Annual production of grass silage for biogas: effects of fibrolytic enzyme additives on ensilage efficiency and specific methane yields

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Abstract: In the grass-to-biogas process, there is an opportunity to increase total CH_4 yield per hectare (ha) of grasslands by applying fibrolytic enzymes to partially hydrolyse the grass fibre during ensilage. The aims of this study were to quantify the effects of fibrolytic enzyme additives applied to each of four consecutive cuts of unwilted grass at ensiling on ensilage characteristics and specific CH_4 yields per unit mass and per unit land area. Considering the importance of the primary growth yield, the effects of the timing of Cut 1 were also investigated. Furthermore, the mass-specific CH_4 yields and area-specific CH_4 yields of any effluent produced during ensilage were determined. At each of four cuts that comprised annual growth, samples from four replicate plots of *Lolium perenne* and of *Phleum pratense* were subjected to one of three treatments, a control and two fibrolytic enzymes (ENZ 1 and ENZ 2) prior to ensiling for 120 days. The mass-specific CH_4 yield of silages and effluents were determined using an *in vitro* batch anaerobic digestion test. Total annual CH_4 yield per ha of grassland was quantified. The effects of altering the timing of Cut 1 were also assessed with the same methods. On average, ENZ 1 and ENZ 2 reduced neutral detergent fibre by 9% and 15%, respectively, compared to the control silages. The fibrolytic effects of added enzymes were successful at aiding silage preservation under some but not all the challenges to ensilage provided in this study. Furthermore, ENZ 1 and ENZ 2 increased effluent outflow by 46% and 96%, respectively. The mass-specific CH_4 yields for silages from each cut or either grass species were not significantly enhanced by enzyme treatments. The area-specific CH_4 yields of silages were numerically negatively affected (P>0.05) by added enzymes (i.e. 4143, 4058, 3944 m^3 CH_4 ha^{-1} a^{-1} for control, ENZ 1 and ENZ 2 treatments, respectively). However, when the effluent was collected and utilised as a feedstock the 6%, 10% and 17% increase in annual area-specific CH_4 yield for the control, ENZ 1 and ENZ 2 treatments, respectively, therefore resulted in total area-specific CH_4 yield values for the ENZ 1 and ENZ 2 treatments that were 101% and 105% of the control treatment, respectively. In conclusion, the enzymes added at ensilage hydrolysed some grass fibre during ensilage, resulting in some improvements to silage fermentation and an increase in silage effluent output. They did not increase mass-specific CH_4 yields and their overall effects on area-specific CH_4 yields were relatively modest.

Keywords: grass yield, silage, fibre, enzymes, effluent, CH_4 yield per unit land area

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1 Introduction

The impetus to increase farm income in Ireland has stimulated interest in numerous non-traditional enterprises, some of which involve renewable energy provision. In this context, anaerobic digestion of grass silage provides an opportunity for additional on-farm revenue creation.

A critical factor in assessing grassland biomass for anaerobic digestion is the CH_4 yield per unit area of land i.e. m^3 CH_4 hectare $(ha)^{-1}$ (McEniry and O'Kiely, 2013; Prochnow et al., 2005). Many factors contribute towards this commercially important metric such as biomass yield and volatile solids content, fermentation during ensilage,

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effluent outflow, total solids and/or energy losses during ensilage and specific CH_4 yield (in L CH_4 kg^{-1} volatile solids) of the silage during anaerobic digestion.

A disproportionately large amount of the annual production from grassland is available to harvest from the primary growth of grass during May to June, and the actual harvest date selected will markedly influence biomass yield and chemical composition (King et al., 2012). Furthermore, successive harvests taken consecutively through the remainder of the annual growth will each have unique compositional characteristics (Keating and O'Kiely, 2000; Conaghan et al., 2008), where all harvests need to be efficiently conserved for use in anaerobic digestion later. Thus, a major objective of ensiling grass is to quantitatively and qualitatively reduce total solids losses, and thus digestible energy losses, that occur during ensilage by maintaining anaerobic conditions and promoting efficient acidification in the ensiled biomass.

There is an opportunity to upgrade biomass during ensilage by applying fibrolytic enzyme additives to partially hydrolyse grass fibre. Since hydrolysis is the primary limiting step in the anaerobic digestion of lignocellulosic biomass such as grass silage (Jagadabhi et al., 2011; Wall et al., 2013) applying fibrolytic enzymes at ensiling may increase the total extent of fibre hydrolysis. Thus, this upgrading step has potential to increase the rate and/or extent of methanogenesis during anaerobic digestion (Suárez Quiñones et al., 2012; Nolan et al., 2018). This, in turn, could influence whether a larger number of lower-yielding higher-digestibility harvests or a smaller number of higher-yielding lower-digestibility harvests were chosen. Furthermore, fibrolytic enzyme activity could potentially improve the preservation of silages by the release of soluble, fermentable sugars (such as pentoses and hexoses) from high lignocellulosic grasses (Kristensen et al., 2007).

There is further scope to increase total CH_4 yield per ha of grassland by collecting effluent produced during ensiling and digesting it for CH_4 production (Abu Dahrieh et al., 2011; Colleran and Barry, 1984). It is postulated in this study that effluent outflow would be increased by the addition of fibrolytic enzymes to unwilted grass at ensiling, but that utilising it in anaerobic digestion would increase total area-specific CH_4 yields.

The aims of this study were to quantify the effects of applying fibrolytic enzyme additives to four consecutive growths of grass produced throughout the growing season on ensilage characteristics and specific CH_4 yields. This was undertaken with each of two grass species. Because of the relative importance of the primary growth yield, the effects of altering the timing of this harvest were also studied. Finally, the mass-specific CH_4 yield of any effluent produced during ensilage was determined and its effects on total CH_4 yield per ha per annum quantified.

2 Materials and methods

2.1 Approach

Two grass species were harvested at three stages in the primary growth, and cut at three consecutive regrowths that followed the intermediate primary growth harvest. These were ensiled alone or with two fibrolytic enzyme treatments, and conservation characteristics were measured. An *in vitro* batch anaerobic digestion test was used to determine the effects of the enzymes applied at ensiling on subsequent specific CH_4 yields of the grass silages and of their effluents.

2.2 Herbage and harvesting

This experiment was conducted at Teagasc Grange (53°30'N, 6°40'W, 83 m above sea level) using three plots (each 10 m × 2 m) of perennial ryegrass (*Lolium perenne* L., an equal mixture of the late diploid varieties Denver, Soriento and Tyrella) and three plots of Timothy (*Phleum pratense* L., an equal mixture of the varieties Comer, Erecta and Promesse) within each of four replicate blocks. Each plot received 120 kg N, 28 kg P and 120 kg K ha^{-1} in mid-March. Immediately prior to each harvest, grass growth stage was determined for 20 randomly selected tillers per plot according to Moore et al (1991). One plot per grass species was harvested within each block on 14 May (early), 28 May (intermediate), and 11 June (late). Consecutive regrowths of the plots harvested on the 28 May (Cut 1) were subsequently harvested on 16 July (Cut 2), 03 September (Cut 3), and 28 November (Cut 4). Each plot received 100, 80 and 60 kg N ha^{-1} immediately after Cuts 1, 2 and 3, respectively,

as well as 10 kg P and 43 kg K ha^{-1} on each of these three occasions. Plots were harvested using a Haldrup forage plot harvester (J. Haldrup, Løgstor, Denmark) cutting to an average 5 cm stubble height and the herbage was weighed and precision chopped (Pottinger Mex VI; Grieskirchen, Austria). A representative sample of harvested herbage was stored at –18°C for chemical analysis (Table 1 and 5).

2.2 Enzyme treatments and ensilage

Representative 5 kg samples of grass from each plot were randomly assigned to three ensiling treatments. These were an untreated control (i.e. none; Tween® 20 solution with no added enzyme) and two fibrolytic enzymes (ENZ 1 and 2). ENZ 1 and 2 enzyme preparations were produced by the manufacture from *Trichoderma longibratum* via solid state fermentation on grain by-products at pH 5.0 and 55°C. Preparations were dissolved in a 1% (v/v) Tween® 20 solution for 16 h prior to application, the role of which is to stabilize the enzyme and prevent adsorption (Kristensen et al., 2007). Enzyme activity was determined by the manufacturer according to the methods of Miller et al. (1960) for cellulase activity (carboxymethyl cellulase units; CMCU) and of Ferreira et al. (1999) for xylanase activity (xylanase unit; XU). ENZ 1 had mainly xylanase activity (11459 XU g^{-1}) with relatively minor side-activity of cellulase (640 CMCU g^{-1}) and ENZ 2 had mainly cellulase activity (6100 CMCU g^{-1}) with relatively minor side-activity of xylanase (150 XU g^{-1}), and these were added at 1.5 g enzyme per kg of herbage total solids.

