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Final Report

Effects of Nonequilibrium Plasmas on Eukaryotic Cells

AFOSR Grant FA9550-06-1-0004

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Abstract

This document is our final report, describing the research activities carried out under AFOSR Grant FA9550-06-1-0004. First, descriptions of our cold plasma generation systems are presented. Two systems, developed with past and present AFOSR support, are available in our laboratory. The first is a pulsed device capable of emitting a cold plasma plume in room air. The second is an air plasma generator the core of which is a dielectric barrier discharge excited by a high AC voltage. Following these brief descriptions we first present the effects of an atmospheric pressure air plasma on four different types of eukaryotic microalgae. Effects on the viability, motility, and morphology of the cells are presented and discussed. Second, we report on the results regarding the effect of plasma on a multi-cellular organism (planaria), to see if plasma alters in any way its growth after suffering a wound. Our preliminary conclusion is that plasma has no apparent negative effect on the cells regeneration process. We then report on our latest work regarding the characteristics of the plume generated by the Plasma Pencil. Finally, we report preliminary results on the effects of direct exposure of plasma to oral borne bacteria. This can lead to applications in dentistry.

Introduction

The past decade has witnessed a burst of research activities in a new and exciting field, the interaction of non-equilibrium plasmas with bacteria in both the vegetative and spore states. Novel applications such as the sterilization of heat-sensitive tools and the decontamination of surfaces are the spin-off of such research. The Air Force Office of Scientific Research (AFOSR) has been a leader in supporting several efforts in this interdisciplinary field. However, until the present, not much effort or support has been dedicated to study the effects of non-equilibrium plasmas with "eukaryotic" cells, such as mammalian cells. In many respects eukaryotic cells are different and have a more complex structure than prokaryotic cells (such as the cells of bacteria). For example, unlike the case with prokaryotes, cellular functions in eukaryotic cells are localized and the cells exhibit membrane-bound sub-cellular structures (organelles). The effects of plasma on eukaryotic cells are therefore expected to be quite different from those on the cells of bacteria. Preliminary work has shown that at low power, short exposures of mammalian cells to "cold" plasma can lead to cell detachment without causing necrotic effects. Under certain conditions, short exposures can lead to "apoptosis", or programmed cell death. Death of cells by apoptosis is not accompanied by lysis, which is a source of inflammation in affected areas. These very preliminary results, although in need of more in-depth investigations, point out that cold plasma might play a role in practical medical applications, such as the removal of dead tissue and the acceleration of wound healing. These "plasma-induced" bioeffects are therefore of military and civilian interest.

In this document, we report on results obtained during a 3-year research program on the effects of cold plasma on some select eukaryotic cells. First, a brief description of our cold plasma generation capability is described. Then, results on the exposure of four different types of eukaryotic microalgae in aqueous environments to cold air plasma will be reported. This is followed by a presentation of a model describing the operation of the plasma pencil, a device capable of generating a cold plasma plume. This plasma plume is used to test if cold plasma has any effect on cells regeneration following a wound (cut). The model organism in our studies is a multi-cellular worm, "planaria", which is capable of regenerating parts of its body in a relatively short period of times (several days). The next section presents our latest work on the experimental characterization of the plasma plume emitted by the plasma pencil. It was our intention to investigate the potential use of the plasma plume as a blood coagulation device. Unfortunately, due to various issues related to experimenting with blood in our lab, we were unable to conduct this investigation. Therefore we took advantage of the time remaining for this project to investigate the effects of cold plasma on oral borne bacteria. This could lead to interesting applications in dentistry.

Cold Plasma Generators

The following are brief descriptions of two cold plasma generation systems developed by our group with bio-applications in mind. The first is the "plasma pencil", which is a pulsed device capable of emitting a cold plasma plume in room air, and the second is an air plasma generator designed for remote exposure of samples.

A. The Plasma Pencil

This device is basically a 1inch diameter dielectric tube (about 5 inches long) that can be hand held and the plasma plume it generates can be directed at will towards a surface to be treated including human skin or dental gums. Unlike other known plasma "jet" devices, which generate very short plumes in the millimeter range, and at temperatures that can reach several tens of degrees above room temperature, this device is capable of producing and maintaining room temperature plasma plumes several centimeters in length.

The plasma pencil operates as follows: Sub-microsecond high voltage pulses at repetition rates in the 1-10 kHz range are applied between two specially designed electrodes through which helium gas is flowing (flow rates in the 1-10 liter/min range). Each of the two electrodes is made of a thin copper ring attached to the surface of a centrally perforated glass disk. The hole in the center of the glass disk is about 3 mm in diameter, while the diameter of the disk is about 2.5 cm. The diameter of the copper ring is greater than that of the hole but smaller than that of the disk. The two electrodes are inserted in a dielectric cylindrical tube of about the same diameter as the glass disks and are separated by a gap the distance of which can be varied in the 0.5-1 cm range. Figure 1 is a schematic of the device. When helium is injected at the opposite end of the dielectric tube and the high voltage pulses are applied to the electrodes, a discharge is ignited in the gap between the electrodes and a plasma plume reaching lengths up to 5 cm is launched through the hole of the outer electrode and in the surrounding room air. Figure 2 is a photograph of the plasma plume. The length of the plume depends on the helium flow rate and the magnitude of the applied voltage pulses. The plasma plume remains at low temperature and can be touched by bare hands without any harm. The device, being basically a 1inch diameter dielectric tube (about 5 inches long) can be hand held and the plume directed at will towards a surface to be treated including human skin or dental gums.

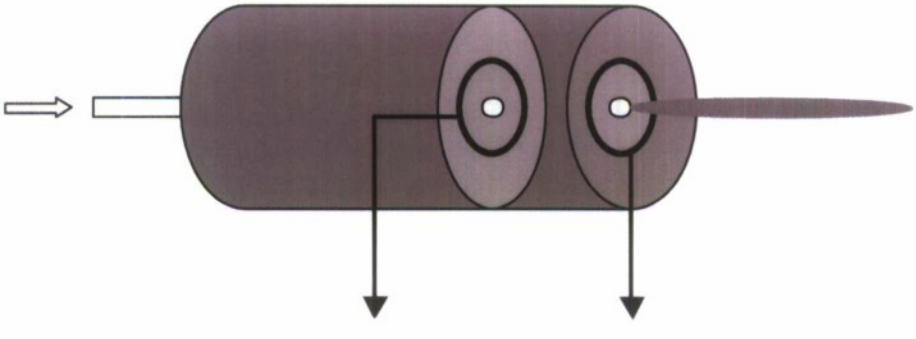


Fig. 1 Schematic of the "Plasma Pencil"

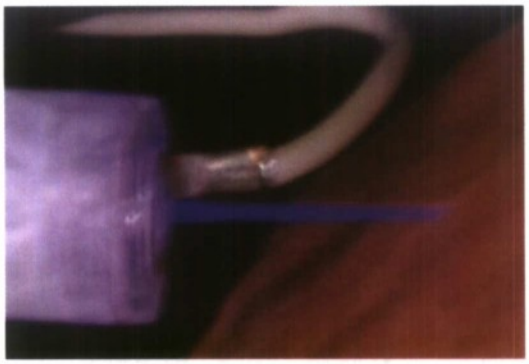


Fig. 2 Plasma pencil showing plume in contact with the PI's hand

B. *Air Plasma Generator*

This plasma generator was built based on prior designs developed by Laroussi and co-workers in the last few years. It uses the dielectric barrier discharge configuration operating in air or similar gas mixture, at atmospheric pressure. Figure 3 shows the basic design concept with biological application in mind.

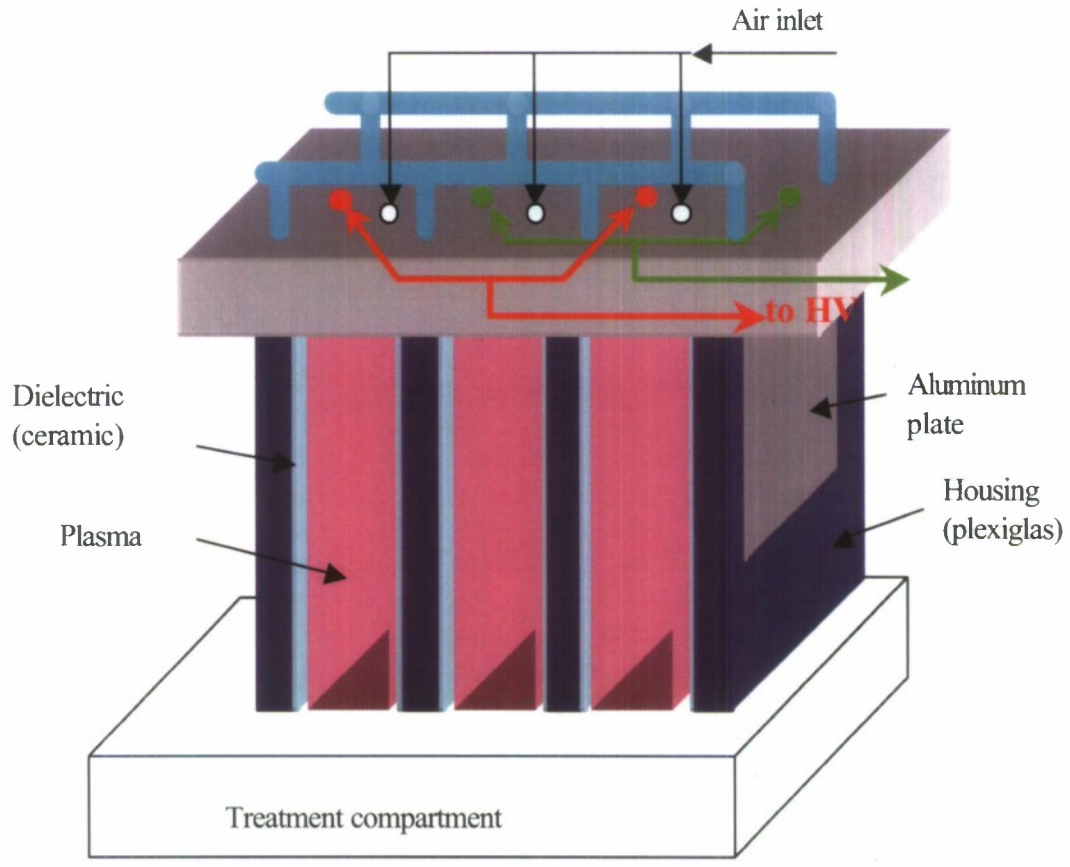


Fig. 3 Air Plasma generator with separate treatment chamber

Figure 4 shows the actual device configuration that was built and tested successfully. The power source that ignites the plasma is a transformer capable of delivering up to 9 kV_{RMS} at the line frequency of 60 Hz. The operating pressure is one atmosphere.

