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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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Abstract

Lemnaceae are being exploited to remediate a variety of different wastewaters. Dairy processing waste is produced in large amounts, and contains a range of valuable plant nutrients, 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 establish growth of *L. minor* on this medium. A lack of growth can be due to a lack of essential plant micro- and macro-nutrients, or the presence of phytotoxic ingredients. In this study we 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 synthetic wastewater, we demonstrated that the skewed Ca:Mg ratio in synthetic dairy industry wastewater is a key obstacle to good growth. Experiments showed that a ratio which favours 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 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, and therefore phytoremediation of dairy industry processing wastewater.

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

Introduction

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

Dairy industry processing wastewater is generated during the production of dairy products such as milk powder, cheese and yogurt from raw milk. Typically, large volumes are produced. In Europe dairy processing is seen as the largest industrial food wastewater source, with 0.5–37 m^3 of effluent per m^3 of processed milk (Kolev Slavov 2017). This processing wastewater is 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; Carvalho et al. 2013; Ryan & Walsh 2016). As expected for a waste product from the food 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.

Duckweed species have been shown to be tolerant of a wide range of conditions and nutrient concentrations (Landolt & Kandeler 1987). Nevertheless, any particular wastewater needs to fulfil minimal criteria to facilitate growth and phytoremediation. An important criterion is the presence of adequate levels of essential plant growth nutrients. Taking nitrogen as an example, *Lemna minor* will grow on either ammonium or nitrate as a nitrogen source and can tolerate concentrations ranging between 0.2 to 150 mM with optimal concentrations varying depending on the nitrogen source (Landolt & Kandeler 1987; Paolacci et al. 2016). Duckweed can tolerate a pH range from 4 – 8 (Landolt & Kandeler 1987), but also the pH is also important in determining the tolerance of duckweed to ammonia (Körner et al. 2003). In the case of phosphate, duckweed tolerates concentrations ranging between 0.001 to 10 mM (Landolt & Kandeler 1987; Paolacci et al. 2016). For calcium and magnesium acceptable concentrations range between 0.2-20 mM and 0.2-10 mM, respectively (Landolt & Kandeler 1987; Van Dam et al. 2010; Paolacci et al. 2016), while the Ca:Mg ratio is also an important determinant of plant growth. Other criteria for plant growth and phytoremediation relate to the presence of, potentially phytotoxic, pollutants. In the specific case of dairy industry processing waste there is a heavy load of organic matter in the wastewater which is measured as biochemical oxygen demand (BOD_5 mg/L), chemical oxygen demand (COD mg/L) and fats (mg/L) (Janczukowicz et al. 2008; Carvalho et al. 2013). Duckweed do not require organic compounds in the medium for survival and growth (Körner et al. 1998), however, they can contribute to the reduction in the amount of organic matter as part of a phytoremediation approach (Körner et al. 1998; Li et 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 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 industry wastewater has been developed (Tarpey 2016; Gil-Pulido et al. 2018). The composition of this synthetic wastewater (Table 1) is based on measurements of the composition of real wastewater. Unfortunately, preliminary experiments showed that 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 dairy wastewater. Synthetic dairy industry wastewater has a Ca:Mg ratio of 1:14.6. It is known that an imbalance in favour of magnesium can have a negative effect on the growth and health of duckweed (Landolt & Kandeler 1987; Paolacci et al. 2016). This antagonistic Ca:Mg relationship was first studied in terrestrial plants (Loew & May 1901). It has been found that in soils magnesium decreases the calcium uptake in the plant, while calcium reduces magnesium uptake to a lesser extent (Halstead et al. 1958). Thus, imbalances in the soil Ca:Mg ratio can potentially aggravate calcium or magnesium deficiencies as well as magnesium toxicity (Brady et al. 2005). Therefore, based on existing literature, we hypothesised that a ratio between calcium and magnesium in favour of magnesium causes acute toxicity in *L. minor*, and that changing the ratio in favour of calcium removes this acute toxicity for *L. minor*. In this study, different levels of this imbalance between calcium and magnesium were tested in short- and long-term experiments in order to identify the concentration of calcium and magnesium most suitable for duckweed in the particular chemical environment of the synthetic dairy wastewater. This study underpins an important aspect of phytoremediation, assessing and amending wastewater composition to facilitate plant growth.

Materials and Methods

Cultivation of stock and experimental plants

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

A synthetic wastewater was used as a growing media (Table 1) for experimental purposes. The composition of this synthetic wastewater was based on analysis of real dairy industry processing wastewater (Tarpey 2016). Control plants were grown in 100 mL of half-strength 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 (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 μmol m^{-2} s^{-1} and a photoperiod of 16 hours light to 8 hours dark. 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}.

