Journal of Medicine, Physiology and Biophysics
ISSN 2422-8427 (Online)
Vol. 10, 2015

Electrochemically Activited Water. Biophysical and Biological Effects of Anolyte and Catholyte as Types of Water

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Abstract

Results of antimicrobial action of electrochemically activated water (anolyte/catholyte) are provided. The two types of water are produced in the anode and cathode chamber of an electrolitic cell, respectively. Under laboratory conditions cell culture and organ suspensions of Classical swine fever virus were treated with anolyte. By inoculating them with cell cultures quality viral presence (presence of viral antigen) was reported using the immunoperoxidase technique. It was found that: anolyte did not affect the growth of the cell culture PK-15; viral growth in the infection of a cell monolayer with a cell culture virus was affected in the greatest degree by anolyte in 1:1 dilution and less by the other dilutions; viral growth in the infection of a cell suspension with cell culture virus was affected by anolyte in dilution 1:1 in the greatest degree, and less by the other dilutions; viral growth in the infection with a virus in organ suspension of a cell monolayer was affected by the anolyte in all dilutions applied.

Unexpectedly, stronger biocidal effect of the catholyte was obsrved when E. coli DH5a strain was treated by anolyte and catholyte, respectively.

In order to provide additional information about the antiviral activity of the electrochemically activated water, and about the distribution of water molecules according to the energies of the hydrogen bonds, the non-equilibrium energy spectrum (NES) and diferential non-equilibrium energy spectrum (DNES) of anolyte and catholyte is evaluated.

Keywords: anolyte, cell culture, CSF virus, disinfection, hydrogen bond, water, structure, clusters, NES, DNES

1. Introduction

Water with its anomalous physical and chemical properties outranks all other natural substances on Earth. The ancient philosophers considered water as the most important component of matter. It performs a vital role in numerous biochemical and metabolic processes occurring in cells with participation of water, being a universal polar solvent for hydrophilic molecules having an affinity for water. Hydroxyl groups (-OH) in H_2O molecule, are polar and therefore hydrophilic. Moreover water acts as a reagent for a big number of chemical reactions (hydrolysis, oxidation-reduction reactions). In chemical processes water, due to its high ionizing ability, possesses strong amphoteric properties, and can act both as an acid and a base in reactions of chemical exchange.

Modern science has confirmed the role of water as a universal life sustaining component, which defines the structure and properties of inorganic and organic objects consisting of water. The recent development of molecular and structural-chemical concepts has enabled to clarify an explanation of the ability of water molecules to form short-lived hydrogen bonds with neighboring molecules and many other chemical substances and to bond them into intermolecular associates.

As each H_2O molecule has four sites of hydrogen bond formation (two non-shared electron pairs at the oxygen atom and two uncompensated positive charges at the hydrogen atoms), one H_2O molecule in a condensed state is capable to form hydrogen bonds with four H_2O molecules (two donor and two acceptor) (Figure 1), which results in forming a tetrahedron crystal structure clearly observed in ice crystals

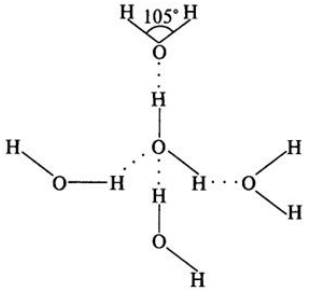

Figure 1. Hydrogen bonding between four individual H_2O molecules. The value of the angle between the covalent H–O–H bond in H_2O molecule is shown.

The most remarkable peculiarity of a hydrogen bond consists in its relatively low strength; it is 5–10 times

weaker than a chemical covalent bond (Pimentel & McClellan, 1960). This leads to the fact that water should be considered as associated liquid composed from a set of individual H_2O molecules, linked together by hydrogen bonds and weak intermolecular van der Waals forces (Liu, Cruzan, Saykally, 1996) thus building clusters of up to dozens of molecules.

Hydrogen bonds are easily disintegrated and re-formed through an interval of time, which makes water structure quite unstable and changeable. External influences can provoke changes in the water structure that will reflect on the number of hydrohen bonds, i.e. on the size of clusters. In 2005 R. Saykally (University of California, USA) calculated the possible number of hydrogen bonds and the stability of water clusters depending on the number of H_2O molecules (Figure 2) (Saykally, 2005). The possible number of hydrogen bonds (100) depending on the number of H_2O molecules (250) in clusters was also estimated (Sykes, 2007). O. Loboda and O.V. Goncharuk provided data about the existence of icosahedral water clusters consisting of 280 H_2O molecules with average size up to 3 nm (Loboda & Goncharuk, 2010).

