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Original Research Article

OZONATION IN WASTEWATER DISINFECTION

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A b s t r a c t

Due to the potential microbiological hazard associated with discharging treated sewage into the receiving body, its disinfection is a key issue to protect ecological safety and human health. Water scarcity and drinking water supply, irrigation, rapid industrialization, use of treated water, protection of water sources, overpopulation and environmental protection force us to look for solutions to ensure safe reuse of wastewater, and this depends primarily on the quality of wastewater disinfection. Many wastewater disinfection methods are commonly used. One of the chemical processes of disinfection sludge is ozonation. Ozonation is widely used in wastewater treatment by oxidation, because ozone is a very strong and effective oxidizing agent. Studies have shown that the effectiveness of ozone in disinfecting water and sewage is up to 50% greater than that of chlorine . An additional advantage of this method is that it also eliminates odors that may be unavailable.

The article presents the results of research on the effectiveness of ozonation treatment in the disinfection of treated sewage, based on indicator bacteria such as coliforms, including *Escherichia coli*, mesophiles, psychrophiles, and spores. The study took into account various effects of time (dose) and temperature. For the purpose of this study, both traditional and modern methods of assessing microbiological quality of wastewater were used. The first one is represented by conventional culture measurements and the second one by using a luminometer (ATP) and flow cytometer (FCM).

Keywords: municipal wastewater, disinfection, ozonation, ATP, FCM

1. INTRODUCTION

Water scarcity currently affects over 40% of the world's population and it is anticipated that this percentage will increase in the coming years. The primary emitter responsible for introducing various types of pollution into the environment is humans. Over 80% of sewage generated by human activities is discharged solely into water reservoirs [4].

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Wastewater refers to water, which quality has been diminished due to human activities. It includes liquid waste discharged from households, commercial properties, industries, and agriculture, containing viruses, bacteria, and parasite eggs. Due to various external factors such as the source of origin (industrial, municipal, domestic), population density, or time of formation, the composition of wastewater can be highly diverse. Sewage is primarily contaminated with carbon, hydrogen, oxygen, nitrogen, and phosphorus compounds, as well as numerous organic and inorganic microelements.

Wastewater treatment, on the other hand, is a process aimed at converting sewage into water suitable for re-introduction into the natural water cycle, considering the pollution level permitted by international and national laws to ensure minimal impact on the environment. In Poland and many other countries, in compliance with relevant regulations, sewage undergoes analysis and control based on fundamental parameters after the treatment process, i.e.: BOD_5, COD, suspended solids, total nitrogen, and total phosphorus. Treated sewage discharged to the receiver is tested only for the previously mentioned indicators. However, it should be remembered that other wastewater contaminants, such as microorganisms, toxic chemicals, radioactive substances, as well as drug residues, and xenoestrogens, may also pose a potential threat. The current sewage treatment methods in use may be inadequate and might not eliminate all hazardous pollutants. Despite a 99% effectiveness rate [5], there is no guarantee that all harmful pathogens will be effectively removed. Moreover, besides posing a threat to the quality of receiving waters, pollutants released into the environment may lead to various health risks, including allergies, toxicity and the potential transmission of infectious diseases.

The largest proportion of microorganisms in wastewater are viruses, of which there are about 140 types, and the number of their particles exceeds the number of bacterial cells five times. The most important viruses are adenovirus, calicivirus, hepatitis E virus, picornavirus (including enterovirus; poliovirus, hepatitis A virus), and rotavirus. Pathogenic bacteria of aquatic origin include*:* *Campylobacter jejuni*, *Campylobacter coli*, pathogenic strains of *Escherichia coli*, *Salmonella spp*. (including *S. typhi* and *S. paratyphi species*), *Shigella spp*., *Vibrio cholerae*, *Yersinia enterocolitica*, *Legionella spp*., *Pseudomonas aeruginosa*, *Aeromonas spp*. Parasite eggs (*Ascaris*, *Trichuris*, and *Toxocara*) can also be a problem [6,7].

