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Optimization of ensiling fermentation of *Elodea* sp. for biogas production

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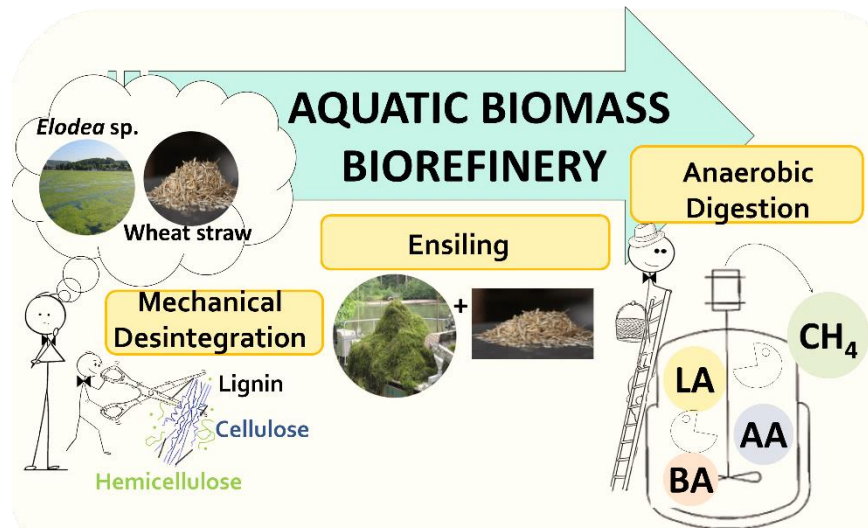
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Abstract

The invasive aquatic *Elodea* species *E. nuttallii* and *E. canadensis* are capable of significant growth and are a cause of considerable environmental issues. The established method to control *Elodea* overgrowth in Germany is mechanical harvesting. Unfortunately, the biomass of *Elodea* remains unused and must be treated in compost plants at considerable expense and effort due to its poor structure, high water content and low carbon-nitrogen (C/N) ratio. Based on a biorefinery concept, anaerobic digestion (AD) for biogas production may prove to be a promising solution for utilizing and adding value to the *Elodea* waste fraction.



Following the harvesting process, the *Elodea* biomass undergoes a rapid microbial decomposition process due to its high moisture content (approximately 95%). Nevertheless, the economic production of biogas via commercial AD, which is typically conducted in a continuous manner, necessitates the provision of a year-round supply of high-quality biomass. It is therefore necessary to consider aspects such as seasonal variations in biomass and the conservation of biomass. The present thesis, presented in three papers, aimed to explore options for developing novel process chains for the AD of aquatic plants and agricultural residues, with a focus on long-term biomass conservation. The work generally followed two biological processes at the laboratory scale: firstly, AD, which produces methane, and secondly, ensiling as a conservation method. This included evaluating the co-ensiling of *Elodea* and wheat straw with and without silage additives, as well as the mono-ensiling of both as controls.

The results demonstrated that the preservation of *Elodea* is a challenging process due to its high moisture content and low water-soluble carbohydrate (WSC) concentration, both of which have a detrimental impact on the ensiling process. Nevertheless, although pure ensiling of *Elodea* is feasible, the option of co-ensiling *Elodea* with wheat straw may prove a more suitable

approach for the development of a method for long-term storage in conventional clamp silos. In this context, the use of wheat straw can provide suitable substrate characteristics for co-ensiling, including sufficient total solids (TS), additional sugars and the consequent production of organic acids. A further assessment was conducted using the BMP test to evaluate the methane yields of the mono-ensiling of *Elodea*, as well as the co-ensiling with wheat straw. The batch of AD of the mono- and co-ensiling of *Elodea* demonstrated the considerable potential of *Elodea* biomass as a suitable substrate or co-substrate for biogas plants. Nevertheless, for practical applications (e.g. conservation in clamp silos for subsequent AD), a mixture of *Elodea* with wheat straw comprising approximately 30% TS is recommended. In the present study, the experiments were conducted exclusively with wheat straw due to the limitations in experimental capacity. It may be feasible to combine *Elodea* with a variety of other drier lignocellulosic materials, and further investigation into this possibility is warranted. The feasibility of ensiling and subsequent AD of wheat straw was also explored, indicating that a pretreatment (e.g. particle size reduction) is necessary due to the recalcitrant structure of the polysaccharides, which impedes their conversion. Initially, the feasibility of ensiling wheat straw was investigated by assessing the impact of particle size reduction on biodegradability. The findings indicated that reducing the particle size enhanced the biodegradability of wheat straw, leading to the production of organic acids. The ensiling of wheat straw also demonstrated that the structure of this lignocellulose is highly complex due to its elevated lignin content, which presents a significant challenge. This hypothesis was also corroborated when wheat straw was subjected to AD without prior ensiling, but previously pretreated by reducing the particle size. In this instance, the reduction of particle size proved to be a pivotal factor in enhancing the bioaccessibility and bioavailability of fermentable sugars, thereby increasing the overall digestibility and hydrolysis of the wheat straw. This resulted in a significant increase in methane yields, reaching up to 26%. Conversely, the utilization of silage additives proved ineffective in the mono-ensiling of both *Elodea* biomass and wheat straw, as well as in the co-ensiling process.

While ensiling *Elodea* and co-ensiling with wheat straw for biogas plants may be a promising avenue, further research is necessary to ensure cost-effective implementation. It is evident that technology along the value chains requires significant technical improvements, including modifications to cutting, collecting, and harvesting techniques. Furthermore, cost estimates must be established in conjunction with the resulting final biogas yield.

Zusammenfassung

Die invasiven aquatischen *Elodea*-Arten *E. nuttallii* und *E. canadensis* weisen ein bemerkenswertes Wachstumspotenzial auf, das in manchen Fällen zu Herausforderungen führen kann. Die mechanische Ernte von *Elodea*-Pflanzen stellt in Deutschland die gängigste Methode zur Bekämpfung des *Elodea*-Wildwuchses dar. Bedauerlicherweise bleibt die *Elodea*-Biomasse ungenutzt und muss aufgrund ihrer spezifischen Eigenschaften mit großem Aufwand in Kompostierungsanlagen behandelt werden. Dazu zählen ihre schlechte Struktur, ihr hoher Wassergehalt sowie ihr geringes Kohlenstoff-Stickstoff-Verhältnis (C/N). Es war zu untersuchen, ob die anaerobe Vergärung (AD) zur Biogaserzeugung auf der Grundlage eines Bioraffineriekonzepts eine vielversprechende Lösung sein kann, um *Elodea*-Biomasse zu nutzen und aufzuwerten.

Nach der Ernte durchläuft die gewonnene *Elodea*-Biomasse aufgrund ihres hohen Feuchtigkeitsgehalts (ca. 95 %) einen relativ schnellen mikrobiellen Abbauprozess. Allerdings stellt die wirtschaftliche Biogaserzeugung aus kommerzieller AD, die in der Regel im kontinuierlichen Betrieb erfolgt, gewisse Anforderungen an die ganzjährige Versorgung mit hochwertiger Biomasse. Daher sollten Aspekte wie die Saisonabhängigkeit der Biomasse und die Konservierung der Biomasse in die Überlegungen mit einbezogen werden. Die vorliegende Arbeit, die in drei Beiträgen vorgestellt wird, zielt darauf ab, potenzielle Prozesskettenstrategien für die AD von Wasserpflanzen und landwirtschaftlichen Reststoffen zu untersuchen. Dabei wird besonderes Augenmerk auf die Entwicklung von Lagermethoden für die Langzeitkonservierung von Biomasse gelegt. Im Rahmen dieser Arbeit wurden zwei biologische Prozesse im Labormaßstab durchgeführt. Einerseits wurde Methan in der Biogasanlage erzeugt, andererseits wurde die Silierung als Konservierungsmethode untersucht. Dabei wurden die gemeinsame Silierung von *Elodea* und Weizenstroh mit und ohne Siliermittel sowie die Monosilierung von beiden als Kontrolle berücksichtigt.

Die erzielten Ergebnisse zeigten, dass die Konservierung von *Elodea* eine Herausforderung darstellt, da sie einen hohen Feuchtigkeitsgehalt und eine geringe Menge an wasserlöslichen Kohlenhydraten (WSC) aufweist, die sich beide negativ auf die Silierung auswirken.

Die reine Silierung von *Elodea* ist grundsätzlich möglich, doch könnte die Option der gemeinsamen Silierung mit Weizenstroh für die Entwicklung einer Methode zur Langzeitlagerung in konventionellen Klemmsilos möglicherweise besser geeignet sein. In diesem Zusammenhang möchten wir darauf hinweisen, dass Weizenstroh geeignete Substrateigenschaften für die Co-Silierung bieten könnte. Dazu zählen beispielsweise eine ausreichende Gesamttrockenmasse (TS), zusätzliche Zucker und die daraus resultierende Produktion von organischen Säuren. Zur Bewertung der Methanausbeute der Monosilierung von *Elodea* sowie der Co-Silierung mit Weizenstroh wurde eine AD nach dem BMP-Test durchgeführt. Die Ergebnisse des AD-Ansatzes der Mono- und Co-Silierung von *Elodea* deuten darauf hin, dass *Elodea*-Biomasse ein vielversprechendes Substrat/Cosubstrat für Biogasanlagen sein könnte. Für praktische Anwendungen, beispielsweise die Konservierung in Klemmsilos für eine nachfolgende AD, könnte eine Mischung von *Elodea* mit Weizenstroh von etwa 30 % TS jedoch eine vielversprechende Option sein. Aufgrund von Kapazitätsgründen konnten in dieser Arbeit leider nur Versuche mit Weizenstroh durchgeführt werden. Es wäre durchaus sinnvoll, weitere Untersuchungen zu Mischungen von *Elodea* mit verschiedenen trockeneren lignozellulosehaltigen Substraten durchzuführen.

Des Weiteren wurde die Silierung und anschließende AD von Weizenstroh untersucht. Dabei zeigte sich, dass aufgrund der widerspenstigen Struktur, die der Umwandlung von Polysacchariden entgegensteht, eine Vorbehandlung, beispielsweise in Form einer Reduzierung der Partikelgröße, erforderlich sein könnte. In einem ersten Schritt wurde die Silierung von Weizenstroh durchgeführt, wobei die Größe der Weizenstrohpartikel bewertet wurde. Die Ergebnisse deuten darauf hin, dass eine Verringerung der Partikelgröße die biologische Abbaubarkeit von Weizenstroh erhöhen und zur Produktion organischer Säuren führen könnte. Die Silierung von Weizenstroh offenbarte zudem, dass die Struktur dieser Lignozellulose aufgrund des hohen Ligningehalts von bemerkenswerter Komplexität ist, was eine beträchtliche Herausforderung darstellt. Diese Einschätzung wurde auch durch die Ergebnisse gestützt, die erzielt wurden, als Weizenstroh ohne Silierung, aber mit vorheriger Vorbehandlung durch Verringerung der Partikelgröße einer AD untersucht wurde. In diesem Zusammenhang hat sich gezeigt, dass eine Verringerung der Partikelgröße einen wichtigen Einfluss auf die Biozugänglichkeit und Bioverfügbarkeit von fermentierbaren Zuckern haben kann. Dadurch könnte sich auch die Verdaulichkeit des Weizenstrohs verbessern lassen, insbesondere durch eine

Hydrolyse des gesamten Weizenstrohs. Dies führte zu einer erfreulichen Steigerung der Methanausbeute um bis zu 26 %. Andererseits konnten wir bei der Monosilierung von *Elodea*-Biomasse und Weizenstroh sowie beim Co-Silierungsprozess mit Siliermitteln keine Vorteile feststellen.

Die Silierung von *Elodea* und die gemeinsame Silierung mit Weizenstroh für Biogasanlagen könnten durchaus eine attraktive Option sein. Um diese jedoch kosteneffizient umzusetzen, wäre weitere Forschung erforderlich. Die Technologie entlang der Wertschöpfungsketten müsste erheblich verbessert werden (z. B. Schneiden, Sammeln, Erntezeit), und die Kostenschätzungen müssten zusammen mit dem daraus resultierenden endgültigen Biogasertrag ermittelt werden.

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List of abbreviations

3G: third generation	NH ₃ : ammonia
a _w : water activity	NDF: neutral detergent fibers
AA: acetic acid	NFC: non-fiber carbohydrates fraction
AD: anaerobic digestion	O ₂ : oxygen
ALC: alcohols	P: phosphorus
BA: butyric acid	pKa: acid dissociation constant
BMP: biochemical methane potential	TS: total solids
C/N: carbon to nitrogen ratio	VS: volatile solids
CH ₄ : methane	VFA: volatile fatty acids
CO ₂ : carbon dioxide	WSC: water-soluble carbohydrates
DBFZ: Deutsches Biomasseforschungszentrum gemeinnuetzige GmbH	
FM: fresh matter	
GHG: greenhouse gases	
H ₂ : hydrogen	
H ₂ O: water	
ISR: inoculum to substrate ratio	
LA: lactic acid	
LAB: lactic acid bacteria	
N: nitrogen	
N ₂ O: nitrous oxide	

1. Introduction

E. nuttallii and *E. canadensis* are characterized by exhibiting high areal productivities because of their rapid growth rates that may result in high-value biomass (Munoz Escobar *et al.*, 2011). However, *Elodea* overgrowth can become a damaging weed if not well managed. Mechanical harvesting has become established in Germany (Zehnsdorf *et al.*, 2015), but despite existing efforts, usage of the plant biomass is still lacking, as current management of aquatic weeds does not consider further utilization concepts.

Invasive *E. nuttallii* and *E. canadensis* play important roles in the functioning of aquatic ecosystems maintaining the structure, function and biodiversity of the water system. They increase the water transparency and water quality parameters by different effects, e.g. enhancing the self-cleaning potential by excreting O₂ into the water body and reducing algae growth by reducing the nutrient overload of eutrophic water bodies and by being a competitor for light. On the other hand, their overgrowth harms some aspects of the water system. It interferes with transportation, recreational and tourist activities. Even for infrastructure, for example, bridge pillars, etc., the biomass is problematic, as it can become clogged with driftwood and then lead to damming, enhanced water pressure and change of water flow. When remained in the water, the degradation causes O₂ depletion and retransfers the nutrients into water, which leads again to eutrophication.

After harvesting, disposal of broken plants is often done by leaving them to rot on the shores of waters, which releases nutrients that re-eutrophicates the water bodies (Zehnsdorf *et al.*, 2015). Due to the ruptured plant fragments because their dispersal is uncontrolled, they resettle in downstream allowing *Elodea* spread to new areas (Bowmer *et al.*, 1995). For all these reasons, management of *Elodea* overgrowth has to incorporate *Elodea* biomass into appropriate valorization routes to design products and alternative processes for its sustainable control management and use.

Based on the principles of advanced biorefinery, AD generating biogas, might be a

promising solution to treat, use and add value to the *Elodea* waste fraction. For a commercial AD, to overcome the issue of biomass seasonality, ensiling, as a previous preservation method in possible agricultural silos (e.g. clamp silos) is proposed. The fact, that *Elodea* biomass exhibits high moisture, complicates storage in clamp silo. Ensiling is based on the lactic acid (LA) fermentation whereby lactic acid bacteria (LAB) convert WSC into organic acids mainly to LA, the primary acid responsible for decreasing pH of silages. In this line, pH of silages is important when assessing silage quality, since $\text{pH} < 4$ restrains the growth of undesirable microorganisms, thus conserving the energy and nutrient content as well as quality of biomass maximally, while keeping it available throughout the year.

A possible co-ensiling with wheat straw may be an alternative to allow its storage prior to AD. The advantage of using wheat straw in the co-ensiling of *Elodea* biomass is that it contains the sufficient TS required for preservation in conventional clamp silos, which will allow the develop of ensiling technology using extremely wet materials. It represents also one of the most abundant low-cost resources and is a nonfood renewable resource. However, its inherent complex structure with recalcitrant characteristics makes it resistant to carbohydrate hydrolysis; therefore, a previous treatment is necessary to avoid low conversion efficiency.

This work was structured in three papers with the aim of exploring options to find novel process chain strategies for AD of aquatic plants and agricultural residues as well as the development of storage methods for long time conservation.

- **Paper I** presents the valorization of wheat straw by assessing the reduction of wheat straw particle size and the adjusted TS content to investigate their effects on organic acids (especially LA formation), drop in silage pH (silage quality) and methane yields.
- **Paper II** shows the development of a method for long time conservation via co - ensiling of aquatic biomass to reach suitability for storage in conventional clamp silos. Therefore, silage mixtures of aquatic plants and wheat straw were tested for silage quality and methane yields.

→ **Paper III** proves the hypothesis, that not only the inclusion of wheat straw but also addition of additives to the ensiled *Elodea* biomass can (i) promote favorable conditions to accelerate the growth of LAB and thus produce greater LA, resulting in a rapid pH drop, (ii) producing high- quality silages to be used in commercial AD and (iii) preserving its methane production potential.

2. Objectives

Invasive *E. nuttallii* and *E. canadensis* are a key cause of concern in infested freshwaters if not well managed. However, they have a remarkable biorefinery potential as a natural resource for providing renewable energy. With a proper management, including harvesting and exploitation of valuable constituents to use in an efficient AD process, this biomass would have an added value; consequently, this renewable biomass would attract considerable attention.

The general objective was to evaluate the feasibility of a sustainable biogas production through two biological processes i) ensiling and ii) AD, by optimizing the main drivers affecting *Elodea* biomass and methane-rich biogas preservation.

Specific objectives

- (i) to promote favorable conditions for ensiling of wheat straw (wheat straw silages), evaluating the effects of wheat straw particle size reduction, TS content and addition of silage additives on enhancing silage quality and methane production (**Paper I**);
- (ii) to evaluate the mono-ensiling of *Elodea* biomass (*Elodea* silages) and the possible co-ensiling of *Elodea* with wheat straw (mixture silages) as an alternative for *Elodea* biomass conservation for a year -round biogas system (**Paper II**);
- (iii) to investigate the mono-ensiling of *Elodea* biomass (*Elodea* silages) for promoting suitable ensiling conditions evaluating the effects of silage additives on *Elodea* biomass conservation for a year -round biogas system (**Paper III**);
- (iv) to assess favorable conditions for the ensiling of *Elodea* biomass evaluating the effects and effectiveness between the inclusion of previously pretreated wheat straw (co-ensiling) and silage additives on enhancing overall silage quality and methane production potential (**Paper III**).

3. Background

3.1 Invasive submerged macrophytes *Elodea* species

Elodea species belong to the family *Hydrocharitaceae*, are perennial and grow permanently or periodically submerged below, or up through the water surface. They are native to North America and develop in almost all types of water bodies within freshwater systems—but especially dominant in shallow waters (e.g. lakes and rivers usually < 5.0 m of depth, growing at shorelines or in slowly flowing water) (Catling and Wojtas, 1986; Nichols and Shaw, 1986). They have a remarkable influence on the physical and chemical condition of water and sediment, nutrient cycle and photosynthetic O₂ production indicating their key role in the functioning of complex freshwater ecosystem processes (Durate and Roff, 1991; Jeppesen *et al.*, 1998). From their Introduction into Europe to the present, they have been considered invasive species (Buldrini *et al.*, 2023). However, the submerged macrophytes *E. nuttallii* and *E. canadensis* are of increasing public and scientific interest because of their rapid spread and lasting invasion in German fresh waters (Hussner, 2010). Especially *E. nuttalli* has been included into the List of invasive alien species of Union concern (European Union (EU) Regulation 1143/2014).

In general, these two species prefer rather warm-waters (between 10-25°C) with different trophic levels (from oligo-mesotrophic to highly eutrophic waters) but are also considered as well adapted, more opportunistic and fast-growing organisms (Rönicke *et al.*, 2005; Zehnsdorf *et al.*, 2017). For this reason, their wide distribution indicating the relatively flexible growth particularly in eutrophic waters, physiological traits and growth characteristics make them further exploration worthwhile. In addition, the adaptive mechanisms evolved by these two species allow them to also optimally response to environmental heterogeneity and inhabit various types of aquatic habitat including wetlands (Rejmánková, 2011). Despite their great benefits such as habitats, water purifiers, increasing water transparency and quality by enhancing redox potential, converting CO₂ to O₂ during photosynthesis and inhibiting algae

propagation (through the competition for nutrients and light), besides removing phosphorous (P) and nitrogen (N) (primary pollutants leading to eutrophication), some studies conducted on macrophyte mappings have revealed these species as a significant threat to native plants (mostly *Potamogeton* species) displacing them through the process of so-called phase shifts (Hilt *et al.*, 2006; Hussner, 2012).

In Germany, studies have recognized water bodies mainly small lakes as extremely sensitive to the current anthropogenic climate change, which has been directly connected with rapid heating of waters and eutrophication and probably the massive proliferation of *E. nuttallii* and *E. canadensis* (Hussner *et al.*, 2010). In addition, *Elodea* massive proliferation is a current troublesome aquatic weed with serious ecological, economic and social consequences, where *E. nuttallii* has become well established in the Erft river in North Rhine-Westphalia (Hussner *et al.*, 2010).

In the infested waters, these species expand rapidly and form large mats that limit their use and outcompete native species. A further problem of these large mats is that they often remain in the waters and decompose after their growth period, causing serious secondary pollution (i.e. eutrophication because of the death plants-associated N and P) and increased deoxygenation by degradation processes. This may result in decreased water quality and changes in the aquatic community structure (Hilt *et al.*, 2006). During *Elodea* extensive growing period (in spring and summer), the plants act as sink for nutrients primarily N and P, thus causing nutrients deficiencies for competing other macrophytes and phytoplankton. Other important aspects related to *Elodea* infestation include energy production, boat traffic and recreational activities, which have been reported to be impaired and thus creating the need of removing these plants from the waters.

3.1.1 *Elodea* structure

Submerged perennial *Elodea* species are in their vegetative phase a "competitive ruderal", with multiple regeneration strategies, which are important to a potentially successful invader. In addition, their phenotypic plasticity which is an essential of *Elodea* invasiveness in

new environments is the result of their higher adaptative strategies. These species are rooting hydrophytes meaning that they complete their life cycle submerged in water, usually root in mud and are capable of enormous growth. *Elodea* species share similarities in appearance and habitat, therefore, they have been much confused in literature (Cook and Urmi-König 1985). However, all of these species have herbaceous stems and oblong to linear leaves in whorls of two to eight (Butkuvienė *et al.*, 2022). *E. canadensis* has different morphological variations from *E. nuttallii*. The width/length ratio of its leaves and the angle at the leaf apex are higher in *E. canadensis* than in *E. nuttallii*, which show the shape of the leaf apex (Figure 1) and are their most important taxonomical features (Kočić *et al.*, 2014).

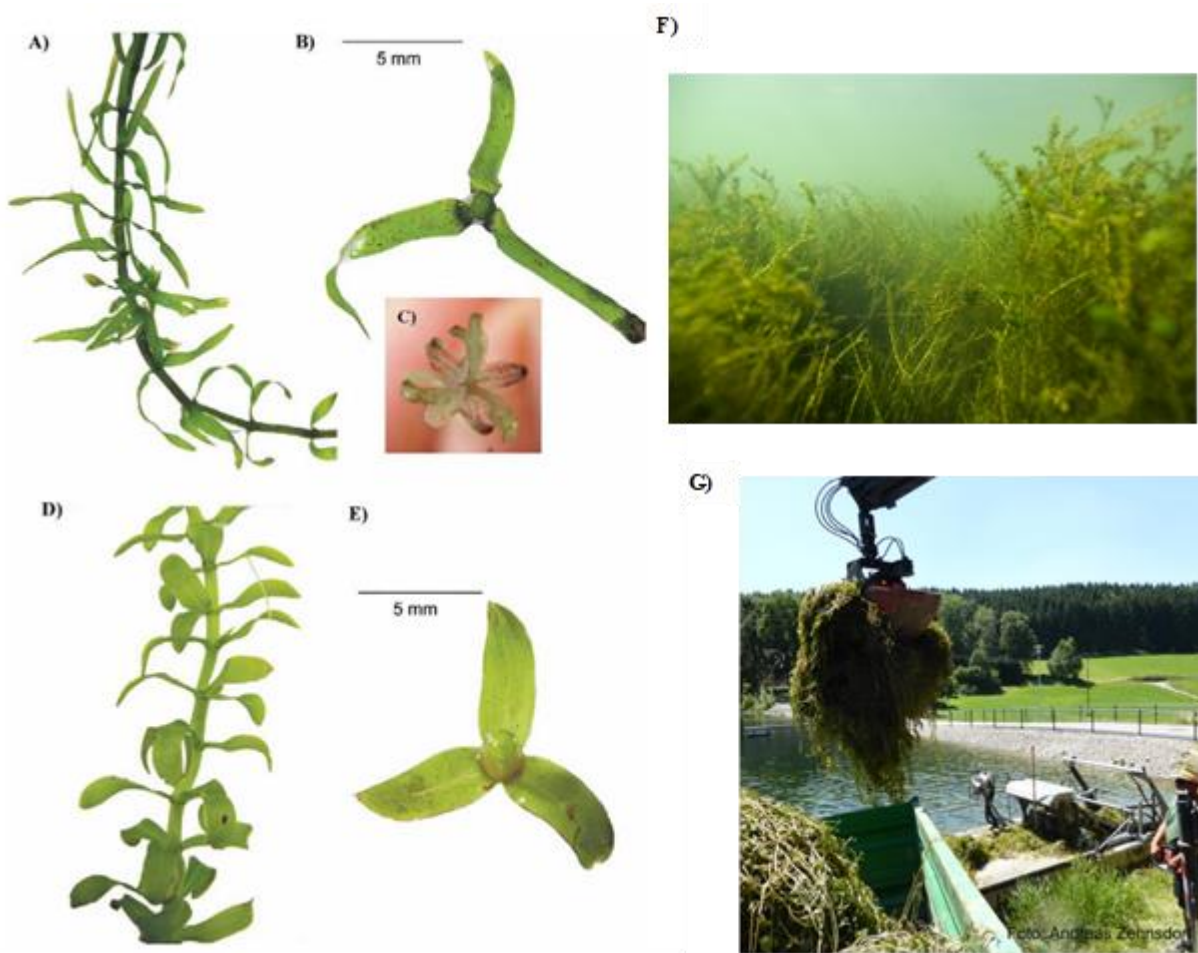


Figure 1. (A–C) *E. nuttallii*: A) part of the plant, B) whorl with the leaves, C) female flower. (D–E) *E. canadensis*: D) part of the plant, E) whorl with the leaves. Photo by A. Kočić (taken from Kočić *et al.*, 2014). (F–G) *E. nuttallii* and *E. canadensis*, photos taken from the archiv AquaMak- Newsletter (<https://www.ufz.de/index.php?de=37401>).

The reproductive biology of these *Elodea* species has been reported to be only vegetative (because only female plants have been found in Europe), involving vegetative fragments and turions that are able to form roots from nodes and start growing (Cook and Urmi-König, 1985).

3.1.2 Aquatic *Elodea* overgrowth control methods

E. nuttallii and *E. canadensis* are invasive macrophytes, which have been proliferating at a phenomenal rate during the last decades in German waterways. In case of overgrowth, the

strong covering of vegetation can cause problems in hydroelectric power plants and leads to limitations in ship and boat traffic as well as bathing and fishing activities. After vegetation period, dead plants can accumulate and then negatively influence flood protection and water engineering works. For this reason, the aquatic biomass has been periodically removed. Several strategies have been proposed to eliminate these plants. The main methods used as management regulations to control and limit overgrowth of invasive species include biological, mechanical and chemical control methods (Zehnsdorf *et al.*, 2015). Biological and chemical methods affect directly the aquatic life and involve, in the case of biological methods, the introduction of other species and the use of herbicides and other toxic chemicals in the case of chemical controls. On the other hand, the mechanical control method or mechanical harvesting using mowing boats is rather the best alternative and the most widely used method that has become established in Germany (Hussner *et al.*, 2014).

