



# **Investigating the Effects of Atmospheric Pressure Plasma Radiation on the Sterilization of *Cladosporium sphaerospermum***

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### **Investigating the Effects of Atmospheric Pressure Plasma Radiation on the Sterilization of Cladosporium sphaerospermum**

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# **Abstract**

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## List of Abbreviations

<b>APP</b>	Atmospheric Pressure Plasma
<b>C. Sphaerospermum</b>	Cladosporium Sphaerospermum
<b>DBD</b>	Dielectric Barrier Discharge
<b>OES</b>	Optical Emission Spectroscopy
<b>UV</b>	Ultraviolet
<b>KIT</b>	Kyoto Institute of Technology
<b>EEDF</b>	Electron Energy Distribution Function
<b>2SP</b>	Second Positive System
<b>ISS</b>	International Space Station

# 1 | Introduction

In various industries, including medical, agricultural fields and aerospace fields, the sterilization of surfaces or equipment is an ongoing challenge that still shows room for improvement. Many sterilization methods rely on the use of chemicals or high temperatures around 120 °C. This can be problematic as it can damage or fatigue materials and leave traces of toxic gases like formaldehyde [8]. Using a plasma instead of chemicals or heat has been shown to be a promising alternative. Especially the use of atmospheric pressure plasmas (APP) is a great general-purpose sterilization method as the plasma can be generated without the need for a vacuum chamber. APPs are generally non-thermal, can be created around room temperature and don't need to be in direct contact with the materials they interact with. This allows for the treatment of sensitive materials and fine adjustment of the intensity by varying distance and time of exposure.

In the past the treatment of bacteria with APPs has been the focus of many studies [8, 10], however another group of microorganisms, fungi, which include moulds, has not been studied as much. Moulds are known to be more resistant to sterilization than bacteria and are ubiquitous in virtually all environments. Many of the conventional methods fall short of effectively eradicating the spores of moulds especially in porous surfaces. This is a problem in the food industry, where moulds can spoil food and cause allergic reactions in humans but also in households [18, 2].

It is known that the sterilization effect that APPs have on microorganisms is related primarily to reactive species that are formed in the plasma. Studies have also proposed that the radiation emitted by the plasma contributes to this, but it is not clear how much of the effect can be attributed to the radiation. The aim of this work is to investigate the effect of APPs on the mould *Cladosporium sphaerospermum* and to dissect the role of the radiation, specifically in the ultraviolet (UV) range, in the sterilization process. To research this the study tries to answer the following three questions:

- Can UV radiation effectively inactivate spores of *Cladosporium sphaerospermum*?
- Does the APP used in this experiment emit UV at wavelengths known to be effective in spore inactivation?
- Does plasma-emitted radiation alone produce a sterilization effect on *Cladosporium sphaerospermum* spores, and how much of the overall effect can be attributed to this radiation?

To investigate these questions multiple experiments are designed and conducted during a 4-week internship at the Kyoto Institute of Technology (KIT) in Japan. They isolate the plasma and the spores at first in two experiments that measure the inactivation of spores by UV radiation and a measurement of the optical spectrum of the plasma. Then the spores are treated with the APP and the effect of the radiation is isolated by using a transparent quartz glass barrier between the plasma and the spores. The results are then compared to the inactivation without the barrier to determine the effect of the radiation. Finally, they are analysed and discussed in the context of prior studies.

Because the mould needs multiple days of incubation time, more literature research was done in parallel. The next chapter provides scientific context on the use of plasmas for sterilization, followed by some theory on the mould *Cladosporium sphaerospermum* and a direct connection to the author's university research on space applications.

## 2 | Scientific Context

There have been many recent studies on the use of plasmas for sterilization and its effects on microorganisms [10, 8, 17, 11, 21]. This chapter aims to provide an overview of the scientific context surrounding this topic, as well as research on the interaction of fungi with radiation.

The use of plasma treatment in the medical field goes back to the 20th century. Its history is outlined well in [13]. While the first plasma treatments were used on skin their antimicrobial<sup>1</sup> properties were soon discovered. In the 1990s Mounir Laroussi demonstrated the use of dielectric barrier discharge (DBD) plasma for sterilization of surfaces [9]. Its first use focussed on the sterilization of medical instruments and equipment where removing bacteria and viruses is crucial. With this, many studies began on the interaction of plasma with bacteria, and it has been shown that DBD plasma can be used very effectively for sterilization [21, 8]. As a result, many devices have been developed and are now commonly used in hospitals and laboratories [13]. Although sterilization has been studied extensively most of the studies have focussed on the deactivation of bacteria exclusively. Recent studies have started to investigate the interaction of plasma with fungi which is relevant for the food industry and the cleaning of environments [2]. While its efficacy has been proven well, it is not yet fully understood which processes carry responsibility for the inactivation of microorganisms especially on fungi. It is believed that the main mechanism of inactivation is the generation of reactive species in the plasma which can diffuse into the cell and damage DNA and proteins [8, 21]. A clear relationship between treatment time, proximity and inactivation rate has been shown. According to prior studies moulds like *C. sphaerospermum* are a lot more resistant to UV radiation than bacteria [8].

Non-thermal plasma can, however, also radiate ultraviolet (UV) light, and the electric field applied through DBD can also have an effect on the microorganisms. A recent study by Li, Yiqian and others [10] has researched the effects of the physical energy of the plasma on bacteria by separating samples with different materials transparent to radiation and electric

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<sup>1</sup>Inhibits the growth of microorganisms.

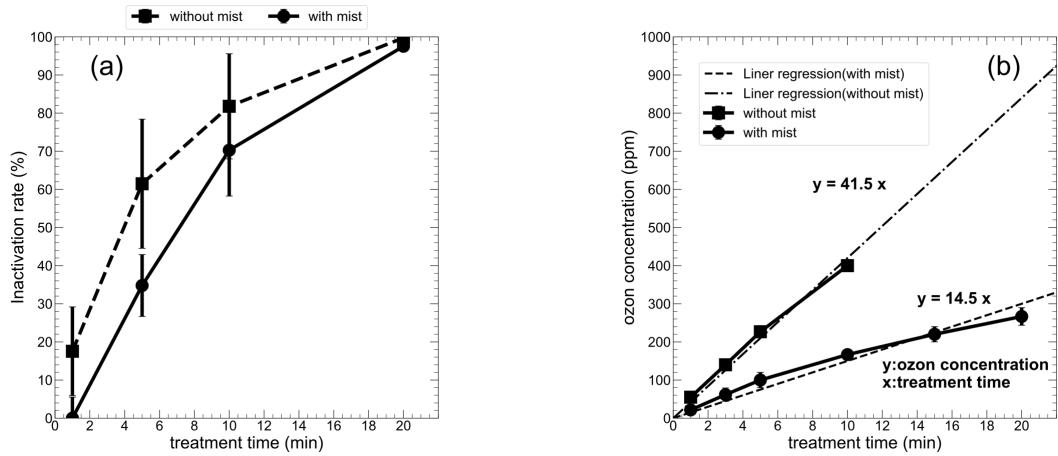


Figure 2.1.: Results of the study by Tomoya Ohara and others [17]. (a) inactivation rates of *C.sphaerospermum* as a function of treatment time in the treatments without and with water mist, and (b) ozone concentration as a function of the time.

fields and found them to have a significant effect. In this work a similar approach is taken to investigate the effects of the radiation on the fungus *C. sphaerospermum*. The basis for which was formed by previous work by the department for plasma physics at the Kyoto Institute of Technology done by Tomoya Ohara and others [17]. They showed that treatment times of 20 minutes are sufficient to inactivate the fungus by more than 99 % and measured the concentration of ozone and hydroxide ions. Figure 2.1 shows the results of their study.

## 3 | Theory and Methods

In this chapter the theoretical background and the methods used in this work are presented. This includes the collection of research on the fungal species *Cladosporium sphaerospermum*, as well as the atmospheric pressure plasma and the different processes that lead to the sterilization of the mould as they interact.

### 3.1. *Cladosporium sphaerospermum*

*Cladosporium sphaerospermum* (*C. sphaerospermum*) is a species of fungus. Fungi are a large group of eukaryotic organisms<sup>1</sup> that include yeasts, moulds and common mushrooms. It is a black mould that is commonly found in indoor environments, especially in areas that are damp or have high humidity. It was first described by a German mycologist, Albert Julius Otto Penzig, in 1886 from decaying citrus plant material in Italy [18]. *C. sphaerospermum* primarily reproduces asexually through the production of conidia, which are non-motile spores<sup>2</sup> formed at the tips of hyphae<sup>3</sup>. These spores are easily dispersed through the air, allowing the mould to rapidly spread into new environments. Moulds like *C. sphaerospermum* require humid conditions because moisture is essential for spore germination<sup>4</sup>. In dry environments, spores usually remain dormant [2].

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<sup>1</sup>Organisms whose cells have a membrane around their nucleus, which includes animals, plants and fungi.

<sup>2</sup>Spores not capable of movement.

<sup>3</sup>Long, branching structures that grow from their tips. They give moulds their furry appearance.

<sup>4</sup>The process by which a spore begins to grow new hyphae.