Grass was ensiled in laboratory silos (13 L volume; a 10.5 kg steel weight was placed on the grass within each silo to simulate compaction in a farm-scale horizontal silo; O'Kiely and Wilson, 1991) at 15°C for 120 days. The effluent outflow was recorded on days 2, 5, 9, 14, 28, 98 and 120 of ensilage and samples were stored at -18°C for subsequent analysis in the *in vitro* batch digestion test where a pooled (weighted mean) single sample of effluent was produced for each silo. On the same days effluent was sampled during the primary growth, silage compaction was measured by the change in length (cm) outside the silo of a nylon monofilament line attached to the steel compaction weight within the silo and exiting the silo via the gas valve. After ensiling, the weight of silage was recorded. A representative sample of each silage and its aqueous extract (15 mL) were stored at –18°C.

2.5 Chemical analysis

For total solids determination, sub-samples that were dried at 98°C (16 h) for grass and at 85°C (18 h) for silage, in an oven with forced air circulation. Silage total solids values were corrected for the loss of volatile organic compounds according to Porter and Murray (2001).

Sub-samples that were dried (40°C) and milled (sieve with 1 mm apertures) were assayed for *in vitro* total solids digestibility, neutral detergent fibre (assayed with a heat-stable amylase and sodium sulphite, and expressed exclusive of residual ash), acid detergent fibre (expressed exclusive of residual ash) and acid detergent lignin (expressed exclusive of residual ash), crude protein, water-soluble carbohydrates, ash (grass samples only) and buffering capacity (grass samples only) as previously described in King et al. (2012). The aqueous extract of silage was used to record pH using a pH electrode (Hanna instruments; H198127; Leighton Buzzard, UK), volatile fatty acids (VFA; acetic, propionic and butyric acids), ethanol, lactic acid and ammonia-N as previously described by Navarro-Villa et al. (2013). Both lactic acid and ammonia-N were measured with an Olympus AU400 Chemistry Analyser (Shizuoka, Japan). Silage total solids recovery rate was calculated as the proportion of silage total solids (corrected for the loss of volatiles) removed relative to the grass total solids ensiled. Volatile solids were subsequently calculated as: volatile solids = total solids – ash.

2.6 In vitro batch anaerobic digestion tests

Prior to the *in vitro* batch anaerobic digestion test, silage sub-samples (approximately 50 g) were frozen in liquid nitrogen and comminuted to pass through a sieve with 1 mm apertures, as reported in Nolan et al. (2014). The specific CH_4 yield from the silages and effluents were assessed separately. *In vitro* batch digestion tests were conducted in duplicate 250 mL incubation bottles per silage or effluent sample using the method previously described in Nolan et al. (2014) according to VDI guidelines (2006). Briefly, a 2:1 inoculum-to-substrate

ratio on a volatile solids basis and final volatile solids concentration of 10 g kg^{-1} total medium was used for this test. Micro- and macro-mineral solutions were added and included $NaHCO_3$ as buffer (3 g L^{-1}; Gonzalez-Gil et al., 2001). The final volume per incubation bottle was adjusted to 120 mL with distilled water. Blank (i.e. inoculum only) and cellulose (Sigma, 22184; positive control) were similarly incubated with nine replicates of each. Nitrogen gas was used to create anaerobic conditions prior to incubating at 38°C for 35 days. The incubation bottles were swirled by hand each day.

Biogas was estimated using a detachable pressure transducer (Tracker 220, Gems Sensors and Controls, Basingstoke, UK; as per VDI (2006) guidelines) and CH_4 concentration in the biogas was determined by gas chromatography using an automated gas chromatograph (Shimadzu GC-17A; Shimadzu Corporation, Kyoto, Japan) with a flame ionisation detector. Biogas data evaluation included corrections for the inert gas in the headspace on the first day of measurements, the CH_4 produced by inoculum-only (i.e. blank samples), and the CH_4 volume corrected to standard temperature and pressure (i.e. 273 K; 1013 hPa) as per VDI (2006) guidelines.

Duplicate analytical estimates from the *in vitro* batch anaerobic digestion test were averaged to give a single value for each of the four replicate silages per treatment and similarly for each of the four replicate effluents per treatment. First and second order kinetics of mass-specific CH_4 yields obtained were estimated according to Wall et al. (2013) using Matlab 2011b software. Subsequently, annual silage (with and without effluent included) area-specific CH_4 yields (m^3 CH_4 ha^{-1}) were calculated from the annual yield of grass total solids per hectare, silage total solids recovery, silage volatile solids total $solids^{-1}$ and mass-specific CH_4 yield (see Table 6). Effluent area-specific CH_4 yields (annual growth) were similarly calculated (effluent total solids yield ha^{-1}, volatile solids total $solids^{-1}$ and mass-specific CH_4 yields) and added to silage area-specific CH_4 yield for total annual area-specific CH_4 yields (Figure 2).

2.7 Statistical analysis

The annual growth and primary growth data were analysed separately. The means and standard deviations (s.d.) were calculated for the grass yield (annual growth) and chemical composition (annual and primary growths). Data for silage composition, silage mass-specific CH_4 yield, digestion kinetics, effluent mass-specific CH_4 yield and area-specific CH_4 yield were analysed as a split-split-split plot design using the MIXED procedure in SAS, Version 9.3, with cuts (annual growth = cuts 1+2+3+4) or harvest (primary growth = early, intermediate and late) as the main plot, species (i.e. PRG and TIM) as the sub-plot, and enzyme additive treatment (i.e. control, ENZ 1 and ENZ 2) as the sub-sub-plot, and with the effects of replicate block being accounted for within the main plot. The Tukey adjustment for multiple comparisons was used in testing the differences between pairs of means. The correlation coefficient between effluent production and silage compaction data was analysed using CORR procedure in SAS, version 9.3.

3 Results

The results are presented separately for the annual growth (section 3.1) and the primary growth (section 3.2) of the grasses.

3.1 Annual growth

3.1.1 Grass yield and chemical composition

Grass was harvested at a similar phenological growth stage for each species and at each cut (Table 1). However, biomass yields were greatest at Cut 1 and lowest at Cut 4. Grass fibre, crude protein and ensilability characteristics varied considerably across the four consecutive cuts whereas the differences between the grass species were generally smaller.

3.1.2 Silage

Cut

The crude protein concentration increased (P<0.001) from Cut 1 through to Cut 4. Compared to the other three cuts, Cut 2 had the greatest (P<0.001) total solids, water-soluble carbohydrates and lactic acid contents (Tables 2 and 3). In contrast, Cut 2 had the lowest (P<0.001) propionic acid and butyric acid values. The values of fermentation products were also greater (P<0.01) for Cut 2 compared to Cuts 1 and 3. Cuts 3 and 4 had higher (P<0.001) acid detergent lignin, pH, acetic

acid and ammonia-N values and lower (P<0.001) lactic acid/fermentation products values than Cuts 1 and 2. The effluent outflow was lowest in Cut 2 (P<0.01) and greatest in Cut 4 (P<0.01) with the other two cuts being intermediate (where Cut 3<Cut 1).

Species

Overall in comparison to PRG, TIM had lower (P<0.01) values of water-soluble carbohydrates and total solids recovery rates and higher (P<0.01) values of effluent outflow.

Enzyme

In comparison to the control, both added enzymes reduced (P<0.001) values of neutral detergent fibre, acid detergent fibre, pH, acetic acid, propionic acid (P<0.01) and ammonia-N yet increased (P<0.001) values of crude protein (P<0.01), water-soluble carbohydrates, lactic acid, lactic acid/fermentation products and effluent outflow. In comparison to the control, only ENZ 2 had a lower (P<0.05) butyric acid and a greater (P<0.01) fermentation products and, only ENZ 1 had greater ethanol (P<0.05). ENZ 2 had the greatest (P<0.001) total solids contents and lowest (P<0.05) butyric acid values.

Cut x species

In comparison to PRG, TIM had: higher (P<0.001) values of total solids digestibility and fermentation products and lower values of neutral detergent fibre in Cut 2; higher (P<0.001) values of crude protein, pH, ammonia-N (P<0.01) and ethanol (P<0.05) and lower (P<0.001) values of fermentation products, lactic acid and lactic acid/fermentation products (P<0.01) in Cut 3; and higher (P<0.001) values of crude protein, pH, acetic acid (P<0.01), propionic acid, butyric acid (P<0.01) and ammonia-N (P<0.01) and lower values of lactic acid (P<0.001) and lactic acid/fermentation products (P<0.01) in Cut 4.

Cut x enzyme

In comparison to the control, both added enzymes, reduced acid detergent fibre (P<0.05) at Cut 1, increased water-soluble carbohydrates (P<0.05) at Cut 2, and increased crude protein (P<0.05) and reduced ammonia-N (P<0.001) at Cut 3. Furthermore, ENZ 2 had the greatest water-soluble carbohydrates (P<0.001) at Cut 1, ethanol (P<0.05) at Cut 2, and the lowest acid detergent fibre (P<0.001) at Cut 4.

Cut x species x enzyme

At Cut 4, the control and ENZ 1 treatments had higher (P<0.05) propionic acid values for TIM compared to PRG. For TIM silages within Cut 4, the control treatment had the greater (P<0.01) propionic acid values than either added enzyme.

