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Sublethal and Killing Effects of Atmospheric-Pressure, Nonthermal Plasma on Eukaryotic Microalgae in Aqueous Media

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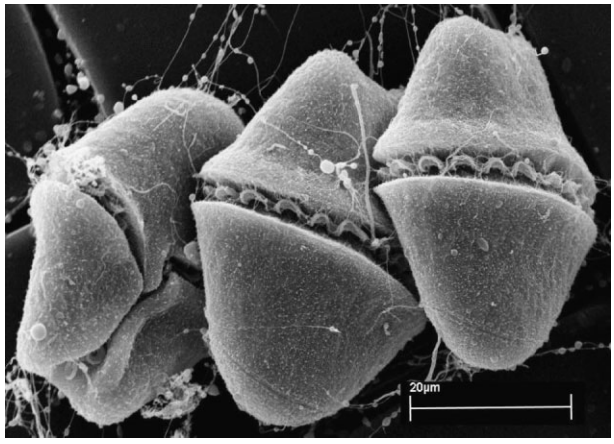
In-depth studies on the interaction of nonthermal plasmas with microorganisms usually focus on bacteria; only little attention has been given to their effects on more complex eukaryotic cells. We report here nonthermal plasma's effects on cell motility, viability staining, and morphology of eukaryotic microalgae, with three marine dinoflagellates and a marine diatom as major targets. The effects on motility and viability staining depended on the time of exposure to plasma and the species of microalgae. We observed a strong pH decrease in aqueous samples (marine and freshwater algal cultures, their culture media, and deionized water) after exposure to plasma, and hypothesized this decreased pH as the principal mechanism by which plasma exerts its deleterious effects on cells in aqueous media. The hypothesis was supported by results of experiments in which decreasing the pH of algal samples (effected by addition of acid) caused the same morphological damage (as determined with scanning-electron microscopy) as did exposure to plasma.



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Introduction

Nonthermal atmospheric-pressure plasmas offer unique features such as simple design, low operational cost, high electron energy, and low operating gas temperatures, features that have suggested their use to sterilize reusable medical tools and to decontaminate biological and chemical warfare agents.[1–5] In-depth studies on the interaction of such plasmas with microorganisms, however, are relatively recent and until now, the focus has been their effects on bacteria.[4,6–12] Very little work has considered effects of nonthermal plasmas on more complex eukaryotic cells.[13–16] In addition, the drive to develop practical means of decontamination has led

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researchers to concentrate on the germicidal effects of plasmas, with little attention given to fundamental biological aspects of their effects on microorganisms, microeukaryotic ones in particular. Previous studies have shown eukaryotic cells are more resistant to plasmas than are bacterial cells, and their exposure to plasma sometimes can elicit beneficial changes, e.g., helping to coagulate blood during surgery.[17] In other instances, however, exposure to cold plasmas at high power levels can damage mammalian cell membranes, leading to necrosis.[18] Lower doses of exposure to plasma can stimulate apoptosis,[17] and at even lower doses, detachment of cells from cultured cell sheets.[17,18]

There have been no reports of plasma effects on eukaryotic microalgal cells, which differ from mammalian cells in their composition and structure; in particular, microalgae have a cell wall, which is lacking in mammalian cells. Furthermore, except for very few cases [7,18], almost all studies of plasma's effects on bacterial or other cells have focused on surface- or air-borne microorganisms.[5,6,8,10] There is very little known concerning whether and how the functional behavior and mechanism of plasma differs when an aqueous medium containing microorganisms is exposed. In investigating the deactivation effect of low-energy pulsed atmospheric electron beam on bacterial cells, Ghomi et al.[19] observed the survivability of *E. coli* increased if a wet medium, instead of a dry one, was used. Kieft et al.[18] reported the thickness of a covering liquid layer was the most important factor influencing the effects of plasma on mammalian cells. Espie et al.[7] worked with a liquid medium and suspected reactive species and UV light caused substantial inactivation of cells, but concluded "much is unknown regarding the inactivation kinetics of the plasma process."

