DOI:10.34918/87339

Oskar TOKARCZYK[1], Robert CICHOROWSKI[1], Krzysztof JANIK[1],
Weronika LOSA[1], Marta PROCHOTA[1], Tomasz ŻYTECKI[1],
Divine Yufetar SHYNTUM[2], Alicja STAŚCZAK[1,2,*], Sebastian ŻABCZYŃSKI[3],
Dariusz CHOIŃSKI[1], Sebastian STUDENT[1,2]

Chapter 13. AUTOMATIC SYSTEM FOR BACTERIAL CELL VIABILITY ANALYSIS OF CONFOCAL MICROSCOPY IMAGES

13.1. Introduction

In recent years, light-emitting diodes (LEDs) have become increasingly popular in the field of research because of their diverse capabilities, including their small size and low energy consumption [1], which make them ideal for use in sterilisation systems. One of the most widely used applications of LEDs is in the field of ultraviolet radiation, specifically UVC irradiation. UVC radiation is a type of ultraviolet wavelength of radiation with a 100 to 280 nm. It is known for its mutagenic effect on microorganisms such as viruses or bacteria [2, 3], which causes damage to their DNA and prevents them from reproducing. This makes UVC irradiation a highly effective tool for sterilisation purposes. When applied to a surface or object, UVC radiation can penetrate the cell walls of microorganisms, disrupting their genetic material and rendering them inactive or dead. The COVID-19 pandemic has drawn increased attention to the use of UVC irradiation for sterilisation purposes. The SARS-CoV-2 virus, which causes COVID-19, is highly contagious and can survive on surfaces for extended periods of time, making it a significant challenge in preventing transmission. However, studies have shown that UVC irradiation can be effective in eliminating the virus on surfaces and in the air.

[1] Faculty of Automatic Control, Electronics and Computer Science, Silesian University of Technology, Gliwice, 44-100, Poland.
[2] Biotechnology Center, Silesian University of Technology, Gliwice, 44-100 Gliwice, Poland.
[3] Environmental Biotechnology Department, Faculty of Energy and Environmental Engineering Silesian University of Technology, Gliwice, 44-100 Gliwice, Poland.
[*] Corresponding author: alicja.stasczak@polsl.pl.

In fact, several hospitals and healthcare facilities have begun using UVC irradiation systems as part of their infection prevention measures for COVID-19. These systems are used to sterilise patient rooms, waiting areas, and equipment, and have been shown to be effective in reducing the risk of transmission. UVC irradiation is also being explored as a potential method to sterilise personal protective equipment (PPE) used by healthcare workers. Due to shortages of PPE during the pandemic, finding ways to safely and effectively sterilise and reuse PPE has become a critical issue. While UVC irradiation has shown promise as a tool for combating COVID-19, it is important to note that it is not a substitute for other measures of infection control, such as social distancing and hand hygiene. However, when used in combination with these measures, UVC irradiation can be a powerful tool to reduce the spread of the virus and achieve sterile conditions in a wide range of applications. To determine the effectiveness of UVC irradiation, authors conducted an experiment in which bacterial cells were exposed to UVC irradiation for a fixed period of time in an isolated space and the survival rate of the bacteria was evaluated. The assessment of bacterial survival is a crucial step in many experiments, but it can be a time-consuming and tedious process, prone to measurement error when performed manually by visual assessment of microscopic images by the researcher. Automated methods for assessing bacterial survival rates are much more efficient and can greatly reduce the chances of measurement errors, while also enabling the analysis of a much larger number of samples. To address this challenge, researchers have developed a computer program that can accurately identify dye signals in confocal microscope images and then automatically count the number of live and dead bacterial cells in the sample. This automated process can significantly reduce the time and effort required to assess bacterial survival rates, making it easier for researchers to obtain accurate and reproducible results.

13.2. Materials and methods

In the experiment, a workstation which can be seen in Fig. 1, equipped with a UVC lamp, a nebuliser was used to spread bacteria. After irradiation, fluorescent markers and a confocal microscope were used for taking images. The UVC lamp emitted ultraviolet radiation in the range of 100 to 280 nm, which was found to be highly effective in killing bacterial cells. The nebuliser was used to evenly spread the bacterial cells across the surface of the sample, ensuring that all cells were exposed to UVC

irradiation. Fluorescent markers were used to identify and differentiate between live and dead bacterial cells, and a confocal microscope was used to capture high-resolution images of the bacterial cells.

