

Title	The importance of the calcium-to-magnesium ratio for phytoremediation of dairy industry wastewater using the aquatic plant <i>Lemna minor</i> L.
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Publication date	2020-01-07
Original Citation	Walsh, É., Paolacci, S., Burnell, G. and Jansen, M. A. K. (2020) 'The importance of the calcium-to-magnesium ratio for phytoremediation of dairy industry wastewater using the aquatic plant <i>Lemna minor</i> L.', <i>International Journal of Phytoremediation</i> . doi: 10.1080/15226514.2019.1707478
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1080/15226514.2019.1707478
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Download date	2024-06-19 22:24:45
Item downloaded from	https://hdl.handle.net/10468/9644

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2 **The importance of the calcium-to-magnesium ratio for phytoremediation of**
3 **dairy industry wastewater using the aquatic plant *Lemna minor* L..**

4

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10 **Abstract**

11 Lemnaceae are being exploited to remediate a variety of different wastewaters. Dairy
12 processing waste is produced in large amounts, and contains a range of valuable plant nutrients,
13 for example, nitrate, ammonium, phosphate, iron and calcium. Our aim was to remediate dairy
14 processing waste with the duckweed plant *Lemna minor*. However, initial trials failed to
15 establish growth of *L. minor* on this medium. A lack of growth can be due to a lack of essential
16 plant micro- and macro-nutrients, or the presence of phytotoxic ingredients. In this study we
17 show that not just nutrient concentrations, but also the ratios between them can be important in
18 facilitating growth. Using lab-scale experiments in which *L. minor* were grown on 100 mL of
19 synthetic wastewater, we demonstrated that the skewed Ca:Mg ratio in synthetic dairy industry
20 wastewater is a key obstacle to good growth. Experiments showed that a ratio which favours
21 magnesium over calcium negatively affects *L. minor* growth and photosynthetic yield, leading
22 to RGRs as low as 0.05 day^{-1} . A change in this ratio to favour calcium, through the addition of
23 calcium sulphate, leads to RGRs of $0.2 - 0.3 \text{ day}^{-1}$. Experiments lead us to conclude that a
24 Ca:Mg ratio of 1:1.6 (by molar concentration) or greater is necessary for *Lemna minor* growth,
25 and therefore phytoremediation of dairy industry processing wastewater.

26

27 *Keywords: phytoremediation, dairy processing, Lemna, duckweed, wastewater, toxicity*

28 **Introduction**

29 Phytoremediation refers to the process whereby plants, and associated microorganisms, are
30 used to remove and/or degrade contaminants from soils and waters. Lemnaceae species,
31 commonly referred to as duckweed (Landolt 1986), have been extensively studied for their
32 phytoremediation potential. This potential relates to fast growth rates, relative tolerance to a
33 range of pollutants, and high pollutant removal rates (Zayed et al. 1998; Cheng & Stomp 2009;
34 Ziegler et al. 2015). Furthermore, the high protein content and good protein quality, i.e.
35 desirable amino acid composition, make Lemnaceae biomass attractive as a potential
36 component in animal feeds (Cheng & Stomp 2009; Anderson et al. 2011; Appenroth et al.
37 2017). Thus, where Lemnaceae are used to remediate uncontaminated agricultural waste
38 streams, a circular economy approach can be considered. In this scenario, nutrients (most
39 importantly N- and P-containing compounds) present in wastewater are recycled into animal
40 feed. Re-using plant nutrients, such as phosphate, nitrate and ammonia, present in wastewater
41 can generate income from waste, reduce the costs associated with storage and tertiary
42 wastewater treatments, and prevent environmental damage (i.e. eutrophication) associated with
43 release of nutrient-rich waste on to surface waters (Diaz & Rosenberg 2008; Conley et al.
44 2009).

45 Dairy industry processing wastewater is generated during the production of dairy products such
46 as milk powder, cheese and yogurt from raw milk. Typically, large volumes are produced. In
47 Europe dairy processing is seen as the largest industrial food wastewater source, with 0.5–37
48 m³ of effluent per m³ of processed milk (Kolev Slavov 2017). This processing wastewater is
49 rich in nitrogen and phosphorous as well as other essential plant nutrients such as calcium,
50 potassium and magnesium (Ince 1998; Demirel & Yenigun 2004; Goyal & Gandhi 2009;
51 Carvalho et al. 2013; Ryan & Walsh 2016). As expected for a waste product from the food
52 industry, dairy industry processing wastewater contains only low concentrations of

53 contaminants such as heavy metals (Ince 1998; Demirel & Yenigun 2004). Therefore, dairy
54 industry processing wastewater is well suited to remediation using a circular economy
55 approach.

56 Duckweed species have been shown to be tolerant of a wide range of conditions and nutrient
57 concentrations (Landolt & Kandeler 1987). Nevertheless, any particular wastewater needs to
58 fulfil minimal criteria to facilitate growth and phytoremediation. An important criterion is the
59 presence of adequate levels of essential plant growth nutrients. Taking nitrogen as an example,
60 *Lemna minor* will grow on either ammonium or nitrate as a nitrogen source and can tolerate
61 concentrations ranging between 0.2 to 150 mM with optimal concentrations varying depending
62 on the nitrogen source (Landolt & Kandeler 1987; Paolacci et al. 2016). Duckweed can tolerate
63 a pH range from 4 – 8 (Landolt & Kandeler 1987), but also the pH is also important in
64 determining the tolerance of duckweed to ammonia (Körner et al. 2003). In the case of
65 phosphate, duckweed tolerates concentrations ranging between 0.001 to 10 mM (Landolt &
66 Kandeler 1987; Paolacci et al. 2016). For calcium and magnesium acceptable concentrations
67 range between 0.2-20 mM and 0.2-10 mM, respectively (Landolt & Kandeler 1987; Van Dam
68 et al. 2010; Paolacci et al. 2016), while the Ca:Mg ratio is also an important determinant of
69 plant growth. Other criteria for plant growth and phytoremediation relate to the presence of,
70 potentially phytotoxic, pollutants. In the specific case of dairy industry processing waste there
71 is a heavy load of organic matter in the wastewater which is measured as biochemical oxygen
72 demand (BOD₅ mg/L), chemical oxygen demand (COD mg/L) and fats (mg/L) (Janczukowicz
73 et al. 2008; Carvalho et al. 2013). Duckweed do not require organic compounds in the medium
74 for survival and growth (Körner et al. 1998), however, they can contribute to the reduction in
75 the amount of organic matter as part of a phytoremediation approach (Körner et al. 1998; Li et
76 al. 2017). All compounds mentioned are present in dairy processing wastewaters but there is a
77 high degree of variability in their concentrations, and this relates to different factories and

78 processes (Demirel & Yenigun 2004; Goyal & Gandhi 2009; Carvalho et al. 2013; Tikariha &
79 Sahu 2014), as well as strong seasonal influences on milk production.