In Poland, there is no mandatory testing of microbiological indicators in wastewater after the treatment process. Additionally, there is no obligation to disinfect wastewater, with certain exceptions for wastewater discharged from infectious hospitals. While wastewater treatment processes facilitate the removal of a considerable number of microorganisms, including pathogens, there is limited or no available data regarding the extent of bacterial contamination in treated wastewater discharged into receiving bodies. Moreover, there is only minimal data on the contamination of raw wastewater [8] .

The article presents the results of research on the effectiveness of ozonation treatment in disinfection of treated sewage, based on indicator bacteria such as coliforms, *Escherichia coli*, mesophiles, psychrophiles and spores. The study took into account various effects of time (dose) and temperature. For the purpose of this study both traditional and modern methods of assessing microbiological quality of wastewater were used. The first one represented by conventional culture measurements and the second one by using luminometer (ATP) and flow cytometer (FCM).

2. METHODOLOGY OF THE RESEARCH

2.1. Material and its initial preparation

The research used purified sewage from municipal sewage treatment plants in Rzeszów and Tarnobrzeg. Both wastewater treatment plants use the same treatment technology – activated sludge, but have different treatment efficiencies. Since the sewage was collected in February, the temperature was around 12°C. The tests were performed immediately after the sewage was sent to the laboratory.

2.2. The course of the experiment

The disinfection process of the tested wastewater was carried out in a glass, sterile bottle with a capacity of 1 liter. Ozonation of sewage was performed by dissolving gaseous ozone produced by the ZY-H170 ozonator, with a capacity of 7 g/h (117 mg/min), and then by introducing sewage into it using a system of stones (diffusers).

The total amount of ozone introduced into the sewage was expressed in mg O_3 /min. The following ozonation times were used, which is related to the appropriate concentrations of ozone introduced:

- 1 min - 117 mg O_3;
- 3 min - 351 mg O_3;
- 5 min - 585 mg O_3;
- 7 min - 819 mg O_3;
- 9 min - 1053 mg O_3.

After the disinfection process was carried out, the stability of the obtained disinfection effect was tested. Sewage after 9 minutes of ozonation was selected for microbiological stability tests. Samples of wastewater (150 ml) were poured into sterile 250ml bottles and placed in incubators under the following conditions:

1. in a refrigerator at 4°C
2. in an incubator at 15°C
3. in an incubator at 28°C

Analyses were performed on the day of wastewater disinfection and after 24, 48, 72 hours, and 7 days.

2.3. Methodology of microbiological determinations

The effectiveness of the sewage disinfection processes was evaluated based on selected microbiological quality indicators (Table 1.).

Table 1. Indicators of the studied sewage

1.	Number of psychrophilic bacteria on the R2R agar media	Culture method by standard PN-EN ISO 6222:2004
2.	Number of mesophilic bacteria on the R2R agar media	Culture method by standard PN-EN ISO 6222:2004
3.	Number of coli and *Escherichia coli* bacteria	Culture method on chromogenic medium
4.	FCM	Sysmex – Partec protocol for CytFlow Cube 6
5.	Luminometry determination of ATP concentration *	PROMEGA protocol for 20/20 luminometer
6.	Color – spectrophotometric method	PN-EN ISO 7887:2012 - Water quality - Color testing and determination

* at work given as the RLU value - general and extracellular

The determination of the total number of microorganisms present in the tested wastewaters was conducted using flow cytometry. Cells were counted using a Partec Cube 6 flow cytometer (Sysmex-

Partec) equipped with a 488 nm blue laser, forward scattering detector (FSC), side scattering detector (SSC), and three fluorescence detectors (FL1- 536 ± 20 nm, FL2-590 ± 25 nm, FL3N 615 nm). The TM BacCount Viable reagent kit was used for the determinations, which is intended for non-specific counting of all bacteria (total number of cells) and counting live bacteria in water samples. A fluorescent dye, which is perfect for excitation by the blue line of the argon laser at 488 nm: SYBRGreen I nucleic acid stain (10,000 x diluted in DMSO) [9] was used for the determinations. CyStainTM Green (SYBRGreen) is a membrane-permeable dye that non-specifically stains all bacteria in water samples that emit green fluorescence. Both living and dead cells will be stained. Additionally, in accordance with the methodology, the CySteinTM Red dye was used, which, unlike the green fluorochrome, is not permeable through the cell membrane and stains nucleic acids. It is used as an indicator of dead cells. Due to the large number of loads in the tested sewage, the samples were diluted to a concentration of 10^{-1} ml. Each time, the sample volume was 30 μl, the flow time was 60 sec, the flow rate was 0.5 μl/sec, and the load, thanks to the dilutions used, ranged from 1 to 3 kPart/sec.

