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Seed Viability of *Heracleum mantegazzianum* (Apiaceae) Is Quickly Reduced at Temperatures Prevailing in Biogas Plants

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Abstract: *Heracleum mantegazzianum* is an invasive plant species with enormous effect on ecosystems and human health. Mechanical weed management often results in large amounts of biomass. Fermentation in biogas plants can be used for disposal of this biomass contaminated with seeds and for energetic utilization, if spreading of viable seeds with fermentation residues is prevented. Our aim is to quantify the risk of seed survival in mesophilic biogas plants. Seeds were harvested at three ripening stages in central Germany. They were incubated for 0, 0.5, 1, 2, 4, and 8 days at 35 and 42 °C in water baths. Thereafter, seed viability was assessed by a tetrazolium test. Furthermore, germinative capacity of seeds which had passed an incubation of 48 h at 35 °C were tested. After eight days in water bath none of the 1199 tested seeds were viable anymore. The time until half of the seeds died (ED_{50}) ranged from 9 to 65 h, whereby high temperature accelerated the mortality. Germinative capacity was similar to the seed survival rate. The results suggest that fermentation of *H. mantegazzianum* biomass poses only a low risk of viable seed spread, if the operating temperature of the biogas plant achieves 42 °C and a high retention time is ensured.

Keywords: giant hogweed**;** biogas reactor; biomass disposal; germinative capacity; invasive plant management; water bath

1. Introduction

Heracleum mantegazzianum Sommier & Levier, giant hogweed, is one of the most successful invader weed species in Europe [1], causing biodiversity loss and reduced ecosystem functioning [2–4]. Due to its rapid, competitive growth and reproduction potential giant hogweed species have a high potential for further infestation and strong survival [5–7]. Preserving biodiversity is part of the Convention on Biodiversity, particularly with regard to taking precautions against alien species and, if necessary, controlling them [8]. In the vicinity of human settlements, control of *H. mantegazzianum* is necessary due to health hazards, because it contains photosensitive furanocoumarins that can cause skin burns and blindness [9]. In a large spatial landscape context, long-term strategic approaches are required to repress the invasion [10]. At a local scale, management tools for *H. mantegazzianum*, in particular cutting, result in a huge amount of biomass [11]. Contamination of the biomass with germinable seeds poses a risk of dispersal during disposal and promotes further infestation.

The native distribution area of the herbaceous umbellifer *H. mantegazzianum* is the western Caucasus [12,13]. After being introduced as a garden ornamental in Europe from the 1800s, it spread quickly [14–18]. The use as a pollen and nectar supplying plant [19] and to a lesser extent as material for silage in the twentieth century has led to further distribution [16]. Today, it is widespread across

temperate Europe and has also invaded North America and Australia [14,20]. In the research area of our study, central Europe, this perennial plant germinates early in the year from January to March [21]. It grows rapidly up to 5 m high [14], forming stands where it dominates other vegetation. Usually, flowering and seed production start in the third year of growth, when sufficient reserves are stored in the roots [22]. Once accomplished, flowering begins at the end of June up to the beginning of July [23] and ripening of the seeds begins in the second half of July [24]. *Heracleum mantegazzianum* is monocarpic and dies after flowering [24]. It is exclusively seed-propagated. On average, the plant produces 10,000 to 20,000 flat, elliptical seeds (Figure 1) [22,23]. The seed bank type is classified as short-term persistent [21]. In the first year, the survival rate in the seed bank was found to range about 9% [25]. After five years, the seed bank survival never exceeded 1% [26]. The seeds contain an underdeveloped embryo and are morpho-physiological dormant [21]. Before germination, the seeds need to be stratified by cold and wet conditions during winter [22]. Seed dispersal vectors are mainly water, wind, wildlife, and human activities [21]. The seeds are able to float for three days and spread quickly along rivers [27]. When growing along the riverside, it promotes the erosion potential of river banks [17]. Other common habitats are margins of woodlands and grasslands [1], ruderal places, and rubbish dumps [28].