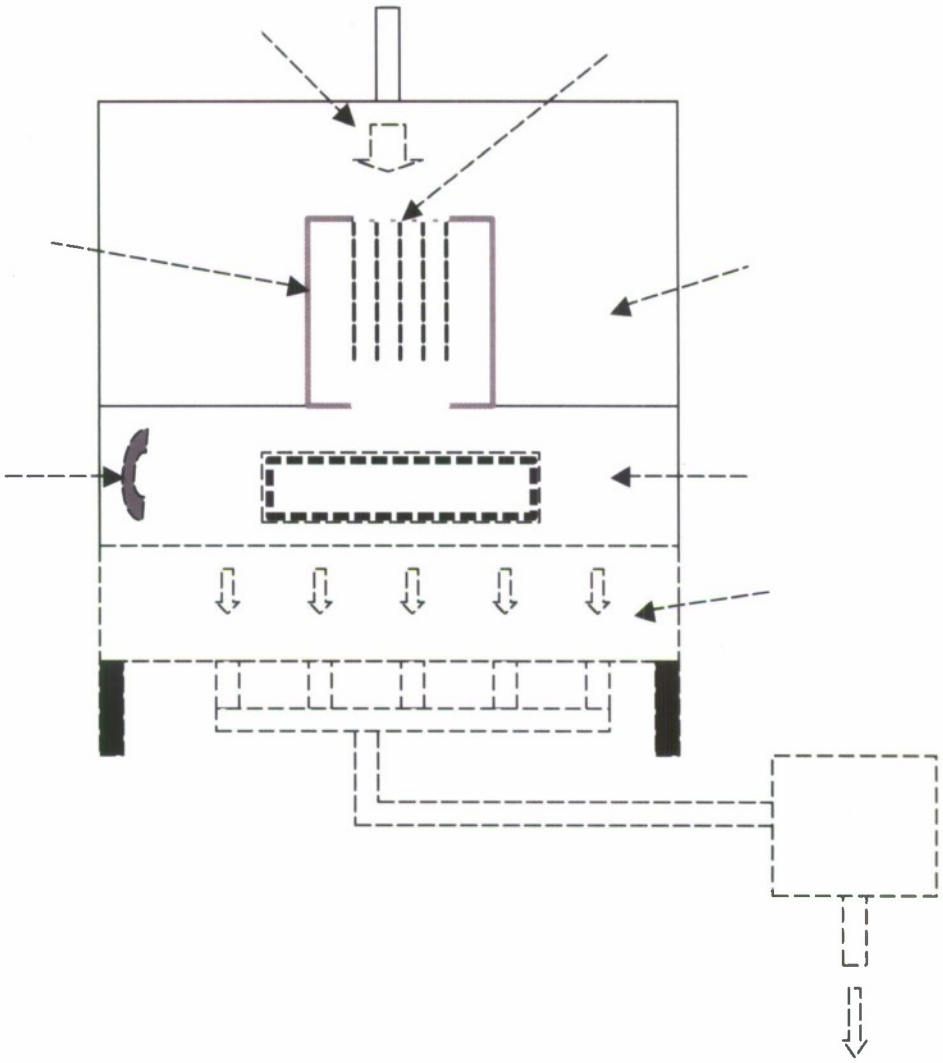


Fig. 4 Configuration of the the Plasma sterilization chamber

Plasma Chemistry & Diagnostics

Of great interest to this work are the concentrations of active species generated by the air plasma since chemical reactive species are suspected to play a major role in interactions with organic materials. Since the operating gas is air, the most likely species in the discharge are O_3, and NO_x (NO, NO_2,...). A relation between these species can be described by the following equations:

$$NO + O_3 \leftrightarrow NO_2 + O_2 \qquad (1)$$
$$NO + Radical \leftrightarrow NO_2 + ... \qquad (2)$$
$$NO_2 + O_2 + h\nu \leftrightarrow O_3 + NO \qquad (3)$$

In case of humid air, OH will be generated as well. This can be described by the equation:

$$H_2O + O_3 \leftrightarrow O_2 + 2\,OH \qquad (4)$$

Interaction with organic material (presence of C) may cause oxidation products like CO and CO_2.
The following reactions are possible:

$$NO_2 + C \leftrightarrow NO + CO \qquad (5)$$
$$C + 2O_3 \leftrightarrow CO_2 + 2O_2 \qquad (6)$$
$$C + O_3 \leftrightarrow CO + O_2 \qquad (7)$$

Ozone is also generated by the following pathway:

$O + O_2 + O_2 \rightarrow O_3^* + O_2 \rightarrow O_3 + O_2$,
where atomic oxygen is produced via electron impact with oxygen molecules.

Ozone and NO_2 were found to be the dominant species produced by the air plasma. As illustrations, the following figures show examples of concentration of O_3 and NO_2 generated in our laboratory by a similar single DBD stage, however excited at a higher frequency than used in our bio-experiment. Note that the generator that we used in our bio-experiments contained 4 DBD stages in cascade. The two electrodes of each DBD were spaced by about 4 mm.

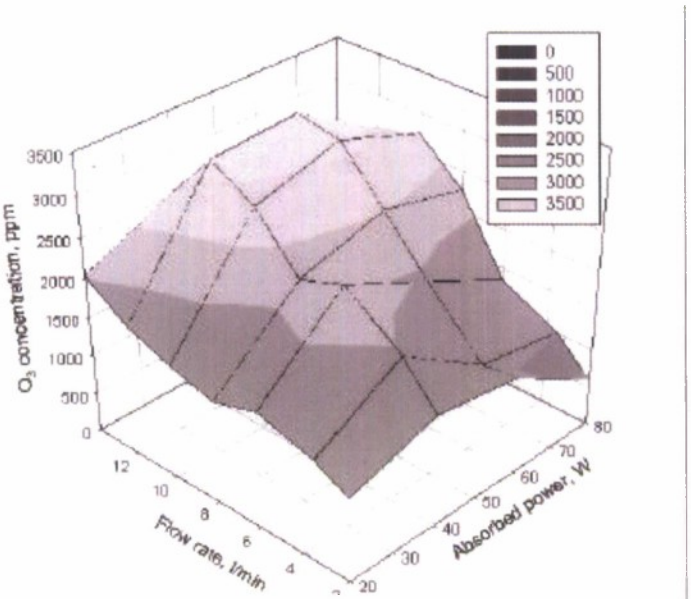


Fig. 5 Ozone concentration versus power and airflow rate

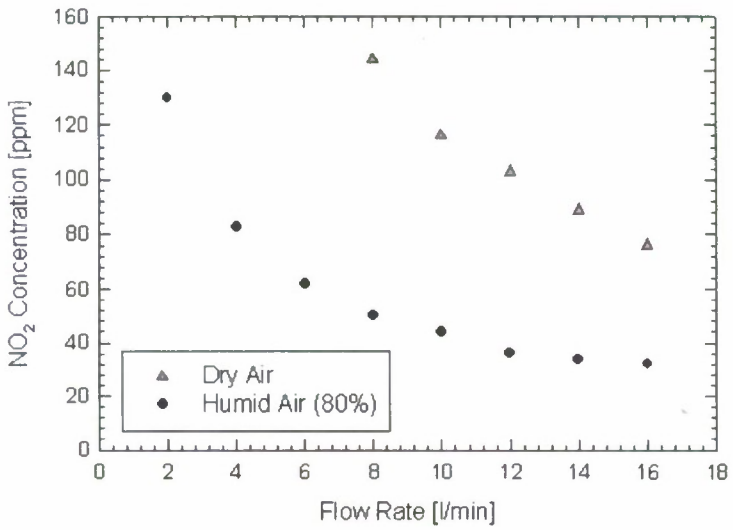


Fig. 6 NO_2 concentration versus flow rate (single DBD stage). Plasma dissipated power is 3 W.

The power density of the UV radiation emitted by an air plasma was also previously measured for a single stage in the 200-310 nm wavelength range. Figure 7 shows that the measured total power density was much below 1 mW/cm^2.

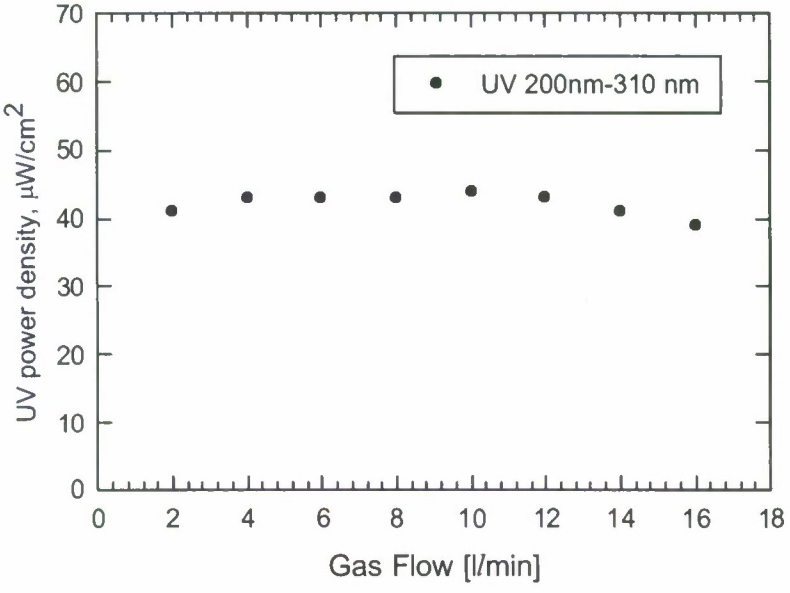


Fig. 7 UV power density versus airflow rate, from a single DBD stage. UV detector was placed about 70 mm from plasma. Plasma dissipated power is 10 W.

Effects of Air Plasma on Microalgae

Here we report our observations on the effects of a non-thermal air plasma on the cell motility, viability, and morphology of a variety of eukaryotic microalgae, with three dinoflagellates (unarmored and armored) and a diatom as major targets, in aqueous environments. The effects on motility and viability depended on the time of exposure to plasma and the species of microalgae. More importantly, a strong decrease of pH in a series of aqueous samples (algal cultures, marine and freshwater culture media, and deionized water) was observed after exposure to plasma, and was hypothesized as the mechanism of killing cells by plasma. The hypothesis was then further strongly supported by observations using scanning-electron microscopy, in which a decreased pH in algal samples (effected by addition of acid) caused the same morphological damages as did exposure to plasma.

