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LETTER TO THE EDITOR

Induction of a viable-but-non-culturable state in bacteria treated with gas discharge plasma

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Sir,

The article by Cooper *et al.* (2010) deals with the use of plasma to inactivate micro-organisms and concludes that plasma induces bacteria to enter a viable-but-non-culturable (VBNC) state, probably due to oxidative stress. The authors correlate viability results with morphological ones, and they propose that longer exposure times may be needed to ensure complete cell death. Their approach is interesting, but the paper does not report novel aspects. In fact, their approach and conclusions are almost identical to previous contributions from our group, although we are not referenced in their paper.

The authors state that 'the assessment is made employing a comparison of the methods of culturability, membrane integrity, bacterial morphology and respiration in present studies on the responses of bacteria under plasma induced stress'. Their assessment techniques are identical to the methods we previously used and reported, being the only difference that instead of Atomic Force Microscopy these authors used another type of microscopy. We are glad that researchers and the scientific community in general are now on our same track, putting the accent on cell viability and physiology instead of focusing only on culturability determinations.

Before our group started looking at viability issues, the plasma community used to believe that plasma was effective based solely on culturability results. Our group was a pioneer when in 2006, we hypothesized that plasma induced a VBNC state (Abramzon *et al.* 2006). Further work published in 2008 and 2009 plus dozens of presentations to meetings supported these ideas (Vandervoort *et al.* 2008; Joaquin *et al.* 2009). Our work paved the way to think that viability tests have to be always carried out before concluding that plasma inactivates cells. We were the first discussing these issues, and it took us many international meetings to 'convince' the plasma community about it. Therefore, Cooper *et al.* propose hypotheses that were already proven valid several years ago.

Cooper *et al.* hypothesize that the loss of cell culturability after plasma treatment is induced as one of the responses to oxidative stress. This hypothesis was already proposed in 2006 by Abramzon *et al.*, who demonstrated that although plasma-treated bacteria yielded a very low colony-forming unit counting, other experiments such as colorimetric assays and a spectrophotometric measurement of bacterial extracellular fluid showed that cells were still alive at short exposure times. These authors hypothesized that plasma-assisted cell removal proceeded through a first step in which bacterial cells were not culturable, but entered a (VBNC) stage followed by a second step in which cells were actually killed. Abramzon *et al.* stated that bacteria enter into this dormant state in response to one or more environmental stresses including oxidative stress and radicals.

Cooper *et al.* stated that correlation of culturability results, with membrane integrity and respiration activity, is indicative of the VBNC state. These authors showed changes in cellular morphology to support their point. The approach from our 2006–2009 papers is supported by the authors in their recent contribution. In 2008, Vandervoort *et al.* showed that 'combining the AFM results with data from several other analysis techniques including fluorescence microscopy, cell viability studies, and ATP and DNA assays, a more complete picture emerges and shows that the plasma-treatment process produces interactions that render bacterial cells nonculturable after short plasma-exposure times (e.g. 5 min), but with little change in cell morphology, whereas longer exposure times (e.g. 60 min) result in major cell damage.' (Vandervoort *et al.* 2008). Cooper *et al.* reported the presence of a heterogenous population of plasma-treated *Bacillus stratosphericus* cells comprising of predominantly elongated larger cells, flattened cells and normal cells. Similar results were reported by Vandervoort *et al.*, showing a heterogenous population of plasma-treated *Chromobacterium violaceum* cells including mostly intact cells and a few cells exhibiting defects such as flattened cells and rough surfaces.

In Cooper's paper, their assessment is made employing a comparison of the methods of culturability, membrane integrity, bacterial morphology and respiration of bacteria under plasma-induced stress. In their 2009 contribution, Joaquin *et al.* used an almost identical approach to demonstrate that plasma-treated bacteria remained alive, although nonculturable at short exposure times and later succumb to oxidative stress. These authors demonstrated that at short exposure times, bacteria respond either by increasing respiration and thus, ATP production, or by uncoupling ATP production from respiration as a way of coping with the stress. At longer plasma exposure times, bacteria succumb to oxidative stress and are no longer able to respond to it, resulting in loss of viability (Joaquin *et al.* 2009).

Finally, Cooper *et al.* concluded that to ensure the death of bacteria, relatively longer plasma treatment time is advisable. Their advice coincides with our previous statements showing that cells undergo minor damage during the first 5 min of plasma exposure and that 60 min was required in our case, to ensure complete death (Abramzon *et al.* 2006).

We believe that based on the evidence submitted, we deserve proper credit in Cooper's contribution.

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