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Using microalgae in the circular economy to valorise anaerobic digestate:
challenges and opportunities
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31 Abstract:

32 Managing organic waste streams is a major challenge for the agricultural industry. Anaerobic 33 digestion (AD) of organic wastes is a preferred option in the waste management hierarchy, as this process can generate renewable energy, reduce emissions from waste storage, 34 35 and produce fertiliser material. However, Nitrate Vulnerable Zone legislation and seasonal restrictions can limit the use of digestate on agricultural land. In this paper we demonstrate 36 the potential of cultivating microalgae on digestate as a feedstock, either directly after 37 38 dilution, or indirectly from effluent remaining after biofertiliser extraction. Resultant microalgal biomass can then be used to produce livestock feed, biofuel or for higher value 39 bio-products. The approach could mitigate for possible regional excesses, and substitute 40 41 conventional high-impact products with bio-resources, enhancing sustainability 42 within a circular economy. Recycling nutrients from digestate with algal technology is at an early stage. We present and discuss challenges and opportunities associated with developing 43 44 this new technology.

46 Keywords:

47 Anaerobic digestion, algae, nutrient recycling, livestock feed, circular economy

48

49 **1.0 Introduction:**

50 Current agricultural approaches to organic waste management can result in large losses of 51 nutrients, particularly nitrogen (N) and phosphorus (P), to the atmosphere and local aquatic 52 ecosystems (Carpenter et al. 1998; Smith et al. 2001a & 2001b; Misselbrook et al. 2010), affecting water and air quality (Withers & Lord, 2002; Erisman et al. 2008). Current 53 54 agricultural activities also result in the emission of greenhouse gases (GHGs), both directly as a result of organic waste management approaches (Chadwick et al. 2011), and indirectly as a 55 consequence of land use change, driven by changing patterns in animal product consumption 56 57 (Tilman & Clark, 2014).

58 By 2050, consumption rates of meat and livestock products are predicted to double (Steinfeld 59 et al. 2007). The global increase in demand for meat products will result in a rise in demand for protein for animal feed, particularly soya, which is likely to drive land-use change in the 60 form of deforestation (Gasparri et al. 2013). This activity is a major contributor to global 61 62 anthropogenic GHG emissions, and has been estimated to account for ~20% of global CO₂ emissions (Van der Werf et al. 2009). European dependence on the import of protein for 63 64 animal feed also has implications for food security, due to large potential for future supply chain volatility (de Visser et al. 2014). Increased global demand and competition, coupled 65 with reductions in supply as a consequence of climate change, are likely to drive price 66 increases and reduce availability (Osborne et al. 2013). 67

68 Reducing GHG emissions from agriculture is an essential component in the UKs national strategy for CO₂ equivalent emission reduction, necessary in order to meet the obligations of 69 70 the Paris climate agreement (Wollenberg et al. 2016). Managing GHG emissions from 71 manure can be achieved through improved infrastructure, such as covered slurry lagoons, or 72 with technology such as anaerobic digesters. These harvest the produced methane in a controlled environment for the purposes of energy production (Hopkins & Del Prado, 2007). 73 74 Due to the financial opportunities offered by energy production, food and farm waste is 75 increasingly being converted to biomethane via anaerobic digestion (AD). Recognised for its 76 potential pollution abatement qualities, the AD process also yields a typically nutrient rich 77 digestate. Digestates, when applied onto agricultural land, can provide benefits such as waste stabilisation and reduction in GHG emissions, odour reduction and the provision of low 78 79 carbon nutrients and biostimulants that support crop growth (e.g. Möller & Müller, 2012; Walsh et al. 2012; WRAP, 2012; Scaglia et al. 2017; Sigurnjak et al. 2017). Digestates can be 80 81 rich in a number of macro nutrients (e.g. N, P, K, S, Mg, Ca, Fe, and Na) and may contain a 82 number of trace elements (e.g. Co, Fe, Se, Mo and Ni) either as a result of the original 83 feedstock used (Marcato et al. 2008), or due to supplementation as part of a trace element 84 addition for improved digester performance (Williams et al. 2013). Digestate can be 85 separated into solid and liquid fractions. Liquid digestate typically has a high nutrient status, 86 intermediate in strength between livestock manures and inorganic fertiliser (Nkoa, 2014). 87 Digestate contains significantly more available N than cattle slurry (80 - 90% of N in whole)or liquor digestate – AHDB, 2017). Whilst the compound form of N in digestate is more 88 89 readily available for uptake by plants, environmental losses can occur after land application, 90 posing particular risks in regions where N is in excess.

91 Under the EC Nitrates Directive (91/676/EEC) and Nitrate Vulnerable Zone (NVZ)

92 legislation, the amount of N that can be returned to land is restricted. Phosphate land

93 overloads are now also significant in numerous European regions and land usage restrictions 94 are being implemented (Sigurnjak et al. 2017). Regional and seasonal restrictions on the use 95 of digestates, either due to crop non-growth periods or limitations on nutrient loadings to 96 agricultural land in particular for N and P, the resulting long periods of storage required and 97 the restricted local farm land availability, are becoming significant barriers to AD 98 deployment and for digestate use (Passanha et al. 2013). In order to support a continual 99 growth in AD technology deployment and mitigate for overloads of nutrients potentially 100 causing a negative environmental impact, new markets and novel uses for digestates are 101 required.

102 Alternative uses for digestates have started to be investigated and results seem promising in particular within biorefining platforms, such as enhancing ethanol production by using 103 104 digestate effluents instead of freshwater and nutrients (Gao and Li, 2011); enhancing polyhydroxyalkanoate production by using digestate as fermentation nutrient media 105 106 (Passanha et al. 2013), and for increasing the yields of carboxylic acids from acid phase 107 anaerobic fermentations when thermally treated and filtered digestate was used as bacterial 108 stimulant (Kumi et al. 2016). Another option to valorise digestate is to establish a microalgae biorefining platform and further mitigate environmental impacts in terms of avoiding excess 109 110 nutrient loads discharged onto environmental receptors and at the same time drive a low 111 carbon protein production industry.