Organisms: We used three marine dinoflagellate species (*Akashiwo sanguinea*, *Scrippsiella trochoidea*, and *Heterocapsa triquetra*) isolated from the Elizabeth and Lafayette Rivers, Norfolk, Virginia, USA), and a marine diatom (*Corethron hystrix*) (Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Maine, USA) to observe the effect of exposure to plasma in aqueous environment. We also used 11 cultures of freshwater microalgae including 7 green algal cultures (Volvocales mixed culture, *Carteria*, *Chlamydomonas*, *Chlorella*, *Gonium*, *Hydrodictyon*, *Micrasterias*), a euglenoid (*Euglena acus*), two golden-brown algae (*Ochromonas*, *Synura*), and a freshwater dinoflagellate (*Peridinium*) (Carolina Biological Supply Company, North Carolina, USA) to survey the possible physiological and morphological changes in response to plasma exposure. The marine species were maintained in either GSe (Doblin et al. 1999) or f/10 medium in a light incubator (20±1° C, 12:12 h L:D photoperiod, approximately 80 μEin m^2

s^{-1} light intensity), while the freshwater species were maintained in Alga-Gro freshwater medium or Soil-Extract medium (both from Carolina Biological Supply Company).

Exposure procedure: To test the effect of plasma exposure on motility (for algal species with flagella) and viability, 1 mL of algal culture was added into a well of a 12-well culture plate (liquid depth in the well 5 mm), the culture plate was placed (without cover) into the plasma 'chamber', with the well containing algae placed directly below the source of plasma (distance of the liquid surface from the plasma is 25 mm), and the sample was exposed to plasma for 80 to 640 seconds. As control, a plate containing algal culture as above was placed in the chamber for 80 seconds with the power off. After treatment, 0.12 mL of the sample was taken from one well, put into a 0.1-mL glass counting chamber, covered with a cover slip, and examined using a Nikon light microscope (total magnification 100×) to count all motile and non-motile cells. The rest of the sample in the well (0.88 mL) was stained with neutral red (NR) for 5-10 min (0.001% w/w final conc.), then examined to determine the number of stained (red) and non-stained cells. Neutral red is taken up by living cells and stains them red under bright field of light microscope (Conn 1961). Therefore, the viable cells are defined hereafter as cells stained red by NR, while motile cells are referred to as viable regardless whether they stained or not.

To test the possible recovery of motility and viability for algal cells after plasma exposure, algal cultures were treated as above (including controls). The treated samples were inoculated into fresh culture medium contained in 6-well plates (0.3 mL into 3 mL) within 0.5 h, incubated in the same light incubator as for culture maintenance, and their growth state was checked after 24, 48, or 72 h.

Measurement of pH in algal cultures and media after exposure to plasma: pH values in 1 mL cultures (with cell concentrations approximately the same or a bit higher than those used for plasma exposures) of *A. sanguinea*, *S. trochoidea*, and *H. triquetra*, 1 mL of GSe medium (marine, salinity 30 PSU), Alga-Gro medium (freshwater), Soil-Extract medium (freshwater), and deionized water (18.2 MΩ) were measured before and immediately after exposure to plasma in a way as above. A MI-710 microelectrode (Microelectrodes, Inc., New Hampshire, USA) connected to a ORION pH meter (model 290A, Thermo Electron Co., Massachusetts, USA) was used as the pH probe. The samples were exposed to plasma for 40 to 640 seconds.

Scanning electron microscopy (SEM): The possible fine morphological changes in algal cells caused by exposure to plasma were observed with SEM. Since the considerable decreases of pH in algal samples were observed after plasma exposure, algal cultures in which pH levels were reduced by addition of 0.1 M HCl were also observed with SEM, in order to test whether merely decreasing pH would lead to the same morphological changes associated with exposure to plasma. Cultures unexposed to plasma or having no pH adjustment were used as controls. Samples were prepared for SEM as follows: One milliliter each of the original algal cultures (*A. sanguinea*, *S. trochoidea*, and *H. triquetra*), the cultures treated with plasma (320 seconds for *A. sanguinea*, 480 seconds for both *S. trochoidea* and *H. triquetra*), and the cultures with pH adjusted (pH 3.1 for *A. sanguinea*, 2.7 for *Scrippsiella trochoidea*, and 2.8 for *H. triquetra*, corresponding to 320, 480, and 480 seconds plasma exposure, respectively) were fixed with OsO_4 at 2% final concentration (dissolved in GSe medium of 4% concentration) for 30 to 60 min, filtered onto a 0.2 μm NUCLEPORE Track-Etch or 10 μm nylon membrane, dehydrated with an acetone series, critical-point dried, coated with gold, and observed with a LEO 435VP SEM (England).

Results and Discussion

Effect of plasma exposure on motility and viability of algal cells. For all marine algal species tested, both motility (for dinoflagellates) and viability (as defined by neutral red staining) decreased with increasing exposure time, and the exposure time at which all cells were killed differed from species to species (Fig. 8). No obvious morphological change was observed under light microscopy for algal cells of *S. trochoidea*, *H. triquetra*, and *C. hystrix*, even after the longest exposures (320 or 640 seconds) rendering all cells non-motile, while many dead cells (not stained by neutral red) of *A. sanguinea* were damaged or broken and all cells were broken after exposing to plasma for 480 seconds. This difference in morphological damage among algal species was presumably due to differences in cell structure and chemical composition of cell coverings: both *S. trochoidea* and *H. triquetra* are armored dinoflagellates, i.e. with cellulosic plates under their cell membrane, the diatom *C. hystrix* has a siliceous cell wall and organic layer, while *A. sanguinea* is an unarmored (naked) dinoflagellate, i.e. without the cellulosic plates (see SEM micrographs below).

Some non-motile cells recovered their motility and viability within 24 or 48 h under normal culturing conditions following inoculation into fresh culture media, if the exposure time was below certain levels (e.g. 320 seconds for *A. sanguinea,* and 480 seconds for *C. hystrix*; however, about 50% of *S. trochoidea* and 10% of *H. trochoidea* cells, exposed to plasma for 480 seconds, recovered viability within 48 h). The recovery of viability of cells under normal culturing condition will be explained below. The above mentioned effects of plasma exposure on cell motility and viability were also observed in all the freshwater species of microalgae; some cells or colonies of those fragile species (Volvocales and *Ochromonas*) were broken under longer exposure time, while the cells or colonies of other species were kept intact under the longest exposure time (data not shown).

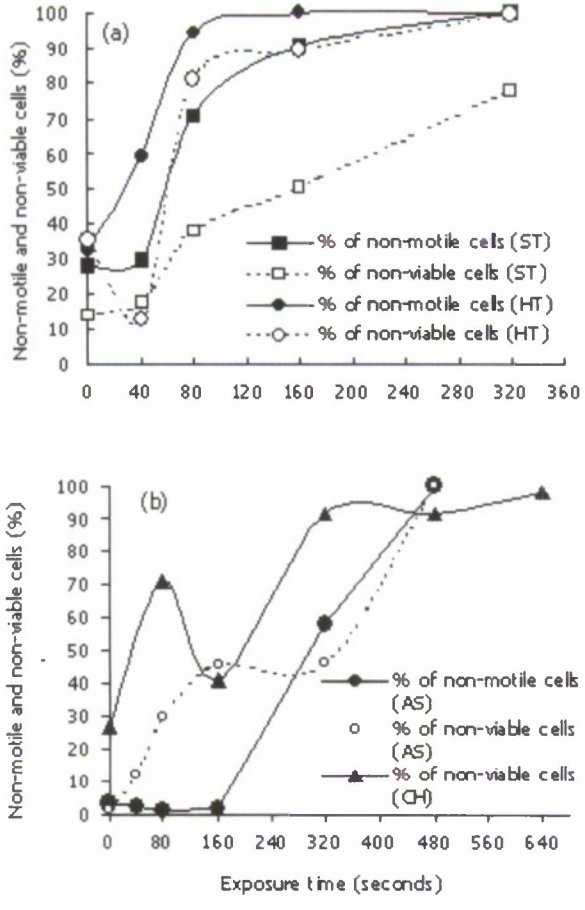


Fig. 8 Effects of exposure to plasma on motility and viability of (a) *Scrippsiella trochoidea* (ST), *Heterocapsa triquetra* (HT), (b) *Akashiwo sanguinea* (AS), and *Corethron hystrix* (CH). The cell concentrations of ST, HT, AS, and CH were 2,000, 5,880, 11,000, and 2,700 cells/mL, respectively.

Effect of plasma exposure on pH in algal cultures and culture media. We observed considerable decrease of pH in algal cultures, culture media, and deionized water after plasmas treatment, and the values of decrease increased with the length of exposure time (Fig. 9). As extremes, the pH in cultures of *H. triquetra*, *S. trochoidea*, and *A. sanguinea*, media of GSe, Alga-Gro, and Soil-Extract, and deionized water decreased from 8.45 to 2.88, 8.18 to 2.75, 8.36 to 2.47, 8.10 to 2.23, 9.03 to 2.37, 8.06 to 1.83, and 7.49 to 2.22, respectively, after 640 seconds of exposure. Further, the decrease in pH appeared to be affected by the buffering capacity of the samples when exposure time was below certain levels, because a more drastic pH decrease was observed in the deionized water and two freshwater culture media (Alga-Gro freshwater medium and Soil-Extract medium) when exposure time was below 320 seconds. When the exposure time was 320 seconds or longer, pH in all cultures, media and deionized water was less than 3, which is no doubt unsuitable for all algal species used in the study to survive. Given that the decreased pH provides explanation for the killing effect of plasma on microalgae as shown before, it will also explain the above-shown recovery of viability for cells immobilized or 'killed' by plasma,

because the low pH in plasma-treated algal samples was diluted by a relatively large volume of fresh culture medium (1:10 v/v) and was buffered by the marine culture medium (a 0.3 mL GSe medium with pH adjusted to be 2.7 was added into 3 mL GSe medium with a pH of 8.0, and the pH in the mixture was measured to be 6.9, which falls within the tolerant range of pH to the algae).

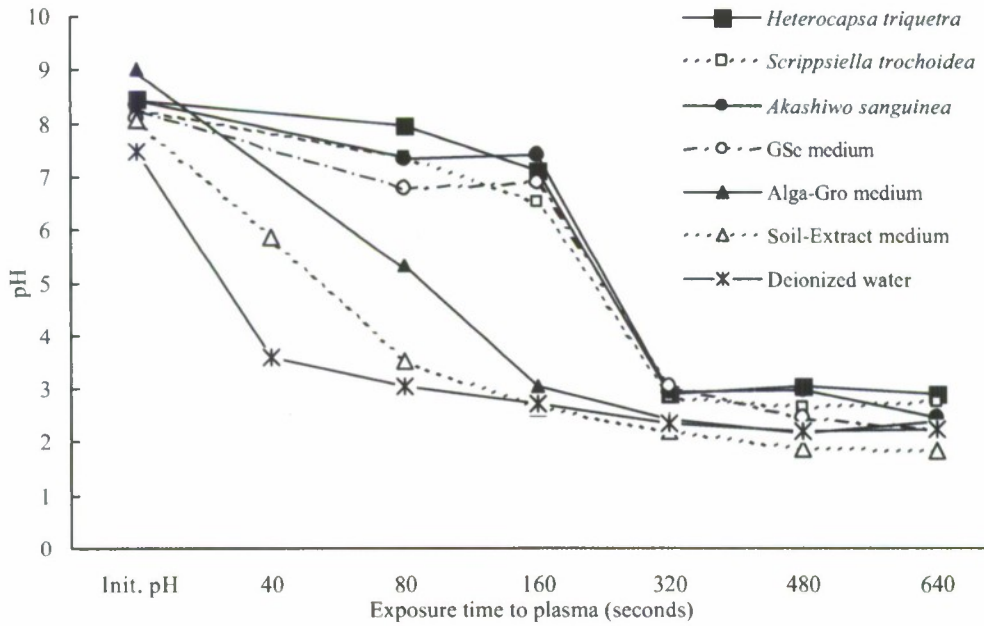


Fig. 9 Effects of exposure to plasma on pH of algal cultures, culture media, and deionized water.

