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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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Abstract: 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

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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, nonhardseeded (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 insight 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. Materials and Methods

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 lambs quarters, 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, appelswilde.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.

2.2. Treatments

2.2.1. Anaerobic Digestion in a Commercial Biogas Reactor

The full-scale, commercial biogas reactor (CR, Figure 1) 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 1. However, the mean reactor temperature (42 $\pm$ 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 (Table A1). After each run, seeds were rinsed with water, transported to the laboratory in Rostock and processed within five hours after removal from the reactor.

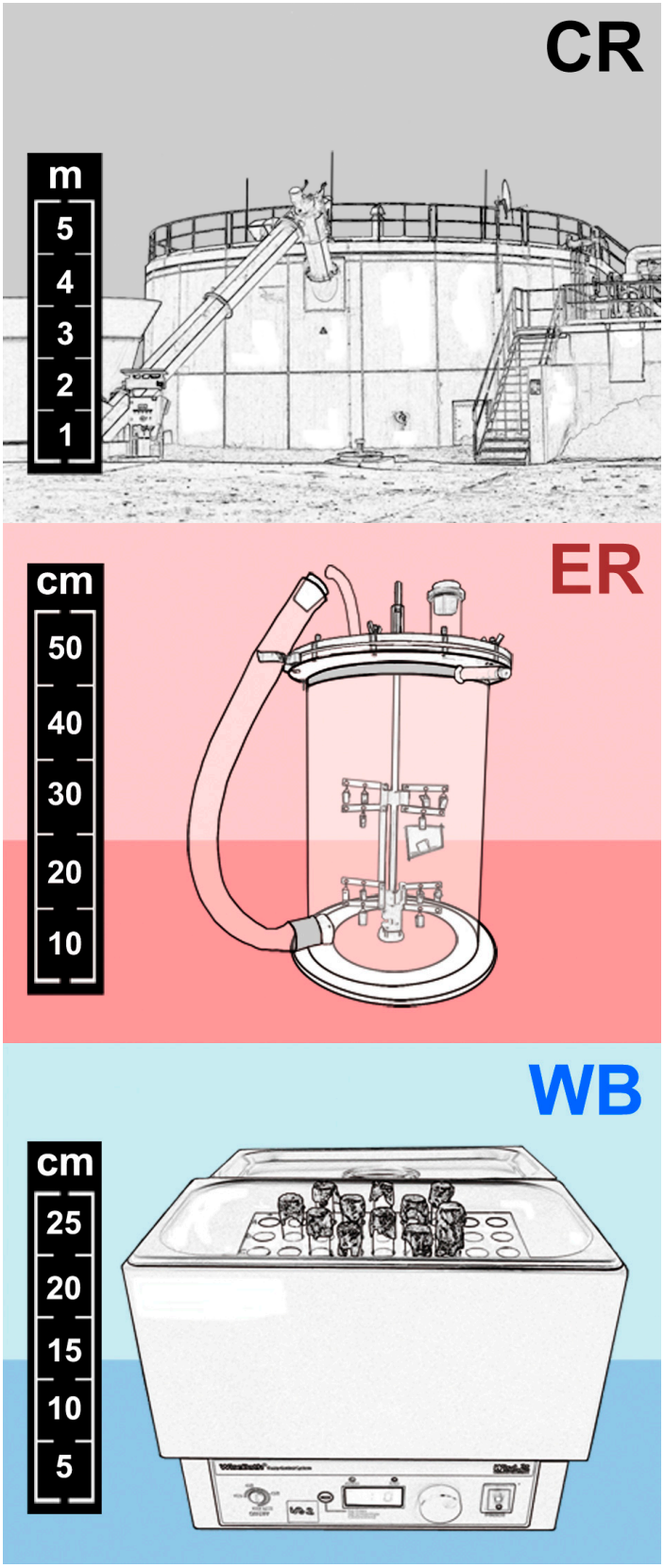

Figure 1. Schematic representation of the scales of the three treatments in which plant seed survival was tested: (CR) full-scale, commercial biogas reactor (800 m^3); (ER) lab-scale, experimental biogas-reactor (8 dm^3) with stirrer; (WB) test tubes (20 cm^3) in water baths. For details see Materials and Methods.

Table 1. 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			
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.

2.2.2. Anaerobic Digestion in Experimental Biogas Reactors

Exposure of the seeds to mesophilic AD in lab-scale, experimental biogas reactors (ERs, Figure 1) 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 1).

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 (Table A1). 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

2.2.3. 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 1) 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 (Table A1).

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) 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. 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. 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 (1)). Models were fitted specieswise, 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 Chi^2-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.

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

$V(t)$: 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), 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.

