Grassification

D2.3.1

Assessment of the potential of protein extraction and protein production (insects and microalgae) from the liquid fraction

Document Control Page

Title	D2.3.1 Assessment of the potential of protein extraction and protein production from the liquid fraction
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Nature	Document
Dissemination Level	Public
Version number	2.0
Planned Delivery Date	01 12 2019
Version date	06 07 2020
Abstract	

Version	Date	Modified by	Comments
1.0	28.01.2020		Initial version
2.0	06.07.2020	Marcella Souza	Revision for dissemination

Contents

1. Introduction

1.1 General background

The overall objective of the Grassification project is to apply a multi-dimensional approach to roadside grass clippings refining in order to optimize it into a viable value chain for the bio-based and circular economy. The project commits itself to optimize logistics and technical aspects of the grass clippings supply chain and processing, demonstrate its market potential as well as formulate policy and legal recommendations to create a more supportive framework for the recycling of this renewable resource. These actions will increase the volume of usable material, lower costs, and generate a higher added-value for this so called 'waste' streams, which eventually will result in a higher market value of the industry. In this way, the use of roadside grass clippings as a renewable resource for the production of bio-based products and hence the circular economy will become more attractive. Roadside grass clippings refining thus facilitates transition towards a circular economy.

One of the main value-chains currently investigated for grass, also contemplated in the Grassification project, is the production of materials from the fiber fraction, such as building materials, insulation panels, biocomposites, and others. This may entail a first fractionation step, where the solid and liquid fractions are separated. The obtained liquid fraction can account up to 60% of the total fresh weight of the initial biomass; therefore, its valorisation is important for developing an economically viable value-chain from grass. In the Grassification project, the characterization of the liquid fraction was carried out and three main value-chains are investigated for valorizing the liquid fraction of grass:

- recycling minerals in the liquid fraction for organic mineral fertiliser production
- production of energy through anaerobic digestion
- protein production (direct extraction or insect/microalgae growth)

This report describes the results of direct extraction of proteins or producing protein-rich insects and microalgae using the liquid fraction as nutrient source. The research was carried out by Avans University, Inagro and Ghent University.

1.2 Goal of this study

The goal of this study was to determine the feasibility to use the liquid fraction of pressed grass from roadsides for protein production.

1.3 Reading guide

Chapter 2 describes the composition of the liquid fraction of pressed grass. Chapter 3 presents a small literature study of protein production from grass juice and Chapter 4 presents the possibilities to concentrate the protein in this liquid. The application of liquid for insect feed and microalgal cultivation is described respectively in chapter 5 and 6. Chapter 7 gives the main conclusions.

2. Composition of the liquid fraction

This chapter gives an overview of the composition of the liquid from pressed roadside grass. The main focus was on the protein content in order to see if the liquid from pressed grass is suitable as an added component for insect feed and pig feed.

2.1 Origin of the liquid from pressed grass

The liquid from pressed roadside grass originated from 2 different mowing sessions coordinated by ProNatura. **Table 1** gives an overview of the characteristics. The liquid fraction was stored in a (kitchen) freezer at -18°C to prevent biological and chemical conversion processes.

***Table 1**: Origin of liquid from pressed roadside grass*

Characteristics	Session 1 (autumn 2018)	Session 2 (Spring 2019)
Location	Roadside cuttings from highway of the municipality in Utrecht.	Roadside cuttings from the municipality of Maldegem
Date	Mowing 8/11/2018 Screw press: 8/11/2018	Mowing 18/06/2019 Screw press: 18/06/2019
Mowing machinery	Rotary mower	Flail mower
Pressing machinery	Screw press ('tegendruk schroefpers') adapted for biobased resources at Rhinetech	Screw press ('tegendruk schroefpers') adapted for biobased resources at Rhinetech
Liquid fraction	42.8% of the fresh material	±40 % of the fresh material
Fibre fraction	57.4% of the fresh material	±60% of the fresh material

2.2 Material & methods

Table 2 gives an overview of the parameters that were measured and the methods that were used.

***Table 2:** Parameters that were measured in the liquid from pressed roadside grass and methods used*

Parameter	Equipment / method used
pH	Metrohm 827 pH Lab, room temperature, stirring sample with magnet
EC	Metrohm 827 pH Lab, room temperature, stirring sample with magnet
Dry matter	Moisture Analyser He 73 Mettler Toledo Approximately 5g of liquid at 100°C
Total organic matter (TOC)	Sievers INNOVOX Lab TOC analyser, model PRD 68000-01
Protein	- N Kjeldahl (see appendix I) - SDS Page gel (see appendix I)
Amino acids	LC-MS

2.3 Composition of the liquid fraction

General parameters

Table 3 gives an overview of the dry matter content, the pH, the conductivity and the density of the liquid from pressed roadside grass. The sample derived from Session 1 had a much higher pH. This may be due to the handling of the sample of Session 2 (left at room temperature in first hours after pressing), resulting in fermentation of part of the organic carbon into organic acids. The difference in EC and in the dry matter content may be due to the mowing heads used, which result in different fibre lengths and, therefore, different extraction efficiencies of the intracellular contents.

Table 3: *Dry matter content, pH, EC and density of liquid from pressed roadside grass*

		Session 1 (November 2018)	Session 2 (June 2019)
pH	-	6.2	4.3
EC	µS/cm	49	155
Dry matter	%	5.80 ± 0.1	11.76 ± 0.04
Density	g/ml	0.98	0.98

Total organic carbon (TOC)

Table 4 gives an overview of the total organic carbon content of the liquid from pressed roadside grass before and after centrifugation. The results show that the organic carbon is mainly present in the soluble fraction of the liquid. Almost no carbon is present in the solid fraction of the liquid, which mainly consists of minerals (sand, silt). The reason for the presence of sand/silt is that, during mowing, not only grass is collected but also part of the top soil layer and soil that is attached to the grass. The amount of sand present in the clippings depends on the mowing method. More information about this can be found in D1.1.2 (Testing & comparison of performance new type of mowing head against existing mowing heads). The organic carbon content of the liquid of Session 2 is much higher than for Session 1. This difference can be explained by differences in soil composition, climatic factors and species composition between the different roadsides (van Vuuren & van den pol, 2006). The difference could also be (partly) explained by the mowing method used.

Table 4: *Total organic carbon content of non-centrifugated liquid, centrifugated liquid and solid fraction from pressed roadside grass*

		Session 1 (November 2018)	Session 2 (June 2019)
Non-Centrifugated liquid	mg TOC/ml	17.7 ± 0.2	53.5 ± 0.5
Centrifugated liquid	mg TOC/ml	17.6 ± 5	50.1 ± 1.5
Solid fraction	mg TOC/ml	0.1	3.4

Protein content

Table 5 gives an overview of the protein content of the non-centrifugated liquid and centrifugated liquid from pressed roadside grass measured with N Kjeldahl. These concentrations are comparable to concentrations that were found in a study by Anderson & Kiel (2000), of 9.4 g protein/kg liquid for Rye grass and of 15.7 g protein/kg liquid for Clover grass.

Table* 5: Protein content of non-centrifugated liquid, centrifugated liquid and solid fraction from pressed roadside grass*

	Session 1 (November 2018)		**Session 2 (June 2019)**	
	%protein	**g protein/kg liquid**	**%protein**	**g protein/kg liquid**
Non-Centrifugated liquid	1.49 ± 0.26	14.90 ± 2.6	2.46 ± 0.49	24.57 ± 4.94
Centrifugated liquid	0.67 ± 0.07	6.69 ± 0.70	1.49 ± 0.26	14.90 ± 2.61
Solid fraction	0.82 ± 0.3	8.21 ± 3.0	0.97 ± 0.5	9.67 ± 4.9

In line with the results of the TOC measurements, the protein concentration in the liquid from Session 2 is higher (24.6 g protein/kg liquid) than in the liquid from Session 1 (14.9 g protein/kg liquid). Also here, this difference can be explained by differences in soil composition, climatic factors and species composition between the different roadsides (van Vuuren & van den pol, 2006). The difference could also be (partly) explained by the mowing method used. When using the N Kjeldahl technique, a certain amount of protein was also measured in the solid fraction; however, this is not possible because the TOC measurements showed that there is almost no organic matter present in the solid fraction (and thus no protein). The N Kjeldahl technique actually measures nitrogen, which is then converted into protein content by using a conversion factor. In the solid fraction, ammonium salts are present and these are most likely the origin of the nitrogen being measured with N Kjeldahl.

To validate the presence of protein in the liquid fraction, also SDS page gel was performed on the liquid from roadside grass from Session 2. **Figure 1** shows the results. On the right part of the SDS Page gel, a protein molecular marker (17-190 kDa, 1kDa equals to 9 amino acids) helps to determine the molecular weight of identified proteins. From the samples of the liquid fraction of roadside grass, the results of the 4x and 8x dilution cannot be used because of the bleeding from the (control) marker. However, the 1.3x and 2x dilution show clear protein bands at 25 kDa and at about 30 kDa. Studies on soluble proteins in plants indicate that the most abundant soluble protein in plant leaves is Rubisco, with a molecular weight between 46-57 kDa (approx. 414-513 amino acids) (Ma et al., 2009). It is possible that the 2 observed protein bands result from split-up of Rubisco as a result of the handling of the liquid. Further research is needed to confirm this.

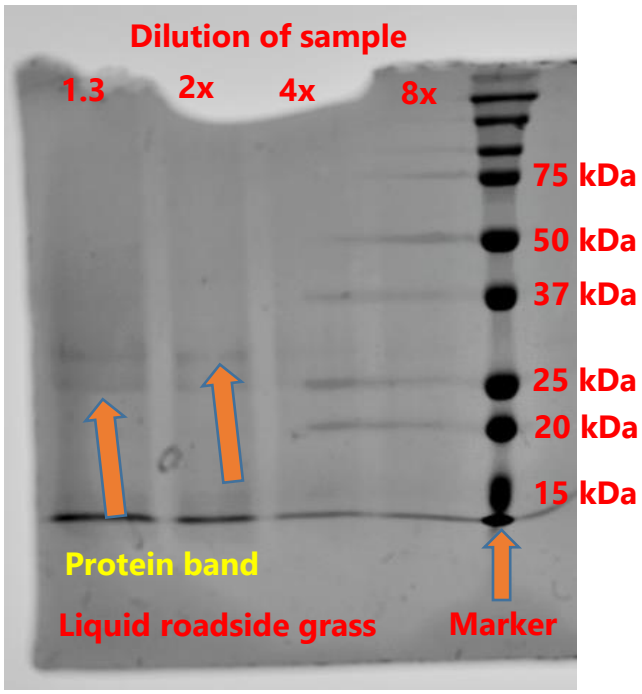

***Figure 1**: results of SDS page gel for liquid from roadside grass from session 2 at different dilutions (1.3x; 2x; 4x; 8x).*

Amino acids

With LC-MS, the amino acid composition of the liquids was measured, even though not all amino acids could be determined. Due to too much disturbance of the matrix (grass liquid), it was not possible to measure the amino acid concentration in the liquid from roadside grass of Session 2. However, it was possible to detect the presence of amino acids. **Table 6** shows the results. Tryptophan, threonine and possibly methionine are present. Cysteine and Lysine were not detected. Further research needs to be done on this topic.

