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Viability of Plant–Pathogenic Fungi Reduced by Anaerobic Digestion

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Abstract Feedstock of anaerobic digestion infected with phytopathogens could enhance the risk of spreading those pathogens to uninfested field through digestate. The viability of *Fusarium proliferatum*, *Fusarium verticillioides*, *Sclerotinia sclerotiorum*, and *Rhizoctonia solani* was investigated in anaerobic digestion experiments using infected plant material of sorghum (*Sorghum bicolor*), sugar beet (*Beta vulgaris* subsp. *vulgaris* var. *altissima*), and potato (*Solanum tuberosum* L.). Results from lab-scale reactors were confirmed in full-scale biogas plants. Anaerobic digestion under mesophilic conditions (35–42 °C) reduced most of the phytopathogens of feedstocks investigated. Thus, *S. sclerotiorum* and *R. solani* lost their viability within 6 h. In the case of sorghum, however, *Fusarium* spp. infected feedstock required a maximum of 138 h for sanitation. Thus, the risk of spreading plant pathogens with the digestate can only be decreased when the feedstock would undergo an additional treatment before anaerobic digestion or of the resulting digestate.

Keywords Biogas · Energy crops · Silage · Phytopathogens · Risk reduction

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Introduction

Anaerobic digestion technology is commonly employed as an integrated part of farming in many regions in Europe to generate methane as a sustainable and versatile renewable energy carrier. In Germany, approximately 20 % of animal wastes and biomass harvested from 1.1 million ha were processed in biogas plants last year resulting in approximately 65.5 million m^3 digestate [11]. The increasing trend towards co-digestion of slurry, dung, and crop material is closely linked to the return of treated biomass as organic fertilizer to arable land. Questions, however, arise on the potential dissemination of pathogens to uninfested fields. Current research in this area mainly focuses on human and animal pathogens [3, 7, 14, 22], whereas the behavior of phytopathogens within the biogas process have been given little attention yet [20]. Crop biomass as feedstock for anaerobic digestion can be infested by many different organisms capable of causing viral, bacterial, or fungal infections. These phytopathogens have very different physical properties with regards to their ability to survive under adverse environmental conditions. Thus, phytopathogen viability can vary between species and within the same species under different conditions of anaerobic digestion. Phytopathogenic fungi can play an important role in anaerobic digestion systems in that they are able to form resting structures enabling them to survive long periods without a suitable host.

Species of *Fusarium* are probably the most prevalent toxin-producing fungi in plants, and they are commonly found infesting cereals grown in all areas of the world. The mycotoxins they form, fumonisins and moniliformin, can also be harmful to humans and domestic animals [10]. *Fusarium verticillioides*, which can produce high levels of fumonisins, and *F. proliferatum*, which produces both fumonisins and moniliformin, are known to infest sorghum as well as other cereals [4, 23].

Sclerotinia sclerotiorum causes significant yield losses worldwide on more than 400 crop species, including bean,

peanut, rape, sunflower, and sugar beet. The fungi survive mainly as sclerotia in soil. The sclerotia germinate carpogenically forming apothecia that release airborne ascospores, which form the primary inoculum source that ultimately infect the host plant [26]. The sclerotia have high levels of resistance both to chemical and biological degradation, which permits this fungus to survive in the absence of a host for several years. Since ascospores play a critical role in the plant disease epidemics, their stability during anaerobic digestion is of particular interest due to potential spread to fields after digestion.

Rhizoctonia solani is a soil-borne fungal pathogen associated with severe diseases in potato and sugar beet. The fungus infects stems, stolons, roots, and tubers or all below ground plant organs. Early infection of seed stock causes stem canker and can significantly delay plant emergence, whereas stolon infection affects tuber number and size [25]. The sclerotia cause the tuber blemish disease called black scurf that develops on progeny tubers. Beside losses in quality, particularly for fresh market potatoes, sclerotia act as an inoculum source for subsequent crops [2, 13]. Thus, disease incidence increases with increasing soil inoculum level and potato cropping frequency [1, 6].

Crops currently preferred for the production of bioenergy in Central Europe are maize (*Zea mays*) and cereals like rye (*Secale cereale*), triticale (*Triticum*× *Secale*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and sorghum (*Sorghum bicolor* spp.) [8]. To maintain a year round supply of feedstock for biomethanation, the entire green plants are harvested, chopped, and then preserved by ensiling [9]. Owing to economic and practical reasons, most of the agricultural biogas plants run continuously as complete stirred tank digesters at mesophilic temperatures (35–42 °C) [24].

