Applied and Environmental Microbiology, Nov. 2010, p. 7662–7664 Vol. 76, No. 22
0099-2240/10/$12.00 doi:10.1128/AEM.01615-10

Combined Effects of Long-Living Chemical Species during Microbial Inactivation Using Atmospheric Plasma-Treated Water▽

Murielle Naïtali,[1]* Georges Kamgang-Youbi,[1,2,3,4] Jean-Marie Herry,[4] Marie-Noëlle Bellon-Fontaine,[1] and Jean-Louis Brisset[2]

AgroParisTech, UMR1319 Institut Micalis, Equipe Bioadhésion, Biofilms et Hygiène des Matériaux, 25 Avenue de la République, 91300 Massy, France[1]; Université de Rouen, UFR Sciences, Département de Chimie, 76821 Mont Saint Aignan, France[2]; Université de Yaoundé I, Département de Chimie Inorganique, Laboratoire de Chimie Minérale, P.O. Box 812, Yaoundé, Cameroon[3]; and INRA, UMR1319 Institut Micalis, Equipe Bioadhésion, Biofilms et Hygiène des Matériaux, Domaine de Vilvert, 78350 Jouy-en-Josas, France[4]

Received 7 July 2010/Accepted 21 September 2010

Electrical discharges in humid air at atmospheric pressure (nonthermal quenched plasma) generate long-lived chemical species in water that are efficient for microbial decontamination. The major role of nitrites was evidenced together with a synergistic effect of nitrates and H_2O_2 and matching acidification. Other possible active compounds are considered, e.g., peroxynitrous acid.

Nonthermal plasma gases are currently under study as potential alternatives to conventional sterilization techniques in numerous settings (the food industry, hospitals). Atmospheric nonthermal plasmas of the gliding-arc type (Glidarc) (9, 25) were found to be efficient against microorganisms for treatments performed under burning discharge (12, 13, 22, 26), and the inactivation of cells in water could continue after the discharge had been switched off (13). Microbial cells were also killed by contact with water that had first been activated by discharges (and so-called plasma-activated water [PAW]) without being themselves subjected to the plasma plume (14, 15). Studies performed hitherto using Glidarc in the context of microbial decontamination have aimed to test the influence of biological (i.e., population level, planktonic or adherent state [14]) and physical parameters on decontamination efficiency. Little is known of the mechanisms of action, especially when PAW is used.

UV radiation, charged particles, and temperature are some of the principal factors governing microbial inactivation under plasma technology (20), but they are not relevant for PAW decontamination because the burning discharge is switched off during treatment. It is likely that reactive-nitrogen- and -oxygen-based species play an important role in the lethal effect of nonequilibrium atmospheric air-based plasma (10, 20). DNA, RNA, proteins, and lipids are the principal targets of these oxidants (4, 8). The main radical species present in the Glidarc plasma plume have been identified as $^{\cdot}OH$ and $NO^{\cdot}$ when humid air is the working gas (1). These radicals are precursors of other active species in water, such as nitrates, nitrites, and hydrogen peroxide (3), which endow the medium with high and sustainable reactivity. The efficiency of these long-lived chemical species in removing chemical pollutants was yet evidenced (24), but their implication in microbial inactivation by PAW was demonstrated for the first time here. Chemical species are also responsible for acidification (2) which role in the antimicrobial activity was also considered during the present study.

