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Effect of the growth medium composition on nitrate accumulation in the novel protein crop Lemna minor

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ABSTRACT

Duckweed is a potential alternative protein source for food and feed. However, little is known about the nitrate accumulation in this plant. A high nitrate level in vegetables can indirectly lead to an elevated intake of nitrites and N-nitroso compounds, increasing the risk of diseases for humans and animals. This research hypothesizes that the nitrate accumulation of *Lemna minor* differs between growing media. Additionally, it evaluates whether legal safety levels of nitrate for human and animal intake are exceeded. The duckweed was grown on (i) rainwater, and (ii) three synthetic media containing different nutrient levels. Furthermore, (iii) biological effluent of swine manure treatment and (iv) aquaculture effluent from pikeperch production were used, as these are potential media for closing nutrient loops in the agriculture sector. It was found that nitrate levels increased with the increasing availability of macronutrients in the water, and pH showed a particularly strong negative correlation with the nitrate levels in the plant. Nevertheless, nitrate content never exceeded 530 mg NO_3 kg^{-1} fresh weight. To conclude, *Lemna minor*'s nitrate content was below safety limits for human consumption in all tested growing media; however, a potential risk for ruminants was observed as these are more sensitive to nitrate conversions in their gastro-intestinal track.

1. Introduction

The growth of the world population and the improvement of living standards have increased the demand for animal-derived protein (Boland et al., 2013; United Nations, 2015), which has considerable environmental implications (de Beukelaar et al., 2019). In particular, feed production has been identified as the key contributor to the environmental impact of pork production. More specifically, land use change of rain forests and pastures into soybean fields is one of the most detrimental consequences of the increased feed proteins demand (Reckmann et al., 2016). As a result, there is an increasing interest in plant-based protein alternatives to substitute meat in the human diet, but also to substitute soybean proteins in feed by local and land use efficient sources (de Beukelaar et al., 2019; Reckmann et al., 2016).

One potential protein alternative is duckweed (*Lemna minor*). This is a highly productive plant that has been intensively investigated for its value as a protein ingredient in food and feed (Appenroth et al., 2017; Culley and Epps, 1973; Putra and Ritonga, 2018). These small floating macrophytes occur all over the world and are the most rapidly growing Angiosperms, following a quasi-exponential growth rate (Ziegler et al., 2015). The estimated production in outdoor pilot ponds in Europe is between 7 and 22 tonnes dry weight (DW) ha^{-1} yr^{-1} (Landolt and Kandeler, 1987). To the authors knwoledge, the maximal outdoor productivity reported is 68 tonnes ha^{-1} yr^{-1} and this was found in an outdoor pilot scale experiment containing *Lemna punctate* and which was executed in the Santa Catarina State in southern Brazil (Mohedano et al., 2012). In addition to an excellent productivity, duckweed's key advantage is its high protein content of up to 45% DW (Landolt and Kandeler, 1987). Finally, the low amount of fibre makes it readily digestible for monogastric animals and fish (Aslam et al., 2017).

Besides proteins, plants can also contain other nitrogen-rich components such as nitrate. Nitrate by itself is relatively nontoxic and small quantities can have a beneficial health effect (Ashworth et al., 2015; Butler, 2015). When consumed, however, it can be endogenously

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transformed into nitrite, which can react with amines and amides to produce N-nitroso compounds (Santamaria, 2006; Yordanov et al., 2001). These compounds have been related to an increased risk of diseases (Choi et al., 2007; Santamaria, 2006). For this reason, nitrate levels in food ingredients are regulated in Europe by the commission regulation (EC) No 1881/2006 (supplementary material A). For example, 'Iceberg' lettuce should have a nitrate content below 2 g NO_3/kg fresh weight (FW).

It can be hypothesized that duckweed is sensitive to nitrate accumulation for two reasons. First, in non-leguminous crops, excessive concentrations of nitrate tend to be found in leaves. For this reason, leafy vegetables like spinach and lettuce are considered as prominent nitrate-accumulators (Colla et al., 2018; Maynard et al., 1976; Santamaria, 2006). As duckweed predominantly consists of leaf-like tissue, it can be hypothesized that this plant reacts similarly to leafy vegetables. Second, a positive correlation between available macro-nutrients and the nitrate content in a diverse group of plants has been previously found (Colla et al., 2018; Kyriacou et al., 2019; Maynard et al., 1976). Unfortunately, nutrient availability is also positively correlated with duckweed's protein content (Mohedano et al., 2012; Zhao et al., 2014). Thus, conditions favouring high protein production might also favour nitrate accumulation.

Until now, duckweed production has been assessed on a diverse range of growing media by several researchers, including both synthetic media and contaminated wastewaters (Mohedano et al., 2012; Skillicorn et al., 1993; Tonon et al., 2017; Ziegler et al., 2015). However, the influence of the medium on the nitrate accumulation by duckweed from feed/food safety has not been investigated yet, even though an elevated nitrate content may hamper its use as a protein source. To assess this hiatus, *Lemna minor* was cultivated on different growing media with varying nutrient availability to test nitrate accumulation in different situations. The gathered data were used to discuss the risk of *Lemna minor* consumption for human and animal health and to identify the driving factors that might facilitate nitrate accumulation in this plant.

2. Material and methods

2.1. Set-up

Six different growing media with 4 parallel replicates were tested: (i) rainwater (R); (ii) A Synthetic "N medium" (SN), as described in the duckweed ISCDRA forum volume 3 (Appenroth et al., 1996; Appenroth and Sree, 2015); (iii) the same synthetic medium containing a 3 times higher concentration of macronutrients compared to SN (Concentrated Synthetic "N medium" or CSN); (iv) a synthetic medium containing a 3 times lower concentration of macronutrients compared to SN (Diluted Synthetic "N medium" or DSN); (v) biological effluent from a swine manure treatment facility (BE) at the site of Ivaco, Ichtegem, Belgium; (vi) effluent of an aquaculture facility that produces pikeperch (*Sander lucioperca* L.) at Inagro vzw, Roeselare-Beitem, Belgium (PE). The starting concentrations of these media are listed in supplementary material B.

Medium SN was made by mixing the following salts: 809 mg l^{-1} KNO_3, 246 mg l^{-1} $MgSO_4.7H_2O$, 236 mg l^{-1} $Ca(NO_3)_2.4H_2O$, 136 mg l^{-1} KH_2PO_4, 9,2 mg l^{-1} FeNaEDTA, 2.6 mg l^{-1} $MnCl_4.H_2O$, 0.31 mg l^{-1} H_3BO_3 and 97 μg l^{-1} $Na_2MO_4.2H_2O$. The CSN and DSN contained respectively three times more and three times less of the following salts: KNO_3, $MgSO_4.7H_2O$, $Ca(NO_3)_2.4H_2O$ and KH_2PO_4. This was preferred because adding HNO_3 would change the pH and selecting only KNO_3 or $Ca(NO_3)_2.4H_2O$ could introduce effects from nutrient imbalances which can be detrimental (Walsh et al., 2020). All other salts, containing mostly micro-nutrients, were kept constant over the three treatments to reduce the effect of micronutrient shortage. Additionally, instead of distilled water and ultrapure salts, the synthetic media was formulated with rainwater and artificial fertilisers. The recipes can also be found in the supplementary material C.