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

[Table 1 near here]

Synthetic wastewater modifications

A reduction in the concentration of chloride was achieved by replacing ammonium chloride in the synthetic wastewater with ammonium sulphate, thus removing the majority of chloride. A reduction in the concentration of sodium was achieved by the removal of sodium bicarbonate. Iron and manganese concentrations were reduced through the addition of less iron sulphate heptahydrate and manganese chloride tetrahydrate, respectively. Potassium was increased through the addition of potassium bicarbonate. Sulphate was increased through the substitution of ammonium chloride for ammonium sulphate and the addition of potassium sulphate. The calcium concentration was increased by adding calcium sulphate ($CaSO_4$) to the synthetic wastewater. The magnesium concentration was increased or decreased by altering the concentration of magnesium sulphate heptahydrate ($MgSO_4.7H_2O$). A secondary impact of altering the calcium and magnesium concentrations is that sulphate concentration is also affected. The highest concentration to which sulphate is increased, 8.3 mM, is still significantly below the maximum concentration of sulphate tolerated by *L. minor*, 60 mM (see Table 2 for 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 similar to that of half-strength Hutner's medium, to ensure differences observed were not due to differences in pH.

Measured parameters

On day seven of the short-term experiments mass (fresh biomass), colony number, frond 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):

$$RGR = \frac{ln\,(\frac{W_2}{W_1})}{\Delta T}$$

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

Chlorophyll *a* fluorescence was measured using pulse amplitude modulated chlorophyll *a* fluorometry (WALZ Imaging fluorometer, Effeltrich, Germany). For chlorophyll *a* fluorescence analysis, plants were dark adapted for 15 minutes immediately before measurements. Then, three random colonies from each Magenta were taken for analysis; the measured values of these three colonies were averaged together and treated as one replicate. The chlorophyll fluorescence analysis procedure is as follows; first, a low intensity modulated measuring light was turned on to measure F_0 on the dark-adapted plant, and secondly a saturating pulse of light (2700 µmol/m^2/s) was applied to obtain the maximum fluorescence F_m. Subsequently, actinic light (photosynthetically active light of 186 µmol/m^2/s) was applied to the plants and at 20 second intervals saturating pulses were applied to measure F_m', the maximum fluorescence under light-adapted conditions. F_t is the value of fluorescence 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 efficiency of PSII under steady state light conditions were calculated according to Maxwell and Johnson (2000) using the following equations:

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

$$Y(II) = (F_m{}' - F_t)/F_m{}'$$

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. RGR was calculated based on growth over seven days.

Data analysis

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

Results and Discussion

Investigation of components of synthetic dairy wastewater

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

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

[Table 2 near here]

Increasing concentrations of calcium relative to magnesium

Synthetic dairy processing water has a low concentration of calcium (0.014 mM), notably lower than the optimal range of concentrations (0.2 – 20 mM). Furthermore, it was noted that the Ca:Mg ratio in the synthetic wastewater was 1:14.6, and this relative lack of calcium compared to magnesium might potentially also cause growth problems (Landolt & Kandeler 1987; Van Dam et al. 2010). To explore the roles of calcium, magnesium and the Ca:Mg ratio in controlling growth, an initial experiment was conducted in which *L. minor* plants were grown in medium containing increasing amounts of calcium, whilst the magnesium concentration was kept constant at 0.2 mM. The calcium concentrations ranged from 0.014 to 1.21 mM, translating to Ca:Mg ratios of 1:14.6 to 6.1:1, respectively. As a control, plants were grown on 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 was modified through the addition of calcium, RGR values increased significantly. In modified synthetic wastewater with a concentration of calcium greater than or nearly equal to that of 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 of 1:14.6, RGR increased up until a Ca:Mg of 1:1.6 ratio, where RGR-values plateaued (Figure 1). Analysis of variance (ANOVA) between treatments (excluding Hutner) showed significant 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 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 from each other. The RGR of plants on synthetic waste of Ca:Mg ratio 1:14.6 differed 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 RGR of plants on synthetic wastewater of Ca:Mg ratio of 1:8.2 differed significantly from that of plants on a Ca:Mg ratio of 1:3.3, 1:1.6, 1.2:1, 3:1 and 6.1:1, $p<0.05$.

