

Title	The importance of the calcium-to-magnesium ratio for phytoremediation of dairy industry wastewater using the aquatic plant Lemna minor 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 Lemna minor L.', International Journal of Phytoremediation. doi: 10.1080/15226514.2019.1707478
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1080/15226514.2019.1707478
Rights	© 2020, Taylor & Francis Group. This is an Accepted Manuscript of an article published by Taylor & Francis in International Journal of Phytoremediation on 7 January 2020, available online: http:// www.tandfonline.com/10.1080/15226514.2019.1707478
Download date	2025-08-03 21:56:35
Item downloaded from	https://hdl.handle.net/10468/9644



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

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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 processing waste with the duckweed plant Lemna minor. However, initial trials failed to 14 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 facilitating growth. Using lab-scale experiments in which L. minor were grown on 100 mL of 18 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 to RGRs as low as 0.05 day⁻¹. A change in this ratio to favour calcium, through the addition of 22 23 calcium sulphate, leads to RGRs of 0.2 - 0.3 day⁻¹. Experiments lead us to conclude that a Ca:Mg ratio of 1:1.6 (by molar concentration) or greater is necessary for *Lemna minor* growth, 24 and therefore phytoremediation of dairy industry processing wastewater. 25

26

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

28 Introduction

29 Phytoremediation refers to the process whereby plants, and associated microorganisms, are used to remove and/or degrade contaminants from soils and waters. Lemnaceae species, 30 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 range of pollutants, and high pollutant removal rates (Zaved et al. 1998; Cheng & Stomp 2009; 33 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 feed. Re-using plant nutrients, such as phosphate, nitrate and ammonia, present in wastewater 40 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. 2009). 44

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 m³ of effluent per m³ of processed milk (Kolev Slavov 2017). This processing wastewater is 48 49 rich in nitrogen and phosphorous as well as other essential plant nutrients such as calcium, potassium and magnesium (Ince 1998; Demirel & Yenigun 2004; Goyal & Gandhi 2009; 50 Carvalho et al. 2013; Ryan & Walsh 2016). As expected for a waste product from the food 51 52 industry, dairy industry processing wastewater contains only low concentrations of contaminants such as heavy metals (Ince 1998; Demirel & Yenigun 2004). Therefore, dairy
industry processing wastewater is well suited to remediation using a circular economy
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 & Kandeler 1987; Paolacci et al. 2016). For calcium and magnesium acceptable concentrations 66 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 high degree of variability in their concentrations, and this relates to different factories and 77

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

In order to facilitate reproducible laboratory phytoremediation studies, a synthetic dairy 80 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 composition of real wastewater. Unfortunately, preliminary experiments showed that 83 84 duckweed did not grow well in this synthetic dairy wastewater. The aim of the present study was to identify the reasons responsible for the poor performance of duckweed on the synthetic 85 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 μ mol m⁻² 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 wastewater. For all experiments plants were grown in their respective media in magenta vessels 111 112 (Magenta GA-7 Plant Culture Box) for 7 days in a growth room at a constant temperature of 21°C, light intensity of 80.82 μ mol m⁻² s⁻¹ and a photoperiod of 16 hours light to 8 hours dark. 113 114 This light intensity is lower than that used in standard toxicological protocols, thus growth rates were moderately lower than standard, not exceeding an RGR of 0.3 day⁻¹. 115

116 Experimental design

This study contains two types of experiments; short-term (7 days) and long-term (42 days). In both types of studies *L. minor* plants were grown on a number of differently modified versions of synthetic dairy wastewater (Table 1) with half-strength Hutner's medium as a control. At the start of each experiment, three three-frond colonies were added to each magenta. On day zero of each experiment, the starting mass (fresh weight), colony number and frond number were determined. The synthetic wastewater was not changed during the 7-day experiments. In 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 through the addition of potassium bicarbonate. Sulphate was increased through the substitution 133 134 of ammonium chloride for ammonium sulphate and the addition of potassium sulphate. The 135 calcium concentration was increased by adding calcium sulphate (CaSO₄) to the synthetic wastewater. The magnesium concentration was increased or decreased by altering the 136 137 concentration of magnesium sulphate heptahydrate (MgSO₄.7H₂O). 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 naturally around 8. However, for experiments the pH was reduced to between 4.5 - 5, a pH 142 143 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 counted by eye. Before weighing, plants were wrapped in tissue paper to remove excess water.
The Relative Growth Rate (RGR) was calculated based on fresh weight measurements using
the formula below (Connolly & Wayne 1996):

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

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 fluorometry (WALZ Imaging fluorometer, Effeltrich, Germany). For chlorophyll a 155 156 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 saturating pulse of light (2700 μ mol/m²/s) was applied to obtain the maximum fluorescence 161 F_m . Subsequently, actinic light (photosynthetically active light of 186 μ mol/m²/s) was applied 162 to the plants and at 20 second intervals saturating pulses were applied to measure Fm', the 163 164 maximum fluorescence under light-adapted conditions. Ft is the value of fluorescence 165 immediately before the saturating pulse is applied, i.e. the steady-state value of fluorescence. F_v/F_m, the maximum quantum efficiency of photosystem II (PSII), and Y(II), the quantum 166 167 efficiency of PSII under steady state light conditions were calculated according to Maxwell 168 and Johnson (2000) using the following equations:

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

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

For the long-term 42-day experiment, mass (fresh biomass), colony number and frond number
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.