Microalgae are increasingly being researched and used globally to remediate nutrients in organic waste, and as a source of biomass, products and energy (Sivakumar et al. 2012; Abinandan et al. 2015). Microalgae need a source of nutrients to grow and can therefore be used to recycle nutrients in digestate (Wang et al. 2010; Uggetti et al. 2014). The resultant microalgae crops, which are high in protein, can be used as a feed source for livestock or aquaculture industries (Becker, 2007; Yaakob et al. 2014). This system presents an

opportunity to establish a circular economy solution for organic waste streams, which would limit the impact of agriculture and organic waste management on the environment, by reducing nutrient pollution, GHG emissions, and the requirement for land use change to enable animal feed production, and increase the potential for food security in the European Union and beyond.

123 **1.1 Background**

124 The potential of using algae to remediate waste, including nutrients, metal, carbon dioxide and organic pollutants, has been recognised over many decades. Pioneering work in the 125 1950s by William Oswald established the potential of microalgae in domestic sewage 126 treatment and, in particular, that consortia rather than unicellular culture were the most 127 128 effective (Oswald et al. 1953). The drive for secure energy in the US led to the National 129 Renewable Energy Laboratory's Biofuel Program and the Aquatic Species Program (Sheeman et al. 1998). This program undertook screening of microalgae for lipids and 130 131 cultivation, which established the foundation for further studies. This coupled with a renewed drive for renewable energy production in the early 2000s, culminated in a series of Roadmaps 132 (Fishman et al. 2010; Parker and Schlarb-Ridley, 2013; Barry et al. 2016). More recently, 133 134 improved 'omics techniques and better understanding on algal genomes has re-invigorated algal biotechnology research. In addition, there is now an increasing recognition that we need 135 to reduce and recycle waste and reduce consumption of finite resources including nutrients 136 working towards a circular economy approach. The ability to cultivate algal biomass from 137 waste nutrients, of which digestate is an excellent source, and then use biomass either whole 138 or fractionated as a commodity is an attractive proposition. Algae are a rich source of protein 139 140 and lipids and many other useful compounds with bioactive properties. In addition to the food, feed and fuel industries, algal bioactives have proven application in the pharmaceutical 141

and cosmetics industry (Singh et al. 2017). Algae, particularly cyanobacteria, can also be
applied as a soil treatment and a slow release fertiliser (Sharma et al. 2012).

144 It has been suggested at a global level that the contribution of microalgae protein to human 145 nutrition is limited due to the small scale of production. Within the EU, factors including current legislation, unfavourable climatic conditions for growth, and insufficient consumer 146 147 demand, are the cause of this adverse effect on production (Vigani et al. 2015). Nevertheless, the growing need for a stable and reliable domestic supply of protein for animal feed from 148 149 within the EU (de Visser et al. 2014) makes this a key area for research. In addition, the 150 production of microalgae has the potential to generate essential nutritional compounds, such 151 as omega-3, where the current source of supply (fish-oils) is becoming increasingly costly and rare (Vigani et al. 2015). This may have significant implications for human nutrition 152 153 globally. Thus, the global market application for microalgae products is increasing. The EU has the potential to become a market leader in the next decade due to its dominant position in 154 155 the global agri-food markets.

156

157 2.0 Challenges

158 2.1 AD Technology Infrastructure and Digestate Separation

AD technology infrastructure differs depending on the plant design, which is influenced by feedstock characteristics, their processing and temporary storage of feedstocks, types of digesters, the level of processing and use of the biogas and also according to the level of processing and storage of digestates. Figure 1a shows a schematic of typical AD technology infrastructure.

164 Detailed schematics of a variety of AD plants have been previously presented in Monson et 165 al. (2007). Digestates can be utilised without any further processing directly after digestion,

166 or they can go through a number of separation and processing techniques. Whilst the majority 167 of digestate from digesters is currently applied to land as whole digestate, some digestates are 168 separated into the solid or fibre fraction and the liquor fraction. In the case of crop-based 169 digestates including animal slurries, separation is used to ensure that the liquor fraction can 170 be applied to land using precision equipment (digestate shallow injection) without blockages. 171 Separation or 'dewatering' is the preliminary step in a host of digestate enhancement 172 techniques, which include ammonia stripping, micro, ultra, nanofiltration and reverse osmosis. Dewatering tends to represent a substantial investment with potentially high 173 174 operational costs, but can dramatically reduce transport costs if a chosen outlet can be found 175 for the liquor fraction. Dewatering can be achieved by the use of centrifuges and belt filter presses. The efficiency of dewatering depends upon the nature of the digestate and the 176 177 characteristics of residual particles digestates' chemical and microbial matrices following the 178 AD process. For example, the presence of polysaccharides or cellular intracellular water typically provides difficulties in dewatering and coagulant/flocculants are used to support the 179 180 task (e.g. Oliveira et al. 2016). The ability to sterilise digestates, recover, separate and 181 concentrate various nutrients residual in digestate utilising membrane systems for further 182 utilisation is receiving considerable attention. Recent developments in membrane separation technologies have made it possible to separate and recover products from digestates, with 183 184 these technologies being more cost efficient (Fuchs and Drosg, 2010).

185 **2.2 Challenges of applying anaerobic digestate as a feedstock**

Digestates are typically rich in two essential nutrients, N (primarily NH₃) and P (primarily
PO₄), which are essential for the growth of photosynthetic organisms such as microalgae.
However, digestate may also contain other potentially toxic elements (PTEs) or compounds
such as lead (Pb), zinc (Zn) and copper (Cu) (Coelho et al. 2018). Essential nutrients and PTE

190 concentrations present in the digestate vary depending on feedstock composition in AD191 plants.

192 Metals and phosphates bind strongly to solids during the digestion process, but this will be 193 affected by digestate sludge pH, as solubilisation will happen at low pH statuses. Thus, 194 acidifying the digestate sludge can release metals and P into a soluble form. Microfiltration 195 coupled with acidification can then be applied to remove metals and produce a material of 196 different N:P compositions (from 34 to 8), by varying the P component (Gerardo et al. 2013). 197 In order to optimise the digestate and prepare the medium that will be used during the 198 microalgae biomass production process, a suitable system must be established (Figure 1b). Here, the flow of the digestate is presented in two main parts: upstream and downstream. 199 200 During the upstream process, the digested liquor (digestate) is collected from the main 201 digester and put in the settling tank. This is necessary because digestates collected from AD plants have typically mesophilic temperatures ranging from 27 to 42°C, and pH mainly in the 202 203 alkaline region (typically between 7.4 - 8.2) (Coelho et al. 2018). Both these abiotic 204 parameters are above the optimal values for the common microalgal strains such as Chlorella or Scenedesmus (e.g. 25°C and neutral pH). 205

After a Hydraulic Retention Time (HRT) of >8 hours in a settling tank to allow solid matter precipitation, the upper layer of the digestate from the tank should be passed through microfiltration (0.2 μ m) in order to retain the remaining solids in the digestate. Membrane technology (micro/ultrafiltration) is a well known technology that recently has been applied to the upstream and downstream process in microalgae production (Gerardo et al. 2014; Mayhead et al. 2018).