***Table 6**: Presence of amino acids in the liquid from roadside grass*

	Detected	Not detected
Session 1	Tryptophan: 4.2 µg/mL Methionine: 16.6 µg/mL Threonine: 192 µg/mL	Cysteine Lysine
Session 2	Threonine Tryptophan	Cysteine Lysine Methionine

3. Application of liquid for feed

This task focussed on a small literature study on the appropriateness of grass juice towards feed application. Several studies have already looked at this opportunity, although focussed on nature grass or cultivated grass, while this project looks at the possibilities of roadside verges.

In Grassa (Sanders et al., 2016), the grass juice was warmed up and diluted with lactic acid to obtain clotted proteins. These high valued proteins were then used as alternative to soya-proteins for cattle feed. The high amino acid content makes it also interesting for pig feed if it is possible to make it directly available. Norsvin (cooperative business owned by Norwegian pig farmers) and other partners are now looking at the possibilities to use grass juice as a component of smoothies for pigs[1]. Indeed, they state that protein-rich materials should be added to the pig feed, because barley and fodder do not contain enough proteins for the ideal pig feed. Examples of such protein-rich raw materials are soy beans, oilseeds and field beans. In the study case, grass juice is added as example. Because pigs cannot utilize nitrogen compounds (e.g. ammonia and urea), grass juice is more interesting compared to dried grass or ensiled grass.

Figure 2 describes the refining process producing the liquid and fibre fraction. **Figure 3** shows a comparison of aminoacids between soybean meals, rape seed meal and grass protein products.

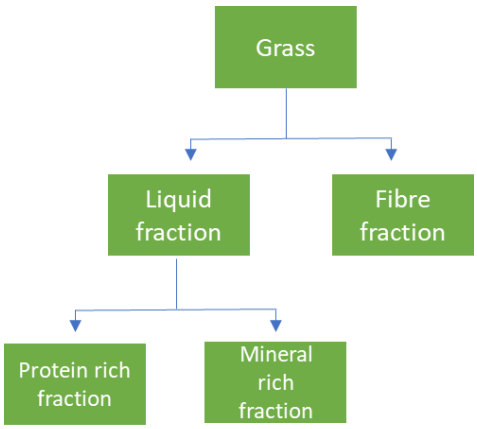

Figure 2: *Refining process for the separation of grass in liquid and fibre fractions*

[1] https://orkel.no/newsletter-dealer/smoothies-for-pigs/

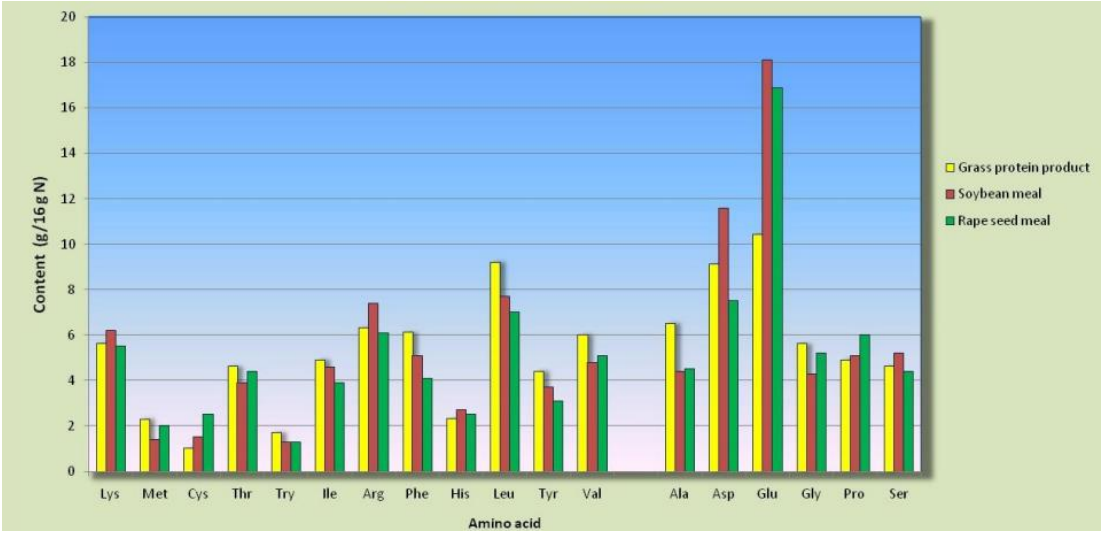

Figure 3: *The aminoacids contents for grass proteins compared to soybean and rape seed meal (Sanders et al. 2016)*

A challenge for roadside verges, compared to nature grass or agricultural grass, is the possible concentration of heavy metals. The presence of heavy metals in the liquid fraction would hamper the application towards feed. Quality of the grass depends on mowing moment and location. However, results so far from this project and previous ones have not indicated the heavy metal content to be high in roadside grass.

Based on results obtained by Avans in Spring 2019 (Table 5), following observations are made:

- The protein content in the total liquid fraction is very low, but similar to other (unfiltered) grass juices
    - Rye grass: 9.4 g protein/kg liquid
    - Clover grass: 15.7 g protein/kg liquid
- About 45% of the protein content is situated in the non-solids fraction (supernatant)

The low protein content makes the grass juice, as such, not interesting enough for feed production. Therefore, a process for up concentrating the proteins on grass juice has been tested by Avans.

4. Increasing the protein concentration

The protein concentration in the liquid fraction from pressed roadside grass is very low. This makes the liquid not very suitable as a source of feed for insects and/or pigs. Therefore, the possibilities to increase the protein concentrations in the liquid fraction were studied. In a first step, a literature study was conducted to see which methods are currently used on lab scale and on full scale in industries that produce or recover proteins. The following methods were found (Goldring, 2019; Burgess, 2009; Luo et al., 2015; Sari et al., 2015; Arlabosse et al., 2011):

- Evaporation
- Precipitation using:
  - HCl
  - Ammonium sulphate
  - Organic solvents + TCA
- Membrane ultrafiltration
- Heat coagulation
- Ultracentrifugation

The method chosen to test the possibility of increasing the protein content of the liquid from pressed roadside grass was evaporation using a Rotary Evaporator (Rotavap).

Method for evaporation

The rotary evaporator (Heidolph Hei-VAP Value Digital rotary evaporator) was chosen as the method for concentrating the proteins. In the rotating flask, 100 ml of the liquid sample was added and lowered into a 60°C water bath. The temperature inside the flask was set to 40°C.

The pressure for the liquid of Session 1 was set to 100 mbar and the one for Session 2 was set to 140 mbar. The pressure of the Session 2 sample was set higher to guarantee that the sample would be sprayed into the collection flask at 100 mbar. To determine the effects of evaporation, both the protein concentration and the TOC concentration were measured after 10, 20, 40 and 60 minutes.

Results

The results for TOC and protein are shown in **Figure 4** and **Figure 5**, respectively. The detailed results can be found in Appendix 2. As can be seen, the TOC and protein concentration increased with time. For the liquid from pressed roadside grass from Session 1, after 60 minutes in the Rotavap, the TOC concentration increased from 27 to 44 mg TOC/kg liquid. The protein concentration increased from 17 to 37 mg P/kg liquid. This corresponds to a concentration by a factor 1.6 - 2.2.

For the liquid from pressed roadside grass from Session 2, after 60 minutes in the Rotavap, the TOC concentration increased from 63 to 243 mg TOC/kg liquid. The protein concentration

increased from 17 to 70 mg P/kg liquid. This corresponds to a concentration with a factor 3.9 – 4.1.

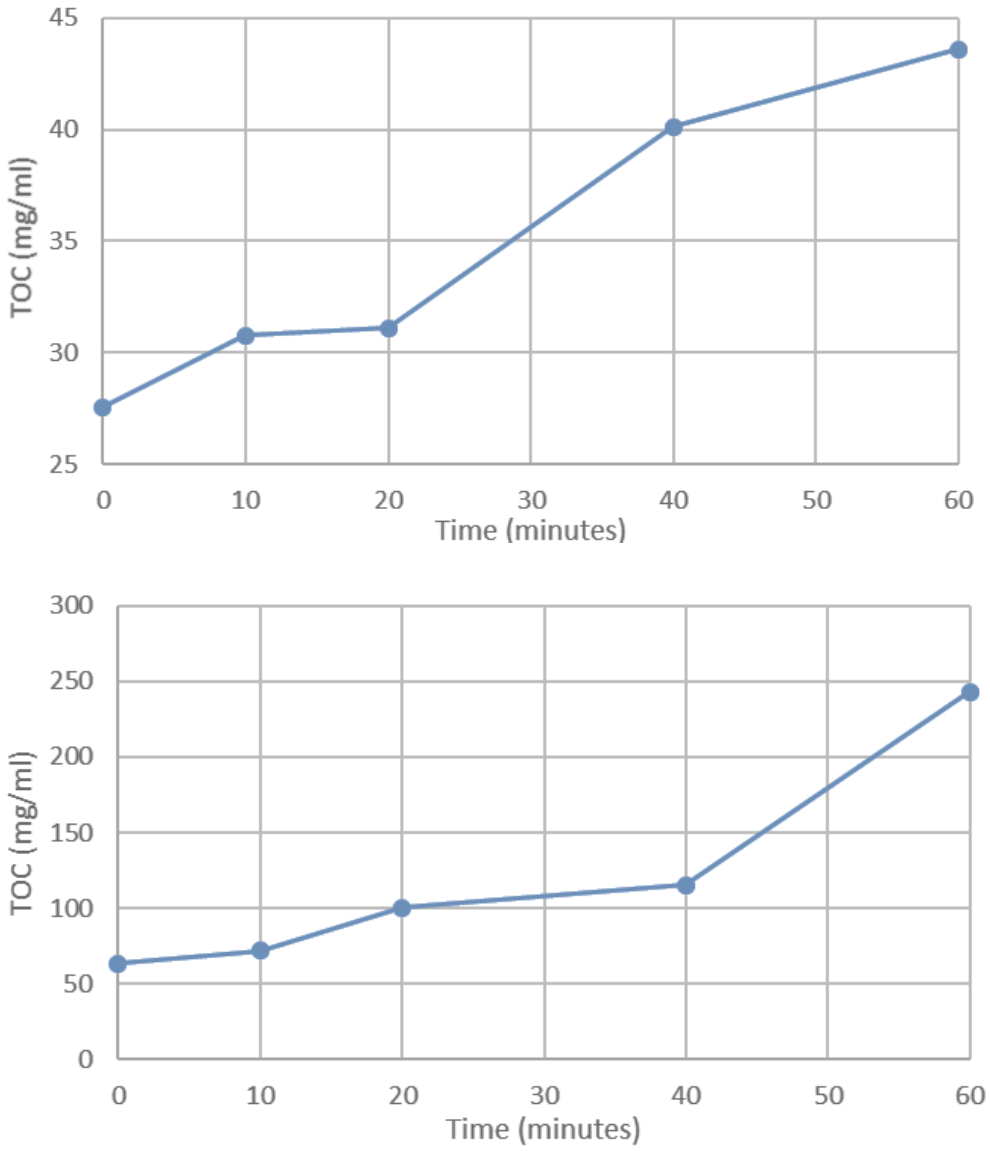

***Figure 4**: Increase of the TOC concentration in the liquid fraction (after centrifugation) of Session 1 (top) and Session 2 (bottom) as a result of evaporation with a Rotavap. Note: the y-axis is NOT the same for both graphs.*

