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# Survival of Plant Seeds in Anaerobic Digestion and Ensilage

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# Summary

Plant biomass is a promising and increasingly used feedstock for energy and material production in a circular, bio-based economy. Today, seed-bearing wild plant species complement the feedstock portfolio, for example in biogas production. Plant seeds can, however, be very resistant to numerous environmental factors. Therefore, it is possible that seeds survive the biogas production chain and are unintentionally dispersed with the intermediate or end products such as the digestate from biogas plants. This could lead to weed problems, the control of which would compromise sustainability and cause additional costs and labor.

This study investigated the extent to which seeds of wild plants can survive processes in the biogas production chain, namely anaerobic digestion (AD) and ensiling. A total of 16 species (22 seed lots) were examined, most of them selected from a commercially available wildflower mixture designed for biogas production. The determination of seed viability by a combination of germination test and tetrazolium staining and the modeling of seed viability as a function of exposure time proved valuable to gain insight into the wide diversity of viability responses. The responses differed between species and seed lots. Hardseedness (HS), i.e., physical dormancy, was confirmed to favor survival, and depth and degree of dormancy may play a role. The basis of resistance in surviving non-hard-seeded (NHS) species remains to be investigated, as do possible relationships between resistance potential and taxonomic affiliation.

AD was found to reduce seed viability, with the extent of reduction being determined by an interplay of seed characteristics and process parameters. Responses of seed viability ranged from complete inactivation within 24 hours, to a steep decline after a lag phase, to an initial increase in viability (hormesis). After the maximum exposure time of 36 days in the lab- or full-scale biogas reactors at mesophilic temperatures between 35 and 42 °C, the average seed-killing efficacy was about 53% and 100% for HS and NHS species, respectively. Seeds of some species survived all treatments. *Malva sylvestris*, *Melilotus albus* and *Melilotus officinalis* were particularly AD-resistant and were the only species to exhibit biphasic viability curves. However, for most species, prolonged exposure and higher temperatures resulted in greater seed inactivation. Compared to the experimental lab-scale reactors, the full-scale commercial reactor killed seeds more effectively. The comparison of water-baths and reactors revealed that seed thermoresistance is not yet suitable as a reliable proxy of survival in AD, as temperature was an important but not the only factor determining seed inactivation. Estimates of seed survival in AD will become more accurate if future studies record complete viability curves, i.e. up to inactivation of dormant and non-dormant seeds. On this basis, the transferability of results from lab- to full-scale could be quantified and the suitability of thermoresistance as a survival proxy in AD re-evaluated.

Lab-scale ensiling over a period of up to eight months did not completely inactivate any of the HS species tested, but did inactivate all NHS species. The seed-killing efficacy for the HS species ranged from 5 to 60 %. No consistent effect of silage types differing in the ensiled feedstock, the ensiling conditions and the resulting biochemical composition of the silages on seed survival was observed. The factors underlying the killing of seeds in silages therefore still need to be determined.

Finally, it was proven that the biomass of the wildflower mixture is suitable for ensiling, preferably mixed with maize. This increases the probability of this biomass - and its seeds - entering the biogas production chain. However, whether the use of such seed-bearing wild plant biomass as a biogas feedstock carries the risk of contaminating silage and digestate with viable seeds depends both on the risk of seed survival and on whether seeds enter the biogas chain in sufficient quantity and quality. The seed-killing efficacies of AD and silage and the seed-borne resistance potential determined in this thesis pave the way for estimating this contamination risk and can thus help to ensure the sustainable use of wild plant biomass.

# Zusammenfassung

Pflanzliche Biomasse ist ein vielseitiger Rohstoff, der zunehmend in der Energie- und Materialproduktion der kreislauforientierten, biobasierten Wirtschaft Anwendung findet. Heute ergänzen auch samenbildende Wildpflanzenarten das Biomasseportfolio, zum Beispiel in der Biogasproduktion. Allerdings können Pflanzensamen sehr widerstandsfähig gegenüber zahlreichen Umweltfaktoren sein. Es ist daher möglich, dass Samen die Biogasproduktionskette überleben und unbeabsichtigt mit den Zwischen- oder Endprodukten, wie z. B. dem Gärrest aus Biogasanlagen, ausgebracht werden. Dies könnte zu Unkrautproblemen führen, deren Bekämpfung die Nachhaltigkeit beeinträchtigen und zusätzliche Kosten und Arbeit verursachen würde.

In dieser Arbeit wurde untersucht, inwieweit Samen von Wildpflanzen Prozesse der Biogasproduktion, nämlich die anaerobe Vergärung (AV) und die Silierung, überleben können. Insgesamt wurden 16 Arten (22 Samenchargen) untersucht, von denen die meisten aus einer im Handel erhältlichen Wildblumenmischung für die Biogaserzeugung ausgewählt wurden. Die Bestimmung der Lebensfähigkeit der Samen durch eine Kombination aus Keimtests und Tetrazoliumfärbung sowie die Modellierung der Samen-Lebensfähigkeit als Funktion der Expositionszeit erwiesen sich als wertvoll, um einen Einblick in die große Vielfalt der Lebensfähigkeitsreaktionen zu erhalten. Diese Reaktionen unterschieden sich zwischen den Arten und den Saatgutpartien. Es konnte bestätigt werden, dass Hartschaligkeit (HS), d. h. physikalische Dormanz, das Überleben begünstigt, wobei Tiefe und Grad der HS eine Rolle spielen könnten. Die Grundlage der Resistenz bei den überlebenden, nicht-hartschaligen (NHS) Arten muss noch ermittelt werden, ebenso wie mögliche Beziehungen zwischen Resistenzpotenzial und taxonomischer Zugehörigkeit.

Es wurde festgestellt, dass die AV die Lebensfähigkeit der Samen reduzierte, wobei das Ausmaß der Reduktion durch ein Zusammenspiel von Sameneigenschaften und Prozessparametern bestimmt wurde. Die Reaktionen der Samen-Lebensfähigkeit reichten von einer vollständigen Inaktivierung innerhalb von 24 Stunden über einen steilen Rückgang nach einer *lag*-Phase bis hin zu einem anfänglichen Anstieg der Lebensfähigkeit (Hormesis). Nach der maximalen Expositionszeit von 36 Tagen in den Labor- oder kommerziellen Biogasreaktoren bei mesophilen Temperaturen zwischen 35 und 42 °C betrug die durchschnittliche Wirksamkeit der Samenabtötung etwa 53 % bzw. 100 % für HS- und NHS-Arten. Die Samen einiger Arten überlebten alle Behandlungen. *Malva sylvestris*, *Melilotus albus* und *Melilotus officinalis* waren besonders AV-resistent und wiesen als einzige Arten biphasische Lebensfähigkeitskurven auf. Bei den meisten Arten führten jedoch eine längere Verweildauer und höhere Temperaturen zu einer stärkeren Inaktivierung der Samen. Im Vergleich zu den Versuchsreaktoren im Labormaßstab tötete der kommerzielle Reaktor die Samen effektiver ab. Der Vergleich von Wasserbädern und Reaktoren zeigte, dass sich die Thermoresistenz von Samen noch nicht als zuverlässiger Indikator für das Überleben in AV eignet, da die Temperatur ein wichtiger, aber nicht

der einzig bestimmende Faktor für die Inaktivierung der Samen war. Die Abschätzung des Samen-Überlebens in der AV wird genauer werden, wenn künftige Studien vollständige Lebensfähigkeitskurven aufzeichnen, d. h. bis zur Inaktivierung sowohl von dormanten als auch nicht-dormanten Samen. Auf dieser Grundlage könnte die Übertragbarkeit der Ergebnisse vom Labor- auf den Praxismaßstab quantifiziert und die Eignung der Thermoresistenz als Überlebensindikator in der AV neu bewertet werden.

Die Silierung im Labormaßstab über einen Zeitraum von bis zu acht Monaten hat keine der getesteten HS-Arten vollständig inaktiviert, wohl aber alle NHS-Arten. Die samenabtötende Wirkung für die HS-Arten lag zwischen 5 und 60 %. Es wurde kein einheitlicher Effekt von Silagetypen, die sich im silierten Ausgangsmaterial, den Silierbedingungen und der daraus resultierenden biochemischen Zusammensetzung der Silagen unterschieden, auf das Überleben der Samen beobachtet. Die Faktoren, die der Abtötung von Samen in Silagen zugrunde liegen, müssen daher noch ermittelt werden.

Schließlich wurde nachgewiesen, dass sich die Biomasse der Wildblumenmischung für die Silierung eignet, vorzugsweise gemischt mit Mais. Dies erhöht die Wahrscheinlichkeit, dass diese Biomasse - und ihre Samen - in die Biogasproduktionskette gelangen. Ob die Verwendung solcher samentragender Wildpflanzenbiomasse als Biogasrohstoff das Risiko birgt, Silage und Gärreste mit lebensfähigen Samen zu verunreinigen, hängt jedoch sowohl vom Risiko des Samen-Überlebens als auch davon ab, ob die Samen in ausreichender Menge und Qualität in die Biogaskette gelangen. Die in dieser Arbeit ermittelten Saatgutabtötungseffizienzen von AV und Silage sowie das samenbürtige Widerstandspotential ermöglichen eine Abschätzung dieses Kontaminationsrisikos und können so zu einer nachhaltigen Nutzung von Wildpflanzenbiomasse beitragen.

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# List of Abbreviations

| | |
|---|---|
| AA | acetic acid |
| AD | anaerobic digestion |
| $AD_{CR}$ | anaerobic digestion in a full-scale, commercial biogas reactor |
| $AD_{ER}$ | anaerobic digestion in a lab-scale, experimental biogas reactor |
| ADF | acid detergent fibre |
| ANOVA | analysis of variance |
| BC | buffering capacity |
| BGY | specific biogas yield |
| $CH_4$ | methane |
| $CH_4Y$ | specific methane yield |
| $CO_2$ | carbon dioxide |
| CF | crude fibre |
| CP | crude protein |
| CR | commercial, full-scale biogas reactor |
| DM | dry matter |
| $E_{LS}$ | ensilage in a lab-scale silo |
| EPG | electric power generation |
| ER | experimental, lab-scale biogas reactor |
| EULOS | enzyme-insoluble organic matter |
| FC | fermentability coefficient |
| FM | fresh matter |
| GHG | greenhouse gas |
| GLMM | generalized linear mixed model |
| HS | hardseeded / hardseededness; synonym for physical dormancy |
| $KNO_3$ | potassium nitrate |
| LA | lactic acid |
| n | number of repetitions |
| na | not available |
| nd | below detection limit |
| NDF | neutral detergent fibre |
| $NH_4$-N | ammonium-bound nitrogen |
| NHS | non-hardseeded |
| NMDS | non-metric multidimensional scaling |
| SD | standard deviation of the mean |
| SEM | standard error of the mean |
| SKE | seed-killing efficacy |
| TTC | 2,3,5-triphenyltetrazolium chloride |
| TS | total solids |
| VFA | volatile fatty acids |
| VS | volatile solids |
| WB | water-bath |
| WSCH | water-soluble carbohydrates |

# 1

# Introduction

## 1.1 Why Survival of Plant Seeds in Biogas Reactors and Silos matters

### 1.1.1 Plant Seed Persistence

Plant seeds can be extremely durable and resistant. They can withstand extreme conditions such as fire, freezing and desiccation, or animal digestion (e.g., Traveset, 1998; Gusta et al., 2006; Pausas and Lamont, 2022). Exceptionally long-lived seeds have been found to germinate and grow even after hundreds of years, for example, a 1,300-year-old sacred lotus (*Nelumbo nucifera* Gaertn., Shen-Miller et al., 1995) and a 2,000-year-old date palm (*Phoenix dactylifera* L., Sallon et al., 2008).

The survival potential of seeds is not only fascinating from a biological perspective, but also important in agriculture and conservation. On the one hand, the weed seedbank is a target of (integrated) weed management efforts in agricultural systems, with dormant or long-lived seeds posing a particular challenge (e.g., Buhler et al., 1997; Gallandt, 2006). Artificial seed banking, on the other hand, strives to identify and provide conditions that promote seed longevity, both of rare wild species and important crop species (e.g., Hay and Probert, 2013; Nadarajan et al., 2023).

In the context of circular bioeconomy (e.g., Nagarajan et al., 2021), plant seed survival is also relevant. Namely, when plant biomass is used for energy and material production. In this case, it is possible that seeds that survive biomass processing are unintentionally dispersed with the intermediate or final products - such as digestate from biogas plants. Given our globally changing world, the impact of spreading the surviving seeds cannot be predicted with certainty (Thuiller et al., 2008). Nevertheless, problems could arise, especially with species of known invasive potential or with problematic properties such as allergens. Moreover, even a few seeds could cause a major infestation (Mt. Pleasant and Schlater, 1994). Control of potentially surviving problematic species would compromise sustainability and create undesirable additional costs and labor. Therefore, it seems appropriate to assess the risk of dispersal before the seeds are released into the environment. That is, it should be determined whether the seeds are still viable after biomass processing. It is precisely this interface between plant seed persistence and biomass-utilization that this thesis is devoted to.

### 1.1.2 Plant Biomass in Circular Bioeconomy

The concept of circular bioeconomy attempts to provide an integrated response to recent global challenges such as climate change, depletion of natural resources, and a growing demand for food and energy. A definition that considers all economic, environmental, political, ethical, and social dimensions remains under discussion. However, the overarching principle of circular bioeconomy is to decouple economic growth from dependence on fossil resources by slowing, narrowing, and closing material resource loops built on the foundation of biomass-based feedstocks and biological processes (Scarlat et al., 2015; D'Amato et al., 2017; Stegmann et al., 2020; Nagarajan et al., 2021; Tan and Lamers, 2021).

Sustainable supply with biomass in sufficient quantity and quality is a key factor for the success of (circular) bioeconomy initiatives (Pfau et al., 2014; Lewandowski, 2015; Scarlat et al., 2015; Tan and Lamers, 2021). The sources for meeting the biomass demand are diverse, ranging from animal manure to micro-algae. Nevertheless, the most commonly used feedstocks are plant-based (Nagarajan et al., 2021). However, the sustainability of using (terrestrial) plant biomass raises many concerns already familiar from the bioenergy debate, namely competing claims on land and resources for the production of food, feed, fiber, materials, and energy. Furthermore, (potential) direct and indirect impacts of associated land-use changes are discussed, e.g., displacement of smallholder farmers, land degradation, biodiversity loss, and risks posed by invasive species (Pfau et al., 2014; Lewandowski, 2015; Scarlat et al., 2015; Hadley Kershaw et al., 2021; Muscat et al., 2021).

The consensus of suggestions for sustainable plant biomass production systems is that they should be site-specific, multifunctional, require few inputs, and explore new biomass options (Lewandowski, 2015; Scarlat et al., 2015; Yang et al., 2018; von Cossel et al., 2019a; von Cossel et al., 2019c; Garibaldi et al. 2023). In terms of new biomass options, the spectrum of species, including seed-bearing ones, has been expanding for about a decade (e.g., Bomgardner, 2013; Sharma and Pant, 2019; Papamatthaiakis et al., 2021). An often-overlooked consequence of expanding the spectrum of species, however, is that it simultaneously increases the probability of seeds entering (biomass) processing facilities.

### 1.1.3 Biogas from Plant Biomass

Biogas plants in rural farming communities are a perfect example of the application of circular bioeconomy (Yadav et al., 2021; Schulte et al., 2022). This is because the anaerobic digestion in biogas reactors serves a multitude of functions when converting biomass into an energy carrier, biogas, and a material product, digestate, both of which can be used in a variety of ways (Fagerström et al., 2018; Pavičić et al., 2022; Burg et al., 2023). For example, the methane from the biogas can be used as climate-neutral fuel or to generate electricity and heat. The digestate can be used as a fertilizer, thereby recycling nutrients and organic carbon, and for soil improvement and amendment (Weiland, 2010; Walsh et al., 2012; Scarlat et al., 2018; Kovačić et al., 2022). In addition, liquid, solid or whole digestate can be bio-refined to obtain other products, such as biochar, composites or biochemicals (e.g., Wang and Lee, 2021; Gebhardt et al., 2022; Weckerle et al., 2023).

How sustainable the production of biogas actually is depends, among other things, on the feedstock. For optimal resource cycling, only wastes and residues would be used (e.g., Theuerl et al., 2019). Currently, however, in addition to municipal biowastes, animal manure, and crop residues, sequential crops and dedicated energy crops are used as feedstock. The specific mix of feedstocks used

depends on their availability, the size, type and operation of the biogas plants, and the policies of the individual countries (Guo et al., 2015; International Energy Agency, 2020).

Europe is the world's largest producer of biogas, with agricultural substrates (28%) and energy crops (25%) being the main feedstocks (Pavičić et al., 2022). Two-thirds of Europe's biogas plant capacity is located in Germany (International Energy Agency, 2020). In 2019, German biogas plants used about 46% renewable resources including energy crops, 49% manure and slurry, 3% municipal biowaste, and 2% residual materials from industry, commerce, and agriculture (Fachagentur für Nachwachsende Rohstoffe e.V. (FNR), 2021). The dominant crop among renewable resources in Germany was and is maize, whose dry matter and methane yield potential is higher than that of any other crop due to its low nutrient demand, high water-use efficiency, and high digestibility (Herrmann, 2013; Deutsches Maiskomitee e.V., 2023). However, maize is representative of numerous negative aspects of the so-called first-generation energy crops. These include intensification of agriculture with high use of fertilizers and pesticides, leading to surface and water pollution, soil erosion, GHG emissions, and damage to the health of humans and herbivores (Altieri, 2009; Herrmann, 2013). In addition, the often-continuous cultivation of maize in large-scale monocultures affects the spread of pests, rodents (Arnold, 2011; Deuker et al., 2012), and weeds (Redwitz and Gerowitt, 2018) and can lead to serious habitat losses for wildlife in arable landscapes (Vollrath, 2009).

Sustainable, future energy crops should not only provide satisfactory methane yields and high value digestates, but also ecosystem services. Ecologically focused approaches would integrate biodiverse mixtures of perennial species into crop rotations on low-productivity or environmentally sensitive farmland, where they could promote pollination, regulate diseases and pests, and regenerate soils by increasing soil organic matter content and retaining water and nutrients (Holland et al., 2015; Englund et al., 2020b; Schulte et al., 2022). In fact, there are already numerous studies exploring the cultivation, utilization, and monetization of (polycultures of) various herbaceous perennial plant species grown on marginal lands (e.g., Voigt et al., 2012; Carlsson et al., 2017; Jones, 2017; Mehmood et al., 2017; Weißhuhn et al., 2017; von Cossel, 2020; Englund et al., 2020a; Freund Saxhaug et al., 2020; Kiefer et al., 2023). More economical approaches aim to use biogas feedstocks combining maize and biodiversity enhancing partners, e.g., row cropping of maize with flowering species (Schulz et al., 2020) or strip cropping with mixtures of wild plant species specifically developed for biogas use (Vollrath et al., 2016; von Cossel et al., 2021).

In summary, an increasing number of plant species is currently entering biogas production, which is a system that closes material loops. Consequently, in the case of using seed-bearing species - or biomass containing weed seeds – it is highly probable that seeds that survive the biogas production process will be released into the environment.

### 1.1.4 Seeds in Biogas Production

The biogas production chain commonly comprises the steps of feedstock supply, feedstock storage, anaerobic digestion, and storage and utilization of biogas and digestate (Fröschle et al., 2015; Hijazi et al., 2016). Seeds can occur in all of these steps (**Figure 1-1**).

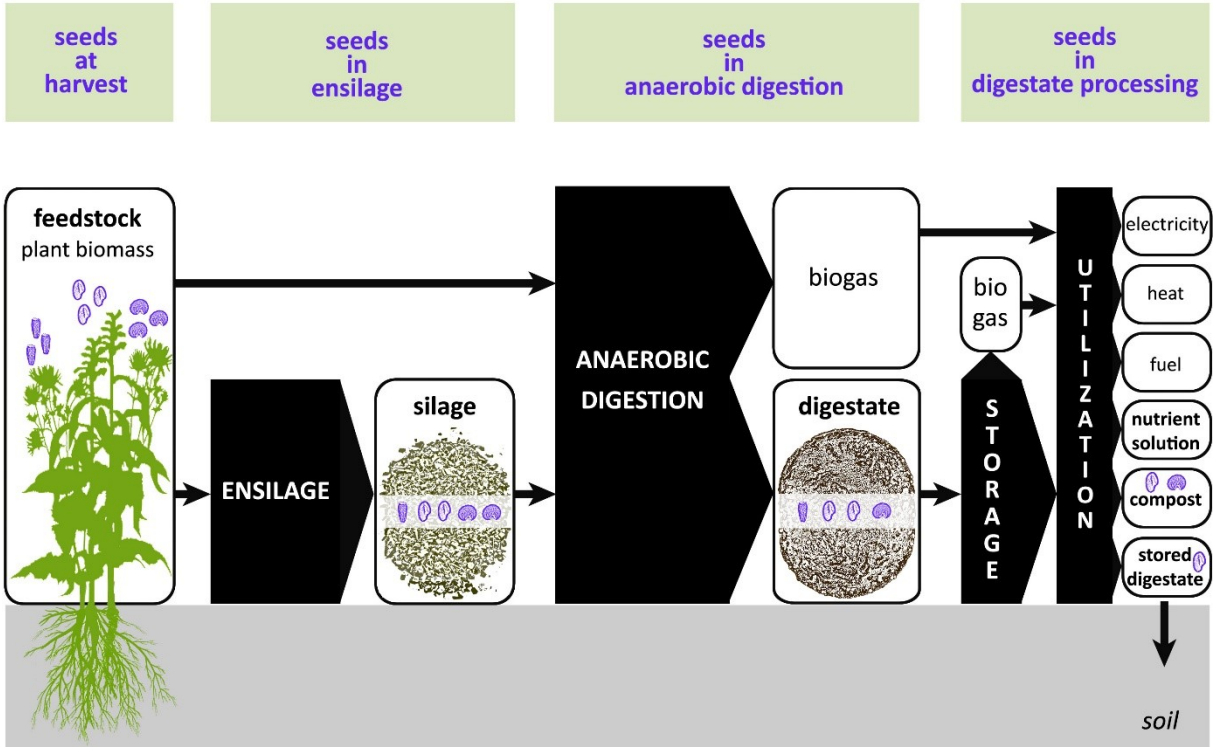


**Figure 1-1 | Potential occurrence of viable plant seeds (purple) in processes (black boxes) and products (boxes outlined in black) of the biogas production chain using plant biomass (green) as feedstock. Image sources in chapter 1.5.1.**

Main entry port for seeds to the biogas production chain is plant biomass, which is harvested around the time of seed maturity. It can come from energy crops, but also weeds (e.g., Westerman and Gerowitt, 2012). In addition, seeds could enter via animal manure or machinery (Christoffoleti et al., 2007).

The harvested biomass, including seeds, can be fed directly into the biogas reactor and thus into anaerobic digestion (AD). However, it is usually preserved and stored beforehand by means of ensilage (**Figure 1-1**; Hijazi et al., 2016; Wendt and Zhao, 2020). Ensilage, i.e., the anaerobic storage of plant biomass by means of lactic acid fermentation, originates from forage production. When used for biogas purposes, it aims to provide a non-seasonal supply and to conserve the methane formation potential. In general, relatively low moisture contents, high contents of available carbohydrates, and low buffer capacities are considered prerequisites for stable silages rich in lactic acid with dry matter contents above 30% and pH-values between 4.1 and 5.0 (Kalač, 2011; Thaysen, 2011; Teixeira Franco et al., 2016). Maize meets these biochemical requirements in an ideal way (McDonald et al., 1991). However, the

complex technique for producing good maize silage cannot be readily transferred to other species (Teixeira Franco et al., 2016). Given the diversification of biogas feedstocks, the need for research is considerable. For example, although commercial wild plant flower mixtures have been developed and are grown specifically for biogas production (Vollrath and Werner, 2012; von Cossel and Lewandowski, 2016), at the time of research for this thesis, there was exactly one peer-reviewed publication on the ensilability of such mixtures (Oh et al., 2010).

The available studies on seed survival in silage have focused on weeds. They predominantly concluded that ensiling can reduce seed viability (e.g., Shevkenek, 1934; Aper et al., 2014; Piltz et al., 2017). However, the extent of seed inactivation varied greatly depending on the species and study, meaning that the results only apply to the seed lots and ensiling conditions tested. In addition, the search for seed traits and silage characteristics that influence seed killing is still ongoing (e.g., Woodward, 1940; Blackshaw and Rode, 1991; Simard and Lambert-Beaudet, 2016; Weller et al., 2016). Therefore, general statements or predictions about survival of seeds of untested species - such as those intended as biogas feedstock - in ensilage are subject to uncertainty.

When entering AD in the biogas reactor, seeds encounter a dynamic biochemical process in the absence of oxygen. A consortium of microorganisms converts complex organic matter into biogas and digestate (Uçkun Kiran et al., 2016). AD process technology aims to create optimal conditions for the microbial community and thus for methane production (Liebetrau et al., 2019). In typical German agricultural biogas plants designed to digest energy crops, AD takes place in continuously stirred tank reactors operated at temperatures of 37 to 42 °C (mesophilic range) and pH-values between 4.5 and 8.2 (Weiland, 2010; Hijazi et al., 2016; Fachagentur für Nachwachsende Rohstoffe e.V. (FNR), 2023). With regard to the wildflower mixtures mentioned above, it was demonstrated that their methane yield per hectare can be increased from 50% to 60.5% of that of maize when undersown under maize. This reduces the trade-off between bioenergy and biodiversity and could make their cultivation more likely (von Cossel et al., 2019b).

Survival of (weed) seeds during AD in biogas plants was reviewed in 2013 by Westerman and Gerowitt. They estimated the risk of AD survival to be high for species with the following characteristics: Hardseededness (HS, physical dormancy), high thermoresistance, a thick seed coat or adaptations to endozoochory. They identified temperature and exposure time as the most important factors for seed inactivation in AD. Subsequent studies have essentially confirmed these findings (e.g., Johansen et al., 2013; Oechsner et al., 2018; Zhou et al., 2020). However, as with ensiling, the results are only valid for the seed lots and biogas reactor types studied. At the time of this thesis, only two studies had explicitly addressed seed survival of wild plants used as biogas feedstock (van Meerbeek et al., 2015; Baute et al., 2016). However, even these cannot be generalized in order to reliably assess seed survival from biogas wild plant mixtures. Moreover, it is not unlikely that wild plant seeds in particular have as yet unexplored responses and resistance mechanisms to AD (Westerman and Gerowitt, 2013). This is because the seeds of wild plants and weeds display a wide variety of physical, chemical, and

biological properties that help them to persist in soil (e.g., Long et al., 2015) - and possibly also in AD. Furthermore, there is no information on how the scale of the AD system and the associated level of process control affect seed survival. After all, the range extends from lab-scale batch tests in glass bottles (Eckford et al., 2012) to full-scale commercial, completely stirred tank reactors (Leonhardt et al., 2010). In other words, it is unclear, to what extent the results from different AD systems are transferable to each other. In this context, the hypothesis of Westerman and Gerowitt (2013) remains to be proven that the determination of sensitivity to thermal inactivation is suitable for determining the minimum seed mortality in biogas reactors. If this was the case, tests in reactors could be replaced by less laborious water-bath tests.

Summed up, there is still a lack of systematic research on seed survival in AD and ensilage. It is therefore unclear whether and to what extent the seeds of wild plant feedstocks can survive these processes of biogas production.

## 1.2 Goals and Objectives

**The overarching goal** of this thesis is to expand the knowledge of seed fate in plant biomass-based processes of the circular bioeconomy. Specifically, the question was whether using the biomass of ecologically valuable wild plants as a feedstock for farm-based biogas production risks contaminating the resulting products with viable seeds. In answering this question, both agricultural and seed biological aspects were addressed. This means that, on the one hand, seed viability was analyzed with regard to response and resistance mechanisms and, on the other hand, seed inactivation was considered with regard to its agricultural and ecological implications.

**Object of study** was the survival of wild plant seeds in ensilage and anaerobic digestion (AD). For AD, the focus was exclusively on seed viability. For ensilage, it was additionally investigated whether the biomass of species-rich, flowering wild plant mixtures is suitable for storage in silage and could thus enter the biogas production chain via this route - together with its seeds.

**In detail**, the focus was on the following:

**I. Seed viability**

a. gain insight into which seed characteristics may favor survival, with emphasis on:
   - physical dormancy, i.e., hardseededness
   - potentially genus- or family-based resistance mechanisms

b. explore the diversity of seed viability responses of different species to AD and ensilage in order to broaden the data base

c. evaluate the suitability of seed thermoresistance as a proxy of survival in AD

**II. Seed inactivation**

a. determine seed-killing efficacy of AD depending on selected process parameters:
   i. exposure time
   ii. operating temperature (mesophilic range)
   iii. level of process control (lab-scale, full-scale)

b. determine seed-killing efficacy of ensilage, more precisely of silage types differing in:
   i. ensiled feedstock
   ii. ensiling conditions
   iii. silage biochemical characteristics

**III. Seed-bearing wild plants as biogas feedstock**

a. determine ensilability of wild plant biomass as a prerequisite for its use for bioenergy

b. assess the results with regard to the risk of seed contamination of silage and digestate

## 1.3 Methodology

Briefly, the methodology of this thesis was to

(1) select relevant plant species,

(2) expose their seeds to treatments in anaerobic digestion (AD) or ensilage,

(3) determine seed viability before and after the treatments, and

(4) statistically analyze seed survival.

### 1.3.1 Species Selection

The focus of this work was on wild plant species, i.e., species whose characteristics, including their resistance potential, have not yet been modified or only slightly modified by breeding. In addition, the focus was on dicotyledonous species because monocotyledonous species, with few exceptions, have been shown to rapidly lose viability during treatment in ensilage and AD (e.g., Blackshaw and Rode, 1991; Leonhardt et al., 2010; Piltz et al., 2017; Zhou et al., 2020).

The first criterion for selection was that the species were either capable of producing HS seeds (HS species) or belonged to different non-hardseeded (NHS) species that had not yet been tested in ensilage or AD. Secondly, species were selected that are used or considered as feedstock for biogas production or are weeds in Europe. In detail, most species were selected from a wildflower mixture specifically designed for biogas production, namely the mixture "BG70" from Saaten Zeller GmbH & Co. KG (Eichenbühl-Guggenberg, Germany, saaten-zeller.de). The weed species studied occurred predominantly in maize. Finally, three species were included that were resistant to AD in previous studies, namely *Abutilon theophrasti*, *Chenopodium album*, and tomato (**Table 1-1**).

In total, 16 different species were exposed to the treatments in AD or ensilage. For some species, different seed lots were used to investigate the influence of seed quality on survival success. Seeds of HS species and *C. album* were subjected to all treatment variants due to their expected high resistance. The remaining NHS species were tested in selected treatments only (**Table 1-1**).

**Table 1-1 | Species and seed lots whose seeds and/or biomass were subjected to the treatments in anaerobic digestion (AD) and ensilage compiled in this thesis. Species are sorted according to their families and the associated ability to produce hardseeded (HS) seeds or not (NHS) (cf. Baskin et al., 2000). 'Relevance' indicates why a species was selected and 'seed lot' summarizes the seed´s harvest year and supplier. Treatments were: mesophilic AD in a full-scale commercial reactor ($AD_{CR}$) or in a lab-scale experimental reactor ($AD_{ER}$), buffer solution in a water-bath (WB), and ensilage in lab-scale silos ($E_{LS}$).**

| Species | Common name | Family | Relevance[1] | Seed lot | | Treatment | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Year | Supply[2] | $AD_{CR}$ | $AD_{ER}$ | WB | $E_{LS}$ |
| **HS species (seeds)** | | | | | | | | | |
| *Abutilon theophrasti* Medikus | velvetleaf | Malvaceae | reference [a,b,c] \| $weed_{MO}$ [d,e,f,g,h] | 2008 | ESP | | x | | x |
| | | | | 2015 | GH | x | x | x | |
| *Malva alcea* L. | rose mallow | Malvaceae | $biogas_F$ | 2014 | AW | | x | x | x |
| | | | | 2015 | AW | x | x | x | |
| *Malva sylvestris* L. | common mallow | Malvaceae | $biogas_F$ \| $weed_{MO}$ [g,h,i,j] | 2014 | GER | | | x | x |
| | | | | 2015 | HE | x | x | x | |
| *Melilotus albus* Medikus | white sweet clover | Fabaceae | $biogas_F$ \| $weed_M$ [h] | 2014 | AW | x | x | x | x |
| *Melilotus officinalis* (L.) Pallas | yellow sweet clover | Fabaceae | $biogas_F$ | 2014 | AW | x | x | x | x |
| **NHS species (seeds)** | | | | | | | | | |
| *Ambrosia artemisiifolia* L. | common ragweed | Asteraceae | $weed_{MO}$ [f,g,h,l,m] | 2016 | USA | | | x | |
| *Chenopodium album* L. | common lambsquarters | Amaranthaceae | reference [b,c,n] \| $weed_{MO}$ [g,h,i,o,p] | 2014 | GER | x | x | x | x |
| *Centaurea nigra* L. | common knapweed | Asteraceae | $biogas_F$ | 2016 | GER | | | x | |
| *Cichorium intybus* L. | blue dandelion | Asteraceae | $biogas_F$ \| $weed_{MO}$ [g,h,i] | 2014 | AW | | x | | x |
| *Cynodon dactylon* (L.) Persoon | bermuda grass | Poaceae | $weed_{MO}$ [g,h,q,r,s] | 2016 | GH | | | x | |
| *Daucus carota* L. | wild carrot | Apiaceae | $biogas_F$ \| $weed_O$ [g,k,t] | 2014 | AW | | | | x |
| | | | | 2015 | HE | | x | x | |
| *Echium vulgare* L. | vipers bugloss | Boraginaceae | $biogas_F$ | 2014 | AW | | | | x |
| | | | | 2015 | HE | | x | | |
| *Fallopia convolvulus* (L.) Löve | wild buckwheat | Polygonaceae | $weed_{MO}$ [g,h,u,v,w] | 2015 | GER | | | x | |
| *Lycopersicon esculentum* Miller | tomato | Solanaceae | reference [b,c] | 2014 | CU | | x | | |
| | | | | 2015 | BH | | x | x | |
| *Polygonum aviculare* L. | common knotgrass | Polygonaceae | $weed_{MO}$ [g,h,q,u,w] | 2015 | GER | | | x | |
| *Verbascum thapsus* L. | great mullein | Scrophulariaceae | $biogas_F$ | 2014 | AW | | x | | x |
| **HS and/or NHS species (biomass)** | | | | | | | | | |
| wildflower mixture[3] | - | - | $biogas_F$ | - | - | | | | x |
| *Zea mays* L.[4] | maize | Poaceae | $biogas_O$ [x,y,z] | - | - | | | | x |
| *Melilotus* sp.[5] | sweet clover | Fabaceae | $biogas_F$ | - | - | | | | x |

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**Table 1-1 | footer**

[1] **Relevance**: **biogas$_F$** – species contained in wildflower mixture designed for biogas production ("BG70", Saaten Zeller GmbH & Co. KG, Eichenbühl-Guggenberg, Germany, saaten-zeller.de); **biogas$_O$** – species used or suggested as feedstock for biogas production (x – Herrmann and Rath, 2012; y – Hofmann et al., 29/2017; z – Fachagentur für Nachwachsende Rohstoffe e.V. (FNR), 2021); **weed$_M$** – weed in maize in Europe (d - Sattin et al., 1992; e - Follak et al., 2014; f – Follak et al., 2017; g – Weber and Gut, 2005; o - Głowacka, 2011); **weed$_O$** – weed in other culture crops in Europe (h – Jensen et al., 2011; i – European and Mediterranean Plant Protection Organization, 2023; j – Fried et al., 2019; k – Morrison et al., 2021; l – Smith et al., 2013; m – European and Mediterranean Plant Protection Organization, 2021; p – Bajwa et al., 2019; q – European and Mediterranean Plant Protection Organization, 2013; r – Nehring et al., 2013; s – Soares et al., 2023 and references therein; t – Wijnheijmer et al., 1989; u - Meiss et al., 2010; v - Bitarafan and Andreasen, 2020; w – Mehrtens et al., 2005); **reference** – seeds of species survived AD in previous studies (a - Katovich et al., 2004; b - Westerman et al., 2012b; c - Westerman et al., 2012a; n - Šarapatka et al., 1993)

[2] **Supply**: AW - Appels Wilde Samen GmbH, Darmstadt, Germany (appelswilde.de); BH – tomato variety "St. Pierre" from Bingenheimer Saatgut AG, Echzell-Bingenheim, Germany (bingenheimersaatgut.de); CU – tomato variety "paprikaförmige" from Culinaris Saatgut für Lebensmittel, Göttingen, Germany (culinaris-saatgut.de); ESP – collected by Paula R. Westerman in a sunflower field, Vilanova de Bellpuig, Lleida, Spain; GER – collected on experimental plots of the University of Rostock, Rostock, Germany; GH – Seeds from greenhouse trials, Rostock, Germany; HE - Herbiseed Ltd., Twyford, UK (herbiseed.com); USA – collected on maize field margin by Adam Davis, Urbana, Illinois, USA.

[3] mixture "BG70", see [1] "biogas$_F$".

[4] variety "Ronaldinho", KWS SAAT SE & Co. KGaA, Einbeck, Germany (kws.com).

[5] almost pure stands that had developed from the wildflower mixture, see [3].

### 1.3.2 Treatments

#### 1.3.2.1 Anaerobic Digestion incl. water-bath experiments

The treatments in anaerobic digestion (AD) were performed in three systems that differed in size and degree of process control. They included a full-scale commercial biogas reactor ($AD_{CR}$), laboratory-scale experimental reactors ($AD_{ER}$), and experiments in water-baths (WBs). The $AD_{ER}$ treatments were the core of the AD studies. The other two treatments served to complement the results by determining seed survival under practical conditions ($AD_{CR}$) and under sterile conditions (WB) (**Table 1-1**, **Table 1-2, Figure 1-3**).

All AD treatments took place in the mesophilic temperature range. This was chosen instead of the thermophilic temperature range because it is most commonly used in agricultural biogas plants in Europe (Weiland, 2010; Kovačić et al., 2022 and references therein) and carries a higher risk of pathogen and seed contamination (e.g., Fröschle et al., 2015; Lebuhn et al., 2023).

#### 1.3.2.2 Ensilage

For the ensilage treatments, chopped biomass was ensiled in lab-scale silos ($E_{LS}$) (**Table 1-1**, **Table 1-2, Figure 1-3**). The silos were filled by hand and stored up to nine months in the dark. Different silage types were prepared using different biomass compositions and ensiling conditions. Both biomasses and silages were characterized biochemically.

**Table 1-2 | Characteristics of the systems used for anaerobic digestion and ensilage of seeds, namely a commercial biogas reactor (CR), experimental biogas reactors (ER), test tubes filled with buffer in a water-bath (WB) and lab-scale silos (LS). Temperature and pH values are medians.**

| System | Type | Volume | Feedstock(s) | Operating or storage temperature [°C] | pH |
|---|---|---|---|---|---|
| CR[a] | CSTR[d] | 800 m³ | mixture of maize silage, whole grain cereals and pig slurry | 45 | 7.7 |
| ER[b] | CSTR | 8 dm³ | mixture of maize silage and cattle manure | 35<br>42 | 7.6<br>7.7 |
| WB[c] | batch | 20 cm³ | sterile HEPES[e] buffer | 42 | 7.0 |
| LS[c] | batch | 3 dm³ | maize, wildflowers, mixtures of maize and wildflowers | 16 | 3.9 |

[a] biogas plant located in Wildau-Wentdorf, Dahmetal, Saxony-Anhalt, Germany
[b] located at the Department Technology Assessment, Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), Potsdam, Brandenburg, Germany
[c] located at Group Crop Health, University of Rostock, Rostock, Mecklenburg-Western Pomerania, Germany
[d] (single-phase) completely stirred tank reactor
[e] 4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid

#### 1.3.2.3 Seed exposure to treatments

In the biogas reactors and silos, the seeds were introduced dry and cleaned. For the WB treatments, the seeds were surface sterilized beforehand. The AD treatments of seeds were conducted between March 2015 and September 2016. Ensilage of seed samples took place between September 2014 and May 2016.

During treatments, the seeds should be in direct contact with the respective substrate, but still easy to find and remove. Therefore, seeds were exposed to AD and ensilage sewn into fine-mesh polyester bags (mesh size 200 µm). The bags were affixed to the stirrer in the lab-scale reactors (**Figure 2-1** in Chapter 2.1), sewn in larger bags of stronger polypropylene (mesh size 25 µm) and attached to a probe inserted into the full-scale reactor, or stuffed into the lab-scale silos. In the WB treatments, seeds were exposed to the buffer in the test tubes without bags.

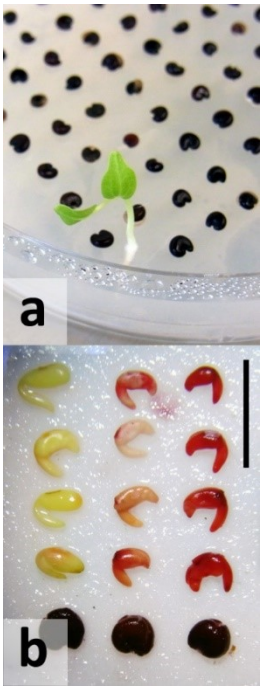
In the AD and WB treatments, samples could be introduced and removed without disturbing the process. Thus, samples could be taken after several exposure times. As seed viability was expected to decrease with increasing exposure time in AD (cf. Westerman and Gerowitt, 2013), the number of seeds was increased with exposure time, ranging from 100 to 300 seeds in one bag. Further, the expected higher resistance of HS species was accounted for by longer exposure times. Unlike the reactors, each lab-scale silo represented a separate unit where opening and air access would have disrupted the ensiling process. Therefore, seeds (and biomass) in ensilage were examined only after one or two exposure times.

### 1.3.3 Determination of Seed Viability

This thesis aimed to determine the viability of all seeds capable of surviving AD or ensiling. Germination tests would not have been adequate for this purpose, as many of the species studied could

produce hardseeded, i.e., physically dormant, seeds. Dormant seeds are viable, but would not germinate even under favorable conditions (Baskin and Baskin, 2004). Therefore, the test procedure used by Westerman et al. (2012a, b) was adopted and seed viability was calculated as:

$$total\ seed\ viability = \frac{\Sigma germinated\ seeds + \Sigma dormant\ seeds}{\Sigma number\ of\ tested\ seeds}$$




In short, the test procedure included the following steps: After treatment, the seed bags were rinsed with tap water. Seeds were surface sterilized and counted to determine the number of seeds that were externally intact. These seeds were subjected to a 21-day germination test on a growth medium ('diaspore agar', **Figure 1-2 a**) under light and temperature conditions designed to break dormancy, promote germination, and prevent pathogen contamination. A seed was considered germinated when the radicle had protruded at least 2 mm. Subsequently, the metabolic activity of all seeds that had not germinated was tested using 2,3,5-triphenyltetrazolium chloride (TTC, **Figure 1-2 b**) (Elias et al., 2012; Vollrath et al., 21/2013; França-Neto and Krzyzanowski, 2019). A seed was judged fully viable but dormant if the embryo – and endosperm, if relevant – was stained red (Association of Official Seed Analysts, 2010).

**Figure 1-2 | Seed viability. The black bar in Figure 1-2 b equals 10 mm. Image sources in Chapter 1.5.1.**

### 1.3.4 Statistical Analysis of Seed Survival

Depending on the study, total viability, percentage of dead seeds and/or percentage of germinated seeds were analyzed. The frequency of sampling in the treatments determined the type of data analysis: For AD and WB treatments, seed viability was modeled as a function of exposure time. For ensilage treatments, seed inactivation and the effects of (en-)silage properties were analyzed based on (generalized) linear mixed effect models.

Finally, seed-killing efficacies (*SKE*s) were calculated to compare the effect of different treatments. The *SKE*s correspond to the decrease in viable seeds after a certain time of exposure to a treatment compared to untreated controls. Viability of untreated control seed lots was determined several times during the study period to account for changes during storage. Only controls performed at a similar time point as a particular treatment were used to estimate the effect of that treatment.

## 1.4 Outline of Thesis Chapters

An illustrated overview of all chapters is provided in **Figure 1-3**.

**Chapter 2** explores the survival probability of seeds in mesophilic, anaerobic digestion (AD). The focus is on the effects of exposure time, temperature, and scale of the treatment system.

**Chapter 2.1** (Hahn et al., 2022) is a multi-aspect study of seed viability of wildflower species in AD in lab-scale, experimental reactors ($AD_{ER}$). The response of different species and seed lots to AD was investigated for two mesophilic temperatures and after four exposure times. Five of the species were hardseeded (HS), while seven were non-HS (NHS). The effects of AD were considered in terms of both seed viability and percentage of germinated seeds. The aspects studied were discussed in the context of seed resistance potential and the suitability of wildflowers as biogas feedstock.

- **$AD_{ER}$ | max. 36 d | 35°C and 42°C | seeds of 11 species**

**Chapter 2.2** (Hahn et al., 2016) examines the contribution of temperature to seed inactivation in AD relative to other mortality factors. Seed viability of one HS and one NHS species was compared between lab-scale AD and exposure to a buffer solution in water-baths (WBs).

- **$AD_{ER}$ and WB | max. 12 d | 42°C | seeds of 2 species**

**Chapter 2.3** (Hahn et al., 2018) presents a screening for the thermoresistance of eight NHS and three HS species from eight plant families in WBs. Species tested were weeds, tomato, and wildflowers from a biogas mixture. Seeds of selected species were additionally exposed to lab-scale AD in order to test the predictive value of the WB screening. The aim was to expand the database on seed survival by adding responses from new species and potentially discover resistance mechanisms other than HS.

- **$AD_{ER}$ and WB | max. 18 d | 42°C | seeds of 11 species**

**Chapter 2.4** (Hahn et al., 2023) is a comparison of seed inactivation between lab-scale systems, namely experimental reactors and WBs, and a full-scale, commercial biogas reactor ($AD_{CR}$). Five HS and one NHS wild plant species were tested. The intention was to determine the seed-killing efficacy of $AD_{CR}$ and to clarify whether testing thermoresistance in WBs could provide an estimate of seed survival in full-scale AD.

- **$AD_{CR}$, $AD_{ER}$ and WB | max. 36 d | 42°C – 45°C | seeds of 6 species**

**Chapter 3** addresses the ensilability of wild plant biomass and the possibility that its seeds could be killed by ensilage. The focus is on the effects of biochemical characteristics of feedstocks and silages.

**Chapter 3.1** (Müller and Hahn, 2020) investigates whether the species-rich, ecologically valuable biomass of wildflower strips could be ensiled achieving a silage quality that ensures low-loss storage. Biomasses from different wildflower stands and mixtures with maize were tested. Ensilability was (1) estimated from the biochemical characteristics of the harvested biomass and (2) determined from the fermentation patterns of silages prepared in lab-scale silos ($E_{LS}$). On this basis, influences on the ensiling success and suitability of ensiled wildflower biomass for bioenergy use were evaluated.

➢ **$E_{LS}$ | 90 d | 16°C | no seeds**

**Chapter 3.2** (Hahn et al., 2021) assesses the potential of ensilage to inactivate seeds to an extent relevant for weed control. Seeds of five HS and five NHS wild plant species were exposed to silages prepared from different feedstocks and under varying conditions. Seed-killing efficacies determined for different silage types were discussed with regard to the silages' biochemical composition and in the context of integrated weed management.

➢ **$E_{LS}$ | 4 to 9 months |16°C | seeds of 10 species**

**Chapter 4** provides a synthesis of the results of Chapters 2 and 3 and suggests directions for future research on seed survival in the context of the agricultural utilization of seed-bearing (wild) plant biomass.

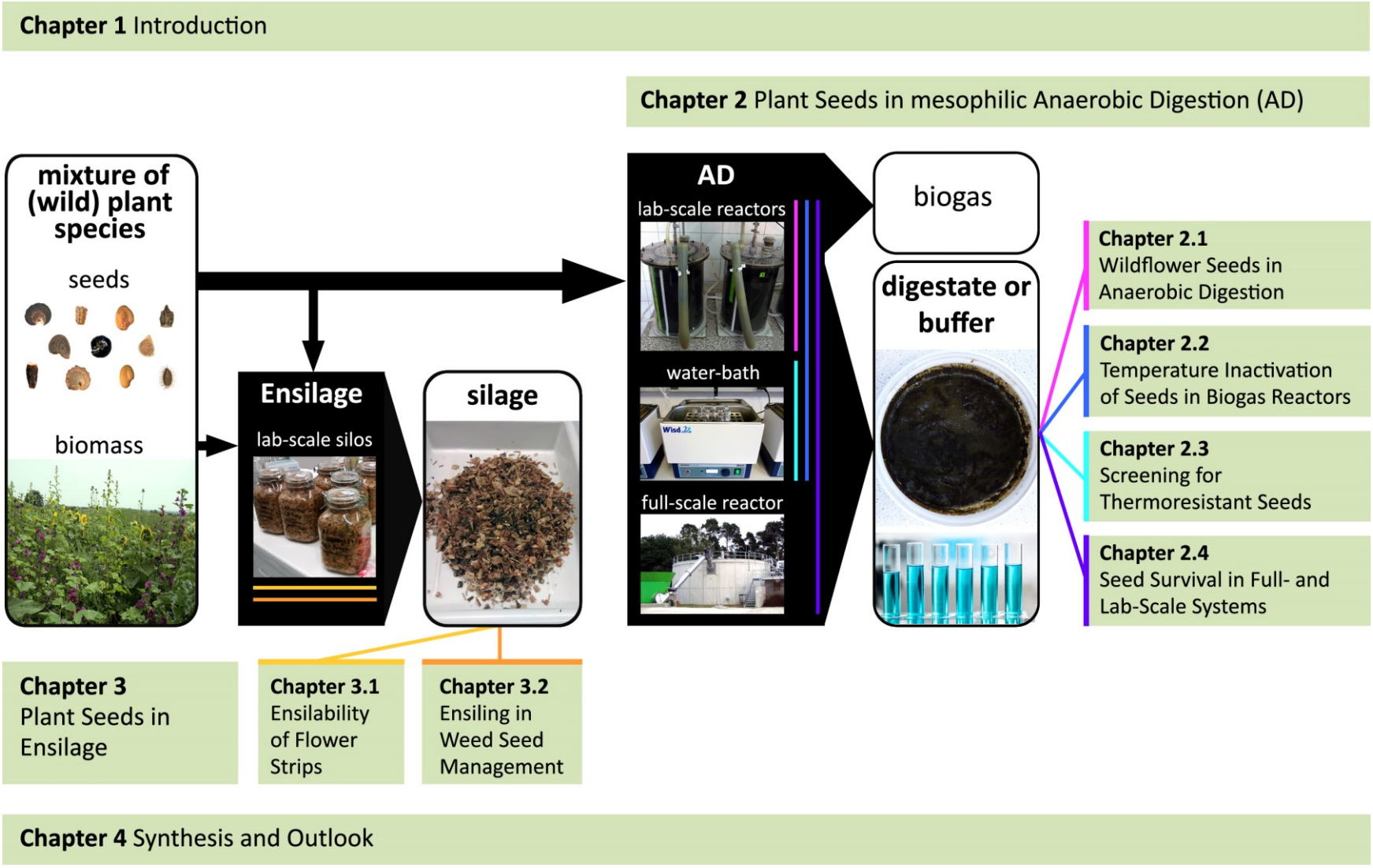



**Figure 1-3 | Illustrated chapter overview. Image sources in Chapter 1.5.1.**

## 1.5 References

### 1.5.1 Images

| | |
|---|---|
| Biomass collage | assembled from adaptations of: Wilken D, Rauh S, Weiß R, Strippel F, Wiesheu M, Luyten-Naujoks K, Kirsch A, Herbes C, Kurz P, Halbherr V, Dahlin J, Nelles M (März 2019) Düngen mit Gärprodukten, p. 6. + https://de.freepik.com/vektoren-premium/faseriges-wurzelsystem-schwarze-silhouette-des-verzweigten-rhizoms-vektorgrafik-des-unterirdischen-teils-von_38951135.htm + https://pixabay.com/de/vectors/zweig-wurzel-pflanze-natur-holz-576680/ + https://pixabay.com/de/vectors/lebensmittel-meerrettich-pflanze-2027539/ (all accessed 05.07.2023) |
| Biomass photo | https://www.jagdverband.de/sites/default/files/Marcus_B%C3%B6rner_MG_9111_0.JPG (accessed 1.07.2023) |
| Buffer test tubes | adapted from https://www.crushpixel.com/de/stock-photo/chemistry-laboratory-glassware-science-laboratory-2962681.html (accessed 20.07.2023) |
| Digestate | © Vincent Plogsties, ATB (03.05.2016) |
| Seed photos | Cappers RT, Bekker RM, Jans JE (2012) Digitale zadenatlas van Nederland. Digital seed atlas of the Netherlands. Barkhuis Publ, Eelde, 502 pp. |
| Seedling on agar | by Juliane Hahn (30.05.2016) |
| Seeds stained with TTC | by Juliane Hahn (30.10.2015) |
| Silage | by Ophélie Rollin (21.07.2015) |
| Silos (lab-scale) | by Ophélie Rollin (02.06.2015) |
| Reactor (full-scale) | © Vincent Plogsties, ATB (2010) |
| Reactors (lab-scale) | © Vincent Plogsties, ATB (15.09.2015) |
| Water-bath | by David Parzych (18.02.2016) |

### 1.5.2 Literature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# 2

# Plant Seeds in mesophilic Anaerobic Digestion

## 2.1 Wildflower Seeds in Anaerobic Digestion

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### Viability of Wildflower Seeds after Mesophilic Anaerobic Digestion in Lab-Scale Biogas Reactors

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The use of wildflower species as biogas feedstock carries the risk that their seeds survive anaerobic digestion (AD) and cause weed problems if spread with the digestate. Risk factors for seed survival in AD include low temperature, short exposure and hardseededness (HS). However, it is not possible to predict how AD will affect seed viability of previously unstudied species. In laboratory-scale reactors, we exposed seeds of eight species from a mixture of flowering wild plants intended as biogas feedstock and three reference species to AD at two mesophilic temperatures. Half of the species were HS, the other was non-HS (NHS). Viability was determined using a combination of tetrazolium and germination tests. Viability and germinability were modeled as functions of exposure time using a dose-response approach. Responses to AD varied considerably among species, and none of the considered influencing factors (time, temperature, HS) had a consistent effect. Seed lots of a species differed in inactivation times and seed-killing efficacy. The HS species *Melilotus officinalis*, *Melilotus albus*, and *Malva sylvestris* were particularly AD-resistant. They were the only ones that exhibited biphasic viability curves and tended to survive and germinate more at 42°C than at 35°C. Viability of the remaining species declined in a sigmoidal curve. Most NHS species were inactivated within a few days (*Cichorium intybus*, *Daucus carota*, *Echium vulgare*, and *Verbascum thapsus*), while HS species survived longer (*Malva alcea*). AD stimulated germination in the HS species *A. theophrasti* and its AD-resistance overlapped with that of the most resistant NHS species, *C. album* and tomato. In all seed lots, germinability was lost faster than viability, implying that mainly dormant seeds survived. After the maximum exposure time of 36 days, seeds of HS species and *Chenopodium album* were still viable. We concluded that viability responses to mesophilic AD were determined by the interplay of AD-conditions and species- and seed-lot-specific traits, of which HS was an important but only one factor. For the use of wildflowers as biogas feedstock, we recommended long retention times and special care with regard to HS species.

**Keywords:** **dose response models, exposure time, flowering wild plant mixtures, hardseededness, physical dormancy, seed survival, seed viability, temperature**

### 2.1.1 Introduction

Anaerobic digestion (AD) of renewable feedstocks in biogas plants is considered one of the most environmentally friendly and energy efficient bioenergy sources. A particular advantage is that the semi-solid leftover of biogas production, the digestate, which is produced in addition to the energy carrier methane, can be used as plant fertilizer, thus tightening nutrient cycles (Weiland, 2010; Guo et al., 2015;

Salnikova et al., 2019). AD feedstocks have included energy crops such as maize (*Zea mays* L.), triticale (× *Triticosecale*) or beets (*Beta vulgaris* L.) for decades (e.g., Venendaal et al., 1997; Herrmann et al., 2016; Hofmann et al., 2017). The sustainability of using energy and agrofuel crops, however, has become increasingly controversial (e.g., Altieri, 2009; Eggers et al., 2009; Meyer-Aurich et al., 2012; Gasparatos et al., 2013). In response, there are now calls for biomass production systems to be multifunctional and adapted to local conditions (von Cossel et al., 2019b; Englund et al., 2020b). For this reason, among others, the portfolio of energy crops continues to expand (e.g., Papamatthaiakis et al., 2021). The focus is on perennial species that offer a variety of ecological benefits at low input (Don et al., 2012; Müller-Stöver et al., 2016; Emmerling et al., 2017; Hofmann et al., 2017; Jones, 2017; Englund et al., 2020a). Probably the most diverse option at present is the cultivation of perennial species mixtures (e.g., von Cossel and Lewandowski, 2016; Carlsson et al., 2017; Weißhuhn et al., 2017; Yang et al., 2018).

