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# Nitrogen dynamics in a mature *Miscanthus x giganteus* crop fertilized with nitrogen over a five year period

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**The objective of this study was to investigate N dynamics and response to N fertilization in a mature crop of *Miscanthus x giganteus*. A crop of *Miscanthus x giganteus* sown in 1994 was fertilized with five N rates (0, 38, 63, 90 and 125 kg N/ha/year) over a five year period (2008–2012) in Carlow, Ireland. Foliar chlorophyll concentrations were directly related to N fertilization level throughout the study and rose after N applications until July before falling with the onset of N remobilisation. Shoot numbers were unaffected by N fertilization until the final years of the study when they increased with N level. Crop height was unaffected by fertilization in the early years of the study but in the final years of the study, it increased with N level until July after which the effect diminished. There was a small but significant stimulation of harvested biomass yields in autumn (average 15 t/ha) with increasing N fertilization, but there was no effect on harvested yields in spring (average 10.5 t/ha). The N concentration in the rhizome network gradually built up during the course of the study and was proportional to N application. Aboveground biomass N content was also proportional to N application. Nitrogen remobilisation between the October and February harvests was small; abscissed leaves accounted for most of the N loss over this period. The deleterious environmental consequences of N fertilizer may outweigh any potential economic benefits if increases in biomass production are small or non-existent.**

*Keywords*: height; *Miscanthus*; nitrogen; remobilisation uptake; yield

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## Introduction

Increasing concern about the impact of rising greenhouse-gas (GHG) concentrations together with rapidly decreasing fossil fuel reserves have stimulated interest in renewable sources of energy including bioenergy. Smith *et al*. (2000) reported that energy crops have great potential to mitigate carbon emissions and are likely to be major contributors to the renewable energy mix in the future. Among the perennial grasses, *Miscanthus x giganteus* is a good candidate energy crop that establishes quickly and produces an annual harvest with low moisture content (Clifton Brown, Bruer and Jones 2007). Fast-growing energy crops offer a means of meeting short-medium term energy targets such as those established by the European Union's Renewable Energy Directive (2009/28/EC). The nitrogen (N) requirement of a crop represents a critical component of both its energy and GHG balance, because the N fertilizer manufacturing process is energy intensive and losses of N both during manufacture and after application can have serious local and global environmental impacts.

There is general agreement that the N requirements of *Miscanthus x giganteus* are low compared to other crops (Lewandowski *et al*. 2000; Heaton, Voigt and Long 2004). It is thought that the N requirements of the crop are low, due to high nutrient absorption efficiency, high nutrient use efficiency, significant nutrient recycling and possible contributions from N fixation (Cadoux *et al*. 2012). However, the N fertilization requirements of *Miscanthus x giganteus* are still unclear as relatively few N fertilizer trials have been conducted on *Miscanthus x giganteus* and some studies have reported yield increases following N fertilization while other studies have demonstrated no effect on yield. Consequently, the exact needs of the crop, particularly in relation to different soils, have still not been defined (Cadoux *et al*. 2012). Studies conducted in Germany (Lewandowski and Schmidt 2006; Boehmel, Lewandowski and Claupein 2008), Italy (Ercoli *et al*. 1999; Cosentino *et al*. 2007), Turkey (Acaroglu and Aksoy 2005), the United Kingdom (Shield *et al*. 2014) and the United States (Wang *et al*. 2012; Arundale *et al*. 2014; Haines *et al*. 2014) have reported that yield is stimulated after the application of N fertilizer. However, other studies conducted in Austria (Schwarz *et al*. 1994), Germany (Himken *et al*. 1997), Greece (Danalatos, Archontoulis and Mitsios 2007), United Kingdom (Beale and Long 1997b; Christian, Riche and Yates 2008), Ireland (Clifton-Brown *et al.* 2007) and the United States (Maughan *et al*. 2012; Davis *et al*. 2014) reported that there was no yield response to N fertilization.

Geography alone would not appear to explain the lack of response to N as studies with no response have a similar geographical spread to those studies which show a response to N application. High yields of 25–30 t/ha DM (tonnes per hectare of dry matter) and 38 t /ha DM have been reported in studies where there was no response to N application at rates of up to 180 kg N/ha (Danatalos *et al*. 1997; Himken *et al.* 1997) which suggests that yield level does not necessarily influence whether there is a response to the application of N fertilizer. The authors of some of the studies with no response to N have suggested that soil factors such as high soil N levels and/or high mineralisation rates contributed to the lack of response (Schwarz *et al*. 1994; Himken *et al*. 1997; Clifton-Brown *et al*. 2007; Christian *et al*. 2008; Haines *et al*. 2014). Cadoux *et al*. (2012) studied the nutrient requirements of *Miscanthus x giganteus* based on a review of past studies and suggested that there was a large contribution from soil N in experiments which demonstrated no response to N application. Miguez *et al*. (2008) conducted a meta-analysis of the effects of various management factors on *Miscanthus x giganteus* biomass production and concluded that N fertilizer did not have an important effect during the first three growing seasons but that it did seem to have a relatively small effect on biomass production in the long term. However, Christian *et al*. (2008) applied N fertilizer to *Miscanthus x giganteus* over 14 successive harvests but found no response to N application.

Several studies have investigated the dynamics of N during the annual growing season of *Miscanthus x giganteus*. Beale and Long (1997a) reported that nutrient flow from the rhizome network to aboveground biomass increased until July when maximum levels were attained in aerial biomass and minimum levels were attained in the rhizome network. Nutrient flow was then reversed as the nutrient content in aboveground biomass declined and the rhizome nutrient content increased. Accumulation of N in aboveground biomass during the early part of the growing season can be dependent on belowground N reserves while autumn remobilisation is linked to aboveground N treatments (Strullu *et al*. 2011). Furthermore, Strullu *et al*. (2013) demonstrated that the growth rate of leaf area index is dependent on belowground N stocks before regrowth. Heaton, Dohleman and Long (2009) looked at the N dynamics of *Miscanthus x giganteus* crops grown at northern, central and southern locations in Illinois and found that N remobilization was essentially complete by December. Himken *et al*. (1997) reported that N fertilization had no effect on the N concentration in shoots although the N content in rhizome dry matter increased with N fertilization. In contrast, Strullu *et al*. (2011) found that N fertilization increased N concentration in both aboveground and belowground biomass. Thus, while there is either no response or a minor response of biomass yield to N fertilization, N recycling in the plant throughout the year is highly dynamic.

