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Atmospheric-pressure, nonthermal plasma sterilization of microorganisms in liquids and on surfaces*

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Abstract: Gas discharge plasma inactivation of microorganisms at low (close to ambient) temperature is a promising area of investigation that is attracting widespread interest. This paper describes atmospheric-pressure, nonthermal plasma (NTP) methods for cold sterilization of liquids and thermal sensitive surfaces. These methods are based on the use of direct current (DC) gas discharge plasma sources fed with steady-state high voltage. Parameters characterizing the plasma sources used (plasma-forming gas, gas flow rate, electric power consumed, etc.) are given. The results for plasma sterilization of different microorganisms (vegetative cells, spores, fungi, biofilms) are presented. An empirical mathematical approach is developed for describing NTP inactivation of microorganisms. This approach takes into account not only the destruction of different components of the cells, but their reparation as well.

Keywords: plasma inactivation; nonthermal plasma; cold sterilization; direct current gas discharge plasma sources; nonthermal plasma inactivation of microorganisms.

INTRODUCTION

Development of effective and energy-efficient approaches for the destruction of biologically dangerous contaminants (pathogens and toxic chemicals) at low (close to ambient) temperature in gases, liquids, and on the surface of bodies is a challenge for modern science. In addition to sterilization, a complimentary application is a protection of different industrial materials, equipment, and electronic devices against biocorrosion and biodegradability. Indeed, corrosion of metals speeds up manifold when induced by thin films of microorganisms deposited on their surface. Usually, microorganisms forming such biofilms are very resistant to traditional sterilization methods.

Sterilization of objects consists of destruction or removal of the microorganisms, including vegetative cells, spores, viruses, etc. Traditional sterilization and disinfection methods use heating in dry and humid environments, filtration, radiation, and strong chemicals (biocides). These methods are

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labor- and time-consuming and expensive as well (e.g., sterilization of biofilms with strong biocides takes more than 24 h). Besides, using biocides does not provide environmental safety.

Gaseous nonthermal plasma (NTP) has unique characteristics because it contains numerous biochemically active agents like UV photons, OH radicals, O atoms, electronically and vibration excited molecules, etc. A distinguishing property of NTP is that all the foregoing agents mentioned can be generated in gas or liquid without heating, close to ambient temperatures. By now, a lot of information devoted to the inactivation of microorganisms by various plasmas created at low and atmospheric gas pressure has been published (see, e.g., refs. [1–7] and the abundant literature cited therein). Many attempts have also been made to determine specific mechanisms responsible for plasma inactivation of microorganisms [2–5]. Nevertheless, from a scientific point of view this problem is still open for discussion.

This paper presents new results on cold sterilization at atmospheric pressure. One of the reasons why we have given particular attention to the development of plasma methods that work at atmospheric pressure is that this approach allows us to inactivate microorganisms not only on surfaces but in liquids as well. Another advantage of NTP inactivation at atmospheric pressure is the avoidance of expensive vacuum equipment required for plasma processing at low pressure. The paper also offers an empirical mathematical approach to describing the plasma inactivation process. The approach developed here takes into account not only the destruction of different elements of the cells but their reparation as well.

EXPERIMENTAL PLASMA DEVICES USED

We used two types of atmospheric-pressure plasma generators. One of them was used for sterilization (disinfection) of liquid media, another for processing of surfaces. Effective generation of biochemically active species (O, OH, etc.) inside a liquid is of great interest for many scientific and practical applications (water purification, biomedical applications, etc). In many cases, short-pulsed discharges forming streamers in the bulk of liquid or in gas above the liquid surface are used to do that [8–13]. Another promising approach allowing us to generate a lot of radicals in liquid is an electric discharge in the water filled with chaotically moving gas bubbles [14–17]. In such cases, active species are produced by cold plasma generated inside small bubbles (but not in a liquid itself), and after that they are transported due to diffusion from bubbles into the liquid.

Our device used to generate intensive NTP in bubbling water is shown in Fig. 1. The length of the glass tube in this figure is 50 cm, and the inner diameter of the tube is 3.6 cm. Cold plasma enables both the UV radiation and active species like O and OH radicals to be generated within the gas bubbles, which effectively destroys biologically harmful contaminants (not only pathogens, but toxic chemicals as well) in the treated water without attendant heating.

Three views in Fig. 2 show plasma jets in pure nitrogen, N_2 + 2 % O_2 mixture and air at atmospheric pressure used for sterilization of surfaces. The length of plasma jet is equal to 10–5 cm (a), 2–3 cm (b), and 1–2 cm (c). All plasma devices described above are fed by direct current (DC) high-voltage power suppliers.

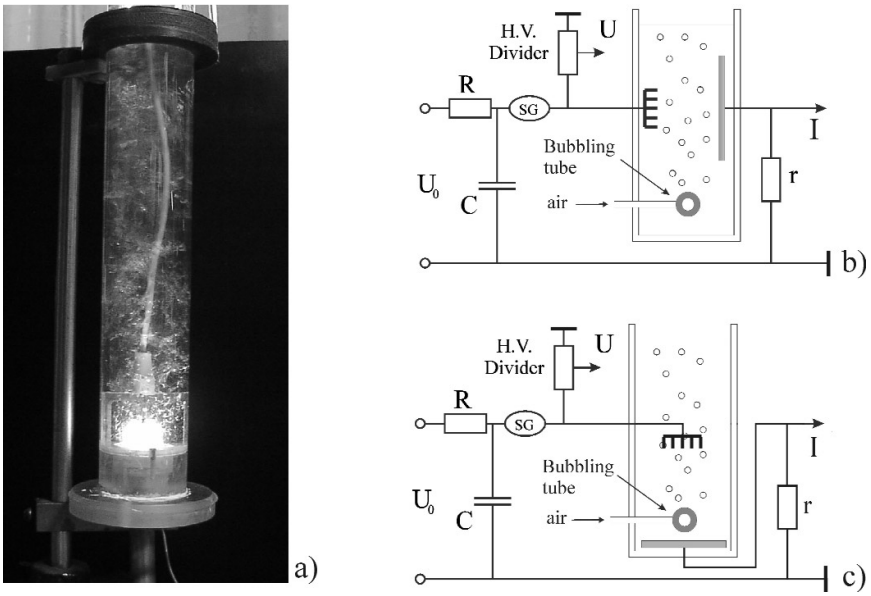


Fig. 1 Illustration and scheme of the experimental set-up used for generation of plasma in liquid. (a) The glass tube filled with water activated by gas bubbles; (b and c) Electrical circuits used for generation of self-running pulsed-repetitive streamer-spark discharge between multi-pin and plane electrodes in water with intensive flow rate of air bubbles. The electric current is directed perpendicular to the tube axis (b) or parallel to the tube axis (c); R is the ballast resistor, SG is the spark gap, C is the capacitor, and r is the current shunt. The gap between the multi-pin electrode and plane is 10–15 mm.

