#### UDC 616:579.253:537.5:579.695

### EFFECTS OF ELECTRON BEAM AND OZONE COMBINED PROCESSING ON VARIABILITY OF THE RESIDUAL MICROFLORA

### Babych E.M., Sklyar N.I., Ryzhkova T.A., Kalinichenko S.V., Semerenskaya E.I.

### State Establishment «I.I. Mechnikov Institute of Microbiology and Immunology of AMS of Ukraine», Kharkov

The problems of the physical-chemical agents' influence on the microbial world and the mutagenesis conditioned by this affect attract one's attention because of the intensive study of the effectiveness of the use of various physical factors and oxidizers for disinfecting natural waters and sewages [1].

It is experimentally proved that dynamics of DNA synthesis in prokaryotic and eukaryotic cells is sensitive to the electron beam and ozone influence [2, 3]. Probably, by applying different amount of energy, these agents can cause damage or just desadaptation in the genetic apparatus. This fact is not only of a general biological interest but there also arises a problem of possible imbalance in ecological microbial interactions, which can conduce to biocenosis' formation suppressing, appearance of bacteria populations characterized by heightened virulent properties, thus have indirect influence on human health. Therefore, in the connection with the problems of water purification from biological objects, one must understand the biological effects produced by the electron beam and ozone.

The goal of the work is to determine the nature of adaptive variability of the sanitary-model microflora and frequency of the mutants' appearance stimulated by the electron beam and ozone influence in various regimes of processing samples of natural water and sewages.

## Materials and methods

As the test-objects, we chose representatives of sanitary-model microflora with diametrically opposite ability to resist to the influence of the physical and chemical agents. Our 24 tests indicated that Escherichia coli strains (E.coli ATCC №25922, E.coli B №116), Citrobacter freundii (C.freundii) *№15*, Serratia marcescens (S.marcescens) №184 and Staphylococcus aureus (S.aureus) ATCC №25923 were the most sensitive to the electron beam and ozone combined influence and similar in their quantitative characteristics. On the contrary, test cultures of Klebsiella (K.pneumoniae № №32, 159 and K.oxytoca №180) were characterized by a heightened resistance to the influence of these agents. The places of sampling natural waters and sewages were defined in the previous reports [4].

Procedure of water disinfection were performed on the laboratory facility developed in National Scientifical Center "Kharkov Physical Technical Institute", which consists of the powerful emitter, the high power-saving ozonizer, both flowing and landlocked system of new type with the ion formation cabinet of disinfection with ion formation [4].

The mutagen properties of antimicrobial agents were examined under the standard conditions with the model samples, samples of test cultures in natural waters and purified sewages.

The model samples were prepared in the form of a microbial suspension of each representative of the sanitary-indicative microflora.

The test cultures' biochemical properties were determined according to "Bergey's Manual of Determinative Bacteriology", 1997 by application of the standard microbiological techniques.

The antilyzocyme and antiinterferon activities were investigated in the cup-plate technique by detection of delayed-action antagonism between the test cultures *Micrococcus luteus var. Lysodeiktikus №2665* and *Corynebacterium xerosis №181* respectively (test-strains obtained from the Museum of microorganisms of SE «I.I. Mechnikov Institute of Microbiology and Immunology of AMS of Ukraine» collection ) [5, 6].

The test-culture anticomplementary activity was determined by the kinetic method proposed by Y.A. Brudastov, which consists in measuring the level of haemolysis in sensitized erythrocytes of a ram [7].

Adhesive properties of bacteria were investigated according to the technique by V.I. Brylis and the coauthors [8]. For estimating the microbe's adhesive properties, we used the criteria of the adhesion average index (AAI), the adhesion coefficient (AC) and the microorganisms' adhesion index (MAI) – the latter is the average number of microbial cells, adhered on one erythrocyte that participates in the adhesion process.

The test cultures' sensitivity to antimicrobial agents was studied by Bauer-Kirbi's method with the use of ready-made commercial disks [9].

The experiments were made three-four times. Statistical analysis was performed using computer programs Microsoft Excel 2003 and "Biostat-4". Parametric tests were used by calculation of mean and its standard deviation. P values of <0,05 were considered significant.

# **Results and discussion**

The choice of objects of the investigations was conditioned by the following circumstances. On the one hand, the given technique stipulates the water disinfection with the help of extremely unstable agents ( $H^+$ ,  $OH^-$ ,  $H_2$ ,  $H_2O_2$ ). On the other hand, combined with the electron beam effect, the activity of heavy radicals is so high that mutagenesis processes can develop among bacteria.