Matching N fertilizer application rates to crop demand can maintain or increase biomass production and thus bring environmental benefits in terms of increased GHG mitigation. Additionally, economic benefits may accrue to the farmer. However, excessive applications of N may lead to increased emissions of nitrous oxide ($N_2O$) (Crutzen *et al*. 2008; Davis *et al*. 2014; Roth *et al*. 2014) and increased leaching of N to groundwater after application (Christian & Riche 1998; Davis *et al*. 2014). Furthermore, excessive uptake of N by *Miscanthus x giganteus* can lead to increased concentrations of N in harvested biomass (Strullu *et al*. 2011). Emissions of $N_2O$ during combustion increase with increasing biomass N concentration (Houshfar *et al*. 2012a; Houshfar, Lovas and Skreiberg 2012b). Additionally, emissions of oxides of nitrogen ($NO_x$) increase with increasing fuel N content (Sommersacher, Brunner and Obernberger 2012). Although the radiative forcing of $NO_x$ is considered neutral, $NO_x$ is an aerial pollutant which causes damage to human health while N emissions to air can also indirectly cause eutrophication.

It is thus important to continue to define and understand the N requirements of *Miscanthus x giganteus*. Almost all previous N studies have been conducted on *Miscanthus x giganteus* crops in the first few years of their life cycle and only one previous study (Christian *et al*. 2008) applied N to *Miscanthus x giganteus* crops up to 14 years old. Thus, the objective of this study was to study N dynamics and yield response to N fertilization in a mature stand of *Miscanthus x giganteus* that had remained unfertilized during the first 13 years of its life cycle.

## Methodology

*Experimental layout*

The experiment was conducted at Oak Park Research Centre, Carlow, Ireland (52.86° N, 6.90° W) on a Eutric Cambisol soil (FAO 1998). The soil has one true soil layer which extends down to 35 cm below the surface and is a sandy loam (Conry and Ryan 1967).

The N trial was laid out in 2008 on a crop of *Miscanthus x giganteus* established in 1994. The target plant density was 10,000 plants/ha, a planting density of 11,188 plants/ha was measured in October 1994. The crop was established from microplantlets and remained unfertilized until 2007 when 70 kg N/ha were applied to the experimental area. Weeds were controlled periodically by spraying Roundup® (Glyphosate) during late March before shoot emergence. Meteorological data was collected from a synoptic weather station located at the research centre.

The experimental design was a randomised complete block with four replications. Plots measured 5 m by 25 m. Treatments were 0, 38, 63, 90 and 125 kg N/ha of N fertilizer (calcium ammonium nitrate) applied in May/June of each year (Table 1). A calibrated seed drill with coulters removed was used to apply the N fertilizer. All plots received an annual base dressing of 30 kg phosphorus (P)/ha, 120 kg potassium (K)/ha and 20 kg sulphur (S)/ha.

*Leaf chlorophyll concentration*

Leaf chlorophyll concentration was used as an indicator of leaf nitrogen concentration. Evans (1989) reported a strong linear relationship between leaf nitrogen content and chlorophyll within species, and leaf chlorophyll meters (SPAD method) have been successfully used to assess leaf N status in many crops (Minotti, Halseth and Sieczka 1994; Chapman 1997; Weih and Ronnberg-Wastljung 2007). Leaf chlorophyll concentration was measured on 30 stems in each plot using a Minolta SPAD 502 meter (Minolta Camera Company, 3-13, 2-Chome, Azuchi-Machi, Osaka 541, Japan). Measurements of leaf chlorophyll concentration were carried out in the middle of the first fully expanded leaf i.e., the highest leaf on the stem with a ligule. The chlorophyll concentration of the first three fully expanded leaves was also measured on the same day during the final three growing seasons on either one date (2010, 2012) or on two dates (2011).

*Stem numbers and plant height*

Stem numbers and stem height were measured at regular intervals to ascertain the influence of N application on the primary components of biomass yield. Stem numbers were counted in all years (except 2010) in 6 quadrats in each plot, each quadrat measuring 1 $m^2$. Stem height was measured on several occasions during each growing season. Height was

**Table 1. Dates on which N fertilizer was applied and harvesting operations were carried out**

| | Fertilizer application | Autumn harvest | Spring harvest | Rhizome harvest |
|---|---|---|---|---|
| 2008 | 6th May | 20th Oct | – | |
| 2009 | 12th May | 14th Oct | – | |
| 2010 | 2nd June | 8th Nov | – | |
| 2011 | 12th May | 26th Oct | 28th Feb | |
| 2012 | 9th May | 25th Oct | 12th Feb | 26th Feb |

measured to the highest leaf ligule on 50 stems on each plot.

### *Aboveground and belowground biomass*

Biomass harvest dates are given in Table 1. In 2008, three 1 $m^2$ quadrats were harvested from each plot during October. In 2009 and 2010, the plots were divided in two at harvest and a swath was harvested from each side of the plot (total area, 1.25 m × 20 m). The fresh weight of the harvested material was determined in the field using a Salter Brecknell WB6200 (1000 Armstrong Drive, Fairmont, MN, 56031, USA) weighing system before 10 stems were taken from each plot and separated into leaf and stem for dry matter analysis. After the autumn harvests in 2008, 2009 and 2010, the remaining plot areas were harvested during the following March using a self-propelled forage harvester.

During 2011 and 2012, the plots were divided into two parts for harvesting purposes. Half of the plot (2.5 m × 20 m) was harvested in October. The other half of the plot was harvested during the following February. Fresh weight of the harvested material and dry matter content was determined as described above.

At the end of the experiment, rhizomes and root material were harvested from three 1 m by 1.4 m quadrats from each plot using a small tracked mechanical digger. Rhizomes were washed and weighed to determine fresh weight before a sample was taken for dry matter analysis. All samples for dry matter analysis were dried at 50 °C until constant weight was reached.

### *Nitrogen concentration*

Three samples of rhizome per plot were dug up on 4th November 2008, on January 12th 2011 and on February 12th 2012. Samples of rhizome (three per plot) were also taken from the rhizome harvest on February 26th 2013. Rhizome samples were washed before being dried at 50 °C. Dried samples were ground through a 1 mm screen before N content was measured with a LECO FP-238 (LECO Corporation, 3000 Lakeview Avenue, St Joseph, Michigan 49085-2396).