PAW was produced by application of Glidarc (5 min) over 20 ml of sterile distilled water. The design of the device and the procedure for gas discharge have been described previously (23), as well as the operating conditions (14). PAW contained 0.01 ± 0.01 mmol liter^{-1} H_2O_2, 0.13 ± 0.02 mmol liter^{-1} nitrates (evaluated using Spectroquant hydrogen peroxide cell test and Spectroquant nitrate cell test kits [Merck, Darmstadt, Germany]), and 1.6 ± 0.2 mmol liter^{-1} nitrites (Griess reagent; VWR, Fontenay-sous-Bois, France). Its pH value was 3.0 ± 0.1. No major change in PAW characteristics was detected 30 min after the treatment (corresponding to the maximum period of disinfection). The contributions of nitrites, nitrates, and H_2O_2 to the lethal effect of PAW were tested by evaluating the disinfection potential of acidified (by HCl) solutions prepared using these compounds alone or in a mixture at the concentrations found in PAW. Inactivation was performed as previously described (14). Briefly, the suspension (0.1 ml) of *Hafnia alvei* (a Gram-negative bacterium belonging to the *Enterobacteriaceae* family, selected as a bacterial model) was added to the disinfecting solutions (9.9 ml) and left in contact for increasing periods of time. After neutralization, survivors were evaluated by plating.

More than 50% of the logarithmic abatement by PAW could be explained by the mixture in the acidic medium of nitrites, nitrates and, H_2O_2 (Table 1, lines 1 and 9). When a 20-min application period was considered, the chemical mixture tested explained 75% of the logarithmic reduction achieved by PAW and 99.99% of the number of the dead bacteria. The important role of acidified nitrites in this death rate was shown. They were the only compounds that caused a significant lethal effect (Table 1, line 5). When nitrite formation was prevented by the use of sulfamic acid (21), no lethal effect of PAW was noted (Table 1, line 6). Although acidified nitrates and H_2O_2 were not lethal when utilized alone, their addition to nitrites enhanced the lethal effect (Table 1, lines 3, 4, and 7 to 9). The mixing of several chemical compounds could lead to the cre-

* Corresponding author. Mailing address: AgroParisTech, INRA, UMR1319 Micalis, Equipe B2HM, 25 Avenue de la République, 91300 Massy, France. Phone: (33) 1 69 53 64 73. Fax: (33) 1 69 93 51 44. E-mail: murielle.naitali@agroparistech.fr.

▽ Published ahead of print on 1 October 2010.

TABLE 1. Implication of nitrites, nitrates, hydrogen peroxide and pH in the disinfection capacity of PAW against *H. alvei*

Disinfecting solution	Log_{10} $(N_0)^a$	Mean (± SD) Log_{10} $(N)^a$ after contact with disinfecting solution for:			
		5 min	10 min	20 min	30 min
PAW	7.9 ± 0.1	6.8 ± 0.3	5.7 ± 0.3	2.5 ± 0.9	<2
Acidified water[b]	8.0 ± 0.1	7.9 ± 0.1	7.7 ± 0.1	7.6 ± 0.0	7.6 ± 0.0
Acidified H_2O_2[c]	8.0 ± 0.1	7.8 ± 0.1	7.8 ± 0.1	7.7 ± 0.1	7.6 ± 0.1
Acidified NO_3^-[c]	8.0 ± 0.1	7.8 ± 0.2	7.7 ± 0.0	7.6 ± 0.0	7.5 ± 0.1
Acidified NO_2^-[c]	7.9 ± 0.1	7.6 ± 0.1	7.1 ± 0.2	5.5 ± 0.4	4.0 ± 0.3
PAW + sulfamic acid[d]	7.9 ± 0.0	7.8 ± 0.1	7.7 ± 0.0	7.4 ± 0.2	6.9 ± 0.5
Acidified NO_2^- + H_2O_2[c]	7.9 ± 0.1	7.5 ± 0.0	7.0 ± 0.3	5.0 ± 0.8	3.4 ± 0.7
Acidified NO_2^- + NO_3^-[c]	7.9 ± 0.1	7.4 ± 0.1	6.8 ± 0.3	4.7 ± 0.8	2.9 ± 1.0
Acidified NO_2^- + NO_3^- + H_2O_2[c]	7.9 ± 0.1	7.3 ± 0.1	6.7 ± 0.3	3.8 ± 0.9	2.2 ± 0.3
Neutralized PAW[e]	7.9 ± 0.1	7.8 ± 0.1	7.8 ± 0.0	7.8 ± 0.0	7.7 ± 0.0
Buffered PAW[f]	7.8 ± 0.1	7.8 ± 0.1	7.7 ± 0.3	7.6 ± 0.3	7.6 ± 0.3