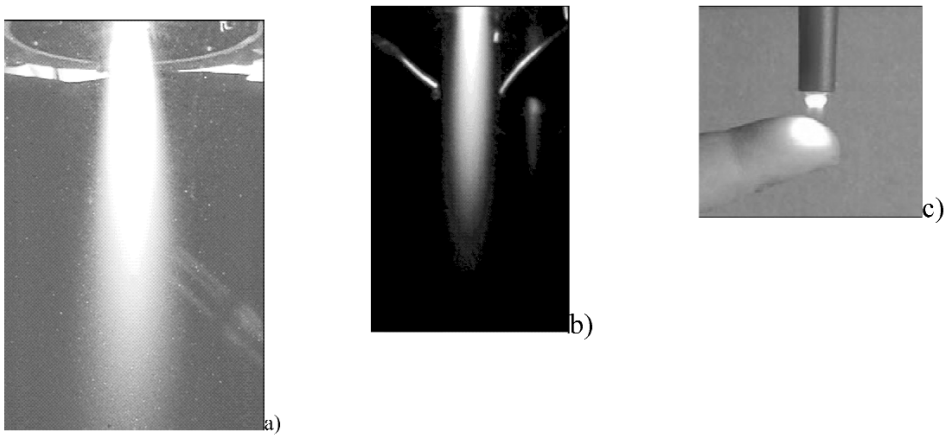


Fig. 2 DC source generating cylindrical cold plasma jet for remote 2D and 3D treatment. Electrical power of this plasma source is 15–75 W, and gas velocity at the outlet is about 15 m/s. Variable parameter is gas mixture: (a) pure N_2; (b) N_2 + 2 % O_2; (c) N_2 + 20 % O_2 (air).

TYPES OF MICROORGANISMS AND METHODS USED

General information

In our work we used bacteria (prokaryotes) and fungi (eukaryotes). Bacteria are classified as gram-negative (*Escherichia coli* and *Serratia marcescens*) and gram-positive (*Bacillus subtilis* and

Mycobacterium flavescens) microorganisms. They differ primarily in their cell wall structure. The main component of the cell wall is a netted structure, peptidoglycane (murein). The latter forms so-called "murein sacculus", a rigid structure of the cell wall, making the cells stronger (see, e.g., [18]). Besides, gram-positive bacteria have a thicker cell wall than that of gram-negative microorganisms.

Both vegetative cells and spores of *B. subtilis* were used. A spore-forming vegetative cell produces a highly tolerant spore protected with a multilayer comprising the cortex and protein envelopes. The resulted spore acquires thereby higher resistance to the bactericidal action of various chemical and physical factors.

Fungi tested were *Candida lypolitica* and *Aspergillus niger*. They belong to higher (imperfect) fungi attributed to the eukaryotes. The latter differ markedly in their structures from prokaryotes. The composition and structure of the fungal cell walls differ significantly from those of bacteria. Natural multiple-species biofilms enhancing the steel corrosion were also used in our experiments.

Thus, the set of microorganisms selected here gives us a unique opportunity to check the efficiency of low-temperature plasma in the inactivation of microorganisms essentially different from each other in their properties.

Specific information

B. subtilis MPV 7095 #1997, and *E. coli* ATCC 25922 #2393 were provided by the State Research Center for Applied Microbiology & Biotechnology (SRCAMB). *S. marcescens* str.#1, *M. flavescens* E-91, *C. lypolitica* XT, and *A. niger* 1H were borrowed from the collection (Laboratory of Biotechnological Ecology, SRCAMB).

A medium for culturing the bacteria was fish meal hydrolyzate (FMH) (SRCAMB, Obolensk). By its composition, this medium is similar to the meat extract. M9 medium was selected as a minimum synthetic medium. Its composition was as follows: (10 ml) 20 % glucose solution, (1 ml) 0.1 M ($MgSO_4$), (1 ml) 0.01 M ($CaCl_2$), (10 ml) salt concentrate, (up to 100 ml) sterile water, (6 g) sodium phosphate disubstituted anhydrous, (3 g) sodium phosphate monosubstituted anhydrous, (0.5 g) NaCl, (1 g) NH_4Cl, and water (up to 100 ml). Czapek medium was used to work with fungi. The API medium has been developed by the American Petroleum Institute [19].

Microorganisms and methods used for surface plasma sterilization

The *B. subtilis*, *E. coli*, *S. marcescens*, *M. flavescens*, and *C. lypolitica* were cultured either on medium M9 added with 1 % glucose or on the FMH for two hours. an average microbial density of the lawn produced on Petri dishes was $2.4–7.5 \times 10^6$ cells/cm^2.

The *A. niger*, the spore-forming culture, was grown on Czapek medium at 37 °C over 7 days. Produced spores were suspended with a saline solution. Suspension of 100 μL in volume was evenly spread over the surface of freshly prepared Czapek agar. The average spore density was 10^6 spores/cm^2.

In the case of surface sterilization, the jet of plasma was directed perpendicularly to the middle of a Petri dish located at the distance of 1 cm away from a plasma nozzle. The plasma treated dishes were incubated overnight at 37 °C in an incubator (for 3 days in the case of fungi). Diameters of the inactivated zones were measured.