[Figure 1 near here]

To complement growth rate measurements, key photosynthetic parameters were measured in parallel. In particular Fv/Fm and Y(II) were analysed. Both of these parameters test if, and to what degree, a stressor is affecting photosystem II in the plant. The former refers to the plant in a dark-adapted state, while the latter refers to plants during photosynthesis at steady-state conditions. The control plants on half-strength Hutner's medium displayed good photosynthetic activity, as shown by average values of 0.65 for Fv/Fm and 0.39 for Y(II) (Figure 2). On synthetic wastewater with Ca:Mg ratios of 1:14.6 and 1:8.2, Fv/Fm and Y(II) 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 those of plants on the half-strength Hutner's control. As the calcium concentration was increased, Fv/Fm and Y(II) values increased up until a Ca:Mg ratio of 1.2:1 where values started to plateau. For Fv/Fm, ANOVA showed significant differences between synthetic wastewater treatments, F(6, 14) = 7.843, $p<0.01$, indicating that there was a positive association between changing the Ca:Mg ratio and Fv/Fm, an indicator of the health of *L. minor*. A post-hoc Tukey test showed that the significant variance (shown as letters above the bars in Figure 2) between the groups ($p<0.01$) was between plants growing on a Ca:Mg ratio of 1:8.2 and 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 differences between treatment groups, F(6, 14) = 22.62, $p<0.001$. This indicates, similarly to Fv/Fm, there was a positive association between the Ca:Mg ratio and Y(II), an indicator of the photosynthetic efficiency of the plant. A post-hoc Tukey test shows this significant variation was between plants growing on synthetic wastewater of Ca:Mg ratios 1:14.6 and 1:8.2, which 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, which had higher Y(II) values.

[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.

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 ratios of 1:1.6 through to 1:41.2, respectively. It can be seen (Figure 3) that RGR progressively 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} at a Ca:Mg ratio of 1:41.2. The *L. minor* plants at the high magnesium concentration (4.99 mM) were characterised by both poor growth and chlorosis. An analysis of variance (ANOVA) 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 significant differences between the RGR of plants growing on a Ca:Mg ratio of 1:1.6 and that of plants in Ca:Mg ratios 1:16.5 ($p<0.05$) and 1:41.2 ($p<0.01$).

[Figure 3 near here]

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

[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).

Simultaneous increase of calcium and magnesium concentrations

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

[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 half-strength Hutner's medium, the control.

[Figure 6 near here]

A comparison of figures 3 and 4 with figures 5 and 6 shows that a magnesium concentration of 1 mM or more can have a significantly negative impact on *L. minor* growth and 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 high magnesium concentrations have only a minor effect on RGR, and no effect on photosynthesis, when the ratio of Ca:Mg is kept at 1:1.6 (Figures 5 and 6). Thus, the data emphasise the antagonism between calcium and magnesium, and the relative importance of the Ca:Mg ratio.

In Lemnaceae, antagonistic interactions between calcium and magnesium have been observed, and these impacted upon the aquatic toxicity of magnesium sulphate (Van Dam et al. 2010), the degradation process of starch (Appenroth & Gabrys 2003) and the germination of turions (Appenroth et al. 1999). Van Dam *et al.* (2010) observed the toxic effects of magnesium on *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 that a high magnesium concentration, in a near calcium-free environment, inhibited turion germination in *Spirodela polyrhiza*. Furthermore, these authors also observed that the 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 calcium-dependent processes (for example turion germination).

Thus, for phytoremediation approaches the calcium concentration, the magnesium concentration and the Ca:Mg ratio in wastewater all need to be considered. This conclusion is important in the context of remediation of dairy industry processing waste which can have a 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 mM) in milk processing waste while Goyal and Gandhi (2009) found a Ca:Mg ratio of 5:1 (7.26:1.48 mM) from cheese whey. Thus, an unfavourable Ca:Mg ratio is a fact that needs to be considered when developing a protocol for the phytoremediation of dairy processing waste.

Long-term growth on modified synthetic diary wastewater

Notwithstanding the importance of the Ca:Mg ratio, we note a small decrease in RGR at higher magnesium concentrations, even where the Ca:Mg ratio is constant (Figure 5). It is possible that this is "pure" magnesium toxicity, which is slowly building up over time. To explore this in more detail, short term (7 day) experiments were complemented by 42-day experiments, in which the Ca:Mg ratio was kept at 1:1.6, but at two different concentrations of calcium and magnesium. Under these conditions, healthy growth of *L. minor* was observed for the full length of the experiment, and growth rates were very similar to those obtained on half-strength 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 1:1.6, and those grown on Hutner's throughout the 42-day experiment (Figure 7). Neither was there a difference between plants on low concentrations (0.12 mM Ca and 0.2 mM Mg) of 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 42, and this is presumably due to the frequent replacement of medium.