Since 2008, mixtures of flowering wild plant species were introduced in Germany to supplement silage maize as a biogas feedstock (Vollrath, 2012; Vollrath et al., 2016; von Cossel and Lewandowski, 2016). These flowering mixtures are of interest mainly because they significantly improve ecosystem services such as habitat functioning, soil protection and landscape aesthetics (von Cossel, 2020; Janusch et al., 2021), while their methane yield is rather low (von Cossel et al., 2019a, 2021; Lask et al., 2020). However, a sustainability issue rarely considered is that wildflower mixtures and other energy crops can spread undesirably into new habitats. When used as a biogas feedstock, propagules such as seeds that survive the biochemical processes during AD enter the digestate. There is a risk that these establish as weeds on fields fertilized with this digestate. Of course, seed persistence in soil is only one of many criteria for weediness (Baker, 1974), however, according to Harper (1977) it is important for the success of plants in farmed fields. The weed control measures required upon seed survival would compromise sustainability and cause undesirable, additional costs and labor. Non-native or quarantine species not yet widespread are particularly problematic in this context (Raghu et al., 2006; Simberloff, 2008; Westerman and Gerowitt, 2013). In this regard, the biogas wildflower mixtures should be evaluated with care, as they contain various poorly cultivated (wild) species. However, whether seeds of species from biogas wildflower mixtures survive AD has not yet been the subject of investigations. In general, studies on seed susceptibility to AD are scarce and systematic studies on the ability of seeds from different taxonomic and functional groups to survive AD are lacking (Westerman and Gerowitt, 2013). Thus, reliable predictions of seed viability of previously unstudied plant species in AD are not possible.

Most available studies on seed survival in AD dealt with weeds (e.g., Jeyanayagam and Collins, 1984; Šarapatka et al., 1993; Schrade et al., 2003; Eckford et al., 2012; Westerman et al., 2012a; Johansen et al., 2013; Zhou et al., 2020). Of the plants whose biomass is (intended to be) used as biogas feedstock, only 14 species have been studied to date (Heiermann et al., 2010; Strauß et al., 2012; van Meerbeek et al., 2015; Baute et al., 2016; Sölter et al., 2016; Starfinger and Sölter, 2016; Hassani et al., 2021). Based on weeds studied through 2012, Westerman and Gerowitt (2013) identified plant groups whose seeds

might have a higher probability of surviving AD than usual. They comprised species that are either hardseeded (HS), i.e., form physically dormant seeds with one or more impermeable layers in the seed or fruit coat (Baskin et al., 2000), and species adapted to dispersal by endozoochory, e.g., by thick seed coats. HS is common in the Fabaceae and occurs in members of the Malvaceae (Baskin et al., 2000), both of which are families of interest for biogas flowering mixtures (Vollrath, 2012). HS as a risk factor for high AD resistance potential and consequently for seed dispersal with the digestate has been explicitly mentioned by Leonhardt et al. (2010), Westerman et al. (2012a,b), and Hassani et al. (2021). However, not all species resistant to AD are HS, so it is suspected that other seed traits may aid seed survival in AD as well (Westerman and Gerowitt, 2013).

In addition to characteristics of the seeds themselves, temperature and exposure time were found to be the most important factors driving seed inactivation in AD. In general, seed viability decreases exponentially with time, with the seeds remaining unaffected by AD during an initial lag phase (Westerman and Gerowitt, 2013). In addition, higher temperatures result in a greater decrease in seed viability (reviewed by Westerman and Gerowitt (2013) and confirmed by Johansen et al. (2013); Oechsner et al. (2018), and Zhou et al. (2020). In particular, ADs under thermophilic conditions (approx. 45–55°C) appear to be significantly more effective in killing seeds than mesophilic ones (approx. 30–45°C) (Šarapatka et al., 1993; Lorenz et al., 2001; Schrade et al., 2003; Westerik and Kleizen, 2006; Leonhardt et al., 2010; Johansen et al., 2013; Zhou et al., 2020). This implies that ADs in the mesophilic temperature range pose a higher risk of unintended seed spread – as pointed out by Westerman and Gerowitt (2013); Alsanius et al. (2021), and Hassani et al. (2021). With regard to the use of wildflower species as biogas feedstock, this could be problematic, as they are to be grown mainly in Germany, where 84% of biogas plants are mesophilic (vTI, 2009).

Finally, existing studies differ in which seeds they consider viable. Many studies on the effects of AD on seed viability have based their conclusions solely on germination tests (Engeli et al., 1993; Lorenz et al., 2001; Ryckeboer et al., 2002; Schrade et al., 2003; Marcinisyn et al., 2004; Westerik and Kleizen, 2006; Strauß et al., 2012; Johansen et al., 2013; Miloti´c and Hoffmann, 2016; Oechsner et al., 2018; Zhou et al., 2020). In doing so, they did not consider that dormant seeds may have survived andcould germinate once dormancy is broken. However, HS species and wild plant species in general can exhibit different classes, levels, and types of dormancy (Baskin and Baskin, 1998, 2004). Therefore, to determine the actual risk of spreading viable seed with the digestate, the (total) viability must be determined as the sum of germinable and dormant seeds. This procedure has only been used in some studies on seed survival in AD (Jeyanayagam and Collins, 1984; Eckford et al., 2012; Westerman et al., 2012a,b; Baute et al., 2016).

The objective of this study was to evaluate the effects of mesophilic AD on seed viability of wildflower species intended as biogas feedstocks. In addition, three species that have already been investigated in similar studies were included as references. The focus was on the impact of AD-process control parameters on seed viability of hardseeded (HS) or non-hardseeded (NHS) species. Seed viability was

explored as a function of exposure time in AD at two mesophilic temperatures. We hypothesized that in AD, (1) seed viability of species with HS would be reduced less than that of NHS species, (2) seed viability would decrease more at higher incubation temperatures, and (3) seed viability would decrease with increasing exposure time. In addition, for both HS and NHS species, we examined whether seeds that survived AD were germinable or dormant. Finally, we discussed the implications of the results with respect to the use of wildflower species as biogas feedstocks.

## 2.1.2 Materials and Methods

### 2.1.2.1 Plant Species

#### 2.1.2.1.1 Species Selection

Seed vitality after mesophilic anaerobic digestion (AD) was studied in eleven different species. Five of the species were hardseeded (HS) the others not (NHS). The majority of species were selected from a wildflower mixture that has been specifically designed for biogas production ("BG70" by Saaten Zeller GmbH & Co. KG, Eichenbühl-Guggenberg, Germany)[1]. From this mixture, *Malva alcea* L. (rose mallow, Malvaceae), *Malva sylvestris* L. (common mallow, Malvaceae), *Melilotus albus* MEDIK. (white sweet clover, Fabaceae) and *Melilotus officinalis* (L.) PALL. (yellow sweet clover, Fabaceae) were selected to represent HS species. NHS representatives were *Cichorium intybus* L. (Blue dandelion, Asteraceae), *Daucus carota* L. (wild carrot, Apiaceae), *Echium vulgare* L. (viper's bugloss, Boraginaceae) and *Verbascum thapsus* L. (great mullein, Scrophulariaceae). In selecting NHS species, emphasis was placed on ensuring that they were from diverse families whose response to mesophilic AD has been poorly investigated.

In addition to the eight flowering species from the biogas mixture, this study included one HS and one NHS weed species that were found to be relatively resistant to mesophilic AD. *Abutilon theophrasti* MEDIK. (Malvaceae, velvetleaf) is a HS species whose seeds that survived AD with relatively high probability (Katovich et al., 2004; Westerman et al., 2012a,b). The NHS species *Chenopodium album* L. (Amaranthaceae, common lambsquarters) and tomato (*Lycopersicon esculentum* Mill., Solanaceae) were among the best surviving NHS species in several AD-treatments (Engeli et al., 1993; Šarapatka et al., 1993; Lorenz et al., 2001; Schrade et al., 2003; Katovich et al., 2004; Westerik and Kleizen, 2006; Leonhardt et al., 2010; Strauß et al., 2012; Westerman et al., 2012a,b; Johansen et al., 2013; Baute et al., 2016; Zhou et al., 2020).

#### 2.1.2.1.2 Seed Lots, Seed Acquisition, and Storage

The following species were tested using only one seed lot: *D. carota*, *E. vulgare*, and *M. sylvestris* that were propagated in 2015 and obtained from "Herbiseed" (Twyford, United Kingdom, herbiseed.com). Seeds of *C. intybus*, *M. albus*, *M. officinalis* and *V. thapsus* were propagated in 2014 by "Appels Wilde

[1] saaten-zeller.de

Samen GmbH" (Darmstadt, Germany)[2]. Seeds of *C. album* were harvested in 2014 from *C. album* plants grown at the University of Rostock (Germany).

Two seed lots each were examined of *M. alcea*, *A. theophrasti* and tomato because one lot ran out during the course of the experiments, so a second lot was needed to obtain results for each species in all AD-treatments. Seeds of "*M. alcea* – 2 years" and "*M. alcea* – 1 year" were ordered from "AppelsWilde Samen" (see above) and were propagated in 2014 and 2015, respectively. The seed lot "*A. theophrasti* – 7 years" was propagated and collected in 2008 in a sunflower field in Vilanova de Bellpuig, Lleida (Spain, collector PW). In 2015, the younger lot "*A. theophrasti* – 1 year" was propagated from seeds of "*A. theophrasti* – 7 years" in a greenhouse at the University of Rostock (Germany). The seed lots of tomato came from two different varieties: "paprikaförmige" ['tomato – PAPRIKA', propagated in 2014, "Culinaris – Saatgut für Lebensmittel" (Göttingen, Germany)][3] and "St. Pierre" ['tomato – PIERRE,' propagated in 2015, "Bingenheimer Saatgut AG" (Echzell, Germany)][4].

Until the beginning of and during the experiments in 2015, seeds were stored at room temperature in the dark. The seeds of "*A. theophrasti* – 7 years" harvested in 2008 had previously been stored at 7°C.

#### 2.1.2.2 Anaerobic Digestion of Seeds

##### 2.1.2.2.1 Lab-Scale Reactors

From March 2015 until September 2016 seeds were exposed to mesophilic AD in eight lab-scale continuously stirred biogas reactors at the ATB in Potsdam (Germany) (**Figure 2-1**). The reactors were run according to German Standard Procedure VDI 4630 (VDI-Fachbereich Energietechnik, 2006). They had a working volume of 8 l each and were operated at a constant organic loading rate of 3 $g_{VS}\ l^{-1}\ d^{-1}$ fed with maize silage and cattle slurry. Half of the reactors were run at 35°C, while the other half was run at 42°C, representing the lower and upper mesophilic temperature range of agricultural biogas plants (Weiland, 2010). In order to check the stability of the anaerobic digestion process the biogas produced was continuously measured with a milligascounter type TGC1/5 (RITTER, Bochum, Germany). Biogas volume measured was normalized to standard conditions: dry gas, $t_0 = 273$ K, $p_0 = 1{,}013$ hPa. The biogas composition was determined online using a gas analyzer SSM 6000 (PRONOVA, Berlin, Germany). To evaluate the stability of the biological process, samples of process liquid of each reactor were taken once a week and analyzed for total solids, volatile solids, ammonium nitrogen, total nitrogen, volatile fatty acids and pH (Terboven et al., 2017; **Supplementary Data Sheet 1**).

Continuous biogas production was monitored at both temperatures during seed exposure experiments. The average biogas production in the twelve reactors was $13.1 \pm 2.61\ l_N\ d^{-1}$ and the methane content was 57 ± 2% (data not shown). The actively proceeding biomethanation process was not disturbed by insertion and removal of the seeds (**Supplementary Data Sheet 1**).

[2] appelswilde.de
[3] culinaris-saatgut.de
[4] bingenheimersaatgut.de/de

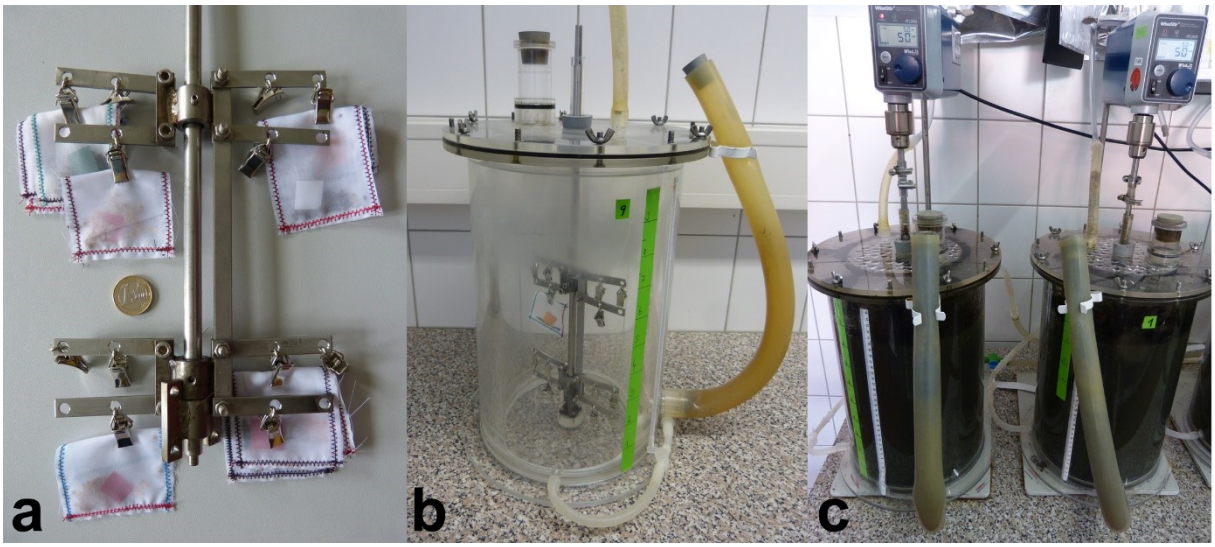


**Figure 2-1 | Experimental setup for anaerobic digestion of seeds in lab-scale reactors. (a) Central paddle stirrer with attached fine mesh polyester bags enclosing seeds and identification markers. (b) Lab-scale reactor consisting of a double-walled cylinder for temperature control via a water jacket, a gas-tight lid with tubes for feeding, gas collection, and attachment of the stirrer, and a separate outlet for the digestate at the bottom. (c) Running reactors connected to thermostat, stirrer drive, gas meter and gas analyzer.**

#### 2.1.2.2.2 Exposure of Seeds to Anaerobic Digestion

Seeds were placed in fine-mesh polyester bags attached to the reactors' stirrer for exposure to AD and subsequent removal (**Figure 2-1**). Seeds were sampled after four different exposure times, and the duration of exposure differed between HS and NHS species. The seeds of NHS species were exposed to AD for 1, 3, 6, and 9 days at both 35 and 42 °C. The species *C. album* and tomato were additionally exposed to 35 °C for 18 and 36 days. Seeds of the HS species were exposed to AD at both temperatures for 3, 9, 18, and 36 days (**Supplementary Table 2-2**). However, since the seeds of *A. theophrasti* – 1 year and *M. alcea* – 1 year were harvested after the experiments with exposure times up to 36 days at 35 °C had already started, they were exposed to AD at 35 °C for only 1, 3, 6, and 9 days (**Supplementary Table 2-2**).

For most species, four replicates were taken at each combination of exposure time and temperature. As seed survival is known to decrease with increasing exposure time (Westerman and Gerowitt, 2013), the number of seeds was increased with exposure time in order to optimize the discriminative power of the assay: Depending on the exposure time, 100, 200, or 300 seeds of a species were anaerobically digested per replicate (**Supplementary Table 2-2**).

#### 2.1.2.2.3 Determination of Seed Viability

After AD, seeds were rinsed with water, transported to the laboratory in Rostock and processed within 5 h after removal from the reactor. Seed viability was determined by testing the germination and metabolic activity of all seeds that did not germinate within 21 days.

In detail, surface sterilized seeds were incubated on plates with "diaspore agar" (agar 13.0 g $l^{-1}$, $KNO_3$ 2.0 g $l^{-1}$, gibberellic acid 0.5 g $l^{-1}$, ampicillin 0.1 g $l^{-1}$, streptomycin 0.1 g $l^{-1}$, benzimidazole 0.02 g $l^{-1}$) at 20/4°C day/night temperatures with a 16 h photoperiod. The number of germinated seeds on the plates was recorded at regular intervals during 21 days. A seed was considered germinated if the radical protruded at least 2 mm from the seed. The viability of all remaining non-germinated seeds was tested using 2,3,5-triphenyltetrazolium chloride (TTC) that indirectly determines the metabolic activity and thus viability of seed tissue cells (França-Neto and Krzyzanowski, 2019). To enable the TTC molecules to enter the seed, seed coats were carefully punctured with a needle or scalpel without inflicting injuries to the embryo (Elias et al., 2012). The punctured seeds were placed between two filter papers, soaked with 3 ml of 1.0% TTC solution and incubated in the dark at 35°C for 20–22 h. Based on the guidelines in the Tetrazolium Testing Handbook (Association of Official Seed Analysts, 2010) seeds were judged fully viable but dormant if the embryo – and endosperm, if relevant - was stained red. Embryos that were not stained (white), poorly stained (light pink), or lacked staining in areas critical for normal seedling development were classified as non-viable. Likewise, seeds whose embryo had rotted or which had already been degraded in the reactor and therefore could not be recovered (lost) also fell into the non-viable category. Ultimately, this test procedure provided the number of seeds that (1) germinated during the 21-day germination test after AD treatment, (2) remained dormant but metabolically active, and (3) were not viable.

The same procedure was used to determine the germination and viability of control seeds that had not been exposed to AD (minimum of three replicates of 300 seeds per species, **Supplementary Table 2-2**). In preparation for the germination tests, the previously dry stored control seeds were exposed to a water-saturated atmosphere for 2 days in the dark.

#### 2.1.2.3 Statistical Analyses

##### 2.1.2.3.1 Seed Viability Models

Both germinated and dormant seeds were viable after the different exposure times in mesophilic AD. For each replicate, the proportion of viable seeds, V, was calculated by dividing the cumulative number of germinated seeds after 21 in the germination test plus the number of dormant seeds by the total number of evaluated seeds (**eq. 2-1**).

$$V = \frac{\sum \text{germinated seeds} + \sum \text{dormant seeds}}{\sum \text{total number of evaluated seeds}} \quad \textbf{(2-1)}$$

With $V$, proportion of viable seeds observed in a replicate after a certain exposure time to AD; $\sum$germinated seeds, cumulative number of germinated seeds from the 21-day germination test;

∑dormant seeds, number of seeds viable in tetrazolium testing after the germination test; ∑total number of evaluated seed; total number of evaluated seeds in the replicate.

All statistical analyses were carried out using the software environment R (version 4.1.2) (R Core Team, 2021). Seed viability as a function of exposure time, *V(t)*, was modeled with a dose-response approach using the R-package "drc" (version 3.0.1, Ritz et al., 2015). The response variables, *V* or *V(t)*, mean the "observed *V*" of the samples or its values modeled over time. Log-logistic models with a lower limit of zero were fitted to the observed proportions of viable seeds (**eq. 2-2**, **Supplementary Figure 2-5 A**). Models were fitted species-wise for both temperatures simultaneously by setting temperature as a grouping variable. The 35°C- and 42°C-models shared the upper horizontal asymptote, i.e., the maximum proportion of viable seeds, $V_{max}$. The data type was "binomial" and the total number of evaluated seeds was set as weights. The model fit was evaluated both by a $Chi^2$-test and visually (**Supplementary Table 2-3**). In case all or almost all seeds had lost viability even after the shortest exposure time to AD (1 day or 3 days), no model was fitted.

$$V(t) = \frac{V_{max}}{1 + e^{SLP(\log(t) - \log(MIT))}} \quad \textbf{(2-2)}$$

With *V(t)*, proportion of viable seeds as a function of the time of exposure in AD (*t*); $V_{max}$, maximum proportion of viable seeds (upper asymptote); *SLP*, parameter proportional to the slope of *V(t)* in the inflection point; *MIT* (median inactivation time), the time after which *V(t)* reaches 50% of $V_{max}$.
Due to viability increases in AD, the log-logistic models did not provide a good fit for *M. sylvestris*, *M. albus*, and *M. officinalis*. To improve their fit, one parameter was added to the log-logistic models using the Brain-Cousens modification (Ritz and Streibig,2016, **eq. 2-3**). In these models, the lag-phase at the beginning of exposure is replaced by a sigmoid curve describing hormesis, and thus, turning a monotonically decreasing dose-response relationship into a biphasic one (Cedergreen et al., 2005; Kendig et al., 2010; **Supplementary Figure 2-5 B**).

$$V(t) = \frac{V_{max} + H}{1 + e^{SLP(\log(t) - \log(E))}} \quad \textbf{(2-3)}$$

With *V(t)*, proportion of viable seeds as a function of the time of exposure in AD (*t*); $V_{max}$, maximum proportion of viable seeds (upper asymptote); *H*, size of the hormesis effect, i.e., stimulation of viability at *t* close to zero; *SLP*, parameter changing the slope of the model curve; *E*, parameter shifting and stretching the model curve.

From the viability models the median inactivation times (*MIT*s) and decimal reduction times (*DRT*s) were estimated, i.e., the times required to reduce viability to 50 or 10% of the initial viability, respectively (**Supplementary Figure 2-5**). If models could be fitted to the data obtained for both 35 and

42°C, parameter estimates, *MIT* and *DRT* were compared between the two temperatures species-wise using the "drc"-built-in functions *compParm* and *EDcomp* (Ritz and Streibig, 2016). The level of significance, α, was set to 0.05.

#### 2.1.2.3.2 Seed-Killing Efficacy

To assess how much AD reduced seed viability, the viability models were used to estimate the mean $V$ and standard error after 36 days for AD at both 35 and 42°C. Based on these data and the approach of Hahn et al. (2021), the seed-killing efficacies of 36 days in AD were calculated as follows (**eq. 2-4**):

$$seed-killing\ efficacy\ (\%) = 100 \times \left(1 - \frac{V(36\ days)}{V(0\ days)}\right) \quad \textbf{(2-4)}$$

#### 2.1.2.3.3 Cumulative Germination and Dormant Seeds

In addition to total viability, its components, i.e., the proportions of germinated and dormant seeds, were analyzed individually. Cumulative germination after the 21-day germination test, *cG*, was modeled as a function of exposure time, *t*, similar to *V(t)*. Due to the skewness of the data, however, an asymmetric, three-parameter Weibull type 1 function with a lower limit of zero was chosen for most seed lots (**eq. 2-5**). The only exception to this was *A. theophrasti – 7 years* for which a log-logistic model including hormesis was used (compare **eq. 2-3**). The model fit was evaluated by a $Chi^2$-test and visually (**Supplementary Table 2-4**).

From the germination models decimal reduction times for *cG* (*DRT*(*cG*)) were estimated and compared to *DRT*(*V*) for 35°C and 42°C. In addition, the proportion of dormant seeds, *D*, was calculated from the difference between the models for *V(t)* and *cG(t)*. Finally, the percentages of germinated, dormant, and inactive seeds were calculated in the untreated controls and after 36 days in AD at 35°C and 42°C, respectively.

$$cG(t) = cG_{max}\ e^{-e^{SLP(IFT-\log(t))}} \quad \textbf{(2-5)}$$

With *cG(t)*, proportion of cumulative germination at the end of the 21 days germination test, *cG*, as a function of the time of exposure in AD (*t*); $cG_{max}$, maximum proportion of *cG* (upper asymptote); *SLP*, parameter proportional to the slope of *cG(t)* in the inflection point; *IFT* (inflection time): the time after which the curve of *cG(t)* changes its flection.

## 2.1.3 Results

### 2.1.3.1 Seed Viability

#### 2.1.3.1.1 Responses During Anaerobic Digestion

Viability responses to mesophilic AD varied among species and among seed lots of a species (**Figure 2-2**). The species most resistant to mesophilic AD were *M. officinalis*, *M. albus*, and *M. sylvestris* (**Figures 2 - 2 a–c** and **Table 2 - 1**). The species whose seeds were inactivated most rapidly was *V. thapsus* (**Figure 2-2 n** and **Table 2-1**).

For all species except *M. sylvestris*, *M. albus*, and *M. officinalis*, seed viability continuously decreased with increasing exposure time in the lab-scale reactors. A more or less pronounced lag-phase was followed by an exponential decrease in viability, which led either to complete seed inactivation or a plateau. If a plateau was reached, it was lower at 42°C than at 35°C. In addition, the viability curves dropped more steeply in AD at 42°C than at 35°C
(**Figures 2-2 d–n**).

The viability curves of the particularly AD-resistant HS species *M. officinalis*, *M. albus*, and *M. sylvestris* were biphasic: modeled viability initially increased during the first days of AD (**Figures 2-2 a–c**). This means that the observed viability of samples treated with AD was higher than that of the untreated controls. This observed increase was most pronounced in *M. sylvestris*, with some individual values exceeding the maximum of the controls. Modeled viability of *M. sylvestris* reached its maximum of 149 and 136% of untreated controls, respectively, in AD at 35°C after 2.5 days and in AD at 42°C after 8.9 days.
Subsequently, viability decreased, but after 36 days in AD at 35°C,it was as high as in the untreated controls and even 23% higherat 42°C (**Figure 2 - 2 c**). In the two *Melilotus* species, the increasein viability was lower than in *M. sylvestris*, remaining belowthe maximum value of the untreated controls. Furthermore, the increase occurred after a shorter time and was stronger in AD at 42°C than in AD at 35°C. Maximum viability values at 42°C were 108% for *M. albus* (4.3 days) and 103% for *M. officinalis* (0.9 days) (**Figures 2-2 a,b**). In addition, *M. albus* tended to lose viability more rapidly in AD at 35°C than at 42°C (**Figure 2-2 b**). In tendency, this was also observed in *M. officinalis* and *M. sylvestris* (**Figures 2-2 a,c**).

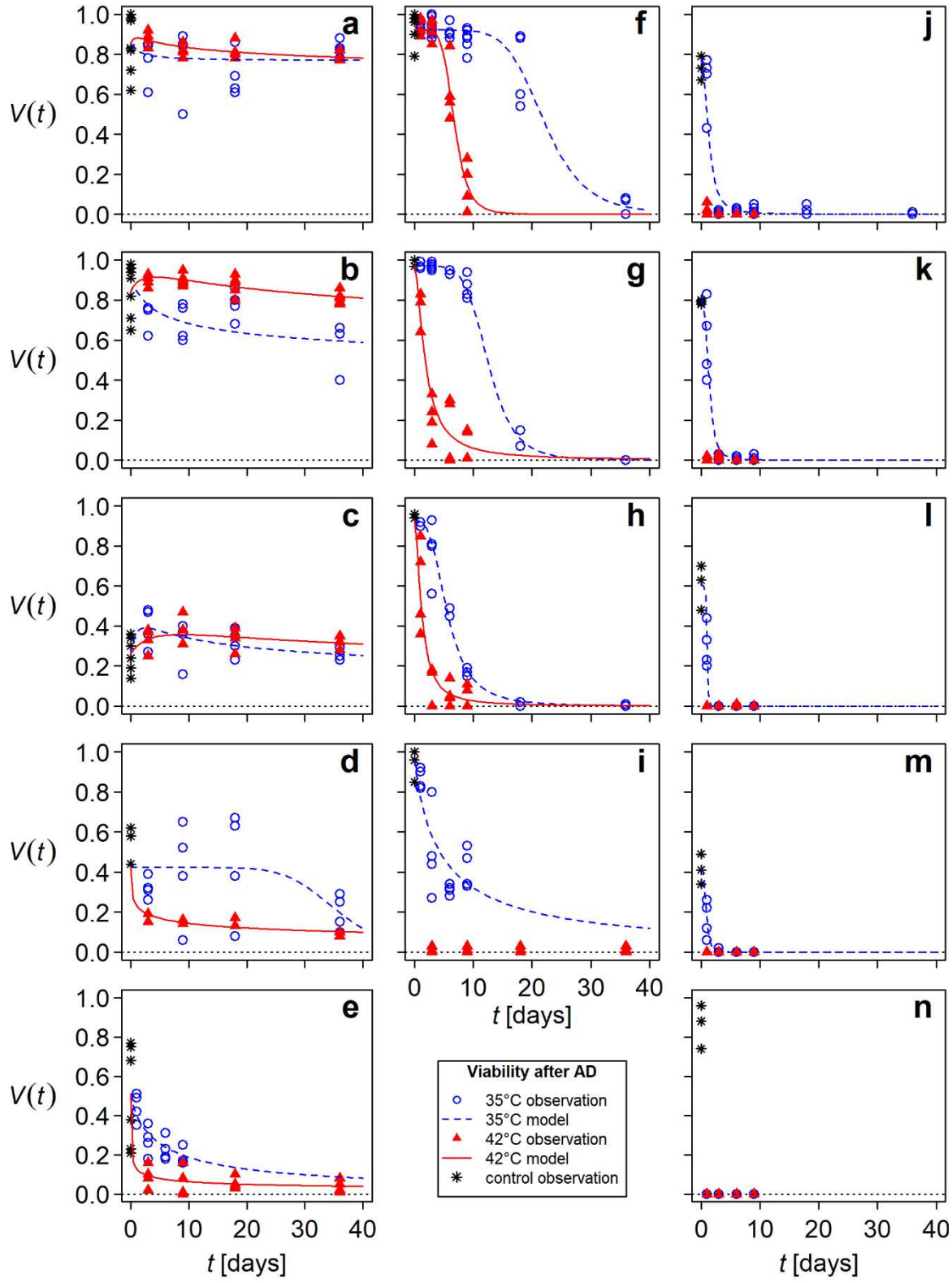


**Figure 2-2 | Modeled seed viability, *V*, of flowering wild plant species and tomato during anaerobic digestion (AD) in lab-scale reactors at 35°C (blue, dashed lines) and 42°C (red, solid lines). Symbols present observations, each containing a minimum of 100 seeds: asterisks for untreated controls, blue open circles for AD at 35°C and red filled triangles for AD at 42°C. (a) *Melilotus officinalis*, (b) *Melilotus albus*, (c) *Malva sylvestris*, (d) *Malva alcea – 2 years*, (e) *Malva alcea – 1 year*, (f) *Chenopodium album*, (g) tomato – PIERRE, (h) tomato – PAPRIKA, (i) *Abutilon theophrasti – 1 year*, (j) *Abutilon theophrasti – 7 years*, (k) *Daucus carota*, (l) *Cichorium intybus*, (m) *Echium vulgare*, (n) *Verbascum thapsus*. For better comparability, panels were arranged according to the species' viability after 36 days of exposure to AD.**

#### 2.1.3.1.2 Seed-Killing Efficacies

Averaged over all species and both temperatures, the mean seed-killing efficacy (*SKE*) of 36 days in mesophilic AD was 76 ± 40% (n = 28). However, the values for NHS species, particularly AD-resistant HS species, and the remaining HS species (*A. theophrasti* and *M. alcea*) differed greatly (**Table 2-1**). According to the viability models, NHS species were completely inactivated after 36 days.The only exception was *C. album*, of which 4 ± 1% of the seeds remained viable after 36 days in AD at 35◦C (**Table 2-1**). Of theNHS species, only *Chenopodium album* and tomato were able to survive AD beyond 9 days of exposure at both temperatures (**Figures 2-2 f–h**). *Daucus carota* seeds were only 1% viable after 9 days when anaerobically digested at 35°C (**Figure 2-2 k**). Survival of all other NHS species was poorer: *C. intybus*, *E. vulgare*, and *V. thapsus*, survived less than 3 days at 35°C and less than lday at 42°C (**Figures 2-2 l–n**). The mean *SKE* on the HS species*A. theophrasti* and *M. alcea* was 86 ± 16% (n = 8). The lowest mean *SKE* was determined for the particularly AD-resistant HS species. It was only 5 ± 19% (n = 6) with a range of −23 to 36% (**Table 2-1**).

**Table 2-1 | Estimated median inactivation times (*MIT*s) and decimal reduction times (*DRT*s) of seeds of flowering wild plant species and tomato after anaerobic digestion (AD) at 35 or 42°C in lab-scale reactors, and corresponding seed-killing efficacy of AD.**

| | MIT | | | | DRT | | | | Seed-killing efficacy [%] of 36 days in | |
|---|---|---|---|---|---|---|---|---|---|---|
| | AD 35°C | | AD 42°C | | AD 35°C | | AD 42°C | | AD 35°C | AD 42°C |
| **HS species** | | | | | | | | | | |
| *Abutilon theophrasti – 7 YRS* | **1.3** | *(0.06)* | **< 1** | | **3.4** | *(0.14)* | **< 1** | | **100** | **100** |
| *Abutilon theophrasti – 1 YR* | **4.7** | *(0.24)* | **< 1** | | **51.9** | *(8.27)* | **< 3** | | **86** | **99** |
| *Malva alcea – 2 YRS* | **35.4** | *(0.65)* | **1.7** | *(1.36)* | **46.9** | *(6.29)* | **>365** | | **52** | **76** |
| *Malva alcea – 1 YR* | **5.1** | *(0.68)* | **0.1** | *(0.06)* | **76.7** | *(27.70)* | **18.4** | *(4.98)* | **82** | **92** |
| *Malva sylvestris* | **>365** | | **>365** | | **>365** | | **>365** | | **0** | **-23** |
| *Melilotus albus* | **>365** | | **>365** | | **>365** | | **>365** | | **36** | **2** |
| *Melilotus officinalis* | **>365** | | **>365** | | **>365** | | **>365** | | **9** | **7** |
| **NHS species** | | | | | | | | | | |
| *Chenopodium album* | **22.1** | *(0.30)* | **6.8** | *(0.07)* | **31.1** | *(0.60)* | **9.8** | *(0.17)* | **96** | **100** |
| *Cichorium intybus* | **1.0** | *(0.02)* | **< 1** | | **1.2** | *(0.60)* | **< 1** | | **100** | **100** |
| *Daucus carota* | **1.3** | *(0.06)* | **< 1** | | **2.7** | *(0.13)* | **< 1** | | **100** | **100** |
| *Echium vulgare* | **0.9** | *(0.06)* | **< 1** | | **1.6** | *(0.13)* | **< 1** | | **100** | **100** |
| *Verbascum thapsus* | **< 1** | | **< 1** | | **< 1** | | **< 1** | | **100** | **100** |
| tomato - PAPRIKA | **5.6** | *(0.18)* | **1.3** | *(0.07)* | **11.5** | *(0.37)* | **4.6** | *(0.22)* | **100** | **100** |
| tomato - PIERRE | **12.6** | *(0.20)* | **1.9** | *(0.09)* | **18.4** | *(0.51)* | **7.3** | *(0.33)* | **100** | **100** |

Species are grouped according to their potential to exhibit hardseededness (HS) in their seeds or not (NHS). Standard errors of the mean are given in parentheses.

Standard errors were not calculated when models could not be fitted because seeds were completely inactivated even after the shortest exposure time ("<1" or "<3"), or when estimated values exceeded 1 year (365 days, ">365").

#### 2.1.3.1.3 Inactivation Times

Inactivation times showed a wide range, with the longest determined for *M. officinalis*, *M. albus*, and *M. sylvestris*. To inactivate 50% (*MIT*) or 90% (*DRT*) of their originally viable seeds, it was estimated that more than 1 year would have been required. The *DRT*s for the NHS representatives from the wildflower biogas mixture ranged from only a few hours to2.7 ± 0.13 days (*D. carota*). The inactivation times for the other species fell between these values. With 31.1 ± 0.60 days and 9.8 ± 0.17 days at 35°C and 42°C, respectively, *DRT* values for *C. album* were in a similar range to those of the HS species, *A theophrasti* (**Table 2-1**).

For most species, inactivation times were shorter in AD at 42°C than in AD at 35°C. Exceptions were three particularly AD-resistant species and *M. alcea – 2 years* (**Table 2-1**). In addition, inactivation times differed between the seed lots of a species. The tomato variety PAPRIKA was inactivated faster than the variety PIERRE (**Figures 2-2 h,g** and **Table 2-1**). The batch *A. theophrasti –1 year* had a 15-times longer *DRT* at 35°C than the 7-year old batch and even a few viable seeds remaining after 36 days in AD at 42°C (**Figures 2-2 i,j** and **Table 2-1**). The difference between the two seed lots of *M. alcea* was most apparent when comparing their response to AD at 35°C: inactivation in *M. alcea – 2 years* was preceded by a 21-day lag phase, whereas it started immediately in*M. alcea – 1 year* (**Figures 2-2 d,e**). As a result, it took considerably longer to reduce the viability of the older *M. alcea* by 50% than the younger one (*MIT*: 35 days instead of 5 days), but it took less time to reduce it by 90% (*DRT*: 47 days instead of 77 days) (**Table 2-1**).

### 2.1.3.2 Germinable and Dormant Seeds

#### 2.1.3.2.1 Before Anaerobic Digestion

Untreated controls of HS and NHS species differed in the contribution of germinable and dormant seeds to viability (**Figure 2-3**, left). In NHS species, an average of 98 ± 3% (n = 7) of all viable seeds germinated. Dormant seeds occurred only in *D. carota*, *E. vulgare*, and tomato – PIERRE (**Figure 2-3**, left, bottom). In contrast, in the untreated samples of HS species, only 29 ± 20% (n = 7) of the viable seeds had germinated, while the rest remained dormant (**Figure 2-3**, left, top).

#### 2.1.3.2.2 During Anaerobic Digestion

The contribution of germinable (cumulative germination, *cG*) and dormant seeds, *D*, to (total) viability, *V*, differed between HS and NHS species during the different exposure periods in AD (**Figure 2-4**). In NHS species, the majority of viable seeds germinated, and the loss of *V* corresponded to an approximately equal loss of *cG* (e.g., *C. album* in **Figures 2-4 a,b**). This nearly simultaneous loss of *cG* and *V* was reflected in the fact that *DRT*s of *cG* were at most 5 days shorter than those of *V* (**Supplementary Table 2-5**). In all HS species except *A. theophrasti*, most viable seeds remained dormant during exposure to mesophilic AD. The viability curve paralleled that of the proportion of dormant seeds (e.g., *M. albus* in **Figures 2-4 c,d**, *M. sylvestris* in **Figures 2-4 e,f**).

The time interval between 90% loss of *cG* and *V* was not days as in NHS species, but weeks or months (**Supplementary Table 5**).

Only in *A. theophrasti* *cG* peaked during the first 3 days of exposure and not in the untreated controls as in the other species. For instance, in the *A. theophrasti – 7 years* seed lot, after 1 day in AD at 35°C, *cG* was five-times higher than in the untreated control. This peak of germinable seeds was paralleled by a local minimum of dormant seeds. However, *D* increased again as soon as the *cG*-peak exceeded its maximum (**Figure 2-4 g** and **Supplementary Figure 2-6 J**).

In four species, namely *A. theophrasti*, *C. album*, *D. carota* and tomato, a peak of dormant seeds was observed after *cG* and *V* began to decline. In the most extreme case, *C. album*, these *D*-peaks could account for up to one-third of *V* and last up to 30 days (**Figure 2-4 a**). After longer exposure times, the curves of *D* and *V* finally coincided. Thus, the proportion of *D*, accounted for the sum of all seeds that were still viable after AD.

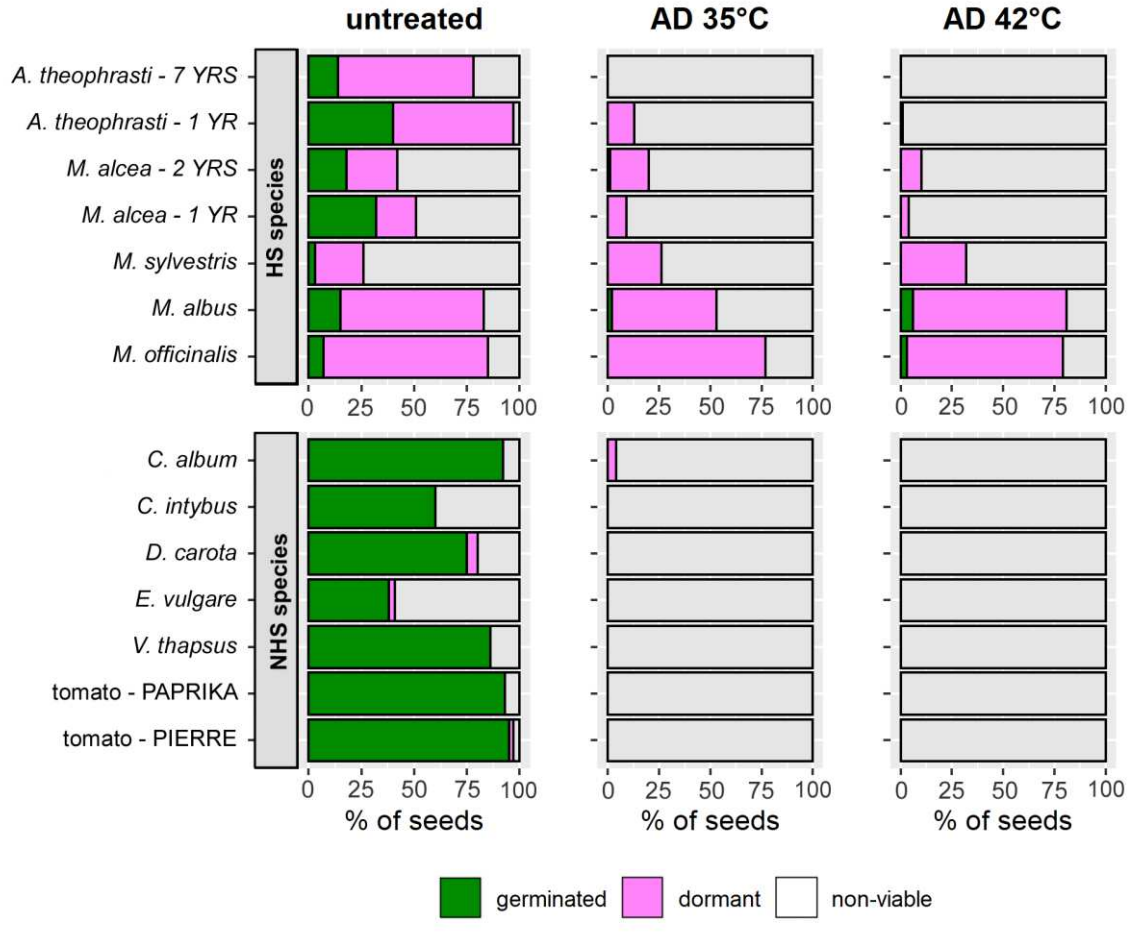


**Figure 2-3 | Mean percentages of germinated (green), dormant (pink), and non-viable seeds (gray) in batches of flowering wild plant species and tomato that were either untreated (left) or exposed to AD at 35°C (center) or 42°C (right) for 36 days. The top row shows the values for species with hardseededness (HS), the bottom row those for non-hardseeded (NHS) species. The percentage of germinated seeds equals the cumulative germination (*cG*) after completion of the 21-day germination test after AD-treatment. Values were predicted from models for *V(t)* and *cG(t)*.**

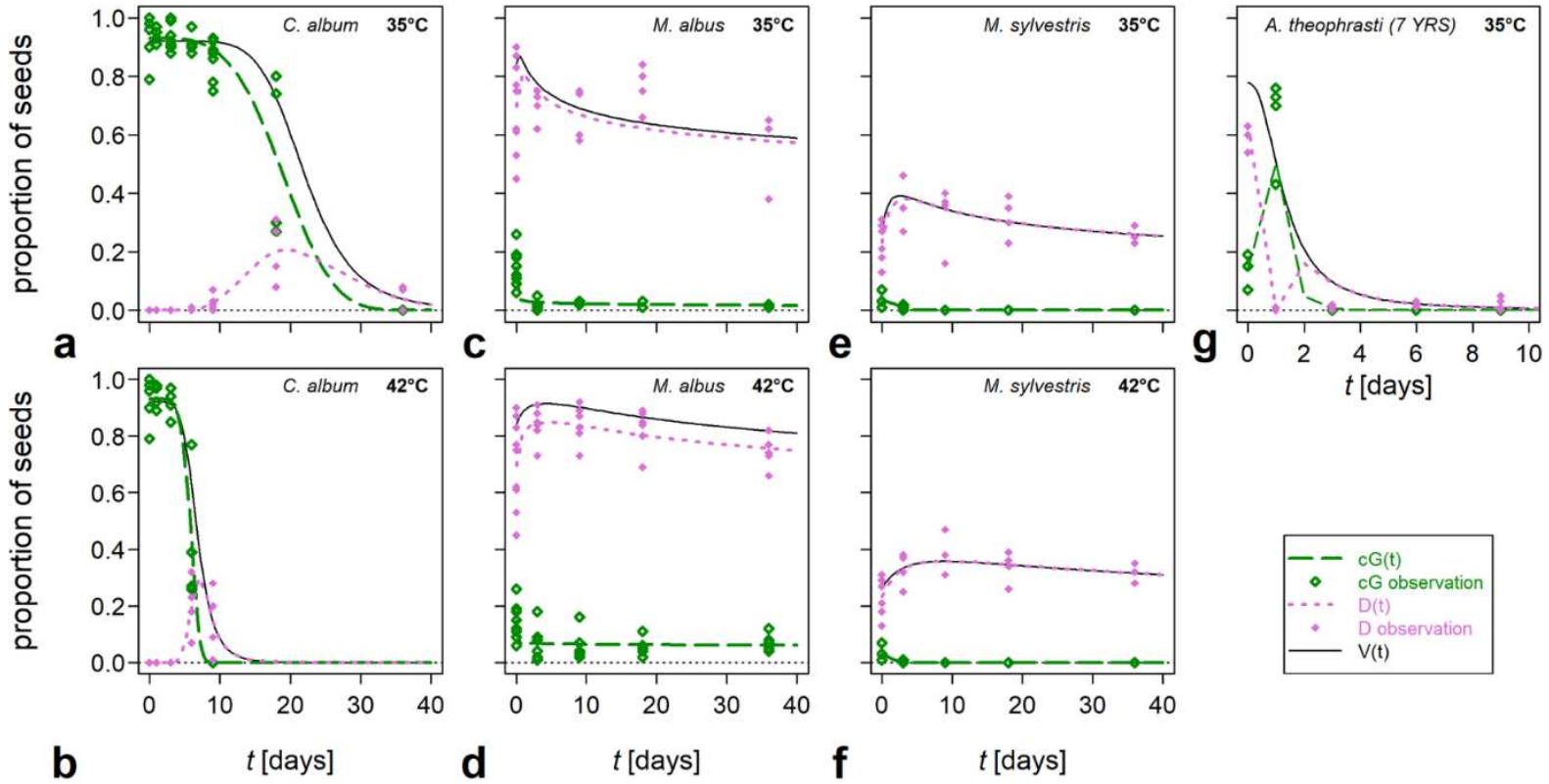


**Figure 2-4 | Modeled proportions of germinated (cumulative germination, *cG*, green long-dashed lines) and dormant seeds (*D*, pink dotted lines) out of all viable seeds (*V*, black solid lines, compare** Figure 3**) of selected flowering wild plant species after mesophilic, anaerobic digestion. *D(t)* was calculated from the difference between the models for *V(t)* and *cG(t)*. Symbols represent observations, each based on a minimum of 100 seeds: green open diamonds for *cG* and pink closed diamonds for *D*. (a,b) *Chenopodium album* at 35 and 42°C, respectively; (c,d) *Melilotus albus* at 35 and 42°C, respectively; (e,f) *Malva sylvestris* at 35 and 42°C, respectively; (g) *Abutilon theophrasti – 7 years* at 35°C. Please note the different scaling of the x-axis in (g).**

#### 2.1.3.2.3 After 36 Days in Anaerobic Digestion

Seeds that were still viable after 36 days in mesophilic AD were almost exclusively dormant. This was also the case for the only surviving NHS species, *C. album* (**Figure 2-3**, middle + right). Small proportions of germinated seeds were only found in *M. alcea*, *M. albus* and *M. officinalis* (**Figure 2-3**, middle + right, top). The highest percentages of germinated seeds were observed for *M. albus* and *M. officinalis*: 0.06 and 0.03, respectively, after 36 days of AD at 42°C (**Figure 2-3**, right, top). Furthermore, only in the *Melilotus* species did more seeds germinate after AD at 42°C than at 35°C. Finally, only in *Melilotus* species were bare, i.e., seed coat-less, embryos frequently found in the sample bags in addition to seeds that were recognizable as viable or non-viable. When some of the bare embryos were subjected to a germination test, it became apparent that they were able to develop and grow.

### 2.1.4 Discussion

Mesophilic AD affected seed viability of the species studied to varying degrees, and none of the potentially modulating factors had a consistent effect. We rejected the hypotheses: mesophilic AD, reduced seed viability of one of the HS species more than that of two of the NHS species (hypothesis 1). Seed viability of three species did not decrease markedly more at the higher incubation temperature than at the lower one (hypothesis 2). The observed seed viability of three species increased when exposure time increased up to a certain point (hypothesis 3). Instead, we report a more complex response of the wildflower seeds studied: more factors than HS affected AD-resistance (see section "Anaerobic Digestion-Resistance of Hardseeded and Non-hardseeded Species"). Nevertheless, some of the HS species were particularly AD-resistant (see section "Particularly Anaerobic Digestion-Resistant Hardseeded Species"). Furthermore, responses were dependent on both germinable and dormant seeds (see section "Diversity of Viability Responses"). These findings have implications for the use of flowering wild plants as biogas feedstocks (see section "Flowering Wild Plants as Biogas Feedstocks").

#### 2.1.4.1 Anaerobic Digestion-Resistance of Hardseeded and Non-hardseeded Species

In this study, seed-killing efficacies tended to be lower and inactivation times longer in HS species than in NHS species. However, inter- and intraspecific variation was high. Considering together viability curves, seed-killing efficacies, and inactivation times, it appears that the HS and the NHS species studied overlapped in AD-resistance potential, with NHS species tending to be at the "low" end of the scale and HS species at the "high" end. The HS species *A. theophrasti* and the NHS species *C. album* and tomato mainly caused this overlap. The most AD-resistant NHS species in this study was *C. album*, and its maximum survival times were in the upper ranges of values from comparable studies (Schrade et al., 2003; Katovich et al., 2004; Leonhardt et al., 2010; Westerman et

al., 2012a,b; Johansen et al., 2013; Oechsner et al., 2018; Zhou et al., 2020). The same, although less pronounced, was true for tomato (Engeli et al., 1993; Lorenz et al., 2001; Schrade et al., 2003; Marcinisyn et al., 2004; Westerik and Kleizen, 2006; Strauß et al., 2012; Westerman et al., 2012a,b; Baute et al., 2016). Whether this was due to seed lot characteristics or different AD-conditions cannot be determined. However, several other NHS species survived mesophilic ADtreatments for more than 1 week: *Amaranthus retroflexus* L.(common tumbleweed, Šarapatka et al., 1993; Katovich et al.,2004), *Digitaria sanguinalis* (L.) SCOP. (purple crabgrass (Engeliet al., 1993), *Echinochloa crus-galli* (L.) P. BEAUV (commonbarnyard grass, Šarapatka et al., 1993), *Panicum dichotomiflorum* MICHX. (autumn millet, Jeyanayagam and Collins, 1984), *Rumex obtusifolius* L. (broadleaf dock, Engeli et al., 1993; Šarapatka et al., 1993) and *Sorghum halepense* (L.) PERS. (Jeyanayagam and Collins, 1984). The survival of these NHS species supports the suggestion that HS is not the only mechanism by which seeds can gain resistance to AD (Westerman and Gerowitt, 2013). In the case of *C. album* and tomato a physically hard (but not HS) or thick seed coat has been discussed as the basis of their AD resistance (Blackshaw and Rode, 1991; Strauß et al., 2012; Aper et al., 2014). The protection provided by this hard seed coat would be lost during AD by microbial degradation processes; first slowly (lag-phase) and then increasingly rapidly. However, it is unclear whether this mechanism alone is sufficient to explain the remarkably high AD-resistance of *C. album* and tomato in this study.

Resistance differences within HS species were likely related to the fact that both the degree and depth of HS varies among species and seed lots (Baskin and Baskin, 2004). A good example of this is the 7-year-old seeds of *A. theophrasti* used in this study, which have already been tested in two other studies. Two years after harvest, about half of *A. theophrasti* seeds survived 30 days of AD in lab-scale batch reactors at 37°C (Westerman et al., 2012b) and all seeds were inactivated after 9 days of AD in commercial fermenters at 41°C (Westerman et al., 2012a). Now 7 years old, this seed lot was completely inactivated after 36 days in AD at both 35 and 42°C, and *DRT* was shorter at 42°C (<1 day) than at 41°C (2 days, Westerman et al., 2012a). Since other differences can be excluded here, AD-resistance must have decreased due to seed aging during storage (Sano et al., 2016). Aging and loss of AD-resistance appeared to be associated with a reduction in the depth of HS, as the degree of HS was higher at older ages (82% of "hard" seeds in the seed lot) than at younger ages (about 65%, Westerman et al., 2012b). A lower HS depth would also be consistent with the observation that the older seed lot germinated more and was less AD-resistant than the 1-year-old *A. theophrasti* lot used in this study, which had an even lower degree of HS (59%). However, unlike the first comparison, these two seed lots did not differ only in storage duration. This implies that differences in their AD-resistance could also be due to other factors leading to variations in HS, such as genetic differences, weather and site conditions, seed maturity, endogenous dormancy rhythms, and conditions of storage (Rolston, 1978; Baskin and Baskin, 1998; Hilhorst, 1998; Hay and Probert, 2013; Jaganathan et al., 2016). In this context, Westerman et al. (2012b) noted that genetic factors or environmental conditions during seed filling and maturation could be responsible for the difference in survival probability of mesophilic AD between populations of *A.*

*theophrasti* harvested in different years and/or locations. Similarly, the higher AD-resistance of the older *M. alcea* batch in this study can be explained by the fact that its seeds were more mature and therefore had greater depth (Goldberg et al., 1994) and higher degree of HS (58% versus 38%) than the younger batch.

The influence of different seed lots on AD-resistance is not limited to HS species. Different seed lots of NHS species responded differently to AD as well (Traveset, 1998; Westerman et al., 2012b; Zhou et al., 2020). Consequently, AD-resistance seems determined by both species-specific traits and characteristics of the respective seed lots. This raises the question of (1) what factors affecting seed lot quality lead to differences in AD-resistance and (2) how large the differences between seed lots are compared to the differences between species. In addition, the relative importance of seed traits and AD-conditions on seed-killing efficacy would need to be determined.

#### 2.1.4.2 Particularly Anaerobic Digestion-Resistant Hardseeded Species

Compared to the other species examined in this study, the HS species *M. officinalis*, *M. albus*, and *M. sylvestris* were particularly resistant to mesophilic AD, tended to lose viability more slowly and to germinate more after AD at 42°C than at 35°C, and showed an initial increase in observed viability.

The seed-killing efficacy of AD on *M. officinalis*, *M. albus* and *M. sylvestris* was very low compared to other members of the Fabaceae and Malvaceae. The four other Fabaceae species studied to date, namely *Glycine max* (L.) MERR. (soybean), *Lupinus polyphyllus* LINDLEY (garden lupin), *Trifolium pratense* L. (red clover) and *Vicia tetrasperma* (L.) Schreb. (smooth vetch) had lost substantially more than 9% – 36% viability when exposed to similar AD-conditions (35–38°C, 7–30 days) (Leonhardt et al., 2010; Strauß et al., 2012; Westerman et al., 2012b; Hassani et al., 2021). Among the three members of the genus *Malva* studied, seed-killing efficacy was higher in *M. alcea* (this study) and *Malva neglecta* Wallr. (dwarf mallow) (Westerman et al., 2012a,b) than in *M. sylvestris*. It is particularly noteworthy that after 36 days of AD, the measured values for seed viabilities of *M. albus* and *M. officinalis* were still in the range of the values of the untreated controls. Values of *M. sylvestris* were even higher than in the controls, resulting in negative *SKE*s. The high AD-resistance distinguishes *M. officinalis*, *M. albus* and *M. sylvestris* from all other species.

The second unique characteristic of the particularly AD-resistant HS species was their tendency to lose viability faster and more severely at 35°C than at 42°C. Additionally, the range of responses was wider at 35°C. This was in contrast to all other species studied to date. However, it must be put into perspective that (a) this response was prominent only in *M. albus* and (b) other Fabaceae and Malvaceae were each exposed to only one temperature in previous studies (Katovich et al., 2004; Leonhardtet al., 2010; Strauß et al., 2012; Westerman et al., 2012a,b; Hassani et al., 2021). Some of our observations in *Melilotus* sp.suggest that fatal germination has played a role in the unexpected and rather counterintuitive response to temperature increase. First, we found bare but germinable embryos of *M. albus* and*M officinalis* in the sample bags, indicating that AD triggered

germination in these species. As a result, seeds would die due to thermosensitivity (Westerman and Gerowitt, 2013) and degrade, unless germination was triggered just before samples were removed from the reactor. Second, fewer *Melilotus* seedsgerminated after AD at 35°C than after AD at 42°C, which may indicate that AD at 35°C caused more seeds to germinate already in the reactor and then die. In this way, viability wouldddecline more at 35°C than at 42°C. It also cannot be ruled out, however, that the tendency of higher survival probability at the higher temperature was related to HS or the increase in observed viability.