BE was obtained after sequential (i) mechanic separation of pig manure, (ii) biological treatment (nitrification/denitrification) of the obtained liquid fraction, and (iii) dilution with rainwater to a final concentration of 22% (v/v). PE was the aquaculture effluent from a closed recirculated production which was obtained after mechanic separation to remove feed and manure from the aquaculture effluent followed by a biological treatment (nitrification) of the liquid fraction.

Plastic trays (45 × 34.5 × 25.5 cm) of 25 L and a cultivation area of 0.114 m^2 were filled with 16 L of each growth medium. To compensate for evaporation loss, rainwater was added in the middle of the experiment (16/07/2019) to return the trays to their original weight.

2.2. Plant material

Duckweed was sampled from nature and was pre-grown for six months in a cubicontainer (SM COMPOSITE IBC, Mauser, Brühl, Germany) with a cultivation area of 1 m^2. The cubicontainer was initially filled with SN. Until the start of the experiment, artificial fertilisers were added sporadically, and plant material was harvest monthly to maintain optimal growth. These conditions lead to a starting nitrate content of 10 g NO_3/kg dry weight (DW) of 454 mg NO_3/kg fresh weight (FW).

The species were identified using molecular barcoding based on plastidic markers. The DNA for species identification was isolated from 15 mg oven-dried tissue using the CTAB method (Doyle and Dickinson, 1987; Doyle and Doyle, 1987) and quantified spectrophotometrically at 260 nm. The plastidic region of the atpF-atpH intergenic spacer was amplified in a total PCR reaction volume of 10 μl containing one-fold buffer B, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer (Wang et al., 2010), 0.05 U/μl hot start Taq polymerase (all reagents from Axon Labortechnik GmbH, Kaiserslautern, Germany) and 75 ng of genomic DNA. Amplification was carried out at 95 °C for 12 min, followed by 35 cycles at 95 °C for 45 s, at 55 °C for 45 s, at 72 °C for 45 s, and a final extension step at 72 °C for 10 min. Purification of the PCR product was done using a 1:5 enzyme mix of Exonuclease I (20 U/μl) and Alkaline Phosphatase (1 U/μl) (both Thermo Fisher Scientific Inc., Waltham, MA, USA). Subsequently, Sanger sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA). The sequencing product was precipitated with ethanol and loaded on a 3130 capillary Genetic Analyser (Applied Biosystems). The query sequence was compared with sequences from a reference database for all Lemna species of one of the authors (MB).

The identification showed that the used duckweed species was *Lemna minor* or *Lemna Japonica*. However, as it not probable to find *Lemna japonica* strains in Belgium, these analyses affirmed that *Lemna minor* was used in the experiment. Moreover, the population did not contain other duckweed species.

2.3. Climatic conditions

The experiment was conducted in a greenhouse at ambient climatic conditions. Air temperature, solar irradiance and relative humidity were monitored every 5 min during the experiment (Priva-meetbox, Priva, the Netherlands). These parameters amounted respectively on average 22 °C, 308 W m^{-2}, and 85%. Furthermore, an average daylength of 16 h was observed. In Supplementary Material D and E, a more detailed description is given.

2.4. Growth rate

On 5th July 2019, each tray was inoculated with 60 g FW of duckweed with an average DW of 4.5%, resulting in a density of 525 g FW/m^2 or 24 g DW/m^2. All trays were inoculated before 16h00 and were harvested on 12th July 2019 before 10h30, resulting in a growing period of 6.7 days.

FW was weighed after rinsing the plants with rainwater and air drying them in a net for 10 min. Dry weight was measured after drying

duckweed for 48 h at 80 °C. To determine a representative dry weight percentage (DW%) at the start of the experiment, five sub-samples of 60 g FW were taken at the start (DW_{start}). At the end of the experiment all fresh duckweed was harvested and dried (DW_{end}). First, biomass productivity or the linear growth rate (LGR) was calculated as follows (Xu et al., 2019):

$$LGR = \frac{(DW_{end} - DW_{start})}{time^{*}surface} \left[gm^{-2}d^{-1}\right] \quad (1)$$

Secondly, relative growth rates (RGR) were calculated as follows (OECD, 2006):

$$RGR = \frac{(\ln(DW_{end}) - \ln(DW_{start})}{time} \left[d^{-1}\right] \quad (2)$$

2.5. Plant analysis

After drying, the plant material was analysed for nitrate (NO_3. duckweed) content. Nitrate was extracted from 1 g of dried plant material with hot water that was saturated with tetraborate, as described in ISO 6635:1984. The NO_3 content of the extract was measured with a segmented flow analyser (Primacs SNC-100, Skalar, the Netherlands).

Total N content (T-N) was determined according to the procedure of Dumas using a CNS analyser (Primacs SNC-100, Skalar, the Netherlands), described in the guideline NEN- EN16168:2012 presented by the Royal Dutch Normalisation Institute (NEN). In this method, 200 mg of dried plant material is combusted and the produced N_2 is measured with a thermal conductivity sensor. As all nitrogen forms are combusted, this analysis gives the sum of organic, nitrate, nitrite, and ammonium nitrogen.

Kjeldahl nitrogen (Kj-N) was measured according to Van Ranst et al. (1999), without the addition of a reduction agent, using a distiller (Büchi auto Kjeldahl Unit K-370, Büchi, Switzerland), a destructor (Büchi digest automat K438, Büchi, Switzerland), a sampler (Büchi Kjeldahl sampler type K-371, Büchi, Switzerland) and a scrubber (Büchi scrubber B414, Büchi, Switzerland). This method measures organic and ammonium nitrogen. Additionally, protein content was calculated by multiplying Kj-N with the factor 6.25 (Casal et al., 2000).