Stem and leaf samples from the autumn biomass harvests in 2008, 2010, 2011 and 2012 and the spring biomass harvests in 2011 and 2012 were dried at 50 °C and ground through a 1 mm screen before N content was measured as described above.

### *Nitrogen content in plant parts*

Nitrogen content is defined here as the quantity of N contained in plant parts. Nitrogen content was calculated by multiplying biomass quantity expressed on an area basis by the corresponding N concentration. The change in the N content between autumn and spring harvests was calculated by subtracting the value of leaf and stem N contents at the spring harvests from the value of leaf and stem N contents at the corresponding autumn harvests. The decrease in leaf N content was further divided into N loss through remobilisation and nitrogen loss from leaves dropping onto the ground. Nitrogen lost through remobilisation was calculated by subtracting the N content in the leaves at the spring harvest from the nitrogen content in the same mass of leaves at the autumn harvest. Loss of N content from leaves dropping to the ground was calculated by subtracting the N content lost through remobilisation from the overall decrease in leaf N content.

### *Soil analysis*

Three soil cores to a depth of 25 cm were sampled at random from each plot in February 2013. Organic matter was determined by the loss on ignition method (Ball 1964), total nitrogen and total carbon were determined using an elemental analyser (LECO Corporation, 3000 Lakeview

Avenue, St Joseph, Michigan 49085-2396). Potassium, phosphorus and magnesium were analysed using Morgan's method (Gallagher, Ryan and Brogan 1961).

### *Statistics*

The results were analysed by analysis of variance using Proc GLM procedure (SAS 2009). Pairwise differences between treatments were evaluated using Tukey's test.

## Results

### *Meteorological data*

The 2009 growing season (April to March) was particularly wet (1147 mm) but was followed by two relatively dry years [2010 (763 mm) and 2011 (666 mm)], the rainfall in the first and last years of the study was intermediate between these two extremes. Highest maximum temperatures were recorded during the 2010 (33.3 °C) and 2011 (30.9 °C) growing seasons whereas the lowest minimum temperatures were recorded during the winters which followed the 2009 (–12.1 °C) and 2010 (–12.9 °C) growing seasons. Average temperatures were highest during the 2011 growing season (10.4 °C).

### *Chlorophyll concentrations*

Leaf chlorophyll concentration in the first fully expanded leaf increased with increasing N application (selected data shown in Figure 1). With some exceptions, this relationship was statistically significant throughout the growing season in each year of the study. Measurements made on each date are not directly comparable as measurements were not necessarily made on the same leaf. However, there was a clear trend of decreasing leaf chlorophyll concentrations in all treatments from early-mid July until the end of the growing season in each of 2009, 2010, 2011 and 2012. Chlorophyll measurements were not made during the latter part of the 2008 growing season. Leaf chlorophyll concentrations throughout the study ranged from 30 to 50 SPAD units and chlorophyll concentrations in the leaves of the treatment which received the highest N fertilization rate were generally about 5 SPAD units higher than chlorophyll concentrations in the leaves of the control (Figure 1).

Measurements of leaf chlorophyll concentration in the first three fully expanded leaves are shown in Table 2. On each occasion, there was a highly significant effect ($P < 0.0001$) of N application with leaf chlorophyll concentration increasing with N application rate. There was also a clear gradient in chlorophyll concentration from the oldest leaf (leaf 3) where the chlorophyll concentration was highest to the youngest leaf (leaf 1) where the chlorophyll concentration was lowest ($P < 0.0001$). The only exception to this was during 2012 when the highest chlorophyll concentration was measured in leaf 2 (Table 2). However the differences in the chlorophyll concentrations of the three different leaves were small, typically 2–3 SPAD units.

### *Plant height and daily growth rate*

The addition of N fertilizer had no significant effect on height during the 2008, 2009 and 2010 growing seasons. Similarly, there were no significant differences in daily growth rate between treatments in these three years (data not shown). However, during the 2011 growing season, height increased with N rate during the early part of the growing season (Table 3) and there was a statistically significant effect of N fertilizer on height on 27/6/2011 ($P < 0.01$) and 20/7/2011 ($P < 0.05$). Thereafter, the differences in height between the different