Table 1 Mean (s.d.) yield and chemical composition (g kg^{-1} TS, unless indicated otherwise in the footnotes) of two grass species at four consecutive cuts through the annual growth

Cut	1		2		3		4	
Species	PRG	TIM	PRG	TIM	PRG	TIM	PRG	TIM
GS	2.5	2.5	2.5	2.6	2.6	2.7	2.7	2.7
Yield	6841 (1464.8)	6766 (2102.6)	3583 (684.8)	2480 (199.2)	2356 (259.0)	2492 (156.1)	1626 (368.3)	1102 (243.7)
TS	138 (1.2)	135 (18.4)	181 (11.0)	213 (11.1)	147 (4.4)	142 (2.2)	130 (23.8)	133 (16.0)
TSD	679 (21.4)	670 (35.4)	725 (12.7)	669 (54.7)	762 (8.3)	726 (28.6)	791 (7.3)	776 (7.9)
NDF	645 (41.8)	627 (22.4)	596 (4.4)	662 (26.8)	509 (6.1)	582 (20.8)	523 (15.8)	514 (31.7)
ADF	360 (19.4)	353 (14.1)	327 (6.0)	370 (7.6)	279 (7.0)	326 (10.1)	253 (12.4)	185 (12.3)
ADL	35 (3.5)	31 (1.4)	33 (6.4)	48 (8.3)	30 (9.1)	41 (9.7)	29 (9.1)	22 (14.8)
Ash	86 (8.9)	91 (3.8)	101 (3.2)	74 (4.2)	101 (3.4)	91 (3.4)	108 (3.9)	112 (7.8)
CP	128 (9.9)	117 (24.9)	165 (13.2)	140 (7.4)	197 (17.3)	222 (6.0)	262 (36.5)	259 (31.5)
WSC	57 (47.3)	89 (40.6)	64 (8.2)	44 (22.5)	100 (8.9)	36 (6.3)	75 (29.2)	81 (21.4)
WSCaq	9 (7.7)	15 (8.2)	14 (8.1)	12 (6.4)	17 (2.0)	6 (1.2)	12 (5.9)	13 (5.0)
BC	357 (22.1)	344 (56.5)	555 (44.5)	393 (35.1)	517 (42.1)	452 (9.6)	455 (32.6)	453 (31.9)

Note: Cut 1 (28 May), 2 (16 July), 3 (03 September), 4 (28 November); Species, PRG, perennial ryegrass, TIM, timothy; GS, growth stage according to Moore et al. (1991); Yield (kg TS ha^{-1}); TS, total solids (g kg^{-1}); TSD, total solids digestibility (g kg^{-1}); NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; CP, crude protein; WSC, water-soluble carbohydrates; WSC*aq*, in aqueous phase (g L^{-1}); BC, buffering capacity (m Eq kg^{-1} TS).

Table 2 Effects of cut, species and enzyme treatments on the chemical composition (g kg^{-1} TS, unless indicated otherwise in the footnotes) of silages from two grass species at four consecutive cuts through the annual growth

Cut	Species	Enzyme	TS c	TSD	NDF	ADF	ADL	CP	WSC
1	PRG	Control	162	623	675	414	49	110	12
1	PRG	ENZ 1	178	635	560	335	30	120	17
1	PRG	ENZ 2	189	633	560	318	31	104	21
1	TIM	Control	166	603	659	417	41	115	12
1	TIM	ENZ 1	170	601	594	370	40	117	15
1	TIM	ENZ 2	191	614	569	336	42	118	19
2	PRG	Control	213	660	611	359	35	157	15
2	PRG	ENZ 1	215	653	568	327	38	164	18
2	PRG	ENZ 2	225	646	513	355	33	161	18
2	TIM	Control	192	693	571	340	30	160	14
2	TIM	ENZ 1	199	690	536	314	58	158	18
2	TIM	ENZ 2	223	661	501	281	48	163	19
3	PRG	Control	145	726	531	350	70	182	14
3	PRG	ENZ 1	163	720	492	318	71	206	15
3	PRG	ENZ 2	175	742	448	271	76	210	15
3	TIM	Control	143	661	607	406	91	148	13
3	TIM	ENZ 1	152	647	563	365	81	188	14
3	TIM	ENZ 2	168	636	561	352	78	190	14
4	PRG	Control	156	719	480	318	57	227	15
4	PRG	ENZ 1	167	707	458	309	77	235	16
4	PRG	ENZ 2	186	698	411	249	56	239	16
4	TIM	Control	144	709	477	331	58	252	13
4	TIM	ENZ 1	158	666	430	288	71	270	14
4	TIM	ENZ 2	200	670	370	228	60	294	15
Standard error of the mean									
Cut (C)			4.1	6.9	6.3	5.6	4.6	3.3	0.4
Species (S)			2.9	4.9	4.4	3.7	3.2	2.3	0.3
Enzyme (E)			3.5	5.9	5.4	4.5	3.9	2.9	0.3
C × S			5.8	9.7	9.1	8.8	6.9	4.7	0.6
C× E			7.1	11.9	11.4	10.5	8.2	5.7	0.7
S × E			5.0	8.4	7.8	6.7	5.6	4.1	0.5
C × S × E			10.0	16.8	17.3	17.1	12.4	8.1	1.1
Levels of significance									
C			***	***	***	***	***	***	***
S			NS	**	NS	NS	NS	NS	**
E			***	NS	***	***	NS	**	***
C × S			NS	***	***	***	NS	***	NS
C × E			NS	NS	NS	*	NS	*	***
S × E			NS	NS	NS	NS	NS	NS	NS
C × S × E			NS	NS	NS	NS	NS	NS	NS

Note: Cut 1 (28 May), 2 (16 July), 3 (03 September), 4 (28 November); Species, PRG, perennial ryegrass, TIM, timothy; Control, no enzyme added, ENZ 1, mainly xylanase, ENZ 2, mainly cellulase; TSc, (g kg^{-1}) total solids corrected for the loss of volatile compounds according to Porter and Murray (2001); TSD, (g kg^{-1}) total solids digestibility; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; CP, crude protein; WSC, water-soluble carbohydrates; * P<0.05, ** P<0.01, *** P<0.001, NS=not significant.

Table 3 Effects of cut, species and enzyme treatments on the conservation characteristics (g kg^{-1} TS, unless indicated otherwise in the footnotes) of silages from two grass species at four consecutive cuts through the annual growth

Cut	Spp.	Enzyme	pH	LA	AA	PA	BA	Eth	NH_3-N	FP	LA/FP	TSrr	Eff.
1	PRG	Control	4.49	36	20	5.0	20.3	16	257	97	0.34	0.94	178
1	PRG	ENZ 1	3.59	113	12	1.0	2.9	11	84	141	0.81	0.93	220
1	PRG	ENZ 2	3.49	109	8	0.3	2.7	10	83	130	0.84	0.88	345
1	TIM	Control	4.56	40	28	4.4	23.8	13	258	109	0.26	0.78	237
1	TIM	ENZ 1	4.19	89	20	2.2	20.7	15	157	124	0.55	0.85	329
1	TIM	ENZ 2	3.70	105	11	1.1	6.1	10	90	133	0.74	0.82	407
2	PRG	Control	4.33	67	23	2.5	3.5	17	155	112	0.62	0.82	26
2	PRG	ENZ 1	3.95	98	21	2.0	1.4	12	88	135	0.75	0.98	77
2	PRG	ENZ 2	3.72	125	12	0.6	0.7	24	68	162	0.77	0.96	182
2	TIM	Control	3.81	131	15	0.8	0.6	10	94	158	0.83	0.85	77
2	TIM	ENZ 1	3.71	147	18	0.8	1.0	13	85	181	0.82	0.82	159
2	TIM	ENZ 2	3.68	143	13	0.5	1.2	29	73	187	0.77	0.77	240
3	PRG	Control	5.01	52	37	5.9	15.1	5	402	115	0.35	0.84	117
3	PRG	ENZ 1	4.46	110	25	3.5	9.4	15	168	163	0.61	0.83	196
3	PRG	ENZ 2	3.94	163	19	2.2	3.6	11	122	199	0.82	0.83	224
3	TIM	Control	5.64	4	31	4.8	18.2	19	698	78	0.05	0.87	116
3	TIM	ENZ 1	5.40	7	30	2.9	9.5	24	332	74	0.10	0.84	190
3	TIM	ENZ 2	5.12	28	22	2.6	8.2	15	306	75	0.35	0.84	263
4	PRG	Control	4.77	78	30	3.9	9.5	16	262	137	0.44	0.86	256
4	PRG	ENZ 1	4.34	98	35	3.1	3.1	18	156	158	0.55	0.83	354
4	PRG	ENZ 2	4.04	133	16	1.3	8.0	14	105	172	0.73	0.80	444
4	TIM	Control	5.55	10	59	17.1	25.0	13	432	125	0.08	0.76	295
4	TIM	ENZ 1	5.48	8	47	10.3	37.8	29	334	132	0.06	0.72	378
4	TIM	ENZ 2	5.11	51	33	5.5	25.3	13	218	128	0.32	0.81	451
Standard error of the mean													
Cut (C)			0.094	9.2	2.0	0.35	2.05	1.5	22.0	7.8	0.047	0.022	24.8
Species (S)			0.067	6.4	1.4	0.24	1.43	1.0	15.6	5.5	0.033	0.016	13.8
Enzyme (E)			0.082	7.9	1.7	0.30	1.76	1.3	19.1	6.7	0.041	0.019	16.2
C × S			0.133	13.3	2.9	0.52	2.98	2.1	31.2	11.0	0.069	0.033	31.6
C × E			0.163	16.7	3.7	0.62	3.74	2.7	38.2	13.5	0.086	0.041	39.8
S × E			0.115	11.4	2.5	0.42	2.55	1.8	27.0	9.5	0.059	0.028	21.2
C × S × E			0.231	25.3	5.5	0.93	5.67	4.0	54.0	19.0	0.131	0.053	53.5
Levels of significance													
C			***	***	***	***	***	NS	***	**	***	NS	***
S			***	**	**	***	**	**	***	*	***	**	**
E			***	***	***	***	*	*	***	**	***	NS	***
C × S			***	***	**	***	**	*	**	***	**	NS	NS
C × E			NS	NS	NS	**	NS	***	*	NS	NS	NS	NS
S × E			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
C × S × E			NS	NS	NS	**	NS	NS	NS	NS	NS	NS	NS