80 In order to facilitate reproducible laboratory phytoremediation studies, a synthetic dairy
81 industry wastewater has been developed (Tarpey 2016; Gil-Pulido et al. 2018). The
82 composition of this synthetic wastewater (Table 1) is based on measurements of the
83 composition of real wastewater. Unfortunately, preliminary experiments showed that
84 duckweed did not grow well in this synthetic dairy wastewater. The aim of the present study
85 was to identify the reasons responsible for the poor performance of duckweed on the synthetic
86 dairy wastewater. Synthetic dairy industry wastewater has a Ca:Mg ratio of 1:14.6. It is known
87 that an imbalance in favour of magnesium can have a negative effect on the growth and health
88 of duckweed (Landolt & Kandeler 1987; Paolacci et al. 2016). This antagonistic Ca:Mg
89 relationship was first studied in terrestrial plants (Loew & May 1901). It has been found that
90 in soils magnesium decreases the calcium uptake in the plant, while calcium reduces
91 magnesium uptake to a lesser extent (Halstead et al. 1958). Thus, imbalances in the soil Ca:Mg
92 ratio can potentially aggravate calcium or magnesium deficiencies as well as magnesium
93 toxicity (Brady et al. 2005). Therefore, based on existing literature, we hypothesised that a ratio
94 between calcium and magnesium in favour of magnesium causes acute toxicity in *L. minor*,
95 and that changing the ratio in favour of calcium removes this acute toxicity for *L. minor*. In this
96 study, different levels of this imbalance between calcium and magnesium were tested in short-
97 and long-term experiments in order to identify the concentration of calcium and magnesium
98 most suitable for duckweed in the particular chemical environment of the synthetic dairy
99 wastewater. This study underpins an important aspect of phytoremediation, assessing and
100 amending wastewater composition to facilitate plant growth.

101 **Materials and Methods**

102 *Cultivation of stock and experimental plants*

103 The duckweed strain used in this study was *Lemna minor* - Blarney (international strain ID
104 number 5500). A stock of sterile *L. minor* was kept in optimised growing conditions on half-
105 strength Hutner's medium (Hutner 1953) in a growth room at a constant temperature of 22°C,
106 light intensity of 52.66 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a photoperiod of 14 hours light to 10 hours dark.

107 A synthetic wastewater was used as a growing media (Table 1) for experimental purposes. The
108 composition of this synthetic wastewater was based on analysis of real dairy industry
109 processing wastewater (Tarpey 2016). Control plants were grown in 100 mL of half-strength
110 Hutner's medium and experimental plants were grown in 100 mL of synthetic dairy processing
111 wastewater. For all experiments plants were grown in their respective media in magenta vessels
112 (Magenta GA-7 Plant Culture Box) for 7 days in a growth room at a constant temperature of
113 21°C, light intensity of 80.82 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a photoperiod of 16 hours light to 8 hours dark.
114 This light intensity is lower than that used in standard toxicological protocols, thus growth rates
115 were moderately lower than standard, not exceeding an RGR of 0.3 day^{-1} .

116 *Experimental design*

117 This study contains two types of experiments; short-term (7 days) and long-term (42 days). In
118 both types of studies *L. minor* plants were grown on a number of differently modified versions
119 of synthetic dairy wastewater (Table 1) with half-strength Hutner's medium as a control. At
120 the start of each experiment, three three-frond colonies were added to each magenta. On day
121 zero of each experiment, the starting mass (fresh weight), colony number and frond number
122 were determined. The synthetic wastewater was not changed during the 7-day experiments. In
123 the case of long-term experiments (42 days), the 100mL of synthetic wastewater was replaced

124 weekly. Furthermore, the density of plants in the long-term experiment was returned to a
125 constant amount, three colonies, at the start of each week.

126 [Table 1 near here]

127 *Synthetic wastewater modifications*

128 A reduction in the concentration of chloride was achieved by replacing ammonium chloride in
129 the synthetic wastewater with ammonium sulphate, thus removing the majority of chloride. A
130 reduction in the concentration of sodium was achieved by the removal of sodium bicarbonate.
131 Iron and manganese concentrations were reduced through the addition of less iron sulphate
132 heptahydrate and manganese chloride tetrahydrate, respectively. Potassium was increased
133 through the addition of potassium bicarbonate. Sulphate was increased through the substitution
134 of ammonium chloride for ammonium sulphate and the addition of potassium sulphate. The
135 calcium concentration was increased by adding calcium sulphate (CaSO_4) to the synthetic
136 wastewater. The magnesium concentration was increased or decreased by altering the
137 concentration of magnesium sulphate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$). A secondary impact of
138 altering the calcium and magnesium concentrations is that sulphate concentration is also
139 affected. The highest concentration to which sulphate is increased, 8.3 mM, is still significantly
140 below the maximum concentration of sulphate tolerated by *L. minor*, 60 mM (see Table 2 for
141 maximum tolerated and ‘optimal’ concentrations). The pH of synthetic dairy wastewater is
142 naturally around 8. However, for experiments the pH was reduced to between 4.5 – 5, a pH
143 similar to that of half-strength Hutner’s medium, to ensure differences observed were not due
144 to differences in pH.

145 *Measured parameters*

146 On day seven of the short-term experiments mass (fresh biomass), colony number, frond
147 number and chlorophyll *a* fluorescence were measured. Frond and colony numbers were

148 counted by eye. Before weighing, plants were wrapped in tissue paper to remove excess water.
149 The Relative Growth Rate (RGR) was calculated based on fresh weight measurements using
150 the formula below (Connolly & Wayne 1996):

$$151 \quad RGR = \frac{\ln \left(\frac{W_2}{W_1} \right)}{\Delta T}$$

152 Where W_1 is initial fresh biomass (Day 0), W_2 is final fresh biomass (Day 7), ΔT is length of
153 the experiment in days and \ln is the natural logarithm.

154 Chlorophyll *a* fluorescence was measured using pulse amplitude modulated chlorophyll *a*
155 fluorometry (WALZ Imaging fluorometer, Effeltrich, Germany). For chlorophyll *a*
156 fluorescence analysis, plants were dark adapted for 15 minutes immediately before
157 measurements. Then, three random colonies from each Magenta were taken for analysis; the
158 measured values of these three colonies were averaged together and treated as one replicate.
159 The chlorophyll fluorescence analysis procedure is as follows; first, a low intensity modulated
160 measuring light was turned on to measure F_0 on the dark-adapted plant, and secondly a
161 saturating pulse of light ($2700 \mu\text{mol}/\text{m}^2/\text{s}$) was applied to obtain the maximum fluorescence
162 F_m . Subsequently, actinic light (photosynthetically active light of $186 \mu\text{mol}/\text{m}^2/\text{s}$) was applied
163 to the plants and at 20 second intervals saturating pulses were applied to measure F_m' , the
164 maximum fluorescence under light-adapted conditions. F_t is the value of fluorescence
165 immediately before the saturating pulse is applied, i.e. the steady-state value of fluorescence.
166 F_v/F_m , the maximum quantum efficiency of photosystem II (PSII), and $Y(II)$, the quantum
167 efficiency of PSII under steady state light conditions were calculated according to Maxwell
168 and Johnson (2000) using the following equations:

$$169 \quad F_v/F_m = (F_m - F_0)/F_m$$

$$170 \quad Y(II) = (F_m' - F_t)/F_m'$$

171 For the long-term 42-day experiment, mass (fresh biomass), colony number and frond number
172 were measured every seven days before media were replaced with fresh synthetic wastewater.
173 RGR was calculated based on growth over seven days.

174 ***Data analysis***

175 Statistical analyses were conducted using R software (R 3.4.3). Numbers of independent
176 replicates were 3 to 4, as stated in legends. One-way ANOVA and Welch's ANOVA were used
177 to examine whether there were significant differences in RGR, Y(II) and Fv/Fm between
178 treatment groups (excluding the Hutner's treatment group). Post-hoc tests Tukey and Games-
179 Howell were used in pairwise comparisons of treatment groups (also excluding the Hutner's
180 treatment group). Welch's ANOVA and Games-Howell tests were used when a dataset was
181 not homoscedastic thus failing one of the assumptions of an ANOVA and Tukey test. In the
182 42-day experiment average RGR between treatment groups was compared each week, Hutner
183 being included in this analysis.