SEM observations. SEM observations demonstrated striking morphological effects of plasma exposure on the microalgae, although these effects differed among species examined, and were related to the cellular structure and chemical nature of cell coverings (Fig. 10). The most prominent effect was found in *A. sanguinea* (compare Fig. 10, a and b) exposed to plasma for 320 seconds, at which time the cell membranes were dissolved, some of the cell contents were released, cell shape changed, and the remaining fibrils formed a somewhat reticulate or porous structure as a cell covering. In the *A. sanguinea* sample where pH was adjusted to 3.0 (corresponds to the pH value in sample exposed to plasma for 320 seconds), the same morphological changes as those caused by plasma were observed (compare Fig. 10, b and c). For *S. trochoidea*, because there is a resistant cellulose layer (composed of a number of plates) under cell membrane (the membrane is intact in the cells of control, as shown in Fig. 10d), the major effect of the plasma exposure was to strip off the outer cell membrane thus expose the plates (Fig. 10e). For some cells, however, the overall shape shrunk and cell contents were released, thus cells were seen with a porous surface; and cells with a small portion were even broken (micrographs were not shown). Because of the cellulose composition, the plates of *S. trochoidea* for most cells could keep intact under plasma exposure (Fig. 10e). Again, the same cellular damages were also observed when pH in the sample of *S. trochoidea* was decreased to 2.7, which corresponds to the value in the

sample exposed to plasma for 480 seconds (Fig. 10f). In addition, the same morphological damages as in *S. trochoidea* were also observed in cells of another armored dinoflagellate, *H. triquetra*, which were treated with plasma exposure (480 seconds) or pH adjustment (pH 2.8) (SEM micrographs were not shown).

Taken all together, therefore, the SEM observations provided solid evidence to support our hypothesis that the decreased pH produced by plasma exposure is the killing effect of plasma on microalgae in aqueous environments. Although the killing effect caused by UV production (Lu and Laroussi 2005 JAP) could not be completely excluded, it should not be the primary mechanism, because of rapid attenuation of UV with depth of liquid and minimal UV production in the plasma generating system used in the present study (see also Katsuki et al 2003). Since oxygen- and nitrogen-based reactive radicals are known to cause oxidation of the cell membrane lipids and proteins (Laroussi and Lu 2005 APL), we are not certain whether the observed morphological alterations are caused by simple dissolution of cellular components in the highly acidified media only, or by the oxidation reactions between membrane lipids and proteins and the freshly produced radicals as well. Because of the aqueous environment of algal cells, there is only limited direct contact between the reactive radicals and cell surfaces, however, we cannot discount a secondary role for the oxidation reaction.

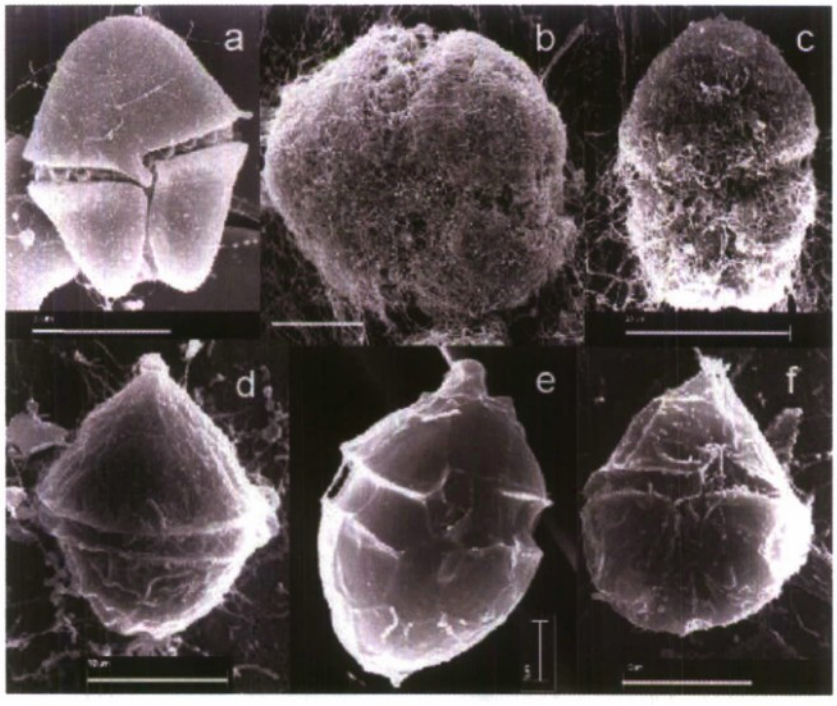


Fig. 10 Scanning electron microscope micrographs showing effects of plasma exposure and pH decrease on cellular morphology of (a-c) *Akashiwo sanguinea* and (d-f) *Scrippsiella trochoidea*: (a) control; (b) cell exposed to plasma for 320 s; (c) cell in medium with pH decreased to 3.0; (d) control; (e) cell exposed to plasma for 480 s; (f) cell in medium with pH decreased to 2.7. Scale bars: 20 μm (a, c), 10 μm (b, d, f), 3 μm (e)

Investigations of the Physics and Dynamics of the Plasma Pencil

In the latest period of performance of this research program the plasma pencil was used to investigate the effects that a cold plasma plume may have on multi-cell organisms (planaria). This is motivated by our quest to see if plasma can be used to assist in wound healing. Before presenting the results of this work, a summary of the main characteristics of the plasma pencil is presented. In addition, since it was discovered that the plume emitted by the plasma pencil is actually a plasma "bullet" propagating at very velocity, a model describing the propagation process of the plasma bullet is discussed.

Current Voltage Characteristics

The current-voltage characteristics of the discharge are shown in Figure 11 and Figure 12. Figure 11 shows the applied voltage V_a, displacement current I_{no} (without He flow: no plasma) and total current I_{tot} (with plasma on). It should be mentioned that the voltage waveform remains the same when the plasma is on or off. To find out the actual discharge current, the displacement current I_{no} is subtracted from the total current I_{tot}. Figure 12 shows the applied voltage V_a and actual discharge current I_{Dis} versus time. The first pulse of the discharge current starts after the applied voltage reaches its plateau value. The delay between the start of the applied voltage and that of the first discharge current pulse is about 60 ns. The first current pulse (between point 1 and 3) lasts about 100ns. Immediately after the first current pulse, the discharge current has another increase. The peak value of this second current pulse is about one fourth that of the first current pulse. But it lasts much longer (between point 3 and 6), about 375 ns. As can be seen, this second current pulse rises to a peak value of about 1.2 A in 100ns (between point 3 and 4), then slowly decreases to zero in 275ns (between point 4 and 6). It is interesting to note that this second current pulse was not observed in our planar dielectric barrier discharge driven by the same power supply (this particular device did not emit a plume). The mechanism of this second current pulse, which is directly linked to the launch of the plume, will be discussed in the next section of this paper. After point 6, which is about 25 ns after the arrival of the falling front of the applied voltage (point 5), a third current pulse starts. This corresponds to a new breakdown of the gap. This discharge ignites because of the voltage induced by the charges, which have accumulated on the surface of the dielectric disk during the previous discharge. This current pulse lasts about 100ns then decays to zero.

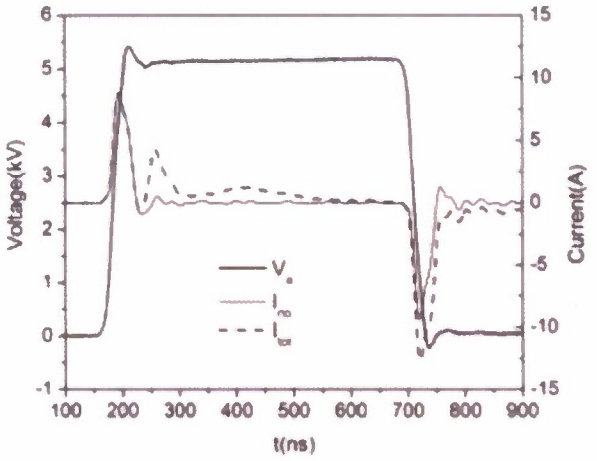


Fig. 11 Applied Voltage, V_a, total current, I_{tot}, and displacement current, I_{no}, versus time.

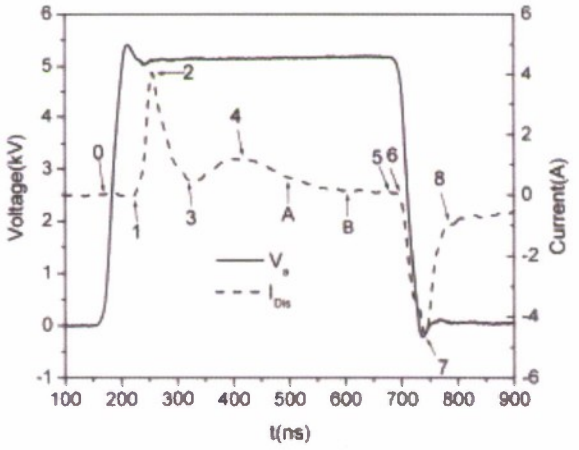


Fig. 12 Applied voltage, V_a, and discharge current, I_{Dis}, versus time

Plume propagation dynamics

As we reported earlier, to better understand the dynamics of the plasma plume, a high-speed ICCD camera with an exposure time of 50 ns was used to capture the temporal emission behavior of the plasma. Figure 13 shows that the plume is actually a small volume of plasma traveling at a very high velocity. This bullet-like plasma is launched from the exit aperture of the device and into open air. Its velocity, in the order of 10^5 m/s is far higher than the gas velocity which only about 8 m/s. An attempt to explain this interesting process is presented in this report. It is based on a photo-ionization model.