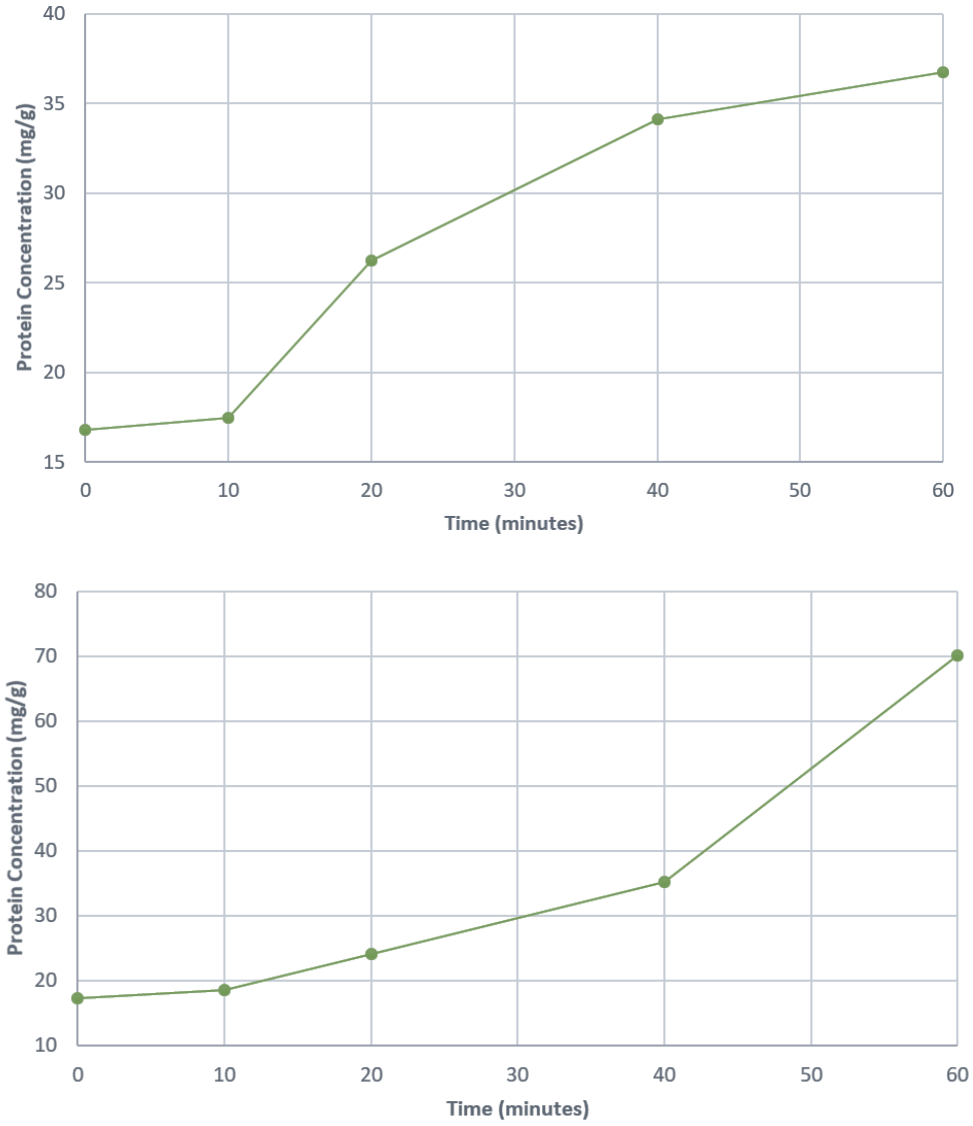

Figure 5: *Increase of the protein concentration (based on N Kjeldahl) in the liquid fraction (after centrifugation) of Session 1 (top) and Session 2 (bottom) as a result of evaporation with a Rotavap. Note: the y-axis is NOT the same for both graphs.*

Even with the concentration step, the protein concentration was found to be too low to result in an economically viable process for protein recovery from the liquid of roadside grass. The next chapters deal with the production of nutrient-rich organisms fed with the liquid of roadside grass as an alternative approach to produce protein with this feedstock.

5. Application of liquid for insect feed

The goal of these experiments was to assess if it is beneficial to use the liquid fraction of grass as the moisture source in the feed for insects compared to the use of tap water. The experiment was performed with two different insect species with a distinct life cycle: (i) yellow mealworm (*Tenebrio molitor*), a beetle species that lives on a dry feed but needs a daily input of wet feed; and (ii) black soldier fly (*Hermetia illucens*), a fly species that needs high moisture content feeds (30% dry matter).

Abbreviations:

GL	Grass liquid fraction
AG	Agar
DF	Dry feed
NS	Non-significant
S	Significant
CF	Chicken feed
SH	Spelt husks
WB	Wheat bran
W	Water
DM	Dry Matter

5.1. Material and methods

Mealworm experiments

In natural conditions, mealworms do not drink, but eat feed with a high moisture content (e.g. carrots). For that reason, a comparison was made between agar (2%) added to boiling water or GL, resulting in a solid structure. This can be stored in the fridge for 7 days and can be eaten by mealworms in a natural way. However, because it involves a boiling step that may result in a denaturation of the proteins or other nutritional changes, a comparison had also to be made between water and GL as raw material, even if this is less ideal as wet feed administration for the mealworms. Additionally, a comparison was made between two dry feeds: wheat bran and a commercial feed 'Insectus'. This was done to assess if the use of GL has a different effect when feeding a nutritionally poor-quality feed (wheat bran) compared to a high-quality feed. Therefore, a total of 8 different treatments were assessed. The order of the list below is also the order used in the graphs of the results section.

1) Insectus + water
2) Insectus + grass liquid
3) Insectus + Agar with water
4) Insectus + Agar with grass liquid
5) Wheat bran + water
6) Wheat bran + grass liquid
7) Wheat bran + Agar with water
8) Wheat bran + Agar with grass liquid

The mealworms used for this experiment are being bred in the Inagro Insect research center (Rumbeke-Beitem, Belgium) since 2013. They were kept in 60x40 cm plastic crates (inner surface area of ± 2000 cm^2) at a temperature of 26-27 °C, a relative humidity between 60 and 70% and a CO_2 concentration below 1500 ppm. The parental animals were fed at libitum with wheat brand (dry feed) and chicory roots (wet feed). The parental beetles were allowed to lay eggs for 1 week and thereafter the eggs were harvested and allowed to grow for 4 weeks on Insectus (without administration of wet feed). After 4 weeks, the total amount of larvae was estimated and redistributed at a density of 7000-7500 mealworms per crate (60*40) and 150 g of dry feed per 1000 mealworms was added (insectus or wheat bran). Wet feed was added 5 times a week with equal amounts in each crate with a total average amount of 110 mg water/larvae. Samples were taken from the fresh dry feed and wet feed for N, P and K analysis.

The larvae were harvested when visually no feed was left in one or more treatments. The content of each crate was then divided into mealworms, frass (fraction smaller than 0.5 mm) and leftover material. The following parameters were determined:

1) Total harvestable weight
2) Average mealworm weight
3) Amount of frass
4) Dry weight and N-P-K concentration of the mealworm and frass
5) FCR (Food conversion ratio)[2] as Total feed added/(mealworm harvest – initial mealworm weight) all on a dry weight basis.

There were four replicates per treatment, but to ensure that there was no population or time effect, the replicates were not set-up simultaneously, but sequentially in time.

Statistical analysis was performed in R using a three-way ANOVA with backward selection of the following full model:

Estimate = Dryfeed + Agar + Wetfeed + Dryfeed*Agar + Dryfeed* Wetfeed + Agar* Wetfeed + replicate

A Bonferonni correction was applied to reduce the risk of Type I errors (therefore the P-value limit was 0.007 instead of 0.05). The replicate was added as dummy variable.

Black soldier fly experiments

The larvae of the black soldier fly prefer a solid, moist rearing substrate (containing 30% dry matter). Grass juice is a liquid (7% DM), meaning it cannot be fed in its pure, unprocessed form. Therefore, it was fed in a mixture with dry feedstocks with varying nutritional values (spelt husks as a low nutritious feedstock, wheat bran as an intermediate feedstock and chicken feed as a nutrient-rich feedstock).

Black soldier fly (BSF) eggs were collected from Inagro's BSF breed stock. The eggs started on a nutritious chicken feed (FARM 1 Crumble) mixed with water (chicken feed : water, 30 : 70) as

[2] The FCR for insects measures the efficiency to convert insect feed into weight

a standard practice. Per gram of eggs, 0.5 kg of wet feed was supplied. After 7 days on this starter feed, larvae were divided per 20,000 and placed on the experimental feed.

Experimental feeds were formulated in such a way that all larvae received equal amounts of DM (aimed at 30% on fresh matter basis), as shown in **Table 7**. However, not all feedstocks were analysed for DM at the moment of formulation. The DM content of GL was assumed equal to the one of an earlier batch, which turned out to be an underestimation of the real value. This resulted in the fact that larvae fed with diets containing GL were given more dry matter than their counterparts in the control diet containing water.

Table 7: *Composition of experimental diets on fresh basis (kg) (CF: chicken feed; spelt husks: SH; wheat bran: WB; water: W; grass liquid: GL).*

	CHICKEN FEED	SPELT HUSKS	WHEAT BRAN	WATER	GRASS LIQUID
CF : GL	2.95				7.05
CF : W	3.26			6.74	
WB : GL			3.00		7.00
WB : W			3.33	6.67	
SH : GL		2.88			7.12
SH : W		3.19		6.81	

All diets were mixed at the start of the experiment and tested in triplicate. The experimental conditions were the following:

- a box size of 60 x 40 cm,
- 10 kg of wet feed, all fed at the start,
- a density of 20,000 7 day old larvae (DOL) per box,
- a starting weight of 4 mg per larva (7 DOL),
- an ambient climate of 27 °C at 60% RH.

After 8 to 10 days, all boxes had finished eating (the substrate was dry and cooling down). The larvae were separated from the substrate by mechanical sieving. The fresh weight of the larvae and the residual substrate were determined.

5.2. Results

Mealworm experiments

The average weight of the mealworms was 4.0 mg (± 0.5 mg) at the start of the experiment and 73.5 mg (± 15 mg) at harvest after 3 to 4 weeks, with an average DM content of 35%.