The content of organisms in the tested sewage, including the number of microorganisms (both living and dead), was determined using flow cytometry:

LNA – low nucleic acid, means the number of microorganisms with low nucleic acid content, e.g. viruses or fragments of the genetic material of microorganisms.

HNA – high nucleic acid, means the number of microorganisms with a high content of nucleic acid, mainly bacteria [10] .

3. RESULTS

3.1. The effectiveness of the disinfection process

3.1.1. Changes in the number of bacteria

Studies have indicated that as the duration of ozonation increased, there was a decrease in the abundance of mesophilic bacteria, psychrophilic bacteria, and coliforms in wastewater. However, even with an extensive ozonation period, not all microorganisms were completely removed from the disinfected wastewater. The count of *E. coli* bacteria significantly decreased after just 1 minute of ozone disinfection. Within the initial minutes of the test, it showed an effectiveness of nearly 78%. This effectiveness continued to increase with prolonged ozonation time. (Table 2).

Table.2. The influence of ozonation time on the disinfection effect

	1 min	**3 min**	**5min**	**7min**	**9 min**
E. coli bacteria	78%	94%	96%	98%	99%
Coliform bacteria	40%	89%	97%	99%	99%
Mesophilic bacteria	23%	73%	85%	97%	98%
Psychrophilic bacteria	34%	94%	98%	98%	98%

The amount of bacteria in the treated wastewater was on average:

- *E. coli* bacteria: 3,5 x 10^4 cfu/100 ml;
- *Coliform* bacteria: 3,8 x 10^4 cfu/100 ml;
- Mesophilic bacteria: 8 x 10^4 cfu/ ml;
- Psychrophilic bacteria: 110 x 10^4 cfu/ ml.

No *coli* and *E.coli* were found in the samples (9 min./L) after ozon disinfection. The obtained results indicate that ozonation of wastewater for 9 minutes is not sufficient to completely remove microorganisms.

3.1.2. Changes in ATP values during the ozon disinfection process

ATP serves as a reliable marker for living cells due to its role as a byproduct of cellular metabolism. When cells die, ATP synthesis ceases, and the existing ATP molecules are broken down by intracellular enzymes such as ATPase and phosphatases. The efficacy of ozone in disinfecting treated wastewater was confirmed. The control sample exhibited a high content of adenosine triphosphate (total ATP). Ozonation resulted in a decrease in the total ATP value. During the disinfection processes, bacteria in the wastewater were damaged, causing the escape of adenosine triphosphate due to the disruption of the cytoplasmic membrane [11,12], thus resulting in an increase in extracellular ATP levels (Fig.1.).