Depending on the location, *H. mantegazzianum* can be controlled by manual and mechanical measures (cutting, ploughing), grazing [29], or with herbicides [29–31]. Cutting the root or the whole plant takes advantage of nutrient depletion in order to prevent accumulation of energy for flowering and reproduction. Early and repeatedly done, it is an effective but labor-intensive method. Umbel removal terminates the monocarpic lifecycle and is effective but risky if ripe seeds shed early. All actions have to be executed until the seed bank is empty and the root system is dead [1].

The treatment of the resulting biomass is crucial for most of the control measures. In tests, *H. mantegazzianum* was suited as feedstock for biogas production, similar to current agricultural crops [32]. Using biogas plants for disposal of the accrued biomass can generate an additional use and reduce costs. However, the introduction of a large number of seeds into the biogas plant is risky: a new seed dispersal pathway via fermentation residues must be prevented.

During the anaerobic biogas process, seeds are affected by pH value (6.8 to 8), by the operating temperature (20 to 40 °C in mesophilic fermenters), and by microorganisms and chemicals like enzymes and acids [33]. High temperatures and a long exposure time to such high temperatures are regarded as main factors for inactivation of seeds [33,34]. The time until half of the formerly viable seeds are inactivated varies between hours and weeks depending on the species [35]. After 40 days in a mesophilic digester at 37 °C, all *H. mantegazzianum* seeds had died [32]. However, a batch system does not correspond to conditions in usually used continuous flow-through biogas plants. The average retention time varies between 20 and 40 days in such systems [33], but one percent of the material leaves the biogas plant after just one day [36]. Apart from external factors, survival of seeds is influenced by the seed characteristics (e.g., traits of the seeds, like hard seed coats) and metabolic (in)activity of the embryo, like the dormancy state. Seed resistance against conditions prevailing in biogas plants may; therefore, depend on the maturity of the seeds. From the perspective of invasive weed management; therefore, it is important to determine the right time for plant cutting.

In this study, *H. mantegazzianum* seeds were sampled in central Germany at different ripening stages. Laboratory water baths at different temperatures were used for seed incubation. This incubation method has been proven to be a good proxy for conditions common in biogas plants [37]. Because microorganisms, acids, and enzymes are missing in water baths, seed survival will be overestimated rather than underestimated, which is reasonable in the context of risk assessment. Seed viability was tested after different exposure times to determine the rate of inactivation. Additionally, germination tests were run to assess the performance of incubated seeds if released in the environment.

The overall objective of our study was to assess the risk of distributing viable *H. mantegazzianum* seeds with fermentation residues. We hypothesized that:

i. Seed survival will increase when the incubation temperature decreases,
ii. Seed survival will increase when seeds are more mature,

iii. Viable seeds will germinate after stratification, independently from previous incubation.

Based on the results, we suggested management principles using biogas plants for *H. mantegazzianum* biomass disposal in order to prevent further spread, to facilitate the eradication efforts and to generate an additional biomass use.

2. Materials and Methods

2.1. Seed Sampling

Heracleum mantegazzianum seeds were collected in Meiningen, Germany (50°32′36.9″ N 10°23′54.2″ E), from July to August 2018. On 1 July 2018, two weeks after the begin of flowering of the main umbel, transparent perforated and air-permeable polyethylene bags (Crispac Bag, 330 × 500 mm, pores 2 mm) were put over the terminal flower to avoid seed losses (Figure A1). Some umbels were trimmed beforehand to fit into the bags. At the time of bagging, all bagged flowers had already set seeds. Seeds were harvested at three different times: two (early), five (intermediate), and eight (late) weeks after flowering begin. Harvested seeds were air-dried at room temperature and kept dark until the beginning of the experiment.