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

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 and 3). 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, Tables 3 and 4). 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 3, Tables 3 and 4). The species that was inactivated most rapidly was *A. theophasti*. At each exposure time, only individual seeds were still alive in some replicates. Therefore, no model was fitted for *A. theophasti* (Figure 2 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 3, Tables 3 and 4).

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 4). 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 and 3). 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 and 3). 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 1), no consistent effect on seed viability was observed (left column in Figures 2 and 3).

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 3).

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). 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, 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, 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 3). Seeds of the two *Melilotus* species lost the least of their viability in ER (*SKE* after 36 days < 10%, Table 4). 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 4, Figure 3). Finally, seed viability of *M. sylvestris* was almost unresponsive to ER and WB. Estimated *SKE*s were zero after 36 days of treatment (Table 4). 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 3, top row).

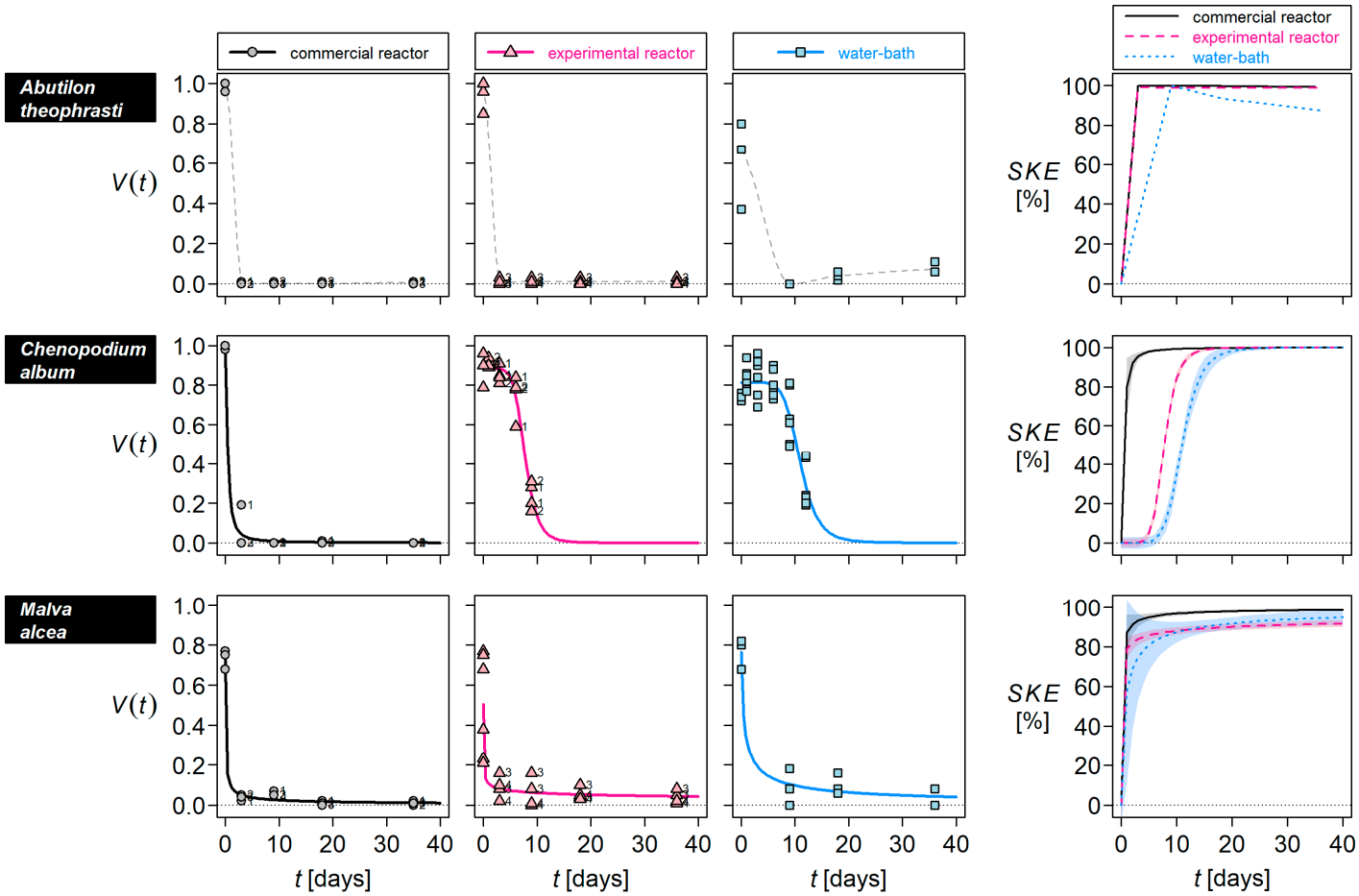

Figure 2. Proportion of viable seeds (*V*, columns on the left) and percent seed-killing efficacy (*SKE*, right column) during anaerobic digestion in a commercial biogas reactor (black), an experimental biogas reactor (pink) and in a buffer solution in a water bath (blue) for the species *Abutilon theophrasti* (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 1). *p*-values of the viability model fits (Chi^2-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.