184 **Results and Discussion**

185 ***Investigation of components of synthetic dairy wastewater***

186 As part of a phytoremediation approach for dairy industry wastewater, *L. minor* was grown
187 under laboratory conditions on synthetic dairy wastewater. However, it was found that growth
188 rates were poor, and that colonies displayed extensive chlorosis. Chlorosis occurred relatively
189 fast (within days) indicating toxicity rather than deficiency symptoms. Thus, a systematic desk-
190 top study of all individual chemical components present in the synthetic medium was
191 conducted. The concentration of each element in the synthetic wastewater was first calculated
192 and then compared with the minimum required, the maximum tolerated and the optimal range
193 of values (Table 2). Based on the data in table 2, a number of elements were selected that were
194 present in non-optimal concentrations and that could potentially have a negative impact on *L.*

195 *minor* growth. The elements iron, manganese, sodium, chloride, potassium and sulphate were
196 identified as falling into this category. Iron, chloride and manganese were present at
197 concentrations at upper end of their optimal ranges. Potassium and sulphate were both present
198 in concentrations at the lower end of their respective optimal ranges. Reductions of 100% in
199 chloride, or sodium concentrations did not improve growth. Reductions of some 70% in iron
200 or manganese concentrations did not improve growth, and neither did 10-fold increases in
201 potassium or sulphate (data not shown). These experiments did not reveal any candidate to
202 explain observed growth impairment on unmodified synthetic wastewater.

203 [Table 2 near here]

204 ***Increasing concentrations of calcium relative to magnesium***

205 Synthetic dairy processing water has a low concentration of calcium (0.014 mM), notably lower
206 than the optimal range of concentrations (0.2 – 20 mM). Furthermore, it was noted that the
207 Ca:Mg ratio in the synthetic wastewater was 1:14.6, and this relative lack of calcium compared
208 to magnesium might potentially also cause growth problems (Landolt & Kandeler 1987; Van
209 Dam et al. 2010). To explore the roles of calcium, magnesium and the Ca:Mg ratio in
210 controlling growth, an initial experiment was conducted in which *L. minor* plants were grown
211 in medium containing increasing amounts of calcium, whilst the magnesium concentration was
212 kept constant at 0.2 mM. The calcium concentrations ranged from 0.014 to 1.21 mM,
213 translating to Ca:Mg ratios of 1:14.6 to 6.1:1, respectively. As a control, plants were grown on
214 half-strength Hutner's medium which has a Ca:Mg ratio of 1:1.

215 Under control growth conditions, plants on half-strength Hutner's medium displayed vigorous
216 growth (average RGR of 0.226 day⁻¹) and had a healthy appearance. In contrast, plants growing
217 on unmodified synthetic wastewater (Ca:Mg ratio of 1:14.6) had an average RGR of just 0.061
218 day⁻¹ (Figure 1). These plants appeared chlorotic. When the synthetic dairy processing medium

219 was modified through the addition of calcium, RGR values increased significantly. In modified
220 synthetic wastewater with a concentration of calcium greater than or nearly equal to that of
221 magnesium, RGR values were 0.164 (Ca:Mg – 1:1.6), 0.163 (Ca:Mg – 1.2:1), 0.163 (Ca:Mg –
222 3:1) and 0.148 (Ca:Mg – 6.1:1) day⁻¹. From a low growth rate on medium with a Ca:Mg ratio
223 of 1:14.6, RGR increased up until a Ca:Mg of 1:1.6 ratio, where RGR-values plateaued (Figure
224 1). Analysis of variance (ANOVA) between treatments (excluding Hutner) showed significant
225 variance among them, $F(6, 14) = 7.606$, $p < 0.001$, indicating that there is a positive association
226 between the Ca:Mg ratio and the RGR (day⁻¹) of *L. minor*. A post-hoc Tukey test revealed
227 which differences in RGR were significant. The RGR values of plants grown on synthetic
228 wastewater of a Ca:Mg ratio of 1:3.3, 1:1.6, 1.2:1, 3:1 and 6.1:1 did not differ significantly
229 from each other. The RGR of plants on synthetic waste of Ca:Mg ratio 1:14.6 differed
230 significantly from that of plants on a Ca:Mg ratio of 1:1.6, 1.2:1 and 3:1, $p < 0.05$. While the
231 RGR of plants on synthetic wastewater of Ca:Mg ratio of 1:8.2 differed significantly from that
232 of plants on a Ca:Mg ratio of 1:3.3, 1:1.6, 1.2:1, 3:1 and 6.1:1, $p < 0.05$.

233 [Figure 1 near here]

234 To complement growth rate measurements, key photosynthetic parameters were measured in
235 parallel. In particular Fv/Fm and Y(II) were analysed. Both of these parameters test if, and to
236 what degree, a stressor is affecting photosystem II in the plant. The former refers to the plant
237 in a dark-adapted state, while the latter refers to plants during photosynthesis at steady-state
238 conditions. The control plants on half-strength Hutner's medium displayed good
239 photosynthetic activity, as shown by average values of 0.65 for Fv/Fm and 0.39 for Y(II)
240 (Figure 2). On synthetic wastewater with Ca:Mg ratios of 1:14.6 and 1:8.2, Fv/Fm and Y(II)
241 values were considerably lower than those of the control plants. In synthetic media with Ca:Mg
242 ratios of 1:3.3, 1:1.6, 1.2:1, 3:1 and 6.1:1, Fv/Fm and Y(II) values were similar, or higher, than
243 those of plants on the half-strength Hutner's control. As the calcium concentration was

244 increased, Fv/Fm and Y(II) values increased up until a Ca:Mg ratio of 1.2:1 where values
245 started to plateau. For Fv/Fm, ANOVA showed significant differences between synthetic
246 wastewater treatments, $F(6, 14) = 7.843$, $p < 0.01$, indicating that there was a positive association
247 between changing the Ca:Mg ratio and Fv/Fm, an indicator of the health of *L. minor*. A post-
248 hoc Tukey test showed that the significant variance (shown as letters above the bars in Figure
249 2) between the groups ($p < 0.01$) was between plants growing on a Ca:Mg ratio of 1:8.2 and
250 those on a ratio of 1:3.3, 1:1.6, 1.2:1, 3:1 and 6.1:1. For Y(II) ANOVA also showed significant
251 differences between treatment groups, $F(6, 14) = 22.62$, $p < 0.001$. This indicates, similarly to
252 Fv/Fm, there was a positive association between the Ca:Mg ratio and Y(II), an indicator of the
253 photosynthetic efficiency of the plant. A post-hoc Tukey test shows this significant variation
254 was between plants growing on synthetic wastewater of Ca:Mg ratios 1:14.6 and 1:8.2, which
255 had low Y(II) values, and plants growing on Ca:Mg ratios of 1:3.3, 1:1.6, 1.2:1, 3:1 and 6.1:1,
256 which had higher Y(II) values.

257 [Figure 2 near here]

258 These observations are important from a management perspective and show that the addition
259 of calcium turns synthetic dairy industry wastewater in a suitable medium for growth of *L.*
260 *minor*. However, the observations do not inform whether the positive effects of calcium on *L.*
261 *minor* growth and photosynthesis are due to a rise in calcium concentration and/or an increase
262 in the Ca:Mg ratio.