Exclusively in *M. officinalis*, *M. albus*, and *M. sylvestris*, there was no lag-phase at the beginning of the AD-treatment, but an increase in observed seed viability. The resulting biphasic viability curves extend the spectrum of known responses to AD because, previously, such increases in seed viability in AD have only been reported for NHS species (Schrade et al., 2003; Westerik and Kleizen, 2006; Leonhardt et al., 2010; Baute et al., 2016; Zhou et al., 2020). In NHS species, the initial viability increase in AD was associated with the breaking of dormancy and initiation of germination (Westerik and Kleizen, 2006; Leonhardt et al., 2010; Zhou et al., 2020). However, in these three studies, germinability was equated with seed viability, which unfortunately does not allow conclusions to be drawn about total viability, i.e., germinating plus dormant seeds. Combining germination and TTC tests, we found that the increase in observed viability of the highly AD-resistant HS species was not due to an increase in germinating seeds, but to that of dormant seeds. This means that the total observed viability of the seed lot increased compared to the untreated control. The only study we know of that attempts to explain a similar result examined *Heracleum mantegazzianum* SOMMIER & LEVIER (giant hogweed) in a water bath at 35°C (Tanke et al., 2019). The authors attributed the increase in seed viability after 12 h to insufficient hydration of previously dry-stored control seeds. Hence, the TTC assay failed to capture the full respiratory potential of the seeds under these conditions (Elias et al., 2012; Miller, 2014). In other words, the observed viability increase was assumed to be an artifact. However, the controls in our study were well hydrated due to the pretreatment and germination test before the TTC test. Moreover, unlike the NHS species *H. mantegazzianum*, the HS species *M. officinalis*, *M. albus* and *M. sylvestris* in this study were not inactivated shortly after the viability peak, but their observed viability remained at a high level. Therefore, we hypothesize that the increase in observed viability was due to AD-induced metabolic stimulation of seeds whose metabolic activity was not detectable by TTC staining before AD. If this was the case, the increase in seed viability would be a form of hormesis, i.e., a dose-response phenomenon in which low doses of a stressor - here, brief exposure to AD - have a stimulatory effect, whereas high doses cause inhibition (e.g., Calabrese and Baldwin, 2002; Mattson, 2008; Kendig et al., 2010). To date, hormesis has been demonstrated in over 15,000 experimental studies (Kozumbo and Calabrese, 2019). And biogas reactor conditions, e.g., heat, enzymes, amino and organic acids, alcohols, hydrogen sulfide, ammonia, and cyanides (Westerman and Gerowitt, 2013), resemble stressors known to induce hormesis (e.g., Calabrese et al., 2007). The assumed stimulation by AD would have to activate processes that can repair (oxidative) damage to DNA, membranes, proteins, etc., that occurred before

the AD treatment, i.e., during maturation and storage of the seed. Examples of such processes include activation of DNA repair mechanisms and antioxidant (enzyme)systems, expression of growth factors, anti-apoptotic proteins and heat shock proteins, and de-novo synthesis of cellular components. These processes occur in established, conserved hormetic pathways (Calabrese et al., 2007; Mattson, 2008) and enhance seed vigor during pre-germinative metabolic events utilized in seed priming (Paparella et al., 2015; Lutts et al., 2016). However, the observed viability increases in *M. officinalis*, *M. albus* and *M. sylvestris* may also be due to limitations of TTC testing that were detectable only in these three species. It is conceivable that the metabolic activity of (AD-)microorganisms attached to the seeds resulted in an apparent increase in seed viability. With the same result, AD may have facilitated TTC uptake in these HS species. Furthermore, TTC tests are invasive, so the viability of the AD-treated samples in the untreated state may have varied more than that of our controls, even though all samples were from the same seed lot. Then, there is the question of the extent to which the timing and mechanisms of an increase in viability differ between HS and NHS species. The HS species in this study took between 6 hours and 9 days longer to reach the viability maximum than NHS species in other studies (Schrade et al., 2003; Westerik and Kleizen, 2006; Leonhardt et al., 2010; Baute et al., 2016; Zhou et al., 2020). Thus, the shortest sampling interval of 24 h could have been too long to detect viability increases in all species studied here. Finally, given the particular AD-resistance of these HS species, it is of particular interest to determine whether or not the hypothesized metabolic stimulation is due to processes that require imbibition. This aspect is important because repair processes are normally associated with (partial) rehydration of seeds (Weitbrecht et al., 2011; Powell and Matthews, 2012; Paparella et al., 2015; Sano et al., 2016), but the protective effect of HS against AD is irreversibly lost once the water impermeability of the seed coat is broken and seeds absorb water (Westerman and Gerowitt, 2013). However, it is also possible that metabolic stimulation was triggered by factors other than rehydration, because unimbibed, dry seeds can have low-level metabolic activity (e.g., Weitbrecht et al., 2011; Sano et al., 2016) and even priming effects are not necessarily related to seed imbibition (Lutts et al., 2016). In summary, to clarify whether hormesis can be triggered by AD in seeds, future studies should include measurements with a higher resolution in the range of the increase in observed viability and be complemented by molecular analyses.

#### 2.1.4.3 Diversity of Viability Responses

Observed responses during mesophilic AD ranged from complete inactivation to viability increases. Furthermore, responses differed between germinable and dormant seeds. Germinability was lost faster than viability in all species, indicating that mainly dormant seeds survived AD. In contrast to the NHS species, the HS species lost their germinability very quickly compared to their viability. And at the end of the measurement period, almost all of their surviving seeds were active but not germinating, thus, most probably physically dormant. These observations confirm that HS makes survival in AD more likely (Leonhardt et al., 2010; Westerman et al., 2012b; Jaganathan et al., 2016; Hassani et al., 2021). It

remains unclear to what extent, however, as the HS species had lost so little viability at the end of the measurements. It appeared as if the remaining hard seeds could survive AD for even longer periods (viability plateau). The exceptionally long estimated inactivation times for the particularly AD-resistant HS species reflected this. However, they are unlikely to survive longer than a year in mesophilic AD, as the most resistant species studied to date survived only about 30 days (e.g., Jeyanayagam and Collins, 1984; Westerman et al., 2012b), and up to 155 days in extreme cases (Hassani et al., 2021). Moreover, seeds can be inactivated in AD despite intact physical dormancy (Westerman et al., 2012b) and viability plateaus can end quite abruptly, as observed in *M. alcea – 2 years* (**Figure 2-2 d**). For these reasons, a realistic assessment of AD-resistance in HS species requires that measurements continue until both the dormant and non-dormant seeds are fully inactivated.

A special case with regard to its responses was *Abutilon theophrasti*. First, it was the only HS species to be completely (7 years old seed lot) or nearly completely (1 year old) inactivated at the end of the measurement period. Possible reasons for this have already been discussed (see section "Anaerobic Digestion-Resistance of Hardseededness and Non-hardseeded Species"). Second, mesophilic AD stimulated germination in it, which has previously been observed only in NHS species (Schrade et al., 2003; Westerik and Kleizen, 2006; Leonhardt et al., 2010; Baute et al., 2016; Oechsner et al., 2018; Zhou et al., 2020). Third, it was the only HS species in which peaks of metabolically active seeds (*D*-peaks) occurred when cumulative germination decreased. Otherwise, these *D*-peaks occurred only in *C. album*, tomato, and *D. carota*.

It remains to be clarified how the *D*-peaks are to be evaluated in terms of seed viability. Based on our investigations, we can either assume that the *D*-peaks represented the parts of the seed lots no longer protected by the seed coat. These would have been damaged, losing their ability to germinate and retaining only some metabolic activity, which we would have mistakenly interpreted as dormancy. If we follow this line of reasoning, we would agree with Eckford et al. (2012). They assumed that AD-treated seeds of *Fallopia convolvulus* L. (wild buckwheat) with pink-stained and thus theoretically viable embryos were not capable of normal growth and development, because a TTC test does not measure the capacity for normal cell division, growth speed, or dormancy (Copeland, 1976; Miller, 2014). However, for AD-treatments of perennial biomass species and tomato Baute et al. (2016) found that Petri-dish germination with TTC staining resulted in similar viability estimates as glasshouse germination with cold-moisture stratification. The overestimation by TTC staining was only 5%. Moreover, a TTC test may well determine the number of seeds that would develop normal seedlings in a germination test when all available viability indicators are included in the evaluation (Elias et al., 2012). We did this by classifying only the red stained, physically intact embryos as viable. Thus, the *D*-peaks could represent fully viable, in principle germinable but dormant seeds. This would mean that secondary dormancy was induced by AD. Possible triggers such as heat, high moisture and low oxygen levels (Hilhorst, 1998; Bentsink and Koornneef, 2008) occur in biogas reactors. Overud (2002) previously speculated that the low oxygen content was responsible for the induction of secondary

dormancy in *Rumex crispus* L. (curled dock) in ensiling, another anaerobic fermentation process. This reasoning, together with the observation that *D*-peaks occurred only in those NHS species that were relatively resistant to AD, raises the question of whether dormancy mechanisms other than HS contribute to AD-resistance in seeds. Considering that chemical and biological processes contribute to seed inactivation in addition to temperature (Westerman and Gerowitt, 2013; Zhou et al., 2020), any dormancy-related defenses against microbial or chemical attack (e.g., Fuerst et al., 2011; Chen et al., 2018) could increase the probability of seed survival in AD.

In summary, the way in which species and seed lot traits impact seed viability responses to AD seems multifaceted. Future studies should include physiological and molecular aspects in order to develop a more mechanistic view that allows to better predict seed viability in AD.

#### 2.1.4.4 Flowering Wild Plants as Biogas Feedstocks

Plant seeds that survive AD in a commercial biogas plant can get particularly widely distributed because the digestate is also traded between farms. In addition, repeated AD of seed-bearing biomass and its fertilization with digestate containing (viable) seeds can lead to the selection for AD-resistant biotypes (Westerman and Gerowitt, 2013). Under these conditions, a few surviving seeds would be sufficient for a new weed flora to emerge. Our finding that a portion of all tested HS species was still viable after 36 days of mesophilic AD suggests that these risks exist when using these species or the flowering mixture as a biogas feedstock. This is especially true for mesophilic biogas plants, which operate with shorter hydraulic retention times (HRTs) than in this study. In Germany, for example, 15% use HRTs between 15 and 35 days (vTI, 2009). In addition, approximately 1–10% of the freshly fed feedstock passes through the reactor after only 6–24 h (Turner et al., 1983; Baier et al., 2010; Eckford et al., 2012). Especially, complete mix, one-stage reactors suffer from this short-circuiting (Ward et al., 2008). After such shortened retention times in AD, seeds that are either not yet inactivated or possibly even activated (hormesis, germination stimulation) can enter the digestate. Consequently, it would be safest to exclude HS species in the composition of biogas mixtures - and as a biogas feedstock in principle.

Excluding HS (wild plant) species as biogas feedstock, however, would mean losing their socio-ecological benefits (e.g., Kuhn and Vollrath, 2010; von Cossel, 2020), e.g., the nitrogen fixation of Fabaceae or the attractive flowering offer of Malvaceae. Furthermore, it is still unclear whether the risk of spread found in experiments applies in agricultural practice. In most commercial biogas plants, the proportion of surviving seeds is likely to be lower because the average HRT is longer than that tested, e.g., 91 days in Germany (vTI, 2009). The other steps in the biogas production chain can contribute to seed inactivation as well, namely cultivation, harvest, ensiling, storage, pre-treatment and digestate storage (Fröschle et al., 2015). On farms, biomass is usually ensiled prior to AD. In general, ensiling reduced the viability (e.g., Aper et al., 2014; Simard and Lambert-Beaudet, 2016; Piltz et al., 2017), including that of seeds from the flowering wild plant mixture (Hahn et al., 2021). When the same seed lot was both ensiled and anaerobically digested in biogas reactors (Westerman et al., 2012b) or in the

rumen of cattle (Blackshaw and Rode, 1991; Mayer et al., 2000; Stanton et al., 2012; Aper et al., 2014; Piltz et al., 2017), seed-killing efficacy was mostly higher in ensiling than in AD. However, there was considerable variation between and within species. The same picture emerged when comparing the seed-killing efficacies of 36 days in mesophilic AD (**Table 2-1**) with those obtained for 8 months of ensiling by Hahn et al. (2021). The same seed lots of *C. album*, *C. intybus*, *V. thapsus*, *A. theophrasti – 7 years*, *M. alcea – 2 years*, *M. albus* and *M. officinalis* were used in both studies. In NHS species and *M. officinalis*, a greater proportion of seeds was killed by ensiling than by AD. In contrast, ensiling was less effective on *A. theophrasti – 7 years* and *M. alcea – 2 years*, inactivating only 5 and 23%, respectively, while AD killed 100% and about 60%, respectively. Consistent with this, Westerman et al. (2012b) found that their batch of *A. theophrasti* and the species *Malva neglecta* were more resistant to ensiling than to AD. Piltz et al. (2017), studying *Malva parviflora* L. (small-flowered mallow) and referring to *Malva pusilla* SM. (small mallow) studied by Blackshaw and Rode (1991), even suggested that resistance to ensiling may be characteristic of the Malvaceae genus. Finally, for *M. albus*, our results showed that seed-killing efficacy did not differ per se between the processes of ensiling and AD, but depended on the respective conditions: AD at 35°C killed 36% of *M. albus* seeds, ensiling killed 23% and AD at 42°C killed only 2% (**Table 2-1**; Hahn et al., 2021). Regarding a combined seed-killing efficacy of ensiling and AD, the few available sources indicate that it is about the same as that of the individual processes, but tends to kill more seeds (Blackshaw and Rode, 1991; Stanton et al., 2012; Westerman et al., 2012b; Piltz et al., 2017).

We conclude that it is not necessary to categorically exclude HS species to avoid unwanted seed spread when mixtures of flowering wild plant species are used as biogas feedstock. Rather, we recommend that conditions during AD be designed to increase the seed-killing efficacy. This can be achieved, for example, by sufficiently long HRTs in the biogas reactor without short-circuiting. Furthermore, HS species whose seeds are easy to inactivate should be identified. Considering that AD is only one step within the biogas process chain, seed-killing efficacy of AD may have been underestimated in our experiments conducted with mature, vigorous, dry-stored seed, because the seed in practice may be immature and damaged by upstream treatments, making it more susceptible to AD (Westerman and Gerowitt, 2013; Zhou et al., 2020). Therefore, the influence of cultivation parameters on seed quality, e.g., harvest timing, and the influence of feedstock storage conditions, pre-treatment and digestate processing technologies (Monfet et al., 2018; Alsanius et al., 2021) on seed viability remain to be explored. And even though our reactors were comparable to a full-scale fermenter in terms of operating parameters (Heiermann and Plogsties, 2018), our laboratory results require validation on the practical scale. Finally, seed establishment studies under field conditions are needed to realistically assess the dispersal risk of seeds that survive AD.

**Data Availability Statement.** The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher on request.

**Author Contributions.** JH organized the project, designed the study, analyzed the viability of the seeds, and drafted and edited the manuscript. PW analyzed the viability of the seeds and edited the manuscript in coordination with JH. FM contributed to performance and description of the statistical analyses in coordination with JH. MH administrated the operation of the lab-scale reactors and edited the manuscript in coordination with JH. BG led the project and contributed to the conception and design of the study. All authors contributed to manuscript revision, read, and approved the submitted version.

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### 2.1.7 Supplementary Material

**Supplementary Data Sheet 1: Process control of lab-scale biogas reactors**

A total of 12 continuously stirred laboratory-scale reactors were operated for the anaerobic digestion (AD) experiments (**Supplementary Table 2-1**). The anaerobic digestion in the first reactors was initiated with a start-up phase on 29.01.2015. Feeding was carried out manually on six days per week. After reaching the intended organic loading rate (ORL = 3 $g_{VS}$ $l^{-1}$ $d^{-1}$) and a stabilization phase, the insertion of bags containing wildflower plant seeds started on 10.03.2015. Although the reactors were opened frequently, operation was stable according to the monitored time courses of biogas yield, methane content and further process parameters (total solids, volatile solids, pH, ammonium nitrogen, total nitrogen, volatile fatty acids, pH) (**Supplementary Table 2-1**, **Supplementary Figure 2-1 to 2-4**). Fluctuations in gas production resulted mainly from non-feeding on Sundays.

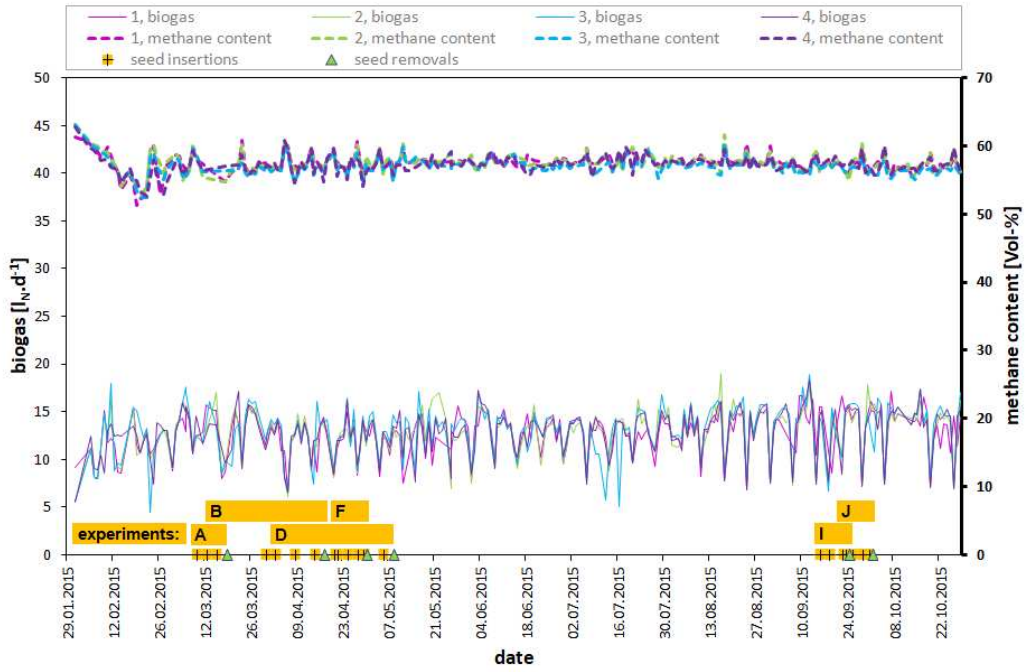
**Supplementary Table 2-1 | Chemical characteristics of the reactor-specific process fluid during the monitored time periods.**

| Reactor | T [°C] | n | | TS [% FM] | VS [% DM] | pH | $NH_4$-N [$g \cdot l^{-1}$] | $N_{tot}$ [$g \cdot l^{-1}$] | AA [$g \cdot l^{-1}$] | PA [$g \cdot l^{-1}$] | VFA [$g \cdot l^{-1}$] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 35 | 11 | mean | 7.6 | 79.3 | 7.5 | 1.6 | 4 | 0.35 | 0.07 | 0.41 |
| | | | range | (6.8-8.6) | (77.0-81.7) | (7.3-7.7) | (1.4-1.8) | (3.4-4.8) | (0.16-0.49) | (0.00-0.33) | (0.18-0.73) |
| | | | sd | 0.6 | 1.4 | 0.1 | 0.2 | 0.3 | 0.09 | 0.1 | 0.15 |
| 2 | 35 | 11 | mean | 7.7 | 79.5 | 7.5 | 1.6 | 4 | 0.37 | 0.06 | 0.42 |
| | | | range | (6.9-8.4) | (76.9-81.8) | (7.4-7.6) | (1.4-1.9) | (3.2-4.8) | (0.21-0.49) | (0.00-0.27) | (0.23-0.72) |
| | | | sd | 0.6 | 1.5 | 0.1 | 0.2 | 0.4 | 0.09 | 0.08 | 0.15 |
| 3 | 35 | 11 | mean | 7.5 | 78.6 | 7.6 | 1.7 | 4 | 0.34 | 0.05 | 0.38 |
| | | | range | (6.7-8.5) | (76.3-80.9) | (7.4-7.7) | (1.4-1.9) | (3.1-4.8) | (0.18-0.45) | (0.00-0.20) | (0.18-0.58) |
| | | | sd | 0.6 | 1.5 | 0.1 | 0.2 | 0.4 | 0.08 | 0.06 | 0.12 |
| 4 | 35 | 11 | mean | 7.5 | 78.8 | 7.6 | 1.7 | 4 | 0.34 | 0.05 | 0.39 |
| | | | range | (6.8-8.2) | (76.2-81.1) | (7.4-7.7) | (1.4-1.9) | (3.3-4.8) | (0.21-0.47) | (0.00-0.31) | (0.22-0.73) |
| | | | sd | 0.5 | 1.6 | 0.1 | 0.2 | 0.3 | 0.08 | 0.09 | 0.15 |
| 5 | 42 | 6 | mean | 6.5 | 75.1 | 7.7 | 1.9 | 4.2 | 0.31 | 0 | 0.31 |
| | | | range | (6.3-6.7) | (73.3-76.2) | (7.6-8.0) | (1.8-1.9) | (3.9-4.8) | (0.26-0.43) | (0.00-0.02) | (0.26-0.43) |
| | | | sd | 0.2 | 1 | 0.2 | 0.1 | 0.3 | 0.06 | 0.01 | 0.06 |

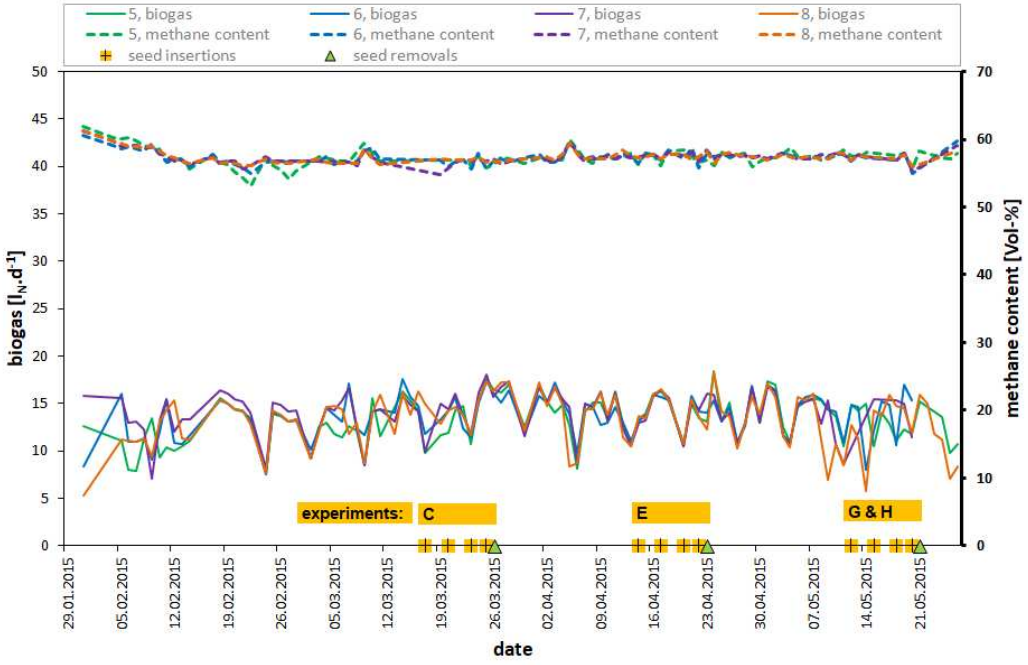
**Supplementary Table 2-1 | continued**

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | mean | 6.4 | 75.3 | 7.7 | 1.8 | 4.2 | 0.31 | 0 | 0.31 |
| 6 | 42 | 6 | range | (6.2-6.6) | (74.5-76.0) | (7.6-7.7) | (1.8-1.9) | (3.8-4.8) | (0.26-0.42) | (0.00-0.02) | (0.26-0.42) |
| | | | sd | 0.1 | 0.7 | 0 | 0.1 | 0.3 | 0.06 | 0.01 | 0.06 |
| | | | mean | 6.4 | 74.9 | 7.7 | 1.9 | 4.2 | 0.31 | nn | 0.31 |
| 7 | 42 | 6 | range | (6.3-6.5) | (73.9-76.1) | (7.6-7.8) | (1.8-1.9) | (3.9-4.8) | (0.26-0.43) | --- | (0.26-0.43) |
| | | | sd | 0.1 | 0.8 | 0.1 | 0.1 | 0.3 | 0.06 | --- | 0.06 |
| | | | mean | 6.5 | 74.7 | 7.7 | 1.8 | 4.1 | 0.31 | 0.01 | 0.32 |
| 8 | 42 | 6 | range | (6.4-6.8) | (73.9-76.4) | (7.6-7.8) | (1.8-1.9) | (3.8-4.8) | (0.24-0.44) | (0.00-0.02) | (0.24-0.45) |
| | | | sd | 0.2 | 0.9 | 0.1 | 0.1 | 0.3 | 0.07 | 0.01 | 0.08 |
| | | | mean | 5.5 | 75.8 | 7.7 | 1.7 | 3.6 | 0.22 | 0.04 | 0.26 |
| 9 | 42 | 20 | range | (4.4-6.2) | (70.0-79.0) | (7.4-8.1) | (1.3-1.9) | (3.1-3.9) | (0.05-0.81) | (0.00-0.13) | (0.05-0.93) |
| | | | sd | 0.4 | 2.2 | 0.2 | 0.2 | 0.3 | 0.18 | 0.04 | 0.21 |
| | | | mean | 5.6 | 75.6 | 7.7 | 1.8 | 3.7 | 0.19 | 0.04 | 0.23 |
| 10 | 42 | 18 | range | (5.2-5.9) | (73.2-78.0) | (7.4-8.1) | (1.6-1.9) | (3.5-3.9) | (0.04-0.82) | (0.00-0.13) | (0.04-0.94) |
| | | | sd | 0.2 | 1.6 | 0.2 | 0.1 | 0.1 | 0.19 | 0.04 | 0.22 |
| | | | mean | 6.2 | 76.2 | 7.8 | 1.9 | 4 | 0.31 | 0.06 | 0.35 |
| 11 | 42 | 10 | range | (5.9-6.4) | (72.7-77.5) | (7.7-8.0) | (1.8-2.0) | (3.9-4.1) | (0.12-0.48) | (0.00-0.18) | (0.14-0.52) |
| | | | sd | 0.1 | 1.4 | 0.1 | 0.1 | 0.1 | 0.78 | 0.05 | 0.86 |
| | | | mean | 5.7 | 75.4 | 7.9 | 1.8 | 3.9 | 0.24 | 0.04 | 0.35 |
| 12 | 42 | 11 | range | (5.0-6.0) | (72.2-76.9) | (7.7-8.0) | (1.8-1.9) | (3.7-4.1) | (0.00-0.49) | (0.00-0.17) | (0.13-0.67) |
| | | | sd | 0.3 | 1.4 | 0.1 | 0.1 | 0.1 | 0.17 | 0.05 | 0.17 |
| | | | **mean** | **6.6** | **76.6** | **7.7** | **1.8** | **4.0** | **0.30** | **0.04** | **0.34** |
| **average** | | **-** | **range** | **(4.4-8.6)** | **(70.0-81.8)** | **(7.3-8.1)** | **(1.3-2.0)** | **(3.1-4.8)** | **(0.00-0.82)** | **(0.00-0.33)** | **(0.04-0.94)** |
| | | | **sd** | **1.3** | **4.8** | **0.5** | **0.5** | **1.0** | **0.87** | **0.19** | **0.98** |

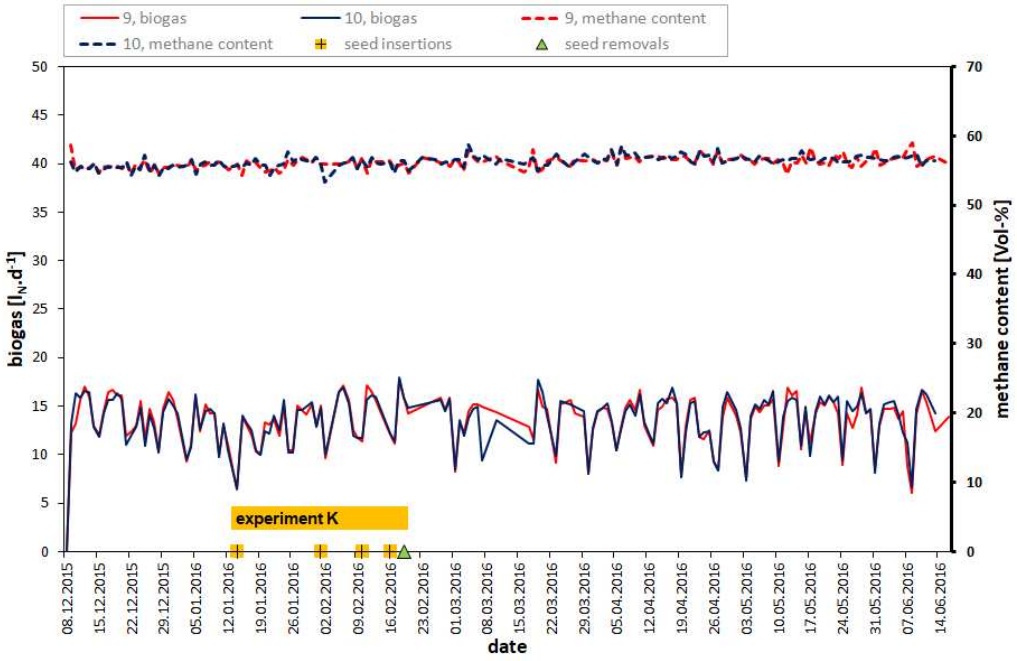
T: operating temperature; n: number of weeks (monitored period), one analysis per reactor and week; mean: mean value; sd: standard deviation; TS: total solids; FM: fresh matter; DM: dry matter; VS: volatile solids; $NH_4$-N: ammonium-bound nitrogen; $N_{tot}$: total nitrogen, AA: acetic acid; PA: propionic acid; VFA: volatile fatty acids (sum of acetic acid, propionic acid and butyric acid comprising butyric, iso-butyric, caproic, valeric, and iso-valeric acid; total acids concentration is expressed as acetic acid equivalent)




**Supplementary Figure 2-1 | Biogas yield (solid lines) and methane content (dashed, thick lines) during the operation of the lab-scale reactors 1-4 at 35°C. Above the x-axis, it is indicated when seeds were inserted or removed from the reactors (labeled as experiments A, B, D, F, I, and J).**




**Supplementary Figure 2-2 | Biogas yield (solid lines) and methane content (dashed, thick lines) during the operation of the lab-scale reactors 5-8 at 42°C. Above the x-axis, it is indicated when seeds were inserted or removed from the reactors (labeled as experiments C, E, G, and H).**




**Supplementary Figure 2-3 | Biogas yield (solid lines) and methane content (dashed, thick lines) during the operation of the lab-scale reactors 9 and 10 at 42°C. Above the x-axis, it is indicated when seeds were inserted or removed from the reactors (labeled as experiment K).**


**Supplementary Figure 2-4 | Biogas yield (solid lines) and methane content (dashed, thick lines) during the operation of the lab-scale reactors 11 and 12 at 42°C. Above the x-axis, it is indicated when seeds were inserted or removed from the reactors (labeled as experiment L).**

**Supplementary Table 2-2 | Number of replicates and number of seeds per replicate for hardseeded (HS) and not hardseeded (NHS) species exposed mesophilic, anaerobic digestion (AD) at 35°C or 42°C for different durations (1 day – 36 days). The numbers of seeds per replicate are indicated by: # = 100 seeds, ## = 200 seeds, ### = 300 seeds.**

| | Untreated control | AD 35°C | | | | | | AD 42°C | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 d | 3 d | 6 d | 9 d | 18 d | 36 d | 1 d | 3 d | 6 d | 9 d | 18 d | 36 d |
| **HS species** | | | | | | | | | | | | | |
| *Abutilon theophrasti – 7 YRS* | 3 # | 4 # | 8 # | 4 ## | 8 ## | 4 ## | 4 ### | 8 # | 8 # | 8 ## | 8 ### | - | - |
| *Abutilon theophrasti – 1 YR* | 4 # | 4 # | 4 # | 4 ## | 4 ### | - | - | - | 4 # | - | 4 # | 4 ## | 4 ### |
| *Malva alcea – 2 YRS* | 3 # | - | 4 # | - | 4 # | 4 ## | 4 ### | - | 2 # | - | 2 # | 2 ## | 2 ### |
| *Malva alcea – 1 YR* | 6 # | 4 # | 4 # | 4 ## | 4 ### | - | - | - | 4 # | - | 4 # | 4 ## | 4 ### |
| *Malva sylvestris* | 6 # | - | 4 # | - | 4 # | 4 ## | 4 ### | - | 4 # | - | 4 # | 4 ## | 4 ### |
| *Melilotus albus* | 9 # | - | 4 # | - | 4 # | 4 ## | 4 ### | - | 6 # | - | 6 # | 6 ## | 6 ### |
| *Melilotus officinalis* | 9 # | - | 4 # | - | 4 # | 4 ## | 4 ### | - | 4 # | - | 4 # | 4 ## | 4 ### |
| **NHS species** | | | | | | | | | | | | | |
| *Chenopodium album* | 6 # | 4 # | 8 # | 4 ## | 8 ## | 4 ## | 4 ### | 4 # | 4 # | 4 ## | 4 ### | - | - |
| *Cichorium intybus* | 3 # | 4 # | 4 # | 4 ## | 4 ### | - | - | 4 # | 4 # | 4 ## | 4 ### | - | - |
| *Daucus carota* | 3 # | 4 # | 4 # | 4 ## | 4 ### | - | - | 4 # | 4 # | 4 ## | 4 ### | - | - |
| *Echium vulgare* | 3 # | 4 # | 4 # | 4 ## | 4 ### | - | - | 4 # | 4 # | 4 ## | 4 ### | - | - |
| *Verbascum thapsus* | 3 # | 4 # | 4 # | 4 ## | 4 ### | - | - | 4 # | 4 # | 4 ## | 4 ### | - | - |
| tomato – PAPRIKA | 3 # | 2 # | 4 # | 2 ## | 4 ## | 2 ## | 2 ### | 4 # | 4 # | 4 ## | 4 ### | - | - |
| tomato – PIERRE | 3 # | 4 # | 6 # | 4 ## | 6 ## | 2 ## | 2 ### | 4 # | 4 # | 4 ## | 4 ### | - | - |

**Supplementary Table 2-3 | Model type and fit (Chi²-test) and parameter estimates (*standard errors in parentheses*) obtained from the log-logistic (LL) and log-logistic models modified to capture hormesis (HLL) used to describe seed viability, *V*, during exposure to anaerobic digestion (AD) at 35°C and 42°C. The lower asymptote was set to zero for all models. Asterisks (*) indicate significant differences in parameter estimates between AD at 35°C and 42°C (p<0.05).**

| | model | | $V_{max}$ | | *SLP* | | | | | *MIT* or *E* | | | | | *H* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | type | p-value | | | 35°C | | | 42°C | | 35°C | | | 42°C | | 35°C | | 42°C | |
| **HS species** | | | | | | | | | | | | | | | | | | |
| *Abutilon theophrasti – 7 YRS* | LL | <0.0001 | **0.78** | *(0.02)* | **2.31** | *(0.10)* | | **-** | - | **1.30** | *(0.06)* | | **-** | - | ***nd*** | | ***nd*** | |
| *Abutilon theophrasti – 1 YR* | LL | <0.0001 | **0.96** | *(0.01)* | **0.92** | *(0.06)* | | **-** | - | **4.74** | *(0.24)* | | **-** | - | ***nd*** | | ***nd*** | |
| *Malva alcea – 2 YRS* | LL | 0.0015 | **0.42** | *(0.01)* | **7.77** | *(3.92)* | | **0.37** | *(0.12)* | **35.38** | *(0.65)* | | **1.67** | *(1.36)* | ***nd*** | | ***nd*** | |
| *Malva alcea – 1 YR* | LL | 0.8024 | **0.51** | *(0.02)* | **0.81** | *(0.12)* | * | **0.38** | *(0.07)* | **5.10** | *(0.68)* | | **0.06** | *(0.06)* | ***nd*** | | ***nd*** | |
| *Malva sylvestris* | HLL | 0.9893 | **0.26** | *(0.02)* | **1.23** | *(0.07)* | | **1.21** | *(0.21)* | **1.89** | *(1.51)* | | **7.16** | *(10.23)* | ***0.27*** | *(0.23)* | ***0.06*** | *(0.09)* |
| *Melilotus albus* | HLL | <0.0001 | **0.84** | *(0.01)* | **1.10** | *(0.01)* | | **1.10** | *(0.02)* | **0.75** | - | | **5.41** | *(0.82)* | ***1.16*** | - | ***0.18*** | *(0.03)* |
| *Melilotus officinalis* | HLL | 0.1823 | **0.85** | *(0.01)* | **1.00** | *(0.02)* | * | **1.05** | *(0.02)* | **1.57** | *(1.23)* | | **1.52** | *(4.84)* | ***0.49*** | *(0.39)* | ***0.60*** | *(1.99)* |
| **NHS species** | | | | | | | | | | | | | | | | | | |
| *Chenopodium album* | LL | 0.9965 | **0.92** | *(0.00)* | **6.41** | *(0.28)* | * | **5.94** | *(0.29)* | **22.09** | *(0.30)* | * | **6.77** | *(0.07)* | ***nd*** | | ***nd*** | |
| *Cichorium intybus* | LL | 1.0000 | **0.60** | *(0.03)* | **11.27** | *(28.91)* | | **-** | - | **1.00** | *(0.02)* | | **-** | - | ***nd*** | | ***nd*** | |
| *Daucus carota* | LL | <0.0001 | **0.80** | *(0.02)* | **3.03** | *(0.16)* | | **-** | - | **1.32** | *(0.06)* | | **-** | - | ***nd*** | | ***nd*** | |
| *Echium vulgare* | LL | 0.9999 | **0.41** | *(0.03)* | **3.90** | *(0.62)* | | **-** | - | **0.90** | *(0.06)* | | **-** | - | ***nd*** | | ***nd*** | |
| *Verbascum thapsus* [a] | - | - | **-** | - | **-** | - | **-** | **-** | - | **-** | - | | **-** | - | ***nd*** | | ***nd*** | |
| tomato – PAPRIKA | LL | <0.0001 | **0.93** | *(0.01)* | **3.07** | *(0.18)* | * | **1.70** | *(0.08)* | **5.64** | *(0.18)* | * | **1.26** | *(0.07)* | ***nd*** | | ***nd*** | |
| tomato – PIERRE | LL | 0.0136 | **0.97** | *(0.00)* | **5.79** | *(0.27)* | * | **1.63** | *(0.07)* | **12.56** | *(0.20)* | * | **1.89** | *(0.09)* | ***nd*** | | ***nd*** | |

[a] No model was fitted for *V. thapsus* because all seeds were inactivated before sampling at the first exposure time.

$V_{max}$: maximum proportion of *V*; defined to be identical for both temperatures.

*SLP*: a parameter proportional to the slope of the curve in the inflection point.

MIT: mean inactivation time; i.e., the time after which the LL curve changes its flection and *V* is reduced to 50% of the initial *V*. In HLL, *E* is not directly interpretable.

*H*: hormesis effect size, which is not determined in LL models ("nd").

-: parameters could not be estimated, mostly due to inactivation of seeds during the shortest exposure time.

**Supplementary Table 2-4 | Model type and fit (Chi²- test) and parameter estimates (*standard errors in parentheses*) of the Weibull (W) and log-logistic models modified to capture hormesis (HLL) used to describe cumulative germination, *cG*, during exposure to anaerobic digestion (AD) at 35°C and 42°C. The lower asymptote was set to zero for all models. Asterisks (*) indicate significant differences in parameter estimates between AD at 35°C and 42°C (p<0.05).**

| | model | | $cG_{max}$ | | *SLP* | | | | *IFT* or *E* | | | | *H* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | type | p-value | | | 35°C | | | 42°C | 35°C | | | 42°C | 35°C | | 42°C | |
| **HS species** | | | | | | | | | | | | | | | | |
| *Abutilon theophrasti – 7 YRS* | HLL | <0.0001 | **0.14** | *(0.02)* | **6.09** | *(0.51)* | | - - | **1.10** | *(0.10)* | | - - | ***0.85*** | *(0.21)* | - | - |
| *Abutilon theophrasti – 1 YR* | W | 0.7318 | **0.39** | *(0.02)* | **7.11** | *(4.71)* | | - - | **2.61** | *(0.24)* | | - - | ***nd*** | | ***nd*** | |
| *Malva alcea – 2 YRS* | W | 0.9166 | **0.18** | *(0.02)* | **0.46** | *(0.09)* | | **0.38** *(0.14)* | **2.37** | *(1.08)* | | **0.34** *(0.45)* | ***nd*** | | ***nd*** | |
| *Malva alcea – 1 YR* | W | 0.9602 | **0.32** | *(0.02)* | **0.53** | *(0.08)* | | **0.30** *(0.08)* | **2.27** | *(0.45)* | | **0.07** *(0.08)* | ***nd*** | | ***nd*** | |
| *Malva sylvestris* | W | 1.0000 | **0.03** | *(0.01)* | **2.21** | *(10.88)* | | **1.46** *(10.60)* | **2.88** | *(0.89)* | | **1.61** *(7.30)* | ***nd*** | | ***nd*** | |
| *Melilotus albus* | W | 0.7972 | **0.15** | *(0.01)* | **0.10** | *(0.02)* | | **0.04** *(0.03)* | **0.02** | *(0.03)* | * | **1059.30** *(2713.96)* | ***nd*** | | ***nd*** | |
| *Melilotus officinalis* | W | 0.0272 | **0.07** | *(0.01)* | **0.12** | *(0.02)* | | **0.10** *(0.07)* | **0.01** | *(0.00)* | * | **740.02** *(1091.97)* | ***nd*** | | ***nd*** | |
| **NHS species** | | | | | | | | | | | | | | | | |
| *Chenopodium album* | W | 0.9995 | **0.93** | *(0.00)* | **3.76** | *(0.24)* | * | **6.34** *(1.22)* | **20.86** | *(0.35)* | * | **6.23** *(0.07)* | ***nd*** | | ***nd*** | |
| *Cichorium intybus* | W | 1.0000 | **0.60** | *(0.03)* | **5.00** | *(302.21)* | | **-** - | **1.02** | *(1.17)* | | **-** - | ***nd*** | | ***nd*** | |
| *Daucus carota* | W | 1.0000 | **0.75** | *(0.03)* | **3.10** | *(46.16)* | | **-** - | **1.15** | *(2.43)* | | **-** - | ***nd*** | | ***nd*** | |
| *Echium vulgare* | W | 1.0000 | **0.38** | *(0.03)* | **2.24** | *(2.60)* | | **-** - | **0.99** | *(0.06)* | | **-** - | ***nd*** | | ***nd*** | |
| *Verbascum thapsus*[a] | - | - | **-** | - | **-** | - | - | **-** - | **-** | - | | **-** - | **-** | | **-** | |
| tomato – PAPRIKA | W | <0.0001 | **0.94** | *(0.01)* | **1.79** | *(0.11)* | * | **0.97** *(0.05)* | **6.19** | *(0.16)* | * | **1.19** *(0.07)* | ***nd*** | | ***nd*** | |
| tomato – PIERRE | W | <0.0001 | **0.95** | *(0.01)* | **3.26** | *(0.19)* | * | **1.03** *(0.05)* | **11.81** | *(0.19)* | * | **1.33** *(0.07)* | ***nd*** | | ***nd*** | |

[a] No model was fitted for *V. thapsus* because all seeds failed to germinate before sampling at the first exposure time.

c$G_{max}$: maximum proportion of *cG;* defined to be identical for both temperatures.

*SLP*: a parameter proportional to the slope of the curve in the inflection point.

*IFT*: inflection time; i.e., the time after which the W curve changes its flection. In HLL, *E* is not directly interpretable.

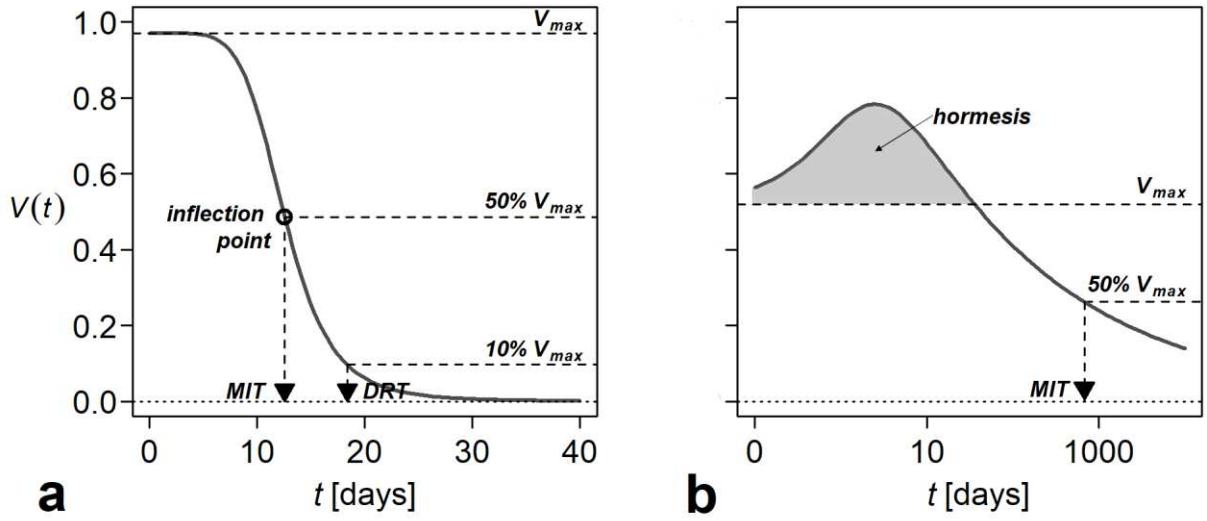
*H*: hormesis effect size, which is not determined in W models ("nd").

-: parameters could not be estimated (-), mostly due to failure of germination during the shortest exposure time.

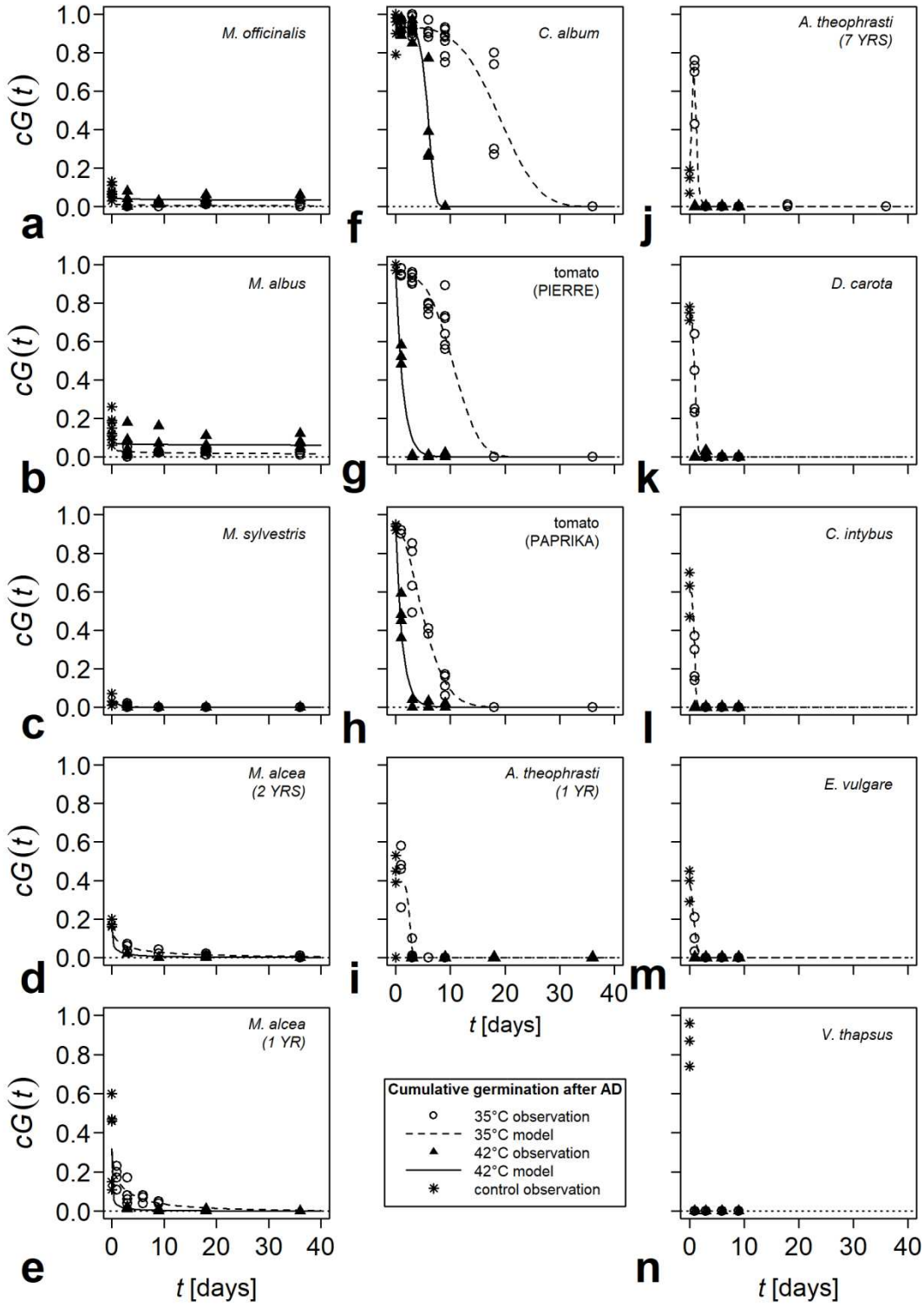
**Supplementary Table 2-5 | Estimated Decimal Reduction Times for cumulative Germination, *DRT(cG),* of flowering wild plant species and tomato after anaerobic digestion (AD) at 35°C or 42°C and difference between *DRTs* of *cG* and overall viability, *V* (see Table 1). Species are grouped according to their potential to exhibit hardseededness (HS) in their seeds or not (NHS). Standard errors of the mean are given in parentheses.**

| | ***DRT(cG)* [days]** | | | | ***DRT(cG)*-*DRT(V)* [days]** | |
|---|---|---|---|---|---|---|
| | **AD 35°C** | | **AD 42°C** | | **AD 35°C** | **AD 35°C** |
| **HS species** | | | | | | |
| *Abutilon theophrasti – 7 YRS* | **2.6** | *(0.2)* | **<1** | | -0.8 | nd |
| *Abutilon theophrasti – 1 YR* | **2.9** | *(0.1)* | **<1** | | -48.9 | nd |
| *Malva alcea – 2 YRS* | **14.7** | *(2.9)* | **3.2** | *(1.8)* | -32.2 | -(365)·n |
| *Malva alcea – 1 YR* | **11.0** | *(1.5)* | **1.0** | *(0.6)* | -65.7 | -17.3 |
| *Malva sylvestris* | **4.2** | *(7.0)* | **2.9** | *(1.3)* | -(365)·n | -(365)·n |
| *Melilotus albus* | **61.6** | *(56.4)* | **>365** | | -(365)·n | -(365)·n |
| *Melilotus officinalis* | **7.1** | *(5.9)* | **>365** | | -(365)·n | -(365)·n |
| **NHS species** | | | | | | |
| *Chenopodium album* | **26.0** | *(0.7)* | **7.1** | *(0.2)* | -5.1 | -2.7 |
| *Cichorium intybus* | **1.2** | *(13.5)* | **<1** | | 0 | nd |
| *Daucus carota* | **1.5** | *(9.2)* | **<1** | | -1.2 | nd |
| *Echium vulgare* | **1.4** | *(0.6)* | **<1** | | -0.2 | nd |
| *Verbascum thapsus* | **<1** | | **<1** | | nd | nd |
| tomato – PAPRIKA | **9.9** | *(0.3)* | **2.8** | *(0.1)* | -1.6 | -1.8 |
| tomato – PIERRE | **15.2** | *(0.4)* | **3.0** | *(0.1)* | -3.2 | -4.3 |

<1: seeds failed to germinate even after the shortest exposure time; no models fitted, no standard errors calculated.
>365: estimated *DRT* values exceeded one year (365 days); no models fitted, no standard errors calculated.
nd: The difference between *DRT(cG)* and *DRT(V)* was not determined if no models could be fitted for *cG*, *V* or both.
-(365)·n: Differences between *DRT(cG)* and *DRT(V)* exceeding one year.




**Supplementary Figure 2-5 | Schematic representation of dose-response models used to describe seed viability, *V*, in anaerobic digestion (AD). (a) *V* as log-logistic function of exposure time in AD, *t*. (b) *V* as log-logistic function of *t* including hormesis. Here, the x-axis was log-scaled and the function extrapolated to 10,000 days to visualize the parameters. $V_{max}$ = maximum proportion of viable seeds, *MIT* = median inactivation time, *DRT* = Decimal Reduction Time, hormesis = increase of *V* at short exposure to AD. For details see section 2.1.2.3.1 "Seed Viability Models".**




**Supplementary Figure 2-6 |** Cumulative germination, *cG*, of flowering wild plant species and tomato after 21 days of a germination test following anaerobic digestion (AD) in lab-scale reactors at 35°C (dashed lines) and 42°C (solid lines). Symbols present observations, each containing a minimum of 100 seeds: asterisks for untreated controls, open circles for AD at 35°C and filled triangles for AD at 42°C. (**a**) *Melilotus officinalis*, (**b**) *Melilotus albus*, (**c**) *Malva sylvestris*, (**d**) *Malva alcea (2 YRS)*, (**e**) *Malva alcea (1 YR)*, (**f**) *Chenopodium album*, (**g**) tomato (PIERRE), (**h**) tomato (PAPRIKA), (**i**) *Abutilon theophrasti (1 YR)*, (**j**) *Abutilon theophrasti (7 YRS)*, (**k**) *Daucus carota*, (**l**) *Cichorium intybus*, (**m**) *Echium vulgare*, (**n**) *Verbascum thapsus*. For better comparability, panels are arranged according to the species' viability after 36 days of exposure to AD.

## 2.2 Temperature Inactivation of Seeds in Biogas Reactors

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# Die Bedeutung der Temperatur für die Inaktivierung von Samen im Biogas-Reaktor

*The importance of temperature in the inactivation of seeds in biogas reactors*

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**Zusammenfassung |** Samen von Unkräutern können mit der Ernte von Biomasse oder Zufuhr von Dung in den Biogas-Reaktor gelangen. Alle Unkrautsamen, die auf diese Weise in die Biogas-Prozesskette gelangen und die anaerobe Vergärung überleben, können mit der Ausbringung des Gärrestes verbreitet werden. Die Inaktivierung der Samen im Biogas-Reaktor erfolgt hauptsächlich über die Temperatur. Im Vergleich von Labor-Biogas-Reaktor und Wasserbad haben wir den Einfluss der Temperatur auf das Überleben der Samen von einer hartschaligen und einer nicht-hartschaligen Art überprüft. Von den Tomatensamen, die auch als Indikator für die Hygienisierung von Vergärungsanlagen genutzt werden, überlebten im Mittel nur 20 % die maximale Expositionszeit im Reaktor und im Wasserbad. Die Samen verloren ihre Vitalität im Reaktor schneller als unter ausschließlichem Temperatureinfluss. Die Vitalität der hartschaligen Art, *Melilotus albus*, sank bis zur maximalen Expositionszeit (12 Tage) auf etwa 70 % ab. Die Abnahme der Vitalität erfolgte gleichermaßen in Wasserbad und Reaktor. Die Inaktivierung der Samen von *M. albus* beruhte hauptsächlich auf der Wirkung der Temperatur. Bei der Tomate waren auch andere Faktoren beteiligt. Die Tomate ist kein geeigneter Indikator-Organismus für die Inaktivierung von (hartschaligen) Pflanzensamen im Biogas-Reaktor.

**Stichwörter: Biogas-Reaktor, Hartschaligkeit, Temperatur, Tomate, Wasserbad**

**Abstract |** Weed seeds can enter the biogas reactor by the harvest of biomass or by animal manure. All seeds that enter the biogas process chain and survive anaerobic digestion can be spread with the digestate. The inactivation of seeds in the biogas reactor is mainly due to temperature. In comparison of a laboratory-scale biogas reactor and a water bath experiment, we tested the contribution of temperature in the inactivation of seeds from one hardseeded and one non-hardseeded species. On average, as few as 20 % of the tomato seeds, which are used as an indicator species for the sanitation of fermentation plants, survived the maximum exposure time in the reactor and water bath. In the reactor the seeds lost their viability quicker than could solely be explained by temperature. Viability of the hardseeded species, *Melilotus albus*, declined to 70 % after the maximum exposure time of 12 days. The decline was similar in water baths and reactor. Inactivation of *M. albus* seeds was mainly due to temperature. For tomato seeds, factors other than temperature must have contributed to inactivation. Tomato appears to be no appropriate indicator for inactivation of (hardseeded) seeds in biogas reactors.

**Keywords: Biogas reactor, hardseededness, temperature, tomato, water bath**

### 2.2.1 Einleitung

Mit der Ernte von Biomasse oder der Zufuhr von Dung gelangen auch Samen von Unkräutern in den Biogas-Reaktor. Mais für die Biogasproduktion wird gewöhnlich so früh geerntet, dass nicht alle Unkräuter die Möglichkeit zur Vermehrung haben. Dennoch fanden WESTERMAN et al. (2012A) neben Arten, die unterhalb der Schnitthöhe des Maises wuchsen und nur wenige Samen produzierten, auch hochwachsende, viele Samen produzierende Arten, deren Fruchtstände größtenteils oberhalb der Schnitthöhe lagen. Es handelte sich hauptsächlich um *Chenopodium album* (L.) und *Echinochloa crus-galli* (L.) P. BEAUV. Alle Unkrautsamen, die auf diese Weise in die Biogas-Prozesskette gelangen und die anaerobe Vergärung überleben, können mit der Ausbringung des Gärrestes verbreitet werden.

