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ORIGINAL RESEARCH

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A new carbohydrate retaining variety of Miscanthus increases biogas methane yields compared to *M x giganteus* and narrows the yield advantage of maize

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Abstract

Anaerobic digestion (AD) currently relies heavily on crop feedstocks to maintain a constant output. A major annual crop used is *Zea mays* (maize), but this practice presents significant concerns because high energy inputs and food-growing land are required for cultivation. The autumn harvest date of maize exposes soils over winter resulting in erosion and runoff into waterways. Miscanthus is physiologically and morphologically similar to maize. It is also of interest for biogas generation. As a perennial grass, Miscanthus requires far less input and can be grown on land that is unsuitable for food crops. It is typically harvested in late winter to early spring. Maize produces higher biogas yields than the most commonly grown commercial variety of Miscanthus (Mxg), because it has a higher nonstructural carbohydrate (NSC) concentration that facilitates the AD process. We aimed to investigate whether a new Miscanthus hybrid ("GNT-14") that was bred from a high carbohydrate accumulating parental type can improve biogas yield from Mxg. Comparisons were made on biogas yields at two time points, October and January; the NSC, cellulose, and lignin concentrations were quantified; and the contribution of the NSC to biogas yield was determined by comparing intact and washed samples. The NSC concentrations of GNT-14 were fivefold higher than Mxg in January, and a 28% increase in methane was observed. While Mxg showed a reduction in methane yields (L/kg) from biomass harvested in January compared to October, GNT-14 showed no such decline. Although the potential methane yields of GNT-14 were only 70% that of maize, the energy input (GJ ha^{-1}) required for cultivation was 26% of maize. Our results demonstrated that GNT-14 could be harvested later than maize for biogas generation, offering soil protection over winter. We encourage Miscanthus breeding efforts to focus on NSC concentration as well as yield.

KEYWORDS

anaerobic digestion, cellulose, energy, lignin, Miscanthus, nonstructural carbohydrate

1 | INTRODUCTION

Increased awareness of the role of fossil fuels in driving climate change has led to a growing demand for sustainable energy solutions (IPCC, 2014; UNFCC, 2015). The increased consumption of renewable energy since 2005 allowed the European Union (EU) to cut consumption of fossil fuels by 130 Mtoe (Million tonnes of oil equivalent) in 2015, which is comparable to the entire fossil fuel use of Italy (EEA, 2017). Electricity generation from solid biomass grew from 4.5 Mtoe in 2005 to 10 Mtoe in 2017 (Moorkens et al., 2019). However, anaerobic digestion showed a faster rate of growth in the same time period, and in 2018, 5.6 Mtoe were generated in the EU through biogas (EEA, 2017; Moorkens et al., 2019).

Anaerobic digestion (AD) is the decomposition of organic matter in an oxygen-depleted environment to produce biogas, typically at around 60% methane and 40% carbon dioxide (DEFRA, 2011; Toledo-Cervantes, Estrada, Lebrero, & Muñoz, 2017; Whittaker, Hunt, Misselbrook, & Shield, 2016). Biogas can be used for process heat or for both heat and electricity generation using a combined heat and power unit. Alternatively, the biogas can be upgraded to clean methane by removal of the carbon dioxide and cleaning/conditioning the gas for use in transport applications or injection into the gas grid (Amon et al., 2007; Department for Business, 2012; Peterson & Wellinger, 2009). Substrates for digestion can include waste streams such as animal manure or municipal solid waste; however, in the UK and several parts of Europe, maize (*Zea mays*) is increasingly being grown specifically as a feedstock for biogas production (Amon et al., 2007; Bruni, Jensen, Pedersen, & Angelidaki, 2010). The advantages of maize include ease of cultivation, high carbohydrate contents and yields, and high digestibility (Vervaeren, Hostyn, Ghekiere, & Willems, 2010). The European Commission State of Play report in 2014 (European Commission, 2014) raised environmental concerns about the sustainability of biogas systems specifically when utilizing annual crops such as maize. The report highlighted issues around a significant increase in the planted area of these crops, which was often at the expense of food production. In the UK, the area of maize grown specifically for AD increased from 29 Kha in 2014 to 52 Kha in 2016, prior to 2014 UK government figures suggested no land area of maize as being used for energy production (DEFRA, 2017). The use of maize as an energy crop can be controversial due to a high energy input requirement in terms of annual cultivation and agro-chemicals that lower the net energy gain (Felten, Fröba, Fries, & Emmerling, 2013) and increase its negative environmental impact. Typically harvesting at the end of the growing season (late autumn) means that soils can be exposed over winter, which can lead to erosion and sedimentary runoff into water courses during heavy rain, this is particularly problematic on even gently sloping land (ADAS and Ricardo Energy & Environment, 2016; European Environmental Agency, 2006; Palmer & Smith, 2013). If AD is going to play a major role in the European energy mix, alternative, more environmentally sustainable crop species are required to replace maize as a primary feedstock.

Related to maize, sugarcane and Sorghum at the family level (Poaceae), *Miscanthus spp*., is a C4 genus covering a range of giant grass species (Greef, Deuter, Jung, & Schondelmaier, 1997). The naturally occurring sterile hybrid *M x giganteus* (hereafter Mxg) is already planted widely across the EU as a second-generation commercial biomass crop (Clifton-Brown et al., 2017; Clifton-Brown, Stampfl, & Jones, 2004). Unlike maize, Mxg is perennial grass with very high water and nutrient use efficiencies and effective overwinter recycling of nutrients to belowground rhizomes (Clifton-Brown & Lewandowski, 2000). *Miscanthus* spp. in general have several environmentally favorable attributes, particularly the ability to produce high yields of stem biomass on agriculturally marginal land that is less suitable for food production with very little demand for chemical inputs (McCalmont et al., 2017). Land can be described as marginal for a variety of reasons that render it unsuitable for food production, examples include bio-physical properties such as stone and clay content, waterlogging or other factors resulting in low workability (Wagner et al., 2019).

Maize currently has commercial advantages over the standard Mxg as an AD feedstock, particularly in terms of a higher nonstructural carbohydrate content (BSPB, 2015; Kiesel, Nunn, et al., 2017; Kiesel, Wagner, & Lewandowski, 2017), which leads to a higher biogas yield of 313–366 L CH_4 kg^{-1} DM (dry matter) (Amon et al., 2007) compared to Mxg at 172–186 L/kg DM (Whittaker et al., 2016), while Kiesel, Nunn, et al. (2017) and Kiesel, Wagner, et al. (2017) found the range of 250–300 L/kg DM depending on winter or autumn harvest, respectively. Biogas yield per area of plantation is determined by biomass yields (Mg/ha DM), and this maize also currently shows an advantage over the commercial standard Mxg in the UK where it typically yields higher biomass (BSPB, 2015; Hastings et al., 2017). However, in Germany, a major producer and consumer of AD-derived biogas, biomass yields per hectare for Mxg have been shown to exceed those in the UK, and as a result, specific methane yields per hectare (~5.5 million L/ha) can exceed maize (5.3 million L/ha) (Mayer et al., 2014).