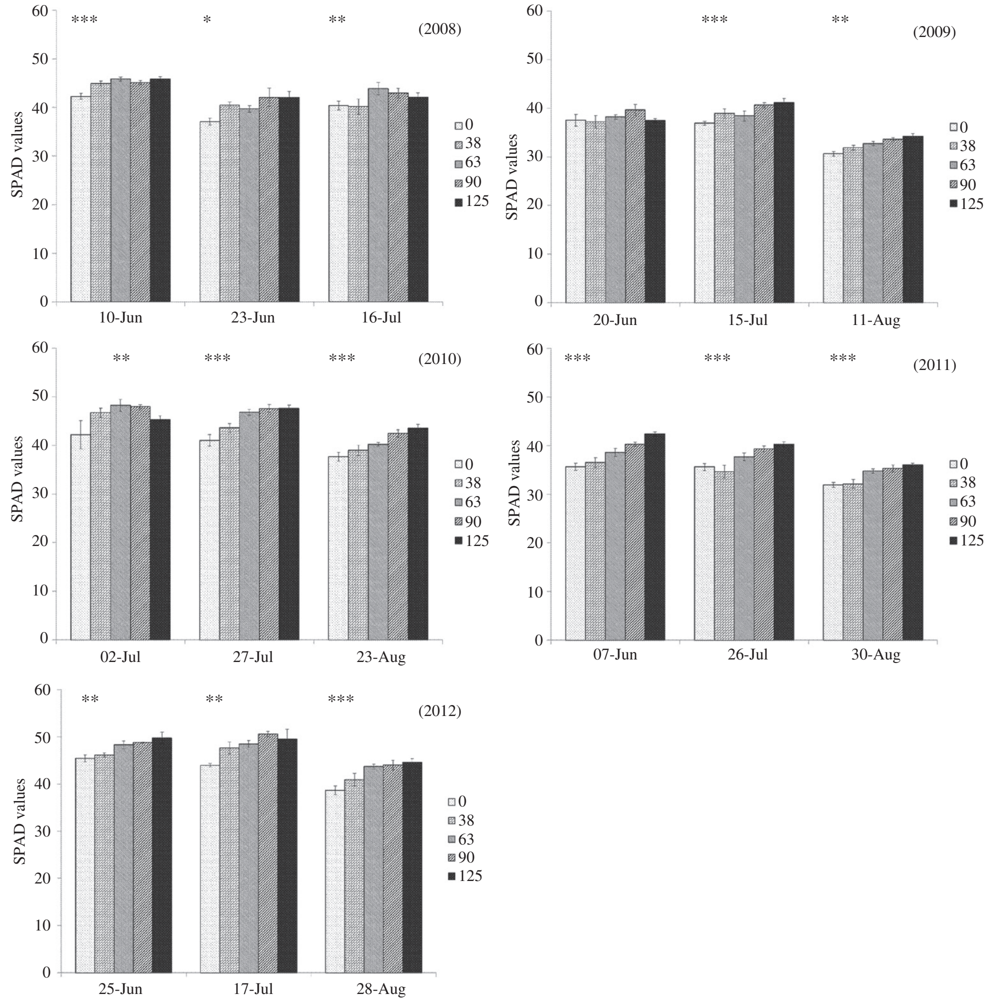

*Figure 1. Leaf chlorophyll measurements in the first fully expanded leaf of* Miscanthus x giganteus *during the growing seasons of 2008, 2009, 2010, 2011 and 2012. Measurements are presented in SPAD values. Error bars illustrate standard error of the mean.*

treatments gradually diminished until the original relationship between N fertilization and height was reversed with plant height decreasing with increasing N fertilization rate. This effect was statistically significant on 1/11/2011 ($P < 0.05$) when plant height decreased with increasing N fertilization rate (Table 3). On this date, plants in the unfertilized control plots were 30 cm taller than those in the treatment which received the highest level of N application.

In the 2012 growing season, plant height also increased with N fertilization during the early part of the growing season. There was a significant effect of fertilization on plant height on 18/6/2012 ($P < 0.01$) as well as on 9/7/2012 ($P < 0.05$). However,

**Table 2. Leaf chlorophyll concentrations in leaves at different positions on the stems of *Miscanthus x giganteus*, and the effect of nitrogen level. Leaf 1 is the fully expanded leaf at the top of the stem. Measurements are presented in SPAD units. Means followed by the same letter are not significantly different**

| Date | Chlorophyll concentrations | | | |
|---|---|---|---|---|
| | 27/7/10 | 26/7/11 | 29/08/11 | 17/7/12 |
| *Leaf position* | | | | |
| Leaf 1 | 45.3$^{b}$ | 37.5$^{c}$ | 34.0$^{c}$ | 48.1$^{b}$ |
| Leaf 2 | 48.0$^{a}$ | 38.9$^{b}$ | 36.0$^{b}$ | 51.9$^{a}$ |
| Leaf 3 | 49.0$^{a}$ | 40.1$^{a}$ | 37.3$^{a}$ | 48.5$^{b}$ |
| *Nitrogen level (kg N/ha)* | | | | |
| 0 | 43.0$^{d}$ | 37.3$^{c,d}$ | 34.3$^{b}$ | 43.9$^{c}$ |
| 38 | 45.1$^{c}$ | 36.5$^{d}$ | 34.5$^{b}$ | 48.7$^{b}$ |
| 63 | 48.3$^{b}$ | 38.9$^{b,c}$ | 36.5$^{a}$ | 49.8$^{a,b}$ |
| 90 | 49.9$^{a,b}$ | 40.2$^{a,b}$ | 36.6$^{a}$ | 52.1$^{a}$ |
| 125 | 50.7$^{a}$ | 41.3$^{a}$ | 37.1$^{a}$ | 52.6$^{a}$ |
| *Statistical analysis* | | | | |
| Leaf | *** | *** | *** | *** |
| Nitrogen | *** | *** | *** | *** |
| Leaf*Nitrogen | | | | |

there was no significant difference in height between any of the treatments for the rest of the growing season (Table 3).

N fertilization increased daily growth rate during the early part of both the 2011 and 2012 growing seasons but this effect diminished later in the growing seasons (data not shown). In 2011, there were no significant differences in growth rate between treatments up until 20th July. Thereafter, daily growth rate decreased with increasing N fertilization rate, this relationship was most evident between 13th August and 31st August ($P < 0.0001$) and between 31st August and 12th September ($P = 0.0001$). Daily growth rate increased with N fertilization ($P = 0.0451$) at the start of the 2012 growing season between 18th June and 29th June. However, there was no significant effect

**Table 3. The effect of N fertilization on stem height of *Miscanthus x giganteus*, 2011–2012. Means followed by the same letter are not significantly different**

| | Stem height measurements (cm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Date | 27/6/11 | 20/7/11 | 5/8/11 | 25/8/11 | 1/11/11 | 18/6/12 | 9/7/12 | 31/7/12 | 31/8/12 | 12/9/12 |
| N level | | | | | | | | | | |
| 0 | 85.3$^{c}$ | 152.9 | 183.9 | 204.5 | 248.2$^{a}$ | 42.5$^{b}$ | 100.3$^{b}$ | 155.4 | 208.0 | 225.2 |
| 38 | 87.0$^{b,c}$ | 148.9 | 180.5 | 195.5 | 216.5$^{a,b}$ | 41.7$^{b}$ | 105.9$^{a,b}$ | 158.4 | 212.8 | 224.9 |
| 63 | 99.1$^{a,b}$ | 166.3 | 202.1 | 214.0 | 233.1$^{a,b}$ | 45.7$^{a,b}$ | 110.6$^{a,b}$ | 166.9 | 220.5 | 235.7 |
| 90 | 98.6$^{a,b,c}$ | 164.1 | 195.8 | 203.4 | 215.0$^{a,b}$ | 48.2$^{a}$ | 115.8$^{a,b}$ | 162.7 | 215.5 | 232.4 |
| 125 | 106.3$^{a}$ | 167.2 | 192.0 | 199.1 | 217.9$^{b}$ | 49.2$^{a}$ | 112.7$^{a}$ | 164.3 | 217.4 | 230.5 |
| *Statistical analysis* | | | | | | | | | | |
| Nitrogen | ** | * | | | * | ** | * | | | |

