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Biomethane Yield from Different European *Phragmites australis* Genotypes, Compared with Other Herbaceous Wetland Species Grown at Different Fertilization Regimes

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Abstract: *Phragmites australis*, *Typha latifolia*, *T. angustifolia* and *Arundo donax* are tall wetland graminoids with the potential to replace fossil fuels under sustainable cultivation conditions. We investigated the biomethane (CH_4) production of these four species, including four different genotypes of *P. australis*, which represent the high intraspecific diversity of European reed. All plants were grown under three different macronutrient supplies (no nutrients added, an equivalent of 75 kg N ha^{-1} year^{-1} added and an equivalent of 500 kg N ha^{-1} year^{-1} added). Biomethane production was measured in four independent batch digestion tests. Across all experiments, fertilization regime had little effect on CH_4 yield, which was on average 222 ± 31 L kg^{-1} volatile solids (VS). The lowest yield was produced by *T. angustifolia* (140 L kg$_{VS}$$^{-1}$) receiving no nutrients, while the highest yield was produced by *A. donax* (305 L kg$_{VS}$$^{-1}$) in the highest nutrient treatment. The intraspecific diversity of *P. australis* did not affect biomethane production. All *P. australis* genotypes produced on average 226 ± 19 L CH_4 kg$_{VS}$$^{-1}$, which, although high, was still lower than conventional biogas species. The biomass production of *P. australis* was less increased by fertilization than that of *Typha* sp. and *A. donax*, but all species had similar biomass without fertilization.

1. Introduction

Biomethane is a sustainable and renewable alternative to fossil fuels, produced from organic material, e.g., from highly productive plants. Biomethane can be used as vehicle fuel, as fuel in combined heat and power systems, or to produce electricity and heat, and the digested effluent (digestate) can be utilized as fertilizer [1–3]. In the face of the global climate and energy crisis, it is of utmost importance to identify plants that can serve as biomass feedstock for biomethane production. Species cultivated on arable land, however, compete with food and fodder production for the cropland and raw materials, such as fertilizers, required for cultivation. Alternative cultivation methods are urgently needed to produce sustainable, renewable resources for bioenergy. A promising option is

paludiculture, the cultivation of flooding-tolerant plants on re-wetted peat soil. Peatlands drained for multiple purposes are prone to subsidence and emit greenhouse gases due to high decomposition rates under oxidized conditions [4]. After re-flooding, these formerly drained organic soils will once again contribute to C-sequestration, and thereby climate change mitigation, as well as potentially intercept nutrient run-off from agricultural land and thereby contribute to decreased eutrophication [5,6]. Not all re-flooded peatlands will be feasible for nature restoration, if heavily degraded. This will especially be true for former agricultural land under prolonged and extreme drainage. Further, to avoid a complete loss of cultivation areas by flooding, paludiculture can compensate for loss of conventional agriculture. Paludi-crops can potentially replace terrestrial bioenergy crops, which conflict with food crops. Moreover, crops for biomethane production are harvested at peak biomass production, when the plants have allocated most of the available nutrients from the soil to the shoots. Summer harvest is a common procedure to remove nutrients from soils over a short time span. Harvest during late summer would ecologically be the most sustainable, since breeding birds would not be disrupted [5,6].

The area potentially available for paludiculture in Europe differs between countries [7]. About 80% of all German peatlands, for example, are located in northern Germany. Of those, between 102,200 ha (Mecklenburg-Vorpommern) and 207,500 ha (all northern federal states) have been assessed to be suitable for paludiculture, which is about half of all peatlands in that region [7,8]. This area may become even higher if nature conservation of certain peatlands will be impossible to implement. Although drained peatlands may constitute a comparably low percentage of the total agricultural area (e.g., 7.3% in Germany), they are responsible for considerable amounts of the total agricultural greenhouse gas emissions (38% in Germany) [9].

A promising paludi-crop is the perennial wetland grass *Phragmites australis* (common reed). *Phragmites australis* has a wide geographic distribution and includes several distinct populations with a plethora of genotypes [10–12]. The species often dominates fresh and brackish marshes and is highly productive, due to its clonal growth form and tall dense stands that shade potentially competing species. Constructed wetlands are often planted with *P. australis*, benefiting from its high nutrient and pollutant removal efficiency as well as high biomass production promoting the removal of nitrogen through denitrification [13–15]. However, different genotypes respond very differently to nutrient availability, which will affect their tissue nutrient accumulation, physiology and growth potential, and must therefore be taken into account when choosing a plant for a specific purpose [16–20]. It has been estimated that a minimum of 20 ton dry mass of *P. australis* could be harvested from paludiculture in northern Germany, a considerable amount for feeding several power plants of 20 MW capacity [8].