It is highly advisable to use the same technology to perform the digestate pre-treatment
during the upstream process. Using this technology will allow mechanical sterilisation of the

214 digestate, avoiding the inclusion in the microalgae culture of the main pathogens present in digestates, such as Eschericia coli (0.5 -2.0 µm) and Salmonella spp. (2.0-5.0 µm). Also, 215 216 using micro/ultrafiltration (filtration with a low molecular weight cut off) can help to adjust 217 N:P ratio of the digestate to an optimum level, as suggested above. This will be different for 218 each strain of microalgae, but a ratio of 7:1 for N:P has been suggested as suitable for 219 balanced nutrients in algae (Fenton & O'hUallachain, 2012). Managing the digestate to 220 achieve an optimum ratio for N:P is vital for a successful microalgae culture. This is necessary because high ammonia concentrations (> 2.3μ M) can inhibit microalgae growth 221 222 (Cho et al. 2013). Furthermore, the presence of solid matter will have a direct impact on 223 microalgae growth, by reducing the potential for light availability, resulting in a lower growth rate (Mayhead et al. 2018). Further research is necessary in order to improve the potential of 224 225 ultra/diafiltration technology for the removal of PTEs that potentially can inhibit microalgae growth. Special attention should be paid to Cu, since it is one of the most toxic elements for 226 photosynthetic organisms. 227

228 **2.3 Algal species selection**

Amongst the many thousands of microalgal species present in nature, there are only a few 229 230 commonly occurring species currently studied and known to be robust survivors in wastewater or in digestate. These include species belonging to the genera Chlorella, 231 232 Scenedesmus, and Desmodesmus, with key species being Chlorella vulgaris and Scenedesmus obliquus. Algal consortia and algal-bacteria consortia are more suitable for large-scale 233 cultivation on wastewater than unicellular culture, by acting symbiotically, especially in 234 235 terms of preventing contamination and enabling long-term cultivation (González-Fernández, 2011; Medina and Neis, 2007; Gonçalves et al. 2017). In this symbiosis, the O₂ released by 236 algal photosynthesis is utilized by aerobic-heterotrophic bacteria to mineralize organic 237 compounds, and bacterial respiration provides CO_2 as a carbon (C) source to the algae. 238

239 Uptake of nutrients from digestate has been shown to be more efficient in mixed algal and bacterial consortia systems than for unicellular systems (Kerckhof et al. 2014; Mahapatra et 240 241 al. 2014; Lahel et al. 2016; Vulsteke et al. 2017). In mixed algal-bacterial consortia systems, 242 growth increases the pH and allows precipitation of phosphorus, promoting the remediation process (Kang et al. 2018). Furthermore, cultures cultivated under mixotrophic conditions, 243 have been shown to have higher growth rates compared to when cultivated under 244 245 heterotrophic or autotrophic conditions (Lalucat et al. 1984). 246 There are a number of challenges in large-scale cultivation of algae on digestate. A key challenge in mixed consortia and mixotrophic systems, especially where there is a source of 247 248 dissolved C present (e.g. glycerol or organic acids), is to ensure that bacteria do not dominate the consortia system causing the algal cells to crash. Another challenge in large-scale algal 249 250 cultivation on digestate is the dynamic nature of the algal-bacterial consortia. Successful large-scale cultivation of algae particularly on wastewater and digestate requires close 251 monitoring and regulation of biotic and abiotic conditions (Van Den Hende et al. 2014; 252 253 Silkina et al. 2017). The ability to maintain a functional and reproducible stock culture of a

255 (Silkina et al. 2017).

254

256 **2.4 Optimising digestate feedstock for algal growth**

To understand the influence of digestate on algal metabolic processes, flux balance analysis
(FBA) (Orth, 2010) was used to model growth potential in *C. vulgaris*, *i*CZ843 (a standard
model organism – Zuñiga et al. 2016), using different dilutions of swine with crop fed
digestate (Figure 2a), with a key focus on docosahexaenoic acid (DHA) production (Figure
2b). Robustness analyses were then performed to identify optimal conditions for growth and
DHA production. The model was first validated with experimentally measured growth rates

mixed algal consortia is beneficial and has been demonstrated through cryopreservation

(Table 1). All simulations were conducted using the COBRApy toolbox using Python and
Gurobi solver, version 7.5.2 (Ebrahim, A., 2013).

The constituents of swine and arable crop digestate streams at various dilutions have been measured elsewhere (ammonia and acetic acid - Zulini et al. 2016; phosphate, nitrate, magnesium, and iron - Levine et al. 2010). These values were used to model microalgae growth rates under mixotrophic, phototrophic and heterotrophic growth conditions for different dilution factors (Figure 2a). As per Orth (2010), growth rate is expressed as hr⁻¹ and metabolite fluxes, such as that of DHA, is expressed as mmol per gram of dry weight growth (mmol gDW⁻¹ hr⁻¹).

272 Thirty-fold dilutions of digestate resulted in the highest rate of predicted growth for each

273 growth regime (Figure 2a), which is in agreement with the results presented by Zuliani et al.

274 (2016). The highest growth rate was observed with a 30-fold dilution with heterotrophic

275 metabolism (0.111 hr⁻¹) followed by mixotrophic growth and phototrophic growth (both

predictions were 0.042 hr^{-1}). This trend was consistent across all dilutions bar the 200-fold

277 digestate dilution, where the mixotrophic regime yielded the highest growth rate.

Heterotrophic growth of microalgae to produce biotechnologically important metabolites is
cheaper and simpler than mixotrophic growth (Perez-Garcia, et al. 2011). The capacity of
potential production of DHA was therefore explored for each growth regime and dilution
using Flux Variability Analysis (FVA).