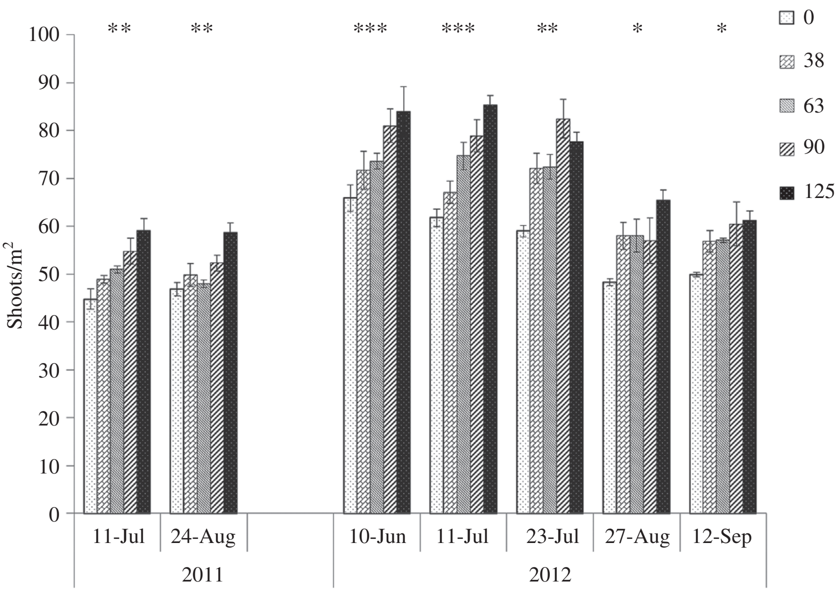

*Figure 2. Shoot numbers per m$^2$ of* Miscanthus x giganteus *in the years 2011–2012. Error bars indicate standard error of the mean.*

of N fertilization rate on daily growth rate during the remainder of the 2012 growing season.

*Shoot density measurements*

Nitrogen fertilization had no significant effect on shoot numbers in 2008 or in 2009, and there were no measurements of shoot density in 2010. Measurements of shoot numbers in 2011 and 2012 are shown in Figure 2. In 2011, there was a significant effect of N fertilization on shoot numbers during the early part of the growing season (11/7/2012; $P < 0.01$) as well as towards the end of the growing season (24/8/2011; $P < 0.01$). Fertilizer application increased shoot numbers by approximately 25% on each of these dates. In 2012, shoot density was measured on five occasions from early in the growing season (10/6/2012) until late in the growing season (12/9/2012). N fertilization had a significant effect on shoot numbers on all of these occasions with shoot numbers increasing with N application. At the end of the growing season, shoot numbers in the 125 kg/ha N treatment were 35% higher than those in the control.

*Biomass yield*

During the 2008 autumn harvest, N application had no significant effect on biomass yield. Fertilization had a significant effect on total biomass yield harvested in autumn during the 2009 to 2012 period ($P < 0.05$) (Table 4). There was a significant effect of year from 2009 to 2012 ($P < 0.0001$), yields from 2011 were lowest of all four years probably because this was a relatively dry growing season with low rainfall. There was no significant interaction between year and N response on total biomass yield. Nitrogen application had no significant effect on either stem or leaf biomass yields harvested in autumn, although the trend was for stem and leaf yields to increase with N application.

**Table 4. The effect of N fertilization on autumn biomass yields (2009–2012) and spring biomass yields (2011–2012) of *Miscanthus x giganteus*. All figures are in tonnes of dry matter per hectare. Means followed by the same letter are not significantly different**

| | Autumn stem yield | Autumn leaf yield | Autumn total yield | Spring stem yield | Spring leaf yield | Spring total yield |
|---|---|---|---|---|---|---|
| *N level (kg/ha N)* | | | | | | |
| 0 | 10.8 | 3.7 | 14.5 | 9.5 | 1.2 | 10.7 |
| 38 | 10.7 | 3.7 | 14.3 | 8.0 | 1.1 | 9.1 |
| 63 | 11.3 | 4.0 | 15.3 | 10.1 | 1.3 | 11.4 |
| 90 | 11.7 | 4.2 | 15.8 | 9.6 | 1.2 | 10.8 |
| 125 | 11.6 | 4.0 | 15.5 | 9.6 | 1.3 | 10.9 |
| *Year* | | | | | | |
| 2009 | 11.9$^{a}$ | 4.0$^{a}$ | 15.9$^{a}$ | – | – | – |
| 2010 | 12.0$^{a}$ | 4.3$^{a}$ | 16.3$^{a}$ | – | – | – |
| 2011 | 10.0$^{b}$ | 2.9$^{b}$ | 12.9$^{c}$ | 7.6$^{b}$ | 1.3$^{a}$ | 9.1$^{b}$ |
| 2012 | 10.9$^{a,b}$ | 4.3$^{a}$ | 15.2$^{a}$ | 10.9$^{a}$ | 1.2$^{b}$ | 12.1$^{a}$ |
| *Statistical analysis* | | | | | | |
| Nitrogen | | | * | | | |
| Year | *** | *** | *** | *** | * | *** |
| Nitrogen*Year | | | | | | |

Nitrogen application had no significant effect on spring harvested stem yields, leaf yields or total yields in the two final years of the study (Table 4). There were significant differences between years as yields in 2011 were significantly lower than in 2012 but there was no significant interaction between year and N response. Total biomass yields declined by 30% between the autumn and spring harvests in 2011 and by 20% in 2012. Stem yields declined by 25% and leaf yields by 55% between autumn and spring harvests in 2011. There was no difference in stem yields between autumn and spring harvests in 2012 whereas leaf yields declined by 72%.

### *Stem and leaf nitrogen – autumn harvests*

There was no effect of treatment on N concentrations in stem and leaf at the end of the first growing season (stem N = 0.5%; leaf N = 1.6%). However, stem and leaf N concentration at the autumn harvest during 2010 to 2012 increased with N application ($P < 0.0001$). Year had a significant effect on stem and leaf N concentration ($P < 0.0001$) as N concentration increased from year to year during this period (Table 5). The response of stem N concentration to treatment was dependent on year ($P < 0.05$). Leaf N concentration was over twice that of stem N concentration. In contrast, stem and leaf N content at the autumn harvest expressed as kg N/ha were similar reflecting the higher proportion of stem in total biomass. Stem, leaf and total N content all increased with N application ($P < 0.0001$). Year had a significant effect on N content in plant parts. N content at the autumn harvest increased from 2010 to 2012 although N content in 2011 was lower than the other two years reflecting lower yields in 2011. At the autumn harvest, the highest N treatment had 33 kg N/ha additional to that of the control.