More fundamentally, deactivation or sterilization mechanisms of plasma have not been well resolved[3,6] and the responsible mechanisms are highly dependent on the configuration of the plasma-producing apparatus, exposure mode, and gas types used, because the composition of plasma species changes accordingly.[3,9] In many cases, explanations for the observed effects of plasma have been either descriptive[6,9] or speculative.

Here, we report the effects of nonthermal plasma on cell motility, viability staining, and morphology in a variety of eukaryotic microalgae in aqueous environments. These algae, and others like them, can cause a variety of concerns for human populations, ranging from taste and odor problems in drinking-water supplies[20] to noxious blooms of so-called "red tides", responsible for fish deaths, beach closures, and shellfish poisoning.[21] Based on systematic measurements of pH and comparative observations of algal cell morphology using scanning electron microscopy, we concluded that a strong pH decrease in samples following plasma exposure is the principal mechanism responsible for its deleterious effects on algal cells.

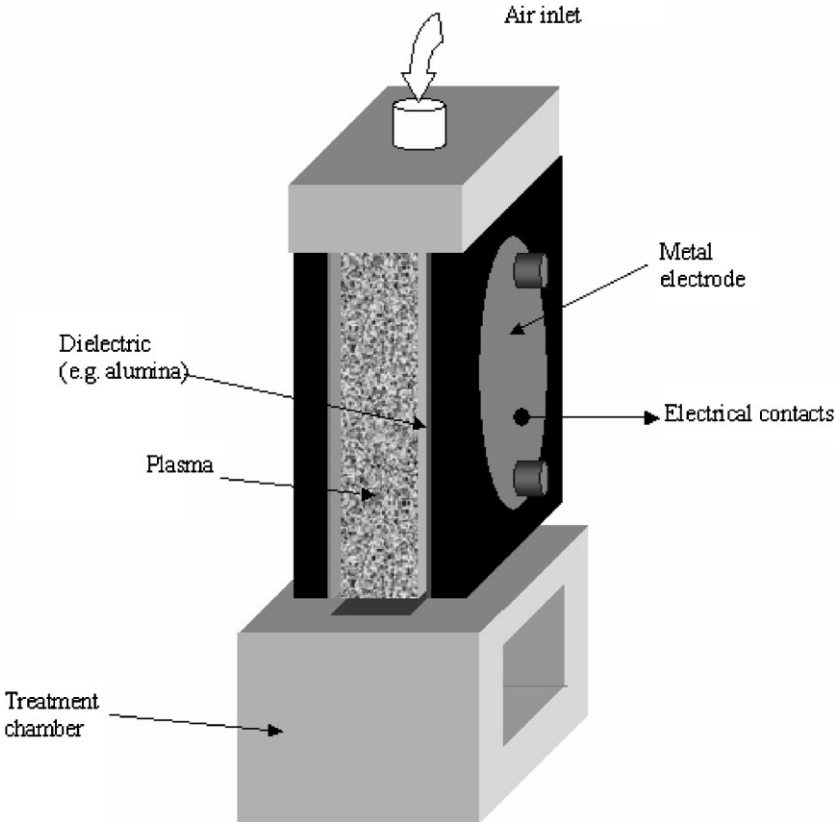

Figure 1. General configuration of one stage of the DBD system used to expose cultures of microalgae to nonthermal, atmospheric-pressure plasmas.

Experimental Part

Plasma Apparatus

The plasma generator used in this investigation was based on the Dielectric Barrier Discharge (DBD) concept.[22] DBDs use two parallel plates or concentric cylindrical electrodes separated by a variable gap. At least one of the two electrodes is covered by a dielectric material such as glass or alumina (Al_2O_3). Figure 1 shows the parallel plate DBD system used in our studies. DBDs are usually driven by sinusoidal voltages with frequencies in the 50 Hz – few kHz range. When the discharge is ignited, charged particles are collected on the surface of the dielectric. This charge build-up creates a voltage drop, which counteracts the applied voltage, and greatly reduces the voltage across the gap. The

discharge subsequently extinguishes. As the applied voltage increases again (at the second half cycle of the applied voltage) the discharge re-ignites. Depending on the operating parameters, DBDs can generate either filamentary or diffuse mode plasmas.