Plasma sterilization efficiency of the spore-contaminated surfaces was characterized with use of the standard *B. subtilis* spore-immobilized test strips (about 10^6 spores per each strip). The strips to be treated were stood edgewise toward the jet. After the treatment, each strip was aseptically transferred to an individual tube containing the test medium with added tryptone and phenol red dye. The tubes were incubated at 37 °C in an incubator. The effect of plasma sterilization was judged from the changes in the medium color in 7 days of the incubation. The absence of changes in color (still red) meant that the sample was sterile, while a change in color from red to yellow indicated the presence of the living microorganisms in the sample.

Microorganisms and methods used for plasma sterilization in liquid

Suspensions of vegetative cells and spores of *B. subtilis*, as well as of vegetative cells of *E. coli*, were used. To produce the vegetative cells, *B. subtilis* and *E. coli* were grown in 1 % glucose-added the M9 medium at 37 °C for 18 h, with shaking (200 rpm). To produce spores, *B. subtilis* was cultured on the FMH agar at 37 °C for 4 days. The produced spores were suspended with a saline solution. Before being treated with plasma, the cell or spore suspensions were 100–1000-fold diluted to adjust to the final concentration of 10^5–10^6 CFU/ml. For treating suspensions, we used a glass tube of 1 L in volume (Fig. 1). After plasma processing, the samples were taken to determine an amount of the survived microorganisms. The rate of survival was determined by culturing on 1 % glucose-added M9 and FMH agar.

Biofilms and methods used for plasma sterilization in ambient air and liquid

The multiple-species natural biofilms were grown on surfaces of coupons made either of mild steel or polyethylene. The sizes of the coupons were $20 \times 7 \times 1$ mm. The coupons were submerged into the tubes containing the API medium followed by culturing at 28 °C under microaerophilic conditions. The growth was slow, and it took therefore 14 days to produce the mature biofilm with superficial density of 10^7 colony-forming units (cfu)/cm^2 maximum. The biofilms were grown under aseptic conditions excluding the contamination with the exterior microflora. Inoculate was a natural microbial community isolated from soils of petroleum exploration sites (Tatarstan). The community comprised predominantly aerobic heterotrophs and sulfate-reducing bacteria (SRB). The coupons were subjected to low-temperature plasma in two ways:

(i) Surfaces of the coupons were treated by plasma jet in ambient air. The samples to be treated were located at the distance of 7 mm away from the nozzle of the plasma jet.
(ii) Surfaces of the coupons were treated by plasma in a saline solution (9 g of NaCl per 1 L of water). In this case, the coupons were submerged into a saline solution. This liquid was blown out with gas bubbles at the rate of 100 cm^3/s. Electric discharge was excited inside each bubble. This discharge generated numerous chemically active plasma agents which were transported due to diffusion into the liquid and reacted with biofilms.

After plasma treatment of the coupons, biofilms were washed out with a saline solution. Concentrations of the aerobic heterotrophs and sulfate-reducing bacteria in the rinses were determined. The number of heterotrophs was determined by culturing cells on the FMH agar. The amount of sulfate-reducing bacteria was determined by 10-fold titration method using the API medium. The 10-fold sample was analyzed for the presence of iron sulfide, a metabolite of sulfate-reducing bacteria, which usually forms a black sediment.

EXPERIMENTAL RESULTS

Plasma surface sterilization

In these experiments, the NTP jet was directed to the center of the Petri dish perpendicularly to its surface. The distance between surface of agar and outlet edge of plasma nozzle was 7 mm. The typical power deposited in the plasma jet was 15 W.

Bacteria

Some results on bacteria inactivation are presented in Figs. 3, 4, and 5. Figure 3 presents photos of Petri dishes of 90 mm diameter, showing the distribution of bacteria population of *E. coli* and *B. subtilis* on agar. These pictures were taken after incubation over 24 h at 37 °C. Before incubation, *E. coli* and *B. subtilis* were treated with plasma jet under exposure time of 10 and 90 s, respectively. Figure 4 shows

Fig. 3 Bacteria population distribution of *E. coli* (a) and *B. subtilis* (b) on the FMH agar after plasma jet treatment. Exposure times are 10 and 90 s, respectively.

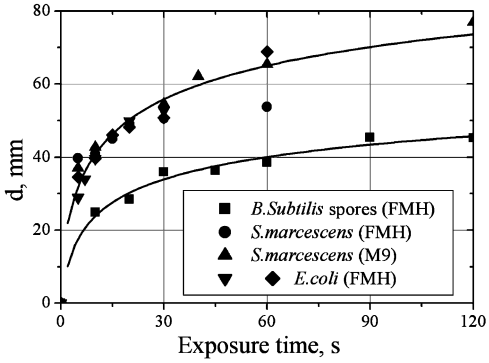
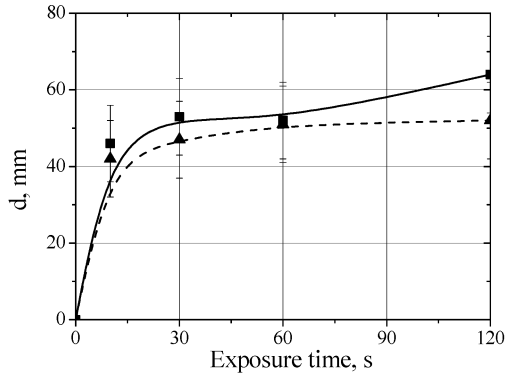


Fig. 4 Dependence of diameter d of the inactivated area on the agar surface vs. exposure time for different kinds of bacteria. Strains of bacteria and mediums are presented in the legend. Electrical power of plasma jet is about 20 W. Initial diameter of plasma jet striking the surface is about 3 mm.

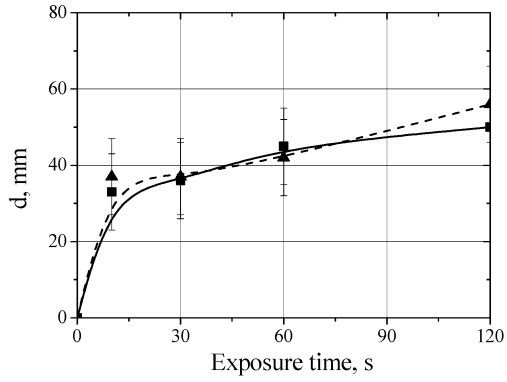
that the NTP jet having initial diameter about 3 mm is able to kill the bacteria over the surface up to 75 mm in diameter. This effect is associated with wide spreading of plasma over agar due to striking thin jet with the surface.