Note: Cut 1 (28 May), 2 (16 July), 3 (03 September), 4 (28 November); Species, PRG, perennial ryegrass, TIM, timothy; Control, no enzyme added, ENZ 1, mainly xylanase activity, ENZ 2, mainly cellulase activity; LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid; Eth, ethanol; NH_3-N, ammonia-N (g kg^{-1} N); FP, total fermentation products (LA + AA + PA + BA + Ethanol); LA/FP, lactic acid as a proportion of total fermentation products; TSrr, total solids recovery rate g g^{-1} = (silage weight x silage TS/1000)/(fresh grass weight x fresh grass TS/1000); Eff., Effluent (g kg^{-1} grass ensiled); * P<0.05, ** P<0.01, *** P<0.001, NS=not significant.

3.1.3 In vitro batch anaerobic digestion test

Cut

Cut 4 had the greatest (P<0.001) silage mass-specific CH_4 yield with the differences between the other three cuts also significantly different from each other in the ranking of Cut 1>2>3 (Table 4). Cut 1 had the greatest (P<0.001) silage area-specific CH_4 yield values, while Cut 3 had the lowest (P<0.001) first order decay constant (k) values. The maximum daily CH_4 production rate (u) was greatest (P<0.001) in Cut 4 and lowest (P<0.001) in Cut 3, with the other two cuts being intermediate. The lag phase (Δ) was longest (P<0.001)

in Cut 2 and shortest (P<0.001) in Cut 1, with the other two cuts being intermediate. The half-life times (T_{50}) consecutively increased (P<0.001) from Cut 1 to Cut 3 (6.8 to 9.7 days, respectively) and decreased (P<0.001) in Cut 4 (8.1 days). The effluent mass-specific CH_4 yield was greater (P<0.01) at Cut 2 compared to Cuts 3 and 4.

Species

Overall, PRG silages had higher (P<0.05) area-specific CH_4 yield than TIM silages.

Table 4 Effect of cut, species, and enzyme treatments on silage mass- and area-specific CH_4 yield, kinetics and effluent mass-specific CH_4 yield, from two grass species at four consecutive cuts through the annual growth

Cut	Species	Enzyme	Mass-SMY	Area-SMY	K	u	Δ	T_{50}	Effluent mass-SMY
1	PRG	Control	436	2616	0.10	43.6	1.7	6.7	382
1	PRG	ENZ 1	363	2167	0.10	34.0	0.8	6.9	525
1	PRG	ENZ 2	393	2254	0.10	37.8	1.6	6.9	342
1	TIM	Control	396	1829	0.10	38.6	1.5	6.7	528
1	TIM	ENZ 1	375	2066	0.10	36.3	2.1	7.3	338
1	TIM	ENZ 2	395	2058	0.10	39.8	1.7	6.6	555
2	PRG	Control	351	965	0.10	36.2	4.4	9.1	485
2	PRG	ENZ 1	348	1157	0.10	34.1	3.8	9.0	611
2	PRG	ENZ 2	315	1024	0.10	31.6	3.7	9.0	604
2	TIM	Control	339	758	0.10	33.2	4.0	9.0	588
2	TIM	ENZ 1	330	559	0.10	32.2	4.0	9.3	540
2	TIM	ENZ 2	300	569	0.09	26.6	3.0	8.8	412
3	PRG	Control	258	463	0.08	21.5	3.8	10.0	382
3	PRG	ENZ 1	268	490	0.09	23.5	3.0	9.0	493
3	PRG	ENZ 2	219	402	0.08	17.5	3.6	10.0	323
3	TIM	Control	304	626	0.08	24.9	3.6	9.8	369
3	TIM	ENZ 1	273	537	0.08	22.5	3.3	9.6	441
3	TIM	ENZ 2	267	517	0.08	22.0	3.5	9.7	320
4	PRG	Control	478	622	0.10	47.5	3.2	8.2	466
4	PRG	ENZ 1	565	739	0.10	55.1	2.9	8.0	449
4	PRG	ENZ 2	497	619	0.10	48.3	3.1	8.3	350
4	TIM	Control	501	406	0.10	48.6	2.9	8.1	306
4	TIM	ENZ 1	526	400	0.10	53.7	2.7	7.6	473
4	TIM	ENZ 2	518	444	0.09	47.4	2.7	8.2	343
Standard error of the mean (s.e.m.)									
	Cut (C)		16.2	109.2	0.002	1.98	0.12	0.12	36.4
	Species (S)		10.3	69.9	0.001	1.11	0.08	0.08	24.9
	Enzyme (E)		12.9	88.2	0.002	1.44	0.12	0.11	26.6
	C × S		23.5	157.5	0.003	2.52	0.20	0.18	54.5
	C × E		31.2	207.2	0.004	3.47	0.28	0.27	61.1
	S × E		18.9	118.2	0.003	1.99	0.18	0.16	39.3
	C × S × E		48.2	321.1	0.006	5.09	0.46	0.42	97.5
Levels of significance									
	C		***	***	***	***	***	***	*
	S		NS	*	NS	NS	NS	NS	NS
	E		NS	NS	NS	NS	NS	NS	NS
	C × S		NS	NS	NS	NS	NS	NS	NS
	C × E		NS	NS	NS	NS	NS	NS	NS
	S × E		NS	NS	NS	NS	NS	NS	NS
	C × S × E		NS	NS	NS	NS	NS	NS	NS

Note: Cut 1 (28 May), 2 (16 July), 3 (03 September), 4 (28 November); Species, PRG, perennial ryegrass, TIM, timothy; Control, no enzyme added, ENZ 1, mainly xylanase activity, ENZ 2, mainly cellulase activity; Mass SMY, mass specific CH_4 yield (L CH_4 kg^{-1} volatile solids), note average (s.d.) of cellulose control 390 (77.4) L CH_4 kg^{-1} volatile solids; Area SMY, area specific methane yield (m^3 CH_4 ha^{-1}), accounting for storage losses during ensiling, not accounting for field, harvesting losses; k, first order decay constant per day, the average (s.e.m.) coefficient of determination for all k values was R^2 =0.997 (0.0003); u, maximum specific methane production rate (ml CH_4 g^{-1} volatile solids d^{-1}); Δ, lag phase (days); T_{50}, half-life defined as the time (days) taken to produce 0.50 of the methane; * P<0.05, ** P<0.01, *** P<0.001, NS=not significant.

3.2 Primary growth

3.2.1 Grass chemical composition

Grass phenological growth stage increased as the primary growth progressed, and the values for both grass species were similar to one another at each stage of the primary growth (Table 5). With advancing grass growth stage the contents of total solids, neutral detergent fibre, acid detergent fibre, acid detergent lignin and water soluble carbohydrates generally increased whereas of the values for total solids digestibility, crude protein and buffering capacity decreased. There were no consistent differences between the two species.

3.2.2 Silage

Harvest

With advancing maturity within the primary growth, there was a general increase (P<0.001) in values of neutral detergent fibre and acid detergent fibre with a corresponding decrease (P<0.001) in total solids digestibility (Tables 6 and 7). In comparison to the later two harvests, the Early harvest had higher (P<0.001) crude protein, propionic acid and fermentation products (P<0.01) yet had lower (P<0.001) water-soluble carbohydrates. Generally, in comparison to both earlier harvests, the Late harvest had greater (P<0.01) values of total solids and acid detergent lignin. Furthermore, the Late harvest had a lower pH (P<0.05) yet a higher lactic acid/fermentation product (P<0.01) compared to the Early harvest.