Clearly, yield improvements are a major target in terms of increased gas production from Miscanthus plantations (Kiesel & Lewandowski, 2017; Wagner et al., 2019; Whittaker et al., 2016), but biomass compositional improvements may

also provide significant gains, particularly considering non-structural carbohydrates (NSCs). Attempts have been made to determine whether increased NSC content could be a target for Miscanthus breeding programs or whether hybrids already exist that could rival maize for use in anaerobic digestion systems. A previous study, Purdy et al. (2017), quantified NSC across 38 diverse genotypes of green-cut Miscanthus biomass harvested at two time points, mid-summer (July) and autumn (October–November), and revealed considerable variation between both genotype and harvest date. NSC is not the only driver of AD however; access to cellulose in the plant cell wall (Azman et al., 2017; Golkowska & Greger, 2013) is a major driver of biogas generation, and this can be influenced heavily by the lignin content (da Costa et al., 2014). With Miscanthus genotypes showing large variations in all these components, it seems reasonable to suggest that there may be target hybrids within breeding programs that could outperform maize in anaerobic digestion for biogas. Currently, Mxg is used commercially, but for use in AD it relies on early "green" harvesting when the biomass has a higher NSC content and greater overall biomass, due to the avoidance of overwinter leaf drop (Purdy et al., 2017). However, this early harvesting (compared to the more usual overwinter ripening and spring harvest) can have implications on future yields due to incomplete senescence and restricted nutrient cycling to the belowground rhizome (Mayer et al., 2014; Yates et al., 2015). Early harvesting may also result in the removal of leaf material, which naturally falls over the winter. The fallen leaves from Miscanthus form a natural layer of mulch on the soil surface that may protect it from the harshest winter elements. This may be particularly relevant in immature plantations with larger gaps between plants. Therefore, in addition to the targets of NSC content and biomass yield, genotype selection needs also to consider impacts of harvest timing on biomass quality and yields in subsequent years.

A novel hybrid "GNT-14" with a parental type that was previously identified as a high carbohydrate accumulating genotype (Purdy et al., 2017) was selected from the Miscanthus breeding program at Aberystwyth University and compared to the current commercially grown variety, Mxg. Owing to the parental pedigree, we hypothesized that this genotype may be able to outperform the current commercial cultivar (Mxg) for methane production from AD.

To that end, this paper is set out to investigate the following:

- To demonstrate a more productive Miscanthus cultivar for methane production
- To establish the possible cause of the difference in methane production through biomass compositional analyses
- To calculate the energy and carbon (CO_2) emission cost of methane production between maize and the tested Miscanthus cultivars.

2 | MATERIALS AND METHODS

2.1 | Sample harvest and preparation

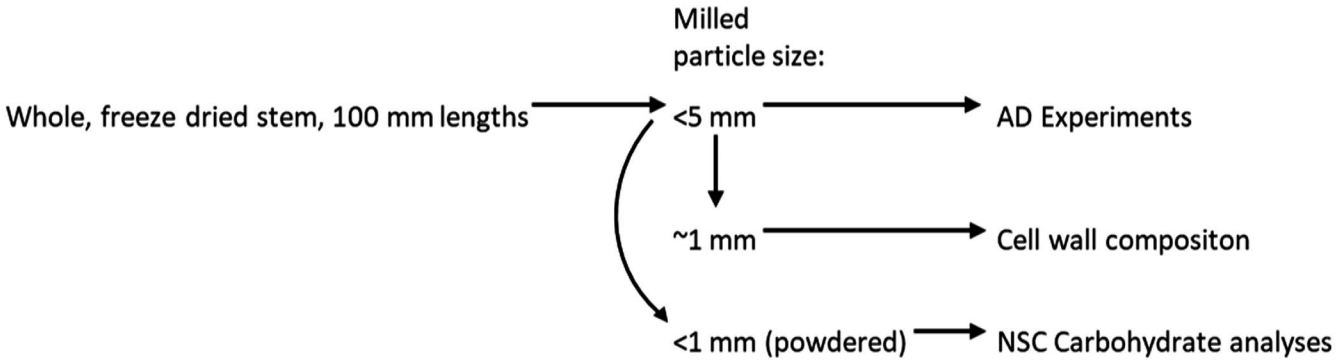
2.1.1 | Plant material

To test the effect on anaerobic digestion of selecting a potentially high carbohydrate type, the genotype "GNT-14" was selected from the Miscanthus breeding program because it was the progeny of a diploid Chinese *M. sacchariflorus x M. sinensis*. The *M. sacchariflorus* parent was genetically similar to one identified in a previous study as being a high carbohydrate type (Purdy et al., 2017). In 2014, a field trial was established in a randomized block design near the coastal town of Aberystwyth (52°25′29.4″N 4°03′05.3″W) on a hill site, 132 m above sea level. The soil type is freely draining slightly acidic, loamy soil with low top soil carbon (Cranfield University, 2019) and a high stone content; soils in this area are classified as moderate quality agricultural land best suited to grazing (Welsh Government, 2017). Synthetic crosses were planted in three blocks each containing 10 plots of inter- and intraspecific hybrids from the breeding program and individually spaced plants of *M. sinensis* ("Goliath") and Mxg. As Miscanthus is an outcrossing species, all plants of GNT-14 in each plot were full siblings but genetically unique (i.e., not clonal). The Mxg and Goliath plants were grown from cloned rhizome material.

2.1.2 | Harvests and sample preparation

In October 2016 and January 2017, two and three plants, respectively, that were closest to average plot height (measured with a telescopic measuring pole), were selected and harvested from each plot of GNT-14 and Mxg plants spaced within each of the three blocks. The increased harvest in January was to compensate for the reduction in biomass that occurs between Autumn and Spring. A single stem, that was representative of canopy height, was cut from each plant at 5 cm above ground. The whole stem, including leaves, was then cut into 10-cm pieces, flash-frozen in liquid nitrogen, and freeze-dried on the same day of the harvest. Rough-ground samples were ground into <5-mm pieces with a Retsch mill (SM100, Retsch, Haan, Germany). The decision to use a 5-mm particle size was based on previous reports (Kiesel, Nunn, et al., 2017; Kiesel, Wagner, et al., 2017; Whittaker et al., 2016; Yancey, Wright, & Westover, 2013) and the practical consideration of being able to accurately measure the desired amount of material into a 125-ml AD bottle with a neck opening of 13 mm. Fine-ground samples were then achieved with a Tecator Cyclotec 1,093 sample mill. The filter was set to 1 mm for cell wall composition analysis. Rough-ground

samples were further processed by cryomill (6870 Freezer Mill, Spex, Sampleprep, Stanmore, UK) for nonstructural carbohydrate (NSC) analysis. The milling and sample to analysis process is summarized below:



2.1.3 | Yield assessment of GNT-14 and Mxg

In 2014, a randomized complete block design (RCBD) trial to assess yield of four promising hybrids GNT-8, GNT-9, GNT-10, GNT-14, and Mxg was planted at Hackthorn, Lincolnshire (52°25N, 00°23W) (HCK). Soil texture (0-30 cm) was SZL (sandy silt loam), prior to planting the field was used for combinable crop rotation.