263 ***Increasing concentrations of magnesium relative to calcium***

264 To explore in more detail the importance of the Ca:Mg ratio, the ratio was changed in favour
265 of magnesium at adequate calcium concentrations. Calcium concentrations were kept constant
266 at 0.12 mM, which was previously shown to accommodate good growth (Figure 1). *L. minor*
267 was grown with magnesium concentrations ranging from 0.2 to 4.99 mM, yielding Ca:Mg

268 ratios of 1:1.6 through to 1:41.2, respectively. It can be seen (Figure 3) that RGR progressively
269 decreased as the concentration of magnesium was increased in the synthetic wastewater. The
270 average RGR at a Ca:Mg ratio of 1:1.6 was 0.21 day⁻¹. RGR decreased to a value of 0.035 day⁻¹
271 at a Ca:Mg ratio of 1:41.2. The *L. minor* plants at the high magnesium concentration (4.99
272 mM) were characterised by both poor growth and chlorosis. An analysis of variance (ANOVA)
273 showed significant differences in RGR between groups, $F(3, 12) = 7.996$, $p < 0.05$, indicating
274 the significant influence of magnesium on plant growth. A post-hoc Tukey test showed
275 significant differences between the RGR of plants growing on a Ca:Mg ratio of 1:1.6 and that
276 of plants in Ca:Mg ratios 1:16.5 ($p < 0.05$) and 1:41.2 ($p < 0.01$).

277 [Figure 3 near here]

278 Fv/Fm and Y(II) values reflect the same trend seen in the RGR data in Figure 3. As the
279 magnesium concentration increased in the synthetic wastewater the Fv/Fm and Y(II) values
280 were reduced (Figure 4). At a Ca:Mg ratio of 1:1.6, the average Fv/Fm was 0.752 and Y(II)
281 was 0.426. The lowest photosynthetic efficiencies were found at a ratio of Ca:Mg of 1:41.2,
282 where Fv/Fm was 0.099 and Y(II) was 0.007. A Welch's ANOVA test (used because of
283 heteroscedasticity in the dataset) showed that there was a significant difference between the
284 treatment groups, $p < 0.01$. A Games-Howell post-hoc test (also used because of
285 heteroscedasticity in the dataset) showed that there were significant differences in Fv/Fm and
286 Y(II), shown as letters above bars in Figure 4. The Fv/Fm of plants growing in Ca:Mg ratio of
287 1:41.2 differed significantly from that of plants growing in Ca:Mg ratio of 1:1.6 ($p < 0.05$) and
288 of 1:8.2 ($p < 0.05$). Similarly, the Y(II) of plants growing in Ca:Mg ratio of 1:41.2 differed
289 significantly from that of plants growing in a ratio of 1:1.6 ($p < 0.001$) and 1:8.2 ($p < 0.05$).

290 [Figure 4 near here]

291 The observed negative effects of magnesium additions on both RGR and photosynthetic
292 parameters can be attributed to the rise in magnesium concentration and/or the decrease in the
293 Ca:Mg ratio. High concentrations of magnesium have been reported to be toxic to Lemnaceae,
294 although this effect can be countered by increasing the Ca:Mg ratio (Van Dam et al. 2010). To
295 further explore this point, an experiment was conducted in which the concentrations of both
296 calcium and magnesium were increased, so as to maintain an optimised 1:1.6 Ca:Mg ratio,
297 while using magnesium concentrations that were earlier found to be toxic (Figures 3 and 4).

298 ***Simultaneous increase of calcium and magnesium concentrations***

299 *L. minor* was grown in synthetic dairy wastewater with a Ca:Mg ratio of 1:1.6 and a range of
300 calcium (0.12 – 3.12 mM) and magnesium (0.2 – 5.14 mM) concentrations. The RGR for plants
301 growing on synthetic wastewater was slightly lower than that of the control on half-strength
302 Hutner's medium, across all concentrations. The highest RGR for plants growing on synthetic
303 waste came from those growing on a Ca:Mg ratio of 1:1.6 at the lowest concentration. As the
304 absolute concentrations of calcium and magnesium increased plant RGR decreased moderately
305 (Figure 5). An ANOVA showed a significant difference between the RGR of *L. minor* in
306 different synthetic wastewater treatments, $F(4,15) = 3.89$, $p < 0.05$ indicating that increased
307 concentrations of both calcium and magnesium had a significant impact on *L. minor* RGR. A
308 post-hoc Tukey test revealed significant differences in RGR between plants growing on
309 wastewater of calcium and magnesium concentrations of 0.21 and 0.2 mM, respectively, and
310 that of plants growing on the two highest concentrations ($p < 0.05$ for both). Once the
311 concentrations of calcium and magnesium were increased to 2.5 and 4.1 mM, respectively, or
312 above, RGR significantly decreased.

313 [Figure 5 near here]

314 Fv/Fm and Y(II) measurements revealed non-significant differences between plants that were
315 grown in synthetic wastewater containing different concentrations of calcium and magnesium
316 (Figure 6). When treatments were compared using an ANOVA test no significant differences
317 were found; Fv/Fm, $F(4, 15)=0.524$, $p=0.72$; Y(II), $F(4, 15)=0.809$, $p=0.538$. Furthermore,
318 Fv/Fm and Y(II) values for plants grown on synthetic wastewater were similar to those on half-
319 strength Hutner's medium, the control.

320 [Figure 6 near here]

321 A comparison of figures 3 and 4 with figures 5 and 6 shows that a magnesium concentration
322 of 1 mM or more can have a significantly negative impact on *L. minor* growth and
323 photosynthesis (Figures 3 and 4) when calcium concentrations are low. A magnesium
324 concentration of 4.99 mM (Figures 3 and 4) can even cause death of plants. However, the same
325 high magnesium concentrations have only a minor effect on RGR, and no effect on
326 photosynthesis, when the ratio of Ca:Mg is kept at 1:1.6 (Figures 5 and 6). Thus, the data
327 emphasise the antagonism between calcium and magnesium, and the relative importance of the
328 Ca:Mg ratio.

329 In Lemnaceae, antagonistic interactions between calcium and magnesium have been observed,
330 and these impacted upon the aquatic toxicity of magnesium sulphate (Van Dam et al. 2010),
331 the degradation process of starch (Appenroth & Gabrys 2003) and the germination of turions
332 (Appenroth et al. 1999). Van Dam *et al.* (2010) observed the toxic effects of magnesium on
333 *Lemna aequinoctialis* (as well as on algae and other freshwater species), and the alleviation of
334 these toxic effects through the addition of calcium. Similarly, Appenroth *et al.* (1999) observed
335 that a high magnesium concentration, in a near calcium-free environment, inhibited turion
336 germination in *Spirodela polyrhiza*. Furthermore, these authors also observed that the
337 inhibiting effect of magnesium could be abolished by either adding calcium or by reducing the

338 concentration of magnesium. The explanation for this observed antagonism is that magnesium
339 is capable of competing with and inhibiting calcium uptake while also affecting calcium-
340 dependent processes (for example turion germination).

341 Thus, for phytoremediation approaches the calcium concentration, the magnesium
342 concentration and the Ca:Mg ratio in wastewater all need to be considered. This conclusion is
343 important in the context of remediation of dairy industry processing waste which can have a
344 highly variable Ca:Mg ratio. For example, standardised synthetic dairy wastewater has a
345 Ca:Mg ratio of 1:14.6 but Demirel and Yenigun (2004) found a Ca:Mg ratio of 1:1.5 (1.37:2.06
346 mM) in milk processing waste while Goyal and Gandhi (2009) found a Ca:Mg ratio of 5:1
347 (7.26:1.48 mM) from cheese whey. Thus, an unfavourable Ca:Mg ratio is a fact that needs to
348 be considered when developing a protocol for the phytoremediation of dairy processing waste.