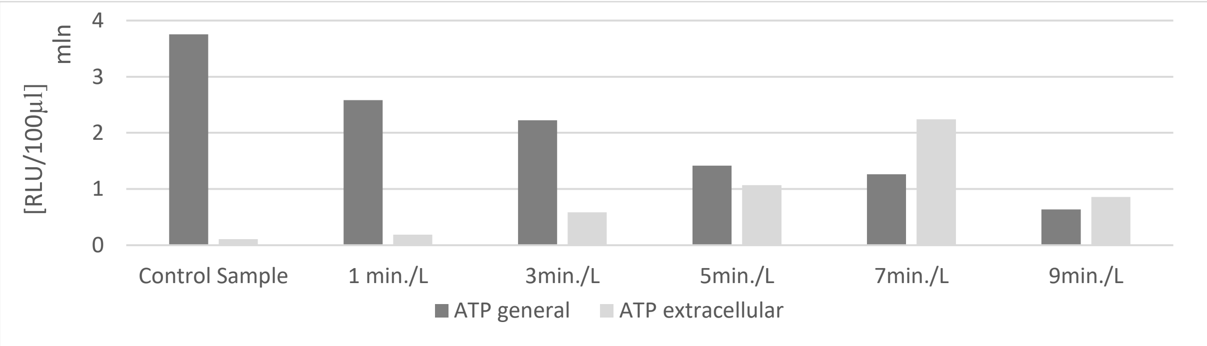
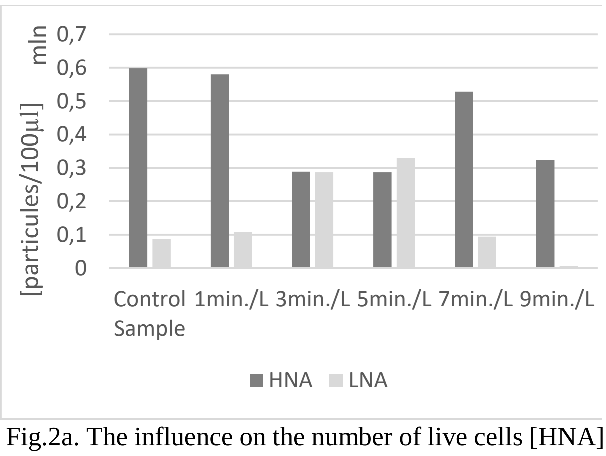


Fig.1. RLU values for individual ozon disinfection rates in subsequent time/dose of tests ATP

3.1.3. Changes in the number of particles during the ozon disinfection process (FCM analysis)



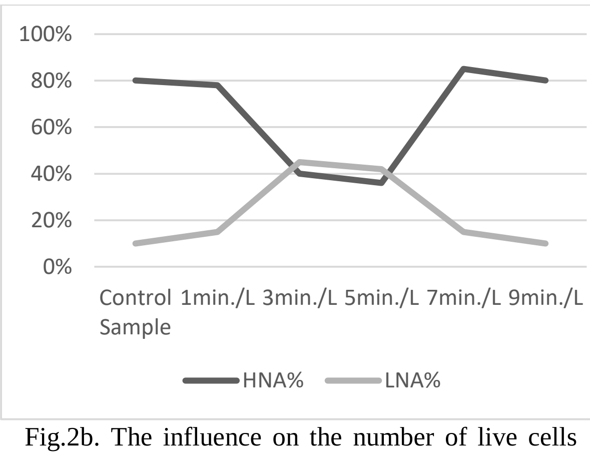


Fig.2a. The influence on the number of live cells [HNA] and dead cells [LNA] during ozon disinfection

Fig.2b. The influence on the number of live cells [HNA] and dead cells [LNA] - relationship in percentages

The results of flow cytometer determinations confirmed the effectiveness of disinfection of treated wastewater using ozonation. A high number of live particles (HNA) compared to low nucleic acid

(LNA) particles is characteristic of the control sample (Fig. 2a, Fig. 2b). This tendency persists until the sample is ozonated for 5min/L, then it breaks down and the number of HNAs increases again. In the initial phase of ozonation, when the ozone dose is low, most of the bacteria with high DNA content (HNA) e.g. gram-positive bacteria, are destroyed, while bacteria with low DNA content (LNA) e.g. gram-negative bacteria remain intact. As a result, the ratio of HNA to LNA decreases. This may also be due to the fact that some microorganisms, including bacteria, have defense mechanisms against DNA damage, such as catalase and peroxidase enzymes, which allow them to survive in harsh conditions. However, with increasing ozone dose and ozonation time, further destruction of LNAs occurs, resulting in a decrease in their number. In samples ozonated for 9 minutes, a decrease in both HNA and LNA values can be observed.

3.1.4. Color

The influence of the time of contact with ozone on the color of wastewater was observed. The longer the contact time, the greater the reduction in their color (Fig. 3).

