Grassification

O 4.1

Characterization of the liquid fraction of grass and its valorisation potential

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Document Control Page

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Abstract	The liquid fraction of grass accounts to about 60% of the total fresh weight of the initial biomass and, therefore, its valorization is important for developing an economically viable value-chain from grass. The Grassification project has, up to now, investigated three different valorization routes for this stream: production of fertilizer, biogas, and protein – recovered directly from the liquid or as a result of using the liquid as a feed for insects or microalgae. The initial results obtained are presented in this report, together with the characterization of this liquid stream.

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Contents

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1. Introduction

Roadside grass needs to be cut at least twice per year for safety reasons and several EU member-states legislation impose a "cut-and-collect" regime, where the grass clippings have to be collected in order to improve biodiversity of the roadside verges[1]. This generates a significant amount of biomass that is currently seen as waste according to the EU Waste Framework Directive (2008/98/EC), with only a small part being valorized into compost, a low-value product. Nevertheless, this biomass could be used as a valuable feedstock, contributing to the European Commission's Bioeconomy Strategy, in which renewable biological resources are understood as essential for achieving the goals of the UN's 2030 Agenda for Sustainable Development.

The Grassification project aims at valorizing this untapped source of renewable feedstock and changing its waste status, following the European Commission end-of-waste criteria. For achieving this goal, the material currently considered as waste must have a proven use and must not cause negative environmental and health impacts. Therefore, the proposal and evaluation of value-chains from grass is of utmost importance to prove that this biomass has valuable applications without adverse consequences.

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[2]. The obtained liquid fraction can account up to 60% of the total fresh weight of the initial biomass[3]; therefore, its valorization 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 were investigated for valorizing the liquid fraction of grass: (i) fertilizer production, i.e., use of the liquid fraction as a tomato feed; (ii) anaerobic digestion for biogas production; and (iii) protein production. The latter was subdivided into three strategies: (i) direct use of the liquid fraction as a source of protein; (ii) growth of protein-rich insects using

1 Noordijk J, Delille K, Schaffers AP, Sýkora KV. Optimizing grassland management for flower-visiting insects in roadside verges. 2009. Biological Conservation 142 (10), 2097-2103

2 Mandl MG. Status of green biorefining in Europe. 2010. Biofuels, Bioproducts & Biorefining 4, 268–274

3 Sharma HSS, Carmichael E, Muhamad M, McCall D, Andrews F, Lyons G, McRoberts C, Hornsby PR. Biorefining of perennial ryegrass for the production of nanofibrillated cellulose. 2012. RSC Advances 2, 6424–6437

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the liquid fraction as feed; and (iii) production of protein-rich microalgae (cyanobacteria) using the liquid fraction as growth medium. In the present report, the fist value-chain, i.e., fertilizer production will not be covered, as negative results were obtained. A report on these results can be found in deliverable D 2.1.1 from the Grassification project and additional experiments are planned for assessing the impact of different treatments on the fertilizer potential of this stream. Only the main findings of the other tested value-chains are reported here; for the complete results, please refer to deliverables D 2.2.1 and D 2.3.1 from the Grassification project.

2. Characterization of the liquid fraction of grass

2.1 Origin of the liquid fraction of grass

The liquid fraction was originated from pressed roadside grass from 2 different mowing sessions. 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 and pressing: 8/11/2018	Mowing and pressing: 18/06/2019
Mowing machinery	Rotary mower	Flail and rotary mower
Pressing machinery	Screw press ('tegendruk schroefpers') adapted for biobased resources at Rhinetech	Screw press S22 from ECO Grondstoffen
Liquid fraction	42,8% of the fresh material	±30 % of the fresh material
Fibre fraction	57,4% of the fresh material	±70% of the fresh material

A video of the pressing process for Session 2 can be seen at: https://youtu.be/RXgS2IxCeYk.

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2.2 Composition of the liquid fraction

General characteristics and nutrients

Liquid fractions obtained from the pressing of different grass clippings were characterized according to their pH, EC, TOC, and N, P, K contents. Characterizations were conducted with the untreated samples and also after filtration, as the liquid fractions had a high suspended solids content. Results can be seen in Table 2.