The collection of our experimental data obtained on cold plasma-jet treatment of different bacteria on agar surface is presented in Figs. 4 and 5.

Experimental curves in Fig. 4 can be approximated with analytical expression (solid curves): $d \cong a\sqrt{\ln b(t+t_0)}$, where a, b, and t_0 are the fitting parameters depending on type of bacteria, t is the exposure time. This expression demonstrates that the square of the inactivated area increases with exposure time much slower compared with the linear dependence.



a)



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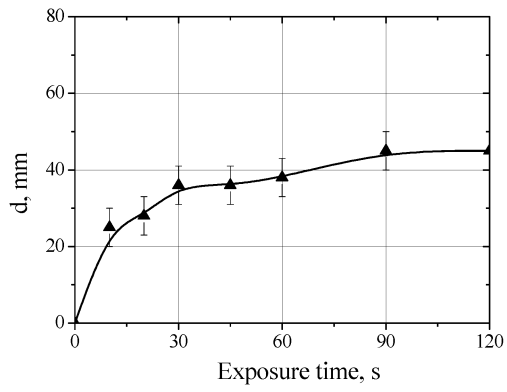

c)

Fig. 5 Diameter *d* of the inactivated area on agar vs. exposure time for different microorganisms. Variable parameter is sort of the medium (dashed curve – M9, solid curve – FMH). Power of plasma jet is 20 W. (a) *S. marcescens*; (b) *M. flavescens* E-91; (c) *B. subtilis*.

Fungi

Experimental data on plasma-jet inactivation of fungi grown on agar surface are presented in Fig. 6. One can see that the NTP jet allows us to inactivate fungi as well.

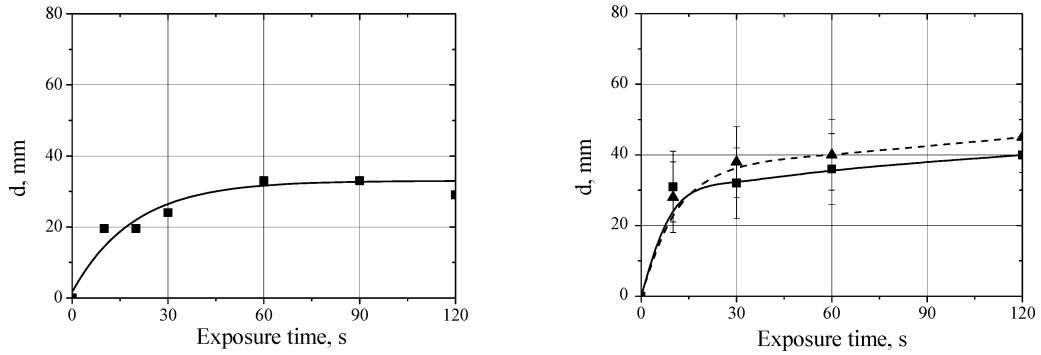

Fig. 6 Inactivated area diameter *d* on agar vs. exposure time. Tested microorganisms are fungi: (a) *A. niger* spore; (b) *C. lypolitica*. Variable parameter is sort of medium (dashed curve – M9, solid curve – FMH). Power of plasma jet is 20 W.

B.subtilis spore-immobilized test strips (biological indicators)

Some results on plasma jet sterilization of microorganisms on the spore-immobilized test strips are shown in Table 1. The initial number of cells was 10^6 cfu/strip. These data demonstrate the real availability of deep and fast sterilization by cold plasma jet even with such resistant microorganisms as spores impregnated into the paper strip. One can see that addition of oxygen in plasma-forming gas results in much stronger inactivation of spores. After processing by oxygen-containing plasma jet, spore growth was not observed even in 7 days, which is a good result for many practical applications.

Table 1 Data on sterilization of *B. subtilis* spore-immobilized strips by plasma jet.

Process gas-forming cold plasma jet	Plasma exposure time, min 5	7	10
	Number of days after which the spore growth was observed		
Air	4	n/g	n/g
N_2 + 2 % O_2	2	4	n/g
N_2	no data	no data	2

Note: n/g means no growth of spores after 7 days of incubation.

Biofilms—sterilization by plasma jet in ambient air

It is well known that traditional methods of destroying biofilms by biocides is environmentally unfriendly and takes about 24 h. In contrast, Figs. 7 and 8 show that NTP jet can destroy biofilm by a much shorter exposure time (tens of seconds in the case of treatment in ambient air, and hundreds of seconds in the case of treatment in liquid). Data presented in Fig. 7 show that aerobic heterotrophs have higher resistance against active plasma agents compared with the sulfate-reducing bacteria. Taking this into account, we focused mainly on studying the survival of the aerobic heterotrophs. In the case of biofilms

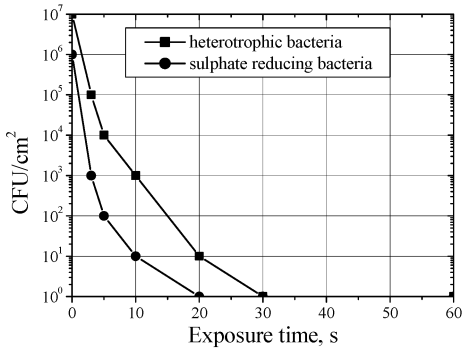

Fig. 7 Survival of microorganisms in biofilms on metallic coupons after plasma jet treatment. The lower curve corresponds to the sulfate-reducing bacteria; upper curve corresponds to the heterotrophic bacteria. P = 15 W.

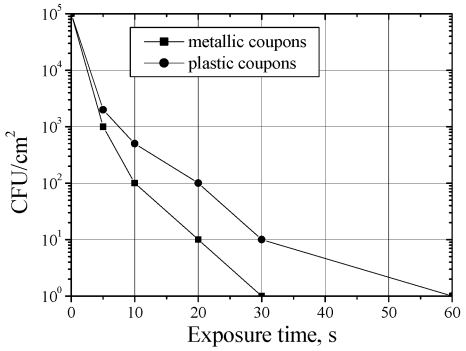

Fig. 8 Survival of aerobic heterotrophs in biofilms on metallic (the lower curve) and plastic (the upper curve) coupons after plasma jet treatment. P = 15 W.

grown on the plastic coupons, our results demonstrate that aerobic heterotrophs have stronger survival rates compared with those grown on the metallic coupons.

