hours of cultivation. (\*) means non-significant differences  
733 among the reference medium (Reference) and the new culture media. Significant  
734 differences were set at  $p < 0.05$  by using Fisher's Least Significant Difference (LSD) test



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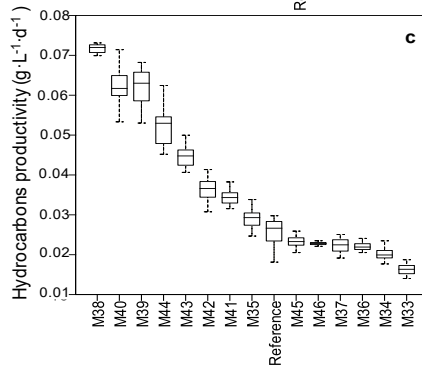
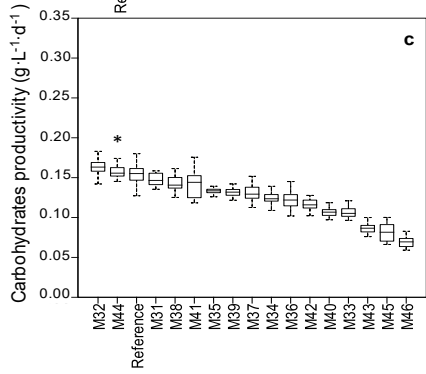
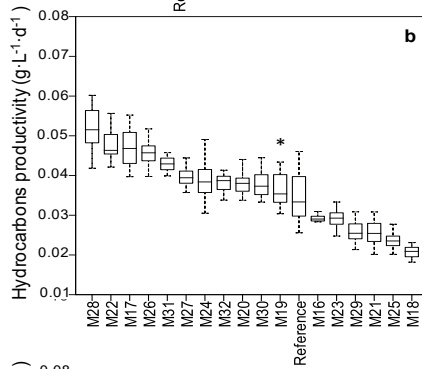
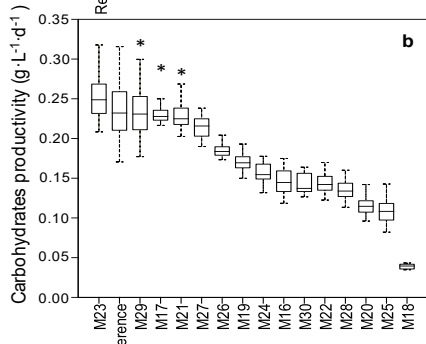
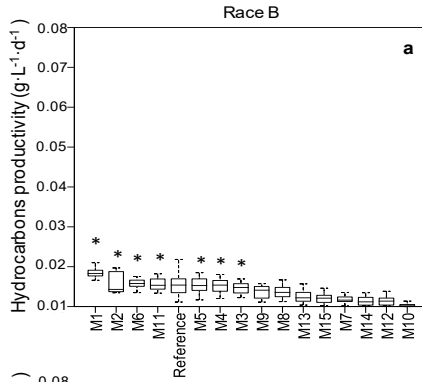
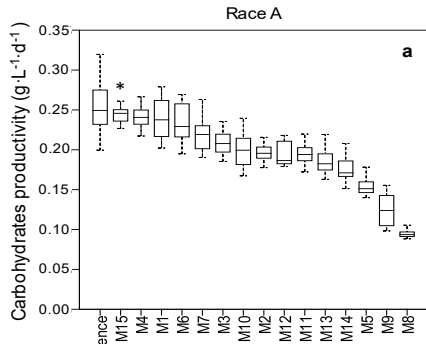
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740 **Fig. 3** Box plots of carbohydrates and hydrocarbons productivity data, expressed as grams  
741 of biomass produced per liter of culture broth and per day ( $\text{g L}^{-1} \text{d}^{-1}$ ) of each culture media  
742 in experiment 1 (a), experiment 2 (b) and experiment 3 (c), for strains CCALA-778 (left  
743 column) and AC761 (right column). (\*) means non-significant differences among the  
744 reference medium (Reference) and the new culture media. Significant differences were  
745 set at  $p < 0.05$  by using Fisher's Least Significant Difference (LSD) test



