1. Introduction

The honeybee is of major importance for the pollination of many crops. In 2005, the worldwide economic value of insect pollination was estimated to be £105 billion (€153 billion) [1]. A decrease in the pollination activity of honeybees would have deleterious economic effects and would lower crop yield. Additionally, the direct sale of bee products is an important financial factor. According to the Food and Agriculture Organization of the United Nations (FAO), the global Gross Production Value of honey in 2012 was £4.2 billion ($6.6 billion).

Honeybees are threatened by various factors such as diseases, parasites, pesticides, environmental, and socio-economic factors [2]. One of the most severe bee diseases is the American Foulbrood (AFB) [3], the only notifiable bee disease. It is caused by infection of bee larvae with spores of the gram-positive bacterium Paenibacillus larvae. Spores of this organism are found in high numbers in an infected hive and are highly resistant to physical and chemical inactivation methods. The procedures to rehabilitate affected apiaries often result in the destruction of beehive material. In this study we assess the suitability of a double inductively coupled low pressure plasma as a non-destructive, yet effective alternative inactivation method for bacterial spores of the model organism Bacillus subtilis on beehive material. Plasma treatment was able to effectively remove spores from wax, which, under protocols currently established in veterinary practice, normally is destroyed by ignition or autoclaved for sterilization. Spores were removed from wooden surfaces with efficacies significantly higher than methods currently used in veterinary practice, such as scorching by flame treatment. In addition, we were able to non-destructively remove spores from the highly delicate honeycomb wax structures, potentially making treatment of beehive material with double inductively coupled low pressure plasma part of a fast and reliable method to rehabilitate infected bee colonies with the potential to re-use honeycombs.

Keywords: inductively coupled plasma, bacterial spores, American Foulbrood, Bacillus subtilis, honey bee, sterilization

(Some figures may appear in colour only in the online journal)
disease in many countries. The causative organism of this devastating disease is the gram-positive, facultative anaerobic bacterium *Paenibacillus larvae* (formerly *Bacillus larvae*). *P. larvae* is able to form spores, which are highly resistant to hostile conditions like nutrient-poor environments, aridity, radiation, acidity etc.

Less than 10 of these spores are sufficient for the successful infection of bee larvae during the first 24–36 h of their life [4, 5]. For the infection of larvae at an age of 48 h, already millions of spores are needed, and adult bees are not affected by *P. larvae* at all [6]. The spores are typically fed to the young larva with royal jelly, pollen and honey provided by the worker bees as food to the brood [7]. The spores then germinate and the vegetative bacteria colonize the midgut of the larva. The vegetative bacteria eventually breach the epithelial barrier of the gut and digest the larva from within. The majority of larvae are fully decomposed, before even reaching the pupa stage. Finally, the vegetative bacteria form once again spores [3].

These spores then form, in a mixture with the remains of the larva, so-called scales at the bottom of the cell. Worker bees then attempt to remove the scales, often without success [8, 9]. This cleaning behavior of the worker bees is thought to play an important role in the spreading of the disease throughout the hive [7, 9]. Scales—together with the decomposed bodies of larvae and sunken in or perforated cell lids—are clinical signs for AFB infection of a hive [10]. If not treated, an infected hive typically collapses.

AFB is a notifiable disease in many countries [2]. As soon as a beekeeper discovers AFB infestation of a hive in theses countries, an official veterinarian has to be informed. The veterinarian will then initiate provisions to prevent the spreading of the disease. Antibiotics, such as Oxytetracycline, have proven ineffective due to the appearance of resistant AFB strains [11–13] and their use in the beekeeping industry is strictly regulated in many countries, for example in the European Union [14]. Control of AFB by physical or chemical inactivation is complicated due to the resistant nature of the causative agent. Spores are notoriously difficult to inactivate [15], especially in conditions as they are found in the field. Some countries therefore have established drastic methods to control the disease. In New Zealand, an infected bee colony, including all adult bees, brood, and beehive material is typically destroyed by ignition under the New Zealand American Foulbrood Pest Management Strategy (AFB NPMS)/Biosecurity Act (appendix 1). In Germany, usually an artificial swarming procedure is applied, which removes adult bees from the infected brood and thus enables the survival of the adult bees [16]. Infectious beehive material and brood is then inactivated chemically by boiling in 3% sodium hydroxide (lye), physically through scorching by flame treatment, or by ignition. The killing efficiency of chemical inactivation methods is typically low and even scorching of wood by flame treatment is not able to reliably remove spores to prevent re-infection [17, 18]. Additionally, the outcome of these methods is often hard to control, due to the working conditions typically encountered in the field. Alternative methods, such as gamma irradiation for sterilization have been developed and are commercially available in some countries [19–21], but, to our knowledge, are usually not used in procedures ordered by official veterinarians.