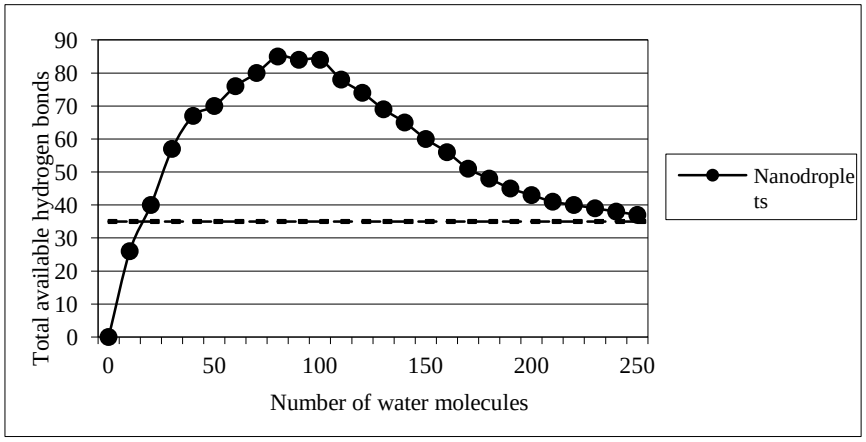

Figure 2. The total number of hydrogen bonds depending on the number of water molecules in clusters.

It was noticed that under the influence of electric power the hydrogen bonds are easily disrupted and the cluster size is diminished to up to a few molecules but not more than 20. This restructered water called electrochemically activated (ECA) water possesses interesting properties that have been studied during the last decades.

An interesting measure called NES (Non-equilibrium Energy Spectrum) for the estimation of hydrogen bonds was suggested by A. Antonov (Antonov 1990, Antonov, 1995, Ignatov, 1998, Ignatov, Mosin, 2014). It is based on the evaluation of energy of hydrogen bonds in electro-volts (eV) and is designated by the spectrum of energy distribution. NES is a measure of changes in the structure of water as a result of external influences.

A current trend in the development of modern science and practice of disinfection is not the creation of new disinfectants but the search of methods to enhance the antimicrobial activity in order to reduce or

completely eliminate the adverse effects on humans, animals, the environment, and to expand the range of action of the existing ones. In full accordance with this trend is the electrochemical activation of aqueous solutions with low mineralization, and especially aqueous solutions of sodium chloride to a concentration of 0.5%. By applying modern nanotechnology, disinfectants (anolyte) with outstanding antimicrobial activity will be received by electrochemical activation. It is based on the nascent oxygen, chlorine dioxide, hydrogen peroxide, ozone and others contained therein. Nascent oxygen is produced only in the reaction mixture and it reacts at once with the reducing agent. Along with that, the electrochemically obtained disinfectants are distinguished by their full safety for humans, animals and the environment as they rapidly decompose to water and trace amounts of sodium chloride.

To study the effect of anolyte and catholyte on different types of bacteria a number of experiments have been performed (Kirkpatrick, 2009). It was noticed that catholyte with REDOX in the range (-700, -100) favorizes the development of anaerobs, while anolyte with REDOX in the range (+200, +750) supports the growth of aerobs.

Studies about the virucidal effect of anolyte are few and of insufficient depth, making research on the possibilities of applying anolyte in the implementation of effective control of viral diseases in humans and animals and especially on particularly dangerous viral infections. One of them is the classical swine fever, which is prevalent in different regions of the world, inflicting heavy economic losses. It is caused by enveloped viruses belonging to the genus Pestivirus of the family Flaviviridae. The resistance and inactivation of the virus of CSF virus are subject of extensive research. Although it is less resistant to external stresses other than the non-enveloped viruses, it retains for a long period of time its virulence: in frozen meat and organs – from a few months up to one year; in salted meat – up to three years; in dried body fluids and excreta – from 7 to 20 days. In rotting organs it dies for a few days and in urine and faeces – for about 1-2 days. In liquid fertilizer it can withstand 2 weeks at 20 ℃, and over 6 weeks at 4 ℃. Its thermal resistance may vary depending on the strain, but the inactivation is dependent mostly on the medium containing the virus. Although the CSF virus loses its infectivity in cell cultures at 60 ℃ for 10 minutes, it is able to withstand at least 30 minutes at 68 ℃ in defibrinated blood. It is relatively stable at pH 5-10, and the dynamic of the inactivating process below pH 5 depends on the temperature.

According to Linton et al., (1987), Sands et al., (1979) and Springthorpe et al., (1990), effective disinfection with viruses whose infectivity is associated with the elements of the casing is achieved by disinfectants dissolving fats, surfactants, disinfectants or fatty acids, organic solvents (ether and chloroform), detergents, proteases, and common disinfectants. It is still believed that a 2% solution of sodium hydroxide is most suitable for the disinfection of spaces contaminated with them. According to Wittmann (1967), to achieve effective disinfection it is necessary to irreversibly damage their nucleic acid.

Investigations in this respect have been carried out on E. Coli using as desinfectant anolyte with ORP equal or greater than +1100 mV and pH = 5.5 (Zinkevich et al., 2000). The authors investgated the action of anolyte Sterilox obtained via electrolysis of diluted NaCl solution on planktonic cells of E. coli JM109. Using atomic force microscope (AFM) they established that within 5 min of influence all cells inflated and burst. Also, experiments have shown a full destruction of proteins, DNA and RNA. It is supposed that anolyte enters the cells provoking structural and functional damages on the cell‘s membrane and cell‘s wall. Similar work was done by S. V. Kumar et al. (1999). They evaluated the inactivation efficacy of anolyte of

pH = 2.7 and ORP = + 1100 mV on Escherihia coli O157:H7, Salmonela enteritidis and Lusteria monocytogenes. The following five strains of Escherihia coli E06 (milk), E08 (meat), E10 (meat), E16

(meat) and E22 (calf feces) were used. All patogens have been significantly reduced (7.0 logCFU/ml) or fully destroied (8.0logCFU/ml) after 2 to 10 minutes inactivation in the whole temperature range from 4^0 C to 23^0 C. It is supposed that the low level of pH of the anolyte makes sensitive the outer cell's membrane thus helping the hypochlorous acid to enter the cell and destroy it.