The main objective of this study was to gain insight into the viability of some selected fungal plant pathogens in sorghum, sugar beet, and potato while being processed by anaerobic digestion. The results should contribute to risk assessment of reintroducing these pathogens on farmland through application of contaminated digestates. The particular effect of anaerobic digestion was investigated in lab-scale experiments comprising the entire range of plant material and phytopathogens named above. The results obtained in lab-scale reactors were to be confirmed in a full-scale digester with a selection of plant materials and phytopathogens.

Materials and Methods

Feedstock

Investigations were conducted with a broad range of materials including sorghum, potatoes, and sugar beet. Sorghum was inserted as fresh matter as well as ensiled material.

Fresh material and silages were stored at −18 °C in small portions until usage. Liquid cattle manure and digestate from a full-scale biogas plant was used as basic feedstock for the anaerobic digestion process. Whole crop sorghum was chopped and ensiled as material for feeding the reactors. In order to ensile the material properly, it was mechanically compressed [9]. Material for feeding the lab-scale reactors was ensiled in 120 l barrels holding approximately 75 kg per barrel. The containers were sealed and stored outside at ambient temperature (frost-protected) for at least 60 days.

Infected Plant Material

Plant material used was infected with *Fusarium proliferatum*, *F. verticillioides*, *R. solani*, or *S. sclerotiorum*, respectively. Sorghum (*S. bicolor*) "Super Sile" was either cultivated in the greenhouse or under field conditions and inoculated with the pathogens 28 days before being inserted into the lab-scale reactors and full-scale digester. Inoculation was conducted by stem injection of 0.5 ml spore suspension/plant (for lab-scale reactor trials: *F. proliferatum*, 3.4×10^7 spores/ml, and *F. verticillioides*, 1.8×10^7 spores/ml; for full-scale digester trials: *F. proliferatum*, 3.56×10^6 spores/ml, and *F. verticillioides*, 2.35×10^6 spores/ml).

Silage of diseased sorghum was gained by ensiling the infected plant material 14 days postinoculation in small glass vessels of 0.5–1.5-l volumes. The jars were sealed and stored at 25 °C for 60 days (lab-scale reactor) or 21–70 days (full-scale biogas plant).

Potato tubers "Jelly" and sugar beet "Ricardo" were inoculated by applying small pieces of *R. solani* or *S. sclerotiorum* colonized agar to the tubers. The tubers and beets were incubated in a humidity chamber at 25 °C for 28 days. The agar pieces were removed prior to insertion into the reactors.

Detection of the Phytopathogens in Plant Material and Digestate

Occurrence and viability of the fungal phytopathogens was determined microscopically as well as with biological assay methods (Fig. 1).

Basic raw material was analyzed for infection in 15 aliquots prior to ensiling or insertion into the anaerobic digestion process. The samples were divided into nine samples for lab-scale and five to ten samples for full-scale biogas reactors, respectively. After digestion, inactivation of the plant pathogens was evaluated based on 25 aliquots of each sample carrier after removal from anaerobic digestion.

To detect fungal infestations, aliquots of the plant material or the digestates, respectively, were placed on a special nutrient-poor agar (SNA), according to Nirenberg [12], and evaluated by light microscopy after 14 days incubation. Identification was based on morphological characteristics

