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SOME DISCREPANCIES OF LIVE CELLS COUNT DURING STAINING WITH DEAD/LIVE BACTERIAL VIABILITY KIT

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ABSTRACT
PURPOSE: In this work we studied a toxic effect of thin ZnO films, prepared by magnetron sputtering and sol-gel dip coating methods on Pseudomonas putida. METHODS : Bacterial suspension in exponential phase of cells division is washed and stained with Dead/Live kit. It is spread on thin ZnO films and the survived cells are counted every 2 hours. Two microbiological methods are used: direct counting with epifluorescent microscope and cultivation in nutrient medium. RESULTS: The results are processed with different statistical methods and show bactericidal effect of the ZnO thin films. CONCLUSIONS : The surface relief of the thin nano-structured film is important for the antibacterial effect and the strongest bactericidal effect demonstrates the ZnO film obtained by sol-gel dip coating. The cultivation method has shown that microscopic cell count underestimate the quantity of viable microbial cells.

Key words: ZnO thim films, sol-gel dip coating, magnetron sputtering, Pseudomonas putida

INTRODUCTION

It has long been established that cultivable bacteria represent only a small fraction of all bacteria present in natural habitats (1). Therefore, different methods for precise cells count are used, one of which is epifluorescent microscopy and fluorescent dyes. Since acridine-orange staining is not reliable enough to distinguish live and dead cells in the environment (2a, 2b), a DEAD/LIVE kit of Promega Company (USA) is used. The kit consists of two dyes. The bacterial cell wall and membrane have different permeability to them – the red stained cells are counted as dead and the green ones are considered as live. Stocks (3) determines the ratio of propidium jodide to DNA quantity as 0,4:1. We used this ratio in our experiment to observe the changes in the number of live and dead cells in interaction with ZnO thin films. ZnO nanoparticles and thin films have broad application in biology, medicine, etc. (4, 5). Franklin and co-authors, (6) have studied the toxicity of ZnO nanoparticles to *P. subcapitata* and have determined the concentration of the dissolved Zn ions derived from ZnO. It is found that the toxicity of ZnO particles and ZnCl2 is due to the dissolved Zn2+ ions (6). Heinlaan and co-authors established lethal/effective concentrations L(E)C50 for bulk ZnO, nano-ZnO and ZnSO4.7H2O in amount of 1.8, 1.9, 1.1 mg/l respectively, for *Vibrio fischeri* (7). They report that the toxicity is due to the dissolved Zn ions as proved with recombinant Zn-sensitive bacteria. However, other groups have reported that the reason for

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the toxic effect is the damaging of the cell wall and the membrane (8). Their conclusions are that ZnO nanoparticles are effective for inhibition of both Gram-positive and Gram-negative bacteria.

In this work we investigate the antibacterial effect of undoped ZnO thin films obtained by different methods using a direct counting and cultivation method. Both methods show differences in quantity of viable cells and ratio of live/dead cells, and possible reasons are discussed.

MATERIALS AND METHODS

1. ZnO deposition methods

Magnetron sputtering (MS)

The thin films are deposited by r.f. magnetron sputtering (MS) of a ZnO ceramic target (100 mm disc) in atmosphere of Ar (0.5 Pa) at substrate temperature of 500°C and r.f. power of 180 W. The thickness of the deposited films is about 600 nm. SEM pictures are obtained by JSM-840A JEOL with LaBa6 cathode to study the structure of the films (9).

Sol-gel dip coating (SGDC)

The precursors are zinc acetate, 2-methoxyethanol and monoethanolamine. The film deposition was carried out on glass substrates (ISO-LAB-Germany). Total five layers were deposited. Each deposited layer was dried up in air at 80°C for 15 min. The final annealing was carried out at 500°C for 1 hour and the film thickness is about 1 µm. The films were prepared and investigated by SEM - JSM-5510 JEOL operating at accelerating voltage of 10 kV, as described in (10).