Während der mesophilen Vergärung im Biogasreaktor herrschen ein dunkles, feuchtes und anaerobes Milieu bei Temperaturen zwischen 20 und 40°C sowie pH-Werte, die zwischen 6,8 und 8 liegen. Neben Wasser, Methan und Kohlenstoffdioxid tritt eine Vielzahl von Substanzen in Biogasreaktoren auf z. B. Enzyme, organische Säuren, Alkohole, Schwefelwasserstoff-Verbindungen, Cyanide und Ammoniak. Zusätzlich werden die Samen bei ihrem Eintritt in den Reaktor von einem Biofilm aus Bakterien, Archen und Protisten besiedelt. Pflanzensamen im Biogasreaktor können demzufolge auf thermischem, biologischem und chemischem Wege inaktiviert werden (WESTERMAN und GEROWITT, 2013).

Pflanzensamen unterscheiden sich stark in ihrer Fähigkeit, die extremen Bedingungen in Biogas-Anlagen zu überleben (WESTERMAN et al., 2012). Die Temperatur gilt hierbei als der wichtigste Parameter, von dem das Überleben der Samen abhängt (WESTERMAN und GEROWITT, 2013). Die sogenannte Thermoresistenz der Samen ist daher von besonderer Bedeutung. Die Thermoresistenz hängt stark vom Wassergehalt der Samen ab. In der mesophilen, anaeroben Vergärung liegt die Temperatur bei 20°C oder höher und die Samen sind vollständig wassergesättigt (Wassergehalt >20 %). Solange die Temperaturen nicht zu hoch sind (T=20 – 35°C), können vollständig wassergesättigte Samen einige Zeit überleben, wenn sie nicht keimen oder verrotten (z.B. VILLIERS, 1974; MURDOCH und ELLIS, 2000). Bei höheren Temperaturen (T>35°C) nimmt ihre Lebensfähigkeit exponentiell mit der Zeit ab (ECONOMOU et al. 1998; DAHLQUIST et al., 2007; WESTERMAN et al., 2012C). Im Allgemeinen gilt: je höher die Temperatur, desto kürzer die Zeitspanne bis zur thermischen Inaktivierung (WESTERMAN und GEROWITT, 2013).

Es gibt allerdings Mechanismen und Bestandteile, die den Effekt von hohen Temperaturen auf die Sameninaktivierung modifizieren können. In einer Literaturstudie identifizierten WESTERMAN und GEROWITT (2013) Unkrautarten mit harten Samen (physikalische Dormanz), hoher Thermoresistenz, einer dicken Samenschale oder mit Anpassungen an Endozoochorie als Hochrisiko-Arten für das Überleben in Biogasreaktoren. Einen Spezialfall stellen hier solche Samen dar, die über eine wasserundurchlässige Schicht in ihrer Samenschale verfügen (ROLSTON, 1978) und nachweislich ungewöhnlich widerstandsfähig gegenüber anaerober Vergärung sind. Diese Samen werden als

„hartschalig“ bezeichnet und sind weniger anfällig für Hitze-Stress, weil sie kein Wasser aufnehmen, nicht quellen und nicht weich werden wie „nicht-hartschalige“ Samen (WESTERMAN und GEROWITT, 2013). Hartschaligkeit findet sich oft bei den Fabaceae, wurde aber auch bei Convolvulaceae, Geraniaceae, Malvaceae und Solanaceae beobachtet (ROLSTON, 1978; BASKIN et al., 2000; MURDOCH und ELLIS, 2000).

In dieser Pilotstudie wollten wir den Einfluss der Temperatur auf die Inaktivierung von Pflanzensamen im Biogasreaktor quantifizieren. Dazu haben wir die Überlebenswahrscheinlichkeit der Samen von zwei Arten während der anaeroben Vergärung bei 42°C im Labormaßstab bestimmt. Um den alleinigen Einfluss der Temperatur auf das Überleben der Samen zu ermitteln, wurden sie zusätzlich in einem Wasserbad bei 42°C inkubiert.

## 2.2.2 Material und Methoden

### 2.2.2.1 Pflanzensamen

Als Testorganismen haben wir die Tomate und eine Art aus einer Pflanzenfamilie mit bekannter Hartschaligkeit gewählt. Das erlaubte uns, zusätzlich zu überprüfen, wie gut die Samen der Tomate als Indikator für die Phytohygienisierung in der mesophilen anaeroben Vergärung geeignet sind.

Tomatensamen (Sorte St. Pierre) (*Lycopersicon esculentum* (L.)) wurden als Präzisionssaatgut von Bingenheimer Saatgut AG (Echzell-Bingenheim, Deutschland) bezogen. Als Beispielart für hartschalige Samen diente in dieser Studie ein Vertreter der Familie der Fabacea, der Weiße Steinklee (*Melilotus albus* (L.)). Die Samen von *M. albus* wurden von Appels Wilde Samen GmbH (Darmstadt, Deutschland) bezogen.

### 2.2.2.2 Überlebenswahrscheinlichkeit bei 42°C in Biogas-Reaktoren im Labormaßstab

Die kontinuierlich durchmischten Laborreaktoren (Arbeitsvolumen 8l) wurden mit einer Mischung aus Maissilage und Rindergülle betrieben. Die Vergärungstemperatur in den Reaktoren lag mit 42°C im oberen mesophilen Bereich. Abhängig von der Expositionszeit wurden 100, 200 oder 300 Samen pro Art in feinmaschige Polyester-Beutel eingenäht (WESTERMAN et al., 2012B) und am Rührer der Reaktoren befestigt. Die Samen wurden der anaeroben Vergärung bei 42°C in vier Replikaten für 1, 3, 6 oder 9 Tage ausgesetzt. Nach den unterschiedlichen Expositionszeiten wurde die Lebensfähigkeit der Samen wie bei WESTERMAN et al. (2012B) beschrieben durchgeführt. Zusammengefasst: Die Samen wurden für 2 min mit 1 % NaOCl-Lösung oberflächensterilisiert, drei Mal in destilliertem Wasser gespült und auf „Diasporen-Agar“ ausgelegt. Die Keimungsraten der Samen wurden 21 Tage lang überprüft. Die Lebensfähigkeit der Samen, die in den 21 Tagen nicht keimten, wurde mittels Tetrazolium-Färbung getestet. Als Kontrolle wurde die Keim- und Lebensfähigkeit der Samen, die nicht der anaeroben Vergärung ausgesetzt wurden, bestimmt. Dazu wurden sie zwei Tage vor Beginn der Tests im Dunkeln angequollen. Die Anzahl der gekeimten und lebensfähigen Samen wurde zur Bestimmung des Anteils vitaler Samen addiert.

#### 2.2.2.3 Überlebenswahrscheinlichkeit bei 42°C im Wasserbad

Um den Einfluss der Temperatur auf die Überlebenswahrscheinlichkeit der Samen während der anaeroben Vergärung zu quantifizieren, wurden Samen in Präzisionswasserbädern (WB-6, Firma witeg Labortechnik GmbH, Wertheim, Deutschland) bei 42°C inkubiert. Die Wasserbäder waren auf 0.1°C genau regelbar. Die Samen wurden für 2 min mit 1 % NaOCl-Lösung oberflächensterilisiert, drei Mal in destilliertem Wasser gespült und mit 2 ml 0.5M Puffer (HEPES, pH 7.0) in Reagenzgläser gegeben. Die Reagenzgläser wurden für 1, 3, 6, 9 und 12 Tage inkubiert. Pro Art und Expositionszeit wurden je acht Replikate mit 50 Samen untersucht. Nach den verschiedenen Expositionszeiten wurde die Lebensfähigkeit der Samen mittels Tetrazolium-Färbung bestimmt.

#### 2.2.2.4 Statistische Analyse

Für die Modellierung des nicht-linearen Zusammenhangs zwischen Expositionszeit („dose“) und Vitalität der Samen („response“), wurde das Paket „Dose-response-curves“ (drc, Version 2.5-12 (RITZ und STREIBIG, 2015) für R! (Version 3.2.1) verwendet.

Mittels der Funktion „mselect“ wurde ein Ausgangsmodell mit verschiedenen anderen Modellen verglichen. Die Art wurde als Gruppenvariable gesetzt. Als das bestangepasste Modell wurde das gewählt, welches den kleinsten AIC-Wert und Standardfehler der Residuen, sowie den größtmöglichen lack-of-fit-Wert aufwies. Signifikante Unterschiede zwischen den einzelnen Parametern wurden mit Funktion „compParm“ festgestellt. Außerdem wurden ED50- und ED90-Werte berechnet, die die Zeit angeben, nach der laut Modellierung 50 % bzw. 90 % der Samen bei 42°C abgestorben wären.

### 2.2.3 Ergebnisse

#### 2.2.3.1 Tomatensamen

Die Vitalität der Tomatensamen im Biogasreaktor sank innerhalb der ersten drei Tage rapide auf 20 %. In den folgenden sechs Tagen verblieb sie entweder auf diesem Niveau oder die Samen starben vollständig ab. Im Wasserbad war der Verlauf umgekehrt: in den ersten drei Tagen nahm die Vitalität kaum ab und sank bis zur maximalen Expositionszeit von 12 Tagen auf Werte zwischen 0 % und 40 % (***Abb.1 A*** **= Figure 2-5 A**).

Die Überlebenswahrscheinlichkeit der Tomatensamen in Abhängigkeit von der Expositionszeit bei 42°C wurde mit der vier-parametrigen Weibull-Funktion modelliert, die definiert ist als: $f(x) = c + (d - c)\, exp(-exp\left(b(log(x) - log(e))\right))$. Die einzelnen Parameterwerte sind in ***Tab.1*** (= **Table 2-2**) angegeben. Im Vergleich der Modellparameter zwischen Wasserbad und Reaktor unterschieden sich c (untere Asymptote) und e (Wendepunkt der Kurve) signifikant. Entsprechend der Modellierung lagen die $ED_{50}$-Werte bei 8,7 Tagen im Wasserbad und 1,6 Tagen im Reaktor. Die $ED_{90}$ Werte lagen bei 13,9 Tagen im Wasserbad und 11,8 Tagen im Reaktor.

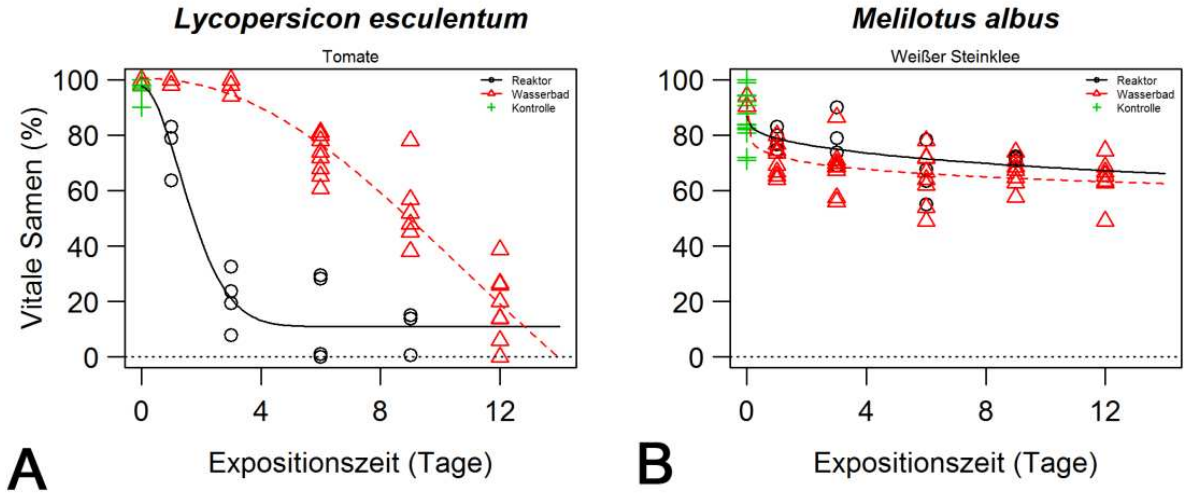


***Abb. 1*** *Überlebenswahrscheinlichkeit der Samen von* L. esculentum *(A) und* M. albus *(B) bei 42°C über 12 Tage im Wasserbad (Dreiecke) oder 9 Tage in der anaeroben Vergärung im Biogas-Reaktor im Labormaßstab (Kreise). Die gestrichelte und die durchgezogene Linie sind die gefitteten dose-response-Modelle.*

**Figure 2-5 | Probability of survival of seeds from *L. esculentum* (A) and *M. albus* (B) at exposure to 42°C for 12 days in water baths (triangles) and 9 days at laboratory-scale anaerobic digestion (circles), respectively. The dotted and solid lines are the fitted dose-response-models.**

***Tab. 1*** *Mittlere geschätzte Parameterwerte (± Standardfehler) der Weibull-Modelle für die Überlebenswahrscheinlichkeit von Tomate (*L. esculentum*) und Weißem Steinklee (*M. albus*) in Abhängigkeit von der Expositionszeit bei 42°C im Wasserbad und im Labor-Biogas-Reaktor.*

**Table 2-2 | Mean parameter estimates (± standard errors) of the Weibull models for the survival probability of tomato (*L. esculentum*) and White sweet clover (*M. albus*) as a function of exposure time at 42°C in water baths and in laboratory-scale biogas reactors, respectively.**

| Art | Behandlung | Modell | Parameterwerte | | | |
|---|---|---|---|---|---|---|
| | | | b | c | d | e |
| *L. esculentum* | Wasserbad | W1.4 | 2,10 ± 0,4 | -69,04 ± 104,6* | 100,54 ± 2,7 | 14,69 ± 6,4* |
| | Reaktor | W1.4 | 1,84 ± 0,3 | 10,89 ± 3,1 | 97,72 ± 2,8 | 1,98 ± 0,2 |
| *M. albus* | Wasserbad | W1.3 | 0,18 ± 0,1 | 0 | 91,95 ± 5,6 | 2566,8 ± 3258,7 |
| | Reaktor | W1.3 | 0,40 ± 0,3 | 0 | 86,76 ± 2,1 | 371,38 ± 1035,8 |

W1.4 = vier-parametrige Weibull-Funktion; 4-parameter Weibull function
W1.3= vier-parametrige Weibull-Funktion mit fester unterer Asymptote (c); 4-parameter Weibull function with fixed lower limit (c)
b= Steigung; slope
c= untere Asymptote; lower limit
d= obere Asymptote; upper limit
e= Wendepunkt der Kurve; inflection point
* signifikante Unterschiede zwischen den Parametern für Tomatensamen in Wasserbad und Biogas-Reaktoren; indicates significant differences between the parameter estimates for tomato seeds in water baths and biogas reactors

#### 2.2.3.2 Samen von *M. albus*

Die Überlebenswahrscheinlichkeit der Samen von *M. albus* war im Reaktor und Wasserbad verhältnismäßig ähnlich. Bis zur maximalen Expositionszeit von 12 Tagen sank sie auf ca. 70 % ab (***Abb. 1B*** **= Figure 2-5 B**).

Das beste Modell zur Berechnung der Überlebenswahrscheinlichkeit von *M. albus* war ebenfalls die Weibull-Funktion mit drei Parametern und mit fester unterer Asymptote (c) bei 0 % (***Tab.1*** = **Table 2-2**). Die Koeffizienten beider Modelle unterschieden sich nicht signifikant voneinander. Die Modellierung ergab eine $ED_{50}$ von 83 Tagen im Reaktor und 171 Tagen im Wasserbad. Als $ED_{90}$ Werte wurden 7 Jahre im Reaktor und 547 Jahre im Wasserbad berechnet.

### 2.2.4 Diskussion

#### 2.2.4.1 Temperatureffekt und Modellierung

Die Samen der Tomate verloren ihre Vitalität im Reaktor innerhalb der ersten drei Tage schneller als im Wasserbad. Auch im Vergleich der Modelle war der Unterschied zwischen reinem Temperatureinfluss (= Wasserbad) und Reaktor sichtbar: Die Wendepunkte waren signifikant verschieden voneinander. Die Reduktion der Vitalität setzte unter alleinigem Einfluss der Temperatur später ein, was sich auch in den $ED_{50}$-Werten niederschlägt (1,6 Tage im Reaktor vs. 8,7 Tage im Wasserbad). Dies könnte ein Hinweis darauf sein, dass bei der Inaktivierung von Tomatensamen im Biogas-Reaktor die Temperatur nicht der Hauptfaktor ist, sondern auch chemische und/oder biologische Prozesse eine Rolle spielen (WESTERMAN und GEROWITT, 2013). Der $ED_{90}$-Wert lag im Labor-Reaktor mit 11,8 Tagen viel höher als die 2,0±1,8 Tage, die in Praxis-Biogas-Anlagen bei 41°C ermittelt wurden (WESTERMAN et al., 2012C). Allerdings betonen WESTERMAN et al. (2012C) schon in ihrer Studie, dass Labor-Reaktoren nicht notwendigerweise ein gutes Modellsystem für Praxis-Biogas-Anlagen darstellen.

Die Abnahme der Vitalität der Samen von *M. albus* erfolgte fast deckungsgleich in Reaktor und Wasserbad. Auch die Modelle zur Überlebenswahrscheinlichkeit in Abhängigkeit von der Expositionsdauer unterschieden sich nicht signifikant voneinander. Offenbar war hier die Temperatur der entscheidende Faktor für Inaktivierung der Samen im Reaktor.

Wir möchten erwähnen, dass die Extrapolation der genutzten Modelle über den Messzeitraum hinaus fraglich ist. Deutlich wird diese Limitation in den errechneten $ED_{90}$-Werten für *M. albus*. Laut des Modells, das mit einer maximalen Expositionszeit von 12 Tagen erstellt wurde, würde es 547 Jahre dauern, um die 90 % der *M. albus* Samen im Wasserbad bei 42°C zu inaktivieren. Das ist unrealistisch. Selbst in der Bodensamenbank, wo sie nicht permanent hohen Temperaturen ausgesetzt sind, bleiben nur wenige Samen weniger Arten länger als 100 Jahre keimfähig (THOMPSON et al., 1997). Mehr Messwerte in der Zeit wären notwendig, um die für hartschalige Arten zu erwartende, doppelt exponentielle Absterbedynamik (WESTERMAN et al., 2012C) plausibel modellieren zu können.

#### 2.2.4.2 Tomate als Indikator-Organismus

In den Untersuchungen von WESTERIK und KLEIZEN (2006) und STRAUß et al. (2012) waren die Samen der Tomate widerstandsfähiger gegenüber der mesophilen, anaeroben Vergärung als die meisten der getesteten Arten. Dies führte zu der Annahme, dass Tomatensamen sich als Indikator-Organismus für die Phytohygiene von Gärresten eignen würden. In Deutschland wird zum Nachweis der phytohygienischen Unbedenklichkeit von Vergärungs- und Kompostierungsanlagen - laut der BioAbfV 1998 - die Tomate als Hygiene-Leitorganismus genutzt. Der Grenzwert im Biotest beträgt ≤ 2 % keimfähige Samen je Prüfbereich. In unserer Studie überlebten die Samen von *M. albus* sowohl die Vergärung im Labormaßstab als auch die Exposition im Wasserbad viel besser als die der Tomate. Zudem entwickelte sich die Überlebenswahrscheinlichkeit beider Arten in Abhängigkeit von der Expositionszeit unterschiedlich (unterschiedliche Modelle). Während *M. albus* im Reaktor vermutlich vorrangig durch den Einfluss der Temperatur inaktiviert wurde, waren die Tomatensamen auch für andere Faktoren anfällig. Damit ist die Tomate - in Übereinstimmung mit SCHRADE et al. (2003) und WESTERMAN et al. (2012B, C) - laut unseren Ergebnissen kein geeigneter Indikator-Organismus für die Phytohygiensierung des Gärrestes im Biogas-Prozess. Um die Wirkung der Temperatur im Biogasreaktor auf die Inaktivierung von Pflanzensamen und damit das Risiko einer Kontamination des Gärrestes abschätzen zu können, sollte die systematische Forschung zur Überlebensfähigkeit von Unkräutern aus verschiedenen taxonomischen und funktionellen Gruppen in der anaeroben Vergärung fortgesetzt werden (WESTERMAN und GEROWITT, 2013).

### 2.2.5 Danksagung

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## 2.3 Screening for Thermoresistant Seeds

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# Wildpflanzen-Samen in der Biogas-Anlage: Screening des Überlebensrisikos verschiedener Arten

*Wildflower seeds in the biogas reactor: Screening the risk of survival of different species*

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**Zusammenfassung |** Wenn Pflanzen-Samen in eine Biogas-Anlage gelangen, besteht das Risiko, dass sie die anaerobe Vergärung überleben und mit Ausbringung des Gärrestes verbreitet werden. Neuerdings kommt durch den Einsatz von Blühmischungen eine Vielzahl von Wildpflanzen-Samen mit dem Biogas-Prozess in Kontakt. Das von diesen Arten ausgehende Kontaminationsrisiko lässt sich derzeit nicht verlässlich abschätzen, da systematische Forschung zur Überlebensfähigkeit von Pflanzensamen fast vollständig fehlt. Um dem abzuhelfen bietet sich die Überprüfung der Thermoresistenz der Arten an, da Temperatur und Verweildauer die wichtigsten Parameter sind, von denen das Überleben der Samen in Biogas-Anlagen abhängt. Wir haben das Überleben von 11 Wildpflanzen-Arten aus verschiedenen Familien unter den Temperatur-Bedingungen einer mesophilen Biogas-Anlage mit einem Screening im Wasserbad untersucht. Die Samen wurden bei 42°C und pH 7 in einer wässrigen Lösung inkubiert und ihre Lebensfähigkeit über einen Zeitraum von bis zu 18 Tagen bestimmt. Zur Validierung der Ergebnisse wurden Samen ausgewählter Arten einer anaeroben Vergärung bei 42°C im Labormaßstab ausgesetzt. Die mittlere Inaktivierungszeit unterschied sich bei 8 der 11 untersuchten Wildpflanzen-Arten signifikant, wobei 6 Arten nur sehr langsam bzw. nicht vollständig inaktiviert wurden. Bei diesen Arten besteht das Risiko, dass sie mit dem Gärrest verbreitet werden. Mit Hilfe des Wasserbad-Screenings ließ sich das Überlebensrisikos der Samen in der mesophilen, anaeroben Vergärung verlässlich abschätzen.

**Stichwörter: Biogas-Reaktor, Temperatur, Thermoresistenz, Überlebens-Risiko, Wildpflanzen-Arten**

**Abstract |** If plant seeds enter a biogas reactor there is the risk of surviving anaerobic digestion and spreading with the digestate application. Recently, a large number of wildflower species can enter the biogas chain due to the use of wildflower seed mixtures for the production of biogas. The contamination risk associated with these species cannot be reliably estimated as there is a lack of systematic research on the survival of seeds from different plant species. As seed survival in biogas plants mainly depends on temperature and exposure time, the investigation of the species´ thermoresistance is a first step to close this gap of knowledge. We investigated the survival of 11 wildflower-species from different families in a waterbath at 42°C and pH 7, conditions that are usually encountered in a mesophilic biogas plant. Seeds were incubated in a buffer solution and their viability was determined during 18 days of exposure. Additionally, seeds of selected species were exposed to anaerobic digestion at 42°C in an experimental reactor. The mean inactivation time differed significantly between 8 species. Inactivation was very slow or even lacking for 6 species, suggesting that these species could be dispersed with the digestate. The waterbath-screening of thermoresistance allowed for reliable estimation of the probability of seed survival in mesophilic, anaerobic digestion.

**Keywords: biogas reactor, probability of survival, temperature, thermoresistance, wildflower species**

### 2.3.1 Einleitung

Mit der Ernte von Biomasse gelangen auch Pflanzensamen in den Biogas-Reaktor. Wenn sie die anaerobe Vergärung überleben, besteht das Risiko, dass sie mit Ausbringung des Gärrestes verbreitet werden. In einer Literaturstudie identifizierten WESTERMAN und GEROWITT (2013) Wildpflanzen mit harten Samen (physikalische Dormanz), hoher Thermoresistenz, einer dicken Samenschale oder mit Anpassungen an Endozoochorie als Hochrisiko-Arten für das Überleben in Biogasreaktoren. Die Mechanismen hinter dieser erhöhten Widerstandsfähigkeit sind allerdings zumeist unbekannt. Studien umfassen nur wenige Arten. Systematische Forschung zur Überlebensfähigkeit von Pflanzensamen fehlt fast vollständig (WESTERMAN und GEROWITT, 2013). Dies ist vor dem Hintergrund der zunehmenden Zahl an Herbizidresistenzen von (Mais-) Unkräutern (GEROWITT, 2012) und dem Einsatz von artenreichen Blühmischungen für die Biogasproduktion (z.B. www.saaten-zeller.de) problematisch, wenn man das Ziel verfolgt den Einsatz von Herbiziden zu verringern und Florenverfälschung zu vermeiden. Denn im Moment ist vollkommen unklar, ob das Risiko besteht, dass diese Arten überleben mit dem Gärrest verbreitet werden.

Für den Einstieg in die systematische Forschung zur Überlebensfähigkeit von Wildpflanzen-Samen im Biogas-Prozess bietet sich die Überprüfung der Widerstandsfähigkeit der Arten gegenüber der Temperatur an. Temperatur und Verweildauer sind die wichtigsten Parameter, von denen das Überleben der Samen in Biogas-Anlagen abhängt (WESTERMAN und GEROWITT, 2013). Aufbauend auf den Experimenten zur „Bedeutung der Temperatur für die Inaktivierung von Samen im Biogas-Reaktor“ (HAHN et al., 2016) haben wir in dieser Studie, die Thermoresistenz mehrerer Wildpflanzen-Arten aus verschiedenen Familien mit einem kostengünstigen und leicht reproduzierbaren Screening im Wasserbad verglichen.

### 2.3.2 Material und Methoden

#### 2.3.2.1 Pflanzenmaterial

In Anlehnung an die Temperatur in mesophil betriebenen Biogasanlagen (HAHN et al., 2016) wurde das Überleben von Samen bei 42°C am Beispiel von 11 verschiedenen Pflanzenarten untersucht (***Tab.* 1 = Table 2-3**). Die Auswahlkriterien waren, dass (1) ein Eintrag in die Biogas-Prozesskette wahrscheinlich war, (2) möglichst viele verschiedene Pflanzen-Familien vertreten waren und (3) Arten mit Eigenschaften, die eine erhöhte Widerstandsfähigkeit gegenüber einer anaeroben Vergärung vermuten lassen.

#### 2.3.2.2 Screening bei 42°C im Wasserbad

Für das Screening des Überlebensrisikos bei 42°C wurden die Samen aller Arten in einer Pufferlösung in Präzisions-Wasserbädern inkubiert, die auf 0,1°C genau regelbar waren (WB-6, Firma witeg Labortechnik GmbH, Wertheim, Deutschland). Die Samen wurden nach einer Oberflächensterilisation

in Reagenzgläsern mit 5 ml 0,5 M HEPES Puffer (pH 7,0) versetzt und für 1, 3, 6, 9, 12 und 18 Tage bei 42°C inkubiert. Pro Art und Expositionszeit wurden mindestens drei Replikate mit je 50 Samen untersucht. Nach den verschiedenen Expositionszeiten wurde die Lebensfähigkeit der Samen mittels Tetrazolium-Färbung bestimmt.

#### 2.3.2.3 Anaerobe Vergärung bei 42°C im Labormaßstab

Zur Überprüfung der Ergebnisse aus dem Wasserbad-Screening wurden Samen der Arten *C. album*, *D. carota*, *L. esculentum*, *M.alcea* und *M. officinalis* einer anaeroben Vergärung bei 42°C im Labormaßstab ausgesetzt wie in HAHN et al. (2016) beschrieben. Zusammengefasst wurden die Samen in feinmaschige Polyester-Beutel eingenäht und in vier Replikaten in die mit Maissilage und Rindergülle betriebenen Laborreaktoren eingebracht. Die Samen von *C. album*, *D. carota* und *L. esculentum* wurden für 1, 3, 6 oder 9 Tage, die von *M. alcea* und *M. officinalis* für 3, 9, 18 und 36 Tage vergoren. Nach den unterschiedlichen Expositionszeiten wurden zunächst die Keimungsraten der Samen 21 Tage lang überprüft. Anschließend wurde die Lebensfähigkeit der Samen, die in den 21 Tagen nicht keimten, mittels Tetrazolium-Färbung getestet (WESTERMAN et al., 2012B). Aus der Summe der gekeimten und der laut Tetrazolium-Test lebensfähigen Samen wurde der Anteil vitaler Samen errechnet. Als Kontrolle wurde die Keim- und Lebensfähigkeit von unbehandelten Samen bestimmt. Dazu wurden sie zwei Tage vor Beginn der Tests im Dunkeln angequollen.

***Tab.1*** *Untersuchte Pflanzenarten mit Familienzugehörigkeit, Eintragspfad in die Biogas-Anlage und Herkunft des Saatgutes für diese Studie.*

**Table 2-3 | Examined plant species, plant family, pathway to enter a biogas-plant, and origin of seeds used in this study.**

| Familie | Art | | Eintragspfad | Herkunft |
|---|---|---|---|---|
| **Amaranthaceae** | *Chenopodium album* | Weißer Gänsefuß L. | Mais-Unkraut | Aufs. D [a] |
| **Apiaceae** | *Daucus carota* | Wilde Möhre L. | Blühmischung [1] | HS [b] |
| **Asteraceae** | *Ambrosia artemisiifolia* | Beifußblättrige Ambrosie L. | | Aufs. USA [c] |
| **Asteraceae** | *Centaurea nigra* | Schwarze Flockenblume L. | Blühmischung [1] | SZ [d] |
| **Fabaceae** | *Melilotus officinalis* | Gelber Steinklee (L.) PALL. | Blühmischung [1] | AW [e] |
| **Malvaceae** | *Malva alcea* | Rosen-Malve L. | Blühmischung [1] | AW [e] |
| **Malvaceae** | *Malva sylvestris* | Wilde Malve L. | Blühmischung [1] | SZ [d] |
| **Poaceae** | *Cynodon dactylon* | Bermudagras (L.) PERS. | | Kult. D [f] |
| **Polygonaceae** | *Fallopia convolvulus* | Windenknöterich (L.) Á. LÖVE | Mais-Unkraut | Aufs. D [a] |
| **Polygonaceae** | *Polygonum aviculare* | Vogelknöterich L. | Mais-Unkraut | Aufs. D [a] |
| **Solanaceae** | *Lycopersicon esculentum* | Tomate (L.), Sorte St. Pierre | Hygiene-Indikator [2] | BH [g] |

[1] Biogas-Blühmischung „BG70" von Saaten Zeller[d]; [3] Nachweis der phytohygienischen Unbedenklichkeit von Vergärungs- und Kompostierungsanlagen laut BioAbfV (BMU, 1998)

[a] Aufsammlung auf Versuchsflächen der Universität Rostock (Deutschland) während der Kultivierung der Blühmischung „BG70" 2014-2015; [b] Herbiseed Ltd., Twyford, UK; [c] Aufsammlung in Urbana, Illinois, USA, Oktober 2016 Illinois; [d] Saaten Zeller GmbH & Co. KG, Eichenbühl-Guggenberg, Deutschland; [e] Appels Wilde Samen GmbH, Darmstadt, Deutschland; [f] Ernte von Pflanzen im Treibhaus der Universität Rostock.; [g] Bingenheimer Saatgut AG, Echzell-Bingenheim, Deutschland

#### 2.3.2.4 Statistische Auswertung

Der Anteil vitaler Samen während der Expositionszeit wurde mit Hilfe des Pakets „drc“ (RITZ und STREIBIG, 2015) für R (R CORE TEAM, 2017) modelliert. Grundlage der Modelle war eine log-logistische Funktion mit einer unteren Grenze bei 0: $F(t) = d/(1+exp[b(log(t)-log(MIZ)])$, mit „b“ – Parameter, der proportional zur Steigung von „F“ bei der Inkubationszeit „t“ ist; „d“ – maximaler Anteil an vitalen Samen; „MIZ“ – mittlere Inaktivierungszeit (d.h., Zeit nach der die Hälfte der ursprünglich vitalen Samen inaktiviert wurde). Es wurde ein Modell für das Screening aller Arten in der wässrigen Lösung erstellt. Daneben wurde für jede der fünf Arten, die zusätzlich der anaeroben Vergärung ausgesetzt wurden, je ein Modell berechnet, dass die Inkubation in wässriger Lösung und in der anaeroben Vergärung umfasste. Das Signifikanz-Niveau für den Vergleich der Modellparameter zwischen den Arten oder den Inkubationsvarianten wurde auf $\alpha < 0,05$ festgelegt.

### 2.3.3 Ergebnisse

In der anaeroben Vergärung erfolgte die Inaktivierung der Samen von *C. album*, *L. esculentum* und *M. alcea* signifikant schneller als in der wässrigen Lösung (***Tab. 2*** = **Table 2-4**). Auch bei *D. carota* war eine tendenziell schnellere Abnahme im Reaktor zu erkennen. Einzig das Modell für *M. officinalis* ergab eine um eine Größenordnung höhere mittlere Inaktivierungszeit in der Vergärung.

***Tab. 2*** *Mittlere Inaktivierungszeit (MIZ) und Standardfehler (SF) von Wildpflanzen-Samen bei Inkubation in einer Pufferlösung (pH 7) und in der anaeroben Vergärung in einem Laborreaktor bei jeweils 42°C. Sterne geben signifikante Unterschiede in der MIZ einer Art zwischen Pufferlösung und Vergärung an (α<0,05).*

**Table 2-4 | Mean inactivation time (MIZ) and standard errors (SF) of wildflower seeds that were incubated at 42°C in a buffer solution (pH 7) or in an anaerobic digestion in an experimental reactor. Asterisks indicate significant differences in MIZ of a species between buffer solution and digestion (α<0.05).**

| | Puffer | | | Vergärung | |
|---|---|---|---|---|---|
| **Art** | **MIZ** | **±SF (Tage)** | | **MIZ** | **±SF (Tage)** |
| ***C. album*** | 10,8 | ± 0,2 | * | 7,1 | ± 0,1 |
| ***D. carota*** | 1,2 | ± 0,1 | | 0,0 | ± 0,0 |
| ***L. esculentum*** | 8,5 | ± 0,2 | * | 1,8 | ± 0,1 |
| ***M. alcea*** | 2,8 | ± 0,2 | * | 0,6 | ± 0,1 |
| ***M. officinalis*** | 319,6 | ± 261,8 | * | 3160,0 | ± 1289,0 |

Im Wasserbad-Screening bei 42 °C verloren die Samen der verschiedenen Wildpflanzen-Arten ihre Vitalität unterschiedlich schnell (***Abb. 1*** = **Figure 2-6**), wobei sich die mittlere Inaktivierungszeit (MIZ) bei 8 der 11 Arten signifikant unterschied (***Tab. 3*** = **Table 2-5**). Vertreter derselben Pflanzenfamilie zeigten einen ähnlichen Inaktivierungsverlauf (***Abb.1 c,e,g*** = **Figure 2-6 c,e,g**), was sich auch in einem gleichen „b“-Parameter der Modelle zeigte (Daten nicht gezeigt). Allerdings unterschied sich die MIZ zwischen den beiden Vertretern einer Familie signifikant (***Tab. 3*** = **Table 2-5**). Mit MIZ von mehr als 1 Woche waren *L. esculentum*, *C. album*, *C. dactylon* und *M. officinalis* (***Tab. 3*** = **Table 2-5**) am

widerstandsfähigsten gegenüber der Inkubation bei 42°C in wässriger Lösung. Zudem wurden die Samen dieser vier Arten und der beiden Malvaceen im Versuchszeitraum nie vollständig inaktiviert.

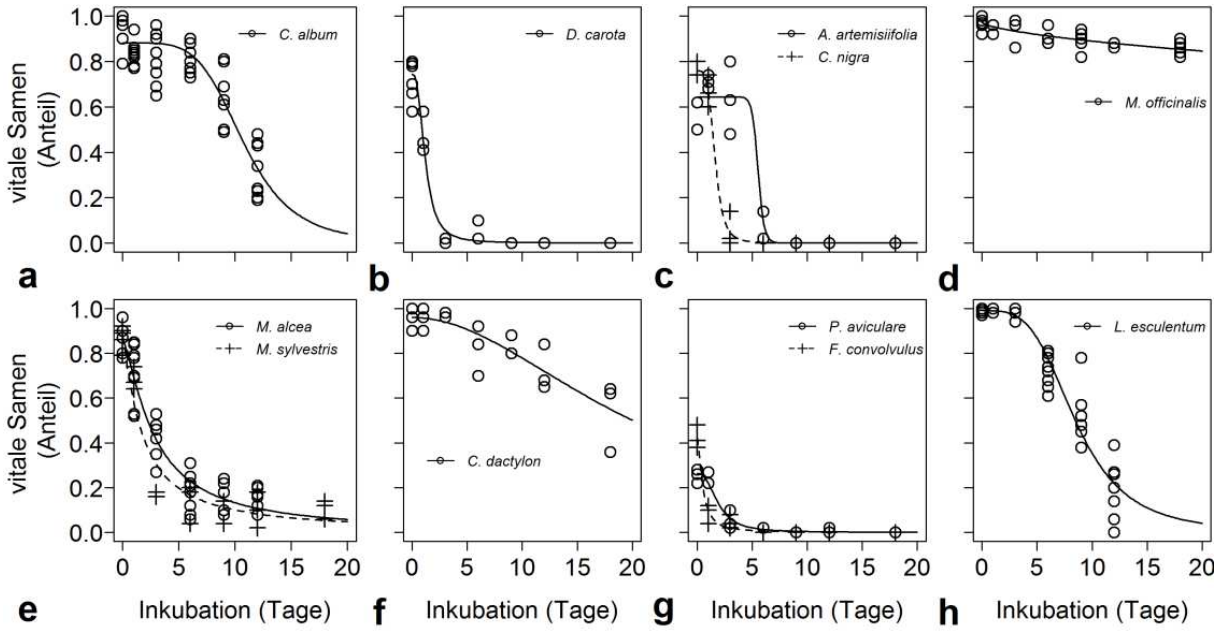


***Abb. 1*** *Anteil vitaler Samen von Arten aus verschiedenen Pflanzenfamilien während der Inkubation in einer wässrigen Lösung mit pH 7 bei 42°C. (**a**) Amaranthaceae, (**b**) Apiaceae, (**c**) Asteraceae, (**d**) Fabaceae, (**e**) Malvaceae, (**f**) Poaceae, (**g**) Polygonaceae, (**h**) Solanaceae.*

**Figure 2-6 | Proportion of viable seeds from species from different plant families during the incubation in a buffer solution with pH 7at 42°C. (a) Amaranthaceae, (b) Apiaceae, (c) Asteraceae, (d) Fabaceae, (e) Malvaceae, (f) Poaceae, (g) Polygonaceae, (h) Solanaceae.**

***Tab. 3*** *Mittlere Inaktivierungszeit (MIZ) und Standardfehler (SF) von Wildpflanzen-Samen bei Inkubation in einer wässrigen Lösung mit pH 7 bei 42°C. Kleinbuchstaben geben signifikante Unterschiede zwischen Pflanzenarten an (α<0,05).*

**Table 2-5 | Mean inactivation time (MIZ) and standard error (SF) of wildflower seeds that were incubated in a buffer solution with pH 7 at 42°C. Lowercase letters indicate significant differences between plant species (α<0.05).**

| Familie | Art | MIZ (Tage) | SF (Tage) | |
|---|---|---|---|---|
| **Polygonaceae** | *F. convolvulus* | 0,5 | 0,2 | a |
| **Apiaceae** | *D. carota* | 1,2 | 0,1 | a |
| **Asteraceae** | *C. nigra* | 1,6 | 0,2 | b |
| **Malvaceae** | *M. sylvestris* | 1,7 | 0,2 | b |
| **Polygonaceae** | *P. aviculare* | 1,9 | 0,4 | bc |
| **Malvaceae** | *M. alcea* | 2,8 | 0,2 | c |
| **Asteraceae** | *A. artemisiifolia* | 5,6 | 1,1 | d |
| **Solanaceae** | *L. esculentum* | 8,5 | 0,2 | e |
| **Amaranthaceae** | *C. album* | 10,8 | 0,2 | f |
| **Poaceae** | *C. dactylon* | 20,9 | 1,9 | g |
| **Fabaceae** | *M. officinalis* | 319,6 | 261,8 | h |

## 2.3.4 Diskussion

### 2.3.4.1 Aussagekraft des Screenings in wässriger Lösung für Überleben in anaeroben Vergärungsprozessen

Die Ergebnisse aus dem Wasserbad-Screening wurden im Laborreaktor insofern bestätigt, als dass die Inaktivierung der Samen im Reaktor gleich schnell oder sogar beschleunigt verlief. Das Screening in wässriger Lösung stellt demnach eine konservative Schätzung des Überlebensrisikos der Samen einer Wildpflanzen-Art in anaeroben Vergärungsprozessen bei 42°C dar. Durch die über den Messzeitraum hinausgehende MIZ und die sehr großen Standardfehler bei *M. officinalis* wurde allerdings deutlich, dass bei Arten mit Hartschaligkeit (ROLSTON, 1978) ein Versuchsdesign mit einer höheren Anzahl von Probenahmen in der Zeit notwendig ist. So könnte auch der bei hartschaligen Arten vermutete zweiphasige Inaktivierungsverlauf (HAHN et al., 2016; WESTERMAN et al., 2012C) sinnvoll in einem (biphasischen) Modell abgebildet werden (BECKON et al., 2008).

### 2.3.4.2 Überlebensrisiko verschiedener Wildpflanzen-Samen bei 42°C

Die Samen von fünf der untersuchten Wildpflanzen-Arten starben innerhalb von 18 Tagen bei 42°C ab, während die Samen der sechs anderen nur langsam (MIZ > 7d) oder nicht vollständig inaktiviert wurden.

Die nur langsam abnehmende Lebensfähigkeit der Samen von *C. album* könnte in Kombination mit den in Praxisanlagen gelegentlich auftretenden sog. „Kurzschluss"-Stoffströmen dazu führen, dass geringe Prozentsätze von *C. album*-Samen die Vergärung überleben und mit dem Gärrest verbreitet werden. Verschärfend kommt hinzu, dass zum Teil große Mengen an Samen von *C. album* in Biogas-Anlagen gelangen (WESTERMAN et al., 2012A). Dies unterstreicht auch aus phytohygienischer Sicht die Notwendigkeit der Prozess-Kontrolle mit einer ausreichend langen Verweildauer des Substrates vor allem durch die Prävention von Kurzschluss-Strömen, um die ungewollte Verbreitung dieser und anderer Wildpflanzen zu verhindern. Ferner wurde auch in dieser Studie deutlich (vgl. HAHN et al. 2016), dass die Tomate sich nicht als Hygiene-Leitorganismus für die phytohygienischen Unbedenklichkeit von Vergärungsanlagen (laut BioAbfV 1998) eignet. Das Überlebensrisiko der Samen von *L. esculentum* lag unter dem von *C. album* und war damit nicht höher als das der meisten anderen Arten.

Die höchste Temperaturresistenz zeigte *M. officinalis*, ein Vertreter der Fabaceen, der ebensowenig vollständig inaktiviert wurde, wie die beiden untersuchten Malvaceen. In beiden Familen tritt das Phänomen der Hartschaligkeit auf (ROLSTON, 1978; BASKIN et al., 2000), das zu einer ungewöhnlichen Widerstandsfähigkeit gegenüber anaerober Vergärung führen kann (WESTERMAN und GEROWITT, 2013). Es würde sich demnach empfehlen, solche Arten aus Biogas-Blühmischungen auszuschließen. Neben der Hartschaligkeit identifizierten WESTERMAN und GEROWITT (2013) auch Anpassungen an Endozoochorie als Hochrisiko-Faktor für das Überleben von Unkrautsamen in Biogasreaktoren. Dies könnte die überraschend langsame Inaktivierung der Samen der nicht-hartschaligen, aber endozoochor verbreiteten Poacee *C. dactylon* (BURTON und ANDREWS, 1948;

ANDERSON et al., 2014) erklären. In anderen Studien wurden Samen von Poaceen schnell und vollständig inaktiviert (BAUTE et al., 2016; LEONHARDT et al., 2010; JEYANAYAGAM und COLLINS, 1984). Beim derzeitigen Kenntnisstand kann eine verlässliche Abschätzung des Überlebensriksikos daher nur auf Artebene erfolgen. Um in Zukunft Aussagen auf höheren taxonomischen oder funktionellen Ebenen zu ermöglichen, sollten sich weiterführende Studien mit der Art und Verbreitung der der Temperaturresistenz zugrundeliegenden Mechanismen befassen.

### 2.3.5 Danksagung

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## 2.4 Seed Survival in Full- and Lab-Scale Systems

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### Mesophilic, Anaerobic Digestion in a Full-Scale, Commercial Biogas Reactor Kills Seeds More Efficiently than Lab-Scale Systems

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When plant biomass is anaerobically digested, seeds may survive the energy production process and contaminate the digestate. Hard-seeded (HS), i.e., physically dormant, species were found to be difficult to inactivate. Here, we aimed to verify this finding from lab-scale experimental reactors (ERs) in a full-scale commercial reactor (CR). In addition, we tested seed survival in a pH-buffered water bath (WB). Seeds were exposed to CR, ER and WB treatments at 42 °C for a maximum of 36 days. The viability of seeds was checked by measuring germination and response to tetrazolium staining and modeled as a function of exposure time using a dose–response approach. CR killed seeds more effectively than ER and WB treatments. The non-HS reference species, *Chenopodium album*, was completely inactivated by all treatments. Responses of the HS species ranged from complete inactivation to complete insensitivity. The most resistant was *Malva sylvestris*. The least resistant species were inactivated mainly by temperature, while additional mortality factors were effective in the more resistant species. We concluded that mesophilic AD in CRs can reduce the risk of seed contamination in the digestate for non-HS but not for HS species. Moreover, WB treatments seem suitable to estimate the minimum mortality of non-HS species in CR.

**Keywords:** **CSTR, digestate valorization, dose response models, exposure time, hardseededness, physical dormancy, seed inactivation, seed survival, water bath, weed spread**

### 2.4.1 Introduction

The sustainability of anaerobic digestion (AD) of renewable feedstocks in biogas plants is based on generating not only methane as an energy carrier but also digestate and derived products that can be used for various purposes [1–3]. In (organic) agriculture, digestate is considered an environmentally friendly alternative to mineral fertilizers to close nutrient cycles (e.g., [4,5]). Furthermore, digestate is being discovered for material recovery and use, such as peat replacement in growing media [6], fiber for composites [7,8] and biochemical production [9,10]. Beyond feedstock selection, effective digestate valorization must become a priority to successfully integrate AD into a circular bioeconomy in which biogas plants process residues from other production systems and return them to the biomass cycle. Regardless of the material flow in which the digestate is then to be utilized, it must be free of toxic

substances and other potentially harmful components [11]. This includes living organisms such as pathogenic microbes and plant seeds [12,13]. The latter could establish undesirably, i.e., as weeds, in potting soil or in fields fertilized with digestate. Any such contamination will result in additional costs and labor that will compromise the sustainability of digestate use.

Results on the sanitation status of digestate after AD are ambiguous with respect to pathogens and plant seeds: both complete sanitation and surviving pathogens or seeds are reported (e.g., [14–16]). However, if plant biomass is used as feedstock for AD, the digestate may contain viable seeds. Survival potential has already been demonstrated for seeds of several weeds, but also for other species (e.g., [17–20]). Non-native or quarantine species that are not yet widely established and form many seeds with high AD-resistance potential are particularly problematic in terms of digestate contamination [20–23]. The AD-resistance potential of seeds seems to be determined by species-specific traits and characteristics of the seed lot [20,23]. One species-specific trait that has been identified as a risk factor for seed survival in AD is hardseededness (HS), which is a type of physical dormancy based on the formation of one or more impermeable layers in the seed or fruit coat [24]. Leonhardt et al. [25], Westerman et al. [19], Westerman et al. [26], Hassani et al. [27], and Hahn et al. [20] have reported HS species that can survive AD. However, non-hardseeded (NHS) species can also survive AD exposure. Therefore, endozoochory adaptations, such as thick or physically hard seed coats, as well as yet-to-be-identified seed traits, are also discussed as risk factors for survival in AD [23].

Despite the above findings, it is not yet possible to make reliable predictions about the survival of seeds in AD. Knowledge is still limited and fragmentary. Systematic studies on the ability of seeds from different taxonomic and functional groups to survive AD are lacking [23]. One reason is that introducing seeds into operating biogas reactors is difficult, expensive and time-consuming. In addition, determining seed viability is mostly manual work, so only random samples of specific seed lots can be tested. Finally, the reactors in which the seeds were exposed to AD differed in their process technology and operation mode (see [23] for a review). Widely varying types and concepts are classified according to scale (micro, small, medium, large), feedstock (wet < 15% TS or dry > 15% TS), feeding pattern (batch, continuous, semicontinuous), number of process stages (e.g., single or two stages), process temperature (i.e., mesophilic, thermophilic), and the fluid dynamic (i.e., plug flow, completely stirred) [1,28]. These differences are relevant because it is suspected that, in addition to exposure time and temperature, the mode of operation of the reactor affects seed survival [23].

Most studies of seed survival in AD have used lab-scale, experimental reactors, or similar systems. Methods ranged from bottles with a capacity of about 0.5 L operated in batch mode (e.g., [29,30]) to 400 L completely stirred tank reactors (e.g., [31,32]). In full-scale, commercial biogas reactors, providing facilities and farm activities for potential seed contamination under real conditions, nine seed survival studies have been conducted to date [14,15,17,25–27,33–35]. Where indicated, sizes of these commercial reactors ranged from 260 $m^3$ to 6000 $m^3$, and batch and completely stirred systems were represented. Process temperatures varied from 30 to 55 °C, and seeds were exposed to AD for

between 1 h [34] and 155 days [27]. In addition to tests in reactors or reactor like systems, there are studies that have estimated seed survival in AD using lower-cost water-bath experiments (e.g., [36]). They are based on the premise that seeds survive AD mainly due to thermoresistance (cf. [23]). Finally, six studies have compared the effects of two or more AD systems on seed survival [14,15,25–27,32]. However, even in these, the mode of operation and process temperature often differed between the systems compared.

In summary, the existing data on seed survival in AD have been obtained at different scales and using quite different systems. Moreover, data on more seed lots are needed to predict seed survival in AD more reliably. This data could best be obtained if it were possible to replace expensive and laborious trials in full-scale biogas reactors by less complex tests in lab-scale systems. A prerequisite for this is to determine the extent to which the results are representative of real conditions in practice.

The objective of this study was to determine the survival of seeds in full-scale, commercial biogas reactors using the six species that had best survived mesophilic, anaerobic digestion in lab-scale experimental reactors [20]. Five of the species were hardseeded and one was not. Seed survival was explored as a function of exposure time and additionally tested in pH-buffered water baths. By comparing the three systems, we aimed to gain in-sight into the dynamics of seed inactivation and evaluate whether experimental reactors and water baths are suitable as less complex and less costly options for estimating seed survival in commercial biogas reactors.

## 2.4.2 Materials and Methods

### 2.4.2.1 Species and Seed Collection

The six species that best survived 36 days of mesophilic AD in experimental reactors in a previous study [20] were examined. These were five HS species, namely *Abutilon theophrasti* (velvetleaf, Malvaceae), *Malva alcea* (rose mallow, Malvaceae), *Malva sylvestris* (common mallow, Malvaceae), *Melilotus albus* (white sweet clover, Fabaceae), and *Melilotus officinalis* (yellow sweet clover, Fabaceae) and one NHS species, *Chenopodium album* (common lambsquarters, Amaranthaceae).

Seeds of *M. sylvestris* were propagated in 2015 and obtained from “Herbiseed” (Twyford, UK, herbiseed.com). *M. albus* and *M. officinalis* seeds were propagated in 2014 and those of *M. alcea* in 2015 by “Appels Wilde Samen” (Darmstadt, Germany, ap-pelswilde.de). Seeds of *C. album* and *A. theophrasti* were harvested in 2014 and 2015, respectively, from plants grown at the University of Rostock (Rostock, Germany). Until the beginning of the treatments, seeds were stored at room temperature in the dark.

### 2.4.2.2 Treatments

#### 2.4.2.2.1 Anaerobic Digestion in a Commercial Biogas Reactor

The full-scale, commercial biogas reactor (CR, **Figure 2-7**) was the biogas plant Wildau-Wentdorf located in Dahmetal, Sachsen-Anhalt, Germany [37]. This reactor has special modifications that allow the introduction of samples and has already been used by Westerman et al. [26] to study seed survival in mesophilic anaerobic digestion (AD). The reactor is a single-phase completely stirred tank reactor (CSTR) of 800 $m^3$ effective volume, equipped with an inclined stirring mixer in addition to an a-centric vertical stirring mixer. The daily fed-in ration was composed of 10 tons of maize silage, 1 ton of whole grain cereals and 10 $m^3$ of pig slurry based on the volatile solids added to the reactor. During seed treatments from May until September 2016, the CR was stably operated, as characterized by the parameters given in **Table 2-6**. However, the mean reactor temperature (42 ± 2°C) slowly increased from about 43°C in May to 46°C in August 2016 due to the high outdoor temperature.

Similar to Westerman et al. [26], the seeds were exposed to AD in CR inside fine-mesh polyester bags. In order to be able to retrieve the bags, they were sewn into compartmentalized bags made of stronger polypropylene (Polynova 93430 FF; mesh size 25 µm), which could be attached to the end of a 2 m long probe or "sword" that was lowered into the CR via an inlet from the top. The 'sword' was fixed into position on the reactor lid and disposed 6 m from the centre and 2 m from the edge of the container. The sword tip was located at a depth of 0.5 m from the liquid surface and 3.5 m from the bottom, such that the seed bags were directly in the flow of mixing devices installed.

There were three runs in CR: the 1st from 27 May to 1 July 2016, the 2nd from 1 July to 5 August 2016, and the 3rd from 5 August to 9 September 2016. There were 2 replicates per run, resulting in a total of 6 replicates for each species. Depending on the exposure time of 3, 9, 18 and 35 days, the bags of the 4 times contained 100, 100, 200 and 300 seeds of a single species, respectively (**Supplementary Table 2-6**). After each run, seeds were rinsed with water, transported to the laboratory in Rostock and processed within five hours after removal from the reactor.