PLASMA STERILIZATION IN LIQUID

Bacteria suspended in physiological solution

Results from the destruction of different types of bacteria in a liquid (physiological solution) filled with gas bubbles activated by NTP are presented in Figs. 9 and 10. The varied parameters in these experiments were discharge power (30 and 60 W) and the type of substratum (poor for the M9 medium and rich for the FMH medium). One can see the vegetative cells *E. coli* are inactivated under exposure time only of several minutes for poor and rich medium, and it is being discovered that this time diminishes with power of gas discharge. For instance, inactivation time equals 7 min for discharge power of 30 W and 3 min for 60 W. However, spores are more resistant to active agents compared with vegetative cells and require a longer time for inactivation. Indeed, Fig. 10 shows that exposure time of 10 min is not enough for complete inactivation of spores. There is a general rule for bacteria—incubation of the plasma-treated microorganisms in rich medium results in a higher number of surviving cells.

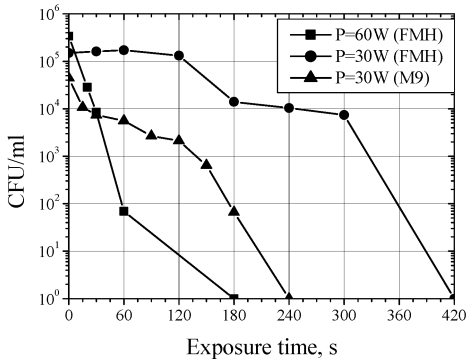

Fig. 9 Survival of *E. coli* in suspension (physiological solution) under activation by NTP generated in gas bubbles.

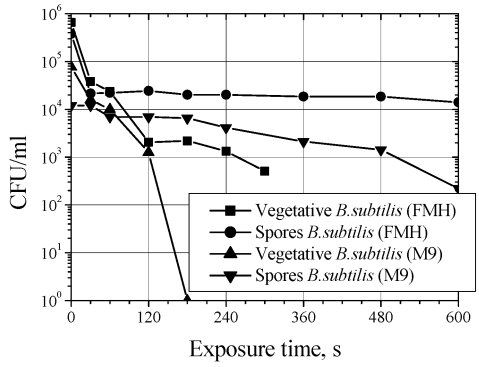

Fig. 10 Survival of spores and vegetative cells *B. subtilis* in suspensions (physiological solution) under activation by NTP generated in gas bubbles. Power of discharge is 60 W.

Biofilms immersed in physiological solution

It is well known that traditional methods for destroying biofilms by biocides is environmentally unfriendly and takes a lot of time—several tens of hours. In contrast, Fig. 11 shows that NTP can destroy biofilm by a much lower exposition time (hundreds of seconds). In the case of biofilms grown on the plastic coupons, aerobic heterotrophs have stronger survival rate compared with those grown on the metallic coupons.

In total, the experimental data presented demonstrate that NTP exhibits itself as an excellent instrument for destroying not only individual microorganisms but biofilms as well.

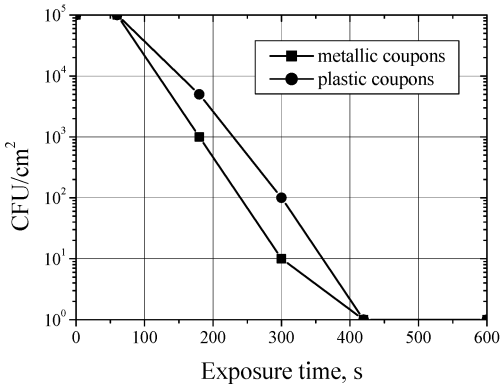

Fig. 11 Survival of aerobic heterotrophs in biofilms on metallic (the lower curve) and plastic (upper curve) coupons treated in physiological solution by NTP generated in gas bubbles. P = 60 W.

Remarks on biofilm destruction

Biofilm is a highly organized complex microbial community. In this community, bacteria are involved in the complicated system of informational and energetic relationships, maintaining vital functions of the community and each separate cell as well. Such collaborative activity improves significantly the resistance of biofilm. On the other hand, functions of the highly organized community depend strongly on the functioning of its individual components. In such a case, disfunction of some of them can bring a lethal outcome to the community.

Keeping this in mind, we suppose that the high efficiency in the NTP-inactivation of biofilm can be associated with the destruction of its vitally important components easily injured by active plasma agents. For instance, in our case biofilm has the upper layers predominantly consisting of aerobic heterotrophs. Microorganisms of the upper layers consume oxygen from the environment, providing the conditions for functions of anaerobic microorganisms (e.g., sulfate-reducing bacteria) in inferior layers. We assume that plasma influences first of all the upper layers. As a result, ambient oxygen penetrates into inferior layers of biofilm and kills anaerobes.

Another reason for the high rate in the NTP inactivation of biofilm can be attributed with small sizes of activate plasma agents like O, OH, O_3, etc., which are tiny particles. In contrast to biocides, small plasma particles can easily enter the biofilm matrix, affecting the microorganisms constituting deeper layers.

EMPIRICAL MATHEMATICAL MODEL OF THE BACTERIA PLASMA INACTIVATION

Basic ideas underlying the mathematical model

For experimental conditions typical for the NTP sterilization of microorganisms, it is reasonable to suppose that all bacteria are isolated each from other, i.e., they are not in a form of bioconsortium or bioaggregates (so, biofilms are not the subject of our mathematical modeling). In such a case, complete sterilization of the bacterial colony can be reached only at individual inactivation of each cell. To simplify the theoretical consideration, we assume that cells in the colony to be sterilized do not shield each other from active agents of the NTP. In other words, the flux of active agents attacking each cell is more or less the same. Chemical reactions of active plasma agents with different cell components result in local disturbance/destroying of their normal structures. We will call a consequence of such chemical reaction "injury" for the cell. Energy released by the isolated active agent in the elementary chemical reaction is small enough (roughly about 5 eV) [20]. We assume, therefore, that a single injury given to the cell by an isolated active agent in itself has no lethal outcome for the cell, but accumulation of injuries can re-

sult in inactivation of the cell either immediately under NTP processing or later. So, the probability of the cell inactivation due to single chemical reaction attributed with isolated active agent is assumed to be negligible.