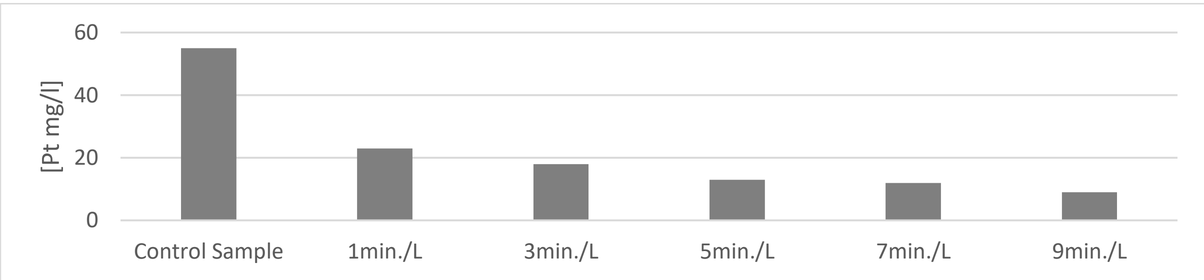


Fig. 3. The influence of ozonation length/doses on color of wastewater

3.2. Results of studies on the stability of wastewater after the ozon disinfection process

The wastewater following the ozonation process lacked microbiological stability. Observations revealed a reconstitution of the microflora and a considerable impact of temperature on the microorganisms within the wastewater (Fig.4. - Fig.8.). Raising the temperature in samples after ozone disinfection led to an increase in the bacterial count. The highest amounts were observed in samples placed in a greenhouse at 28 ℃. In contrast, the opposite trend was observed in control (non-ozone-treated) samples. In this case, the increase in temperature resulted in the inhibition of microorganisms in the wastewater. The highest number of bacteria was observed in the control sample placed at 4 ˚C. This trend was noted for both coliforms and meso and psychrophilic bacteria.

Re-growth of microorganisms occurs after 24 h (for samples placed in a greenhouse at 28 ℃), but the greatest increase in abundance occurred between 24 h and 48 h after the disinfection process.