### 3.1.1. Growth and Morphology<sup>5</sup>

*C. sphaerospermum* has a darkly-pigmented mycelium<sup>6</sup> that can appear black or dark green. The colonies of the mould are typically flat and have more of a powdery appearance than other moulds. It is typical for fungi of the *Cladosporium* family to have branching, tree-like hyphae on whose ends conidia are formed in chains. The spores themselves are round to oval in shape and measure a few micrometers in length. They are very resilient and can stay alive even in conditions not favourable for growth. Due to their small size they are invisible to the naked eye. In Figure 3.1 the morphology of *C. sphaerospermum* is shown.

In addition to a humid environment optimal conditions for the growth of *C. sphaerospermum* include a temperature of 25 °C. It is however a psychrophilic fungus<sup>7</sup> and can grow at temperatures as low as -5 °C [18]. It nourishes through saprotrophic nutrition, which is the process of using decaying or dead organic matter as a source of nutrients. This is why it is commonly found in decaying plant material, where it was also discovered. The conversion of starch, cellulose and other compounds such as carbon dioxide provides the energy needed for growth.

### 3.1.2. Ecological Role and Occurrence

Like many fungi *C. sphaerospermum* plays an important role in the ecosystem as a decomposer. It breaks down dead organic matter, recycling nutrients back into the soil, where it thrives. This process is important as it fertilizes the soil and allows for the growth of new plants, enabling the life cycle. Because of its resilience to different conditions it is able to grow in many different environments which include anthropogenic places like indoor environments. In humid areas and on porous surfaces, such as wood or concrete walls it can build mycelium and produce new spores. The easily dispersed spores reach virtually everywhere and are even found in orbiting spacecraft [5].

While fungi don't perform photosynthesis and therefore do not convert CO<sub>2</sub> into oxygen they still contribute to the carbon cycle. By digesting plant material and binding carbon dioxide in their biomass they play an important role in storing carbon and reducing the amount of carbon dioxide released into the atmosphere when plants decay. Because of their abundance they are

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<sup>5</sup>The study of the structure of organisms.

<sup>6</sup>A network of branching hyphae.

<sup>7</sup>An organism that is able to grow in very low temperatures.

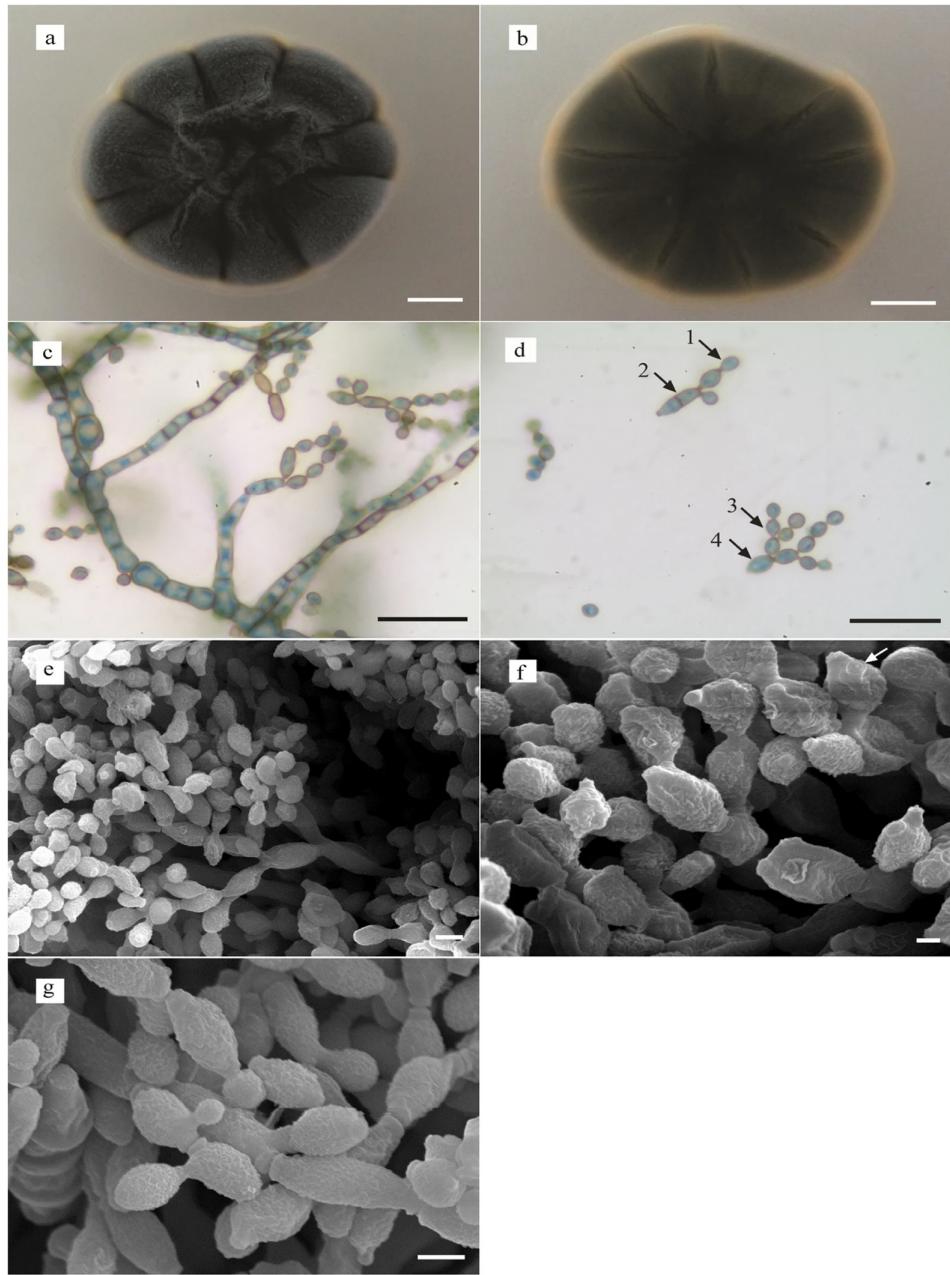


Figure 3.1.: Morphology of *C. sphaerospermum* from [25]. Colonial morphology front (a) and reverse (b) of *C. sphaerospermum* UM 843 on SDA after 7-day incubation. Light micrograph showing conidia (d 2 and d 4).  $\times 630$  magnification, bars 20  $\mu\text{m}$ . Observation under scanning electron micrograph showing (e,f,g) conidiophores bearing conidium (e,  $\times 2000$  magnification, bar 3  $\mu\text{m}$ ), pericinal rim<sup>8</sup>. (f,  $\times 5000$  magnification bar 1  $\mu\text{m}$ ) and verruculose surface of conidia (g,  $\times 5000$  magnification, bar 2  $\mu\text{m}$ ).

able to store large amounts and help build stable compounds for long-term soil carbon storage.

### 3.1.3. Effects on Human Health

Because of its ubiquity *C. sphaerospermum* surrounds humans in their daily lives. The small size of the spores allows them to be inhaled deeply into the lungs where they can lead to allergenic reactions as well as pathogenic infections<sup>9</sup>, particularly when the immune system is weakened. Compared to other airborne moulds it is however not considered a serious health risk as it does not produce mycotoxins<sup>10</sup>. Fungi in general can cause respiratory problems, especially when inhaling them in large quantities over a long time. They are however also essential in medical science as they are used to produce antibiotics, such as penicillin. While working with them in the lab clean benches can be used to provide sufficient ventilation but they generally don't pose any danger to a healthy person.

### 3.1.4. Response to Radiation

UV radiation is commonly used to sterilize surfaces and is very effective in killing most microorganisms like bacteria and fungi. *C. sphaerospermum* however shows a higher resistance to different types of radiation including UV and even high energy ionizing radiation. It is able to not only survive doses of ionizing radiation that would kill most other organisms but also to thrive in these conditions. This was discovered in 1990s as the fungus is able to grow in the high radiation environment of the destroyed Chernobyl nuclear power plant [5], even on the highly radioactive reactor walls. The mould does not only survive in these conditions but was proposed to be radiotrophic - a process where fungi potentially use ionizing radiation as a source of energy analogous to photosynthesis - growing faster.

Its tolerance to radiation both ionizing and non-ionizing is attributed to the pigment molecule Melanin. Pigment molecules such as Melanin absorb visible light and other radiation which can protect cells, can be used to absorb energy. It gives the mould its dark colour and protects its from UV radiation. Melanin is also able to absorb ionizing radiation and potentially convert it into chemical energy usable to the fungus.

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<sup>8</sup>The outer edge of a cell wall layer formed parallel to the spore surface.

<sup>9</sup>A pathogen is any organism that can cause disease in a host.

<sup>10</sup>Toxins produced by certain fungi that can cause severe disease in humans and animals.

To further research this discovery the mould was sent to the International Space Station (ISS) in 2018 where it was exposed to cosmic radiation [15]. The study found that the mould was able to survive the radiation and even thrive in the microgravity environment while absorbing some of the energy.

### 3.1.5. Relation to Space Exploration

Although it's not the main focus of this study, the connection of *C. sphaerospermum* to space exploration is briefly explored, as the author's university program focuses on space applications.