There were significant differences observed in average weight, total harvest, FCR and DM content. The average weight (**Figure 6**) and DM harvest (**Figure 7**) significantly increased, while the FCR (**Figure 8**) significantly decreased with the use of agar or Insectus and even more when the two were combined (significant interaction). It is important to highlight that a lower FCR is better, as it indicates a good conversion of feed into insect mass. When focusing on the nitrogen (protein) FCR, the trends change (**Figure 9**) as a significantly lower N-FCR is observed

when using wheat bran with agar. The dry matter content of the mealworms was also affected by the dry feed and the use of agar, but not to a significant extent (**Figure 10**). The use of water or GL did not change any of the parameters significantly. In **Table 8**, a summary is made for the different models and for the % variance each parameter explains.

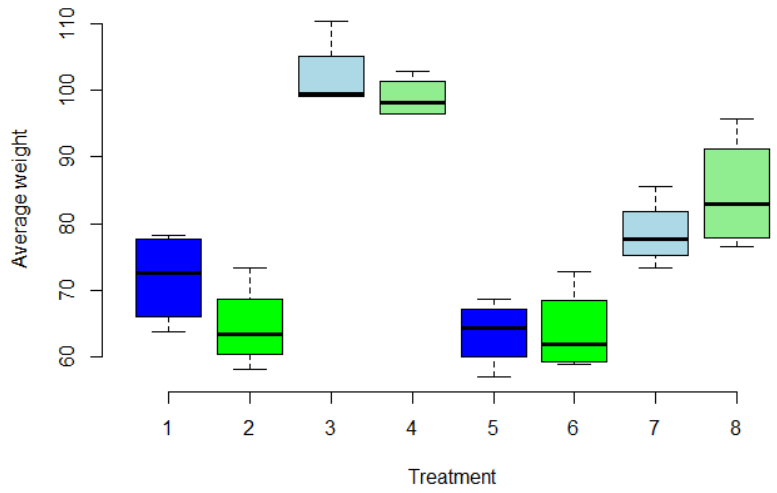

***Figure 6**: Boxplot of the average weight at the time of harvest. 1-4: insectus; 5-8: wheat bran; green (2, 4, 6, 8): Gras liquid present; 3, 4, 7, 8: agar present*

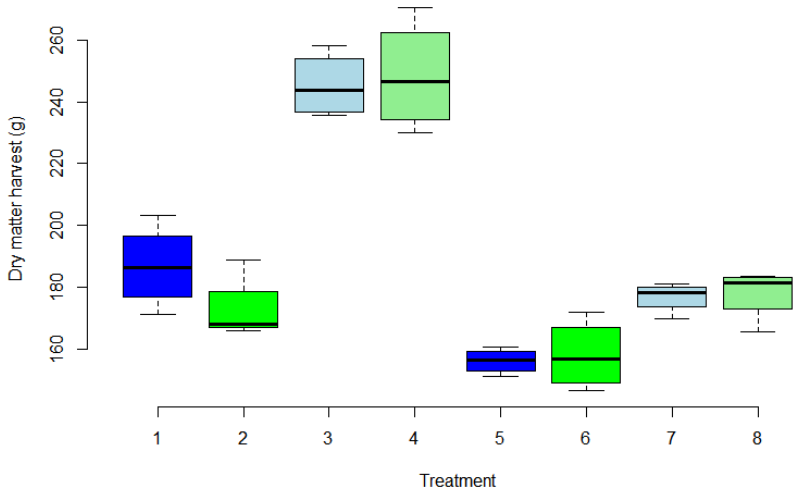

***Figure 7**: Boxplot of the dry matter harvest. 1-4: insectus; 5-8: wheat bran; green (2, 4, 6, 8): Gras liquid present; 3, 4, 7, 8: agar present*

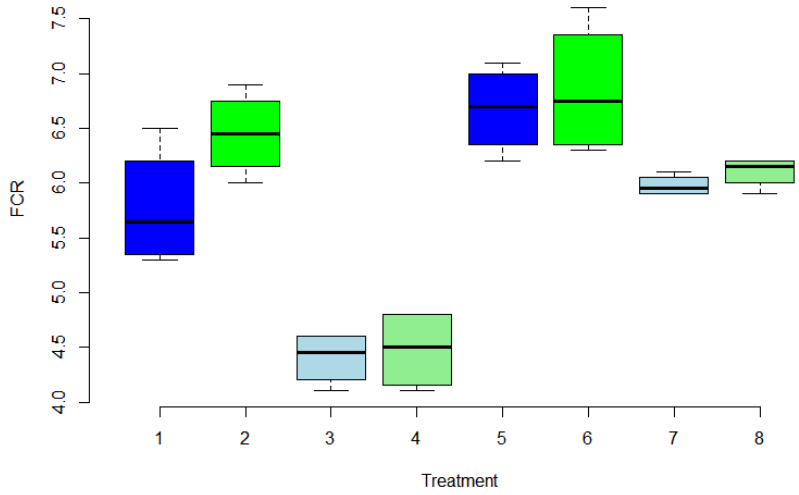

Figure 8: *Boxplot of the FCR. 1-4: insectus; 5-8: wheat bran; green (2, 4, 6, 8): Gras liquid present; 3, 4, 7, 8: agar present*

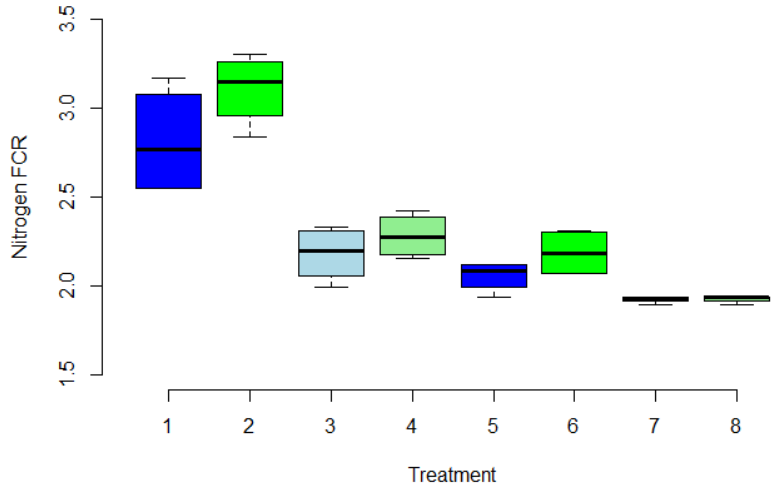

Figure 9: *Boxplot of the nitrogen FCR. 1-4: insectus; 5-8: wheat bran; green (2, 4, 6, 8): Gras liquid present; 3, 4, 7, 8: agar present*

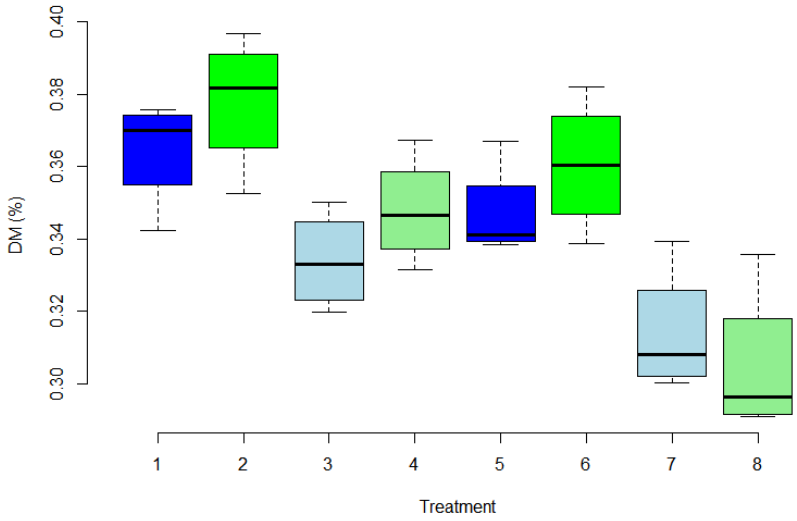

Figure 10: *Boxplot of the mealworm dry matter content (%). 1-4: insectus; 5-8: wheat bran; green (2, 4, 6, 8): Gras liquid present; 3, 4, 7, 8: agar present*

Table 8*: Summary of the results on the weight, harvest, FCR and DM content. % indicate the amount of variation explained by this parameter. NS = not significant*

	Dry feed	**Agar**	**GL**	**DF*Agar**	**DF*GL**	**Agar*GL**	**Replicate**
Average weight	14 %	66 %	NS	6 %	NS	NS	7 %
Total harvest	42 %	38 %	NS	11 %	NS	NS	3 %
FCR	36 %	41 %	NS	6 %	NS	NS	7 %
N-FCR	44 %	30 %	3 %	11 %	NS	NS	6 %
DM content	20 %	46 %	NS	NS	NS	NS	13 %

The protein (N) content of the mealworms was only significantly affected by the use of agar (**Figure 11**). The phosphorus concentration increased significantly with the use of wheat bran and if no agar was used (**Figure 12**). There was no significant influence on the potassium concentration (**Figure 13**).

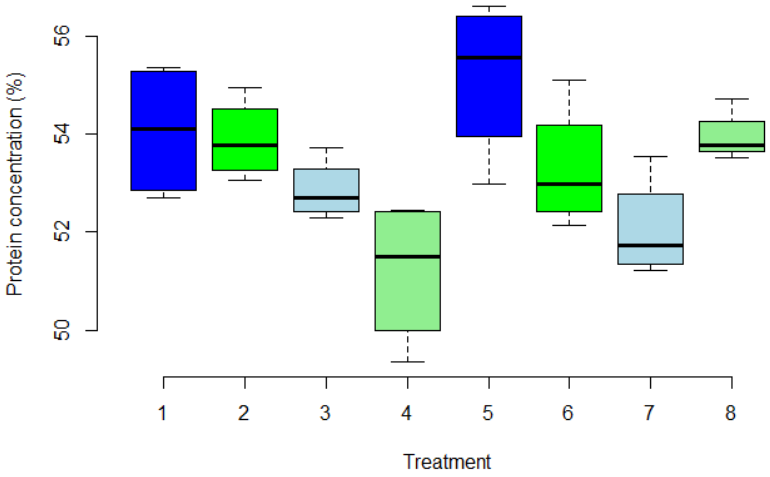

***Figure 11**: Protein concentration in the mealworms (dry weight basis), protein calculated via N concentration * 6.25.*