[a] N_0 and N, the total numbers of cultivable cells (CFU) in the disinfecting solution before and after disinfecting treatment, respectively. The limit of detection was 100 CFU. The results shown are experimental data obtained from at least three independently grown cultures.
[b] Acidified water is distilled water acidified to pH 3.
[c] The concentrations of the chemical species in the acidified solutions were those measured in PAW directly after treatment.
[d] Sulfamic acid (40 mg $liter^{-1}$) was added to distilled water before plasma treatment in order to trap the nitrites formed. No nitrates were detected. The pH of the solution was 2.1.
[e] PAW was neutralized by NaOH (0.1 mol $liter^{-1}$). The pH of neutralized PAW was 6.
[f] Distilled water was buffered (Na_2HPO_4/KH_2PO_4 at 1:15 mol $liter^{-1}$) before activation by Glidarc. The pH of buffered PAW was 6.

ation of other active species with a synergistic lethal effect. The combination of H_2O_2 and nitric oxide (which may result from the disproportionation of acidified nitrites, as discussed below) appears to have potent antibacterial activity (28). One might also refer to peroxynitrous acid, an oxidant known as germicidal (11, 17, 19, 29) that was evidenced during the treatment of water by Glidarc (24). It can be the product of the reaction between acidified nitrites and H_2O_2: $H_2O_2 + H^+ + NO_2^- \rightarrow ONO_2H + H_2O$. In addition, it may also form during reactions between plasma primary active species (2): $NO^{\cdot} + HO_2 \rightarrow ONO_2H$ and $ONO + {}^{\cdot}OH \rightarrow ONO_2H$. It may thus be transiently encountered and biologically active in PAW. Because the plasma primary active species were absent from the chemical mixtures tested, one might explain the greater efficiency of PAW than of the mixtures by a decrease in peroxynitrous acid formation.

This study also underlined the need for an acidic pH to ensure the efficacy of PAW, in line with the recent results on the antimicrobial activity of plasma-treated liquids (5, 27). No lethal effect was observed during the application of either neutralized PAW or buffered PAW to bacterial suspensions for periods of up to 30 min (Table 1, lines 10 and 11). An acidic pH did not exert a lethal action because of its absolute value (Table 1, line 2). It is important to obtain molecules in biologically active forms, as weak acids penetrate bacterial membranes in a nondissociated form (18). Moreover, the pH value governs the production of other active compounds. At an acidic pH, nitrites are converted into nitrous acid (pK_a of $HNO_2/NO_2^- = 3.3$), an unstable acid that disproportionates to nitrates and nitric oxide. The latter is endowed with antimicrobial activity (7). It is a potent oxidant. It could readily diffuse across biological membranes (6). It could also synergistically act with H_2O_2, as referred to above.

In conclusion, this study demonstrates the action of long-lived chemical species in the lethal effect of PAW on *H. alvei*. This can also probably be considered for other microorganisms, with efficiency depending on microbial structures. Acidified nitrites and H_2O_2 are known to be less efficient versus yeast than versus bacteria (16, 30), and PAW was found to be more efficient against *H. alvei*, *Staphylococcus epidermidis*, and *Leuconostoc mesenteroides* than against *Saccharomyces cerevisiae* (15). Furthermore, the chemical species are probably implicated in the lethal effect of Glidarc during treatments under burning discharge followed (or not) by temporal postdischarges, for as long as these treatments are applied to cells suspended in an aqueous medium.

We thank Helene Lagrave for her help with the measurement of chemical species in PAW.

Georges Kamgang-Youbi received financial support from the SCAC at the French Embassy in Cameroon.

This paper is dedicated to the memory of the late Avaly Doubla, who was the codirector of Georges Kamgang-Youbi's thesis.