**Table 2-6 | Process fluid and performance parameters of the commercial full-scale reactor, the experimental lab-scale reactor, and the buffer solution in the water bath.**

| Parameter | Commercial Reactor | | | | Experimental Reactor | | | | Water Bath | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | run | min | mean | max | run | min | mean | max | min | mean | max |
| **T** | **∅** | **41.3** | **44.6** | **46.1** | **∅** | **40.3** | **42.1** | **43.3** | **41.9** | **42.0** | **42.1** |
| **[°C]** | 1 | 41.3 | 43.0 | 44.6 | 1 | 41.9 | 42.5 | 42.9 | | | |
| | 2 | 44.7 | 45.1 | 46.0 | 2 | 42.1 | 42.5 | 42.7 | | | |
| | 3 | 45.4 | 45.7 | 46.1 | 3 | 41.1 | 41.5 | 42.0 | | | |
| | | | | | 4 | 40.3 | 42.0 | 43.3 | | | |
| **pH** | **∅** | **7.7** | **7.7** | **7.8** | **∅** | **7.4** | **7.7** | **8.0** | **-** | **7.0** | **-** |
| | 1 | - | 7.7 | - | 1 | 7.6 | 7.6 | 7.7 | | | |
| | 2 | - | 7.7 | - | 2 | 7.6 | 7.6 | 7.6 | | | |
| | 3 | - | 7.8 | - | 3 | 7.4 | 7.7 | 8.0 | | | |
| | | | | | 4 | 7.7 | 7.8 | 7.9 | | | |

**Table 2-6 |** continued

| Parameter | Commercial Reactor | | | | Experimental Reactor | | | | Water Bath | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | run | min | mean | max | run | min | mean | max | min | mean | max |
| **TS** | **∅** | **9.0** | **9.2** | **9.7** | **∅** | **5.6** | **6.0** | **6.8** | | **na** | |
| **[%FM]** | 1 | - | 9.7 | - | 1 | 6.5 | 6.5 | 6.5 | | | |
| | 2 | - | 9.0 | - | 2 | 6.5 | 6.6 | 6.8 | | | |
| | 3 | - | 9.0 | - | 3 | 5.6 | 5.7 | 5.8 | | | |
| | | | | | 4 | 5.8 | 6.0 | 6.4 | | | |
| **VS** | **∅** | **84.4** | **84.4** | **84.5** | **∅** | **70.0** | **74.5** | **77.4** | | **na** | |
| **[%TS]** | 1 | - | 84.4 | - | 1 | 75.9 | 76.1 | 76.2 | | | |
| | 2 | - | 84.5 | - | 2 | 76.1 | 76.2 | 76.4 | | | |
| | 3 | - | 84.4 | - | 3 | 70.0 | 74.5 | 77.4 | | | |
| | | | | | 4 | 71.7 | 73.8 | 75.8 | | | |
| **$NH_4$-N** | **∅** | **3.2** | **3.3** | **3.5** | **∅** | **1.5** | **1.8** | **1.9** | | **na** | |
| **[$g \cdot L^{-1}$]** | 1 | - | 3.2 | - | 1 | 1.8 | 1.8 | 1.9 | | | |
| | 2 | - | 3.3 | - | 2 | 1.8 | 1.8 | 1.9 | | | |
| | 3 | - | 3.5 | - | 3 | 1.5 | 1.7 | 1.8 | | | |
| | | | | | 4 | 1.8 | 1.8 | 1.9 | | | |
| **AA** | **∅** | **0** | **0.1** | **0.2** | **∅** | **0.1** | **0.3** | **0.8** | | **na** | |
| **[$g \cdot L^{-1}$]** | 1 | - | 0.2 | - | 1 | 0.3 | 0.3 | 0.3 | | | |
| | 2 | - | 0.1 | - | 2 | 0.3 | 0.3 | 0.3 | | | |
| | 3 | | nd | | 3 | 0.1 | 0.3 | 0.8 | | | |
| | | | | | 4 | 0.2 | 0.3 | 0.4 | | | |
| **VFA** | **∅** | **0.1** | **0.1** | **0.2** | **∅** | **0.1** | **0.4** | **0.9** | | **na** | |
| **[$g \cdot L^{-1}$]** | 1 | - | 0.2 | - | 1 | 0.3 | 0.3 | 0.3 | | | |
| | 2 | - | 0.1 | - | 2 | 0.3 | 0.3 | 0.3 | | | |
| | 3 | | nd | | 3 | 0.1 | 0.4 | 0.9 | | | |
| | | | | | 4 | 0.3 | 0.4 | 0.7 | | | |
| **Biogas** | | | **na** | | **∅** | **5.8** | **13.9** | **17.2** | | **na** | |
| **[$l_N \cdot d^{-1}$]** | | | | | 1 | 8.0 | 13.3 | 17.0 | | | |
| | | | | | 2 | 5.8 | 13.2 | 15.9 | | | |
| | | | | | 3 | 9.9 | 12.1 | 14.0 | | | |
| | | | | | 4 | 8.1 | 14.6 | 17.2 | | | |
| **$CH_4$** | **∅** | **50.0** | **51.7** | **53.0** | **∅** | **51.7** | **54.7** | **58.2** | | **na** | |
| **[Vol.%]** | 1 | 51.0 | 51.5 | 52.0 | 1 | 55.0 | 57.2 | 58.2 | | | |
| | 2 | 51.0 | 51.9 | 52.0 | 2 | 55.2 | 57.1 | 57.9 | | | |
| | 3 | 50.0 | 51.9 | 53.0 | 3 | 54.4 | 55.7 | 56.7 | | | |
| | | | | | 4 | 51.7 | 53.6 | 56.2 | | | |
| **EPG** | **∅** | **1.2** | **5.8** | **6.0** | | | **na** | | | **na** | |
| **[$MWh_{el}$]** | 1 | 5.5 | 5.9 | 6.0 | | | | | | | |
| | 2 | 4.7 | 5.9 | 6.0 | | | | | | | |
| | 3 | 1.2 | 5.7 | 6.0 | | | | | | | |

T: operating temperature; TS: total solids; FM: fresh matter; VS: volatile solids; $NH_4$-N: ammonium-bound nitrogen; AA: acetic acid; VFA: volatile fatty acids (sum of acetic acid, propionic acid and butyric acid comprising butyric, iso-butyric, caproic, valeric, and iso-valeric acid; total acids concentration is expressed as acetic acid equivalent); $CH_4$: methane; EPG: Electric Power Generation; nd: below detection limit; -: no data, as only one measurement was taken; na: not available; ∅ Mean value if parameter was determined in different runs.

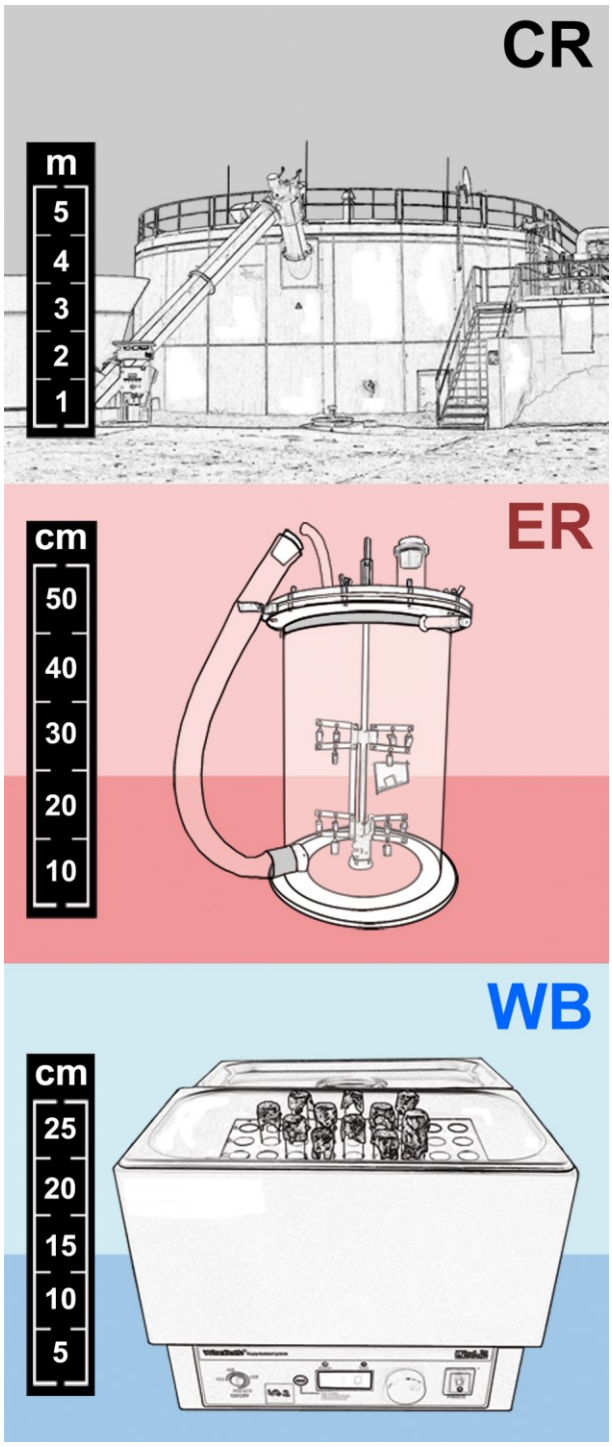


**Figure 2-7 | Schematic representation of the scales of the three treatments in which plant sees survival was tested: (CR) full-scale, commercial biogas reactor (800 m³); (ER) lab-scale, experimental biogas reactor (8 dm³); (WB) test tubes (20 cm³) in water baths. For details see Materials and Methods.**

#### 2.4.2.2.2 Anaerobic Digestion in Experimental Biogas Reactors

Exposure of the seeds to mesophilic AD in lab-scale, experimental biogas reactors (ERs, **Figure 2-7**) at the ATB in Potsdam (Germany) is described by Hahn et al. [20]. In brief, the continuously stirred reactors had a volume of 8 L and were fed on a mixture of maize silage and cattle slurry. Seeds were exposed to AD in ER in fine-mesh polyester bags attached to the centric vertical reactors' stirrer. The present study considers only the seed treatments in the two reactors operated at 42°C during the period

from 12 May 2015 to 23 September 2016. During this time, process parameters indicated a stable performance at lab scale under controlled conditions (**Table 2-6**).

There were three runs in ER: the 1st and 2nd from 12 to 21 May 2015, the 3rd from 1 January to 19 February 2016, and the 4th from 18 August to 23 September 2016. Each species was exposed to ER in two runs with at least one replicate, resulting in a minimum of 4 replicates per species. The species *M. albus* was digested with three replicates in the 3rd run, resulting in a total of 6 replicates. In ER, the duration of exposure was longer for HS than NHS species. Seeds of HS species were exposed to AD for 3, 9, 18, and 36 days. The seeds of NHS species *C. album* were exposed for 1, 3, 6, and 9 days. Depending on the exposure time, the number of seeds was 100, 100, 200, and 300, respectively (**Supplementary Table 2-6**). Just as in CR, the seeds were rinsed with water, transported to the laboratory in Rostock and processed within five hours after removal from the reactor.

#### 2.4.2.2.3 Buffer Solution in a Water Bath

From 28 July 2015 to 5 January 2016, seeds of the six species were exposed to water-bath treatments (WB, **Figure 2-7**) at 42°C in sterile 0.5 M HEPES buffer at pH 7.0 (Carl Roth GmbH & Co KG, Karlsruhe, Germany). The temperature in the precision water baths (‘wisebath” WB6, Witeg Labortechnik GmbH, Wertheim, Germany) could be set with a deviation of 0.1°C.

Prior to incubation in buffer, seeds were exposed to a water-saturated atmosphere in the dark for two days to prevent cracking during surface sterilization in 1% NaOCl solution. Under sterile conditions, 50 surface sterilized seeds of one species were added to 7 mL of buffer in a test tube for each exposure time and run. These samples were then placed in randomized positions in the water bath. Seeds of the HS species were subjected to the water-bath treatment for 9, 18, and 36 days in 3 replicates. To capture the more rapid inactivation of *C. album* compared with HS species (preliminary experiments, data not shown), seeds of this species were sampled after shorter and more exposure times, namely at 1, 3, 6, 9, and 12 days. For *C. album*, 8 replicates were run (**Supplementary Table 2-6**).

### 2.4.2.3 Seed Viability

Seed viability after AD in the commercial and experimental reactor was determined by the combination of a germination test and subsequent test of metabolic activity by tetrazolium staining described by Hahn et al. [20]. The viability of seeds after the water-bath treatment was determined by tetrazolium staining, only. In the germination test, a seed was considered germinated and viable if the radical protruded at least 2 mm from the seed. In the tetrazolium test, a seed was judged fully viable if the embryo—and endosperm, if relevant for the respective species—was stained red. Seed viability, $V$, of a sample was calculated as the proportion of viable seeds to the total number of seeds.

Viability of untreated seeds for each treatment (controls, 0 days exposure, **Table 2-7**) was determined in the same manner as that of treated seeds. Prior to the viability tests, however, the control seeds, which had previously been stored dry, were exposed to a water-saturated atmosphere in the dark for two days.

**Table 2-7 | Sample sizes (n) and mean proportion *(standard error of the mean)* of viable to total seeds, *V*, and germinated (*G*) to viable seeds, *G/V*, in untreated controls for the treatments in a commercial reactor (CR), experimental reactor (ER) and buffer solution in a water bath (WB).**

| Species | Control for | n | *V* | | *G/V* | |
|---|---|---|---|---|---|---|
| *Abutilon theophrasti* | CR | 3 | 0.99 | *(0.01)* | 0.46 | *(0.04)* |
| | ER | 4 | 0.95 | *(0.04)* | 0.46 | *na* |
| | WB | 3 | 0.61 | *(0.13)* | na | |
| *Chenopodium album* | CR | 3 | 0.99 | *(0.01)* | 1.00 | *0* |
| | ER | 3 | 0.88 | *(0.05)* | 1.00 | *0* |
| | WB | 5 | 0.75 | *(0.01)* | na | |
| *Malva alcea* | CR | 3 | 0.73 | *(0.03)* | 0.70 | *(0.06)* |
| | ER | 6 | 0.50 | *(0.11)* | 0.58 | *(0.06)* |
| | WB | 3 | 0.77 | *(0.04)* | na | |
| *Malva sylvestris* | CR | 3 | 0.29 | *(0.03)* | 0.11 | *(0.01)* |
| | ER | 6 | 0.26 | *(0.04)* | 0.11 | *(0.02)* |
| | WB | 3 | 0.40 | *(0.06)* | na | |
| *Melilotus albus* | CR | 3 | 0.97 | *(0.01)* | 0.10 | *(0.03)* |
| | ER | 9 | 0.85 | *(0.04)* | 0.18 | *(0.03)* |
| | WB | 3 | 0.97 | *(0.02)* | na | |
| *Melilotus officinalis* | CR | 3 | 0.98 | *(0.01)* | 0.06 | *(0.02)* |
| | ER | 9 | 0.86 | *(0.04)* | 0.08 | *(0.01)* |
| | WB | 3 | 0.93 | *(0.02)* | na | |

na: not available.

#### 2.4.2.4 Data Analyses

Data analyses were carried out using the software environment R (version 4.2.1) [38].

Seed viability as a function of exposure time, *V(t)*, was modelled with a dose–response approach using the R-package 'drc' (version 3.0.1) [39] and compared between the treatments in the water bath and in the experimental and commercial biogas reactors. Log-logistic models with a lower limit of zero were fitted to the observed proportions of viable seeds (**Equation 2-6**). Models were fitted species wise, with treatment set as a grouping variable. The data type was "binomial" and the total number of evaluated seeds was set as weights. The model fit was evaluated both by a Chi²-test and visually. In case all or almost all seeds had lost viability even after the shortest exposure time (1 day or 3 days), no model was fitted.

$$V(t) = \frac{V_{max}}{1 + e^{SLP(\log(t) - \log(MIT))}} \quad \textbf{(2-6)}$$

*V(t)*: proportion of viable seeds as a function of the time of exposure in AD (*t*);
$V_{max}$: maximum proportion of viable seeds (upper asymptote);
*SLP*: parameter proportional to the slope of *V(t)* in the inflection point;
*MIT* (median inactivation time): the time after which *V(t)* reaches 50% of $V_{max}$.

From the viability models, the median inactivation times (*MIT*s) and decimal reduction times (*DRT*s) were estimated, i.e., the number of days required to inactivate 50% or 90% of the initially viable seeds.

The parameter estimates *MIT* and *DRT* were compared between the three treatments specieswise using the 'drc' built-in functions *compParm* and *EDcomp* [39]. The level of significance α was set to 0.05.

For a direct comparison of the seed-killing effect of the three treatments, the percent seed-killing efficacy (*SKE*) was calculated as a function of exposure time. Viability models were used to estimate viability and 95% confidence intervals for 0 to 36 days of treatment. Using **Equation 2-7**, the viability values were converted to *SKE*s. Since it was not possible to fit a model for *A. theophrasti*, the *SKE*s for this species were calculated from the mean measured values.

$$SKE\ [\%] = 100 \times \left(1 - \frac{V(t)}{V(0\ \text{days})}\right) \qquad \textbf{(2-7)}$$

### 2.4.3 Results

Seed viability of the six species was lost at different rates and to different degrees during treatment in the commercial reactor (CR), the experimental reactor (ER), and the water bath (WB) (**Figures 2-8** and **2-9**). The species most affected by the treatments were *A. theophrasti*, *C. album* and *M. alcea*. Their decimal reduction times (*DRT*s) ranged from a few hours to three weeks and their seed-killing efficacies (*SKE*s) exceeded 80% after 36 days of treatment (**Figure 2-8**, **Tables 2-8 and 2-9**). The other three species, *M. sylvestris*, *M. albus*, and *M. officinalis*, had lost a maximum of 34% of their viability after 36 days regardless of treatment type, and the *DRT* estimates were longer than one year, with one exception (**Figure 2-9**, **Tables 2-8** and **2-9**). The species that was inactivated most rapidly was *A. theophrasti*. At each exposure time, only individual seeds were still alive in some replicates. Therefore, no model was fitted for *A. theophrasti* (**Figure 2-8** top row). *Malva sylvestris* proved least sensitive to all three treatments. It was also the species for which curves, inactivation times and *SKE*s differed least between treatments (**Figure 2-9**, **Tables 2-8** and **2-9**).

Comparing treatments, there was a trend for seed viability to be lost most rapidly and severely in CR. After 36 days of treatment, *SKE*s averaged across all six species were 64 ± 39% in CR, 51 ± 50% in ER and 54 ± 45% in WB (**Table 2-9**). Except for *M. sylvestris*, a steep decline in viability occurred in all species during the first 3 days of exposure to CR (left column in **Figures 2-8** and **2-9**). In numbers, this steep decline corresponded to *SKE*s of 99%, 96%, 93%, 27% and 21% for *A. theophrasti*, *C. album*, *M. alcea*, *M. albus*, and *M. officinalis*, respectively (see also right column in **Figures 2-8** and **2-9**). This means that in CR, most of the *SKE* of the entire exposure time (36 days) was reached in the first 3 days. For *M. sylvestris*, the exception, the *SKE* was only 0.3% during these days. With respect to the varying temperatures during the three runs in CR (**Table 2-6**), no consistent effect on seed viability was observed (left column in **Figures 2-8** and **2-9**).

The estimated *DRT*s in CR were about 2 days for *C. album* and *M. alcea*, an order of magnitude lower than in the other two treatments (*DRT*s of about 2 weeks). *DRT* estimates for *M. sylvestris*, *M. albus*, and *M. officinalis* had extreme ranges and standard deviations in all three treatments (**Table 2-8**).

Species responses to treatment in ER and WB were more diverse than those in CR. Only *A. theophrasti* and *M. alcea* showed a steep decline in seed viability in ER and WB, similar to that observed in CR. Their further inactivation was also very similar in CR, ER and WB (**Figure 2-8**). The only difference was that the observed viability of *A. theophrasti* in WB increased steadily after it had been completely lost by 9 days of exposure (**Figure 2-8**, top row). Seeds of *C. album* were completely inactivated by all three treatments, but a lag phase occurred in ER and WB, in contrast to CR. This lag phase was longer in WB than in ER (**Figure 2-8**, middle row). Further, *C. album* was the only species for which inactivation times differed significantly between all treatments, with inactivation being fastest in CR and slowest in WB. *DRT*s of *C. album* were 1.7 ± 0.4 days, 10.8 ± 0.2 days and 15.3 ± 0.7 days in CR, ER and WB, respectively (**Table 2-8**). Seeds of the two *Melilotus* species lost the least of their viability in ER (*SKE* after 36 days < 10%, **Table 2-9**). For *M. officinalis*, inactivation in WB proceeded similarly to that in ER. For *M. albus*, however, viability decreased more rapidly in WB than in ER, yielding a *SKE* of 30% in 36 days, which was comparable to that in CR (**Table 2-9**, **Figure 2-9**). Finally, seed viability of *M. sylvestris* was almost unresponsive to ER and WB. Estimated *SKE*s were zero after 36 days of treatment (**Table 2-9**). In the measured values, a slight increase in viability was observed in ER after 9 days, but this was not reflected in the model (**Figure 2-9**, top row).

**Table 2-8 | Parameter estimates (*standard error of the mean*) for maximum viability, $V_{max}$, slope parameter, *SLP*, and median inactivation time, *MIT*, as well as estimates of decimal reduction time, *DRT*, obtained from the seed viability models. Lowercase letters indicate significant differences (alpha < 0.05) of estimates between treatments in the commercial biogas reactor (CR), the experimental biogas reactor (ER) and the water bath (WB). Standard errors for inactivation times were not calculated when estimated values exceeded one year (365 days, >365).**

| Species | Treatment | $V_{max}$ | | SLP | | MIT [days] | | DRT [days] | |
|---|---|---|---|---|---|---|---|---|---|
| *Abutilon theophrasti* [x] | all | - | | - | | - | | - | |
| *Chenopodium album* | CR | **0.99** *(0.01)* | a | **1.52** *(0.29)* | a | **0.40** *(0.19)* | a | **1.7** *(0.4)* | a |
| | ER | **0.88** *(0.01)* | b | **6.71** *(0.41)* | b | **7.75** *(0.09)* | b | **10.8** *(0.2)* | b |
| | WB | **0.81** *(0.01)* | c | **6.66** *(0.84)* | b | **10.97** *(0.20)* | c | **15.3** *(0.7)* | c |
| *Malva alcea* | CR | **0.73** *(0.03)* | a | **0.64** *(0.16)* | ab | **0.05** *(0.07)* | a | **1.6** *(0.8)* | a |
| | ER | **0.51** *(0.02)* | b | **0.29** *(0.06)* | b | **0.01** *(0.01)* | a | **20.1** *(7.2)* | ab |
| | WB | **0.76** *(0.04)* | a | **0.70** *(0.35)* | a | **0.64** *(1.02)* | b | **14.6** *(4.1)* | b |
| *Malva sylvestris* | CR | **0.29** *(0.02)* | a | **1.77** *(2.19)* | a | **83.14** *(78.65)* | a | **287.6** *(707.9)* | a |
| | ER | **0.32** *(0.01)* | ab | **2.99** *(120.08)* | a | **>365** - | a | **>365** - | a |
| | WB | **0.36** *(0.02)* | b | **2.09** *(15.14)* | a | **>365** - | a | **>365** - | a |
| *Melilotus albus* | CR | **0.97** *(0.01)* | a | **0.15** *(0.04)* | a | **>365** - | a | **>365** - | a |
| | ER | **0.88** *(0.01)* | b | **9.86** - | ab | **46.45** - | a | **58.0** - | b |
| | WB | **0.97** *(0.01)* | a | **1.03** *(0.26)* | b | **80.44** *(27.02)* | a | **>365** - | ab |
| *Melilotus officinalis* | CR | **0.98** *(0.01)* | a | **0.23** *(0.03)* | b | **>365** - | a | **>365** - | a |
| | ER | **0.86** *(0.01)* | b | **0.76** *(0.74)* | b | **>365** - | a | **>365** - | a |
| | WB | **0.93** *(0.02)* | c | **0.70** - | b | **>365** - | a | **>365** - | a |

[x] For *A. theophrasti* no viability model could be fit.

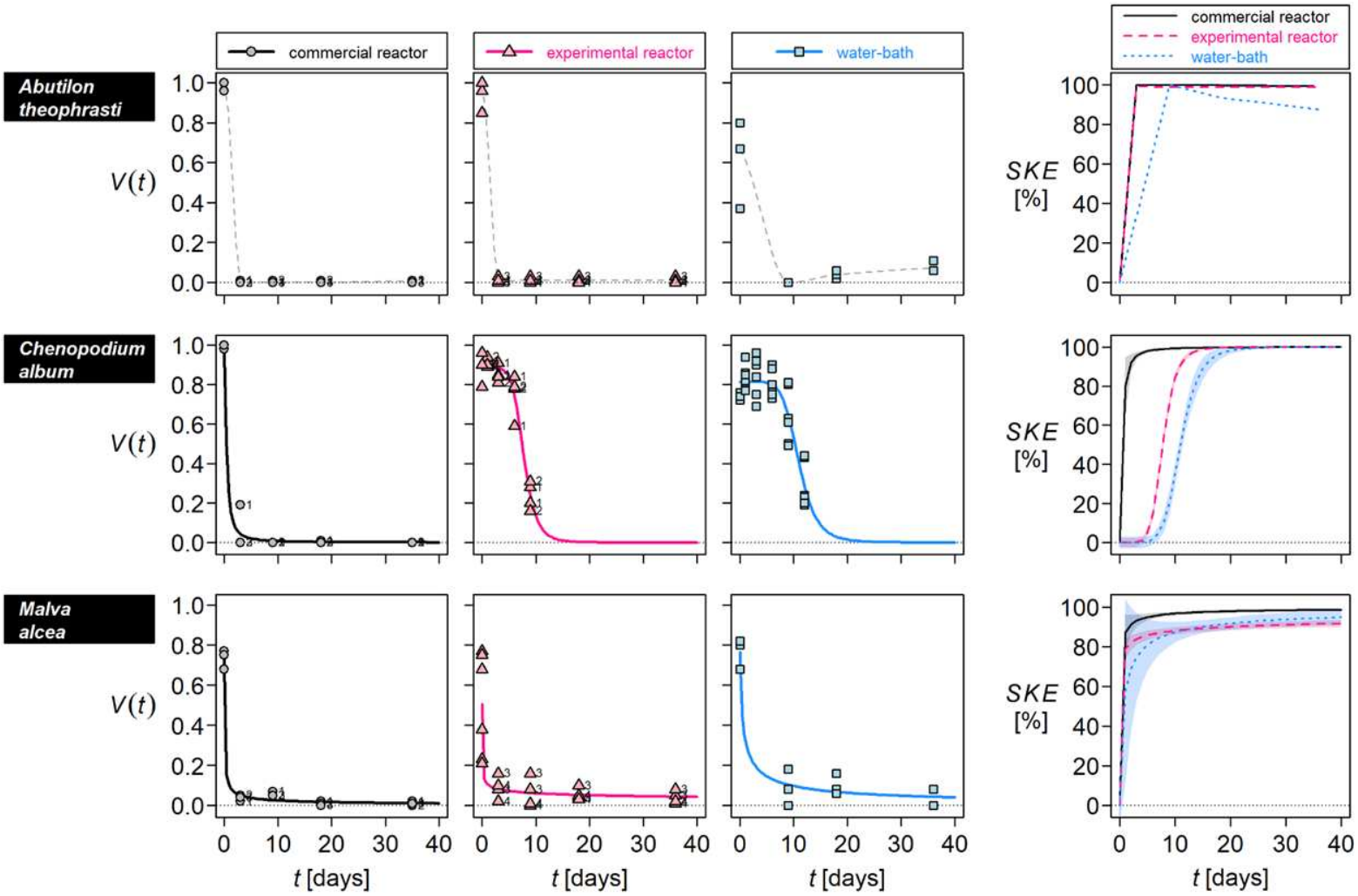


**Figure 2-8 | Proportion of viable seeds (*V*, columns on the left) and percent seed-killing efficacy (*SKE*, right column) during anaerobic digestion in a commercial biogas reactor (black), an experimental biogas reactor (pink) and in a buffer solution in a water bath (blue) for the species *Abutilon theophrasti* (top row), *Chenopodium album* (middle row) and *Malva alcea* (bottom row). In the viability plots, solid lines represent viability, *V*, as a function of exposure time, *t*, and symbols represent observations containing at least 50 seeds each. The grey dashed lines for *A. theophrasti* display trend lines since no viability model could be fit. Numbers next to the observations in the reactors indicate the respective run (1–4, see** Table 2-6**). p-values of the viability model fits (Chi²-test) were 0.3632 for *C. album* and 0.0173 for *M. alcea*. In the *SKE* plots, shaded areas display 95% confidence intervals. *SKE*s for *A. theophrasti* were calculated from the mean measured values.**

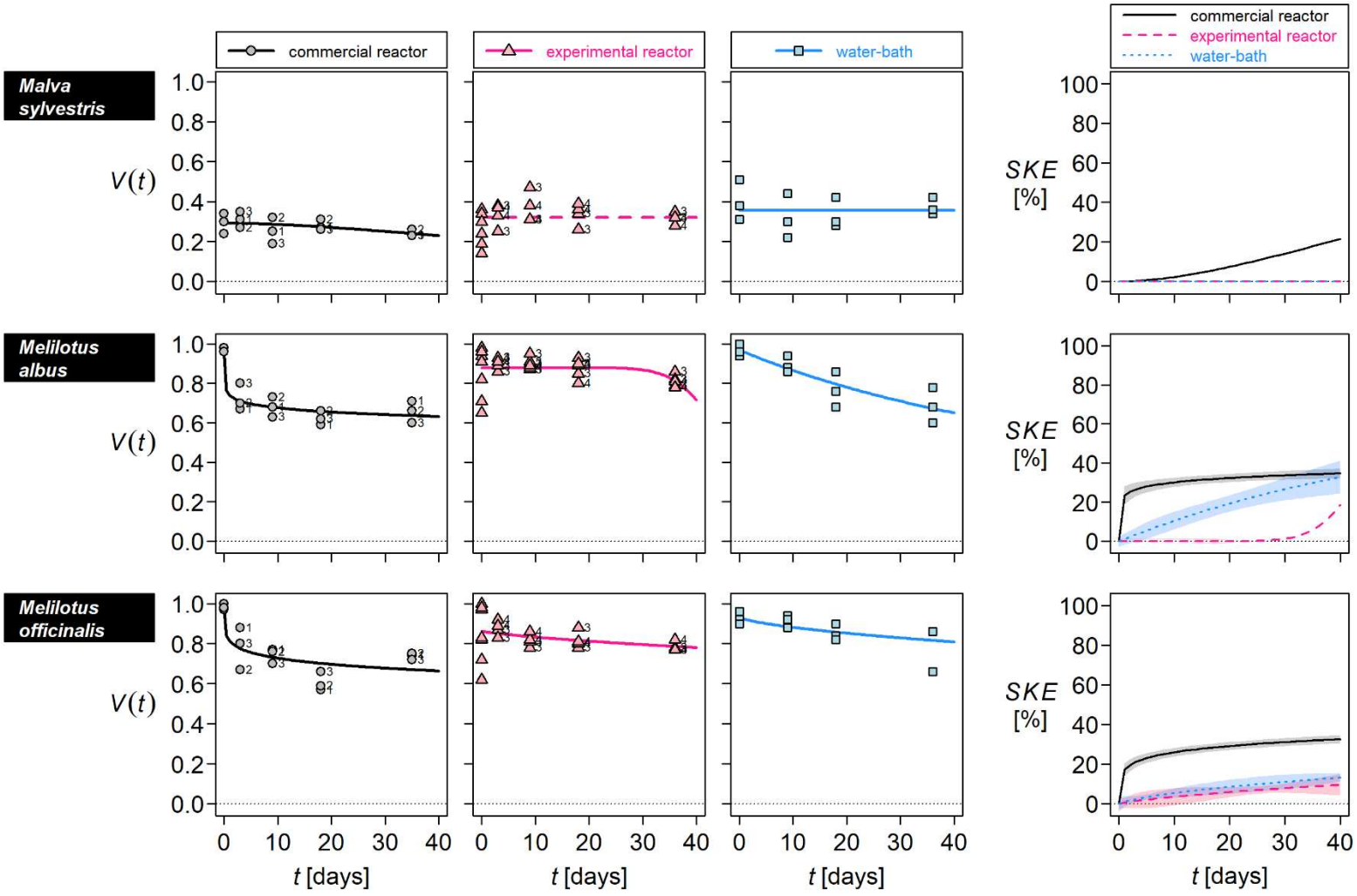


**Figure 2-9 | Proportion of viable seeds (*V*, columns on the left) and percent seed-killing efficacy (*SKE*, right column) during anaerobic digestion in a commercial biogas reactor (black), an experimental biogas reactor (pink) and in a buffer solution in a water bath (blue) for the species *Malva sylvestris* (top row), *Melilotus albus* (middle row), and *Melilotus officinalis* (bottom row). In the viability plots, lines represent viability, *V*, as a function of exposure time, *t*, and symbols represent observations containing at least 50 seeds each. Numbers next to the observations in the reactors indicate the respective run (1–4, see** Table 2-6**). p-values of the viability model fits (Chi²-test) were 0.0955 for *M. sylvestris*, 0.8700 for *M. albus* and 0.0708 for *M. officinalis*. In the *SKE* plots, shaded areas display 95% confidence intervals.**

**Table 2-9 | Predicted seed-killing efficacy of 36 days in a commercial biogas reactor, an experimental biogas reactor and in a water bath on six species.**

| Species | Seed-Killing Efficacy [%] of 36 days in | | |
|---|---|---|---|
| | Commercial Reactor | Experimental Reactor | Water-Bath |
| *Abutilon theophrasti* | 99 | 98 | 88 |
| *Chenopodium album* | 100 | 100 | 100 |
| *Malva alcea* | 99 | 91 | 94 |
| *Malva sylvestris* | 19 | 0 | 0 |
| *Melilotus albus* | 34 | 7 | 30 |
| *Melilotus officinalis* | 32 | 9 | 12 |

## 2.4.4 Discussion

### 2.4.4.1 Seed-Killing Efficacy of the Commercial Reactor

Seed survival varied among the three treatments and among the six species. However, the seed-killing efficacy (*SKE*) of the commercial reactor (CR) treatment was at least equal to, but mostly higher than, that of the experimental reactor (ER) and water-bath (WB) treatments. After 36 days in CR, *C. album* was completely inactivated while the HS species retained 1 to 81% of their initial viability. The two species *C. album* and *A. theophrasti* had been previously studied in full-scale CRs at comparable mesophilic temperatures (38–45°C) [25,26], and the determined extent of seed inactivation due to CR was in a similar range.

For the NHS species *C. album*, seed inactivation in the CR of this study was almost identical to that reported by Leonhardt et al. [25]. They found that *C. album* seeds were killed after three days in a CR run at 45°C and that mean germination was reduced by 26% in another CR at 45°C and by 99% in a CR at 42°C. However, after one week of exposure, *C. album* seeds were completely inactivated in all CR treatments by Leonhardt et al. [25]. In the CR used by Westerman et al. [26], which ran at 41°C, *C. album* was killed more slowly. The decimal reduction time (*DRT*) was 19.7 days, which was 18 days more than in the CR at 44°C in this study. The only other NHS species that survived anaerobic digestion (AD) in full-scale, stirred CRs for three days in this temperature range were *Fallopia convolvulus* (wild buckwheat, Polygonaceae), *Persicaria lapathifolia* (willow weed, Polygonaceae), *Panicum virgatum* (switchgrass, Poaceae), *Phalaris arundinaceae* (reed canary grass, Poaceae), *Phragmites australis* (common reed, Poaceae), and *Lycopersicon esculentum* (tomato, Solanaceae) [25,26,35]. Therefore, it seems that biogas reactors that are completely stirred and operated in the upper mesophilic temperature range can anaerobically digest the biomass of these NHS species without risking contamination of the digestate with seeds. However, it should be emphasized that this requires the prevention of short circuits [40] and, thus, a sufficiently long exposure of the seed to AD, e.g., for the mean hydraulic retention time of approx. 91 days in Germany (calculated from [41]). This is because if the number of seeds entering the reactor is high (e.g., [42]), even low percentages of surviving seeds, such as those observed by

Westerman et al. [26] for *C. album* after 9 days in CR, may correspond to a large number of viable seeds in the digestate.

Of the species with HS, which is considered a risk factor for AD survival [23], only three were tested in CR treatments prior to our study: *A. theophrasti* and *Malva neglecta* (dwarf mallow, Malvaceae) [26] as well as *Lupinus polyphyllus* (garden lupin, Fabaceae) [27]. The results for *A. theophrasti* were very similar, as mentioned above. In both this study and the study by Westerman et al. [26], the 1- and 2-year-old seed lots were almost completely inactivated. That is, few, if any, seeds were viable per exposure time tested. The 5-year older seed lot had a higher resistance potential, with maximum 10% viable seeds after 9 days of exposure to CR at 41°C [26]. The survival rates of *M. neglecta* [26] and *M. alcea* (this study) were also within this range. However, it cannot be said with certainty that these species or other members of the Malvaceae pose little risk of digestate contamination after CR treatment. First, *Malva sylvestris* was the best-surviving species in this study, with only 19% of its seeds killed by CR treatment. Second, the seed lot-dependent expression of HS plays a role in AD-resistance potential [20]. For example, the germination rates of *A. theophrasti* and *M. alcea* were high compared to that of the other three HS species in this study. This indicates a lower proportion of fully mature, hardseeded, and thus, likely AD-resistant seeds in the lots of *A. theophrasti* and *M. alcea*. Third, under unfavorable conditions, even a few surviving seeds can cause problems, such as weed infestations, which can become established years later from the seed bank [43,44]. These differentiating considerations are not necessary for the representatives of the Fabaceae: *Melilotus albus* and *M. officinalis* clearly survived the CR treatment and would be present in the digestate after 36 days, having lost only about one-third of seed viability. This is in line with the extreme resistance potential of *L. polyphyllus*, of which 2 to 50% were still alive after 155 days in a batch reactor at 37°C [27]. In summary, it has now been confirmed on the basis of seven instead of three studied representatives of HS species that HS is a risk factor for seeds to survive AD in full-scale CRs. Consequently, the NHS species tomato used so far in Germany [45] is not suitable to evaluate the phytohygiene of biogas plants. Instead, HS species should be considered as indicators for the sanitation of digestate.

#### 2.4.4.2 Factors Inactivating Seeds

All species that survived lab-scale AD at 42°C in Hahn et al. [20], which is the same as the ER treatment in this study, survived the CR treatment as well. The ranking of AD resistance also remained largely the same, i.e., the most resistant species in ER were the most resistant in CR. However, the course of inactivation differed between ER and CR, with stronger and faster seed inactivation in CR: seed-killing efficacies, if not close to 100% in both reactors, were higher and inactivation times, if less than one year, i.e., could be meaningfully interpreted [20], were shorter in CR. Similarly, Leonhardt et al. [25] found that their 10 species studied survived better in experimental batch reactors at 35°C than in full-scale CSTRs at 42–45°C. Other studies, however, found that a species survived in CR but was killed in ER (35°C, [14]), or that 30 days in ER killed a comparable amount of seeds as 155 days in CR (37°C, [27]).

Further, it was reported that the ranking of AD resistance changed between species depending on whether they were digested in batch or continuous reactors [19,26]. Differences in AD conditions, exposure times, and initial seed viability have been suggested as reasons for the varying responses of the same species in different treatments or studies [23]. In our study, however, seed survival of a species differed between CR and ER, although we obtained high similarity in exactly these parameters. Both the ER and the CR were completely stirred, operated at nearly the same temperature and fed continuously on a mixture of maize silage and slurry. The resulting level of process stability, and thus system comparability, is rarely achieved when full-scale reactors are involved. Seed sampling was done at the same exposure times, admittedly with a higher resolution in the first week for the NHS species. In addition, we used exactly the same seed lots in all treatments and converted the results to *SKE*s to account for differences in initial viability. With this in mind, the differences in the inactivation curve, *SKE*, and *DRT* between the ER, CR, and WB treatments should be attributable to factors or combinations of factors related to the AD process and seed lot characteristics.

In the three least resistant species, *A. theophrasti*, *C. album* and *M. alcea*, seed killing in the reactors seemed to be largely due to thermal inactivation. In the ER and CR treatments, the inactivation curves had a similar shape as in the WB treatment and were only shifted towards faster inactivation. The shifts occurred particularly in *C. album* and were also found by Zhou et al. [32] when comparing the survival of *Digitaria sanguinalis* (purple crabgrass, Poaceae) in anoxic water baths and lab-scale reactors. These shifts in the course of inactivation between WB and reactor treatments indicate the involvement of additional mortality factors in the reactors, which include microorganisms contributing to AD and biochemicals such as organic acids, enzymes and alcohols [23]. Differences in AD chemism and microbial consortia might also explain why *C. album* and *M. alcea*, as well as the more resistant species (see below), lost viability faster in CR than in ER. According to our measurements, CR and ER differed in two chemical factors: In CR, the concentration of ammonia was higher than in ER, while the concentration of volatile fatty acids was slightly lower (cf. **Table 2-6**). High ammonia concentrations contribute to inactivation of pathogens such as viruses, bacteria and protozoa in AD [46,47]. It can cause genome loss, seems to be able to penetrate the cell membrane and is toxic to methanogens ([47] and references therein). Ammonia inhibition levels of AD processes vary widely due to the adaptable balance between acidogenic and methanogenic microorganisms, as well as differing substrates, temperatures, pH-values, etc. However, generally, concentrations greater than 3000 mg $NH_4$-N $L^{-1}$ are considered to inhibit the AD process [48]. This concentration was exceeded in our CR, indicating an adapted AD process [48], possibly with a microbial community that differed greatly from that in the ER and affected the seeds more. In addition, the higher ammonia concentration in CR may have affected members of the seed microbiome, which is an intense exchange with the seed [49]. Furthermore, ammonia is toxic for seed germination and seedling growth (e.g., [50–52]), but can have beneficial effects as a gaseous signalling molecule [53]. Thus, it might directly affect (imbibed, germinable) seeds in AD. Regardless of how ammonia concentrations may have affected the seeds in this study, it

highlights how diverse the effects of a single mortality factor in AD can be. Further research involving metagenomic analyses and extended monitoring of chemical parameters may reveal the interplay of factors leading to seed death in AD.

To put the effect of the AD-related mortality factors into perspective, it is important to emphasize that just as differing types of pathogens play an important role [46], so did the different plant species. The individual species responded very differently even to the WB treatment, which in principle, determines the contribution of only one factor, temperature, to seed inactivation. Moreover, surprisingly, an increase in observed viability occurred in two of the less resistant species when exposed to WB: in *A. theophrasti* at the end of exposure and in *C. album* at the beginning. Similar increases in viability or germinability were observed for other species in WB [15,32,36,54] and ER treatments [20,25,29,31,32]. Increased germinability was explained by breaking the dormancy and initiation of germination [25,29,32]. However, this explanation is not feasible for our data because we recorded total viability, i.e., the sum of germinable and non-germinable but viable seeds. Therefore, we follow the reasoning of Hahn et al. [20] and suggest that the increase in observed viability in the WB treatment is due to either metabolic stimulation (hormesis) of seeds whose metabolic activity was not detectable by TTC staining before treatment or facilitated TTC uptake into the seeds. In addition, *M. alcea* provides evidence that seed changes over the course of the study may have contributed to the stronger inactivation in CR. CR treatments took place at the end of the study. By this time, the proportion of germinating seeds had tended to increase in the seed lot of *M. alcea* compared to the beginning of the study. Presumably, then, fewer seeds of *M. alcea* were hardseeded. Seeds that have lost their HS imbibe water. Once their moisture content exceeds 15%, they become more sensitive to temperatures above 35°C and, thus, more likely to be inactivated by AD [23].

Regarding the influence of higher temperatures, the unplanned temperature difference of 2.5°C between CR and ER might have increased seed mortality in CR. Temperature is the most important factor affecting seed survival in AD, and in general, a higher temperature is associated with a higher proportion of dead seeds [23]. However, temperature varied by the same order of magnitude (maximum 2.7 °C) between runs in the CR, and there was no consistent effect on seed survival between them. Similarly, in Leonhardt et al. [25], the seed survival between the CRs operated at 45°C did not differ from that operated at 42 °C. In fact, seeds survived longer in one of the 45°C-CRs than in the 42°C-CR. Therefore, the temperature difference might have contributed to the greater inactivation of the seed in CR, but in interaction with the chemical and biological factors mentioned above and others that remain to be determined.

For the more resistant species, the seed-inactivating factors indicate even more interactions challenging to interpret. In *M. officinalis*, temperature seemed to cause seed killing in the ER, but to be enhanced by additional factors in the CR. In *M. sylvestris*, only factors present in the CR seemed to be able to trigger seed inactivation at all. In *M. albus*, the most complex case, factors in the CR appeared to enhance thermal inactivation, whereas factors in the ER dampened it. It would be interesting to find

out what (combination of) factors in the CR caused even the most resistant species in this study to lose viability. The possible involvement of ammonia and higher temperature has already been discussed above. Another indication that in CR different factors inactivate seeds than in ER is given by the response of *M. albus*. The seed of *M. albus* had a slightly higher proportion of viable, nongerminating, i.e., hardseeded, seeds at the time of the CR treatment compared to the ER treatment. Nevertheless, more seeds died in CR than in ER. So, it is possible that factors were active in CR that could kill seeds without prior imbibition. This could also be indicated by the fact that *C. album* is killed in CR without a preceding lag phase. The lag phase is the period during which seeds are initially unaffected by AD, for example, because the seed coat is still intact and prevents imbibition, which makes the seeds less susceptible to thermal inactivation [23]. Then, there is the question of what in the ER treatment caused *M. albus* to survive better than in the WB treatment and caused an increase in observed viability in *M. sylvestris*. Involvement of microbial activity is conceivable if, for instance, the slightly higher concentration of volatile fatty acids and the production of methane in the ER than in the CR are expressions of a different microbial community. Direct protective effects by microbes are conceivable. Chen and Nelson [55] reported that seed-colonizing microbes from municipal sewage sludge compost suppressed the pathogenic *Pythium ultimum* in several plant species. In addition, Westerman and Gerowitt [23] discussed mechanisms and compounds potentially protecting seeds in biogas reactors, e.g., heavy metals that might prevent imbibition and, consequently, inactivation of seeds. Finally, if the observed increase in viability of *M. sylvestris* after brief exposure to ER is not an artifact but a hormetic response (cf. [20]), there would definitely be factors in AD that have a positive effect on seed viability—even if only for a short time. However, all these are hypothetical considerations which require confirmation via the inclusion of seed biological and biochemical methods.

Last but not least is the factor time, which was effective in all treatments, interacted with all factors and strongly influenced our results and their extrapolation. The residence time of contaminated substrate in the reactor is also considered crucial for the inactivation of pathogens in AD [47]. In this study, seeds of the more resistant species were not yet completely inactivated at the maximum exposure time of 36 days. Moreover, the observed inactivation was not linear for any species. That means that extrapolations beyond 36 days are subject to a high degree of uncertainty. This is reflected in the very long inactivation times estimated in half of the cases using the models in this study. Not only did they exceed the previously reported maximum survival time of 155 days [27] by several orders of magnitude but they were also well beyond the operational range of retention times in biogas plants [41] in Germany. Therefore, measurements should continue until all seeds are completely inactivated in order to realistically evaluate the survival probability of seeds of a species in AD. This applies equally to full-scale and lab-scale systems.

#### 2.4.4.3 Estimating Seed Survival in Commercial Reactors

Estimating the probability of seed survival in full-scale CRs using lab-scale systems would have the advantage of being less laborious, less costly, and more amenable to standardization. However, in this study, seed inactivation in CR, ER and WB treatments was comparable only to a limited extent. Although the CR treatment was most effective in killing seeds, there were differences among species, and the ratio of seed inactivation between CR and ER and between CR and WB was also variable. Thus, we disagree with Leonhardt et al. [25], who found that sanitation in full-scale CRs can be reproduced comparably in lab-scale ERs. Given the diversity and ambiguity regarding factors that might cause differences in seed inactivation in different AD systems, we instead agree with Westerman et al. [26], who advised extreme caution when extrapolating results.

If lab-scale systems are to be used to estimate seed killing, a "transfer formula" for the higher kill rate to be expected in CR must be specified. In the case of the rapidly inactivating NHS species like *C. album*, the inactivation curves in CR and WB were parallel. Thus, screening in WB would determine the minimum mortality of a species in CR, as suggested by Westerman and Gerowitt [23]. In our study, the relationship between decimal reduction times in CR and WB could be expressed as $DRT_{CR} = DRT_{WB} - 13.6$ [days] or as $DRT_{CR} = DRT_{WB}/9$ [days]. Which formulation of the relationship is appropriate for transfer to CR and whether it is valid for other feeding patterns needs to be clarified. Moreover, keeping in mind that each biogas plant is an individual [56], the applicability to other plants must also be verified before WB treatments can be used as a low-cost screening option. For HS species, it is currently not possible to transfer the results of WB treatment to a full-scale CR due to their diverse responses and the limited knowledge of their inactivation dynamics. To address this issue, further studies could (1) record complete inactivation curves, i.e., until all seeds are killed in WB and CR, (2) simultaneously check seed dormancy, and (3) ideally monitor chemical and microbiological parameters in CR. It is likely that the results are species-dependent, but it may be possible to reveal general inactivation mechanisms for HS species in AD.

Finally, it should be emphasized that AD, especially in the circular economy, is not an isolated process. It is part of the biogas process chain, which includes upstream and downstream processes that can influence seed survival (e.g., [12,57]). The synergy of the seed-inactivating effect of AD with that of other processes should provide comprehensive insight into the risk of digestate contamination with seeds.

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## 2.4.7 Supplementary Material

**Supplementary Table 2-6 | Number of replicates and number of seeds per replicate for species exposed to mesophilic, anaerobic digestion in commercial (CR) or experimental reactors (ER) or to buffer solution in a water bath (WB) (1–36 days). The numbers of seeds per replicate are indicated by + = 50 seeds, # = 100 seeds, ## = 200 seeds, ### = 300 seeds, - = no seeds.**

| Treatment | Exposure Time [days] | *A. theophrasti* | *C. album* | *M. alcea* | *M. sylvestris* | *M. albus* | *M. officinalis* |
|---|---|---|---|---|---|---|---|
| CR | 0 | 3 # | 3 # | 3 # | 3 # | 3 # | 3 # |
| | 3 | 3 # | 3 # | 3 # | 3 # | 3 # | 3 # |
| | 9 | 3 # | 3 # | 3 # | 3 # | 3 # | 3 # |
| | 18 | 3 ## | 3 ## | 3 ## | 3 ## | 3 ## | 3 ## |
| | 35 | 3 ### | 3 ### | 3 ### | 3 ### | 3 ### | 3 ### |
| ER | 0 | 4 # | 3 # | 6 # | 6# | 9 # | 9 # |
| | 1 | - | 4 # | - | - | - | - |
| | 3 | 4 # | 4 # | 4 # | 4 # | 6 # | 4 # |
| | 6 | - | 4 ## | - | - | - | - |
| | 9 | 4 # | 4 ### | 4 # | 4 # | 6 # | 4 # |
| | 18 | 4 ## | - | 4 ## | 4 ## | 6 ## | 4 ## |
| | 36 | 4 ### | - | 4 ### | 4 ### | 6 ### | 4 ### |
| WB | 0 | 3 + | 5 + | 3 + | 3 + | 3 + | 3 + |
| | 1 | - | 6 + | - | - | - | - |
| | 3 | - | 6 + | - | - | - | - |
| | 6 | - | 6 + | - | - | - | - |
| | 9 | 3 + | 6 + | 3 + | 3 + | 3 + | 3 + |
| | 12 | - | 6 + | - | - | - | - |
| | 18 | 3 + | - | 3 + | 3 + | 3 + | 3 + |
| | 36 | 3 + | - | 3 + | 3 + | 3 + | 3 + |

# 3

# Plant Seeds in Ensilage

## 3.1 Ensilability of Flower Strips

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### Ensilability of Biomass from Effloresced Flower Strips as Co-substrate in Bioenergy Production

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Flower strips are grown to an increasing degree in order to enhance the ecological value of agricultural landscapes. Depending on their profitable life span and the crop sequence, the strips' biomass must be mulched after flowering to enable repeated tillage. A promising alternative is the use of the flower strips' biomass as a co-substrate for biomethanisation - thereby contributing to the climate-friendly generation of energy. This potential bioenergy substrate occurs only seasonally and is commonly produced only in limited quantities at a farm scale. To realize the additional benefit of flower strips as energy suppliers, stock piling of the strips' biomass is required. However, information about the ensilability of flower strip biomass is still rare. We conducted a 2-year study to analyze the ensilability of pure biomass from effloresced flower strips and mixtures of flower strip biomass with 33 and 67% whole crop maize, respectively. Ensiling took place in 3 l model silos at laboratory scale after chopping the substrate. Before ensiling several chemical characteristics of the biomass stock were determined to assess the substrate's biochemical ensilability potential (dry matter content, water-soluble carbohydrates, buffering capacity, nitrate content). The process-engineered ensiling success after 90 days was determined based on fermentation patterns. The ensilability potential of the pure flower strip substrates reached modest levels (fermentability coefficients according to Weißbach vary around the threshold of 45). Nevertheless, acceptable silage qualities were achieved under the laboratory conditions (pH ranging from 4.2 to 4.7). Compared to pure flower strip biomass, the addition of maize noticeably improved both the substrate's biochemical ensilability potential and the quality of real fermented silage. We conclude that a mixture of 33% biomass from flower strips with 67% whole crop maize can be regarded as a recommendable ratio if proper ensiling technology is applied.

**Keywords:** **ensiling, biomass, field margins, buffer strips, preservation success, substrate composition, fermentation pattern, biomethanisation**

### 3.1.1 Introduction

Two developments characterize the current situation in the agricultural sector: the increasing demand for food (Davis et al., 2016) and the growing importance of bio-based energy production (Hennig et al., 2016). Both developments are linked via their respective land requirements and are held responsible for the negative effects of intensive land use on biodiversity (Robertson et al., 2012; Tilman and Clark, 2015). To counteract these adverse tendencies and to enhance the ecological value of agricultural landscapes, buffer strips along field margins (Mante and Gerowitt, 2006; Fritch et al., 2011) and

vulnerable waterbody zones (Buckley et al., 2012) are growing in importance. For ecological and esthetic reasons, these buffer strips mostly contain a broad mixture of flowering annuals, biennials (Jacot et al., 2007) and perennials (Carlsson et al., 2017). In Europe, the support measures under the so-called second pillar of the EU Common Agricultural Policy (CAP) framework have led to a significantly increasing area of flowering strips in many regions (Haaland et al., 2011) recently.

Depending on their profitable life span and the crop sequence in which they are integrated, the strips' biomass must be mulched after flowering in late summer in order to enable repeated tillage in early autumn. Since many species, such as mallows, can form enormous biomasses, mulching, and tilling are associated with a great deal of effort. A promising alternative is the use of the flower strips' biomass as a source of renewable bioenergy (Christen and Dalgaard, 2013; Golkowska et al., 2016). This kind of biomass is especially appreciated as it does not compete with food production (Dauber et al., 2012; Gelfand et al., 2013) and has numerous ecological benefits, e.g., providing habitats for insects and birds. Although other conversion routes of tall herb biomass to energy like combustion (Ciesielczuk et al., 2016) are conceivable, biomethanisation is of the greatest importance (van Meerbeek et al., 2015). This technology does not require expensive drying and is most widespread in European rural areas (Capodaglio et al., 2016).

At farm scale, the biomass from effloresced flower strips crops up only seasonally and in limited quantities (Ferrarini et al., 2017). Therefore, stock piling is required if the strips' biomass is supposed to be used as a substrate for the production of bioenergy. A well-founded knowledge of the storage capability of the biomass is essential for several reasons: (1) to avoid energy losses (Einfalt, 2017; Towey et al., 2019), (2) to prevent the entry of substances that interfere with the conversion processes, e.g., ammonia N (Poggi-Varaldo et al., 1997), (3) to make targeted use of the advantages of any preliminary conversion effects, e.g., ensiling as methane potential booster before anaerobic digestion (Teixeira Franco et al., 2016), caused by degradation processes and an increase in volatile fatty acids (VFA) (Corno et al., 2016), and thus, to design an economically efficient storage process. Expertise in the storage capability of flower strip biomass would not only be useful for the ensiling and energetic use of the flower strips, but also for harvests from perennial wild flower stands, as found in increasing numbers in restoration projects across Europe (von Cossel and Lewandowski, 2016) and North America (Voigt et al., 2012).

However, information about the ensilability of flower strip biomass is still rare. Despite an extensive literature research, only one peer-reviewed source (Oh et al., 2010) on the topic could be found. Further information stems from gray literature such as conference contributions and non-peer-reviewed technical contributions, e.g., Kalzendorf (2011). In addition, a wide range of possible seed mixtures and varieties makes it actually impossible to assume a generalizable composition of the flower strip biomass and thus, of the substrate for ensiling. Multispecies mixtures containing effloresced dicots that were neither bred nor intended for the purpose of biomass utilization and stock piling may hold

some surprises regarding their carbohydrate composition, their secondary metabolites, their epiphytic population and further factors that potentially influence the ensiling success significantly.

Against the background of scarce knowledge, it seemed reasonable to determine the ensilability of effloresced flower strip biomass using an approach based on the biochemical characteristics of the biomass stock. From the substrate properties of the flower strip substrates, we intended to calculate estimates of their ensiling capability based on known biochemical principles of fermentation and to check these estimates in laboratory experiments. With this approach, we aimed for conclusions that potentially could be applied to ensiling of a wide range of wild flower substrates. In detail, we wanted to answer the following questions:

i. What are the substrate characteristics of the biomass from effloresced flower strips? Are there peculiarities compared to well-known forage substrates?
ii. Does the standing year play a role in the substrate characteristics?
iii. How to evaluate the substrate characteristics with regard to ensilability?
iv. Are the results of characteristic-based ensilability assessments reflected by measured qualities of corresponding silages?
v. Is a mix of flower strip biomass with whole crop maize a contribution to the ensiling success?

## 3.1.2 Materials and Methods

### 3.1.2.1 Substrates

The flower mix substrates originated from plots of a field trial in Rostock (Germany, 54°04′04.1′′ N 12°04′055.7′′ E). The perennial flower mixture used, “BG 70” (Saaten Zeller GmbH & Co. KG), was developed especially for the use as biomass substrate in biogas plants and contains 23 species. The first sowing took place in 2014. In 2015, the experiment was repeatedly established at the same location. In this way, comparable variants could be sampled in 2015 both from the first and second main standing year after establishment. The mixed flower stands received no fertilizer. Further details on the field experiment, the seed mixture and their botanical development are given in (de Mol et al., 2018).

The growths from the effloresced flower mixture were mowed with a Haldrup parcel harvester on September 12, 2014 (first standing year after establishment) and September 16, 2015 (first and second standing years) at a stubble height of 8–10 cm. With increasing population age, we observed the tendency of the dominance of individual competing species such as melilot (*Melilotus* ssp.). Since melilot is recommended as a biogas substrate (Bull, 2014), we included a representative of the genus Melilotus, yellow sweet clover (*Melilotus officinalis*), in our investigation in 2015. Nearly pure stands of yellow sweet clover from field plots of the same project in Malchow (Germany, 53°59′08.8′′ N 11°28′22.1′′ E) were used for this purpose on September 18, 2015.

Immediately before the harvest, the yield shares of the main species components were estimated. The estimates were validated using three subsamples per variant, divided by species and weighed

separately. The degree of senescence was also estimated and validated in the same way. Botanical compositions and selected field characteristics of the evaluated substrate variants are shown in **Table 3-1**.

As a reference substrate, fresh chopped whole crop maize from a neighboring field (variety "Ronaldinho," breeder KWS®) harvested at early silage ripening stage was used. With the help of the BBCH scale (Weber and Bleiholder, 1990), the harvest stages were specified in terms of developmental physiology to BBCH 82 in 2014 and BBCH 87 in 2015. The maize biomass was used to prepare different mixtures with the flower strip biomass for ensiling (see section "Ensiling Procedure").