The methods described above will inevitably lead to the loss of the delicate honeycomb structure of the wax and often cause the destruction, or structural weakening of wooden or plastic parts of the beehive material. Although honey can be extracted from the infected beehives and is marketable, and beeswax can be molten and then autoclaved for effective sterilization, a beekeeper affected by AFB is often faced with high economic losses. These losses can be due to the total loss of bee colonies, or significant losses in productivity of a colony when it is rehabilitated by artificial swarming, the loss of beehive material, and the substantial amount of work spent rehabilitating affected beehives.

Thus, it would be desirable to expand existing rehabilitation procedures with a non-destructive, fast and efficient inactivation method that could be used in the field to sterilize affected beehive material.

We recently used a double inductively coupled plasma reactor (DICP) to sterilize several thermally sensitive surfaces. The DICP is a low pressure reactor and conditions of the plasma treatment can be controlled in a way that the temperature of sensible surfaces stays below material-damaging temperatures during treatment [22]. We could already show that 10⁶ CFU of *Bacillus atrophaeus* spores sprayed on glass slides can be effectively killed in this reactor in a 40 s treatment in an argon/hydrogen plasma [23]. Therefore, we hypothesized that this plasma reactor could potentially be of use in the non-destructive sterilization of beehive material.

In this study, we use the DICP reactor to effectively remove spores from beehive material. In our study, we use spores of the model organism *Bacillus subtilis*. *B. subtilis* is a close relative to *P. larvae* and products derived from it have GRAS (generally regarded as safe) status. In contrast to the highly infectious *P. larvae*, *B. subtilis* can therefore be used in field studies. Our data demonstrates that the DICP reactor can be operated at temperatures, which do not affect the beeswax structure of honeycombs, while still being effective in reducing the spore count of spore treated wax and wood surfaces. We conclude that plasma treatment could provide a viable alternative or expansion to sterilization regimens currently used in veterinary practice for beehive materials during outbreaks of AFB.

2. Materials and methods

2.1. Sample preparation

2.1.1. Beeswax specimens. Beeswax was autoclaved and subsequently dried for a 5 d period at 80 °C to remove residual water from the autoclaving process. Subsequently, approximately 6 ml of the wax was poured under sterile conditions into 60 mm diameter petri dishes and allowed to cool. Wax plates were then cut in half and the halves labeled as matched pairs.
2.1.2. Wood specimens. 15 × 2 cm wooden tongue depressors (VWR, Darmstadt, Germany) were cut into 4 cm pieces. Round end pieces were discarded, fully rectangular pieces marked as matched pairs, autoclaved and dried over night at 60 °C.

2.1.3. Glass slides. Standard microscope glass slides were cleaned with dust-free tissues and 100% ethanol. They were labeled as matched pairs with a waterproof marker, autoclaved and dried over night at 60 °C.

2.1.4. Honeycomb specimens. Frames made of 1 × 2 cm milled pinewood bars measuring 9.5 × 9.8 cm were constructed by nailing 9.5 cm end bars to 7.8 cm top and bottom bars. Ponal PUR glue (Henkel AG & Co. KGaA, Düsseldorf, Germany) was used to seal and stabilize the contact surface of the joints. This wooden frame was then strung with stainless steel crimp wire through two brass eyelets reinforced holes in each sidebar. Beeswax foundation (cell size 5.4 mm) was embedded into the frames with the help of an electric embedder (figures 1(a) and (b)). Eight of these miniature frames were then fitted into standard beehive frames. The super was separated from the brood nest by a queen excluder. During a honey flow the bees constructed a drawn comb filled with honey on the beeswax foundation in the frame. Honey was removed with an extractor and the empty combs then placed in the super for another 2 d, during which residual honey was removed from the frames and cell walls damaged by the honey extraction process repaired by the bees (figure 2).