All hybrids were established from clonally produced material, and GNT-8 and GNT-14 were produced using in vitro tillering and established from plug plants. Mxg was planted from commercially available rhizome (Terravesta Ltd.). Plots were covered with Samco "Grey" mulch film with "pin hole 20" aeration (Samco Agricultural Manufacturing Ltd., Adare, Limerick, Ireland) immediately after planting. Each plot contained 50 plants and planted at a competitive density (2 plants per m^2); harvest area per plot was $12m^{-2}$ (24 plants). Yield data used herein were from the 2017 harvest in the 3rd complete year of growth.

Dry matter yield was estimated as described in Clifton-Brown et al. (2001) where harvested samples were oven dried at 80°C until constant weight. The yield reflects the dry mass of harvested Miscanthus from the third established year (mature crop). Plots were hand harvested with a hedge trimmer in early February in line with current commercial harvesting time. Studies had shown from modeled and measured biomass change that a consistent 30%–40% higher biomass is achieved in Autumn when compared to winter (Lewandowski, Clifton-Brown, Scurlock, & Huisman, 2000; Nunn et al., 2017; Purdy, Cunniff, et al., 2015). In particular, Nunn et al. (2017) had shown a consistent multiplying factor of 1.3 for October harvest (for both Mxg and other Sacc x Sin hybrids) when compared with the winter harvest. This multiplying factor of 1.3 was applied to both Mxg and GNT-14 winter harvest values from the Hackthorn trial to obtain estimated October dry matter yields. Dry matter per ha for maize was taken from the published BSPB/NIAB 2016, 2017 and 2018 forage maize descriptive lists (BSPB, 2015, 2016, 2017).

2.2 | Soxhlet extraction

Soxhlet extraction was performed by placing a recorded weight of fine-ground or rough-ground samples ($Samples_{before}$) into weighed Whatman extraction thimbles 603 (Cat# 10350220), then covered with weighed Whatman glass microfiber filters GF/D (CAT# 1823025) and secured with cotton wool. Thimbles with the sample were then placed into a Quickfit soxhlet extractor (EX5/53/60) connected to a Quickfit condenser (C11/23) and a Quickfit glass bulb (FF500/3S) holding the washing solvent. The glass bulb sat on a heating mantle (Electromantle) that brought the solvent to a boil. The steam was then condensed by the condenser cooled with running tap water. The sample was first extracted with water until the water in the soxhlet chamber became clear, and then, the process was repeated with absolute ethanol.

After the glassware had cooled down to ~40°C, the thimble was retrieved and placed in a drying oven at 40 °C until a constant weight was reached. The dry matter ($Sample_{after}$) was recorded to determine wash-off content ($\%_{WO}$), which was adjusted by calculation into a proportion of dry matter. Samples were then stored in airtight container.

To calculate % of washed off material:

$$\%_{WO} = \left(Samples_{before} - Sample_{after}\right) \times 100 \div Samples_{before}$$

2.3 | Klason Lignin analysis

Klason lignin analysis was conducted according to Hatfield, Jung, Ralph, Buxton, and Weimer (1994) with minor modifications. Approximately 250 mg of dried and washed sample (WS) was dissolved in 3 ml 72% H_2SO_4 (Fisher, Cat# R819160025D) for 2 hr at room temperature. The mixture was agitated every 15 min with a glass rod. After 2 hr,

43 ml of deionized water was added before the container was sealed and put into an autoclave at 121 °C for 1 hr. After the temperature cooled to below 60 °C, the sample was poured through a preweighed Pyrex Gooch crucible (Pyrex, 3650/02M) lined with Whatman glass microfiber filters GF/A (Cat# 1820-050). After washing off the content with deionized water, the crucible was drained of liquid. The whole crucible was then placed in drying oven at 105 °C for 16 hr (overnight). The weight of the Klason Lignin sample (KL) was recorded the next day after cooling down in a desiccator for 20 min. Then, the crucibles with dried sample inside were ashed in a Eurotherm 91e (CSF110) furnace at 550°C for 2 hr. Once cooled to ~60°C, the crucible was placed in the desiccator to cool. Once the crucible had cooled to room temperature, the content was weighted to obtain the Ash weight.

To calculate cell wall lignin content:

$$\%\text{Lignin}_{\text{cw}} = (\text{KL} - \text{Ash}) \times 100 \div (\text{WS} - \text{Ash})$$

To calculate lignin content in dry matter:

$$\%\text{Lignin}_{\text{dm}} = \text{Lignin}_{\text{cw}} \times \left(1 - \%_{\text{WO}}\right)$$

2.4 | Cellulose determination

Cellulose was determined by a modified assay assembled from various publications (Foster, Martin, & Pauly, 2010; Updegraff, 1969; Ververis, Georghiou, Christodoulakis, Santas, & Santas, 2004). Approximately 5 mg ± 0.2 mg of purified (Soxhlet extracted and enzyme digested) and fine-ground cell wall material was weighed into a 2-ml screw cap tube and 1.8 ml of Updegraff reagent (acetic acid: nitric acid: water, 8:1:2 v/v) was then added. The tube was then capped, vortexed, and heated in a heating block at 100°C for 30 min. The samples were then cooled on ice to room temperature or cooler. Samples were centrifuged at 10,000 rpm for 15 min. 1.6 ml of supernatant was removed with a pipette to ensured that the pellet was not disturbed. 1.5 ml water was added, vortexed and spun as above, and 1.5 ml supernatant was discarded as above. The pellet was then washed 3 times with 1.5 ml acetone. The pellet was then air-dried overnight in the fume hood. The purified cell wall material and 5 mg of cellulose (Sigma, C8002) as a separate control were then hydrolyzed with 50 µl of 72% H_2SO_4 on the next day. The sample was mixed and incubated at 30°C, shaking at 200 rpm for an hour. After incubation, samples were diluted with 1.4 ml of deionized H_2O with tubes, then capped and autoclaved at 121°C for 1 hr. Once cooled, an aliquot of 0.43 ml was added into 20 mg $CaCO_3$ to be neutralized. The pellet was then centrifuged at 10,000 rpm for 15 min. Supernatant (200 µl) was removed to a fresh tube with the same amount (200 µl) of deionized water added. 33.2 µl of the supernatant was then added with 1 ml of GOPOD reagent from Megazyme (K-GLUC), and assay was carried as instructed by manufacturer. GOPOD assay with 1 mg/ml, $^2/_3$ mg/ml, $^1/_3$ mg/ml glucose standard and deionized water blank was run parallel with the samples to determine standard curve for glucose content in every assay. The assay was read at 510 nm in a µQuant spectrometer (BIO-TEC instruments, INC). The measured glucose content was directly implicated as cellulose content (Foster et al., 2010) and checked against the cellulose standard.