The plasma generator used in this work comprised four DBD stages connected in cascade. The dielectric material used for the DBDs consisted of 1 mm thick alumina squares (10×10 cm^2). The gap between the two electrodes of each DBD was about 4 mm. The electrical power was supplied by a step up transformer that could deliver voltages up to 9 kV_{RMS} at the line frequency of 60 Hz. Room air was the operating gas and was circulated through the DBD gaps at flow rates of 4 $L \cdot min^{-1}$. Samples containing microalgae to be treated were placed in a second chamber below the plasma generation chamber (Figure 1). The reactive species generated by the plasma diffused down to the "treatment chamber" through a wide slit located just under the DBD plasma units. This mode of treatment is referred to as "remote exposure" (as opposed to "direct exposure", in which the plasma comes in direct contact with the items under treatment), and consequently, thermal effects are negligible, charged particles recombine before reaching the sample, and neither UV radiation nor short-lived (order of microseconds or less) neutral reactive species are of consequence.[3]

Eukaryotic Microalgae

We used three marine dinoflagellate species (*Akashiwo sanguinea*, *Scrippsiella trochoidea*, and *Heterocapsa triquetra*) isolated from the Elizabeth and Lafayette Rivers, subestuaries of the Chesapeake Bay in Norfolk, Virginia, USA, and a marine diatom (*Corethron hystrix*), purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Maine, USA. To assess the phylogenetic extent of physiological and morphological changes in response to plasma exposure, we also tested 11 cultures of freshwater microalgae, including 7 green algal cultures (Volvocales mixed culture, *Carteria olivieri*, *Chlamydomonas* sp., *Chlorella* sp., *Gonium pectorale*, *Hydrodictyon reticulatum*, *Micrasterias* sp.), a euglenoid (*Euglena acus*), two golden-brown algae (*Ochromonas danica*, *Synura* sp.), and a dinoflagellate (*Peridinium* sp.) (all from Carolina Biological Supply Company, North Carolina, USA). The marine species were maintained either in GSe[23] or f/10 medium in a lighted incubator provided with cool fluorescent light (20 ± 1 °C, 12 h-12 h light-dark photoperiod, light intensity approximately 80 μEinstein $m^{-2} \cdot s^{-1}$), while the freshwater species were maintained in Alga-Gro freshwater medium or Soil-Extract medium (both from Carolina Biological Supply Company) in the lighted incubator. The cultures used for experiments were at exponential or late-exponential growth stage.

Exposure Procedure

To test the effect of plasma exposure on cell motility (for those algal species having flagella) and viability staining, 1 mL of algal culture was added to a well of a 12-well culture plate (depth of liquid in the well was 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 closest edge of the plasma was 25 mm), and the sample was exposed to plasma for predetermined times ranging from 40 to 640 s. Fresh aliquots of algal cultures were used for each exposure time. The algae used (and their initial concentrations expressed as cells $\cdot$ mL^{-1}) were: *Scrippsiella trochoidea* (2 000); *Heterocapsa triquetra* (5 880); *Akashiwo sanguinea* (11 000); *Corethron hystrix* (2 700). To determine viability of cells not exposed to plasma, cells were added to culture plates and placed in the chamber as described and held there for 80 s, but the power was not turned on. This treatment was regarded as "0 seconds" of exposure to plasma.