Species

Overall, in comparison to PRG, TIM had higher values of neutral detergent fibre (P<0.05), acid detergent fibre (P<0.01), acid detergent lignin (P<0.01) and acetic acid (P<0.05).

Enzyme

In comparison to the control, both added enzymes similarly increased (P<0.001) values of total solids (P<0.01), water-soluble carbohydrates, lactic acid, fermentation products and lactic acid/fermentation products and reduced pH (P<0.001). Compared to the control, ENZ 1 had reduced values of acid detergent lignin (P<0.05) and ENZ 2 had reduced values of propionic acid (P<0.05) and butyric acid (P<0.01).

Harvest x species

For TIM silages only, values of ethanol were higher (P<0.01) at the Early harvest compared to the Late harvest and the total solids recovery rates were lower (P<0.01) for the Intermediate harvest compared to the Late harvest.

Harvest x Enzyme

In comparison to the control treatment, both enzymes reduced neutral detergent fibre (P<0.05) and acid detergent fibre (P<0.01) in the Early and Intermediate harvests. In contrast, compared to the control, ENZ 2 and ENZ 1 reduced neutral detergent fibre (P<0.01) and acid detergent fibre (P<0.001), respectively, in the Late harvest.

Compared to the control treatment, ENZ 2 reduced (P<0.05) acetic acid contents in the Intermediate harvest. In comparison to the control, ammonia-N values were reduced (P<0.01) by both enzymes during Early harvest and ENZ 2 during Intermediate harvest. In comparison to the control, the outflow of effluent was higher (P<0.01) for both enzymes in the Early harvest, but only for ENZ 2 in the Intermediate harvest.

Overall, there was a positive correlation coefficient between effluent outflow (g kg^{-1} fresh grass ensiled) and total silage compaction (cm; Figure 2) for primary growth silages (r = 0.82, n = 70, P<0.001).

3.2.3 In vitro batch anaerobic digestion test

Harvest

The silage mass-specific CH_4 yields were higher (P<0.05) for the Early harvest than the two later harvests (Table 8). The first order decay constant (k) values were greatest (P<0.01) for the Early harvest and lowest for the Intermediate harvest (mean values of 0.114 and 0.099, respectively). The maximum CH_4 production rate per day (u) was greater (P<0.001) for Early harvest compared to Intermediate and Late harvests. The lag phase (Δ; P<0.001) and half-life time (P<0.001) increased with advancing stage of maturity in the primary growth.

The mass-specific CH_4 yield of silage effluent ranged from 302 to 622 L CH_4 kg^{-1} volatile solids and there were no significant treatment effects.

Species

Generally, the half-life time was greater (P<0.05) for TIM silages (7.0 days) compared to PRG silages (6.7 days).

Table 5 Mean (s.d.) chemical composition (g kg^{-1} TS, unless indicated otherwise in the footnotes) of two grass species at three stages in the primary growth

Harvest	Early		Intermediate		Late	
Species	PRG	TIM	PRG	TIM	PRG	TIM
Growth stage	2.3	2.2	2.5	2.5	2.8	2.8
TS	116 (11.4)	119 (5.4)	138 (1.2)	135 (18.4)	181 (18.4)	183 (8.7)
TSD	722 (14.4)	731 (5.0)	679 (21.4)	670 (35.4)	620 (19.8)	616 (38.2)
NDF	602 (14.4)	628 (24.0)	645 (41.8)	627 (22.4)	674 (23.4)	718 (10.6)
ADF	355 (8.3)	354 (7.0)	360 (19.4)	353 (14.1)	390 (22.4)	401 (15.8)
ADL	36 (2.4)	38 (4.6)	35 (3.5)	31 (1.4)	40 (7.2)	44 (3.6)
Ash	108 (9.8)	93 (9.6)	86 (8.9)	91 (3.8)	82 (7.3)	78 (6.2)
CP	166 (11.3)	174 (9.5)	128 (9.9)	117 (24.9)	110 (11.3)	112 (10.6)
WSC	46 (9.3)	29 (19.5)	57 (47.3)	89 (40.6)	76 (12.1)	38 (19.7)
WSCaq	6 (1.9)	4 (2.8)	9 (7.7)	15 (8.2)	17 (3.9)	9 (4.8)
BC	558 (81.9)	472 (17.8)	357 (22.1)	344 (56.5)	286 (35.0)	280 (11.5)

Note: Harvest (primary growth), Early = 14 May, Intermediate = 28 May, Late = 11 June; Species, PRG, perennial ryegrass, TIM, timothy; Growth stage according to Moore et al. (1991); TS, total solids (g kg^{-1}); TSD, total solids digestibility (g kg^{-1}); NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; CP, crude protein; WSC, water-soluble carbohydrates; WSC*aq*, in aqueous phase (g L^{-1}); BC, buffering capacity (m Eq kg^{-1} TS).

Table 6 Effects of harvest, species and enzyme treatments on the chemical composition (g kg^{-1} TS, unless indicated otherwise in the footnotes) of silages from two grass species at three stages in the primary growth

Harvest	Species	Enzyme	TSc	TSD	NDF	ADF	ADL	CP	WSC
Early	PRG	Control	140	647	642	398	35	140	9
Early	PRG	ENZ 1	161	663	539	312	31	158	12
Early	PRG	ENZ 2	184	675	485	305	31	153	16
Early	TIM	Control	145	668	622	392	43	148	9
Early	TIM	ENZ 1	192	664	530	315	34	159	12
Early	TIM	ENZ 2	204	685	519	294	33	163	14
Inter.	PRG	Control	162	623	675	414	49	110	12
Inter.	PRG	ENZ 1	178	635	560	335	30	120	17
Inter.	PRG	ENZ 2	189	633	560	318	31	104	21
Inter.	TIM	Control	166	603	659	417	41	115	12
Inter.	TIM	ENZ 1	170	601	594	370	40	117	15
Inter.	TIM	ENZ 2	191	614	569	336	42	118	19
Late	PRG	Control	174	582	675	403	38	117	16
Late	PRG	ENZ 1	186	613	618	370	36	116	18
Late	PRG	ENZ 2	201	574	605	351	40	100	21
Late	TIM	Control	197	613	699	417	55	124	13
Late	TIM	ENZ 1	200	565	667	400	49	108	16
Late	TIM	ENZ 2	213	551	624	365	49	112	18
Standard error of the mean									
Harvest (H)			5.1	8.1	6.3	4.7	1.8	2.9	0.6
Species (S)			4.1	6.6	5.1	3.8	1.5	2.3	0.5
Enzyme (E)			5.1	8.1	6.3	4.7	1.8	2.9	0.6
H × S			7.2	11.4	9.2	6.9	2.8	4.0	0.9
H × E			8.8	14.0	11.6	8.7	3.5	4.9	1.1
S × E			7.2	11.4	9.2	6.9	2.8	4.0	0.9
H × S × E			12.4	19.8	17.5	13.1	5.2	7.0	1.6
Levels of significance									
H			**	***	***	***	**	***	***
S			NS	NS	*	**	**	NS	NS
E			**	NS	***	***	*	NS	***
H × S			NS	NS	NS	NS	NS	NS	NS
H × E			NS	NS	*	**	NS	NS	NS
S × E			NS	NS	NS	NS	NS	NS	NS
H × S × E			NS	NS	NS	NS	NS	NS	NS

Note: Harvest (primary growth), Early = 14 May, Inter., Intermediate = 28 May, Late = 11 June; Species, PRG, perennial ryegrass, TIM, timothy; ENZ 1, mainly xylanase activity, ENZ 2, mainly cellulase activity; TSc, total solids (g kg^{-1}), corrected for the loss of volatile compounds according to Porter and Murray (2001); TSD, total solids digestibility (g kg^{-1}); NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; CP, crude protein; WSC, water-soluble carbohydrates; * P<0.05, ** P<0.01, *** P<0.001, NS=not significant.