According to the Waste Compost and Incineration Regulation (Abfallkompost- und Verbrennungsverordnung- AbfKompVbrV) and the Circular Economy Act (Kreislaufwirtschaftsgesetz- KrWG), Law on the Promotion of the Circular Economy and Ensuring the Environmentally Sound Management of Waste, the resulting aquatic biomass, which is classified as organic waste, has to be treated in compost plants. However, due to the high moisture content and low C/N-ratio there is a high risk of greenhouse gas emissions, especially CH₄, N₂O and the indirect greenhouse gas NH₃. An adequate composting management would then require with high demand on other material like wood chips for optimizing poor structure and C/N-ratio with regard to the kinetics of C- and N-rich components. The idea of a sustainable management of *Elodea* species must take into consideration the utilization of the plant biomass (i.e. harvested plant/ aquatic biomass) to make it economically attractive. Furthermore, harvesting would be a measure to remove N and P from waterbodies thereby reducing eutrophication.

3.2 Wheat straw: a lignocellulosic biomass with a recalcitrant native form

Monocotyledonous grass species like wheat are one of the high-yield crops in the world and wheat straw is the by-product of wheat after harvest of grain. Wheat straw is classified as a waste and/or lignocellulosic biomass, agriculture residue and/or nonfood renewable resource because it is not used for food or feed (the latter seldom due to very low feed efficiency). Moreover, straw in general, is not in competition with other food, feed or energy crops as no additional agricultural area is needed for its production. Parts of the wheat straw are commonly used for mulching or as bedding material for livestock or horses, while the main amount is left in the field to decay or incinerated. Biomass like wheat straw is the most abundant natural renewable source because it is considered as produced from atmospheric CO₂ and H₂O using the sunlight energy through the photosynthesis process. For that, this lignocellulosic biomass may provide a means to economize sustainable biofuels such as biogas production. Among lignocellulosic biomasses, wheat straw has been recently regarded as a sustainable alternative for 2nd generation (2G) cellulosic biofuel production that may allow a greener and more sustainable future (Brosowski *et al.*, 2016). In Germany, for example, the average yield of wheat straw is 0.8 kg/kg grain. Therefore, significant potential exists for sustainable biofuels and/or biomass from this lignocellulose. Especially, if this biomass is being used for regional production of biogas and the digestate is used regionally as organic fertilizer, this potential can largely be used with regard to sustainability issues.

Polysaccharides present in lignocellulosic biomass are of great interest because they are a potential source of fermentable sugars for biorefining technologies. Most lignocellulose consists of three major polymers that are interwoven with each other forming a complex three-dimensional structure (Figure 2): the two polysaccharides, cellulose (33%–51%) a homopolymer of glucose and hemicellulose (19%–34%) a heteropolymers of various pentoses and hexoses and one aromatic unsaturated polymer lignin (20%–30%) constructed from three basic phenylpropanoid monomers, *p*-hydroxyphenyl (*H*), guaiacyl (*G*) and syringyl (*S*) units, derived from three monolignols, *p*-coumaryl, coniferyl and sinapyl alcoholic precursors, respectively (Lapierre *et al.*, 1995).

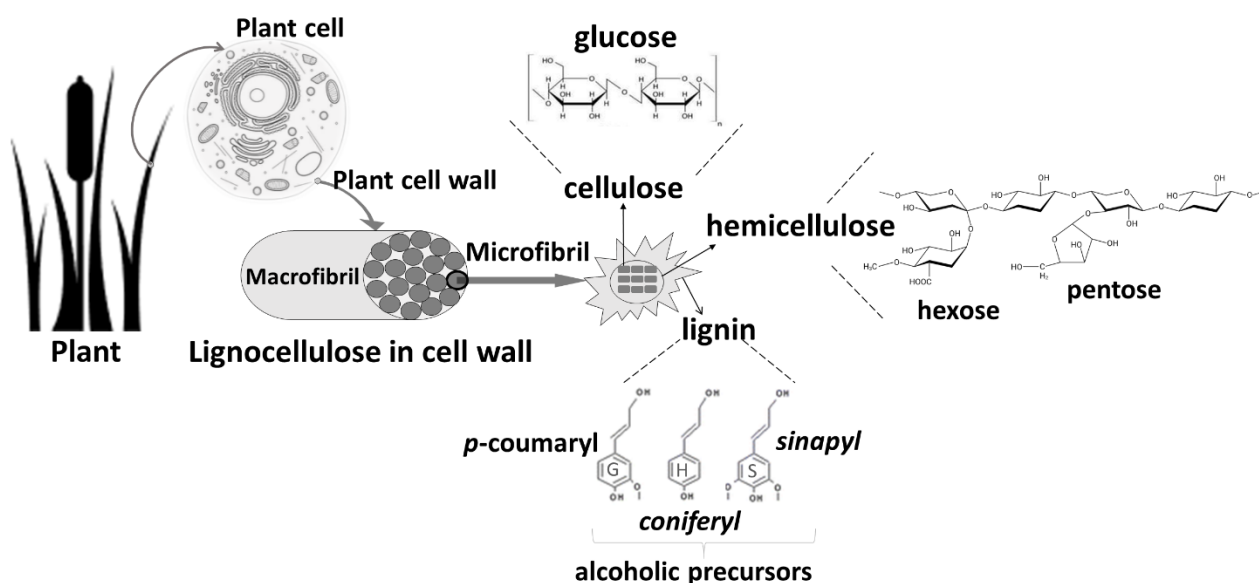


Figure 2. Inherent complex structure of lignocellulose. Cellulose, hemicellulose and lignin form structures called microfibrils, which are organized into macrofibrils that mediate structural stability in the plant cell wall. Adapted and modified from Hasanov *et al* (2020).

In wheat straw, however, the structure of the lignin additionally contains a *p*-hydroxyphenyl-guaiacyl-syringyl unit and interunit bonds β -O-4' ether linkage (del Río *et al.*, 2012). This complex structure with recalcitrant and heterogeneous characteristics is resistant towards degradation. It limits saccharification of carbohydrates to fermentable reducing sugars

via hydrolysis and then fermentation of these available sugars by microorganisms. Therefore, to enhance the hydrolysis of carbohydrates a pretreatment is needed to modify the lignin structure and bioavailability of wheat straw for its efficient valorization and utilization (Hendriks and Zeeman, 2009). In general, to develop a lignocellulose-based biofuel - system, polysaccharides must be, in large part, converted to monomeric substrates through biodegradation procedures to be available for their utilization in the production of “green” energy or green chemicals, for instance, biogas (methane) production. Several studies have demonstrated that pretreatment or preconditioning of biomass is an essential process to enhance the efficiency of enzymatic hydrolysis (Kratky and Jirout, 2020). The most common pretreatments for biomass degradation, specifically the cellulose and hemicellulose components, are (i) physical (milling, microwave, extrusion, sonication), (ii) chemical (alkali, acid, oxidising agents) and/or physico-chemical (steam pretreatment, catalyst, hydrothermolysis) and, (iii) biological (fermentation, use of fungi, enzymes and /or ensiling) (Galbe and Wallberg, 2019). Nevertheless, the optimization of the process parameters is also crucial, because the cost of a pretreatment remains a key barrier to economical production of value- added products.

3.3 Pretreatment technologies

Under the concept of advanced biorefinery, lignocellulosic biomass offers the possibility of renewable alternatives to fossil fuels as (crude oil, natural gas etc.) in marketable bio-based products (chemicals, materials) and bioenergy (biofuels, power, heat). However, the presence of lignin plays a major structural role in the efficiency of the hydrolysis of carbohydrates. The breakdown of lignocellulosic biomass involves the release of long-chain polysaccharides (hemicellulose and cellulose) followed by their hydrolysis into the C₅- and C₆ fermentable sugars (hexose and pentose, respectively). In this line, a pretreatment is essential to breakdown recalcitrant biomass to enhance biodegradability – digestibility. In the current study, the principles of two pretreatments are presented: (i) the reduction of the size of particles assessed as a physical/mechanical pretreatment and (ii) the long-term storage through ensiling fermentation as a biological pretreatment. Both of them with the aim of improving digestibility

of wheat straw causing a degradation of the cell wall polymers that is, cellulose, hemicellulose and lignin.

3.3.1 Reduction of the particle size (physical/mechanical pretreatment)

Particle size reduction has been identified as the very initial step that alters the structure of any material to a greater contact surface area allowing the mass to become more homogeneous and can therefore be used more efficiently. It is well known that the reduction of the size of particles is commonly applied to solid biomass (for a simpler handling) and also to highly lignocellulosic biomass (Kratky and Jirout, 2020). Besides, it is a key part of the development of advanced biorefinery processes for the valorization of biomass into platform chemicals, value-added products and/or energy carriers. Particle size reduction may also be crucial and plays a key role for the conversion of lignocellulosic biomass into biofuels such as biogas via biochemical and thermochemical pathways, as it enables effective biodegradability.

3.3.2 Ensiling process (long-term storage)

Ensiling is a long-term storage, based on a natural fermentation under anaerobic conditions with the formation of mainly LA, the primary acid responsible for decreasing the pH in silages. The four main principles of the ensiling process (Figure 3) based on its biochemical and microbiological events (Weinberg and Ashbell, 2003) are: (1) *aerobic processes*, where enzymatic activity, plant cells and aerobic microflora consume the oxygen entrapped and use fermentable carbohydrates, which is detrimental for subsequent anaerobic lactic acid fermentation, (2) *fermentation*, it starts when the ensiled mass has become anaerobic and respiration terminates. The gradual acidification due to mainly LA accumulation promotes the development of acid-tolerant LAB species and pH of silage decreases to below 5.0, (3) *storage*, the silage is sealed and no air penetrates, it may last several months and as long as the pH is sufficiently low and anaerobiosis preserved, few changes occur (4) *unloading*, the silage is opened and reexposed to air, this may cause the growth of undesirable aerobic microorganisms initially present in the silage (e.g., yeasts and molds) elevating the pH and consuming easy degradable sugars, which result in mass and energy losses.

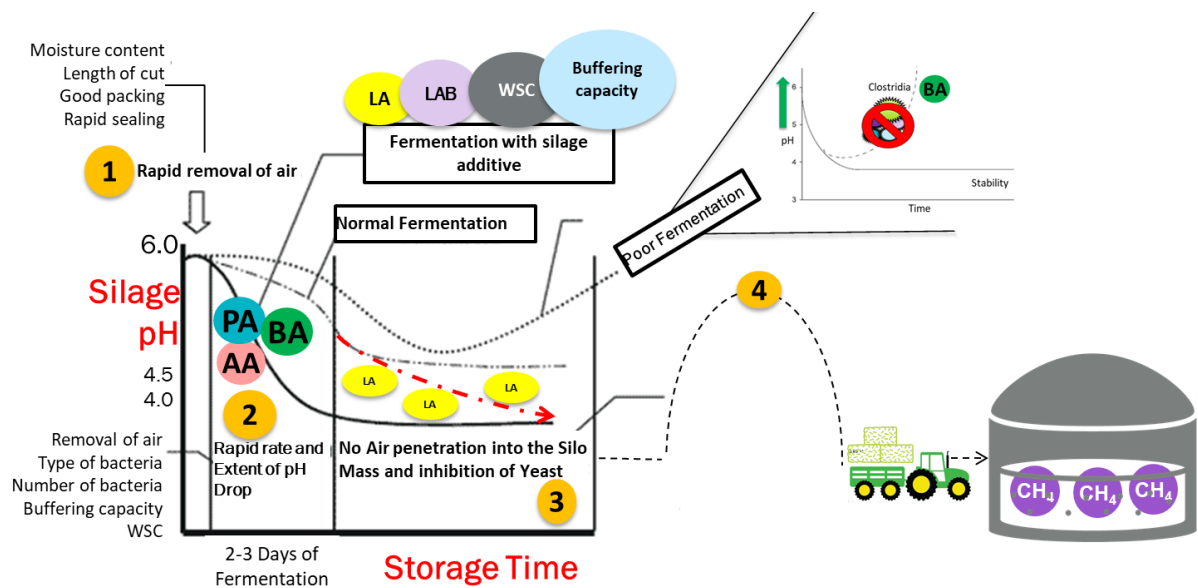


Figure 3. The four main steps of the ensiling process. (1) Switching from aerobic to anaerobic conditions, (2) Fermentation, (3) Storage and (4) Unloading for feeding. Lactic acid (LA), acetic acid (AA), butyric acid (BA), propionic acid (PA), lactic acid bacteria (LAB), water soluble carbohydrates (WSC), methane (CH₄). Adapted and modified from Dunière *et al* (2013).

Upon ensiling, if fermentation failed, clostridial fermentation would occur (Figure 3, Poor Fermentation), resulting in poorly preserved silages. Clostridial fermentation may cause anaerobic deterioration of silages by conversion of either fermentable carbohydrates or LA into BA. Contrary, lactic acid bacteria (LAB) convert the available substrate almost exclusively into LA (homofermentative pathway) or LA, acetate, ethanol and CO₂ (heterofermentative pathway). The LAB is divided into two main groups, depending on their fermentation products. One group include those bacteria that convert carbohydrates essentially into lactate, while the other group include those producing, in addition, substantial quantities of volatile acids and CO₂ (McDonald *et al.*, 1991). The energy balance according to McDonald *et al* (1991) suggests that LA formed by the homofermentative pathway results in no TS and negligible energy losses, while the heterofermentative pathway producing LA and AA accounted for 4.8 and 1.0% TS and energy losses, respectively. Clostridia fermentation producing BA results in 51.1 and 18.8% TS and

energy losses, respectively, while producing CO₂ and H₂. Therefore, the fermentation pathway is crucial to conserve the energy and nutrient value maximally, while keeping the silage available as feedstock throughout the year. An important aspect is the characteristics of the ensiled materials, indicating those with high TS and WSC and low buffering capacity as suitable for ensiling to ensure lactic acid fermentation. Poorly ensilable material (usually with relatively low TS <300 Kg⁻¹ g, low WSC and high buffer capacity) often result in silages with pH higher than 5 and subsequent TS and energy losses (Borreani *et al.*, 2009).

3.4 Silage additives

The use of natural or industrial silage additives with different modes of action is an option to convert poor quality substrates to silages with adequate quality. Silage additives have the particular purpose of ensuring, that LAB dominate the fermentation and produce LA during the fermentation process in quantities high enough for well-preserved silages. In general, when pH reduces to about 4, silages can be well preserved. pH is highly related to LA and LA exhibits the lowest acid dissociation constant (pKa, 3.86) among all the produced organic acids. Therefore, LA plays the major role in the rapid acidification and the growth inhibition of detrimental microorganisms to preserve the silage. Silage additives can be classified into five categories: (i) fermentation stimulants, (ii) fermentation inhibitors, (iii) aerobic deterioration inhibitors, (iv) nutrients and (v) absorbents (McDonald *et al.*, 1991). The first and second categories act by reducing storage losses, by either promoting rapid fermentation or by inhibiting partially or completely microbial growth. The third category acts by improving bunk life of silage (increase aerobic stability). The fourth and fifth categories are used to improve nutrient composition of silage or added to low TS crops to reduce nutrient losses by effluent (Yitbarek and Tamir, 2014). Overall, ensiled materials with high buffering capacity and low WSC content suggest a need for an additive to ensure a satisfactory fermentation.

3.5 Anaerobic digestion (AD)

AD is widely accepted as one of the most convenient final valorization steps in biorefinery

scenarios as it allows effective conversion of biodegradable matter into biogas and digestate used for energy production and replacement of agricultural fertilizers. It is also considered as an efficient waste management practice applied to a wide variety of biodegradable materials. The microbiology and chemistry involved in the AD process start with a group of facultative and anaerobic bacteria “acid formers” (acidogens), where complex materials (carbohydrates, proteins and fats) are hydrolyzed, fermented and biologically converted to volatile fatty acids (VFA) in the hydrolysis stage. In the second stage (dehydrogenation and acetogenesis) the volatile acids longer than C_2 are then oxidized by β -oxidation and converted to acetate and H_2 by the obligate hydrogen producing acetogens through the hydrogenotrophic pathway. Finally, the acetate and H_2 are converted to CH_4 and CO_2 by the “methane formers” (methanogens) via the aceticlastic pathway.

AD efficiency in biogas production highly relies on the biodegradation process and operating parameters. Operating parameters, that influence biogas production are temperature, hydraulic retention time, organic loading rate, inoculum, feeding pattern, pH and pretreatment, which is a vital step in the AD process of lignocellulose. Pretreatment of lignocellulosic biomass will accelerate the hydrolysis stage of the AD process, which is reflected in significantly higher synthesis of the precursors of methane, acetate and H_2 from simple carbohydrates. A pretreatment will then encourage solubilization/hydrolysis of complex materials and will, perhaps, indicate the yield of the hydrolysis/acetogenic biomass and the impact on microbial kinetics (Speece, 1996).

4. General methods

4.1 Raw materials and pretreatment

4.1.1 *Elodea* biomass

Elodea biomass was a mixture of the *Elodea* species *E. nuttallii* and *E. Canadensis*, which was collected at the Parthe river in Leipzig, Germany (51° 21' 53.2" N, 12° 24' 51.2" E) during the autumn of 2015. Immediately after arrival at the laboratory, the whole plant biomass was manually washed with cold tap water to remove adherent soil and impurities. The whole *Elodea* plant was cut by hand to a particle size of approximately 3–5 cm and intermediately stored at 4°C for later use.

4.1.2 Wheat straw

Wheat straw was provided by the Department of Thermochemical Conversion of the DBFZ. Prior to ensiling, wheat straw was treated using the following techniques: mechanical disintegration by chopping using a milling machine (Strohmühle, Hirlinger Landtechnik GmbH, Burladingen, Germany) and by grinding using a Bioextruder (Lehmann-UMT GmbH, Pöhl, Germany) to particle sizes of 2.0 and 0.2 cm, respectively. This disintegration technology was used with the aim of investigating the effect of the particle size reduction. The resulting chopped and ground wheat straw samples were placed in sealed plastic barrels and stored at 4°C for later use.

4.2 Inoculant

The inoculum was collected from a full-scale mesophilic anaerobic biogas plant at the DBFZ and utilized for all the BMP experiments carried out in **Paper I**, **Paper II** and **Paper III**. The substrates utilized onsite were corn silage and cattle manure. Digestate derived from the primary digester was sieved (mesh size of 5 mm) and degassed at room temperature for five days to reduce specific biogas production before BMP experiments.

4.3 Silage preparation

Experimental silages were prepared using *Elodea* biomass and wheat straw and were generated in different ways as described in Table 1. The storage period for all the silages was 180 days. In **Paper I**, only wheat straw, previously pretreated at two particle sizes, was used. The aim of using only straw was to evaluate the effects of the particle sizes and moisture content, alone and in combination. This was regarded as a control experiment, to use wheat straw in the co-ensiling with *Elodea*, which aimed to enhance the suitability (substrate characteristics) of *Elodea* biomass to be preserved via ensiling. The complexity of factor interaction in mixture silages of *Elodea* and straw was even higher with further influencing factors (especially buffering capacity, C/N-ratio). It showed the effect of particle size and moisture to improve wheat straw digestibility (affecting the complex lignin structure). In this way, lignocellulose-rich substrates like straw as an abundant source with currently small competition for use can be included in the biogas process. More detailed, the wheat straw silages were investigated at two particle sizes, with and without addition of silage additives and at two TS contents, which were adjusted to 30 and 45% with tap water (Table 1). The adjustment of moisture was necessary as wheat straw shows a low moisture content (< 5 %FM), which is an important biochemical factor. Moisture has an effect on promoting microbial activity, swelling of microfibrils, solution and dilution of soluble carbohydrates, enzymes as well as organic acids.

Paper II presents the mono-ensiling of *Elodea* (*Elodea* silages) and the co-ensiling of *Elodea* with wheat straw (mixture silages). *Elodea* silages were prepared only with *Elodea* biomass and the mixture silages were a mixture of *Elodea* and wheat straw (Table 1). Moreover, the TS content of the mixture silages was adjusted to 30 and 45% using the water containing *Elodea* biomass. The paper focus on the mixture rates (which defines also moisture content) and evaluates the particle size of straw. **Paper III** also shows and evaluates the preparation of *Elodea* silages and mixture silages, as previously described in **Paper II**, but the effect of addition of silage additives was investigated. Within all the mixture silages, the TS content was adjusted to 30 and 45% using the water containing *Elodea* biomass as described in **Paper II** and **Paper III**.

Table 1. Experimental silages at different particle size, total solids (TS), with and without wheat straw. Silages additives used alone or in combination.

Silages	Wheat straw particle size (cm)		TS (%FM)	
	0.2	2.0	30	45
<i>Elodea</i>	-	-	-	-
SC1 ^a	-	X	X	-
SC2		X	-	X
SG1	X	-	X	
SG2	X	-	-	X
Estc30 ^b	-	X	X	-
ESTc45	-	X	-	X
ESTg30	X	-	X	-
ESTg45	X	-	-	X

^aSC1 to SG2, refers to the mono-ensiling of wheat straw (wheat straw silages).

^bESTc30 to ESTg45, refers to the co-ensiling of *Elodea* with wheat straw (mixture silages).

Silage additives

	Control	Ez	Ch	Ch+Ez	Ch+He	Ch+He+Ez	He+Ez	He+Sg	Total
<i>Elodea</i>	*	-		-	-				15
SC1	*								24
SC2	*								24
SG1	*								24
SG2	*								24
Estc30	*								24
ESTc45	*								24
ESTg30	*								24
ESTg45	*								24

Ez = Enzymes; Ch = chemical additive; Ch+Ez = combination of enzymes and chemical additive; Ch+He= combination of LAB inoculant and chemical additive; Ch+He+Ez = combination of LAB inoculant, enzymes and chemical additive; He+Ez = combination of enzymes and enzymes; He+Sg = combination of LAB inoculant and sugar solutions. All experimental silages were performed in triplicate.

Three commercial silage additives (Schaumann Forschung GmbH, Pinneberg, Germany) included chemical (Ch), enzymes (Ez) and inoculant (He) were used for treating the experimental silages as described in **Paper I** and **Paper III**. In addition, a solution of sugars (Sg) at 10% (on TS basis) was used as a source of fermentable carbohydrates. The three commercial silage additives as well as the sugar solution were added alone or in combination (Table 1) as presented in **Paper I** and **Paper III**.

4.4 Analysis of chemical composition and fermentation quality

In **Paper I**, **Paper II** and **Paper III**, the TS content and volatile solid (VS) content of fresh *Elodea* and wheat straw as well as of the *Elodea* and mixture silages were determined. All terms related to TS and VS were presented as the correction of TS and VS for loss in volatile components. Furthermore, opened mini silos were also evaluated for fermentation quality, which included the determination of concentrations of VFA (i.e. acetic acid (AA) and butyric acid (BA)), organic acids (i.e. lactic acid (LA)), alcohols (ALC) and silage pH as described in **Paper I**, **Paper II** and **Paper III**.

4.5 Biochemical methane potential (BMP) tests

After 180 days of ensiling, mini silos were opened and analyzed for methane yield through the BMP tests conducted at lab-scale using eudiometer devices as described in **Paper I**, **Paper II** and **Paper III**. The procedure was as follows: each AD reactor contained 450 g of inoculum and approximately 2.5 g of the substrate (on VS basis) and were kept in a water bath at mesophilic conditions ($37 \pm 2^\circ\text{C}$) for 35 days following VDI (2006). The inoculum to substrate (ISR) ratio was approximately 2.3:1 (on VS basis). Before the batch experiments, the AD reactors' headspace was flushed with N-gas for about 2 min to assure anaerobic conditions. The headspace correction was carried out as described in the method description VDI (2006). All reactors were stirred manually once a day during AD and analyzed in triplicate. The methane concentration in biogas was measured using a landfill gas monitor (GA2000, Ansyco, Karlsruhe, Germany). The methane yield was standardized to 273.15 K and 101.32 kpa. Additionally, only for fresh *Elodea*, methane yield was obtained using an automatic methane potential Test system II (AMPTS II,

Bioprocess Control, Sweden) and it was also performed at mesophilic conditions ($37 \pm 2^\circ\text{C}$) for 35 days.

5. Outcomes

In this section, three main points from the results from **Paper I**, **Paper II** and **Paper III** will be discussed. Firstly, the chemical characteristics of the *Elodea* and wheat straw biomass were evaluated to find the suitability of these substrates to be preserved via ensiling. Secondly, the effect of the size reduction of particles of wheat straw was investigated as a pretreatment prior to ensiling and the effect of adding silage additives on the quality of all the experimental silages. Thirdly, the assessment of the role of wheat straw particle size reduction through its effects on ensiling for an improved *Elodea* biogas system.

5.1 Chemical characteristics of *Elodea* and wheat straw biomass

The chemical composition of both *Elodea* and wheat straw biomass used in **Paper I**, **Paper II** and **Paper III** was very heterogeneous. The *Elodea* biomass chemical composition showed moderate high protein and a very high ash content (236.9 and 402.5 g Kg⁻¹ TS basis, respectively). Wheat straw was characterized with low ash and protein, but high lignin contents (67.7, 36.8 and 121.3 g Kg⁻¹ TS basis, respectively) as it is basically composed of neutral detergent fibers (NDF) of which the hemicellulose and cellulose contents in wheat straw were much higher (283.0 and 494.0 g Kg⁻¹ TS, respectively) compared to those of *Elodea* biomass (46.6 and 175.1 g Kg⁻¹ TS). TS of wheat straw was determined to be 86.5 % on FM basis, far higher than that of *Elodea* biomass (6.7% on FM basis), as expected for terrestrial plants at advanced maturity, where the cell wall components (i.e. the NDF: cellulose, hemicellulose and lignin as the major components) are supposed to increase, while decreasing the non-fiber carbohydrates (NFC) fraction (i.e. fermentable sugars). In general, based on these biochemical characteristics, it appears, at least that both substrates suggested a need for a pretreatment for their efficient valorization and utilization.

Concerning the C/N-ratio, optimal values for the AD process are reported within the range of 15 to 30. The C/N-ratio of *Elodea* was 10 as typically found for aquatic plants (between

10-20) (Müller *et al.*, 2018), while for wheat straw the C/N-ratio was of about 46 (Jaffar *et al.*, 2016). However, it is well known that considerably high or low C/N-ratios could inhibit methane production, favoring accumulation of VFA or ammonia and thus resulting in toxic inhibition of methanogenic archaea (Yang *et al.*, 2015). Therefore, the results recommended, in the case of *Elodea* biomass and also because of its relatively high moisture, a co-digestion with wheat straw (if used semi-continuous systems without pretreatment or ensiling) or with commonly available carbon-rich substrates (e.g. sorghum, maize) to provide a more suitable substrate C/N-ratio for stabilizing the digestion system and increasing the process stability and biogas production.

5.2 Mechanical pretreatment: particle size reduction and its influence on fresh *Elodea* and wheat straw

The reduction of the size of particles of wheat straw and *Elodea* biomass was investigated in **Paper I** and **Paper II**. Particle size reduction is a mechanical pretreatment disintegration technology in bioprocess engineering that has an impact mainly on the degradation of biomass recalcitrance (Schumacher *et al.*, 2014). The mechanical size reduction enlarges the accessible surface area to a greater microbial and/or enzymatic hydrolysis of polysaccharides to monosaccharides. The results from **Paper I** suggested that the size reduction of wheat straw particles was effective in increasing surface area and could improve access to a possible higher conversion of the additional sugar components. On the other hand, **Paper II** described that for *Elodea* biomass the reduction of the size of particles was not possible in the lab due to its very high content of moisture ($\approx 95\%$), which was unfavorable for milling. Therefore, it is suggested that the characteristics and the physical structure among these two heterogeneous biomasses could be possible factors influencing the application of particle size reduction or another pretreatment method within the disintegration technologies.