2.6. Water analysis

Water samples were taken and analysed at the start and end of the experiment. The measured parameters were EC, pH, nitrate (NO_3. water), nitrite (NO_2^-), ammonium (NH_4^+), total phosphorus (T-P), chloride (Cl^-), sulphate (SO_4^{2-}), bicarbonate ($H_2CO_3^-$), Ca, Mg, Na, K, B, Mn, Fe, Cu, and Zn. Electrical Conductivity (EC) was measured with a conductivity tester (ProfiLine Cond 3110, WTW, Weilheim, Germany), and pH with a pH-meter (ProfiLine pH 3110, WTW, Weilheim, Germany). The concentrations of the nitrogen compounds were determined with a continuous flow analyser (SFA type 4000, Skalar Analytical B.V., Breda, The Netherlands) following ISO 13395:1996 for NO_3^- and NO_2^-, and ISO 11732:2005 for NH_4^+. Subsequently, the total dissolved nitrogen (T-DIN) was defined as the sum of NO_3^-, NO_2^- and NH_4^+ concentrations. After a microwave destruction (MARS6, CEM, Matthews, USA) in an *aqua regia* solution (1 HNO_3: 3 HCl), concentrations of P, Ca, Mg, Na, K, B, Mn, Fe, Cu, and Zn were determined by inductively coupled plasma-optical emission spectroscopy (optima 8300, PerkinElmer, Zaventem, Belgium). Cl^- and SO_4^{2-} were determined by liquid chromatography (850 Professional IC anion, Metrohm, Antwerpen, Belgium) in a 150 mm column (Metrosep A SUPP 5–150/4.0, Metrohm, Antwerpen, Belgium), following the ISO 10304–1:2007 method. Finally, $H_2CO_3^-$ was determined by titration following the ISO 9963–1:1994 method.

2.7. Statistical analyses

All statistical analyses were performed in R (R Core Team, 2014). All hypotheses were evaluated on a 5% significance level ($p < 0.05$). Normality was tested using the Shapiro-Wilk test. Homoscedasticity was analysed by a modified Levene test. When the requirements were met, Tukey's range test was used to compare treatments; otherwise, the non-parametric Dunn's test was performed. The correlations between parameters were determined using a Pearson test. Principal component analysis (PCA) was performed to evaluate the relationships and correlations between the water composition and duckweed's nitrate content. A strong influence of the variables is represented by a large distance from the origin. Variables with a similar direction are strongly correlated and those with an opposite sense explain for a negative correlation (Perendeci et al., 2019).

3. Results and discussion

3.1. Nitrate composition of the growth medium during the growing season

The most abundant inorganic N source in the growing media is nitrate, however, also in the two waste streams a small amount of ammonium is present, Table 1 and Supplementary Material C. As expected, the nitrate content of the rainwater is negligible, and after one week it decreases below the detection limit. Between the synthetic media (DSN, SN, CSN), the nitrate content is significantly different and increases with an approximate factor of three. There is no significant difference in the nitrate content of DSN and BE, and of DSN and PE, but the nitrate content of PE is significantly lower than that of BE.

Remarkably, the nitrate content of SN, CSN, and PE does not significantly decrease after one week, although nitrate uptake and denitrification of nitrate into nitrogen gas by bacteria is expected. This can be explained by evaporation of water. Rainwater was added in the middle of the experiment to reduce the effect of evaporation, but at the end of the experiment the containers had a mass of 13.7 ± 0.1 kg, which is an evaporation loss of 2.8 ± 0.1 kg of the growing medium. Due to evaporation, the concentration of nitrates increases. Additionally, nitrification of ammonia can increase the nitrate content. Only in BE this could lead to a considerable increase of 0.44 mM of N. In other media, only traces of ammonia were found. Nevertheless, nitrate content in the medium was more or less constant, but for BE and DSN there is a considerable concentration decrease which might have affected the plant's nitrate content.

Table 1
Nitrate concentration of the water before and after cultivation in the 6 media, i.e., R = rainwater, SN = a synthetic medium as described as "N medium" in the duckweed ISCDRA forum volume 3 (Appenroth et al., 1996; Appenroth and Sree, 2015), DSN = a diluted SN medium of which the amount of macronutrients are 3 times lower, CSN = a concentrated SN medium of which the amount of macronutrients are 3 times higher, BE = biological effluent of pig manure treatment, and PE = pikeperch effluent.

	Before	After		
R	0.01 ± 0.00^{a}	LOD	<	mM
DSN	$3.6 \pm 0.3^{b,c}$	2.2 ± 0.2^{a}	<	mM
SN	9.8 ± 1.8^{d}	10.3 ± 1.8^{b}	=	mM
CSN	26.9 ± 0.2^{e}	24.8 ± 2^{c}	=	mM
PE	2.2 ± 0.0^{b}	1.6 ± 0.4^{a}	=	mM
BE	4.3 ± 0.1^{c}	2.2 ± 0.6^{a}	<	mM

The symbols = , < or >mean that a specific component has at the end of the experiment a significant equal, lower, or higher content than before the experiment.
[a, b, c, d, & e]: The significance letters indicate the results from a Tukey HSD test and are interpreted as followed. When there is no significant difference between the nitrate content of two different media, than both media will have the same letter of significance. These letters can only be read vertically and thus do not compare the concentrations before and after.

3.2. Influence of the growth medium in Lemna minor production

Nitrate accumulation in plants results from an imbalance between the uptake of nitrate and its assimilation into organic nitrogen forms. Excessive amounts are likely to occur in plants grown under stress conditions (Maynard et al., 1976). Therefore, the dry weight obtained was measured to identify if any of the used media lacked nutrients and hence would result in plant stress. RGR and LGR of duckweed are visualised in Fig. 1 (A and B).

Even though all media had a varying nutrient availability (supplementary material C), there was no significant dry weight production difference between treatments. This indicates that the composition of the growing media used was not a limiting factor for duckweed growth. This is especially surprising for the R treatment, as rainwater would be expected to have limited availability of nutrients and hence have a reducing effect. However, this phenomenon of sustained growth on nutrient-depleted water has been described in the past. Due to luxury consumption, N and P can be taken up and stored within plant tissue before the experiment, and the stored nutrients can be mobilised to sustain growth during the experiment (Kufel et al., 2012). A prolonged experiment, or pre-cultivating the duckweed on each particular growing medium would limit the effect of luxury consumption. This would most likely lead to growth differences, which might also affect the plants composition.

Also, the climatic conditions did not strongly inhibit *Lemna minor* growth, with an average temperature of 22 °C, relative humidity of 85%, daylength of 16 h and solar irradiance of 308 W m^{-2}.

The productivity was, on average, 5.8 ± 0.6 g DW m^{-2} d^{-1} in this experiment. This is similar to yet unpublished results, where duckweed was grown on SN, PE, and BE with an average productivity of respectively, 4.7, 5.2 and 5.7 g DW m^{-2} d^{-1} for a 175 consecutive days and with a weekly harvest (Devlamynck et al., unpublished data). Also, in literature, similar productivities are found. A study by Tonon et al. (2017) recorded productivities of 5.72 g DW m^{-2} d^{-1} on municipal treatment water under a sub-temperate climate. The slight difference in growth can be explained by the timing and length of the experiment. Because herein the data was collected during one week during summer where daylength was longer, and both temperature and solar irradiance, higher than compared to a year-long monitoring.