The purpose of this study is to establish the virucidal effect: 1) of anolyte in different dilutions on Classical swine fever virus in cell culture and organ suspensions; 2) of anolyte/catholyte on E. Coli DH5a, and 3) to see how the virocidal effect relates to the maxima in the spectrum of anolyte ant catholyte[1].

The paper is organized as follows. Section 2 describes methods and materials; section 3 icludes experimental results; section 4 contains concluding remarks and future work is shortly outlined.

[1]Such a dependence has been established between the local extremum at (– 0.1387 eV; 8.95 μm) in the spectrum of catholyte solution and suppressing the development of tumor cells (Ignatov & Mosin, 2014).

2. Materials and methods

Figure 3 demonstrates a laboratory setting for a membrane electrolysis method for the preparation of an acid (anolyte) and alkali (catholyte) solution in electrochemical activation of sodium chloride.

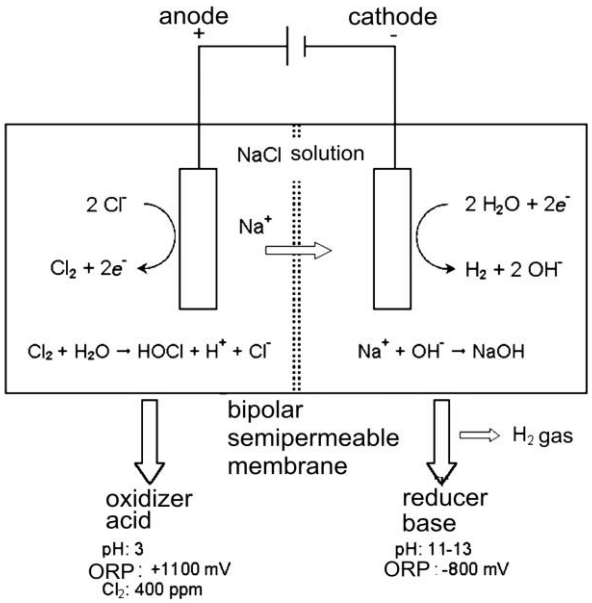

Figure 3. Laboratory setting for a membrane electrolysis method for the preparation of an acid

(anolyte) and alkali (catholyte) solution in electrochemical activation of sodium chloride

The electrochemical activation (ECA) is based on the standard principles of the electrolysis where the application of current leads to chemical changes in the electrolyte. Near the cathode the current provokes decomposition of water molecules according to the formula

$$2H_2O + 2e^- \rightarrow H_2 + 2OH^- \quad (1)$$

Hydrogen will set off apart and strong active reductants OH^- , $H^-_3O^-_2$, H_2 , HO_2^-, HO^-_2 , O^-_2. will be generated. The water will become alcaline due to the hydroxides. As a result ORP, the surface tension and contents of the dissolved oxigen and nitrogen will diminish while the concentration of hydrogen and oxides will increase. Mineral nanoparticals and hybrides of them will be formed. The heavy metals will be transformed to unsolvable hydrooxides and the electrical coductivity will diminish (Prilutsky and Bahir, 1997). The solution will have high pH. This is a reducible solution with strong absobtion and catalytic properties. It has abnormal ORP that can not be obtained via other physical or chemical action.

In the opposite, the anode action leads to a significant activity, increase in ORP and formation of stable and non-stable acids. Here the water is transformed as follows

$$2H_2O \rightarrow 4e^- + 4H^+ + O_2 \quad (2)$$

The initial chlorine will be transformed as

$$2Cl^- \rightarrow 2e^- + Cl_2 \quad (3)$$

Clorious gase will form highly active oxidants Cl_2O, ClO_2, ClO^-, $HClO$, Cl^-, O_2, O_3, HO_2. As a result the surface tension will increase toghether with the electrical conductivity (Hsu, 2005; Prilutsky and Bahir, 1997). The obtained solution has low pH and high ORP. For a long period of time water obtains new qualities reffering to kinetics and chemical reaction of the molecules.

The parameters pH, REDOX and conductivity of the anolyte/catholyte depend on different factors including the ratio of water volumes in the two chambers, electrodes, NaCl concentration, the initial temperature, voltage and processing time.