2.2. Seed Survival in Water Baths

Seed survival of five individual plants per harvest time was tested in water baths at 35 and 42 °C, corresponding to often prevailing operating temperatures in mesophilic digesters (accuracy: 0.1 °C; WB-6, witeg Labortechnik GmbH, Wertheim, Germany). Twenty-four hours prior to the experiment the seeds swelled in containers with water-saturated air to increase the metabolic activity of all seeds, both untreated and later treated. Then the seeds were surface sterilized with 1% NaOCl for two minutes and washed three times with distilled water under sterile conditions. Tubes were filled with 20 seeds each and 7 mL 0.5 M HEPES buffer (pH 7.0) was added. Seeds were incubated 0, 0.5, 1, 2, 4, and 8 days, respectively. For each temperature and incubation period, two replicates per individual were tested. After incubation, the viability of the seeds was determined by tetrazolium chloride staining (TTC) [38]. The seeds were pricked and incubated with 1% TTC solution at 35 °C for 24 h. Finally, the seeds were cut, and the color of the embryos was evaluated (Figure 1). Embryos were considered as viable if they had a red staining. Embryos were classified pink if radicle or cotyledon were not stained or if the whole embryo was light pink stained. We considered embryos with pink staining as "damaged". White embryos without any staining were considered as dead. Within the last category "rotten," the embryo structures were no longer detectable.

(**A**) (**B**)

Figure 1. (**A**): *Heracleum mantegazzianum* seeds, harvested 2 weeks (early), 5 weeks (intermediate), 8 weeks (late) after flowering. (**B**): Embryos stained with tetrazolium chloride for different viability categories: viable (red), damaged (pink), dead (white).

2.3. Germination after Stratification

Seeds were treated as in the seed viability test and incubated for 48 h at 35 °C in the water bath. Three individuals of each harvest group were tested with twelve replicates of 25 seeds (900 seeds per harvest group in total), except intermediate (three individuals and one mixture of different individuals with nine replicates), in total 2700 seeds. The 2700 untreated seeds (control treatment) were only surface-sterilized and washed with distilled water. Each 25 seeds were sown in 5 × 5 cm pots and covered with 0.5 cm soil. The soil was a mixture of loamy sandy soil and potting soil with peat as a main component. The pots were buried completely randomized surface flush for a 60-day-stratification in the field of the experimental station of Rostock University (54°04′04.1″ N 12°04′55.7″ E). During stratification between 21 December 2018 and 15 February 2019, precipitation was 100.9 mm, the mean temperature was 1.9 °C (20 cm above ground), and there were 27 frost nights (data from Institute for Water Management, University of Rostock). From 15 February 2019 to 01 April 2019 pots were exposed to 12 h day/12 h night intervals at 20 and 5 °C, respectively, in a climate chamber (Type KBWF-720, Binder GmbH, Tuttlingen, Germany) in a completely randomized design. Seedlings were counted and removed from the pots weekly.

2.4. Statistical Evaluation

For each temperature, at all harvest times, time-response curves were fitted simultaneously using the "drc" package (version 3.0-1) [39] provided in R (version 3.5.0) [40]. Separate models were fitted for viable embryos (red stained) and pooled viable and damaged embryos (red and pink stained). Parameters were estimated for the four-parameter log-logistic model [41]:

$$f\big(x,(b,c,d,e)\big) = c + \frac{d-c}{(1+\exp\big(b(\log(x)-\log(e))\big))} \quad (1)$$

where f is the response proportion of viable and damaged embryos depending on incubation time x, c and d are the lower and upper limits of the response, b is the steepness of the curve, and e is the effective time ED_{50}, the time until half of former viable seeds are affected. ED_{50} values were compared with t-tests, and differences were considered as significant if the p-value was <0.05.

For the statistical analysis of the germination test, the factors treatment (untreated vs. incubated) and maturity stage of the seeds (early, intermediate, late) were combined to six independent variables. The effect of these variables on the germination rate was analyzed with a general linear model with binomial distribution and a logit link function. Pairwise comparisons as implemented in the R package "multcomp" [42] were used to test the hypotheses that there was no difference in germination rates between untreated and incubated seeds within single maturity groups, and additionally that there was no difference between maturity groups within the two treatments.

3. Results

3.1. Seed Viability after Incubation

After eight days in a water bath, no viable (red stained) embryos from 1199 tested seeds were left at any temperature or harvest (Table 1). At 35 °C, only 1% survived the four-day incubation. Already after two days at 42 °C no embryos were viable anymore. At both temperatures within the intermediate and late harvested seeds there were damaged (pink stained) embryos at every time. In particular, there were still three damaged embryos after eight days at 42 °C in intermediate and late harvest, while in early harvested seeds at 42 °C there were no more viable or damaged embryos after two days.