2.2. Preparation and maintenance of endospores of B. subtilis

A suspension of spores of B. subtilis 168 was prepared using a modification of Schaeffers sporulation media by a protocol of Harwood and Cutting [24]. One liter of this 2 × SG media contains 16.0 g Difco nutrient broth, 2.0 g KCl and 0.5 g MgSO₄·7 H₂O. The pH was adjusted to 7.0. Following the autoclaving process, sterile solutions were added to the 55 °C cooled media to a final concentration of 1 mM Ca(NO₃)₂, 0.1 mM MgSO₄, 1 µM FeSO₄, and 0.1% (w/v) glucose. 5 ml of this medium was inoculated with a single colony of B. subtilis 168 from an LB agar plate (10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, 10 g l⁻¹ NaCl, 1.5% agar). This culture was grown overnight in a roller drum at 37 °C and 400 µl were used the next day to inoculate 400 ml 2 × SG medium, which was aerobically incubated at 37 °C. After 48h, the mixture of vegetative cells and spores was harvested by centrifugation. The resulting pellet was washed three times with cold, sterile water. The pellet was then resuspended in 20 ml cold,
sterile *Aqua destillata* (*A. dest.*.) and stored rotating at 4 °C. The water was renewed every workday for three weeks. The content of endospores in this solution was determined by a 1 h incubation in 50% ethanol (v/v) at room temperature [25]. In parallel, a control sample was incubated in sterile *A. dest.*. Both spore samples (50% ethanol and water) were then serially diluted 1:10 in sterile *A. dest.*. 10 µl of the 10⁻¹–10⁻⁶ dilutions were pipetted on an LB agar plate. After drying, plates were incubated overnight at 37 °C and the number of colonies were counted. The amount of ethanol tolerant CFUs (=spores) and total CFUs (=vegetative cells + spores) was compared to calculate the spore content. After three weeks, the suspension was free of vegetative cells.

2.3. Application of spore suspension to surfaces

An argon driven calibrated spray nozzle (Schlick, Coburg, Germany) mounted on a scaffold was used to evenly distribute the *B. subtilis* spores on different substrates. A sample volume of 100 µl containing 1.35 · 10⁶ spores was aspirated into a filtered pipette tip and mounted to the liquid supply support of the spraying machine. The density of spores applied to surfaces was 1.35 · 10⁴ cm⁻². Used substrates were glass slides, beeswax plates, wooden specimens, and honeycombs. After each sample application, the spraying device was washed over night at 37 °C and the number of colonies were counted. The amount of ethanol tolerant CFUs (spores) and total CFUs (vegetative cells + spores) was compared to calculate the spore content. After three weeks, the suspension was free of vegetative cells.

2.4. Plasma reactor

Figure 3 shows the DICP. It consists of a stainless steel cylinder which is enclosed by two quartz plates. The inner diameter is 0.4 m and the height is 0.2 m resulting in a volume of 25 l. The reactor was evacuated by a turbomolecular pump in combination with a rotary vane pump to a base pressure of 10⁻⁴ Pa. During plasma operation, a combination of a roots pump and rotary vane pump was used to maintain a pressure of 5–10 Pa at a gas flux of up to 200 sccm. Process gases (argon, hydrogen, nitrogen and oxygen) were controlled through mass flow controllers and entered the reactor through a gas shower. The gas composition used was 125 sccm Ar, 2.5 sccm O₂, 6 sccm N₂ at 10 Pa and 20 sccm H₂ at 5 Pa. Electric power of up to 5 kW at 13.56 MHz was coupled through two copper coils at the top and bottom of the reactor. A matching network was used for equal distribution of power to each coil and to electrically match the plasma impedance to the generator. Several flanges allowed plasma diagnostics (e.g. Langmuir probe measurements). A comprehensive characterization of the reactor and more detailed description can be found elsewhere [22, 23].