Hitherto there does not exist any universal testsystem that could detect all principal types of genetic injuries. All such installations were designed for finding out the adaptive changes. The latter can either heighten the microorganisms' pathogenic potential (generation of toxins, stimulation of ferments' activity) or stimulate the development of new properties, due to which microbial populations would become more resistant to the influence of environment (sensitivity to fags, mobility). The phenotypic changes were estimated by temporal appearance of microbial cells of untypical forms and sizes, short-term disturbances in the colony morphology and in the test culture biochemical characteristics. The irradiation and ozonizing influence is more minutely investigated by detecting the frequency of appearance of the dissociation of various types (R-forms). Besides, we have examined mutations inherent in one, several and scores of genes: alteration in an individual biochemical feature; disturbance of two or more of the typical characteristics; the bacterium mobility and sensitivity to fags, respectively.

The physical-chemical agents' application resulted in appearance of modifications among the sanitary-indicative bacteria in 64,2% of the cases. Particularly high adaptive variability was detected for the C.freundii test-strain. The number of modifications for *C.freundii* isolates was 94,4% as compared with 55,6-61,1% for other representatives of examined bacteria.

The frequency of emergence of the induced microbes modifications depended on the sample processing intensity. If the energy expenditures were low (the ozonizing during 1 min and irradiation with the dose 0,8 kGy), the formation of subpopulations characterized by alterations in certain biological features was registered just in 26,7% of the cases. At the same time, after the disinfection in more intensive regimes, the specific gravity of modified sanitary-model bacteria reached 45,8-

In the study of test-cultures' biochemical properties before and after the processing with the physical and chemical agents it was indicated that the most frequently modifications arose with the disturbance of just one fermentative feature –  $(70,2 \pm 4,7)$  %. Modifications with the disturbance of two fermentative features were detected in  $(14,9 \pm 3,6)$ % of the cases. In other tests changes in three or more of biochemical functions were fixed.

As phenotypic nonheritable defects in just a bacterium's single feature or in several ones are widely spread in natural environment, while determining the after-effects of the technique in question, it is important to know not only the modification emergence frequency but also a tendency in the population changes.

Later on it is suggested to study antilyzocyme, anticomplementary and antiinterferon activities of the residual microflora. Such features give the bacteria an advantage in colonization of the man's intestinal tract [10-13].

As Table 1 indicates, if the water samples were processed in such regimes that the representatives of enterobacteria, staphylococcus and enterococcus could survive in large quantities, in certain cases (10,0%) this circumstance stimulated the appearance of modifications with the above-mentioned features. As a rule, such variants were detectable when higher energies were spent on the water processing.

 Table 1. Frequency of appearance of modifications concerning antilyzocyme, anticomplementary and antiinterferon features in various regimes of the water disinfection

Test culture	The number of		Modifications of individual features in the regimes of the water sample processing											
i est culture	tests		Nº1			<u>N</u> •2			N <b></b> 23					
		L	C	Ι	L	C	Ι	L	С	Ι				
K.pneumoniae №32	27	0	0	0	0	0	0	0	1	1				
E.coli ATCC 25922	27	0	0	0	1	0	0	1	2	0				
S.aureus ATCC 25923	27	0	0	0	0	0	0	2	1	0				
E.faecalis №183	27	0	0	0	0	0	0	0	1	0				
In all	108	0	0	0	1	0	0	3	5	1				

Notes:  $\mathbb{N}_1$  – the disinfection regime: ozonizing during 1 min (15mg/l  $O_3$ ) and the irradiation dose 0,8 kGy;  $\mathbb{N}_2$  – the disinfection regime: ozonizing during 2 min (15mg/l  $O_3$ ) and the irradiation dose 1,6 kGy;  $\mathbb{N}_3$  – the disinfection regime: ozonizing during 4 min (15mg/l  $O_3$ ) and the irradiation dose 3,2 kGy. L – the antilyzocyme activity; C – the anticomplementary activity; I – the antiliterferon activity.

It should be noted that the bacteria' ability to colonize the mucosa is conditioned not only by the aboveenlisted signs. At the first stage in the colonization process development, first of all, those populations that are characterized by high adhesive indices have the advantage [11-14]. Due to the microbial cells' strong ability to form connections with the corresponding receptors of eukaryotes mucous coat, one or another subpopulation gets the dominant position in the man's biological niche. The physical-chemical agents' influence on the adhesive properties of different representatives of the sanitary-model microflora was tested on enterobacteria (*K.pneumoniae*  $N \ge 159$ ; *E.coli* ATCC 25922; *C.freundii*  $N \ge 116$ ) and staphylococcus (*S.aureus* ATCC  $N \ge 25923$ ).