As seen in Figure 2b, *i*CZ843 predicted that heterotrophic growth on digestate diluted 30 times would result in optimal production of DHA (1.49 x 10⁻⁴ mmol gDW⁻¹ hr⁻¹). At each dilution factor tested, heterotrophic metabolism resulted in more DHA production than mixotrophic and phototrophic growth. At a 200-fold dilution, *C. vulgaris* cells grown

286 mixotrophically are predicted to be completely incapable of synthesising DHA. Thus, these

simulations suggest that optimal production of DHA can be obtained from heterotrophicgrowth on digestate diluted 30 times.

289 Biomass and DHA production were predicted with the model (Figure 2a & b), and used to 290 investigate which nutrients limit or increase biomass. Robustness analyses were also conducted for acetate, NH₄ and NO₃. For NH₄ uptake, an optimal growth rate of 0.103 hr⁻¹ 291 was achieved with uptake of 2 mmol gDW⁻¹ hr⁻¹, after this, biomass decreased. For NO₃, a 292 detrimental effect on biomass was observed with increasing uptake, suggesting NH₄ alone 293 294 can provide almost all of the N requirements to sustain a heterotrophic algal cell grown on digestate diluted 30-fold (original growth rate of heterotrophic grown cell on 30-fold diluted 295 digestate sample was predicted to be 0.111 hr⁻¹). 296

Since heterotrophically grown cells rely on an inorganic C source to grow, a robustness analysis was performed to investigate how acetate uptake affects growth rate. Increasing acetate uptake resulted in greater heterotrophic growth rates, even beyond the predicted flux presented in Figure 2a (0.111 hr⁻¹), to a high of 0.837 hr⁻¹. This result indicates the optimal acetate uptake rate is 35 mmol gDW⁻¹ hr⁻¹, which corresponds with an 8-fold increase in algal biomass. After this point, any increase in acetate has an adverse effect on cell biomass.

Digestate diluted 30 times contains 3.33 mg L⁻¹ of acetate. The analysis conducted suggests 303 the acetate concentration of digestate can be increased by a factor of 10 when acid anaerobic 304 305 fermentations are targeted, with other conditions remaining the same for optimised cell growth. The ratio of C:N is accepted to be a key factor governing plant and microalgae 306 growth (Commichau et al. 2006; Zheng, 2009; Fait et al. 2018). This was also explored 307 308 further in the analysis. The reduction in the growth rate that was observed when NH₄ uptake exceeds 2 mmol gDW⁻¹ hr⁻¹ can be explained by the impact of C limitation. In the same 309 310 respect, the reduction in growth rate observed when acetate uptake was greater than 35 mmol

gDW⁻¹ hr⁻¹, was explained by N limitation. To test this hypothesis, a robustness analysis was 311 312 performed to predict the biomass of heterotrophic cells grown in conditions of 30-fold digestate dilution, with acetate constrained to an optimal uptake of 35 mmol gDW⁻¹ hr⁻¹, as 313 314 determined by the above analysis. 315 The optimised heterotrophic growth rate was revealed to be a function of acetate and NH₄ uptake. Optimal uptake bounds of NH₄ are determined at 15 mmol gDW⁻¹ hr⁻¹ and any excess 316 beyond this inhibits cell growth, confirming the need to dilute digestate. Furthermore, at an 317 uptake rate of 35 and 15 mmol gDW⁻¹ hr⁻¹ for acetate and NH₄ respectively, algal cells were 318 shown to more than double their production of DHA from 0.149 x10⁻³ gDW⁻¹ hr⁻¹ to 1.106 319 $x10^{-3}$ gDW⁻¹ hr⁻¹. To achieve this optimised production of DHA, using a metabolic 320 reconstruction of C. vulgaris, model predictions suggest digestate diluted 30 times should be 321 supplemented with acetate to a final concentration of 35 g L⁻¹ and NH₄ should be reduced to 322 15 g L⁻¹. All other nutrients can be kept at 30 fold dilutions. 323

324 2.5 Implementation

Commercial scale algae cultivation is currently a relatively immature sector and the techno-325 326 economic challenges of integrating this process with AD have to be addressed. However, in order to catalyse wider adoption of these systems we also need a better understanding of the 327 328 scope and scale of potential market opportunities from a bioremediation perspective as well 329 as from the perspective of high value products. This requires a foundation of knowledge and data/information from across the whole value chain, which can be translated and transferred 330 to stakeholders (particularly project developers and investors). This information may be 331 332 complex technical, economic and regulatory information or tacit knowledge (experience and 'know how' of expert and non-expert stakeholders). Current research around implementation 333 of Algal-AD systems is delivered by multi-disciplinary teams working transnationally with a 334

wide range of stakeholder groups. In order to provide coherent and consistent support to
stakeholders the data and information generated through research needs to be synergised and
harmonised.

Standard methodologies from knowledge based engineering can be utilised to collate and 338 integrate data and information from a wide range of sources and translate and represent it via 339 340 user friendly online decision support tools. These tools can then be used to explore aspects 341 such as technical feasibility, economic viability, and environmental sustainability. Traditionally, knowledge based engineering has been applied to mature sectors such as 342 343 aerospace and automotive where data and information is explicit and can be stored easily as 344 facts and rules, however, research across the biobased industries is still evolving and this can make knowledge capture, integration and representation far more challenging. Translating 345 346 tacit knowledge into machine-readable data enables greater accessibility, consistency and less error (Farazi et al. 2018). This can enable project developers to reduce the risk of a project 347 earlier in the project life cycle. For example, one of the challenges of implementing AD 348 projects is the security and consistency of biomass supply. Tools have been developed which 349 350 integrate geographical data (identifying the location of bioresources) and local infrastructure 351 (roads, rail etc.) with supplier information relating to availability of supply and biomass 352 characteristics. This enables project developers to undertake a bioresource assessment prior to 353 project implementation. This technique can also be used to identify current land use (e.g. 354 agricultural), existing facilities (e.g. AD plants) as well as protected areas such as Nitrate Vulnerable Zones (NVZs). 355

These map based applications represent complex data in a more accessible way. They enable stakeholders to evaluate potential opportunities and connect with other stakeholders thereby improving supply chain integration.

Tools have also been developed that enable end users to understand process performance for a given technology and explore multiple valorisation pathways according to their specific resources or requirements. This would have traditionally required consultation with various experts; however, by capturing this knowledge within an online tool, users can conduct preliminary feasibility assessments. For example, growth modelling tools can be used to explore the potential of a given technology based on design or on process inputs (e.g. light, nutrients, water, etc.).