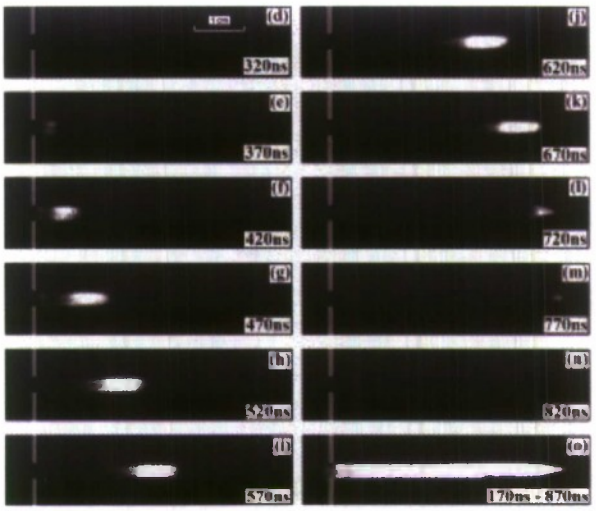


Fig. 13 ICCD pictures of the plasma plume

Plume/bullet propagation model

Until now there has not been many attempts to investigate the physical processes that can elucidate the way jets/plume are initiated and maintained in an atmospheric pressure environment that is, in most cases, simply the surrounding air. Most of the jets/plumes that were developed to date are ejected from a plasma source and into a region (room air) where the electric field can be very weak or even non-existent. So how low temperature plasmas can be maintained in such experimental conditions is a question worth delving into. We recently proposed the following model to explain how the plume emitted by their plasma jet (the plasma pencil, a device driven by nanoseconds high voltage pulses) is produced. This model may apply to many of the cold plasma jets/plumes used today.

The velocity at which the plasma plume travels was measured and found to be several order of magnitude greater than the gas flow velocity in the device, which is about 8 m/s. To have a plume travel at high speed under very low electric field, photo-ionization has to play an important role. In his photo-ionization-based model (Lu & Laroussi, 2006; Dawson & Winn, 1965) the head of a cathode-directed streamer is a assumed to be a sphere of radius r_0, containing n^+ positive ions. As the streamer head moves forward, it leaves behind a quasi-neutral ionized channel with a very low conductivity; the head is not connected to the anode and only the streamer head is measurably luminous. This was also observed experimentally (Lu & Laroussi, 2006). Therefore, the following sequence of events was proposed:

Assume that at given instant of time, the streamer head consists of a small sphere, which has a radius r_0 and a space charge n^+; Because of photons emission from the streamer, suppose that a single photoelectron is created at a suitable distance r_1 from the center of the sphere. Under the influence of the field set up by the space charge, the electron is accelerated towards the sphere and an avalanche is initiated. In moving towards the sphere, from r_1 up to some point r_2, the electron forms an avalanche of multiplication

$$n = \exp \int_{r_2}^{r_1} \alpha dr , \qquad (1)$$

and of diffusion radius

$$r_0 = \left(6 \int_{r_2}^{r_1} \frac{D}{v_d} dr \right)^{1/2}, \qquad (2)$$

where α is Townsend's first ionization coefficient, D is the diffusion coefficient, and v_d is the electron drift velocity. If the multiplication up to the sphere is sufficient, the electrons neutralize the positive charge but leave behind a new positive region. The best value of r_1 could in principle be obtained from an exact knowledge of the number and type of photons emitted from the sphere within a particular solid angle, their absorption coefficients, and ionizing efficiency. Since complete data on these quantities is not available, r_1 is taken as the distance at which the electric field strength is such that ionization and attachment rates become equal. For air, this occurs when $E/p=30\,{}^{V}/_{cm \cdot mmHg}$ (Dawson & Winn, 1965).

According to Dawson & Winn, the following three requirements must be fulfilled in order for the streamer propagation under low or zero field to occur: (1) The number of new positive ions created by the avalanche must be equal to n^+, the number of ions in the original sphere; (2) The diffusion radius of the avalanche head must not become larger than r_0; and (3) the avalanche must reach the required amplification before the two charge regions begin to overlap, i.e., $2r_0 \leq r_2$.

Next, r_2 and r_0 are calculated for different values of positive charge n^+. The procedure is as follows: First, a value of n^+ is given. Second, the electric field as a function of r from simple electrostatics is calculated according to:

$$E = \frac{Q}{4\pi\varepsilon_0 r^2} \qquad (3)$$

In atmospheric pressure air conditions the main ion is N_2^+ even if the jet/plume contains a carrier gas (such as helium) (Laroussi & Lu, 2005). In addition, the attachment process is dominated by O_2, also from air, which diffuses to the plume. The distance r_1 is determined as the distance at which the reduced electric field is equal to $30\,{}^{V}/_{cm \cdot mmHg}$ (Dawson & Winn, 1965). Then, r_2 is calculated according to Eq. 1 when n is equal to n^+. The Townsend's first ionization coefficient α is calculated according to (Yuri, 1991):

$$\alpha = 15p \cdot \exp(-365\,{}^{p}/_{E}) \text{ cm}^{-1} \qquad (4)$$

where p is the pressure and air is assumed to be the background gas. Finally, by using Eq. 2, r_0 is calculated, where D and μ_e were determined according to (Yuri, 1991):

$$D = \frac{2\times10^5}{p[Torr]}\,{}^{cm^2}/_{s} \qquad (5)$$

$$\mu_e = \frac{0.86\times10^6}{p[Torr]}\,{}^{cm^2}/_{V \cdot s} \qquad (6)$$

Table I shows the calculated r_2 and r_0 for different n^+ [23]. In these calculations, the values of D and v_d were determined for the case of helium gas. The three requirements for the streamer self-propagation are met only if $2r_0$ is smaller than r_2. From Table I, it can be seen that when n^+ is less than 2×10^9, $2r_0$ is greater than r_2. This means that the streamer head can't self propagate under this condition. However, when n^+ is larger than 3×10^9, $2r_0$ is smaller than r_2, meaning that the streamer head can self propagate under low or zero external electric

field. Therefore, the plume velocity can reach values as high as 10^6m/s and it can travel up to several centimeters without the presence of an external electric field. This is in agreement with our experimental observations.

Table I Calculated radii (r_0 and r_2) for different original space charge number [17]

n^+ (number of original positive charges) (10^9)	1	2	3	4	5
r_2 (cm)	0.02	0.1	0.17	0.23	0.3
r_0(cm)	0.056	0.068	0.075	0.080	0.085

Effects of the Plasma Pencil on Planaria

In this part of the project, we shifted our attention to multicellular organisms. We used planaria (flatworms), known for more than two centuries to have remarkable regenerative capabilities (Newmark and Alvarado, 2002) and currently a focus of studies hoping to identify a molecular strategy for metazoan wound healing. These organisms are easily held in culture and provide an abundant, inexpensive experimental model.

We purchased several species of flatworms from Carolina Biological Supply (North Carolina), and after maintaining them in the laboratory, determined that the species best suited for our research was *Dugesia tigrina*. Once a week, worms were fed liver and hardboiled egg and their water changed using filtered, local pond water.

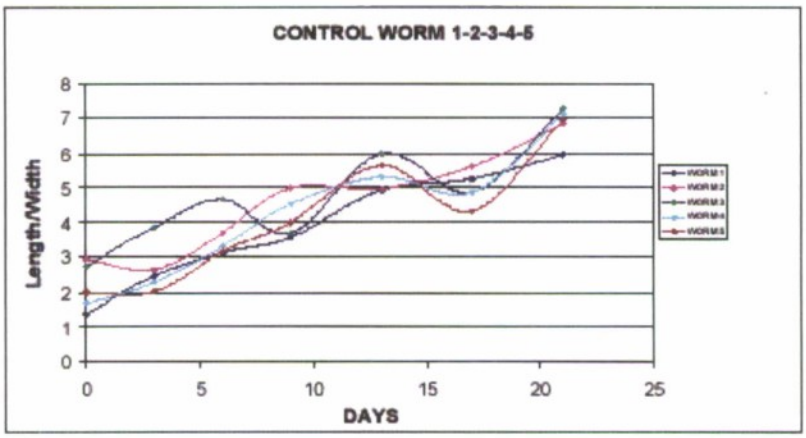
Our hypothesis was that exposure to plasma would have an impact on wound healing in these planaria. We did not know whether the effect would be negative or positive. We performed several experiments in this regard, all with the common design of completely slicing the worm laterally, then exposing its anterior portion to a range of plasma doses, and subsequently measuring the extent of the worm's regeneration over time. Controls consisted of sliced worms treated identically in all ways save that when they were placed in the plasma pencil's plume the power was "off". Regeneration was assessed using image-analysis software applied to photomicrographs taken with an Olympus DP-70 digital camera mounted on a Olympus BX-50 stereomicroscope. We compared the regeneration in plasma-exposed worms and those of the controls.

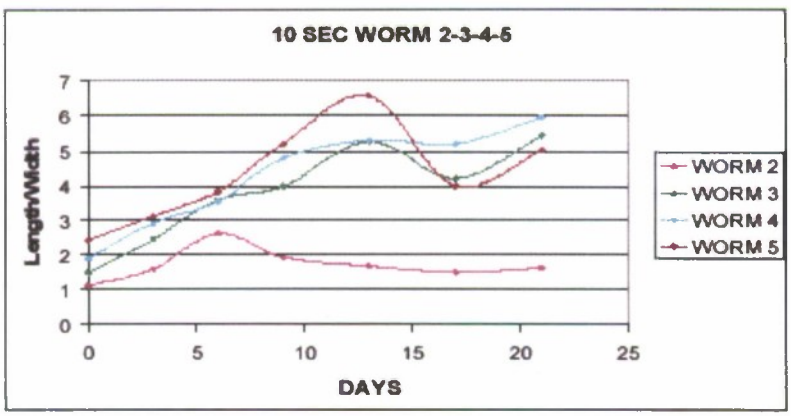
Procedure

The worms were decapitated (pre-pharyngeal cuts) and the heads were placed individually in 6-well culture plates containing a small volume of filtered pond water. Following transport to the plasma lab, as much water as possible was removed from a well, the culture plate was placed on a platform, and the worm was exposed to the plasma pencil for 0 seconds (controls), 10, 20, and 40 seconds. For each exposure time, five replicate worms were used. Immediately following exposure, filtered pond water was added to the well. The worms were returned to Dobbs' lab and photographed. To do so, worms were first removed from their wells and placed on a depression slide full of water. The depression was covered with a cover slip and excess water was wicked away using a tissue. The slide was then positioned under the Olympus SZX-12 stereomicroscope to obtain the best focus possible at 7x magnification. Multiple digital images were taken of each worm and from

image analysis, its mean length and width were determined. This measurement procedure was repeated at 3- or 4-day intervals for 21 days.

Regeneration of the worms in the various treatments is shown in the following figures. Some treatments have fewer than five worms represented, indicating that worms died during the 3-week course of measurement. In no case did we see evidence for regeneration of worms exposed to plasma treatment being different than regeneration in control worms.