[Figure 7 near here]

Conclusions

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 and phytoremediation. Here we confirm the hypothesis that the Ca:Mg ratio can be a major 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 feasible in a commercial setting. An addition of calcium to the synthetic wastewater to reach a 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 medium, which achieve RGRs between 0.226 – 0.330 day^{-1}. It is acknowledged that calcium can act as an antagonist for other elements, such as iron, manganese and potassium. However, initial experiments showed that a reduction in the concentrations of iron and manganese did not reverse the acute toxicity seen in plants. Thus, higher concentrations of calcium in later experiments acting as an antagonist towards these elements would not have reversed acute toxicity either. The data presented in this paper show convincingly that it is the ratio between calcium and magnesium, which is causing acute toxicity to *L. minor* in this case. A Ca:Mg ratio of 1:1.6 or greater is necessary for *Lemna minor* growth, and therefore phytoremediation of the dairy industry processing wastewater.

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Declaration of interest statement

The authors wish to disclose no conflict of interest.

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Tables

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

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

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

Table 2. Compound concentrations in half-strength Hutner's medium and synthetic wastewater 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)
[d](Landolt & Kandeler 1987)
[e](Sree et al. 2015)
[f](Liu et al. 2017)
[g](Wang 1986)
[h](Lahive et al. 2012)
[i]NP – Not present

Figure captions

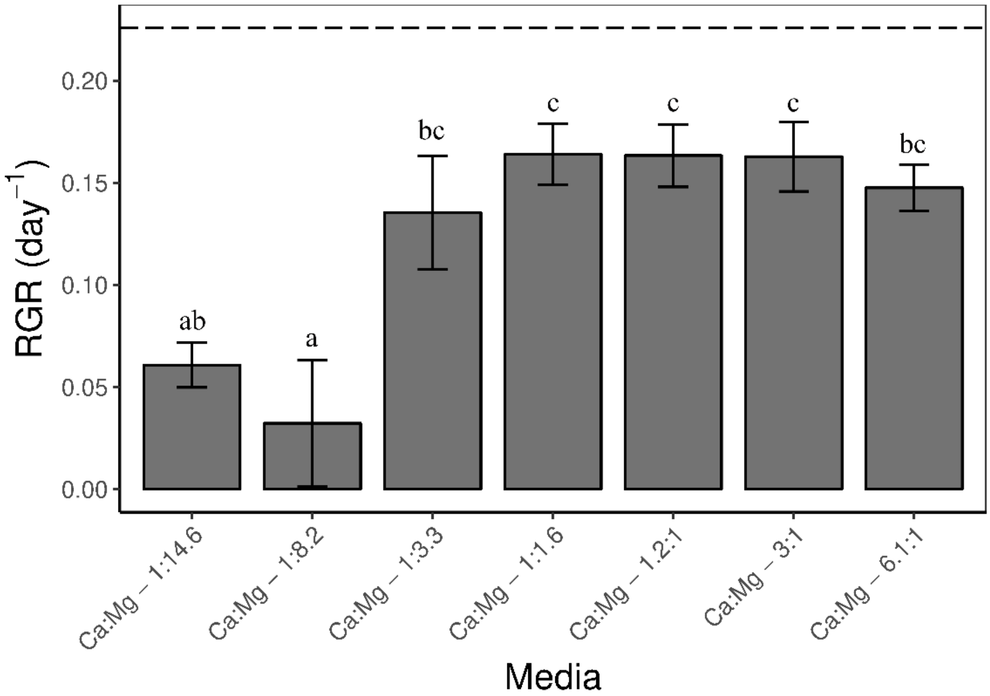

Figure 1. Biomass RGR of *L. minor* growing on synthetic wastewater medium with various Ca:Mg ratios (bars) and on half-strength Hutner's medium (dashed line)**.** The unmodified synthetic waste medium has a Ca:Mg ratio of 1:14.6. Error bars represent standard error (n=3). ANOVA shows a positive association 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.