To calculate cellulose content in cell wall material:

$$A_1 = 510\,\text{nm absorbance from } 1\,\text{mg/ml glucose standard}$$

$$A_{\text{blk}} = 510\,\text{nm absorbance from deionised water blank}$$

$$A_{\text{sample}} = 510\,\text{nm absorbance from for samples}$$

$$\%\text{Cellulose}_{\text{CW}} = \left(A_{\text{sample}} - A_{\text{blk}}\right) \times 2 \times 1.45 \times 100 \div \left(A_1 - A_{blk}\right) \div 5$$

To calculate cellulose content in total dry matter:

$$\%\text{Cellulose}_{\text{dm}} = \%\text{Cellulose}_{\text{CW}} \times \left(1 - \%_{\text{WO}}\right)$$

2.5 | Nonstructural carbohydrate (NSC) analysis

Soluble sugars and starch were analyzed as previously described (Purdy, Cunniff, et al., 2015; Purdy, Maddison, Cunniff, Donnison, & Clifton-Brown, 2015). Soluble sugar extraction: Approximately 20 mg (actual weight recorded) of each cryo-milled plant tissue sample was weighed into 2-ml screw cap microcentrifuge tubes. Sugars were extracted four times with 1 ml of 80% (v/v) ethanol and the resulting supernatants pooled; two extractions were at 80°C for 20 min and 10 min, respectively, and the remaining two extractions at room temperature. A 0.5 ml aliquot of soluble sugar extract and the remaining pellet containing the insoluble fraction (including starch) were dried down in a centrifugal evaporator (Jouan RC 1022, Saint Nazaire, France) until all the solvent had evaporated. The dried-down residue from the soluble fraction was then resuspended in 0.5 ml of distilled water. Samples were stored at −20°C for analysis.

Soluble sugar analysis: Soluble sugars of samples extracted in the previous step were quantified enzymatically by the stepwise addition of hexokinase, phosphoglucose isomerase, and invertase (Jones, Outlaw, & Lowry, 1977). Samples were quantified photometrically (Ultraspec 4000, Pharmacia Biotech, Sweden) by measuring the change in wavelength at 340 nm for 20 min after the addition of each enzyme. Sucrose, glucose, and fructose were then quantified from standard curves included on each 96-well plate.

Starch quantification: Starch was quantified using a modified Megazyme protocol (Megazyme Total Starch Assay Procedure,

AOAC method 996.11, Megazyme International, Ireland). Briefly, the dried pellet was resuspended in 0.4 ml of 0.2 M KOH, vortexed vigorously, and heated to 90°C in a water bath for 15 min to facilitate gelatinization of the starch. A total of 1.28 ml of 0.15 M NaOAc (pH 3.8) was added to each tube (to neutralize the sample) before the addition of 20 µl α-amylase and 20 µl amyloglucosidase (Megazyme International, Ireland). After incubation at 50°C for 30 min and centrifugation for 5 min, a 20 µl aliquot was combined with 0.6 ml of GOPOD reagent (Megazyme). A total of 0.2 ml of this reaction was assayed photometrically (Ultraspec 4000, Pharmacia Biotech) on a 96-well microplate at 510 nm against a water-only blank. Glucose was quantified from known standard curves on the same plate. Each sample and standard was tested in duplicate. Each plate contained a Miscanthus control sample of known concentration for both soluble sugars and starch analysis.

2.6 | Anaerobic digestion

A modified anaerobic digestion (AD) was carried out as described in Corton, Toop, Walker, Donnison, and Fraser (2014). Digestate used was supplied by Harper Adams University (originated from Cog Moor, UK). Volatile solid content of both sample and digestate (VS) was determined by dry matter. Dry matter of biomass samples (DM_{sample}) was described earlier, while the dry matter of digestate ($DM_{digestate}$) was determined by evaporating 100 ml of digestate in 80°C until constant weight. Then, 1 g of dried sample and digestate (1 g) was ashed in a furnace at 550°C over night. The weight of sample (Ash_{sample}) and digestate ($Ash_{digestate}$) was obtained after cooling the sample to room temperature in a desiccator.

Volatile solid (VS):

$$VS_{digestate}\ (\text{per ml}) = \left(DM_{digestate} - Ash_{digestate}\right) \div 100$$

$$VS_{sample} = DM_{sample} - Ash_{sample}$$

$$\%VS_{sample} = VS_{sample} \div DM_{sample} \times 100$$

A ratio of digestate: Rough-ground samples with volatile solid of 3:2 were placed into a 125-ml Wheaton bottle (22748) with 70 ml of digestate. The bottles were sealed with 20mm × 9mm butyl plug (Fisher Scientific 11598190) crimped with an aluminum top seal (Fisher Scientific, 10270322). The head space was purged with oxygen free nitrogen (BOC) for 60 s per bottle. Positive controls consisted of 150-mg cellulose (Sigma, C8002) powder added into the reaction. Bottles with only 70 ml of digestate were used as negative control as well as blank for subtracting methane that was produced by digestate alone.

The bottles for AD reactions were then placed in an incubator at 37°C (Stuart Scientific hybridization oven) as this temperature was consistent with previous reports (e.g., Whittaker et al., 2016). Gas contents were read with an ADC 5000 series gas analyzer to determine CO_2 and CH_4 content. The reaction was allowed to continue for 21 days as per Corton et al. (2014). With the digestate source being fresh (within 3 weeks of procurement), most of the gas would have been produced within this period. The analyzer was calibrated with 80% CO_2 and 80% CH_4 every time before assaying the samples. The samples were manually shaken regularly. Gas readings (total, CO_2, and CH_4) were collated for the full 21 days to reflect the total gas produced in the 21-day period. The methane production was presented as liter per kilogram dry matter (l/kg) based on the methane yield on the amount of dry matter.

To accounted for errors such as leaking seals, the interquartile range (IQR) was used to calculate the range where the majority of the value should lie in each group in an attempt to reduce the noise. The values were tested for outliers separately according to their genotype/line and treatment (washed or different harvest time). Those values that lay outside the IQR were regarded as outliers. Outlier values were omitted from calculation.

2.7 | Energy and CO_2 emission analysis

The carbon emission and energy cost for maize and Mxg were calculated and described by Felten et al. (2013) who used values for end of season (brown) harvested Miscanthus. However, because GNT-14 is a new hybrid no published values were available. A major difference in the energy inputs required for GNT-14 compared to Mxg lies in the method of propagation because Mxg is usually propagated by rhizome pieces but the new generation of Miscanthus hybrids (including GNT-14) is propagated from plug plants, which shorten the time to produce mature yields (Clifton-Brown et al., 2017). Hastings et al. (2017) compared the carbon emissions between rhizome propagated and plug plant propagated Miscanthus, so we used their values to calculate the energy difference between rhizome and plug plant propagation, to adjust the estimated carbon and energy cost for GNT-14 compared to Mxg:

$$AnCO_2 = [CO_{2(Mxg)} \times YR - Est_{(Mxg)}] \div YR$$

$AnCO_2$: Annual additional CO2 emitted to produce Miscanthus for both Mxg and GNT-14 (Mg ha^{-1} yr^{-1})

$CO_{2\,(Mxg)}$: Average CO_2 emission every year over 16 years (Mg ha^{-1} yr^{-1}) (Felten et al., 2013)

YR: 16 years of Miscanthus growth (Felten et al., 2013).