**Table 3-1 | Main species composition and field characteristics of the flowering mixture´s substrate stocks to be ensiled.**

| Substrate | Standing age (year of harvest) | Main species | Percentage share | Senescent biomass in % FM | Harvest DM content in % FM |
|---|---|---|---|---|---|
| Flower mix | 1 (2014) | *Chenopodium album* | 26% | 14.2 | 40.2 (.90) |
| | | *Malva ssp.* | 24% | | |
| | | *Tanacetum vulgare* | 17% | | |
| | | *Artemisia vulgaris* | 13% | | |
| | | *Other species* | 20% | | |
| Flower mix | 1 (2015) | *Malva ssp.* | 28% | 16.8 | 42.1 (6.19) |
| | | *Chenopodium album* | 23% | | |
| | | *Tanacetum vulgare* | 18% | | |
| | | *Centaurea nigra* | 13% | | |
| | | *Other species* | 18% | | |
| Flower mix | 2 (2015) | *Tanacetum vulgare* | 23% | 17.8 | 42.8 (3.40) |
| | | *Artemisia vulgaris* | 20% | | |
| | | *Malva ssp.* | 18% | | |
| | | *Melilotus ssp.* | 16% | | |
| | | *Other species* | 23% | | |
| Yellow sweet clover | 2 (2015) | *Melilotus officinalis* | 99% | 0.5 | 25.0 (0.46) |

Dry matter (DM) contents are presented as means with standard deviation of the mean in brackets.

### 3.1.2.2 Ensiling Procedure

The harvested biomasses from the flower strip mixtures and from the yellow sweet clover were chopped to a length of 2–4 cm. The chopping length of the whole crop maize was 0.5–1.5 cm. All substrates were used for ensiling as pure substrates (100% flower mix substrate = FM100; 100% maize = ZM100; 99% yellow sweet clover = YSC99; see **Table 3-1**). In addition, mixed substrates from the flower strip's biomasses with maize were prepared. The mixing ratios were 1:2 (33% flower mix, 67% maize = FM33) and 2:1 (67% flower mix, 33% maize = FM67). Proportions are based on fresh weights immediately before ensiling. In terms of dry matter, this would correspond to a flower biomass:maize – mixing ratio of 2.9:1 in 2014 and 3.6:1 in 2015 for FM67, and a ratio of 0.7:1 (2014) and 0.9:1 (2015) for FM33, respectively.

The feedstock substrates were ensiled in at least three replicates in 3 l glass jars. The jars were washed and sterilized (180°C, 8 h) before the substrates were filled in and compressed in layers by hand. The resulting final packing densities ranged from 0.35 to 0.60 g $cm^{-3}$ DM. The filled jars were closed airtight with a rubber-lined lid that was fixed by clips. Glass jars of all treatments were stored in a dark, tempered room (16°C) for 90 days. After ensiling the silages were removed from the glass jars, sealed airtight in plastic bags and stored at -40°C prior to the analyses of fermentation profiles.

Furthermore, subsamples from each substrate (ca. 500 g FM) were dried in a temperature-controlled range of <45°C and thereafter grounded to a sieve mesh of 1 mm wide. The four field repetitions were reduced to two test repetitions for lab capacity reasons using a sample splitter. This pooled material was used for the determination of the substrate's biochemical properties in both test years.

#### 3.1.2.3 Biochemical Analyses

Several biochemical parameters which are suitable to estimate the ensilability and the fermentation success were determined from the substrates immediately before ensiling and from the fermented substrates after ensiling, respectively. In the study period 2015, the analysis spectrum could be extended to nitrate, buffering capacity (BC) and NDF (see subsection "Parameters Characterizing Substrate's Ensilability").

##### 3.1.2.3.1 Parameters Characterizing Substrate's Ensilability

DM content of the feedstock immediately before ensiling was determined by oven drying at 45°C to a constant weight. BC was analyzed by titration with lactic acid (0.1 mol $l^{-1}$) to a pH of 4.0 according to (Weißbach, 1992). We analyzed the sum of water-soluble carbohydrates (WSCH) and the enzyme-insoluble organic matter (EULOS) by Near Infrared Reflectance Spectroscopy (NIRS, Bruker® MPA, Bruker, Germany) with the photometrical Anthron method according to Naumann and Bassler (2012) as the reference for WSCH and the enzymatic method according to de Boever (de Boever et al., 1986) as the reference for EULOS. Dry combustion technique (ElementarR Analyzer, Vario Max CNS, Elementar, Germany) has been adapted to determine crude protein contents (CP, N × 6.25). Nitrates were analyzed by continuous-flow analysis (CFA Analyzer AA3, SealR, Germany). Neutral detergent fibre (NDF), acid detergent fibre (ADF), and crude fibre (CF) were determined by wet chemical analyses using a Fibretherm, GerhardtR, Germany. Hemicellulose contents have been estimated as the difference between NDF and ADF concentrations.

In order to characterize fermentability in a more holistic manner, the two parameters DM and WSCH/BC were combined to the fermentability coefficient (FC) according to Weißbach and Honig (1996):

$$FC = DM[\%] + 8WSCH/BC \quad \textbf{(3-1)}$$

Feedstocks with FC<35 are considered as "difficult-to-ensile," whereas those with FC > 45 are referred to as "easy-to-ensile."

#### 3.1.2.3.2 Fermentation Characteristics of Ensiled Substrates

After thawing of the frozen silage samples at room temperature, silage extracts were prepared from 50 g silage and 200 mL deionized water. The pH values of these extracts were measured potentiometrically by a calibrated pH analyzer (precision 0.01). Between each measurement of pH, a cleaning of the probe was carried out with distilled water. Fermentation products were analyzed in the filtrated extracts thereafter. Lactic acid was determined by HPLC (Aminex HPX-87H, Bio-radR, United States) with a flow rate of 0.60 ml $min^{-1}$ at the UV detector. Short-chain fatty acids and ethanol were quantitatively separated by gas chromatography (GC-14A, CLASS-VP, ShimadzuR , Kyoto, Japan). The ammonium content in the silage extracts was determined according to the method of Voigt and Steger (1967). Silage DM was determined by drying to a constant weight (105°C, 24 h) and was corrected for the loss of volatiles during drying as described by Weißbach and Strubelt (2008a,b). Ashing followed after drying at 600°C for at least 4 h in a muffle furnace until obtaining a light gray ash color and led to the parameter crude ash content (CA).

#### 3.1.2.3.3 Potential Biogas Yield Estimation

The potential for methane formation was estimated using practice-proven estimation equations based on biochemical parameters of the substrates before ensiling (Weißbach, 2009).

$$\text{ZM100:}\quad VS = 984 - (CA) - 0.47(CF) - 0.00104(CF)^2 \qquad \textbf{(3-2)}$$

$$\text{FM100, YSC99:}\quad VS = 1000 - (CA) - 0.62(EULOS) - 0.000221(EULOS)^2 \qquad \textbf{(3-3)}$$

The substrate's amount of fermentable organic substances (VS g $kg^{-1}$ DM) was estimated for pure maize using **Eq. (3-2)** and for all other pure substrates using **Eq. (3-3)**. Mixed substrates were assessed by weighted means of **(3-2)** and **(3-3)** according to the mass proportion of the single substrates. Substrate-specific biogas (BGY) and methane ($CH_4Y$) yield potentials of the tested feedstock substrates were derived from VS as follows:

$$BGY = 0.80(VS) \qquad \textbf{(3-4)}$$

$$CH_4Y = 0.42(VS) \qquad \textbf{(3-5)}$$

BGY and $CH_4Y$ are given in norm liter per kg (Nl kg $DM^{-1}$) and are corrected of VFA.

### 3.1.2.4 Data Analysis

Biochemical composition data are presented as averages and standard deviation of the mean (sd) with n = 2 replicates. In the absence of real local repetitions, the effects of the standing age on substrate's biochemical properties were analyzed including the flower-maize-mixtures FM67 and FM33 as

replicates. The parameters whose values were below the detection limit ("not detected") in most samples were not included in studying the differences in the biochemical compositions of the silages.

All evaluation-relevant data records were first tested for normal distribution using the Shapiro–Wilk test. For a given normal distribution, analysis of variance (ANOVA) was applied to investigate the effects of the factors "substrate" (2014 and 2015) and "standing age" (2015 only). If the values were not normally distributed and neither log nor sqrt transformations achieved a normal distribution, mixed linear models were applied with "substrate" and "standing age" as a fixed factors and "year" as random variable. Modeled parameters were estimated with an ANOVA of type III and a Satterthwaite's adjustment.

The substrate specific patterns of the fermentation products were visualized with non-metric multidimensional scaling (NMDS) based on Bray–Curtis distances. The influence of substrate properties on fermentation profile was additionally tested with a goodness-of-fit permutation test using the squared correlation coefficient as test statistics.

All statistical analyses were performed by scripts using the R environment version 3.3.2 (R Development Core Team, 2016). The R-package "lme4" was used to calculate the mixed linear models (Bates et al., 2015), and the "vegan" package to perform NMDS (Oksanen et al., 2018).

## 3.1.3 Results

### 3.1.3.1 Substrates' Biochemical Properties

The substrates' properties with a known or reasonably suspected influence on the ensiling capability were determined from substrates immediately before ensiling in 2015 (**Table 3-2**). With more than 40%, the dry matter content was highest in the pure flower strip mixture substrate (FM100). The lowest DM content was found in the silage maize, which was not yet fully silage-ripened. The blends of flower strip mixture and maize reached intermediate values. No trend in DM content could be discerned with regard to the feedstocks of different standing ages. The ash contents of the flower mixture substrates were very low (<7% DM). However, it should be noted that the mixtures were harvested using plot technology.

Pure substrate from effloresced flower mixtures were characterized by very high crude fiber contents (>45% DM) and low crude protein contents (<8% DM) reflecting the late ontogenetic state of the dominating plant species at the harvest time. In contrast to this, yellow sweet clover (YSC99), which has the potential to dominate perennial flower strips after several years of use, had CF (25.6% DM) and CP (22% DM) values that resemble legume forage plants such as alfalfa. The nitrate content was the only characteristic that has been significantly influenced by the age of the flower strips stand ($F = 7.78$; $P = 0.049$). In the second year after the establishment of the mixtures, the nitrate content of the harvested biomass decreased by 1.4 to only 0.1 g per kg DM. A tendency toward higher WSCH values could not be statistically confirmed.

**Table 3-2 | Chemical characterization of the tested feedstock variants before ensiling (experimental year 2015, means from two laboratory repetitions with standard deviations in parentheses).**

| Type of feedstock substrate [1] | FM100 | | FM67 | | FM33 | | ZM 100 | YSC 99 |
|---|---|---|---|---|---|---|---|---|
| Standing year | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 1 |
| **Parameter [2]** | | | | | | | | |
| Dry matter content | 426.7 | 400.9 | 363.8 | 380.7 | 325.5 | 315.0 | 266.6 | 268.6 |
| (g $kg^{-1}$) | (8.0) | (1.1) | (6.4) | (2.2) | (2.2) | (1.9) | (3.8) | (4.0) |
| Crude ash | 63.8 | 65.3 | 66.0 | 62.0 | 53.8 | 52.0 | 32.6 | 89.3 |
| (g $kg^{-1}$ DM) | (1.7) | (0.9) | (1.7) | (0.5) | (0.4) | (1.2) | (0.4) | (0.8) |
| Crude protein | 61.1 | 55.3 | 69.2 | 69.0 | 75.2 | 81.2 | 74.1 | 213.7 |
| (g $kg^{-1}$ DM) | (6.3) | (3.9) | (4.2) | (2.2) | (0.4) | (2.5) | (11.2) | (5.1) |
| Crude fibre | 460.7 | 426.6 | 399.7 | 388.1 | 330.3 | 282.2 | 222.5 | 242.9 |
| (g $kg^{-1}$ DM) | (39.9) | (23.7) | (25.6) | (49.3) | (50.1) | (50.2) | (4.4) | (13.0) |
| Hemicellulose | 160.6 | 215.4 | 184.9 | 215.5 | 182.2 | 187.0 | 215.2 | 110.6 |
| (NDF-ADF, g $kg^{-1}$ DM) | (4.1) | (2.5) | (1.3) | (1.8) | (2.5) | (1.8) | (2.7) | (1.6) |
| Water-soluble carbo- | 3.2 | 9.9 | 15.1 | 36.1 | 85.1 | 115.5 | 182.9 | 49.9 |
| hydrates (g $kg^{-1}$ DM) | (0.6) | (0.3) | (0.4) | (0.2) | (0.3) | (3.4) | (1.5) | (0.8) |
| Nitrate content | 1.5 | 0.1 | 0.7 | 0.1 | 0.6 | 0.2 | 0.3 | 0.3 |
| (g $kg^{-1}$ DM) | (0.01) | (0.06) | (0.21) | (0.05) | (0.16) | (0.07) | (0.04) | (0.05) |
| Buffering capacity | 9.2 | 6.8 | 8.5 | 8.0 | 6.8 | 6.9 | 10.4 | 22.4 |
| (g LA $kg^{-1}$ DM) | (0.14) | (0.10) | (0.11) | (0.01) | (0.16) | (0.05) | (0.65) | (0.28) |

[1] FM100 - 100% flower mix; FM67 - 67% flower mix, 33% maize; FM33 - 33% flower mix, 67% maize; ZM100 - 100% maize; YSC99 - 99% yellow sweet clover

[2] DM - dry matter; LA - lactic acid; WSCH - water-soluble carbohydrates

### 3.1.3.2 Substrates' Ensilability Assessment

The FC of the pure flower mix substrate was not significantly influenced by its standing age ($F = 0.216$; $P = 0.666$). This fact allowed us to average the FC values over the levels of this factor (**Figure 3-1**) and find a significant effect of the substrate type on the FC (ANOVA, $F = 17.98$; $P = 0.020$*).

The substrate-specific characteristics of FC in the study are shown in **Figure 3-1**. All substrates containing maize as a component clearly exceeded the FC > 45 threshold and thus indicate good conditions for low-loss preservation. The unusually high values of the maize-dominated test variants FM33 and ZM100 are due to their very high content of WSCH, which is also reflected in high WSCH/BC-ratios (see also **Figure 3-2**). In contrast to mixtures with maize, the two pure flower mix substrates FM100 and YSC99 had FCs that are within the limits of good conservation suitability.

Below 28% DM, an increasing risk of leachate from the feedstock must be expected. However, the effloresced stands had sufficiently high (>30%) contents of DM without wilting efforts (**Figure 3-2**). This finding does not apply to the dominant stocks of yellow sweet clover (YSC99) whose biomass was still vital at the time of harvest and contained little senescent material. On the other hand, dry matter contents of the pure flower mixture in the first cropping year exceeded the recommended DM-range of 30–40% and reached a level that is only suited as a metabolic substrate for very osmotolerant lactic acid producers.

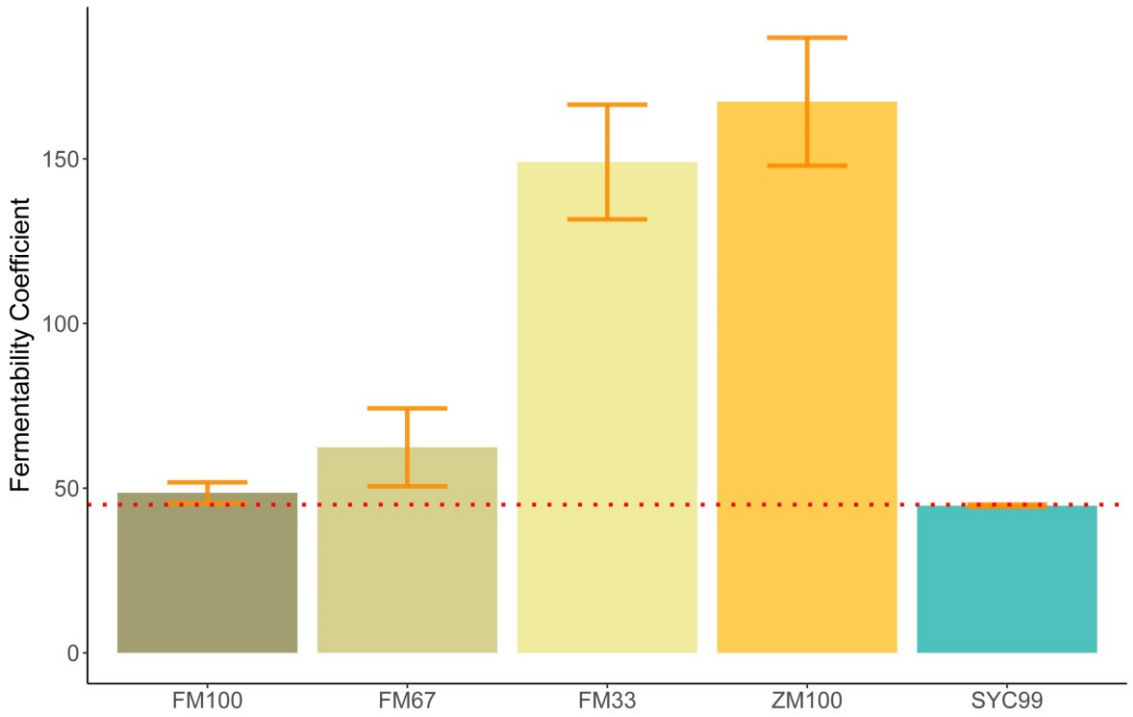


**Figure 3-1 | Mean Fermentability Coefficients (FC) of the tested feedstock substrates. Error bars indicate standard deviations of the mean. (Sample size: FM100, FM67, FM33 n = 4; ZM100, YSC99 n = 2). The red dotted line indicates the FC threshold according to Weißbach and Honig (1996).**

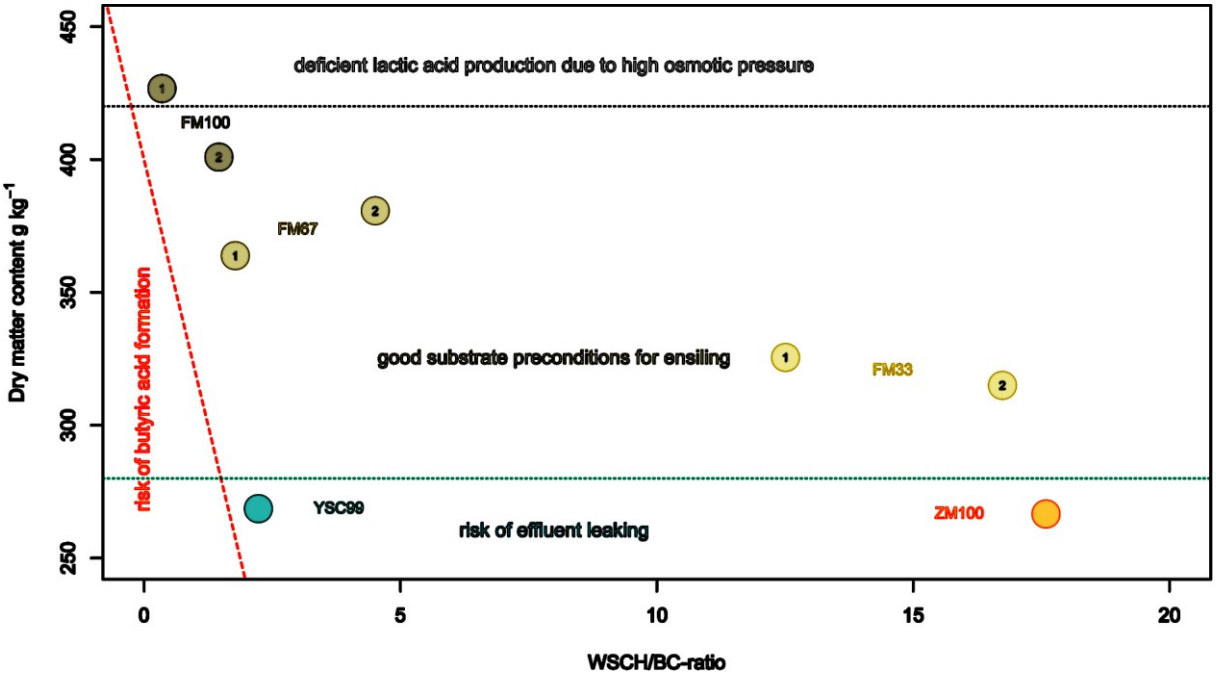


**Figure 3-2| Arrangement of the tested feedstocks in the estimation frame according to Weißbach and Honig (1996). The digits 1 and 2 within the location points indicate the standing year. The dashed orange line reflects the critical dry matter content as a function of the WSCH/BC-ratio. The dotted light-gray line shows approximately the beginning of the range of limited metabolic activity of natural epiphytic lactic acid bacteria population due to forced osmotic pressure. THE COLORS OF THE FIGURE HAVE BEEN REVISED FOR PRESENTATION IN THIS THESIS IN ORDER TO ENSURE LEGIBILITY.**

### 3.1.3.3 Realized Silage Quality

Silage fermentation patterns varied according to substrate, year, and standing age. ANOVA after fitting GLMM models revealed significant effects of substrate types on silage characteristics for most of the main fermentation products (**Table 3-3**), namely pH, lactic acid, acetic acid, and ethanol. The only exceptions were butyric acid and propionic acid, since their contents were partly below the detectability threshold and thus escaped the biostatistical model estimations.

Despite trends in feedstock analysis before ensiling (see "Substrates' Ensilability Assessment"), standing age caused only minor variation in the main silage characteristics leading to non-significant effects in the mixed models. Only lab-silages containing maize fell below the pH value threshold of four (**Table 3-3**). Undesirable butyric acid was found only in the variants of the pure flower mix substrates with DM contents of more than 40% in the harvested substrate. In order to allow a better comparison of the silage with the properties of the harvested substrate, which was investigated only in 2015, relevant fermentation parameters of the results from 2015 are shown separately in **Figure 3-3**.

When comparing the amount of lactic acid formed (**Figure 3-3 A**) with the corresponding pH values (**Figure 3-3 B**), it is noticeable that yellow sweet clover did not follow the common trend of decreasing pH values at higher lactic acid concentrations. Since acetic acid and ethanol are metabolites of the same bacterial group (coli-erogenic), their contents in the laboratory silos were compared (**Figures 3-3 C,D**). The comparison revealed that during ensiling of effloresced flower mixture biomass, less alcohol was formed in relation to acetic acid.

**Table 3-3 | Main fermentation products of the tested lab-scale ensiled feedstock after a storage period of 90 days (2 year means with standard deviations in parentheses).**

| Feedstock Substrate[1] | Standing age (years) | pH | Lactic acid (g $kg^{-1}$ DM) | Acetic acid (g $kg^{-1}$ DM) | Butyric acid (g $kg^{-1}$ DM) | Propionic acid (g $kg^{-1}$ DM) | Ethanol (g $kg^{-1}$ DM) |
|---|---|---|---|---|---|---|---|
| FM100 | 1 | 4.54 (0.09) | 4.57 (1.69) | 1.45 (0.23) | 0.62 (0.217) | 0.09 (0.006) | 0.38 (0.17) |
| | 2 | 4.30 (0.24) | 4.09 (0.84) | 1.07 (0.12) | 0.02 (0.003) | 0.04 (0.004) | 0.11 (0.05) |
| FM67 | 1 | 3.92 (0.06) | 6.22 (0.53) | 1.57 (0.29) | n.d. | 0.01 (0.002) | 0.75 (0.55) |
| | 2 | 3.86 (0.02) | 6.69 (0.32) | 1.34 (0.24) | n.d. | 0.01 (0.002) | 0.70 (0.26) |
| FM33 | 1 | 3.90 (0.23) | 6.60 (0.32) | 1.80 (0.49) | n.d. | n.d. | 0.51 (0.07) |
| | 2 | 3.72 (0.02) | 6.84 (0.21) | 2.30 (0.44) | n.d. | n.d. | 0.64 (0.06) |
| ZM100 | 1 | 3.64 (0.09) | 7.80 (0.43) | 2.22 (0.14) | n.d. | 0.12 (0.015) | 1.43 (0.44) |
| YSC99 | 1 | 4.61 (0.03) | 9.66 (1.27) | 1.88 (0.32) | n.d. | 0.03 (n.f.) | 1.25 (0.25) |
| **ANOVA results (F; P)** | | | | | | | |
| | Substrate | $F = 112.69$; $P < 0.001$ | $F = 33.81$; $P < 0.01$ | $F = 10.24$; $P < 0.01$ | n.f. | n.f. | $F = 19.09$; $P < 0.01$ |
| | Standing age | $F = 3.90$; $P = 0.055$ | $F = 0.59$; $P = 0.446$ | $F = 0.28$; $P = 0.599$ | n.f. | n.f. | $F = 0.17$; $P = 0.684$ |

n.d., not detectable; n.f., not feasible.

[1]FM100 – 100% flower mix; FM67 – 67% flower mix, 33% maize; FM33 – 33% flower mix, 67% maize; ZM100 – 100% maize; YSC99 – 99% yellow sweet clover.

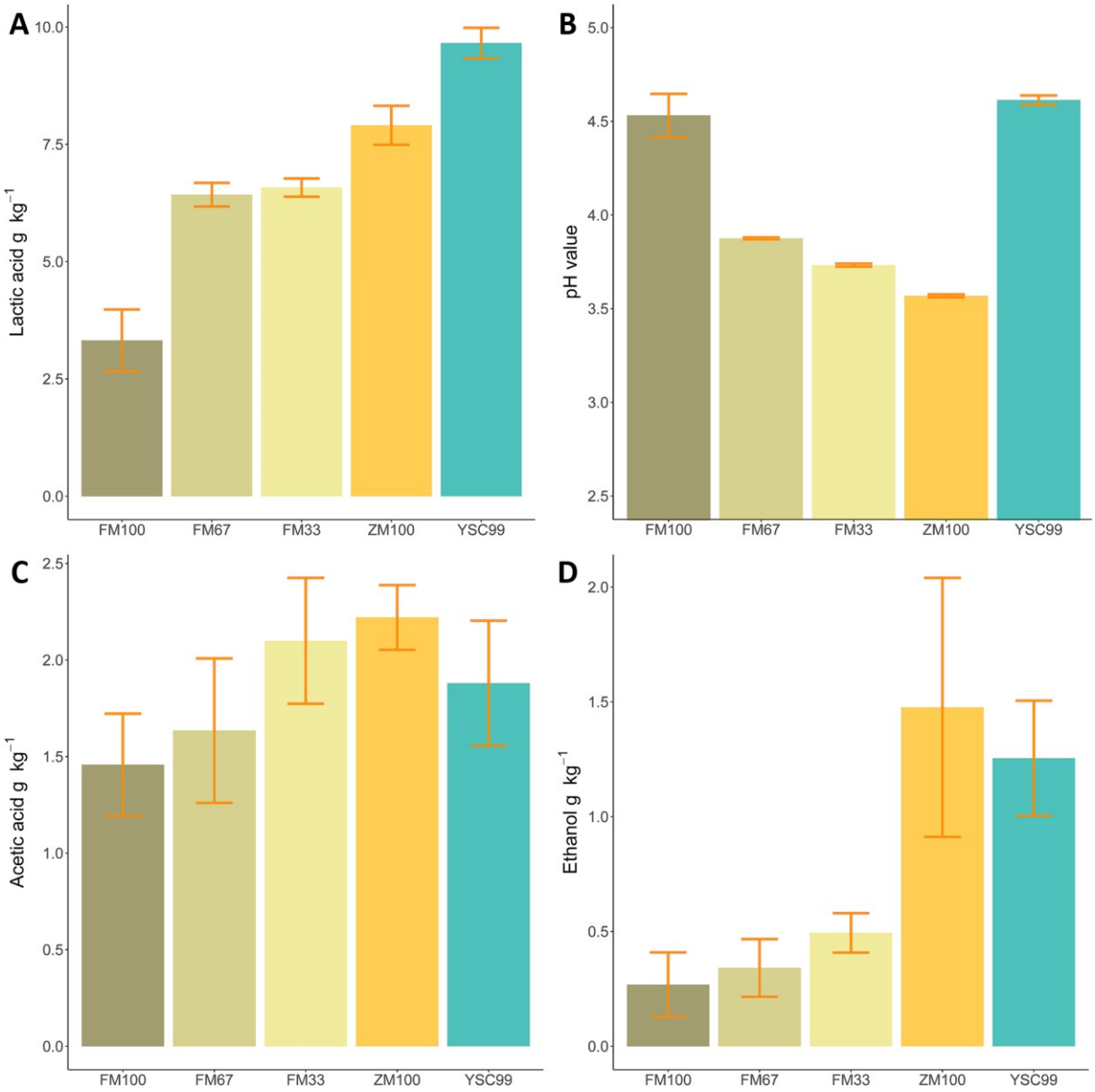


**Figure 3-3 | Mean fermentation products of the tested feedstock substrates in 2015. (A) Lactic acid content, (B) pH value, (C) acetic acid content, (D) ethanol content. Error bars indicate standard deviations of the mean (Sample size: FM100 n = 4, FM67 n = 4, FM33 n = 5; ZM100 n = 5, YSC99 n = 3).**

#### 3.1.3.4 Relationship Between Substrate Properties and Fermentation Profiles

In order to make relationships between substrate biochemical characteristics and fermentation patterns visible, a complex multivariate analysis was carried out. We applied a NMDS which allowed us to include the whole range of characteristics in the analysis and to represent them graphically (**Figure 3-4**). The goodness of fitting the multidimensional data to the reduced dimensioned NMDS was good (see **Supplementary Figure 3-1** for details).

The plot contains a table presenting the results of the vector fitting procedure additionally. The data on the expression of the substrate characteristics before ensiling served as vectors. The substrate

characteristics of this figure-integrated tabular list were arranged according to the closeness to the matrix of fermentation characteristics, expressed by the squared correlation coefficient. These are also the vectors with a relatively high gradient length, which can be seen from the length of the arrows.

On the one hand, it is noticeable that the individual substrates always form well-defined clusters if they are 1-year stocks. On the other hand, there is a trend toward splitting into subgroups, as in the case of the pure flower mixture variant FM100, shown on the left side of the plot.

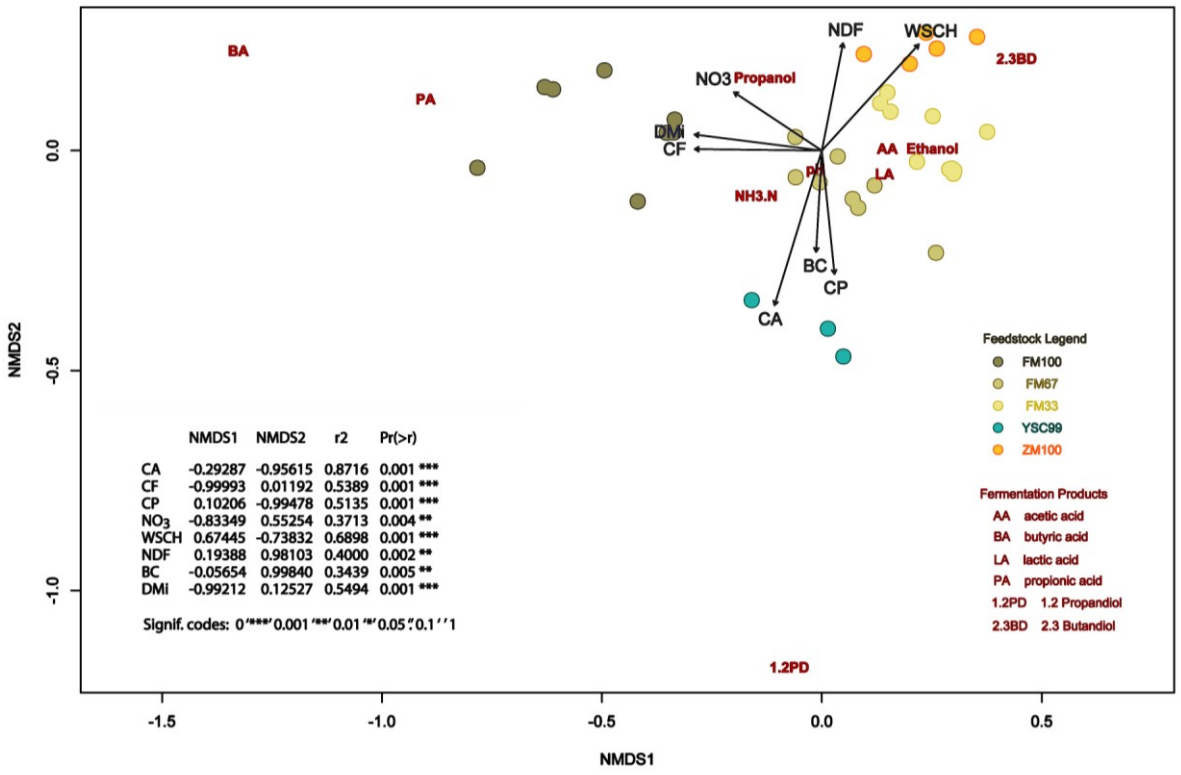


**Figure 3-4 | Non-metric multidimensional scaling ordination plot showing the position of the fermentation characteristics (dark red colored abbreviations) in relation to the initial biochemical substrate properties (darkgray colored arrows including abbreviations). The location of the corresponding substrates is additionally point-plotted and explained in a legend. Nomenclature of the biochemical characteristics: CA, crude ash; CF, crude fiber; CP, crude protein; NDF, neutral detergent fiber; NO3, nitrate; WSCH, water soluble carbohydrates; BC, buffering capacity; DMi, initial dry matter content of the substrates (before ensiling). THE COLORS OF THE FIGURE HAVE BEEN REVISED FOR PRESENTATION IN THIS THESIS IN ORDER TO ENSURE LEGIBILITY.**

## 3.1.4 Discussion

### 3.1.4.1 Substrate Characteristics and Fermentation Patterns

To our knowledge, this study is the first exploring the ensilability of effloresced flower strip's biomass. Regarding the scarcity of data concerning biomasses from wildflower mixtures, we consider the description of the substrate characteristics valuable as well; especially since the botanical composition

of the stock is known and adequately described. With the inclusion of melilot, the 2-year study shows quite a wide range of possible substrate compositions despite limited numbers of variants.

The high fiber contents found in the growths of the flowering strips together with the high percentages of senescent foliage, low protein and sugar contents are characteristics of fast-growing, high-flowering dicotyledons with a low tendency to vegetative regeneration and persistence. Such substrate constellations offer poor conditions for successful ensiling due to a lack of readily available sugars for the lactic acid formation (Pitt, 1990) and a high stock of harmful molds and yeasts (Dunière et al., 2013). Consequently, a low lactic acid content of only 4 g $kg^{-1}$ was formed in the pure flowering mixture silage (FM100). Nevertheless, this was sufficient to lower the pH value to below 4.7, which is necessary for stable storage at a dry matter content of 40% (Kalač, 2011). The occurrence of butyric acid indicates that the reduction of the pH value was slow, so that the preservative acidification effect was not yet present in the initial storage phase. A certain contribution of fiber degradation to low molecular saccharides could also have contributed to continued lactic acid formation. Unfortunately, the fiber fractions of the silages after fermentation were not analyzed again, which could have helped to verify this thesis by comparing pre-ensiling with post-ensiling results. If we recall the ordination (**Figure 3-4**, left pointing arrows), we can see that the characteristics CF, DM, and $NO_3$ have the greatest influence on the fermentation patterns of pure FM100-silages. However, it is not very likely that the contribution of crude fiber to the explanation of fermentation profiles is related to the carbohydrate donors. If that was the case the NDF arrow would rather point in the direction of the FM100 positions. Instead, it seems to be the effect of an intercorrelation with the dry matter content: the older the plants in the stand, the drier and more fibrous they become. It is therefore obvious to assume that the ontogenetic development of the flower mixture stands is the significant background-variable and responsible for variations in silage quality. Obviously, the known Clostridia-suppressive effect of nitrate (Kaiser et al., 1999) is rather important in the limit range of fermentability.

For the fermentation acid patterns of the maize-dominated silages, the height of the WSCH and the NDF fraction played an important role (**Figure 3-4**, right pointing arrows), although there was no lack of easily fermentable saccharides. Nonetheless, the ratio of the fermentation products lactic acid, acetic acid, ethanol and 2,3-butanediol might have been influenced by these ingredients in a way which has not been recognized as random.

#### 3.1.4.2 Substrate's Ensilability Assessment

The prediction of ensiling success on the basis of the substrates' biochemical properties is both a promising and a difficult undertaking, as not only biochemical, but also physical and microbiological processes are involved (Müller and Bauer, 2006). Assuming a proper ensiling technology and an average lactic acid bacteria (LAB) stocking on the phyllosphere is given, the existing estimation framework can be successfully applied for the major forage crops (Weißbach and Honig, 1996). The few authors dealing with the fermentability of herbs or herb-rich growths (Daniel and Opitz von Boberfeld, 1997) found that

some of these species oppose specific effects on fermentation processes and attribute this to secondary metabolites (Weißbach, 1998). Consequently, the conservation results could not be reliably predicted with the existing substrate-based estimation frameworks. In our study, the flower strip mixtures also contained plants with notable amounts of antimicrobially active secondary metabolites like *Melilotus* (coumarins) or *Tanacetum* (flavonoids, terpenes, coumarins). Nevertheless, we can state that the results of the ensilability classifications prior to ensiling (**Figures 3-1**, **3-2**) are sufficiently consistent with the fermentation profiles of the silages obtained from them. Therefore, our resultsdo not argue against the application of the existing estimation frames (developed for forages) for the ensiling of flower strip mixtures. However, one should be aware that particularly high concentrations of antimicrobial active metabolites, similar to variations in nitrate contents in the feedstock, could modify the ensiling success. In order to expand the still rare knowledge in this respect, further targeted investigations are necessary, both on the laboratory level and in practice.

#### 3.1.4.3 The Effect of Standing Age on Ensilability of Biomass from Flowering Strips

In our analyses, the factor "standing age" proved to be of little influence on ensilability. However, this was also partly due to differences in the degrees of freedom (one degree for factor "standing age" against five degrees for the factor "substrate") and thus, due to the study design. The short rotation type of flower stripe examined here represent the most frequently occurring option of buffers in European arable landscapes due to designated support schemes and administrative regulations. The effect of the year of use on ensilability has two aspects: the changes in the soil nutrient pool and the botanical shifts in the mixed stands. In the comparison of the first year with the second standing year, both processes left their imprints on the biochemical characteristics of the grown substrates. The significant decrease in the nitrate content is a sign of N-limitation that is already beginning in the second year after establishment. Although there were no serious shifts in the abundance of the dominating species, higher contents of WSCH and lower CF concentrations indicate physiologically younger plant material in the second standing year. This finding could also be explained by more restrained growth due to N-depletion. From the point of view of ensilability assessment, this results in advantages for the availability of monosaccharides for lactic acid formation, but also in disadvantages for butyric acid inhibition with increasing standing age. According to Kaiser et al. (1997), a minimum content of 1.5% $NO_3$ should be targeted in order to achieve sufficient safety against butyric acid formation under field conditions. In our experiment, the advantages and disadvantages of the age-affected substrate pattern apparently compensated each other, so that there were no significant deviations with regard to the fermentation profiles.

In the case of perennial flowering mixtures, experience has shown that the age of the crop stand can have a major influence on substrate characteristics, especially if there is a stronger shift from annuals to perennials (de Cauwer et al., 2006). Under humid climate conditions, grass coverage increases with increasing standing age (de Cauwer et al., 2005). This stand development can lead to an improvement

in ensilability if at least two cuts are made. However, this development reduces many ecosystem services of flowering strips. In addition, nitrogen fertilization would also be required to maintain a level of biomass production, which justifies mowing and transport.

#### 3.1.4.4 Further Implications for the Storage of Biomass from Flowering Strips

In accordance with Teixeira Franco et al. (2016), we consider classical measures of production engineering measures such as short chopping lengths and good compaction to be more important than additives in order to ensure a low-loss storage of biomass – also from flower mixtures designed for energy recovery. However, the use of additives to increase storage safety or energy yield (Herrmann et al., 2011) is widespread. Based on our investigations of the substrate composition, it seems that if an application of additives was considered for late harvested flower strips, enzyme application would be more promising than inoculation with LAB. Generally, late summer growths have a high content of natural epiphytes (Filya et al., 2007), including LAB, so that LAB-inoculations are not necessary to guarantee the desired lactic acid formation. In fact, there is a risk that the inoculant LAB are overwhelmed by the natural epiphytes and do not affect fermentation significantly (Muck, 1989). Moreover, contents of less than one percent WSCH are not sufficient for an economically justifiable LAB-application (Bolsen et al., 1996). On the other hand, hemicellulose contents up to 21% DM are a promising pool for successful depolymerization by suitable enzyme products (Schimpf et al., 2013) that have the potential to enhance biogas yield if biomethanisation was chosen as conversion path for biomass from flower stripes.

Gravimetrically determined mass losses of laboratory silos like jars are not really suitable to describe the storage losses of biomass to be expected under real conditions of a field storage pile (Wendt et al., 2018). The individual weighings carried out as part of our study showed losses on the order of 0.5% and essentially reflected the fermentation activity as a whole. The latter, in turn, is strongly dependent on the DM content. Therefore, approaches such as those of Goeser et al. (2015) to draw conclusions about the expected losses under practical conditions from the fermentation patterns appear more successful. Following this logic, the mixture FM33 has to be recommended, since the proportion of fresh maize is sufficient to form an adequate amount of lactic acid for butyric acid-free storage, but the advantage of the higher dry matter content from the flowering strip biomass – another precondition of low storage loss – is still evident.

#### 3.1.4.5 Technological Aspects of Realizing the Bioenergy Potential of Biomass from Flowering Strips

For the energetic utilization of biomasses rich in lignocellulose, such as that of flower strips, a number of conversion routes are possible, e.g., combustion (van Meerbeek et al., 2015), ethanol (Chen et al., 2011) or biogas production (Vollrath et al., 2016). For the latter two techniques, ensiling is an important component of the production process (Chen et al., 2007) facilitating storage (Emery et al., 2015) and pre-treatment of the substrate (Essien and Richard, 2018).

Economically and logistically, the way of utilization is to be preferred, that not only copes best with the substrate's qualities but also enables short routes of transport. Regarding the routes of transport biomethanisation is the preferred way to process biomass from flowering strips in the rural areas of Europe due to the large number of decentralized biogas plants (Capodaglio et al., 2016). The question of the substrate quality, however, cannot be answered independently of the specific type of biogas plant. Certainly, very few plant operators would rely on a substrate that delivers significantly lower methane yields than maize. In the present study it became obvious that using maize as a cosubstrate is essential to realize the bioenergy potential of flower strip biomass. On the one hand maize proved to be an excellent mixing substrate to ensure low-loss ensiling of the flower strip biomass, especially in the case of the variant FM33. On the other hand, the mixing with maize optimized the specific methane yield of the flower strip biomass. The pure flower mixture (variant FM100) only had a specific methane yield of approx. 180 Nl $CH_4$ $kg^{-1}$, while the mixed substrate (FM33) with 67% fresh maize content yielded approx. 300 Nl $CH_4$ $kg^{-1}$ representing nearly 90% of the reference yield of pure maize (see **Supplementary Figure 3-2**). Thus, the FM33 variant did not only have the best storage properties, but also promises high acceptance as substrate by the operators of biogas plants.

The production of a mixed substrate, however, remains a challenge at the commercial scale. The optimum crop for mixing would be maize that has not yet matured too far with DM contents of 22–28% on a whole plant basis; in particular if the biomass of the effloresced flower mixture is no longer vital and exceeds DM contents of more than 40%. The maize would supply the substrate with high contents of WSCH and moisture to lower the osmotic pressure and to enhance the compactability of the feedstock during ensiling. In practice silage maize is harvested at DM contents of 30–36%. Therefore, it may be a good idea to apply the widespread practice to use maize from the field edges and from hunting corridors as an early mixing substrate that is harvested before the actual silage maize campaign starts.

### 3.1.5 Conclusion

The use of increasing amounts of flower strips' biomass as a source of renewable bioenergy is a promising option to reconcile economic and environmental concerns. A primary challenge associated with the realization of this alternative is to store the feedstock in a way that losses are minimized. Due to the similarity of the biomass with delayed harvested forage, ensiling offers a cost-effective form of storage. Since there is little experience with the ensiling capability of flower strip mixture's substrates, we studied the ensilability of botanically classified and composition-related described feedstock from late harvested flower strips as pure substrate or blended with whole-crop maize. This study showed that existing frameworks developed for roughages could be successfully applied to predict the ensiling success on the base of the substrates' biochemical properties. This knowledge is important in order to make the right preparations and process-related decisions that lead to low-loss storage of this largely unknown feedstock. Pure biomass from effloresced flowering strips is set on a certain risk of

misfermentation if not blended with a favorable feedstock like maize. We conclude that a mixture of 33% biomass from flower strips with 67% whole crop maize can be regarded as a recommendable ratio for low-loss storage. In addition, the multivariate approach used in this study to uncover the relationship between characteristics of the initial substrate and the fermentation pattern seems applicable for further investigations of substrate storage as a basis for the production of bioenergy.

**Data Availability Statement.** All relevant data is contained within the manuscript. In addition, raw data from processed data will be made available by the authors, without undue reservation, to any qualified researcher on request.

**Author Contributions.** JM and JH contributed to the conception and the design of the study; JH organized and administrated the project; JM wrote thefirst draft of the manuscript; JH supplemented and improved the manuscript; JM performed the statistical analysis in coordination with JH. Both authors contributed to manuscript revision, read and approved the submitted version.

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**Conflict of Interest.** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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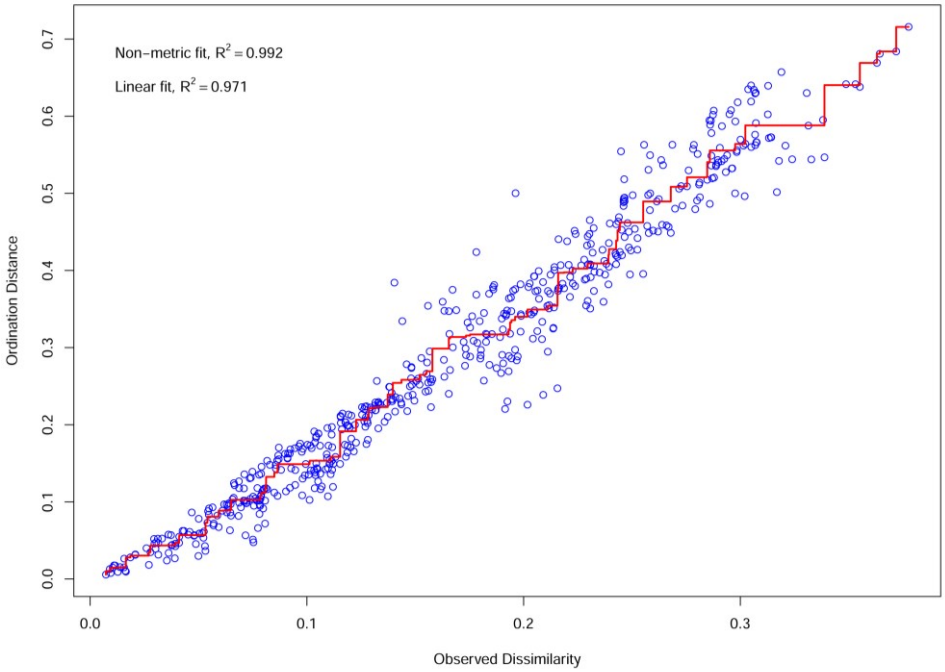
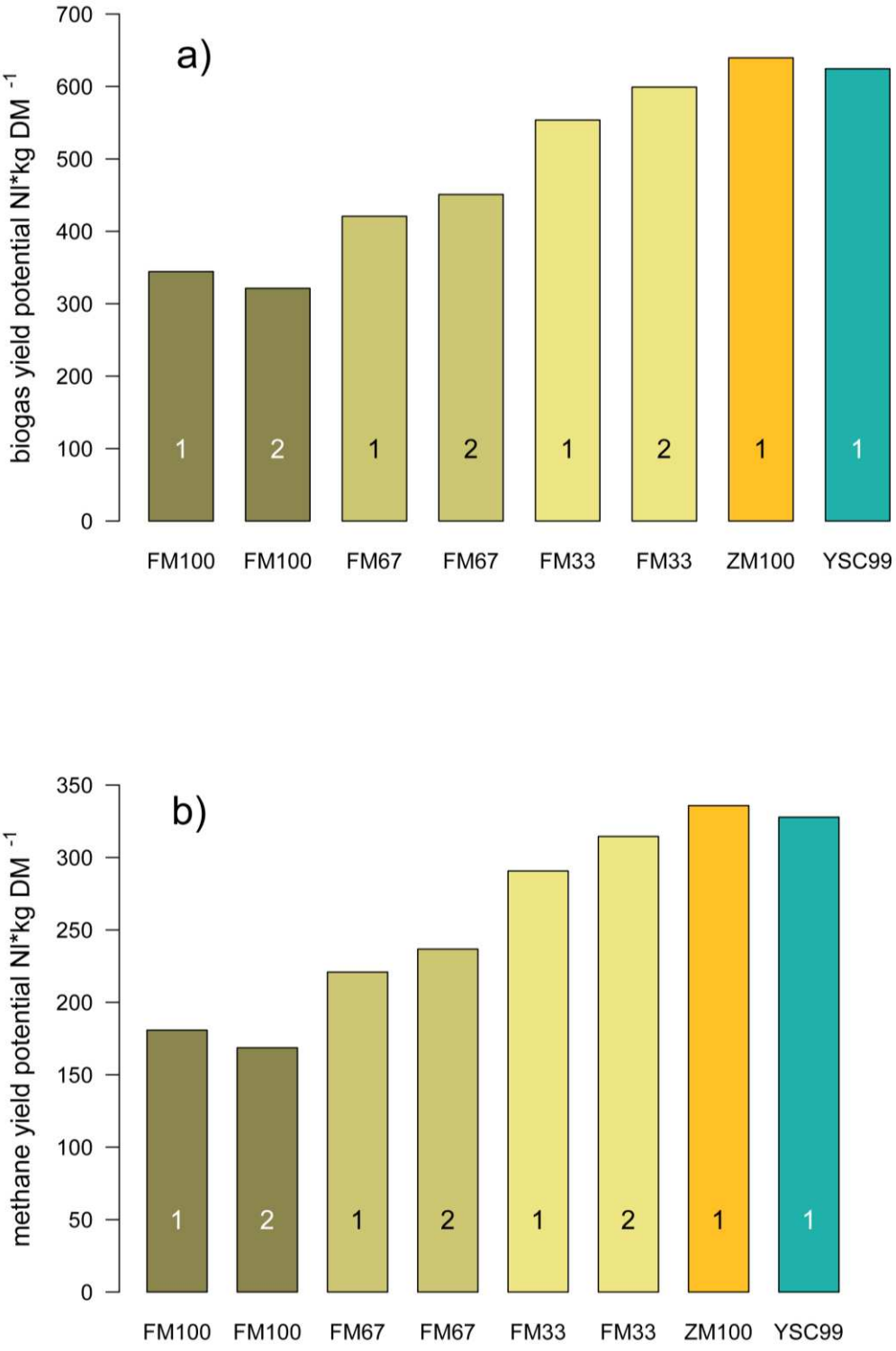
Wendt, L. M., Murphy, J. A., Smith, W. A., Robb, T., Reed, D. W., Ray, A. E., et al. (2018). Compatibility of high-moisture storage for biochemical conversion of corn stover: storage performance at laboratory and field scales. Front. Bioeng. Biotechnol. 6:30. doi: 10.3389/fbioe.2018.00030

### 3.1.8 Supplementary Material




**Supplementary Figure 3-1 | Shepard plot showing scatter around the regression between the interpoint distances in the final NMDS configuration against their original dissimilarities.**




**Supplementary Figure 3-2 | Substrate-specific biogas (a) and methane (b) yield potentials of the tested feedstock substrates calculated according to Weißbach (2009). Calculations are based on ash content, crude fiber content, and enzyme solubility of harvested substrates before ensiling. Numbers in the bar indicate the standing age of the biomass stock. Substrates nomenclature: FM100 = pure biomass from flowering stripes, FM67 = mixture of 67% flower stripe's biomass and 33% silage maize, FM33 = 33% flower stripe's biomass and 67% silage maize, ZM100 = pure silage maize, YSC99 = 99% yellow sweet clover.**

## 3.2 Ensiling in Weed Seed Management

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### Ensiling Reduces Seed Viability: Implications for Weed Management

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Ensiling, a lactic acid fermentation process, is mainly used to preserve biomass. In addition, it has been shown to affect seed viability of some plant species. The extent to which this makes ensiling suitable as a weed control measure, however, has not yet been determined. Both the range of controllable species and the parameters of an ensiling process that safely kills seeds are still undefined. We aimed to determine the effect of varying substrate and ensiling conditions on the seed viability of 10 species selected to represent a wide range of different seed traits. Five different types of silages were made from maize or mixtures of wildflower and maize biomass and ensiled in lab-scale silos for 8 months. The pure maize silages were prepared under conditions either ideal or suboptimal for ensiling forage. Seeds of important weeds (*Chenopodium album*, *Abutilon theophrasti*) and of species from a wildflower mixture suitable for ensiling and biogas production (*Cichorium intybus*, *Daucus carota*, *Echium vulgare*, *Malva alcea*, *Malva sylvestris*, *Melilotus albus*, *Melilotus officinalis*) were tested. Seed viability was determined using a combination of tetrazolium and germination tests. Ensiling reduced seed viability across all 10 species significantly. Seed-killing efficacies of ensiling, however, differed widely among the species studied, largely related to whether the species could produce hard (physically dormant) seeds. Seeds from species without hardseededness were completely inactivated by ensiling, while the seed-killing efficacies for hardseeded species ranged from 5 to 60%. Variation in ensiled substrate and ensiling conditions had no consistent effect on seed survival. We concluded that ensiling has the potential to sustainably reduce seed viability of a wide range of species and therefore should be adopted as a component of integrated weed management in organic agriculture.

**Keywords:** **silage substrate, ensiling condition, integrated weed management, seed survival, hard seeds**

### 3.2.1 Introduction

In order to meet the growing demand for food in a sustainable way (El Bilali et al., 2019), it is essential that environmentally friendly approaches such as organic farming also achieve high and secure yields (Reganold and Wachter, 2016). Weeds have the potential to jeopardize these goals especially in organic cropping systems that have to manage without chemical herbicides (Stonehouse et al., 1996; Hatcher and Froud-Williams, 2017). Today, there is a broad consensus among agronomists worldwide that integrated weed management is needed to control weeds efficiently (Merfield, 2019; MacLaren et al., 2020). Tillage, crop rotation, intercropping, and increase of crop competitiveness are repeatedly referred to as tools of integrated weed management in organic farming (Barberi, 2002). Ensiling, i.e., the process

of conserving biomass by promoting lactic acid fermentation, however, seems to be hardly perceived as a weed control measure in the current discussion. This is surprising, since ensiling was recognized very early as a measure for weed seed reduction, along with other agricultural fermentation processes such as bovine digestion and farmyard manure rotting (Atkeson et al., 1934; Shevkenek, 1934; Tildesley, 1937; Zahnley and Fitch, 1941). In addition, organic mixed farms in particular offer good conditions for the integration of such fermentation cascades, as they aim to close biomass and nutrient cycles (Barker, 2021). Furthermore, the contribution of agricultural fermentation processes to reducing the spread of weed seeds is also relevant in light of decades of herbicide use. Consequently, the weed-reducing potential of fermentation processes is receiving attention from various scientific communities such as forage scientists and seed biologists still (Takabayashi et al., 1975; Pleasant and Schlather, 1994; Michael et al., 2006). Particularly with regard to the germinability of weed seeds, there has been a recent resurgence of interest in the effects of ensiling (Mayer et al., 2000; Overud, 2002; Westerman et al., 2012).

With regard to weed seed load, nowadays, especially in extensively managed systems, a larger amount of seeds than in the past enters the biomass streams and thus the silos. In intensive forage production, early cutting dates hardly allow for any significant weed seed formation. One of the reasons for the higher seed load in extensive agriculture is the high proportion of leys in the forage areas designated for ensiling in organic farming systems (Döring et al., 2017). Especially during dry periods during the ley establishing phase, many seed-forming weeds emerge (Brainard et al., 2011) and lead to notable weed contamination of the forage. Another cause of the increasing weed seed load is the growing number of agri-environmental measures such as field-margin protection schemes and the integration of flowering mixtures into farming systems (Marshall, 2005) that are being adopted by organic farms (Hald And Nielsen, 2007). Furthermore, the new trend toward the carryover of green manures as a substitute for farmyard manuring on organic farms with low livestock density (Notaris et al., 2018; Toleikiene et al., 2020) contributes to a renewed interest in the effects of ensiling and other anaerobic digestion processes on weed seed viability.

Ensiling technology is optimized to ensure that the energy content of fresh organic substrates is retained to the greatest extent possible (Müller and Bauer, 2006). This is mainly achieved by lowering the pH value through lactic acid fermentation. However, depending on the ensiled substrate and the ensiling conditions, other fermentation products are produced as well (Pahlow, 2007). It is currently completely unknown what influence the divergent, different fermentation profiles of different silage types have on seed viability. This is also due to the fact that there is no systematic research on seed survival in silages, yet. Many of the studies on ensiling seeds have dealt with only one weed species (Overud, 2002; van Eekeren et al., 2006; James et al., 2011; Trolove and Dowsett, 2015; Weller et al., 2016) or with only one type of silage (Mayer et al., 2000; Koarai et al., 2015; Piltz et al., 2017). In addition, both silage types and methods used to determine seed viability often differed. Therefore, the statement made in several studies that ensiling has the potential to reduce seed viability must always be

related to the respective plant species and ensiling environment (e.g., Aper et al., 2014; Simard and Lambert-Beaudet, 2016; Piltz et al., 2017). Similarly, the interaction of species and ensiling environment in relation to seed viability parameters is poorly understood.