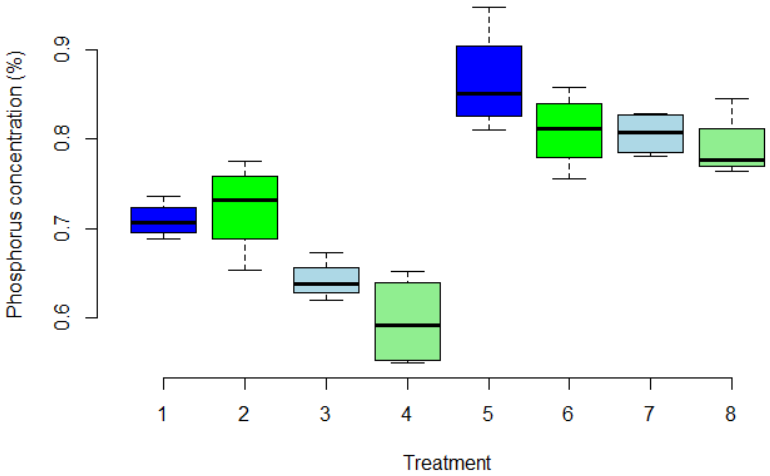

***Figure 12:** Phosphorus concentration in the mealworms (dry weight basis)*

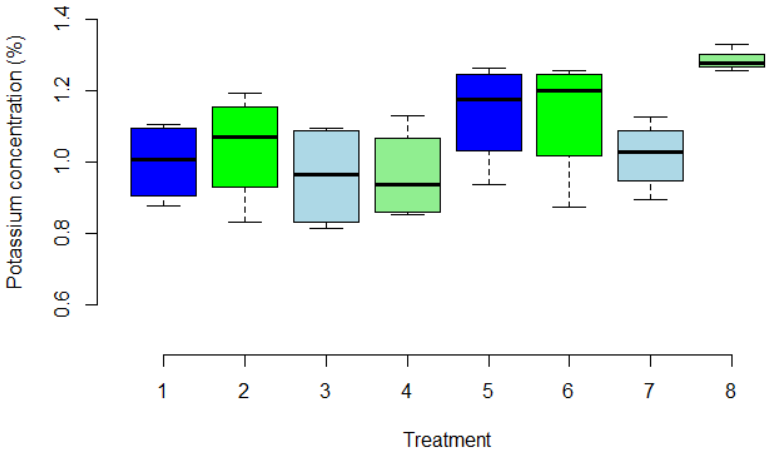

***Figure 13**: Potassium concentration in the mealworms (dry weight basis)*

The protein (N) content of the frass was significantly affected by the type of dry feed and the use of agar (**Figure 14**). The phosphorus concentration increased significantly with the use of wheat bran and if no agar was use. 1% of the variation was also explained by the interaction between GL and the dry feed (**Figure 15**). GL had a strong positive effect on the concentration of potassium in the frass in all but treatment 8 (**Figure 16**).

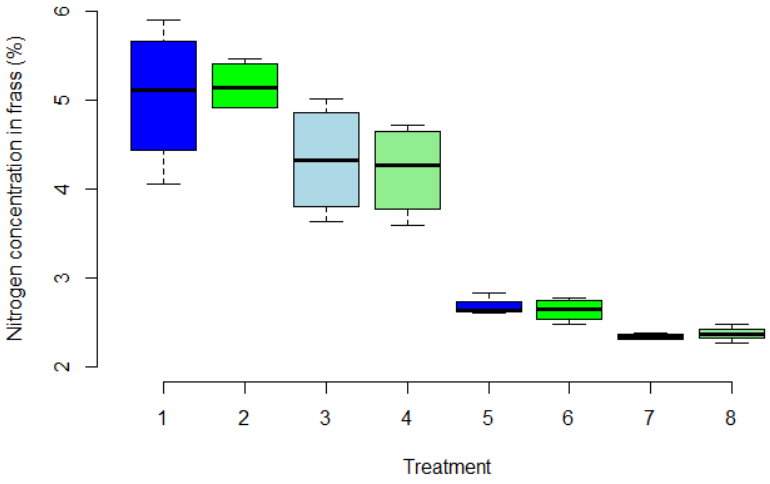

***Figure 14:** Nitrogen concentration in the frass (dry weight basis)*

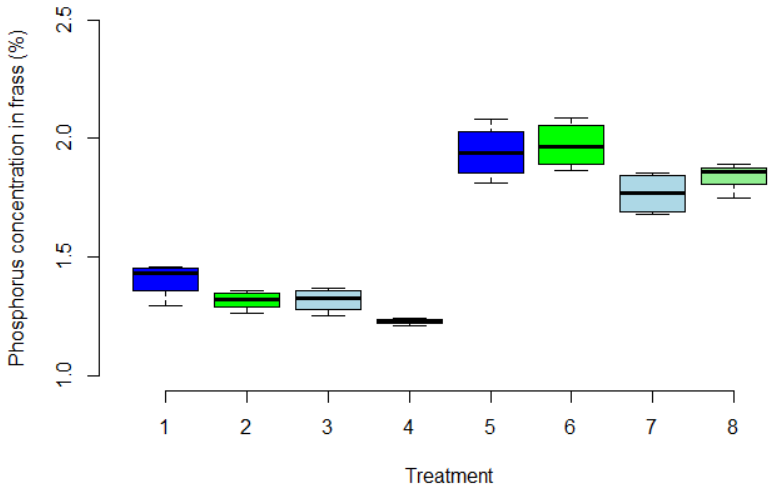

Figure 15: *Phosphorus concentration in the frass (dry weight basis)*

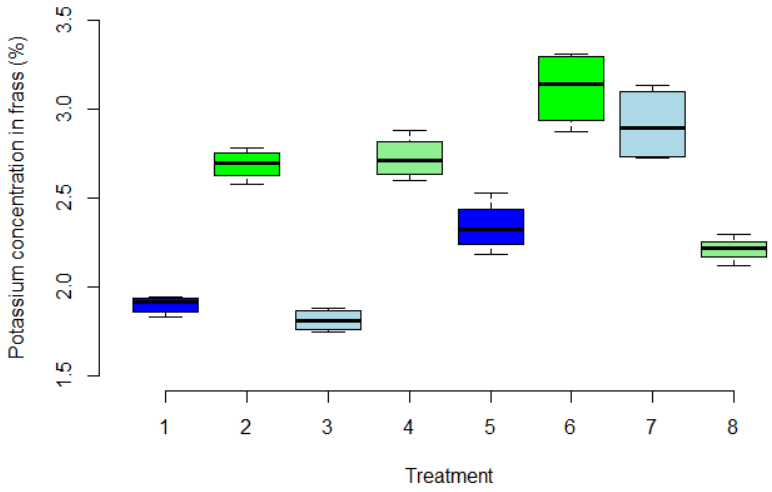

Figure 16: *Potassium concentration in the frass (dry weight basis)*

Black Soldier Fly

Larval growth and composition

Significant differences were observed for average larval weight and dry yield per diet between different diets (**Figure 17**). Most of the variation could be explained by the dry feedstock: chicken feed resulted in significant better growth than wheat bran, which was in its turn significantly better than spelt husks (**Table 9**). Replacing water by grass juice had a significant impact on growth as well, and for the better. However, the influence was less pronounced than that of the dry feed.

Table 9: *Summary of the results on average weight per larva, dry yield per diet, FCR and N efficiency. % indicate the amount of variation explained by this parameter. NS = not significant.*

	Dry feed	Moisture	Dry feed*Moisture	Unexplained
Average weight	96.2 %	2.2 %	NS	1.6 %
Dry yield per box	96.6 %	2.9 %	0.2 %	0.3 %
FCR	84.8 %	6.2 %	9.0 %	0.1 %
N efficiency	78.1 %	14.0 %	6.0 %	1.9 %

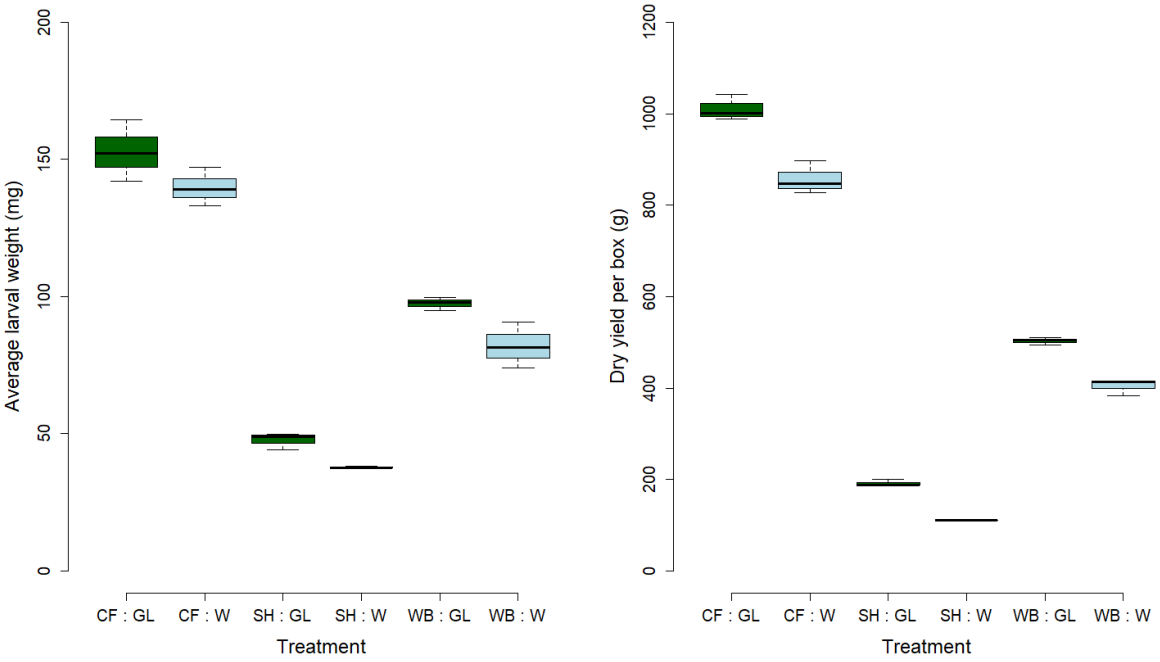

Figure 17*: Boxplot representation of the average larval weight (left) and dry yield per box (right) (n = 3).*

Feed conversion and nitrogen uptake improved as well with the addition of grass juice (**Figure 18**). However, the more nutritious the dry feed, the less pronounced the difference was; for chicken feed CF:W did not differ significantly from CF:GL.

The diet also had a significant impact on the composition of the larvae (**Figure 19** and **Figure 20**). DM content varied from 18% in SH:W to 32.5% in CF:GL; this has a significant impact on the amount of water harvested. The nutritious diets resulted in lower protein concentrations in the dry larvae (probably due to a higher fat content, which dilutes the protein). Phosphorus and potassium were determined as well. Phosphorus was less depending on the diet, except for a remarkable high concentration in the WB:W-diet (for which there is currently no explanation). Potassium was significantly lower in the diets containing chicken feed.