13.2.1. Equipment and reagents

Custom-made chamber with nebulisation system, UVC lamp composed of six LiteON LTPL-G35UVC275GM UV light emitting diodes and LEDiL VIOLET-12X1 lenses, fluorescent markers kit: LIVE/DEAD® BacLightTM Bacterial Viability Kit, microscope slides, coverslips, Eppendorf and confocal microscope.

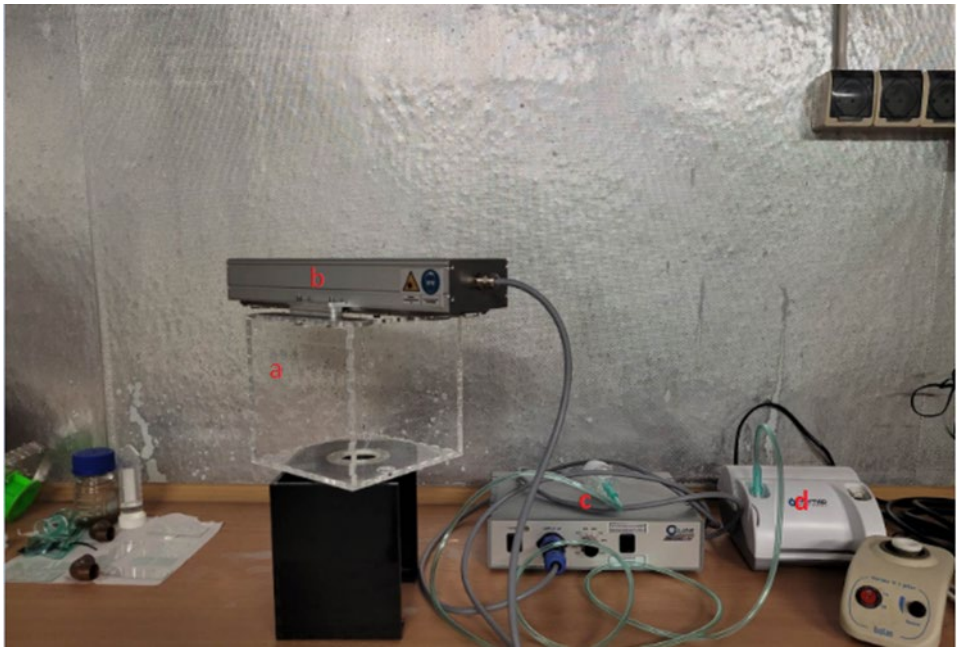

Fig. 1. Bacteria irradiation station equipped with sample chamber (a), UV lamp (b), controller to control lamp power (c), and nebuliser to distribute bacteria in chamber (d)
Rys. 1. Stanowisko do napromieniania bakterii wyposażone w komorę na próbki (a), lampę UV (b), sterownik do sterowania mocą lampy (c) oraz nebulizator do rozprowadzania bakterii w komorze (d)

13.2.2. Fluorescent markers kit

The fluorescent markers used in the experiment were the LIVE/DEAD® BacLightTM Bacterial Viability Kit. They enable dead bacteria to be distinguished from live bacteria for a wide range of strains such as *Bacillus cereus*, *B. subtilis*, *Clostridium perfringens*, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Mycobacterium phlei*, *Pseudomonas aeruginosa*, *P. syringae*, *Salmonella oranienburg*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus* and *Streptococcus*

pyogenes. The LIVE/DEAD BacLight Bacterial Viability Kits utilise mixtures of two solutions: SYTO® 9 green-fluorescent nucleic acid stain and red-fluorescent nucleic acid stain, propidium iodide. These stains differ in their spectral characteristics and ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 stain generally labels all bacteria in a population – those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the fluorescence of the SYTO 9 stain when both dyes are present. Thus, with an appropriate mixture of the SYTO 9 and propidium iodide stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red [4].