2. Surface morphology

The surface morphology of all tested films was investigated by scanning electron microscopy (SEM) on a JEOL JSM-6500F microscope, using an electron beam energy of 5–10 keV and an emission current of 64 mA. Prior to investigation, the ZnO thin films were coated with a uniform goldlayer to ensure good electrical conductivity. The morphology of thin films is presented in **Figures 1a and 1b.** Magnetron sputtered ZnO films have columnar structure and relatively smooth surface, while the films obtained by sol-gel dip coating have a very wrinkled and uneven surface **(Figure 1b)**.

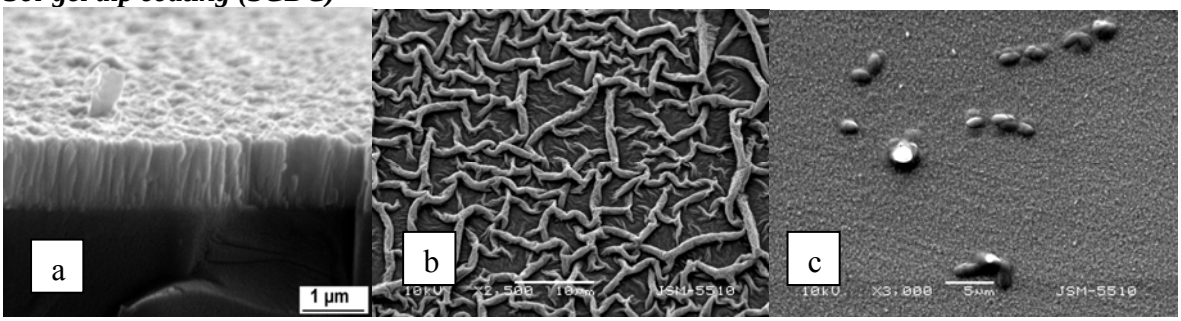

Figure 1. Cross section SEM pictures of the deposited thin films by magnetron sputtering - ZnO (a) and sol-gel dip coating (b) and SEM picture of ZnO surface of the film obtained by magnetron sputtering with *Pseudomonas* cells (c).

Microorganisms and materials

Gram-negative bacterium *Pseudomonas putida* (ATCC 12633) is used, supplied from Bulgarian National bank of industrial microorganisms and cell cultures; LIVE/DEAD BacLight Bacterial Viability Kit L-7007 (Promega) is applied .

Microscope counting

Flame sterilized thin ZnO films are placed on object glass in sterile Petri dish. Bacterial culture in exponential phase is obtained by pre-culturing from rich nutrient medium in synthetic broth and cultivation at 180 RPM and 25°C over night. The second inoculation is made in the morning in the same synthetic broth (ISO10712:1995), and the third after the 4th h of the pre-culturing. To minimize the influence of the nutrient medium the cells are collected with centrifugation (10000RPM for 8 minutes), supernatant is thrown out, pellet is re-suspended gently in sterile physiological solution and the procedure is repeated 3 times. As prepared bacterial suspension (1 ml), with density of about 10^8CFU/ml, is mixed with 200 µl Dead/Live bacterial viability kit (Promega) for staining according (3). 10 µl from bacterial stained suspension are evenly distributed on different thin ZnO films covered with sterile coverslip glass. The control is sterilized in the same way on object glass (the same size)