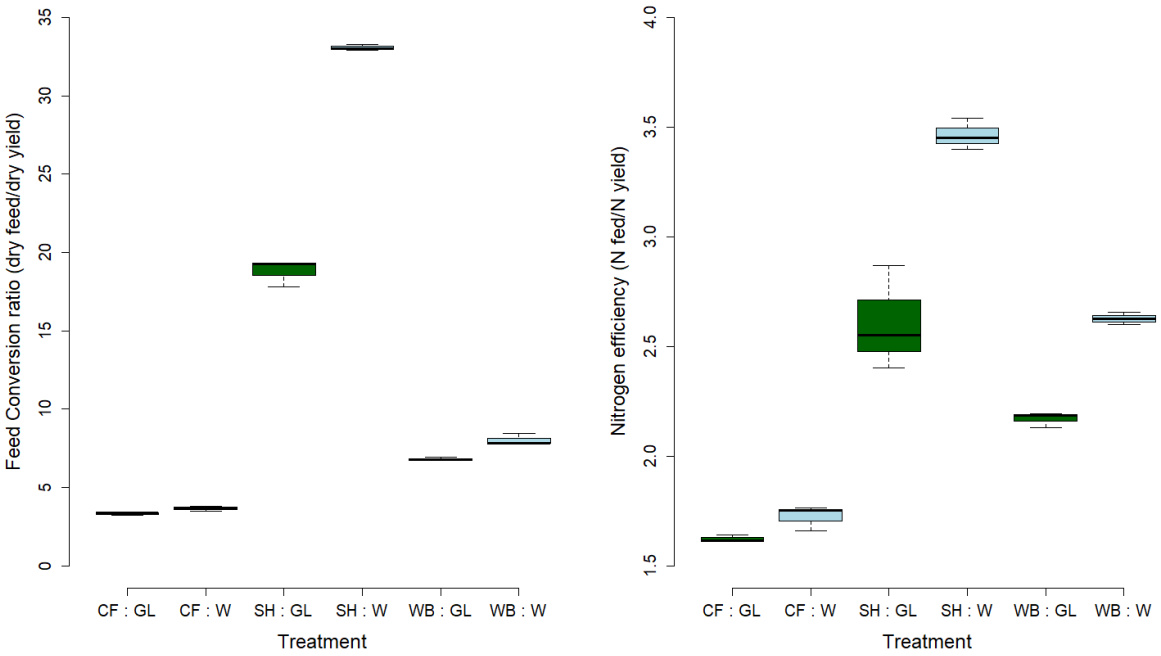

***Figure 18**: Boxplot representation of feed conversion ratio (left) and nitrogen efficiency (right) (n = 3).*

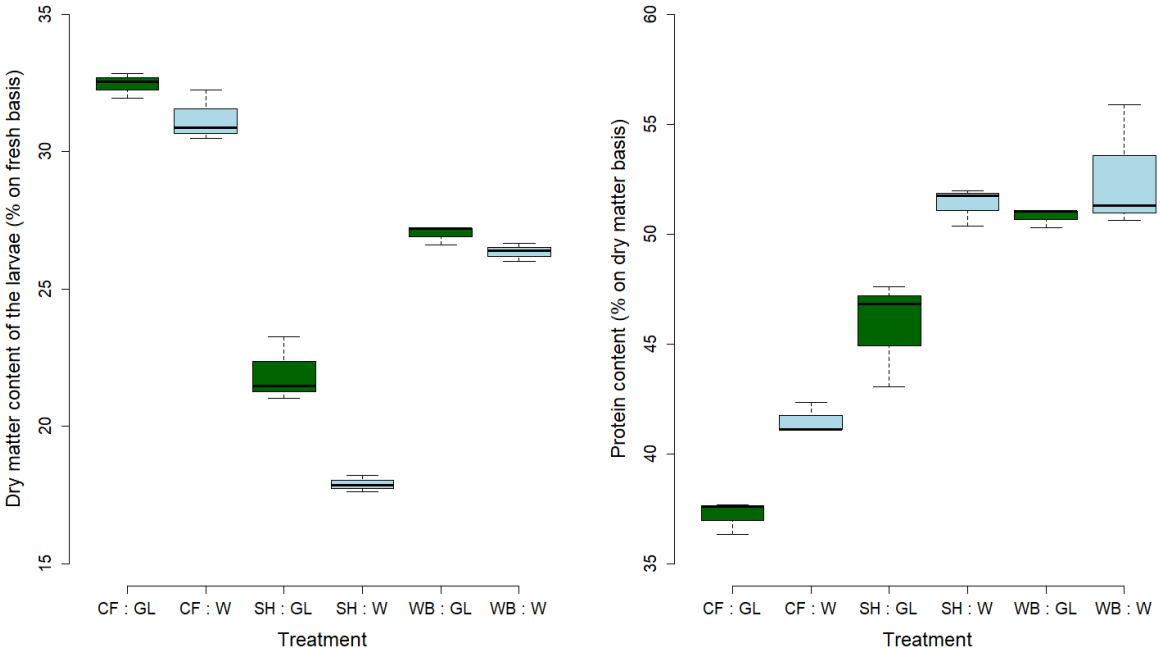

***Figure 19:** Boxplot representation of dry matter content of the larvae (left) and protein content with Kjeldahl factor 6.25 (right) (n = 3).*

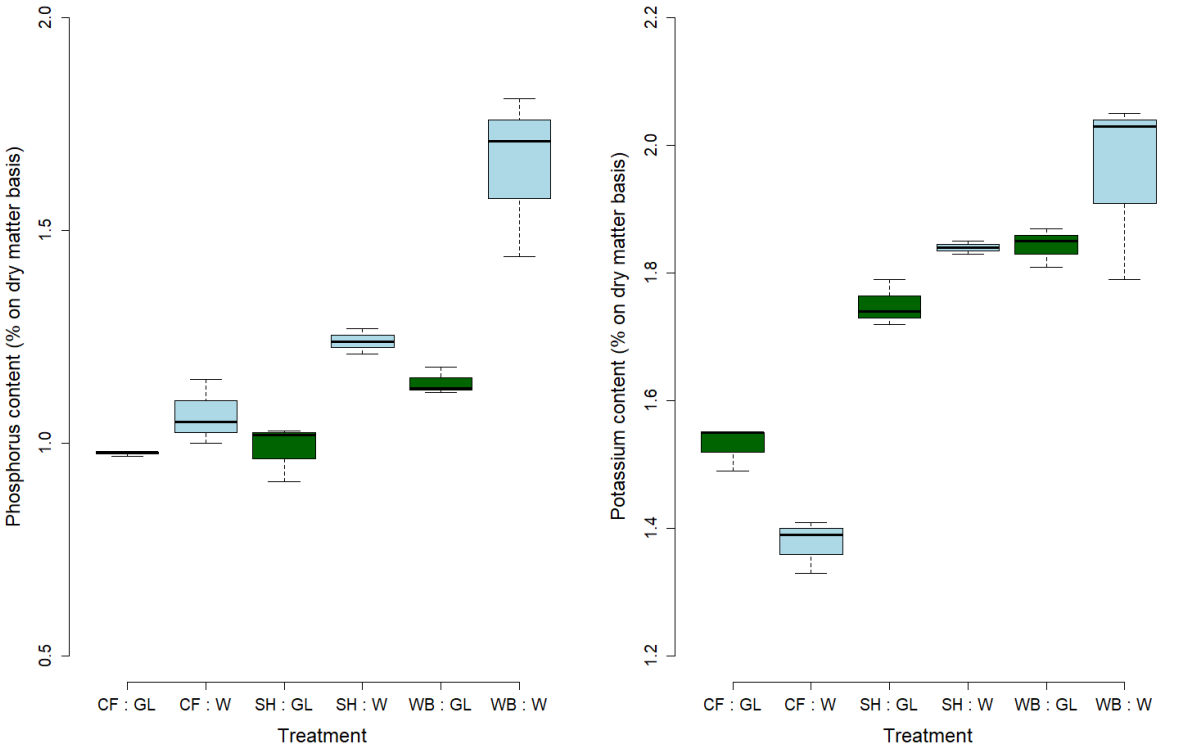

Figure 20: *Boxplot representation of phosphorus content of the larvae (left) and potassium content (right) (n = 3).*

Frass

The composition of the frass depends heavily on the composition of the dry feedstock (**Figure 21**). Only for potassium, grass juice has a large contribution, because grass juice in itself is rich in potassium.

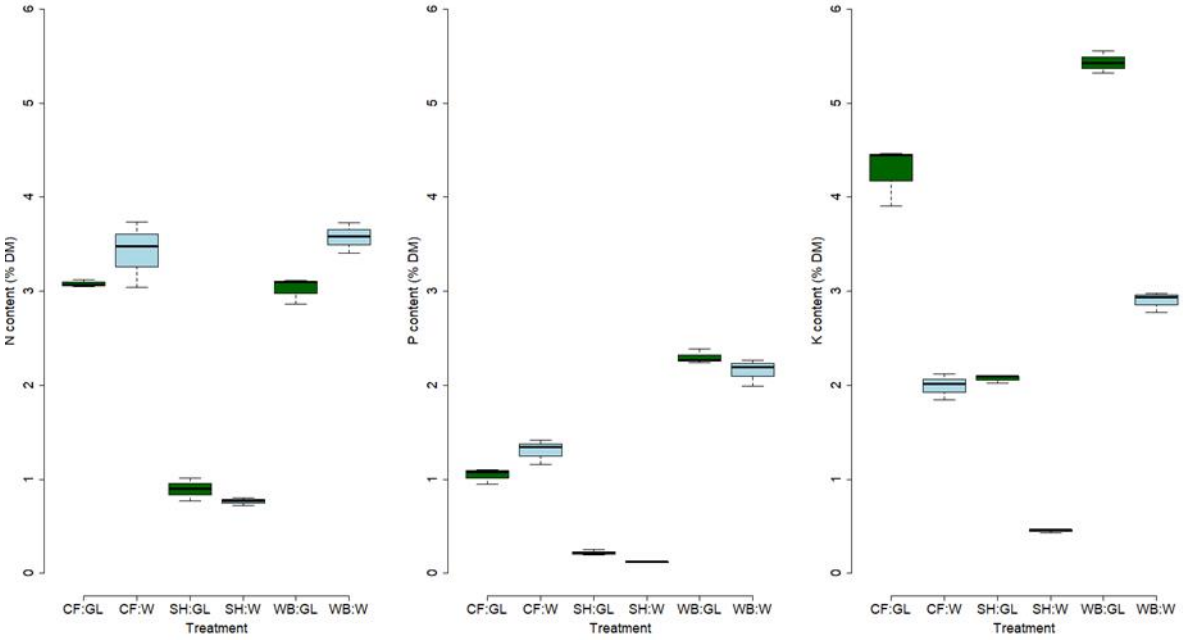

Figure 21: *Boxplot representation of the composition of black soldier fly frass (n=3). Nitrogen (left), phosphorus (middle) and potassium (right).*

5.3. Discussion and conclusion

Mealworms

Overall the mealworms grew better on Insectus and agar (and especially on the combination). However, if the overall goal of the rearing facility is to optimize the protein conversion (N-FCR), then the combination wheat bran with agar is better. The nutritional composition, estimated here with the N, P and K concentration, did not differ much between the different treatments and probably has no practical influence in a rearing facility. There are fairly large influences on the composition of the frass, although frass application will determine the best combination. If a high N/P ratio is needed, the mealworms should be fed with Insectus, while wheat bran should be fed when a low ratio is preferred.