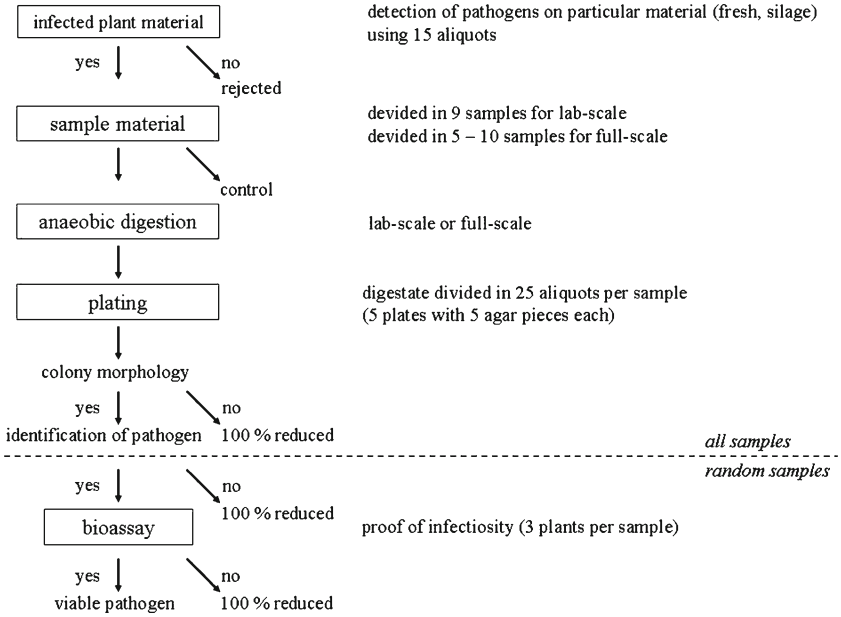

Fig. 1 Experimental set-up to determine the occurrence and viability of the fungal phytopathogens in residues of anaerobic digested plant material focusing on tracing the pathogens

of mycelia, septum, shape, and size of conidiophores and spores [5]. The pathogenicity of isolated fungal pathogens was examined by biological means with the fungal pathogens that survived the digestion process. The phytopathogens after reisolation were propagated on SNA media, inoculated on a suitable host plant, and again reisolated from the plant tissue.

Complete inactivation of particular phytopathogen was assumed when none of the aliquots reveal mycelial growth or the reisolated fungal isolates failed to be pathogenic.

Lab-Scale Reactor and Experimental Design

Continuous anaerobic digestion tests were conducted according to German Standard Procedure VDI 4630 [21]. Plant material was processed in four individual reactors each destined for a specific feedstock: potato, sugar beet, fresh sorghum, and ensiled sorghum, respectively.

Description of Lab-Scale Reactors

Tests were conducted with 10-l reactors, filled to 8 l. These reactors comprise a double-walled cylinder, which enables the maintenance of mesophilic (37 °C) conditions, and a gas tight lid, which contains a feeding tube, a tube for gas collection, and a central tube for fixing the impeller. The feeding was carried out manually on 6 days per week. Mixing was done with a central paddle impeller at a speed of approximately 80 rpm for 15 min every hour. The daily feeding amounted was approximately 100 g fresh matter equivalent to an organic loading rate of 3 kg ODM m^{-3} day^{-1} resulting in a hydraulic retention time of 80 days. Experiments were conducted under conditions characterized by the parameters given in Table 1.

Insertion of Infected Material Into Anaerobic Digestion

Up to nine samples of 0.5 g dry matter of the infected plant material were inserted into the digesters using cylindrical germ carriers made of polypropylene (Fig. 2). The carriers had a capacity of approximately 10 ml and had two openings that were covered by a polytetrafluoroethylene membrane (pore size, 1 μm; Omnipore, Millipore, Schwalbach/Ts., Germany) to avoid leakage of the phytopathogens. Germ carriers were inserted into the prepared paddles of the impeller. Each impeller could carry up to nine carriers (Fig. 2). The introduction of the germ carriers containing the diseased plant feedstock was completed following the recapping and sealing of the lid. Simultaneous removal of treated carriers and insertion of fresh carriers minimized disturbance of the digestion process.

The germ carriers were exposed for 6, 24, and 138 h, removed, and immediately analyzed or stored for 28 and 183 days at room temperature, respectively. There were three replicates of each phytopathogen, incubation time, and storage duration. The experiment was carried out at least twice. This approach resulted in exposure of as many as 54 sample carriers per phytopathogen.

Table 1 Process parameters in lab-scale reactors and full-scale biogas plant

Parameter	Lab-scale	Full-scale	
		First run	Second run
Temperature (°C)	37±1	40–41	40–41
pH	7.4–8.1	7.6	7.6–7.7
NH_4–N (g kg_{FM}^{-1})	0.9–2.5	2.3–2.6	3.2–3.3
Acetic acid (g kg_{FM}^{-1})	0.02–1.01	0.4–0.5	0.8–1.0