2.5. Determination of surface temperature

The surface temperature of samples was determined by a PT-100 resistance thermometer. The temperature probe was soldered to a screened cable and connected to an electrical feedthrough. Resistance was measured every 300 ms. Direct temperature measurement during continuous plasma operation was not possible due to coupling of electromagnetic waves into the cable. The measurements were performed immediately after the plasma was switched off. Additionally, materials were inspected manually for noticeable changes in appearance due to thermal damage.

2.6. Sterilization in the plasma reactor

Spore coated samples were treated for specified times under different plasma conditions in the DICP reactor. To account for differences in spore coating, specimens were paired. Wax plates were marked with sterile tweezers and plates were cut
in half using a sterile scalpel before spore coating. Wood pieces and glass slides were placed right next to each other as marked pairs before spore coating. One half of these pairs was used as an untreated control sample, whereas the other half was exposed to a plasma treatment. The number of recovered spores from plasma-treated samples was compared to the number of spores from the paired, untreated sample.

2.7. Recovery of spores from glass slides and direct recovery from wax plates

The spore coated glass slides and wax plates were applied to LB agar plates for a 10 min period with subsequent incubation at 37 °C overnight.

2.8. Recovery of spores from wax surfaces

Spore count on wax surfaces was determined based on a procedure published by Bzdil [26]. Three wax pieces with a total surface of 37.7 mm² exposed to the spray were removed from each wax plate or honeycomb with the help of a sterilized 4 mm diameter cork borer. The wax pieces were transferred into an Eppendorf tube (1.5 ml) with 850 μl sterile A. dest heated to 70 °C. After adding 50 μl of 70 °C pre-warmed Tween 80, the samples were incubated for 30 min at 70 °C and 1000 rpm in a Thermomixer. During incubation, samples were thoroughly mixed on a vortex mixer every 10 min. Subsequently, a 2–4 h incubation time at room temperature followed and CFUs were determined from the aqueous phase by serial dilution as described above. Spore contamination was calculated as CFU per mm² spray exposed surface and CFU per mg beeswax, respectively. From an untreated wax sample, we typically recovered around 10⁶ CFU per mm².

2.9. Recovery of spores from wooden surfaces

Wooden samples were transferred into a 50 ml Falcon tube, together with 8.5 ml sterile A. dest and 500 μl 70 °C pre-warmed Tween 80. The incubation was carried out for 1 h, rotating at 37 °C. All samples were serial diluted 1:10 with sterile A. dest and treated as outlined above to determine the quantity of spores.

2.10. Determination of spore count on honeycomb specimens

Wax samples with a weight of approximately 100 mg each were taken as described. Honeycombs were disassembled into individual pieces to assess the wooden samples. As initial control, to account for spores present naturally on honeycombs, the spore counts of untreated honeycomb specimens were determined. For comparison, matched pairs of combs were sprayed simultaneously with a B. subtilis spore suspension. All samples were additionally treated with ethanol as described, to determine the amount of endospores. All experiments were performed in triplicates.

3. Results

3.1. Sample temperature in the DICP stays below the melting point of beeswax during conditions typical for sterilization runs

The sterilization of beehive material is challenging, but strictly required after the outbreak of bee diseases such as the AFB. Under sterilization regimens currently used in veterinary practice (i.e. thermal inactivation by ignition, autoclaving, or chemical inactivation in heated lye) the delicate wax structure of the honeycomb is inevitably destroyed. We hypothesized that a sterilization using ‘cold’ plasma generated in the DICP reactor could provide a viable, less destructive alternative.

To test this, we first determined the final sample surface temperatures reached during typical sterilization runs in this plasma reactor using a PT-100 temperature sensor, placed at the location of the sample. The starting points for our investigation were based on previous experiments performed on less challenging surfaces such as glass slides [23]. The melting point of beeswax is in the range of 62–64 °C. We, therefore, tested several conditions, aiming for a maximum of energy transferred to contaminating microbes, while in parallel avoiding the thermal deformation of beeswax containing samples. To keep the surface temperature below 62 °C, two different procedures were used: a pulsed plasma at 1 kHz and several cycles of 5 s continuous plasma with cooling times in between. In both cases an argon/oxygen/nitrogen (125 sccm Ar, 2.5 sccm O₂, 6 sccm N₂) plasma at 10 Pa and 1000 W electrical power was used. This gas mixture was shown to be effective for sterilizing B. atrophaeus spores before [27]. For the pulsed plasma, a pulse frequency of 1 kHz and a pulse duty cycle from 5–40% was used. A 10% duty cycle, which creates a series of short plasma pulses of 10 μs duration with intermittent gaps of 90 μs, heats the surface temperature to 51 °C after 450 s, when starting from room temperature. This was well below the melting range of beeswax, while higher duty cycles exceeded the melting range (figure 4(a)).