The study of fungi, like *C. sphaerospermum*, is not only interesting for terrestrial applications but also for use in space exploration. Because of its ability to survive in high radiation environments and the potential to use radiation as an energy source it might be useful in long-term space missions. NASA's Artemis program is currently the most prominent research projects that aims to send humans back to the Moon and eventually to Mars [14]. This proposes many novel challenges surrounding the long-term survival and sustainability of human life in space. One of the biggest challenges is the radiation environment in space and on the moon, which is much harsher than on Earth. The radiation from cosmic rays and solar flares can cause severe damage to human cells and DNA, leading to cancer and other health problems [22]. The results of the study conducted on the International Space Station [15] suggest that *C. sphaerospermum* could potentially serve as a highly sought-after solution for creating a self-replicating biological radiation shield, as discussed in [22]. While shielding can be achieved with materials like polyethylene or aluminium, they are always a trade-off between weight and efficacy. Ideally, the shielding would be able to reproduce, be grown in situ and be combinable with on-site resources such as moon rock or soil [22]. This way, the shielding could be produced locally and would not need to be transported from Earth, making it possible to dramatically increase the thickness, as weight is not a concern. The use of radiotrophic fungi for this purpose still requires much more research, but the results of the study on the ISS are very promising.

The sterilization of surfaces in space is also a major concern. This is important for experiments to prevent contamination, especially those that involve taking samples from the surface of other celestial bodies. If it is possible to develop a portable plasma sterilization device using APPs, it might prove useful for on-mission sterilization of equipment and surfaces. This would be a great advantage over current methods that rely on chemicals or heat which are not always

available or safe to use in spacecraft.

## 3.2. Atmospheric Pressure Plasma

A plasma is a state of matter that consists of ionized gas and free electrons. The particles behave collectively in a highly dynamic way because they are closely coupled through electromagnetic fields. The state is also conductive, and the plasma can be influenced by external electric and magnetic fields [4]. Although the particles carry charges locally, a plasma is electrically neutral when observed as a whole. Plasmas can be created by applying energy in the form of heat or electromagnetic fields to a gas. To ignite a plasma at low temperatures, a discharge is needed, the conditions for which - such as pressure, voltage and distance - are described by Paschen's law. This law states that it is generally easier to create a plasma in a low-pressure environment, as the mean free path (the distance between particle collisions) is larger, allowing the electrons to gain more energy before colliding with other particles, so they can exceed the ionization threshold. This is why many plasmas are created in vacuum chambers. However, with a sufficiently high voltage, it is possible to create plasma at atmospheric pressure also. Such a plasma is known as an atmospheric pressure plasma (APP). APPs have the advantage of being comparatively simple to handle as no vacuum vessel is required to contain them. This also makes it easier to bring them into the proximity of samples for various applications such as sterilization, surface treatment or material processing [4]. To sustain the plasma an alternating current in the Kilohertz range is used, so that the changing electromagnetic field is able to accelerate electrons continuously. The much heavier ions are accelerated only minimally in comparison. Although electron temperatures commonly reach around  $10^4$  K, it is possible to sustain a plasma at room temperature if the energy provided is controlled [3]. Such plasmas are referred to as cold plasmas or non-thermal plasmas. Because of the high voltage required to ignite APPs arc discharges can occur between the electrodes which significantly reduce the plasma stability. This makes it very hard to predict their behaviour and would heat them up to much higher gas temperatures which needs to be prevented for many experiments. Dielectric barriers can be used to achieve a controlled discharge and energy transfer.

Many processes occur in a plasma besides ionization. The gas atoms are also frequently excited which leads to the emission of photons as these states are non-stable which gives plasmas their characteristic glow. Molecules can also be dissociated by collisions which leads to the formation of radicals and other chemical bonds that can have varying lifespans. The

radiation as well as the reactive species produced in the plasma allow the plasma to interact with surfaces and materials in its proximity.

### 3.3. Dielectric Barrier Discharge

To achieve a controlled non-thermal plasma at atmospheric pressure a dielectric barrier discharge (DBD) can be used. This means that the two electrodes that carry the high voltage to ignite and sustain the plasma are separated by a dielectric barrier. There are different configurations to realize this. Some setups use only one dielectric barrier at one of the electrodes while commonly both electrons - planar or cylindrical - are covered with a dielectric material. The dielectric barrier is a non-conductive material that prevents the formation of an arc discharge between the electrodes but can be polarized by the electric field. Often glass, quartz or ceramics are used for this purpose. While the plasma is ignited the dielectric barrier prevents the electrons from flowing freely between the electrodes and charges are built up on the surface of the dielectric. This stops the discharge very quickly and thereby limits the current flow, an alternating current is needed. By controlling the voltage and frequency of this current the discharge can be sustained in a stable way. The dielectric barrier also helps to create a uniform electric field similar to its use in capacitors.

DBD is used widely in different applications that require a non-thermal plasma like the semiconductor or medical industry. It is used for surface treatment, cleaning and sterilization, like in this work, where a cold plasma is needed that does not destroy the surfaces it interacts with. It is interesting

### 3.4. Optical Emission Spectroscopy

Optical emission spectroscopy (OES) uses the electromagnetic radiation emitted by a plasma to analyse its composition. This can be done non-intrusively meaning that the plasma does not need to be disturbed and remains unaffected by the measurement. That is a major advantage when working with small volumes of plasma like in this work where probes would significantly change the conditions [3]. OES measures a spectrum of the emitted light which gives the intensity at different wavelengths. Because the photons originate from distinct transitions between energy levels sharp peaks are observed in the spectrum. Their wavelength can be

used to identify the species that are present in the plasma as well as the processes that occur. This also allows an estimate of the plasma temperature and the concentration of the different gases in the plasma, although more information like cross-sections for the different processes is needed [3].

In OES one needs to account for the spectrometer's spectral response, which is the sensitivity of the detector per wavelength. This is usually done by measuring a calibration spectrum and correcting the measured spectrum accordingly.

### 3.4.1. Boltzmann Plots

Boltzmann Plots are often used to derive the electron temperature  $T_e$  from a measured spectrum. They underlie the assumption that the probability for transitions follows a Boltzmann distribution, with the intensity of the emission line proportional to the Einstein coefficient for spontaneous emission. The relationship between intensity and temperature is given by:

$$\frac{I_j}{g_j} \propto A_{ij} \exp\left(-\frac{E_j}{k_B T_e}\right), \quad (3.1)$$

where  $I_j$  is the intensity of the emission line from an excited state  $j$ ,  $g_j$  is the degeneracy of that state,  $E_j$  is the energy of the state,  $A_{ij}$  is the Einstein coefficient for spontaneous emission, and  $k_B$  is the Boltzmann constant.

When the intensities are put in relation in a plot of  $\ln\left(\frac{I_j}{g_j A_{ij}}\right)$  over the energy  $E_j$  they result in a straight line. The slope  $m$  of this line provides an estimate for the electron temperature  $T_e$ :

$$m = -\frac{1}{k_B T_e}. \quad (3.2)$$

However, it is also assumed that the plasma is in local thermal equilibrium which is not the case in non-thermal plasmas. The assumption that the electron temperature is equal to the gas temperature is not valid and the Boltzmann plot can not be used to get an absolute temperature value in this work [3].

### 3.4.2. Other methods

A very interesting study by Hiroshi Akatsuka [7] was able to show that the electron temperature and density can be derived from the OES spectrum of a non-equilibrium plasma. To do so the continuum spectrum of the plasma was measured and its absolute emissivity per wavelength  $\lambda$  was calculated. The continuum spectrum is caused mainly by BREMSSTRAHLUNG emitted by decelerating electrons in the plasma [7]. It is much less intense than the peaks formed by electron transitions but is closely related to the electron temperature and density. The following relationship describes how the emissivity  $\varepsilon_\lambda$  and the intensity  $I_\lambda$ , found through OES, are related and the optical length through the plasma  $L$  is known:

$$I_\lambda = \int_0^L \varepsilon_\lambda(l) dl. \quad (3.3)$$

By then fitting an electron energy distribution function (EEDF) to the measured spectrum the electron temperature and density can be derived as fit parameters. The EEDF describes the distribution of the electron energy in the plasma and is a function of the electron temperature. A Maxwellian distribution and a Druyvesteyn distribution were used in the study to obtain accurate results [7]. While the results from this study are used as a reference for comparison the method itself could not be applied in this work because of limited resources and time.

## 3.5. Sterilization Mechanism

APPs have been used for sterilization of surfaces in many studies as outlined in chapter 2. Although the plasma is not in direct contact with the sample two main mechanism have been found to be responsible for the sterilization effect. The first is the radiation emitted by the plasma, which can be UV or visible light. The second is the reactive species that are produced in the plasma and interact with the surface. Additionally, heat as well as electric fields can also play a role in the sterilization process. The exact mechanism is still subject to research and what process dominates depends on the specific setup and sample. This study aims to test and isolate the effects of the radiation and reactive species on the inactivation of *C. sphaerospermum*. The following sections will discuss what is known about the different processes and how contribute to the sterilization.