The methodologies for developing these tools are continually being developed. Working closely with stakeholders (across the value chain and also data providers) enables knowledge engineers to understand requirements and optimise the tools' design and functionality. The architecture of these tools is modular and therefore flexible and adaptable. This means they can be expanded and updated as further data is generated over time.

371

372 **3.0 Opportunities**

373 **3.1 Commercial Applications**

The production of microalgae has been demonstrated for numerous applications, including the production of cosmetics (Spolaore et al. 2006), biofuels (Suganya et al. 2016), human or animal feed (Becker, 2007), or as a soil treatment and slow release fertiliser (Mulbury et al. 2004). Of key interest here is the potential for this material to provide a solution to the burgeoning problem of protein production for livestock feed (de Visser et al. 2014).

379 Protein and lipid substitutes for the animal feed sector represent the most obvious use of the

380 cultivated biomass, either used as a whole biomass or fractionated into bulk constituents.

381 Further refinement of the biomass to produce higher value products including pigments,

niche fatty acids and peptides present a more convincing economic LCA. A key challenge

here is the regulatory and legislative requirement associated with the use of algae in feed and food and with the use of a waste to produce feed. Currently only a handful of species are generally recognised as safe (GRAS). Although the commercial scale algal industry has been active for several decades, there are still only a handful of species cultivated on a large scale and for only a small range of products. Wider acceptance of algae across more species, and for a wider range of products, requires a shift in legislation and regulation on the use of these valuable organisms.

390 **3.2 Microalgae for animal or aquaculture feed**

391 Cultivated microalgae play an important role in the early rearing of farmed marine shellfish and finfish. In intensive hatcheries, individual strains of microalgae are cultivated in separate 392 393 reactors and administered regularly to the farmed species. Algae biomass is also incorporated 394 in formulated animal feeds, both for aquaculture species and terrestrial livestock. To date, feed formulators have mainly focused on algae as a supplement to provide specific functional 395 396 benefits rather than gross nutrients such as protein. Algae have been credited with improving the immune system (Turner et al. 2002), lipid metabolism (Nakagawa, 1997), improved gut 397 function (Michiels et al. 2011) and stress resistance (Nath et al. 2012; Sheikhzadeh et al. 398 399 2012), as well as providing an organic source of carotenoids (Gouveia et al. 2002; Choubert and Heinrich 1993). The reason only a few studies evaluate algae as a major feed ingredient 400 401 for farmed animals is typically due to the large amounts of biomass needed.

402 Nevertheless, the demand for meat and fish is rising worldwide and so is the need for animal 403 feeds and ingredients. Historically, aquaculture has depended heavily on fishmeal, and fish 404 oil as the main source of protein and lipids, but these sources are finite. Consequently, there 405 is a growing interest in partial or complete replacement of fishmeal by alternative protein 406 sources of either animal or plant origin. The main challenge in reducing fishmeal use is to

find alternatives that maintain acceptable growth rates, and support animal health and quality
of the final product. Furthermore, alternative feed sources must have nutritional
characteristics such as a medium to high protein level, a balanced amino acid profile, high
digestibility, palatability as well as low levels of antinutritional factors.

Several suitable protein substitutes are commercially available such as soybean meal, pea 411 412 seed meal, corn gluten, poultry by-product meal (Table 2). However, none of them contains the long chain polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic 413 414 acid (DHA). Without DHA and EPA in the aquafeed, the end product would also lack these long chain omega 3 fatty acids, which are an important nutritional element of fish and 415 416 seafood for humans. Freshwater algae such as Chlorella and Spirulina lack DHA and EPA but may still have good potential as protein sources (Table 2), whereas marine microalgae 417 418 such as Nannochloropsis, Tetraselmis, Pavlova or the heterotroph Schitzochytrium are the fundamental sources of EPA and DHA. As fish oil supply is limited, marine lipid rich algal 419 420 biomass is being considered as an alternative ingredient especially in aqua feeds. In order to evaluate the suitability of a novel feed ingredient, determination of the 421 digestibility is crucial in order to assess the overall nutritional value. In a digestibility trial 422 423 using mink (Mustela vison), reported by Skrede et al. (2011), three algal species Nannochloropsis oceanica, Phaeodactylum tricornutum and Isochrysis galbana were 424 425 included at graded levels up to 24% (dry weight) in the feed. The protein digestibility determined for N. oceanica, P. tricornutum and I. galbana were found to be 35.5%, 79.9% 426 and 18.8%, respectively, which is rather low. The authors hypothesized that the cell wall of 427 the diatom *P. tricornutum* may be have been more easily broken down by digestive processes 428

than the others, thus resulting in higher digestibility. Other authors have noted the negative

430 effects of a tough algal cell wall on digestibility. Jarynsk et al. (2007) tested the digestibility

431 of *Chlorella* biomass in rats using three treatments such as spray-dried, electroporated and

432 ultrasonicated. Ultrasonication was found to increase the protein digestibility of Chlorella from 53% (spray-dried) to 63%. In another study by Blake and Lupatsch (2012), using spray-433 dried and freeze-dried *Chlorella* in tilapia, the process of freeze drying improved protein 434 435 digestibility from 63% to 69%. Digestibility coefficient of solar dried Spirulina biomass has also been tested for Arctic char and Atlantic salmon at 30% dietary inclusion level (Burr et al. 436 2011). Protein digestibility ranged between 82% and 84.7% for the two fish species 437 438 respectively. These relatively high digestibility coefficients compare favourably with terrestrial plant ingredients, confirming the high potential of *Spirulina* as a protein source for 439 440 farmed fish.

441 Unlike terrestrial crops, marine algae can directly produce HUFA such as arachidonic acid

442 (AA, 20:4n-6) (Porphyridium), eicosapentaenoic acid (EPA, 20:5n-3) (Nannochloropsis,

Phaeodactylum, Nitzschia, Isochrysis, Diacronema) and docosahexaenoic acid (DHA, 22:6n3) (*Crypthecodinium, Schizochytrium*). Whilst most of these algae are not suitable for direct
human consumption, they might indirectly boost the nutritional value for humans if added to
animal feeds.