The influence of the liquid fraction of grass (GL) as wet feed is limited. The N-FCR is higher and therefore negatively affected when using GL. This indicates that the proteins present in the GL cannot be used as effectively by the larvae when compared to the proteins from the dry feed. The main effect of GL was seen on the potassium concentration of the frass. This was not surprising considering the high initial potassium concentration in GL.

Black soldier fly

Black soldier fly larvae are an efficient way to dry and process grass juice if it is properly combined with a dry feedstock. Especially if the dry feedstock is of low nutritional quality, growth will benefit from replacing water with grass juice. For nutritious feedstocks such as chicken feed, grass juice was not significantly beneficial, but, more important, no adverse effects could be observed despite the fact that 10% less dry feedstock was used in the diet. Efficiency and productivity between larvae on chicken feed with water and on chicken feed with grass juice were comparable. Only the protein concentration of the larvae on grass juice was lower (probably due to the fact that the larvae contained more fat).

An attempt was made to rear larvae on pure grass juice by adjusting the dry matter content by evaporating excess water in the juice (going from 7% to 30%). This resulted in a sticky fluid with a high viscosity in which the larvae died fast, forcing us to abandon this idea.

6. Production of protein-rich microalgae (cyanobacteria) using the liquid fraction as growth medium

The interest in using the liquid fraction of grass for microalgae cultivation lies in the potential of these microorganisms to be used in the food and feed industry as novel protein sources[3]. With a growing world population, food security is an important issue, being one of the UN's Sustainable Development Goals. In order to meet the increasing food demand, agricultural practices need to be changed to enhance productivity while reducing environmental impact. Microalgae are a promising alternative, as they do not need arable land or freshwater for growing, can be harvested several times in a year, and have a high nutritional value, with several species being able to provide all the essential amino acids required in the human diet. In the present study, *Arthrospira platensis* (Spirulina) was chosen for its high protein content and for its existing commercialization as a nutrient supplement.

A first experiment was conducted with mineral medium (Zarrouk medium) for knowing the growth curve of the microalgae and determining the duration of the cultivation that would give maximum productivity. The results are presented in **Figure 22**. According to the data obtained, the maximum productivity was reached at the 7th day of cultivation, when the cells entered the stationary growth phase.

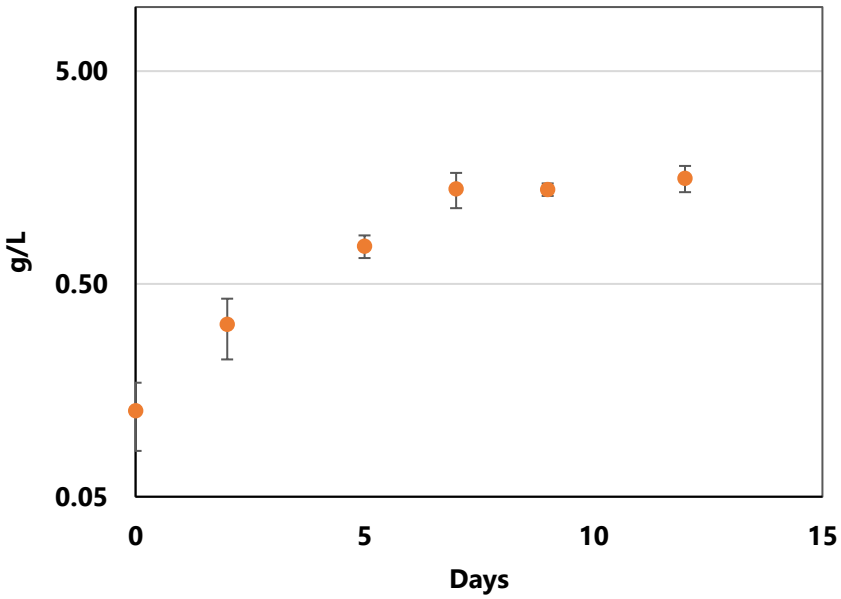

Figure 22: *Growth curve for Arthrospira platensis cultivated in mineral medium*

In order to determine the maximum amount of LFG that would not have a negative impact on the algal growth, the mineral medium was supplemented with increasing concentrations of filtered liquid fraction of grass (LFG) and the cells were grown for 7 days. The results obtained can be seen in **Figure 23**. It was possible to add up to 20% of LFG to the mineral medium

[3] Bleakley S, Hayes M. Algal Proteins: Extraction, Application, and Challenges Concerning Production. Foods 6(5), 33

without any negative impact on the production of microalgal biomass. The addition of higher amounts of LFG inhibited algal growth; this can be due to the dark color of the medium, which restricts light penetration, or to the presence of some toxic compounds that reach inhibitory levels when adding higher concentrations of LFG to the growth medium.

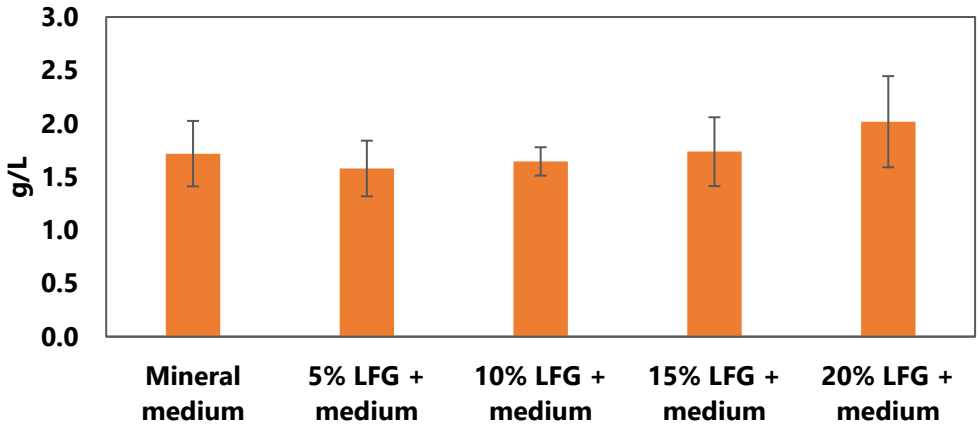

Figure 23: *Biomass concentration obtained after growing the cells for 7 days in mineral medium or in mineral medium supplemented with different concentrations of the liquid fraction of grass (LFG)*

Another experiment was carried out to verify if the LFG could be used as a sole nutrient source for microalgal cultivation, without the need for supplementation with mineral nutrients. As a first step, the microalgae grown on mineral medium (Zarrouk medium) and the filtered liquid fraction of grass (LFG) were characterized for their elemental composition to determine if any of the essential nutrients need by the algae were missing in the LFG. **Figure 24** shows the amount of nutrients found in the microalgae, converted from mg/g of biomass to mg/L of growth medium considering a biomass concentration of 2 g/L, and the elemental composition of the medium containing different concentrations of LFG.

For most of the macronutrients, in general even the lowest LFG concentration, of 5%, would give enough content for supporting a healthy algal growth. However, the nitrogen content of the LFG was too low with the lowest concentrations, only reaching the needed levels with the 15% and 20% concentrations.

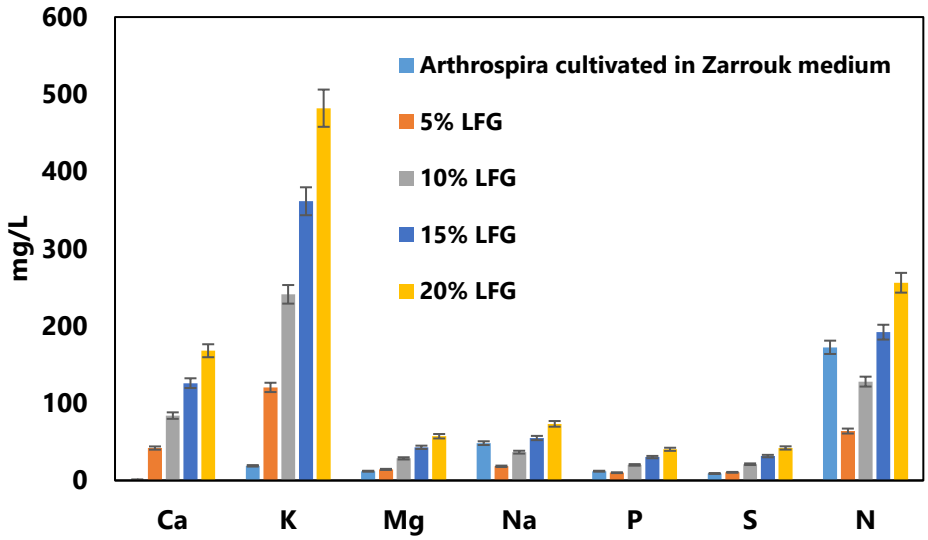

Figure 24: *Macronutrients composition of Arthrospira platensis grown on mineral medium, converted from mg/g of biomass to mg/L of growth medium considering a biomass concentration of 2 g/L, and of the medium containing different concentrations of LFG.*

Regarding the micronutrient concentrations, the algal biomass had much higher concentrations than those found in the LFG. However, mineral media is usually composed of excess micronutrients to guarantee a minimal concentration that will be perceived and absorbed by the cells, and microalgal cells have been reported as hyper-accumulating metals when these are abundant. Therefore, the apparent lack of micronutrients might not impact negatively in the growth of the cells when using only LFG as a nutrient source. For testing this hypothesis, new experiments conducted only with LFG were performed.

As described before, LFG has a low pH, while *A. platensis* usually requires an alkaline pH. Nevertheless, a test was made without any pH adjustment to assess if the cells would be able to survive in this condition. No growth was perceived in any of the tested conditions without pH adjustment, confirming that the low pH of LFG was indeed inhibiting the algal growth. Therefore, two strategies were envisioned to adjust the pH of the growth medium: addition of NaOH or $NaHCO_3$, which is already included in the Zarrouk medium and also serves as an additional carbon source. As can be seen in **Figure 25**, the use of NaOH resulted in very little cell growth; therefore, the addition of $NaHCO_3$ was chosen as the preferred method for pH adjustment.