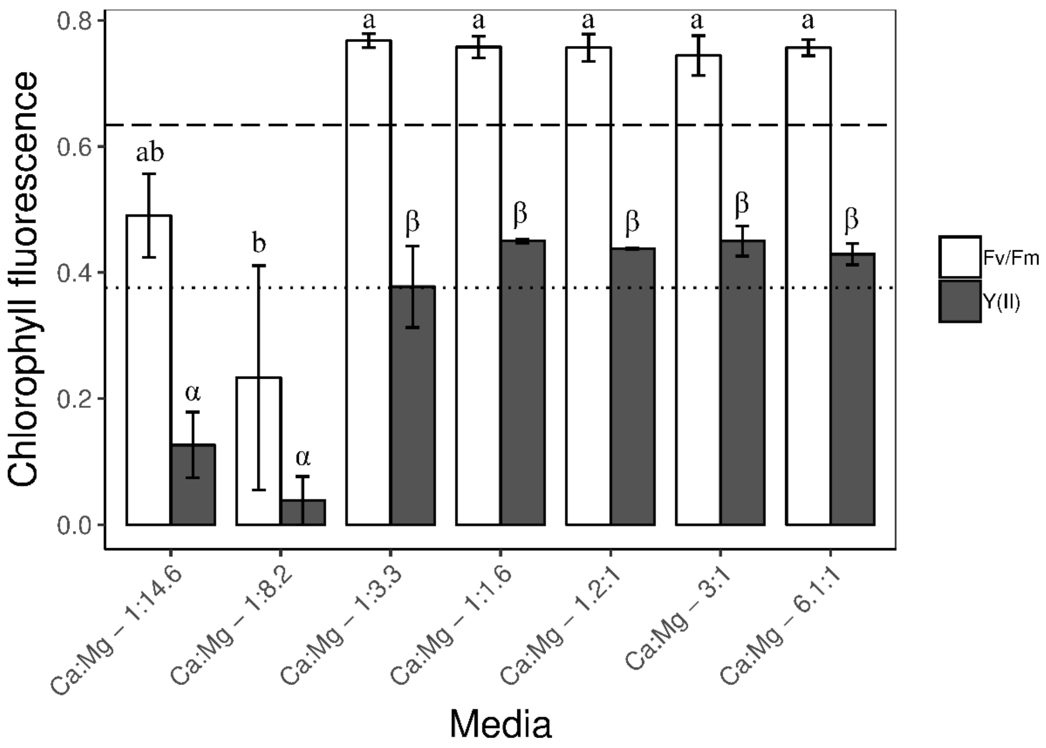

Figure 2. Fv/Fm and Y(II) values of *L. minor* growing on synthetic wastewater with different Ca:Mg ratios (bars) and on half-strength Hutner's medium (dashed and dotted lines, Fv/Fm and Y(II), respectively). Error bars represent standard error (n=3). ANOVA shows a positive association between Ca:Mg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