In this study, we aimed to determine the effect of varying substrate and ensiling conditions on seed viability. As model species we chose known weeds and species from a wild plant flowering mixture. Wildflower mixtures have gained agronomic importance in the context of enhancing the ecological value of agricultural landscapes through flowering strips (Fritch et al., 2011). In addition, their biomass is intended to be used as a source of renewable bioenergy (Cossel and von Lewandowski, 2016; Lask et al., 2020). Since they have gained importance only recently, information on the survivability of their seeds in ensiling is lacking. "Using this model species, we hypothesized that ensiling had the potential to reduce plant seed viability to an extent relevant to integrated weed-management. Furthermore, we tested the hypotheses that differences in seed survival rates after ensiling could be attributed to seed species and/or certain seed characteristics. Finally, we tested the hypothesis that different types of silage affected seed viability in specific ways."

## 3.2.2 Materials and Methods

### 3.2.2.1 Plant Species

#### 3.2.2.1.1 Species Selection

The potential of ensiling to reduce plant seed viability was tested on 10 different dicotyledonous plant species. We did not study monocotyledonous species because other studies have already demonstrated that, with few exceptions, they quickly lose their viability during ensiling (e.g., Blackshaw and Rode, 1991; Lück, 2012; Koarai et al., 2015; Piltz et al., 2017). Our selection should include species that are important weeds, and species that have a high probability of entering silages and represent a wide range of different seed traits. In addition, half of the species should exhibit the so-called hardseededness (HS) in their seeds, i.e., physical dormancy (Baskin et al., 2000), as seeds from such species are considered particularly resistant to degradation in fermentation processes (Westerman and Gerowitt, 2013).

The important weeds examined in this study were *Chenopodium album* L. (common lambsquarters, Amaranthaceae) and *Abutilon theophrasti* Medik. (velvetleaf, Malvaceae), with seeds of *A. theophrasti* exhibiting HS (LaCroix and Staniforth, 1964). Both weeds are problematic in several regions of the world (e.g., Follak et al., 2014; Bajwa et al., 2019) and spread via abundant seeds that are relatively resistant to degradation in soil and fermentation processes (Toole and Brown, 1946; Bassett and Crompton, 1978; Warwick and Black, 1988; Westerman et al., 2012). In addition, *C. album* has been shown to have the potential to contaminate maize biomass and thus maize silage with large numbers of its seeds (Westerman and Gerowitt, 2012). The other eight species studied are highly likely to enter silages as well, because they are included in a wildflower mixture that was developed for bioenergy use and is also suitable for ensiling (Cossel and von Lewandowski, 2016; Vollrath et al.,

2016; Lask et al., 2020; Müller and Hahn, 2020). This means that seeds that survive ensiling can be spread with the silage and potentially become weeds. An additional motivation to study species from this wildflower mixture was that they are already slightly influenced by breeding, which improves their germination ability, which in turn lowered the variance in our experiments. Four HS species were selected from the mixture, namely *Malva alcea* L. (rose mallow, Malvaceae), *Malva sylvestris* L. (common mallow, Malvaceae), *Melilotus albus* Medik. (white sweet clover, Fabaceae), and *Melilotus officinalis* (L.) Pall. (yellow sweet clover, Fabaceae). Regarding the non-hardseeded species, we ensured that they were from different plant families and represented as much variation in seed morphology as possible. We selected *Cichorium intybus* L. (blue dandelion, Asteraceae), *Daucus carota* L. (wild carrot, Apiaceae), *Echium vulgare* L. (viper‘s bugloss, Boraginaceae), and *Verbascum thapsus* L. (common mullein, Scrophulariaceae). Of the selected wildflower species, only *M. officinalis* had previously been studied in ensiling (Woodward, 1940).

##### 3.2.2.1.2 Seed Acquisition and Storage

The seeds of most species were obtained from “Appels Wilde Samen” [Darmstadt, Germany (appelswilde.de)], where they were harvested in 2014. The seeds of *C. album* and *M. sylvestris* were also collected in 2014, but from plants grown on test areas of the University of Rostock. The seeds of *A. theophrasti* had already been harvested in 2008 from a sunflower (*Helianthus annuus* L.) field in Vilanova de Bellpuig, Lleida, Spain. Since then they had been stored in the dark at 7 °C. After harvest and between the ensiling treatments, seeds of all species were stored in paper bags at room temperature.

### 3.2.2.2 Ensiling Treatment

#### 3.2.2.2.1 Silage Preparation

Five types of silage were prepared, which differed in their substrate composition and/or the ensiling conditions. Substrates used were silage maize as a whole crop substrate and biomass from a wildflower mixture designed for use in bioenergy (Vollrath et al., 2016). Substrates were harvested and ensiled in two experimental series, which corresponded to two consecutive growing seasons. The silage maize was grown on the same site (Rostock, Germany, 54°04′04.1″N 12°04′55.7″E) in the same variety (“Ronaldinho,” breeder KWS®) in both trial years (2014 and 2015). In both years, maize was harvested at early silage ripening stage, but different weather conditions had led to varying maturation. According to the BBCH scale (Weber and Bleiholder, 1990), the developmental physiology of the maize corresponded to BBCH 82 in the first and BBCH 87 in the second trial year. The dry matter (DM) content was 28.1% in the first and 23.9% in the second year, respectively. The wildflower biomass was used for ensiling only in the second trial year, but there were two variants of it: wildflower biomass from the first year of standing and from the second year of standing, which differed due to changes in species dominance patterns. In the second standing year, the biomass was less fibrous but had a higher content of senescent material. At harvest, DM content of the wildflower biomass varied from 40.1% in the

first to 42.8% in the second standing year. Further information on the species composition and the biochemical parameters of the initial substrates before ensiling is available from Müller and Hahn (2020), as the same biomass stock was used in this study.

For ensiling the maize was chopped to a length of 0.5–1.5 cm with a forage harvester, while the wild flower biomass was chopped to 2–4 cm with a commercial garden shredder. Ensiling was conducted immediately after the chopping and, if required, mixing of the substrates. We used 3 l glass jars as lab-scale silos. The jars were washed and sterilized (180°C) before the substrates were filled in layers and compressed by hand. We filled the jars to the brim so that there was no cavity under the lid. Afterwards the filled jars were closed with a rubber-lined lid that was fixed by clips. This simple but standardized method prevented air infiltration but allowed the escape of gases formed during fermentation processes. Four silage types were prepared using this optimized method: "maize 82, ideal" from 100% maize harvested at BBCH 82, "maize 87, ideal" from 100% maize harvested at BBCH 87, as well as mixtures from 67% (fresh weight) maize biomass harvested at BBCH 87 and 33% (fresh weight) wildflower biomass harvested in the first ("wildflower blend 1") or second standing year ("wildflower blend 2"). In the first trial year, an aliquot of maize harvested at BBCH82 was deliberately exposed to stressful ensiling conditions in order to provoke suboptimal but possibly more practical fermentation acid patterns. This silage type is hereafter referred to as "maize 82, stress." To prepare this silage of lower quality, the substrate was stuffed less tightly, and inoculated with a soil-suspension containing *Clostridia* of a misfermented field silage pile. Additionally, aerobic stress was induced by lifting the lid for 24 h at the beginning, after 28 and after 42 days. This stress treatment and ensiling under ideal, optimized conditions form the two levels of the factor "ensiling condition," namely "stress" and "ideal."

All silage types were ensiled in at least three replicates and stored in the dark at 16°C. Substrates should be ensiled for at least 3 months to ensure that all fermentation processes have been completed and the growth of detrimental microbial populations such as yeasts has been limited (Müller and Bauer, 2006; Wyss and Pradervand, 2017).

#### 3.2.2.2.2 Silage Characteristics

After ensiling and removal of the ensiled seeds (see section Ensiling of seeds), silages were sealed airtight in plastic bags and stored at −40°C before analyzing their biochemical composition, including fermentation profiles. The silage characteristics were determined as described in Müller and Hahn (2020). The biochemical composition of the silage types was visualized with non-metric Multi-Dimensional Scaling (n-MDS) based on Bray-Curtis distances using R package "vegan" (Oksanen et al., 2002). A PerManova ["adonis" function in R by Anderson (2001)] using the Bray–Curtis dissimilarity index (Bray and Curtis, 1957) and including a permutation test with 1,000 permutations was applied to test to what extent the type of silage explained the variance in the biochemical composition of the silages.

#### 3.2.2.2.3 Ensiling of Seeds

The survival of seeds of all 10 species was tested in the silages "maize 82, ideal" and "maize 82, stress." Seeds of the HS species *M. alcea*, *M. albus*, and *M. officinalis* were additionally tested in the silage types "maize 87, ideal," "wildflower blend 1," and "wildflower blend 2". Both experimental series were accompanied by untreated controls, referred to as "untreated A" and "untreated B." For ensiling, 100–300 seeds of one species were placed into fine-meshed polyester bags. One bag for each species was stuffed into the middle part of a silage jar together with the substrate. The number of replicates and the total number of seeds examined varied among silage types and plant species. See **Supplementary Table 3-1** for a detailed record.

The specific ensiling duration depended on the analytical capacities and averaged 8 months, ranging from 4 to 9 months. Seeds were retrieved after 239 days from "maize 82, ideal" and after 281 days in "maize 82, stress." From "maize 87, ideal," "wildflower blend 1," and "wildflower blend 2" one replicate was opened after 128 days, and the seeds from the other replicates were retrieved after 237 days.

#### 3.2.2.2.4 Seed Viability Tests

After the bags containing the seeds were removed from the silage, they were rinsed with tap water. The seeds were then surface sterilized with 1% NaOCl, rinsed three times with sterilized water and placed on plates with "diaspore agar" (agar agar 13.0 g $l^{-1}$, KNO3 2.0 g $l^{-1}$, gibberellic acid 0.5 g $l^{-1}$, ampicillin 0.1 g $l^{-1}$, streptomycin 0.1 g $l^{-1}$, benzimidazol 0.02 g $l^{-1}$). In the following 21 days, the seeds were incubated at 20/4°C day/night temperatures with a 16 h photoperiod and germination was checked after 3, 7, 11, 16, and 21 days. A seed was considered germinated if the radical protruded at least 2mm from the seed. The viability of all remaining non-germinated seeds was tested using 2,3,5-triphenyl tetrazolium chloride (TTC). For this purpose, the seed coats were carefully punctured with a needle or scalpel without inflicting injuries to the embryo and the punctured seeds were placed between two filter papers, soaked with 3ml of 1.0%TTC solution and incubated in the dark at 35°C for 20–22 h.

The same procedure was used to determine the viability of untreated control seeds that had not been exposed to ensiling (minimum of 3 replicates of 300 seeds per species, **Supplementary Table 3-1**). However, the previously dry-stored control seeds were exposed to a water-saturated atmosphere for 2 days in the dark before the viability test.

Based on their response in the viability test, the seeds were classified as either dead or potentially viable. All seeds whose embryo was unstained (white) or rotten were considered "dead." Seeds that were completely decomposed during ensiling were assigned to the "dead" category as well. All seeds that showed metabolic activity in the form of germination or staining in the tetrazolium test were considered potentially viable.

### 3.2.2.3 Statistical Analysis of Viability Data

Statistical analysis was performed only for species that had not completely lost their viability due to ensiling. Various generalized linear mixed effect binomial models with a logit link function were used to estimate the proportion of dead seeds among all seeds. Ensiling (yes or no), different ensiling treatments, plant species, and duration of ensiling were considered fixed effects. The glass jar was used as random effect in each model. Additional random variables were introduced to reflect the variance structure. In all models, the respective number of seeds under study was used as a weight in the fitting process. **Table 3-4** provides an overview of the research questions and the specified models.

**Table 3-4 | Overview of research questions on seed persistence under different ensiling conditions and the related structure of generalized linear mixed models. The dependent variable was either the proportion of dead seeds to examined seeds (prop. dead) or the proportion of germinated seeds to viable seeds (prop. germ).**

| **Research question** [a] | **A** | **B** | **C** | **D** | **E** | **F** |
|---|---|---|---|---|---|---|
| *Dependent variable* | prop. dead | prop. dead | prop. dead | prop. dead | prop. dead | prop. germ |
| **Fixed Effects** | | | | | | |
| ensiled (yes/no) | x | x | | | | x |
| silage type (2-5 levels) [b] | | | x | x | | |
| species (5 levels) | | x | x | x | | x |
| duration (2 levels) | | | | | x | |
| ensiled x species | | x | | | | x |
| treatment x species | | | x | x | | |
| **Random effects** | | | | | | |
| glass jar | x | x | x | x | x | x |
| species | | | | | | |
| species x year | x | | | | | |
| species x silage type | | | | | x | |
| species x year x silage type | | x | | | | x |
| species x glass jar | | | x | x | | |
| **Subset of data** [c] | all | all | first year | second year | second year (ensiled, only) | all |

[a] Research questions: (A) Was the proportion of dead seeds affected by ensiling? /// (B) Was the proportion of dead seeds of a species affected by ensiling? /// (C) Was the proportion of dead seeds of a species affected by the ensiling conditions? /// (D) Was the proportion of dead seeds of a species affected by the composition of the ensiled substrate? /// (E) Was the proportion of dead seeds affected by the duration of ensiling? /// (F) Was the ratio of germinated to viable seeds of a species affected by ensiling?

[b] Levels of silage type depend on experimental year.

[c] All, all data; First year, data from first experimental year; Second year, data from second experimental year.

Starting from the respective null model, the deviance of the fixed effects was analyzed by sequentially adding main and interaction effects. Likelihood-ratio tests were used for pairwise comparisons of the increasingly complex models. Pairwise comparisons of the fixed effect levels were performed based on log odds ratios using Tukey's method for P-value adjustment.

If ensiling was used as a component of integrated weed management, it would affect the seed stage in the cycle of population dynamics. Against this background, the seed-killing efficacy of ensiling was calculated as follows:

$$seed-killing\ efficacy\ (\%) = 100 \times \left(1 - \frac{1 - proportion\ of\ dead\ seeds_{ensiled}}{1 - proportion\ of\ dead\ seeds_{unensiled}}\right) \quad \textbf{(3-6)}$$

All analyses were carried out using R software (R Core Team, 2020). The models were fitted using the R package "lme4" (Bates et al., 2015). The R package "emmeans" provided functions to estimate marginal means and odd ratios, and to test for significance (Lenth, 2021). All models were checked for the appropriateness of the chosen binomial distribution, for over- and under-dispersion, and for outliers with the respective test routines of the R package "DHARMa" (Hartig, 2020).

### 3.2.3 Results

Ensiling generally increased the proportion of non-viable, i.e., dead, seeds (**Figure 3-5**). However, a substantial difference existed between hardseeded (HS) and non-hardseeded (NHS) plant species.

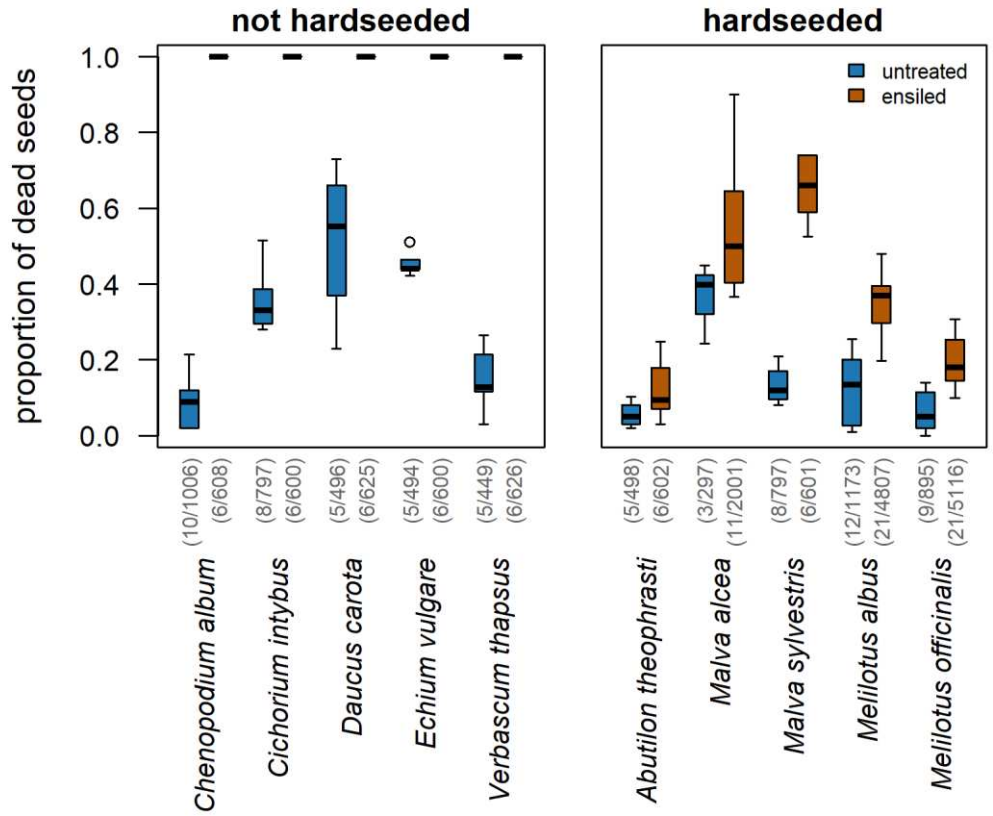


**Figure 3-5 | Ensiling increased the proportion of non-viable weed seeds from both NHS and HS species compared to untreated controls. Numbers in parentheses indicate replicates/total numbers of seeds studied.**

Regarding the NHS species, ensiling was 100% effective. That means that all seeds of the NHS species were dead after ensiling, while on average 75% of them were viable without ensiling. Of the HS species, an average of 36% was dead after ensiling. Since 11% had already been dead before the treatment, ensiling resulted in a highly significant seed-killing efficacy of 28% across all five HS species tested [$P(Chi^2) < 0.0001$, **Supplementary Table 3-2**]. Among the individual HS species, the seed-killing efficacy ranged from 5 to 60%. In *M. sylvestris*, *M. albus*, and *M. officinalis* ensiling significantly increased the proportion of non-viable seeds compared to untreated controls (**Table 3-5**; **Figure 3-5**).

**Table 3-5 | Effect of ensiling on the proportion of dead seeds and seed killing efficacy of ensiling in five HS species.**

| Species | untreated *vs.* ensiled (odds ratio) | standard error | P(z-ratio) | seed killing efficacy (%) |
|---|---|---|---|---|
| *Abutilon theophrasti* | 0.49 | 0.26 | 0.183 | 5 |
| *Malva alcea* | 0.56 | 0.25 | 0.188 | 23 |
| *Malva sylvestris* | 0.08 | 0.03 | <0.0001 | 60 |
| *Melilotus albus* | 0.27 | 0.09 | 0.0002 | 23 |
| *Melilotus officinalis* | 0.21 | 0.08 | 0.0001 | 15 |

Across all HS species, all silage types had a highly significant seed-killing effect compared to the un-ensiled control (**Figure 3-6**, bottom). Also referring all HS species, ensiling "maize 82" under ideal conditions multiplied the proportion of dead seeds by a factor of 4.3 on average compared to un-ensiled controls, while suboptimal (stress) ensiling conditions increased the proportion of dead seeds by a factor of 4.1 (**Table 3-6**; **Figure 3-6**, left bottom). This corresponded to seed-killing efficacies of 29 and 27%, respectively. Ensiling seeds of HS species in three different substrates ("maize 87," "wildflower blend 1," "wildflower blend 2") under identical, ideal conditions caused a 4.9–5.6-fold increase of the proportion of dead seeds compared to un-ensiled controls, corresponding to a seed-killing efficacy of 23–26% (**Figure 3-6**, right). The seed-killing efficacy did not differ significantly between silages with different substrate compositions (**Figure 3-6**, right bottom; **Tables 3-6**, **3-8**). However, it should be mentioned here that across all HS species "maize 82, ideal" killed 1.5-times as many seeds as "maize 87, ideal" (**Table 3-6**). In addition, the response of the individual HS species did differ between the silage types (**Figure 3-6**, top) and even significantly with respect to the ensiling conditions (**Table 3-7**). For *A. theophrasti* and *M. alcea* the seed-killing efficacy of "maize 82, ideal" was significantly higher than that of "maize 82, stress," while it was the opposite for *M. sylvestris* and *M. albus*. In *M. officinalis*, the percentage of dead seeds was the same for both ensiling conditions (**Figure 3-6**, left).

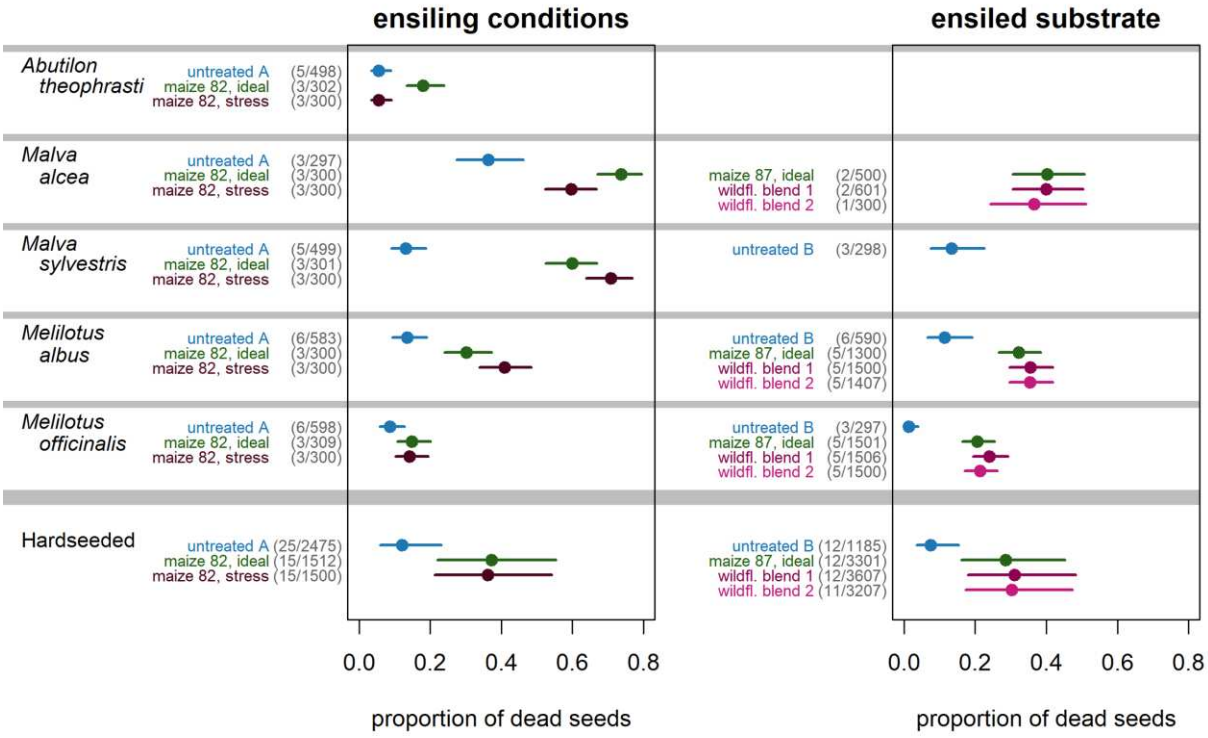


**Figure 3-6 | Effect of ensiling conditions and ensiled substrate on the proportion of non-viable seeds of HS weed species. Numbers in parentheses indicate replicates/total numbers of seeds studied.**

**Table 3-6 | Pairwise comparisons of the effect of different silage types and untreated controls on the proportion of dead seeds. Upper right triangle: odds ratios (column to row); lower left triangle: corresponding P (z-ratio).**

| | Un-treated A | Maize 82, ideal | Maize 82, stress | Un-treated B | Maize 87, ideal | Wildflower blend 1 | Wildflower blend 2 |
|---|---|---|---|---|---|---|---|
| **Untreated A** | | 0.23 | 0.24 | 1.69 | . | . | . |
| **Maize 82, ideal** | *<0.001* | | 1.05 | . | 1.48 | . | . |
| **Maize 82, stress** | *<0.001* | *0.76* | | . | . | . | . |
| **Untreated B** | *<0.001* | . | . | | 0.20 | 0.18 | 0.19 |
| **Maize 87, ideal** | . | *0.01* | . | *<0.001* | | 0.88 | 0.92 |
| **Wildflower blend 1** | . | . | . | *<0.001* | *0.39* | | 1.04 |
| **Wildflower blend 2** | . | . | . | *<0.001* | *0.59* | *0.76* | |

Ensiling durations differed among silage samples due to the capacities available for seed viability analyses. According to the statistical evaluation, the duration of ensiling had a significant effect on successfully killing the seeds. If the duration of ensiling was shortened from almost 8 to 4 months, the proportion of dead seeds decreased by a factor of 0.8 [P(z-ratio) = 0.0478, **Supplementary Table 3-3**]. Furthermore, significant year-to-year differences were observed in the proportion of dead seeds in the untreated control samples, being 1.7 higher in the first experimental year (**Table 3-6**).

**Table 3-7 | Analysis of deviance of the generalized linear mixed effects model for the proportion of dead seeds of HS species as influenced by ensiling conditions ("ideal" and "stress" in maize 82), plant species (*A. theophrasti*, *M. alcea*, *M. sylvestris*, *M. albus*, *M. officinalis*), and their interaction.**

| Added variable | Degrees of freedom | AIC | Chi² | P(Chi²) |
|---|---|---|---|---|
| (no fixed effects) | | 272.80 | | |
| Ensiling condition | 1 | 274.60 | 0.1963 | 0.6577144 |
| Species | 4 | 222.54 | 60.0596 | 2.818e-12 *** |
| Silage quality × species | 4 | 210.03 | 20.5130 | 0.0003954 *** |

***P-values < 0.001.

**Table 3-8 | Analysis of deviance of the generalized linear mixed effects model for the proportion of dead seeds of HS species as influenced by composition of ensiled substrate ("maize 87, ideal," wildflower blends 1 and 2), plant species (*M. alcea*, *M. albus*, *M. officinalis*), and their interaction.**

| Added variable | Degrees of freedom | AIC | Chi² | P(Chi²) |
|---|---|---|---|---|
| (no fixed effects) | | 334.81 | | |
| Ensiled substrate | 2 | 338.18 | 0.625 | 0.7316 |
| Species | 2 | 315.62 | 26.564 | 1.705e-06 *** |
| Ensiled substrate × species | 4 | 322.88 | 0.741 | 0.9462 |

***P-values < 0.001.

The germination experiments revealed that the proportion of germinated to viable seeds was lower after ensiling, by a factor of 3 to a factor of 26 [P(z-ratio) < 0.002, **Figure 3-7**; **Supplementary Table 3-4**]. Only *A. theophrasti* was an exception to this, with the same proportion of germinating seeds regardless of ensiling. It is worth mentioning that in two of the three samples of "maize 82, ideal", most of the germinable seeds of *M. albus* (about 70%) had already started to germinate in the silage itself. This means that they were already recognizable as seedlings when the silage jars were opened.

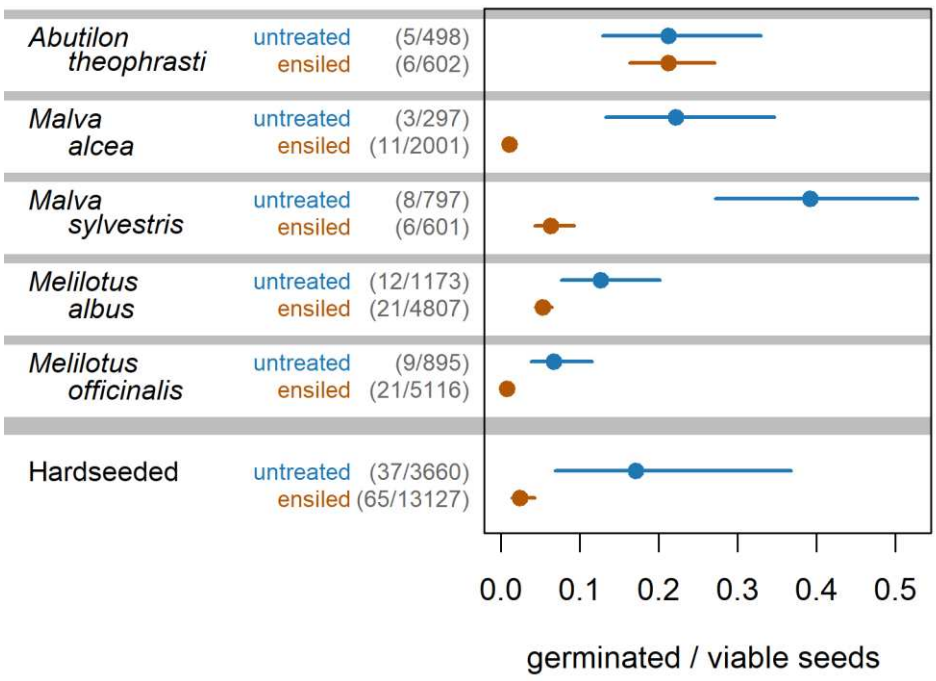


**Figure 3-7 | Proportion of germinated to viable seeds of HS species before (untreated) and after ensiling.**

In terms of their biochemical characteristics, the five silage types produced differed significantly [P(F-value) = 0.0010, **Table 3-9**; **Figure 3-8**; **Supplementary Table 3-5**]. The factor "silage type" explained 95.6% of the variance in the biochemical composition of the silages (**Table 3-9**).

**Table 3-9 | Effect of silage type on the biochemical composition of silages estimated by PerManova.**

| | Degrees of freedom | Sums of squares | Fisher model | explained variability | P(F-value) |
|---|---|---|---|---|---|
| Silage type | 4 | 0.1097 | 86.781 | 0.95594 | 0.0010 *** |
| Residuals | 16 | 0.0051 | | 0.04406 | |
| Total | 20 | 0.1148 | | 1 | |

The following biochemical parameters were included: dry matter content, content of crude ash, total nitrogen, ammonium bound nitrogen, pH-value, lactic acid, acetic acid, propionic acid, ethanol, propanol, and 2,3-butanediol.
***P-values < 0.001.

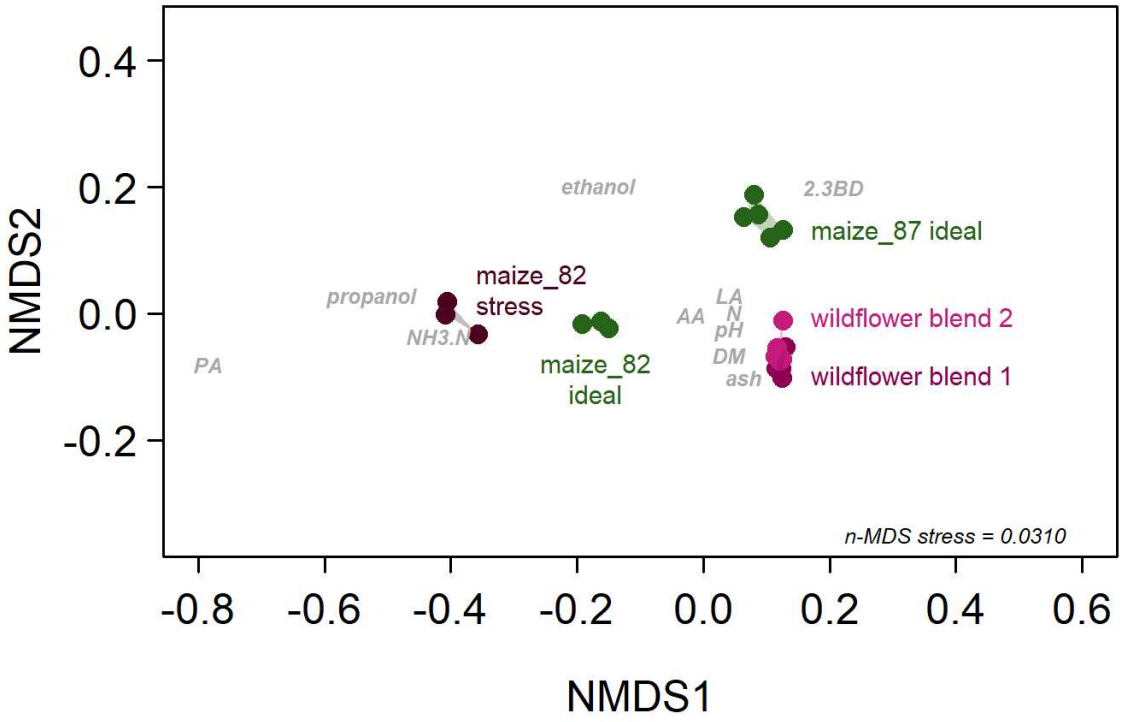


**Figure 3-8 | Differences in the biochemical composition of the five silage types displayed in non-metric Multi-Dimensional Scaling (n-MDS). Points indicate replicates and polygons mark replicates belonging to the same silage type. Nomenclature of the biochemical characteristics (gray): AA, acetic acid; DM, dry matter content; LA, lactic acid; N, total nitrogen; NH3.N, ammonium bound nitrogen; PA, propionic acid; 2.3BD, 2,3-butanediol.**

## 3.2.4 Discussion

### 3.2.4.1 Seed-Killing Efficacy of Ensiling

Lab-scale ensiling for 8 months decreased the proportion of viable seeds of the hardseeded (HS) species studied and completely inactivated the non-hardseeded (NHS) ones. These results are consistent with

the findings of other studies ensiling seeds on a long-term basis, i.e., for more than 3 months (Shevkenek, 1934; Woodward, 1940; Mayer et al., 2000; Overud, 2002; Lück, 2012; Westerman et al., 2012; Aper et al., 2014; Koarai et al., 2015; Simard and Lambert-Beaudet, 2016). However, regarding the HS species the seed-killing efficacies of our silages were low compared to other long-term studies (Mayer et al., 2000; Westerman et al., 2012; Koarai et al., 2015; Simard and Lambert-Beaudet, 2016). Instead, the killing efficacy in our experiments was more in the range of studies that ensiled HS seeds for a maximum of 3 months reducing the viability of HS species by about half on average (Woodward, 1940; Mayer et al., 2000; Stanton et al., 2012; Simard and Lambert-Beaudet, 2016; Piltz et al., 2017).

The seed-killing efficacies found here are nonetheless within the range of previous studies (highlighting the immense range of responses of HS species in ensiling). In fact, the viability losses of the HS species studied so far varied between 0 and 100%. In individual samples of *A. pelecinus* (L.) Barneby (biserrula, Fabaceae) the mean viability was even higher than in untreated controls (Stanton et al., 2012). Viability loss also varied, sometimes greatly, within HS species of which several batches were examined, e.g., *A. theophrasti* (Westerman et al., 2012; Simard and Lambert-Beaudet, 2016; this study), and *M. officinalis* (Woodward, 1940; this study). For example, our one-year-old seed batch of *M. officinalis* originating from Germany lost about 15% of its initial viability in our lab-scale, maize-based silages, while a batch of unknown age from the U.S. was almost completely inactivated in various silages of alfalfa (Woodward, 1940). The differences between studies could be due to possible effects of the surrounding silage substrate as well as to different initial seed qualities. Seed lot quality can vary strongly depending on factors such as seed age, plant population, and storage conditions (e.g., Hay and Probert, 2013). For example, the viability of our untreated seeds differed significantly between the two experimental years.

Regarding the differing survival rates of HS species in ensiling it might be relevant that the degree and the depth of HS differ between species and seed lots (Baskin and Baskin, 2004). In general, the coat of young seeds of HS species in the early stages of ripening is still permeable to water (Jaganathan et al., 2016 and references therein), and thus possibly also to harmful fermentation products from ensiling. Fully mature seeds of HS species, on the other hand, are likely to be protected by their hard, water-impermeable seed or fruit coat. The different killing efficacies for different aged batches of A. theophrasti from three different studies fit this theory well: After more than 3 months of ensiling, batches of freshly harvested *A. theophrasti* seeds were completely inactivated (Simard and Lambert-Beaudet, 2016), while a 3-year-old batch had lost only one-fifth (Westerman et al., 2012) and our 6-year-old batch had lost as few 5% of its initial viability. Whether seeds of HS species actually become more resistant to ensiling due to increasing maturity and depth of HS could be demonstrated by ensiling different batches of a HS species with known characteristics under identical conditions. Transferred into farming practice, increasing insensitivity of HS species to ensiling over time would mean that the seed-killing efficacy of ensiling would tend to be higher than that determined in our study. This is because in practice, only (young) weed seeds still on the harvested plants would enter the silo.

However, HS is not the only mechanism that can make seeds resistant to ensiling. This is evident, for example, from the fact that not all NHS species studied so far were completely inactivated by ensiling, although none survived in our study. For instance, *Rumex crispus* L. (curled dock, Polygonaceae) and *Spergula arvensis* L. (stickwort, Caryophyllaceae) had lost only a maximum of 36 and 47% of their initial viability after 3 and 8 months in ensiling, respectively (Mayer et al., 2000; Overud, 2002). The specific mechanisms by which species without HS survived ensiling are not yet clear. But seed coat thickness (Simard and Lambert-Beaudet, 2016), seed maturity (Piltz et al., 2017), and induction of dormancy (Overud, 2002) have been suggested as factors that might increase their resistance toward ensiling.

In addition to killing seeds, ensiling appeared to have weakened seed vigor because the proportion of germinated to viable seeds was lower after our ensiling. This makes it unlikely that these viable seeds will develop into seedlings. We assume the same for the seeds of *M. albus* that have already started to germinate in the silage, as seedlings are too unstable to survive the mechanical stresses associated with application or further processing. Other studies did not report germination in silage, but morphological changes in seeds indicating damage and, thus, limited survivability (Simard and Lambert-Beaudet, 2016; Weller et al., 2016).

Although the properties of the plant seeds themselves are important, their survival in silages is mainly influenced by the conditions of the particular ensiling process. In this regard, the influence of ensiling duration on seeds was studied and—as in our study—it was found that longer ensiling was equivalent to a higher seed-killing efficacy (Mayer et al., 2000; van Eekeren et al., 2006; Trolove and Dowsett, 2015; Simard and Lambert-Beaudet, 2016). In addition to the longer time the ensiled biomass can act on the seeds, the different biochemical phases of the ensiling process may play a role here. During these phases, various fermentation products are formed due to microbiological activity, which depends on properties of the ensiled biomass and the respective ensiling conditions until finally a relatively stable (storage) state is reached (Müller and Bauer, 2006). The biochemical characteristics of a silage thus depend on factors in which all studies on seed survival in ensiling differed to a greater or lesser extent.

#### 3.2.4.2 Effect of Silage Type on Seed-Killing Efficacy

In agreement with other studies that have tested different silage types on more than one species (Woodward, 1940; Anonymus, 1959–1960; Simard and Lambert-Beaudet, 2016) we did not find a consistent effect on the seeds of all weed species studied. When the silage types were distinguished by substrate composition, the seed-killing effect of the types did not differ in our study or that of Woodward (1940), but it did species-specifically in Simard and Lambert-Beaudet (2016) and Anonymus (1959–1960). However, when distinguished according to ensiling conditions (ideal and stressed), the effect of our silage types differed, but again depending on the weed species. A comparison of seed-killing efficacy between silage types that differ in ensiling conditions, i.e., factors that are important to prepare

a high-quality forage silage (Müller and Bauer, 2006) (high/low compaction, extent of air inflow, contamination with soil bacteria), has not been done in any other study that we are aware of. Rather, differences between the silage types were based on the variation in individual factors such as substrate composition (Anonymus, 1959–1960; Simard and Lambert-Beaudet, 2016), manipulation of DM (Overud, 2002; van Eekeren et al., 2006), addition of inoculants (Weller et al., 2016), and varying depths of incubation in the silo (Shevkenek, 1934; Tildesley, 1937; James et al., 2011; Trolove and Dowsett, 2015). Only Woodward (1940) varied three factors, namely substrate, moisture content, and addition of molasses, for his silage types.

Even though the silages studied so far have been classified into types based on certain properties, they remain largely "black boxes" and thus hardly comparable between studies because little is reported on their biochemical characteristics. Data on selected biochemical factors collected in some studies led to different assumptions about the mechanisms of action behind the seed-killing effect of ensiling. So far, moisture or DM content, changes in temperature, pressure, and pH value, $CO_2$ content, lactic acid content and the activity of proteolytic enzymes have been discussed as possible inactivation mechanisms (Woodward, 1940; Blackshaw and Rode, 1991; van Eekeren et al., 2006; Simard and Lambert-Beaudet, 2016; Weller et al., 2016). In terms of moisture or DM, there is some evidence that silages with higher moisture and consequently lower DM content are more efficient in killing seeds (Woodward, 1940; Overud, 2002; van Eekeren et al., 2006). However, this trend is contradicted by our data: Our silage with the lowest DM content had a significantly lower seed-killing efficacy than that with the highest DM content ("maize 87, ideal": 26.2 ± 0.31% fresh weight vs. "maize 82, ideal": 34.4 ± 0.49% fresh weight, **Supplementary Table 3-5**). Furthermore, we can add to the discussion by noting that a significant difference in silage biochemistry does not necessarily translate into a significantly different seed-killing efficacy because, unlike other studies, we have determined the biochemical characteristics including fermentation acid patterns of our silages. The variance of the fermentation products in our silages reflects their range quite well in practice silages produced according to the current state of knowledge; although there would be a considerable amount of butyric acid in misfermented practice silages. From the fact that we found no consistent differences in seed-killing efficacy within the biochemical spectrum we studied, we conclude that neither substrate choice nor manipulation of ensiling conditions (within good practice) are effective measures to increase weed seed killing during ensiling.

#### 3.2.4.3 Comparison With Other Pathways Reducing Seed Viability

To assess the potential of ensiling as a measure of integrated weed management, it seems useful to compare it with the better-known pathways of seed reduction in organic farming. In the field itself, seed predation in particular (e.g., Westerman et al., 2003) is similar to weed seed removal by harvesting and ensiling biomass, although animal predators additionally reduce seed depots on the soil surface. Furthermore, diverse tillage operations directly affect the pool of weed seeds by burying them (Grundy et al., 1999). The extent to which the weed seed load can be reduced by seed predation and tillage

depends on management practices and environmental conditions, but most importantly on the weed species and their characteristics [e.g., Nichols et al. (2015) and references therein]. For example, *Galium aparine* L. (catchweed bedstraw, Rubiaceae) and *Lolium rigidum* Gaudin (rigid ryegrass, Poaceae) lost 49 and 62% of their seeds, respectively, to predation and 33 and 54% to 2-month burial (Baraibar et al., 2017). Comparatively low were the mean seed predation rates reported by Navntoft et al. (2009) of 17 and 10% for organically and conventionally managed fields in New Zealand, respectively. Despite this wide range of seed reduction by on-field processes, the average seed-killing potential of our silages was higher, at least for NHS species. For the HS species *A. theophrasti*, however, the annual predation rate of 20–80% determined by Westerman et al. (2005) was higher than the seed-killing efficacy of our silages.

Another important aspect when evaluating ensiling as a weed control measure is the further use of the silages produced, which, after all, still contain viable weed seeds in some cases. Usually the silage is fed, i.e., subjected to animal digestion, and the resulting manure is stored until it is used as fertilizer. All of these fermentation processes have the potential to inactivate seeds (e.g., Aper et al. 2014). However, seed inactivation does not necessarily increase when seeds are exposed to them sequentially (Blackshaw and Rode, 1991; Stanton et al., 2012; Piltz et al., 2017). Rather, the process step which weed seeds are exposed to first is crucial for weed control because (HS) seeds that survive the initial fermentation process often survive subsequent processes (Edwards and Younger, 2006). In terms of weed control, ensiling is likely to be more suited as a first process step than feeding, since many seed-producing weed species have evolved adaptations to survive herbivory (Blackshaw and Rode, 1991).

#### 3.2.4.4 Ensiling and the Use of Flowering Plant Mixtures

When the biomass of wild plant flowering mixtures is used (Cossel and von Lewandowski, 2016) their seeds can be unintentionally spread and possibly become weeds. According to our study, ensiling can help reduce this risk of weed spread by killing the seeds of NHS and reducing the viability of HS wildflower species. Especially compared to direct use of biomass as in the newly emerging practice of "cut and carry" (Benke et al., 2017), in which mulched biomass is transferred from field to field as an organic nutrient source, ensiling would contribute greatly to integrated weed management. Of course, the HS species that have survived ensiling still pose a risk as potential weeds. However, this risk would presumably be lower than after direct use in a biogas plant. Westerman et al. (2012) reported that weed seed viability was generally more affected by ensiling than by anaerobic digestion. Therefore, upstream processing of wild plant biomass via ensiling would improve weed management compared to immediate biomethanization.

### 3.2.5 Conclusions

Ecological weed management aims to subject weeds to multiple, temporally variable stresses, for which Liebman and Gallandt (1997) coined the term "many little hammers." Based on the results of this study,

we conclude that ensiling is an often overlooked yet effective way to reduce weed seed loads in a sustainable manner. By reducing number and vigor of weed seeds, ensiling helps exclude weeds from fields, reducing their density and delaying their emergence before they can interfere with crop growth or reproduction. Thus, ensiling can be one of the "little hammers" in ecological weed management.

Ensiling unfolds its full seed reduction potential primarily with NHS weeds. However, it has a gap in effectiveness when it comes to reducing the viability of HS species, especially if they have reached full maturity and/or have additional dormancy mechanisms. It remains to be investigated whether this effectiveness gap can be closed by specifically varying silage properties or by extending the duration of ensiling beyond the usual storage times of forage or energy substrates.

As an on-farm integrated weed management tool ensiling targets only seeds that are part of the biomass stream intended for whole-crop storage. Also in terms of biomass streams a tendency toward specialization in organic farming can currently be observed ("conventionalization debate"). This may include a marginalization of animal husbandry in favor of well-marketable cash crops. In this context, our results suggest that the traditional and proven integration of cropping and livestock with their characteristic material flows and biomass processing steps (ensiling, manure storage) is not only beneficial for soil nutrient supply, but is also an essential element of integrated weed management in organic agriculture.

**Data Availability Statement.** The original contributions presented in the study are included in the article/SupplementaryMaterial, further inquiries can be directed to the corresponding author/s.

**Author Contributions.** All authors contributed to the conception and the design of the study, the preparation of the silages, and writing and revision of the manuscript. JH edited the manuscript, organized the project, analyzed the viability of the seeds, and performed the multivariate statistical analyses. FdM performed the statistical analysis in coordination with JH and JM.

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**Conflict of Interest.** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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### 3.2.8 Supplementary Material

**Supplementary Table 3-1 | Number of replicates (rep) and total number of seeds (seed) per species exposed to the different types of silage and in the untreated controls.**

| | untreated A | | maize 82 ideal | | maize 82 stress | | untreated B | | maize 87 ideal | | wildflower blend 1 | | wildflower blend 2 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | rep | seeds | rep | seeds | rep | seeds | rep | seeds | rep | seeds | rep | seeds | rep | seeds |
| *Abutilon theophrasti* | 5 | 498 | 3 | 302 | 3 | 300 | - | - | - | - | - | - | - | - |
| *Chenopodium album* | 10 | 1006 | 3 | 305 | 3 | 303 | - | - | - | - | - | - | - | - |
| *Cichorium intybus* | 8 | 797 | 3 | 300 | 3 | 300 | - | - | - | - | - | - | - | - |
| *Daucus carota* | 5 | 496 | 3 | 321 | 3 | 304 | - | - | - | - | - | - | - | - |
| *Echium vulgare* | 5 | 494 | 3 | 300 | 3 | 300 | - | - | - | - | - | - | - | - |
| *Malva alcea* | 3 | 297 | 3 | 300 | 3 | 300 | - | - | 2 | 500 | 2 | 601 | 1 | 300 |
| *Malva sylvestris* | 5 | 499 | 3 | 301 | 3 | 300 | 3 | 298 | - | - | - | - | - | - |
| *Melilotus albus* | 6 | 583 | 3 | 300 | 3 | 300 | 6 | 590 | 5 | 1300 | 5 | 1500 | 5 | 1407 |
| *Melilotus officinalis* | 6 | 598 | 3 | 309 | 3 | 300 | 3 | 297 | 5 | 1501 | 5 | 1506 | 5 | 1500 |
| *Verbascum thapsus* | 5 | 449 | 3 | 309 | 3 | 317 | - | - | - | - | - | - | - | - |

**Supplementary Table 3-2 | Analysis of deviance of the generalized linear mixed effects model for the proportion of dead seeds of hardseeded species as influenced by ensiling (yes/no), plant species (*A. theophrasti*, *M. alcea*, *M. sylvestris*, *M. albus*, *M. officinalis*) and their interaction.**

| Added variable | degrees of freedom | AIC | Chi² | P(Chi²) |
|---|---|---|---|---|
| (no fixed effects) | | 1002.77 | | |
| ensiled | 1 | 994.54 | 10.228 | 0.001383 ** |
| species | 4 | 969.20 | 33.342 | 1.017e-06 *** |
| ensiled × species | 4 | 965.94 | 11.258 | 0.023816 * |

**Supplementary Table 3-3 | Analysis of deviance of the generalized linear mixed effects model for the proportion of dead seeds of hardseeded species as influenced by duration of ensiling.**

| Added variable | degrees of freedom | AIC | Chi² | P(Chi²) |
|---|---|---|---|---|
| (no fixed effects) | | 410.04 | | |
| duration | 1 | 408.12 | 3.917 | 0.04779 * |

**Supplementary Table 3-4 | Analysis of deviance of the generalized linear mixed effects model for the proportion of germinated to viable seeds of hardseeded species as influenced by ensiling (yes/no), plant species (*A. theophrasti*, *M. alcea*, *M. sylvestris*, *M. albus*, *M. officinalis*) and their interaction.**

| Added variable | degrees of freedom | AIC | Chi² | P(Chi²) |
|---|---|---|---|---|
| (no fixed effects) | | 688.97 | | |
| ensiled | 1 | 672.55 | 10.543 | 0.001167 ** |
| species | 4 | 642.53 | 38.026 | 1.107e-07 *** |
| ensiled × species | 4 | 610.23 | 40.302 | 3.749e-08 *** |

**Supplementary Table 3-5 | Biochemical characteristics of the different silage types. The silages prepared from the substrate "maize 82" were replicated three times, the other ones five times. Duration of ensiling was 239 days for "maize 82, ideal", 281 days for "maize 82, stress" and 128 - 237 days for "maize 87, ideal" and both wildflower blends. Presented are arithmetic means (standard deviation). See below for parameter abbreviations and units.**

| | maize 82 ideal | | maize 82 stress | | maize 87 ideal | | wildflower blend 1 | | wildflower blend 2 | |
|---|---|---|---|---|---|---|---|---|---|---|
| DM | 34.4 | (0.49) | 29.8 | (1.39) | 26.2 | (0.31) | 31.8 | (0.34) | 32.0 | (0.19) |
| ash | 3.9 | (0.09) | 4.3 | (0.43) | 3.4 | (0.12) | 5.2 | (0.11) | 4.7 | (0.17) |
| N | 1.5 | (0.20) | 1.5 | (0.06) | 1.6 | (0.02) | 1.6 | (0.03) | 1.5 | (0.04) |
| NH3.N | 9.5 | (0.72) | 10.4 | (0.54) | 1.7 | (1.23) | 1.3 | (0.12) | 1.3 | (0.11) |
| pH | 3.8 | (0.03) | 3.8 | (0.04) | 3.6 | (0.01) | 3.7 | (0.01) | 3.7 | (0.02) |
| LA | 7.3 | (0.19) | 7.1 | (0.40) | 7.9 | (0.41) | 6.6 | (0.19) | 6.8 | (0.21) |
| AA | 2.1 | (0.13) | 3.3 | (0.05) | 2.2 | (0.17) | 2.1 | (0.33) | 2.3 | (0.44) |
| BA | 0 | | 0 | | 0 | | 0 | | 0 | |
| PA | 0.1 | (0.02) | 0.2 | (0.01) | 0 | | 0 | | 0 | |
| VA | 0 | | 0 | | 0 | | 0 | | 0 | |
| ethanol | 1.3 | (0.10) | 2.2 | (0.48) | 1.5 | (0.56) | 0.5 | (0.09) | 0.6 | (0.07) |
| propanol | 0.2 | (0.03) | 0.4 | (0.10) | 0.1 | (0) | 0.1 | (0) | 0 | (0) |
| butanol | 0 | | 0 | | 0 | | 0 | | 0 | |
| 1.2PD | 0 | | 0 | | 0 | | 0 | | 0 | |
| 2.3BD | 0.1 | (0.02) | 0.1 | (0.02) | 0.5 | (0.06) | 0.3 | (0.04) | 0.3 | (0.07) |

The biochemical characterization of the five types of silage studied was based on the analysis of the following parameters: dry matter in % of fresh weight (abbreviated as: DM), content of crude ash in % of DM (ash), total nitrogen % of DM (N), ammonium bound nitrogen % of N (NH3.N), pH-value (pH), lactic acid % of DM (LA), acetic acid % of DM (AA), butyric acid % of DM (BA), propionic acid % of DM (PA), isovaleric acid % of DM (VA), ethanol % of DM, propanol % of DM, butanol % of DM, 1,2-propanediol % of DM (1.2PD), and 2,3-butanediol % of DM (2.3BD). For the respective analyses see "Materials and Methods".

# 4

# Synthesis and Outlook

## Opening Remarks

Whether wild plant seeds survive anaerobic digestion (AD) and ensilage cannot be answered with a general "yes" or "no" on the basis of this thesis. The responses of seed viability varied widely between treatments, species, and seed lots. This is perhaps not surprising, considering that the seeds, with their species- and individual-specific arsenal of resistance mechanisms, encountered biochemical processes that are themselves shaped by various parameters.

In the following, the insights gained in this thesis are presented in a condensed form and approaches for further research are outlined. The structure follows that set out in the "Goals and Objectives", starting with seed viability responses (Chapter 4.1), continuing with the potential of seed inactivation through AD and ensilage (Chapter 4.2) and ending with considerations on the implications of seed survival for the use of seed-bearing wild plants as biogas feedstock (Chapter 4.3)[5].

## 4.1 Seed Viability in Anaerobic Digestion and Ensilage

The first focus of this thesis was on the diverse responses of seed viability to AD or ensilage treatments. And in this context, the ability of different plant species to maintain their seed viability despite these treatments. Aspects of seed biology were considered, as very little is known about the response mechanisms of seeds to AD and ensilage. In fact, nothing was known about those of the wildflower seeds studied here. However, identifying traits that confer resistance to AD and/or ensilage in seeds could facilitate research, e.g., if the traits in question could be measured by testing in water-baths.

### 4.1.1 Seed Characteristics potentially favoring Survival

To gain insight into which seed characteristics may favor survival, this thesis placed emphasis on physical dormancy, i.e., hardseededness (HS), and a variety of species to potentially detect resistance mechanisms based on higher taxonomic levels than species.

Regarding the survival probability of species with HS compared to non-HS (NHS) species, it could be confirmed that HS species tended to be more resistant to AD and ensilage (see Westerman and Gerowitt, 2013). It should be emphasized that, thanks to investigations in this thesis, it can now be stated on the basis of seven instead of previously three species that HS increases the probability of seed survival even in full-scale commercial biogas reactors operated at mesophilic temperatures (Chapter 2.4). Furthermore, the survival-enhancing effect of HS was quite obvious in the silage treatments. In AD, however, a much more differentiated picture emerged. Given the overlap in survival probability between

[5] The goals and objectives listed in the introduction are underlined for ease of reference.

some NHS and HS species, the discovery of the particularly AD-resistant species *Malva sylvestris*, *Melilotus albus* and *M. officinalis*, and the remarkable resistance of the endozoochorously distributed $C_4$-grass *Cynodon dactylon*, it became clear that more traits than the mere ability to form HS seeds affected the AD-resistance potential (Chapter 2.1, 2.3). On the one hand, the degree and depth of HS seemed to modulate survivability. On the other hand, the level of resistance of HS, which is based on impermeable layers in the seed or fruit coat (Baskin et al., 2000), may possibly also be achieved or enhanced by other dormancy classes, secondary metabolites, photosynthetic metabolism, or morphological structures (e.g., Fuerst et al., 2014; Long et al., 2015; Chen et al., 2018; de Mol et al., 2020).

The search for relationships between resistance potential and taxonomic affiliation revealed that species belonging to the same family or genus may or may not respond similarly to AD or ensilage. For four of the ten families examined, more than one species was tested (**Table 1-1**). The representatives of the NHS families Asteraceae and Polygonaceae proved to be not very resistant with inactivation times of less than one week (**Table 4-1**). This is in line with most of the earlier findings on these families (Shevkenek, 1934; Woodward, 1940; Šarapatka et al., 1993; Schrade et al., 2003; Katovich et al., 2004; Leonhardt et al., 2010; Westerman et al., 2012a; Westerman et al., 2012b; Johansen et al., 2013; van Meerbeek et al., 2015; Simard and Lambert-Beaudet, 2016; Starfinger and Sölter, 2016; Oechsner et al., 2018). Together with the similarity of viability curves within the families Asteraceae, Polygonaceae and Fabaceae (Chapters 2.1, 2.3, 2.4), there were indications that the resistance potential of the seeds could be family-based. However, this assumption must clearly be regarded as conjecture. Firstly, the database of investigated species and genera is still too small to reliably confirm it. Secondly, there were exceptions. For example, in some studies Asteraceae survived ensilage (Anonymus, 1959-1960; Mayer et al., 2000). Furthermore, the AD-resistance potential of the genus *Melilotus* (Chapters 2.4, 3.2) exceeded the previously known, already high values for Fabaceae (Leonhardt et al., 2010; Strauß et al., 2012; Westerman et al., 2012b; Hassani et al., 2021). Only in two ensilage studies were they in a similar range (Stanton et al., 2012; Koarai et al., 2015). In addition, the responses of the Malvaceae representatives differed greatly. Admittedly, all three species were quite resistant to ensilage (Chapter 3.2); a response that has been assumed to be characteristic of Malvaceae (Piltz et al., 2017). However, regarding AD, the least and one of the most resistant HS species in this thesis were Malvaceae, namely *Abutilon theophrasti* and *M. sylvestris*, (Chapters 2.1, 2.4). Moreover, as the intra-species variation in responses was particularly high in the Malvaceae, the question arose as to the influence of the individual seed lots.