According to the literature [1–3] and our own opinion, the most probable inactivation mechanisms related to the NTP sterilization can be: (1) partial damage or irreversible alteration of the cell wall and membrane(s) that results in an increase of the substance transport and eventually in a metabolic disturbance up to lysis and in a disturbance of cell division; (2) partial damage or irreversible alteration of different intracellular components that results in a disturbance of different important functions of the cell (biosynthesis, energy, reparation, etc.); (3) damage of the DNA mainly due to breaking of its chains (single-fiber nick breakings are repaired effectively by the cell itself but duplex nick breakings are frequently a reason of the cell inactivation).

All mechanisms listed above assume the following (step-by-step) sequence of the events occurring in average with isolated cell under the NTP sterilization. At the beginning of this process, the most part of active agents attacking the cell influences predominantly the cell wall and membrane(s), resulting in wall erosion, membrane porosity, and a disturbance of functions of the membrane receptors controlling the substance transport. Note that active plasma agents like O, OH, O_3, etc. are tiny particles which are much smaller in sizescompared with biomolecules. In contrast to huge biomolecules, there is therefore finite probability for active species to penetrate into the cell even through the undestroyed cell wall and membrane. It is evident that the penetration ability will grow with an increase in the number of injuries got by both the cell wall and membrane. Thus, the flux of active agents penetrating into the cell increases. They migrate chaotically within the cell and damage (partially or irreversibly) different intracellular components. Because the number of active agents diminishes due to interactions with intracellular components, only a small part of the agents is able to reach the DNA. At this stage, however, the damaged cell is weakened strongly and its ability for reparation is also diminished. In such a case, even a small number of active agents can be enough to destroy the DNA irreversibly.

Of course, we understand clearly that the notions formulated above are too simplified compared with a real phenomenon of the NTP sterilization and do not take into account many complicated biochemical aspects of this problem. Nevertheless, in order to stimulate discussion of this issue, we would like to offer the phenomenological approach based on formal analogy of the elementary cell-active particle interactions with those in chemistry kinetics. Taking this in mind, let us consider the following set of dynamic equations, the variables of which depend on time t and describe the parameters of the isolated cell attacked by NTP-active agents:

$$\frac{dP_1}{d\mathrm{t}} = I_1$$

$$\frac{dP_2}{d\mathrm{t}} = I_2(1 - e^{-kP_1^2}) - \frac{P_2}{\tau_2}$$

$$\frac{dP_3}{d\mathrm{t}} = I_3(1 - e^{-kP_1^2}) - \frac{P_3}{\tau_3}$$

$$\frac{dP_4}{d\mathrm{t}} = I_4(1 - e^{-kP_1^2})\frac{P_4}{P_{4\mathrm{cr}}} - \frac{P_4}{\tau_4}$$

$$W_{\mathrm{in}}(P_{\mathrm{i}}) = e^{-\left(\frac{P_{\mathrm{icr}}}{P_{\mathrm{i}}}\right)^2}$$

$$N_{\mathrm{surviv}} = N_0\left[1 - W(P_1)\right]\cdot\left[1 - W(P_2)\right]\cdot\left[1 - W(P_3)\right]\cdot\left[1 - W(P_4)\right]$$

- Here, P_1, P_2, P_3, P_4 are the number of injuries associated with the cell wall and membrane(s), numerous and easily destroyed intracellular components, scanty but heavily destroyed intracellular components, DNA due to duplex nick breaking of its chain, respectively (for simplicity, all intracellular components are divided only into two groups having different abilities to react with active agents);
- P_{1cr}, P_{2cr}, P_{3cr}, P_{4cr} are the critical number of injuries for cell wall and membrane(s), easily and heavily destroyed intracellular components and DNA determining alone the probability of the cell inactivation $W_{in}(P_i)$ equal to about 30 %;
- N_{surviv}/N_0 is the part of cells surviving after getting P_1, P_2, P_3, P_4 injuries;
- τ_2, τ_3, τ_4 are the average times for reparation of easily and heavily destroyed intracellular components and DNA (it is assumed that typical exposure time under NTP sterilization is shorter compared with reparation time of the membrane(s) structures and cell wall);
- k is the empirical coefficient describing an increase in total (cell wall and membrane(s)) penetrability $\prod = [1 - \exp(-kP_1^2)]$ for active agents due to accumulation of the injuries P_1;
- I_1, I_2, I_3, I_4 are the intensities of interaction between active agents and cell wall and membrane(s) components, easily and heavily destroyed intracellular components, DNA, respectively (these intensities depend on both the sorts of NTP agents and specific properties of cell components, and for most cases they have a ranking of $I_1 > I_2 > I_3 > I_4$).

COMPARISON BETWEEN EXPERIMENTAL AND CALCULATED RESULTS

Some results on the fitting of numerical calculations to our experimental data and data from paper [5] are presented in Figs. 12 and 13. Table 2 contains the set of the fitting parameters corresponding to the best coincidence between results of numerical simulations and experimental data.

Table 2 Set of the fitting parameters corresponding to the best coincidence between simulations and experiment.

Fig.	I_1	I_2	I_3	I_4	τ_2	τ_3	τ_4	k	P_{1cr}	P_{2cr}	P_{3cr}	P_{4cr}
12a	1500	400	100	30	30	100	30	10^{-6}	$3{\cdot}10^5$	$3{\cdot}10^3$	$1{\cdot}10^4$	400
12c	700	300	100	25	20	300	35	10^{-6}	10^6	$2{\cdot}10^3$	10^5	300
12e	1000	300	100	7	30	30	30	10^{-6}	10^6	$2{\cdot}10^3$	10^5	300
12g	1000	115	100	20	20	500	26.5	10^{-6}	$2{\cdot}10^6$	$2{\cdot}10^3$	$2{\cdot}10^4$	400
13a	1700	150	70	7.3	100	300	89	10^{-6}	$2{\cdot}10^6$	$3{\cdot}10^3$	10^4	600
13c	1200	350	100	8.7	100	300	89	10^{-6}	$5{\cdot}10^5$	$6{\cdot}10^3$	$2{\cdot}10^4$	600
13e	1000	300	200	9.5	100	300	89	10^{-6}	10^6	$5{\cdot}10^3$	$8{\cdot}10^4$	600
13g	1000	250	150	8.5	100	300	89	10^{-6}	10^6	$6{\cdot}10^3$	$3{\cdot}10^4$	600

In spite of simplifications mentioned above, the empirical model describes adequately the survival curves obtained in the experiments. This model allows us to make an important conclusion on the crucial role of the reparation of different cell components under plasma sterilization—if the reparation is "switched off" in the model, the calculated exposure time needed for complete sterilization ($N_{surv}/N_0 \leq 10^{-6}$) is much lower compared with experimental one (dashed curves in Figs. 12 and 13). It means the cell is not a passive object but the cell fights intensively for its own life immediately in the course of sterilization process.