Duckweed follows, however, an exponential growth rate (Ziegler et al., 2015). In this experiment, the RGR ranged between 0.132 and 0.169 d^{-1}. In laboratorial conditions, Ziegler et al. (2015) reported a RGR from 0.153 to even 0.519 d^{-1}. The lower RGR in our experiment can be explained by the suboptimal conditions, and the high initial mat density of duckweed. Herein, the water surface area was fully covered, and a density of 24 g DW m^{-2} was inoculated. From Monette et al. (2006), it is clear that RGR can be maximised if initial mat density is reduced. However, LGR is maximal at 45 g DW m^{-2}, and only slight variations (less than 11%) were found between for densities ranging from 24 to 80 g DW m^{-2}.

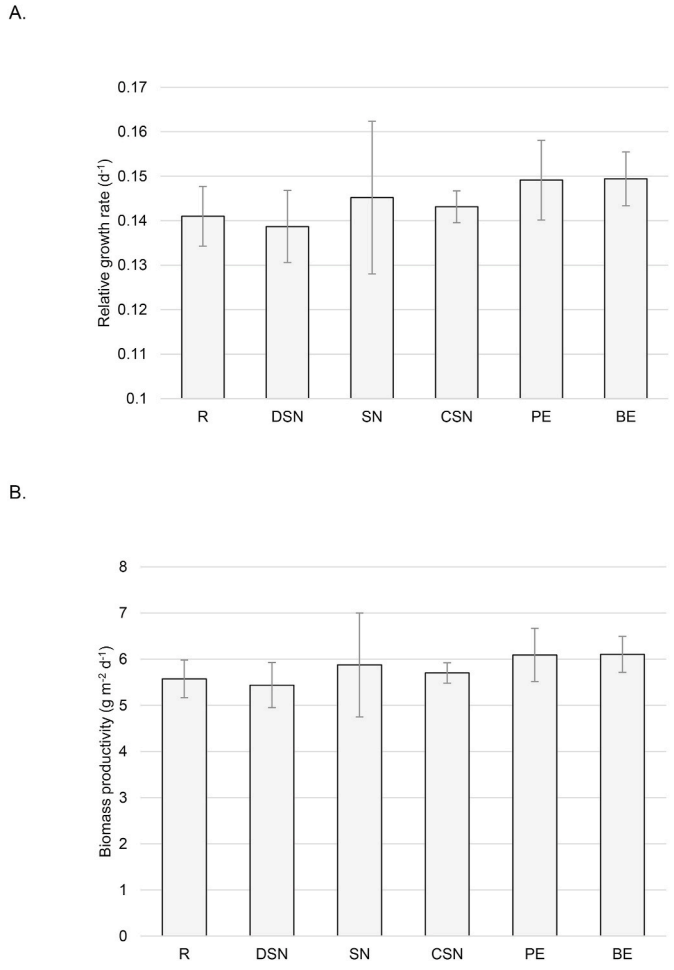

Fig. 1. A. Relative growth rate and B. Linear growth rate of *Lemna minor* grown under greenhouse conditions for 6.7 days on 6 growing media (with R = rainwater, SN = a synthetic medium as described as "N medium" in the duckweed ISCDRA forum volume 3 (Appenroth et al., 1996; Appenroth and Sree, 2015), DSN = a diluted SN medium of which the amount of macronutrients are 3 times lower, CSN = a concentrated SN medium of which the amount of macronutrients are 3 times higher, BE = biological effluent of pig manure treatment and PE = Pikeperch effluent).

3.3. Influence of the growth medium on Lemna minor's nitrate content

In contrast to what was observed for the dry weight, nitrate contents differed significantly amongst all treatments (Fig. 2). One exception was the difference between SN and DSN, which was only nearly significant (p = 0.068). Furthermore, *Lemna minor*'s nitrate content in all treatments decreased compared to the starting condition (ST); except for CSN, in which it increased; and except for SN, in which it remained the same (Fig. 2). The latter could be explained by the small composition difference between the SN treatment and the medium used for stock

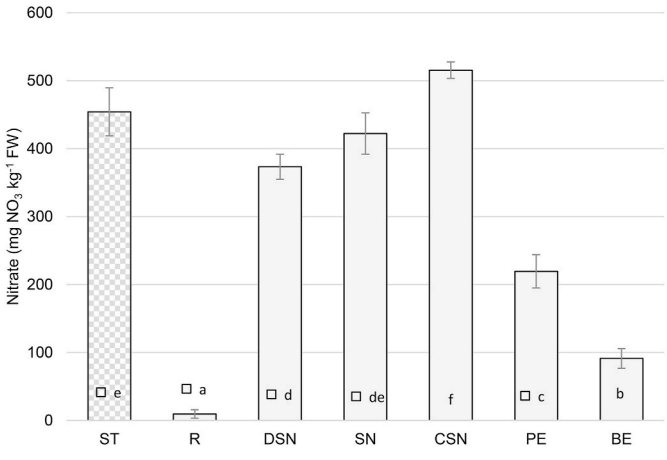

Fig. 2. Nitrate content of fresh *Lemna minor* grown for 6.7 days under greenhouse conditions on 6 growing media (with ST = starting concentration, R = rainwater, SN = "N medium" as described in the duckweed ISCDRA forum volume 3 (Appenroth et al., 1996; Appenroth and Sree, 2015), DSN = a diluted SN medium of which the amount of macronutrients are 3 times lower, CSN = a concentrated SN medium of which the amount of macronutrients are 3 times higher, PE = pikeperch effluent, and BE = biological effluent of pig manure treatment) (the alphabetic order of the significance letters, a, b, c, …, coincides with an ascending order).

cultivation.

Within the synthetic media, a clear upward trend is visible from DSN to SN to CSN. As all the salts containing macronutrients were equally increased by design, the effect of any macronutrient can't be distinguished from another. As follows, it can only be concluded that a higher availability of macronutrients favours nitrate accumulation in *Lemna minor*. This positive correlation has also been demonstrated in other plants (Colla et al., 2018; Kyriacou et al., 2019; Maynard et al., 1976).

Although the design was not intended to identify the effect of individual parameters, the inclusion of the two waste streams yielded sufficient variation to identify several significant correlations (Fig. 3). It could be expected that the more nitrogen available, the higher the chance of nitrate accumulation. Most research on various plants has confirmed this positive correlation, including the present study, in which both NO_3 and T-DIN had positive correlations with the duckweed's nitrate content. However, it is also mostly shown that T-DIN does not solely determine the nitrate accumulation of terrestrial plants, but also by the availability of macronutrients and trace elements such as P, K, Ca, Mg, and Mo (Colla et al., 2018). That nitrate is not the sole driving force was also observed in our research. There was a negative correlation of pH with the duckweed's nitrate content, while there was a positive correlation of Mn, Mg, T-P, and K, in descending order. Plotting these variables in Fig. 4 clearly shows these correlations.