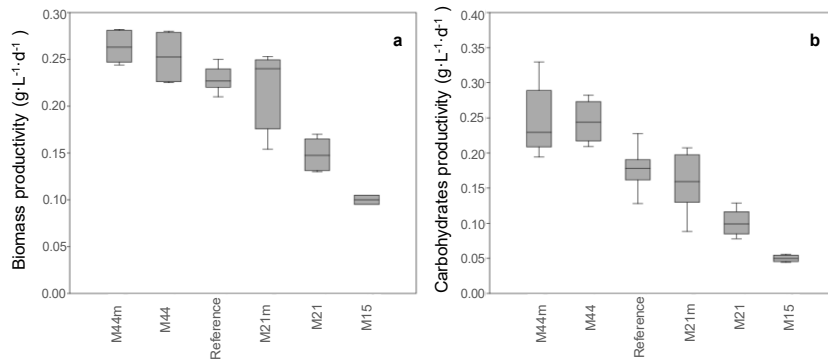
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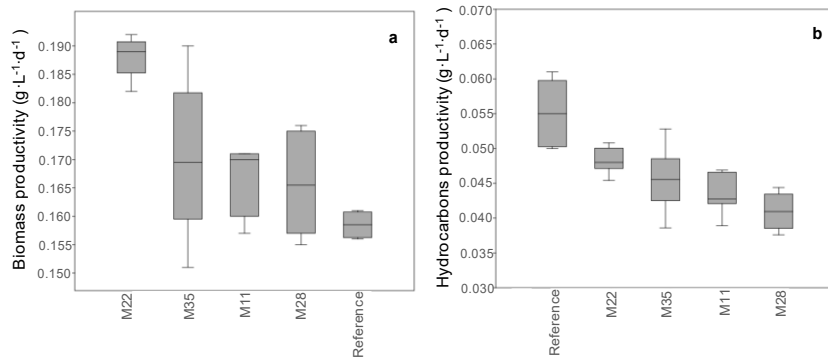
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750 **Fig. 4** Boxplot of biomass (a) and carbohydrates (b) volumetric productivity data,  
 751 expressed as grams of biomass produced per liter of culture broth and per day ( $\text{g L}^{-1} \text{d}^{-1}$ ),  
 752 after 170 hours of cultivation of each culture media for *B. braunii* CCALA-778



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 755 **Fig. 5** Boxplot of biomass (a) and hydrocarbons (b) volumetric productivity data,  
 756 expressed as grams of biomass produced per liter of culture broth and per day ( $\text{g L}^{-1} \text{d}^{-1}$ ),  
 757 after 330 hours of cultivation of each culture media for *B. braunii* AC761



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761 **Table 1 Nutrients concentration, calculated as the average value for the different**  
 762 **media groups.** General media refers to media used for other green microalgae; *B. braunii*  
 763 media summarizes the 26 media recipes found for that specie; Reference medium is the  
 764 medium to be optimised (modified Chu 13) and Proposed study range is the selected  
 765 concentration range used in the experimental design

Nutrients	General media	<i>B. braunii</i> media	Reference medium	Proposed study range
<b>Nitrogen</b>	7.4 mM	4 mM	4 mM	4 – 40 mM
<b>Phosphorus</b>	0.6 mM	0.3 mM	0.6 mM	0.6 – 6 mM
<b>Iron</b>	34 µM	13.7µM	54.5 µM	10 – 70 µM
<b>Molybdenum</b>	2.1 µM	0.3µM	0.25 µM	0.2 – 2 µM
<b>Selenium</b>	0 µM	4 µM	50 µM	0 – 50 µM

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