5.3 Ensiling evidenced the positive influence of particle size reduction

Particle size reduction and silage quality may perhaps be related when considered silage quality as measured by the pH at opening ($\text{pH} \leq 4$). However, to reach this low value most of the fermentable sugars must be converted by LAB into LA, which accumulates and reduces pH. Interestingly, the release of sugars through wheat straw plant cell rupture yielded a higher acid production, thus reducing and stabilizing silage pH. In this section the positive influence of reduction of wheat straw particle size on the mono-ensiling of wheat straw and the co-ensiling of *Elodea* with wheat straw is discussed. Furthermore, mono-ensiling of *Elodea* and the main challenges that has to face in obtaining high quality silages are also discussed.

5.3.1 Mono-ensiling of *Elodea* biomass: The *Elodea* silages

Results from **Paper II** showed that *Elodea* can be stored by ensiling. However, with a water content of about 95%, the storability of this material is challenging. pH value of the *Elodea* silages was found to be higher (6.5) than that required for well-fermented silages (< 4). In addition, higher AA than LA was produced (7.5 and < 0.1 %TS, respectively) as well as considerable formation of BA (2.1 %TS), which indicates metabolic activity from clostridial organisms (Borreani *et al.*, 2009). The increase in pH might have been caused by the relatively high buffering capacity of the raw *Elodea* biomass related to high ash contents. In comparison to straw, *Elodea* biomass has much higher water, protein and ash contents (**Paper II**, Table 1). Also, the acid dissociation constant (pK_a) value of organic acids, is 3.86 for LA and 4.75 for AA.

From this, it was observed that these differences between silages are mainly attributed to the water content of *Elodea* biomass as compared with straw biomass. This does not only affect the chemical composition of the silage, but also the activity of the microorganisms involved in the conservation process. The ensiling of fresh *Elodea* led to a liquid silage material which was storable under laboratory conditions for 180 days without major energy losses. However, for practical applications, materials with high TS content should be used as co-substrates to

investigate the stability of the silages for storage in conventional agricultural bunker silos.

5.3.2 Mono-ensiling of wheat straw: the wheat straw silages

The results indicated that the smaller the size of particles is, the better the quality of wheat straw silages. Here, silage quality was based upon two indicators, pH value and LA content. From the results of the wheat straw silages, **Paper I** showed that particles at sizes of 0.2 cm led to lower pH (4.7 and 5.2 on average, respectively) and consistent LA content (1.4 and 1.8 %TS on average, respectively) when compared with those at 2.0 cm, being favorable for wheat straw conservation and within a wheat straw silage preservation range (Ni *et al.*, 2014). In general, ensiling of wheat straw is challenging as it cannot be directly ensiled because of its unfavorable physicochemical properties. Primarily, the presence of the crosslinked lignified matrix, which provides resistance to microbial access to the carbohydrate fraction and reflects the high TS content and subsequent low WSC. And also, its manageability, implying that a pretreatment is required. In the ensiling environment, for example, sugar components (i.e. the NFC fraction, mainly the WSC) are required for LAB to reduce the pH by a rapid formation and accumulation of organic acids. As plants mature, the NDF fraction tends to increase; consequently, the sugar components represented by the NFC fraction declines. Therefore, it is assumed that the degradation of hemicellulosic components by the reduction of particles allowed for increased bioaccessibility and bioavailability of fermentable carbohydrates and thus digestibility/hydrolysis of the overall wheat straw biomass.

Moreover, the low moisture content of wheat straw (< 5%FM), another important biochemical factor, did not facilitate the natural lactic acid fermentation and addition of water was necessary to ensure optimal water activity (a_w) (or metabolic water available) for growth of LAB (Kung *et al.*, 2018). In this line, however, adding water increases water consumption in an already particle size manufacturing process and to make the process economically feasible alternatives to replace the added water becomes another challenge (see section 5.5).

5.3.3 Co-ensiling of *Elodea* and wheat straw: the mixture silages

Mixture silages were performed in **Paper II** and **Paper III** and the results also showed a

significant improvement in silage quality. The mixture silages with wheat straw at 0.2 and 2.0 cm of particles showed no differences among the LA mean values of the two sizes (1.9 and 0.5 %TS, respectively), but consistently higher pH in those silages with much smaller sizes (5.4 and 4.9, respectively). However, these values are still considered ideal for well-fermented silages when wheat straw was used in a co-ensiling process (Sieborg *et al.*, 2020).

On the other hand, **Paper II** and **Paper III** also demonstrated that the *Elodea* silages resulted in poorer preserved silages when compared with the mixture silages, resulting in high pH (≈ 6), negligible LA and formation of BA ($> 2\%$ TS). However, the reduction of wheat straw particle size could have been beneficial for the co-ensiling of *Elodea* probably due to the efficient utilization of additional sugars. Also, the increased TS concentration promoted by addition of wheat straw.

Therefore, it is suggested that if mechanically pretreated wheat straw is used in the co-ensiling of *Elodea*, *Elodea* biomass could be preserved and used for biogas production. Moreover, this would also overcome the *Elodea* biomass seasonality. For this, it may conclude that the co-ensiling of *Elodea* with wheat straw would then suggest a feasible option that could contribute to increase the scale of available *Elodea* substrate production throughout the year.

5.4 The effect of silage additives on wheat straw, *Elodea* and mixture silages

Results from **Paper I** and **Paper III** suggested, that addition of additives did not benefit the ensiling fermentation of the investigated silages. LA values of wheat straw silages with and without additives ranged from 0.1 to 4.0 %TS and pH values varied from 4.3 to 6.4 and no significant effect on their increase were observed. However, it is important to note from the results that the reduction of wheat straw particles size considerably affected its physical structure and helped the ensiling process to hydrolyze wheat straw whether silage additives were used or not (**Paper I**, Table 1 and **Paper III**, Figure 1). In addition, **Paper III** showed, that the use of additives in the *Elodea* silages resulted in even poorer quality (Table 3 and Figure 1).

Elodea biomass has been characterized in this work as a poorly ensilable material due to the low WSC, high buffering capacities and relatively high moisture contents. Therefore, results appeared to indicate, that increased silage quality was strongly associated with inclusion of wheat straw rather than the addition of silage additives.

5.5 Synergies between *Elodea* and wheat straw biomass

The results of **Paper II** and **Paper III** suggested that wheat straw would have the potential to play the major role in the preservation of *Elodea* through the co-ensiling process, for example,

- high water, ash and protein contents present in *Elodea* biomass directly affected its buffering capacity; consequently, acidification (drop of pH) during ensiling was not achieved. However, wheat straw seemed to have helped counteracting this effect by absorbing excess of liquid from *Elodea* biomass (**Paper II**, Table 2),
- the capacity of wheat straw of absorbing excess of moisture also prevented clostridia fermentation which is a common indicator of insufficient preservation and results in large losses of TS
- its positive effect was reflected in the increased TS, consequent LA and stabilization of silage pH (in the mixture silages, wheat straw was a source of TS and indirect fermentable carbohydrates, while *Elodea* biomass provided an adequate moisture level,
- from a productive point of view, wheat straw would maximize the utilization of *Elodea* biomass, for example, if pretended to store in large-scale, co-ensiling could be carried out using surface-walled clamp silos (bunker silos) to promote suitable substrate characteristics (mainly the solids concentration) and also to avoid effluent production and pollution of water courses,
- for energy purposes and costs, drier ensiled substrates are preferred because they are

easier to handle and a higher quantity of TS can be carried per trailer load.

In addition, the very high moisture content of *Elodea* biomass was, in particular, the main challenge in the mono-ensiling of *Elodea* demonstrating the following:

- wheat straw functioned as absorbing excess moistures avoiding dilution of organic acids, osmotic active substances, proteins and also cations from the ash of *Elodea* biomass that dilutes with water and buffers the silage pH and could also prevent dilution of silage additives
- a more efficient use of *Elodea* biomass resulted with moistures of 70% than 55%. Therefore, the option of co-ensiling would imply a low cost-effective manufacturing process and would ensure a more efficient use of these two biomasses simultaneously.

5.6 Ensiling as an efficient biological pre-step and its possible positive effects on methane yield of *Elodea* and wheat straw

In **Paper I**, methane yields of wheat straw silages exceeded methane yields of corresponding wheat straw without ensiling and their increase was higher for ground straw (0.2 cm) than for chopped straw (2.0 cm) (Table 4). Subsequent digestion in batch test showed that ensiling significantly improved wheat straw methane yields of up to 36% (from 179 to 244 mL CH₄ g⁻¹ VS). The results also showed that methane yields of wheat straw were increased by ensiling fermentation without silage additives by 11–24% when compared to the non-ensiled control sample. In contrast, the addition of silage additives led to a minimal increase of methane yields between 1 and 8%. This implies that silage additives do not have any economic benefit to the process, considering the comparable low effect on the methane yield, the silage product costs and the additional effort for the product application during silage preparation.

In **Paper II**, results showed that highest methane yields were achieved by the fresh *Elodea* (242.2 mL CH₄ g⁻¹ VS) and that a decrease of 10% was observed when *Elodea* was ensiled (218.6 mL CH₄ g⁻¹ VS). However, similar methane yields were found when *Elodea* was ensiled with

wheat straw at 2.0 cm and at 0.2 cm (228.3 and 224.5 mL CH₄ g⁻¹ VS). This may suggest that changes in methane yields after grinding the substrate prior to ensiling might be attributed to the digestibility of lignocellulosic biomass. Therefore, *Elodea* biomass could be a potential feedstock for a year-round biogas system if considered the benefits of the co-ensiling with wheat straw and/or with biomasses with relatively high TS as possible co-substrates.

In general, particle size reduction was a key factor that benefited the ensiling process and could increase the hydrolysis rate (**Paper III**, Table 4) and biodegradability of wheat straw. In addition, it has been documented, that the organic acids, produced during ensiling, can act as catalysts (due to prolonged storage/long reaction time) decreasing biomass recalcitrance and breaking down the ester bonds of the biopolymers facilitated by the reduction of the pore size and degree of polymerization (Larsen *et al.*, 2017). In this line, this has a positive effect on methane formation. Furthermore, it has also been reported, that the acids produced during ensiling (mainly LA and AA) solubilize hemicellulose, a reason for increased accessibility to cellulose (Hansen *et al.*, 2011).

6. Conclusions & future perspectives

6.1 Co-ensiling or mono-ensiling of *Elodea* biomass?

To enable the energetic use of the *Elodea* biomass, development and optimization of storage methods for an aquatic plant-based feedstock for biogas production are required. However, several challenges exist in obtaining preserved *Elodea* biomass with minimal losses. Primarily, its chemical composition: the very high moisture content, low WSC and high buffering capacity and secondly, if pretended to be ensiled in large volumes in conventional clamp silos. To adapt substrate characteristics, a mixture of *Elodea* biomass with wheat straw prior ensiling is an adequate and practicable measure. Mixture silages from *Elodea* and wheat straw show suitable substrate characteristics for ensiling and biogas production and can achieve high biogas yields. Although the experiments have only been done at a laboratory scale, for practical applications, the mixture of *Elodea* with straw at a TS of ~30% is recommended.

In principle, mixtures of aquatic plants with different lignocellulosic substrates are possible. Typical harvesting times for *Elodea* plants are in summer for recreational water bodies and in autumn for rivers (protection of water constructions against aggradations). For this reason, different mass flow combinations (e.g. in autumn, a combination with maize straw) and efficient process chains need to be further investigated.

6.2 A distributed biorefining network

Optimally, a distributed biorefining network would be capable of a sustainable utilization of *Elodea* biomass while generating value, if considered that:

- *Elodea* biomass is produced by growing in freshwaters mainly in eutrophic water bodies, but *Elodea* could also be cultivated in municipal wastewater systems, wetlands or constructed (artificial) wetlands and/or open and closed (photobioreactors) cultivation

systems.

- After demonstrating the effectiveness of the co-ensiling of *Elodea* biomass with wheat straw, the question, “What markets can be satisfied with the produced goods?” is raised. So far, two options would be under discussion, the production of short chain fatty acids during ensiling for chemical processing purposes and the use of the resulting silage for a year-round biogas system.

6.3 Combining ensiling and particle size reduction as a pretreatment for wheat straw

Wheat straw has a low digestibility, which is reflected by very slow rates of hydrolysis. In an eventual large-scale application, the previous ensiling of wheat straw would be a good option to improve the hydrolysis step in the AD process. Organic acids are the result upon ensiling for prolonged periods and they may cause the degradation of fractions of wheat straw not available for subsequent AD.

Most limitations of wheat straw are, as previously discussed, low rates of hydrolysis and the high TS content as well as nutritional deficiencies. Great benefits of lignocellulosic pretreatment (combining particle size reduction and ensiling) on velocities of degradation and biogas yields at laboratory scale were observed in this study. However, it is still unclear how a pretreatment can provide economic benefits for practical applications and therefore further investigations are recommended.

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Theses

Optimization of ensiling fermentation of *Elodea* sp. for biogas production

Presented by Daniela Gallegos Ibáñez

Background and aims of the PhD doctoral work

- ❖ After harvesting, *Elodea* biomass remains unused and it must be treated in compost plants at considerable expense and effort due to its poor structure, high moisture content and low C/N ratio. The AD of this biomass for biogas production may prove to be a promising solution for utilizing and adding value to the *Elodea* waste fraction. Nevertheless, the economic biogas production from commercial AD, usually conducted in continuous mode, requires a year-round supply of high-quality biomass. It is therefore necessary to consider aspects such as seasonal variations in biomass and its preservation.
- ❖ Several challenges exist in obtaining preserved *Elodea* biomass with minimal losses:
 - i) Biochemical factors mainly the very high moisture content ($\approx 95\%$) has negative effects on the ensiling process as it dilutes organic acids, osmotic active substances, proteins and also cations from the ash of *Elodea* biomass that buffers the silage pH, resulting in higher values (> 6). In addition, the high moisture can also dilute silage additives.
 - ii) The low WSC content may result in negligible LA formation, which is necessary to counteract the high buffer capacity of *Elodea* biomass, reducing pH and thus enhancing the preservation of silages.

- iii) It was demonstrated that *Elodea* biomass can be preserved via ensiling (in this work in plastic bags), however, for a year-round biogas production the high moisture complicates storage in conventional clamp silos and requires therefore a co-ensiling process with dry materials to promote suitable substrate characteristics, mainly the solids concentration.
- ❖ The optimization of storage methods for an aquatic plant-based feedstock for biogas production can consider the use of wheat straw in the co-ensiling of *Elodea* biomass, which simultaneously will allow lignocellulosic and aquatic biomass utilization in the biogas technology. In this line, the use of wheat straw could be a useful strategy for the development of the silage clamp technology that requires sufficient dry matter content.
 - ❖ The main reasons for the low-uptake of wheat straw digestion are the challenges on rheology and hydrolysis, as wheat straw mainly consists of lignocellulosic components is less suitable for biogas production than energy crops and results in lower gas yields. Therefore, a pretreatment is required to increase degradation rates, higher biogas yield and technical processing problems.

Main research results

- ❖ The use of wheat straw in the co-ensiling of *Elodea* biomass demonstrated to be a promising solution to overcome aspects such as seasonal variations in biomass and its preservation. In addition, co-ensiling of *Elodea* with wheat straw showed suitable substrate characteristics for ensiling (improved TS content, formation of organic acids and stabilization of silage pH) and biogas production, achieving high biogas yields. For practical applications, the mixture of *Elodea* biomass with straw to a TS of $\approx 30\%$ can be recommended.
- ❖ Reduction of the size of wheat straw particles could be an alternative to improve ensiling by altering wheat straw recalcitrant structure to a greater contact surface area and the release of additional fermentable sugars. In this line, the AD of wheat straw is also improved since degradation rates (i.e. the hydrolysis step of anaerobic fermentation) may also be increased, resulting in an efficient conversion into methane, while in the ensiling

process because of prolonged times and the organic acids produced may act as catalysts solubilizing hemicellulose.

❖ Silage additives are commonly used to ensure that LAB dominate the fermentation and produce LA during the fermentation process in quantities high enough for well-preserved silages. In this work, however, the effect of silages additives was limited: i) in the *Elodea* silages, silages additives did not increase the LA content and silages resulted in similar pH values when compared to silages without additives. Also, the formation of BA, an indicator of clostridial activity, was observed in *Elodea* silages with additives, this may elevate pH and imply a failed fermentation. ii) in the mono-ensiling of wheat straw as well as in co-ensiling process, the effect of addition of additives was also limited and the LA content and pH values were similar to those of silages without additives. In addition, the addition of silage additives led to a minimal increase of methane yields between 1 and 8%. This may imply that silage additives did not have any economic benefit to the process, considering the comparable low effect on the methane yield, the silage product costs and the additional effort for the product application during silage preparation.

❖ On the other hand, increases in methane yield due to the size reduction of wheat straw particles without and with ensiling were by up to 26 and 36%, respectively. In addition, similar methane yields were found when *Elodea* was ensiled with wheat straw at different particle sizes (i.e. 2.0 cm and 0.2 cm). This may suggest that wheat straw played a key role in the storage of *Elodea* biomass for biogas production. In the present study, the experiments were conducted exclusively with wheat straw due to the limitations in experimental capacity. However, it may be feasible to combine *Elodea* with a variety of other drier lignocellulosic materials, and further investigation into this possibility is warranted.

Proof of individual contribution

1 st publication	Author (Gallegos Daniela)	Co-authors
Gallegos, Daniela.; Wedwitschka Harald, Moeller Lucie, Zehnsdorf Andreas, Stinner Walter. <i>Effect of particle size reduction and ensiling fermentation on biogas formation and silage quality of wheat straw.</i> Bioresource Technology; 245 (2017) 216-224.	- collected and prepared raw materials; - carried out analytical analysis; - recorded the data; - performed statistical analysis and interpretation of the data; - performed visualization of the data; - wrote the manuscript.	Wedwitschka Harald (designed and performed the experiment, carried out the ensiling process and batch test, supervised the study, edited and reviewed the manuscript); Moeller Lucie (reviewed the manuscript); Zehnsdorf Andreas (reviewed the manuscript); Stinner Walter (designed and performed the experiment, supervised the study, edited and reviewed the manuscript).

2 nd publication	Author (Gallegos Daniela)	Co-authors
Gallegos Daniela.; Wedwitschka Harald, Moeller Lucie, Weinrich Sören, Zehnsdorf Andreas, Nelles Michael, Stinner, Walter. <i>Mixed silage of Elodea and wheat straw as a substrate for</i>	- collected and prepared raw materials; - carried out analytical analysis; - carried out the ensiling process and batch test - recorded the data; - performed statistical analysis and interpretation of the data; - performed visualization of the data; - wrote the manuscript.	Wedwitschka Harald (designed and performed the experiment, carried out the ensiling process and batch test, supervised the study, edited and reviewed the manuscript); Moeller Lucie (reviewed the manuscript); Weinrich Sören (performed the kinetic analysis reviewed the manuscript); Zehnsdorf Andreas (reviewed the

<i>energy production in anaerobic digestion plants.</i> Energy, Sustainability and Society; (2018) 8:7.		manuscript); Nelles Michael (reviewed the manuscript); Stinner Walter (designed and performed the experiment, supervised the study, edited and reviewed the manuscript).
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3 rd publication	Author (Gallegos, Daniela)	Co-authors
Gallegos, Daniela.; Jurado-Molina, Jesus,; Wedwitschka, Harald.; Delgado, Efren,; Nelles, Michael, Stinner, Walter <i>Ensiling of .invasive Elodea sp., a Novel Aquatic Biomass Feedstock for the Sustainable Biogas Production: Effects of Wheat Straw and Silage Additives on Silage Quality and Methane Production</i> ACS Agricultural Science and Technology; 2023, 3, 6, 477-486	- collected and prepared raw materials; - carried out analytical analysis; - carried out the ensiling process and batch test - recorded the data; - performed statistical and kinetic analysis and interpretation of the data; - performed visualization of the data; - wrote the manuscript.	Jurado_Molina Jesus (contributed in the kinetic analysis and interpretation and reviewed the manuscript); Wedwitschka Harald (designed and performed the experiment, carried out the ensiling process and batch test, supervised the study, edited and reviewed the manuscript); Delgado Efren (reviewed the manuscript); Nelles Michael (reviewed the manuscript); Stinner Walter (designed and performed the experiment, supervised the study, edited and reviewed the manuscript).

Curriculum Vitae

Title: Master of Science in Biochemical Engineering
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Education

- 2016- present **PhD studies to obtain the title of Doctor of Engineering**, University of Rostock Faculty of Agricultural and Environmental Sciences, Department Waste Management and Material Flow, under the PhD students programme of Rostock University and DBFZ (Deutsches Biomasseforschungszentrum gGmbH). Department of Biochemical conversion.
Dissertation: “Optimization of ensiling fermentation of *Elodea* sp. for biogas production”
- 2010-2012 **Master of Sciences in Biochemical Engineering**, Durango Institute of Technology (Instituto Tecnológico de Durango, ITD), Durango, Mexico, working together with the University of Bonn, Germany. Institute of Nutritional and Food Sciences, Department of Food Technology and Food Biotechnology.
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- 2002-2008 **Bachelor: Biochemical Engineering**, Durango Institute of Technology (Instituto Tecnológico de Durango, ITD). Durango, Mexico.
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Working experience

- 2024 Guest lecture on topic “Design of experiments (DoE)” to students from the bioprocess technology department at Zittau Görlitz University of Applied Sciences.
- 2022 Exchange of visiting scientists between DBFZ and the Instituto Tecnológico de Durango (ITD), Durango, Mexico Department of Biochemical Engineering, in

	the field of anaerobic residue treatment and the production of alternative protein sources based on insect biotechnology.
2015-2016	MASISA, Durango, Mexico. Support for instalation of machinary and equipment for the manufacturing of wood boards products such as MDF (medium-density fiberboard) and particleboard as well as fiberboard and related productes in Latin America. Support for translations of work and instruction manuals from English and German into Spanish (not professional and/or certificated translator).
2014-2015	Product engineer (R&D) at Leoni Wiring System and Mercedes Benz (MB) support for start- up projects, implamentation and training for FORS. Analysis of customer drawings and engineering changes, Langfang, China.
2012-2015	Product engineer (R&D) area Mercedes Benz (MB) project “non-JIT”. Analysis of customer drawings and engineering changes to detect possible new components and processes that affect production. Elaboration of bill of materials (BOM’s) and implementation of engineering changes submitted by the customer. Documentation of the technical change history of the Harness. Coordination of engineering change implementation meetings for the client. Supervision of engineering departments involved in the design of assembly boards, clip test assembly boards, clip test table, electrical testing and any other electrical testing and any other tools for harness manufacturing.
2012	Guest at the University of Alberta, Edmonton, Canada Department of Agricultural, Food and Nutritional Science.
2012	Lab assistant participating in the research project AiF 16014 N “Development of microencapsulated polyphenol extracts derived from fruit and vegetable by products for utilisation as food additive”. University of Bonn, Germany. Institute of Nutritional and Food Sciences Department of Food Technology and Food Biotechnology.

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Declaration of primary authorship

I declare, that I have written the present thesis for doctorate and without help of others. Other than the presented references were not used and quoted results were always marked with relevant reference. The present thesis was never either abroad or in Germany submitted for examination in the present or similar version.

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Daniela Gallegos Ibáñez

Publications

- I. Daniela Gallegos, Harald Wedwitschka, Lucie Moeller, Andreas Zehnsdorf, Walter Stinner (2017): Effect of particle size reduction and ensiling fermentation on biogas formation and silage quality of wheat straw. *Bioresource Technology* 245:216-224.

- II. Daniela Gallegos, Harald Wedwitschka, Lucie Moeller, Soeren Weinrich, Andreas Zehnsdorf, Michael Nelles, Walter Stinner (2018): Mixed silage of *Elodea* and wheat straw as a substrate for energy production in anaerobic digestion plants. *Energy, Sustainability and Society* 8, 7.

- III. Daniela Gallegos, Jesus Jurado-Molina, Harald Wedwitschka, Efren Delgado, Michael Nelles, Walter Stinner (2023): Ensiling of Invasive *Elodea* sp., a Novel Aquatic Biomass Feedstock for the Sustainable Biogas Production: Effects of Wheat Straw and Silage Additives on Silage Quality and Methane Production. *ACS Agric. Sci. Technol* 3, 6, 477–486.

PAPER I

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Effect of particle size reduction and ensiling fermentation on biogas formation and silage quality of wheat straw

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ABSTRACT

The effect of ensiling fermentation and mechanical pretreatment on the methane yield of lignocellulosic biomass was investigated in order to determine the optimum pretreatment conditions for biogas production. Wheat straw was treated using the following techniques: mechanical disintegration by chopping and extruder-grinding to particle sizes of 2.0 and 0.2 cm, respectively, and ensiling by 30% and 45% total solids with addition of enzymatic, chemical and biological silage additives individually and in combination. The total and volatile solid content, biochemical methane potential and products of silage fermentation of 32 variants were tested. The results indicate that the methane potential increased by 26% (from 179 to 244 mL CH₄ g^{−1} VS) by reducing particle size. The maximum methane potential of 275 mL CH₄ g^{−1} VS was obtained from silage with 30% total solids and extruder grinding. However, the effect of the addition of silage additives on the methane potential was limited.

1. Introduction

Cereal straw is an agricultural residue material which is generated in great quantities each year around the world. The average yield of wheat straw is 0.8 kg/kg grain in Germany, which results in a considerable amount of surplus straw. For other grains, and cropping systems, the harvest index is even higher (Dai et al., 2016). Around 30 million metric tons of cereal straw are produced annually in Germany, which represents 14% of the total amount of all agricultural residues, such as animal manure and dung (Brosowski et al., 2016; Weiser et al., 2014). As straw is a residue material, no additional agricultural area is needed for its production. This substrate is thus not in competition with other food, feed or energy crops. The environmental impact for the production of straw is comparably low, as fertilizer, pesticide and diesel fuel only counts for the production of the main product (i.e. grain or rape seed) (Ghosh et al., 2017; Senghor et al., 2017). Bioenergy potential studies such as Brosowski et al. (2016), Giannoccaro et al. (2017) and Palmieri et al. (2017) reveal the high potential of agricultural residues such as cereal straw for bioenergy production. In addition, several lab studies show the suitability of straw for biogas production (Pohl et al., 2013). However, the utilization of straw as a feedstock for agricultural biogas production in Germany is negligible (Weiser et al., 2014). Wheat straw is commonly used for mulching or as bedding material for livestock or horses. Large

quantities of cereal straw are left without further use on field, even considering that a certain percentage of straw left on fields is necessary for soil remediation, if humus reproduction is not enabled by digestate, crop rotation and other organic fertilizers. The main reasons for the low up-take of straw digestion technology are the challenges on rheology and hydrolysis, the relative lack of experience with practical biogas application and the need for substrate pretreatment. As straw mainly consists of lignocellulosic components, untreated straw is less suitable for biogas production than energy crops and results in lower gas yields without pretreatment (Schumacher et al., 2014). Several state-of-the-art disintegration technologies are available on the market (Wellinger et al., 2013). These can be subdivided into mechanical, physical, chemical and biological methods according to Schumacher et al. (2014). These methods are applied either alone or in combination.

Most of the mechanical methods rely on the reduction of particle size leading to increased degradation rates, higher biogas yield and reduced technical processing problems (Bolado-Rodríguez et al., 2016). The high costs due to invest, maintenance and energy input for mechanical pretreatment mostly hamper the cost-effective use of straw for biogas production in practice. Biological pretreatment of straw is the topic of numerous studies (Tian et al., 2017; Zhong et al., 2011) but the practical application of this approach is still in its infancy and is often not economically feasible.

Several studies have been carried out that focus on the physical,

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chemical and biological pretreatment of cereal straw (Schumacher et al., 2014; Romero-Güiza et al., 2017). However, limited information is available up to now on the effect of ensiling fermentation as a pre-treatment of straw. The novel approach of our research is the utilization of ensiling fermentation of wheat straw as a biological pretreatment method aiming on improved substrate degradability. The current study was performed to test the hypothesis that silage conditions such as high moisture content, fermentation products (e.g. volatile fatty acids), enzyme activity and low pH value could affect the lignocellulosic bond of wheat straw. The long reaction time of ensiling processes with common storage times of 2–3 to several months could increase this effect.