The most tested driving factor for nitrate accumulation is the availability of N in the soil or growth medium (Colla et al., 2018; Kyriacou et al., 2019; Maynard et al., 1976). Also, in this study, T-DIN and nitrate content of the water are positively correlated with the plant's nitrate content. This is in line with the theory that nitrate accumulation is favoured if there is an excess availability of nitrogen. Furthermore, ammonium has been shown to inhibit nitrate accumulation in vegetables (Maynard et al., 1976), but this was not observed in the dataset. Perhaps the ammonium cations in the water were too low and too quickly removed to have an influence (supplemental material B). Finally, also the nitrite concentration in the water did not show a significant correlation with duckweed's nitrate content.

Besides N, Wright and Davison (1964) found that available K^+ induces the nitrate accumulation in vegetables. The presented rationale is that, for every nitrate ion, a cation such as K^+ is taken up to preserve electrical neutrality. Indeed, a positive correlation between K and nitrate content was found in our experiments, as shown in Figs. 3 and 4. However, this does not hold for every treatment. BE had the second highest K concentration in our experiment (Fig. 4); nevertheless, nitrate accumulation in duckweed grown in this medium was the lowest.

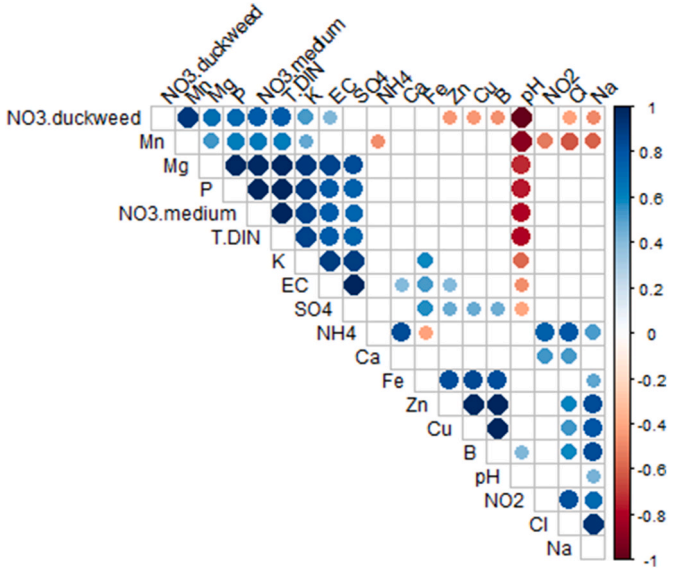

Fig. 3. Correlation matrix of different parameters of the growing medium with the nitrate content of duckweed (NO3.duckweed). All non-significant correlations are removed, and the size of the correlation is indicated by colour (Blue = positive and Red is negative).

Electrical neutrality can also be achieved by protons, as NO_3^-/H^+ symporters are involved in regulation of cellular pH and ion homeostasis in all plants and also duckweed (Feng et al., 2020; Ulrich, 1987). Strikingly, there is a very high negative correlation with pH. Hence, it can be argued that H^+ are more readily available for aquatic plants than K^+. These insights in the uptake mechanism suggest that pH can be a key variable to manipulate duckweed's nitrate content.

In addition, a strong correlation between Mn and the nitrate content of duckweed has been observed. It is recognized that Mn is essential in nitrate assimilation in plants (Heenan and Campbell, 1980; Jones et al., 1949; Mchargue and Calfee, 1932). However, it has been shown that an excessive Mn concentration inhibits the nitrate reductase enzyme activity of soybeans, which leads to nitrate accumulation (Heenan and Campbell, 1980). It is possible that this inhibition would also explain the increased nitrate content in DSN, SN, and CSN, where Mn content is highest and above 10 μM.

Additionally, trendlines were drawn between R and the synthetic media to give insight on the response curve of duckweed to the composition, Fig. 4. The duckweed's nitrate content shows a clear logarithmic fit with the T-N, Nitrate, K, P and Mg concentrations in the water. The fit (R^2) is consistently higher than 0.97, suggesting a strong correlation. These trends indicate that at first there is a strong reaction towards an increasing concentration of previous mentioned elements, but this response flattens as it goes on.

Strikingly, the datapoints on BE lie always under the trendline of T-N, Nitrate, K, P and Mg, Fig. 4. This means that if there is an equal presence of respective nutrients in BE and synthetic media, the nitrate concentration in *Lemna minor* will be lowest on BE. This suggests a lower sensibility towards nitrate accumulation. Similarly, PP lies under the trendline drawn for T-N, Nitrate, and Mg, however not for P and K. Actually, there are only two trendlines that seem to describe accurately the reaction of the duckweed's nitrate content on the two wastewater and that is that of pH and Mn. This observation supports the importance of the elements like previously reported herein.

To determine the importance of previously mentioned variables, principal component analysis (PCA) was performed. The analysis shows that PC 1 and PC 2 explain respectively 50.4 and 30% of the variation, which can be considered as an acceptable level. PC 3 contributed an additional 13% to the variation. T-DIN, P, NO3.water, pH, NO3.Duckweed, Mg, and Mn contributed most to PC1 in a respective decreasing order. Fig. 5 shows that NO_3.plant, pH has a similar direction, but an opposite sense to nitrate content in the plant, affirming the strong negative correlation in Fig. 4. This indicates that pH is a key variable explaining the nitrate content in *Lemna minor*. Further research, in which pH is the only variating factor, should be conducted to prove if pH can indeed reduce the nitrate content of duckweed when macronutrient levels are kept equal. If so, pH can be a tool to prevent hazardous nitrate accumulation in *Lemna minor*, including media with high nutrients.

A comparison with results from nitrate accumulation in duckweed is difficult, as only one other study that reported the nitrate content of duckweed was found in literature. In the study of Lehman et al. (1981), nitrate was determined on the root and frond of individual duckweed plants and the results varied between 22 and 354 mg NO_3/kg DW. These concentrations are similar to the results from R, BE, and PE. This can be explained by the low nitrogen content of the growth medium used by Lehman et al. (1981), which was modified to contain a NO_3_N content of only 20 mg N l^{-1}. Most likely, the K content was also reduced in the modified Hoagland solution as KNO_3 is the prominent salt in the solution, which also would explain a low NO_3 content in the duckweed.