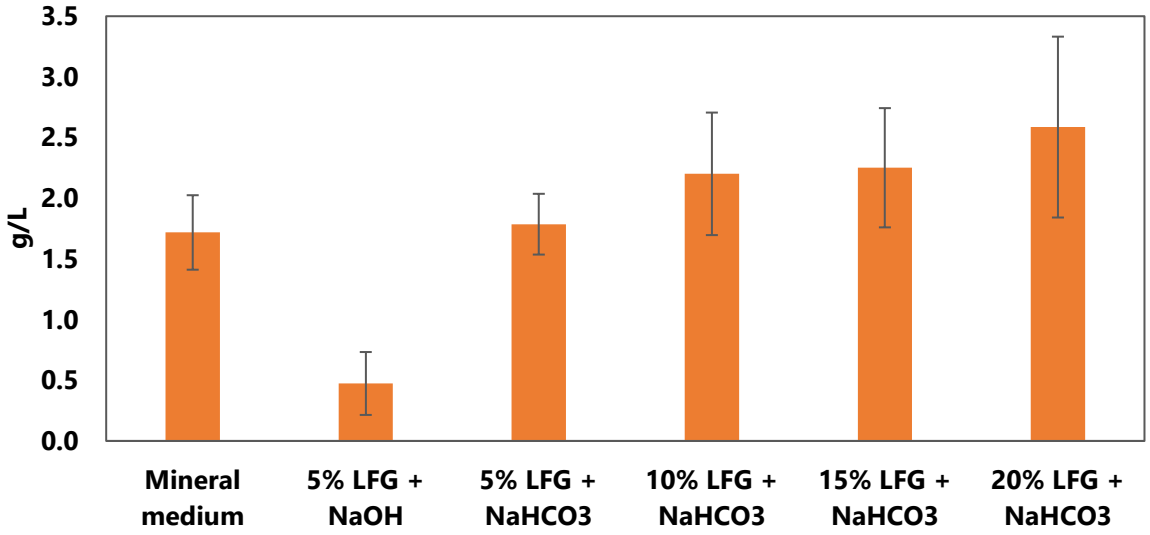

Figure 25: *Biomass concentration obtained after growing the cells for 7 days in mineral medium or in different concentrations of the liquid fraction of grass (LFG) supplemented with NaHCO3*

Good biomass production was perceived in all the tested conditions, even if the amount of nitrogen in the more diluted concentrations was theoretically insufficient for sustaining adequate growth. However, the biomass grown in the more diluted LFG had a different color, indicating some changes in pigment production (**Figure 26**). Since algal pigments are rich in nitrogen, this suggests that the cells were redirecting the nitrogen from pigment to protein production in order to sustain cell growth.

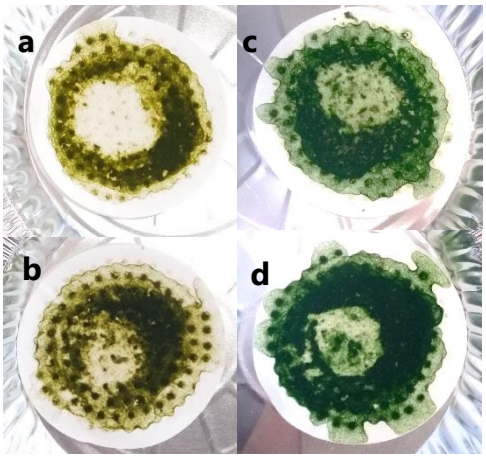

Figure 26*: Cells harvested after 7 days of growth in a)5% LFG, b)10% LFG, c)15% LFG, and d)20% LFG, all supplemented with* $NaHCO_3$

7. Conclusions

- The liquid from pressed roadside grass has a protein content between 6.7 and 14.9 g protein / kg liquid. This is comparable to the content found in other studies.
- This content is very diluted to envision using the grass juice as a direct protein source.
- The protein content is not sufficient to enhance growth of meal worms.
- Black soldier fly benefit from replacing water with grass juice, especially if the dry feedstock is of low nutritional quality.
- The liquid can be used as a nutrient source for *Arthrospira platensis* growth after pH adjustment with $NaHCO_3$.

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Appendix I: Methods for analysis liquid fraction from pressed roadside grass

N-Kjeldahl

The method is divided into three steps:

1. Digestion

The first step of the Kjeldahl method is the digestion of the sample. The sample is prepared by adding 1g of the sample in the tube, 1 Kjeldahl Titanium tablet and 8 ml H_2SO_4. The temperature was set to 300 °C and was gradually increased to 390 °C.

Materials
Liquid grass sample
Kjeldahl Titanium tablet Product number: 11057980 Comp.: $K_2SO_4 + CuSO_4 * 5\ H_2O + TiO_2$
Hydrogen Sulfide H_2SO_4

During the digestion process the nitrogen that is organically bonded in the sample is converted into ammonium ions. Part of this process is similar to an incineration process because organic carbon and hydrogen form carbon dioxide and water. The organic material in the sample begins to carbonize and the sample is transformed into black foam. This foam decomposes during the digestion and finally becomes a clear green liquid indicating the completion of the chemical reaction.

The following chemical equation shows how the nitrogen in the sample was mineralized to dissolved ammonium ions: Sample + H_2SO_4 → CO_2 + SO_2 + H_2O + NH_4^+

2. Distillation

After the digestion the sample, now an acidic mixture, cools down and becomes a light blue liquid. The digestion tube is ready to be transferred to the distillation unit. Preceding the distillation process, is the neutralization of the acidic sample by adding concentrate sodium hydroxide as shown in the following equation: H_2SO_4 + 2 NaOH → 2 Na+ + SO_4^{2-} + 2 H_2O

Materials
Digested Sample
Sodium Hydroxide (NaOH)
Boric Acid (H_3BO_3)

During the distillation process, the solvated ammonium ions react with hydroxyl-ions of the sodium hydroxide and are converted into ammonia gas according to the following equation: $NH4^+$ + OH^- → NH_3(gas) + H_2O. By steam distillation the ammonia is removed from the glass tube and condensed with water into the receiving vessel.

The receiving vessel for collecting the ammonia contains boric acid dissolved in water. The ammonia is captured by the boric acid solution forming solvated ammonium ions. This goes according to the following equation: $B(OH)_3$ + NH_3 + H_2O → NH_4^+ + $B(OH)_4$.

3. Titration

Determining the concentration of the captured ammonium ions in the boric acid required an acid base titration using sulfuric acid. The pH is reduced from its initial pH of 6-7 down to a pH 4.65 using sulfuric acid. Due to an expected low concentration of ammonium ions present in

the sample and therefore the sulfuric acid solution was diluted to 0.01 M. For this titration the Metrohm 848 Titration was used to the measure the sample.

Calculation

After the titration, what is left is to calculate the nitrogen content and protein content in the tested samples using the formula. This formula first calculates the amount of nitrogen in the sample by using the volume of the consumption of diluted sulfuric acid and subtract the consumption volume of a blank sample. This is then multiplied with a molar reaction factor of 2, the concentration of the diluted sulfuric acid (0.01M, the factor of the titrant: 1, and the molar weight of N (14,007 g/mol). The result is then divided by the sample weight (1 g) multiplied by 1000 and then multiplied by 100 to obtain the result in percentage of nitrogen. The nitrogen percentage is then multiplied by the protein factor of 6.25 which then results in the estimated protein percentage in the sample.

Calculation Result:

$$\%N = \frac{(V(1)-V(BI))*F*c*f*M(N)}{m*1000} * 100\%$$

$$\%P = \%N * PF$$

V1 = consumption of titrant sample (mL)
V(BI) = av. Consumption titrant blank (mL)
F = molar reaction factor (1=HCl, 2=H_2SO4)
c = concentration titrant
f = factor of titrant
M(N) = molar weight of N (14,007 g/mol)
m = sample weight
PF = protein factor
%N = % of weight of N
%P = % of weight of protein

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This method is used to separate proteins in order to detect the types of proteins found in the sample. The set up contains two glass plates held together by a plastic stand and frame. First the running gel is produced and then the stacking gel and these are connected to an electrode to pull the sample through the gels.

Gel and Sample Preparation

The running gel and stacking gel are produce in that exact order because the running gel is the first one to be poured between the glass plates. Once the running gel is completely polymerized, the stacking gel is poured on top of that and a comb is put in to form the well where the samples will be injected. Table 1 shows the chemicals and volumes used to make both the running gel and stacking gel. The samples were mixed at different volumes with demi water and the MB buffer, this was then heated at 100 °C for 10 minutes to denature the proteins. The chosen volumes are shown in Table 2.

Table 1 Chemical and volumes used for gels

Running Gel	**Stacking Gel**
2.05 ml D.H_20	2.1 ml D.H_2O
1.25 ml Tris 8.8	0.85 ml Tris 6.8
50 µl SDS	33.5 µl 10% SDS
1.65 Acryl	333 µl Acryl
43.5 µl APS	30 µl APS
3 µl TEMED	3 µl TEMED

Table 2 Different volumes created for the mixture volume

Dilutions	**Liquid sample**	**d.H2O**	**MB**
1.3x	60 µl	-	20 µl
2x	40 µl	20 µl	20 µl
4x	20 µl	40 µl	20 µl
8x	10 µl	50 µl	20

Set-up and Electrophoresis

On the second day of the experiment the gel had the time to properly settle and polymerize. The gel was then ready for injection in its wells. As the gel cassette in first placed in the electrode box, the inner chamber is filled with electrode buffer until the indicated and the wells are then filled using a pipette with gel loading tips. These wells were injected in the order shown in Table 3.

Table 3 Sample mixtures for injection in the wells

Dilutions	1.3x	2x	4x	8x		1.3x	2x	4x	8x	
Gel slots	1	2	3	4	M	4	3	2	1	M
Volume per slot	10 µl				5 µl	20 µl				5 µl

After injection, the rest of the buffer is added to the box to the indicated amount. Once the lid closes the box, the electrodes and the power pack are connected to the box. The powerpack is turned on for approximately 60 minutes or until the blue line reaches the bottom of the gel. After the electrophoresis is done, the gel is removed from its casing and put in Coomasie Blue dye to enhance the proteins retained in the gel. The longer the gel remains in the dye, the clearer it becomes.

Appendix II: results protein concentration

Table II.1: results TOC measurements

	0 min	10 min	20 min	40 min	60 min
Autumn	141 ppm	155 ppm	163 ppm	201	222 ppm
	141 ppm	159 ppm	158 ppm	208	220 ppm
	140 ppm	157 ppm	155 ppm	205	225 ppm
%	2,81%	3,14%	3,17%	4,09%	4,45%
mg/ml	27,57	30,77	31,1	40,12	43,58
	0 min	10 min	20 min	40 min	60 min
Spring	320 ppm	356 ppm	512 ppm	149 ppm	309
	324 ppm	371 ppm	512 ppm	148 ppm	306
	326 ppm	372 ppm	515 ppm	145 ppm	314
%	6,47%	7,33%	10,26%	11,79%	24,77%
mg/ml	63,37	71,8	100,55	115,51	242,78

Table II.2: results N Kjeldahl measurements

After Rotavap						
	T (min)	V1 (ml)	**N%**	**mg N/g**	**P%**	**mg P/g**
Autumn	0	10,64	0,26907447	2,69	1,681715438	16,81
	10	11	0,27915951	2,79	1,744746938	17,45
	20	16,03	0,42006993	4,2	2,625437063	26,25
	40	20,53	0,54613293	5,46	3,413330813	34,13
	60	22,03	0,58815393	5,88	3,675962063	36,76
	T (min)	V1 (ml)	**N%**	**mg N/g**	**P%**	**mg P/g**
Spring	0	10,86	0,27523755	2,75	1,720234688	17,2
	10	11,57	0,29512749	2,95	1,844546813	18,45
	20	14,77	0,38477229	3,85	2,404826813	24,05
	40	21,13	0,56294133	5,63	3,518383313	35,18
	60	41,06	1,12126035	11,21	7,007877188	70,08