13.2.3. Methodology of the experiment

At the beginning the functioning of the UV lamp was verified with a UVC sensor. The reads from the sensor showed that the lamp was operating in the UV. Before starting the experiment, the dye solution was prepared. To do this, a sterile 1.5 ml Eppendorf tube was carefully wrapped in aluminium foil to limit the availability of light, since the dyes decompose when exposed to light. The tube was then added 1 ml of PBS, along with 3 µl of green fluorescent dye and 3 µl of fluorescent red dye. The first step of the experiment was to prepare the bacteria slides. Five millilitres of activated sludge solution was added to the nebuliser from a large bioreactor located at the Silesian University of Technology. The nebuliser tip was placed in a copper tube that led to a container made of transparent plastic with lined basal slides on the bottom. After the nebuliser was switched on, the bacteria were sprayed for 2.5 minutes and then the UV lamp was immediately turned on. During operation, the lamp and chamber were wrapped in aluminium foil to shield the equipment operators from UVC radiation. The irradiations were carried out in 2 cohorts. In the first cohort, bacteria were exposed to UVC for 7 minutes, 3.5 minutes and 1 minute and in the second cohort, bacteria were exposed for 15 minutes, 10 minutes and 7 minutes, respectively. Three plates were exposed at a time in each group and control plates with bacteria that were not exposed were also left in each cohort. After removal from the chamber, 40 µl of the previously prepared fluorescent dye solution described above was added to the slides and coverslips were applied. The slides prepared in this way were incubated in the dark for 60 minutes. After incubation time, the slides were observed on an Olympus FV3000 confocal fluorescent microscope. Example results can be seen in Fig. 2.

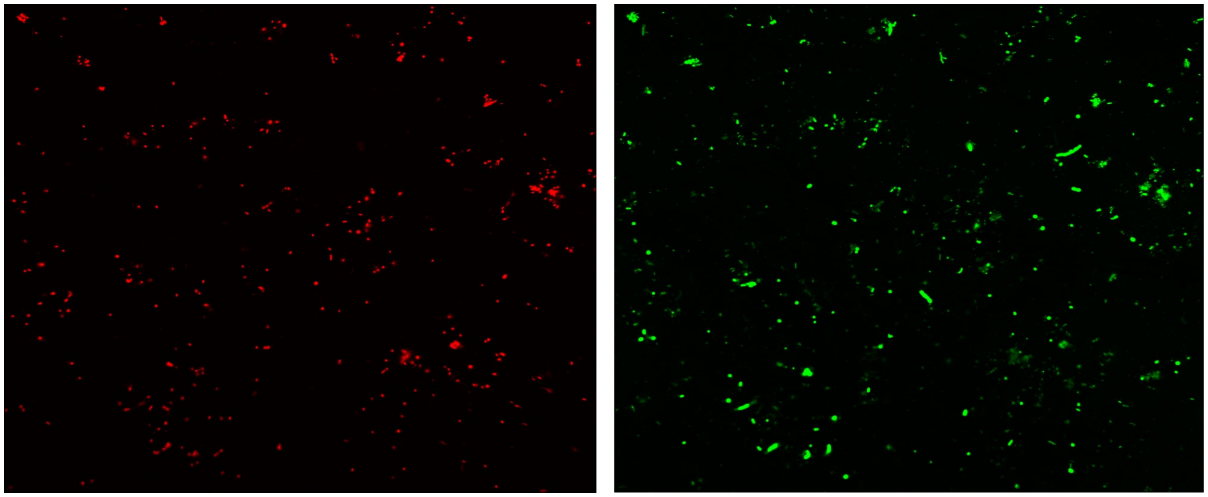

Fig. 2. Sample images obtained through microscopic observations using 60x magnification using oil immersion. Bacteria exposed to UVC irradiation for 10 minutes (green colour stains live cells, red only dead cells)

Rys. 2. Obrazy próbek uzyskane w wyniku obserwacji mikroskopowych przy użyciu powiększenia 60x z zastosowaniem immersji olejowej. Bakterie wystawione na działanie promieniowania UVC przez 10 minut (kolor zielony barwi żywe komórki, czerwony martwe komórki)

13.2.4. Exposure time formula

The formula of exposure time was derived from the lamp parameters [5] and information about the sufficient energy density to eradicate the entire population of microorganisms. To model the exposure time, the work of Kheyradish A. and Mohseni M. [6] was used to properly calculate the illumination angles. The formula is as follows:

$$t = \frac{U}{\eta \cdot P} \cdot \frac{h^2 tan^3 \alpha}{\sqrt{1+tan^2 \alpha}-1} [s] \quad (1)$$

where:

U – value of sufficient energy density to eradicate the whole microorganisms population [J/m^2]

P – power of single diode [W/Sr]

η – single diode efficiency [%]

h – distance between the diode and the exposure surface [m]

α – exposure angle [°]

t – exposure time [s]

With six LiteON LTPL-G35UVC275GM UV light emitting diodes and a LEDiL VIOLET-12X1 lens, the energy density was 40 mJ/cm^2 and, according to the above formula (1) and at the maximum lamp power setting, the exposure time is approximately 15 minutes.