According to a recent study by Gbadamosi and Lupatsch (2018), Nannochloropsis added as 447 448 the sole protein and lipid source in the diet outperformed a soybean only based diet. In addition, feeding tilapia the EPA rich algae resulted in a considerable boost of the EPA levels 449 450 in the fish. The growth performance and feed conversion efficiency of European seabass (Dicentrarchus labrax) were also unaffected when fish were fed a mixture of Tisochrysis 451 lutea and Tetraselmis suecica freeze-dried biomass, which replaced 45% crude protein and 452 36% lipid in the diet. Moreover, including the dried microalgae in the diet resulted in a 453 higher nutritive value than that of a high-soybean meal control feed (Cardinaletti et al. 2018). 454

455 Several studies evaluated the DHA-rich algal meal derived from Schizochytrium, as a replacement for fish oil in Atlantic salmon. Salmon fed 11% algal biomass in their diet had 456 457 similar DHA levels in their filet compared to fish oil fed fish (Sprague et al. 2015). Including 458 5% of Schizochytrium in salmon feed can successfully replace fish oil as source of n-3 LC-PUFA without compromising fish growth rate, feed conversion efficiency and flesh quality 459 (Kousoulaki et al. 2016). The replacement of fish oil with a DHA-rich Schizochytrium also 460 461 significantly decreased both dietary and flesh fillet organic pollutants levels such as dioxin and PCBs compared to fish oil based treatments (Sprague et al. 2015). 462

In order for algal biomass to become a readily available ingredient, algae producers and feed 463 manufacturers will need to take into account the potentially large variations in approximate 464 composition (proteins, lipids, fatty acids, minerals, etc.) and digestibility encountered among 465 466 different algal strains and growing conditions. Effort is needed to ensure a more consistent composition of algal biomass, a consistent supply so that manufacturers can readily 467 incorporate this new feedstuff in formulated feeds. Possible means of increasing the 468 nutritional value of some algal species would be to break down the cell wall fragments by 469 470 mechanical treatment or even removal of most of the fibre, although such additional processing steps would add further to their cost. As several suitable protein sources are 471 472 available, marine algae would be most attractive as a source of long chain polyunsaturated 473 fatty acids such as EPA and DHA.

474 **3.3 Economic potential of nutrient recycling technologies**

The profitability of an AD plant of any size depends on a combination of the organic waste
disposal/utilisation cost, current local renewable energy incentives, and fossil fuel energy
prices. An AD plant running on selected farm wastes and sized to produce at least a 1MWe
costs in the region of £3.5M to construct. In the UK, a biomethane AD plant would also

typically include a 499 kWe Combined Heat and Power (CHP) plant, with the remaining
biogas, a little over 5000 m³ day⁻¹ or approximately 22.1 GWh year⁻¹, diverted to biomethane
upgrading.

The CHP plant would provide heat to the AD plant/algal production system, as well as electricity to carry out necessary biorefinery processes, such as those outlined in Figure 1c. A 484 499 kW_e CHP plant operating for 8100 hours year⁻¹ (92.5% load factor), at 40% electrical 485 efficiency and 56% thermal efficiency, could produce 4.04 GWh year⁻¹ of electricity and 5.7 486 GWh year⁻¹ of heat for on-site utilisation. Thus, the economics of the system can be improved 487 by maximising the on-site utilisation of CHP heat and electricity; this would also mitigate 488 some environmental burdens associated with algal production.

Biogas production and digestate nutrient levels vary considerably, depending upon the quality 489 490 and quantity of the feedstock input into the digester. Feedstocks and biogas production figures were derived from the BORRG AD Assessment Tool (ADAT, 2015) for a potential 1 491 MW_e equivalent digester configuration are shown in Table 3. These three agricultural 492 493 feedstocks are considered typical for the purpose of this study, due to wide availability. However, many AD suppliers prefer to limit the inclusion of poultry litter to less than 10% of 494 total feedstock, due to its propensity to produce ammonia within the process, which can 495 potentially inhibit biogas production. 496

497 The value of whole digestate is shown in Table 4. The value of ammonium N, P₂O₅ Triple

498 Super Phosphate (TSP) and Muriate of Potash have been derived from AHDB (2018),

499 respectively and converted to a value per kg. The two digestate values of ± 9.53 t⁻¹ and ± 5.52

 t^{-1} were derived from these specific AD feedstocks using the ADAT nutrient levels from

501 Table 3 above and standard 'agricultural AD' RB209 values (AHDB, 2017). The NNFCC

502 model (NNFCC, 2010) values digestate on the availability of the nutrients, using 70%, 60%

and 90% respectively for N, P and K availability. Valuing digestate based on this nutrient availability would reduce the value to ± 7.07 t⁻¹ using ADAT nutrient levels and ± 4.14 t⁻¹ using RB209 nutrient levels – these figures, however, are not comparable with fossil fuel fertilisers, which are valued on nutrient levels and not nutrient availability.

507 If the whole digestate is separated into a liquid and fibre fraction, the nutrient level and value 508 in each fraction will be dependent upon the type of separator (Lukehurst et al. 2010), and be 509 dictated by the requirements for the other biorefinery processes.

510 The use of digestate as a biofertiliser is often compared against the economic cost of applying 511 manufactured fertiliser. Table 4 demonstrates manufactured fertilisers are much more concentrated (34.5% ammonium N), compared with digestate (~0.3% - RB209) and other 512 513 organic fertilisers. Therefore, the cost of transportation of these materials to farm or field can 514 be high, offsetting the savings against manufactured fertilisers. Upstream processing of digestate utilised in algal technology, using membranes and de-nitrification technology, 515 516 separates both solid and liquid fractions, and further processing of the liquid removes N via volatilisation of gaseous ammonia. Capturing this ammonia as ammonium can allow it to be 517 reintroduced to the solid fraction sludge to produce a dewatered digestate. Increasing the 518 519 concentration of the digestate nutrient value increases the distance which digestate can be utilised as a biofertiliser, before the cost of fuel in transportation outweighs the cost of 520 521 manufactured fertiliser equivalents. For some digestates, the dewatering and modest removal of N also has the potential to create a favourable balance of NPK for crops such as grass 522 silage, by increasing the proportion of phosphate and potassium applied per unit of applied N. 523

524 **3.4 Environmental potential of nutrient recycling technologies**

525 The manure-to-digestate-to-microalgae-to-animal-feed value chain proposed in this paper
526 involves multiple diversions of waste streams and product substitutions compared with

business-as-usual (BAU). Assessing the net environmental outcomes, e.g. GHG emission
abatement, of such value chains requires a life cycle approach. Life cycle assessment (LCA)
is the evaluation of inputs, outputs and potential environmental impacts of systems, expressed
in relation to a unit of product or service ("functional unit") delivered by those systems
(Finkbeiner et al. 2006). The delivery of multiple products through a circular value chain
requires careful definition of goal, scope and system boundaries prior to any LCA study.
Full evaluation of the environmental effects of manure-to-animal feed value chains may

associated with product substitution. Alternatively, consequential LCA (Weidema, 2000;

require application of expanded system boundaries to account for environmental "credits"