Other tall wetland graminoids that can replace fossil fuels under sustainable cultivation conditions are *Typha latifolia*, *T. angustifolia* and *Arundo donax*. These species have moderate to high flooding tolerance with sustained productivity in waterlogged soils [21–23], spread clonally by belowground shoots and have high biomass productivity. All three species are perennial, with aboveground shoots dying off in autumn but belowground shoots and roots that overwinter and produce new shoots the following growing season. Sowing will therefore largely be unnecessary in their cultivation.

There are many advantages in using perennial wetland plants as biomethane and bio-combustion feedstock. For example, the dead standing biomass can be dry harvested in winter for combustion and stored for a longer period, while avoiding biomass degradation. Moreover, most wetland perennials are highly productive and immobilize large amounts of nutrients in their tissue, nutrients which are removed from the soil and water body and therefore increase the quality of the surrounding ecosystem. Depending on the utilization purpose, different nutrient concentrations in the biomass may be suitable for different applications. Bio-combustion, for example, requires low tissue concentrations of Cl, S, K and Si, while a C:N ratio of plant material between 20:1 and 30:1 is optimal for biogas production. Therefore, a late summer harvest of biomass is more appropriate for biogas production, as more nutrients are still retained in green tissue compared to winter tissue [24–26]. Since nutrient uptake is at least partly determined by the mineral amounts available in the soil, the nutrient regime and possible fertilization are aspects highly important for the desired biomass properties.

Typha sp., *A. donax* and *P. australis* have considerably increased growth rates when supplied with ample nutrients. However, their tissue characteristics also change with the stored minerals, posing a potential challenge to the intended utilization purposes of the biomass. For example, very low nutrient inputs may result in very high C:N ratios in grasses, which are often associated with harder to digest lignin and cellulose concentrations, leading to lower biogas production [27]. We therefore investigated the biomethane (CH_4) production of four tall, perennial wetland species grown under three different macronutrient supplies (no nutrients added, an equivalent of 75 kg N ha^{-1} year^{-1} added and an equivalent of 500 kg N ha^{-1} year^{-1} added). These nutrient regimes were chosen, respectively, to represent a control on pure peat soil, an equivalent of the average global N fertilizer use of 70–80 kg ha^{-1} year^{-1} [28] and an equivalent of the very high nutrient availability expected in most eutrophic re-flooded peat soil. Furthermore, as *P. australis* has tremendous intraspecific variability, which is also mirrored in genotypes displaying highly different growth rates, nutrient uptake efficiencies and tissue properties, we included a comparison of four different genotypes of the species, which represent the high genetic, cytological and morphological diversity of European reed.

2. Materials and Methods

2.1. Plant Growth and Experimental Setup

The plants selected for this study were *Typha latifolia* (TL), *Typha angustifolia* (TA), *Arundo donax* (AD) and four genotypes of *Phragmites australis*. The four genotypes were chosen based on their different morphology and productivity. Here, they are named according to their sampling origin, but they do not represent the overall population of reeds from their original location. The genotypes were IT (from Italy, very tall, thick but few stems), NL (from the Netherlands, very productive, tall, many thin stems), RO (from Romania, very productive, very tall, thick stems) and DK (from Denmark, very short and thin phenotype with low productivity and many stems). The four genotypes of *P. australis* were propagated vegetatively from rhizomes of a live collection of the grass grown in a common environment at the Aarhus University, Denmark (56°13′ N; 10°07′ E) [10]. *Typha latifolia* and *T. angustifolia* were propagated by seeds from wild populations from Denmark (Randers Fjord) and Germany (Aalbude, Lake Kummerow), respectively. *Arundo donax* was vegetatively propagated by culturing stems originating from Italy. Healthy shoots of *A. donax* were layered horizontally in shallow water and adventitious shoots with roots were propagated from the stem nodes. After 40 days, the new shoots of the seven taxa reached similar height (ca. 30 cm) and one shoot per taxon per treatment was transplanted into 60 L containers (0.3 m length × 0.3 m width) containing a commercial peat substrate (Pindstrup), which contained the following nutrient concentrations: Nitrate-N 70 g m^{-3}, Ammonium-N 50 g m^{-3}, P 60 g m^{-3}, K 200 g m^{-3}, Mg 14 g m^{-3}, B 0.4 g m^{-3}, Mo 2.0 g m^{-3}, Cu 1.7 g m^{-3}, Mn 2.9 g m^{-3}, Zn 0.9 g m^{-3}, Fe 8.4 g m^{-3}. One replicate of each of the seven taxa was placed in a 1000 L tank (length 1.2 m, width 1 m, height 1 m). The tank was filled with tap water immediately under the soil surface in the planted pots, so that all pots were water-saturated, but never flooded with standing water. The water table was kept stable throughout the experiment. Each tank represented one of four replicates of three levels of fertilization treatments (Table 1). A nutrient stock solution was prepared using tap water with added 97.2 g L^{-1} Pioneer NPK Macro $19 - 2 - 15 +$ Mg Green salt, 2 mL L^{-1} micronutrient solution and 0.34 g L^{-1} Pioneer Fe Chelate EDDHA (6% Fe), which contained 18.76 g L^{-1} N, 2.24 g L^{-1} P, 14.97 g L^{-1} K, 2.92 g L^{-1} Mg, 3.79 g L^{-1} S and 0.46 mL L^{-1} B, 0.28 mL L^{-1} Cu, 2.64 mL L^{-1} Fe, 1.00 mL L^{-1} Mn, 0.10 mL L^{-1} Mo and 0.36 mL L^{-1} Zn. The three different fertilization treatments were achieved by adding either 0 mL (treatment A), 10 mL (treatment B), or 70 mL (treatment C) of this stock solution to 500 mL tap water. Each individual pot was fertilized weekly for 10 weeks in total, from the beginning of May until August in 2016.