without ZnO nanoparticles. Every variant (control, thin ZnO films and microbial suspension) is placed in a wet chamber (Petri dish with water soaked filter paper) and sign over it about the film type and timing and is put in a dark at room temperature to minimize the color bleaching of kit. 8 replicas from the controls are prepared and six from the every variant of thin ZnO films, obtained by different methods. Two of the control variants are photographed immediately to quantify the live and damaged cells at the zero hour. Every two hours samples and controls are removed sequentially and 20 images of different ZnO thin films and control glass are photographed with camera of Leika DM55000 epifluorescence microscope with UV filter with magnification of 400 times. The cells counting is implemented by two independent operators, the quantity of cells is determined using standard formula involving the size of the image, the average number of bacteria per field and the quantity of suspension. After photography each variant is rinsed in sterile microbiological box and washed with 1 ml of sterile water to remove the bacteria from the thin films and from coverslip glass side in contact with the bacterial suspension. The obtained bacterial suspension is used for the preparation of successive decimal dilutions and 100 µl of them are sowed in a nutrient medium without nanoparticles for quantification of the bacteria survived on the ZnO films. The sowed plates are kept in dark at room temperature (23-25°C) and the numbers of bacterial colonies are recorded on 24th and 48th hours. The quantity of live cells in the pictures is defined as the amount of yellow and green cells together, the red cells are counted as dead; and damaged cells - as the orange ones.

Biostatistical analysis

In our analysis is applied a descriptive statistics. The main sample statistics (minimum, maximum, mean, standard error of mean, standard deviation and median) are computed for the live/dead ratio for all groups. Comparison between the replicas is made by means of Student t-test or Mann-Whitney U-test, when normality test (Kolmogorov-Smirnov and Shapiro-Wilk) failed. The change in the live/dead ratio during the experiment is analyzed with Kruskal-Wallis ANOVA on Ranks. The same test is used for investigation of the differences between the antibacterial effect of the films. The Pairwise Multiple Comparison Procedures is applied - Tukey Test or Dunn's test, for groups with unequal size.

RESULTS AND DISCUSSION

Magnetron sputtering **(MS) Figure 1** shows cross section SEM picture of thin ZnO films. The surface topography of the films is flat and without sharp edges. On the SEM pictures the *Pseudomonas putida* cells look intact - no deformed cells and no cellular material as can be seen in **Figure 1c.**

Cells count Microscopic counting of *Pseudomonas* cells suspension, stained with Dead/Live bacterial kit® on usual object glass without ZnO films is performed as a control. The green and yellow cells counted as live are more than the red cells and their ratio is 2.7 at the start of the experiment (zero hours at **Figure 2)**. At the second hour the same ratio is higher (3.5) and this can be explained by formation of stable cell wall around the new formed daughter cells, as the bacteria are in exponential phase. The ratio between live and dead cells in the control variants show a normal distribution in the zero and second hour in both tested replicas. At the fourth hour the red cells are more than green and yellow and their ratio is 0.9. This can be explained by a processes of loss of the integrity of the cell membrane occurring during the cell division and penetration of propidium iodide in dividing cells. At the sixth hour there is another increase of the number of green cells, but not as high as in the second hour, may be as a result of continuous second partition of the cells. Using T-test at the 4th hour we established that differences between the two replicas in control variant are not significant. Similar are the results for the sixth hours. From the results concerning the survived cells in nutrient medium at Figure 6 it could be seen, that the number of the cells in the control variant is close to that of the green, yellow and orange cells together or even higher. So, at the start of the experiment the quantity of the live cells forming colonies on the nutrient agar is between $5x10^5$ and $6x10^5$ CFU/ml. At the 4^{th} hour the quantity of the live cells is the same, but at the 6^{th} hour the live cells, which form colonies on nutrient agar are $8x10^6$ CFU/ml. The results in Figure 2 and **Figure 4a** prove that the glass used as a control is inert and the surrounding conditions are suitable for the cell multiplication till 6^{th} hour (end of the experiment).