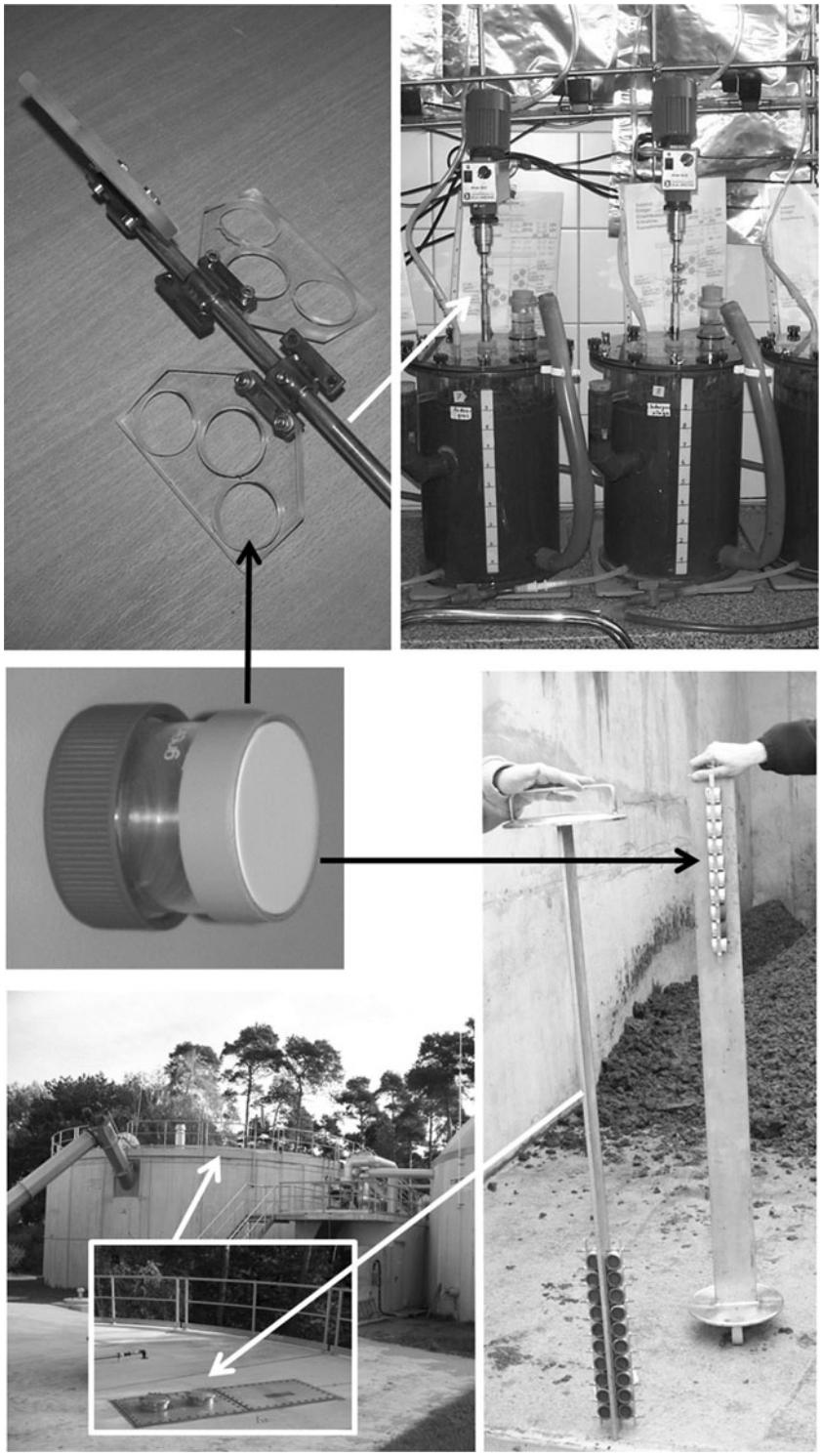

Fig. 2 Insertion of the germ carriers into the anaerobic digestion process. *Top* Impeller with three paddles (*left*) and lab-scale reactor (*right*). *Middle left* Germ-carrier container. *Bottom* Full-scale digester with inlet on the digester concrete lid (*left*) and holder with germ-carrier container (*right*)

Full-Scale Biogas Plant and Experimental Design

The digester used was located at the biogas plant Wildau-Wentdorf, Dahmetal, Germany. The digester is a single phase completely stirred tank reactor of 800 m^3 effective volume operated at a temperature of 40-41 °C and equipped with an inclined stirring mixer as well as an acentric vertical stirring mixer. It is fed with a mixture of 10 tons of maize silage, 1 ton of whole grain cereals, and 10 m^3 of pig slurry per day. Percentage of dry matter in this mixture is >30 %, which qualifies this material as a high solid content feedstock. The hydraulic retention time is 35 days.