Table 7 Effects of harvest, species and enzyme treatments on the conservation characteristics (g kg^{-1} TS, unless indicated otherwise in the footnotes) of silages from two grass species at three stages in the primary growth

Har.	Spp.	Enzyme	pH	LA	AA	PA	BA	Eth	NH_3-N	FP	LA/FP	TSrr	Eff.
Early	PRG	Control	5.02	12	27	6.6	28	11	460	84	0.12	0.85	287
Early	PRG	ENZ 1	3.86	134	26	4.8	4	16	133	184	0.73	0.80	405
Early	PRG	ENZ 2	3.93	102	16	3.0	10	60	177	186	0.49	0.74	434
Early	TIM	Control	4.84	34	28	7.0	14	22	336	105	0.21	0.91	242
Early	TIM	ENZ 1	3.90	117	30	7.4	15	44	107	193	0.56	0.92	419
Early	TIM	ENZ 2	3.78	108	25	5.5	7	42	91	171	0.57	0.93	440
Inter.	PRG	Control	4.49	36	20	5.0	20	16	257	97	0.34	0.94	178
Inter.	PRG	ENZ 1	3.59	113	12	1.0	3	11	84	141	0.81	0.93	220
Inter.	PRG	ENZ 2	3.49	109	8	0.3	3	10	83	130	0.84	0.88	345
Inter.	TIM	Control	4.56	40	28	4.4	24	13	258	109	0.26	0.78	237
Inter.	TIM	ENZ 1	4.19	89	20	2.2	21	15	157	124	0.55	0.85	329
Inter.	TIM	ENZ 2	3.70	105	11	1.1	6	10	90	133	0.74	0.82	407
Late	PRG	Control	4.15	50	5	0.9	15	10	99	79	0.54	0.91	68
Late	PRG	ENZ 1	3.71	121	11	0.6	6	20	113	158	0.76	0.94	78
Late	PRG	ENZ 2	3.49	117	10	0.5	3	20	82	150	0.78	0.91	169
Late	TIM	Control	4.31	35	10	1.9	16	9	142	71	0.44	0.99	104
Late	TIM	ENZ 1	4.05	66	10	1.2	12	9	143	98	0.66	0.96	100
Late	TIM	ENZ 2	3.68	113	10	0.7	9	12	103	144	0.78	0.92	152
Standard error of the mean													
Harvest (H)			0.090	8.4	1.7	0.46	2.8	1.5	20.3	7.5	0.050	0.024	24.3
Species (S)			0.071	6.8	1.3	0.37	2.3	1.1	16.6	5.7	0.040	0.019	17.0
Enzyme (E)			0.090	8.4	1.6	0.45	2.8	1.4	20.3	7.3	0.050	0.024	16.5
H × S			0.131	12.1	2.5	0.69	4.1	2.1	28.8	10.9	0.072	0.035	30.3
H × E			0.165	15.2	3.1	0.82	5.2	2.7	35.2	14.9	0.091	0.044	30.7
S × E			0.131	12.1	2.3	0.65	4.8	2.0	28.8	10.9	0.072	0.035	22.0
H × S × E			0.250	23.1	4.4	1.25	7.9	3.8	49.8	23.3	0.137	0.066	44.3
Levels of significance													
H			*	NS	***	***	NS	NS	**	**	**	*	***
S			NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS
E			***	***	*	**	*	NS	***	***	***	NS	***
H × S			NS	NS	NS	NS	NS	**	NS	NS	NS	**	NS
H × E			NS	NS	*	NS	NS	NS	**	NS	NS	NS	**
S × E			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
H × S × E			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Note: Har., harvest (primary growth), Early=14 May, Inter., Intermediate=28 May, Late=11 June; Spp., species, PRG, perennial ryegrass, TIM, timothy; ENZ 1, mainly xylanase activity, ENZ 2, mainly cellulase activity; LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid; Eth, ethanol; NH_3-N, ammonia-N (g kg^{-1} N); FP, total fermentation products (LA + AA + PA + BA + Ethanol); LA/FP, lactic acid as a proportion of total fermentation products; TSrr, total solids recovery rate=(silage weight x silage TS/1000)/(fresh grass weight x fresh grass TS/1000) g g^{-1}; Eff., Effluent (g kg^{-1} grass ensiled); * P<0.05, ** P<0.01, *** P<0.001, NS=not significant.

Table 8 Effects of harvest, species and enzyme treatments on the mass- and area-specific CH_4 yields, kinetics and effluent mass-specific CH_4 yields, of silages from two grass species at three stages in the primary growth

Harvest	Species	Enzyme	Silage mass-SMY	k	U	Δ	T_{50}	Effluent mass-SMY
Early	PRG	Control	441	0.12	50.2	1.3	5.6	428
Early	PRG	ENZ 1	417	0.11	45.6	1.2	5.7	622
Early	PRG	ENZ 2	432	0.11	48.4	1.3	5.8	384
Early	TIM	Control	435	0.12	51.4	1.6	5.8	305
Early	TIM	ENZ 1	480	0.11	54.2	1.7	6.1	464
Early	TIM	ENZ 2	430	0.11	46.0	1.3	6.0	529
Inter.	PRG	Control	436	0.10	43.9	1.7	6.7	382
Inter.	PRG	ENZ 1	362	0.10	34.3	1.6	6.8	525
Inter.	PRG	ENZ 2	393	0.10	38.0	1.6	6.8	342
Inter.	TIM	Control	395	0.10	39.0	1.5	6.6	528
Inter.	TIM	ENZ 1	375	0.10	36.5	2.1	7.2	369
Inter.	TIM	ENZ 2	395	0.10	40.0	1.7	6.6	555
Late	PRG	Control	388	0.10	39.4	1.9	6.8	362
Late	PRG	ENZ 1	350	0.10	34.9	2.7	7.7	392
Late	PRG	ENZ 2	364	0.11	38.8	3.6	8.2	414
Late	TIM	Control	330	0.11	35.1	3.2	7.8	302
Late	TIM	ENZ 1	328	0.11	36.0	3.9	8.5	401
Late	TIM	ENZ 2	399	0.11	41.6	3.5	8.2	473
Standard error of the mean (s.e.m.)								
Harvest (H)			12.8	0.002	0.96	0.07	0.04	37.8
Species (S)			10.4	0.002	0.84	0.10	0.07	30.6
Enzyme (E)			12.8	0.002	1.10	0.24	0.17	37.3
H × S			18.1	0.003	1.46	0.18	0.12	56.5
H × E			22.2	0.004	1.90	0.41	0.30	71.6
S × E			18.1	0.003	1.59	0.35	0.25	51.6
H × S × E			31.3	0.006	2.75	0.60	0.43	112.1
Levels of significance								
H			*	**	***	***	***	NS
S			NS	NS	NS	NS	*	NS
E			NS	NS	NS	NS	NS	NS
H × S			NS	NS	NS	NS	NS	NS
H × E			NS	NS	NS	NS	NS	NS
S × E			NS	NS	NS	NS	NS	NS
H × S × E			NS	NS	NS	NS	NS	NS

Note: Harvest (primary growth), Early = 14 May, Inter., Intermediate = 28 May, Late = 11 June; Species, PRG, perennial ryegrass, TIM, timothy; ENZ 1, mainly xylanase activity, ENZ 2, mainly cellulase activity; Mass SMY, mass specific methane yield (L CH_4 kg^{-1} volatile solids), note average (s.d.) of cellulose control 390 (77.4) L CH_4 kg^{-1} volatile solids; k, first order decay constant per day, the average (s.e.m.) coefficient of determination for all k values R^2 =0.996 (0.0004); u, maximum specific methane production rate in ml CH_4 g^{-1} volatile solids d^{-1}; Δ, lag phase in days. T_{50}, half-life defined as the time taken in days to produce 0.50 of the methane. * P<0.05, ** P<0.01, *** P<0.001, NS, not significant.

4 Discussion

4.1 Grass yield and chemical composition

The four-cut annual harvest schedule applied similarly to both grass species in this study resulted in all harvests being at the same grass phenological growth stage (Moore et al., 1991). The mean annual total solids yields of 14,406 and 12,840 kg ha^{-1} a^{-1} for PRG and TIM, respectively, were at the higher end of yields currently achieved in Ireland (McEniry et al., 2013), and their distribution among the four consecutive cuts (Cuts 1-4: 48%, 25%, 16% and 11% for PRG; 53%, 19%, 19% and 9% for TIM) was similar to relative values previously obtained for PRG (Keating and O'Kiely, 2000) and TIM (Seppälä et al., 2009).

Although both grass species were repeatedly harvested at the same growth stage, their total fibre content declined from Cuts 1 to 4 (636 to 519 g kg^{-1} total

solids), with cellulose (i.e. acid detergent fibre - acid detergent lignin) being the main constituent at Cuts 1 to 3 but with hemicellulose (i.e. neutral detergent fibre - acid detergent fibre) dominating at Cut 4. Thus, the four cuts provided a range of fibre characteristics under which to test the efficacy of the fibrolytic enzyme additives.

The grass fermentation coefficient developed by Weissbach and Strubelt (2008) categorises the expected ease with which herbage will preserve satisfactorily as silage based on its total solids, water-soluble carbohydrates and buffering capacity values. The fermentation coefficients of the four cuts of both species ranged from 14 to 22 and, being below the critical value of 45, indicates a predicted shortage of substrate for satisfactory lactic acid dominant silage fermentations (Weissbach and Strubelt, 2008). Thus, the various cuts within the annual growth provided a range of conditions within which the effects of fibrolytic enzyme additives on silage preservation could be assessed.

Since Cut 1 contributed almost as much biomass to annual yield as the remaining three cuts combined, and in recognition of the rapid rates of increase in total solids yield and fibre components during the primary growth in late May and early June, this study additionally assessed the effects of advancing or deferring Cut 1 by a fortnight. The changes recorded in chemical composition of the grasses were similar to those reported by King et al. (2012) and Nolan et al. (2018).