### *Rhizome and root biomass, nitrogen concentration and nitrogen content*

There was no effect of N application on rhizome N concentration at the end of the first growing season (rhizome N = 0.92%).

**Table 5. The effect of N fertilization on N concentration and N content in stem and leaf material of *Miscanthus x giganteus* measured in October over a three-year period (2010–2012). The effect of N fertilization on rhizome N concentrations measured in early spring over the same three-year period together with underground biomass and nitrogen content in February 2013. Means followed by the same letter are not significantly different**

|  | Stem N (%) | Leaf N (%) | Stem nitrogen content (kg N/ha) | Leaf nitrogen content (kg N/ha) | Total nitrogen content (kg N/ha) | Rhizome N (%) | Under-ground biomass February 2013 (t/ha DM) | Under-ground nitrogen content February 2013 (kg N/ha) |
|---|---|---|---|---|---|---|---|---|
| *Treatment* |  |  |  |  |  |  |  |  |
| *N level (kg N/ha)* |  |  |  |  |  |  |  |  |
| 0 | 0.34$^{c}$ | 1.00$^{c}$ | 35.1$^{c}$ | 35.7$^{b}$ | 70.8$^{c}$ | 0.73$^{c}$ | 16.9 | 120.0$^{a,b}$ |
| 38 | 0.38$^{b,c}$ | 1.10$^{b,c}$ | 41.5$^{b,c}$ | 40.7$^{a,b}$ | 82.2$^{b,c}$ | 0.81$^{b,c}$ | 15.6 | 128.4$^{a,b}$ |
| 63 | 0.44$^{a,b}$ | 1.17$^{a,b}$ | 46.4$^{a,b}$ | 46.3$^{a}$ | 92.7$^{a,b}$ | 0.87$^{b}$ | 14.3 | 115.8$^{b}$ |
| 90 | 0.47$^{a}$ | 1.18$^{a,b}$ | 54.7$^{a}$ | 47.9$^{a}$ | 102.6$^{a}$ | 1.04$^{b}$ | 14.6 | 145.3$^{a}$ |
| 125 | 0.50$^{a}$ | 1.27$^{a}$ | 56.4$^{a}$ | 47.3$^{a}$ | 103.7$^{a}$ | 1.14$^{a}$ | 12.8 | 143.8$^{a,b}$ |
| *Year* |  |  |  |  |  |  |  |  |
| 2010 | 0.34$^{b}$ | 0.84$^{c}$ | 41.6$^{b}$ | 36.9$^{b}$ | 78.5$^{b}$ | 1.03$^{a}$ | – | – |
| 2011 | 0.39$^{b}$ | 1.23$^{b}$ | 39.2$^{b}$ | 35.3$^{b}$ | 74.5$^{b}$ | 0.84$^{b}$ | – | – |
| 2012 | 0.54$^{a}$ | 1.37$^{a}$ | 59.7$^{a}$ | 58.6$^{a}$ | 118.2$^{a}$ | 0.89$^{b}$ | – | – |
| *Statistical analysis* |  |  |  |  |  |  |  |  |
| Nitrogen | *** | *** | *** | *** | *** | *** |  | * |
| Year | *** | *** | *** | *** | *** | *** | – | – |
| Nitrogen * Year | * |  |  | * | * |  | – | – |

**Table 6. The effect of N fertilization and year of sampling on N concentration and N content in stem and leaf material of *Miscanthus x giganteus* measured at the spring harvest (February) after the 2011 and 2012 growing seasons. Means followed by the same letter are not significantly different**

| | Stem N (%) | Leaf N (%) | Stem nitrogen content (kg N/ha) | Leaf nitrogen content (kg N/ha) | Total nitrogen content (kg N/ha) |
|---|---|---|---|---|---|
| *Nitrogen level (kg N/ha)* | | | | | |
| 0 | $0.34^{b}$ | $0.78^{b}$ | $33.7^{b}$ | 10.0 | $43.7^{b,c}$ |
| 38 | $0.37^{b}$ | $0.94^{a}$ | $31.4^{b}$ | 10.1 | $41.5^{c}$ |
| 63 | $0.39^{b}$ | $0.94^{a}$ | $43.1^{a,b}$ | 12.2 | $55.3^{a,b}$ |
| 90 | $0.53^{a}$ | $0.90^{a,b}$ | $52.3^{a}$ | 10.6 | $62.9^{a}$ |
| 125 | $0.52^{a}$ | $0.96^{a}$ | $51.5^{a}$ | 12.5 | $63.9^{a}$ |
| *Year* | | | | | |
| 2011 | $0.30^{b}$ | $0.81^{b}$ | $23.3^{b}$ | 10.6 | $33.9^{b}$ |
| 2012 | $0.56^{a}$ | $0.99^{a}$ | $61.6^{a}$ | 11.5 | $73.1^{a}$ |
| *Statistical analysis* | | | | | |
| Nitrogen | *** | ** | *** | | *** |
| Year | *** | *** | *** | | *** |
| Nitrogen*Year | | * | | | |

However, there was a significant effect of N application on rhizome N concentration over the 2010 to 2012 period ($P < 0.0001$). Rhizome N concentration increased with N application during this period (Table 5). Year had a significant effect on rhizome N