After treatment, 0.12 mL of the sample was put into a 0.1 mL glass counting chamber, covered with a cover slip, examined using a Nikon light microscope (total magnification 100×), and intact cells, motile and nonmotile, were counted. Dinoflagellates "swim" with a distinctive, whirling motion, so it is straightforward to determine their motility. The motility of the diatom used in these experiments is not so easily assessed and it was not evaluated in this regard. The rest of the sample in the well (nominally 0.88 mL but less in samples exposed to plasma for long periods) was stained with neutral red (NR, 3-amino-7-dimethylamino-2-methylphenazine, a lipophilic free base, 0.001% w/v final concentration) for 5 to 10 min, then examined to determine the number of stained and nonstained cells. Neutral red is a so-called "vital stain", i.e. it is taken up by cell walls and vacuoles of living cells, which subsequently appear red under bright-field microscopy.[24] Therefore, cells stained red by NR and motile cells (whether stained red or not) were functionally defined as viable, while non-motile cells that did not stain were considered to be dead. For each combination of alga and time of exposure, between 150 and 300 cells were examined and categorized. Overall, more than 10 000 cells were evaluated in this part of the study.

In casual observations, some cells exposed to plasma and ostensibly dead as a result were seen to recover when transferred to fresh medium. To some extent then, lack of motility and inability to be stained apparently reflected a level of temporary inactivation. A separate set of experiments was performed to test cells' ability to recover after plasma exposure. Algal cultures were treated as above, then inoculated separately into fresh culture medium contained in 6 well plates (0.3 mL samples into 3 mL of medium) within 0.5 h, incubated as for culture maintenance, and examined for motility and ability to be stained after 24, 48, 72, and for freshwater algae only, 96 h. Because these microalgae can reproduce as frequently as once per day under optimal conditions, results were more useful in determining the degree of plasma exposure resulting in incontrovertible death of all cells in the population, rather than quantifying the recovery of inactivated cells.

Measurement of pH in Algal Cultures and Media after Exposure to Plasma

pH values were measured before and immediately after exposure to plasma in 1 mL cultures of *A. sanguinea*, *S. trochoidea*, and *H. triquetra* (with cell concentrations approximately the same as those used for plasma exposures); 1 mL of GSe medium (marine,

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salinity 30 PSU); Alga-Gro medium (freshwater); Soil-Extract medium (freshwater); and deionized water (18.2 MΩ). A MI-710 microelectrode (Microelectrodes, Inc., New Hampshire, USA) connected to an ORION pH meter (model 290A, Thermo Electron Co., Massachusetts, USA) was used to measure the samples' pH. Samples were exposed to plasma for periods between 40 and 640 s.

Microscopy

Both light and scanning electron microscopy (SEM) were used to investigate whether exposure to plasma induced any gross morphological changes in algal cells. In addition, since considerable pH decreases were observed in samples after plasma exposure, algal cultures in which pH levels were reduced by addition of 0.1 M HCl also were examined to test whether morphological changes associated with exposure to plasma were elicited merely by decreasing the samples' pH. Cultures unexposed to plasma or having no pH adjustment were used as controls.

Samples were prepared for SEM as follows: 1 mL each of untreated algal cultures (*A. sanguinea*, *S. trochoidea*, and *H. triquetra*), cultures treated with plasma (320 s for *A. sanguinea*, 480 s for both *S. trochoidea* and *H. triquetra*), and cultures with lowered pH (3.1 for *A. sanguinea*, 2.7 for *S. trochoidea*, and 2.8 for *H. triquetra*, corresponding to 320, 480, and 480 s of plasma exposure, respectively) were fixed with OsO_4 at 2% (final concentration, w/v, dissolved in GSe medium) 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