### 3.5.1. Reactive Species

As discussed in chapter 2 the main mechanism for the inactivation of microorganisms in prior studies appeared to be the reactive species produced in the plasma. Because of the high electron temperatures in the plasma some electrons gain enough energy to dissociate molecules and create radicals. When working with an APP the main gases present are oxygen and nitrogen as well as water vapour. The most important reactive species produced are hydroxyl radicals ( $\text{OH}$ ) and ozone ( $\text{O}_3$ ). Radicals are missing an electron and are therefore highly reactive chemicals. They are widely used in water treatment and sterilization because they can react with organic compounds and degrade them [23]. Studies such as [24] have demonstrated that the concentration of ozone can be correlated with the rate of microbial inactivation directly. These reactive species can penetrate cells and cause damage to vital biomolecules, including DNA and proteins. This leads to the inactivation of the microorganism as it is unable to reproduce or perform its normal functions and was proven to be effective against the spores of *C. sphaerospermum* [17]. By using chemical probing methods the concentration of reactive species can be measured. For example the concentration of OH radicals can be quantified by their fluorescence when they are reacted with a chemical probe such as sodium terephthalate (NaTA).

### 3.5.2. Radiation Effects

In addition to the radicals produced by the plasma it emits radiation that interacts with the sample. In APP visible light as well as UV light in the UV-A and UV-B range is typically emitted [3]. UV radiation is used a lot for sterilization and is standard in many clean benches used in laboratories but slightly higher frequencies in the UV-C range are used. A study by Li, Yiqian and others [10] found that plasma emitting UV-A and UV-B light can have a significant effect on the inactivation rate of bacteria when using a quartz-glass separation but that the shorter wavelengths are more effective.

The main reason why UV radiation is effective at killing microorganisms is its photochemical interaction with DNA. DNA is very susceptible to absorption of UV light which can alter its structure leading to the breaking of bonds and the formation of mutations [12]. DNA contains four bases: adenine (A), thymine (T), cytosine (C), and guanine (G). These are all aromatic molecules. Aromatic molecules are ring-shaped compounds with delocalized  $\pi$ -electrons, meaning their electrons are not confined to individual bonds but can move freely

within the molecule [12]. This makes them highly stable, but the electrons can efficiently be excited by UV light, especially around 260 nm. The transition



describes the excitation of an electron from a bonding<sup>11</sup>  $\pi$ -orbital to an anti-bonding<sup>12</sup>  $\pi^*$ -orbital. This significantly weakens the bonds in the molecule and can lead to the formation of dimers<sup>13</sup>. Fungi just like bacteria rely on the integrity of their DNA as it is the basis for their reproduction of cells, which makes any damage to the DNA highly effective in killing them.

As mentioned in section 3.1.4 *C. sphaerospermum* is more resistant to UV radiation than bacteria because it is a melanized fungus. Melanin helps to protect the DNA from UV radiation by absorbing some photons before they reach the DNA. This means higher doses are necessary to achieve the same inactivation rate.

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<sup>11</sup>A bonding orbital results from constructive interference of atomic orbitals, lowering electronic energy.

<sup>12</sup>An anti-bonding orbital results from destructive interference of atomic orbitals, raising electronic energy.

<sup>13</sup>In DNA, dimers can form when two adjacent bases bond abnormally due to UV exposure [12].

# 4 | Experimental Setup

In this chapter the setup as well as the methods by which the *C. sphaerospermum* samples are prepared and treated are described. All experiments were conducted in the laboratory of the group for plasma physics at the Kyoto Institute of Technology led by Assoc. Prof. Kazou Takahashi. The work was done in collaboration with Shinano Kinoshita and based on previous research [17] conducted by Tomoya Ohara and her.

## 4.1. APP Setup

To create an atmospheric plasma a DBD is used. The setup consists of two pairs of isolated electrodes. The electrodes are separated by a dielectric material which prevents a continuous discharge. A diagram of entire setup is shown in figure 4.1 and a photograph of its implementation is shown in figure 4.2. The experiment is contained with a transparent box to prevent contamination and control the atmosphere around the plasma and mould. Through a tube dry or mist, generated by an ultrasonic mist generator, can be introduced into the chamber. While the effect of humidity has been studied in the past [17] it is not the focus of this work. Therefore, the mist generator is not used in experiments.

### 4.1.1. Dielectric Barrier Discharge

For the discharge four electrodes are used. They are coated in ceramics. Figures 4.3 and 4.4 show the exact discharge setup. The DBD is powered by a high voltage DC power supply that is then converted to an AC voltage with a blocking oscillator. An oscilloscope is used to measure the voltage and current of the discharge. Figure 4.5 shows the voltage waveform of the DBD. The discharge area has a small volume and radiation is only emitted between the electrodes.

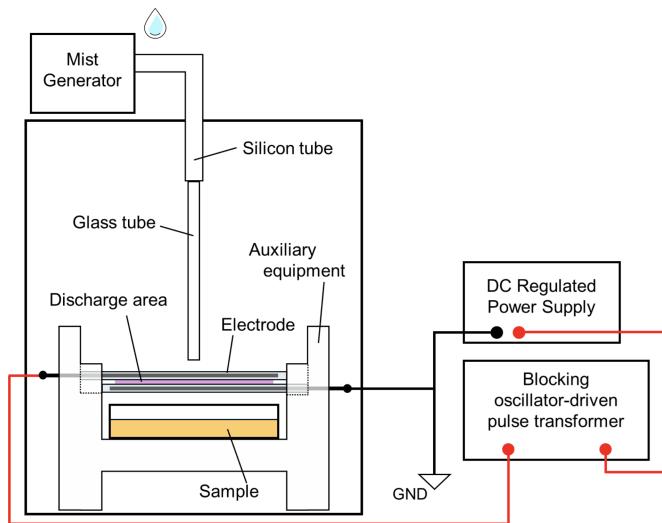


Figure 4.1.: This figure shows the experimental setup. The APP is generated between two pairs of electrodes. The entire setup is placed in a box to prevent contamination. The sample is placed underneath the discharge area. A tube can provide water mist and dry air to control the humidity in the chamber. A power supply and a blocking oscillator provide an oscillating voltage to the electrodes.



Figure 4.2.: Photograph of the experimental setup.

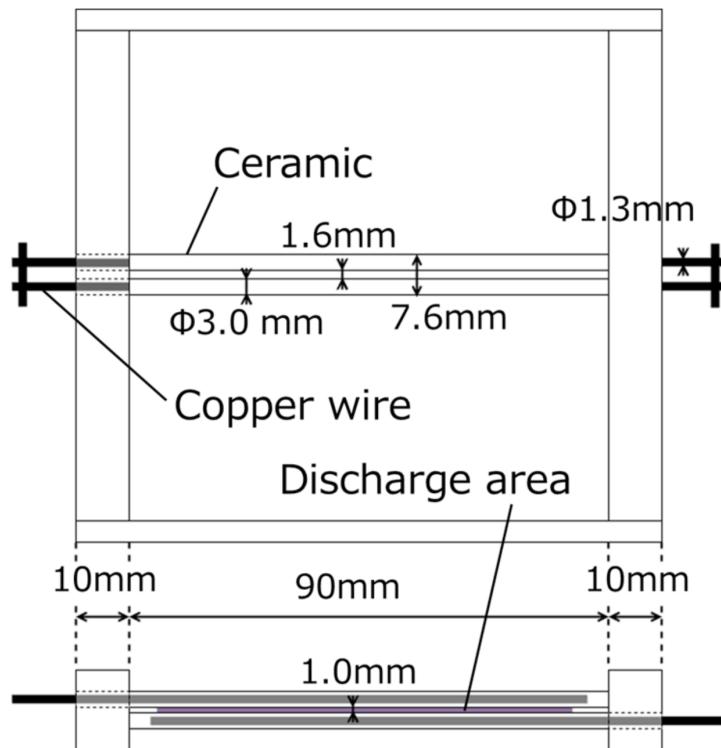


Figure 4.3.: Technical sketch of the DBD setup. On the top the view from above the DBD setup is shown, on the bottom the side view. The APP is generated by a DBD with a voltage of 13 kV. This diagram has kindly been provided by Shinano Kinoshita.

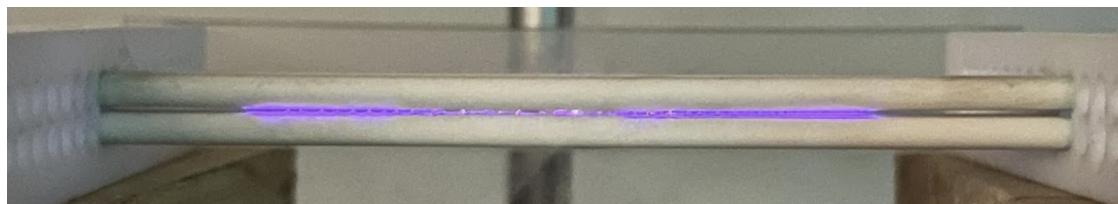


Figure 4.4.: Image of an ignited plasma at atmospheric pressure between the electrodes.