13.3. Data analysis

To analyse the data obtained, in order to avoid measurement errors and tedious counting of cells by hand, it was decided to create a computer programme. The raw microscope images contain three channels. Two of them contain pixels coloured in one colour. The “green channel” only contains live bacteria, and the “red” only dead bacteria. Sometimes there are common pixels/bacteria between the channels. This is due to the imperfectly selected proportions of dyes in the live/dead set. In the further part of the text “Green cells” mean cells in the modified green channel of the image, i.e. alive and dead, and “Red cells” mean cells in the red channel of the image, i.e. dead, this terminology is used because of the fact how the algorithm works and to be easier to understand for reader. The algorithm summarises all pixels from green channel and red channel in “Green cells” channel and then artefacts resulting from bad proportions of dyes can be cleared.

Cell detection software was developed as a plugin for Fiji / ImageJ2, allowing users to process cell viability data for a single image or an entire directory. The graphic user interface allows the user to change the parameters of the algorithm, which allows for more precise data analysis. The plugin can be easily installed in the same way as other imageJ2 plugins. After receiving the input data, depending on the method, the algorithm is performed once or many times, counting the number of cells. It consists of several steps. First, it separates channels (colours) of the input image and gets the green and red channels. Then it modifies channels (merging/deleting pixels), all cells should be in the green channel and only dead cells in the red channel. After this Gaussian filter is used to blur the channels, the channels are binarized using the Otsu method. Finally, blobs/spots (cells) are created in both channels. To analyse the results, the numbers of red and green cells were calculated. The output of the algorithm consists of images with detected cells as can be seen in Fig. 3 and a text file with the number of cells detected.

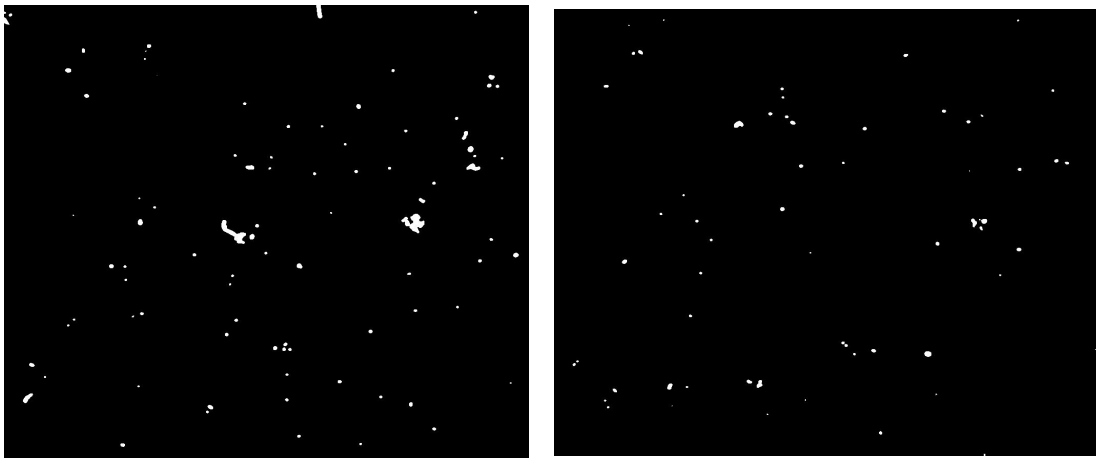

Fig. 3. Example images obtained using the algorithm
Rys. 3. Przykładowe obrazy uzyskane za pomocą algorytmu

The percent of living cells is determined as the difference between the green and red cell numbers, divided by the number of green cells. It can be defined as:

$$\text{LIVE CELLS} = \left(\frac{GREEN\ CELLS - RED\ CELLS}{GREEN\ CELLS}\right) \cdot 100\% \quad (2)$$

where:
GREEN CELLS – all live and dead bacterial cells
RED CELLS – dead bacterial cells
LIVE CELLS – living bacterial cells

The result of these calculations (2) is the viability of bacterial cells, making it possible to produce cross-sectional data showing the effectiveness of the UVC lamp at different exposure times.