Effects of Plasma Exposure on Motility and Viability Staining of Algal Cells

In each of the four species of marine microalgae tested, there was an overall decrease in two measures of cell viability (neutral-red staining and for dinoflagellates only, motility) with increasing exposure time (Figure 2). For *H. triquetra*, loss of motility exceeded loss of viability staining; this pattern was even more pronounced for *S. trochoidea* (Figure 2a). In the latter case, about 25% of the cells stained after 320 s, an exposure time for which no motility was noted. The inverse of this pattern, however, was seen in the third dinoflagellate species tested, *A. sanguinea*, for which percent loss of motility exceeded percent loss of staining ability only at 320 s and thereafter (Figure 2b). No such direct comparison of viability metrics was possible with the diatom *C. hystrix*, as it is not motile in the easily discerned manner of the dinoflagellates. Finally, it is worth noting that a significant percentage of cultured cells (range ca. 5% to 35%; varied with species) were categorized as nonmotile or nonstaining at time = 0,

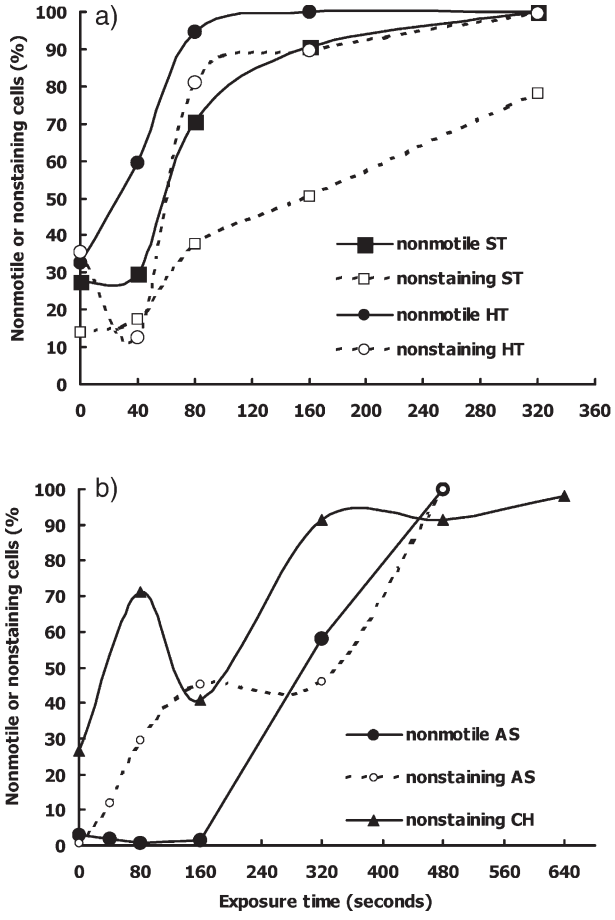

Figure 2. Time-dependent effects of exposure to plasma on viability of a) the dinoflagellates *Scrippsiella trochoidea* (ST) and *Heterocapsa triquetra* (HT), and b) the dinoflagellate *Akashiwo sanguinea* (AS) and the diatom *Corethron hystrix* (CH). Results are presented as percent nonmotile cells or percent cells not stained by neutral red. No motility data for *C. hystrix* were collected, as it is not motile in the easily discerned manner the dinoflagellates are. Each point on the graph represents inspection and categorization of 150 to 300 cells in a single sample.

i.e., even before exposure to plasma. The efficiency of NR staining varies among microalgal species and with their growth stage.[25]

In the recovery experiments, nonmotile or nonstaining cells were placed into fresh culture media and incubated under normal culture conditions. To varying degrees, the four marine species recovered motility or ability to be stained within 24 or 48 h following inoculation. Cells that had not recovered by 48 h did not do so given another 24 h (total of 72 h). The critical exposure time, after which no recovery was seen, was 320 s for *A. sanguinea* and 480 s for *C. hystrix*. Roughly half of *S. trochoidea* and ca. 10% of *H. triquetra* cells, however, recovered within 48 h after 480 s of exposure. We hypothesize these interspecific differences in recovery are related to the different cell

structure and coverings of the various algae (see below, "Effects of plasma on morphology of microalgae").