536 Weidema and Schmidt, 2010) may be applied to account for significant indirect

consequences incurred in other systems as microalgae value chains develop. This approach
requires prospective evaluation of changes associated with the deployment of new microalgae
value chains, usually informed by economic models or trade data to predict indirect changes
in marginal production and consumption driven by market signals (Ekvall and Weidema,

541 2004). Consequential LCA is associated with higher levels of uncertainty compared with

standard "attributional" LCA (Zamagni et al. 2012), but can potentially highlight unintended

543 consequences associated with deployment of new innovations and management practises

545 system interactions within the market economy. In Figure 1c and the text below, an indicative 546 approach for evaluating the environmental balance of the digestate-micro-algae value chain is

(Weidema and Schmidt, 2010; Tonini et al. 2012; Styles et al. 2018) by capturing (some)

547 described.

544

534

548 The first stage in the digestate-to-microalgae value chain is the production of biogas and

549 digestate in an AD plant (Figure 1a). If the AD and microalgae production systems are part of

an integrated biorefinery, then the AD stage may be included in the LCA, accounting for,

551 *inter alia*, fossil energy replaced by biomethane (Budzianowski, 2016). If, however,

552 microalgae production is regarded as an add-on to an existing AD system, then evaluation of 553 the environmental consequences of microalgae production begins with an assessment of 554 conventional (pre-existing) management of the liquid digestate (LD) fraction after digestion 555 and separation (stage 2 in Figure 1c). Taking an expanded boundary approach, products and processes involved in this stage are considered to be avoided, leading to environmental 556 "credits". These credits may be substantial, given that LD storage and spreading can give rise 557 558 to large emissions of ammonia (NH₃), nitrous oxide (N₂O) and methane (CH₄) (Nicholson et al. 2013; Misselbrook et al. 2015; Rodhe et al. 2015), alongside leaching of N and P, 559 560 contributing towards global warming, acidification and eutrophication burdens (Rehl & 561 Müller, 2011; Styles et al. 2016). Microalgae may be produced directly from heavily diluted LD, or from liquid effluent arising from the chemical extraction of biofertilisers (Rehl & 562 563 Müller, 2011; Vázquez-Rowe et al. 2015), in each case avoiding emissions arising from the 564 storage and spreading of digestate. Biofertiliser extraction processes include struvite precipitation and ammonia stripping (stage 3 of Figure 1c), generating process effluent 565 566 containing almost 60% of the K, 30% of the total N and 8% of the NH₄-N contained in the original LD (Styles et al. 2018). Microalgae may be used to treat such effluent, at 567 considerably reduced dilution factors compared with unprocessed LD, avoiding burdens and 568 costs associated with treatment e.g. in an integrated constructed wetland (Figure 1c). 569 570 Liquid digestate is a valuable bio-fertilizer, rich in readily available nutrients (Vaneeckhaute 571 et al. 2013). Therefore, in addition to the aforementioned burdens, agronomic use of LD can 572 generate significant environmental credits through the avoidance of fertiliser manufacture and 573 spreading (stage 4 in Figure 1c). These credits will no longer arise if microalgae are used to 574 directly treat diluted LD. However, the economic propensity for larger AD plants and short-575 distance transport of LD (FNR, 2012) can lead to over-application of LD close to large AD 576 plants (Fedorniak, 2017), asynchronously to plant uptake, leading to low nutrient use

577 efficiency (Nkoa, 2014; AHDB, 2017) and a poor environmental balance (Styles et al. 2016). The extraction of biofertilisers from LD can avoid most of the emissions associated with LD 578 handling in stage 2, whilst considerably enhancing synthetic fertiliser substitution credits in 579 580 stage 4 (Figure 1b), although at the expense of heat, electricity and chemical (e.g. sodium 581 hydroxide and potassium chloride) inputs - overall helping to close nutrient loops and improve the environmental balance of LD management (Styles et al. 2018). Microalgae could 582 583 help to further close nutrient loops and improve the environmental balance of LD management by mopping up surplus nutrients contained in process effluent from stage 3. 584 585 Microalgae production requires considerable inputs of infrastructure, energy and water for processes including cultivation in photoreactors, filtration and centrifuging algae, and 586 fractionation into valuable constituent products (Figure 1c) (Xu et al. 2015), leading to 587 588 significant global warming, abiotic and fossil resource depletion burdens (Mata et al. 2010). The key question to be answered in future LCA studies is whether these burdens are 589 590 outweighed by the environmental credits associated with substitution of high-value products 591 including aquaculture feed, pharmaceutical and cosmetic ingredients, and the avoidance of 592 LD or biofertiliser effluent management (Figure 1c). Calculation of credits arising from 593 microalgae value chains may be complicated by the wide range of products and production 594 pathways substituted by microalgae (Mulbury et al. 2004; Spolaore et al. 2006; Becker, 2007; 595 de Visser et al. 2014; Suganya et al. 2016). There may be trade-offs across impact categories, 596 given the significant eutrophication and acidification credits likely to arise from closing 597 nutrient loops. The latter credits are becoming increasingly highly weighted (implicitly or 598 explicitly) owing to the increasing attention being paid to nutrient leakage and NH₃ emissions 599 in the context of sustainability (Steffen et al. 2015), external pollution costs (Sutton et al. 600 2011; Sutton et al. 2013), and phosphorous cycling in the context of finite resource depletion

601 (Cordell et al. 2009; Schipper, 2014). Closing nutrient cycles and minimising losses is
602 imperative if the bioeconomy is to be sustainably expanded.