The first studies of antiviral activity of anolyte were performed at the National Reference Laboratory "Classical and African Swine Fever," section "Exotic and Especially Dangerous Infections" of the National Diagnostic and Research Veterinary Medical Institute, Sofia, Bulgaria. Experiments were conducted with anolyte obtained by the electrolysis apparatus "Wasserionisierer Hybrid PWI 2100" equipped with a 4 titanium electrodes coated with platinum. The disinfectant has a pH 3.2 and ORP 1070 mV. A 0.3% solution of chemically pure sodium chloride in distilled water was used to obtain it. The interaction of the anolyte with the virus suspension is carried out at a solution temperature of 22 0C. A cell culture of porcine origin that is sensitive to the CSF virus is used: a continuous cell line PK-15. Contamination of cell cultures is carried out with cell culture test virus 2.3 Bulgaria with titre 107,25 $TCID_{50}$/ml and organ suspension of internal organs (spleen, kidney, lymph node) of wild boar originating from the last outbreak of CSF in Bulgaria in 2009. The titer of the established virus in the suspension is $10^{4,75}$ $TCID_{50}$ ml. To establish virucidal activity, the prepared for contamination of cell culture inocula (cell culture virus) were treated

with the following dilutions of anolyte in sterile distilled water 1:1 (50%), 1:2 (33.33%), 1:3 (25%), 1:4 (20%). These dilutions were mixed with inocula in proportion 1:1 (100 µl of virus suspension and 100 µl of the appropriate anolyte concentration). The time of action was conformed to the period, which is

methodologically necessary to "capture" any virus present on the cell culture. Upon infection of a cell monolayer, the mixture was removed after the end of the exposure period of 1 h. Upon infection of a cell suspension, the mixture was not removed. To establish the virucidal activity of the anolyte on the CSF virus in organ suspension, a different formulation was used: the inoculum was mixed directly with concentrated anolyte in anolyte-inoculum ratios respectively 1:1; 3:1; 7:1 and 15:1. It is known that the growth of the virus does not cause a cytopathic effect, therefore, for demonstration of its presence, immunoperoxidase plates dyeing is used. The cells were fixed and the viral antigen was detected after binding to a specific antibody labeled with peroxidase. The organs exude 1 cm^3 of tissue, which is homogenized in a mortar with 9 ml of cell cultures medium containing antibiotics, in order to obtain a 10% organ suspension. Sterile sand is added to improve the homogenization. Preparations were left at room temperature for 1 h. They were centrifuged for 15 min at 2500 g. The supernatant was used to infect the cells. In case of cytotoxic effect, parallel dilutions of the homogenates were prepared in proportions 1:10 and 1:100. From the suspensions into multiwell (24-well) plates were added 200 µl of inoculum to cells with coverage of 50-80%. Cell cultures were incubated at 37 ℃ for 1 h in order to "capture" an eventual virus if present, they were rinsed once with PBS and fresh media was added. Alternatively, the plate was filled directly (in cell suspension), since the preliminary studies have found that anolyte did not induce a cytotoxic effect. Cell cultures were incubated for 72-96 h at 37 ℃ in a CO_2 incubator. The procedure with the positive and negative control samples was similar. The positive control sample was a reference strain of the virus of CSF. The immunoperoxidase technique was used. The fixation of the plates was carried out thermally for 3 h at 80 ℃ in a desiccator. In the processing was used a primary monoclonal antibody C 16 diluted 1:50, and secondary antibody RAMPO diluted 1:50. For immunoperoxidase staining was used 3% H_2O_2 and AEC (Dimethylformamide and 3 - Amino - 9 - ethylcarbazole) in acetate buffer. The antibody-antigen complex was visualized by the reaction of the peroxidase with the substrate.

A polymerase chain reaction in real time was carried out. Cell culture and organ suspensions were examined for the presence of CSF viral genome by polymerase chain reaction in real time (real-time RT-PCR, one step, TagMan), one-step according to Protocol of the Reference Laboratory for CSF of EU. For RNA extraction was used the test QIAamp Vital RNA Mini Kit, Qiagen, Hilden, Germany. The initial volume of the material was 140 µl, and the volume of elution – 60 µl. For amplification was used the test Qiagen OneStep RT-PCR Kit in a total volume of 25 µl, and template volume of 5 µl. In the reaction were included primers A 11 and A14, and probe TaqMan Probe - FAM - Tamra. Research was carried out with a thermocycler machine Applied Biosystems 7300 Real Time PCR System with temperature control for reverse transcription 50 ^oC – 30:00 min, inactivation of reverse transcriptase and activation of Taq 95 ^oC - 15:00 min, denaturation 95 ^oC - 00:10 min, extension 60 ^oC - 00:30 min at 40 cycles.

The second study about the antimicrobial activity of anolyte/catholyte was performed at the Institute of Molecular Biology at the Bulgarian Academy of Sciences (BAS). The two solutions have been prepared

using the Activator-I, developed at the Institute of Information and Communication Technologies at BAS. For this, drinking water without aditional quantity of NaCl was used. This has led to pH = 3.0 and ORP = 480 for the anolyte, and pH = 9.8 and ORP = -180mV for the catholyte.

Bacterial strain used in these experiments was *E. coli* DH5α, with genotype: *fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17.*

The assay Colony Forming Units (CFU) was used in this study to assess cellular viability. The conditions for bacterial cultures cultivation were as in (Maniatis et al., 1982). In short Bacterial cells were cultivated in LB medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl – pH 7.5) at 37 ^{o}C. After overnight cultivation of the bacteria 100 µl samples of the cultures were taken, centrifuged for 1 min at 10000 x g and the pellet of bacterial cells was resuspended in 100 µl of anolyte or catholyte. As controls were used bacterial samples resuspende in non-activated water. Different dilutions of cells were spread on LB-agar petri dishes. After overnight incubation at 37 ^{o}C the appeared bacterial colonies were counted. The viable cells were calculated as a percentage from the CFU. The CFU obtained from cultures treated with non-activated water were accepted for 100 %.