Table 1. Quantity of macronutrients N, P, K within three fertilization treatments added to each pot weekly.

Treatment	Fertilization (kg N ha^{-1} year^{-1})	N (g per pot per Week)	P (g per pot per Week)	K (g per pot per Week)	Amount of Stock Solution Added Per Pot Per Week (mL)
A	0	0	0	0	0
B	75	0.1876	0.0224	0.1497	10
C	500	1.3132	0.1568	1.0479	70

The experimental setup was a fully-factorial, 3 (fertilization treatments: A, B and C) × 7 (taxa − species and genotypes: *P. australis* DK, *P. australis* IT, *P. australis* NL, *P. australis* RO, *A. donax*, *A. angustifolia*, *T. latifolia*) × 4 (replicates), experimental design. The experiment was conducted outdoors in Denmark (56°13′ N; 10°07′ E) from April to August 2016.

2.2. Biomethane Production Experiments and Data Analyses

Green shoots of *P. australis* genotypes and the other three species were harvested in August 2016 and dried in an oven at 60 °C for 24 h. A part of the shoot material was combusted at 550 °C for 2 h in a muffle oven, to determine the ash content. The percentage of ash was subtracted from the dry weight to obtain the CH_4 yield per VS (volatile solids, equivalent to ash-free dry weight), which is usually used for reporting CH_4 yield per amount of substrate. Biomethane yield was measured in four different experimental setups, to allow for different combinations of production methods and treatments. The biogas production measurements included four batch digestion tests that were performed in lab scale using 1 L glass bottles as anaerobic batch digesters. Each test was conducted with digesters with plant biomass as well as controls without plant biomass. All the biomass samples were cut to small pieces (5–10 mm long) and wetted for 24 h prior to the batch test, to assure that the inoculum mixed well with the samples. Inoculum (500 g wet weight) and 10 g dried biomass sample (i.e., substrate) were added to all digesters, while controls only received inoculum. The inoculum was collected from a nearby biogas plant and stored at 37 °C for at least 4 days before the experiment to reduce residual CH_4 production. The inoculum's total solids (TS) and VS were analyzed at three occasions during the experiments. Average TS was 2.74% (minimum (min) 2.48%, maximum (max) 3.07%) and average VS was 1.60% (min 1.45%, max 1.81%). The digesters were randomly distributed in an incubator with a constant temperature (37 °C) and stirred daily. The digesters were sealed with gas-tight rubber stoppers with a septum where gas samples were taken with a syringe. Each digester was connected, through a plastic tube, to a glass U-tube, which was partly filled with water. Due to the increased pressure from produced biogas entering the first part of the U-tube, the water surface was elevated in the second part until it reached an Infrared-photo sensor, after which an "event" corresponding to 45 mL gas production was registered. Simultaneously, water levels in the two parts of the U-tube were reset through an interconnecting pipe.

Biogas composition (CH_4 and CO_2) in all digesters were determined at 6–10 occasions during each experiment by taking 20 μL gas samples using a Hamilton 50 μL syringe. The samples were analyzed in a Varian CP-3800 gas chromatograph using a Thermal Conductivity Detector and CP-Porabond Q capillary column. The experiments were terminated after 15–34 days when daily gas production in the digesters had decreased to 0.5 events, corresponding to less than 10% of the maximum daily production.

For each digester, the daily (24 h) production of CH_4 was calculated by multiplying the produced gas volume with daily concentrations. Daily CH_4 concentrations were obtained by interpolating between measurements. Total CH_4 production for each digester was calculated as the sum of the daily values and the amount of CH_4 remaining in the gas space at the end of the experiment. The latter was calculated by multiplying final CH_4 concentration with the volume of the gas space in the digester (0.5 L). Finally, the CH_4 yields from plant biomass were calculated by subtracting the mean CH_4 production of the control digesters (without biomass) from the CH_4 production for each digester with added plant biomass. The amount of CH_4 per produced volume applied to 25 °C and 1 atm

(the conditions in the U-tubes). For all statistical analyses, Statgraphics Centurion XVII (Statpoint Technologies, Inc., Warrenton, VA, USA) was used.