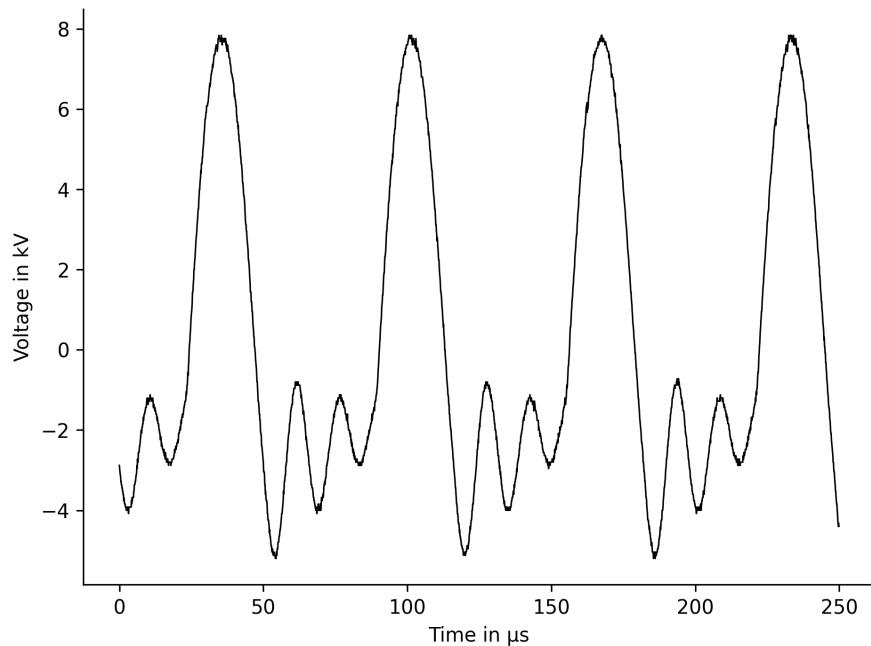


Figure 4.5.: Waveform of the DBD Voltage. The Peak-to-peak voltage is 13 kV and the frequency is 15.150 kHz.

#### 4.1.2. Measurement of the Plasma Spectrum

To measure the optical spectrum of the plasma a photonic multichannel analyser, the PMA-12 by Hamamatsu, is used. It is able to measure spectra down to 200 nm and up to 1200 nm. The spectrometer is connected to a computer via USB and the data is recorded with a software that can automatically correct for its spectral response. Figure 4.6 shows a spectrum of the plasma recorded with the spectrometer. It is later analysed in section 5.1.

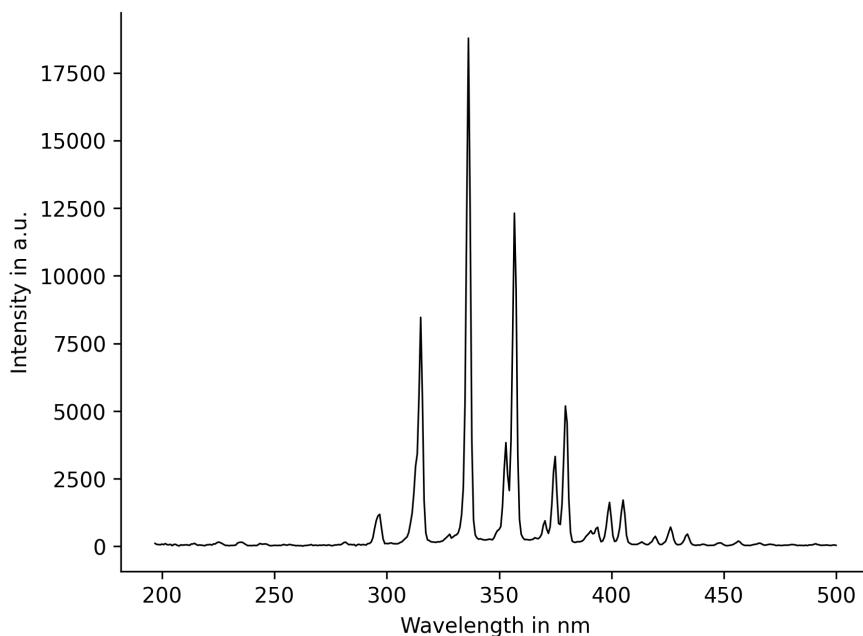


Figure 4.6.: Raw OES spectrum of the plasma at atmospheric pressure with ambient air as the working gas. The peaks are analysed in section 5.1.

## 4.2. Preparation of Samples

The samples are grown in Petri dishes on an agar medium. The medium is prepared in the lab beforehand. Table 4.1 shows its composition. To create new samples spores of the fungus *C. sphaerospermum* are collected from thriving colonies. They are dissolved in sterilized water and then scraped off the surface by using a cotton swab. The solution containing the spores is then diluted in two or three steps of 1:10 to reach a concentration of around  $3.5 \times 10^4$  or  $3.5 \times 10^3$  spores per ml. This is then verified using a cell counting plate under a microscope. 100 µl of the diluted solution are then spread on fresh agar media and are ready for treatment.

Table 4.1.: Composition of the agar medium. Percentage values are expressed as weight per volume (w/v %).

Substance	Amount	Amount in w/v %
Sterilized Water	500 ml	100
DAIGO (Casein soy digest broth)	7.5 g	1.5
Agar (TYPE BA-10)	15 g	3

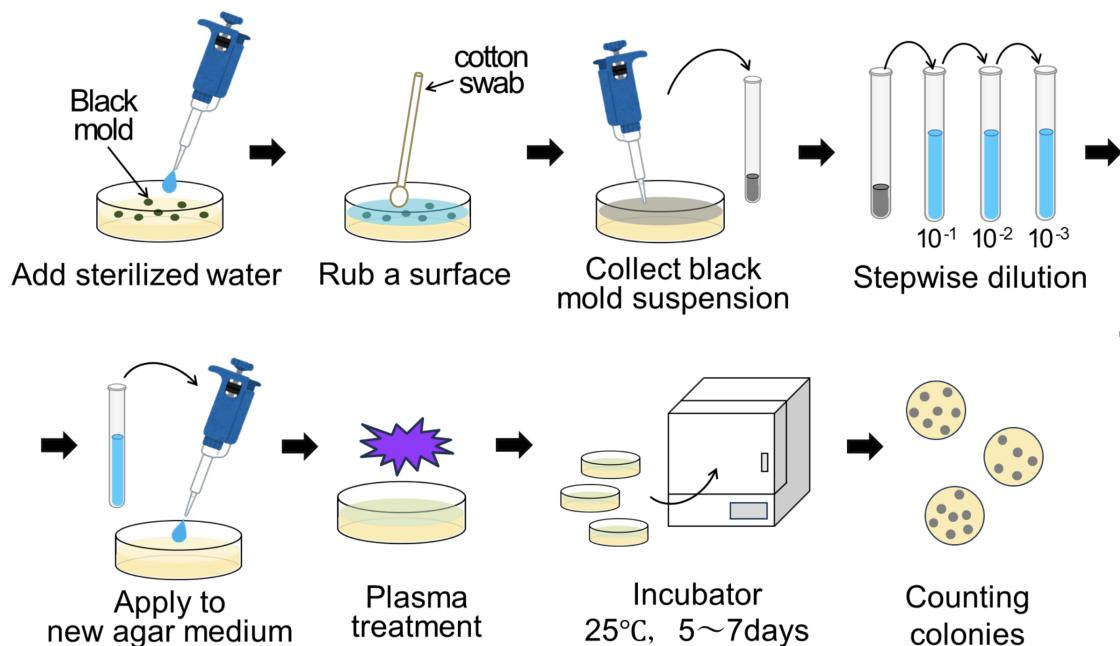


Figure 4.7.: Diagram of the experiment process and the preparation of samples. The fungus is cultivated on an agar medium and stored in Petri dishes. Spores are collected by dissolving them in sterilized water and then their concentration is diluted in steps to the desired concentration. The samples are then treated with the APP and the inactivation rate is measured by counting the colonies on the agar medium.

After treating the spores with plasma, UV or none the samples are incubated in an incubator for multiple days at their ideal growing temperature of  $25^{\circ}\text{C}$ . When the incubation period is over the samples are taken out and the number of colonies is counted to get an estimate of the inactivation percentile. Each experiment contains at least two control samples that are not treated and can be used as a reference.

### 4.3. UV Exposure

To test the effects of UV radiation on the samples before exposing them to the radiation emitted by the plasma, a UV lamp is used. The lamp is inside a clean bench to allow exposure to the UV light while preventing contamination. Figure 4.8 shows the spectrum of the UV lamp. It emits light in the range of 200 nm to 450 nm with its main peak at 254 nm. The data sheet<sup>1</sup>

<sup>1</sup>The lamp used is the sterilization lamp GL15 by Toshiba

of the lamp states that it emits radiation with an irradiance of  $51 \mu\text{W}/\text{cm}^2$ . This is measured at the radius of the cylindrical glass tube that encloses the lamp. To estimate the dose of radiation that the samples are exposed to, the effective irradiance  $I_{\text{eff}}$  at a given distance from the lamp must first be determined. For this, the distance  $r$  from the lamp to the sample is measured, and the irradiance is calculated using the formula:

$$I_{\text{eff}} = I_0 \cdot \frac{r_0}{r}, \quad (4.1)$$

where  $I_0$  is the irradiance at the radius  $r_0$  of the lamp. Here it is assumed that the lamp acts more like a thin line source of light than a point source, which only applies for short distances from the lamp. Once the effective irradiance is known, the dose  $D_{\text{UV}}$  can be calculated as:

$$D_{\text{UV}} = I_{\text{eff}} \cdot t, \quad (4.2)$$

where  $t$  is the time of exposure. This provides an estimate of the UV dose received by the samples during irradiation. In the setup used for the experiments the effective irradiance computes to  $1.14 \mu\text{W}/\text{cm}^2$  at a distance of 58 cm from the lamp.