NES method was used for the estimation of energy bonds of anolyte, catholyte and deionized water in order to make a supposition about their antimicrobial activity and spectrum peculiarities.

3. Results and discussions

3.1. Research of the effects of electrochemical aqueous sodium chloride solution (anolyte) on the virus of classical swine fever virus

As shown in Figure 4 the cytoplasm of the infected cells (positive reaction) was stained in dark reddish brown color, while in the uninfected cells it was colorless. Table 1 summarizes the results of different experiments. Upon treatment of the viral inoculum with anolyte in a 1:1 dilution, there is no virus growth in the 4 infected wells of the plate, upon 1:2 dilution there is no growth in 2 of the wells, the other 2 we reported as positive. Upon treatment with anolyte at dilutions 1:3 and 1:4, the result is identical: no growth in one of the contaminated wells of the plate, and poor growth – in the other three. The results obtained by infection of a cell monolayer and cell suspension are identical.

Contamination of CC with:	**Dilutions of anolyte (100 µl)**	**Total volume of the inoculum (µl)**	**Concentration of anolyte in %**	**Number of wells:**	**Result: positive/negative:**
Virus 200 µl	-	200		4	4/0
Virus 100 µl	1:1	200	25	4	0/4
Virus 100 µl	1:2	200	16,51	4	2/2
Virus 100 µl	1:3	200	12,5	4	3/1
Virus 100 µl	1:4	200	10	4	3/1

Table 1. Virucidal action of anolyte on the cell culture suspension of the CSF virus upon infecting cell monolayer PK-15.

Table 2 summarizes the results of studies aimed at the evaluation of the virucidal effect of anolyte on organ suspension containing CSF virus upon infecting a cell monolayer PK-15. Upon treatment of the viral inoculum (organ suspension) with anolyte in all dilutions, there is no viral growth in the 4 infected wells of the plate.

Contamination of CC with:	**Dilutions of anolyte (100 µl)**	**Total volume of the inoculum (µl)**	**Concentration of anolyte in %**	**Number of wells:**	**Result: positive/negative:**
Virus 200 µl	-	200		4	4/0
Virus 100 µl	1:1	200	50	4	0/4
Virus 50 µl	3:1	200	75	4	0/4
Virus 25 µl	7:1	200	87	4	0/4
Virus 12,5 µl	15:1	200	94	4	0/4

Table 2. Virucidal action of anolyte on organ suspension containing CSF virus upon infecting cell monolayer PK-15.

In all likelihood anolyte has a destructive influence on the envelope of the CSF virus, where the main antigens (proteins) are. Studies of the viral inocula used in the tests by means of polymerase chain reaction in real time demonstrate the presence of a genome (a nucleic acid) in them, also after treatment with anolyte. Some shortening of the time is proved / decreased number of amplification cycles/, required for the formation of a fluorescent signal, respectively, a positive reaction for genome, closely correlated with the exposure in the treatment of the viral inocula. The longer the exposure of processing with anolyte, the sooner the presence of an RNA virus in the reaction is shown. According to one of the co-authors (Karadzhov), this is an indirect indication that anolyte destroys the virus envelope, which, in turn, facilitates the extraction of nucleic acid and its more rapid reading by the fluorescent signal. There is still no sufficient convincing evidence on the impact of different concentrations of anolyte on viral particles. Experiments carried out by Russian and German researchers are mainly with concentrated anolyte. The full virucidal effect obtained from them confirms our opinion for a strong virucidal action of electrochemically activated aqueous solution of sodium chloride. The differences in results obtained by us are due to the use of lower concentrations of active substances in our experiments. We attribute essential significance to the fact that we were given to determine the concentration limit (25%) of a well demonstrated virucidal activity. Further studies to reduce the time of action, and the conducting of experiments in the presence of biofilm that protects viruses would be interesting.

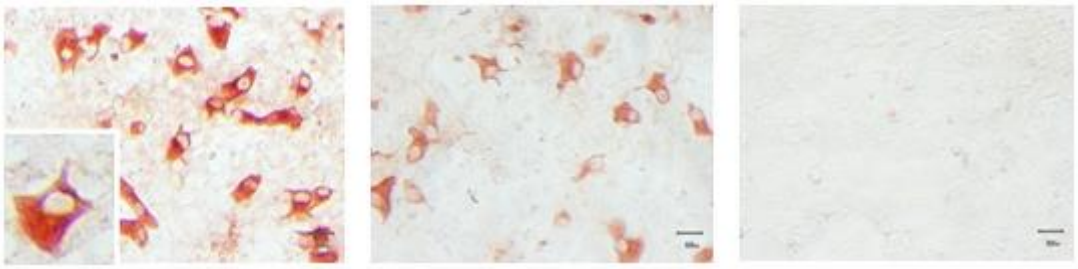

Figure. 4. Established presence of viral antigen (left) and a negative control (right).