2.2.1. Batch Digestion Test 1

Biomethane yield was analyzed for one sample per fertilization treatment of each taxon. Since the plants in fertilization treatment A grew poorly, there was not enough biomass for replicated analyses. The effect of fertilization treatment was therefore tested by two-way analysis of variance (ANOVA) without replication and hence, without factor interaction. The controlled factors were "Fertilization treatment" and "Taxon". Bartlett's test confirmed homoscedasticity for both factors. Tukey's honestly significant differences (HSD) post-hoc test was used to find significant differences between means within each pooled factor.

2.2.2. Batch Digestion Test 2

To test if pre-processing procedures have an effect of CH_4 yield, the shoots were either chopped (5–10 mm long pieces) or ground (mesh size 1 mm) before digestion. Biomethane yield was analyzed in three replicates of all species, where *P. australis* yield was analyzed as a single taxon for biomass of NL, RO and IT. Only plants from the highest fertilization treatment were included in this experiment. Biomethane yield was analyzed by two-way analysis of General Linear Models with type III Sums of Squares and the categorical factors "Species" and "Pretreatment" and their interaction. Bartlett's test confirmed homoscedasticity, and Tukey's HSD post-hoc test was used to find significant differences between means.

2.2.3. Batch Digestion Test 3

Fertilization treatments B and C (middle and high nutrient availability) were compared for two replicates of *A. donax*, *T. latifolia*, *T. angustifolia* and *P. australis* NL and IT. The CH_4 yields were analyzed by two-way analysis of General Linear Models with type III Sums of Squares and the categorical factors "Taxon" and "Fertilization treatment" and their interaction. Bartlett's test confirmed homoscedasticity, and Tukey's HSD post-hoc test was used to find significant differences between all pairwise means.

2.2.4. Batch Digestion Test 4

Biomethane yield for leaves of fertilization treatment B was analyzed for three replicates of *A. donax*, *T. latifolia*, *T. angustifolia* and *P. australis* NL, RO and IT. Data were analyzed by one-way ANOVA with "Taxon" as factor. Bartlett's test confirmed homoscedasticity, and Tukey's HSD post-hoc test was used to find significant differences between means.

2.3. Biomass and Data Analysis

All shoots were cut by the end of the growing season in October 2016. The harvested biomass was dried in an oven at 80 °C until constant dry weight. Since the data were heteroscedastic, a two-factorial analysis of variance was performed with rank-transformation [29]. The two factors were "Fertilization treatment" and "Taxon", and their interaction was analyzed as well. Non-parametric Games–Howell post-hoc test was conducted to determine differences between the groups.

3. Results

Across all experiments, the average CH_4 yield was 222 ± 31 L kg^{-1} volatile solids (VS). The lowest yield produced was 140 L kg_{VS}^{-1} by *T. angustifolia* receiving the lowest fertilization treatment, while the highest yield was 305 L kg_{VS}^{-1}, produced by *A. donax* receiving the highest fertilization treatment. *Phragmites australis* (all genotypes and fertilization treatments) produced on average over all batch digestion tests 226 ± 19 L kg_{VS}^{-1}. The ash content of shoots varied from 4.2% to 11%.

3.1. Batch Digestion Test 1

Biomethane yield differed significantly between species ($p < 0.001$), where *T. angustifolia* had lower yield than all other taxa, and all *P. australis* types had similar yield (Table 2). Fertilization regime also significantly affected biomethane yield ($p < 0.05$), where the highest fertilization resulted in the highest biomethane yield, and the two lower fertilizer levels did not produce detectable yield differences on average. However, there was no replication of the single taxon and differences may be more subtle than detected here, if the analyses were repeated including replication. We thus observed that *A. donax* produced the highest biomethane yield under all fertilizer regimes, compared to all other taxa, and that the increase in biomethane yield with higher fertilizing regime seemed to depend on taxon. The largest increase in biomethane yield production with increasing fertilizer was observed in *T. angustifolia* (19% higher in level B than A, 16% higher in level C than B), *P. australis* DK and NL (8% and 11% higher in C than B, respectively) and *T. latifolia* (18% higher in C than B). In contrast, the other taxa showed very little effect of fertilization regime on biomethane yield.

Table 2. Biomethane yield (L kg_{VS}^{-1} (VS = volatile solids)) of different wetland plant species and genotypes, grown at three different fertilization treatments (A: equivalent to 0 kg N ha^{-1} $year^{-1}$; B: equivalent to 75 kg N ha^{-1} $year^{-1}$; C: equivalent to 500 kg N ha^{-1} $year^{-1}$). Different lowercase letters ([a], [b]) indicate statistically significant ($p < 0.05$) differences between means within one factor, based on Tukey's honestly significant differences (HSD) post-hoc test. SD = standard deviation. IT, Italian genotype *P. australis*; NL, Dutch genotype *P. australis*; RO, Romanian genotype *P. australis*; DK, Danish genotype *P. australis*.