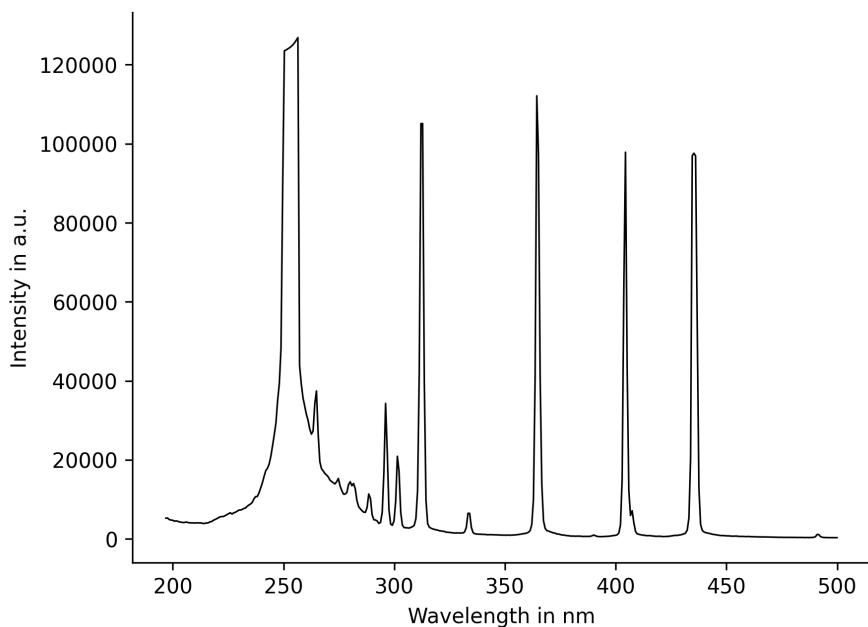


Figure 4.8.: Spectrum of the UV lamp. The main peak is at 254 nm. The spectrum was measured with the same spectrometer from section 4.1.2.

## 4.4. Plasma Treatment

### 4.4.1. Isolation of Radiation

To isolate the effects of the radiation emitted by the plasma from the reactive chemicals a transparent barrier is used. The barrier is made of quartz glass and is also transparent to visible light. It is 8 mm thick. To hold the barrier in place a mount is designed and 3D printed from PLA. The mount is designed to hold the barrier in place and to allow for easy removal.

To verify the transparency of the quartz glass, the spectrum of the UV lamp shown in Figure 4.8 is measured with and without the barrier. The results are found to be nearly identical in the range that is relevant for this experiment, so no correction is needed.

### 4.4.2. Chemical Probe Method

To measure the concentration of hydroxyl radicals ( $\text{OH}$ ) in the plasma a chemical probe method is used. The probe consists of a NATA which is added to the agar medium. It is then placed

in a Petri dish and exposed to the plasma for varying exposure times. After the exposure, the medium is analysed with a spectrometer while shining light onto it to measure the concentration of OH. This is done through its fluorescence by which it emits photons at a specific wavelength of roughly 425 nm. The intensity of the fluorescence is proportional to the concentration of OH in the medium. The probe is used to measure the concentration of OH in the plasma and to verify if the plasma is producing OH and if it is affected by the barrier.

# 5 | Results

In this chapter the results of the experiments are presented. They are divided into three sections that were conducted consecutively to answer the research question that were posed in the introduction. The first section shows an analysis of the OES spectrum of the APP and confirms the plasma composition. The second section describes the effect of UV radiation had on the spores of *C. sphaerospermum* in the experiment. In the third section results of the APP treatment on the spores of *C. sphaerospermum* with and without reactive species are shown.

## 5.1. OES Analysis

In the following the OES spectrum of the plasma first shown in section 4.1.2 is analysed. To do so, the peaks of the spectrum are found using PYTHON and the SCIPY library. They are then compared to possible different species that could be present, and their wavelengths matched to identify the transitions. Figure 5.1 shows the spectrum with the peaks marked using [16, 19, 1]. The spectrum confirms the presence of the main species in the plasma, which are as expected mostly nitrogen, oxygen and water. Most oxygen is dissociated and makes new compounds with hydrogen or nitrogen leaving nitrogen molecules to be dominant in emission. Almost all visible peaks were found to correspond to the Second Positive System (2PS) of nitrogen. The 2PS is a system of transitions between the vibrational levels of the first excited state of nitrogen:



and consists of many bands. Figure 5.2 shows a plate of the 2PS of molecular nitrogen.

Using data from [6] listed in Table A.1 (Appendix) and the OES spectrum of the plasma a Boltzmann plot is created. It is shown in Figure 5.3. By calculating the slope of the plot from relative intensities the electron temperature can be estimated. As mentioned in section 3.4.1 this can not be used as an absolute measurement in this work, because the plasma is

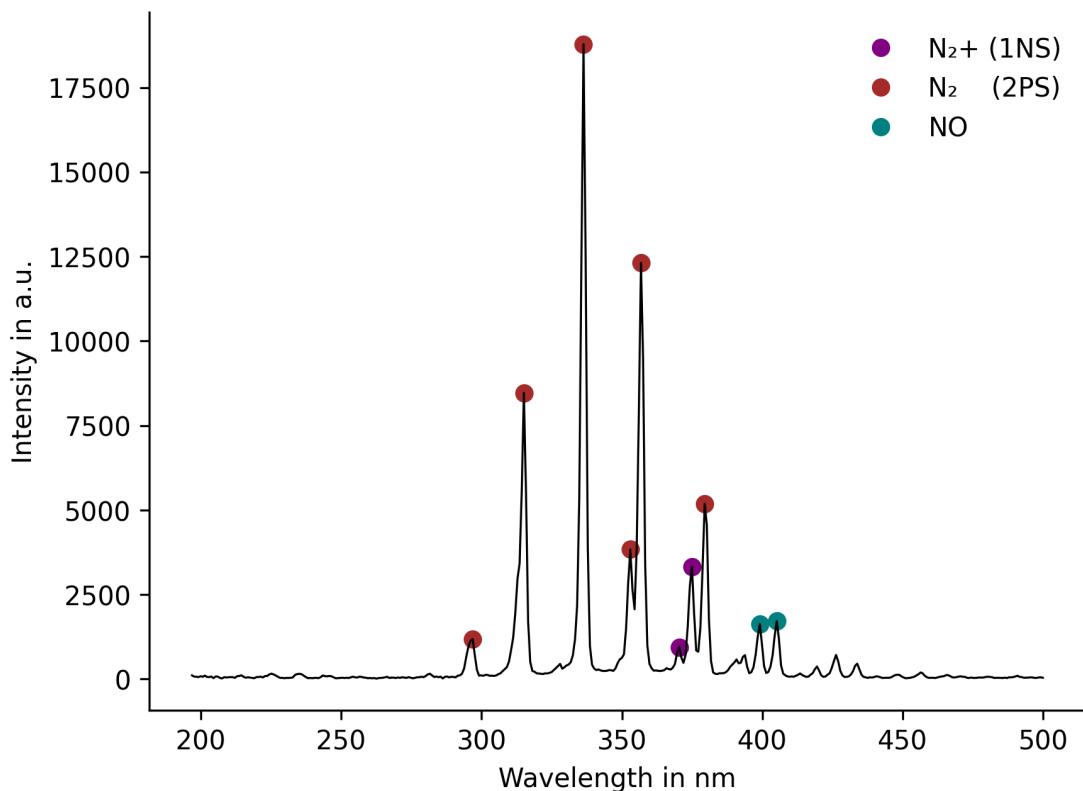


Figure 5.1.: OES spectrum of the plasma with the peaks identified. The Second Positive System of molecular nitrogen is dominant.

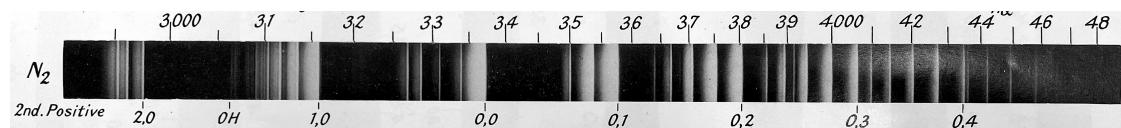


Figure 5.2.: Plate of the Dinitrogen Second Positive System from [20]. The wavelengths are displayed above the photograph in  $\text{\AA}$ .