The viability declined within the 192 h period and approached zero (Figure 2) at both temperatures but more rapidly at 42 °C. The later the seeds were harvested, the less viable they were at the start of the experiment (Table 1, Figure 2). The proportion of rotten seeds in the intermediate and late harvest was much higher than in the early harvest. Late harvested seeds contained more than half rotten embryos (Table 1). Generally, the log-logistic model fitted counted values well (Figure 2). However, the proportion of viable embryos increased after 12 h, which cannot be fitted by the log-logistic model.

Table 1. Number of embryos for each staining, harvest, duration in water bath, and temperature after tetrazolium chloride staining (TTC) solution testing.

		Early						Intermediate						Late					
Untreated		**Red**	**Pink**	**White**	**Rotten**	***n***	**Untreated**		**Red**	**Pink**	**White**	**Rotten**	***n***	**Untreated**		**Red**	**Pink**	**White**	**Rotten**	***n***
		204	120	13	63	400			47	112	74	167	400			29	97	64	210	400
12 h	35 °C	122	47	2	28	199	12 h	35 °C	25	59	29	87	200	12 h	35 °C	5	60	28	107	200
	42 °C	25	83	57	35	200		42 °C	9	45	59	88	201		42 °C	10	33	54	103	200
1 d	35 °C	87	64	12	37	200	1 d	35 °C	21	55	29	95	200	1 d	35 °C	6	47	25	122	200
	42 °C	1	19	141	40	201		42 °C	3	22	71	103	199		42 °C	4	12	72	110	198
2 d	35 °C	20	45	94	41	200	2 d	35 °C	14	38	38	110	200	2 d	35 °C	8	22	57	114	201
	42 °C	0	0	158	42	200		42 °C	0	3	91	106	200		42 °C	0	1	98	101	200
4 d	35 °C	2	12	153	33	200	4 d	35 °C	4	8	84	104	200	4 d	35 °C	0	4	86	110	200
	42 °C	0	0	163	37	200		42 °C	0	2	101	98	201		42 °C	0	4	73	123	200
8 d	35 °C	0	0	164	36	200	8 d	35 °C	0	1	90	109	200	8 d	35 °C	0	2	83	114	199
	42 °C	0	0	161	39	200		42 °C	0	3	87	110	200		42 °C	0	3	74	123	200