The germ carriers were attached to the end of a 2-m long holder that was lowered into the digester from the top via an

inlet that was especially constructed for this purpose (Fig. 2). The holder was fixed into position on the digester concrete lid. It was placed 6 m from the centre and 2 m from the edge of the digester, with the tip of the holder at a depth of 0.5 m from the liquid surface and 3.5 m from the bottom, such that the membranes of the sample carriers were perpendicular to the direction of the fluid stream. The experiments with phytopathogens exposures were conducted during a stable operation of the biogas plant characterized by the parameters given in Table 1.

The germ carriers inserted into the digesters contained either sugar beet infected with *S. sclerotiorum* or fresh sorghum infected with either *F. proliferatum* or *F. verticillioides*, respectively. To determine the effect of the duration of silage production on phytopathogen viability, infected sorghum was ensiled 21, 35, 49, or 70 days prior to the insertion experiment. The effect of storage time was not tested. Additional incubation times of 48, 72, and 96 h were used for *F. proliferatum* and *F. verticillioides* infected sorghum. At least five sample carriers of each pathogen and incubation time were exposed at a time. The experiments were performed twice.

Data Statistical Analysis

Statistical analysis of infectious propagules of plant pathogens was done using the GLIMMIX procedure of SAS (SAS Version 9.3, Cary, NC, USA). For the response, binomial distribution was assumed. Multiple comparison adjustment for the p values was done by Holm–Sidak.

Results and Discussion

Anaerobic Digestion of Infected Plant Material

The first reference to the ability of anaerobic digestion to inactivate or cause mortality of phytopathogens in infested plant residue or other waste material was published in the 1980s [19]. In order to evaluate the viability of soil-borne phytopathogens during anaerobic digestion, we studied four plant pathogens that form persistent survival structures: *F. proliferatum* and *F. verticillioides* forming chlamydospores and *S. sclerotiorum* and *R. solani* forming sclerotia.

In our studies, anaerobic digestion of infected plant material led to a reduction or even complete inactivation of the pathogens tested. The degree of depletion of the pathogens during anaerobic digestion mainly depends on pathogen species and pretreatment of the crop material. This observation is complementary to that of Termorshuizen et al. [18]. They analyzed the suitability of batch anaerobic digestion, which they called anaerobic composting, for the inactivation of human and phytopathogens in fruit, vegetable, and garden waste. Whereas the human pathogenic bacteria *Salmonella typhimurium* and the phytopathogenic fungi *F. oxysporum* and the phytopathogenic bacteria *Ralstonia solanacearum* decreased below detection limit, the survival of others such as *Sclerotium cepivorum* causes significant phytosanitary problems.

It is obvious that required exposure times tend to be significantly longer in biogas plants than in small stirred tank reactors. In our case, only *S. sclerotiorum* mortality was attained within 6 h in reactors as well as in large-scale digesters. The mortality of other pathogens such as species of *Fusarium* were reached within 138 h (Fig. 3). This difference probably results from (1) type of colonizing of fungal pathogen and (2) consistence of infected feedstock. *F. proliferatum* and *F. verticillioides* colonize the plant endogenous, while *S. sclerotiorum* lives on the surface of the plant. Utilized infected sorghum plant material inserted in the digester derived from field plots and contained more lignin due to its advanced physiological age. Thus, *Fusarium* sp. propagules are longer protected from degradation and inactivation than *S. sclerotiorum* colonizing the surface of plant material. Prolonged exposure time resulted in a high reduction of infectious *Fusarium* spp. propagules (Fig. 3).

The time required to ensure phytosanitary safety of the digestates differed according to the pathogen species and pretreatment of the feedstock via ensiling. Nevertheless, one has to consider that the hydraulic retention time of digesters