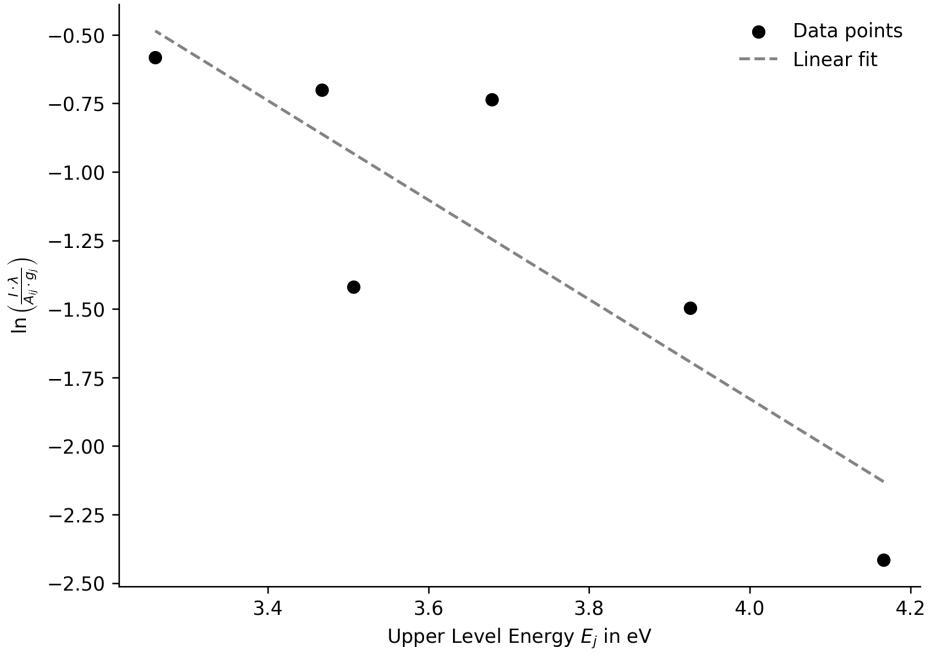


Figure 5.3.: Boltzmann Plot to estimate electron temperature using data from [6] listed in Table A.1.

non-thermal and is not in a local thermal equilibrium. From the slope obtained by fitting the data a temperature value of around 6500 K is found which appears to be in the expected order of magnitude. It lies between the values (3365 K – 9168 K) that were found in [7] for an Argon plasma with a similar setup. This result also supports the correct identification of the observed emission peaks as transitions of the 2PS of molecular nitrogen.

## 5.2. Effect of UV Exposure

To confirm the effect of UV radiation on the spores of *C. sphaerospermum*, a control experiment is performed. First the spores are exposed to the UV lamp on agar medium for different times. After 15 min of exposure full deactivation was achieved. To control for the effect of the UV light on the agar medium, a second experiment is performed where the agar medium and spores are exposed to the UV light separately. To expose the spores they are instead put into the UV light in water and added to untreated agar media later. In Figure 5.5 the petri dishes after incubation are shown. Table 5.1 shows the number of colonies in a matrix. It becomes clear from this data that the UV light has no significant effect on the agar medium while it has

Table 5.1.: Number of colonies after UV exposure as a matrix. The results after 15 minutes are shown with an estimated dose of ca.  $1.03 \text{ mJ/cm}^2$ .

# of colonies	Spores no UV	Spores UV
Agar no UV	185 (cntrl)	0
Agar UV	178	0

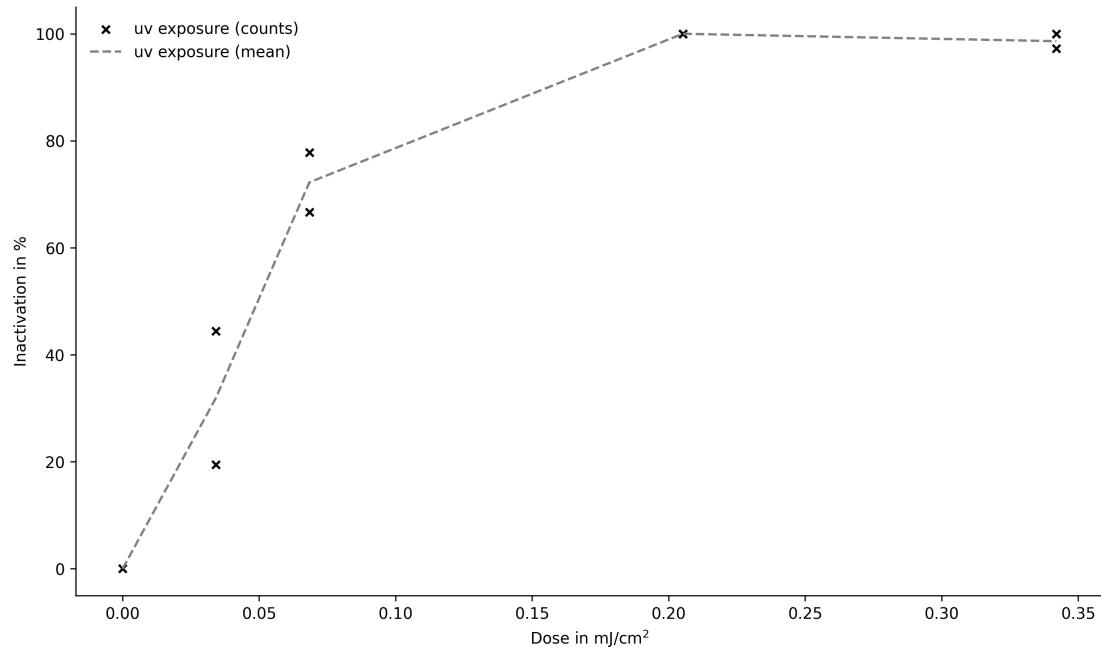
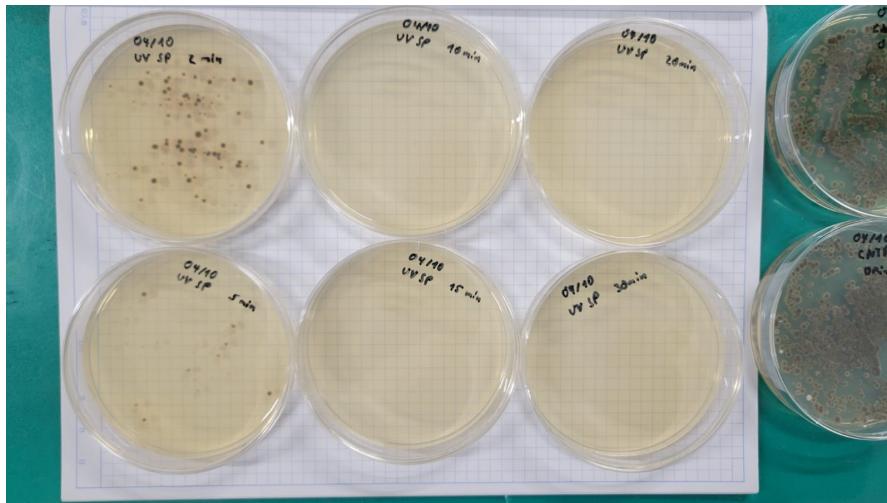


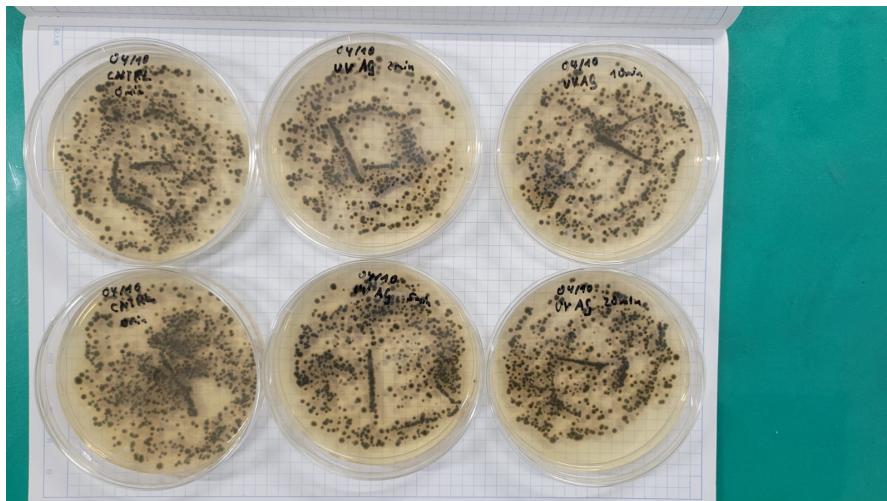
Figure 5.4.: Inactivation of spores after increasing exposure times to UV light

a strong effect on the spores after an exposure time of 15 minutes. Using equation 4.2 the dose of UV radiation can be estimated and equates to  $1.03 \text{ mJ/cm}^2$ .

Since any effects of the UV light on the agar medium can be neglected and the spores are fully deactivated after 15 minutes another experiment treating the spores on the medium can be performed. Figure 5.4 shows the results of different exposure times. Here a clear relationship between the exposure time and the inactivation of spores can be observed. The UV light emitted by the lamp proves to be effective in inactivating the spores. While much more intense and in a different UV band than the UV light emitted by the APP, it provides a good reference and the basis for the treatment of spores with the radiation of the APP.