It should be noted that these results hold for an experiment with a duration of 6.7 days and after a stock cultivation. More importantly, in this experiment, the equal productivity over the treatments indicate that

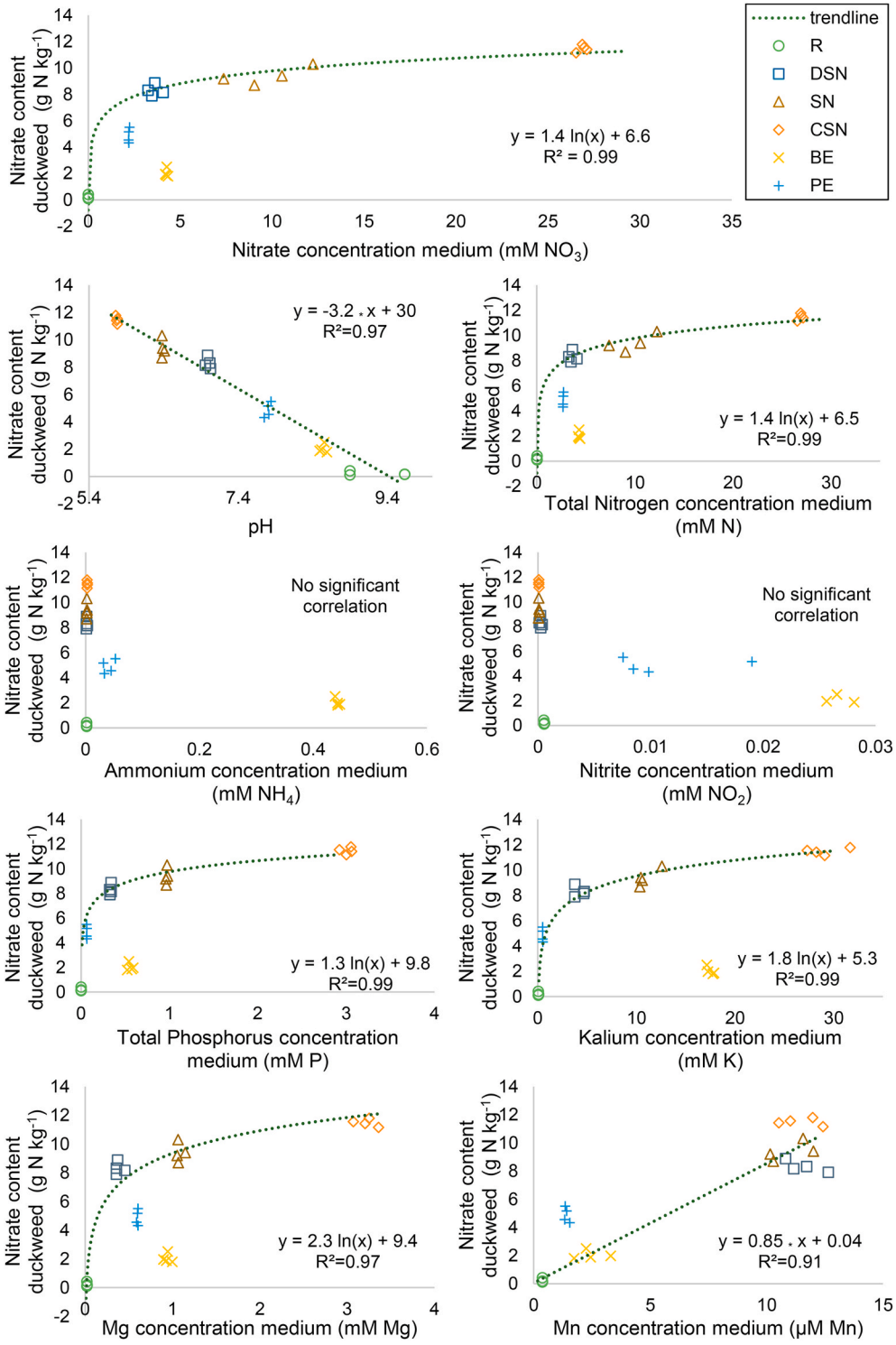

Fig. 4. Scatterplot of several important composition parameters in relation to duckweed's nitrate content. Furthermore, a trendline with the highest fit (R^2) between R and the synthetic media is presented. (R = rainwater, SN = "N medium" as described in the duckweed ISCDRA forum volume 3 (Appenroth et al., 1996; Appenroth and Sree, 2015), DSN = a diluted SN medium of which the amount of macronutrients are 3 times lower, CSN = a concentrated SN medium of which the amount of macronutrients are 3 times higher, PE = pikeperch effluent, and BE = biological effluent of pig manure treatment).

luxury consumption was present. The effect of these starting conditions might still be present at the end of the experiment. Although that the difference between the starting and final nitrate content of duckweed suggests that the plant reacted considerably to the growing medium, there is uncertainty if these results are consistent in a longer cultivation. Future research should address this by adding a pre-cultivation step on the treatments or by prolonging the experiment.

3.4. Total N and protein content

Besides their effect on the nitrate content, different media also resulted in different total N content, as can be seen in Table 2. *Lemna minor* grown on rainwater contained significantly less T-N and Kj-N than all others. Regarding the waste streams, there was no significant difference between the N content in *Lemna minor* grown on PE and BE, and both were lower than the N content of the duckweed grown on the synthetic media (DSN, SN, CSN). Within the synthetic media, the differences were subtle; the N content of the plants grown on the N medium

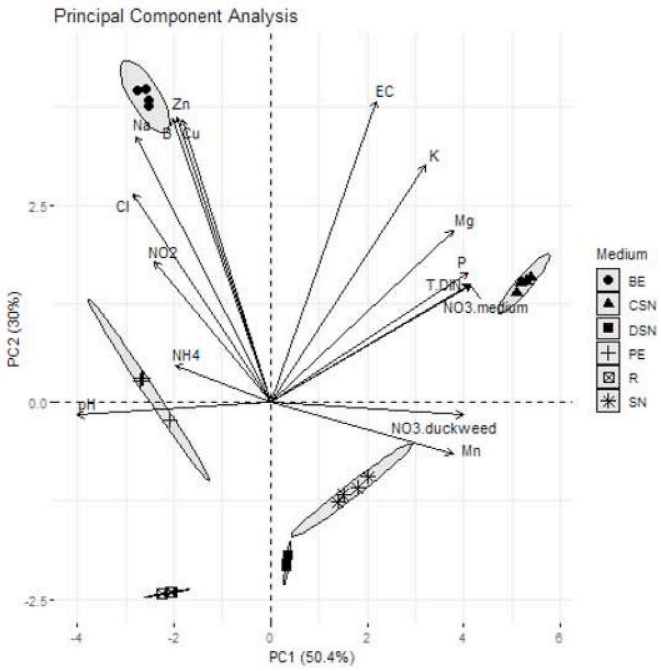

Fig. 5. Variable correlation plot after principle component analyses of different duckweed treatment (R = rainwater, SN = "N medium" as described in the duckweed ISCDRA forum volume 3 (Appenroth et al., 1996; Appenroth and Sree, 2015), DSN = a diluted SN medium of which the amount of macronutrients are 3 times lower, CSN = a concentrated SN medium of which the amount of macronutrients are 3 times higher, PE = Pikeperch effluent, and BE = biological effluent of pig manure treatment).

Table 2
Summary of the Total N measured with the Dumas method and Kjeldahl Nitrogen method and Crude protein, expressed in mean ± standard deviation of the six treatments and the starting concentration (ST = Starting concentration, R = rainwater, SN = "N medium" as described in the duckweed ISCDRA forum volume 3 (Appenroth et al., 1996; Appenroth and Sree, 2015), DSN = a diluted SN medium of which the amount of macronutrients are 3 times lower, CSN = a concentrated SN medium of which the amount of macronutrients are 3 times higher, BE = biological effluent of pig manure treatment, and PE = pikeperch effluent).