Species		Fertilization Treatment			Taxon Mean ± SD
		A	B	C	
P. australis	IT	224.7	195.2	223.3	214 ± 17 [a]
	NL	200.1	208.0	236.1	215 ± 19 [a]
	RO	229.6	233.5	233.1	232 ± 2 [a]
	DK	223.8	223.9	243.7	231 ± 12 [a]
A. donax		245.8	252.1	256.6	252 ± 5 [a]
T. angustifolia		139.5	172.0	204.5	172 ± 33 [b]
T. latifolia		212.6	203.8	249.2	222 ± 24 [a]
Fertilization treatment Mean ± SD		211 ± 35 [a]	213 ± 26 [a]	235 ± 17 [b]	

3.2. Batch Digestion Test 2

Both pretreatment and taxon had a significant effect on CH_4 yield ($p < 0.001$), but no interaction was detected. Ground shoot material resulted in 15% to 26% higher yield than chopped material. *Arundo donax* produced about 11% to 26% more CH_4 than any other taxon, followed by *P. australis*, *T. latifolia* and *T. angustifolia*, which produced least (Figure 1).
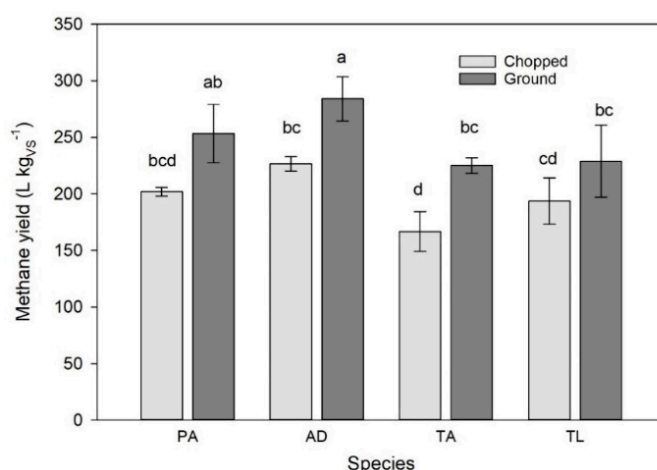
Figure 1. Biomethane yield (mean ± SD) of different wetland plant species (PA: *Phragmites australis*, AD: *Arundo donax*, TA: *Typha angustifolia*, TL: *Typha latifolia*). Shoots were fermented as chopped or ground material. Plants were fully fertilized with an equivalent of 500 kg N ha^{-1} year^{-1}. Different letters indicate statistically significant ($p < 0.05$) differences between means based on Tukey's HSD post-hoc test. VS: volatile solids.

3.3. Batch Digestion Test 3

Biomethane yield differed significantly among taxa ($p < 0.05$; Figure 2), while fertilization treatment or the interaction of the two factors had no effect, possibly due to only two replicates. The lowest CH$_4$ yield on average (± SD) across fertilization regimes B and C was produced by *T. angustifolia* (198 ± 25 L kg$_{VS}$$^{-1}$). The highest yield resulted from *A. donax* (255 ± 7 L kg$_{VS}$$^{-1}$). The other taxa produced CH$_4$ yields that were between these significantly different yields, and *P. australis* genotypes did not differ significantly (*P. australis* IT: 241 ± 12 L kg$_{VS}$$^{-1}$; *P. australis* NL: 213 ± 11 L kg$_{VS}$$^{-1}$; *T. latifolia*: 216 ± 40 L kg$_{VS}$$^{-1}$).
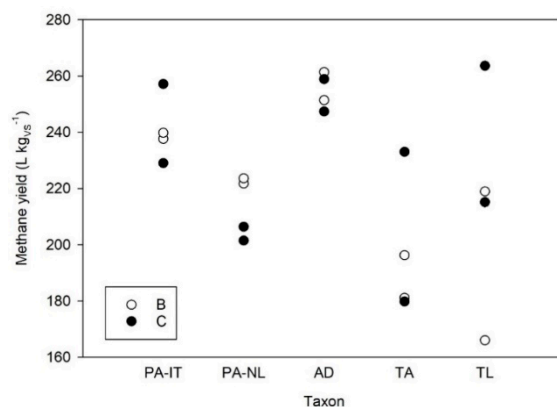

Figure 2. Biomethane yield of two replicates of different wetland plant taxa (PA-IT: *Phragmites australis* Italian genotype, PA-NL: *P. australis* Dutch genotype, AD: *Arundo donax*, TA: *Typha angustifolia*, TL: *Typha latifolia*). Plants were grown at two different fertilization treatments, B (equivalent to 75 kg N ha^{-1} year^{-1}) and C (equivalent to 500 kg N ha^{-1} year^{-1}). VS: volatile solids.