Table 2: *Characterization of the filtered and unfiltered liquid fractions produced from different grass clippings*

	Treatment	pH	EC (mS/cm)	TOC (g/kg)	P_2O_5 (g/kg)	K_2O (g/kg)	Total N (g/kg)
S1 - Rotary	Unfiltered	6.2	11.1	17.7	0.659	2.88	3.53
	Filtered	6.2	11.9		0.406	2.26	1.28
S2 - Rotary	Unfiltered	4.9	16.1	23.6	0.240	3.71	0.846
	Filtered	4.9	15.3		0.240	3.92	0.628
S2 – Flail	Unfiltered	4.9	17.9	29.1	0.376	5.86	1.57
	Filtered	4.9	18.1		0.341	5.82	1.11

S1 – session 1; S2 – session 2 (Table 1)

Liquid fractions obtained with the different mowers in the same session had similar pH values, but the sample derived from Session 1 had a much higher pH, which may be due to the handling of the sample. It was observed for the sample S2 – Flail a lower pH, of 4.3, when immediately frozen after pressing, in contrast with the data presented in Table 1, of 4.9, which was obtained from a sample that was left at room temperature for several hours before freezing. As a result, transformation of part of the organic matter may have occurred, affecting the pH. The same can be said for the TOC content, as the value obtained from the promptly frozen sample was much higher, of 53 g/kg, than the reported in Table 2, which was most probably consumed during the hours that the sample was left at room temperature, as some production of gas was observed. The difference can also be a result of differences in soil composition, climatic factors and species composition between the different roadsides and by the pressing method used.

Regarding the NPK content, it is possible to see that the nutrient concentrations vary not only with the grass origin/mowing season, but also with the type of mower used. The latter

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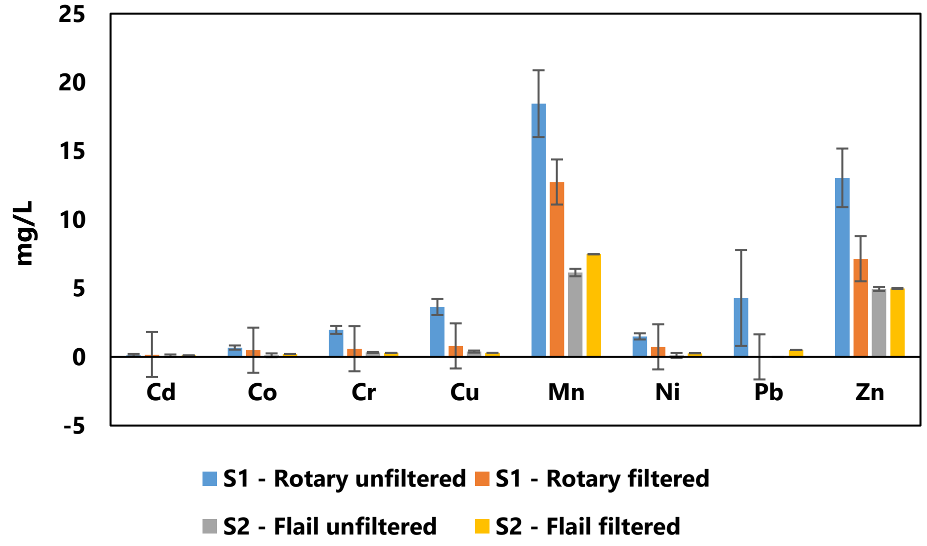
influences the length of the grass fiber, which in turn may affect the efficiency of the pressing step and the transfer of nutrients to the liquid phase.

As the aim of pressing the fibres was to get a nutrient-rich liquid fraction, further characterization was only done with samples from Session 1 and with samples derived from grass clippings obtained with the flail mower in Session 2, which displayed a higher nutrient content than the ones derived from the rotary mower.

Figure 1 shows the heavy metal and macro element contents of the liquid fraction of grass obtained with the flail mower before and after filtration.