349 ***Long-term growth on modified synthetic dairy wastewater***

350 Notwithstanding the importance of the Ca:Mg ratio, we note a small decrease in RGR at higher
351 magnesium concentrations, even where the Ca:Mg ratio is constant (Figure 5). It is possible
352 that this is “pure” magnesium toxicity, which is slowly building up over time. To explore this
353 in more detail, short term (7 day) experiments were complemented by 42-day experiments, in
354 which the Ca:Mg ratio was kept at 1:1.6, but at two different concentrations of calcium and
355 magnesium. Under these conditions, healthy growth of *L. minor* was observed for the full
356 length of the experiment, and growth rates were very similar to those obtained on half-strength
357 Hutner’s medium. ANOVA tests, confirmed this observation, showing no significant
358 difference between the RGR of *L. minor* grown on synthetic wastewater with a Ca:Mg ratio of
359 1:1.6, and those grown on Hutner’s throughout the 42-day experiment (Figure 7). Neither was
360 there a difference between plants on low concentrations (0.12 mM Ca and 0.2 mM Mg) of
361 calcium and magnesium, and those on high ones (3.12 mM Ca and 5.14 mM Mg). The general

362 trend is that the RGR of *L. minor* increased each week up to the end of the experiment at day
363 42, and this is presumably due to the frequent replacement of medium.

364 [Figure 7 near here]

365 **Conclusions**

366 Lemnaceae can be used to clean-up a variety of different wastewaters. However, some waste
367 streams are not suitable for growth of Lemnaceae and need to be modified to facilitate growth
368 and phytoremediation. Here we confirm the hypothesis that the Ca:Mg ratio can be a major
369 determinant of growth and photosynthesis, both in short and long-term trials. Yet, the data also
370 show that growth can be restored by the addition of calcium-sulphate, a procedure that is
371 feasible in a commercial setting. An addition of calcium to the synthetic wastewater to reach a
372 concentration of 0.12 mM, compared to 0.2 mM of magnesium, resulted in RGRs of 0.164 -
373 0.330 day⁻¹. These rates compare well with those of plants growing in half-strength Hutner's
374 medium, which achieve RGRs between 0.226 – 0.330 day⁻¹. It is acknowledged that calcium
375 can act as an antagonist for other elements, such as iron, manganese and potassium. However,
376 initial experiments showed that a reduction in the concentrations of iron and manganese did
377 not reverse the acute toxicity seen in plants. Thus, higher concentrations of calcium in later
378 experiments acting as an antagonist towards these elements would not have reversed acute
379 toxicity either. The data presented in this paper show convincingly that it is the ratio between
380 calcium and magnesium, which is causing acute toxicity to *L. minor* in this case. A Ca:Mg ratio
381 of 1:1.6 or greater is necessary for *Lemna minor* growth, and therefore phytoremediation of the
382 dairy industry processing wastewater.

383 **Acknowledgements**

384 We wish to thank the Irish Environmental Protection Agency (grant 2016-W-LS-11) for
385 funding this study. MJ acknowledges support by WoB.

386 **Declaration of interest statement**

387 The authors wish to disclose no conflict of interest.

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- 488

489 **Tables**

490 Table 1. Composition synthetic dairy wastewater (Tarpey 2016).

Chemical	Concentration mg/L¹	Molar concentration mM¹
Ammonium chloride	167.3	3.13
Urea	129.9	2.16
Disodium phosphate	50	0.35
Potassium bicarbonate	50	0.50
Sodium bicarbonate	130	1.55
Calcium chloride dihydrate	2	0.018
Magnesium sulphate heptahydrate	50	0.20
Manganese sulphate monohydrate	2	0.012
Iron sulphate heptahydrate	35	0.126
Zinc sulphate heptahydrate	2.15	0.007
Cobalt chloride hexahydrate	1.2	0.005
Manganese chloride tetrahydrate	4.95	0.025
Copper sulphate pentahydrate	1.25	0.005
Nickel chloride hexahydrate	0.95	0.004
Sodium molybdate dihydrate	1.1	0.005
Boric acid	0.07	0.001
Sodium selenite	0.49	0.003
EDTA	100	0.342

491 ¹Indicates the final concentration of each compound in the synthetic wastewater media

492 Table 2. Compound concentrations in half-strength Hutner's medium and synthetic wastewater
 493 with duckweed requirements and tolerance concentrations

Compound	Hutner (mM)	Synthetic wastewater (mM)	Minimum Required (mM)	Maximum Tolerated (mM)	'Optimal' Range (mM)
Ammonia/ammonium _{a,b,c}	NP	3.33	0.18	> 5.87	1.2 - 2.9
Nitrate ^a	9.06	NP	0.05	> 16.13	0.05 - 4.8
Urea	NP ⁱ	2.16	ND	ND	ND
Total Nitrogen ^d	9.07	8.17	0.005	149.93	0.2 - 25
Phosphate ^{a,d}	3.00	0.35	0.0003	9.48	0.001 - 0.95
Total Phosphorous ^{a,d}	3.00	0.35	0.0003	9.69	0.001 - 0.97
Potassium ^d	5.96	0.50	0.01	51.15	0.50 - 9.97
Sodium ^{d,e,f}	0.02	1.55	0	217.49	0 - 10.87
Magnesium ^{a,d}	2.97	0.20	0.004	32.92	0.21 - 9.87
Calcium ^{a,d}	3.04	0.014	0.01	39.92	0.20 - 19.96
Sulphate ^{d,g}	3.00	0.35	0.01	59.33	0.52 - 20.82
Chloride ^{e,g}	NP	3.21	0.001	28.21	0.001 - 5.64
EDTA ^d	0.01	0.34	0	> 2.05	0 - 1.71
Iron ^{d,g}	0.004	0.13	0.004	0.39	0.004 - 0.18
Zinc ^{g,h}	0.003	0.007	0.001	0.31	0.002 - 0.09
Cobalt ^d	NP	0.005	0	0.05	0
Manganese ^g	0.001	0.037	0.0005	1.09	0.001 - 0.05
Copper ^{d,h}	0.0001	0.005	0.0001	0.03	0.00001 - 0.01
Nickel ^{d,h}	NP	0.004	0	0.02	0.00 - 0.002
Molybdenum ^{d,h}	0.0004	0.005	0.0002	1.04	0.0002 - 0.10
Boron ^{d,h}	0.02	0.001	0.0001	5.55	0.0001 - 0.46
Selenium ^{d,h}	NP	0.003	0	0.06	0

494 ^a(Paolacci et al. 2016)

495 ^b(Caicedo et al. 2000)

496 ^c(Körner et al. 2001)

497 ^d(Landolt & Kandeler 1987)

498 ^e(Sree et al. 2015)