The objective of this study was to evaluate the effect of mechanical and biological ensiling pre-treatments as well as the use of silage additives on biogas production from wheat straw. Mechanical pretreatment was conducted by chopping and grinding, while biological pretreatment was carried out at laboratory scale by ensiling fermentation at different TS contents. Experiments were conducted without and with the addition of three commercially available silage additives used individually and in combination after a storage period of 180 days. The evaluation of storage losses during ensiling was not part of the scope of this study.

2. Materials and methods

2.1. Raw materials

Wheat straw was gained from the Deutsches Biomasseforschungszentrum gGmbH (DBFZ, Leipzig, Germany) and showed the following characteristics: total solids (TS), 86.5% fresh matter (FM); volatile solids (VS), 95.1% TS; nitrogen (N), 5.9 g kg⁻¹; ash, 67.7 g kg⁻¹; protein (N × 6.25), 36.9 g kg⁻¹; crude fiber, 684.6 g kg⁻¹; crude fat, 10.1 g kg⁻¹; lignin, 121.3 g kg⁻¹; cellulose, 494.0 g kg⁻¹ and hemicellulose, 283.0 g kg⁻¹.

To evaluate the effect of particle size reduction, wheat straw was subjected to physical pre-treatment by means of chopping to a particle size of 2.0 cm (WS1) (Strohmühle, Hirlinger Landtechnik GmbH, Burladingen, Germany) and grinding to a particle size of 0.2 cm (WS2) (Bioextruder, Lehmann-UMT GmbH, Pöhl, Germany) and stored dry at room temperature (20 °C) until its use.

Inoculum for biomethane potential testing (BMP) was gained from the DBFZ research biogas plant. The substrates utilized onsite were maize silage and cattle manure. The organic loading rate of the digestion process was 2.7 g VS L⁻¹ d⁻¹ and the hydraulic retention time was 103 days. Digestate derived from the primary digester was sieved (5 mm) and diluted with tap water and degassed at room temperature for five days to reduce the specific biogas generation before it was used as seeding material in BMP tests. The inoculum average chemical characteristics were pH 7.62, TS 1.62, VS 80.2%, NH₄-N 1.34 g L⁻¹, organic acids 53.01 mg L⁻¹.

2.2. Silage preparation

Ensiling was conducted at laboratory scale by means of ensiling fermentation as previously described by Ashbell et al. (2001). Silages were prepared using the mechanically pretreated wheat straw raw material (WS1 and WS2, respectively) as follows: WS1 was used to generate the chopped straw silages SC1 at 30% TS and SC2 at 45% TS; WS2 was used to generate the ground straw silages SG1 at 30% TS and SG2 at 45% TS. Tap water was added to adjust the TS content of the silages. To treat the material different silage additive were used. Silage additive treatments included one chemical additive, enzymes and one biological additive and used individually or in combination to compare their mode of action. In the case of the biological additive, it was only used in combination. Silage additive treatments were applied as follows:

- (1) Control: Silages without addition of silage additives
- (2) E: An enzyme additive (Schaumann Forschung GmbH, Pinneberg, Germany) containing a mixture of cellulase and hemicellulase was added at a concentration of 200 µL kg⁻¹ FM to increase the accessibility of easily convertible sugars from cellulose and hemicellulose to enhance homo- and heterofermentative lactic acid bacteria products.
- (3) Chem: A chemical silage additive (Schaumacid, Schaumann Forschung GmbH, Pinneberg, Germany) containing formic acid was added at a concentration of 8 µL kg⁻¹ FM. This additive is used to inhibit yeast growth, while homo- and heterofermentative lactic acid bacteria are not affected.
- (4) ChemA: A combination of chemical (8 µL kg⁻¹ FM) and enzymatic (200 µL kg⁻¹ FM) silage additives was tested. The aim of the combined dosing of chemical and enzymatic additives was to support the homo- and heterofermentative fermentation process, leading to fast pre-stabilization of the ensiled material without energy and material loss due to yeast growth.
- (5) ChemB: This variant consisted of a combination of chemical (8 µL kg⁻¹ FM) and biological silage additives (10 g kg⁻¹ FM). The biological silage additive (Schaumalac, Schaumann Forschung GmbH, Pinneberg, Germany) consisted of a combination of hetero- and homofermentative lactic acid bacteria (LAB).
- (6) ChemC: A combination of chemical (8 µL kg⁻¹ FM), enzyme (200 µL kg⁻¹ FM) and biological silage additives (10 g kg⁻¹ FM) was tested to determine the effectiveness of combined additive application.
- (7) He + E: This variant involved the combined addition of the Schaumalac biological (10 g kg⁻¹ FM) and enzymatic silage additives (200 µL kg⁻¹ FM). The aim was to achieve stable homolactic fermentation during ensiling and fast biological stabilization of the ensiled material.
- (8) He + Sg: The combination of Schaumalac biological silage additive (10 g kg⁻¹ FM) and a sugar solution (10% TS) was tested in this variant. Sugar was added in order to increase carbohydrate sources for lactic acid bacteria.

Silage additives were prepared as aqueous suspensions (dissolved or diluted) using tap water at concentrations recommended by the suppliers. After this, a volume of 10 mL of liquid per kilogram of fresh material was applied by spraying onto the substrates using a commercially available hand sprayer. Thereafter, six vacuum sealer bags (30 × 50 cm PA/PE, La.v.a, Germany) were filled with 500 g of each substrate, and the material was vacuum packed (Cookmax Serie 42, Netherlands) and stored in the dark at room temperature (approx. 20 °C). Storage was carried out under anaerobic conditions for all treatments for a period of 180 days. The experiments were performed in triplicate in order to ensure reproducibility.

2.3. Chemical analyses and BMP tests

Total solids (TS) and volatile solids (VS) were determined according to DIN EN 12880 (2001) and DIN EN 12879 (2001). Samples were measured by oven-drying the samples for 24 h at 105 °C (Binder, Germany) and further reducing the organic content to ashes for 30 min at 220 °C and then for 2 h at 550 °C in a muffle furnace (Carbolite, UK). The TS values and all values referring to TS were corrected as described by Weißbach and Strubelt (2008).

The pH value was measured using a Sen Tix 41 pH electrode (WTW, Weilheim, Germany) after homogenizing 5 g of sample with 100 mL of distilled water for a period of 15 min.

The acid spectrum comprises the following organic acids: lactic acid, acetic acid, propionic acid, iso-butyric acid, *n*-butyric acid, iso-valeric acid, *n*-valeric acid, hexanoic acid as well as benzaldehyde and alcohols (ethanol, 2-butanol, 1-propanol, 1-butanol), furfural and 5-methylfurfural (5-HFM). They were determined by using the Headspace GC system, which consisted of a 7890 series II gas chromatograph

Table 1Silage fermentation products of untreated and treated wheat straw (mean $\pm$ standard deviation).

Silage	Treatment	TS ^a (%)	VS ^a (%)	LA (%TS)	AA (%TS)	BA (%TS)	pH
SC1	Control	30.8 $\pm$ 0.4	92.3 $\pm$ 0.7	1.3 $\pm$ 0.0 ^{c-j}	0.6 $\pm$ 0.0 ^{fghi}	0.0 $\pm$ 0.0 ^a	5.3 $\pm$ 0.1 ^{efg}
	E	33.4 $\pm$ 0.2	92.9 $\pm$ 0.0	2.8 $\pm$ 0.1 ^{a-f}	1.7 $\pm$ 0.0 ^{b-h}	0.0 $\pm$ 0.0 ^a	4.7 $\pm$ 0.1 ^{k-n}
	Chem	31.1 $\pm$ 0.8	93.1 $\pm$ 0.1	0.5 $\pm$ 0.0 ^{hij}	0.8 $\pm$ 0.0 ^{fghi}	0.0 $\pm$ 0.0 ^a	4.9 $\pm$ 0.1 ^{h-i}
	ChemA	23.8 $\pm$ 0.4	92.4 $\pm$ 0.2	2.1 $\pm$ 0.0 ^{a-i}	0.9 $\pm$ 0.0 ^{e-i}	0.0 $\pm$ 0.0 ^a	5.1 $\pm$ 0.1 ^{f-i}
	ChemB	28.7 $\pm$ 0.3	91.7 $\pm$ 0.3	0.5 $\pm$ 0.0 ^{hij}	0.5 $\pm$ 0.0 ^{hi}	0.0 $\pm$ 0.0 ^a	5.8 $\pm$ 0.1 ^{cd}
	ChemC	25.0 $\pm$ 0.6	92.8 $\pm$ 0.6	2.6 $\pm$ 0.1 ^{a-g}	2.1 $\pm$ 0.1 ^{a-e}	0.0 $\pm$ 0.0 ^a	n. a
	He + E	23.9 $\pm$ 0.1	91.2 $\pm$ 0.4	2.3 $\pm$ 0.1 ^{a-h}	2.3 $\pm$ 0.0 ^{abcd}	0.0 $\pm$ 0.0 ^a	4.9 $\pm$ 0.1 ^{h-i}
	He + Sg	26.4 $\pm$ 0.3	91.7 $\pm$ 0.1	3.6 $\pm$ 0.1 ^{ab}	1.8 $\pm$ 0.0 ^{a-g}	0.0 $\pm$ 0.0 ^a	4.7 $\pm$ 0.1 ^{j-m}
	Control	32.0 $\pm$ 0.1	91.1 $\pm$ 0.4	0.0 $\pm$ 0.0 ^j	0.6 $\pm$ 0.0 ^{fghi}	0.0 $\pm$ 0.0 ^a	6.2 $\pm$ 0.1 ^{ab}
	E	29.3 $\pm$ 0.1	91.6 $\pm$ 0.1	3.3 $\pm$ 0.1 ^{abc}	2.6 $\pm$ 0.0 ^{abc}	0.1 $\pm$ 0.2 ^a	4.4 $\pm$ 0.1 ⁿ
SG1	Chem	29.2 $\pm$ 0.1	92.2 $\pm$ 0.1	1.1 $\pm$ 0.0 ^{d-j}	0.8 $\pm$ 0.0 ^{fghi}	0.0 $\pm$ 0.0 ^a	4.9 $\pm$ 0.1 ^{h-i}
	ChemA	25.1 $\pm$ 0.1	92.2 $\pm$ 0.0	0.8 $\pm$ 0.0 ^{f-j}	0.6 $\pm$ 0.0 ^{fghi}	0.0 $\pm$ 0.0 ^a	4.9 $\pm$ 0.1 ^{h-i}
	ChemB	26.3 $\pm$ 0.0	92.3 $\pm$ 0.1	1.9 $\pm$ 0.0 ^{a-j}	1.2 $\pm$ 0.0 ^{d-i}	0.0 $\pm$ 0.0 ^a	5.1 $\pm$ 0.1 ^{fgh}
	ChemC	25.8 $\pm$ 0.1	91.4 $\pm$ 0.0	2.6 $\pm$ 2.2 ^{a-g}	0.7 $\pm$ 0.6 ^{fghi}	0.0 $\pm$ 0.0 ^a	4.4 $\pm$ 0.1 ^{mn}
	He + E	25.2 $\pm$ 0.2	82.1 $\pm$ 8.8	0.0 $\pm$ 0.0 ^j	1.9 $\pm$ 0.1 ^{a-f}	0.0 $\pm$ 0.0 ^a	5.4 $\pm$ 0.1 ^{de}
	Control	45.5 $\pm$ 1.4	92.2 $\pm$ 0.6	1.1 $\pm$ 1.0 ^{d-j}	1.3 $\pm$ 1.1 ^{d-i}	< 0.1 $\pm$ 0.1 ^a	5.8 $\pm$ 0.1 ^c
	E	39.9 $\pm$ 0.2	92.6 $\pm$ 0.6	2.9 $\pm$ 0.1 ^{a-e}	1.4 $\pm$ 0.0 ^{b-i}	0.4 $\pm$ 0.7 ^a	4.7 $\pm$ 0.1 ^{k-n}
	Chem	46.0 $\pm$ 2.6	92.6 $\pm$ 0.2	0.3 $\pm$ 0.0 ^{hij}	0.7 $\pm$ 0.1 ^{fghi}	0.1 $\pm$ 0.1 ^a	5.3 $\pm$ 0.1 ^{efg}
	ChemA	52.4 $\pm$ 1.3	92.8 $\pm$ 0.8	0.4 $\pm$ 0.0 ^{hij}	0.7 $\pm$ 0.0 ^{fghi}	0.0 $\pm$ 0.0 ^a	5.4 $\pm$ 0.1 ^{efg}
	ChemB	36.4 $\pm$ 0.2	91.9 $\pm$ 0.2	1.9 $\pm$ 0.0 ^{a-j}	1.0 $\pm$ 0.0 ^{d-i}	0.5 $\pm$ 0.5 ^a	5.9 $\pm$ 0.1 ^{bc}
SC2	ChemC	31.3 $\pm$ 0.1	92.0 $\pm$ 0.7	2.6 $\pm$ 0.0 ^{a-g}	1.4 $\pm$ 0.0 ^{c-i}	0.2 $\pm$ 0.3 ^a	5.0 $\pm$ 0.1 ^{h-k}
	He + E	43.3 $\pm$ 0.4	92.3 $\pm$ 0.4	2.1 $\pm$ 1.8 ^{a-i}	1.3 $\pm$ 1.1 ^{d-i}	1.3 $\pm$ 1.3 ^a	4.9 $\pm$ 0.1 ^{h-i}
	He + Sg	38.2 $\pm$ 0.4	90.7 $\pm$ 0.6	2.1 $\pm$ 0.1 ^{a-i}	1.8 $\pm$ 0.0 ^{a-g}	1.4 $\pm$ 2.4 ^a	5.4 $\pm$ 0.1 ^{ef}
	Control	42.6 $\pm$ 0.2	92.1 $\pm$ 0.1	1.8 $\pm$ 1.6 ^{a-j}	1.3 $\pm$ 1.1 ^{c-i}	0.0 $\pm$ 0.0 ^a	6.3 $\pm$ 0.1 ^a
	E	47.3 $\pm$ 0.0	92.0 $\pm$ 0.1	0.4 $\pm$ 0.3 ^{hij}	0.5 $\pm$ 0.4 ^{hi}	0.0 $\pm$ 0.0 ^a	4.6 $\pm$ 0.1 ^{lmn}
	Chem	41.1 $\pm$ 0.1	92.1 $\pm$ 0.1	0.6 $\pm$ 0.0 ^{ghij}	0.7 $\pm$ 0.0 ^{fghi}	0.0 $\pm$ 0.0 ^a	5.1 $\pm$ 0.1 ^{g-j}
	ChemA	40.6 $\pm$ 0.1	92.1 $\pm$ 0.0	0.9 $\pm$ 0.8 ^{e-j}	0.9 $\pm$ 0.8 ^{e-i}	0.0 $\pm$ 0.0 ^a	4.5 $\pm$ 0.1 ^{mn}
	ChemB	44.9 $\pm$ 0.2	90.8 $\pm$ 0.7	1.8 $\pm$ 0.1 ^{b-j}	0.9 $\pm$ 0.0 ^{fghi}	0.0 $\pm$ 0.0 ^a	5.6 $\pm$ 0.1 ^{cde}
	ChemC	39.0 $\pm$ 0.3	90.9 $\pm$ 0.2	3.0 $\pm$ 0.1 ^{abcd}	2.7 $\pm$ 0.0 ^{ab}	0.0 $\pm$ 0.0 ^a	4.8 $\pm$ 0.1 ^{i-m}
	He + E	39.2 $\pm$ 0.2	93.0 $\pm$ 0.2	3.8 $\pm$ 0.1 ^a	0.3 $\pm$ 0.0 ⁱ	0.0 $\pm$ 0.0 ^a	4.7 $\pm$ 0.1 ^{ijklm}
SG2	He + Sg	40.0 $\pm$ 0.1	93.8 $\pm$ 0.2	0.1 $\pm$ 0.0 ^{ij}	3.0 $\pm$ 0.0 ^a	0.0 $\pm$ 0.0 ^a	n.a

LA = lactic acid; AA = acetic acid, sum of acetic acid and propionic acid; BA = butyric acid, sum of i-butyric, n-butyric, i-valeric, n-valeric and n-caproic acid.

^{a-j} Means that do not share a letter in a column for a given factor are significantly different ($p < 0.05$). LSD. n.a.: not analyzed. TS = Total solids; VS = volatile solids.

(Hewlett Packard, USA) equipped with an HS40 automatic headspace sampler (Perkin Elmer, USA) and a flame ionization detector from Agilent FID Technologies. A method description can be found in Apelt (2016).

Biochemical methane potential (BMP) tests at laboratory scale were conducted in accordance with VDI 4630 (VDI, 2014) using eudiometer devices (Neubert Glas GbR, Geschwenda, Germany) to determine the specific methane yields of the ensiled material after 180 days of storage. The samples consisted of 450 g of inoculum and approximately 2.5 g of volatile solids (VS) of the substrate, each in three replications. The inoculum to substrate rate (ISR) was approximately 2.3:1. In addition to the sample materials, pure inoculum was tested to determine its methane yield in order to subtract this from the sample methane production. To monitor the inoculum performance, microcrystalline cellulose was used as a reference substrate. The headspace was flushed with nitrogen prior incubation. Headspace correction was carried out as described in the method description VDI 4630 (VDI, 2014). The test was operated under mesophilic conditions (38 °C) and was stirred once a day. The gas composition was determined with a land fill gas monitor (GA2000, Ansyco, Karlsruhe, Germany). The BMP test was ended when the daily biogas production had reduced to 1% of the total biogas production over a period of five consecutive days. The specific methane yields were standardized to 273.15 K and 1.01325×10^5 Pa.

2.4. Statistical analyses

Particle size, total solids content and addition of additive were investigated as fixed effects on ensiling after 180 days. Multiple comparisons of the products of silage fermentation were carried out by One-Way Analysis of Variance (ANOVA) to analyze the statistical significance of differences ($\alpha = 0.05$) using SAS v 10.0 software (SAS Institute, Cary, NC, USA). The data was analyzed using Fisher's least

significant difference (LSD).

Response Surface Methodology (RSM) – RSM was used to evaluate the combined effects and the interactions between the independent variables. The independent variables considered were particle size (X_1), TS content (X_2), control (X_3), enzymes: E (X_4), chemical additive: Chem (X_5), combination of enzymes and chemical additive: ChemA (X_6), combination of LAB inoculant and chemical additive: ChemB (X_7), combination of LAB inoculant, enzymes and chemical additive: ChemC (X_8), combination of LAB inoculant and enzymes: He + E (X_9); combination of LAB inoculant and sugar solutions: He + Sg (X_{10}). The range of the independent variables particle size and TS content were 0.2–2.0 cm and 30–45% TS, respectively, and the range of the independent variables without and with addition of additive was 0–1. The response variable was methane yield obtained after anaerobic digestion of all the silages used in the present study and analyzed using commercial software (Design-Expert 7.0.0®, State-Ease Inc., Minneapolis, MN, U.S.A.). The results were analyzed by multiple quadratic regressions (Eq. (1)). Experimental data were adjusted to selected models and regression coefficients were obtained. The statistical significances of the regression terms were examined by variance analysis (ANOVA).

$$\begin{aligned}
 y = & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 \\
 & + \beta_{11} X_1^2 + \beta_{22} X_2^2 \\
 & + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 \\
 & + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 \\
 & + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4
 \end{aligned}
 \quad (1)$$

where y is the variable response, $X_1 \dots X_4$ are the independent variables, and $\beta_1 \dots \beta_4$ are the regression coefficients of the model.

3. Results and discussion

3.1. Silage quality

3.1.1. Effect of particle size

The values for all volatile short chain fatty acids measured in straw silages after 180 days of storage are summarized in Table 1. Results revealed that in most cases reduced particle size can increase the amount of lactic acid (LA), resulting in negligible butyric acid concentrations, which is an indicator of poor silage quality. Analyzing the influence of the particle size by comparing the ground control samples SG1 and SG2 with the chopped control samples SC1 and SC2, it can be observed that numerically but not significantly higher ($p > 0.05$) LA concentration of SG2 were found compared with SC2 and SC1. This numerical difference can be attributed to the recalcitrance of lignocellulosic biomass to hydrolysis. It is expected that an intensified mechanical processing can alter the structure of lignocellulosic biomass to make cellulose more accessible, increasing the digestibility of the biomass. Thus, grinding breaks the lignin seal and disrupts the crystalline structure of cellulose, which causes a larger exposure of the contact surface area to hydrolytic enzymes, converting the carbohydrate polymers into monomeric sugars for a faster and easier LAB fermentation. The results also showed that the activity of the heterofermentative LAB might have predominated in the ground control samples. The acetic acid content of the control samples was numerically lower for the SC1 than that for the other control treatments, but the differences were not significant. This effect can be explained by the LAB, which are classified as homofermentative or heterofermentative based on their by-products of sugar fermentation. Obligate homofermentative LAB convert hexoses into LA via the Embden-Meyerhof pathway, but are unable to ferment pentoses or gluconate. Facultative heterofermentative LAB usually ferment hexoses homofermentatively into LA but under special conditions into equimolar amounts of LA, carbon dioxide and ethanol (or acetic acid). Acetic acid might therefore be formed under conditions where NAD^+ can be regenerated without the formation of ethanol from pentoses via the phosphoketolase pathway (Holzer et al., 2003). The ratios of acids are important in explaining variation in lactic and acetic acid content and are an indicator of the efficiency of silage fermentation. Hence, the facultative homofermentative bacteria can dominate the fermentation, if the desirable lactic to acetic (LA:AA) acid ratio is greater than 3:1 (Li et al., 2016). The LA:AA ratio of the SC1 control was higher (2.1) than that of the SG2 and SC2 control samples (1.4 and 0.9, respectively) (data not shown). The decrease in the production of LA in the ground control when compared to the chopped control is in agreement with results published by Haag et al. (2015). Numerically, but not significantly higher ($p > 0.05$) pH of the SG2 silage were found compared with the SG1 silage, whereas significantly lower ($p < 0.05$) pH of the SC1 silage were found compared with all the silage controls. This might have been due to the higher acetic and lower LA contents in the SG2 and SG1 silages compared with the SC1 silage. The pKa of LA is 3.86, and that of acetic acid 4.77. With the pKa defined as the negative common logarithm of the acid dissociation constant, these values show that LA is twice stronger than acetic acid. The analyzed acid contents presented in Table 1 in non-additive-treated straw silages suggested that the pH might have been affected in two ways. First, the dominance of the epiphytic heterofermentative LAB in the early fermentation stage carried out a heterolactic fermentation, which slightly decreased the pH of the silage. Secondly, this insufficient acidification could not promote the development of acid-tolerant LAB species (mainly homofermentative LAB) so that the heterofermentative bacteria might have utilized more effectively hexoses and some pentoses supplied from the hemicellulose than the homofermentative bacteria, producing in equimolar amounts LA and acetic acid during the fermentation period. Ethanol content suggested the activity of some yeast over the ensiling period. Ethanol was absent or detected in only small amounts (data not

shown) but slightly higher concentrations in SG1 than in SC2. The high pH value and low productions of LA and acetic acid might have stimulated yeasts activity. This was attributed probably to the survival of some yeast throughout the ensilage period. Sufficient fermentable sugars were available for yeasts to maintain their metabolism. Butyric acid was absent or detected in only small amounts, with no differences ($P > 0.05$) among treatments. Acetic acid concentration is desired in silages because of its effects to inhibit yeast and molds and increase the aerobic stability after opening the silo for feeding (Driehuis et al., 1999). In addition, it has been found in literature (Holzer et al., 2003) that during heterofermentative fermentation, dry matter losses due to heterolactic fermenters are 0.5–2.4% whereas energy losses are $< 0.2\%$. Although this can be even much lower than those resulted from a homolactic fermentation owing to the carbon dioxide formation caused by aerobic deterioration. Based on the results of the present study preservation of wheat straw ensiled without silage additive can be recommended since wheat straw silages resulted in well-fermented and stable silage. However, further research on this topic has to be done. The investigated wheat straw silage contained reasonable concentrations of fermentation products such as LA, which can be used as a platform chemical or possible cleaning or conservation agent.

3.1.2. Effect of moisture content

Results of the moisture content are summarized in Table 1. The TS content of the investigated silages in this study were within the appropriate range for ensiling to inhibit undesirable microorganisms (McDonald et al., 1991). It was observed that the pH value significantly increased ($p < 0.05$) in the control sample at a TS content of 45%. This could be attributed to the higher acetic and lower lactic acid contents. When the fermentation is restricted to higher solid contents, leading to lower acidification, good preservation can be achieved at higher pH. The so-called critical pH value, which is necessary for efficient silage preservation can vary between 4.1 and 5.0 at TS of 15% and 50%, respectively (Kalač, 2011). Only those silages where the pH varied from 4.9 to 5.0 complied with these requirements. Borreani et al. (2009) reported that fermentation can be restricted as dry matter content increases, however, clostridial activity is normally minimal, although some growth of lactic acid bacteria occurs, even in ensiled materials to dry matter contents as high as 50% TS. In this study LA was produced in higher amounts in silages at TS content of 30% than in those silages at TS content of 45%. Butyric acid was detected at low amount, with no differences ($p > 0.05$) among treatments. The pH values of the control samples varied between 5.3 and 6.3 with significant positive correlation ($r = 0.58$, $p < 0.01$) (Table 2). This can be explained by the predominant heterofermentative pathway forming the weaker acetic acid, which resulted in a moderate pH drop. In this regard, the pH value was negatively correlated with lactic acid ($r = -0.44$, $p < 0.01$) and acetic acid concentration ($r = -0.26$, $p < 0.05$) (Table 2). In addition, acetic acid was positively correlated with lactic acid production ($r = 0.54$, $p < 0.01$). On the other hand, the addition of enzymes (E) had a negative correlation with the pH value ($r = -0.44$, $p < 0.01$). Cellulose can be hydrolytically broken down into glucose by cellulases, while hemicellulose polymers can be hydrolyzed by hemicellulases to release its component sugars (glucose, galactose and mannose). However pentoses are also hydrolyzed from hemicellulose to xylose and arabinose. Wheat straw shows in its chemical composition (see Section 2.1) higher cellulose content than hemicellulose. Cellulases increased glucose concentration in the silage and therefore raised homofermentative LAB. This trend was evident in SG1 E, SG2 He + E, showing a significant increase in the LA content.

Straw is a comparatively cheap agricultural residue and has very low water content. It can be used for improving material characteristics of very wet substrates prior ensiling in bunker silos.

3.1.3. Effect of addition of additives

LA occurred in all silages at TS of 30% and 45% (Table 1). The LA

Table 2

Pearson linear correlation for the ensiling process of untreated and treated wheat straw.