The data in Table 2 indicate that the sample processing during 1 min did not change the given feature of the test cultures. The ozonizing twice as long and the irradiation dose twice as large resulted to significant reduction of the sanitary-model bacteria AAI and MAI.

 Table 2. Influence of various regimes of water sample processing on adhesive characteristics of representatives of sanitary-model microorganisms

	Values of bacteria adhesive indices in various regimes of water processing										
Adhesion	Ozonizing 1 min + irradiation 0,8 κGy Ozonizing						Ozonizing 2 min + irradiation 1,6 κGy				
index	enterob	iterobacteria staphylococcus			enterol	oacteria	staphylococcus				
	С	E	С	E	С	Е	С	Е			

AC, % (M±m)	92,3±2,2	88,4±2,3	89,2±1,5	87,5±1,7	98,0±1,5	82,6±1,7	91,3±1,7	80,3±1,5
AAI, (M±m)	5,7±0,14	5,8±0,09	5,5±0,12	5,4±0,13	6,16±0,7	3,45±0,4*	5,63±0,4	3,2±0,3*
MAI, (M±m)	5,4±0,19	5,12±0,29	5,3±0,15	5,4±0,12	6,04±0,6	2,78±0,3*	5,3±0,3	2,6±0,25*

Notes: AC –adhesion coefficient; AAI – adhesion average index; MAI – microorganisms' adhesion index; C – the check sample; E – the examined sample; \* – significant difference from control, (p<0,05).

The obtained results indicate that the given technique of the water disinfection sometimes heightens antilyzocyme, anticomplementary and antiinterferon activity of the test cultures. But, notwithstanding this fact, the method does not stimulate the development of adaptive reactions in bacteria which favour formation of microbial populations with heightened persistent potential. On the contrary, a decrease in the sanitarymodel microflora representatives' adhesive features after the sample processing rather indicates the persistent potential reduction.

To estimate a possible after-effects of the given disinfecting technique application on the reservoir microflora, one must know the mode of changes in the sanitary-model bacteria' sensitivity to antimicrobial agents. At present, the problem of formation of pathogenic and opportunistic pathogenic bacteria populations, resistant to antibiotics, has become of special importance all over the world.

As it is known, formation of resistant microbial groups can be stimulated not only by the full-scale application of antimicrobial drugs but also by nonspecific agents influence.

We examined representatives of enterobacteria (K.pneumoniae №№ 32, 159; K.oxytoca №180; E.coli №

 $N_{2}$  3, 158, "B"  $N_{2}$ 116; C.freundii  $N_{2}$ 15; S.marcescens  $N_{2}$ 184), staphylococcus (S.aureus ATCC  $N_{2}$ 25923) and enterococcus (E.faecalis  $N_{2}$ 158) strains.

As Table 3 indicates, after the ozonizing during 2 and 4 min, combined with the irradiation with the beam dose 1,6 and 3,2 kGy respectively, the sensitivity of enterobacteria cultures varied at the same frequency in both regimes of processing (34,8-43,5%). In all tests these reactions were identical and characterized by increase in antibiotics susceptibility of enterobacteria, these data were trustworthy from the viewpoint of statistics.

Enterococcus also often (in  $(56,3\pm7,2)\%$  of the cases) changed their sensitivity to antibiotics after the laboratory sample processing with the physical and chemical agents (see Table 4). As the data indicate, ozonizing and irradiation stimulated an increase in the test culture sensitivity to antibiotics. This phenomenon in single cases was observed even when the original microbial culture was completely resistant to the antimicrobial agent. In other tests, the enterococci growth was on average 1,6 times delayed in comparison with the check-test values (p<0,01), what testifies that the subpopulation's protective properties were essentially weakened by the physical and chemical agents.

Regimes of The number		The changes in sensitivity among the tests		An increase or decrease in the sensitivity to antibiotics													
processing	of tests	Number of		]	K		4		G	]	R		С		S		0
1 8		isolates	%, (M±m)	Ι	D	Ι	D	Ι	D	Ι	D	Ι	D	Ι	D	Ι	D
Ozonizing 2 min + irradiation 1,6 κGy	69	30	43,5±5,9	5	0	7	0	9	0	0	0	3	0	6	0	0	0
Ozonizing 4 min + irradiation 3,2 ĸGy	69	24	34,8±5,7	4	0	5	0	6	0	0	0	4	0	2	0	3	0

|--|

The notes: K – Kanamycin; A – Ampicillin; G – Gentamycin; R – Ryphampicine; C – Chloramphenicol; S – Streptomycin; O – Ofloxacin; I – increase in sensitivity; D – decrease in sensitivity.