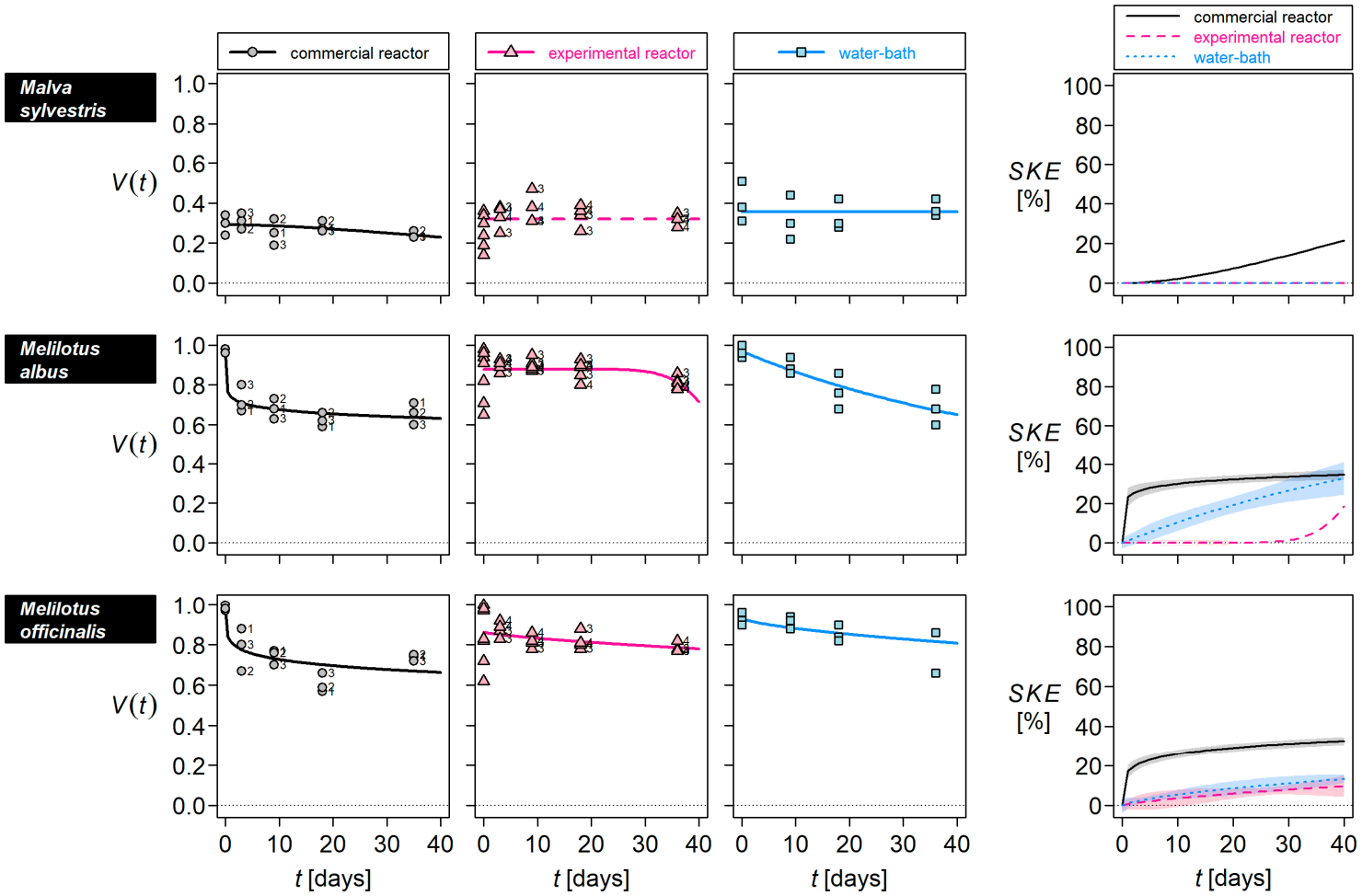

Figure 3. Proportion of viable seeds (*V*, columns on the left) and percent seed-killing efficacy (*SKE*, right column) during anaerobic digestion in a commercial biogas reactor (black), an experimental biogas reactor (pink) and in a buffer solution in a water bath (blue) for the species *Malva sylvestris* (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 1). *p*-values of the viability model fits (Chi^2-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 3. 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.

Table 4. 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

4. Discussion

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.

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 1). 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 signaling 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.

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 need 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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Appendix A

Table A1. 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 +

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