This model reproduces also three slopes (or three stages) in the survival curves observed in the experiments. Based on analysis of the numerical results, we can suppose the scenario for plasma inactivation as follows. The first slope (first stage) is associated predominantly with accumulation of injuries P_2. However, quantitative input of injuries P_1, P_3, P_4 in total inactivation of the cell depends on gas pressure. At low pressure, quantitative contributions of a different sort of injuries correspond to this sequence: P_2, P_3, P_1, P_4. It means that the first stage can be correlated with an accumulation of injuries

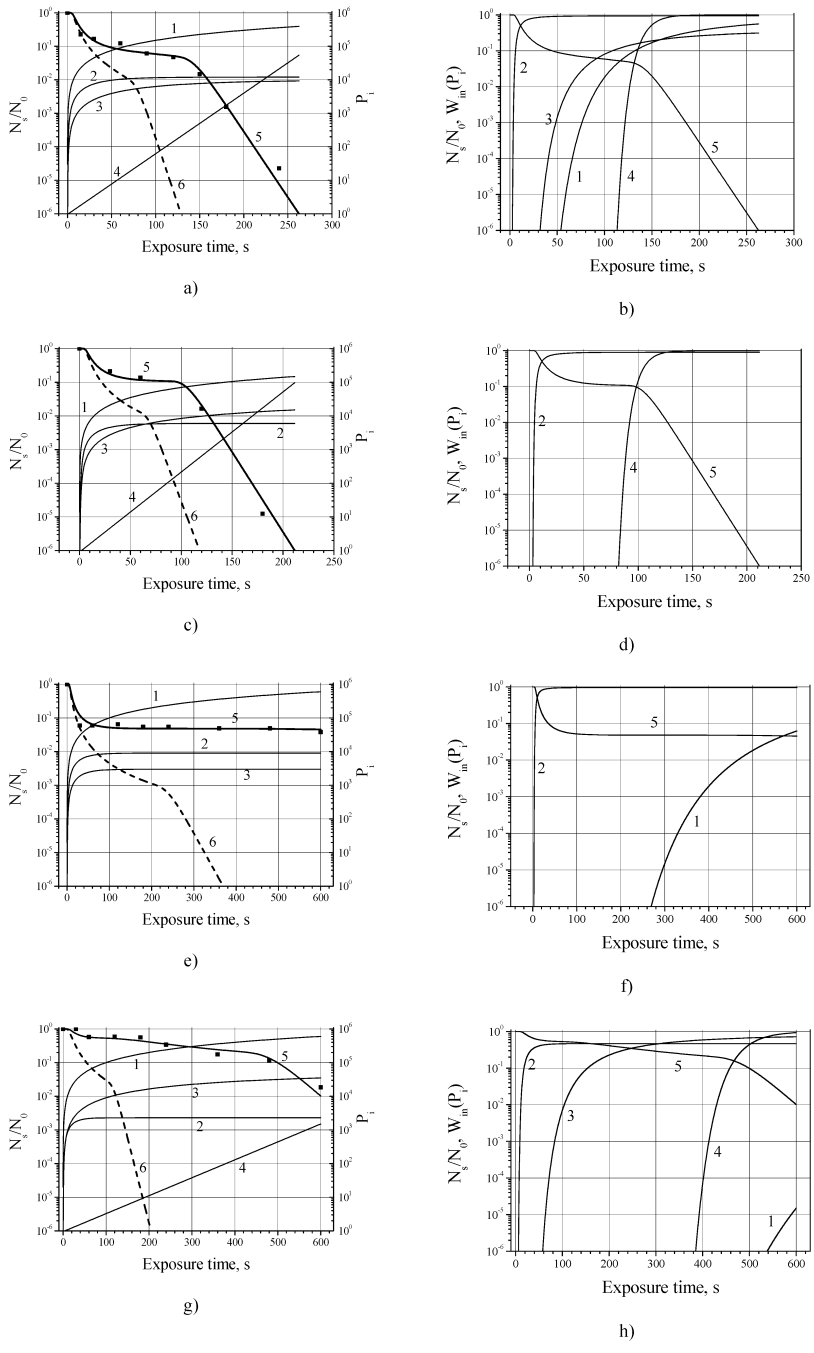

Fig. 12 Results on the fitting of numerical calculations to our experimental data at atmospheric pressure. Discharge power was 60 W. Microorganisms and mediums used were: *E. coli*, physiological solution (a, b); *B. subtilis* vegetative cells, physiological solution (c, d); *B. subtilis* spores, physiological solution, FMH (e, f); *B. subtilis* spores, physiological solution, M9 (g, h). Figures at the curves in Figs. 12a, c, e, g denote: 1, 2, 3, 4 are the numbers of injuries P_1, P_2, P_3, P_4; 5, 6 are the simulated curves for N_s/N_0 with (5) and without (6) taking into account the reparation. Figures at the curves in Figs. 12b, d, f, h denote: 1, 2, 3, 4 are the probabilities $W(P_1)$, $W(P_2)$, $W(P_3)$, $W(P_4)$ of cell inactivation due to injuries P_1, P_2, P_3, P_4; 5 is the simulated curve for N_s/N_0 with taking into account the reparation.