	Particle size	Total solids	Control	E	Chem	ChemA	ChemB	ChemC	He + E	He + Sg	LA	AA	BA	pH	CH ₄
Particle size	1.00														
Total solids	−0.02	1.00													
Control	0.01	0.01	1.00												
E	−0.03	−0.03	−0.17	1.00											
Chem	−0.06	−0.06	−0.16	−0.17	1.00										
ChemA	0.15	0.06	−0.14	−0.14	−0.14	1.00									
ChemB	−0.03	−0.03	−0.17	−0.18	−0.17	−0.14	1.00								
ChemC	−0.14	0.10	−0.14	−0.15	−0.14	−0.12	−0.15	1.00							
He + E	−0.10	−0.02	−0.15	−0.16	−0.15	−0.13	−0.16	−0.14	1.00						
He + Sg	0.27	−0.02	−0.12	−0.12	−0.12	−0.10	−0.12	−0.10	−0.11	1.00					
LA	0.04	0.05	−0.20	0.20	−0.37**	−0.14	−0.09	0.28*	0.15	0.25*	1.00				
AA	0.02	−0.04	−0.14	0.20	−0.27*	−0.14	−0.20	0.20	0.16	0.25*	0.54**	1.00			
BA	0.21	0.20	−0.09	0.00	−0.09	−0.08	0.00	−0.05	0.09	0.28*	0.13	0.16	1.00		
pH	0.12	0.17	0.58**	−0.44**	−0.09	−0.06	0.39**	−0.29*	−0.09	−0.03	−0.44**	−0.26*	0.03	1.00	
CH ₄	−0.58**	−0.33**	0.01	0.06	0.06	−0.46**	0.01	−0.08	0.23*	0.15	0.04	0.10	−0.04	−0.04	1.00

* Significant at $p < 0.05$.** Significant at $p < 0.01$. E = Enzymes; Chem = chemical additive; ChemA = combination of enzymes and chemical additive; ChemB = combination of LAB inoculant and chemical additive; ChemC = combination of LAB inoculant, enzymes and chemical additive; He + E = combination of enzymes and enzymes; He + Sg = combination of LAB inoculant and sugar solutions; LA = lactic acid (%TS); AA = acetic acid (%TS); BA = butyric acid (%TS); CH₄ = Methane yield (mL CH₄ g^{−1} VS).

content of the silage SG2 treated with He + E significantly exceeded LA contents of the control sample ($p < 0.05$), while numerically but not significantly higher LA concentrations of the silages SG1 and SC1 were found with the addition of He + Sg and E. A strong correlation was observed with He + Sg ($r = 0.25$, $p < 0.05$), ChemC ($r = 0.28$, $p < 0.05$) (Table 2). However, LA contents decreased with the addition of chemical additive (Chem), showing a negative correlation ($r = -0.37$, $p < 0.01$) (Table 2). This could be attributed to the different mode of action of the silages additives. Fermentation inhibitors such as formic acid act reducing the pH of the silage to restrict microbial growth. On the contrary, fermentation simulants such as LAB, sugars and enzymes encourage lactic fermentation by supply of substrate, bacteria or enzymes and are more efficient in the anaerobic storage phase than chemical additives. In silages SC at 45% TS the addition of He + Sg numerically increased ($p < 0.05$) the amount of butyric acid compared with the control sample, but no significant differences among the silages were observed. This might have been due to high pH value, despite high TS contents in SC2 silages. With increasing the amounts of lactic acid, the activity of clostridia increased, showing a positive correlation with Sg + He ($r = 0.28$, $p < 0.05$) (Table 2). This is due to clostridial fermentation is mainly based on sugars and LA consumption (Holzer et al., 2003).

The pH value significantly decreased ($p < 0.05$) with the addition of enzymes in the silage SG1 compared with the control sample. The pH value was positively correlated with no addition of additive ($r = 0.58$, $p < 0.01$) and with ChemB ($r = 0.39$, $p < 0.01$), and negatively correlated with E ($r = -0.44$, $p < 0.01$) and with ChemC ($r = -0.29$, $p < 0.05$) (Table 2). Treating the silages with enzymes individually or in combination with LAB inoculant resulted in highest LA contents and lowest pH value.

3.2. Effect of the parameters of ensiling fermentation on methane formation

3.2.1. Effect of particle size

Methane formation is affected by the particle size reduction through its effects on ensiling (Table 3). The correlation coefficient of the fitted model to the experimental data was 0.9761, indicating that the model was significant and that it adequately represents the actual relationship between the parameters studied. The particle size reduction had a significant effect ($p < 0.05$) on the linear term. As the particle size reduction decreases, the methane yield tended to increase (Fig. 1). The particle size reduction from 2.0 to 0.2 cm led to an increase of the methane yield by 26%. There is a strong correlation between particle size and methane yields ($r = -0.58$, $p < 0.01$) (Table 2). This

behavior might be caused by the increase in the specific area of the ensiled material, which in turn results in an increase in the soluble fraction of organic matter due to the shearing of fibers. Similar effects have also been reported by Herrmann et al. (2012), where a nominal length of 4 mm applied to three varieties of maize resulted in highest methane yields. The authors also reported that methane yields decreased with a particle size of 10 mm in maize. The results are also consistent with other studies targeted at obtaining maximum methane yields through thermal pretreatment of lignocellulosic biomass (Ferreira et al., 2013; Schumacher et al., 2014). In addition, Bauer et al. (2009), Theuretzbacher et al. (2015), Bolado-Rodríguez et al. (2016) suggested that the variation of methane yields is probably due to the relevant chemical properties of each species with regard to the seasonal and regional variations.

Methane yields of silages exceeded methane yields of corresponding raw materials without ensiling, and the increase of methane yield was higher for ground materials (0.2 cm) than for chopped materials (2.0 cm) (Table 4). Fig. 2 depicts the methane production of the straw silages without treatment in comparison with non-ensiled dry chopped and ground straw. It can be observed that WS1 showed a much lower methane yield with (179 mL CH₄ g^{−1} VS) in comparison to WS2 (244 mL CH₄ g^{−1} VS). In this regard, the results revealed that methane yield was increased in both non-ensiled and ensiled samples by reducing particle size. However, the chopped straw variant that was ensiled (SC1) showed a similar methane yield (236 mL CH₄ g^{−1} VS) as the dry ground straw WS2 (244 mL CH₄ g^{−1} VS).

The results showed that ensiling fermentation is an effective low-tech substrate pretreatment method. Similar gas yields were achieved from wheat straw after ensiling and energy-intensive straw milling (Table 4). The combination of both technologies showed additional positive effects. In further studies it needs to be assessed, if the testing results are transferable to other straw like biomasses such as corn, rice, barley and rape straw, chaff or bagasse from sugar cane. Especially in comparison to corn and rape straw, wheat straw has different buffering characteristics regarding protein and cation contents.

In addition, the specific methane yield based on fresh matter (FM) show the comparably high methane yield of the dry wheat straw WS1 and WS2 with 142 and 195 mL CH₄ g^{−1} FM (Fig. 3). The straw silage variant with chopped straw SC1 resulted in 68 mL CH₄ g^{−1} FM while the ground straw variant SG2 was tested with 98 mL CH₄ g^{−1} FM. Maize silage was tested as a reference substrate with 102 mL CH₄ g^{−1} FM. It must be considered, for the calculation of material handling, transportation and storage costs, that water content and bulk density characteristics of a substrate material are crucial

Table 3Regression coefficients of models (coded factors) for methane yields $R^2 = 0.9761$ (P of F model 0.0127).

Coefficients											
Intercept	220.91 [*]										
Linear		X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	X ₉	X ₁₀
		−74.0 [*]	−50.8 [*]	0.4	0.2	−0.4	−18.4 [*]	−1.2	−4.8	3.6	−1.1
Interaction		X ₁ X ₂	X ₁ X ₃	X ₁ X ₄	X ₁ X ₅	X ₁ X ₆	X ₁ X ₇	X ₁ X ₈	X ₁ X ₉	X ₁ X ₁₀	
		2.7	−13.5 [*]	−9.5	−12.8 [*]	−5.5	−11.9 [*]	−13.1 [*]	−17.1 [*]	−11.8	
		X ₂ X ₃	X ₂ X ₄	X ₂ X ₅	X ₂ X ₆	X ₂ X ₇	X ₂ X ₈	X ₂ X ₉	X ₂ X ₁₀		
		−11.1 [*]	−9.8	−8.1	−2.8	−10.1	−10.3	−7.3	−10.0		

* indicate significant difference ($p < 0.05$). X₁ = Particle size (cm); X₂ = Total solids content (%); X₃ = Without addition of additive; X₄ = E; X₅ = Chem; X₆ = ChemA; X₇ = ChemB; X₈ = ChemC; X₉ = He + E; X₁₀ = He + Sg; R² = Coefficient of determination.

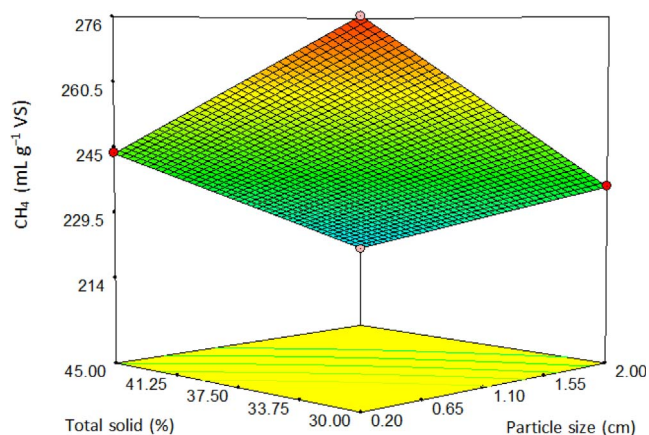


Fig. 1. Experimental (red region) and predicted (slightly red to green zone) Plateau (blue region). Methane yield as a function of particle size and total solid content. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

parameters. Precise cost-analyses are required to determine the economic sustainability of the mechanical pretreatment in comparison to the biological pretreatment with ensiling fermentation or to evaluate combined process chains. Straw harvesting and transportation costs to the biogas plant are not affected by the substrate pretreatment technology onsite of a biogas plant. Mechanical pretreatment is intensive in energy input and maintenance. Costs for substrate and digestate handling and silage storage need to be assessed. At a typical agricultural biogas plant, no additional costs for freshwater occur, as rain water is available, which has to be collected and treated. Alternatively wet co-substrates can be used for straw silage preparation. In both cases no additional costs for digestate distribution occur. However, in a mass balance the water input generates additional digestate. Nevertheless, rainwater also has to be stored and distributed and the transfer of this water into the digestate via silage does not drastically increase the costs.

3.2.2. Effect of moisture content

Methane formation through anaerobic digestion is affected by the TS content of the ensiled material (Table 3). The adjusted regression model had a correlation coefficient value $R^2 = 0.9761$, which indicates that the TS content had a significant effect ($p < 0.05$) on the linear and ($p < 0.05$) on the interaction term ($p < 0.05$). The response surface plot for methane yield as a function of the TS content and particle size is shown in Fig. 1. When the TS content and particle size were at the endpoints (low values, red zone) the methane yield was greater; however, we also observed that at intermediate and high values (green and blue zones) of the TS content and particle size, the methane yield decreased. The methane yields varied between 198 and 280 mL CH₄ g^{−1} VS and decreased significantly with increasing the TS content ($r = -0.33$, $p < 0.01$) (Table 2). This might have been due to

Table 4Methane formation from wheat straw without ensiling and straw silages (mean $\pm$ standard deviation of three replicates).

Silage	Treatment	Specific biogas yield (mL g _{VS} ^{−1})	Specific methane yield (mL g _{VS} ^{−1})	CH ₄ (%)
WS1	untreated	318.2 $\pm$ 2.2 [*]	179.2 $\pm$ 1.2	56.5
WS2	untreated	436.3 $\pm$ 36.0	244.1 $\pm$ 20.1	55.9
SC1	Control	436.0 $\pm$ 19.5	235.9 $\pm$ 10.6	54.5
SC1	E	442.0 $\pm$ 8.7	235.3 $\pm$ 5.6	54.3
SC1	Chem	422.3 $\pm$ 7.6	225.9 $\pm$ 8.5	54.0
SC1	ChemA	351.3 $\pm$ 11.0	197.6 $\pm$ 7.1	56.9
SC1	ChemB	445.7 $\pm$ 13.0	235.0 $\pm$ 5.8	53.9
SC1	ChemC	445.0 $\pm$ 32.5	230.1 $\pm$ 21.7	53.8
SC1	He + E	428.5 $\pm$ 12.0	229.4 $\pm$ 6.5	55.1
SC1	He + Sg	426.7 $\pm$ 30.5	239.8 $\pm$ 14.2	58.4
SG1	Control	525.5 $\pm$ 19.1	275.4 $\pm$ 10.3	53.2
SG1	E	526.0 $\pm$ 7.6	270.4 $\pm$ 4.2	53.0
SG1	Chem	524.0 $\pm$ 5.2	270.4 $\pm$ 3.4	52.1
SG1	ChemB	496.2 $\pm$ 6.5	266.1 $\pm$ 6.8	n.a
SG1	ChemC	471.0 $\pm$ 14.5	257.6 $\pm$ 8.7	54.9
SG1	He + E	534.9 $\pm$ 14.7	279.6 $\pm$ 32.2	55.5
SC2	Control	395.3 $\pm$ 3.2	214.2 $\pm$ 7.7	55.4
SC2	E	432.0 $\pm$ 43.5	230.4 $\pm$ 23.1	54.3
SC2	Chem	396.0 $\pm$ 15.6	223.8 $\pm$ 8.8	57.0
SC2	ChemA	372.7 $\pm$ 25.2	209.4 $\pm$ 13.0	56.7
SC2	ChemB	382.7 $\pm$ 5.5	215.4 $\pm$ 3.8	57.4
SC2	ChemC	362.3 $\pm$ 4.7	199.6 $\pm$ 3.8	56.0
SC2	He + E	398.5 $\pm$ 3.5	219.2 $\pm$ 2.5	56.3
SC2	He + Sg	507.3 $\pm$ 8.3	262.9 $\pm$ 7.1	53.6
SG2	Control	463.4 $\pm$ 14.2	243.8 $\pm$ 6.0	53.8
SG2	E	433.0 $\pm$ 13.7	232.1 $\pm$ 7.2	54.1
SG2	Chem	461.5 $\pm$ 13.4	243.0 $\pm$ 6.8	53.1
SG2	ChemA	421.2 $\pm$ 32.6	206.5 $\pm$ 0.7	52.2
SG2	ChemB	461.6 $\pm$ 18.9	240.1 $\pm$ 7.6	52.6
SG2	ChemC	455.3 $\pm$ 23.4	240.9 $\pm$ 12.0	54.7
SG2	He + E	472.3 $\pm$ 40.5	266.7 $\pm$ 24.0	56.9
SG2	He + Sg	451.6 $\pm$ 5.5	238.1 $\pm$ 3.0	54.7

n.a = not analyzed. E = enzymes; Chem = chemical additive; ChemA = combination of enzymes and chemical additive; ChemB = combination of LAB inoculant and chemical additive; ChemC = combination of LAB inoculant, enzymes and chemical additive; He + E = combination of LAB inoculant and enzymes; He + Sg = combination of LAB inoculant and sugar solutions.

* SD of the mean.

the fact that high moisture contents (30% TS) and lower pH values coupled with prolonged storage durations led to a solubilization and depolymerization of lignocellulosic material which had an positive effect on methane formation.

3.2.3. Effect of addition of silage additives

Methane yields were also affected by the use of silage additive, since they can alter the products of silage fermentation during ensiling (Table 3). The correlation coefficient of the fitted model to the experimental data was (R^2) = 0.9761. The particle size had a significant effect ($p < 0.05$) on the interaction term. In this term model, the effect of the particle size on the methane yields depends on the TS content. The surface plot in Fig. 4a and b showed that the methane yield

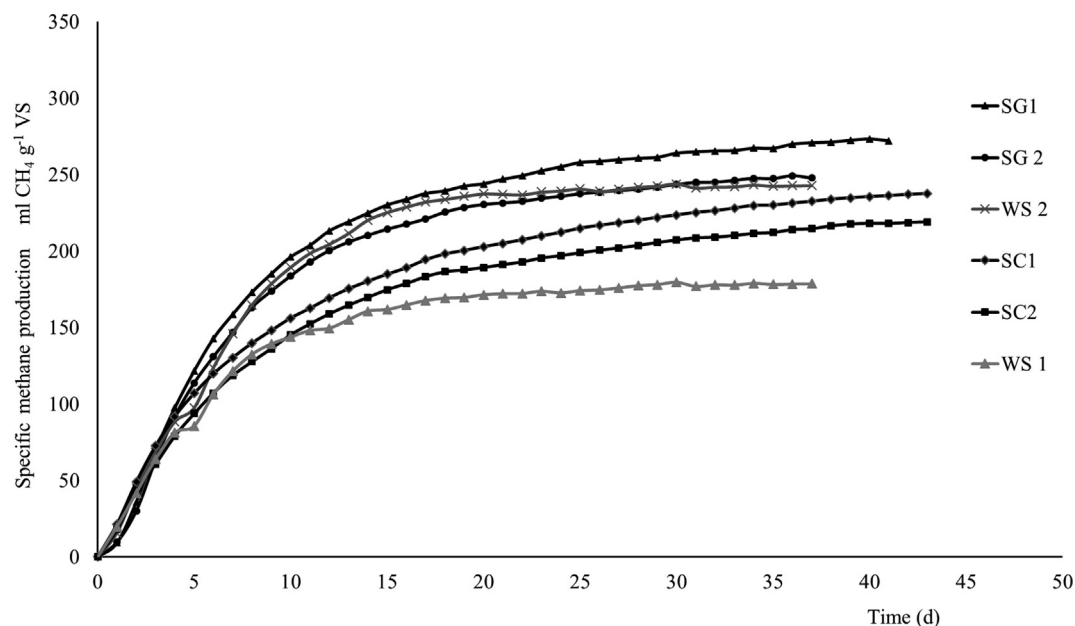


Fig. 2. Specific methane production of SC1: Chopped straw silage (30%TS), SC2: Chopped straw silage (45%TS), SG1: Ground straw silage (30%TS), SG2: Ground straw silage (45%TS), WS1: Chopped wheat straw (86%TS), WS2: Ground wheat straw (87%).

increased when the particle size and the TS content decreased simultaneously while holding all other variables at its low value (code value = 0). Similar effect was observed with reduced particle size and the variable addition of the additives Chem, ChemB, ChemC and He + E ($p < 0.05$). With the addition of He + E, the highest specific methane yield was obtained (Fig. 4c). The Pearson correlation analysis (Table 2) showed that the addition of He + E had a positive correlation ($r = 0.23$, $p < 0.01$). In contrast, when silages were treated with ChemA, the methane formation decreased until its lowest value ($198 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$) (Fig. 4d). The addition of ChemA had a significant effect ($p < 0.05$) on the linear term. Therefore a negative correlation was observed ($r = -0.46$, $p < 0.01$) (Table 2). The effect of combined biological and mechanical pretreatments resulted in higher methane yields, ranging from 198 to $280 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$. Due to an intensified mechanical pretreatment prior ensiling an increase in methane yields of 36% was obtained. In addition, silages treated with the combination of LAB inoculant and enzymes (He + E) only increased methane yields by 3% compared with the control sample SG1.

The results show that the methane yield of wheat straw were increased by ensiling fermentation without silage additives by 11–24% in comparison to the non-ensiled control samples (Table 4). The addition of silage additives led to an additional increase of the methane yield between 1 and 8%. However, silage additives were not required for

ensiling of wheat straw. The results of the study imply that silage additives do not have an economic benefit to the process, considering the comparable low effect on the methane yield, the silage product costs and the additional effort for the product application during silage preparation.

The investigation of ensiling pretreatment of lignocellulose biomass has a high innovation potential due to several reasons. It is a low input technology, leading to a better input/output relation for lignocellulose use in biogas process. The implementation barrier is low as digestion trials in practical scale can be carried out without investments into large machinery. The combination with mechanical pretreatment allows further improvement potential. Further innovative substrate options are opened, enabling the integration of seasonal mass flows with high water contents. As a future outlook, the investigations are a contribution for the creation of strategies for lignocellulose utilization in the biogas process. Such strategies will allow the utilization of one of the largest worldwide biomass flows with small competition for use. The generated digestate allows the closing of nutrient cycles and the soil-humus reproduction. Further research is necessary for optimization of the technique and for effective integrated process chains.

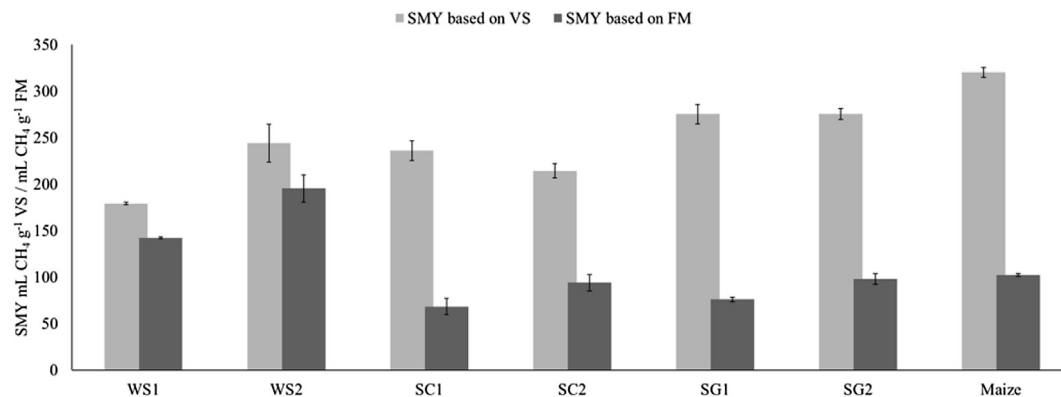


Fig. 3. Specific methane yields of cereal straw based on volatile solids and fresh matter. WS1: Chopped wheat straw (86%TS), WS2: Ground wheat straw (87%), SC1: Chopped straw silage (30%TS), SC2: Chopped straw silage (45%TS), SG1: Ground straw silage (30%TS), SG2: Ground straw silage (45%TS), Maize silage as reference.

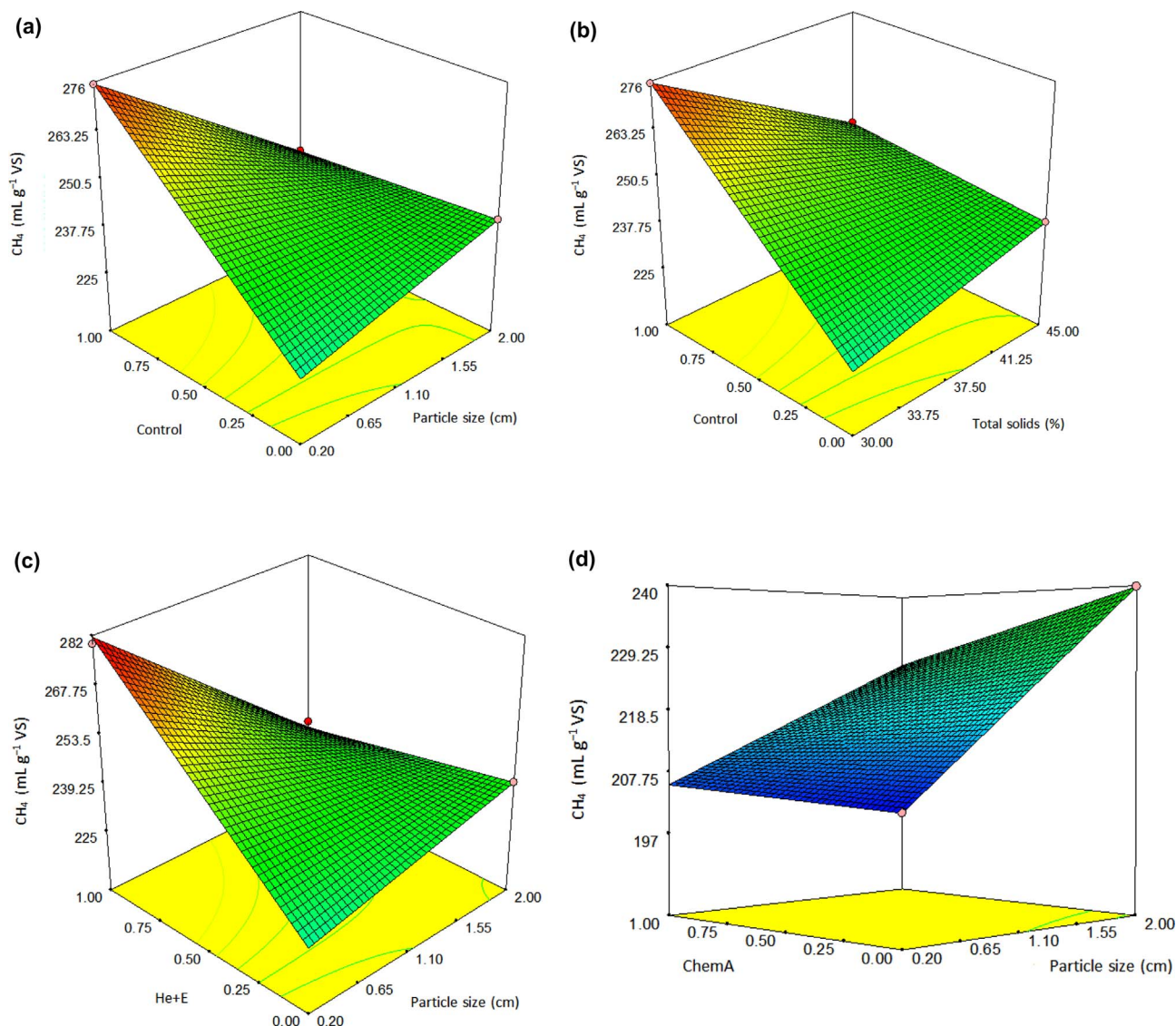


Fig. 4. Experimental (points) and predicted (mesh) methane yields as a function of particle size, total solids contents and silage additive. (a) control sample as a function of particle size, (b) control sample as a function of total solid content, (c) combination of biological additive and enzymes (He + E) and (d) combination of chemical additive and enzymes (ChemA).

4. Conclusions

Physical and biological material pretreatments have a positive effect on the substrate methane potential. Ensiling fermentation was successfully tested as a low tech approach for biological substrate pretreatment of strawlike biomass. Mechanical pretreatment of wheat straw by particle size reduction from 2.0 to 0.2 cm increased methane yields by up to 26%. The combination of grinding and ensiling of wheat straw resulted in the highest increase of the methane potential by up to 36% and the application of silage additives lead to an additional increase of 1%.

Author contributions

The manuscript was written with contributions from all of the authors. All authors have given their approval for the final version of the manuscript.

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PAPER II

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ORIGINAL ARTICLE

Open Access



Mixed silage of *Elodea* and wheat straw as a substrate for energy production in anaerobic digestion plants

Daniela Gallegos^{1,3}, Harald Wedwitschka¹, Lucie Moeller², Sören Weinrich¹, Andreas Zehnsdorf^{2*}, Michael Nelles³ and Walter Stinner¹

Abstract

Background: Waterweeds (*Elodea nuttallii* and *Elodea canadensis*) are invasive neophytes, which have been proliferating at a phenomenal rate during the last decades in German waterways. In case of overgrowth, the strong covering of vegetation can cause problems in hydroelectric power plants and leads to limitations in ship and boat traffic as well as in use for bathing and fishing activities. After vegetation period, dead plants can accumulate and then negatively influence flood protection and water engineering works. For this reason, the aquatic biomass has been periodically removed and disposed without further use. In order to enable the energetic use of this water-containing substrate, the aim of the present study was the optimization of storage methods for an aquatic plant-based feedstock for biogas production. In climatic cold regions, substrate conservation is necessary in order to guarantee a year-round substrate availability. With waterweed (*Elodea*) taken as an example, the ensiling of aquatic plants was studied. The main focus was to develop practical methods for biomass conservation while producing high biogas yields.

Methods: *Elodea* was harvested in the river Parthe in Leipzig-Schönefeld in October 2015. Silage mixtures of *Elodea* and wheat straw were tested after 180 days of storage for pH, volatile fermentation products, and methane potentials. The effect of different silage moisture contents and straw particle sizes on the substrate quality was studied.

Results: Results show that waterweeds can be stored by ensiling and can achieve considerable biogas yields. However, with a water content of about 95%, the storability of the material is challenging. Mixed silage of waterweeds and wheat straw were suitable for storage in clamp silos. The pH values were between 4.9 and 6.5, and the volatile fatty acid content as lactic acid ranged from 0.0 to 1.9% total solid. The mixed silages achieved methane potentials between 166 and 228 mL g⁻¹ volatile solid (VS), which is equivalent to 52 and 72% of maize silage. Considering a methane potential of 228 mL g⁻¹ VS and costs for material pretreatment, the most promising silage variant was mixed silage from waterweeds and ground straw with 30% total solid content.