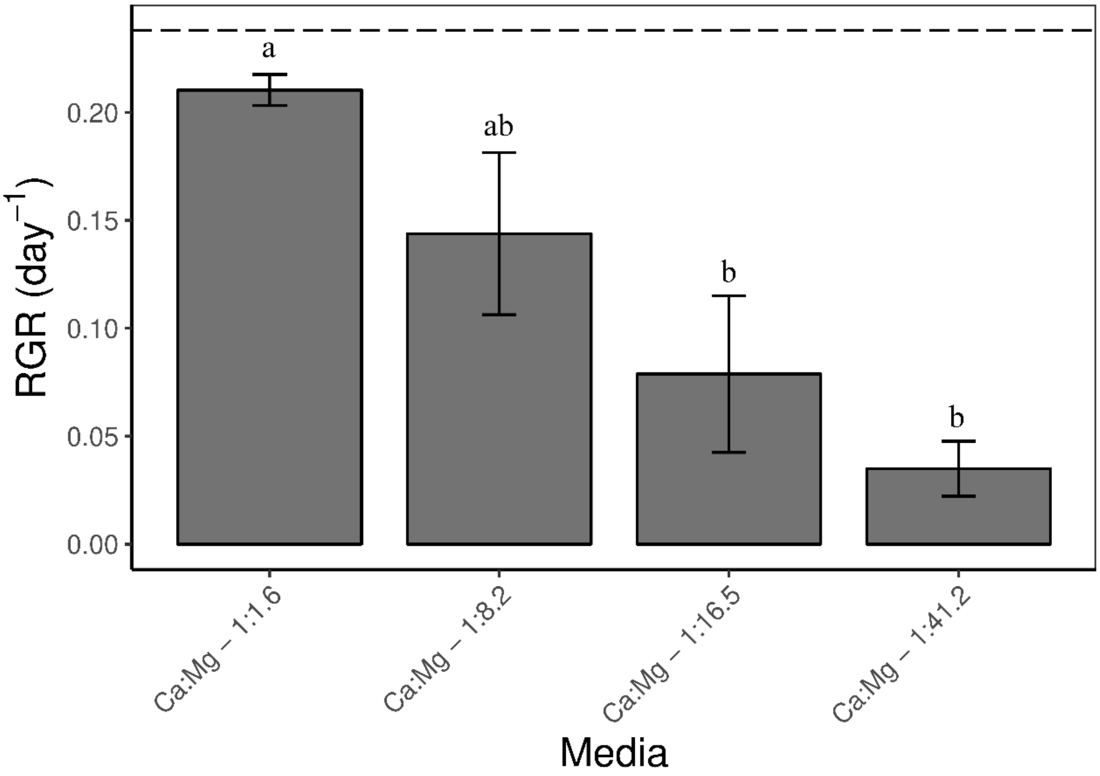

Figure 3. RGR of *L. minor* growing on synthetic wastewater with various Ca:Mg ratios (bars) and on half-strength Hutner’s medium (dashed line). Error bars represent standard error (n=4). ANOVA showed significant differences in RGR between groups, F(3, 12) = 7.996, $p<0.05$. Letters above the bars show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

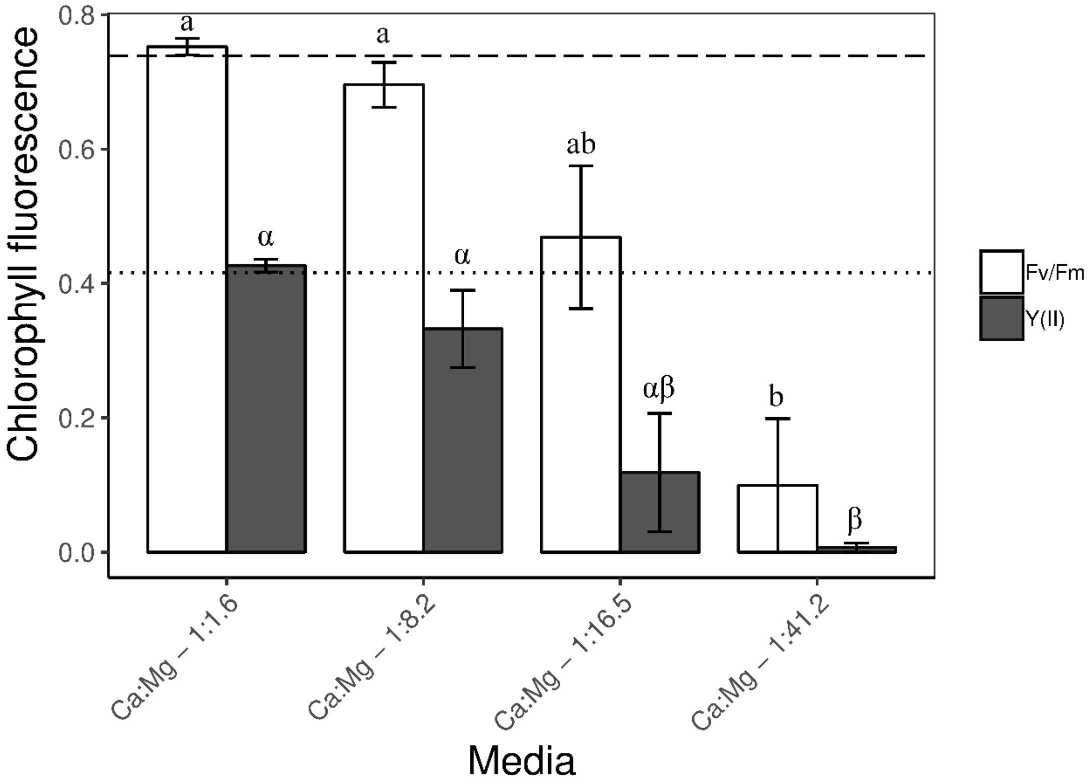

Figure 4. Fv/Fm and Y(II) of *L. minor* growing on synthetic wastewater with various Ca:Mg ratios (bars) and on half-strength Hutner's medium (dashed and dotted lines, Fv/Fm and Y(II), respectively). Error bars represent standard error (n=4). A Welch's ANOVA test showed that there is significant difference 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.