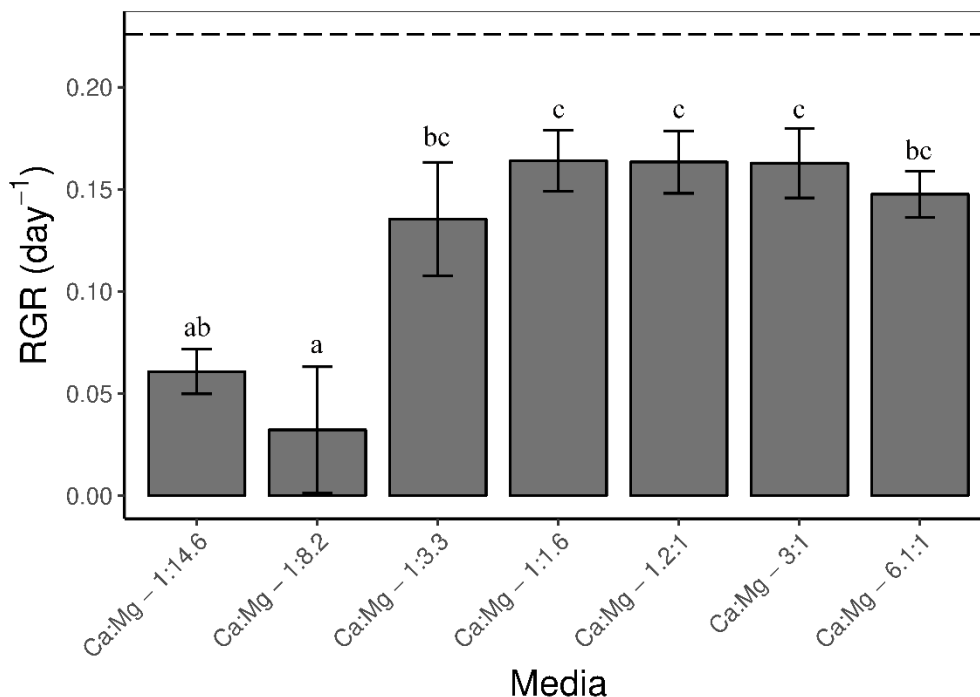
499 ^f(Liu et al. 2017)

500 ^g(Wang 1986)

501 ^h(Lahive et al. 2012)

502 ⁱNP – Not present

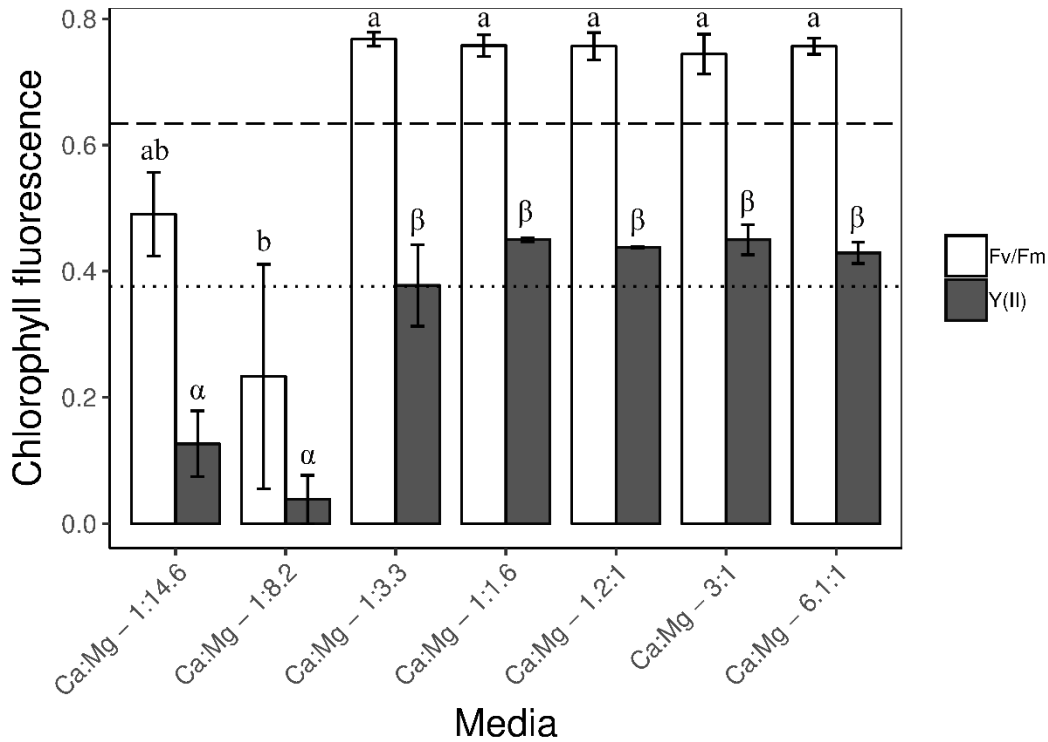
503 **Figure captions**



504

505 Figure 1. Biomass RGR of *L. minor* growing on synthetic wastewater medium with various
506 Ca:Mg ratios (bars) and on half-strength Hutner's medium (dashed line). The unmodified
507 synthetic waste medium has a Ca:Mg ratio of 1:14.6. Error bars represent standard error (n=3).
508 ANOVA shows a positive association between Ca:Mg ratio and RGR (p<0.05). A post-hoc
509 Tukey test which is represented on the graph as letters above the bars, shows the significant
510 variance between groups. Bars that do not share a similar letter differ significantly (p<0.05)
511 from one another.

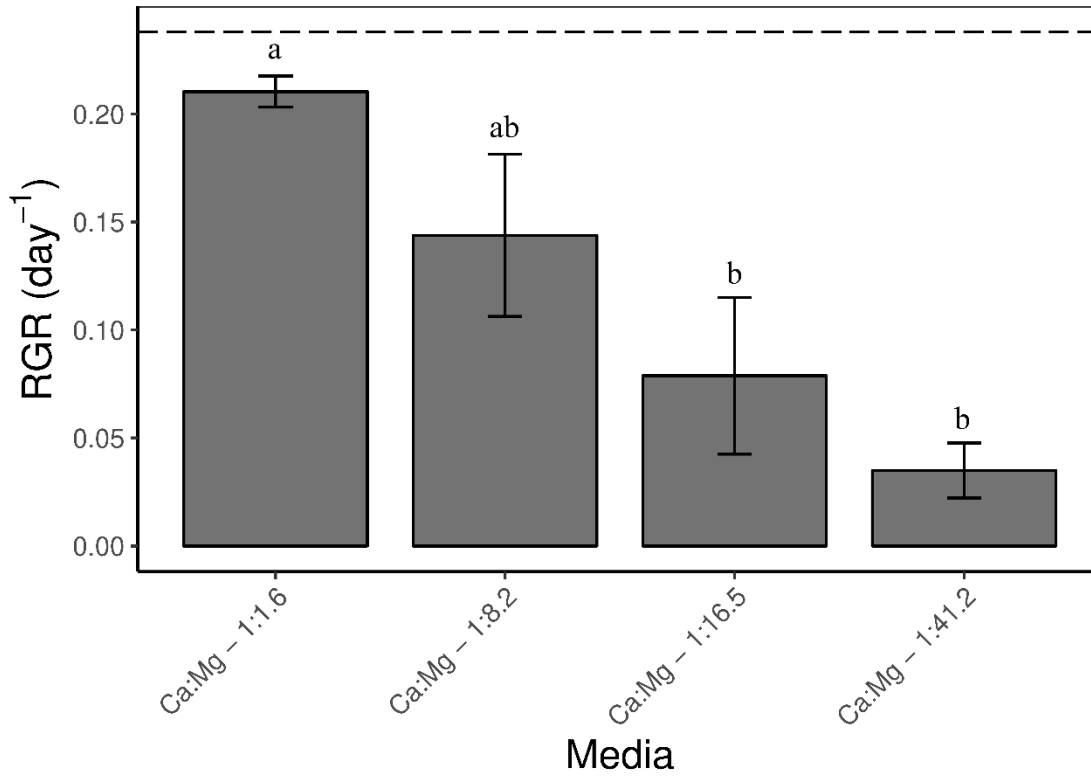
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513