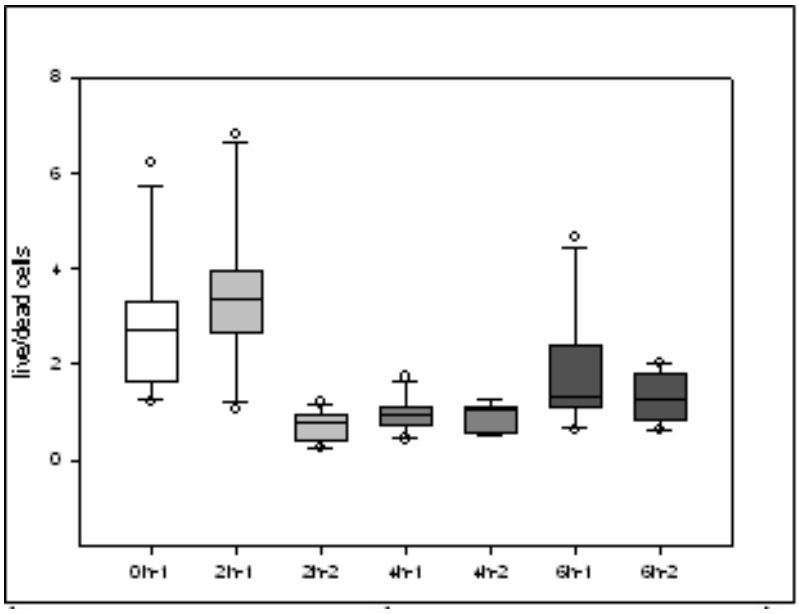

Figure 2. The ratio live/dead cells in the control variant during the experiment

Kruscal Wallis test of the changes in the relationship between live and dead cells in the control variant indicates that the ratio is the greatest for the second hour. Then the ratio of live/dead cells gradually decreases to the value at the 4th hour (0,9) and after that increases slightly to sixth hour(1,77). From a physiological standpoint, the observation can be explained by the state of the bacterial culture. Cells are subjected to the impact of the treatment (centrifugation and washing) in a phase of exponential growth. It can be assumed that most of them duplicate their DNA and are ready to divide. Reduction of the ratio live /dead cells at the 4th hour suggests some changes in the integrity of the cell wall, which is associated with the process of the mother's cell separation into two daughter's cells. The new increase in the ratio live / dead cells at the 6th hours suggests about the complete process of division, separation and formation of the rigid cell wall around the new daughter's cells, impermeable to propidium iodide **(Figure 2, Table 1)**.

Table 1. *Basic statistics of the ratio live/dead cells in the control variant counted by the epifluorescent microscope pictures*

	Size	Min	Max	Mean	Standard Error	Std Dev	Median
control-0h-1	11	1.23	6.20	2.80	0.42	1.38	2.72
control-2h-1	10	1.06	6.82	3.50	0.49	1.54	3.34
control-2h-2	10	0.27	1.19	0.71	0.10	0.32	0.78
control-4h-1	11	0.44	1.72	0.97	0.10	0.34	0.94
control-4h-2	9	0.52	1.25	0.93	0.09	0.28	1.05
control-6h-1	10	0.64	4.67	1.77	0.37	1.17	1.34
control-6h-2	10	0.62	2.03	1.34	0.16	0.51	1.29

The behavior *of Pseudomonas putida* cells on thin ZnO film is very different from the control variant discussed above. The ratio live / dead cells on the thin films looks different in comparison with the values of the control experiment. The mean values of the live and dead cells and the ratio counted from the thin films obtained by sol-gel method (SGDM) show a continuous reduction in the values as can be seen in **Figure 3** and **Table2**.