$Est_{\,(Mxg)}$: CO_2 cost in establishing rhizome propagated Miscanthus (Mxg) (Hastings et al., 2017)

To calculate GNT-14 annual CO_2 emissions, the following formulae were used:

$$CO_{2(GNT-14)} = (AnCO_2 \times YR + Est_{(GNT14)}) \div YR$$

$CO_{2\ (GNT\text{-}14)}$: Average CO_2 emission every year over 16 years (Mg/ha yr^{-1}).

Est $_{(GNT\text{-}14)}$: CO_2 cost in establishing seedling plug propagated Miscanthus (GNT-14) (Hastings et al., 2017)

We made the assumption that the carbon cost is directly related to the energy consumption and calculated the differences between Mxg and GNT-14 energy consumption in proportion to the carbon emission.

2.8 | Statistical adjustment

To account for sources of experimental uncertainties such as bottle seal leakage, digestate variation, and lack of technical replicates, statistical adjustments were employed in data analysis. This was done by calculating the upper and lower quartiles as well as upper and lower bound on each assay and each Miscanthus hybrid to identify and eliminate outliers. All comparisons between samples across harvests were made by analysis of variances (ANOVA), and subsequently, least significant differences (l.s.d. significant level at 5%) were calculated using Genstat 18th Edition, to support the significance of the differences observed.

3 | RESULTS

3.1 | Nonstructural carbohydrate (NSC) content

The concentrations of glucose and fructose showed no significant change in Mxg between the two harvest times (October and January) (Figure 1). However, in GNT-14 both hexoses (glucose and fructose) increased between Oct-16 and Jan-17. Sucrose concentrations declined in Mxg between Oct-16 and Jan-17, while no significant change was observed in GNT-14. Starch concentrations were higher at both time points in GNT-14, but similarly to Mxg it showed a decline over the two periods although the magnitude of decline was larger in Mxg than GNT-14.

The concentration of the analyzed soluble sugars in January accounted for 10% of the DM of GNT-14 and <2% of Mxg. In addition, Mxg harvested in Oct-16 had a higher percentage of the soluble sugars, glucose, fructose, and sucrose (combined = 7.8% DM), compared to GNT-14 (6% DM), but Mxg had approximately half the concentration of starch as GNT-14. In Jan-17, the concentration of total NSC between Mxg and GNT-14 reversed with GNT-14 showing highest carbohydrate content in all tested samples. In particular, the hexoses (glucose and fructose) (Figure 1) in GNT-14 in Jan-17 were ~8-fold higher than Mxg at the same time point.

The total NSC concentration in GNT-14 was 14.14% DM compared with 2.4% DM in Mxg in Jan-17 (Table S1). Therefore, Mxg had only one-fifth of the NSC concentration as GNT-14. No significant differences were observed in Oct-16 between the two genotypes. Though sugar content is lower in GNT-14 when comparing to Mxg at Oct-16, the different was made up by the differences of starch content between the two. Yet, in Jan-17, all aspect of the tested NSC components in GNT-14 surpassed, in quantity, Mxg. For full detail of the data analysis, please consult Table S1.

3.2 | Cellulose content

The cellulose contents for both Oct-16 and Jan-17 are presented as percentage of dry matter (Table S2). A technical replicate was performed to assess the reproducibility of the experiment. The overall cellulose content in this

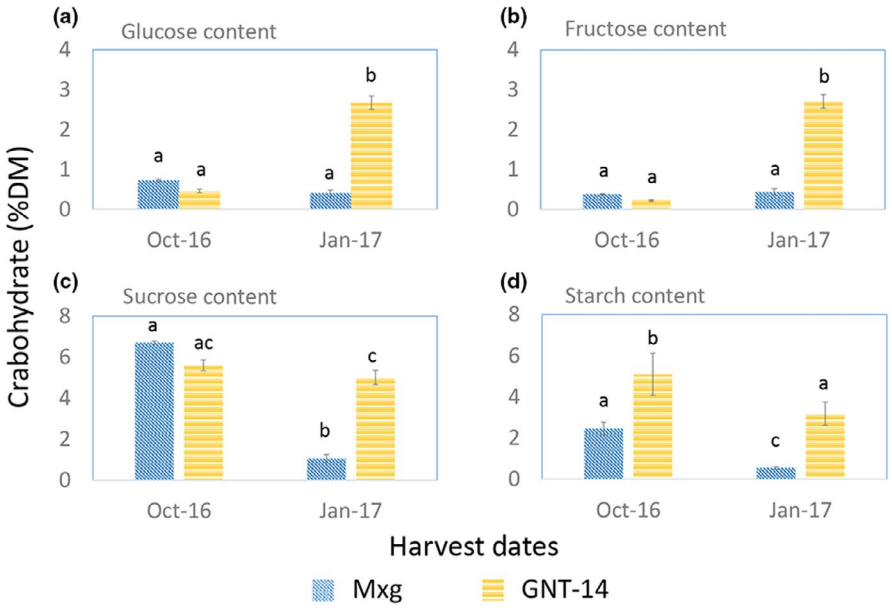


FIGURE 1 Total nonstructural carbohydrate (NSC) composition by % of dry weight from Mxg and GNT-14 Autumn (Oct-16) and Winter (Jan-17) harvest. The NSC examined are (a) glucose content, (b) fructose content, (c) sucrose content, and (d) starch content. Letter above bars denote significant differences ($p < .05$)

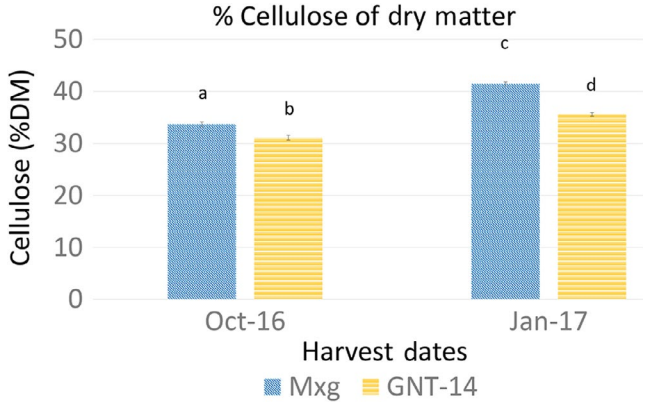


FIGURE 2 Comparison of cellulose content of Mxg and GNT-14 Autumn (Oct-16) and Winter (Jan-17) harvest. Letter above bars denote significant differences ($p < .05$)

analysis ranged between 30% and 42% of the total dry matter (Figure 2). The results showed an increase in cellulose concentration between October and January for both Mxg and GNT-14. Mxg showed an increase of cellulose, on average, 7% dry matter, while GNT-14 showed an increase of 5%. Mxg showed higher cellulose contents by 4% and 5% DM, when compared with GNT-14 at both time points (Oct-16 and Jan-17, respectively).