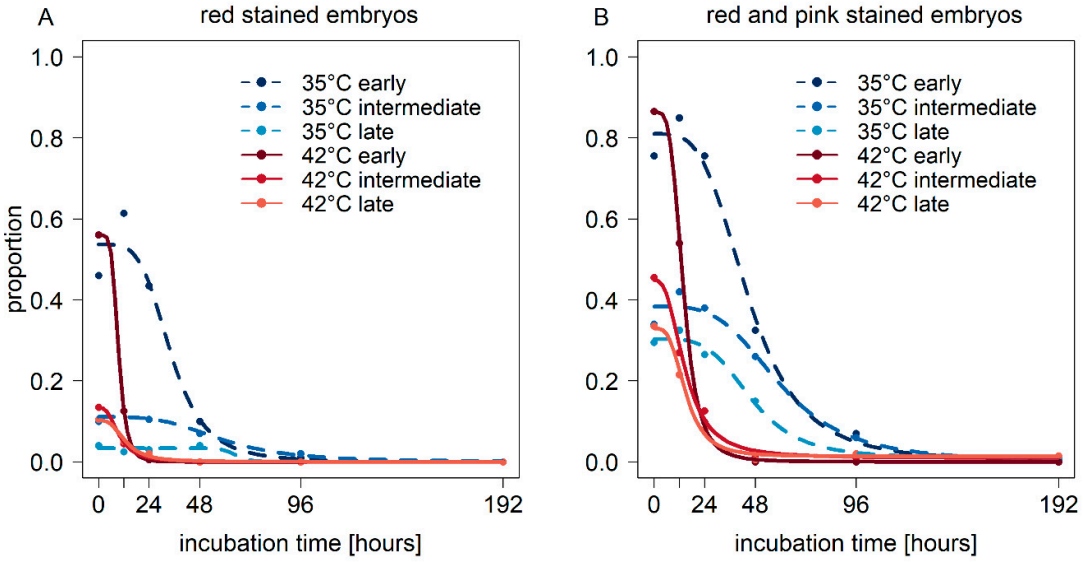

Figure 2. Survival probability of *Heracleum mantegazzianum* seeds as a function of proportion of stained seeds depending on incubation time to water bath at 35 and 42 °C, for harvest times: 2 weeks (early), 5 weeks (intermediate), and 8 weeks (late) after flowering. Survival probability for: (**A**) Red stained embryos, (**B**) Pink and red stained embryos.

After 9 to 12 h at 42 °C, half of the fully viable embryos were negatively affected (Figure 3A). After around 14 h, half of the embryos were dead (Figure 3B). At 35 °C, half of the viable seeds were eliminated after 34 h (early), 58 h (intermediate), and 65 h (late). The ED_{50} value for all seeds (viable and damaged) was reached after 44, 59, and 46 h, respectively. The differences between the two temperatures were highly significant ($p < 0.01$, *t*-test). There were significant differences at 35 °C between early and intermediate harvest ($p < 0.001$ viable (red), *t*-test; $p < 0.003$ viable and damaged (pink and red), *t*-test), while the influence of temperature at 42 °C was independent of the time of harvest.

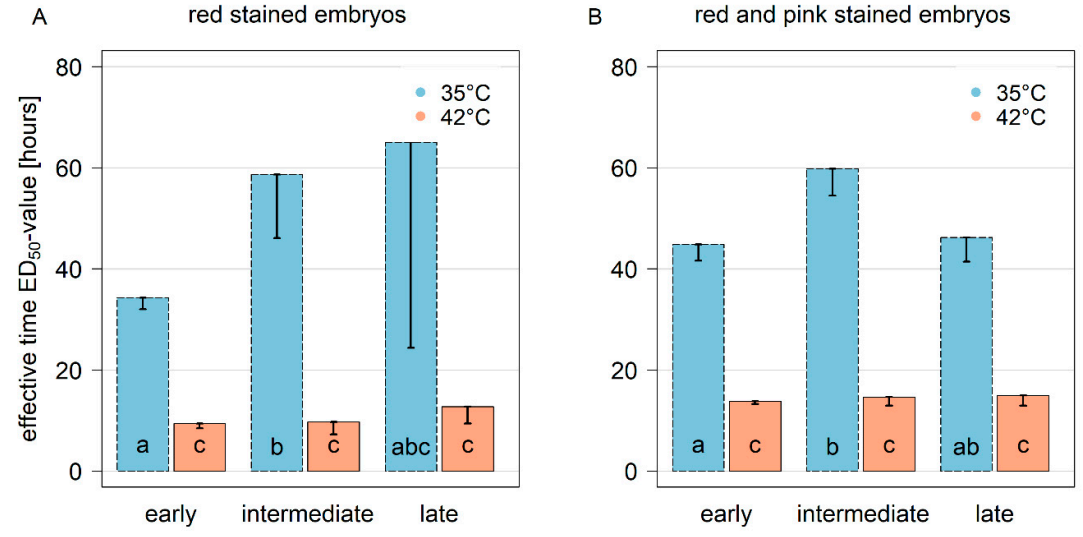

Figure 3. ED_{50} values, the time until half of former viable seeds are negatively affected, of *Heracleum mantegazzianum* seeds in hours of exposure time in water baths at 35 and 42 °C, for harvest times: 2

weeks (early), 5 weeks (intermediate), and 8 weeks (late) after flowering. (**A**) Viable (red) embryos; (**B**) Viable (red) and damaged (pink) embryos. Mean and lower part of the symmetrical standard error. Letters show significant differences between treatments ($p < 0.05$, non-parametric posthoc test).

