Role of nitroxides and peroxides in the inactivation of bacteria: a survey

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In recent years many different plasma sources has been proposed by various research groups, studying their interaction with biological substrates[1–3]. While it is nowadays commonly accepted that a major role in the plasma-substrate interaction is taken by reactive oxygen and nitrogen species[4,5], it is still unclear which specific biochemical pathways are triggered by each source. Although a variety of diagnostics have been applied, the lack of a common protocol does not allow to directly compare the data available in the literature; moreover, custom setups are typically made for each diagnostics, resulting in possible unpredictable variations in plasma conditions between the different measurements.

Figure 1: the micropulsed plasma jet during helium operation

In this work, a preliminary comparison among three different sources for a total of five configurations is reported. The chosen treatment conditions involve a sample of 6 mL of ultrapure water (VWR Puranity TU 3UV/UF+) placed in a small petri dish (Ø 35 mm), in turn placed on a conductive table properly grounded. Above the petri dish a support structure is fixed; each source is equipped with a custom 3d-printed adapter such that, when inserted in the support structure, is perfectly centred with the petri dish, and the distance between the lower extremity of the source (typically, the nozzle) and the water surface is 0.6 mm. An optical fibre is placed on the side of the setup, just above the petri dish and pointing towards the centre of the water surface; given its opening angle, it is able to collect light for the whole length of the plasma plume. All treatment durations are fixed at 10 minutes.

Three different sources have been used: a micropulsed plasma jet, a radiofrequency one and an air-operated source. The micropulsed one, namely the *Plasma Coagulation Controller PCC*[6,7] (fig. 1), is based on a central tungsten electrode covered by a closed gas capillary. Coaxially, a glass nozzle is placed, with an external ground ring. The central rod is excited with micropulsed, 7.4 kV_{peak} high and 0.45 µs wide, with a frequency of 5 kHz; a noble gas (helium or argon) is flown at a fixed rate of 1 L/min trough the nozzle. A plasma plume develops, expanding outside the nozzle and reaching the water surface. The radiofrequency source[8] has a similar layout, but the central electrode is covered in alumina and freely exposed in the last centimetre. The jet is

excited with radiofrequency pulses, 8.8 MHz in frequency and 2.5 kV_{ptp} in amplitude, modulated with a 10% duty cycle over a 2.0 kHz square wave. When a noble gas is flown (1 L/min), a plume develops in the nozzle and propagates in the air. Finally, the air source, called *MibJet*, is based on the concept of DBD: inside an alumina tube (diam. int. 19 mm, ext. 20 mm) a cylindrical grid of stainless steel is inserted and grounded; outside, a band of copper tape is excited with pulses 5.5 kV per 26 μs, at a frequency of 1 kHz. A flow of 0.5 L/min of dry synthetic air carries out the reactive species which are formed inside the tube, on the grid.

The sources have been tested using different diagnostic techniques: with a spectrometer (Avantes) the optical emission spectra are measured *in-situ* during the treatment; the treated samples are tested with oxidation reduction potential (ORP) probe (Metrohm), and their contents in terms of reactive oxygen and nitrogen species are measured (Spectroquant test cells for NO_2^-, NO_3^- and H_2O_2 measured with Prove100 spectrophotometer). Finally, biological effects are assayed suspending *Escherichia Coli* (K-12, MG1655) bacteria, sampled in their exponential phase, suspended in ultrapure water in concentration around 10^7 CFU/mL before the treatment and directly inoculated in Agar dishes after the treatments.

The bactericidal capabilities are quantified in terms of logarithmic abatement (logAbat): the base-10 logarithm of the ratio between the counted colony forming units (CFU) in the (untreated) control and the ones in the treated sample (table 1). The micropulsed source appears to have the best performance, reaching an abatement of more than six orders of magnitude when operated in helium; weaker disinfection effects are obtained also in argon. The radiofrequency jet, instead, does not appear to show valuable bactericidal effects with the used protocol. Finally, the air jet results in limited but stable abatement capability.

	Abatement (logAbat)
Micropulsed/He	> 6
Micropulsed/Ar	3.5 ± 0.2
RF/He	< 0.5
RF/Ar	< 0.5
MibJet	1.3 ± 0.2

Table 1: bactericidal effect of direct treatments of *E. Coli*

To exclude the role of other physical actors and of short living species, the treatments are repeated in indirect form: ultrapure water is first treated with the sources, then bacteria are injected just after the treatment. After 10 minutes, the solution is finally inoculated in Agar dishes, as previously. All the sources display a slight increase in their bactericidal effects in indirect treatments; this can be explained considering the fact that bacteria are suspended in water, which is already enriched in RONS, and confirms that, at least for the considered species and the considered protocol, the role of short-living species is secondary.

Figure 2 reports the measured concentrations of reactive oxygen and nitrogen species inside the

water. A clear difference is present when operating with helium or in argon: the latter, in fact, having a higher mass and a lower ionization energy, results in typically lower electrons temperatures in the plasma[9,10]; this results in a favoured oxygen-based chemistry with respect to nitrogen-based ones, as confirmed by experimental data. Argon jets produce in fact samples richer in peroxides with respect to helium ones, but this does not result in a higher abatement. Similarly, in the air jet the plasma is directly ignited in the air, resulting in a greater amount of nitrogen species; again, this does not result in an improved bactericidal capability.