	T-N g kg^{-1} DW	Kj-N g kg^{-1} DW	CP %
ST	56 ± 1^{e}	53 ± 1^{d}	33 ± 1^{d}
R	25 ± 1^{a}	24 ± 1^{a}	15 ± 0^{a}
DSN	$51 \pm 2^{c,d}$	$47 \pm 3^{c,d}$	$29 \pm 2^{c,d}$
SN	48 ± 1^{c}	45 ± 0^{c}	28 ± 0^{c}
CSN	52 ± 1^{d}	50 ± 1^{d}	31 ± 1^{d}
PE	43 ± 2^{b}	40 ± 2^{b}	25 ± 1^{b}
BE	40 ± 2^{b}	38 ± 1^{b}	24 ± 1^{b}
Average	45 ± 10	42 ± 9	26 ± 6

T-N: duckweed's total nitrogen content analysed by the Dumas method (includes organic, ammonium, nitrate, and nitrite nitrogen).
Kj-N: duckweed's total nitrogen content analysed by the Kjeldahl method (includes organic and ammonium nitrogen).
CP: Crude protein calculated by multiplying Kj-N by 6.25.
$^{a,b,c,...}$ The alphabetic order of the significance letters, coincides with an ascending order and these are the result of a Tukey test that was performed to test the effect of the treatment on the N content.

was significantly, yet slightly, lower than the N content of those on either CSN or DSN. All these differences also hold for the crude protein content of *Lemna minor*, as it is calculated based on the Kj-N (Casal et al., 2000), Fig. 6. As in the synthetic media the concentration of KNO_3, $MgSO_4.7H_2O$, $Ca(NO_3)_2.4H_2O$ and KH_2PO_4 were modified, the results

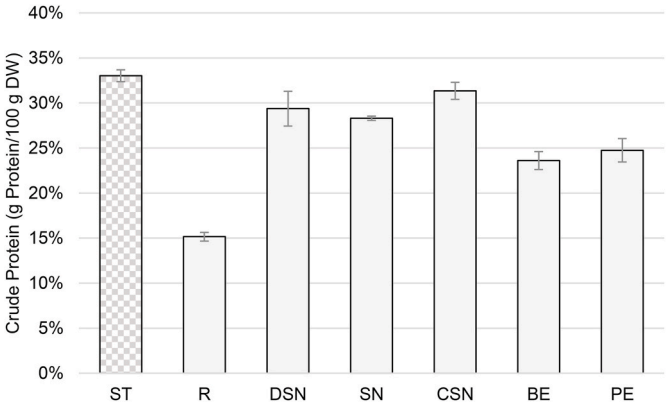

Fig. 6. Crude protein of *Lemna minor* grown under greenhouse conditions for 6.7 days on 6 growing media (with ST= Starting material before the experiment, R = rainwater, SN = a synthetic medium as described as "N medium" in the duckweed ISCDRA forum volume 3 (Appenroth et al., 1996; Appenroth and Sree, 2015), DSN = a diluted SN medium of which the amount of macronutrients are 3 times lower, CSN = a concentrated SN medium of which the amount of macronutrients are 3 times higher, BE = biological effluent of pig manure treatment and PE = Pikeperch effluent).

indicate that there is little benefit of adding more of these artificial fertilisers than available in DSN to increase *Lemna minor*'s protein content. Nevertheless, a combined availability of levels of K, Ca, Mg, NO_3^-, SO_4^{2-}, PO_4^{2-} in the water can lead to nitrate accumulation. Thus, fertilisation should well be balanced, and growing media like DSN seem to be sufficient for a decent protein concentration of *Lemna minor*.

Crude protein content of the synthetic media was around 30 DW%, while that on the waste streams was around 25 DW%. CP of duckweed grown on rainwater was even lower, Fig. 6 and Table 2. This is in the range of commonly found protein concentrations of *Lemna minor* in literature (Iatrou et al., 2018). Higher CP contents are found in growing media of up to 30 mg L^{-1} NH_4–N (Iatrou et al., 2018).

When comparing the two methods used for N determination, the Wilcoxon Rank Sum test indicated that Kj-N was significantly lower than the T-N determined with a CNS analyser. This could be expected, as, in theory, the difference between the measured T-N and Kj-N is the sum of nitrate and nitrite in the plant (McGeehan and Naylor, 1988). However, the measured NO_3 content in the plant is, on average, only 52% of the found difference between these two N determination methods. This is presumably because Dumas destruction is slightly more efficient in breaking down organic nitrogen compounds when compared to the Kjeldahl procedure, as it reaches 1300 °C temperature in the combustion phase (Etheridge et al., 1998). Additionally, Kjeldahl also does not account for azide, azine, azo, cyanide, hydrazone, nitroso, oxime, or semicarbazone forms of nitrogen (Brinkmann et al., 2016), which can also lead to a small underestimation. Strikingly, the gap between T-N and Kj-N does not significantly differ between the treatments.

Finally, the Dumas procedure, when compared to the Kjeldahl method, has several advantages, as it consumes fewer strong reactants, requires less labour, and operates at a more efficient temperature to release the nitrogen from the samples (Etheridge et al., 1998). As a result, this method is more and more preferred to analyse the total N in food and feed samples. However, the present results show that awareness and carefulness are needed when determining N forms in plants, as the Dumas method leads to a significant overestimation of the organic bound nitrogen and is, therefore, less fit for crude protein estimation. A new conversion factor, specific for the Dumas method, should be determined if this method would be used for protein content determination.

3.5. Evaluation of nitrate levels in Lemna minor for food and feed application

Although duckweed contains nitrate, it should not necessarily be considered hazardous. A way to decide on safety, is to compare with legal limits. The European limits for leafy vegetables by the European Commission are provided in supplementary material A. The selected limit for the comparison is 2000 mg NO_3 kg^{-1} FW. As both leafy vegetables and duckweed have an approximate dry weight percentage of 5%, 'Iceberg' type lettuce grown in open air and deep-frozen spinach would normally be suitable plants for comparison. Additionally, these two follow the lowest and thus the strictest limit. The maximal nitrate content in *Lemna minor* observed in this study was 530 mg NO_3 kg^{-1} FW. Hence, all plants had a nitrate content below the maximum allowed levels and could be considered safe for human consumption.