4.2 Ensilage characteristics of control treatment

The main aim of ensiling grasses for biogas production is to quantitatively preserve total solids, and thus to make digestible energy available in a stable state for year-round use (Plöchl et al., 2006). An overall consequence of ensiling grasses (i.e. controls) with low total solids content at each cut, however, were the losses associated with both effluent outflow and a high incidence of secondary clostridial fermentations. These were generally accompanied by increasing concentrations of fibre, but particularly acid detergent lignin, similar to the findings of King et al. (2013) and Nolan et al. (2018). However, the conservation outcomes were not consistent across cuts or grass species. For example, Cuts 1, 3 and 4 generally exhibited elevated concentrations of butyric acid and NH_3-N, indicating that extensive clostridial secondary fermentations occurred (Rooke and Hatfield, 2003). In contrast, Cut 2 and particularly TIM displayed a heterofermentative lactic acid bacteria dominant fermentation (Pahlow et al., 2003).

Altering the timing of the primary growth harvest changed its conservation efficiency. This was partially related to later harvested grass having an elevated total solids content and thus a reduced effluent outflow. Furthermore, the elevated total solids content allied to the reduced buffering capacity resulted in an improvement in FC and consequently in a progressive inhibition of undesirable clostridial fermentation compared to early harvesting. These findings agree with results reported by Conaghan et al. (2008) and King et al. (2013).

4.3 Effect of enzymes on ensilage characteristics

In agreement with Nolan et al. (2018) both enzyme additives, but particularly ENZ 2, significantly reduced silage fibre content compared to the control treatment (9% and 15% reduction in neutral detergent fibre for ENZ 1 and ENZ 2, respectively). These effects were therefore in addition to any hydrolysis of grass fibre components that occurred in the control treatment due to, for example, acid hydrolysis (McDonald et al., 1991).

The mean fibrolytic effect of the added enzymes on silage hemicellulose was a modest 6% reduction in its concentration compared to the control treatment, with ENZ 1 and ENZ 2 each having a more marked impact at only a single harvest (at Cuts 1 and 2, respectively) and with the effects being somewhat more evident with PRG than TIM. In contrast, the fibrolytic effects on cellulose were considerably greater, with mean reductions relative to the control treatment of 14% and 22% for ENZ 1 and ENZ 2, respectively. However, the effects of ENZ 2 appeared quite variable, ranging from a 12% (Cut 2) to a 32% (Cut 4) reduction in cellulose relative to the control treatment. In addition, the relative scale of impact of either added enzyme on each grass species appeared to differ across cuts despite the similar overall effect of ENZ 1 (8%-10% reduction) or ENZ 2 (13%-16% reduction) on the neutral detergent fibre concentration in each species. Thus, the fibrolytic effects were not consistent across added enzymes, grass species or cuts, and there appeared to be specificity between enzymes and substrate, in agreement with the findings of Nolan et al. (2018).

Furthermore, despite the laboratory assays indicating that ENZ 1 and ENZ 2 possessed mainly xylanase and cellulase activities, respectively, both enzyme additives exhibited greater hydrolytic effects on cellulose than hemicellulose under the broad range of conditions prevailing in this study, the reasons for which remains unknown.

The general outcome that enzymes applied to grasses immediately prior to ensiling improved silage fermentation profiles relative to the control treatments (an effect that was generally greater with ENZ 2 than ENZ 1) likely reflects the ability of the added enzymes to increase the supply (and/or possibly the initial rate of availability) of fermentable substrate. Thus, whereas the combined concentration of control silage fermentation products plus water-soluble carbohydrates was on average 2.1 times greater than the concentration of water-soluble carbohydrates in the corresponding grass at ensiling, the equivalent values for the ENZ 1 and ENZ 2 treatments were 2.4 and 2.6, respectively. However, the magnitude of this impact of fermentable substrate provision on the overall fermentation profile was small in circumstances such as Cut 2 TIM, where the control treatment preserved satisfactorily (as per threshold values proposed by Haigh and Parker, 1985) and was entirely inadequate in circumstances such as Cut 3 TIM and Cut 4 TIM, where the control treatments preserved particularly badly (high butyric acid and exceedingly high ammonia-N). However, as the silage preservation challenge became progressively less severe, then firstly ENZ 2 (Cut 1 TIM, Cut 3 PRG and Cut 4 PRG) and then both added enzymes (Cut 1 PRG and Cut 2 PRG) produced well preserved silages. Thus, these added enzymes were capable of successfully aiding silage preservation when the challenge to preservation was mild. It was beyond the ability of these enzymes however to improve silage preservation when the challenge to preservation became more difficult, similar to findings by Dehghani et al. (2012). Under more challenging conditions, alternative effective additive treatment and/or adequate rapid wilting would be necessary to achieve satisfactory silage preservation (McDonald et al., 1991).

The increase in effluent outflow in response to the fibrolytic effects of the added enzymes (from 163 g kg^{-1} control grass ensiled to 238 and 320 g kg^{-1} ENZ 1 and ENZ 2 grass ensiled, respectively) agrees with Nolan et al. (2018). Furthermore, the absence of a grass species effect on the proportional increase in effluent outflow due to added enzymes likely reflects the absence of an overall grass species effect on the proportional reduction in neutral detergent fibre content when the enzymes were applied. However, a disappointing outcome of the large increase in effluent outflow was that enzyme treatments consequently did not improve the recovery rate of total solids ensiled (total solids recovery rate) despite the improvements in silage fermentation efficiency with which they were associated.

By advancing or delaying the primary growth harvest by a fortnight, the many effects of the added enzymes on fibre components, preservation characteristics and effluent outflow reflected the patterns, variability and specificity noted amongst the four cuts comprising the annual growth. The temporal profile of effluent outflow and silage compaction for primary growth silages as shown in Figure 1 suggests the activities of the added enzymes continued from day 2 through to day 98 of the 120 day ensilage duration which confirms a robust durability of the added enzymes during ensilage. These results are similar with findings by Nolan et al. (2018).

4.4 Mass-specific CH_4 yield

The mass-specific CH_4 yield of control silages ranged from 258 to 565 L CH_4 kg^{-1} volatile solids and this is typical of values previously recorded using this *in vitro* anaerobic digestion method (Nolan et al., 2016). The cellulose standard reached 0.94 of it's theoretical CH_4 yield, estimated at 414 L CH_4 kg^{-1} volatile solids via Buswell equations (Wall et al., 2013), suggesting the inoculum was suitable for evaluation of these silages. Few reports have been published on the mass-specific CH_4 yield of silages made from a multi-cut annual grass harvest schedule, and in the current study the considerable differences that occurred across the cuts that comprised annual growth are not readily explained by the corresponding differences in silage composition shown in Tables 2 and 3. In contrast, the quite similar mass-specific CH_4 yields for both species at each cut are in agreement with the species effects reported by Nolan et al. (2018).