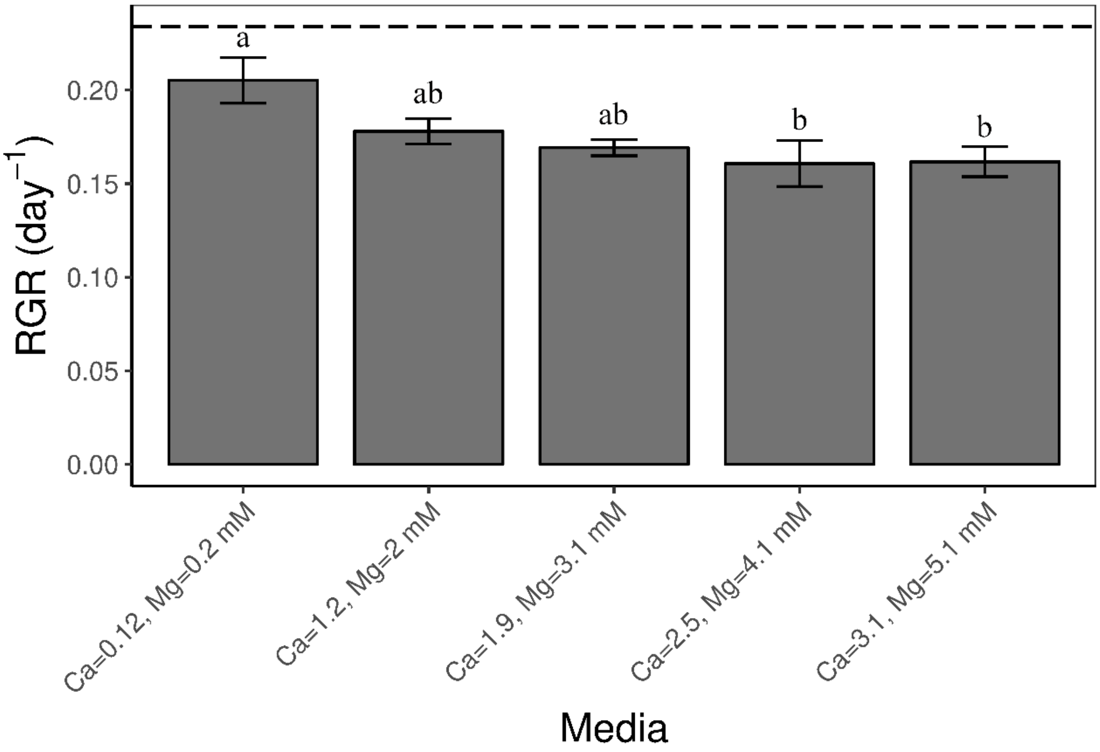

Figure 5. RGR of *L. minor* growing on synthetic wastewater of various concentrations of calcium and magnesium (bars) and on half-strength Hutner's medium (dashed line). Error bars represent standard error (n=4). ANOVA showed a significant difference between the RGR of *L. minor* in different synthetic wastewater treatments, F(4,15) = 3.89, $p<0.05$. A post-hoc 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 those in the two highest concentrations. Bars that do not share a similar letter differ significantly from one another.