Conclusions: Long storability of waterweeds could be possible by ensiling fermentation. Mixed silages from *Elodea* and wheat straw show suitable substrate characteristics for biogas production and can achieve high biogas yields.

Keywords: *Elodea* genus, Waterweeds, Neophyte, Aquatic biomass, Aquatic macrophytes, Biogas production

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Background

Aquatic plants play an important role in the biotope of flowing and standing waters as they are a habitat for small water animals including fries, as they excrete oxygen into the water and take up nutrients. Under good conditions, aquatic macrophytes can spread with phenomenal growth rates gaining large amounts of biomass in a short time. Strong covering of aquatic plant vegetation can lead to problems in the operational use of waterways and negatively influence flood protection and hydroelectric power generation. An overgrowth of aquatic macrophytes can prevent the use of lakes and rivers as recreation areas, in case leisure activities like bathing or fishing become severely impaired [1]. A special role is played by the invasive neophytes such as the *Elodea* genus, which has been proliferating during the last decades in German waterways. In several regions in Germany, *Elodea* needs to be removed from water bodies regularly every year. Most often, the aquatic macrophytes are mowed by use of mowing boats. The biomass obtained is thereby generally disposed without further use, and costs for water operators increase as a result [1]. Instead of an unused disposal of harvested aquatic macrophytes, they could be used as an alternative feedstock for biogas production.

Anaerobic digestion (AD) is a suitable organic waste handling technology based on the biochemical conversion of organic compounds into methane, which can be used for electrical energy and heat production. In comparison to conventional energy crops, water plants are not standing in a competition to animal feed or food. No additional agricultural areas for crop cultivation are necessary as water plants are a residue material produced during water management efforts. Several studies revealed the high biogas potential of aquatic macrophytes. Samples of *E. nuttallii* from five different lakes in Germany showed specific biogas yields of 415–520 L_{biogas} kg⁻¹ volatile solid (VS) [2], which is comparable to hay or cattle dung. However, for practical application, storage methods for aquatic plant-based feedstock need to be developed as in climatic cold regions, substrate conservation is necessary in order to guarantee a year-round substrate availability.

Storage prior to AD of these water plants can be possible by ensiling. Ensiling is a preservation method for wet biomass based on solid-state lactic acid fermentation under anaerobic conditions whereby lactic acid bacteria (LAB) convert water-soluble sugars into organic acids, mainly to lactic acid. The ensiling process involves four phases [3]. During the aerobic processes, the respiration of remaining oxygen occurs until it has been entirely consumed. When the ensiled mass has become anaerobic, the fermentation phase starts, forming lactic acid and organic acids, which accumulate and lower the pH of the silage to below 5.0. It usually lasts for several days or weeks. In well-processed silage, LAB dominate the

fermentation, rapidly producing the low pH conditions that help to preserve the silage, and thus, the growth of detrimental microorganisms, mainly, enterobacteria, clostridia, yeasts, and molds, is inhibited, representing good-quality silage. During the storage period, the silage is sealed and no air penetrates. This may last several months or until the feed-out period as long as the pH is sufficiently low and anaerobiosis preserves. The fourth phase is the unloading phase for feeding, during which silos are opened and reexposed to air. This causes the growth of undesirable aerobic microorganisms initially present in the silage, such as yeasts and molds that may spoil the silage [4].

Energy crops are usually stored in clamp silos. The storage of waterweed is challenging because the material decays rapidly when it is removed from water and gets in contact with air. With a water content of about 95%, the storage of this plant material in agricultural silos is impossible. An ensiling with crops such as corn silage would reduce the silage quality of the more expensive commodities due to inadequate total solid content, enhanced buffer capacities, etc. For this reason, mixed silage of waterweeds and wheat straw was studied as a possible solution for ensilage.

The aim of this study was to develop a method for long time conservation of aquatic plant materials with suitability for storage in conventional clamp silos. Therefore, silage mixtures of water plants and wheat straw as an available agricultural residue material were tested for silage quality and biogas potential. The results of the study can be used to determine optimum conditions for substrate ensiling of aquatic biomass for biogas production.

Methods

Raw materials

Elodea biomass, which was a mixture of *E. nuttallii* and *E. canadensis* (see also Zehnsdorf et al. [5]), was harvested as whole plant in autumn 2015 from the experimental site, the Parthe river in Leipzig, Germany (51° 21' 53.2" N, 12° 24' 51.2" E), and transported in plastic containers to the Deutsches Biomasseforschungszentrum (DBFZ, Leipzig, Germany). Processing and ensiling of *Elodea* were conducted directly after collection. *Elodea* biomass was manually washed with cold tap water to remove adherent soil and impurities. The whole *Elodea* plant was cut by hand to a particle size of approximately 3–5 cm and stored in sealed plastic barrels at 4 °C until further use.

Wheat straw (WS) was provided by the Department Thermochemical Conversion of the DBFZ. Straw samples were chopped to a particle size of 2.0 cm (Strohmühle, Hirlinger Landtechnik GmbH, Burladingen, Germany) and ground to a particle size of 0.2 cm (Bioextruder,

Lehmann-UMT GmbH, Pöhl, Germany). Samples were stored under dry conditions at room temperature (20 °C) in sealed plastic barrels until its use.

The inoculum (average chemical characteristics: pH 7.62, TS 1.62, VS 80.2%, $\text{NH}_4\text{-N}$ 1.34 g/L, organic acids 53.01 mg/L) used for biochemical methane potential (BMP) tests was acquired from the DBFZ research biogas plant, which uses maize silage and cattle manure as substrates. The digestate was sieved (5 mm), diluted with tap water, and degassed at 22 °C for 5 days to reduce specific biogas generation prior to inoculation in the BMP test.

Silage preparation

Ensiling of *Elodea* was carried out in a laboratory scale as described in Gallegos et al. [6]. Silages were prepared with *Elodea* and previously pretreated WS raw material. The moisture content of the mixed silages of *Elodea* and straw was adjusted to 30 and 45% total solid (TS), respectively. The following silages were generated: *Elodea* silage without straw (EN), *Elodea* silage containing chopped straw at a TS content of 30% (ESC1), *Elodea* silage containing chopped straw at a TS content of 45% (ESC2), *Elodea* silage containing ground straw at a TS content of 30% (ESG1), and *Elodea* silage containing ground straw at a TS content of 45% (ESG2).

Five hundred grams of each substrate was filled into vacuum sealer bags (30 × 50 cm PA/PE, La.va, Germany). After filling, the bags were vacuum-packed (Cookmax Series 42, Pentagast eG, Künzel, Germany) to remove air between substrate particles and stored under anaerobic conditions in the dark at room temperature (approx. 20 °C) for a period of 180 days. A total number of 30 silages were prepared: six replicates for *Elodea* biomass and six replicates for each of four mixtures of *Elodea* and WS.

Analytical methods and BMP tests

Total solids (TS) and volatile solids (VS) were determined according to DIN EN 12880 [7] and DIN EN 12879 [8]. The TS content was analyzed by oven-drying the samples for 24 h at 105 °C (Binder, Germany), and the VS content was determined by burning the dried samples to ashes for 30 min at 220 °C and then for 2 h at 550 °C in a muffle furnace (Carbolite, UK). The TS and VS contents of all silages were corrected for volatile organic compounds that were lost during oven-drying including lactic acid, volatile short-chain fatty acids ($\text{C}_2\text{--C}_7$), methanol, ethanol, and propanol according to Weißbach and Strubelt [9]. The method is based on empirical analyses of VS losses during oven-drying of corn silage as reference material. However, the method was applied as silage properties such as total TS, VS, and VFA concentrations of the tested *Elodea* straw and corn silage were similar and there are currently no VS correction methods developed for mixed silages of *Elodea* and straw.

Prior to chemical analyses, the fresh samples were ground to ≤ 1 mm and prepared as previously described by Dittrich-Zechendorf [10]. The approximate composition was carried out only for raw *Elodea* and WS biomass. The content of nitrogen was determined with the Kjeldahl method. Crude protein content was calculated as 6.25 multiplied by the elemental nitrogen content detected. Crude fat was determined gravimetrically after acidic hydrolysis with 3 M hydrochloric acid followed up by extraction with hexane. Crude fiber was determined by boiling the samples in H_2SO_4 and KOH followed up by drying and ashing the samples at 500 °C for 2 h. Acid detergent fiber (ADF), neutral detergent fiber (NDF), and acid detergent lignin (ADL) content were analyzed according to Weende and Van Soest methods using the FIBRE THERM® system based on the FibreBag technology (C. Gerhardt, Germany). ADL was determined gravimetrically after adding 72% sulphuric acid to the bag from ADF analysis for 3 h at a temperature of 20–23 °C and drying for 24 h at 105 °C and finally ashing the sample in a muffle furnace at 500 °C for 2 h. The methods are described in detail in Dittrich-Zechendorf [10].

For the analyses of volatile fatty acids (VFA), alcohols, and pH, 5 g of the ensiled samples were previously diluted with 100 mL of distilled water and homogenized by blending for 15 min. The pH value was measured directly using a pH electrode Sen Tix 41 (WTW, Germany) in aqueous extracts. Lactic acid (LA), volatile fatty acids, and alcohols were analyzed in the ensiled samples as described by Apelt [11]. LA, VFA, including acetic acid, propionic acid, isobutyric acid, *n*-butyric acid, isovaleric acid, *n*-valeric acid, hexanoic acid, and benzaldehyde, and alcohols, including ethanol, 2-butanol, 1-propanol, 1-butanol, furfural, and 5-methylfurfural (5-HFM), were measured using the Headspace GC system, which consisted of a 7890 series II gas chromatograph (Hewlett Packard, USA) equipped with an HS40 automatic headspace sampler (Perkin Elmer, USA) and a flame ionization detector from Agilent FID Technologies.

BMP test at laboratory scale was conducted in accordance with VDI 4630 [12] using eudiometer devices (Neubert Glas GbR, Geschwenda, Germany) to determine the specific methane yields of the ensiled samples after 180 days. The methane potential of fresh *Elodea* plant material was determined using the automated methane potential test system 2 (Bioprocess Control, Lund, Sweden). The samples consisted of 450 g of inoculum and 2.5 g of VS of the substrate, each in three replications. The inoculum to substrate ratio (ISR) was approximately 2.5:1. The pure inoculum was measured to determine its methane yield and to subtract this from the other samples. The test was operated under mesophilic conditions (38 °C). To monitor the inoculum performance, microcrystalline cellulose was used as a reference substrate.

The BMP test was ended when the daily biogas production had reduced to 1% of the total biogas production over a period of five consecutive days. The gas composition was determined with a landfill gas monitor (GA2000, Ansyco, Karlsruhe, Germany). The specific methane yields were standardized to 273.15 K and 101.325 kPa.

Kinetic modeling of the BMP tests

Referring to the different model derivations presented by Brule et al. [13], an exponential two-pool model (model C) was used to evaluate the methane production kinetics of the discontinuous laboratory experiments. This model structure differentiates between a rapidly and slowly degradable fraction (two-pool) of the available substrate. Therefore, four model parameters need to be adjusted to depict the respective experimental results in detail: the total methane potential S (mL g^{-1} VS), the ratio of rapidly degradable substrate to total degradable substrate α (–), and the two first-order reaction constants for the degradation of rapidly degradable substrate k_F (d^{-1}) and slowly degradable substrate k_L (d^{-1}) components. Additionally, the coefficient of determination R^2 (–) was calculated to assess the kinetic modeling results of all individual samples.

The model implementation as well as the numeric parameter estimation is realized in the software environment Matlab as described by Brule et al. [13]. Furthermore, the implementation has been extended to include realistic constraints for all model parameters. Thus, the kinetic constants and biogas potential (k and S) can only accept positive values, whereas the ratio between rapidly and slowly degradable substrate components (α) varies between 0 and 1.

Statistical analyses

All data were analyzed with SAS v 10.0 software (SAS Institute INC., Cary, NC, USA), using a type I error rate (α) of 0.05 by analysis of variance, to determine if there were significant differences between treatments, and if differences existed, post hoc least significant difference (LSD) tests were used to determine where they occurred. The effects of different substrate, straw particle size, moisture content and particle size $\times$ straw biomass interaction on chemical parameters, silage fermentation products, and methane yields were conducted to test for linear relationship using fraction of variance explained by the regression line (r^2) of the SAS statistical package.

Results and discussion

Chemical characteristics of raw materials

Chemical composition of freshly harvested *Elodea* and WS is presented in Table 1. Whole plant *Elodea* biomass showed a high water content, which is typical of aquatic plants [14]. The lignin, cellulose, and hemicellulose content of *Elodea* were found within the range reported for

Table 1 Chemical composition for raw materials

Parameter	WS	<i>Elodea</i>	Units
Total solids (TS)	86.5	6.76	%FM
Volatile solids (VS)	95.1	59.75	%TS
Nitrogen (N)	5.9	37.9	g kg^{-1} TS
Ash	67.7	402.5	g kg^{-1} TS
Protein (N $\times$ 6.25)	36.8	236.9	g kg^{-1} TS
Crude fiber	684.6	245.6	g kg^{-1} TS
Crude fat	10.1	3.2	g kg^{-1} TS
Lignin	121.3	21.56	g kg^{-1} TS
Cellulose	494.0	175.1	g kg^{-1} TS
Hemicellulose	283.0	46.6	g kg^{-1} TS
C:N ratio	95.4 ^a	10.0 ^b	–

FM fresh matter

^aData from M. Solé-Bundó et al. [22]

^bData from Zehnsdorf et al. [5]

fast-growing species [15]. Furthermore, *Elodea* biomass was also characterized by a high ash and protein content compared to wheat straw [5, 16]. WS was characterized by high TS and fiber contents as typically found for lingocellulosic biomasses [17, 18].

Silage characteristics

Silage fermentation of elodea silages without and with the addition of WS

The results of fermentation characteristics in *Elodea* silages without and with the addition of WS are presented in Table 2. The addition of WS, reduced particle

Table 2 Chemical characteristics, pH, and products of silage fermentation of *Elodea* silages without and with addition of WS

Silage	TS (%)	VS (% TS)	LA (%TS)	AA ¹ (%TS)	BA ² (%TS)	pH
EN	6.7 $\pm$ 0.1	63.5 $\pm$ 1.0	0.0 ^b	7.5 ^a	2.1 ^a	6.5 ^a
ESC1	25.9 $\pm$ 0.9	90.0 $\pm$ 0.5	0.5 ^{a, b}	4.4 ^b	0.0 ^b	5.4 ^c
ESC2	49.9 $\pm$ 0.2	90.7 $\pm$ 0.4	1.1 ^{a, b}	1.5 ^c	0.0 ^b	5.8 ^b
ESG1	25.7 $\pm$ 0.3	81.4 $\pm$ 0.9	0.0 ^b	3.0 ^{b, c}	0.0 ^b	5.6 ^b
ESG2	56.7 $\pm$ 0.8	92.0 $\pm$ 0.6	1.9 ^a	1.8 ^c	0.0 ^b	4.9 ^d
S.E. ($n = 3$)			1.04	2.54	1.16	0.55
Pz			*	n.s.	n.s.	**
Pz $\times$ Sb			*	n.s.	n.s.	**
Moisture content			***	n.s.	n.s.	**
EN biomass			n.s.	***	**	***
WS			*	n.s.	n.s.	**

Different superscript letters in the same column indicate significant difference at $p < 0.05$

TS total solid, VS volatile solid, LA lactic acid, Pz straw particle size effect, Pz $\times$ Sb interaction particle size $\times$ substrate, EN *E. nuttallii* biomass effect, WS wheat straw biomass effect, n.s. not significant

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

¹AA acetic acid—sum of acetic acid and propionic acid

²BA butyric acid—sum of isobutyric, *n*-butyric, isovaleric, *n*-valeric, and *n*-caproic acid

size and high TS content correlated positively with LA content ($r^2 = 0.58$, 0.58 , and 0.61 , respectively), whereas EN biomass was positively correlated with butyric acid content ($r^2 = 0.74$).

The LA content of all silages varied between 0.0 and 1.9% TS and increased by addition of WS ($p < 0.05$) and with decreasing the size of straw particles ($p < 0.05$) with significant interaction between these factors ($p < 0.05$). This difference was probably due to the fact that grinding is more effective at reducing the particle size than chopping. This reduction of particle size increases the available specific surface area. Therefore, hitherto inaccessible areas are exposed for enzymatic attack, converting polymers into monomeric sugars for a faster and easier LA fermentation [19]. Moisture content also had a significant effect on LA content ($p < 0.001$). Higher LA was measured in silages composed of straw at low moisture content, while no LA concentration was detected in *Elodea* silage. This might be explained by an increase in butyric acid during storage due to metabolic activity of clostridia. Since 1 M of butyrate is produced from 2 M of lactate, it is thus assumed that *Elodea* silage underwent butyric acid fermentation, consuming LA completely during the storage period. It was clearly observed that these differences between silages are mainly attributed to the water content of *Elodea* biomass as compared with straw biomass. This does not only affect the chemical composition of the silage, but also the activity of the microorganisms involved in the conservation process. These results agree with findings in herbage silages [20]. The authors described the lactic and butyric acid content as a function of TS content. In a range of about 28–50% TS, a continuing increase of LA occurs; however, above 50% TS content might depress lactic acid bacteria (LAB). The pH value was significantly affected by EN biomass ($p < 0.001$). Higher pH was determined in *Elodea* silage than in *Elodea* silages with straw. This might be due to higher butyric acid content in this silage which resulted in a rise in the pH. In addition, this increase in pH might have also been caused by the relatively high buffering capacity of the raw *Elodea* biomass related to high ash contents. In comparison to straw, *Elodea* biomass has much higher water, protein, and ash contents (Table 1). The water dilutes VFA and osmotic active substances and the proteins as well as the cations from the ash, which buffers the silage acids. In silages containing straw, the heterofermentative LAB might have dominated the ensilage process. This can be explained by the different LAB, which are classified as homo- and heterofermentative LAB based on their by-products of sugar fermentation. Homofermenters convert hexoses almost exclusively into LA, while heterofermenters also convert hexoses homofermentatively into LA, but they are able to ferment

pentoses into equimolar amounts of lactic and acetic acid via a phosphoketolase [21]. This may explain the high pH value in ESC1, ESC2, and ESG1 silages. With the pK_a defined as the acid dissociation constant, measures the strength of an acid, the lower or more negative the number, the stronger and more dissociable the acid. The pK_a of the analyzed organic acids ranked in the following order: $3.86 > 4.75 > 4.82$ (lactic, acetic, and butyric acid, respectively). Results also showed that the pattern of silage fermentation was greatly influenced by the chemical and microbiological characteristics of the ensiled materials present before ensiling. High moisture content suggests that proper preservation of *Elodea* biomass by ensiling is challenging. Ensiling of *Elodea* showed that its water content can reactivate undesirable microorganisms, resulting in high pH values. The pH value of *Elodea* silage observed in this study was not different from that reported by Zehnsdorf et al. [14], who studied the ensiling of *E. nuttallii* in combination with corn. The authors assumed that the *Elodea* genus is not suitable for direct ensiling, unless additional material with suitable total solids is added, which agrees with the results of the present study.

Effects of ensiling on methane yield

Table 3 shows the methane formation of *Elodea* and *Elodea*-straw mixture silages. The results indicated that the straw particle size significantly affects the methane yield ($p < 0.001$), whereas the different substrates used for the ensiling experiment showed no consistent trend.

Differences in methane yields were detected between ESG2 and ESC2 ($p < 0.05$). It was noted that the specific methane yield of the *Elodea* silages containing straw tended to increase for the silages composed of ground straw and to decrease for the silages composed of chopped straw; therefore, methane yield exhibited a strong correlation related to the grinding treatment ($p < 0.01$) applied to straw before ensiling. This may suggest that changes in methane yield after grinding the substrate prior to ensiling might be attributed to the digestibility of lignocellulosic biomass. An enhancing effect of grinding treatment on methane yields as compared with methane yields of chopped biomass is in accordance with the findings in literature for straw silages [6]. In addition, the authors characterized wheat straw as a comparatively cheap agricultural residue with a very low water content that can be used for improving material characteristics of very wet substrates prior ensiling. Generally, findings of enhanced methane production by mechanical treatment are in line with the outcome of previous studies [17]. The accumulative methane production shown in Fig. 1 implied that the highest methane yield was derived from fresh *Elodea* silage without straw and showed a 6% decreased methane yield. However, the ensiling of fresh *Elodea* leads to a

Table 3 Methane production from *Elodea* and *Elodea*-straw mixture silages after 180 days of storage

Silage	Specific methane yield (mL g ⁻¹ VS)
EN without ensiling	242.2 ± 36.4 ^a
EN	218.6 ± 12.5 ^a
ESC1	205.7 ± 12.5 ^{a, b}
ESC2	166.1 ± 13.1 ^b
ESG1	228.3 ± 27.6 ^a
ESG2	224.5 ± 9.8 ^a
Pz	***
Pz × Sb	**
EN biomass	n.s.
WS	n.s.
SC1 ¹	235.9 ± 10.6
SC2 ²	214.2 ± 7.7
SG1 ³	275.4 ± 10.3
SG2 ⁴	243.8 ± 6.0

Different superscript letters in the same column indicate significant difference at $p < 0.05$

Pz straw particle size effect, Pz × Sb interaction particle size × substrate, EN *E. nuttallii* biomass effect, WS wheat straw biomass effect, n.s. not significant

¹SC1 chopped straw at 30% TS

²SC2 chopped straw at 45% TS

³SG1 ground straw at 30% TS

⁴SG2 ground straw at 45% TS

^{1, 2, 3, 4}Data from Gallegos et al. [6]

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

liquid silage material which was storable under laboratory conditions for 180 days without major energy losses. *Elodea* silage containing ground straw showed comparable methane yields, while *Elodea* silage containing chopped straw at 30% TS achieved a 10% decreased methane yield.

These results coincide with specific methane yields observed for *E. nuttallii* reported by Escobar et al. [2].

The authors reported that variability in methane yields among *E. nuttallii* biomasses from five different lakes is dependent on the site of biomass origin. Other authors described that these variations originate from differences in chemical composition due to different locations and growing conditions [14]. This aquatic plant provides nutrients and trace elements and can thus be beneficial for the substrate composition [5, 14]. Estimated kinetic parameters of the two-pool model are presented in Table 4. All model configurations can depict the experimental progression of the specific methane yield (Fig. 1) of individual silage compositions in great detail ($R^2 > 0.94$). Furthermore, identical reaction kinetics for ESC2 ($k_F = k_L \approx 0.151 \text{ d}^{-1}$) and ESG1 ($k_F = k_L \approx 0.109 \text{ d}^{-1}$) reveal that the cumulative methane progression of the respective samples can also be approximated with equal accuracy by single first-order reaction kinetics (according to model A in Brule et al. [13]). The estimated total methane potential (S or S*) mainly confirmed the positive effect of adding wheat straw to *Elodea* prior ensiling. Furthermore, the experimental data as well as the model revealed that silages containing high moisture content showed higher methane potential in comparison to silages containing low moisture content. This difference could be related to the fact that high moisture contents (30% TS) and lower pH values coupled with prolonged storage durations during the ensiling process led to a solubilization and depolymerization of lignocellulosic material, which had a positive effect on methane formation. Based on fixed first-order reaction constants, the EN sample was able to accelerate the digestion process, since the ratio between rapidly and slowly degradable substrate components was highest with $\alpha^* = 0.97$, while silages composed of straw showed much lower ratios. In general, the addition of pretreated straw either by chopping or grinding at different moisture contents was not able to

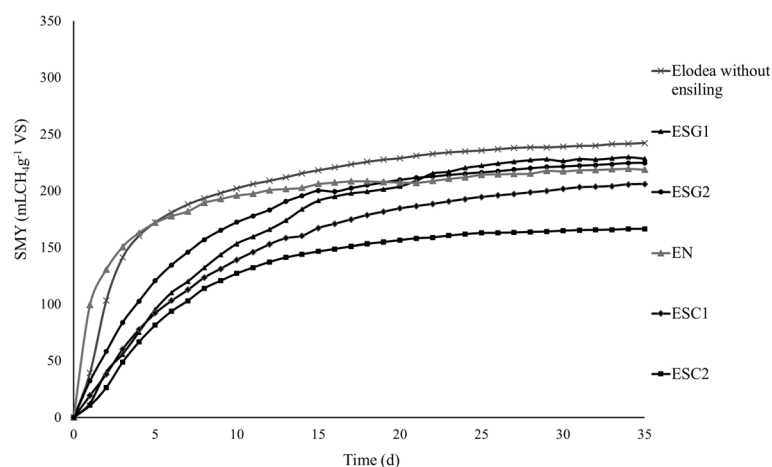


Fig. 1 Cumulative specific methane yield (SMY) of *Elodea* without ensiling; *Elodea* silage without straw (EN), *Elodea* silage containing chopped straw at 30% TS (ESC1), *Elodea* silage containing chopped straw at 45% TS (ESC2), *Elodea* silage containing ground straw at 30% TS (ESG1), and *Elodea* silage containing ground straw at 45% TS (ESG2)

Table 4 Estimated model parameters of the utilized two-pool kinetic model (according to model C in Brule et al. [13])

	Data (mL g ⁻¹ VS)	S (mL g ⁻¹ VS)	α (—)	k_F (d ⁻¹)	k_L (d ⁻¹)	R^2 (—)	S^a (mL g ⁻¹ VS)	α^a (—)	R^{2a} (—)
ESC1	206	260	0.70	0.1263	0.0120	1.00	220	0.19	1.00
ESC2	166	161	0.50	0.1508	0.1506	0.99	180	0.30	0.98
ESG1	228	231	0.46	0.1095	0.1094	1.00	252	0.12	0.99
ESG2	224	275	0.73	0.1707	0.0113	1.00	238	0.40	1.00
EN	219	218	0.59	1.0899	0.1315	1.00	213	0.97	0.94

^aOptimized values (curve fitting) for fixed parameter values of $k_F = 0.3393$ (d⁻¹) and $k_L = 0.0780$ (d⁻¹), respectively. Fixed first-order reaction constants were calculated as the mean value of the individual kinetic parameters (k_F and k_L) over all experiments
Data final experimental value of the discontinuous digestion test (according to VDI 4630 [12]), S estimated total methane potential (at infinite retention time), α ratio of rapidly degradable substrate to total degradable substrate, k_F first-order reaction constant for rapidly degradable substrate components, k_L first-order reaction constant for slowly degradable substrate components, R^2 coefficient of determination

improve the degradation rate for rapidly degradable substrate components (k_F), which might be attributed to the recalcitrance of lignocellulosic biomass to enzymatic hydrolysis, since wheat straw is a highly fibrous substrate [6].

The results of the study showed that laboratory scale produced silage from *Elodea* and straw showed suitable material characteristics for silage storage in conventional agricultural driving silos or also called bunker silos. The results indicate that straw pretreatment had an effect on the methane potential of the mixed silage. However, further economic assessments are necessary to estimate the economic feasibility of straw pretreatment, considering the high energy effort for straw grinding.

Conclusions

Long storability of waterweeds can be achieved by ensiling fermentation. Ensiling has the additional effect to enhance digestibility and biogas yields on lignocellulose substrates. To adapt the characteristics, a mixture of aquatic plants with straw prior ensiling is an adequate and practicable measure. Mixed silages from *Elodea* and wheat straw show suitable substrate characteristics for ensiling and biogas production and can achieve high biogas yields. Although the experiments have only been done at a laboratory scale, for practical applications, the mixture of waterweeds with straw to a TS of approx. 30% can be recommended.

On the experimental level with only small ensiling bags, the stability of the silages under practical conditions could not be investigated. The lower risk of air integration and the higher amount of acetic acid leads to the recommendation for TS of around 30%, but as the silage with lower moisture showed lower pH probably due to reduced dilution, this should be investigated under practical conditions for future optimization.