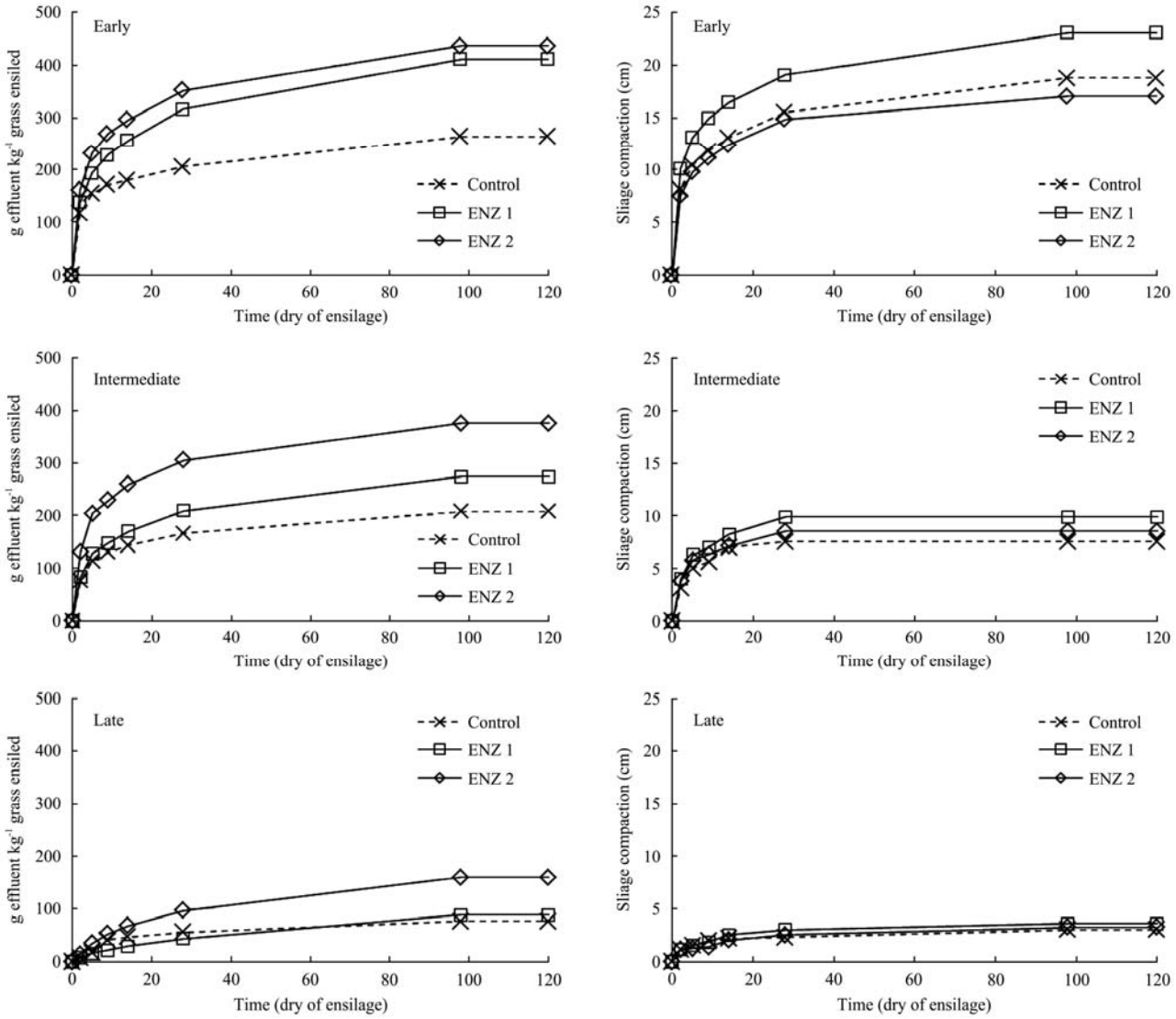

Figure 1 Left: Cumulative effluent production (g effluent kg^{-1} fresh grass ensiled) and, right: cumulative silage compaction (cm) during ensilage of primary growth silages (Early, Intermediate and Late) averaged across the two grass species

Overall the absence of an effect of the added enzymes on mass-specific CH_4 yield for silages from each cut or either grass species was surprising and disagrees with findings previously reported by Nolan et al. (2018). Possible explanations for the lack of an impact of added enzymes on mass-specific CH_4 yield responses in this study are two-fold. Firstly, the added enzymes may have hydrolysed the more easily accessible parts of grass cell wall (hemicellulose and cellulose) that would have been digested anyway during the course of anaerobic digestion. This outcome however is unlikely because the previous study by Nolan et al. (2018) clearly showed an increase in mass-specific CH_4 yield in response to applying these enzymes to comparable grasses. Secondly, the increase in effluent outflow due to enzyme treatment may provide a more tenable explanation. The volatile solids in silage effluent are soluble and fully digestible, and are thus fully accessible for anaerobic digestion. Furthermore, some of the constituents likely to have made an important contribution to many effluents in this study, such as propionic acid, butyric acid and ethanol, have methanogenic potentials considerably greater than, for example, polymers of either pentoses or hexoses (Weissbach, 2009). Thus, the mean mass-specific CH_4 yield for silage effluents in this study was 443 L CH_4 kg^{-1} volatile solids. This contrasts with silage volatile solids (mean mass-specific CH_4 yield of 376 L CH_4 kg^{-1} volatile solids) where the non-digestible nature of lignin allied to its negative impact on the hydrolysis of hemicellulose and cellulose will result in restricted methanogenic potential. Thus, by increasing the effluent outflow the added enzymes likely decreased the mass-specific CH_4 yield

potential of the resultant silages, thereby offsetting any benefits anticipated from hydrolysing fibre during ensilage.

The decrease in mass-specific CH_4 yield with advancing maturity of the primary growth is in agreement with McEniry and O'Kiely (2013) and reflects the negative effects of increased lignification with advancing grass growth stage. There was a similar absence of effect of added enzymes on enhancing mass-specific CH_4 yields in the primary growth as was noted amongst the four cuts of the annual growth, and the enzymes again increased effluent outflow as discussed above.

4.5 Area-specific CH_4 yield

The mean annual area-specific CH_4 yield recorded for the two temperate grass species in this study (control treatments: 4143 m^3 CH_4 ha^{-1}) is at the upper end of the values reported by Prochnow et al. (2009). The contribution of the four cuts to annual area-specific CH_4 yield (54%, 21%, 13% and 12% from Cuts 1 to 4, respectively) largely reflects their relative contributions of biomass. Similarly, the greater annual area-specific CH_4 yield for PRG than TIM (control treatments: 4,666 and 3,619 m^3 CH_4 ha^{-1}, respectively) was primarily a reflection of the greater output of biomass by the former.

In this study, grasses were ensiled without field wilting and this is a common practice in the moist climatic conditions in Ireland. Consequently, the large outflow of digestible, highly methanogenic silage effluent volatile solids means that for such grass silage based anaerobic digestion systems to be technically and economically sustainable it is necessary to collect this effluent and use it as part of the feedstock for CH_4 production (McEniry et al., 2011). This is evident from Figure 2 where it is shown that CH_4 derived from effluent would have increased area-specific CH_4 yield from 4143 to 4412 m^3 CH_4 ha^{-1} a^{-1} (control treatments), from 4058 to 4460 m^3 CH_4 ha^{-1} a^{-1} (ENZ 1 treatments), and from 3944 to 4633 m^3 CH_4 ha^{-1} a^{-1} (ENZ 2 treatments) in response to effluent inclusion.

The enzyme additives used in this study clearly increased hydrolysis of grass fibre and the monosaccharides thereby released contributed to more lactic acid dominant fermentations. However, the concurrent increase in effluent outflow and its associated losses resulted in a 2% and 5% reduction in the area-specific CH_4 yield of ENZ 1 and ENZ 2 silages, respectively compared to control silages. However, when this effluent was collected and utilised as a feedstock it increased annual area-specific CH_4 yield by 6%, 10% and 17% for control, ENZ 1 and ENZ 2 treatments, respectively. Consequently, when effluent was captured and used for methane production ENZ 1 and ENZ 2 treatments had area-specific CH_4 yield values 101% and 105% of the control treatment, respectively. Therefore, the overall assessment of enzymes added at ensiling to enhance methane yields from grass silages where highly dependent on whether effluent was included as a feedstock.

Even though Cut 1 contributed almost half of the biomass in annual yield and earlier harvesting of this cut increased its mass-specific CH_4 yield while later harvesting would be expected to increase biomass yield, area-specific CH_4 yield effects of altering the timing of Cut 1 need to be considered in the context of annual output of CH_4 rather than CH_4 output from Cut 1 in isolation. The optimal harvest timing will therefore depend firstly on the relative impacts of biomass increase and mass-specific CH_4 yield decline associated with deferral of Cut 1, but secondly on the impact of the timing of Cut 1 on the growth, ensilage efficiency and mass-specific CH_4 yield of biomass from subsequent cuts.

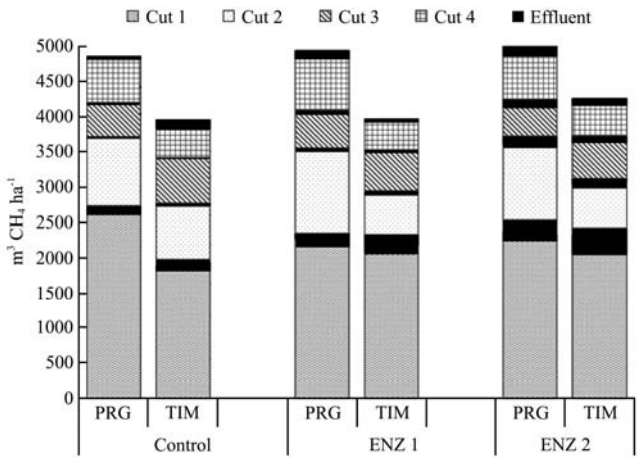

Figure 2 The area-specific CH_4 yields of silages and effluent (in black above each respective cut) for two grass species ensiled with three fibrolytic enzyme treatments, at four consecutive cuts through the annual growth (i.e. Cut 1, 28 May; Cut 2, 16 July; Cut 3, 03 September; Cut 4, 28 November)

5 Conclusion

Although both added enzymes clearly hydrolysed grass fibre, and this contributed to greatly improved preservation in a number of cases, they also significantly increased silage effluent outflow. The scale and nature of this loss resulted in the added enzymes not improving silage mass-specific CH_4 yield with either grass species. The combined effects of grass biomass yield, ensilage efficiency and silage mass-specific CH_4 yield meant that ENZ 1 and ENZ 2 generated annual area-specific CH_4 yield that were 98% and 95% of the control treatment, respectively. However, when the effluent outflow was captured and anaerobically digested the corresponding combined silage plus effluent annual area-specific CH_4 yield were 101% and 105% of the control treatment.

Altering the timing of Cut 1 altered silage mass-specific CH_4 yield. However, any assessment of this effect on area-specific CH_4 yield needs to be considered in the context of annual, rather than solely Cut 1 output.

Area-specific CH_4 yield is a decisive criterion. It is evident from this study that high yields of biomass, high efficiencies of conserving harvested biomass during ensilage and producing silages of high mass-specific CH_4 yield are each important management targets in order to achieve high area-specific CH_4 yield. Capturing and utilising silage effluent is also essential in this context.

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