REFERENCES

1. **Benstaali, B., P. Boubert, B. G. Chéron, A. Addou, and J. L. Brisset.** 2002. Density and rotational temperature measurements of the $NO^{\cdot}$ and ${}^{\cdot}OH$ radicals produced by a gliding arc in humid air and their interaction with aqueous solutions. Plasma Chem. Plasma Proc. **22:**553–571.
2. **Brisset, J. L., D. Moussa, A. Doubla, E. Hnatiuc, B. Hnatiuc, G. K. Youbi, J. M. Herry, M. Naïtali, and M. N. Bellon-Fontaine.** 2008. Chemical reactivity of discharges and temporal post-discharges in plasma treatment of aqueous media: examples of gliding discharge treated solutions. Ind. Eng. Chem. Res. **47:**5761–5781.
3. **Burlică, R., M. J. Kirkpatrick, and B. R. Locke.** 2006. Formation of reactive species in gliding arc discharges with liquid water. J. Electrostat. **64:**35–43.
4. **Cabiscol, E., J. Tamarit, and J. Ros.** 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. Int. Microbiol. **3:**3–8.
5. **Chen, C. W., H. M. Lee, and M. B. Chang.** 2009. Influence of pH on inactivation of aquatic microorganism with a gas-liquid pulsed electrical discharge. J. Electrostat. **67:**703–708.
6. **Denicola, A., J. M. Souza, R. Radi, and E. Lissi.** 1996. Nitric oxide diffusion in membranes determined by fluorescence quenching. Arch. Biochem. Biophys. **328:**208–212.
7. **Fang, F. C.** 1997. Mechanisms of nitric oxide-related antimicrobial activity. J. Clin. Invest. **99:**2818–2825.
8. **Fang, F. C.** 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat. Rev. Microbiol. **2:**820–832.
9. **Fridman, A., S. Nester, L. A. Kennedy, A. Saveliev, and O. Mutaf-Yardimci.** 1999. Gliding arc gas discharge. Prog. Energy Comb. Sci. **25:**211–231.
10. **Gaunt, L. F., C. B. Beggs, and G. E. Georghiou.** 2006. Bactericidal action of the reactive species produced by gas-discharge nonthermal plasma at atmospheric pressure: a review. IEEE Trans. Plasma Sci. **34:**1257–1269.
11. **Hurst, J. K., and S. V. Lymar.** 1997. Toxicity of peroxynitrite and related