Of the 11 cultures of freshwater algae, 7 comprised motile species and they were similarly affected by exposure to plasma, e.g., 100% of *Gonium pectorale* cells lost motility after only 80 s of treatment. Increasing length of exposure was needed to eliminate motility in other cultures: 160 s for *Chlamydomonas* sp., 320 s for Volvocales mixed species, *Ochromonas danica*, *Carteria olivieri*, and *Peridinium* sp., and 400 s for *Euglena acus*.

Viability as determined by NR staining was more difficult to determine with the freshwater algae than with the marine ones. Our principal focus with the freshwater species, however, was to determine whether exposure to plasma resulted in any gross morphological damage to algal cells or colonies. With two exceptions, there was no such obvious effect, even after exposures that caused complete loss of cell motility, e.g., 480 s for *Chlamydomonas* sp., 560 s for *Euglena acus,* and 640 s for *Carteria olivieri*, *Gonium pectorale*, *Hydrodictym reticulatum*, and *Micrasterias* sp. The exceptions were first, a small proportion of the Volvocales cells or colonies were broken after 480 s and second, 99% of the *Ochromonas reticulatum* cells were broken after 320 s.

Finally, critical exposure times, after which no recovery of motility was seen, were determined for a subset of the freshwater algae and varied from 80 s for *Gonium pectorale* to 160 s for *Chlamydomonas* sp. to 320 s for *Carteria olivieri*.

Effects of Plasma Exposure on pH in Algal Cultures and Culture Media

We observed a pronounced, time-dependent pH decrease in algal cultures, culture media, and deionized water after plasma treatment (Figure 3). After 640 s of exposure, decreases ranged from 5.27 pH units in deionized water to 6.66 units in Alga-Gro medium. The pH change apparently was influenced by the buffering capacity of the samples, because an immediate, more precipitous pH decrease was observed in the deionized water and two freshwater culture media (Alga-Gro and Soil-Extract) (Figure 3). When exposure time was 320 s or longer, the pH in all cultures, media, and deionized water dropped below 3, a level highly unsuitable for the microalgae used in this study. Following their exposure to plasma, therefore, the loss of viability observed in the microalgae was associated with, and likely caused by, the physiological rigors of the low pH developed in the medium.

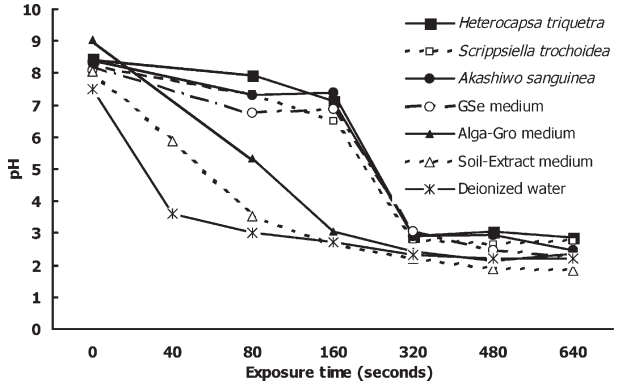

Figure 3. Time-dependent effects of exposure to plasma on pH of algal cultures, culture media, and deionized water.

Assuming that a pH decrease explained the deleterious effects of plasma on microalgae, it also illuminated the recovery of viability measures in cells exposed to plasma, then transferred to fresh medium. The low pH in plasma-treated algal samples presumably was diluted and restored upward by a relatively large volume of culture medium. An after-the-fact experiment, one using volumes equal to those in the recovery experiments, demonstrated this point. The pH of 0.3 mL of GSe medium was lowered to 2.7 using acid, then 3 mL of untreated GSe having pH 8.0 was added; the resultant mixture's pH was 6.9, a value within the range tolerated by the marine microalgae.