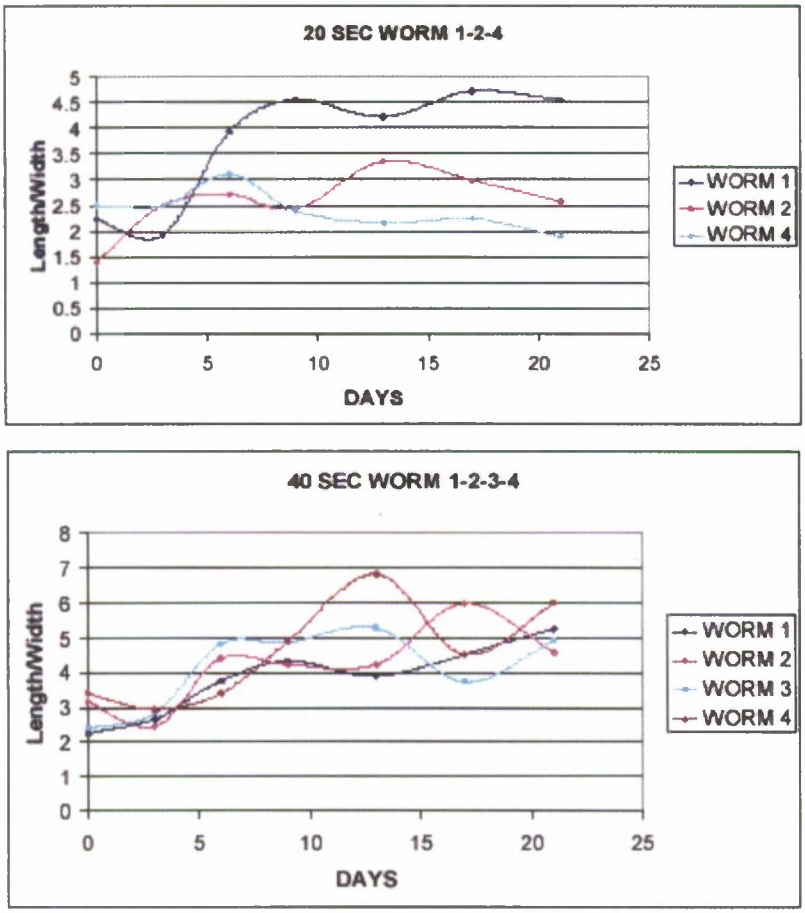


Fig. 14 Length/Width vs. time plots showing no observable difference between controls and plasma exposed planaria (10 to 40 seconds exposure times).

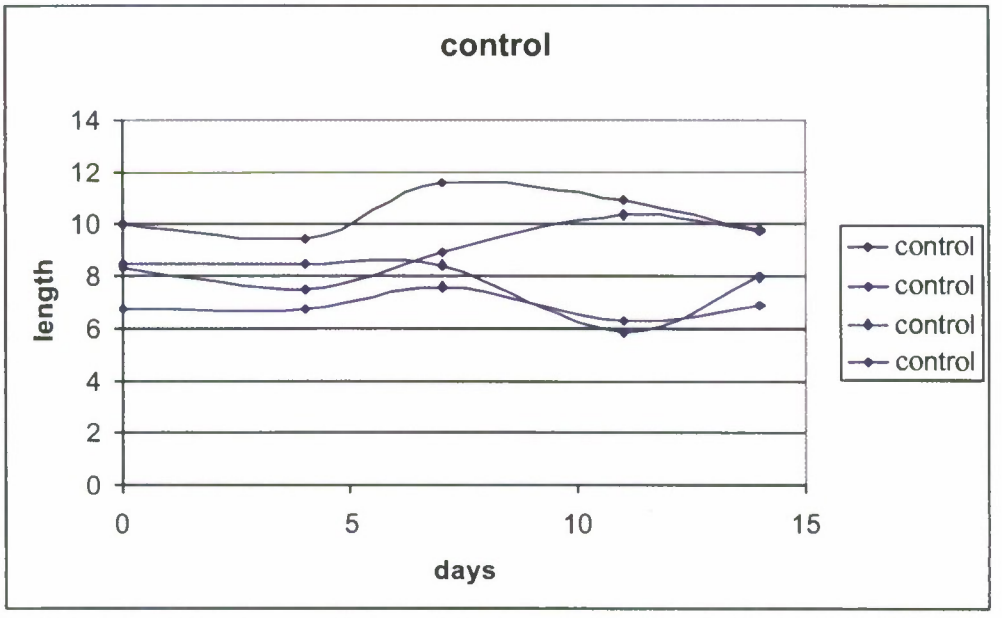
After analysis of the results from this experiment and observing that there was no difference in regeneration between the control and the treated worms we realized that in fact there was very little growth overall. In retrospect, this result seems intuitive. While the worms could regenerate to some extent, they could not feed. Recall it was a pre-pharyngeal cut, so the pharynx would need to regenerate before they regained this function. Therefore, while the worms could form new tissue, it was at the expense of breaking down existing tissue. Although it wasn't clear from the measurements, we think they became longer at the expense of becoming thinner.

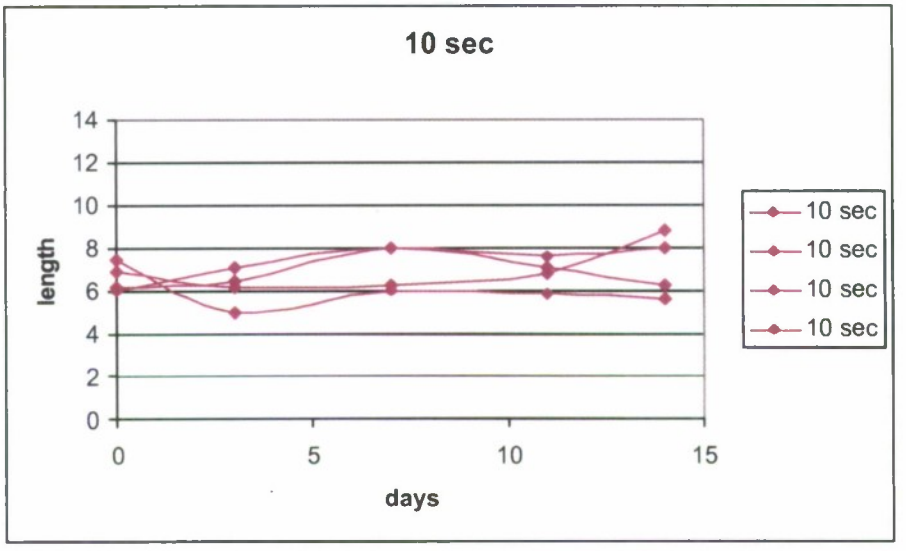
We therefore changed the location of our cut, making instead a post-pharyngeal cut (we sliced off their tails), such that the anterior section of the worm could feed. To maximize potential for regeneration, we fed the worms twice per week, rather than once.

In another change of procedure (due to a series of unsuccessful experiments where desiccation played a role in causing the death of many worms), graduate student Karakas determined he could obtain the multiple microscopic images he needed to measure a worm following its exposure to plasma simply by leaving it in its well and placing the culture dish

on the stereomicroscope. It was not necessary to transfer it to a depression slide, then return it to its well.

Worms were exposed to plasma for 0, 10, 20, and 40 seconds. Nearly all the worms survived the exposure and most lived throughout the following 14 days (see following figures). Then, most died precipitously, including the controls, so we think the water quality of our pond water had deteriorated and caused their death. Up to that point, however, the data are interpretable. As with Expt 1, we saw no evidence that regeneration of worms exposed to plasma treatment differed from regeneration in control worms. Thus, we concluded that exposure to this type of plasma neither helps nor hinders growth/cell regeneration in planaria. These results could have the following interpretations as far as wound healing is concemed: 1. plasma could play a role in assisting wound healing since plasma can kill bacteria (that may infect wounds) but appears not to hinder the natural regeneration of eukaryotic cells; 2. Plasma appears not to have an accelerating effect on cell regeneration (which may be desirable). The planaria model may be too difficult to interpret and another model (organism) and/or approach may have to be considered before we can confidently answer the question about the role of plasma in regeneration processes.

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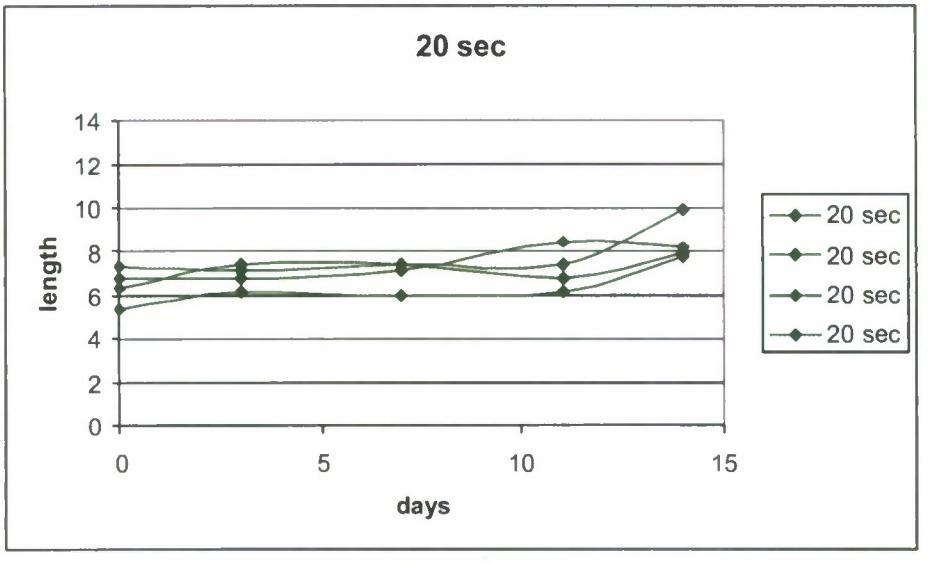
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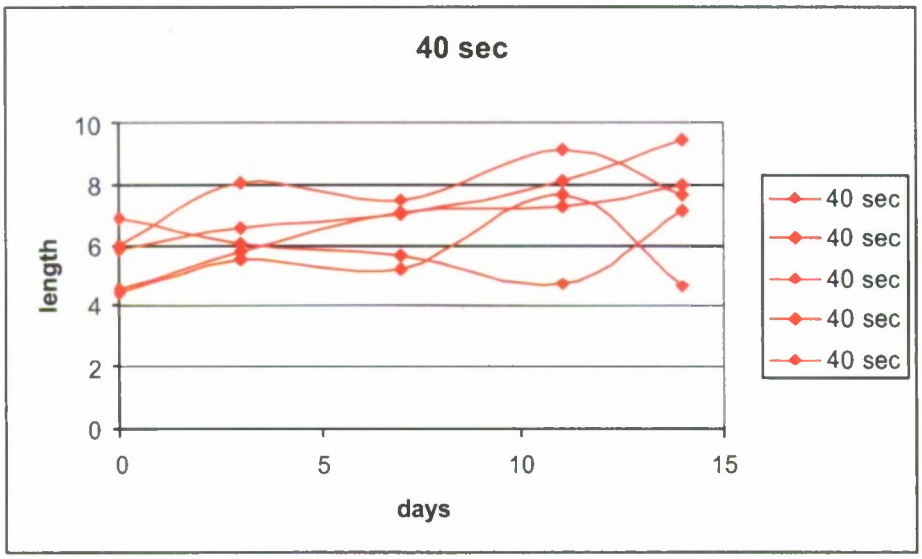