3.3 | Lignin content

Lignin can restrict access to the cellulose microfibrils hindering microbial decomposition (da Costa et al., 2014). Therefore, lignin content was another factor that may have influenced methane production in AD. Lignin contents are presented as percentage of dry matter (Table S3). A technical repeat was performed to assess for reproducibility of the experiment. The overall lignin content in this analysis ranged between 15% and 20% of the total dry matter (Figure 3). The

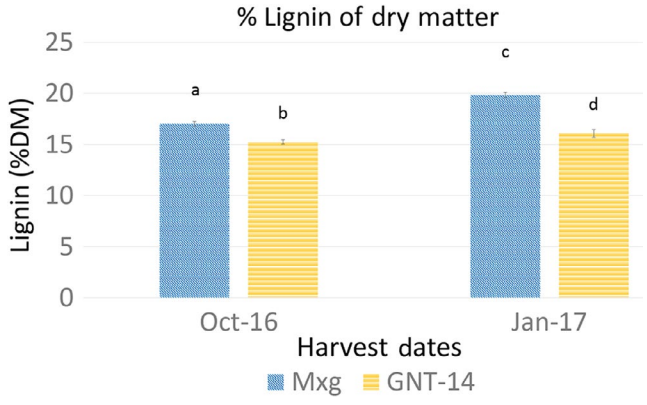


FIGURE 3 Comparison of lignin content of Mxg and GNT-14 Autumn (Oct-16) and Winter (Jan-17) harvest. Letter above bars denote significant differences ($p < .05$)

data showed higher lignin content in Mxg than GNT-14 in both Oct-16 and Jan-17 and also an apparent accumulation of lignin content in both genotypes between Oct-16 and Jan-17.

3.4 | Anaerobic digestion

The result of the two AD experiments of Oct-16 and Jan-17 was calculated as liter of methane gas produced per kilogram of dry matter (L/kg). A comparison was made between the values of two technical repeats from Jan-17 (Mxg and GNT-14) showing no differences, suggesting the results are consistent (data not shown).

A comparison of methane production between the Autumn harvest (Oct-16) and Winter harvest (Jan-17) is recorded in Table S4 and shown in Figure 4. The results showed that while GNT-14 maintained superior methane production in both harvests against Mxg, it was only significantly different in Jan-17. This is because methane production declined in Mxg between the two time points but remained the same in GNT-14. The novel hybrid GNT-14 maintains its methane production consistency while showing superior production when compared to the current commercially available genotype.

Complete Jan-17 samples with no treatment were compared with the same set of samples with the NSC removed by soxhlet extraction (washed sample) before the AD experiment. In agreement with our earlier observations, in the complete samples the results showed that GNT-14 outperformed Mxg in methane gas production (Figure 5). In 3 weeks of anaerobic digestion, the hybrid produced >20% above the commercial variety, a promising improvement in gas production (Table S5). However, no significant differences were found between Mxg and GNT-14 samples when the NSC components were removed by washing. Furthermore, no significant differences were identified between the complete and washed Mxg samples in terms of methane production, while both washed samples were significantly lower than the normal GNT-14 samples. This suggested a component (or multiple components) in the wash-off fraction may have determined the increase in methane production from the complete sample of GNT-14.

3.5 | Energy and CO_2 emission cost in CH_4 production

A summary of the methane yield (L/ha) predicted from this experiment, and the associated energy cost and CO_2 emission is presented in Table 1. In terms of methane yield per hectare, published data suggested that maize outperformed both Miscanthus genotypes at both harvest dates (BSPB, 2015, 2016, 2017; Kiesel, Nunn, et al., 2017; Kiesel, Wagner,

et al., 2017; Whittaker et al., 2016). Interestingly, Miscanthus hybrids potentially outperform maize in terms of dry matter yield when harvested in autumn (Nunn et al., 2017). Maize in UK typically had a yield of ~17 Mg DM ha^{-1} according to the BSPB descriptive list, while both Miscanthus genotypes yielded ~14 Mg DM ha^{-1} in winter. By calculation, Mxg produced 2.5 Ml ha^{-1} from winter harvest; GNT-14 produced 3.2 Ml^{-1} from winter harvest, and Maize produced 5.7 Ml ha^{-1} of methane gas. However, published results demonstrate that maize has a higher energy requirement for production than Miscanthus (Table 1). Based on these published figures for energy and CO_2 emission calculations (Felten et al., 2013), maize needed 20.2 GJ ha^{-1} annually for cultivation. In contrast, Mxg and GNT-14 required only 5.5 and 6.1 GJ ha^{-1} yr^{-1}, respectively, each year over 16 years of cultivation. Similarly to the energy requirement, cultivating maize produced more fossil fuel derived CO_2 than Miscanthus. The process produced 8.4 Mg/ha of CO_2 every year, while Mxg and GNT-14 only produced 0.7 and 0.77 Mg/ha annually over a 16-year period.

A summary of energy costs and CO_2 emission calculations for methane gas per hectare is shown in Figure 6. Methane gas produced by maize required an energy consumption of 3.54 KJ per liter of CH_4, whereas Mxg-derived methane gas only consumed 2.18 KJ per liter of CH_4 from winter harvest. GNT-14-derived methane gas consumed 1.88 KJ per liter of CH_4 from winter harvest. The cultivation of both Miscanthus genotypes showed lower energy requirements and CO_2 emissions.

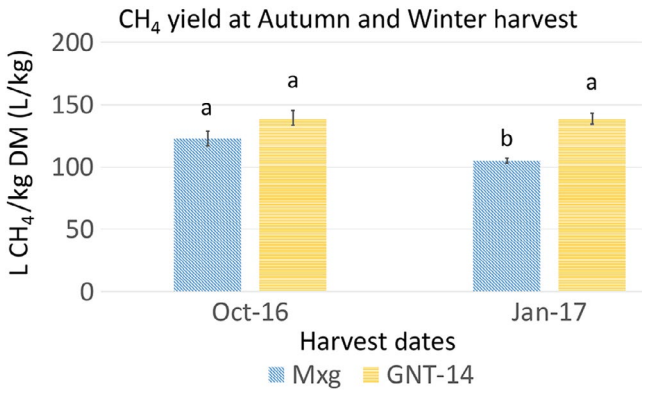


FIGURE 4 Comparison of methane production of Mxg and GNT-14 Autumn (Oct-16) and Winter (Jan-17) harvest. Letter above bars denote significant differences ($p < .05$)

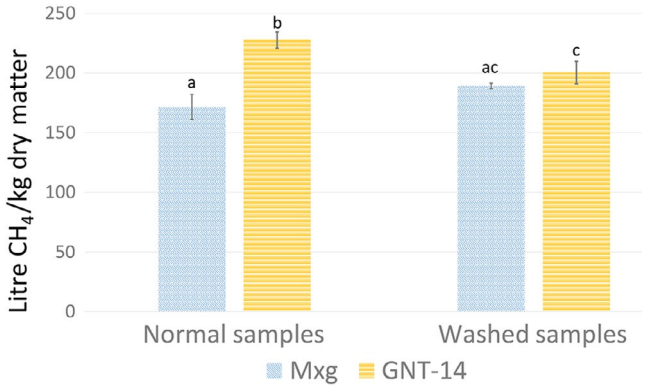


FIGURE 5 Comparison of methane production of washed and complete Mxg and GNT-14 dry matter. Letter above bars denote significant differences ($p < .05$)