Due to capacity restrictions, the experiments were only done with wheat straw. In principle, mixtures of waterweeds with different more dry lignocellulosic substrates are possible. Typical harvest times for aquatic weeds are in summer time for recreation water bodies and in autumn for rivers (protection of water constructions against aggradations). For

this reason, different mass flow combinations (e.g. in autumn, a combination with maize straw) and efficient process chains need to be investigated.

Additional file

Additional file 1: Supplementary information and raw data. (PDF 520kb)

Abbreviations

AA: Acetic acid; AD: Anaerobic digestion; ADF: Acid detergent fiber; ADL: Acid detergent lignin content; BA: Butyric acid; BMP: Biochemical methane potential; C:N: Carbon-nitrogen ratio; EN: *Elodea* biomass; ESC1: *Elodea* silage containing chopped straw at a TS content of 30%; ESC2: *Elodea* silage containing chopped straw at a TS content of 45%; ESG1: *Elodea* silage containing ground straw at a TS content of 30%; ESG2: *Elodea* silage containing ground straw at a TS content of 45%; FM: Fresh matter; k_F : First-order reaction constant for the degradation of rapidly degradable substrate [d⁻¹]; k_L : First-order reaction constant for the degradation of slowly degradable substrate [d⁻¹]; LA: Lactic acid; NDF: Neutral detergent fiber; R^2 : Coefficient of determination; S: Estimated total methane potential [mL g⁻¹ VS]; S^* : Optimized total methane potential [mL g⁻¹ VS]; SMY: Specific methane yield [mL g⁻¹ VS]; TS: Total solids [%FM or g gFM⁻¹]; VFA: Volatile fatty acids; VS: Volatile solids [%TS or g gTS⁻¹]; WS: Wheat straw; α : Ratio of rapidly degradable substrate to total degradable substrate

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Availability of data and materials

The datasets supporting the conclusions of this article are given in Additional file 1.

Authors' contributions

DG performed the study for the article, collected and sorted the publications and the information material, applied the dataset for the calculations, evaluated the statistical analyses, and prepared the manuscript. HW was responsible for the batch tests and ensiling experiments and collected and analyzed the samples. SW carried out the assessment of the biomethane potential test kinetics. AZ, LM, and MN contributed to the consultations and critical reading of the manuscript. WS had the idea for the study and supervised it. All authors edited and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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PAPER III

Daniela Gallegos, Jesus Jurado-Molina, Harald Wedwitschka, Efren Delgado, Michael Nelles, Walter Stinner (2023): Ensiling of Invasive *Elodea* sp., a Novel Aquatic Biomass Feedstock for the Sustainable Biogas Production: Effects of Wheat Straw and Silage Additives on Silage Quality and Methane Production. ACS Agric. Sci. Technol 3, 6, 477–486.

Ensiling of Invasive *Elodea* sp., a Novel Aquatic Biomass Feedstock for the Sustainable Biogas Production: Effects of Wheat Straw and Silage Additives on Silage Quality and Methane Production

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Supporting Information

ABSTRACT: This study investigates for the first time the invasive *Elodea* sp. as a novel aquatic biomass feedstock for the production of renewable energy in the form of methane (CH₄) via anaerobic digestion (AD). For a commercial AD, to overcome the issue of biomass seasonality and the fact that aquatic biomass exhibits high moistures that complicate storage and hence biomass and CH₄ conservation, we propose the ensiling of *Elodea* biomass as an effective preservation method that provides a consistent, reliable, and predictable supply. The effects of inclusion of wheat straw to ensiled *Elodea* (*Elodea* and mixture silages) and silage additives (added alone or in combination) on silage quality and CH₄ production were evaluated. Results revealed that the inclusion of wheat straw played a key role in the *Elodea* biomass and CH₄ preservation and was responsible for any apparent effects of silage additives. Specifically, increased silage quality as evidenced by higher lactic acid (LA) and lower pH correlated strongly with inclusion of wheat straw. In addition, wheat straw impacted CH₄ production through the preservation of *Elodea* biomass. Toward the utilization of this invasive aquatic biomass, ensiling *Elodea* biomass only with wheat straw suggests a significant alternative and is perhaps crucial for its efficient preservation and CH₄ production.

KEYWORDS: *Elodea* sp., aquatic biomass, ensiling, silage additives, methane production

INTRODUCTION

Invasive aquatic macrophytes such as *Elodea* sp. (particularly, *E. nuttallii* and *E. canadensis*) are fast-growing and more opportunistic organisms that can be an ecological and economic problem if not well managed. From their introduction onward, these macrophytes proliferated dramatically, as a response, presumably, to eutrophication resulted from excessive nutrient inputs due to intensified anthropogenic activities and climate change.¹ These plants have great benefits to freshwater systems as food resources and habitats and water purifiers converting CO₂ to O₂ during photosynthesis, besides removing nitrogen in eutrophic waters (one of the primary pollutants leading to eutrophication). However, they are currently problematic aquatic weeds posing potential threats to water bodies.^{1,2} Apparently, *Elodea* sp., when overgrown, become invasive and dominant, rapidly expanding and forming large mats that limit the use of waters and outcompete native species. The problem of these large mats is that they often remain in the waters and disintegrate, leading to a secondary eutrophication and increased deoxygenation by degradation processes. This results in decreased water quality and changes in the aquatic community structure.

Despite existing efforts to manage and reduce *Elodea* sp., utilization of the plant biomass is still lacking as current management of aquatic weeds does not consider further utilization concepts.

Elodea sp. might have, on the other hand, a remarkable biorefinery potential. In this context, *Elodea* biomass can be a

natural and potential feedstock resource for providing renewable energy, and anaerobic digestion (AD) presents a particularly promising approach to such biomass to generate biogas.³ In addition, AD is considered an efficient waste management practice applied to a wide variety of biodegradable materials.⁴ Biogas consists mostly of CH₄ and CO₂, and CH₄ can be integrated into the energy sector with possible uses such as heating and energy generation. However, due to *Elodea* biomass seasonality and its characteristics, availability throughout the year is one of the obvious challenges of CH₄ production from commercial AD. Therefore, an effective preservation method for biomass such as ensiling that has included the ensilage of energy crops and recently *Elodea* biomass might be one way to overcome this.^{5–7}

In the particular case of *Elodea* biomass, one important aspect to be evaluated is its high moisture content (~95% fresh matter; FM) that reflects extremely low dry matter (DM) contents. So that, the use of a dry material like wheat straw (lignocellulosic biomass) to absorb excess moistures or with suitable DM like corn to make silages from *Elodea* biomass possible has been assessed to be positively indicated.

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The mechanisms by which *Elodea* biomass can be well preserved through ensiling are of increasing interest for a sustainable production of CH₄, and little work has been done on *Elodea* sp. Ensiling is used as a preservation method based on the anaerobic fermentation of digestible water-soluble carbohydrates (WSCs) by lactic acid bacteria (LAB) with the formation of mainly LA, the primary acid responsible for decreasing the pH in silages.⁸ In this line, pH of silages seems to be of particular importance when assessing silage quality, since lower pH (<4) restrains the growth of undesirable microorganisms such as clostridia or enterobacteria, thus conserving the energy and nutrient content as well as quality of biomass maximally, while keeping it available throughout the year.

We previously analyzed *Elodea* biomass and found that although it is possible to preserve it in the form of silage with the inclusion of wheat straw, this aquatic biomass is still a poorly ensilable material that results in high pH values (~6). Therefore, we also propose the use of silage additives as an attractive alternative due to their different modes of action known to convert poor quality materials to high-quality silages.⁹

Limited information is available regarding preservation of aquatic biomass based on ensiling fermentation with addition of a dry material and/or silage additives and its effects on CH₄ production. In the present study, we hypothesized that not only the inclusion of wheat straw but also addition of additives to the ensiled *Elodea* biomass can (i) promote favorable conditions to accelerate the growth of LAB and thus produce greater LA, resulting in a rapid pH drop, (ii) produce high-quality silages to be used in commercial AD, and (iii) preserve its CH₄ production potential. In general, exploring and developing advanced biorefinery processes is of great significance, if waste biomasses specifically those originating from aggressively spreading aquatic plants can be used as continually renewable sources. As far as we know, this is the first study that tackles the potential uses of these aquatic plants/biomass providing a valuable scientific basis for improving their preservation via ensiling with wheat straw and the use of silage additives.

MATERIALS AND METHODS

Preparation of *Elodea* and Wheat Straw Raw Materials.

Elodea biomass was collected at the Parthe river in Leipzig, Germany (51° 21' 53.2" N, 12° 24' 51.2" E) during the autumn of 2015. The whole plant was manually washed and cut into approximately 3–5 cm length particles immediately after arrival at the laboratory. Wheat straw was chopped and ground into 2.0 and 0.2 cm of particle size (PZ), respectively. Both materials were placed in sealed plastic barrels and stored at 4 °C for later use. Chemical composition of fresh *Elodea* and wheat straw is reported in Table 1.

Silage Production. Experimental silages were prepared without or with wheat straw and divided into two main silage groups: the *Elodea* silages, which were produced only with *Elodea*, and the *Elodea*-wheat straw silages (mixture silages), which were prepared by using *Elodea* and the chopped and ground wheat straw. Within the mixture silages, four silage groups resulted; EStc30, EStc45, EStg30, and EStg45, where the DM content of all mixture silages was adjusted to 30 and 45% using the water containing *Elodea* biomass as described in Table 2.

Three commercial silage additives (Schaumann Forschung GmbH, Pinneberg, Germany) used for treating the experimental silages included:

Table 1. Chemical Composition of Fresh *Elodea* and Wheat Straw

parameter	wheat straw	<i>Elodea</i> biomass	
dry matter (DM)	86.5	6.76	%FM ^a
organic dry matter (ODM)	95.1	59.75	%DM
nitrogen (N)	5.9	37.9	g/kg DM
ash	67.7	402.5	g/kg DM
protein (N × 6.25)	36.8	236.9	g/kg DM
crude fiber	684.6	245.6	g/kg DM
crude fat	10.1	3.2	g/kg DM
lignin	121.3	21.56	g/kg DM
cellulose	494.0	175.1	g/kg DM
hemicellulose	283.0	46.6	g/kg DM

^aFM, fresh matter.

(i) chemical additive containing formic acid and added at concentrations of 8 μL/kg FM was used as a fermentation inhibitor to reduce pH and restrict microbial growth,

(ii) enzymes containing a mixture of cellulase and hemicellulase and added at concentrations of 200 μL/kg FM were used as fermentation stimulants to increase the accessibility of easily fermentable sugars from cellulose and hemicellulose, and

(iii) inoculant containing *Lactobacillus plantarum* was used as a fermentation stimulant to promote a rapid fermentation producing mainly LA and bringing the pH down rapidly.

Additionally, a solution of sugars (Sg) at 10% on a DM basis was used as a source of fermentable carbohydrates. The three different additives as well as the sugar solution were added alone or in combination to all the experimental silages as shown in Table 2.

Additives were diluted using tap water at concentrations recommended by the producer, and a volume of 10 mL of liquid per kg of fresh matter was sprayed uniformly on fresh materials. After thorough mixing, three replicates of approximately 500 g portions of *Elodea* and also the mixture *Elodea*-wheat straw plus additives were packed manually into vacuum-sealable bags (30 × 50 cm PA/PE, La.v.a, Germany), then sealed quickly using a vacuum sealer (Cookmax Serie 42, Netherlands), and stored in a dark room at ambient temperature for 180 days. The experimental unit for all silages was represented by the mini silo (bags). Accordingly, a total of 111 mini silos were obtained, where each mini silo was treated without additive (WA).

Chemical Composition and Fermentation Quality. DM and organic dry matter (ODM) content were measured for fresh *Elodea* and wheat straw as well as for the *Elodea* and mixture silages following the German standards,^{10,11} respectively. Correction of DM and ODM for loss in volatile components was carried out as determined previously.¹²

After completion of the 180-day ensiling period, mini-silos of each treatment were opened for analyzing fermentation quality. A 25 g FM sample from each mini silo was eluted in 250 mL of distilled water in a shaker at 110 rpm and 20 °C for 24 h, and then filtered through a sieve (mesh size of 1 mm). The pH was immediately measured using a Sen Tix 41 pH electrode (WTW, Weilheim, Germany). 3 mL of the filtrate were put into a Headspace vial, prepared, and analyzed for fermentation products: LA, acetic acid (AA), butyric acid (BA), and alcohols (ALC) as described previously.¹³

Biochemical Methane Potential (BMP) Tests. Opened mini silos were analyzed for CH₄ production following the BMP tests conducted at lab-scale using eudiometer devices (Neubert Glas GbR, Geschwenda, Germany) following the German guideline.¹⁴ Each AD reactor contained 450 g of inoculum and approximately 2.5 g of the substrate (on ODM basis), and was kept in a water bath at mesophilic conditions (37 ± 2 °C) for 35 days. The inoculum to substrate ratio was approximately 2.3:1 (on ODM). Before the batch experiments, the AD reactors' headspace was flushed with nitrogen gas for about 2 min to assure anaerobic conditions. The headspace correction was carried out as described in the method description.¹⁴ All reactors were

Table 2. Experimental Silages at Different Particle Size (PZ), Dry Matter (DM), and with and without Inclusion of Wheat Straw and Silage Additives

silages	PZ (cm)		DM (%FM)		Ez	silage additives ^b							total
	0.2	2.0	30	45		WA	Ch	Ch + Ez	Ch + He	He + Ez	Ch + He + Ez	He + Sg	
Elodea	- ^a	-	-	-	3	0	3	0	0	3	3	3	15
EStc30		x	x		3	3	3	3	3	3	3	3	24
EStc45		x		x	3	3	3	3	3	3	3	3	24
EStg30	x		x		3	3	3	3	3	3	3	3	24
EStg45	x			x	3	3	3	3	3	3	3	3	24

^a“-” not applied. ^bWithout additive (WA), enzymes (Ez), chemical additive (Ch), chemical additive combined with enzymes (Ch + Ez), chemical additive combined with inoculant (Ch + He), chemical additive combined with inoculant and enzymes (Ch + He + Ez), inoculant combined with enzymes (He + Ez), and inoculant combined with sugar solution (He + Sg).

Table 3. Mean of Fermentation Products and pH of the *Elodea* and Mixture Silages with or without Silages Additives at Different Particle Sizes (PZ) and Dry Matter (DM) Contents

item ^a	treatment	silage				
		<i>Elodea</i>	EStc30	EStg30	EStc45	EStg45
LA	WA	0.00 ± 0.00	0.53 ± 0.02	0.00 ± 0.00	1.15 ± 0.89	1.89 ± 1.47
	Ez		2.46 ± 0.06	1.16 ± 1.01	0.00 ± 0.00	0.95 ± 0.82
	Ch	2.21 ± 0.13	3.12 ± 0.03	3.57 ± 0.17	2.21 ± 0.06	1.66 ± 0.05
	Ch + Ez		3.61 ± 0.04	2.87 ± 0.19	1.56 ± 0.04	2.68 ± 0.05
	Ch + He		7.14 ± 0.23	0.15 ± 0.01	2.15 ± 1.86	2.39 ± 2.07
	Ch + He + Ez	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.48 ± 0.05
	He + Ez	0.00 ± 0.00	4.93 ± 0.06	0.00 ± 0.00	4.41 ± 0.03	6.56 ± 0.15
	He + Sg	0.00 ± 0.00	0.00 ± 0.00	4.67 ± 0.26	0.00 ± 0.00	1.80 ± 0.06
AA	WA	7.53 ± 0.32	4.40 ± 0.17	4.49 ± 0.06	1.47 ± 1.14	1.75 ± 0.02
	Ez		2.93 ± 0.06	0.11 ± 0.09	0.23 ± 0.20	1.59 ± 1.38
	Ch	1.64 ± 0.02	1.05 ± 0.01	0.93 ± 0.04	0.91 ± 0.04	0.88 ± 0.77
	Ch + Ez		1.99 ± 0.03	0.00 ± 0.00	2.25 ± 0.00	0.98 ± 0.03
	Ch + He		3.28 ± 0.08	2.24 ± 0.02	0.99 ± 0.86	1.51 ± 0.04
	Ch + He + Ez		4.82 ± 0.03	4.36 ± 0.14	3.53 ± 0.10	1.13 ± 0.98
	He + Ez	13.49 ± 1.42	0.75 ± 0.01	5.37 ± 0.10	1.42 ± 0.01	3.56 ± 0.06
	He + Sg	15.15 ± 1.59	4.44 ± 0.18	3.38 ± 0.11	0.80 ± 0.69	1.81 ± 0.05
BA	WA	2.09 ± 2.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Ez		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Ch	4.77 ± 3.75	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Ch + Ez		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Ch + He		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Ch + He+Ez	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	He + Ez	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	He + Sg	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
pH	WA	6.45 ± 0.13	5.38 ± 0.10	5.63 ± 0.10	5.83 ± 0.10	4.87 ± 0.09
	Ez		5.24 ± 0.10	5.44 ± 0.11	4.81 ± 0.10	4.74 ± 0.09
	Ch	5.39 ± 0.11	n.a	4.96 ± 0.10	5.71 ± 0.11	4.95 ± 0.10
	Ch + Ez		4.71 ± 0.09	4.53 ± 0.09	4.75 ± 0.10	4.93 ± 0.10
	Ch + He		4.70 ± 0.09	5.56 ± 0.11	5.43 ± 0.11	4.77 ± 0.10
	Ch + He+Ez	6.44 ± 0.13	4.50 ± 0.09	5.17 ± 0.10	4.81 ± 0.10	4.96 ± 0.10
	He+Ez	6.67 ± 0.13	5.61 ± 0.11	5.53 ± 0.11	5.76 ± 0.12	4.92 ± 0.10
	He+Sg	5.61 ± 0.11	5.02 ± 0.10	4.64 ± 0.09	4.51 ± 0.09	4.40 ± 0.09

^aLA, lactic acid (% DM); AA, acetic acid (% DM); BA, butyric acid (% DM) and pH. WA, without additive. ± represents standard deviation. n.a, not analyzed. For more abbreviations see Table 2.

stirred manually once a day during anaerobic digestion and analyzed in triplicate. CH₄ production was standardized to 273.15 K and 101.32 kPa. CH₄ concentration was measured using a landfill gas monitor (GA2000, Ansyco, Karlsruhe, Germany). The inoculum was collected from a full-scale mesophilic anaerobic biogas plant at the DBFZ. The substrates utilized onsite were corn silage and cattle manure. Digestate derived from the primary digester was sieved (mesh size of 5 mm) and degassed at room temperature for five days to reduce specific biogas production before BMP experiments.

Kinetic Analyses. We fit two established kinetic models to the CH₄ production data obtained from BMP tests to evaluate and compare CH₄ production kinetics of the *Elodea* and mixture silages. A simple first-order model was applied to describe cumulative methane production based on eq 1

$$B(t) = B_0[1 - \exp(-kt)] \quad (1)$$

where $B(t)$ is the cumulative methane production at time t (mL/g ODM), B_0 represents the maximum cumulative methane production

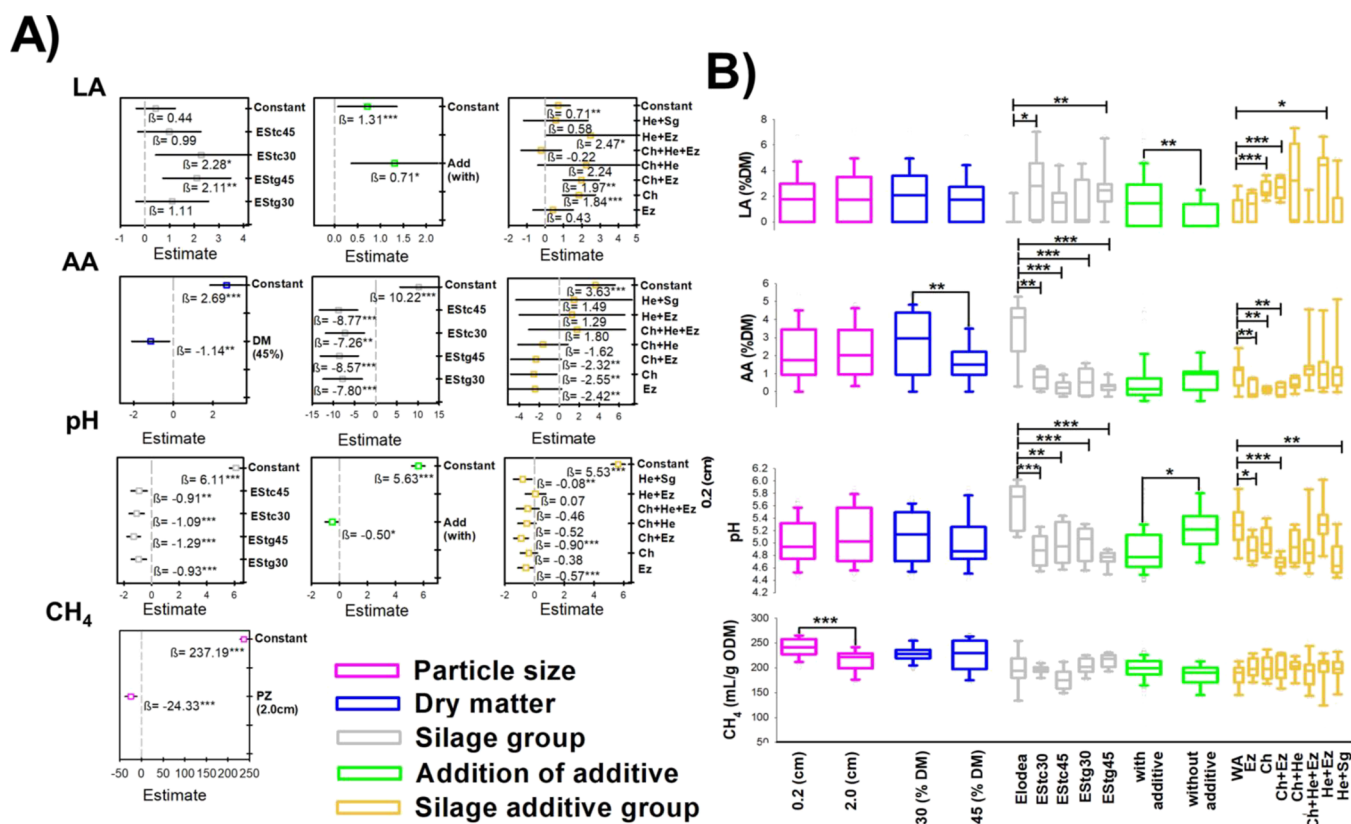


Figure 1. Mixed-effects regression models (A, main effects) for the factors particle size (PZ; 0.2 and 2.0 cm), dry matter (DM; 30 and 45%) content, silage group (i.e., *Elodea*, ESlc30, ESlc45, ESlg30, and ESlg45), addition of additive (Add; with and without), and silage additives (i.e., Ez, Ch, Ch + Ez, Ch + He, Ch + He + Ez, He + Ez, and He + Sg) for lactic acid (LA), acetic acid (AA), pH and methane (CH₄) production. Estimates (β) at 95% confidence intervals. Differences (B) between LA, AA, pH and CH₄ mean values. For abbreviations see Table 2. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

at theoretically infinite digestion time (mL/g ODM), and *k* corresponds to the first-order hydrolysis rate constant (1/day).

Furthermore, the modified Gompertz model, which describes the maximum methane production rate and the duration of a lag phase according to eq 2, was evaluated

$$B(t) = B_0 \cdot \exp \left\{ -\exp \left[\frac{B_m \cdot e}{B_0} \cdot (\lambda - t) + 1 \right] \right\} \quad (2)$$

where *B_m* represents the maximum cumulative methane production rate (mL/g ODM·d) and *λ* is the lag phase duration (days). We estimated model parameters from eqs 1 and 2 and their uncertainties using the negative logarithm of the likelihood LL (eq 3) as the objective function with constant error variance

$$LL = \frac{n \ln(2\pi\sigma^2)}{2} + \frac{\sum_{i=1}^n (B_i^{\text{obs}} - B_i^{\text{est}})^2}{2\sigma^2} \quad (3)$$

where *n* is the total number of experimental data, *i* is an index, *B_i^{obs}* represents the observed cumulative methane production at time *t*, *B_i^{est}* represents the estimated cumulative methane production calculated with eqs 1 and 2, and *σ* is the standard error. Model selection for the best fit to observed data was conducted using the Akaike information criterion (AIC) as described in eq 4

$$AIC = -2 \ln(L_{\text{max}}) + 2P \quad (4)$$

where *L_{max}* is the maximum likelihood and *P* is the number of parameters included in the model. Parameter estimation and their uncertainties were carried out using the subroutine "optim" from the statistical package R (R core team 2018).

Statistical Analyses. Data recorded after 180 days of ensiling and CH₄ production from the BMP test were analyzed by mixed-effects

regression models using Stata 16.1 (StataCorp. 2019, College Station, TX). The experimental *Elodea* and mixture silages were divided into five groups to assess the effect of the inclusion of wheat straw at the two PZ and DM contents, the addition of silage additives and the different silage groups. We also calculated the basic statistics (mean and standard deviation) in each silage group. We defined the fixed effects as addition of additive (Add; with or without additive), particle size (PZ; at 0.2 and 2.0 cm), dry matter (DM; at 30 and 45%) content, silage additives (i.e., Ez, Ch, Ch + Ez, Ch + He, Ch + He + Ez, He + Ez, and He + Sg, see Table 2), and silage groups (i.e., *Elodea*, ESlc30, ESlc45, ESlg30, and ESlg45, see Table 2), while the random effect was the mini silo. The response variables were: LA, AA, BA, ALC, pH, and CH₄ production. Regression models were then estimated separately for the main and interaction effects on each response variable using the general model given as

$$y_{ijk} = \mu + \tau_i + \beta_j + (\tau\beta)_{ij} + \varepsilon_{ijk} \quad (5)$$

where *y_{ijk}* is the value of the response variable, *μ* is the overall mean, *τ_i* is the fixed effect, *β_j* is a random effect, (*τβ*)_{*ij*} is the interaction assumed to be a random effect, and *ε_{ijk}* is the random error.

Differences among means were determined using a least squares linear hypothesis test (*H*₀: *y_{ij}* = *y_{ik}* against *H*_a: *y_{ij}* ≠ *y_{ik}*), with a statistical significance declared at *p* < 0.05. In addition, Principal component analysis (PCA) was used to mainly illustrate and identify associations between the methane production kinetics and fermentation parameters (LA, AA, BA, ALC, and pH) resulted from the ensiling experiment using the packages FactoMiner and factoextra¹⁵ from the statistical software R (R core team 2018).