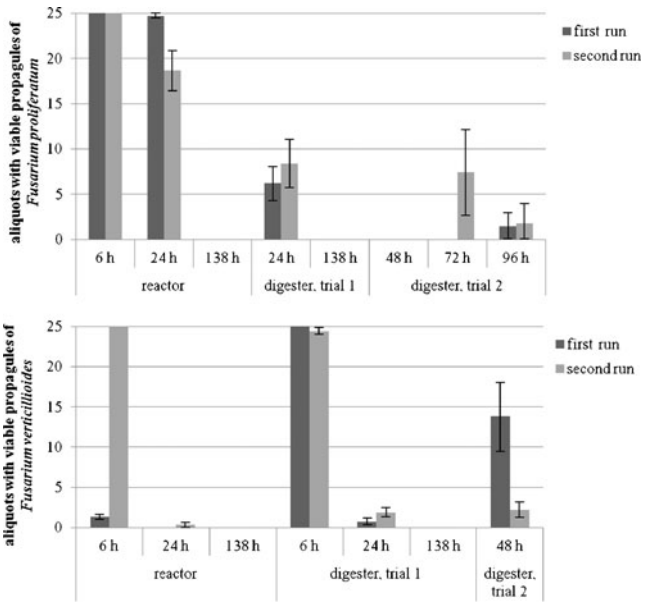

Fig. 3 Aliquots of viable *Fusarium proliferatum* (*top*) and *Fusarium verticillioides* (*bottom*) in digestates after anaerobic digestion of *Fusarium* spp. infected sorghum whole crop material in lab-scale reactors and full-scale digesters, respectively, dependent on exposure time (mean and SD); replication per run, reactor $n=2$; digester, first run $n=10$ replications; digester, second run $n=5$

(35–80 days) is always much longer than the time span that the samples were inserted (several hours to 6 days).

Effect of Pathogen Species

Sanitation of crop material infected with either *S. sclerotiorum* or *R. solani* was obtained within an exposure time of 6 h. These findings match those of Seigner et al. [16] who ascertained an exposure time of 8 h to inactivate the same two pathogens in lab-scale reactors operated at 38 °C. In contrast, sclerotia of *S. cepivorum*, another species of the genus *Sclerotinia*, were, at least in part, viable when recovered from an experimental reactor after 6 weeks of anaerobic composting [18].

Nevertheless, we could demonstrate that species of a genus are affected differently by anaerobic digestion (Fig. 3). Unlike *F. proliferatum*, most of *F. verticillioides* propagules did not survive anaerobic digestion for more than 24 h. In addition, *F. graminearum* mortality was attained within the first 24 h in lab-scale reactors operated at 38 °C [16]. In their tests, pure *F. graminearum* cultures grown on wheat grains were exposed in contrast to our investigations that used infested whole plant biomass. The viability of fungal propagules in pure cultures tends to be shorter because of the absence of surrounding plant tissue that protects them from enzymatic or chemical degradation. Schleusner et al. [15], for example, exposed species of *Fusarium*-infested maize plants to anaerobic digestion. They showed that *F. culmorum* und *F. verticillioides* were inactivated within 24 h in lab-scale reactors. They concluded that the sanitation potential of the anaerobic digestion process is mainly determined by the pathogen species and not by the crop species.

Trial 2 in the biogas plant should facilitate the determination of the exposure time required for complete inactivation. Both *Fusarium* species showed an unexpected increase of propagules after complete or almost complete inactivation in shorter exposure times (Fig. 3). Following a 48-h exposure, *F. proliferatum* could not be detected in any of the analyzed 25 aliquots of the 10 sample carriers, whereas in all five sample carriers exposed for 72 h, at least two of the 25 aliquots harbored infectious propagules of the pathogen in the second run. At an average, seven of the 25 aliquots were contaminated with *F. proliferatum*. However, after 96 h, the pathogen was detectable in only <2 of the 25 aliquots of each sample carrier in both experimental runs. Failure in anaerobic digestion could be excluded because of stable operation of the biogas plant as shown by characteristic parameters (Table 1). As the 25 aliquots investigated represent the whole content of the sample carrier, even an uneven distribution of the pathogens could not eliminate the detection. Pathogens in plant material were definitely infectious when introduced into the digester via sample carriers. It is reasonable to assume that the phenomenon observed is due to fundamental problems when working with biological samples. It is not possible to sample an identical sample carrier repeatedly over the complete duration of the trial. Thus, all carriers represent single individual samples exposed for a defined time. Nevertheless, there was a considerable level of mortality of the *Fusarium* spores during138 h of anaerobic digestion: None of the 32 sample carriers represented by in total 800 aliquots harbor any *F. proliferatum* nor *F. verticillioides* spores.