3.2. Germination after Stratification

The first seedlings germinated two weeks after exposing them to conditions in the climate chamber. Across all harvest times, 14.2% of the untreated and 0.8% of the incubated seeds germinated. These numbers correspond to 23.3% and 7.0% viable seeds, calculated from the viability tests (Table 1). Significant differences ($p < 0.001$) were found between germination rates of incubated and untreated seeds within the same maturity stage. While different maturity stages of untreated seeds revealed significantly different germination rates ($p < 0.001$), no such effect could be detected between maturity stages of incubated seeds ($p > 0.05$). There was a tendency that a higher rate of mature seeds than of premature seeds germinated after incubation, while a higher rate of early harvested seeds germinated, if they were not incubated (Figure 4.)

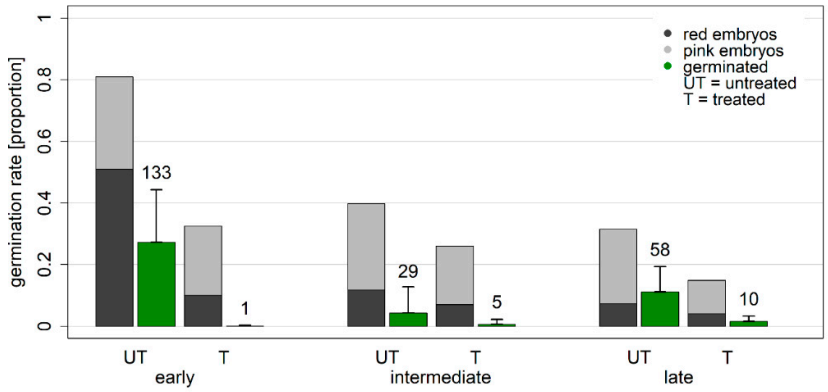

Figure 4. Comparison between actual and potential germination rate of *Heracleum mantegazzianum* seeds. Germination rate for different harvest times (2 weeks (early), 5 weeks (intermediate), 8 weeks (late) after flowering) and treatments (UT: Untreated; T: Incubated in 35 °C water bath for 48 h). Mean number of germinated seeds (green bars, $n = 900$ seeds per harvest time and treatment) and maximum number (whiskers and numbers, $n = 300$ seeds per individual). Potential germination rate calculated from viable (red) and damaged (pink) embryos (grey bars, values of water bath tests for survival after 48 h at 35 °C, Table 1, UT: $n = 400$, T: $n = 200$).

4. Discussion

To our knowledge, this is the first study dealing with the problem of *Heracleum mantegazzianum* seed survival under conditions common in biogas fermenters. Taking into account that most seeds lose their viability faster under anaerobic digestion conditions, than under hot water bath conditions only [37], biogas plants seem to be a possible disposal method for biomass resulting from control measures.

A nonlinear decline of seed viability and germination capacity has been reported for seeds which passed biogas plants under anaerobic digestion conditions [43–50] and for seeds that have been exposed to different temperatures in water baths [34]. After eight-day incubation in hot water no viable seeds occurred. It is known that seed morphology influences the survival of seeds under moist and warm conditions [43]. Hard coated seeds that have a water impermeable layer are more thermoresistant and are inactivated slower. *Chenopodium album* L. seeds for example are hard coated and cannot be fermented at all [35], whereas *H. mantegazzianum* can be classified into the group of species without hard shelled seeds and a quick inactivation rate. With only 14 h under hot water conditions, the effective time until half of the seeds are dead is short compared to other members of

the Apiaceae family. Seeds of *Daucus carota* L., a well-known representative of the Apiaceae, remain alive for about 29 h at 42 °C [43]. Generally, different temperatures affected seed survival more than harvest time in this study. We confirm our hypothesis (i) that the mortality process accelerated with increasing temperature. Other studies have shown that a rapid decrease in vitality is connected with increasing temperature [34,51]. The range of operating temperatures in mesophilic biogas plants is probably the same range which is needed to inactivate many species [44]. Even though the effect of seed inactivation was stronger at 42 °C, seeds of *H. mantegazzianum* were inactivated at low mesophilic temperatures, if they were exposed to such an environment for a longer time.