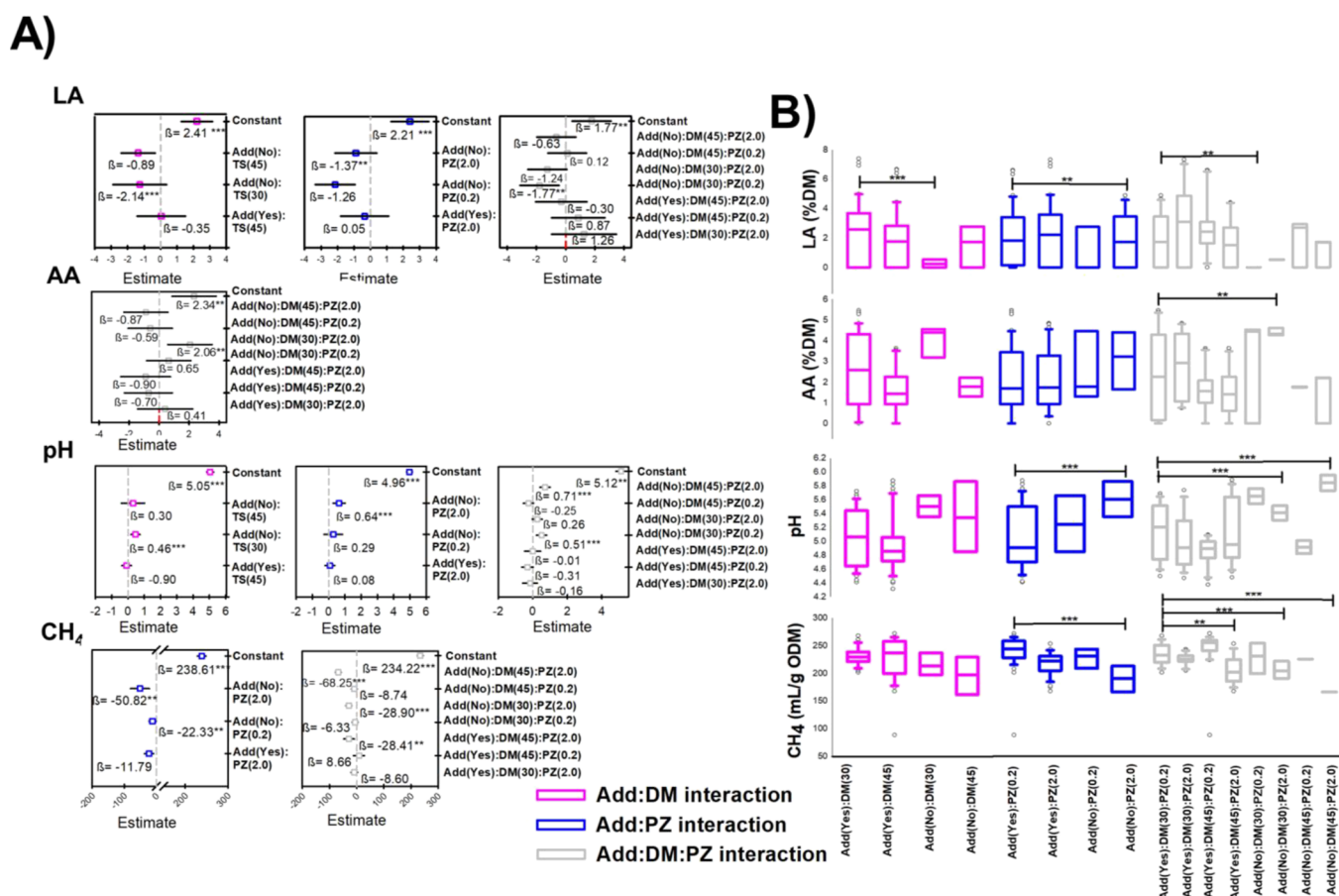


Figure 2. Mixed-effects regression models (A, interaction effects) for the factors particle size (PZ; 0.2 and 2.0 cm), dry matter (DM; 30 and 45%) content and addition of additive (Add; Yes and No) for lactic acid (LA), acetic acid (AA), pH and methane (CH₄) production. Estimates (β) at 95% confidence intervals. Differences (B) between LA, AA, pH, and CH₄ mean values. For more abbreviations see Table 2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

RESULTS

Analysis of the Main and Interaction Effects of Addition of Additive (Add), Particle Size (PZ), and Dry Matter (DM) Content on the Fermentation Quality of the *Elodea* and Mixture Silages. DM and ODM of *Elodea* and mixture silages and fermentation products and pH are reported in Table 3. It was shown that while the LA and pH of the mixture silages ranged from 0.13 to 7.38% DM and 4.3 to 5.9, respectively, those of the *Elodea* silages varied from 0.44 to 2.34% DM and 5.3 to 6.8, respectively, which evidences the increased silage quality due to the inclusion of wheat straw. The AA of *Elodea* silages was 10.22% DM on average, but it was less consistent in the mixture silages (2.12% DM on average). In addition, the BA and ALC tended to be zero in most cases, but BA was present in *Elodea* silages (Table 3).

Figures 1 and 2 illustrate the significant mixed-effects regression models for the main effects: Add, PZ, DM content, silage group (i.e., *Elodea*, EStc30, EStc45, EStg30, and EStg45) and silage additives (i.e., Ez, Ch, Ch + Ez, Ch + He, Ch + He + Ez, He + Ez, and He + Sg), and the interaction effects of Add:DM, Add:PZ, and Add:DM:PZ, respectively, on LA, AA, and pH. Models related to the main and also interaction effects on BA and ALC were not possible to generate due to small values that tended in most cases to be zero. Further details for all the mixed-effects regression models are available in Supporting Information Tables S1 and S2.

Regression analysis revealed no evidence of an association between LA and pH and the factors PZ and DM suggesting a not significant effect of PZ at either 2.0 or 0.2 cm ($P = 0.967$) and at either 30 or 45% DM ($P = 0.830$) (Table S1), respectively. Similarly, there was no evidence of an association between AA and PZ ($P = 0.754$) (Table S1), while an association was found between AA and DM revealing a significant effect of DM ($P = 0.019$) (Figure 1A: AA).

Interestingly, when evaluating the silage groups, estimates (β) of regression models showed significant differences ($p < 0.001$) between LA and pH mean values of the *Elodea* and mixture silages (Figure 1A: LA and pH). EStg45 had the most profound difference, followed by EStc30 on LA (Figure 1A: LA), while all mixture silages showed highest differences in pH (Figure 1A: pH). This suggests that the inclusion of wheat straw had a significant influence and perhaps was the key factor affecting the silage quality.

This analysis also showed a significant effect of the factor Add on LA ($P = 0.007$, Figure 1A: LA) and pH ($P = 0.051$, borderline statistical significance, Figure 1A: pH). Among silage additives, the Ch, Ch + Ez had the highest difference, followed by He + Ez on LA (Figure 1A: LA), whereas the Ch + Ez had the highest difference, followed by He + Sg and Ez on pH (Figure 1A: pH). Here, it is important to note that the addition of silages additives led to significantly higher LA and lower pH in all mixture silages, but not in the *Elodea* silages (Table 3), which may indicate that the inclusion of wheat

Table 4. Observed Methane (CH₄) Production and Estimated Parameters for the Biochemical Methane Production Potential from *Elodea* and Mixtures Silages and Their Uncertainty (± Standard Error) for the First-Order Model^a

			first order model				
			CH ₄ observed	B ₀	k	R ²	AIC
fresh <i>Elodea</i> silage			242.20 ± 36.4	209.08 ± 1.80	0.41 ± 0.03	0.9787	265.85
<i>Elodea</i>	WA	215.10 ± 5.49	215.10 ± 9.51	0.41 ± 0.03	0.9781	265.51	
	Ch	217.22 ± 1.04	217.22 ± 1.80	0.27 ± 0.01	0.9948	223.59	
	He + Ez	246.68 ± 8.46	246.68 ± 14.65	0.44 ± 0.02	0.9862	257.95	
	He + Sg	162.75 ± 7.71	162.75 ± 13.35	0.34 ± 0.01	0.9918	213.37	
	Ch + He + Ez	192.94 ± 1.52	151.52 ± 1.69	0.50 ± 0.05	0.9543	266.34	
EStc30	WA	202.04 ± 5.75	204.31 ± 0.65	0.11 ± 0.00	0.9995	144.96	
	Ez	225.47 ± 5.27	225.27 ± 0.81	0.13 ± 0.00	0.9993	170.15	
	Ch	230.74 ± 2.83	233.63 ± 0.54	0.12 ± 0.00	0.9997	135.29	
	Ch + Ez	211.78 ± 3.62	212.86 ± 0.81	0.12 ± 0.00	0.9993	164.56	
	Ch + He	231.85 ± 1.08	232.93 ± 1.03	0.12 ± 0.00	0.9990	182.64	
EStg45	Ch + He+Ez	215.98 ± 4.19	216.88 ± 0.80	0.12 ± 0.00	0.9992	168.59	
	He+Ez	227.40 ± 5.87	228.03 ± 0.94	0.12 ± 0.00	0.9992	175.90	
	He+Sg	231.16 ± 4.19	229.75 ± 1.12	0.14 ± 0.00	0.9983	199.58	
	WA	225.47 ± 5.65	228.51 ± 1.47	0.13 ± 0.00	0.9979	212.46	
	Ez	238.68 ± 11.01	241.11 ± 2.17	0.12 ± 0.00	0.9967	240.33	
	Ch	255.36 ± 0.67	257.12 ± 1.37	0.15 ± 0.00	0.9983	220.23	
	Ch + Ez	261.48 ± 1.87	266.75 ± 0.75	0.15 ± 0.00	0.9993	178.10	
	Ch + He	242.93 ± 9.44	245.74 ± 1.70	0.15 ± 0.00	0.9972	237.13	
	Ch + He + Ez	247.33 ± 6.61	247.62 ± 0.95	0.17 ± 0.00	0.9989	199.73	
	He + Ez	245.02 ± 2.98	244.46 ± 0.81	0.16 ± 0.00	0.9991	184.61	
EStg30	He + Sg	268.06 ± 5.57	266.75 ± 0.75	0.15 ± 0.00	0.9993	178.10	
	WA	226.46 ± 14.99	245.27 ± 4.49	0.09 ± 0.00	0.9975	236.62	
	Ez	252.89 ± 7.39	253.73 ± 1.35	0.13 ± 0.00	0.9984	208.94	
	Ch	213.76 ± 11.38	216.67 ± 1.42	0.13 ± 0.00	0.9980	211.09	
	Ch + He	238.71 ± 3.96	239.97 ± 1.52	0.13 ± 0.00	0.9980	217.78	
EStc45	Ch + He + Ez	228.91 ± 3.01	239.14 ± 2.64	0.11 ± 0.00	0.9974	224.70	
	He + Ez	210.26 ± .19	219.7 ± 2.55	0.15 ± 0.01	0.9971	215.84	
	He + Sg	224.24 ± 2.94	235.06 ± 3.08	0.15 ± 0.01	0.9868	229.72	
	WA	167.20 ± 6.36	165.45 ± 0.43	0.16 ± 0.00	0.9994	138.84	
	Ez	198.83 ± 6.33	197.23 ± 0.62	0.16 ± 0.00	0.9991	163.74	
	Ch	193.83 ± 5.65	196.88 ± 1.06	0.13 ± 0.00	0.9982	192.10	
	Ch + Ez	187.94 ± 9.63	190.89 ± 0.92	0.13 ± 0.00	0.9984	182.94	
	Ch + He	210.07 ± 14.65	208.72 ± 0.76	0.14 ± 0.00	0.9990	173.73	
	Ch + He + Ez	168.90 ± 3.91	166.13 ± 0.79	0.18 ± 0.00	0.9975	187.88	
	He + Ez	244.59 ± 4.48	240.09 ± 1.70	0.14 ± 0.00	0.9967	228.63	
He + Sg	247.20 ± 5.99	239.42 ± 2.12	0.16 ± 0.01	0.9934	252.22		

^aB₀; maximum cumulative methane production at theoretically infinite digestion time (mL/g ODM), k; hydrolysis rate constant (1/day). AIC; Akaike information criterion, R²; correlation coefficients between the observed and estimated methane production.

straw may also be responsible for any apparent effects of silage additives observed in this study.

ANOVA indicated that overall the inclusion of wheat straw to silages (mixture silages) increased LA by on average 80%, compared to silages without inclusion of wheat straw (*Elodea* silages), irrespective of PZ, DM, and additive (Figure 1B: LA and Table 3). LA was higher ($P < 0.05$) in EStg45 and EStc30 silages compared to *Elodea* silages, but similar to that of EStg30 and EStc45 (Figure 1B: LA). pH significantly decreased ($p < 0.05$) from 6.1 in *Elodea* silages to 5.0 on average in all the mixture silages, but this decrease was consistently lower in mixture silages at decreasing PZ (EStc45, Figure 1B: pH and Table 3). The use of silage additives significantly increased LA ($P < 0.05$, Figure 1B: LA) and decreased pH ($P < 0.05$, Figure 1B: pH); however, significant differences were only found in the mixture silages (Table 3).

Concerning interaction effects (Figure 2), we want to point out, here, that regression models with interactions of the *Elodea* silages were not generated since those silages were without inclusion of wheat straw; therefore, estimates of models were only produced using the data of the mixture silages. The Add:DM, Add:PZ, and Add:DM:PZ interactions had an effect on LA ($P < 0.001$, Figure 2A: LA) and pH ($P < 0.001$, Figure 2A: pH), while only the Add × DM × PZ interaction was significant for AA ($P = 0.006$, Figure 2A: AA).

Analysis of the Main and Interaction Effects of Addition of Additive (Add), Particle Size (PZ), and Dry Matter (DM) Content on CH₄ of the *Elodea* and Mixture Silages. In general, CH₄ values of the *Elodea* silages showed to be similar to those of the mixture silages, suggesting *Elodea* biomass has a great potential for the sustainable production of methane (Table 4). Figures 1 and 2 illustrate the significant mixed-effects regression models for the main effects, Add, PZ,

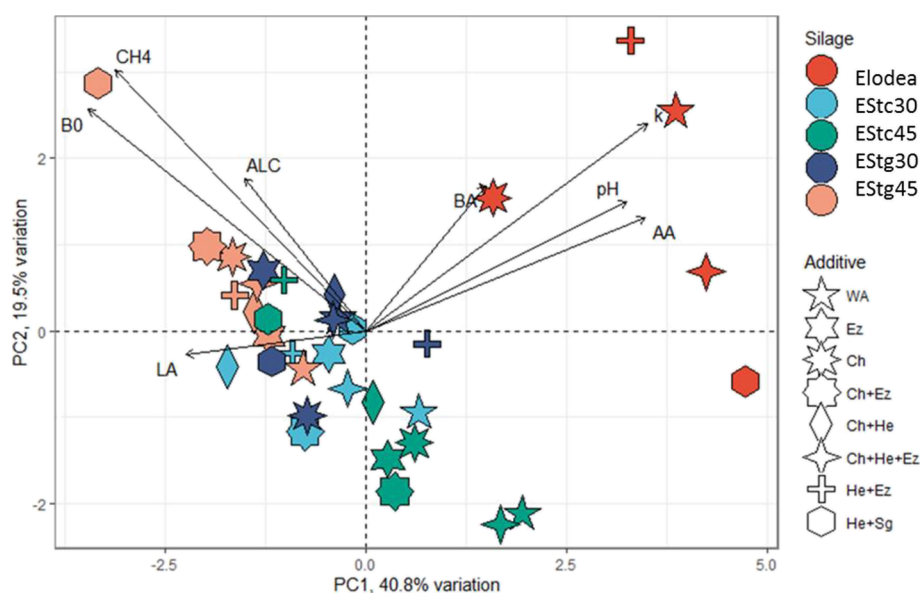


Figure 3. Principal component analysis (PCA) to identify the relationship of kinetic parameters (hydrolysis constant (k), and maximum methane production potential (B_0)). Lactic acid (LA), acetic acid (AA), butyric acid (BA), alcohols (ALC), pH, and methane (CH_4) production. Different colors represent the silage, and different symbols represent the additive. For abbreviations see Table 2.

DM content, silage group and additives, and interaction effects of Add:DM, Add:PZ, and Add:DM:PZ on CH_4 . Regression analysis revealed no evidence of an association between the outcome CH_4 and the factors Add and DM ($P > 0.05$, Table S1). Similarly, when considering the *Elodea* silages as the reference group, no significant differences were found indicating statistically similar CH_4 among the five silage groups ($P > 0.05$, Figure 1B: CH_4). Similarly, when compared among the seven silage additives no significant differences were observed indicating a not significant effect of the addition of any additive used in this study ($P > 0.05$, Figure 1B: CH_4).

Conversely, when investigating the effect of PZ a significant linear relationship was found between the factor PZ and CH_4 ($P = 0.001$, Figure 1A: CH_4). Significant differences were found between the CH_4 mean values of silages at 0.2 and 2.0 cm, where significantly higher CH_4 was on silages at 0.2 cm ($P < 0.05$), Figure 1B: CH_4). Concerning the interaction effect, interactions were only significant when the factor PZ was evaluated ($P < 0.001$) for CH_4 production (Figure 2B: CH_4).

Kinetic Results. Parameter estimates of the applied model structures (eqs 1 and 2) are presented in Table 4 and supplementary Table S3. Except for *Elodea* silages, correlation coefficients (R^2) of the first-order model and the modified Gompertz model were above 0.99, indicating a good predictivity. The Akaike information criterion (AIC) results suggested that the first-order model had the lowest values in all cases. In contrast, the modified Gompertz model consistently had the worst performance (Table S3). Furthermore, parameter estimates of λ (lag phase) in the modified Gompertz model were most often negative. Since the modified Gompertz model is only defined for positive parameter values ($\lambda \geq 0$), negative parameter estimates indicate that the model is not suitable for description of measured CH_4 production. Therefore, the first-order model was selected as the best fit for the observed CH_4 production of the *Elodea* and mixture silages.

Model fitting results revealed that mixture silages achieved overall highest CH_4 production (B_0) compared to the *Elodea* silages (Table 4). Furthermore, a clear positive influence was

observed in the mixture silages at more reduced particle sizes (0.2 cm) at either DM content (EStg30 and EStg45), which produced highest B_0 , irrespective of silage additive. Contrarily, the hydrolysis constant (k) was highest in the *Elodea* silages (about 0.36 (1/day)) compared to the mixture silages (0.14 (1/day)), and similar k values were found among all the *Elodea* silages. In general, neither the inclusion of wheat straw at either particle size (0.2 and 2.0 cm) nor addition of silage additives had an effect on k , but they resulted in a significant increase of B_0 . To clearly summarize and illustrate these results, principal component analysis (PCA) was applied (Figure 3).

According to the model results, composition of easily degradable volatile acids among all the studied silages could likely be a factor affecting kinetic parameters. The first three principal components (PC1, PC2, and PC3) explained 73.72% of the total variation. As depicted in Figure 3, the *Elodea* silages were clearly separated from the mixture silages and identified on the same side of k , AA, BA, and pH, whereas the mixture silages, in particular the EStg45 were identified on the same side of B_0 , CH_4 , and LA. This may indicate that high rates of degradation described by k were found in the *Elodea* silages. Also, high BA and AA as well as high pH were also suggested in the *Elodea* silages. Conversely, CH_4 production was enhanced in the mixture silages by improving their B_0 , and the mixture silages were also related to high LA and low pH.

DISCUSSION

Silage Quality of the *Elodea* and Mixture Silages.

When *Elodea* was ensiled with wheat straw (mixture silages), silage quality improved significantly, as indicated by the increase in LA and the decrease in pH but remained the same when silage additives were used. In contrast, when *Elodea* was ensiled without wheat straw (*Elodea* silages), quality did not improve and even with additives it became worse (Table 3). From this, it appears at least that the inclusion of wheat straw played the major role in the preservation of *Elodea* biomass whether silage additives were used or not.

The fermentation quality of the mixture silages relative to the *Elodea* silages suggested that these silages were well preserved. All mixture silages maintained higher LA and lower pH than *Elodea* silages did and were strongly correlated with the inclusion of wheat straw. Generally, when pH reduces to about 4, silages can be well preserved, but this will occur if sufficient digestible WSCs are available to be converted primarily to LA. This is because the success of the ensilage quality is mainly related to the rate and extent of preferably LA formation to be more than able to compensate for increasing buffering capacity.⁸ Research investigating the production of silages from aquatic biomass has characterized it as a poorly ensilable material due to low WSCs, high buffer capacity, and high moisture contents.¹⁶ In our study, *Elodea* biomass was no exception, the high moisture, protein, and ash contents increased its buffer capacity. In addition, the low LA resulting from low WSCs could not deplete the higher buffer capacity and thus prevented the reduction of pH to a lower value. Since the buffering capacity and the WSCs are characteristics that affect the reduction of pH, we concluded that it is difficult to generate high-quality silages using *Elodea* biomass, unless it is ensiled, at least, with wheat straw. Our results also indicated that the addition of additives to *Elodea* silages had no relevant effects on improving fermentation quality through increasing organic acids, especially LA and hence pH.

Here, we observed that one of the main problems associated with aquatic silages, in this case *Elodea* silages, is the high moisture content, which might have affected the fermentative quality of silages in multiple ways.

First, high moisture contents made *Elodea* biomass unsuitable to be field wilted to increase DM content (to a favorable range of 250–350 g/kg), as it can be done for terrestrial plants.¹⁷ Second, the high moisture led to a dilution of available soluble carbohydrates and organic acids including LA. Third, it affected the fermentative capacity of the microbial community favoring BA formation, an indirect indicator of clostridial activity that elevates pH and implies a failed fermentation.^{18–20} Moreover, it is also known that high moisture contents may dilute any silage additive and produce effluent.^{8,17} In this way, the poorly fermented *Elodea* silages as evidenced by the decrease in LA and increase in pH might have been justified. Therefore, the results of this study appear to indicate that the inclusion of a dry material like wheat straw had a greater potential and is positively indicated to improve fermentation quality of these aquatic silages rather than the use of silage additives. However, to the present time, there has been little research into the ensiling fermentation of aquatic plants, in particular aquatic macrophytes, that supports these findings.

Recently, Herrmann et al.¹⁶ investigated the ensilage of five marine macroalgae (seaweeds, 3rd generation biofuel). In this study, Herrmann found that seaweed silages with moisture contents >75% negatively affected their silage quality as indicated by the low LA and high pH. Herrmann also concluded that further investigation using silage additives is needed to enhance LA fermentation in seaweed silages. So that, we suggest that this study could also be a useful investigation for other research studies into ensiling fermentation of aquatic biomass and/or extremely wet materials.

Silage additives are expected to improve fermentation quality by producing higher LA relative to other organic acids, thus ensuring longevity of silages through their different

modes of action. However, in our study the only apparent effects of additives were strongly associated with the inclusion of wheat straw. In particular, to the factors PZ of which wheat straw was pretreated before ensiling and the adjusted DM content. PZ reduction is identified as the very initial step that alters the lignocellulosic biomass's structure to a greater contact surface area, increasing the hydrolysis of carbohydrates polymers to fermentable sugars.²¹ Apparently, the rupture of a high percentage of plant cells is directly correlated with the increased fermentation quality possibly due to additional fermentable sugars, resulting in more LA and a rapid drop in pH.^{22,23} This together with the fact that DM was the factor that could promote all the microbial activity, especially LAB activity,²⁴ suitable conditions might have been favored. Consequently, any effect of silage additives observed in this study did depend on these two factors.

CH₄ Production and Model Parameters *k* and *B₀* of *Elodea* and Mixture Silages. CH₄ values of fresh *Elodea* and ensiled *Elodea* (with or without additive) were lower than those found by Koyama et al.,²⁵ who evaluated the potential of some *Elodea* sp. for CH₄ production without ensiling. Differences among CH₄ production from aquatic plants may rely on the site of biomass origin and composition due to different locations and growing conditions. Age at time of harvest may also have an influence. DM losses are expected when ensiling fermentation failed, which could likely be one reason for lower CH₄ in this study. Research investigating only the production of CH₄ has indicated that as such this aquatic plant can be used as a source for sustainable CH₄ production. However, no considerations of the availability of feedstock through the year have been made.

The mixture silages EStg30 and EStg45 showed highest methane production (226.46 ± 14.99 and 225.47 ± 5.65 mL/g ODM, respectively) as compared to the ensiled *Elodea* (215.10 ± 5.49 mL/g ODM) but similar to that of *Elodea* without ensiling (242.20 ± 36.4 mL/g ODM). This suggested that the preservation of overall CH₄ production as well as *Elodea* biomass after 180 days storage was significantly enhanced by inclusion of wheat straw. This may be by the fact that ensiling could perhaps have had indirect effects on further AD. Upon ensiling, undesired microorganisms like clostridia produce besides BA, CO₂ and H₂ resulting in mass and DM losses.⁸ Indeed, DM can be lost as CO₂, but if H₂ is lost energy losses would occur, since H₂ may not be longer available for the methanogens in the AD process.²⁶ As previously discussed in the results of silage quality, considerable BA in *Elodea* silages could be the reason for low CH₄ due to losses of DM and energy by clostridial activity. Another possible explanation is the production of effluent that is likely to be occurred in wet materials. Egg et al.²⁶ and Franco et al.²⁷ noted that higher effluent production correlated with lower CH₄ when evaluating silage of wet catch crops. Ensiling *Elodea* without inclusion of wheat straw confirmed that an inefficient preservation affected indirectly, yet negatively CH₄ production. This aligns with Larsen et al.,²⁸ who evaluated wet beet leave silage with and without wheat straw. Overall, results suggested that low DM of *Elodea* biomass, in large part, created the demand for a dry material like wheat straw to be easily ensiled and preserved for subsequent AD process.

The higher LA, negligible BA, and lower pH in the mixture silages compared to *Elodea* silages could be important factors that maintained silage quality and thus CH₄ production. This suggests that ensiling may be a method of organic matter

preservation for biomass energy feedstocks. Sieborg et al.²⁹ found that fresh wheat straw had a k of 0.04 1/day indicating low biodegradability. According to our results, all mixture silages showed generally lower k . In contrast, k of *Elodea* silages were higher suggesting high rates of degradation. This indicated that ensiled *Elodea* with inclusion of wheat straw did not accelerate k but increased the extent of *Elodea* biomass conversion, further confirming that ensiling is above all a method of organic matter preservation.³⁰

Generally, ensiling for prolonged periods is assumed to cause a degradation of structural polysaccharides of plant material into increased amounts of fermentable substrates.²⁶ This also confirmed that the increased CH₄ could possibly be because of the good quality of the mixture silages and the increased degradability of wheat straw due to prolonged ensiling periods.

Overall, the results from this study suggested that *Elodea* biomass ensiled with wheat straw decreased clostridial activity, which is beneficial to obtain well preserved silages for further commercial AD. Although, we found that similar CH₄ was produced at either 30 or 45% DM, however, the silages at 30% DM could imply that more *Elodea* biomass can be stored and thus used.

CONCLUSIONS

Ensiling may prevent the lack of the utilization of *Elodea* biomass. Yet its low DM created the demand for a dry material like wheat straw to efficiently improve biomass and CH₄ conservation. Beyond the benefits of applying additives, inclusion of wheat straw to ensiled *Elodea* was more efficient, since the high moisture of *Elodea* biomass was partly responsible for diluting any effects. In addition, wheat straw at smaller PZ was positively indicated to make *Elodea* biomass suitable for ensiling at prolonged storage periods. In summary, increased silage quality was strongly associated with inclusion of wheat straw, which is beneficial to obtain well preserved silages for a sustainable CH₄ production from commercial AD.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsagstech.3c00043>.

Values of estimates of the factors for the mixed-effects regression models at 95% confidence intervals (in parenthesis) for the main effects: addition of additive (Add), particle size (PZ), dry matter (DM) content, silage group (i.e., *Elodea*, EStc30, EStc45, EStg30, and EStg45) and silage additives (enzymes (Ez), chemical additive (Ch), chemical additive combined with enzymes (Ch + Ez), chemical additive combined with inoculant (Ch + He), chemical additive combined with inoculant and enzymes (Ch + He + Ez), inoculant combined with enzymes (He + Ez), and inoculant combined with sugar solution (He + Sg)) for lactic acid (LA), acetic acid (AA), pH and methane (CH₄) production. LL; log likelihood, AIC; Akaike information criterion, BIC; Bayesian information criterion, N; number of observations; values of estimates of the factors for the mixed-effects regression models at 95% confidence intervals (in parenthesis) for the interaction effects: addition of additive (Add), particle size (PZ), dry matter (DM) content, silage group (i.e., *Elodea*,

EStc30, EStc45, EStg30, and EStg45) and silage additives (enzymes (Ez), chemical additive (Ch), chemical additive combined with enzymes (Ch + Ez), chemical additive combined with inoculant (Ch + He), chemical additive combined with inoculant and enzymes (Ch + He + Ez), inoculant combined with enzymes (He + Ez), and inoculant combined with sugar solution (He + Sg)) for lactic acid (LA), acetic acid (AA), pH and methane (CH₄) production. LL; log likelihood, AIC; Akaike information criterion, BIC; Bayesian information criterion, N; number of observations; and observed methane production and estimated parameters for the biochemical methane production potential from *Elodea* and mixtures silages and their uncertainty ($\pm$ standard error) for the modified Gompertz model (PDF)

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Notes

The authors declare no competing financial interest.

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