3.2. Research on the effects of anolyte and catholyte on Bacteria *Escherichia coli*

In order to assess the effect, if any, of the activated water on bacterial cells we treated cultures of *E. Coli DH5a*. After the treatment of bacterial cells the colony appearing on agar plates were accepted as resulted from survived cells and counted CFU. Therefore, the number of colonies was presented on Figure 5 as percentage of viable cells. It can be seen from the figure that bacterial cells treated with the catholyte hardly survive the treatment. Only approximately 15 % of the cells survived. This clearly shows that the activated water from the cathode possesses a powerful bacteriocidal activity. Notably, the anolyte has also shown slight antibacterial effect. Approximately, 73 % of the bacterial cells had survived the treatment with it. In summary, both types of activated water possess antibacterial effect, however it is obvious that the catholyte is a stroger bacteriocide than the anolyte.

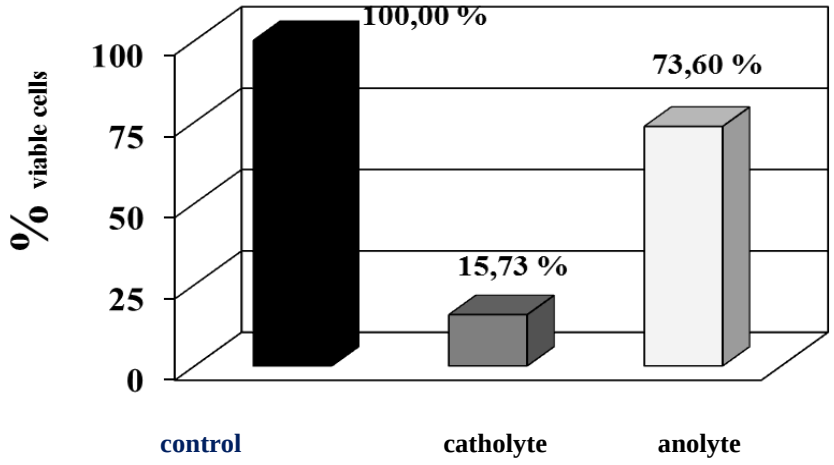

Figure 5. Percentage of viable cells of *E. Coli DH5a* under treatment with catholyte and anolyte relative to the non-activated water.

Figure 6 shows the dependence between acidity and basicity of the solution (pH) and oxidation-reduction potential (ORP). pH in the interval from 3 to 10 and ORP in the interval from -400 to +900 characterize the biosphere of the micro-organisms. Outside these ranges the microorganisms will hardly survive. The disinfecting effect is strengthened by the residual chlorine that decomposes fat and protein of cell membrane.

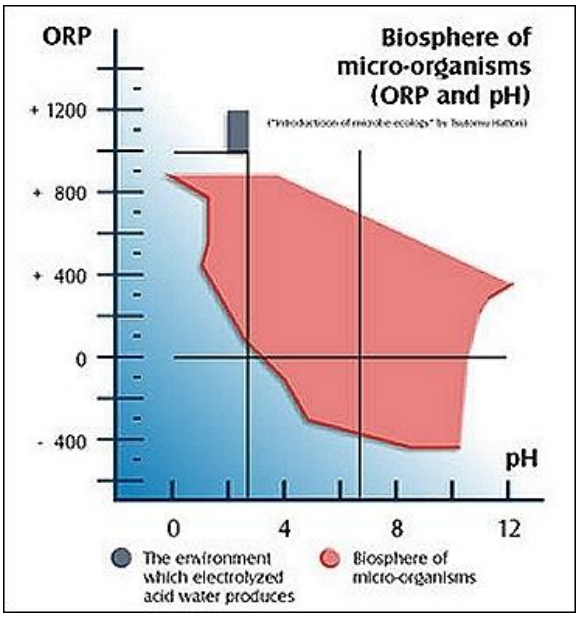

Figure 6. The dependence between acidity and basicity of the solution (pH) and oxidation-reduction potential (ORP). Biosphere of micro-organisms.

3.3. Research of biophysical effects on molecular level of anolyte and catholyte with methods for spectral analysis of liquids – NES and DNES

Other method for obtaining information about the average energy of hydrogen bonds in an aqueous sample is measuring of the spectrum of the water state. It was established experimentally that at evaporation of water droplet the contact angle θ decreases discretely to zero, whereas the diameter of the droplet changes insignificantly (Antonov, 2005). By measuring this angle within a regular time intervals a functional dependence f(θ) can be determined, which is designated by the spectrum of the water state (Ignatov, 2005; Ignatov, 2012; Ignatov & Mosin, 2013). For practical purposes by registering the spectrum of water state it is possible to obtain information about the averaged energy of hydrogen bonds in an aqueous sample. For this purpose the model of W. Luck was used, which consider water as an associated liquid, consisted of O–H…O–H groups (Luck *et al*., 1980). The major part of these groups is designated by the energy of hydrogen bonds (-E), while the others are free (E = 0). The energy distribution function f(E) is measured in electron-volts (eV^{-1}) and may be varied under the influence of various external factors on water as temperature and pressure.

For calculation of the function f(E) experimental dependence between the water surface tension measured by the wetting angle (θ) and the energy of hydrogen bonds (E) is established:

$$f(E) = b\ f(\theta) / 1 - (1 + b\ E)^{2})^{1/2},$$

where $b = 14.33\ eV^{-1}$; $\theta = \arccos(-1 - b\ E)$

The energy of hydrogen bonds (E) measured in electron-volts (eV) is designated by the spectrum of energy distribution. This spectrum is characterized by non-equilibrium process of water droplets evaporation, thus the term "non-equilibrium energy spectrum of water" (NES) is applied.