Cycles of continuous plasma create a more intense plasma, delivering more radiation and particles to the sample surface. We thus argued that a continuous plasma could be more effective in killing spores on the beehive material. However, running a continuous plasma for 45 s (effectively reaching the total plasma time of the 450 s 10% on, 90% off pulsed plasma) leads to unacceptably high sample temperatures. We therefore decided to cycle a 5 s continuous plasma 9 times with intermittent gaps, which allowed the samples to cool. Using a gap time of 20 s, we could keep sample temperatures below 56 °C (figure 4(b)).

3.2. Wax is a challenging surface

To test the applicability of our plasma reactor in the sterilization of beehive materials, we first created beeswax plates by pouring autoclaved, sterilized wax in small petri dishes. We then cut these plates in half, generating a matched pair. These matched pairs were then coated with...
a spray containing a defined amount of *B. subtilis* spores. These spores, while apathogenic, are highly similar to spores of *P. larvae*, the causative agent of AFB. For comparison of sterilization efficiency of different materials, we also coated a matched pair of glass slides with a *B. subtilis* spore suspension. We then subjected one member of the wax plate and the slide pair to a pulsed plasma (125 sccm Ar, 2.5 sccm O2, 6 sccm N2 at 10 Pa, 1 kHz 10% pulse duty cycle, 450 s), while we left the other member of the pair untreated. We then placed the wax plate and slide pairs on LB-plates. Spores will germinate on this medium and form colonies, providing us with an assessment of spores that survived the plasma treatment on the wax or glass surface. While plasma treatment significantly reduced the spore count on both surfaces, the wax plates proved to be a more challenging surface to sterilize than the glass slides (figures 5(a)–(d)).

### 3.3. A cycled, continuous plasma treatment kills spores more effectively than a pulsed plasma treatment

We then wanted to compare the effectiveness in spore killing of a cycled, continuous plasma and a pulsed plasma. We thus used the conditions established during the sample temperature determination. The pulsed plasma was run for 450 s at 125 sccm Ar, 2.5 sccm O2, 6 sccm N2 at 10 Pa, 1 kHz 10% on, 90% off for 450 s. A pulsed plasma (1 kHz 10% on, 90% off for 450 s) did significantly reduce the amount of spores on beeswax ((a) and (b)) but was more effective on glass surfaces ((c) and (d)). Cycled, continuous plasma treatment (5 s on, 20 s off, 9 cycles) kills off more spores on beeswax ((e) and (f)) and glass surfaces ((g) and (h)). Treatment of samples in the low pressure Ar/O2/N2 atmosphere without plasma ignition did not affect spores ((i)–(l)).
5 s with 20 s intermittent gaps at 125 sccm Ar, 2.5 sccm O2, 6 sccm N2 at 10 Pa. While the effective time the samples were exposed to plasma was equal (45 s), the sterilization cycle of the cycled, continuous plasma was less than half the length of the pulsed plasma (205 s versus 450 s). The cycled, continuous plasma was more effective in killing spores on both the beeswax and glass surfaces (figures 5(a)–(h)). Exposing those samples to the same low pressure gas atmosphere without plasma ignition did not affect the spores (figures 5(i)–(l)).