reactive nitrogen species toward *Escherichia coli*. Chem. Res. Toxicol. **1:**802–810.
12. **Kamgang, J. O., R. Briandet, J. M. Herry, J. L. Brisset, and M. Naïtali.** 2007. Destruction of planktonic, adherent and biofilm cells of *Staphylococcus epidermidis* using a gliding discharge in humid air. J. Appl. Microbiol. **103:**621–628.
13. **Kamgang-Youbi, G., J. M. Herry, M. N. Bellon-Fontaine, J. L. Brisset, A. Doubla, and M. Naïtali.** 2007. Evidence of temporal post-discharge decontamination of bacteria by gliding electric discharges: application to *Hafnia alvei*. Appl. Environ. Microbiol. **73:**4791–4796.
14. **Kamgang-Youbi, G., J. M. Herry, M. N. Bellon-Fontaine, J. L. Brisset, A. Doubla, and M. Naïtali.** 2008. Impact on disinfection efficiency of cell load and of planktonic/adherent/detached state: case of *Hafnia alvei* inactivation by plasma activated water. Appl. Microbiol. Biotechnol. **81:**449–457.
15. **Kamgang-Youbi, G., J. M. Herry, T. Meylheuc, M. N. Bellon-Fontaine, J. L. Brisset, A. Doubla, and M. Naïtali.** 2009. Microbial inactivation using plasma activated water obtained by gliding electric discharges. Lett. Appl. Microbiol. **48:**13–18.
16. **King, D. A., M. W. Sheafor, and J. K. Hurst.** 2006. Comparative toxicities of putative phagocyte-generated oxidizing radicals toward a bacterium (*Escherichia coli*) and a yeast (*Saccharomyces cerevisiae*). Free Radic. Biol. Med. **41:**765–774.
17. **Klebanoff, S. J.** 1993. Reactive nitrogen intermediates and antimicrobial activity—role of nitrite. Free Radic. Biol. Med. **14:**351–360.
18. **Konings, W. N., S. V. Albers, S. Koning, and A. J. M. Driessen.** 2002. The cell membrane plays a crucial role in survival of bacteria and archaea in extreme environments. Antonie Van Leeuwenhoek **81:**61–72.
19. **Kono, Y., H. Shibata, K. Adachi, and K. Tanaka.** 1994. Lactate-dependent killing of *Escherichia coli* by nitrite plus hydrogen-peroxide: a role of nitrogen dioxide. Arch. Biochem. Biophys. **311:**153–159.
20. **Laroussi, M., and F. Leipold.** 2004. Evaluation of the roles of reactive species, heat and UV radiation in the inactivation of bacterial cells by air plasma at atmospheric pressure. Int. J. Mass Spectrom. **233:**81–86.
21. **Marouf-Khelifa, K., F. Abdelmalek, A. Khelifa, M. Belhadj, A. Addou, and J. L. Brisset.** 2006. Reduction of nitrite by sulfamic acid and sodium azide from aqueous solutions treated by gliding arc discharge. Sep. Purif. Technol. **50:**373–379.
22. **Moreau, M., M. G. J. Feuilloley, N. Orange, and J. L. Brisset.** 2005. Lethal effect of the gliding arc on *Erwinia* spp. J. Appl. Microbiol. **98:**1039–1046.
23. **Moussa, D., F. Abdelmalek, B. Benstaali, A. Addou, E. Hnatiuc, and J. L. Brisset.** 2005. Acidity control of the gliding arc treatments of aqueous solutions: application to pollutant abatement and biodecontamination. Eur. Phys. J. Appl. Phys. **2:**189–199.
24. **Moussa, D., A. Doubla, G. Kamgang-Youbi, and J. L. Brisset.** 2007. Post-discharge long life reactive intermediates involved in the plasma-chemical degradation of an azoic dye. IEEE Trans. Plasma Sci. **35:**444–453.
25. **Mutaf-Yardimci, O., A. V. Saveliev, A. A. Fridman, and L. A. Kennedy.** 2000. Thermal and nonthermal regimes of gliding arc discharge in air flow. J. Appl. Phys. **87:**1632–1641.
26. **Niemira, B. A., and J. Sites.** 2008. Cold plasma inactivates *Salmonella* Stanley and *Escherichia coli* O157:H7 inoculated on Golden Delicious apples. J. Food Prot. **71:**1357–1365.
27. **Oehmigen, K., M. Haehnel, R. Brandenburg, C. Wilke, K. D. Weltmann, and T. von Woedtke.** 2010. The role of acidification for antimicrobial activity of atmospheric pressure plasma in liquids. Plasma Process. Polym. **7:**250–257.
28. **Pacelli, R., D. A. Wink, J. A. Cook, M. C. Krishna, W. de Graff, N. Friedman, M. Tsokos, A. Samuni, and J. B. Mitchell.** 1995. Nitric oxide potentiates hydrogen peroxide-induced killing of *Escherichia coli*. J. Exp. Med. **182:**1469–1479.
29. **Tecder-Unal, M., F. Can, M. Demirbilek, G. Karabay, H. Tufan, and H. Arslan.** 2008. The bactericidal and morphological effects of peroxynitrite on *Helicobacter pylori*. Helicobacter **13:**42–48.
30. **Weller, R., R. J. Price, A. D. Ormerod, N. Benjamin, and C. Leifert.** 2001. Microbial effect of acidified nitrite on dermatophyte fungi, *Candida* and bacterial skin pathogens. J. Appl. Microbiol. **90:**648–652.