The difference $\Delta f(E) = f$ (samples of water) – f (control sample of water)

– is designated the "differential non-equilibrium energy spectrum of water" (DNES).

DNES calculalated in milielectron volts (0.001 eV or meV) is a measure of changes in the structure of water as a result of external factors. The cumulative effect of all other factors is the same for the control sample of water and the water sample, which is under the influence of this impact. Figure 7 shows average of deionized water from 25 independence measurements done in a period of one year.

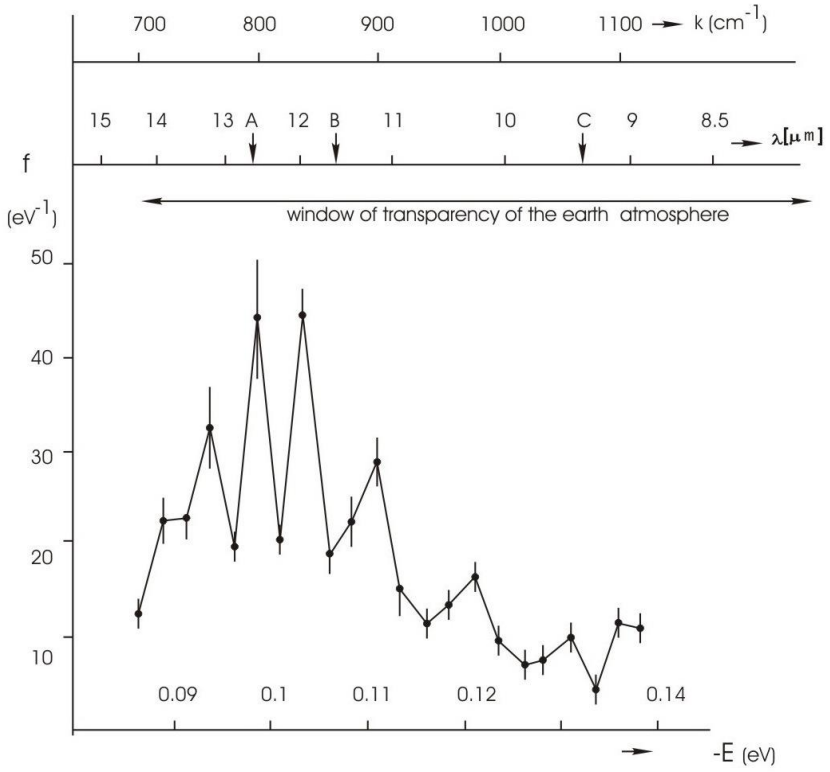

Figure 7. NES-spectrum of deionized water (chemical purity 99.99%, pH = 6.5–7.5, total mineralization 200 mg/l, electric conductivity 10 μS/cm). The horizontal axis shows the energy of the H...O hydrogen bonds in the associates – E (eV). The vertical axis – energy distribution function – f (eV^{-1}). k – the vibration frequency of the H–O–H atoms (cm^{-1}); λ – wavelength (μm).

Data about the anolyte and catholyte as type of water, obtained by non-equilibrium (NES) and differential-equilibrium energy spectrum (DNES) are submitted in this report The average energy

($\Delta E_{H...O}$) of hydrogen H…O-bonds among individual molecules H_2O was calculated for catholyte and analyte by NES- and DNES-methods. We studied the mathematical models and local extremums of catholyte and anolyte. The result for catholyte of NES spectrum is (-0.1285 eV), for anolyte is (-0.1227 eV) and for control sample with deiomnized water is (-0.1245 eV). The calculations of $\Delta E_{H...O}$ for catholyte with using DNES method compiles (-0.004±0.0011 eV) and for anolyte (1.8±0.0011 eV). These results suggest the restructuring of $\Delta E_{H...O}$ values among H_2O molecules with a statistically reliable increase of local extremums in DNES-spectra of catholyte and anolyte (Table 3).

For catholyte the biggest local extremum is at (-0.1387 eV) or 8.95 μm. In 1992 Antonov performed experiments with the impact on tumor cells of a mouse in water. There was a decrease of the spectrum compared with the control sample of cells from a healthy mouse. There was a decrease of local extremum at (-0.1387 eV) or 8.95 μm. In DNES the local extremum at 8.95 μm is with negative value. For catholyte the local extremum is with positive value 133.3 eV^{-1}.

For catholyte the biggest local is at (-0.1312 eV) or 9.45 μm. For the treatment of influenza part of medical drugs is aluminum hydroxide AlO(OH). The local extremu is at (-0.1326 eV) or 9.35 μm.