Table 4. Changes in E.fa	<i>calis (№158)</i> sensitivity to antimicrobial agents after processing with ozone and the beam.

	Growth	delay zone diame	ters before and af	ter the culture pro	cessing			
		Ozonizing 2 m	in + irradiation	Ozonizing 4 min + irradiation 3,2				
Antimicrobial means		1,6	кGy	кGy				
Antimicrobiai means	Check tests	M±m	p (compared with check tests)	M±m	p (compared with check tests)			
Ampicillin	0	10,3±0,58	0,0001	10,3±0,58	0,0001			
Chloramphenicol	14,7±0,58	15,7±0,58	0,1	18,3±0,58	0,001			
Tetracycline	14,7±0,58	19,7±0,58	0,0004	20,3±0,58	0,0002			

Gentamycin	9,7±0,58	10,7±0,58	0,1	10,7±0,58	0,1
Claritromcyn	17,7±0,58	22,3±0,58	0,0004	22,3±0,58	0,0004
Norfloxacin	12,7±0,58	13,3±0,58	0,2	13,3±0,58	0,2
Ofloxacin	9,3±0,58	11,7±0,58	0,01	12,3±0,58	0,002
Cephaclor	12,7±0,58	12,7±0,58	1,0	12,3±0,58	0,6
Ceftriaxon	0	0	0	0	0
Cefepim	0	0	0	0	0

The results obtained at the given stage of detecting the possible phenotypic changes in individual representatives of the sanitary-model microflora in natural water and sewages induced by the electron beam and ozone combined processing permit us to state that the presented technique of the water disinfection does not injure microecology of the environment.

# Conclusions

- 1. 360 tests convincingly demonstrate that the frequency of appearance of modifications among the sanitarymodel microflora representatives (*K.pneumoniae*, *E.coli*, *C.freundii*, *S.marcescens*, *S.aureus*, *E.faecalis*) depends on the disinfection intensity. After the water sample irradiation in the dose 0,8 kGy and ozonizing during 1 min (ozone concentration makes 15 mg/l) the mutants' specific gravity is about 26%; the dose 1,6 kGy and ozonizing during 2 min bring this parameter close to 56,7%.
- 2. The results of 128 tests testify that the changes lead to the weakening of the modified variants ability to occupy people' biological niches. This conclusion is based on the fact that adhesive properties of test cultures become 1,1 times lower after joint processing of water samples with the beam and ozone (p<0,05).
- 3. Water sample processing with the beam and ozone in the above-mentioned regimes favours the formation of the sanitary-model microflora populations characterized by a heightened sensitivity to antimicrobial agents.

### References

1. Effects of Wastewater Disinfection on Waterborne Bacteria and Viruses / Blatchley E., Gong W.-L., Alleman J., Rose J., Huffman D., Otaki M., Lisle J. [Electronic resource] // Water Environment Research. – 2007. – Vol. 79, № 1. – P. 81-92. – Access mode: http://www.ingentaconnect.com/content/wef/wer/2007/00 000079/00000001/art00010

2. An overview of the integration of ozone systems in biological treatment steps / A. Ried, J. Mielcke, A. Wieland [et al.] [Text] // Water science and technology. – 2007. – V. 55. – P. 253-258. – ISSN 0273-1223.

3. Application of accelerated electron beam and microwave irradiation to biological waste treatment / D.I. Martin, I. Margaritescu, E. Cirstea [et al.] [Text] // Vacuum. – 2005. – V. 77, Issue 4. – P. 501-506. – ISSN 0042-207X

4. Synergism of a hight-energy electron beam and ozon on sanitary-indicative microorganisms in sewages /

Ye.M. Babych, T.V. Khirna, N.I. Skliar [et al.] [Text] //

Buk. Med. Herald. – 2007. – № 3, V. 11. – P.111-114. – ISSN 1684-7903

5. Bykharin O.V. The method for microorganisms' antilyzocyme activity detection / O.V. Bykharin, B. Ya. Usavtsev, A.P. Malyshkin [Text] // Journal of microbiology, epidemiology and immunobiology. – 1984. – №2. – P. 27-28. – ISSN 0372-9311.

6. Sokolov V.Yu. Microorganisms' antiinterferon activity. Persistence of bacteria / V.Yu. Sokolov ; ed. O.V. Bykharin. – Ekaterinburg : UrD RAS, 1990. – P. 83-93.