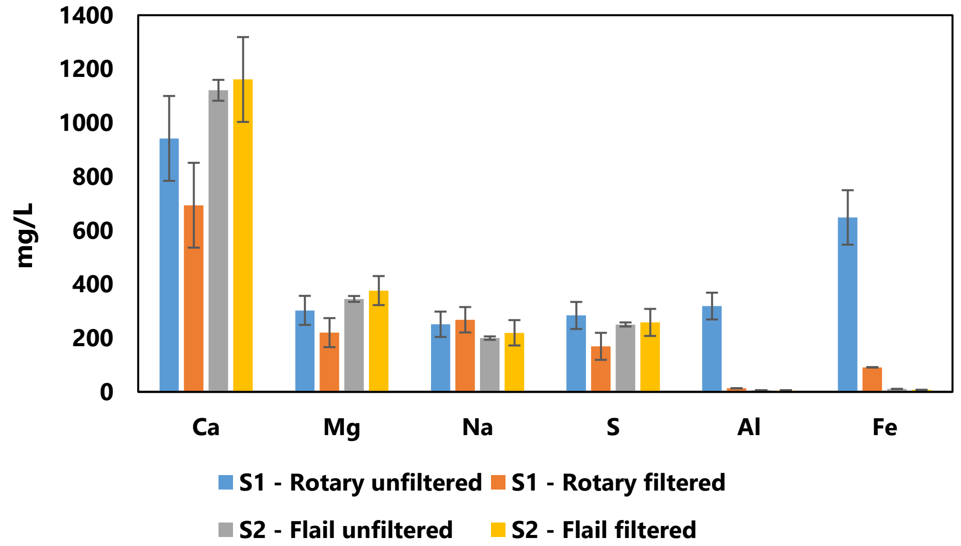


Figure 1: *Elemental composition of the liquid fraction of grass obtained with a flail mower before and after centrifugation*

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It was observed that the heavy metal content of the liquid fraction was in general decreased after filtration, indicating that these elements were solid-bound. However, the macro nutrient content in general did not significantly change with the treatment and, therefore, these nutrients are present in soluble form in the analyzed liquid. Moreover, the samples derived from the rotary mower had higher heavy metal content than the ones from the flail mower, possibly due to higher soil contamination in the clippings.

Protein content

Table 3 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)[4]. They found a concentration of 9,4 g protein/kg liquid for Rye grass and a concentration of 15,7 g protein/kg liquid for Clover grass.

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 a differences in soil composition, climatic factors and species composition between the different roadsides. The difference could also be (partly) explained by the pressing method used.

Table 3: Protein content of non-centrifugated liquid and centrifugated liquid 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

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 2 shows the results. On the right part of the SDS Page gel, a protein molecular marker (17-190 kDa, 1kDa equals to 9 amino

[4] Anderson & Kiel (2000) Integrated utilisation of green biomass in the green Biorefinery, Industrial Crops and Products 11 129–137.

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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 a clear protein band 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.16, 2009)[5]. It is possible that the 2 observed protein bands result from split-up Rubisco as a result of the handling of the liquid. Further research is needed to confirm this.

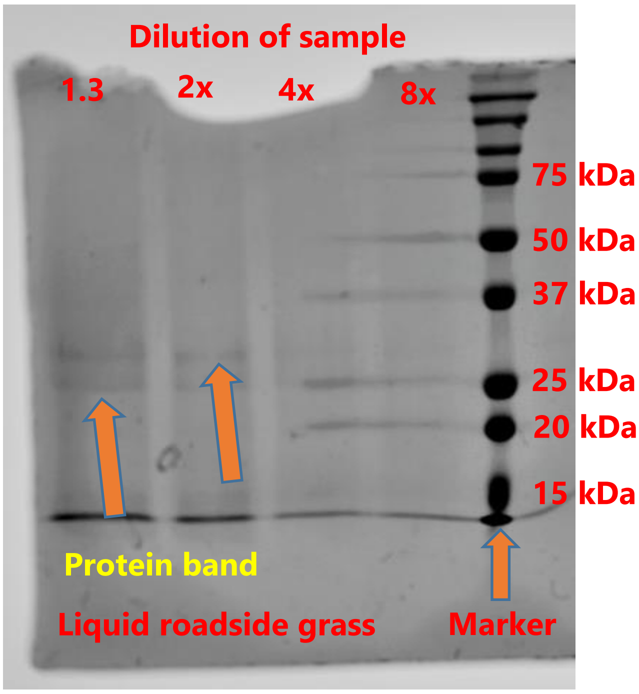

Figure 2: results of SDS page gel for liquid from roadside grass from session 2 at different dilutions (1.3x; 2x; 4x; 8x).

Increasing the protein concentration

The protein concentration in the liquid from pressed roadside grass is very low. Therefore the possibilities to increase the protein concentrations in the liquid fraction were studied. Several techniques are available, such as evaporation, precipitation using (with HCl,

[5] Ma, Z., Cooper, C., Kim, H. J., & Janick-Buckner, D. (2009). A study of rubisco through western blotting and tissue printing techniques. CBE—Life Sciences Education, 8(2), 140-146.

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ammonium sulphate or organic solvents), membrane ultrafiltration, heat coagulation and ultracentrifugation.