Effect of Ensiling

The storage period after ensiling that reflects the disruption of cells, directly corresponded to the inactivation of *Fusarium* spp. during anaerobic digestion. Ensiling of crop material led to increased inactivation of *F. proliferatum* and *F. verticillioides*, respectively, where the former was more strongly affected by the pretreatment (Fig. 4). A significant reduction of *F. proliferatum* viability was observed in silage stored for 35 days compared to fresh sorghum during anaerobic digestion for 24 h. Thus, in fresh sorghum plant material, *F. proliferatum* remains infectious in about one fifth of aliquots. The incorporation of ensiled sorghum

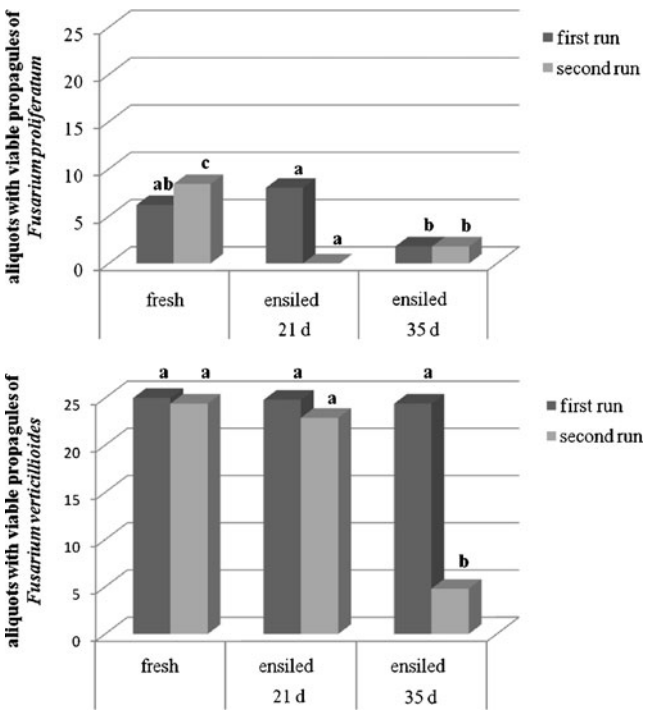

Fig. 4 Aliquots of *F. proliferatum* (*top*) and *F. verticillioides* (*bottom*) in digestates after anaerobic digestion of *Fusarium* spp. in infected sorghum whole crop material in lab-scale reactors and full-scale digesters, respectively, dependent on pretreatment of feedstock by ensiling; replication per run=10, pairwise comparisons (α=0.05) separate for pathogen and run with p value adjustment by Holm–Sidak procedure, assignment of letters per run

decreased this portion to less than one tenth of the original load. This is important as almost 90 % of plant material entering biogas plants is ensiled before [11].

Effect of Storage Period

Storage of digestate accounts for a further significant reduction in viable *Fusarium* spores in case of processing fresh sorghum feedstock (Fig. 5). A storage period of 4 weeks already resulted in a complete inactivation of *F. proliferatum* in sorghum being exposed for 6 h compared to 138 h when waiving storage of the digestate. After storage of 6 months, none of the digestates harbored viable *Fusarium* spp propagules. Comparable results were gained with *Fusarium* spp in infected ensiled sorghum. The exposure time required for a complete inactivation is downgradable to 6 h when digestates are stored for 4 weeks. No effect of storage was visible in regard to the inactivation of *S. sclerotiorum* in sugar beet and *R. solani* in potato as the pathogens lost their infectivity in all sample carries already after the minimal exposure time of 6 h. There are no reports on influence of storage of digestates or residues on the inactivation of plant pathogens, which are based on experimental runs in reactors or digesters. Schnürer and Schnürer [17] pointed to significantly decreased fungal counts of phytopathogenic fungus *Aspergillus* spp. in organic household waste after aerobic storage of anaerobic residues. The authors determined decimal reduction times (*D* value) by investigating batch cultures. Thus, residues of anaerobic reactors were transferred to small serum vials, inoculated with spore suspensions, and incubated at 37 °C. The time required to inactivate 90 % of the fungal population was 1–3 days. To figure out the effect of aerobic storage anaerobic residues from a thermophilic reactor were inoculated with