7. Brudastov Yu.A. Detectoion of bacteria' anticomplementary activity by kinetics of immune haemolysis / Yu.A. Brudastov [Electronic resource] // OSU Herald.  $-2005. - N \ge 12. - P. 51-54. - Access mode: http://vestnik.osu.ru/2005_12/10.pdf$ 

8. The method for adhesive process of microorganisms investigation / V.I. Brylis, T.A Brylene, H.P. Lentser, A.A. Lentser // Laboratory business. -1986.  $-N_{2}4$ . -C. 210-212.

9. Microorganisms' antimicrobial susceptibility testing : Order  $N \ge 167$ . – [valid from 2007-04-05]. – K. : Ministry of Public Health of Ukraine, 2007. – (Normative document Ministry of Public Health of Ukraine.).

10. The influence of chemical analog of extracellular microbial autoregulators on antilyzocyme activity of bacteria / J.V. Bykharin, N.B. Perunova, G.I. El-Registan [et al.] // Journal of microbiology, epidemiology and immunobiology.  $-2007. - N_{\rm D}6. - P. 3-6. - ISSN 0372-9311.$ 

11. Bykharin O.V. The problems of pathogens persistence in infectology / O.V. Bykharin // Journal of microbiology, epidemiology and immunobiology. -2006.  $-N_{2}4$ . -P. 4-8. -ISSN 0372-9311.

12. The role of persistence and virulence factors in microecological disfunctions of human health / B.Ya. Usavtsev, L.M. Hustudinova, L.I. Parshuta [et al.] // Journal of microbiology, epidemiology and immunobiology.  $-2006. - N_{\rm D}4. - P. 58-61. - ISSN 0372-9311.$ 

13. Casadevall A. Host-Pathogen Interactions: The Attributes of Virulence / Arturo Casadevall, Liise-Anne Pirofski // The Journal of Infectious Diseases. – 2001. –V. 184. – P. 337-344. – ISSN: 0022-1899.

**n.** Weak Rolling Adhesion Enhances Bacterial Surface Colonization / B.N. Anderson, A.M. Ding, L.M. Nilsson [et al.] // Journal of Bacteriology. – 2007. – V. 189, №5. – P. 1794-1802. – ISSN: 0021-9193

## UDC 616:579.253:537.5:579.695

## EFFECTS OF OF ELECTRON BEAM AND OZONE COMBINED PROCESSING ON VARIABILITY OF THE RESIDUAL MICROFLORA Babych E.M., Sklyar N.I., Ryzhkova T.A.,

Kalinichenko S.V., Semerenskaya E.I.

The article is devoted to study of possible phenotypic changes in individual representatives of the sanitarymodel microflora in natural water and sewages induced by the electron beam and ozone combined processing. The results obtained at the given stage permit us to state that the presented technique of the water disinfection does not injure microecology of the environment.

**Key words:** variability of microorganisms, disinfection of water, ozone, electron beam, sanitary-indicative microorganisms

#### УДК 616:579.253:537.5:579.695

# ВЛИЯНИЕ СОВМЕСТНОЙ ОБРАБОТКИ ОЗОНОМ И ЭЛЕКТОННЫМ ПУЧКОМ НА ИЗМЕНЧИВОСТЬ ОСТАТОЧНОЙ МИКРОФЛОРЫ

Бабич Е.М., Скляр Н.И., Рыжкова Т.А., Калиниченко С.В., Семеренская Е.И.

Статья посвящена изучению возможных вариантов фенотипических изменений, которые происходят после совместного применения озона и электронного пучка у отдельных представителей санитарнопоказательной микрофлоры природных и сточных вод. Представленные результаты исследования позволяют на данном этапе изучения положительно оценить предложенную технологию обеззараживания воды с микроэкологических позиций.

**Ключевые слова:** изменчивость микроорганизмов, обеззараживание воды, озон, электронный пучок, санитарно-показательные микроорганизмы

## УДК 616:579.253:537.5:579.695

# ВПЛИВ СУМІСНОЇ ОБРОБКИ ОЗОНОМ ТА ЕЛЕКТРОННИМ ПУЧКОМ НА МІНЛИВІСТЬ ЗАЛИШКОВОЇ МІКРОФЛОРИ Бабич Є.М., Скляр Н.І., Рижкова Т.А., Калініченко С.В., Семеренська Е.І.

Стаття присвячена вивченню можливих варіантів фенотипових змін, що відбуваються після сумісного застосування озону та електронного пучка у окремих представників санітарно-показової мікрофлори природних та стічних вод. Приведені результати досліджень дозволяють на даному етапі вивчення позитивно оцінити запропоновану технологію знезараження води з мікроекологічних позицій. Ключові слова: мінливість мікроорганізмів, знезараження води, озон, електронний пучок, санітарно-показові мікроорганізми