514 Figure 2. Fv/Fm and Y(II) values of *L. minor* growing on synthetic wastewater with different
 515 Ca:Mg ratios (bars) and on half-strength Hutner's medium (dashed and dotted lines, Fv/Fm
 516 and Y(II), respectively). Error bars represent standard error (n=3). ANOVA shows a positive
 517 association between Ca:Mg ratio and Fv/Fm, as well as Y(II) (p<0.01). A post-hoc Tukey test,
 518 indicated by letters above the bars, shows significant difference between Fv/Fm of plants at
 519 Ca:Mg ratio of 1:8.2 and all other ratios (excluding 1:14.6), p<0.01. For Y(II), a post-hoc Tukey
 520 test shows significant differences between plants growing at a Ca:Mg ratio of 1:14.6 and 1:8.2
 521 and plants growing in all other ratios (p<0.01). Bars that do not share a similar letter differ
 522 significantly from one another.

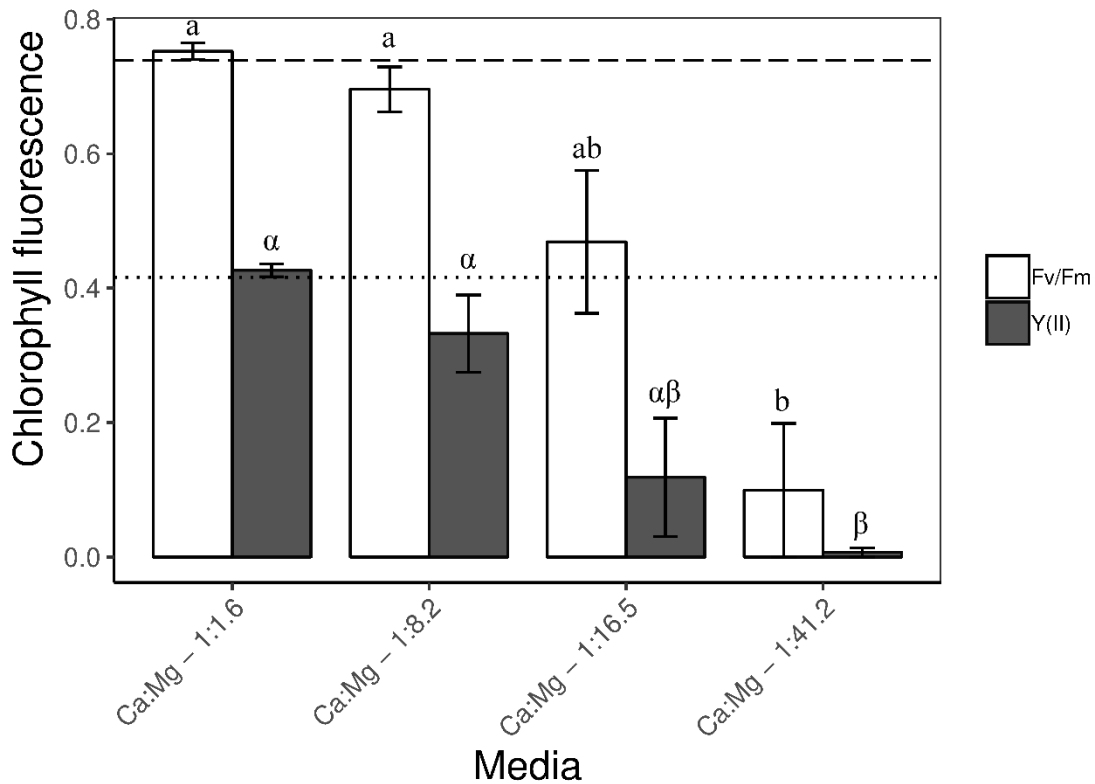
523



524

525 Figure 3. RGR of *L. minor* growing on synthetic wastewater with various Ca:Mg ratios (bars)
 526 and on half-strength Hutner's medium (dashed line). Error bars represent standard error (n=4).
 527 ANOVA showed significant differences in RGR between groups, $F(3, 12) = 7.996$, $p < 0.05$.
 528 Letters above the bars show the results of a Tukey post-hoc test. Bars that do not share a similar
 529 letter differ significantly from one another.

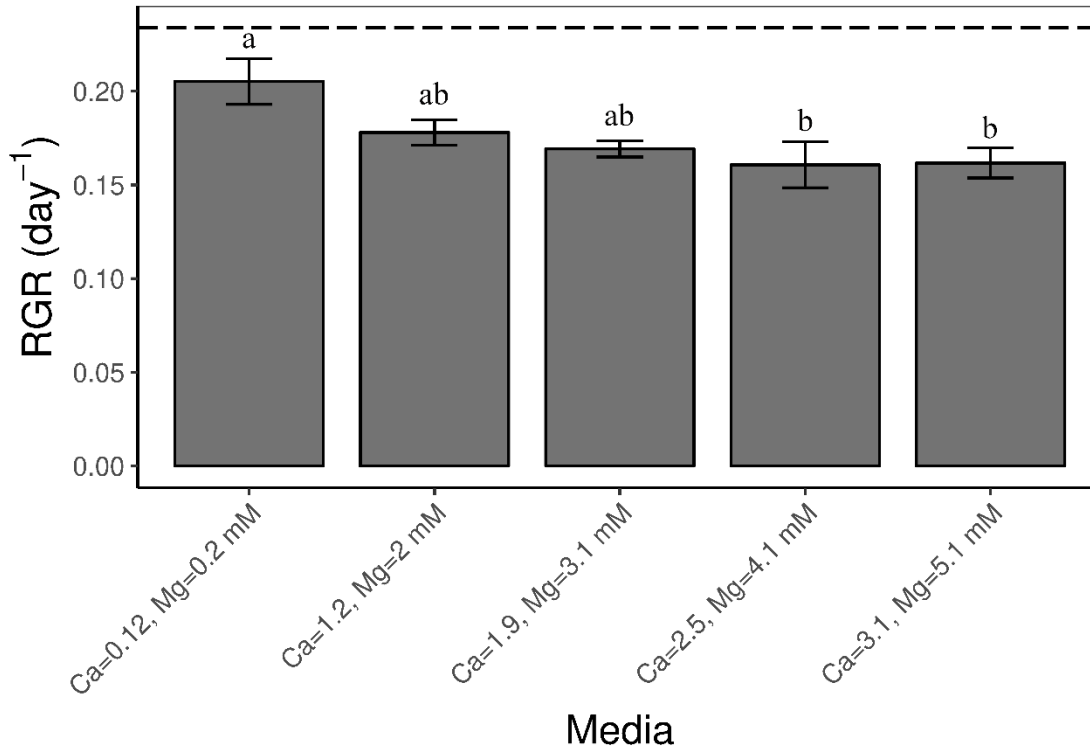
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531

532 Figure 4. Fv/Fm and Y(II) of *L. minor* growing on synthetic wastewater with various Ca:Mg
 533 ratios (bars) and on half-strength Hutner's medium (dashed and dotted lines, Fv/Fm and Y(II),
 534 respectively). Error bars represent standard error (n=4). A Welch's ANOVA test showed that
 535 there is significant difference between the treatment groups, $p < 0.01$. Letters above bars show
 536 significant differences between groups as indicted in Games-Howell post-hoc test. Bars that do
 537 not share a similar letter differ significantly from one another.