-E(eV) x-axis	**Catholyte**	**Anolyte** y-axis (eV^{-1})	**Control Sample** y-axis (eV^{-1})	**DNES Catholyte**	**DNES Anolyte**	**-E(eV)** x-axis	**Catholyte** y-axis (eV^{-1})	**Anolyte** y-axis (eV^{-1})	**Control Sample** y-axis (eV^{-1})	**DNES Catholyte** y-axis (eV^{-1})	**DNES Anolyte** y-axis (eV^{-1})
0.0937	0	0	0	0	0	**0.1187**	0	66.7	66.7	-66.7	0
0.0962	0	0	0	0	0	**0.1212**	66.7	0	0	66.7	0
0.0987	0	0	0	0	0	**0.1237**	0	0	0	0	0
0.1012	66.7	66.7	33.3	33.4	33.4	**0.1262**	0	0	66.7	-66.7	-66.7
0.1037	0	0	33.3	-33.3	-33.3	**0.1287**	0	0	66.7	-66.7	-66.7
0.1062	0	0	0	0	0	**0.1312**	33.3	100	33.3	0	66.7
0.1087	0	0	0	0	0	**0.1337**	33.3	33.3	33.3	0	0
0.1112	0	0	0	0	0	**0.1362**	0	0	0	0	0
0.1137	0	66.7	66.7	-66.7	0	**0.1387**	200	66.7	66.7	133.3	0
0.1162	0	0	0	0	0	–	–	–	–	–	–

Table 3. Local extremums of catholite and anolyte in NES and DNES spectrum. (Ignatov&Mosin, 2014)

Evaluation of the possible number of hydrogen bonds as percent of water molecules with different values of distribution of energies is presented in Table 4. These distributions are basically connected with structuring of H_2O molecules with the same energies. This is mathematical model of anolyte and catholyte (Ignatov,

Mosin, 2012).

-E(eV) x-axis	**Catholyte** y-axis (%((-Evalue)/ (-Etotal value)	**Anolyte** y-axis (%((-Evalue)/ (-Etotal value)	**-E(eV)** x-axis (%((-Evalue)/ (-Etotal value)	**Catholyte** y-axis (%((-Evalue)/ (-Etotal value)	**Anolyte** y-axis (%((-Evalue)/ (-Etotal value)
0.0937	0	0	0.1187	0	16.7
0.0962	0	0	0.1212	16.7	0
0.0987	0	0	0.1237	0	0
0.1012	16.7	16.7	0.1262	0	0
0.1037	0	0	0.1287	0	0
0.1062	0	0	0.1312	8.4	24.8
0.1087	0	0	0.1337	8.4	8.4
0.1112	0	0	0.1362	0	0
0.1137	0	16.7	0.1387	49.8	16.7
0.1162	0	0	–	–	–

Table 4. Energy distribution of alkaline (catholyte) and acid (anolyte) solution in electrochemical activation of sodium chloride in percent.

5. Conclusion

The experimental data obtained during the last years suggest that water is a complex dynamic associative system, consisting of tens and possibly hundreds of individual H_2O molecules bonding by multiple intermolecular hydrogen bonds, being in a state of dynamic equilibrium. Untill now the existence of associative water clusters with general formula $(H_2O)_n$, where n = 3–20 has been scientifically proven. Nevertheless, a satisfactory theory of water has not been developed up to now. Although calculated structural models explain pretty well many anomalous properties of water and being in a good agreement with the experimental data obtained via different techniques and equipement, they face big difficulties when trying to explain the dynamic properties of water – flow, viscosity and short relaxation times, which are measured by picoseconds. The explanation of the very strong influence of different types of water on microbes and viruses will be of great scientific and practicle importance. Experimental results in this respect are peesented in this paper. They are in accordance with results obtained by other researchers and demonstrate the strong biocidal effect of anolyte on the swain virus. Also, interesting results of antibacterial effect were obtained when *E. Coli DH5a* was treated by catholyte and anolyte, respectively. Unexpectidely, catholyte of ORP ≈ -180 mV and pH = 9.8 demonstrated better biocidal effect than anolyte at ORP ≈ +500

and pH = 3.9. The major findings of our research could be formulated as follows.

1. Anolyte did not affect the growth of the cell culture, PK-15.
2. Anolyte administered at a concentration of 25%, exhibits a strong virucidal effect on a cell culture virus and a weaker antiviral activity at concentrations of 16.51%, 12,5% and 10%.
3. Anolyte exerted a strong virucidal effect at a concentration of 50%, 75%, 87% and 94% over in CSF viruses in organ suspension.
4. At concentrations above 50% anolyte can be successfully used to achieve efficient disinfection of surfaces in virological laboratories.
5. Catholyte supresses the growth of *E. coli* to up to 85% while anolyte is at least three times less effective.
6. Local extremum at 9.85 μm in DNES spectrum of catholyte. There is is decreasing of this local extremum in water medium with tumor cells.
7. Local extremum at 9.45 μm in DNES spectrum of anolyte. In 9.35 μm there is effect on inflammation from virus of influenza.
8. Mathematical model of catholyte and anolyte regarding distributation of water molecules according energies of hydrogen bonds.

In this investigation we have tried to relate the antimicrobial anf antivirol action of elrctrolized water with their energy spectrum peculiarities. There is an indication about such a connection but more thorough analysis is needed to prove it.

The inverse biocidal effect between catholyte and anolyte in case of *E. coli DH5a* requires a clear explanation.

Finally, it is still not clear the contribution of differenr ingredients of the activated water to its biocidal effect.

All these problems will be in the focus of our future work.

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