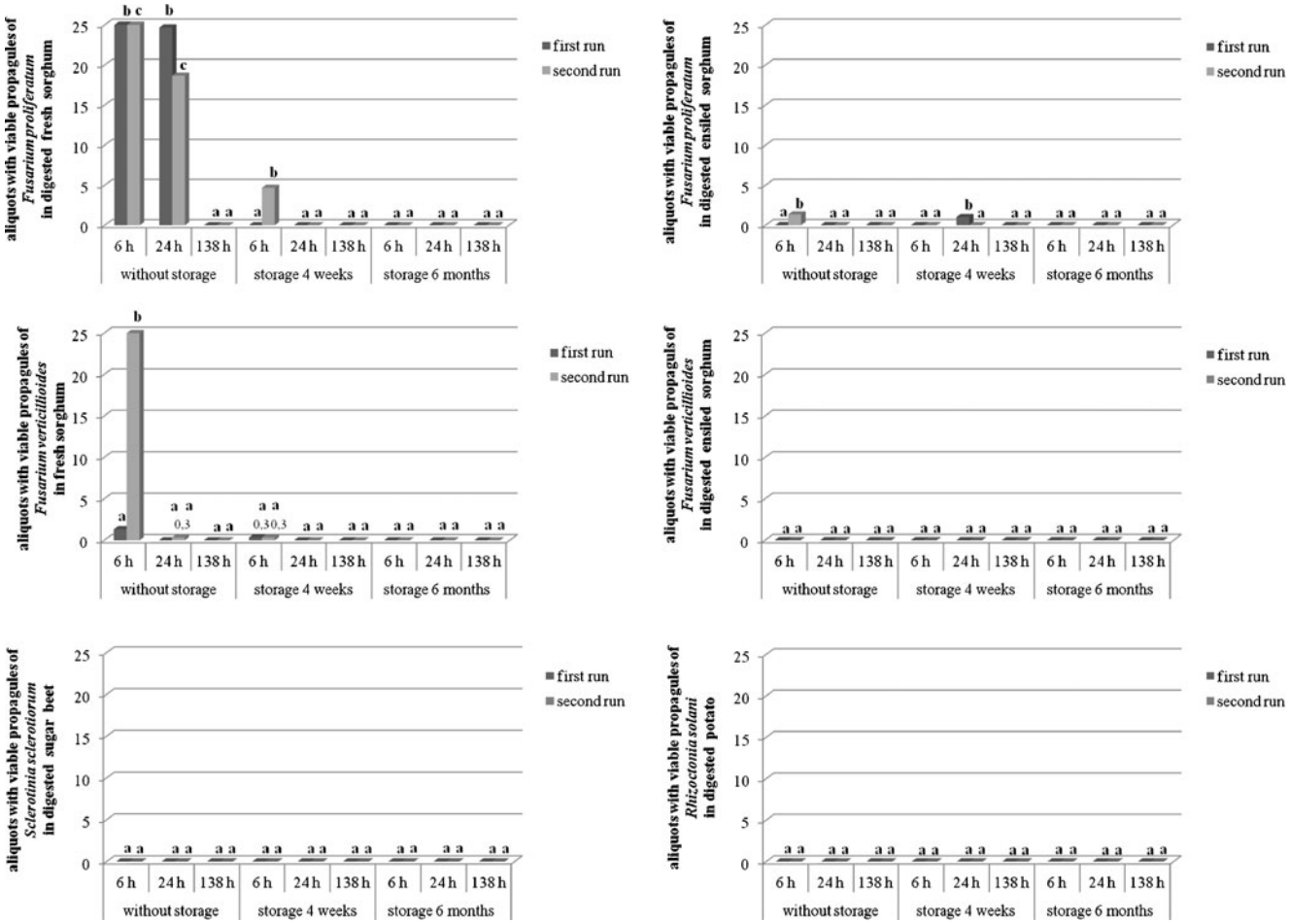

Fig. 5 Aliquots of specific fungal pathogens in digestates after anaerobic digestion of infected plant material in lab-scale reactors dependent on pretreatment of feedstock by ensiling and storage of digestates; replication per run=3, pairwise comparisons (α=0.05) separate for pathogen and run with *p* value adjustment by Holm–Sidak procedure, assignment of letters per run. *Top left* fresh sorghum whole crop material infected with *F. proliferatum*. *Top right* ensiled sorghum whole crop material infected with *F. proliferatum*. *Middle left* fresh sorghum whole crop material infected with *F. verticillioides*. *Middle right* ensiled sorghum whole crop material infected with *F. verticillioides*. *Bottom left* sugar beet infected with *S. sclerotiorum*. *Bottom right* potato infected with *R. solani*

fungal spores and stored for 1 month; *D* value was calculated with 2–5 days.

Conclusions

Beside the disposal of contaminated charges of nonmarketable crops, it is not excludable to use material infected with plant pathogens as feedstock in biogas plants. Hence, digestates from biogas plants have to be sufficiently sanitized prior to application as organic fertilizer on farmland. The time required depends on crop species, pathogen species, and the duration of digestate storage. In most cases, storage of the digestates leads to a further reduction of viability of the pathogens. Modeling of typical loads is necessary to quantify the risk of spreading viable pathogens with digestates from mesophilic operating biogas plants to farmland.

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