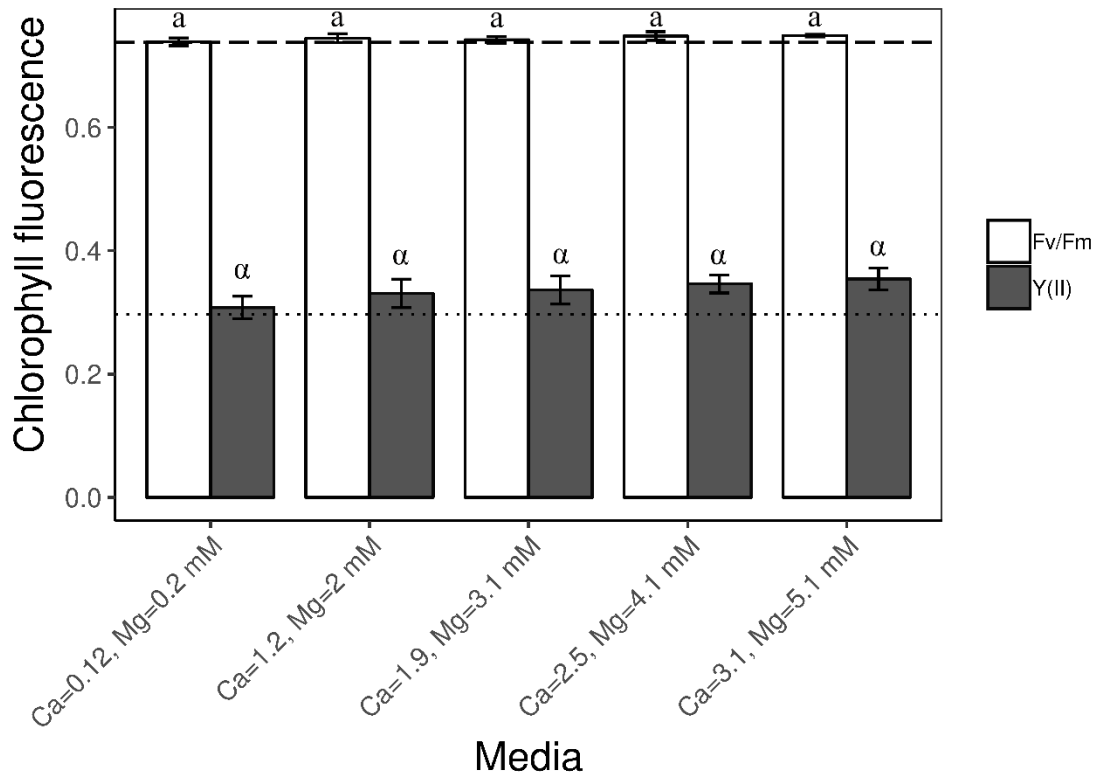
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539

540 Figure 5. RGR of *L. minor* growing on synthetic wastewater of various concentrations of
 541 calcium and magnesium (bars) and on half-strength Hutner's medium (dashed line). Error bars
 542 represent standard error (n=4). ANOVA showed a significant difference between the RGR of
 543 *L. minor* in different synthetic wastewater treatments, $F(4,15) = 3.89$, $p < 0.05$. A post-hoc
 544 Tukey test, indicated by letters above the bars, shows significant differences between the RGR
 545 of *L. minor* in a calcium and magnesium concentration of 0.12 and 0.2 mM, respectively, and
 546 those in the two highest concentrations. Bars that do not share a similar letter differ significantly
 547 from one another.

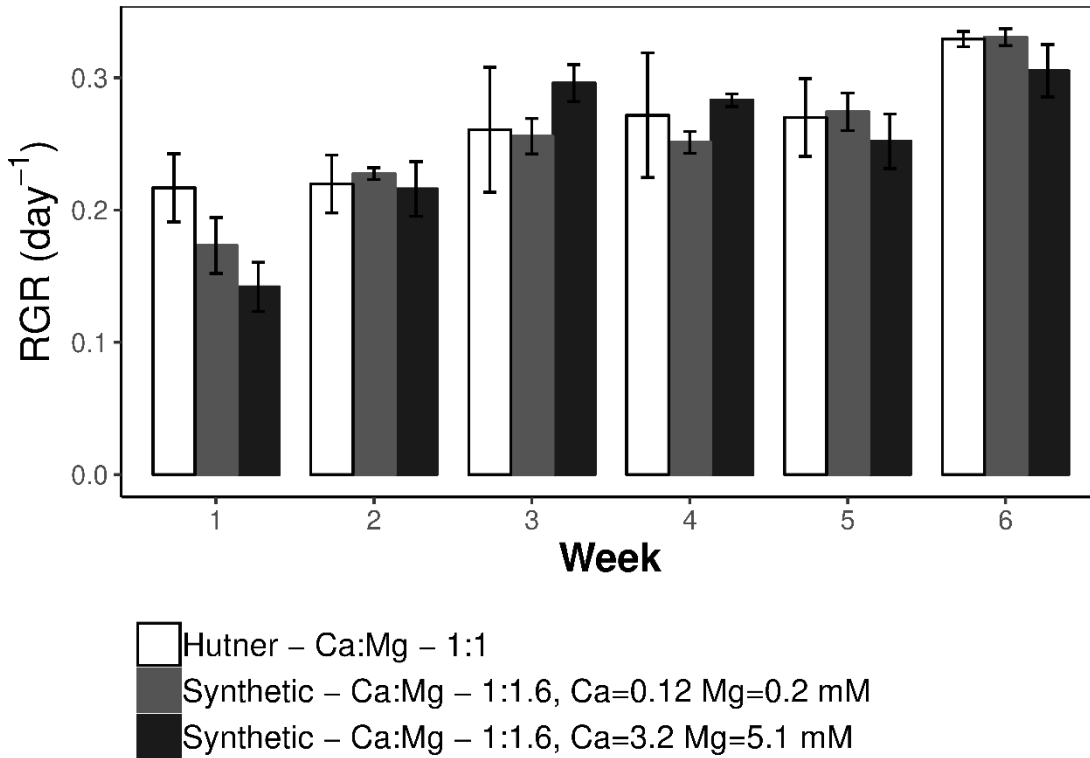
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549

550 Figure 6. Fv/Fm and Y(II) for *L. minor* growing in synthetic wastewater of various
 551 concentrations of calcium and magnesium (bars) and on half-strength Hutner's medium
 552 (dashed and dotted lines, Fv/Fm and Y(II), respectively). Error bars represent standard error
 553 (n=4). ANOVA did not reveal significant effects of concentration of either Fv/Fm or Y(II).
 554 Measurement values of Fv/Fm and Y(II) do not differ significantly between treatments.

555



556

557 Figure 7. Weekly RGR of *L. minor* growing on synthetic wastewater (Ca:Mg ratio of 1:1.6 at
 558 two concentrations) and half-strength Hutner's medium throughout a 42-day experiment.
 559 ANOVA tests showed the RGR between treatments was not significantly different in any week
 560 of the experiment (n=4). Results for week 1-6 respectively: $F(2,9)=2.918$, $p=0.105$;
 561 $F(2,9)=0.112$, $p=0.896$; $F(2,9)=0.554$, $p=0.593$; $F(2,9)=0.337$, $p=0.723$; $F(2,9)=0.28$, $p=0.762$;
 562 $F(2,9)=1.319$, $p=0.314$.

563