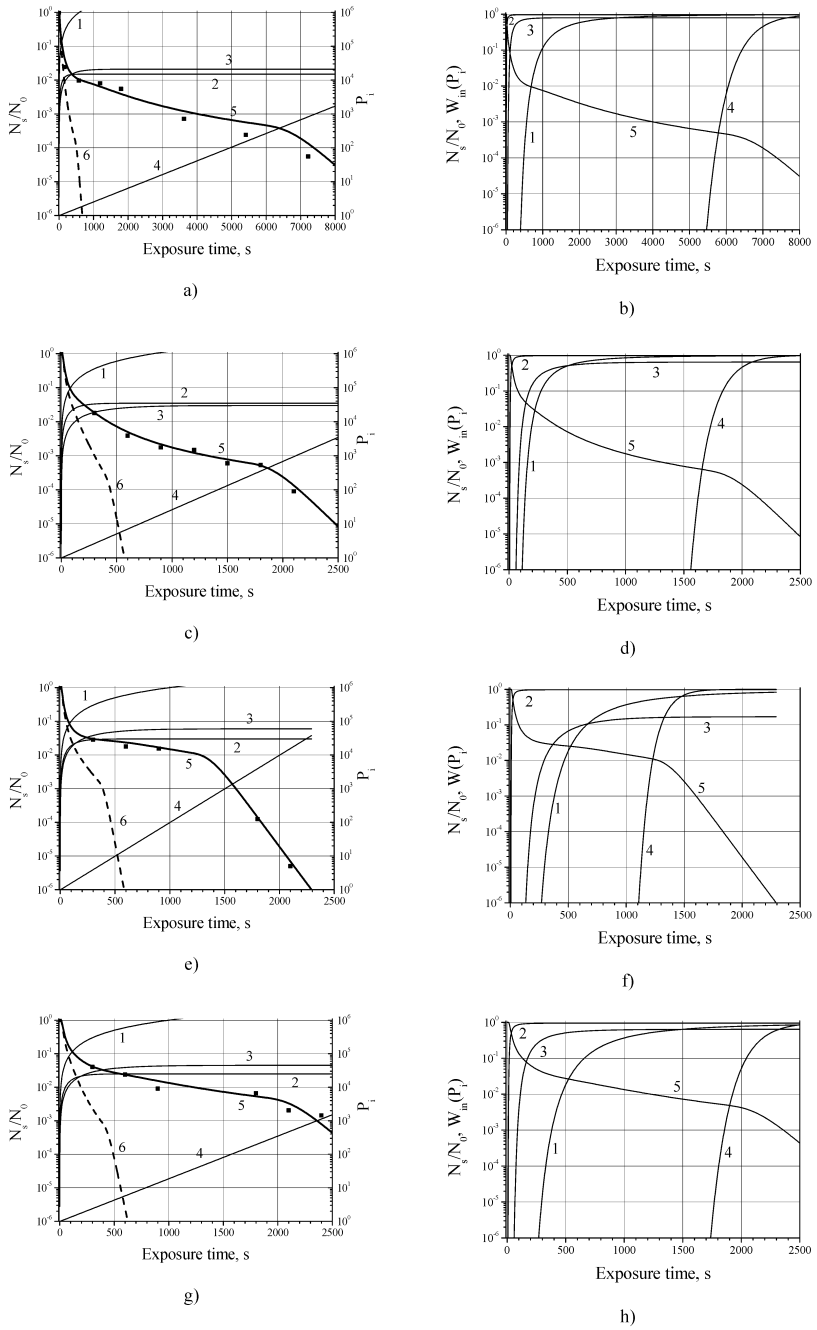

Fig. 13 Results on the fitting of numerical calculations to experimental data from [5] at low pressure. Experiments were performed with use of *B. subtilis* spores. Plasma-forming gases were CO_2 (a, b); 95 % Ar + 5 % O_2 (c, d); 98 % N_2 + 2% O_2 (e, f); 85 % N_2 + 15 % O_2 (g, h). Figures at the curves in Figs. 13a, c, e, g denote: 1, 2, 3, 4 are the numbers of injuries P_1, P_2, P_3, P_4; 5, 6 are the simulated curves for N_s/N_0 with (5) and without (6) taking into account the reparation. Figures at the curves in Figs. 13b, d, f, h denote: 1, 2, 3, 4 are the probabilities $W(P_1)$, $W(P_2)$, $W(P_3)$, $W(P_4)$ of cell inactivation due to injuries P_1, P_2, P_3, P_4; 5 is the simulated curve for N_s/N_0 with taking into account the reparation.

related to easily destroyed intracellular components. Further increase in the number of inactivated cells is associated with accumulation of injuries P_3 (heavily destroyed intracellular components). The third stage can be associated with injuries of the cell wall and membrane(s). Indeed, the experiments of paper [4] show a high number of the destroyed cells after plasma sterilization at low pressure. So, destroying the DNA plays not always an essential role in the cell inactivation (e.g., in our opinion, under low-pressure conditions of the paper [4]).

According to our calculations, under NTP sterilization at atmospheric pressure the sequence for quantitative contributions of injuries is another: P_2, P_3, P_4, P_1. It means that the third stage can be associated predominantly with destroying the DNA. In other words, destroying the cell wall and membrane(s) cannot be essential for inactivation under atmospheric pressure conditions. A possible reason for the dissimilarity mentioned above is a different mixture of active agents generated by NTP at low and atmospheric pressure.

CONCLUSION

Experimental data demonstrate real availability of deep and fast inactivation by NTP at atmospheric pressure such resistant microorganisms as spores, biofilms, and fungi on the surface and in liquids. High efficiency of NTP agents (like O, OH, etc.) in sterilization is associated with their small sizes, which results in easy penetration into the cell compared with biocides controlled actively by membrane receptors (e.g., the typical time for penetration of water molecules into the cell is equal to several tens of milliseconds, which is much smaller compared with that for heavy-weight biocides).

The phenomenological model for the NTP inactivation of bacteria based on formal analogy between elementary cell–active particle interactions and chemical kinetic processes is offered. Analysis of the fitting parameters providing the best approximation of experimental results by numerical calculated curves shows that quantitative input of injuries obtained by cell wall and membrane(s), easily and heavily destroyed intracellular components and DNA in total inactivation of the cell depends on gas pressure.

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