(a) Spore UV exposure



(b) Agar UV exposure and control (left)

Figure 5.5.: Petri dishes with spores (a) and agar (b) treated with UV light. Colonies are visible in the agar media with a high number in the control and exposed agar media. The individual colonies are counted and shown in Table 5.1

### 5.3. Effect of Plasma Treatment

After the radiation of the plasma and the effect of UV light on the spores of *C. sphaerospermum* has been analysed, the effect of the APP treatment on the spores can be further investigated. Because previous experiments [17] have shown that the full APP treatment is effective in inactivating spores, in this experiment it only serves as a control reference for comparison. To keep the experiment as simple as possible, the treatment does not employ the mist generator that has been used in prior work to boost humidity.

Figure 5.6 shows the results of the treatment with and without reactive species. As expected the treatment with reactive species shows a much higher inactivation rate and inactivates more than 99 % of the spores after 20 minutes. While not as effective, the treatment with only the radiation of the APP still shows a significant correlation between the exposure time and the inactivation of spores. After 30 minutes about 50 % of the spores are inactivated.

To confirm that the inactivation in the radiation treatment is really caused by the UV light that the APP emits the intensity of the hydroxyl radicals is measured with and without the glass barrier. Another experiment is conducted where agar media with an added NaTA solution but without spores are treated. The resulting fluorescence can give a good indication of the concentration of hydroxyl radicals that reached the agar medium. The results are shown in Figure 5.7 where the intensities of the fluorescence around the characteristic wavelength (425 nm) of the hydroxyl radicals are plotted. The control group was not exposed to the APP. The results show that, while non-zero, the intensity of the fluorescence is significantly lower when the glass barrier is used. In that case the concentration of hydroxyl radicals also does not show any significant increase over time. It is about 11 % of the intensity of the fully treated media without the glass barrier after 20 minutes. This indicates that the hydroxyl radicals are not the main cause of inactivation in the radiation treatment, but it is also not possible to fully rule out that they play a role also.

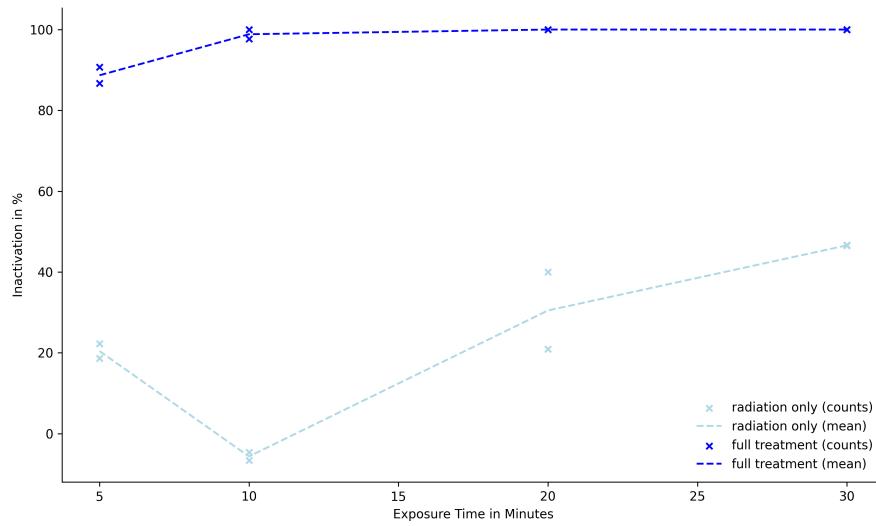


Figure 5.6.: Inactivation of spores after exposure to plasma radiation with and without reactive species. The data for radiation only at 10 minutes seems to be an outlier and may have resulted from an error in the experiment. In general the treatment shows a clear trend of rising inactivation.

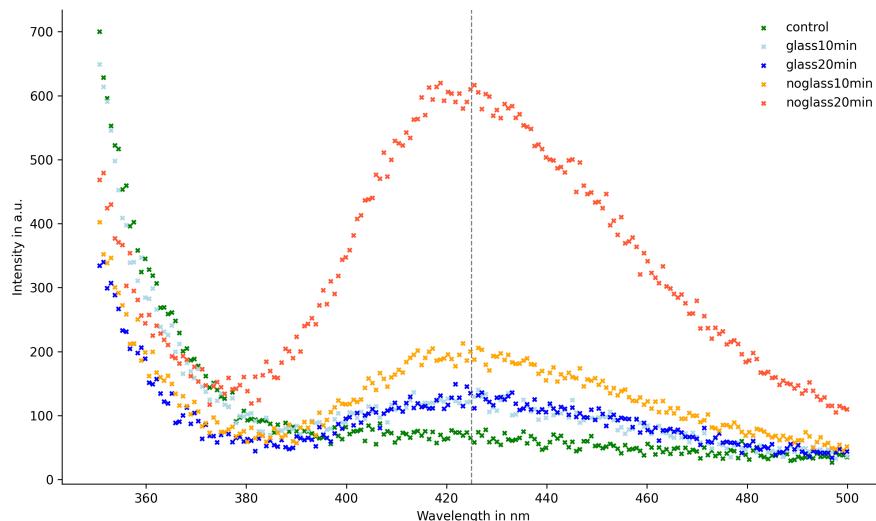


Figure 5.7.: Intensity of fluorescence of hydroxyl radicals with and without the glass barrier. The fluorescence is induced by the chemical probe (NaTA). The intensity of the fluorescence is shown in arbitrary units and can be directly related to OH concentration.

## 6 | Conclusion

This work aimed to isolate the sterilizing effects of radiation in APP treatment on *C. sphaerospermum*. To summarize, this chapter will provide an overview and an assessment of the results that were found. It will also discuss the limitations of the current work and suggest possible outlooks for future work.

The results of the experiments show that the subject of this study is a relevant topic when trying to understand the effects of APP sterilization further and the research question posed were answered. While not perfectly aligning the wavelengths, the two experiments leading up to the full treatment still provided a good basis to support the results of the final experiment. It was very clearly confirmed that radiation in the UV range around 250 nm is capable of sterilizing the spores of *C. sphaerospermum* and the control experiment concluded that its effects on the agar media are negligible at the doses used. It was found that an estimated dose of 0.35 mJ/cm<sup>2</sup> is sufficient to sterilize the spores of *C. sphaerospermum* in this setting. The plasma used in the experiment was found to emit UV radiation mainly in the range of 300 nm to 400 nm. The main species that was found in the OES measurements was Nitrogen and the radiation was primarily attributed to its 2PS. While not very conclusive, a Boltzmann plot was used and found the electron temperature to be around 6500 K or roughly 0.5 eV.

The use of the UV transparent quartz glass enabled a simple experiment, which was able to show that the radiation emitted by the APP alone is capable of sterilizing the spores and might play an important role. While the data is very limited and the number of results are not statistically conclusive, the concept has been proven and a deactivation of approximately 50 % of spores was achieved after 30 minutes. The inclusion of a control experiment that estimates the hydroxyl radical concentration provided support for the assumption that the radiation is the main contributor to the sterilization in the experiment. Gaining a better understanding for the mechanisms, like radiation, that cause APP to be effective at sterilization is crucial for the development and improvement of APP as a sterilization method. It shows potential for treatment of surfaces without touching them directly, which could be a great advantage in.

To improve the results, the concentration of hydroxyl radicals should be lowered to a level that is comparable to that of the control group. This would allow for a more direct comparison of the two experiments and would help to quantify the role of the radiation in the sterilization process. For that a better seal of the quartz glass is needed to prevent the escape of hydroxyl radicals. Also, the possibility of hydroxyl radicals being formed behind the quartz glass by the UV radiation should be considered and investigated. For that a UV blocking layer could be applied to the quartz glass so that the only source of hydroxyl radicals is the APP itself. To further categorize the plasma additional experiments should be conducted and an absolute quantification of the radiation should be performed. As discussed in section 3.4.2 Akatsuka and others [7] have presented a non-intrusive method that could be used to measure the electron temperature and density of the plasma accurately. Additionally, an experiment with a UV lamp capable of emitting the same wavelengths as the APP could be performed to establish a more direct comparison.

Because of the limited time that the author spent at KIT, it was not possible to conduct any more of the proposed experiments. However, the results of the current work seem promising and may lead to further research in this area in KIT's lab.

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# A | Appendix

Table A.1.: Spectroscopic data for selected N<sub>2</sub> Second Positive System transitions.  $A_{ij}$  and  $E_j$  values from Gilmore and others (1992) [6].

Wavelength in nm	Intensity in a.u.	$A_{ij}$ in $s^{-1}$	$g_j$	$E_j$ in $\text{cm}^{-1}$
297	1185.53	$3.94 \times 10^6$	1	33606
315	8464.27	$1.19 \times 10^7$	1	31665
337	18781.21	$1.32 \times 10^7$	1	29671
353	3834.82	$5.60 \times 10^6$	1	28284
357	12313.39	$8.86 \times 10^6$	1	27966
380	5188.45	$3.53 \times 10^6$	1	26290