However, besides the nitrate content of the product, the exposure is a key factor for human health (Haftbaradaran et al., 2018). Currently, it is impossible to assess the daily intake of duckweed. However, in the extreme case where all proteins would be supplied by the consumption of duckweed, the Acceptable Daily Intake (ADI) of nitrate would be exceeded two to eight times. This holds for *Lemna minor* grown in respectively BE and CSN. Furthermore, this calculation used the Dietary Reference Value (DRV) of 0.83 g proteins/kg Body weight/day (EFSA, 2012), and the nitrate ADI of 3.7 mg NO_3/kg Body Weight/day (EFSA, 2008). These extreme conditions are, however, unlikely to occur as proteins are consumed by various sources in the human diet. Nevertheless, animal protein sources are considerably lower in nitrate than vegetable protein sources (Bahadoran et al., 2016). This is relevant for population groups that consume a modest share of animal products and would use duckweed as a key protein source. Hence, the nitrate content should nevertheless be considered in the future when making recommendations of the acceptable daily intake of duckweed.

Astonishingly, previous comparison was also performed for supplying all proteins by *Lemna minor* grown on rainwater (R), and this resulted in an intake of only 30% of the nitrate ADI. Here, the reduced nitrate content is more considerable than the reduced protein content, resulting in a safe consumption even if duckweed is the sole protein source. Therefore, it might be a solution to cultivate duckweed some days, on nutrient deprived waters with a high pH and low Mn to strongly reduce the nitrate accumulation risk.

Regarding animal health, only the nitrite content is legislated in Europe. A safe dose of nitrate in feed isn't clearly established because this depends on the animal type and in which form nitrate is supplied (Lenz, 2018). Ruminants are predominantly more susceptible to nitrate toxicity because they have a higher reduction of nitrate to nitrite and other metabolites previous to absorption (Wright and Davison, 1964). Bradley and Eppson (1940) proposed that an addition of 9300 mg NO_3 kg^{-1} DM (or 420 mg NO_3 kg^{-1} FW) of KNO_3 salt in the feed of calves is safe, assuming a dry matter intake (DMI) of 2.5% of the bodyweight. It should be noted that, generally, the nitrate availability is higher in KNO_3 salt than in plants, and thus more toxic. Yet, *Lemna minor* grown on SN and CSN are respectively equal to and higher than the proposed limit. As

Table 3
Average (±standard deviation) of the observed nitrate content compared with the human and ruminant nitrate limit using a one-sided *t*-test in which '<',' = ' and '>' indicate if the measured concentration is lower, equal to or higher than the proposed limit with the respective p-value next to it. The characters indicated in bold are considered toxic. (ST = Starting concentration, R = rainwater, SN = "N medium" as described in the duckweed ISCDRA forum volume 3 (Appenroth et al., 1996; Appenroth and Sree, 2015), DSN = a diluted SN medium of which the amount of macronutrients are 3 times lower, CSN = a concentrated SN medium of which the amount of macronutrients are 3 times higher, BE = biological effluent of pig manure treatment, and PE = pikeperch effluent).

		human		ruminants
	limit	46 g NO_3 kg^{-1} DW[α]		9.3 g NO_3 kg^{-1} DW[β]
	Duckweed's nitrate content			
	[g NO_3 kg^{-1} DW]	p-value		p-value
ST	10 ± 1	< $1.5*10^{-6}$	>	0.066
R	0.21 ± 0.14	< $3.7*10^{-9}$	<	$4.6*10^{-7}$
DSN	8.3 ± 0.4	< $1.8*10^{-7}$	<	0.0086
SN	9.4 ± 0.7	< $8.7*10^{-7}$	=	0.79
CSN	11 ± 0	< $6.4*10^{-8}$	>	$2.5*10^{-4}$
PE	4.9 ± 0.5	< $3.2*10^{-7}$	<	$2.6*10^{-4}$
BE	2.0 ± 0.3	< $5.4*10^{-8}$	<	$1.2*10^{-5}$

[α] nitrate level of iceberg lettuce described in (EC) No 1881/2006 divided by a dry weight content of 4.36% (USDA, 2018)

[β] Lenz, 2018

a result, these form a risk for nitrate toxicity, as can be seen in Table 3. To conclude, although all other growing media could be considered safe for consumption, there is a potential risk for ruminants.

However, the problem of nitrate in feed is believed to be smaller in monogastrics (Cockburn et al., 2013), so duckweed may be more suitable for pig and fish feeding. However, there is no concrete evidence of nitrate toxicity for these animals. Further research on the nitrate toxicity in monogastric animals is necessary to establish a circular system in which duckweed is not only grown on animal manure but also fed to the same animals. Future research could also focus on nitrite accumulation in duckweed, as there is more known on the toxicity of this N-form for monogastrics.

Additionally, some processes might result in increased nitrate contents and should be avoided when cultivating duckweed for food and feed purposes. Light intensity is inversely correlated to the nitrate content of plants; therefore, diurnal changes in light intensity might cause a diurnal nitrate accumulation pattern (Boroujerdnia et al., 2007; Chowdhury and Das, 2015). It is known that spinach has higher nitrate levels when harvested at low than when harvested at high light intensities (Colonna et al., 2016). In the present study, *Lemna minor* was always harvested before 10 a.m. to minimize nitrate content. Harvesting at dawn or on more cloudy days might, however, increase the nitrate level in duckweed. Finally, only one *Lemna minor* clone was tested in this research, while the use of different species may have a significant impact on nitrate accumulation, as reported for other plants (Razgallah et al., 2016; Reinink et al., 1987).

Notwithstanding that there are some remaining processes to be uncovered, it was shown that it is already possible to sagely cultivate *Lemna minor* for human consumption in terms of nitrates.

4. Conclusions

In synthetic media, increasing availability of macronutrients (supplied by the salts KNO_3, $MgSO_4.7H_2O$, $Ca(NO_3)_2.4H_2O$ and KH_2PO_4) positively affected the nitrate content in *Lemna minor*. In wastewaters, nitrate accumulation was lower compared to synthetic media, although nutrients were sufficiently present. Several parameters have a significant correlation with the plant's nitrate content and can thus account for this difference, but especially pH and Mn expressed a strong linear correlation. PCA indicated that pH can be considered a potential driving force for nitrate accumulation in *Lemna minor* and it should be tested if pH can be a tool to prevent hazardous nitrate accumulation in *Lemna minor*, including media with high nutrients. Additionally, the influence of nitrogen and potassium in the growth medium was confirmed in this experiment. It should, however, be noted that a pre-cultivation step is required in further research in order to estimate the response of *Lemna minor* without the effects of luxury consumption before. Concerning safety, no clear nitrate limits are set for animal safety, but nitrate content in *Lemna minor* could form a risk for ruminants in several growing media. Nevertheless, all treatments resulted in a lower nitrate content than the European limits for leafy vegetables.

Credit author statement

Reindert Devlamynck: Conceptualization, Investigation, Writing – Original Draft. Marcella Fernandes de Souza: Writing – Review & Editing. Manuela Bog: Investigation, Writing – Material & Methods. Jan Leenknegt: Writing – Review & Editing, Supervision. Mia Eeckhout: Writing – Review & Editing, Funding acquisition, Supervision. Erik Meers: Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111380.

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