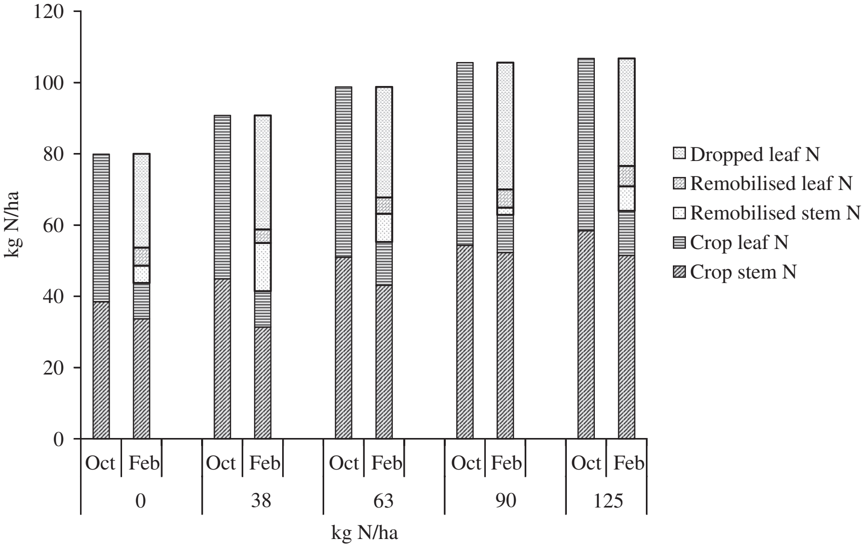

*Figure 3. Nitrogen content in* Miscanthus x giganteus *at October and February, and based on the average of the 2011 and 2012 harvests. The figure shows N content of stem and leaf at each harvest together with the quantity of N lost between October and February harvests divided into N lost through remobilisation and N lost in dropped leaves. Areas of the graph with a shaded background represent N present in the crop whereas areas of the figure with a light background represent N loss between the autumn and spring harvests.*

concentration which generally decreased over the three year period (P < 0.0001). Rhizome and root biomass was quantified in February 2013 at the end of the trial when underground biomass was found to decrease with increasing N application rate. However, the overall N effect was not statistically significant. Rhizome N at the end of the study content was found to increase with N level (P < 0.05), there was an additional 24 kg N/ha in the rhizome network of the 125 kg N/ha treatment compared to that of the control.

### *Stem and leaf nitrogen – spring harvests 2011–2012*

Stem N concentration (Table 6) at the spring harvest increased with N application (P < 0.0001) and was significantly higher in 2012 compared to 2011 (P < 0.0001). Similarly, leaf N increased with N application (P < 0.01) and was significantly higher in 2012 compared to 2011 (P < 0.0001) although there was an interaction between treatment and year (P < 0.05). Leaf and stem N concentrations during the spring harvests were lower than those at the autumn harvests. Stem and total N content (kg N/ha) increased with N application (P < 0.0001) and were higher in 2012 compared to 2011 (P < 0.0001) although there was no effect of either treatment or year on leaf nitrogen content at spring harvest. N treatment had no significant effect on the quantity of N lost between autumn and spring in either stem or leaf (Figure 3). However, there were significant differences between years as more N was lost from stems in 2011 compared to 2012 (P < 0.0001) whereas, in contrast, more N was lost from leaf in 2012 compared to 2011 (P < 0.0001). N treatment had no effect on the quantity of N lost through either remobilisation or leaf loss. Significantly more leaf N was lost from remobilisation in 2011 compared to 2012 (P < 0.05) whereas, in contrast, significantly more leaf N was lost through leaf loss in 2012 compared to 2011 (P < 0.0001). Less than 10% of leaf N was lost from remobilisation; the largest proportion of leaf N was lost through leaf loss (Figure 3). At the spring harvest, N offtakes ranged from 44 kg N/ha in the control to 64 kg N/ha in the 125 kg N/ha treatment.

### *Soil potassium, organic matter, carbon and nitrogen*

Concentrations of soil potassium at the end of the trial decreased with N application (124 mg/L in the control to 98 mg/L in the 125 kg N/ha treatment) but the effect was not significant. Similarly, N application had no significant effect on soil organic matter, soil carbon, nitrogen, phosphorus or magnesium. Average concentrations of these parameters were 6.5% organic matter, 2.8% carbon, 0.3% total nitrogen, 36.5 mg/L phosphorus and 142.9 mg/L magnesium, average soil pH was 6.9.

## Discussion

The objective of this study was to investigate the effect of N application on the growth and yield of a *Miscanthus x giganteus* crop which had remained unfertilized over a thirteen year period. Application of nitrogen had no effect on spring harvested yields although there was a small increase in autumn harvested yields after N application. Miguez *et al*. (2008) and Cadoux *et al*. (2012) concluded that the N requirements of *Miscanthus x giganteus* are low compared to other crops due to a number of factors that include high N use efficiency and efficient recycling.

In this study, leaf chlorophyll concentration was used as an indicator of leaf N concentration (Weih and Ronnberg-Wasljung 2007). On the basis of leaf chlorophyll concentrations, N application was found to be absorbed into the foliage of the crop and leaf N concentrations increased with N level throughout each of the growing seasons of the study. Similarly, Cosentino *et al*. (2007), Wang *et al*. (2012) and Roncucci *et al*. (2014) found that leaf N concentration increased with N application. A vertical gradient in leaf N concentration was found to exist in the upper part of the canopy with the lowest concentrations in the uppermost leaf. In contrast, Weih and Ronnberg-Wastljung (2007) demonstrated that a vertical leaf N gradient existed in willow with the highest concentrations in the uppermost leaves progressively decreasing towards the lower leaves in parallel with the light gradient. They and others have suggested that such a N gradient is optimal as N and leaf area is concentrated at the top of the canopy where light interception is greatest. Wang *et al*. (2012) reported a gradient in leaf nitrogen concentration from the upper to the lower leaves in *Miscanthus x giganteus* after N fertilization, although they found no gradient in chlorophyll concentration.

Leaf N concentrations, measured using leaf chlorophyll as an indicator, declined from early to mid July onwards in all treatments. Cosentino *et al*. (2007) and Strullu *et al*. (2011) have shown a continuous decline in leaf N concentration throughout the growing season. Such effects are often typical of N dilution as plants grow but this effect was not clearly evident in our study when the chlorophyll concentration of the first fully expanded leaf was measured throughout the growing season. In our study, leaf chlorophyll concentrations in the early part of the growing season were relatively stable before declining. Instead, it is more probable that the decline in leaf chlorophyll from July onwards represented the start of autumn N remobilisation. Beale and Long (1997a) and Strullu *et al*. (2011) both demonstrated that the quantity of N present in aboveground biomass reaches a peak around July before declining as N is remobilised to the rhizome network.

Nitrogen application had no effect on shoot density in the early years of the study whereas N application had a very strong effect on shoot density in the final two years of the study. Weisler, Dickmann and Horst (1996) and Cosentino *et al*. (2007) also reported that N application increased shoot density. It is unclear why the effect of N application on shoot density only became obvious in the latter years of this study. The delayed effect may be related to the fact that it appeared to take some time before N levels built up in the rhizome network in proportion to the quantities of N applied. There was no difference in rhizome N concentrations between treatments at the end of the first growing season.