4 | DISCUSSION

The yield of methane gas from Mxg was compared with published data Whittaker et al. (2016) reported 172–186 L/kg dry matter from September harvested material over 45 days, Kiesel, Nunn, et al. (2017) and Kiesel, Wagner, et al. (2017) reported 250–300 L/kg from February harvested biomass over a 35-day digestion, and Mangold, Lewandowski, Hartung, and Kiesel (2019a) reported ~315 L kg DM and ~330 L kg DM from a mid October harvest from non-ensiled and ensiled Miscanthus, respectively. Our yield was 172 L/kg over 21 days, which is at the lower end of previous reports (eg. Whittaker et al., 2016). This could be attributable to the inoculum, which was sourced from a local sewage sludge treatment anaerobic digester (Cog Moors, UK). There was also a difference in particle size used for the AD experiments with ours utilizing a 5-mm milling sieve, whereas Mangold, Lewandowski, Hartung, et al. (2019) and Kiesel and Lewandowski (2017) used a 1-mm sieve. A smaller

TABLE 1 Summary table of methane gas yield and the associated energy cost and CO_2 emission. Miscanthus (GNT-14 and Mxg) is the annual average calculated across 16 years while Maize is the annual average calculated across 5 years

		Estimated biogas produced		Energy expended in feedstock cultivation			
	Yield t/ha	CH_4 L/kg	CH_4 ML/ha	GJ/ha/yr	t CO_2/ha/yr	KJ/L CH_4	CO_2(g)/L CH_4
GNT-14	14.24[a]	228	3.25	6.1[b]	0.8[b]	1.88	0.24
Mxg	14.68[a]	172	2.52	5.5[b]	0.7[b]	2.18	0.28
Maize	16.87	338	5.70	20.2[b]	8.4 [b]	3.50	1.47

[a]Data from Ashman pers. comm. 2018.

[b]Data extracted from Felten et al. (2013) and Hastings et al. (2017).

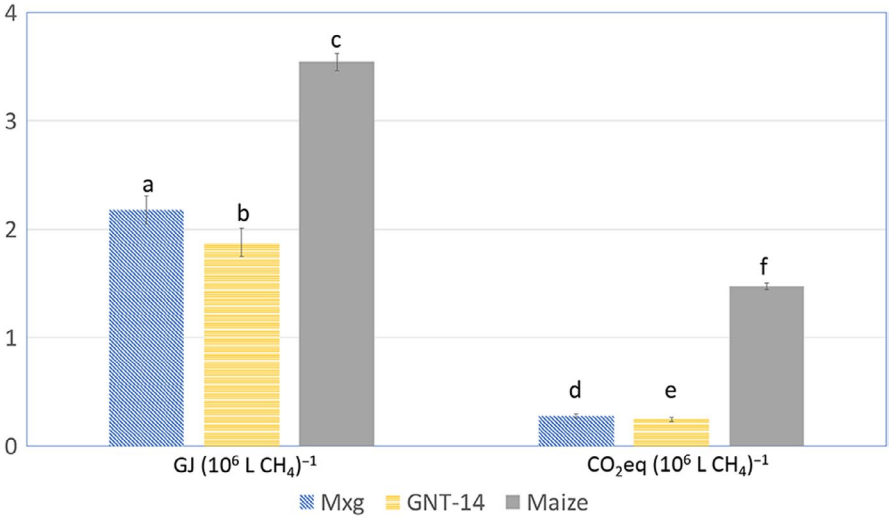


FIGURE 6 Mean over 16 years for Miscanthus (GNT-14 and Mxg) and 5 years of Maize, the energy required and CO_2 emitted per 1 million L of CH_4 gas every year. Letter above bars denote significantly different groups when compared with others ($p < .05$)

particle size would have exposed a larger surface area for microbial access, which is likely to have facilitated digestion. Our digestion time was also shorter, while particle size bigger when compared to that of Mangold, Lewandowski, Hartung, et al. (2019) and Kiesel and Lewandowski (2017), but we found very little gas (below the level of quantification) was produced after 21 days, making this unlikely to explain the differences. Therefore, the potency of the inoculum and/or particle size would seem to be the most likely explanation(s) for our yields being at the lower end of published values.

It has been reported that harvesting Mxg in Autumn (Oct) compared to winter/spring increased methane yield (Kiesel, Nunn, et al., 2017; Kiesel, Wagner, et al., 2017). Our AD results for Mxg agreed with Kiesel, Nunn, et al. (2017) and Kiesel, Wagner, et al. (2017) in that autumn harvests would produce more methane gas (compared to harvest in January). However, GNT-14 maintained its methane yield between October and January allowing the delay of harvesting without compromising methane yields per dry mass. Later harvesting would prevent soil losses and may reduce flood depths and the velocity of overland water flow during winter as the heaviest rainfall events in the UK are October–January (Palmer & Smith, 2013; Rose & Rosolova, 2015; UK MET Office, 2019). Miscanthus and short-rotation coppice willow on floodplains have been demonstrated to act like a "green leaky dam" holding back water and slowing flow into waterways (Rose & Rosolova, 2015). Therefore, the maintenance of stem biomass over the winter period is beneficial to mitigate flooding and protect the soil. Producing biogas from Miscanthus grown on marginal, or waterlogging-prone land would displace the use of maize from prime agricultural land that can then be returned to food or fodder cropping, improving food security.

Besides NSC, anaerobic digestion also draws from other components such as cellulose from the cell wall material (Azman et al., 2017; Golkowska & Greger, 2013). Lignin is known to have a negative effect on biofuel production (Zeng, Zhao, Yang, & Ding, 2014), particularly during enzymatic hydrolysis (Qin et al., 2016). This occurs by restricting access to the cellulose in the cell wall material (Jönsson, Alriksson, & Nilvebrant, 2013). Therefore, it is not surprising that several studies have concluded a negative impact on anaerobic digestion (den Camp, Verhagen, Kivaisi, & de Windt, 1988; da Costa et al., 2014; Koyama, Yamamoto, Ishikawa, Ban, & Toda, 2017; Yin, Seo, Kim, & Lee, 2000). It has also been reported that under similar lignin contents, higher cellulose content was correlated to higher methane potential (Whittaker et al., 2016). Interestingly, in our study when NSC was removed, Mxg showed no higher methane production despite having a higher cellulose content. However, Mxg also had a higher lignin content, suggesting there may have been an antagonistic relationship between available substrate and accessibility. This suggests that neither lignin nor cellulose on their own held the highest determining factor in gas production.