Concentration using evaporation was tested. Within 60 minutes, as increase of the protein concentration with a factor 1.6 to 4.1 was achieved depending on the liquid used. Further research is needed to find out what is the maximum concentration that can be reached and if this concentration is suitable for further application.

Amino acids

With LC-MS, the amino acid composition of the liquids was measured. Not all amino acids could be measured. 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 4 shows the results.

Table 4: 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. 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[6]. 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

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

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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.

As described above, LFG has a low pH, while *A. platensis* usually requires a slightly alkaline pH, indicating that a pH adjustment might be necessary. Indeed, cells were only able to grow once the pH of the LFG was adjusted with the addition of $NaHCO_3$ to the medium, which also serves as a carbon source and helps increase the biomass productivity. After pH adjustment with 16.8 g/L of $NaHCO_3$, microalgal cells grew in concentrations of LFG varying from 5 to 20% (v/v). 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 3). 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.

Therefore, we recommend the use of 15% (v/v) of LFG or a minimum total nitrogen concentration of 180 mg/L for guaranteeing the production of 2.25 ± 0.5 g/L of healthy cells with adequate composition.

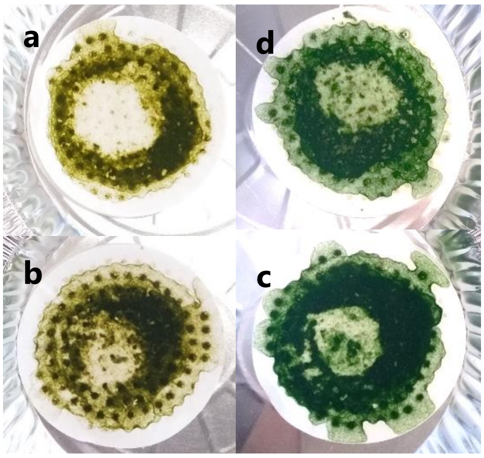

Figure 3: *Cells harvested after 7 days of growth in a) 5% LFG, b) 10% LFG, c) 15% LFG, and d) 20% LFG, all supplemented with* $NaHCO_3$

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4. The liquid fraction as part of insect feed

The FAO estimates that food production needs to increase by 70% by 2050 to feed the world's growing population, with a big focus on higher quality protein diets in developing parts of the world. This is putting pressure on the sustainability credentials of the future animal feed supply chain, given that up to 7% of all greenhouse gases can be attributed to the growing of crops for animal feed. The animal feed markets for aquaculture and poultry represent two of the most important growth sectors and are looking for sustainable and high quality feed alternatives for e.g. soy. Simultaneously, 1/3 of all food/feed produced globally is wasted. This is forcing a fundamental rethink concerning closing cycles. Insects are an opportunity as some of these can cheaply and efficiently transform organic waste into complex proteins and fats in their bodies.

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

For mealworms, the influence of the liquid fraction of grass was limited and the higher N-FCR indicated that the proteins present in the liquid fraction could not be used as effectively by the larvae, compared to the proteins from the classic dry feed.

For Black soldier fly, the grass juice was tested as water alternative and mixed with dry feedstocks of varying nutritional values (spelt husks as a low nutritious feedstock, wheat bran as an intermediate feedstock and chicken feed as a nutrient-rich feedstock). Significant differences were observed for average larval weight and dry yield between different diets. Feed conversion and nitrogen uptake improved with the addition of grass juice. However, the more nutritious the dry feed, the less pronounced the difference was. For nutritious feedstocks such as chicken feed, grass juice was not significantly beneficial compared to water, but, more important, no adverse effects could be observed despite the fact that 10 % less dry feedstock was used in the diet.

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5. Production of energy from the liquid fraction

The interest in using the liquid fraction of grass for energy production lies in easier storage of the liquid fraction, compared to full clippings. One of the actual worldwide challenges is energy production. By producing green energies and searching for alternatives for fossil-based resources, the consortium aims to tackle this challenge.

In the present study, the potential of the liquid fraction was assessed by BMP batch tests for i) fresh material, ii) stored liquid fraction at fridge temperature for 2 months and iii) stored liquid fraction at room temperature for 2 months. Figure 4 summarizes the results obtained for the biogas potential of the different treatments. C:N ratios for the fresh unfiltered liquid fraction varied from 18 to 26. The biogas potential was the highest for the fresh liquid fraction, and decreased with increasing storage temperature. Storage for one (T1) or two months (T2) had no negative or positive impact on the biogas potential. Based on these results, we recommend to use the fresh liquid material, as the energy production is higher and no storage is needed. In case storage is necessary, the liquid fraction should ideally be kept at lower temperatures.