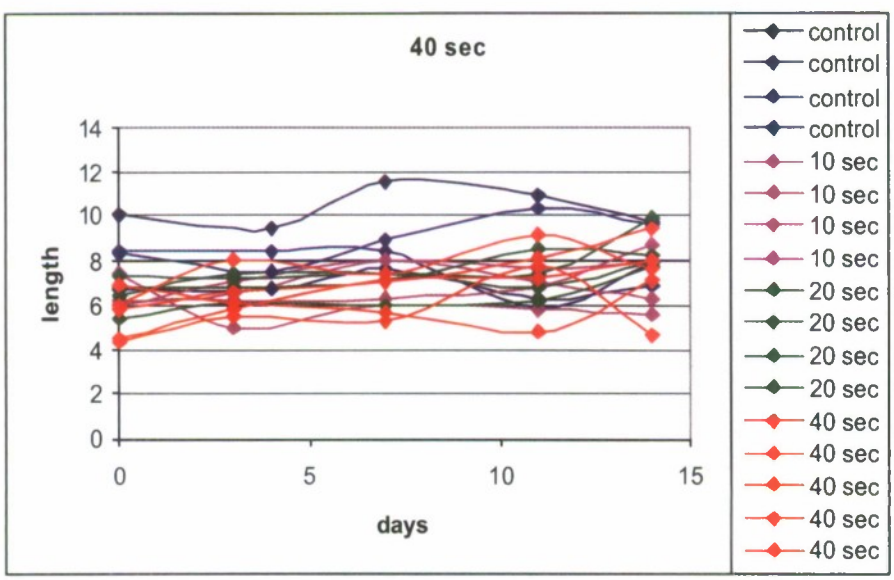

Fig. 15 Length Vs. time of the control and plasma-treated worms. No statistically significant difference can be seen in the growth of the controls and the plasma-treated.

Figure 16 shows photographs of a worm with a post-pharyngeal cut (tail sliced off) and exposed to the plasma plume for 40 seconds. After allowed to grow back under normal feeding conditions, the worm regenerated its tail and grew to an acceptable size. This indicates that the plasma did not have observable deleterious effect on the growth of the worm.

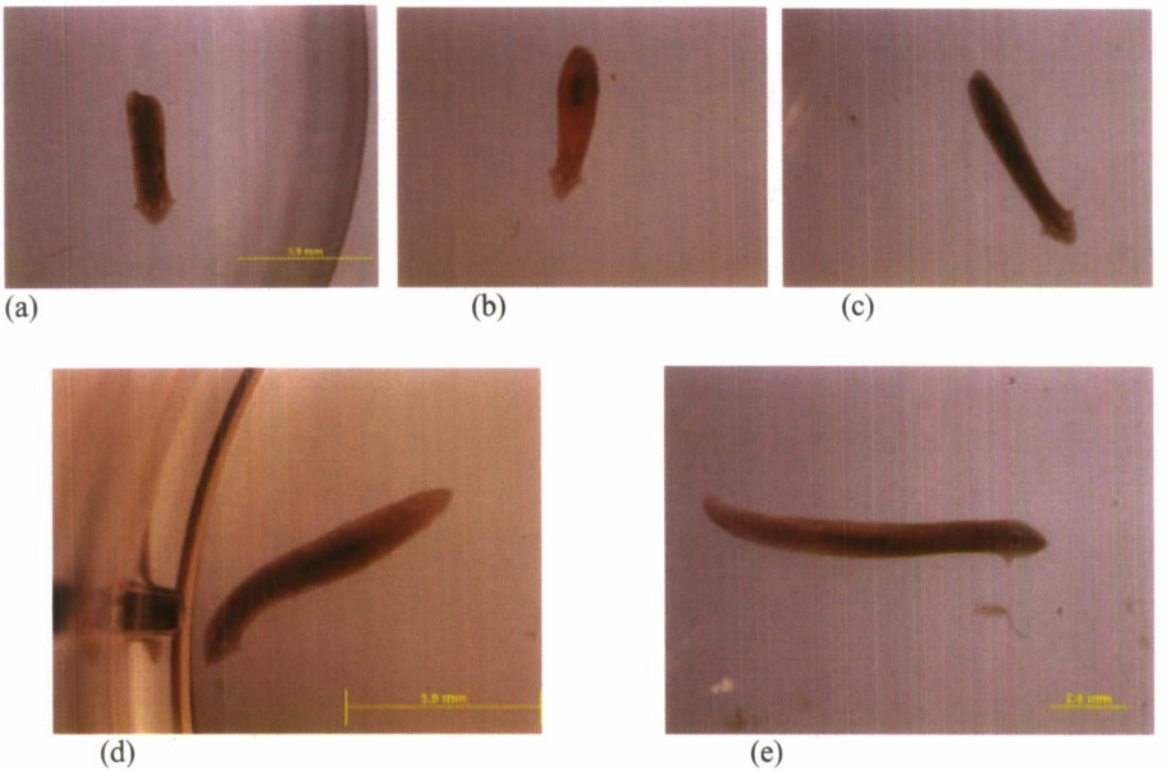

Fig. 16 Photographs of a worm having part of its tail cut, exposed to plasma for 40 seconds, and then left in conditions to grow back. (a) after 1 day, (b) after 4 days, (c) after 7 days, (d) after 11 days, and (e) after 14 days. The photos show that the worm was able to grow back in a normal way.

Investigations of the Plasma Plume Characteristics

Because we intend to use the plasma plume generated by our plasma jet device, the Plasma Pencil", for more biological applications we initiated a study aimed at characterizing the plume and its behavior.

The length of the plume created by the plasma pencil depends mainly of two parameters: the applied voltage (pulse magnitude and width) and the gas flow rate. Although the Plasma Pencil, like most other plasma jets, is electrically driven, the gas flow has an influence on the length and the shape of the plume. The direction at which the gas flow goes through the hole is not important, however, the creation of a gas channel that allows the ionization front to propagate is key. This is why a ratio between the width of the hole and the

gas flow rate has to be found to achieve a laminar flow. In this case, a gas channel is created, which prevents mixing with the ambient air on a longer range.

The voltage is the main parameter affecting the length of the plume. Figure 17 shows the influence of the voltage on the plume length for different gas flow rates. It increases for some range of the voltage magnitude but soon all the curves reach a maximum value. The length remains constant for further increase of the voltage. A mechanism involving chemical reaction rates and diffusion rates is probably the cause of this plateau that leads to equilibrium between the energy input into the plasma by the pulses and the energy lost by diffusion and recombination.

As seen in Figure 17 an optimal value for the gas flow exists, 7.77 l/min, at which value the plume reaches its maximum length.

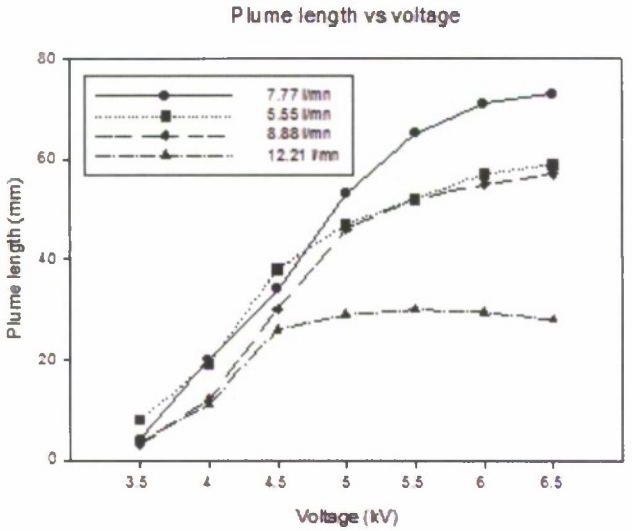

Fig. 17 Length of the plume produced by the plasma pencil as a function of the voltage for different gas flows.

To confirm the existence of an optimum for the gas flow rate, Figure 18 shows the plume length as a function of the gas flow rate for different voltages.

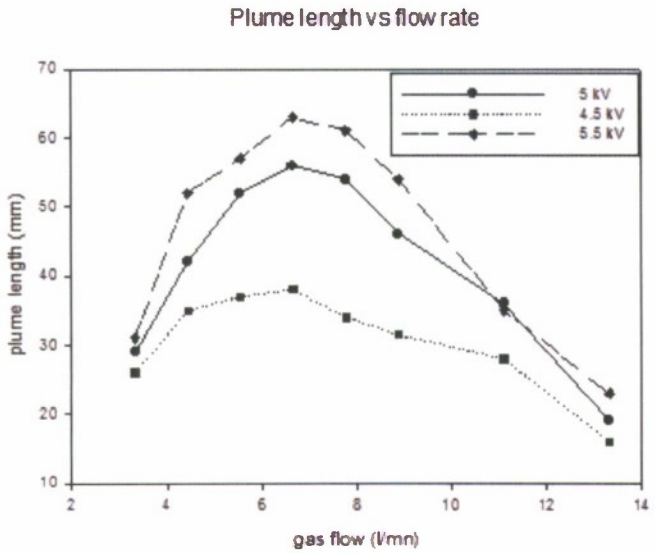

Fig. 18 Length of the plume produced by the plasma pencil as a function of the flow rate for different voltages

The maximum value is obtained with a flow rate around 7 l/mn. The influence of the voltage is not important for very low or very high flow rates. However, to obtain a greater plume length for increasing voltages, the gas flow rate value has to be close to 7 l/mn. At high flow rate values, the plume becomes unstable then begins to decrease when the gas flow is increased. The shape of the plume is modified by the presence of instabilities (Figure 19).

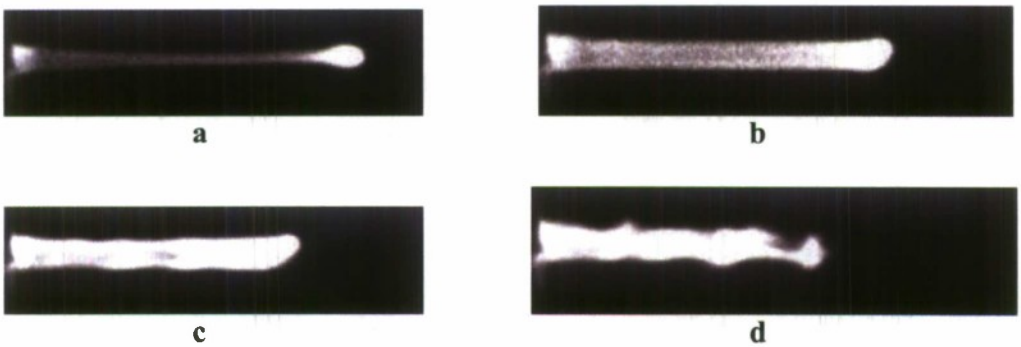

Fig. 19 Shape of the plume for different flow rates a) 6.6 l/mn, b) 8.8l/mn, c) 11 l/mn, d) 13.2 l/mn, (V = 5kV

The turbulence tends to reduce the length of the plume very quickly and are due to fluid dynamics since no change is observed when changing the voltages applied.