Plant height showed a similar trend to that of shoot density in that no trends of N application on plant height became evident until the final years of the study (2011, 2012). Again, the reason for this is unclear although it may have been related to the fact that N concentrations in aerial plant parts built up over time and increased from year to year from 2010 to 2012. Increases in shoot height after N application were also reported by Cosentino *et al*. (2007) in a study conducted in Sicily although the stimulation in height reported in the Sicilian study was greater than those reported here. The initial stimulation of height by nitrogen found in this study (25% in 2011 and 16% in 2012) could have been expected to have had an important effect on biomass yield if sustained. The stimulation in shoot height, however, only lasted until approximately July, after which the relationship between N application rate and plant height diminished. In 2011, the initial stimulation of height by N diminished and then reversed; growth rate was inversely related to N application rate towards the end of the growing season. This time (July) corresponds to the time when leaf N concentration started to decrease in all treatments and presumably to the onset of autumn remobilisation when N started to move from aerial plant parts back to the rhizome network (Beale and Long 1997a; Strullu *et al*. 2011). Thus, the stimulation in growth observed during the final years of the study may have occurred because plant N concentrations, after repeated applications of N, had built up to a level where growth was stimulated but this growth stimulation could not be maintained after the onset of autumn remobilisation once plant N concentrations had started to decline.

Nitrogen application over the course of the study did lead to a significant increase in autumn harvested yields which peaked at an application rate of 90 kg N/ha before declining. Beale and Long (1997a) recommended a similar N application rate of 92 kg/ha for *Miscanthus x giganteus* crops producing an aboveground harvest of 15 t/ha DM. Additionally, Lewandowski and Schmidt (2006) reported that biomass yield in February increased up to a N application rate of 114 kg N $ha^{-1}$ before declining. Shield *et al*. (2014) also reported a yield response to N fertilizer application which peaked at 100 kg N/$ha^{-1}$. A response to N in early (i.e., Autumn) harvested crops is understandable as Strullu *et al.* (2013) reported that growth is impaired under conditions of early harvest and that no fertilizer application leads to N limitation. Total biomass yield in the 90 kg N/ha treatment increased by 1.3 tonnes DM/ha over the control or 0.014 tonnes DM per kg of nitrogen applied. This factor is considerably lower than the factor of 0.232 tonnes DM per kg of N applied that was reported by Miguez *et al*. (2008) for *Miscanthus x giganteus* harvested in winter (after December 21st). In our study, however, N fertilization did not have an effect on biomass yields harvested in early spring. Similarly, Strullu *et al*. (2011) found that N application had no effect on crop yields at late harvest (February) but enhanced crop yields at early harvest (October). Many studies have reported that N application had no effect on biomass yields when crops were harvested in winter/spring (Himken *et al*. 1997; Clifton-Brown *et al*. 2007; Christian *et al*. 2008; Strullu *et al*. 2011). Clifton- Brown *et al*. (2007) concluded that N offtakes could be met by soil reserves and N deposition.

Total N content at the October harvest ranged from 71 kg N/ha in the control to 104 kg N/ha in the 125 kg N/ha treatment. Strullu *et al*. (2011) reported that 42% of aboveground biomass had been remobilised by October. On this basis, maximum N uptake in our study ranged from 122 kg N/ha to 179 kg N/ha, lower than figures for maximum N uptake reported by Himken *et al.* (1997), Beale and Long (1997a), Strullu *et al*. (2011) and Dohleman *et al*. (2012). This result can be explained by the fact that biomass yields were considerably higher in all of these studies in comparison to our study but the N concentration in aerial biomass was similar in all cases. Nitrogen loss between the October and February harvests was lower than those previously reported by Himken *et al*. (1997) (September to March) and Strullu *et al*. (2011) (October to February). Losses from abscissed leaves were similar to those reported by Strullu *et al*. (2011) but lower than those reported by Himken *et al*. (1997). Most of the leaf N was lost through leaf abscission; remobilisation of leaf N was relatively minor and consistent between years. Stem N losses between harvests were non-existent in one of the years and only occurred in the other year because of a reduction in stem biomass between October and February harvests. End of season (February) N offtakes were relatively low and similar to those reported by Himken *et al*. (1997) and Strullu *et al*. (2011) although lower than those reported by Beale and Long (1997a) and Dohleman *et al*. (2012).

The small differences in N content at harvest between the control and the highest N rate treatment (30 kg N/ha, autumn harvest) suggest that uptake of applied N during the study was low. Thus, the poor N fertilizer efficiency found in this study was probably largely attributable to poor N fertilizer uptake efficiency. Nitrous oxide emission factors were measured in one year of the study by Roth *et al*. (2014) and were found to be relatively low (0.5% for the 63 kg N/ha treatment and 0.7% for the 125 kg N/ha treatment). Thus, it is possible that a sizable proportion of applied N was lost by leaching to groundwater or surface water as there were no differences in soil N between treatments at the end of the study. Further environmental implications arise from the N content of harvested biomass which increased with N application. Emissions of $NO_x$ from biomass generally increase with fuel N content (Sommerbacher, Brunner and Obernberger *et al*. 2012). Additionally, $N_2O$ emissions from biomass combustion also increase with fuel N content (Houshfar *et al*. 2012a, 2012b). Thus, in the absence of increased GHG mitigation from higher harvested yields, the environmental implications of applying N fertilizer to *Miscanthus x giganteus* are serious and perhaps counterproductive to the environmental benefits of the crop.

The *Miscanthus x giganteus* crop used in this study only showed a small response to N application even though N offtakes at harvest time were not replaced during the first thirteen years of the life-cycle of the crop. For this crop and in this region, it would appear that the replacement of N offtakes would not necessarily have brought any appreciable benefit to the growth and yield of the crop. End of season N offtakes were smaller than the sum of atmospheric N deposition (Aherne and Farrell 2002) and soil N mineralisation (Herlihy and Hegarty 1979). Additionally, Keymer and Kent (2014) recently provided evidence of nitrogen fixation in *Miscanthus x giganteus*. More research is needed to identify the factors necessary to obtain a response from N application. In the absence of such factors, the environmental benefits of applying N fertilizer may be outweighed by the deleterious environmental consequences of N application.

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