Indeed, the seed lot, i.e. the initial quality of the tested seed, influenced the response to the AD treatments. This was to such an extent that the response or survivability determined on the basis of a single seed lot should not be transferred 1:1 to the entire species. In the studies presented here, the responses and inactivation times of the tomato varieties differed only gradually, but this was different for the HS species. Depending on the lot, *A. theophrasti* survived AD treatments or not, *M. alcea* showed

extremely scattered viability values at AD at 35°C or not, and *M. sylvestris* was particularly AD resistant or not (Chapters 2.1, 2.3, 2.4, see also **Table 4-1**). Such differences in survival between seed lots or populations of a species have been found in several other AD and ensilage studies (Blackshaw and Rode, 1991; Westerman et al., 2012b; Aper et al., 2014; Starfinger and Sölter, 2016; Piltz et al., 2017; Tanke et al., 2019; Zhou et al., 2020; Asaduzzaman et al., 2022). In the case of wild plants in particular, the influence of the seed lot was to be expected. Their adaptation to the local conditions and climate as well as the influence of the mother plant on seed production, maturation and dormancy are already noticeable in germination experiments. Even the intra-population variation of germination responses can be large in some wild populations, e.g. due to different hormonal levels (Baskin and Baskin, 1998; Finch-Savage and Leubner-Metzger, 2006; Bentsink and Koornneef, 2008). These differences could also be effective in AD or ensilage. Therefore, in the future, ideally several seed lots of one species would be examined and results obtained formulated with reference to these seed lots.

In the further search for seed characteristics that favor survival in AD or silage, it could also be investigated whether there are relationships with characteristics that promote seed longevity in other contexts. Based on seed survival in the digestive tract of animals, it has been suggested that, besides HS, the persistence in soil and the thickness of the seed coat could be used to estimate the survivability in AD and silage (Westerman and Gerowitt, 2013 and references therein; Simard and Lambert-Beaudet, 2016). In addition, it would be interesting to compare the resistance potential with traits that are relevant for longevity in seed banking. The focus here is on the interaction of the storage environment and internal characteristics of the seed, including its structure, chemical composition and the range of protection, detoxification, and repair systems (Rajjou and Debeaujon, 2008; Nadarajan et al., 2023). According to the Seed Information Database (Royal Botanic Gardens Kew, 2022) all species tested in this thesis have an orthodox storage behavior, that means they tolerate desiccation. However, there are differences in dispersal type, as well as in the average protein and oil content. As with the information available in the literature on persistence in soil and seed coat thickness, however, no consistent relationships with survivability in AD or silage have yet emerged. For example, the known survival time in soil for the highly AD-resistant *M. officinalis* and the much faster inactivating *Chenopodium album* is around 40 years each (Bassett and Crompton, 1978; Turkington et al., 1978). For *Verbascum thapsus*, which died very quickly in all treatments, there are even reports of viable seeds from 700-year-old soils (Ødum, 1965 in Ansari and Daehler, 2000). Further, the seed coat of the Apiaceae, Asteraceae, and Poaceae, in which the pericarp provides the protective function, is often very thin (Sitte et al., 2002, p. 776). However, the Poaceae *C. dactylon* was considerably more resistant to treatments in WBs (Chapter 2.3), biogas reactors and silos (Lück, 2012) than the other Poacea, Apiaceae and Asteraceae studied. $C_4$ photosynthesis and evolutionary adaptations to warm and humid climates could play a role here (de Mol et al., 2020). There could therefore be relationships between seed survival and seed traits that await discovery. For this purpose, seed lots could be characterized with regard to their structure and chemical composition and then subjected in parallel to AD, ensilage and burial or accelerated ageing treatments.

### 4.1.2 Insights into Seed Viability Responses

The observed viability responses of the 16 plant species studied for this thesis broadened the existing data base on possible responses to AD and ensilage. Eight of these species had never been studied before (*Centaurea nigra*, *Cichorium intybus*, *Daucus carota*, *Echium vulgare*, *Malva alcea*, *M. sylvestris*, *Melilotus albus*, *Verbascum thapsus*), two of them never in an AD treatment (*Melilotus officinalis*, *Polygonum aviculare*). In addition, the number of species examined per study, i.e., under the same conditions, was 11 (AD) and 10 (ensilage), which is in the upper range of what can be found in other studies (median values of 5 and 7 in AD and ensilage, respectively).

The first insight from these data was that the expected diversity of seed viability responses to AD and ensilage was very large. Not only did the speed and extent of seed viability losses vary greatly depending on the species and seed lot, but even increases in observed viability were measured (hormetic response, see below).

The second insight was that modeling seed viability as a function of exposure time as a "dose-response curve" - an approach developed for this thesis and first published in 2016 (Chapter 2.2) - is well suited to capture the different response patterns of species and seed lots to AD. The model type was selected on the basis of several criteria, namely the Akaike information criterion, the estimated standard error of the residuals, the p-value from a lack-of-fit test (function *mselect* in Ritz and Streibig, 2016) and visually. In most cases, models based on a three-parameter log-logistic function with a lower limit of zero were the best fit. In order to establish comparability between the models for the different species and calculate inactivation times, this model was used for the most part (Chapters 2.1, 2.3, 2.4). However, it was not always the best fit. For instance, a Weibull function fitted equally good for the response of the tomatoes (Chapter 2.2). Particularly striking were the discrepancies between the log-logistic models and the observed data for those species exhibiting an increase in viability, i.e., a presumably hormetic response, during AD exposure. "Hormesis" describes the phenomenon that low doses of a stressor can have a stimulating effect, while high doses cause inhibition (e.g., Calabrese and Baldwin, 2002; Mattson, 2008; Kendig et al., 2010). In this thesis, presumably hormetic responses were observed in *M. sylvestris*, *M. albus*, *M. officinalis* (AD, Chapter 2.1) as well as in *A. theophrasti* and *C. album* (WB, Chapter 2.4). Regarding the models, the pure log-logistic function was unsuitable for the resulting biphasic viability curves with viability stimulation during short and inhibition during longer exposure to AD. An acceptable model fit was achieved by adding a parameter indicating the magnitude of the hormesis effect (Chapter 2.1). This raises the question of whether these adaptations of the (empirical) viability models actually indicate hormesis as a previously unrecognized response mechanism to AD. Can short exposure actually induce beneficial adaptive effects in seeds, while prolonged exposure is detrimental? At least it is not unlikely, as hormesis is widespread and an essential part of the response of biological systems to stressors (Agathokleous and Calabrese, 2019). In fact, and although not explicitly mentioned, presumably hormetic increases also occurred in other studies examining seeds in AD or ensilage (e.g.,

Stanton et al., 2012; Baute et al., 2016; Weller et al., 2016). However, irrespective of whether the observed responses were based on hormesis or not, it became clear that it is not yet possible to arrive at a generally applicable model type. It should therefore be avoided to use the simplest theory ("all species respond in the same way") as the basis for modeling given the current state of knowledge, as this could prevent the mechanisms involved from being adequately identified and explained. Instead, it seems advisable to optimize data collection with regard to modelling requirements. For example, viability data should continue to be collected during the exposure period, also in silages, but with more frequent measurements at the beginning and until all seeds are inactivated. This should allow to capture the diversity of possible response patterns and to proceed from well-fitting empirical to mechanistic models.

The third and final insight was that determining seed viability based on germination and metabolic activity was essential for a proper assessment of the survivability of species with dormancy (Chapters 2.1, 2.4, 3.2). It must be emphasized at this point that conclusions from studies based solely on germination tests can only be of limited significance in this context. The combination of germination tests with tetrazolium staining, however, even provided indications of seed biological response mechanisms. In particular, it was found that (physically) dormant seeds survived longer than non-dormant seeds. A high degree and in particular a great depth of (physical) dormancy appeared to be associated with a longer survival time (Chapters 2.1, 3.2). This indicates, that seed maturity affects seed survival in AD and ensilage. Furthermore, AD and ensilage appeared to induce (fatal) germination in some species. In the case of the *Melilotus* species, this may have resulted in them surviving AD treatments at 42°C better than at 35°C (Chapters 2.1, 3.2). Additionally, ensiling seemed to weaken seed vigor (Chapter 3.2). Similar effects, e.g., a delay in germination, are also known from other studies in AD or animal guts (Strauß et al., 2012; Asaduzzaman et al., 2022; Abbas et al., 2023). Moreover, the test method allowed to observe other responses that are not yet fully understood. One example is the aforementioned hormesis. Another is that in some species the proportion of metabolically active seeds reached a peak after the proportion of germinating seeds decreased, which could indicate the induction of secondary dormancy (Chapter 2.1). Finally, both in this thesis and in other studies, it was observed that seeds suffered physical damage during AD and ensilage (e.g., Chapter 2.1; Eckford et al., 2012; Strauß et al., 2012; Simard and Lambert-Beaudet, 2016). All these observations taken together raise the question of whether they are based on the presumed or other seed biological processes and to what extent these affect the establishment of the surviving seeds. Further analysis of the data obtained in this thesis, considering the viability status of all seeds, could help to answer these questions. This would mean, for example, determining the vigor of the seedlings via germination curves, as well as analyzing and interpreting the proportions and fate of seeds that were physically damaged, metabolically damaged (no or pink staining with tetrazolium) or degraded in the treatments. In order to gain deeper insights into seed biology during AD and ensilage, a future methodology should include morphological, physiological, and molecular approaches (see Chapter 4.1.4).

### 4.1.3 Seed Thermoresistance as a Proxy for Survival in AD

Studies on seed survival in AD and ensilage are time-consuming, costly, and labor-intensive. Moreover, each experimental system is unique. Low-cost, easily standardized test systems such as water-bath (WB) experiments would be one way of achieving a higher throughput and thus a greater gain in knowledge. For AD, this seemed possible because temperature was found to be a decisive factor for seed inactivation (Westerman and Gerowitt, 2013). However, it remained to be proven that the resistance of seeds to the thermal inactivation in WBs is indeed predictive of their resistance to AD. In other words, that thermoresistance is a suitable proxy of seed survival in AD.

The studies in this thesis are among the first to systematically compare seed viability between WB and AD treatments. In contrast to the other studies on tomato (Lorenz et al., 2001) and *Digitaria sanguinalis* (Zhou et al., 2020), more than one species was considered in this thesis, namely a total of eight species (ten seed lots) (Chapters 2.2, 2.3, 2.4, the related master thesis by Parzych (2016)). In addition, this thesis is the first to provide comparisons between WB treatments and AD treatments in both experimental ($AD_{ER}$) and full-scale, commercial reactors ($AD_{CR}$) that lasted longer than one day.

Despite the described expansion of available data, it remains difficult to evaluate whether the AD resistance of seeds can be determined on the basis of their thermoresistance. It is true that species that lost much of their viability in WB treatments also did so in the reactors. However, temperature was not always the sole factor determining seed inactivation in the reactors, where additional factors affected seed viability to varying degrees depending on the species (see also Chapter 4.2.1). In fact, thermal inactivation did not always correspond to a “minimum seed mortality” (Westerman and Gerowitt, 2013) in AD (e.g., *M. albus* in Chapter 2.4). Moreover, even in the WBs, the seeds exhibited complex responses (e.g., *A. theophrasti* and *C. album* in Chapter 2.4).

At this state of knowledge, thermoresistance cannot be used as a reliable, meaningful proxy of seed survival in AD. However, screenings in WBs can be carried out to get an idea of whether the seeds of certain species could be resistant to AD. But a reliable, quantitative statement cannot be derived from this. The extent to which temperature contributes to inactivation remains unclear, particularly for HS species, which responded very variably and were not completely inactivated. Comparing WB and $AD_{ER}$, Parzych (2016) hypothesized that the inactivation of HS species is driven by temperature and other factors in a first phase and solely by temperature in a second phase. To prove this, the experimental design and modeling would have to be adapted. Further optimization of WB tests should aim to predict seed survival in $AD_{CR}$, as $AD_{ER}$ and $AD_{CR}$ treatments appear to affect seeds differently and seed survival is particularly important in agricultural practice. It would be useful to develop a "transfer formula" from WB to $AD_{CR}$ results (Chapter 2.4). However, it is questionable whether this is feasible, as this formula is very likely to depend on the species, the seed lot, and the biogas plant. Ultimately, extrapolations from lab- to full-scale systems must continue to be viewed with extreme caution and the question of what the AD resistance of seeds is based on must be answered.

### 4.1.4 Approaches for a Future Methodology

The responses of seed viability to AD and ensilage recorded in this thesis were very diverse and interestingly not limited to loss of viability. The methodology used additionally provided initial indications of seed biological mechanisms. Future studies should aim to be more cost-effective, even more systematic, and more conclusive in terms of causal relationships between seed responses and treatment conditions. In this respect, approaches have already been outlined in the previous chapters 4.1.1 - 4.1.3). Based on the key points of the methodology mentioned in the introduction, some aspects are emphasized again below and others are added.

**Species selection.** An important finding from this thesis was that not only the relevance of the species in the context of biogas production, but also the characteristics of the tested seed lots should be considered. In this regard, it should be noted that the seeds used were dried, cleaned, and stored prior to the treatments in AD and ensilage. In addition, the seed viability changed during storage and seed age played a role in the survival probability. As with Mt. Pleasant and Schlater (1994), the question therefore arises as to what significance the results of this thesis have for the survival of the freshly harvested seed that may enter agricultural AD and ensiling processes. Based on this thesis, it can be assumed - but has yet to be proven - that the resistance of the matured seed used exceeds that of fresh seed. If this holds true, the results obtained could be considered an estimate of the potential maximum survival probability of the species/seed lots tested.

**Treatments.** It is challenging to study and interpret the relative importance of seed traits and treatment conditions for seed survival. For future studies, it would be useful to keep one of these two variables constant. Regarding seed traits, a model species or, based on the results of this thesis, a pair of an NHS and HS model species could be used. Given the wide variety of responses, however, it is difficult to select these - and this thesis cannot provide them due to the limited number of species/seed lots studied and the partly incomplete viability curves. Nevertheless, it must be clearly stated that tomato, which is currently used as the phyto-hygiene indicator organism for digestion and composting plants in Germany (Bundesministerium für Umwelt, Naturschutz, nukleare Sicherheit und Verbraucherschutz, 1998), is definitely not suitable as a model species for AD and ensilage. It was not even the most resistant NHS species in this thesis. With regard to the effects of the treatment conditions, attempts should be made to refine the characterization (and thus also the reproducibility) of the AD and ensiling systems used. The aim would be to find consistent effects of certain factors on seeds despite the individual character of biogas plants (Theuerl et al., 2018) and silos (see Chapters 4.2.1, 4.2.2).

**Determination of seed viability.** Determining seed viability via the combination of germination tests and tetrazolium staining provided first insights into seed biological response mechanisms. However, it is far from clear what actually happens in the seeds and how they maintain their viability when exposed to AD or ensilage. Investigations at various levels can help to answer these questions. First of all, it could be tested whether surviving seeds also establish under real field conditions, i.e. whether the results

obtained by viability determinations are relevant in practice. Secondly, physical characteristics of seeds (e.g. size, shape, seed coat thickness) are described to be related to persistence in soil and survival of predation (e.g., Bekker et al., 1998; Gardarin et al., 2010; Fricke and Wright, 2016). Thus, they may also play a role in AD or silage. Third, non-invasive methods for seed viability assessments would provide the opportunity to record true time series of individual seeds. Suitable techniques measure oxygen influx at the seed surface with micro-optodes, seed chemical composition via Fourier transform near-infrared (FT-NIR) spectroscopy, and volatile compounds via gas-chromatography (reviewed by Nadarajan et al., 2023). However, none of these methods have yet been tested on wild plant species, and implementation into the sampling routine in reactors and silos is likely to be a major challenge. Finally, analyses at the molecular level could provide information on which molecules or cell components are damaged by AD or ensiling, leading to the vigor decline and viability loss. It could also be investigated whether hormesis-associated processes were actually induced in the particularly resistant HS species. However, these analyses can be tedious, as the extraordinary longevity of the seeds is difficult to explain even in soil (cf. Rajjou and Debeaujon, 2008).

**Statistical analysis of seed survival.** The dose-response curve approach used was well suited to analyze seed viability as a function of exposure time in AD. The suitability for ensilage treatments remains to be demonstrated. The further development of the approach aims to capture the full diversity of responses and gain a mechanistic understanding, and includes the optimization of data collection and modelling (Chapter 4.1.2). The possibility of extrapolating the models is addressed in Chapter 4.2.1.

## 4.2 Seed Inactivation through Anaerobic Digestion and Ensilage

The second focus of this thesis was to determine whether seeds of the investigated species could be inactivated by AD or ensilage treatments. More precisely, to what extent, at what time and through which process parameters inactivation may be achieved. This involved confirming, refuting, or identifying relationships between selected parameters and seed inactivation. The intention was to overcome the "black box" perspective of treatments often seen in seed survival studies.

### 4.2.1 Seed-killing Efficacy of AD

The seed-killing efficacy (*SKE*) of AD in the commercial and experimental reactors ($AD_{CR}$ and $AD_{ER}$) and water-baths (WBs) differed considerably between the species and seed lots. They covered the full range from 0 to 100% (**Table 4-1**) - and even exceeded it in the case of *M. sylvestris* (increased viability after 36 days in $AD_{ER}$, Chapter 2.1). As expected, most of the NHS species were inactivated faster and more strongly than the HS species. In fact, almost all NHS species were completely killed within the 36-day exposition to AD treatments. Nevertheless, there were exceptions (*C. album*, *C. dactylon* and *L. esculentum*), and the overlap between more AD-resistant NHS species and less AD-resistant HS species (e.g., *A. theophrasti*, **Table 4-1**) was explicitly mentioned for the first time in this thesis (Chapter 2.1). In addition, the *SKE*s for the particularly AD-resistant HS species *M. sylvestris*, *M. albus* and *M. officinalis* were below 40%, which is the lowest value of all species tested under similar conditions (**Table 4-1**, Chapters 2.1, 2.4).

In terms of process parameters, exposure time and operating temperature were expected to be the main factors for the inactivation of seeds in AD, with longer exposure at higher temperatures having a stronger inactivating effect (Westerman and Gerowitt, 2013). Essentially, these relationships could be confirmed for the species and the mesophilic temperature range investigated in this thesis. However, the effects of time and temperature were not consistent. There was a high variability in the response of the species (Chapter 4.1.2) and at least tendential exceptions to the rule. If the aim is to kill all seeds as reliably as possible, it would be sensible to investigate these exceptions.

Particular attention should be paid to the factor of exposure time. In the analyses of this thesis, the exposure time in AD was the “dose” whose relationship to the response “seed viability” was modeled (dose-response curves, see Chapter 4.1.2). This approach, which was inspired by the work of Westerman et al. (2012a) and Baute et al. (2016) and based on that of Ritz et al. (2015), has been demonstrated to be suitable to track the process of seed inactivation and - within certain limits - predict the likely timing of seed-killing. This is particularly useful with regard to the different retention times (vTI, 2009) and the occurrence of short-circuiting (Ward et al., 2008) in biogas reactors. The viability models can be used to calculate the *SKE* for each exposure time and thus estimate the possibility that viable seeds are present in the digestate. It is therefore recommended that such viability models be used and further developed in future studies. The aim of the further development (see also Chapters 4.1.2, 4.1.4) would

be to improve the predictive power and enable extrapolation beyond 36 days. The latter is particularly relevant with regard to the decimal reduction times (*DRT*s) of the HS species (**Table 4-1**). As these species were not completely killed during the treatments, the models often resulted in extremely long *DRT*s, which indicate the high resistance potential but do not allow a realistic quantitative assessment of the inactivation time. One example is the *DRT* of 547 years for *M. albus* in a WB at 42°C (Chapter 2.2). For comparison, in soil seed banks, only a few seeds of a few species remain viable for more than 100 years (Thompson et al., 1997). For *M. albus*, 40 years have been reported (Turkington et al., 1978). Even gene banks target survival durations of "only" about 100 years in long-term storage under conventional conditions (-20 ± 4°C and 15 ± 3% relative humidity) and a maximum of about 3000 years in cryogenic storage (-170°C to -196°C) (Nadarajan et al., 2023).

Regarding the factor of temperature, *M. albus*, together with the other particularly AD-resistant HS species, was also the exception to the rule. Its example showed for the first time that an increase in (mesophilic) temperature can also reduce seed inactivation. A response that has never been observed before (**Table 4-1**; Chapter 2.1). Furthermore, depending on the species, temperature contributed to seed inactivation to different extents (Chapters 2.2, 2.3, 2.4, discussed in 4.1.3). This means that the comparison of the three test systems, i.e., the process control levels WB, $AD_{ER}$ and $AD_{CR}$, confirmed the assumption of Westerman and Gerowitt (2013) that more factors than time and temperature play a role in seed inactivation in biogas reactors. For example, thermal inactivation seemed to be the main factor for killing the seeds of *A. theophrasti*, *C. album*, *D. carota*, and *M. alcea* in $AD_{ER}$ and/or $AD_{CR}$. However, for *M. sylvestris*, *M. albus*, *M. officinalis* and tomato, seed killing in the reactors exceeded that of thermal inactivation (or was lower in the case of *M. albus* in $AD_{ER}$) (Chapters 2.2, 2.3, 2.4). Unfortunately, considerations on the identity and mode of action of the "additional mortality factors" involved (Westerman and Gerowitt, 2013) had to remain largely hypothetical. Even the unusually high degree of system comparability and number of factors recorded - compared to other studies on seed survival - could not provide any insight into this (Chapter 2.4).

Ultimately, it remains a challenge to gain a truly causal understanding of the "black box" of seed inactivation in AD, including the factors that might affect HS species. However, an important finding of this thesis is that AD in the full-scale commercial reactor killed seeds more effectively than in lab-scale systems. Regardless of whether seed-killing was faster or the inactivation curve was altered, this suggests that general process performance and chemical and micro-biological parameters play a non-negligible role. Future studies could search for differences between comparable experimental systems with different *SKE*s - such as the CR and ER in this study – to explore which factors might be effective. Starting points could be different stirring scenarios (e.g., Heyer et al., 2020) as well as characterizing the microbial community in the reactors, e.g., via molecular fingerprinting (Schwarzenauer and Illmer, 2012; Lim et al., 2018; Theuerl et al., 2018) or combined metagenome, metaproteome and metabolome analyses (Hassa et al., 2018). Suitable plant species would be those that have a certain AD-resistance and show differences between treatments in different reactors and WBs, e.g., *M. albus*.

### 4.2.2 Seed-killing Efficacy of Ensilage

The *SKE*s of the ensilage treatments differed clearly between NHS and HS species: seeds of the former were killed, those of the latter were not. As with the AD treatments, the *SKE*s of ensilage were strongly dependent on the species (**Table 4-1,** Chapter 3.2).

The approach of introducing seeds into different silage types was not new, but the species, feedstock composition and ensiling conditions were unique. It was found that the *SKE*s did not differ between the ensiled feedstocks, but there were species-specific differences between the ensiling conditions. It was confirmed that *SKE*s increased when the ensilage duration increased. However, the *SKE*s were low for the HS species compared to the average viability loss of 96% from other studies that had ensiled HS seeds for long term, i.e. more than three months (Mayer et al., 2000; Westerman et al., 2012b; Koarai et al., 2015; Simard and Lambert-Beaudet, 2016). Furthermore, the *SKE*s differed between the "ideal" silages and the silages "stressed" by lower compaction, contamination with *Clostridia* and oxygen exposure. Unfortunately, it was not possible to attribute these different *SKE*s to the biochemical characteristics of the silages. This was also due to the fact that not all plant species were inactivated to the greatest extent in the same silage type.

In a nutshell, two conclusions can be drawn from the study (Chapter 3.2): (1) ensiling has the potential to reduce the viability of seeds of wild plant species, and (2) the underlying mechanisms seem species-dependent and diverse. This shows how much research is still in its infancy when it comes to what kills which seeds in ensilage and how. However, even though no consistent effects between silage biochemistry and *SKE*s have been found so far, this approach might still help to shed light on this “black box”. This is because the existing studies on seed survival in ensilage have measured the cumulative effect of the biochemical and microbiological milieu, but can only make assumptions about the factors that are actually effective. But what if these factors were analyzed individually? The other two studies, which examined the biochemical characteristics of silage, provide examples of the knowledge that could be gained. Firstly, Weller et al. (2016) found that silages inoculated with *Lactobacillus plantarum* and *Enterococcus faecium* had a higher lactic acid content and killed seeds of *Nassella neesiana* (Chilean needlegrass, Poaceae) faster than non-inoculated silages. Secondly, Piltz et al. (2021) varied moisture contents of (artificial) silages with and without the addition of lactic and acetic acid. They concluded that the extent of inactivation of weed seeds was related to the “…available moisture content per se rather than silage acids ...” (ibid. p. 7). However, it was shown in Chapter 3.2 that in silages with comparatively high moisture contents (66 - 74%)[6], higher moisture does not equate to higher *SKE*s. It therefore appears that there are factors that have a certain probability of killing the seeds of certain plant groups. It seems reasonable to first identify these factors. Based on this, it might be possible to develop a test system that can be used to monitor seed inactivation in agricultural silages.

[6] Range of moisture contents in other studies: 32 to 77% (Woodward,1940; Overud, 2002; van Eekeren et al., 2006; Piltz et al., 2021).

### 4.2.3 Approaches for determining Seed Inactivation in the Biogas Production Chain

As a result of this thesis, it can be stated that AD and ensilage treatments can inactivate seeds of the wild plant species studied, but to very different degrees (**Table 4-1**) and, for the HS species, in a (still) unpredictable relationship with the process parameters investigated. Comparing ensilage and AD, the tendency that the *SKE*s of silages are higher than those of AD treatments observed in other studies (Blackshaw and Rode, 1991; Mayer et al., 2000; Stanton et al., 2012; Westerman et al., 2012b; Aper et al., 2014; Piltz et al., 2017; Piltz et al., 2021; Asaduzzaman et al., 2022; Asaduzzaman et al., 2023) could not be confirmed on the basis of this thesis (**Table 4-1**).

Approaches for further investigation of the effects of process parameters or biochemical factors have already been outlined above. In addition, the next logical step would be to determine the *SKE*s for the other processes in the biogas production chain (Fröschle et al., 2015; **Figure 1-1**). The following appear to be suitable starting points:

When the biomass is harvested and pre-treated for AD or ensilage, it is mechanically processed, e.g., cut, shredded, and compacted. The effects of these processes on seed viability and integrity should be determined; not least because mechanical abrasion of the seed coat (scarification) breaks dormancy in HS species (Rolston, 1978), which presumably affects their resistance to AD and ensilage. Furthermore, the extent to which storage of digestate affects seed viability is still unknown. In this context, the digestate processing practices concerning the solid fraction, such as dewatering, chemical stabilization, composting, and thermal drying (Kovačić et al., 2022), should be considered. Ideally, studies on these topics would also determine the transferability of lab-scale results to processes in agricultural practice. Finally, the interaction of all processes involved in biogas production is important, i.e., the combination of seed inactivation through harvesting, pre-treatment, ensiling, AD, digestate storage and processing. In this context, it is relevant whether the seed-inactivation potentials of the processes add up or whether one of them is decisive (see Chapter 3.2). Ultimately, the proposed investigations are aimed at a comprehensive risk analysis of seed survival in the entire biogas production chain.

**Table 4-1 | Compilation of estimated Seed-Killing Efficacies (*SKE*s) and Decimal Reductions Times (*DRT*s) for all treatments, i.e., mesophilic AD in a full-scale commercial reactor ($AD_{CR}$, Chapter 2.4) or in a lab-scale experimental reactor ($AD_{ER}$, Chapter 2.1), buffer solution in a water-bath (WB, Chapters 2.3 and 2.4), and ensilage in lab-scale silos ($E_{LS}$, Chapter 3.2). Shading highlights *SKE*s below 100% (light grey) and 50% (dark gray) as well as *DRT*s longer 36 days (medium gray).**

| Species | Family | Relevance[1] | Seed lot | | *SKE* [%] | | | | | *DRT* [days][2] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Year | Supply[1] | $AD_{CR}$ 44°C 36 d | $AD_{ER}$ 35°C 36 d | $AD_{ER}$ 42°C 36 d | WB[3] 42°C 36 d | $E_{LS}$ 16°C 240 d | $AD_{CR}$ 44°C 36 d | $AD_{ER}$ 35°C 36 d | $AD_{ER}$ 42°C 36 d | WB[3] 42°C 36 d |
| **HS species** | | | | | | | | | | | | | |
| *Abutilon theophrasti* | Malvaceae | reference \| $weed_{MO}$ | 2008 | ESP | - | 100 | 100 | - | 5 | - | 3 | <1 | - |
| | | | 2015 | GH | 99 | 86 | 99 | 88 | - | <3 | 52 | <3 | <3 |
| *Malva alcea* | Malvaceae | $biogas_F$ | 2014 | AW | - | 52 | 76 | 97 | 23 | - | 47 | >365 | 15 |
| | | | 2015 | AW | 99 | 82 | 92 | 94 | - | 2 | 77 | 18 | 15 |
| *Malva sylvestris* | Malvaceae | $biogas_F$ \| $weed_{MO}$ | 2014 | GER | - | - | - | 97 | 60 | - | - | - | 11 |
| | | | 2015 | HE | 19 | 0 | -23 | 0 | - | 288 | >365 | >365 | >365 |
| *Melilotus albus* | Fabaceae | $biogas_F$ \| $weed_M$ | 2014 | AW | 34 | 36 | 2 | 30 | 23 | >365 | >365 | >365 | >365 |
| *Melilotus officinalis* | Fabaceae | $biogas_F$ | 2014 | AW | 32 | 9 | 7 | 12 | 15 | >365 | >365 | >365 | >365 |
| **NHS species** | | | | | | | | | | | | | |
| *Ambrosia artemisiifolia* | Asteraceae | $weed_{MO}$ | 2016 | USA | - | - | - | 100 | - | - | - | - | 6 |
| *Chenopodium album* | Amaranthaceae | reference \| $weed_{MO}$ | 2014 | GER | 100 | 96 | 100 | 100 | 100 | 2 | 31 | 10 | 15 |
| *Centaurea nigra* | Asteraceae | $biogas_F$ | 2016 | GER | - | - | - | 100 | - | - | - | - | 3 |
| *Cichorium intybus* | Asteraceae | $biogas_F$ \| $weed_{MO}$ | 2014 | AW | - | 100 | 100 | - | 100 | - | 1 | <1 | - |
| *Cynodon dactylon* | Poaceae | $weed_{MO}$ | 2016 | GH | - | - | - | 75 | - | - | - | - | 63 |
| *Daucus carota* | Apiaceae | $biogas_F$ \| $weed_O$ | 2014 | AW | - | - | - | - | 100 | - | - | - | - |
| | | | 2015 | HE | - | 100 | 100 | 100 | - | - | 3 | <1 | 3 |
| *Echium vulgare* | Boraginaceae | $biogas_F$ | 2014 | AW | - | - | - | - | 100 | - | - | - | - |
| | | | 2015 | HE | - | 100 | 100 | - | - | - | 2 | <1 | - |
| *Fallopia convolvulus* | Polygonaceae | $weed_{MO}$ | 2015 | GER | - | - | - | 100 | - | - | - | - | 2 |
| *Lycopersicon esculentum* | Solanaceae | reference | 2014 | CU | - | 100 | 100 | - | - | - | 12 | 5 | - |
| | | | 2015 | BH | - | 100 | 100 | 99 | - | - | 18 | 7 | 16 |
| *Polygonum aviculare* | Polygonaceae | $weed_{MO}$ | 2015 | GER | - | - | - | 100 | - | - | - | - | 5 |
| *Verbascum thapsus* | Scrophulariaceae | $biogas_F$ | 2014 | AW | - | 100 | 100 | - | 100 | - | <1 | <1 | - |

[1] Relevance and Supply: See **Table 1-1** for details and literature sources. / [2] *DRT*s could not be calculated for the $E_{LS}$ treatments because they were only sampled at one time point. / [3] *SKE* and *DRT* values for WB treatments were taken from Chapter 2.4 where available or calculated using the models in Chapter 2.3.

## 4.3 Seed-Bearing Wild Plants as Biogas Feedstock

It is undisputed that the cultivation of wild plant mixtures for biogas production has numerous socio-ecological benefits and the potential to contribute to the success of circular bioeconomy approaches (von Cossel et al., 2019; von Cossel, 2020). In particular, their biodiversity and flowering offerings are valuable in agricultural landscapes (Altieri, 1999; Landis, 2017; Albrecht et al., 2020; Zamorano et al., 2020; Krimmer et al., 2021). In terms of economically and ecologically sustainable use, however, it must be ensured that wild plant biomass fits into the established procedures of the biogas process chain and does not cause any damage or additional costs due to weed infestation caused by surviving seeds.

### 4.3.1 Ensilability of Wild Plant Biomass

The biomass of wild plants differs from that of cultivated crops in several factors that can influence their ensilability, e.g. the content and composition of carbohydrates, the presence of epiphytes and the amount available. Knowledge about the ensilability of wild plant biomass is a prerequisite for its use for bioenergy production. This knowledge is rare, but Chapter 3.1 is one of the few studies that have contributed to it so far. It provided data on the ensilability potential, fermentation patterns and quality of lab-scale silages from a wildflower biogas mixture developed by Saaten Zeller GmbH & Co. KG (saaten-zeller.de/landwirtschaft/biogas-i).

In short, the ensilability potential and silage quality of the pure wildflower biomass were found to be acceptable and could be considerably improved if blended with maize. The quality of the wildflower-maize-silage with the mixing ratio of 33% to 67%, abbreviated FM33, would be suitable for biogas silage. The approximate values for key parameters were within the recommended thresholds, with the exception of the pH value, which was slightly too low (Kalač, 2011; Thaysen, 2011). The latter is based on the acetic acid content of just over 20 % of the total acid content, which would be borderline for forage silage (Galler, 2011), but is not critical in biogas plants (Thaysen, 2011). It may even have contributed to the fact that the specific methane yield estimated for FM33 (300 Nl $CH_4$ $kg^{-1}$) was close to that of the pure maize silage.

The recommended mixing ratio for low-loss silage storage (33% wildflower and 67% maize) could certainly be used in agricultural practice, because the mixture tested was designed to be grown as a supplement to standard biogas crops such as maize (Vollrath et al., 2016). This conclusion is consistent with that from reports from German agricultural extension and advisory services including farmers' statements that they had no difficulties ensiling the wildflower mixture (Vollrath, 2012; Ostertag and Vollrath, 2014; Frick and Pfender, 2019; Messner et al., 2019). However, it remains to be seen whether it is possible to consistently produce high-quality, mixed biogas silage from wildflower biomass in practice. This is because in practical implementation, for example, considerations must be made regarding the time of harvest (dry matter vs. carbohydrates) and the chopping length (storage density)

(Thaysen, 2011; von Cossel, 2020). Here, the discussion about the extent to which the criteria for forage and biogas silage can be clearly separated could also be interesting due to the highly variable composition of wild plant biomass. There are now reports that, in terms of forage technology, misfermented silages with high butyric acid content and a high pH value yielded significantly more biomethane and that the positive effects on biogas production persisted even after storage losses were taken into account (reviewed by Sun et al., 2021). Once these questions have been clarified and provided that a suitable ensiling technique is used, as required in all studies, it seems that ensiled wildflower biomass can indeed be integrated into established agricultural practices. However, this means that together with the biomass, the seeds of the wild plants could enter the biogas production chain.

### 4.3.2 Seed Contamination of Silage and Digestate

Silage and digestate contaminated with viable wild plant seeds could lead to their undesirable spread and possibly to their establishment as weeds. In order to assess the seed-killing efficacies determined in this thesis with regard to the risk of seed contamination of silage and digestate, two aspects must be considered:

1. AD and ensiling reduced the viability of most wild plant seeds. Thus, they counteract seed contamination and could therefore be used as a measure in integrated weed management (see Chapter 3.2). However, the reduction in viability was not synonymous with all seeds being killed. Some species survived the maximum exposure times in all conducted treatments. The species *M. albus*, *M. officinalis*, and *M. sylvestris* proved to be particularly resistant (**Table 4-1**). According to the viability models, they would survive the average retention time in German agricultural biogas reactors (91 days, vTI, 2009) or the usual storage times in silos (several months, e.g., Pahlow et al., 2003; Teixeira Franco et al., 2016).

This leads to 2. If the biomass of precisely these seed-forming wild plants is used as biogas feedstock, species capable of survival enter the biogas process chain, could contaminate the resulting silage and digestate and be spread with them. However, it cannot be concluded from the possibility of their mere survival that there is a risk of contamination of digestate and silage by viable wild plant seeds. This requires knowledge of the probability of seeds entering the biogas process chain. The use of wild plant biomass as a feedstock was of course a premise of this work. However, there is still a need for clarification regarding the quantity and quality of the seeds introduced. With regard to the sustainable use of wild plant biomass as a biogas feedstock, it is also necessary to clarify the risk posed by the surviving seeds if they are released into the environment. A comprehensive analysis of this topic is beyond the scope of this thesis. However, the following sections outline some relevant research and factors that should be considered.

#### 4.3.2.1 Survival and Entry Risk

The risk of silage and digestate containing viable seed only exists if sufficient quantities and qualities of seed enter AD and ensilage. For this seed input, the cultivation probability, biomass yield, seed load and additional seed entry ports play a role.

Cultures of (perennial) wild plants for biomass production can now be found all around the globe (e.g., Mehmood et al., 2017; Englund et al., 2020b). The cultivation of the wild plant biogas-mixture examined in this study has also already been investigated in several projects. Originally developed in southern Germany (von Cossel and Lewandowski, 2016), its cultivation is now being tested throughout Germany (e.g., Emmerling et al., 2017; de Mol et al., 2018; Tamms et al., 2020; Fachagentur für Nachwachsende Rohstoffe e.V. (FNR), 2022). The wild plant cultivation systems tested can differ in terms of their focus and management, e.g. establishing margins of annuals on maize fields or areal stands of perennials (Marzini et al., 2021). Management parameters such as standing time, harvest time and frequency as well as cutting height influence the quantity and quality of the seed entering the biogas process chain. In principle, the mixture is suitable for a standing period of more than five years (Deutscher Jagdverband et al., 2014). Commonly, the biomass is harvested once a year. Depending on the year of establishment, the harvest period ranges from mid-July to mid-September, with later harvests being preferred from an ecological point of view (Krimmer et al., 2021; Marzini et al., 2021; Krimmer et al., 2022). The cutting height is 25 cm (stubble). The regrowth of 80 cm height is included in the following year's harvest (Krimmer et al., 2021). From these parameters, it can be deduced that seeds will enter the harvested biomass. At the time of harvest (after the main flowering), the seeds of several species have already formed and are mature but still on the plant (von Cossel, 2020). The cutting height is similar to that of maize, where it has been shown that weed seeds can enter the cut biomass (e.g., Westerman and Gerowitt, 2012). This means that the seeds of higher-growing (i.e. also biomass-providing) wild plant species can also end up in the harvested biomass. With an annual cut, only young seeds, which are presumably more sensitive than those tested in this thesis (Chapter 4.1.4), will enter the process chain. However, if the plants are left uncut in the field, more mature and therefore possibly more AD-resistant plants can also enter.

Parallel to the studies on which this thesis is based, the same working group (Crop Health, University of Rostock) investigated the cultivation of the wildflower mixture in north-eastern Germany (Mecklenburg-Western, (de Mol et al., 2018; Hahn et al., 2018). In following years, these studies were continued in a second region (Brandenburg, (Tamms et al., 2020, 2021; Tamms et al., 2022). Data on seed quality, establishment of the mixture, weed infestation, biomass yield and seed load are available from these studies and could be incorporated into a risk assessment together with the survival probabilities. Three points should be mentioned here as a brief preview. (a) The species composition of the biomass changed over the standing years, and only few wildflower species became dominant. (b) In addition to the 23 sown wildflower species, a large number of different weed species established on the plots. (c) In Brandenburg, the weed seeds fell from June to mid-August. Wildflower seed rain began

mainly in mid-August, i.e. shortly before harvest in late August/early September. The dominant wildflowers *Artemisia vulgaris*, *Centaurea nigra*, *C. intybus*, and *Tanacetum vulgare* (all Asteraceae) had high seed yields. In Mecklenburg, however, *Malva* and *Melilotus* species also dominated in the second year. Among the weeds, *C. album* was the most common in Brandenburg and also had a very high seed density in the first year. Later, grass seeds (*Anthoxanthum odoratum*, *Holcus lanatus* and *Poa* spp) were more common. Other common weed species were *Elymus repens* (Poaceae), *Apera spica-venti* (Poaceae), *Erigeron canadensis* (Asteraceae) as well as species of chamomile (Asteraceae) and hornworts (Caryophyllaceae). As a first rough estimate based on the findings of this thesis, *Malva* spp. and *Melilotus* spp. and, depending on the input quantity, *C. album* may pose a risk of contaminating silage or digestate when entering the biogas process chain.

Last but not least, it should be noted that wild plant biomass will be co-ensiled or digested. Thus, weed seeds can also enter with the harvested maize biomass and may also include seeds of species capable to survive. One example, would be the HS species *Geranium* spp. (Baskin et al., 2000), which has emerged as a new weed species in maize (Pannwitt et al., 2018) - although it may not grow tall enough to get into the harvest. In addition, as in our experiments (Chapters 2.1, 2.2, 2.4), a large proportion of the biogas plants are fed on a mixture (Pavičić et al., 2022) of plant biomass and manure, i.e., plant biomass that has been pre-digested by animals. Manure can be a source of weed seed and spread itself (e.g., Dastgheib, 1989; Mt. Pleasant and Schlater, 1994; Edwards and Younger, 2006), but has also the potential to reduce weed seed load (MacLaren et al., 2018). Therefore, when quantitatively assessing the risk of seed contamination of digestate, the load of wild plant and weed seeds, the shares of the biomasses used and possible interactions between animal digestion, ensiling and AD should be taken into account.

#### 4.3.2.2 Contamination Risk and Potential Weed Problems

Contamination of silage and digestate with viable wild plant seeds only will become problematic if the seeds really pose a risk when they are released into the environment. This, however, remains to be proven. Specifically, the questions are (i) whether the seeds spread with the contaminated digestate successfully recruit vigorous seedlings, (ii) to what extent the surviving species actually compete with the crops grown on the fields fertilized with digestate, (iii) at what damage threshold they can impair the development of the crops and, if so, whether (iv) they can be controlled. At the moment, none of these questions can be answered with certainty. For this reason, (von Cossel)'s statement from 2020 that the weed potential - including that of HS species – "…that emerges from the wild plant mixture itself […] ...may […] be rather low on the farm ..." (p. 6-7), should be treated with caution. He argues that farmers considered "…the spread of hard-shelled wild plant species such as yellow melilot and common mallow […] not [to be] a serious problem" (ibid., p. 7). Further, that both species "…reach their greatest growth potential only in the second year of vegetation after sowing" (ibid.). And that wild plant mixture

"… cultivation has been and for the foreseeable future will only constitute a small part of the biogas substrate mix" (ibid).

While the points made by von Cossel (2020) are true, they represent only a fraction of the reality of wild plant cultivation, weed biology and seed resistance mechanisms, each of which is influenced by many factors. For example, von Cossel (2020) assessment is based on the reports of farmers from a single cultivation area. However, the location can have an influence on both seed quality (Finch-Savage and Bassel, 2016) and weed control. Knab et al. (2022), for instance, reported that the re-cultivation of former flowering areas can cause problems with weed control in subsequent crops. In this context, it should be noted with regard to the aforementioned HS species *Malva sylvestris* and *Melilotus officinalis* that, even if they only form and shed seeds in their second year, depending on the soil management regime, they can still enter the seed bank on the fields fertilized with the contaminated digestate and establish from there. Especially as they were particularly resistant to AD and survived ensiling (Chapters 2.1, 2.4, 3.2). Furthermore, *Malva* species are considered to be naturally tolerant to glyphosate (Michael et al., 2009; Fried et al., 2019) and *M. sylvestris* is a known weed (**Table 1-1**). In addition, unforeseeable interactions between species may occur in the fields. *Melilotus* species, for example, have allelopathic properties and are sometimes cultivated as weed-suppressing cover crops (Siyar et al., 2019; Mikhailova et al., 2022). This could also impact on the crops growing on the digestate-fertilized fields.

Finally, it should be borne in mind that climate and land use changes could have an impact on the establishment, control and, ultimately, the weed or invasive potential of plants (Walck et al., 2011; Varanasi et al., 2016; Ramesh et al., 2017). Thus, also on species that are able to survive biogas production. For instance, the two highly AD- and ensilage-resistant *Melilotus* species, *M. albus* and *M. officinalis*, were originally introduced to Alaska as forage plants and have been invading large, plant-free regions there since the 2000s (Conn et al., 2008). Further, some species potentially surviving AD and/or ensilage are expected to benefit from climate change, e.g., *Cynodon dactylon* (Chapter 2.3), *A. theophrasti* (Chapters 2 and 3), and *Datura stramonium* (Westerman et al., 2012b) due to their thermophilic nature, as well as *C. album* (Chapters 2 and 3) due to its late emergence (Nehring et al., 2013; Peters et al., 2014). In addition, *C. dactylon*, *A. theophrasti* and *D. stramonium* have reported invasive potential (Warwick and Black, 1988; Way, 2014; Haase et al., 2022). Further, the physiological and morphological adaptations of *Centaurea nigra* to increased $CO_2$ levels, higher temperatures and water stress are thought to confer a competitive advantage that could lead to more severe impacts of this North American weed in the future (Qaderi et al., 2013). Global warming has also been found to drive the invasion history of *Ambrosia artemisiifolia* in Central Europe (Mang et al., 2018). Although these two species were inactivated within a week in water baths at 42°C (Chapter 2.3), the effects described may also apply to surviving species. It is therefore not unlikely that global change-induced shifts in traits, ranges and niches may affect the weed potential of species that survive in the biogas process chain.

In view of the fact that more and more land is being cultivated with wild plants (especially in Germany) and the demand for plant biomass is increasing (e.g., Lewandowski, 2015; Englund et al.,

2020a), it is quite possible that the proportion of wild plant biomass in the biogas substrate mix will no longer be as small as it is today. Against this background, it has hopefully become clear that the question of whether AD- and ensilage-resistant wild plant seeds may pose a risk in agriculture is worth a closer look.

### 4.3.3 Conclusion for Sustainability of Wild Plant Biomass

Wild plant biomass is suitable as a feedstock for both AD and ensilage. The seeds of some species of the wildflower biogas mixture studied can survive both processes. In Europe, and particularly in Germany, the area cultivated for biomass from wild plants is increasing. This means that it is possible and probable that wild plant seeds will enter the biogas process chain and that some will leave it alive. The quantitative assessment of this contamination risk and the question of whether it translates into a weed problem requires further exploration. However, the permanent recirculation of viable, unwanted, potentially weedy seeds in biomass, silage and digestate is unlikely to be beneficial for sustainability (cf. Mancini and Raggi, 2021). The potential follow-up costs could outweigh the numerous beneficial agro-ecosystem services of wild plant cultivation (e.g., Weißhuhn et al., 2017; Janusch et al., 2021). It therefore makes sense to consider the risk of contamination of silage and/or digestate with viable seeds when developing concepts for the integration of wild plants into sustainable agriculture. Ultimately, however, “absolute sustainability is inoperable” (Lewandowski, 2015, p. 40). This means that regional and local factors may result in the "best compromise" (ibid.) for the sustainable use of wild plant biomass being to choose species whose seeds can survive AD and ensilage.

## Closing Remarks

At the beginning of this thesis was the question why the survival of plant seeds in biogas reactors and silos matters (Chapter 1.1). To close, the explanations given there will be briefly supplemented by knowledge gained in this thesis.

**Plant Seed Persistence.** It was found that seeds of some wild plant species have the potential to persist two processes of biomass-utilization, namely AD and ensilage. Seed survival resulted from the interplay of seed response mechanisms and the inactivating factors of the two processes.

**Plant Biomass in Circular Bioeconomy.** If seeds of the AD- and silage-resistant species identified in this thesis enter biogas reactors or silos, some of them could remain viable and contaminate silage and digestate. The possible consequences for cycles in agricultural, biomass-based processes should be considered.

**Biogas from Plant Biomass.** Further research is needed to clarify whether surviving seeds in silage and digestate will cause weed problems if they are released into the environment, e.g. with fertilizers or products derived from digestate.

**Seeds in Biogas Production.** Future research on seed survival during AD, ensilage or similar processes will benefit from the realization that seed responses can vary greatly. They may also benefit from the refinement of the methodology for recording these responses.

With regard to the overall goal of this thesis, it was found that the fate of seeds in plant biomass-based processes of the circular bioeconomy is far from uniform. It seems to be determined by the characteristics of the species and seed lot as well as the conditions in the biomass processing facilities. In view of the large number of possible options for agricultural management, it seems that the knowledge gained can still be expanded considerably.

## 4.4 References


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Zhou, L., Hülsemann, B., Merkle, W., Guo, J., Dong, R., Piepho, H.-P., et al. (2020). Influence of Anaerobic Digestion Processes on the Germination of Weed Seeds. *Gesunde Pflanzen* 72, 181–194. doi: 10.1007/s10343-020-00500-y

# Appendix

# Curriculum vitae

**Juliane Hahn** born 1984 in Rostock, Germany

## Academic and Professional Experience

| | |
|---|---|
| **since 2023** | **Research assistant**<br>Leibniz Institute for Agricultural Engineering and Bioeconomy \| Department Technology Assessment (ATB, Potsdam) |
| **2015-2017** | **Research associate**<br>University of Rostock \| Group Crop Health |
| **2014** | **Research associate**<br>University of Eastern Finland (Joensuu) & Natural Resources Institute Finland (Helsinki) |
| **2013** | **Research assistant**<br>University of Rostock \| Groups Crop Health, Landscape Ecology and Site Evaluation, Grassland and Fodder Science |
| **2012 - 2013** | **Research associate**<br>University of Rostock \| Groups Landscape Ecology and Site Evaluation, Crop Health |
| **2009 – 2012** | **Research associate**<br>University of Rostock \| Group Landscape Ecology and Site Evaluation |
| **2008 - 2009** | **Student Assistant**<br>University of Leipzig & Helmholtz Centre for Environmental Research (Halle (Saale)) |

## Academic Education

| | |
|---|---|
| **2009 - 2015** | **PhD student**<br>University of Rostock |
| **2010** | **International Summer School on Climate Change in the Baltic**<br>IOW Rostock-Warnemünde |
| **2009** | **Diplom Biologie (graduate degree in biology)**<br>University of Leipzig & Helmholtz Centre for Environmental Research (Halle (Saale)) |
| **2006** | **Vordiplom Biologie (undergraduate degree in biology)**<br>University of Rostock |
| **2004** | **Freiwilliges Ökologisches Jahr (voluntary ecological year)**<br>Rostock Zoological Garden |
| **2003** | **Abitur**<br>Gymnasium Reutershagen, Rostock |

# Publication Record

## Peer-reviewed articles

**Hahn J**, Westerman PR, Gerowitt B, Heiermann M (**2023**) Mesophilic, Anaerobic Digestion in a Full-Scale, Commercial Biogas Reactor Kills Seeds More Efficiently than Lab-Scale Systems. Fermentation 9, 1–19. doi: 10.3390/fermentation9050481.

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Koch M, Koebsch F, **Hahn J**, Jurasinski G (**2017**) From Meadow to shallow lake: Monitoring secondary succession in a coastal fen after rewetting by flooding based on aerial imagery and plot data. Mires and Peat 19, 1–17. doi: 10.19189/MaP.2015.OMB.188.

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Baum C, Eckhardt K-U, **Hahn J**, Weih M, Dimitriou I, Leinweber P, et al. (**2013**) Impact of poplar on soil organic matter quality and microbial communities in arable soils. Plant, Soil and Environment 59, 95–100. doi: 10.17221/548/2012-pse.

Glatzel S, Koebsch F, Beetz S, **Hahn J**, Richter P, Jurasinski G (**2011**) Maßnahmen zur Minderung der Treibhausgasfreisetzung aus Mooren im Mittleren Mecklenburg. Telma, 85–106.

Lasak S, **Hahn J**, Jurasinski G, Köhler S, Glatzel S (**2010**) "Methanfreisetzungen im Rahmen des Auftauens eines überfluteten Küstenmoors," in Aktuelle Probleme im Wasserhaushalt von Nordostdeutschland: Trends, Ursachen, Lösungen, eds. K. Kaiser, J. Libra, B. Merz, O. Bens, and R. F. Hüttl (Potsdam), 129–130.

## Presentations

**Writing author**; presenting author

**Hahn J,** Müller J, Westerman PR, Gerowitt B (2019) Weed and wildflower seeds in biogas plants – inactivation of seeds during ensiling. In: Deutsche Botanische Gesellschaft, Abstract Book "Botanikertagung 2019 – International Plant Science Conference", 15-19 September 2019, Rostock, Deutschland, p. 71.

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**Hahn J**, Köhler S, Jurasinski G, Glatzel S, Tuomivirta T, Juottonen H, Fritze H, Schumann R (2013): Greenhouse gas related microbial community of a flooded coastal brackish fen; abundance, diversity, activity. *Invited talk at Geomicrobiological and Geobiological Colloquium, German Research Centre for Geosciences, Section 4.5 Geomicrobiology, Potsdam.*

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**Hahn J** (2011) Von Mikrometer bis Gigatonne – Moore im Fokus der Klimaforschung. *Rostock´s Eleven, Wettbewerb Wissenschaft & Kommunikation, 25.-27.05.2011, Rostock.*

**Hahn J** (2011) It wasn´t me! Prozesse der Freisetzung von klimarelevanten Gasen aus einem Küstenversumpfungsmoor. *Ringvorlesung der Interdisziplinären Fakultät „Kurs auf die Wissenschaft" WS 2010/2011, Universität Rostock, Rostock.*

## Posters

**Writing author**; presenting author

**Hahn J**, Westerman PR, Heiermann M, Gerowitt B (2018) Survival probability of wildflower seeds in water baths at 42°C: A proxy for seed survival in biogas reactors? *In: Kmetijski Institut Slovenije (eds), "18$^{th}$ European Weed Research Society Symposium, EWRS 2018: New approaches for smarter weed management", Book of Abstracts, 17-21 June 2018, Ljubljana, Slovenia, p. 137. Online: http://www.ewrs2018.org/*

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## Author Contributions

The authors' contributions to the publications compiled in this thesis are indicated according to the Contributor Roles Taxonomy (CRediT, https://credit.niso.org/).

| | | CRediT role | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Chapter** | **Author** | **Conceptualization** | **Data curation** | **Formal analysis** | **Investigation** | **Methodology** | **Project Administration** | **Funding Acquisition** | **Resources** | **Software** | **Supervision** | **Validation** | **Visualization** | **Writing – original draft** | **Writing – review and editing** |
| **2.1** | **JH** | X | X | X | X | X | | | | | | X | X | X | X |
| | **PRW** | X | | | X | X | | | | | X | | | | X |
| | **FdM** | | | X | | | | | | | | | | | X |
| | **MH** | X | | | | X | X | X | X | | | | | | X |
| | **BG** | X | | | | | X | X | X | | X | | | | X |
| **2.2** | **JH** | X | X | X | X | X | | | | | | X | X | X | X |
| | **DP** | X | | | X | X | | | | | | X | | | |
| | **PRW** | X | | | X | X | | | | | X | | | | X |
| | **MH** | X | | | | X | X | X | X | | | | | | X |
| | **BG** | X | | | | | X | X | X | | X | | | | X |
| **2.3** | **JH** | X | X | X | X | X | | | | | | X | X | X | X |
| | **DP** | X | | | X | X | | | | | | X | | | |
| | **JS** | X | | | X | X | | | | | | X | | | |
| | **PRW** | X | | | X | X | | | | | X | | | | X |
| | **BG** | X | | | | | X | X | X | | X | | | | X |
| **2.4** | **JH** | X | X | X | X | X | | | | | | X | X | X | X |
| | **PRW** | X | | | | X | | | | | X | | | | X |
| | **BG** | X | | | | | X | X | X | | X | | | | X |
| | **MH** | X | X | | | X | X | X | X | | | X | | | X |
| **3.1** | **JM** | X | X | X | X | | | | | | | X | X | X | X |
| | **JH** | X | | X | X | X | | | | | | | | | X |
| **3.2** | **JH** | X | X | X | X | X | | | | | | | X | X | X |
| | **FdM** | X | X | X | X | X | | | | | | X | X | X | X |
| | **JM** | X | X | X | X | X | | | | | | X | | X | X |

**JH – Juliane Hahn**; BG – Bärbel Gerowitt; DP – David Parzych; FdM – Friederike de Mol; JS - Julia Schulz; JM – Jürgen Müller; MH – Monika Heiermann; PRW – Paula Renate Westerman.

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