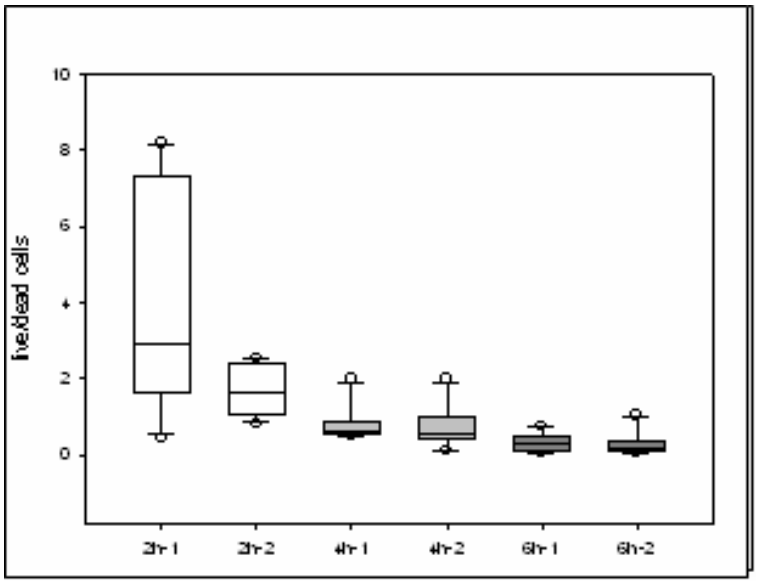

Figure 3. The ratio live/dead cells for the ZnO films obtained by sol-gel deposition method during the experiment.

Table 2. *Basic statistics of the ratio live/dead cells on ZnO thin films obtained by sol-gel method counted by epifluorescent microscope pictures.*

	Size	Min	Max	Mean	Std. Error	Std Dev	Median
SGDC-2h-1	10	0.44	8.19	4.16	0.95	3.01	2.94
SGDC -2h-2	10	0.83	2.54	1.66	0.21	0.67	1.64
SGDC -4h-1	10	0.49	2.00	0.79	0.14	0.45	0.60
SGDC -4h-2	10	0.11	2.00	0.73	0.17	0.54	0.55
SGDC -6h-1	10	0.04	0.75	0.31	0.07	0.23	0.29
SGDC -6h-2	10	0.05	1.03	0.28	0.10	0.31	0.16

The cultivation method shows decreasing of viable cells quantity on SGDM thin ZnO films with the time. The number of the colony-forming units at the second hour is 1x105 CFU/ml and at the 6th hour is 1x104 CFU/ml (ten times less than in the second hour). These results can be attributed to the toxic effects of zinc ions, nanoparticles or free oxygen radicals formed on the surface of the thin films and damages of the cell wall and external membrane during the interaction with the cells. The overall decrease in the quantity of the counted cells can be explained by the fact that damaged cells lyzed and their content can serve as a protecting agent, invisible under UV light, but helpful for other cells **(Figure3, Table 2)**. In visible light around the cells stained with kit, are observed some materials which are in a weak pink color similar to the material from cells' lyses. The cells contents can serve as a helator of zinc ions and disposal of superoxide radicals on the surface of thin films.

The other tested variant is thin ZnO films obtained by magnetron sputtering (MS) deposition. If we consider the interaction of bacteria with the zinc oxide thin films obtained by magnetron sputtering the picture is different. There is a decrease in the proportion live / dead cells between the second and forth hour of the experiment, but between the 4th and 6th h it is considerably lower and the differences are unreliable compared to the cells quantity on the films obtained by sol-gel method **(Figure 4)**. At the second hour the cells quantity is 1,4x105 CFU/ml, at the 4th h is 9,6 x104 CFU/ml, at the 6th h is 8,3 x104 CFU/ml. Decreasing in the number of live cells on the magnetron sputtered ZnO films during the experiment exists, but not so fast as for the cells on the films obtained by sol-gel deposition **(Table 3).**