Under control growth conditions, plants on half-strength Hutner's medium displayed vigorous growth (average RGR of 0.226 day⁻¹) and had a healthy appearance. In contrast, plants growing on unmodified synthetic wastewater (Ca:Mg ratio of 1:14.6) had an average RGR of just 0.061 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 -3:1) and 0.148 (Ca:Mg – 6.1:1) day⁻¹. From a low growth rate on medium with a Ca:Mg ratio 222 of 1:14.6, RGR increased up until a Ca:Mg of 1:1.6 ratio, where RGR-values plateaued (Figure 223 224 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 between the Ca:Mg ratio and the RGR (day⁻¹) of L. minor. A post-hoc Tukey test revealed 226 227 which differences in RGR were significant. The RGR values of plants grown on synthetic 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 228 229 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 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 242 243 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]

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

263 Increasing concentrations of magnesium relative to calcium

To explore in more detail the importance of the Ca:Mg ratio, the ratio was changed in favour of magnesium at adequate calcium concentrations. Calcium concentrations were kept constant at 0.12 mM, which was previously shown to accommodate good growth (Figure 1). *L. minor* 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 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⁻¹ 270 ¹ at a Ca:Mg ratio of 1:41.2. The *L. minor* plants at the high magnesium concentration (4.99 271 mM) were characterised by both poor growth and chlorosis. An analysis of variance (ANOVA) 272 273 showed significant differences in RGR between groups, F(3, 12) = 7.996, p<0.05, indicating the significant influence of magnesium on plant growth. A post-hoc Tukey test showed 274 275 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 Y(II), shown as letters above bars in Figure 4. The Fv/Fm of plants growing in Ca:Mg ratio of 286 287 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]

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

Fv/Fm and Y(II) measurements revealed non-significant differences between plants that were
grown in synthetic wastewater containing different concentrations of calcium and magnesium
(Figure 6). When treatments were compared using an ANOVA test no significant differences
were found; Fv/Fm, F(4, 15)=0.524, p=0.72; Y(II), F(4, 15)=0.809, p=0.538. Furthermore,
Fv/Fm and Y(II) values for plants grown on synthetic wastewater were similar to those on halfstrength 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 concentration of 4.99 mM (Figures 3 and 4) can even cause death of plants. However, the same 324 325 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 these toxic effects through the addition of calcium. Similarly, Appenroth et al. (1999) observed 334 335 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

concentration of magnesium. The explanation for this observed antagonism is that magnesium
is capable of competing with and inhibiting calcium uptake while also affecting calciumdependent 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 Ca:Mg ratio of 1:14.6 but Demirel and Yenigun (2004) found a Ca:Mg ratio of 1:1.5 (1.37:2.06 345 346 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 diary 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 difference between the RGR of L. minor grown on synthetic wastewater with a Ca:Mg ratio of 358 359 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

- 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 streams are not suitable for growth of Lemnaceae and need to be modified to facilitate growth 367 368 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 show that growth can be restored by the addition of calcium-sulphate, a procedure that is 370 feasible in a commercial setting. An addition of calcium to the synthetic wastewater to reach a 371 372 concentration of 0.12 mM, compared to 0.2 mM of magnesium, resulted in RGRs of 0.164 -0.330 day⁻¹. These rates compare well with those of plants growing in half-strength Hutner's 373 medium, which achieve RGRs between 0.226 - 0.330 day⁻¹. It is acknowledged that calcium 374 375 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
- We wish to thank the Irish Environmental Protection Agency (grant 2016-W-LS-11) for
 funding this study. MJ acknowledges support by WoB.

Declaration of interest statement

387 The authors wish to disclose no conflict of interest.

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489 **Tables**

Chemical	Concentration mg/L ¹	Molar concentration mM ¹	
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	

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

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

Table 2. Compound concentrations in half-strength Hutner's medium and synthetic wastewater 492

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

- ^a(Paolacci et al. 2016)
- ^b(Caicedo et al. 2000)
- ^c(Körner et al. 2001)
- 494 495 496 497 498 499 500 501 502 ^d(Landolt & Kandeler 1987)
- ^e(Sree et al. 2015)
- ^f(Liu et al. 2017)
- g(Wang 1986)^h(Lahive et al. 2012)
 ⁱNP Not present

503 Figure captions



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

512



Figure 2. Fv/Fm and Y(II) values of L. minor growing on synthetic wastewater with different 514 515 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.



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



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



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 of L. minor in a calcium and magnesium concentration of 0.12 and 0.2 mM, respectively, and 545 546 those in the two highest concentrations. Bars that do not share a similar letter differ significantly 547 from one another.



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



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