While there were no differences at 42 °C, the effect of the harvest time becomes evident at 35 °C. The seeds harvested later turned out to be more resistant to heat. This may be caused by reduced thermosensitivity that evolves during the ripening process [52]. The differences between the cutting options in time reveal a conflict in the management. On the one hand, an early harvest reduces the inactivation time in comparison to later harvested seeds, and it avoids mature seeds falling out of the flower head during later harvest. On the other hand, the high regeneration potential is problematic, because early cut plants might regenerate and flower again.

The seed viability at the beginning of the experiments and the germination rates were low, especially for the late harvest. During germination tests barely 14.2% of the untreated seeds germinated, although germination rates up to 90% had been reported for *H. mantegazzianum* by Moravcová et al. [53]. The initial seed viability decreased from harvest to harvest. Since some samples from the late harvest were visibly infested by fungi, we assume that the bags had a heat and moisture accumulating effect that promoted fungal growth. This probably promoted the reduction of vitality. This aspect should be explored in further studies. Seed viability could also have suffered from air-drying, although pronounced dry periods are characteristic in the study area at the time of seed ripening too.

Under appropriate germination conditions, a red-stained embryo should sprout a seedling. At the end of the experiment, no red seed occurred. However, pink-stained embryos were present at both temperatures after the eight days at the end of the experiment. During the TTC test, an embryo can be classified as pink for two reasons. Either the embryo has less metabolic activity or some parts of the embryo, such as radicle or cotyledon, are not stained due to lack of activity. If the former is the case, it is possible that a seedling may develop with a time delay in contrast to the other seeds. During standardized germination tests with a limited observation period this could not be confirmed. Consequently, we interpret this as supporting evidence for the assumption that damaged seeds are not able to survive a water bath treatment and following stratification with low temperatures. But as the number of seedlings in untreated late seeds exceeded the number of red-stained embryos, there is a need for further research to investigate the behavior of pink seeds.

The four-parametric log-logistic function fitted the data well, but underestimated the number of viable seeds at 12 h across all samples. The phenomenon that viability increases above the initial viability value has been reported before. An increase up to 105% of vital seeds in *Echinochloa crus-galli* (L.) P. BEAUV. was observed in water bath tests by Oechsner et al. [54]. A reason for this might be that dried seeds, even if they were exposed to water saturated air, need more time to absorb moisture for full metabolic activity.

General specific knowledge about detailed influences on seed survival is missing [33]. In our experiment we were able to show the importance of high temperatures in decreasing seed viability, similar to what has been observed in many studies on other species. So far, the focus has been on weeds in crops. Further research could focus on other invasive plants that are important in landscape conversation and; furthermore, test their potential for disposal in biogas plants. In this way a cheap disposal opportunity could be created as well as an additional use from management of invasive species in the production of gas, energy, and safe fertilizers.

5. Conclusions

For the invasive species management, our results suggest that the disposal of *H. mantegazzianum* biomass contaminated with seeds in biogas plants poses only a low risk. Nevertheless, the risk is a

function of the number of viable seeds introduced in the biogas process. These, in turn, depend on the amount of biomass, the number of seeds per plant, and the viability of seeds. In any case, the fermentation technology should be appropriate: The fermentation process should run at a high mesophilic, up to thermophilic, temperature range to ensure that no viable seeds survive. If the biogas plant cannot be set at a constant high temperature, the biomass should stay longer in the biogas plant. With an average residence time of 20 to 40 days in a flow-through biogas plant, the risk of spreading the seeds through the fermentation residues is largely reduced. Material that has only been in the biogas plant for a short time should be post-treated to ensure a total reduction in viability. Earlier harvest times facilitate seed inactivation. If resources, personal and financial, are restricted to only one measure, this measure should take place at an intermediate maturity stage as a compromise, balancing the benefit of greater inactivation in early cuttings with the danger of seed shattering for delayed cuttings. Nevertheless, it is possible to dispose of later harvested seeds. The consideration of these safety aspects is indispensable because just a few viable seeds would be able to develop a new population.

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Appendix A

Figure A1. Main *Heracleum mantegazzianum* umbel wrapped with polyethylene bag.

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