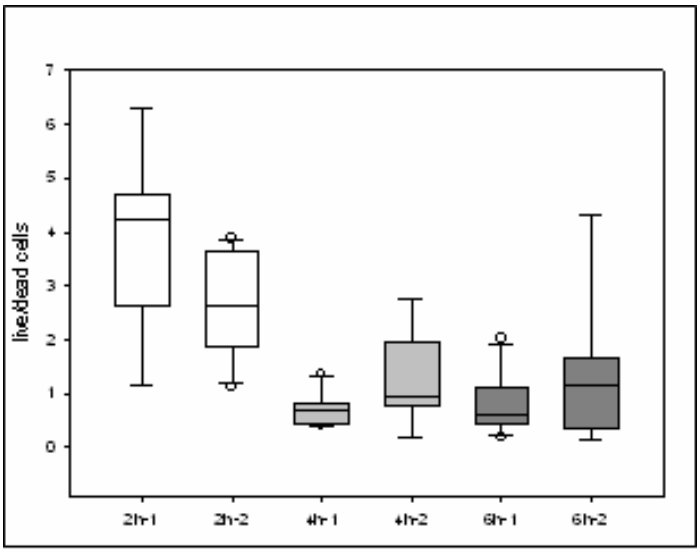

Figure 4. The ratio live/dead cells for the ZnO films obtained by magnetron sputtering deposition method during the experiment. Legend: 2h-1, 2 – two replicas of the second hour;4h-1,2– two replicas of the fourth hour; 6h-1,2 – two replicas of the sixth hour;

Table 3. *Basic statistics of the ratio live/dead cells on thin ZnO films obtained by magnetron sputtering counted by epifluorescent microscope pictures*

	Size	Min	Max	Mean	Std. Error	Std Dev	Median
MS-2h-1	9	1.14	6.29	3.78	0.51	1.52	4.23
MS -2h-2	11	1.11	3.87	2.73	0.29	0.95	2.62
MS -4h-1	10	0.40	1.37	0.69	0.09	0.30	0.68
MS -4h-2	9	0.18	2.76	1.28	0.27	0.80	0.94
MS -6h-1	11	0.19	2.01	0.84	0.17	0.55	0.62
MS -6h-2	9	0.16	4.31	1.26	0.43	1.28	1.13

The Kruskal-Wallis test shows reliable differences between the mean values of ratio live/dead cells for the second, 4th and 6th hours (the means of difference H = 29.31, probability P < 0.001). Repetitions of the different classes are combined. Only at the fourth hour, the test of Mann-Whitney showed differences in the value of P close to the level of significance (U = 20.0, P = 0.045).

The Dune method shows statistically significant differences in the number of live cells between the second and fourth hour and between the second and sixth hour but not in the ratio live / dead cells between the fourth and sixth hour in the experiment with the obtained by magnetron sputtering ZnO films. The result means that the bactericidal effect is stronger at the first 2 hours of interaction and later it weakens. The reason may be the stability of ZnO thin films obtained by magnetron sputtering, thus the quantity of Zn ions around the bacteria is low (Figure 4). After the 4th hour of the presence of bacteria on the ZnO films the effect weakens or disappears.

The effect does not appear in the cells washed from the ZnO thin films, grown in culture medium without Zn ions or nanoparticles.

Comparison between the two methods of counting under the microscope and cultivation on nutrient medium showed that changes in the status of the cells are demonstrated first in the microscopic picture and later in the nutrient medium **(Figure 6)**. Apparently some of the cells with compromised cell wall integrity (injured by hydrogen peroxide) are restored in a supportive environment, free of nanoparticles and give rise to colonies. So the cultivation method does not report dead cells in the fourth hour of the experiment. At the sixth hour there is reliable decrease in the number of live cells enumerated by cultivation method, but according to the statistical program, the differences in the quantity of live and dead

cells counted by the epifluorescent microscopy method between
the fourth and sixth hours are unreliable.
Kruskal-Wallis test shows reliable differences between median values of the ratio live/dead cells for the second, fourth and sixth hours (H = 12.81, P = 0.002).