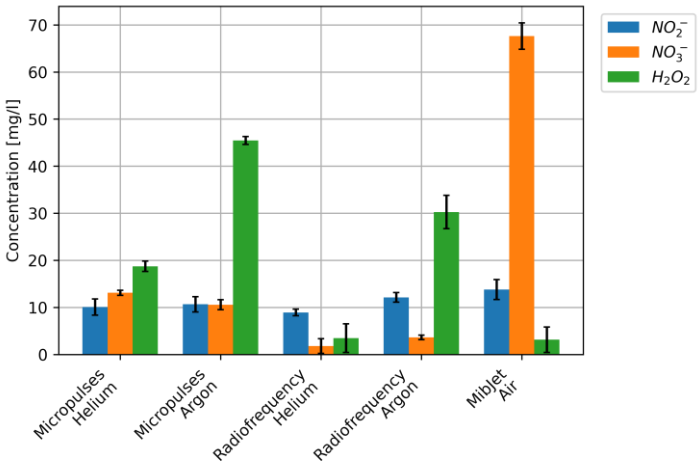

Figure 2: concentration of species in the samples after the treatments.

As a further chemical characterization of the treated sample, ORP has been measured: all the sources appear to increase the ORP from the 300 mV baseline value of ultrapure water. The values of the micropulsed source (439±4 mV in helium and 423±2 mV in argon) appear to be slightly higher than the one of the radiofrequency ones (351±9 mV in helium and 388±10 mV in argon), while the MibJet results in the greatest effect (577±11 mV). A particular consideration should be carried out regarding the pH: measurements using standard probes are not reliable, due to the low conductivity of the tested solution. However, some tests have been carried out using colorimetric strips, suggesting that there are no significant variations from neutrality for all solutions ($6 < pH < 8$).

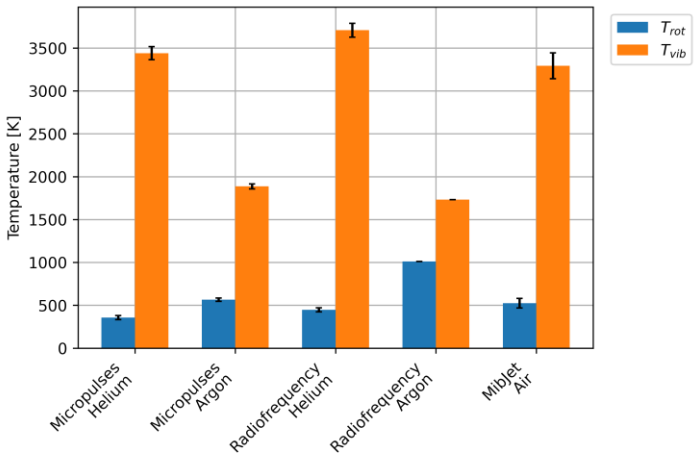

Figure 3: nitrogen second positive system, fitted temperatures

Figure 3 reports the estimated nitrogen vibrational and rotational temperatures, obtaining fitting the nitrogen second positive system using massiveOES simulations[11–13]. Results agree with expectations: considering the less efficient energy transfer in the collisions of argon with nitrogen, the expected nitrogen vibrational

temperature is lower with respect of helium, while the rotational one is expected to be slightly higher. This is fully confirmed by the collected data: helium-operated jets report a vibrational temperature around 3000 K, while argon-operated ones do not overcome 2000 K. In the air jet, having the plasma directly ionizing the air, the temperature reaches again more than 3000 K.

None of the obtained results show a strong correlation with the biological effects: comparing with the micropulsed helium jet, sources having a higher production of peroxides, of nitroxides, with a higher oxidation reduction potential or a higher nitrogen vibrational temperature proven to be less biologically effective, confirming that bactericidal effects of plasma cannot be bounded to an individual actor among the ones analysed in this work.

This works aims to show the potentialities of designing a universal setup which can accommodate multiple sources and different diagnostics. Data produced are fully comparable and the methodology can be easily extended to other plasma sources; moreover, further diagnostics can improve the setup. A complete characterization of multiple sources is fundamental to improve scientific understanding of interactions between plasma and biological substrates, going towards an optimization of the technology.

Acknoledgment

The authors acknowledge COST Action CA20114-PlasTHER “Therapeutical Applications of Cold Plasmas”, supported by COST (European Cooperation in Science and Technology); most of the measurements have been carried out in the framework of a Short Term Scientific Mission (STSM) founded by the COST action itself. This work has been possible thanks to the Italian National Plan for NRRP Complementary Investments (PNC, DL 06/05/2021 n. 59, law n. 101/2021) in the call for the funding of research initiatives for technologies and innovative trajectories in the health and care sectors (Directorial Decree n. 931 of 06-06-2022) - project n. PNC0000003 - AdvaNced Technologies for Human-centrEd Medicine (ANTHEM).

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