603 **3.5 Agronomic nutrient and feed efficiency**

During the digestion process about 20 - 95% of the feedstock organic matter (OM) is 604 605 degraded (Möller & Müller, 2012). Nitrogen is converted to NH₄, but the majority of both N and P are conserved so that the N & P content of the resultant digestate is typically 606 607 comparable to that of the feedstock material (Provenzano et al. 2011). As such, digestate has 608 the potential to offer an organic option for agricultural fertiliser, which could replace some of 609 the demand for inorganic fertiliser (Nkoa, 2014), avoiding burdens associated with energyintensive fertiliser manufacture (Walsh et al. 2012). However, in comparison to undigested 610 611 animal manures, anaerobic digestates have higher rates of NH₃ emission, which presents the 612 potential for comparatively higher rates of pollution. Using direct injection, which is considered best practice for spreading digestate, will reduce gaseous emissions to the 613 614 atmosphere. Nevertheless, whilst this material is readily available for plant uptake, should the digestate be spread at times other than when optimum for crop usage, then environmental 615 losses have the potential to be high, particularly with regard to the pollution of watercourses 616 617 and/or groundwater (Nkoa, 2014; Möller, 2015.).

The production of anaerobic digestate in regions dominated by pastoral agriculture, where organic manure options are often widely available, can lead to a surplus of nutrients in a geographic location least suited for effective use (Hanserud et al. 2017). Farms and regions of intensive livestock production often import animal feeds from predominantly arable areas, but the transfer of these nutrients back to arable areas in the form of slurry or liquid digestate is costly and therefore unlikely to occur. Recycling excess nutrients in such scenarios, to create animal feed products, can reduce the inappropriate land application of anaerobic

digestate, and help to close nutrient cycles in livestock areas, thus curtailing environmental
impact. In addition, the generation of protein for animal feed through this approach may
reduce reliance on soybean imports from tropical regions (de Visser et al. 2014), currently
needed to meet demand for high protein animal feed. This will in turn reduce deforestation
and land-use change as a consequence (Gasparri et al. 2013), which is a major cause of GHG
emissions (Van der Werf et al. 2009).

631 **4.0 Conclusion**

A circular economy solution for organic waste management through the application of microalgae to remediate excess nutrients from anaerobic digestate and create alternative valuable products has real potential. Here it has been demonstrated that an effective system should include mixed algal and bacterial consortia and should optimise digestate feedstock for algal growth by diluting 30 times and supplementing with acetate (to a concentration of 35 g L⁻¹) to avoid C limitation. NH₄ should also be reduced to 15 g L⁻¹. This can be achieved through membrane filtration technology to establish a favourable C:N:P ratio.

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1005 **Figure/table captions:**

1006 Figure 1. Microalgae biorefining system. (a) Typical AD Technology infrastructure (b) diagrammatic representation of proposed system for the upstream/downstream process of 1007 1008 digestates used during microalgae production in closed photo reactors. (c) Products and processes incurred or avoided (green) along the digestate-to-microalgae value chain. DBF = 1009 1010 digestate biofertilizer; ICW = integrated constructed wetland; HVCs = high-value chemicals. 1011 Figure 2. Modelling results: (a) iCZ8473 predictions of C. vulgaris growth rate and (b) DHA 1012 flux when grown under mixotrophic, phototrophic, and heterotropic conditions on different 1013 digestate dilutions.

- 1014 **Table 1**. *i*CZ843 was able to accurately predict experimentally measured growth rates for
- 1015 phototrophic, mixotrophic and heterotrophic growth regimes.
- 1016 **Table 2**. Typical composition of commercially available feed ingredients and selected algal
- 1017 species (per dry matter)
- 1018 **Table 3**. Typical farm waste feedstock characteristics and nutrient values for an example 1
- 1019 MW_e equivalent farm waste digester fed on agricultural feedstocks values derived from
- 1020 ADAT (BORRG, 2015).
- 1021 **Table 4**. Value of nutrient based on ADAT and RB209 nutrient levels and AHDB fertiliser
- 1022 prices



Figure 2:





Table 1.

Growth regime	Predicted growth rate (hr ⁻¹)	Experimentally measured growth rate (hr ⁻¹)
Phototrophic	0.0248	0.014-0.025 (Zuliani et al. 2016)
Mixotrophic	0.0402	0.02-0.06 (Mezzari et al. 2013)
Heterotrophic	0.0168	0.018-0.025 (Zuliani et al. 2016)

Table 2.

	% Crude	% Crude	Crude % Crude		Gross Energy
	Protein	Lipid	Carbohydrate*		MJ/kg
Fish meal	63.0	11.0	-	15.8	20.1
Poultry meal	58.0	11.3	-	18.9	19.1
Corn gluten	62.0	5.0	18.5	4.8	21.3
Wheat gluten	82.0	1.4	15.2	1.4	22.5
Soybean meal	44.0	2.2	39.0	6.1	18.2
Spirulina	58.0	11.6	10.8	13.4	20.1
Chlorella	52.0	7.5	24.3	8.2	19.3
Tetraselmis	27.2	14.0	45.4	11.5	18.0
Nannochloropsis	42.8	16.6	33.9	6.7	22.6
Schizochytrium	12.5	40.2	38.9	8.4	25.6

Table 3.

Feedstock	Quantity	DM	VS	BMP	CH ₄	Ν	Р	К	Ν	P ₂ 0 ₅	K ₂ O
	(t yr ⁻¹)	(% of W/W)	(% of DM)	$(m^3 t^{-1} VS)$	$(m^3 yr^{-1})$	(g kg ⁻¹ TS)	(g kg ⁻¹ TS)	(g kg ⁻¹ TS)	kg year-1	kg year-1	kg year-1
Slurry	48,180	9.0%	83.0%	185	665,824	57	10	48	247,163	99,299	249,765
FYM	26,499	25.0%	80.0%	190	1,006,962	24	6	27	158,994	91,024	214,642
Poultry litter	7,468	30.0%	75.0%	325	546,090	53	8	21	118,740	41,044	56,457
TOTAL									524,897	231,367	520,864

FYM – Farmyard manure; DM – dry matter; VS – volatile solids; BMP – best management practice.

Table 4.

Nutrient	Nutrient	Nutrient	ADAT	Value	RB209	Value
Nutrent	$\pounds t^{-1}$	£ kg ⁻¹	kg t⁻¹	$\pounds t^{-1}$ digestate	kg t ⁻¹	\pounds t ⁻¹ digestate
34.5% ammonium N	242.00	0.70	6.75	4.73	3.6	2.53
46% P ₂ O ₅ Triple Super Phosphate	287.00	0.62	2.97	1.86	1.7	1.06
60% Muriate of Potash (MOP)	263.00	0.44	6.70	2.94	4.4	1.93
Nutrient value of digestate				9.53		5.52