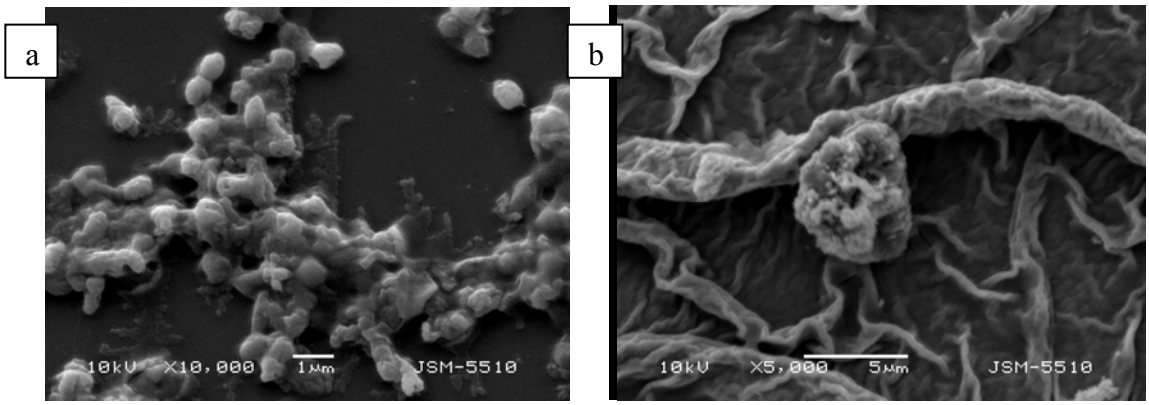

Figure 5. SEM pictures of the control variant glass (a) and ZnO thin films deposited by sol-gel dip coating (b) with Pseudomonas cells on their surface after 9 hours of interaction.

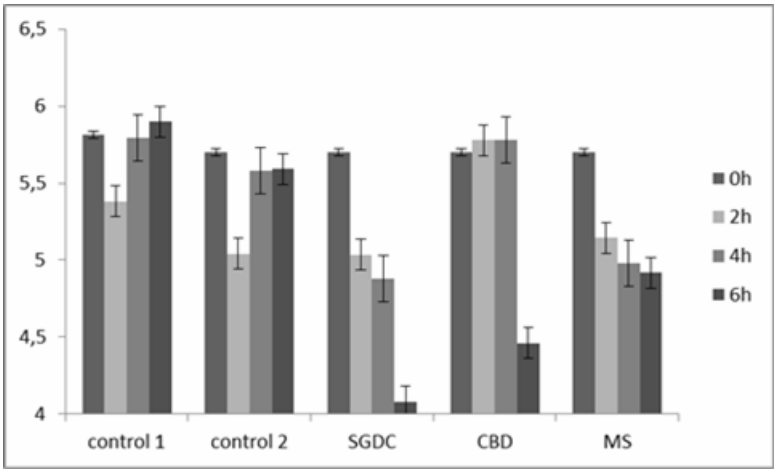

Figure 6. Logaritm CFU/ml of *Pseudomona*s cells during the experiment with ZnO thin films obtained by magnetron sputter deposition.

All the results obtained by two microbiological methods showed that the environmental conditions are crucial for the survival of the bacteria. The difference between the green +yellow cells, counted as live cells, and the red cells, counted as dead cells, and the number of cells in CFU/ml obtained by cultivation method is still considerable. Results from the control variant prove that in exponential phase the fast growing cells have some kind of membrane permeability to propidium iodide (PI). Obviously PI penetrates into the live dividing cells and stains them in red. This is in congruence with the research of Shi L et al. (11) concerning the limits of propidium iodide as a cell viability indicator for the environmental bacteria. M. Berney et al. (12)has supposed that the flow-cytometric pattern can be related to the presence of intermediate cellular states characterized by the degree of damage affecting specifically on the bacterial outer membrane. This hypothesis is supported by the fact that EDTA-treated nonirradiated cells exhibit the same staining properties (12). Our observations proved that some of the red cells are moving actively when they are directly counted on the glass slide. After cultivation on solid rich medium, these cells give the slow growing colonies which are visible after 48 h. These facts suggest that some of the red cells, which have been marked

as dead, are viable and could restore their normal functions, giving colonies that emerge later. The cells number in nutrient media proves the possibility of some sub lethal membrane damages which could bring to red staining and confusion in the results interpretation.