3.4. Batch Digestion Test 4

Different taxa produced significantly different CH$_4$ yields within fertilization treatment B ($p < 0.01$; 75 kg N ha^{-1} year^{-1}; Figure 3), although the *P. australis* genotypes did not differ significantly. *Typha angustifolia* produced significantly (~26%) less CH$_4$ than the *P. australis* genotypes as well as *A. donax* (32% lower yield). Mean CH$_4$ yield of *Typha latifolia* was ~20% higher than *T. angustifolia* but lower than the other taxa, although these differences were not statistically significant.
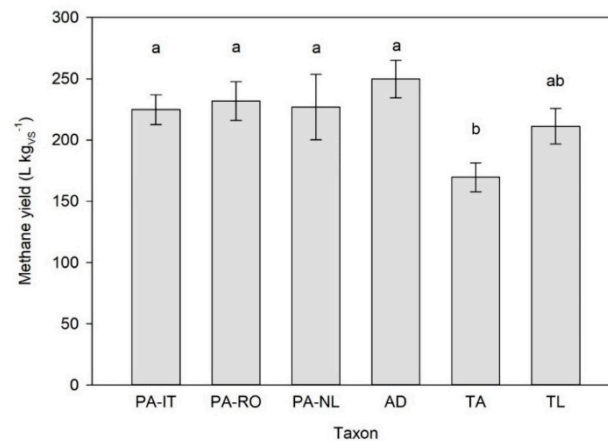
Figure 3. Biomethane yield (mean ± SD) of three replicates of different wetland plant taxa (PA-IT: *Phragmites australis* Italian genotype, PA-RO: *P. australis* Romanian genotype, PA-NL: *P. australis* Dutch genotype, AD: *Arundo donax*, TA: *Typha angustifolia*, TL: *Typha latifolia*). Plants were fully fertilized with an equivalent of 75 kg N ha^{-1} year^{-1}. Different lowercase letters (a, b, ab) indicate statistically significant ($p < 0.05$) differences between means based on Tukey's HSD post-hoc test. VS: volatile solids.

3.5. Biomass

Aboveground biomass was significantly affected by both fertilization treatment ($p < 0.001$), taxon ($p < 0.001$) and their interaction ($p < 0.01$). While increased fertilization affected *A. donax*, *T. latifolia* and *T. angustifolia* biomass in particular, the four *P. australis* genotypes only responded to a minor degree to higher nutrient availability (Figure 4). Although the highest fertilization treatment resulted in three to seven times higher biomass production of *P. australis* compared to the lowest fertilization treatment, the within-treatment variation was very high, which explains the nonsignificant differences for that species. In contrast, *A. donax* significantly increased its biomass about 25 times, while *T. latifolia* had 26 times, and *T. angustifolia* 20 times more biomass production from the lowest to the highest fertilization treatment. While the average biomass produced by *A. donax*, *T. latifolia* and *T. angustifolia* was higher than any *P. australis* genotype, here, also the within-group variation was very high.
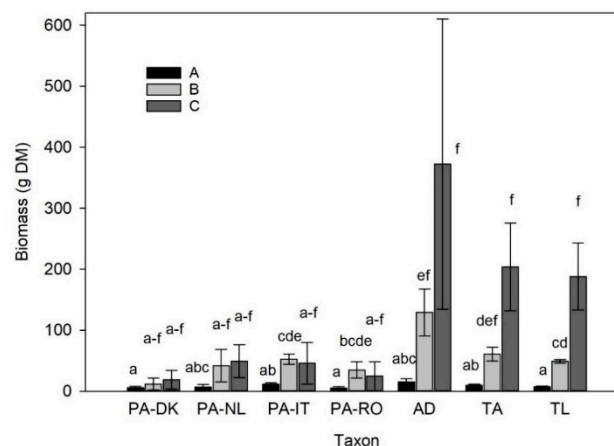

Figure 4. Mean biomass (± SD) of four replicates of different wetland plant taxa (PA-DK: *Phragmites australis* Danish genotype, PA-NL: *P. australis* Dutch genotype, PA-IT: *P. australis* Italian genotype, PA-RO: *P. australis* Romanian genotype, AD: *Arundo donax*, TA: *Typha angustifolia*, TL: *Typha latifolia*). Each plant was grown on a surface area of 0.09 m^2. Plants received different fertilization treatments (A: no nutrients added, B: fertilized with an equivalent of 75 kg N ha^{-1} year^{-1}, C: fertilized with an equivalent of 500 kg N ha^{-1} year^{-1}). Different lowercase letters indicate statistically significant ($p < 0.05$) differences across means. DM: dry mass.