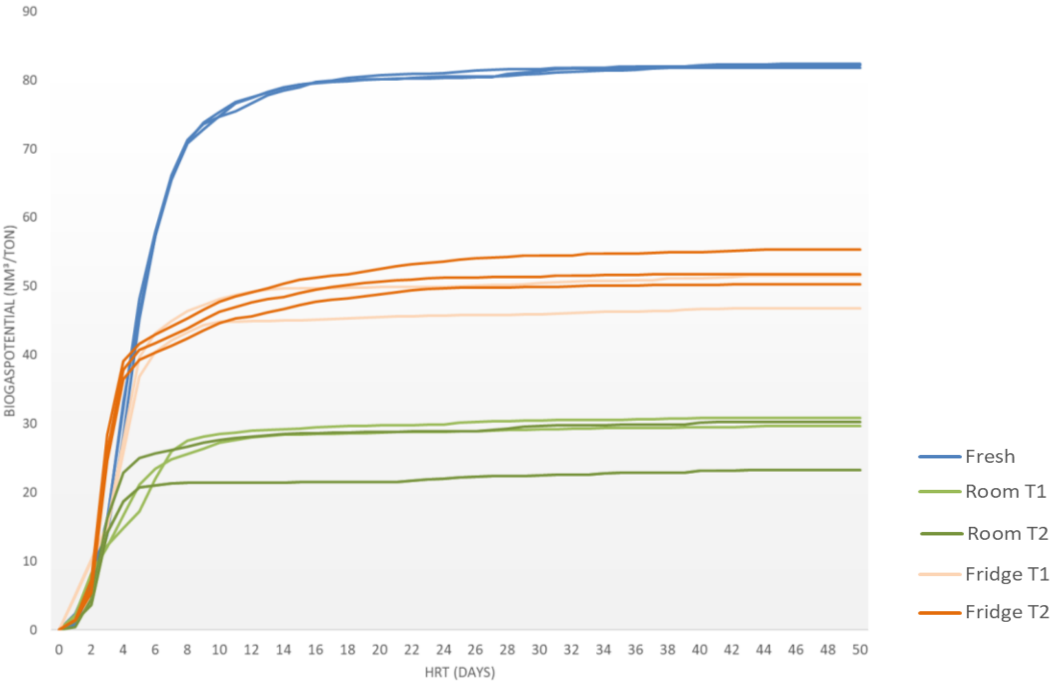

Figure 4: *Evolution of the biogas production [Nm^3/ton] at different hydraulic retention times [days] for different treatments*

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Conclusions and future perspectives

The liquid fraction obtained after pressing roadside grass clippings is a nutrient-rich stream that could be valorised to increase the economically attractiveness of the grass value-chain. In the Grassification project, several valorisation routes were tested, including fertilizer production, anaerobic digestion, and growth of protein-rich microorganisms.

The liquid fraction had a negative impact on tomato growth and was not found suitable to be used as a fertilizer without a previous treatment. Future experiments will focus on understanding the cause of this negative result and will also investigate the possibility of using the liquid fraction as a natural herbicide.

It was possible to produce biogas through the anaerobic digestion of the liquid from pressed grass. However, the amount of biogas produced per ton of liquid was rather low, as this is a diluted stream. Nevertheless, the C:N ratio of the liquid indicates a high quality of this stream as a substrate for biogas production, indicating that it could be co-digested with dry substrates for improving the anaerobic digestion process. This needs to be further investigated.

The direct recovery of protein from the liquid fraction is technologically feasible; however, it might incur in too high processing costs due to the high water content of this stream and it may not result in a representative protein production for entering the market. Nevertheless, the production of protein-rich insects and microalgae yielded more interesting results. Black soldier flies benefitted from the addition of the liquid fraction of grass to less nutritious dry feeds and it was possible to cultivate microalgae in the liquid fraction after pH adjustment without the addition of mineral nutrients. Further analysis should be done to assess the economic feasibility of these value-chains.

One important aspect that needs to be taken into consideration when developing value-chains from the liquid fraction from pressed grass is the seasonal availability of this stream. Grass clippings from roadside are generated only from June to October and this study indicated that the liquid fraction should be used immediately, as storing it resulted in compositional changes. Freezing might be an option, but would incur in a high energy expenditure that may not be economically feasible. Further investigation should be conducted to assess if ensiling of the grass clippings prior to pressing would be a technologically feasible alternative for supplying liquid fraction throughout the year.

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