The evaluation of thin ZnO films by the two methods is presented in Figure 6. Changes in the ratio live/dead cells on the three ZnO thin films are statistically proved and the lowest values are on the film obtained by sol-gel dip coating at the second, fourth and sixth hours **(Figure 6)**. There are reliable differences between the groups.
All results prove the strongest bactericidal effect of ZnO thin films obtained by sol-gel dip coating method.

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REFERENCES

1. Chapelle, F. H., Zelibor, J. L., Grimes, D. J., LeRoy,Jr., Knobel, L. Bacteria in deep coastal plain sediments of Maryland: A possible source of CO2 to groundwater. Water resources research, 23: 1625-1633, 1987.

2a. Boteva S., Kenarova A., Traykov I., Bogoev, V. Vertical distribution of bacterioplankton in Dolnoto Lake – Seven Rila Lakes, Biotechnol and Biotechnol Eq., SE, 23: 365-368, 2009.

2b. Boteva, S., Traykov, I., Kenarova, A., Bogoev, V. Bacterioplankton abundance and spatial dynamics in the Seven Rila Lakes, Bulgaria, Chinese Journal of Oceanology and Limnology, 28 : 451-458, 2010.

3. Stocks, S.M. (2004) Mechanism and use of the commercially available viability stain, BacLight . Cytometry, part A, 61A:189-195.

4. Reddy, K.M., Feris, K., Bell, J., Wingett, D., Hanley, C. and Punnoose, A. Selective toxicity of zinc oxide nanoparticles to prokaryotic and eukaryotic systems, Applied physics letters, 90: 213902-1 -- 213902-3, 2007.

5. Ahamed, M., Akhtar, M.J., Raja, M., Ahmad, I., Siddiqui, M.K., Alsalhi, M.S., Alrokayan, S.A., ZnO nanorod-induced apoptosis in human alveolar adenocarcinoma cells via p53, survivin and bax/bcl-2 pathways: role of oxidative stress. Nanomedicine: Nanotechnology, Biology and Medicine, 1549-9642, 2011.

6. Franklin, N.M., Rogers N.J., Apte, S.C., Batley, G.E., Gadd, G.E., Casey, P.S. Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and ZnCl2 to a freshwater microalga (Pseudokirchneriella subcapitata): the importance of particle solubility. Environ Sci Technol, 41:8484–8490, 2007

7. Heinlaan M., A. Ivask, I. Blinova, H.-C. Dubourguier, A. Kahru Toxicity of nanosized and bulk ZnO, CuO and TiO2 to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus. Chemosphere 71: 1308–1311, 2008.

8. Tam, K., Djurisic, A., Chan, C., Xi, Y., Tse, C., Leung, Y., Chan, W., Leung, F., Au, D. Antibacterial activity of ZnO nanorods prepared by a hydrothermal method. Thin Solid Films 516(18): 6167-6174, 2008.

9. Dimova-Malinovska D., Nichev, H., Georgieva, V., Angelov, O., Pivin, J.C.,Mikli, V. Sensitivity of ZnO films doped with Er, Ta or Co to NH3 at room temperature,
Physica Status Solidi (a), 205: 1993-1997, 2008.

10. Kaneva, N. V., Yordanov, G. G., Dushkin, C. D., Characterization of nanosized ZnO thin films for the photoinitiated decolorization of malachite green in aqueous solutions. Bulletin Mater. Sci., 33: 111-118, 2010.

11. Shi, L, Guenther, S., Huebshmann, T., Wick, L. Y., Harms, H., Mueller, S. Limits of propidium iodide as a cell viability indicator for environmental bacteria Cytometry, part A, 71(A):592-598, 2007.

12. Berney M., Hammes F., Bosshard F., Weilenmann H.U.,and Egli, T. Assessment and Interpretation of Bacterial Viability by Using the LIVE/DEAD BacLight Kit in Combination with Flow Cytometry. Appl. Environ.Microbiol, , 73, 10:3283–3290, 2007.