4. Discussion

We quantified CH$_4$ production of four paludiculture crops, *Typha angustifolia*, *T. latifolia*, *Arundo donax* and *Phragmites australis*, and how it was affected by different fertilization regimes. Since the latter species has a great intraspecific variation, we investigated four different genotypes within *P. australis*, which have very different growth rates and nutrient absorption capacity. Our overall finding was that CH$_4$ production differed between different species, but to a minor degree between nutrient regimes, and the effect of nutrients depended largely on the specific species. While the highest fertilization regime resulted in the highest biomethane productivity in the first batch digestion test (11% higher than the other two fertilization regimes), yield was similar between the high and middle fertilization regime in batch test 3. Batch test 3 was conducted with only two replicates and hence, its results need to be interpreted cautiously. Thus, biomethane yield differences varied from being lower (*A. donax* and *P. australis* NL) to being up to 24% higher (*T. latifolia*) in the high fertilization regime. Most prominently, we found that pretreatment of the biomass had a significant effect on CH$_4$ yield of all treatments and taxa used, with grinding of leaf material resulting in significantly more yield than chopping. Pretreatments such as harvesting time and bioaugmentation have previously been used to raise CH$_4$ yield [30–32]. Effective microbial conversion alleviates digestion of the lignocellulosic biomass, and ground leaf material was finer and more degraded than chopped leaves, increasing the plant substrate digestibility.

Even though we found minor differences in CH$_4$ yield due to fertilization, some of the species increased their aboveground biomass dramatically with higher nutrient regimes, while the taxa barely produced enough biomass to test for biomethane yield without fertilization. *Typha angustifolia* has previously been shown to have extraordinarily high biomass production, higher even than *T. latifolia* [33,34]. In general, *Typha* species are known to thrive in eutrophic rather than oligotrophic systems, which explains the significant increase in biomass production with higher nutrient availability [35]. Interestingly, the biomass of the different *P. australis* genotypes responded less to fertilization, showing a large response variability, in contrast to previous studies [18,36]. This may be partly explained by inherent growth differences, which were likely to occur especially during the first growing season. Compared to aboveground biomass of *Typha* sp., which consists almost solely of leaves, as well as *A. donax*, which has large leaf blades with a very large area and tall thick stems, *P. australis* productivity is lower, even in the tall octoploid type RO. Compared to conventional crops, *P. australis* previously showed lower harvest and biogas yields, and was therefore not recommended for biogas production [37]. However, as this was a short-term study, biomass production was strongly dependent on the initial establishment phase. Annual shoot biomass production in established *P. australis* stands is usually similar to *Typha* sp. stands [38,39].

If paludiculture harvest takes place in late summer/early autumn, when carbohydrates have been translocated to rhizomes, shoot growth in the following year is ensured [40] and a sustainable biomass harvest similar to *Typha* sp. may be possible to achieve for *P. australis*. A late summer harvest may also be favorable to avoid disrupting the breeding season for wetland birds. We found a much stronger effect of nutrient treatment on aboveground biomass production for *Typha* sp. than for *P. australis*, while both taxa had similar biomass without fertilization. Our results therefore suggest that if the paludiculture area is not fertilized, it is possible that *P. australis* may even achieve a higher long-term productivity than *Typha* species. Although peak biomass occurs at the end of the growing season, where we harvested the shoots for a good estimate of potential dry matter productivity, harvest time has previously been shown to affect the biogas production differently in several species [41]. For example, *Miscanthus × giganteus* and *P. australis* had higher protein tissue concentrations when harvested in July than when harvested in October, while *Panicum virgatum* had no differences in protein. Also, hemicellulose concentration varied in these species with harvest time: while *M. × giganteus* had more hemicellulose in October, *P. australis* had more in July [41]. Since the tissue quality affects the decomposition and biogas production to a high degree, the harvest time will have an impact on

biomethane yield. We here harvested tissue in August as a compromise for potential tissue differences in the different taxa from mid to late summer.

Nonetheless, all *P. australis* genotypes produced similar, high CH_4 yields, on average 226 ± 19 L CH_4 kg_{VS}^{-1}. The CH_4 yield was comparable to the biofuel species *A. donax*, and even higher than *T. angustifolia*. *Typha angustifolia* has previously been identified as suitable biomethane crop with yields about 340 L CH_4 kg_{VS}^{-1}, but it has also been emphasized that certain pre-treatments will be needed to enhance biogas yield, since the species has a high cellulose, hemicellulose and lignin content [42]. Other studies have found CH_4 yields for *P. australis* from freshwater ecosystems of 188 L kg_{VS}^{-1} [32] and 260 L kg_{VS}^{-1} [43] or higher [37]. Biomethane yield of *P. australis* from saline marine areas has been measured to be 220 L kg_{VS}^{-1} [44]. *Typha latifolia* produced on average 214 ± 28 L CH_4 kg_{VS}^{-1} in the present study, while CH_4 yields of 105 L kg_{VS}^{-1} have been reported previously [31]. Highly fertilized *A. donax* yielded highest in our study (305 L kg_{VS}^{-1}), but its specific CH_4 yield was previously measured to range from 130 to 170 L kg_{VS}^{-1} [45,46]. Conventional biofuel crops such as maize or sunflower have specific CH_4 yields of 300–400 L kg_{VS}^{-1} and 390 L kg_{VS}^{-1} respectively, and are therefore more productive than the wetland plants studied here [47,48]. We did not analyze tissue elements, but a previous study has shown that especially N, but also P and K concentrations of leaves of different *P. australis* genotypes increased to a similar degree with increasing nutrient availability [36]. This strongly suggests that nutrient allocation among different genotypes varies to a minor degree, which would explain why we did not find larger differences between biomethane yield among the *P. australis* genotypes investigated here.