3.4. A hydrogen-based plasma is more effective in spore killing when compared to an argon/oxygen/nitrogen-based plasma

While we achieved a significant killing of B. subtilis spores, we were not satisfied with the overall sterilization efficiency. We thus changed the gas atmosphere from an argon/oxygen/nitrogen mixture to hydrogen. A hydrogen plasma has some characteristics that make it more favorable to killing microorganisms, namely an increase in vacuum ultraviolet (VUV) radiation [22]. We thus changed the plasma condition to 20 sccm H2 at 5 Pa and 1000 W and used the cycled, continuous plasma treatment. The pressure was reduced in order to gain VUV intensity. Under these conditions, the final surface temperature reached was 54.3 °C and stayed well below 61 °C (figure 4(c)). To qualitatively assess overall spore killing in our previous experiments, we placed the spore coated and plasma treated surfaces on plates containing bacterial growth medium. While convenient, this method also has several shortcomings. Due to the high colony count on untreated surfaces, we were not able to directly quantify spore killing. Furthermore, should spores be covered in beeswax during the plasma treatment instead of becoming inactivated, we would not be able to identify them on the media plates. In order to assess the sterilization efficiency more quantitatively, we decided to extract spores from the wax surfaces for our subsequent experiments. This method involves melting and resuspension of beeswax in appropriate detergents, effectively recovering all spores, even spores that were covered [26]. These spores were then plated in dilutions on LB-medium, providing us with a quantitative assessment of spore killing by direct comparison to the untreated samples. We then could determine that a change of the atmosphere to hydrogen effectively enhanced spore killing by two orders of magnitude (figure 6).

3.5. Most spores on wax surfaces are killed within the first seconds of plasma treatment

Our experiments pointed to a hydrogen-based, cycled continuous plasma as the most effective way to kill bacterial spores on bee hive material, while still keeping the temperature low enough to not melt beeswax. To assess the spore killing efficiency of these plasma conditions, we exposed beeswax samples to varying lengths of continuous plasma, ranging from 0.1 to 5 s. To achieve longer plasma exposure times, we then cycled the plasma with 20 s off times for up to 12 cycles. The spore count on these plasma treated samples was then compared to the count on the matched, untreated sample. Our experiments demonstrate that within the first 5 s, the bulk of the spores (99% ± 1.4) were killed. However, the remaining spores were much more challenging to kill. After 12 cycles of a continuous, 5 s hydrogen-based plasma, we could no longer extract any colony forming spores from our wax samples (figure 7).

3.6. Spores are even more challenging to kill on wooden surfaces, when compared to wax

In addition to wax, wood is another major component of bee hive material. To examine the effectiveness of plasma treatment in the sterilization of wood surfaces, we prepared matched pairs of autoclaved wood pieces, which we coated with B. subtilis spores (figure 8(a)). We then exposed these wood samples to plasma, similar to the beeswax samples (20 sccm H2 at 5 Pa and 1000 W in a cycled, continuous mode). After plasma treatment, spores were extracted from the wood surface in a process similar to the extraction from wax. The spore count of plasma treated samples was then compared to the spore count on the matched wood samples, to assess the killing efficiency. At least 6 cycles were needed to bring the spore count down to 98% ± 1.2. Even after 12 cycles, the spore count was diminished by only 99.75% ± 0.25 (figure 8(b)).

However, during sterilization regimens currently used in veterinary practice (i.e. thermal inactivation by flame treatment), which will destroy the wax structure of the honeycomb, but is less destructive to wood, a less effective spore killing of only 84% is achieved [18].
Encouraged by our results on beeswax and wood samples, we wanted to test the spore-killing efficiency of plasma treatment on complete honeycombs. For this purpose we used miniature honeycombs built by bees in a beehive during a honey flow. Before use in our experiments, the honey was extracted and the honeycombs returned to the beehive for 2 d for cleaning and repair of the structure. Initially, we tested these honeycombs for germ content. We were not able to extract heat-resistant colonies (i.e. spores) from these honeycombs, making them suitable for our sterilization experiments. We coated the honeycombs with spores and subjected them to plasma treatment (20 sccm H₂ at 5 Pa and 1000 W in a cycled, continuous mode). Although the wax did not melt during our plasma treatment in our initial experiments, we observed slight deformation of the honeycomb structure when exposed to more than 4 cycles of a 5 s continuous plasma. Therefore, we aerated the chamber of our plasma reactor every 4 cycles to more effectively cool the honeycomb samples. With our plasma treatment, we were able to significantly decrease the number of spores, leading to a reduction by 99.5% ± 0.14 on the beeswax and 98.8% ± 1.2 on the wooden surfaces of the honeycomb after 16 cycles of a 5 s continuous sample (figure 9). This killing efficiency was higher than the killing efficiency reported during sterilization regimens using chemical disinfectants as well as methods currently used in veterinary practice [17, 18].