13.4. Results and Discussion

The effect of UVC radiation on bacterial cells has been checked using a computer algorithm created, there is a correlation between exposure time and cell viability.

13.4.1. UVC Irradiation

As we can see in Table 1, there is a clear correlation between the viability of bacterial cells and the duration of their UVC irradiation. Low rate of living bacteria with no exposure time is due to the old age of the bacterial culture from which the sample was

taken. From Pearson's correlation coefficient, we know that the value of R is: -0.9548. This is a strong negative correlation. As shown in Fig. 5. below, cells irradiated for 15 minutes show almost 75% less viability than unexposed cells. The study confirmed that the minimum irradiation time to reduce bacterial viability by more than 50% of the bacterial mass is 7 minutes. The results of the studies conducted on the UVC lamp suggest that this method of disinfection is effective in eliminating bacteria, provided it is applied for the right amount of time. Finding the optimal irradiation time is key to achieving the best sterilisation results.

Table 1

Bacterial viability depending on irradiation time

Incubation time [min]	**0**	**1**	**3.5**	**7**	**10**	**15**
Bacterial viability [%]	53.13	46.87	37.04	23.27	21.35	19.35
Standard dev. (σ) [%]	1.64	1.73	1.12	0.4	0.8	0.9

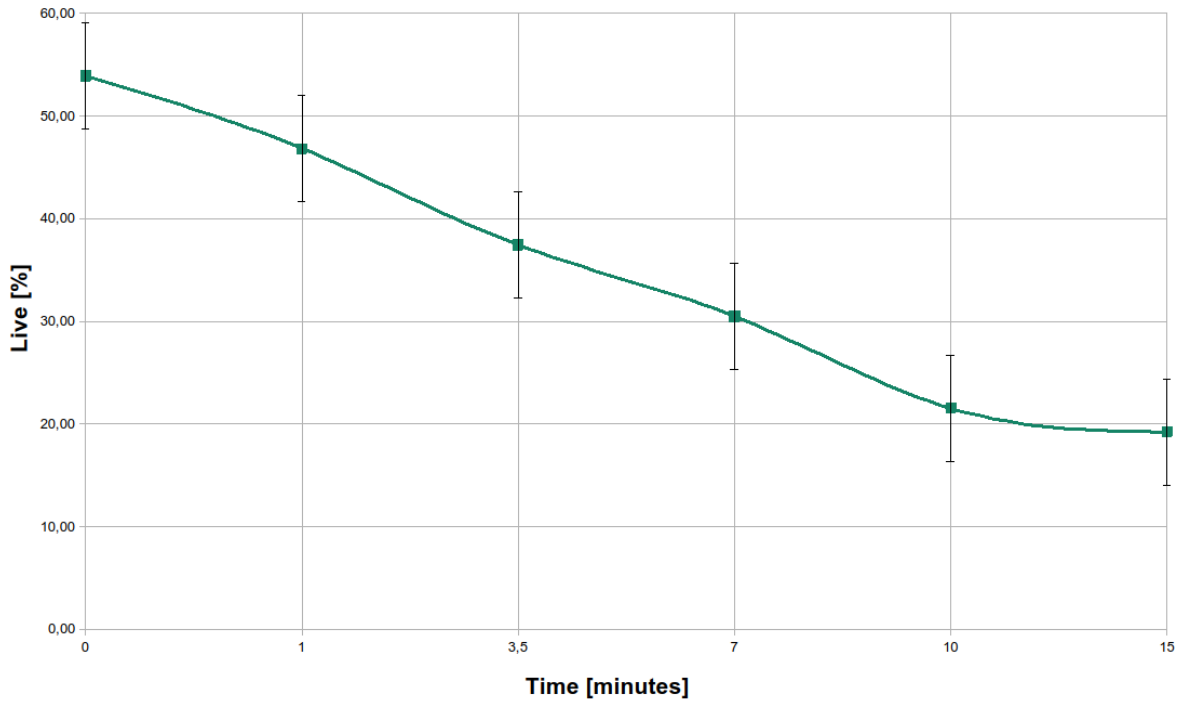

Fig. 5. Bacterial mortality depending on irradiation time
Rys. 5. Śmiertelność bakterii w zależności od czasu napromieniania

The experiment showed that there is a correlation between irradiation time and the viability of the bacteria, however, as can be seen in Table 1, 19% of the bacteria remained alive, so it can be concluded that longer irradiation times would have to be used for such sterilisation to be fully effective. The automated method of assessing bacterial survival provided accurate and reproducible results. These findings are expected to have significant implications for the development of new sterilisation methods and for the use of LEDs in a wide range of applications. More research in this area is needed to explore the potential of UVC irradiation and automated bacterial survival assessment methods in other areas of research and industry.