Pretreating Miscanthus biomass has been reported to facilitate more effective conversion to biofuels (Fu et al., 2018; Hongqiang, Li, Sang, & Xu, 2013; Li, Liu, Nges, & Liu, 2016; Nges et al., 2016; Zhou, Li, Zhang, & Gu, 2017; Zhu, Macquarrie, Simister, Gomez, & McQueen-Mason, 2015). Pretreatments include hydrothermal- and microwave-assisted chemical pretreatment. Out of these reports, Zhu et al. (2015) reported that microwave and chemical pretreatment resulted in 7 times more ethanol production when compared with untreated Miscanthus feedstock. Similarly, Zhou et al. (2017) reported a 50% reduction in digestion time after Miscanthus material had undergone hydrothermal pretreatment. Both these examples demonstrated large improvements in fuel production when biomass was pretreated, but it is important to consider the balance between the energy and environmental cost of pretreatment technologies against the energy gains, especially in "green" energy systems (Carballa, Duran, & Hospido, 2011). As maize is not usually pretreated prior to AD, we would not advocate for the addition of this step in future Miscanthus varieties.

When the NSC was removed during the wash experiment, the superior gas yields of GNT-14 over Mxg disappeared.

NSC is the most readily available form of carbon for microbial metabolism and fermentation (i.e biogas and bioethanol) and, as such, has been recognized as a main contributor to bioenergy (Henry, 2010; Mielenz, Rodriguez, Thompson, Yang, & Yin, 2015; Rooney, Blumenthal, Bean, & Mullet, 2007; Williams, Westover, Emerson, Tumuluru, & Li, 2016). Our study has confirmed that high carbohydrate genotypes of Miscanthus that can outperform the current commercial cultivar are available and increase its competitiveness with maize.

In previous publications (Allison, Morris, Clifton-Brown, Lister, & Donnison, 2011; Maddison et al., 2017), the variation in lignin between diverse genotypes was only ~3% DM, which is consistent with our findings. However, previous reports of cellulose variation showed ~5% range between diverse genotypes, but in our study both genotypes increased in cellulose by 5%–7% between October and January. Cellulose is formed from UDP glucose produced during the metabolism of sucrose by sucrose synthase (SuSy) (Amor, Haigler, Johnson, Wainscott, & Delmer, 1995; Baroja-Fernández et al., 2012; Coleman, Yan, & Mansfield, 2009). Free glucose and fructose are also formed from the metabolism of sucrose via SuSy (fructose and UDP-glucose) and also invertase (glucose and fructose) (Koch, 2004; Ruan, 2014; Smith, Zeeman, & Smith., 2005). The increase in cellulose concentration over winter could be attributable to leaf losses between the two harvest points as the leaf has lower concentrations of cellulose than the stem (Mangold, Lewandowski, Möhring, et al., 2019). Alternatively, it could mean that the sucrose metabolism and cellulose biosynthetic pathways were still functional, which would be remarkable because the average daily minimum temperature in this time period was <6°C (dropping to <4°C in November) (UK Met Office, 2018). In support of the continued functioning of the carbohydrate metabolic pathways was the observation that in GNT-14 the concentration of hexoses (glucose and fructose) also increased. As sucrose did not decline over the same time period in this genotype, there are only two explanations for this increase (a) GNT-14 continued to photosynthesize and fix carbon over the winter period or (b) resources stored in the rhizome were remobilized to the above ground stems. If GNT-14 was drawing from stored rhizome reserves, this could jeopardize future yields owing to the exhaustion of the rhizome before spring, but as the yields of GNT-14 are at least equal to Mxg this does not appear to be the case.

It should be noted that harvested materials for AD are usually ensiled, a process that has been demonstrated to improve the biogas yields from Miscanthus (Mangold, Lewandowski, Hartung, et al., 2019). Both starch and sugar contents of ensiled biomass have been positively correlated with biomethane potential (BMP) but starch more highly than sugar ($r = 0.97$ and 0.74, respectively) (Whittaker et al., 2016). Both NSCs would benefit silage quality and stability. As starch was in greater concentration in GNT-14 than Mxg we would expect it to retain its advantage during ensiling As previously suggested by Purdy et al. (2017), targeting starch for improvement may be a sensible approach to breeding Miscanthus varieties tailored to AD.

When green harvested Mxg was compared with Maize, it was found that maize produced double the amount of methane gas per kg of dry matter (Whittaker et al., 2016). However, the differences have to be considered in light of the biomass that Mxg can produce per area when compared with maize. In the UK, Miscanthus yield is currently still lower than maize (14 Mg/ha vs. 17 Mg/ha) (BSPB, 2015, 2016, 2017; Hastings et al., 2017). So by yield, maize is still superior to both biomass and methane production but the use of GNT-14, instead of Mxg, closed the gap by 17% making GNT-14's methane yields 67% that of maize. Breeding efforts that prioritize NSC composition as well as yield would reduce this gap even further.

Another consideration is to harvest Miscanthus in autumn instead of winter when yield has been shown to be higher (Nunn et al., 2017). Nunn et al. (2017) investigated a number of genotypes including Mxg showing the Autumn (Oct) harvest on average has 1.3 times more dry matter compared with winter harvest. However, even with the estimated increased dry matter per hectare at the autumn harvest, Miscanthus (both Mxg and GNT-14) methane production still fell short when compared with Maize, although the gap was reduced in the case of GNT-14.

When considering the energy balance of biogas generation, the energy cost of producing the biomass should also be factored (Agri-Food & Biosciences Institute, 2017). Fodder maize is currently the choice crop for anaerobic digestion yet the associated cost in energy, which directly related to monetary cost, makes it an expensive energy to produce. Furthermore, the higher CO_2eq. emissions from cultivating maize reduce the mitigation of carbon release, which is one of the goals of renewable energy development (Komor & Bazilian, 2005). In a study comparing the energy balances and CO_2 mitigation potentials of three different energy systems, including maize (for AD) and Miscanthus (for combustion), the overall energy gain was >2.5-fold greater in Miscanthus (Felten et al., 2013). The difference was largely owing to the high-energy fertilizer inputs required for maize cultivation which equated to 2,237 kg CO_2 eq. ha^{-1} per annum (Felten et al., 2013). In contrast, Miscanthus required no additional fertilizer because of the efficiency of the perennial system. Therefore, when considering the amount of energy required and CO_2eq. emitted, Miscanthus is more environmentally sound choice for AD.

While carbon emissions and energy consumption are important parameters to consider, there are other important environmental benefits that Miscanthus can offer. A recent publication by Kiesel, Nunn, et al. (2017) and Kiesel, Wagner, et al. (2017) described a life cycle analysis (LCA)

on Miscanthus, maize and switchgrass for factors including climate change, fossil fuel depletion, terrestrial acidification, freshwater eutrophication, and marine eutrophication. Miscanthus was the best performing of the three species, which supports our finding of better environmental credential for Miscanthus compared to maize.

It should be acknowledged that if the harvest date was brought to forward to October, this would result in a greater loss of nitrogen that would then need to be resupplied the following spring. In a study from Germany, it was found that under field conditions when N was not limiting, there was no benefit of a higher N application, but in a pot study, where N was limited, a positive effect of N fertilization on yield, biomass, and re-growth was observed (Kiesel & Lewandowski, 2017). Therefore, on poorer, marginal soils, replenishment of *N* (ideally sourced from the digestate) is likely to be necessary.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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