**3.7 Spores are killed on honeycombs by non-destructive plasma treatment**

**4. Discussion**

Bacterial spores, such as those formed by *P. larvae*, the causative agent of AFB, are challenging to kill. To control outbreaks of this notifiable bee disease, spores need to be effectively inactivated on bee hive material from affected apiaries. Methods currently in use in veterinary practice to kill AFB spores on beehive material are often destructive and/or labor intensive. These methods include physical inactivation by heat through incineration, or, less destructive, scorching by flame treatment, as well as chemical inactivation in heated lye. Due to the working conditions in the field, where these germ-inactivating countermeasures have to be employed, their results are not always reliable, and even under laboratory conditions spore-killing efficiencies below 85% have been reported [18]. A field-deployable plasma reactor with a well-characterized property to kill spores on these materials could support, speed up, and simplify inactivation efforts in cases of AFB outbreaks. Additionally, it could help preserve the delicate wax structures of the honeycomb. Thus a rehabilitated, but weakened bee colony can be settled, at least partially, on drawn out frames. We used spores of the GRAS organism...
Exposure to a H₂-based, cycled, continuous plasma resulted in a reduction of the spore content on the wax surface by 99.5% — a two- to threefold higher decimal reduction time of 20 ± 1.2 min at 100 °C wet heat at atmospheric pressure [28]. B. subtilis spores are known to have a two- to threefold higher decimal reduction time of 20–30 min under the above mentioned conditions and are thus arguably more resistant [29]. The data we obtained in our experiments suggests, that plasma sterilization could provide a viable alternative or expansion of the methods currently used in AFB control.

Plasma sterilization can be obtained through the use of different reactor concepts. Since other types of non-equilibrium plasma reactors have been shown to effectively sterilize bacterial spores (see e.g. [30]), reactors of these types could potentially be used as well if they can be operated effectively at temperatures low enough.

Nevertheless, low pressure plasma sterilization is unsuitable for the sterilization of liquids and covered surfaces [31]. UV/VUV irradiation needs a direct ‘line of sight’ to the target and can only penetrate surfaces a few micrometers, depending on the wavelength. Radicals and other reactive species, such as oxygen atoms cannot diffuse into bulk material. Therefore, prior to plasma sterilization, honey has to be extracted completely from the honeycombs. The honey is typically still infectious to bee brood, but safe for human consumption and marketable. Similarly, it should be noted that a plasma reactor would most likely not be able to sterilize the brood nest. Thus, the honeycomb structure from frames containing brood will have to be removed and destroyed, or the wax molten out and autoclaved. The wooden frame by itself could then be sterilized with plasma.

Other, non-traditional sterilization techniques for AFB-infected bee hive material have been used, such as gamma irradiation [19–21]. Gamma-irradiation is highly effective in the sterilization of infected bee hive material and even the sterilization of brood-containing and honey-containing honeycombs is technically possible. This, however, leads to killing of the brood and chemical alteration of the honey, such as discoloration, liquefaction, bubble formation, and loss of activity of enzymes typically found in honey [32], which most likely renders the honey unmarketable. Gamma irradiation of bee hive material is commercially available as a service in some countries, but typically performed in specialized facilities and cannot be deployed in the field.

While plasma sterilization potentially could be used in the field, there are still challenges that need to be addressed before a successful deployment. Our experiments were performed with a stationary reactor. The reactor’s chamber was only able to accommodate miniature frames. Future in-the-field usability will require a mobile plasma generator with an optimized chamber geometry, which fits at least standard sized frames and preferably complete hive bodies. We estimate that materials for such a reactor should cost around £ 8000.00, and due to the requirement of a vacuum pump, the needed gas and electrical supply, a van or trailer could be equipped with this reactor for transportability. Existing trailers equipped for methods currently used for the sanitation of apiaries affected by AFB, such as the ‘Bee health mobiles’ deployed by the chamber of agriculture and beekeepers’ associations in some German states, could potentially be retrofitted with this technology.