Since O•, NO•, NO_2, O_3, and OH• are the major components of air plasma for the configuration of experimental setup and exposure mode used in the present study,[3,26] the pH decrease may be attributed to acid-forming reactions such as:

$$2NO + H_2O + O_3 \rightarrow 2HNO_3 \quad (1)$$

$$NO + O_3 \rightarrow NO_2 + O_2 \quad (2)$$

$$NO_2 + OH\bullet \rightarrow HNO_3 \quad (3)$$

where no rank order is implied. Following exposure to plasma, therefore, the algal cultures could be considered a dilute nitric acid.[27]

Effects of Plasma on Morphology of Microalgae

Using light microscopy, no obvious morphological changes were observed in cells of the dinoflagellates *S. trochoidea* and *H. triquetra*, even after plasma exposures (320 or 640 s) that rendered all cells nonmotile. Similarly, there were no gross morphological effects of prolonged plasma exposure on the diatom *C. hystrix*. In contrast, all cells of the dinoflagellate *A. sanguinea* were broken after 480 s.

SEM images demonstrated striking morphological effects of plasma exposure on the dinoflagellates, but effects differed among the three species examined. Consistent with light-microscope observations, the most prominent effect was found in *A. sanguinea* (compare Figure 4a and 4b) exposed to plasma for 320 s. The cell

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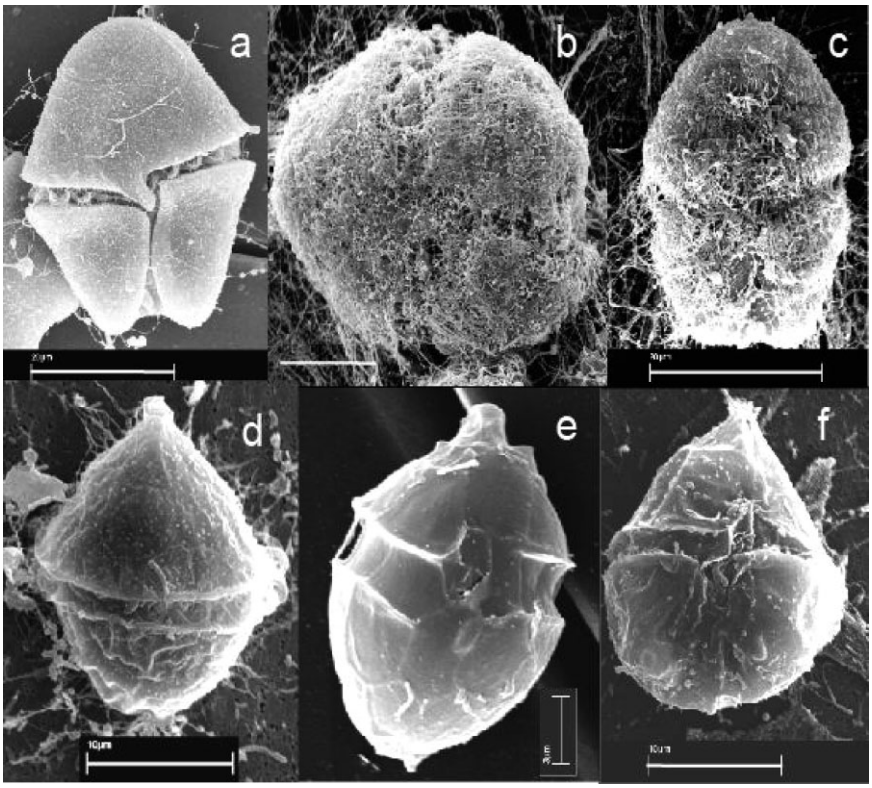

Figure 4. Scanning electron micrographs showing effects of plasma exposure and pH decrease on cellular morphology of (a–c) *Akashiwo sanguinea* and (d–f) *Scrippsiella trochoidea*: a) control; b) cell exposed to plasma for 320 s; c) cell in medium with pH decreased to 3.0; d) control; e) cell exposed 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).

membranes were disrupted, some cell contents were released, cell shape changed, and the remaining fibrils formed a reticulate, seemingly porous structure covering the cell. With *S. trochoidea*, the most commonly observed effect of plasma exposure was to strip off the outer cell membrane, exposing the cellulosic plates underneath (compare Figure 4d and 4e). In some instances, however, the cell shrunk overall, releasing its contents, but leaving the plates intact, giving the effect of a porous surface. To an even lesser extent cells broke open (micrographs not shown). Morphological damage similar to that observed in *S. trochoidea* was also seen in cells of *H. triquetra* exposed to plasma (micrographs not shown).