13.4.2. Algorithm

An algorithm has been successfully created that, on the basis of captured images, distinguishes and quantifies live and dead cells obtained after irradiation. In addition, a relationship has been demonstrated between the time of irradiation and the number of dead cells, which confirms the negative impact of UVC exposure on the bacteria tested. The study has also shown the need to exercise caution and follow safety guidelines when using UVC lamps due to their potentially harmful effects on living organisms. The plug-in was created with the implementation of the algorithm, which will be extended with new possibilities in the future. In addition, we would like to introduce additional functionalities, such as removing elements that are not a point of interest (e.g. inorganic objects that may have been coloured by a fluorescent pigment). We plan to perform additional stages of data processing using standard image processing methods or using neural networks. Once we consider a plugin stable, we will make it publicly available under the “GNU GPL” version 3 licence.

Acknowledgements

The research reported in this paper was co-financed by the European Union from the European Social Fund in the framework of the project “Silesian University of Technology as a Center of Modern Education based on research and innovation” POWR.03.05.00-00-Z098/17.

Bibliography

1. K. Song, M. Mohseni, F. Taghipour, Application of ultraviolet light-emitting diodes (UV-LEDs) for water disinfection: A review. Water Research, 94, 341–349. DOI:10.1016/j.watres.2016.03.003.
2. D.-K. Kim, D.-H. Kang, UVC LED Irradiation Effectively Inactivates Aerosolized Viruses, Bacteria, and Fungi in a Chamber-Type Air Disinfection System. Applied and Environmental Microbiology, 84(17). DOI:10.1128/aem.00944-18.
3. L. Eisenlöffel, T. Reutter, M. Horn, S. Schlegel, U. Truyen, S. Speck, Impact of UVC-sustained recirculating air filtration on airborne bacteria and dust in a pig facility. PloS one, 14(11), e0225047.
4. LIVE/DEAD® BacLight Bacterial Viability Kits Product Information 15-July-2004.
5. https://www.ledil.com/product-card/?product=FN17810_VIOLET-12X1-RS.
6. A. Kheyrandish, F. Taghipour, M. Mohseni, UV-LED radiation modeling and its applications in UV dose determination for water treatment, *Journal of Photochemistry and Photobiology A:Chemistry* 352 (2018) 113–121.

AUTOMATIC SYSTEM FOR BACTERIAL CELL VIABILITY ANALYSIS CONFOCAL MICROSCOPY IMAGES

Abstract

Electroluminescent diodes have gained popularity in the research field due to their wide potential, including their small size, which facilitates their application in sterilisation systems. Ultraviolet radiation with a wavelength from 100 to 280 nm (UVC) is characterised by mutagenic effects against microorganisms. In the above project, the effect of UVC radiation on bacteria contained in the nebulized activated sludge solution from the bioreactor. The methodology focuses on a self-constructed chamber system that allows for the isolation of the tested space. A nebulised solution consisting of activated sludge is aerosolised in the chamber, and exposed to UVC electroluminescent diodes for selected times A software was developed to visualise the power density dependence of UVC radiation. The effect of radiation was verified by evaluating the viability of bacteria using the viability kit. An algorithm has been successfully created that, based on captured images, distinguishes and quantifies live and dead cells obtained after irradiation. A relationship between the time of irradiation and the number of dead

cells has been demonstrated, which confirms the negative impact of UVC exposure on the tested bacteria. The results show that UVC radiation negatively affects bacterial cells, and the automatic system for detecting dead cells as well as live cells works properly. The automatic system we have developed fulfils its purpose and can serve as a commercial tool.

Keywords: UVC, ultraviolet radiation, viability, live and dead cells, algorithm, automatic system, irradiation