Although we only grew the experimental plants for a single growth period and in pots with a surface area of 0.09 m^2, it is useful to cautiously estimate the potential biomethane yield per area. All non-fertilized taxa would yield very little biomethane on an area basis, compared to yield expressed per weight of volatile solids (ranging from 12 L CH_4 m^{-2} in RO *P. australis* to 38 L CH_4 m^{-2} in *A. donax*). Fertilization treatment B, corresponding to 75 kg N ha^{-1} $year^{-1}$, resulted in somewhat higher biomethane yields per area, ranging from 35 L CH_4 m^{-2} in DK *P. australis* in batch digestion test 1 to 468 L CH_4 m^{-2} in *A. donax* in batch digestion test 3. Not surprisingly, the highest yield per area was achieved at the highest fertilization (corresponding to 500 kg N ha^{-1} $year^{-1}$). Although *P. australis* taxa yielded rather low results (45 L m^{-2}, 51 L m^{-2} and 74 L m^{-2} in DK, RO and IT *P. australis*, respectively), *A. donax* would produce up to 1989 L m^{-2} when biomass was ground and up to 1600 L m^{-2} when biomass was chopped. Throughout all tests, *A. donax* would generally yield highest on an area-based biomethane production, followed by *Typha* sp. Clearly, fertilization regime would have a greater impact on biomethane yield per area than per weight of volatile solids, as has been found previously [41]. However, carry-over effects from previous season harvests are likely to affect biomass production, as are harvests after the first growing season. Therefore, long-term experiments are needed to confirm this preliminary indication.

The aboveground biomass used in the present study was harvested in summer when shoots are relatively young and less lignified. Also, in summer, nutrients are still retained in shoots instead of being translocated to rhizomes for overwinter storage [40]. In eutrophic areas, late summer harvesting is therefore encouraged to increase nutrient removal. Biomass harvested in summer coupled with regrown biomass in autumn will possibly produce the highest biomethane yields [30]. Although nutrient availability had effects on CH_4 and biomass yield mainly at the highest fertilization regime, it is likely to have a larger impact after one or several years of cultivation, since nutrients cannot be recycled within the vegetation after the aboveground parts are harvested [49]. The nutrient status of rewetted areas can range from oligotrophic to eutrophic [5]. If the soil fertility is already low at the beginning of the first growing season after sowing or rhizome transplantation, it could be recommended to add nutrients externally. However, next to preserving peat soils, another aim of paludiculture is to remove excess nutrients from the organic substrate, and hence, fertilization is undesirable and should be considered with great caution [5]. Since the very high fertilization regime used here resulted in highly variable biomass production, relatively low fertilizer additions are likely sufficient in the

second and following growing seasons. We merely investigated biomethane production from the first growing season, but future studies should take harvests from several consecutive years into account. For increased sustainability, the leftover biomass can be applied as bio-fertilizer. It has been shown previously that *P. australis* biomass retains most of its N and all of its P after biogas production and can therefore be re-circulated to the harvested land [50].

Paludiculture is preferable to harvesting the investigated species in their natural environment, although they often produce large monospecific stands [5]. There are several reasons for this, however probably most important are the considerable additional benefits of paludiculture. For example, a formerly drained and little productive agricultural field becomes a regained area for cultivation of alternative crops, emissions of CO_2 and CH_4 are minimized under correct management practices and nutrient removal can be increased in paludiculture [5]. Moreover, poorly managed wetland plant harvesting in nature can harm local ecosystems and have a negative impact on biodiversity and the physicochemical characteristics of the environment, which can be avoided by practicing paludiculture [51]. Apart from the overall management and fertilization regime, harvesting techniques and timing of harvest, as well as water table level, may also affect re-growth of paludiculture crops. Although these aspects were outside the scope of this study, they should be considered in future studies.

In conclusion, the intraspecific diversity of *P. australis* was not reflected in biomethane production in our study. Hence, provenance may not be a necessary consideration for biogas yield, and ecological availability and productivity are the preferable issues to consider. All investigated genotypes had fairly high CH_4 yields, but the biomass production when fertilized was low, compared to the two *Typha* species and *A. donax*. Fertilization regime only affected CH_4 yield at the highest nutrient level and seemed to depend on the species. Fertilization affected the aboveground biomass of *Typha* sp. and *A. donax* the most. Overall, however, none of the investigated species had CH_4 yields as high as reports for conventional biofuel crops. More studies are needed regarding pretreatments, which could potentially increase biomethane yields considerably, and tissue quality at different harvest times.

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