This differential morphological damage presumably is related to differences in microalgal cell structure and the chemical nature of their cell coverings. The diatom *C. hystrix* has a siliceous cell wall overlain by organic matter. Both *S. trochoidea* and *H. triquetra* are armored dinoflagellates, i.e., they have cellulosic plates under their cell membrane. In contrast, *A. sanguinea* is an unarmored (naked) dinoflagellate, without such plates. The diatom's siliceous cell wall apparently was little affected by exposure to plasma, and by extension, the armored dinoflagellates had more internal structure, given their plates, and were more resistant to plasma's deleterious effects.

What is the mechanism whereby remote exposure to plasma exerts its deleterious effects on microalgal cells in aqueous environments? Because we observed considerable pH decreases in media after their exposure to plasma, we examined cells to compare plasma-associated morphological changes with those elicited merely by decreasing the sample's pH. In the case of *A. sanguinea,* morphological changes identical to those caused by plasma were observed after acidification (compare Figure 4b and 4c). Essentially, the same result emerged for *S. trochoidea* (compare Figure 4e and 4f) and *H. triquetra* (micrographs not shown). Therefore, SEM observations supported the hypothesis that a decreased pH is the mechanism whereby plasma exerts deleterious effects on microalgae in aqueous environments. In studies of plasma's effects on bacteria, others have reported large pH decreases, but they either merely speculated the rapid reduction in pH contributed to inactivation of cells[28] or in control experiments, showed no pernicious effects of a lowered pH.[27]

What other factors, if any, might contribute to the deleterious mechanism observed in the present study? A "charging effect" between charged particles in plasmas and the cell surface[3,8] should not be directly responsible for the killing effect, because we employed "remote exposure" and the charged particles should be neutralized before reaching the cell surface in the aqueous media. Production of UV light should not be a significant contributor to the process, given the remote-exposure method used[3,13,26] and the rapid attenuation of UV with depth in liquid. Temperature change associated with remote exposure is negligible.[3,26] In a post-facto experiment we performed, the temperature of a water sample increase only 2 °C following exposure to plasma for 5 min. The decreased pH, therefore, remains as the most plausible causative factor. We cannot be certain, however, whether the pronounced morphological alterations of microalgae were caused exclusively by simple disruption of cellular components in highly acidified media, or in addition, by oxidation reactions between membrane lipids and

proteins and freshly produced oxygen and nitrogen radicals.[27] Although there is only limited direct contact between the reactive radicals and cell surfaces in the aqueous environment of algal cells, we do not discount a secondary role for such reactions. In this context, for example, note that after 80 s of plasma treatment that ca. 80% of *H. triquetra* were non-staining (Figure 2A), while the pH had decreased only ca. 0.5 (Figure 3).

Conclusion

This first report of nonthermal plasma's effects on microalgae adds to the scant literature concerning its biological interactions with eukaryotes; previous studies have mainly focused on bacteria (prokaryotes). We determined that remote plasma exposure was deleterious, dose-dependent, and species specific. We attribute the causative mechanism to be a pH decrease of the aqueous media resulting from its exposure to acid-forming components of the plasma. Given conditions inherent in remote exposure, we discount charging effect, UV light, and thermal effects as causative mechanisms, but we cannot fully dismiss a secondary role for oxidative reactions. Finally, whether eukaryotes or prokaryotes are the organisms of focus, we suggest the increased use of vital (and mortal) stains, in addition to culture-based methods, as a tool for increased understanding of plasma's biological effects. As exemplified in this study by comparisons of motility and vital staining, not all metrics of "viability" are fully congruent.

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