Another important parameter for the plume length is the pulse width. The increase of the pulse width results in an increase of the length of the plume as seen in figure 20. The maximum length is reached for a pulse width of 900ns, then the length decreases slightly with longer pulses. Between 200 ns and 800 ns, increase of the plume length is quasi linear which means that the length and the energy deposited into the plume are directly related. The ratio between the length of the plume and the pulse width indicates that the best efficiency is reached for a pulse width of 800ns.

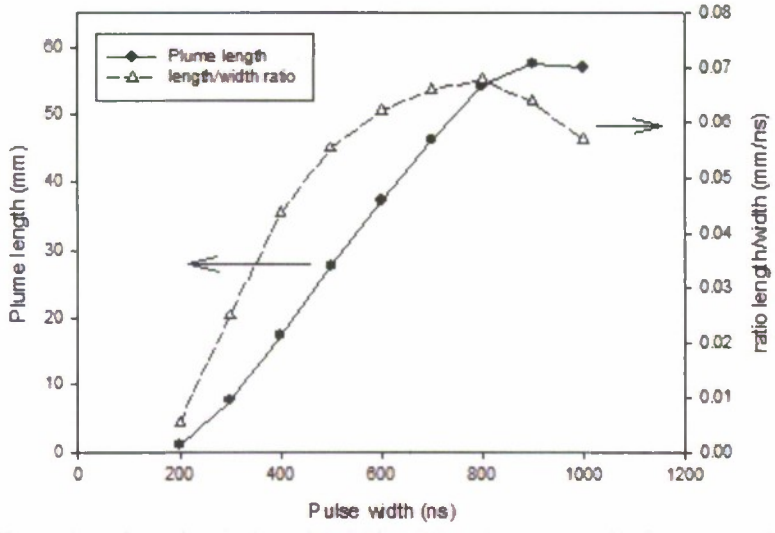

Fig. 20 Plume length and ratio length/width of the plasma pencil plume as a function of the applied voltage width. Helium flow rate = 7.7L/mn, voltage = 5kV

The frequency has little influence over the plume length. No change in the shape of the plume is observed except at 1kHz, where oscillations occur (see Figure 21)

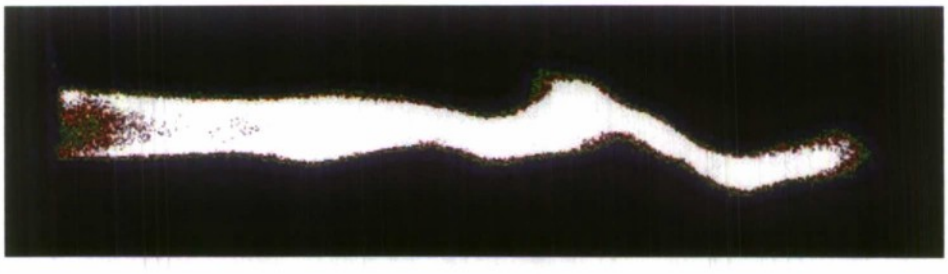

Fig. 21 Oscillation in the propagation of the plume occurring a 1 kHz frequency

Effects of the Plasma Bullets on Oral Borne Bacteria

It was our intention to pursue experiments on blood coagulation by the plasma pencil. Unfortunately, because of various complicated procedures needed to be taken in order to work on blood in our lab (risks of infections, source of safe blood, etc....) and due to the short time left for this project we decided to change course and use the remaining time on a collaboration with ODU's Research Center for Dental Hygiene to study the effects of the plasma pencil on oral borne bacteria.

As we described above, the plasma plume emitted by the plasma pencil is in fact a train of high velocity plasma bullets. These plasma bullets can act as a vehicle transporting chemically reactive species to a target material such as bacterial cells. Here we investigated the effects of the plasma bullets on bacteria of dental relevance, *Streptococcus mutans*, which is implicated in the onset and progression of dental caries. *S. mutans* is a cariogenic organism that contributes to caries in infants, children and adults. *S. mutans* alone are not difficult to destroy; however, combined with other complex oral bacterial and saliva, they are able to form biofilms consisting of high molecular weight glucans in the presence of ingested sucrose.

The experimental design that was utilized during this study is as follows: Samples of *S. mutans* contained in sterile 24-well polystyrene cell culture clusters were exposed to non-thermal atmospheric pressure plasma plume at various time intervals (60, 120, 180, 300 seconds). A total of 90 samples were used. The experimental group consisted of 72 *S. mutans* samples exposed to the plasma at various times (60, 120, 180, 300 seconds) and then inoculated onto MS agar. The control group contained 18 *S. mutans* samples that were not exposed.

Twenty-four hours prior to exposure to the plasma plume, microorganisms were cultured in BHI broth and diluted to 1:100. Experimental and control samples of *S. mutans* were pipetted into sterile wells and exposed to the plasma plume for various time periods (60, 120, 180, 300 seconds). Adding 1 mL of BHI broth to each exposed sample yielded additional samples, which were plated on MS agar using an Autoplate 4000 spiral plater (see Fig. 22 & 23). Plates were inverted to prevent condensation contamination and then incubated for 48 hours at 55°C. Bacterial colonies were then counted and recorded.

Fig. 22 Getting samples from test tube

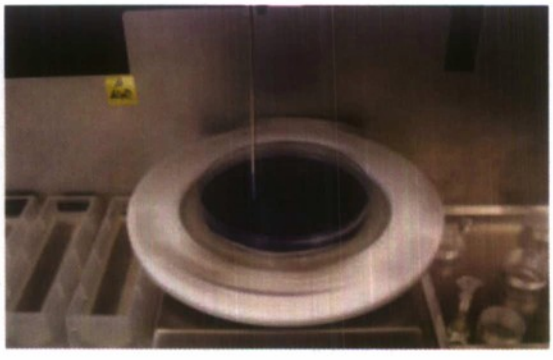

Fig. 23 Dispensing samples on *Mitus salivarius* agar

Figures 24 and 25 show the appearance of the plasma pencil before and during exposure of *S. mutans* samples.

Fig. 24 Plasma plume before exposure of samples

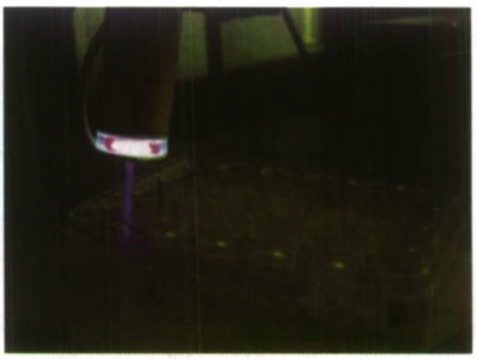

Fig. 25 Plasma treatment of samples

Figure 26 shows the results in a form of a plot of the mean colony forming units (CFUs) per ml versus the time of exposure of the samples to the plasma plume.

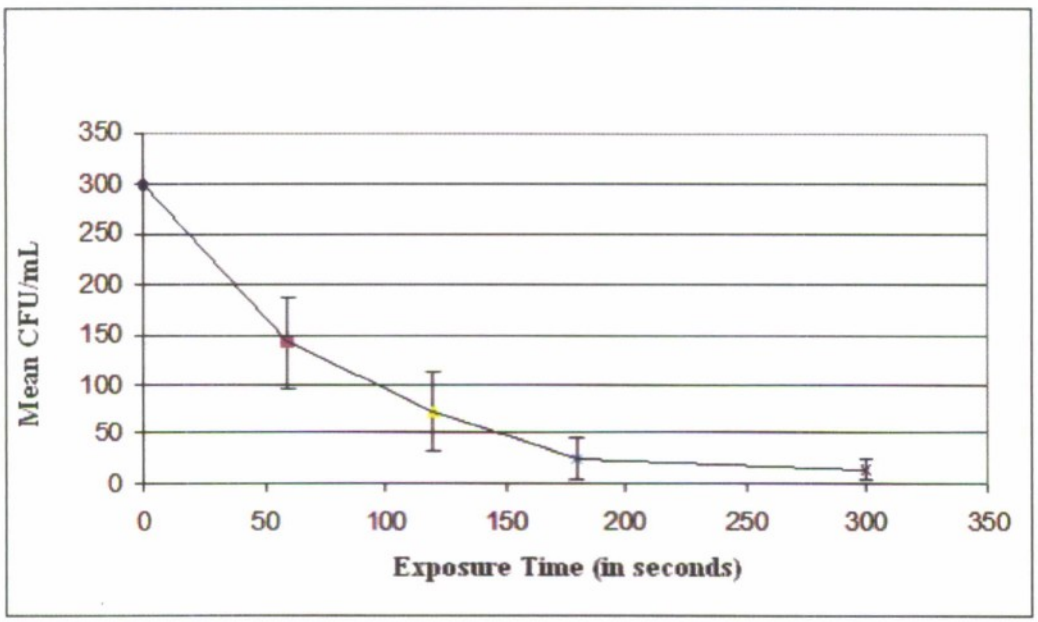

Fig. 26 CFUs of *S. mutans* versus plasma exposure time

As seen in the figure above, the plume emitted by the plasma pencil was capable of inactivating more than 90% of the an initial population of *S. mutans* if the treatment time is longer than 200 seconds. Based on these results we plan to conduct more experiments in a future collaboration with the department of dental hygiene. We plan to test the plasma pencil on other microorganisms implicated in various oral and dental related diseases.

Other Activities of the P.I.

During the years of performance of this project, the PI has been involved in various scholarly activities. He was technical area coordinator as well as session organizer for three International Conferences on Plasma Science (held in Albuquerque, NM, 2007; Karlsruhe, Germany, 2008; and San Diego, CA, 2009). He served as a Guest Editor for the IEEE Transactions on Plasma Science (Vol. 36, No. 2, 2008) and as Guest Editor of two special issues of "Plasma Processes & Polymers" on Plasma Medicine (Vol. 5, No. 6, 2008) and the second to be published in the Fall of 2009. The PI will be the General Chair of ICOPS 2010, which will be held in Norfolk, VA, and is a member of the Scientific Organizing Committee of the International Conference on Plasma Medicine, which will be held in September 2010.

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