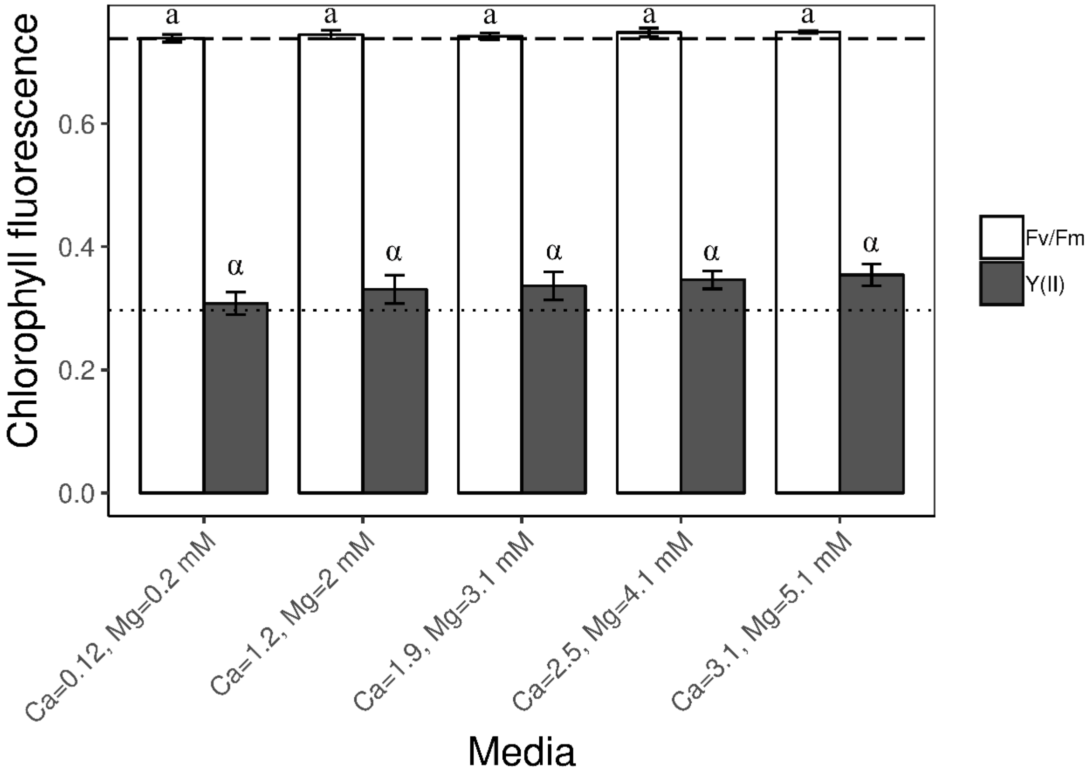

Figure 6. Fv/Fm and Y(II) for *L. minor* growing in synthetic wastewater of various concentrations of calcium and magnesium (bars) and on half-strength Hutner's medium (dashed and dotted lines, Fv/Fm and Y(II), respectively). Error bars represent standard error (n=4). ANOVA did not reveal significant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

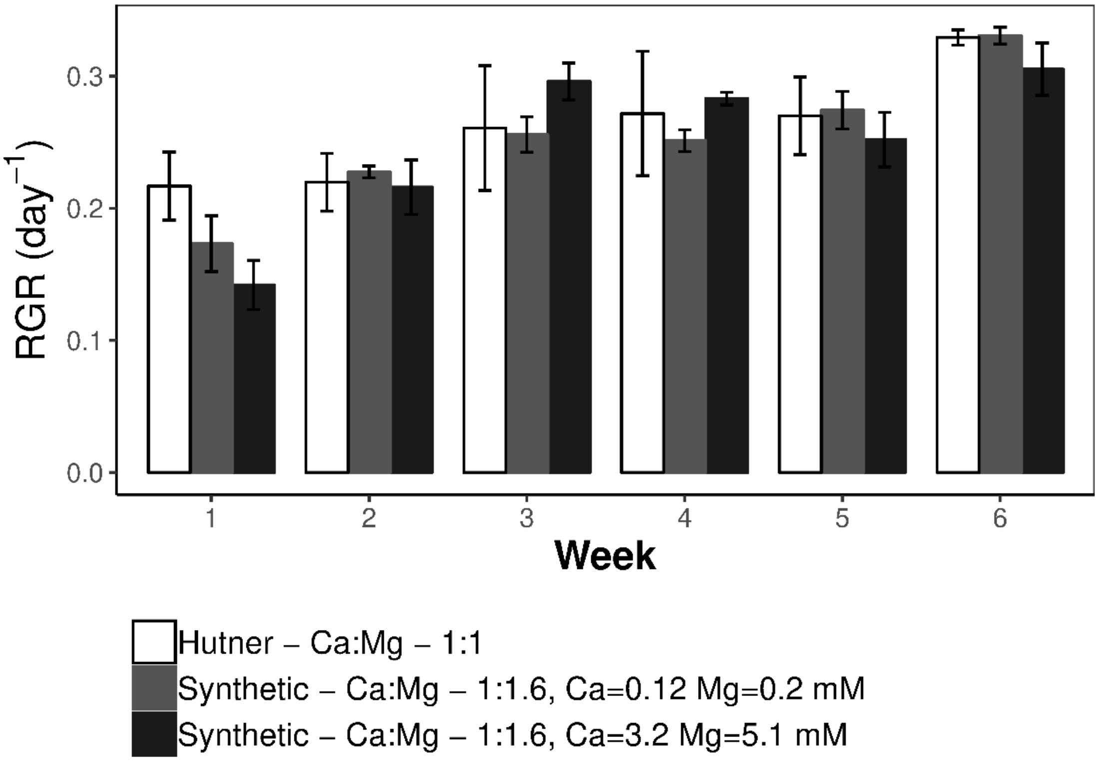

Figure 7. Weekly RGR of *L. minor* growing on synthetic wastewater (Ca:Mg ratio of 1:1.6 at two concentrations) and half-strength Hutner's medium throughout a 42-day experiment. ANOVA tests showed the RGR between treatments was not significantly different in any week of the experiment (n=4). Results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