Our experiments provide some insight into the process of spore inactivation by plasma on bee hive material. We could show that a cycled, continuous plasma leads to better and faster inactivation than a pulsed plasma. A continuous plasma is more intense and can deliver more radiation and particles to a surface. In the same vein, a hydrogen-based plasma is more effective than a plasma based on an argon/oxygen/nitrogen mixture. The higher VUV dose generated by this plasma is most likely causing the more effective killing. Ultraviolet radiation is a potent antibacterial agent and has been identified as a dominant player in plasma inactivation of spores [27, 33]. Nevertheless, synergistic effects between particles and photons in plasma are one of the advantages of this technology over the exclusive use of UV [34, 35].

Our experiments showed that bee hives equipment containing wax and wooden surfaces is a challenging target for effective plasma sterilization. With a continuous, cycled hydrogen-based plasma >90% of the spores are inactivated within the first 5 s on either wax or wooden surfaces, but a more efficient killing of >99% is only achieved after 12 cycles. Wood turned out to be even more challenging than wax. On the latter we were able to completely remove spores, while on the former a small residual number of spores remained even during the longest treatment employed in our experiments. During sterilization by scorching Dobbelaere et al observed a similar distinction between ‘surface’ and ‘internal’ spores on wood [17]. These internal spores are presumably spores buried deeper in wood structures, where they are covered from plasma and UV/VUV irradiation needs a direct 'line of sight' to the target and can only penetrate surfaces a few micrometers, depending on the wavelength. Radicals and other reactive species, such as oxygen atoms cannot diffuse into bulk material. Therefore, prior to plasma sterilization, honey has to be extracted completely from the honeycombs. The honey is typically still infectious to bee brood, but safe for human consumption and marketable. Similarly, it should be noted that a plasma reactor would most likely not be able to sterilize the brood nest. Thus, the honeycomb structure from frames containing brood will have to be removed and destroyed, or the wax molten out and autoclaved. The wooden frame by itself could then be sterilized with plasma.

**Figure 9.** Inactivation of B. subtilis spores on honeycombs. Exposure to a H₂-based, cycled, continuous plasma resulted in a reduction of the spore content on the wax surface by 99.5% ± 0.14 (a) and on the wood surfaces by 98.8% ± 1.2 (b).

B. subtilis to test spore-killing efficiency of a DICP reactor on surfaces typically encountered on bee hive material.

Depending on genotype, the spores of P. larvae differ in their resistance towards wet heat. The most resistant ones have a decimal reduction value of below 10 min at 100 °C wet heat at atmospheric pressure [28]. B. subtilis spores are known to have a two- to threefold higher decimal reduction time of 20–30 min under the above mentioned conditions and are thus arguably more resistant [29]. The data we obtained in our experiments suggests, that plasma sterilization could provide a viable alternative or expansion of the methods currently used in AFB control.

Plasma sterilization can be obtained through the use of different reactor concepts. Since other types of non-equilibrium plasma reactors have been shown to effectively sterilize bacterial spores (see e.g. [30]), reactors of these types could potentially be used as well if they can be operated effectively at temperatures low enough.

Nevertheless, low pressure plasma sterilization is unsuitable for the sterilization of liquids and covered surfaces [31]. UV/VUV irradiation needs a direct ‘line of sight’ to the target and can only penetrate surfaces a few micrometers, depending on the wavelength. Radicals and other reactive species, such as oxygen atoms cannot diffuse into bulk material. Therefore, prior to plasma sterilization, honey has to be extracted completely from the honeycombs. The honey is typically still infectious to bee brood, but safe for human consumption and marketable. Similarly, it should be noted that a plasma reactor would most likely not be able to sterilize the brood nest. Thus, the honeycomb structure from frames containing brood will have to be removed and destroyed, or the wax molten out and autoclaved. The wooden frame by itself could then be sterilized with plasma.

**Figure 9.** Inactivation of B. subtilis spores on honeycombs. Exposure to a H₂-based, cycled, continuous plasma resulted in a reduction of the spore content on the wax surface by 99.5% ± 0.14 (a) and on the wood surfaces by 98.8% ± 1.2 (b).
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