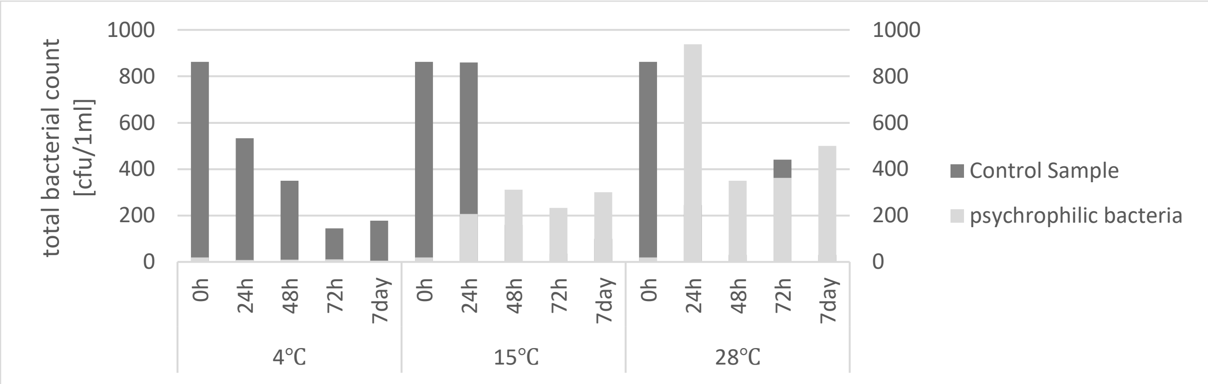


Fig. 4. Changes in the number of psychrophilic bacteria during stability testing

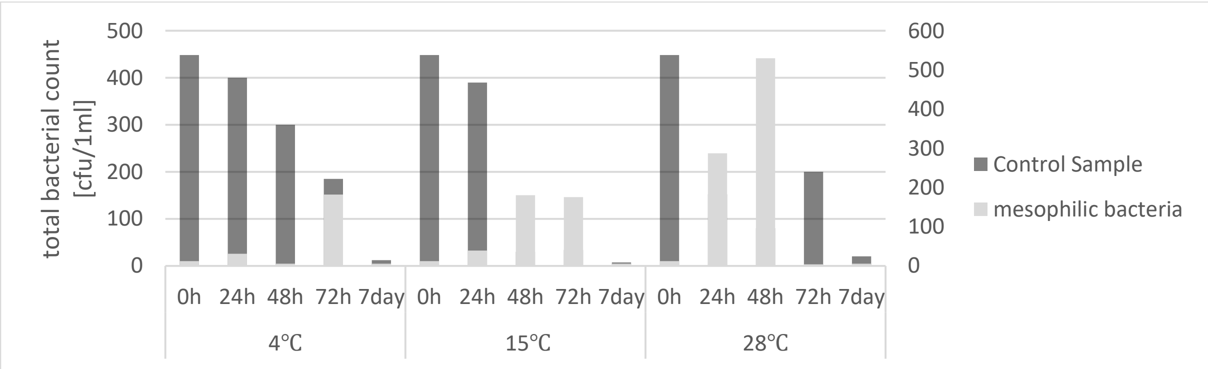


Fig. 5. Changes in the number of mesophilic bacteria during stability testing

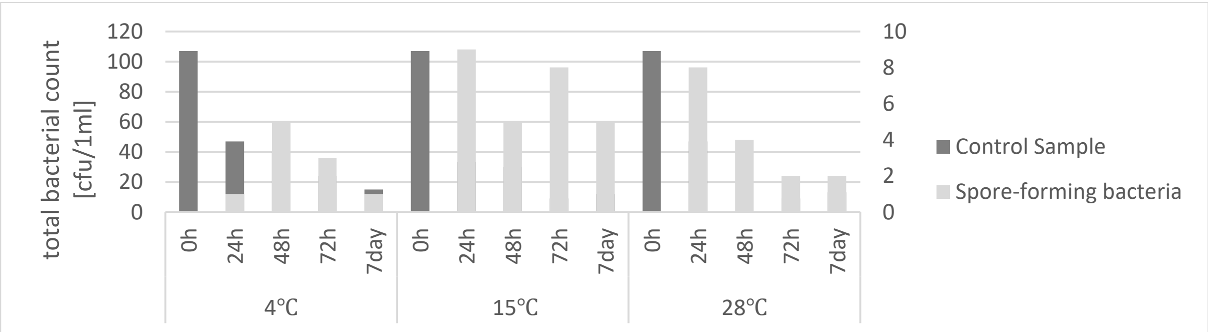


Fig. 6. Changes in the number of spore-forming bacteria during stability testing

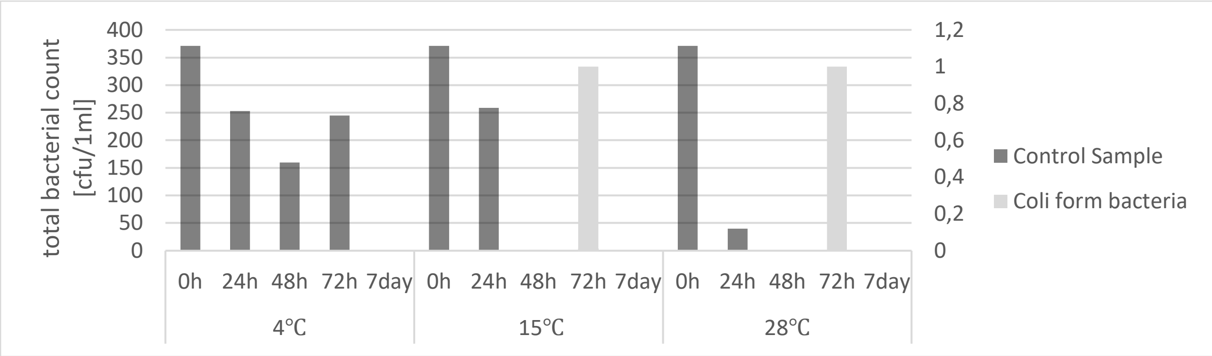


Fig. 7. Changes in the number of *coliform* bacteria during stability testing

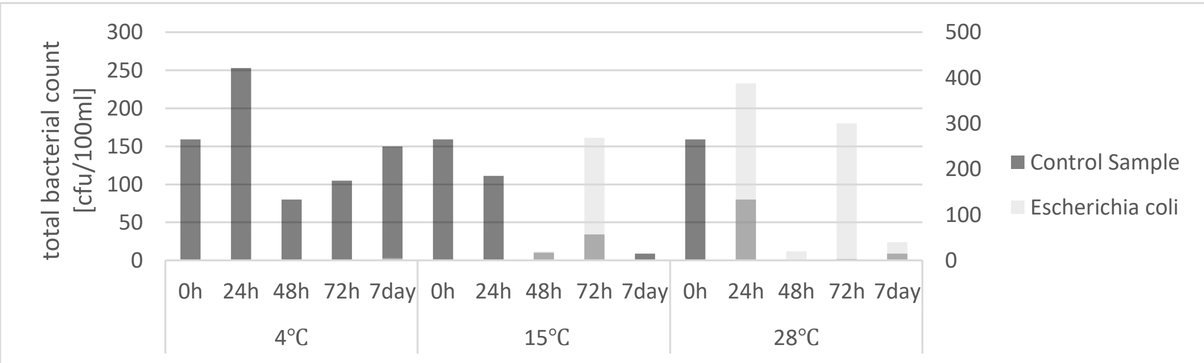


Fig. 8. Changes in the number of *Escherichia coli* bacteria during stability testing

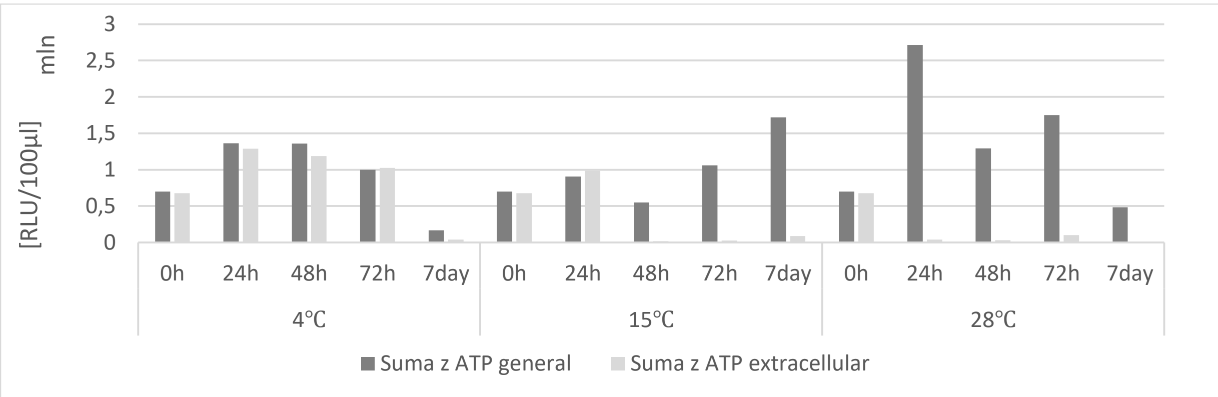


Fig. 9. Results of microbiological stability tests in sewage after ozonation, test at temperatures of 4, 15 and 28 ˚C - luminometry

Concentration ATP measurement confirmed the restoration of the microbial biocenosis in the incubated wastewater samples. The fastest restoration of cells occurred at 28°C. (Fig.9).

4. DISCUSSION

Among the most widely used chemical methods for wastewater treatment is chlorination, valued for its effectiveness and low cost. However, due to the formation of disinfection by-products (DBPs), this method can have adverse effects on public health and aquatic life, resulting in incidents such as fish deaths in water bodies where chlorine-disinfected wastewater was discharged. For this reason, the U.S. Environmental Protection Agency (US EPA) has encouraged research into alternative disinfection methods, such as ozonation, which has shown to be highly effective in treating wastewater [13].

The results presented above indicate that the contact time of sewage with ozone - 9 minutes - was not sufficient to completely eliminate microorganisms. It is possible that, in experimental condition, the concentration of the gas did not reach a sufficient level for the total elimination of *E. coli*, in accordance with a study conducted by the group of Heß [14], which reported that resistance to ozone inactivation probably depends on several factors [15]. Firstly, ozone in water undergoes spontaneous decay, the rate of which depends on pH and temperature. According to literature data, the stability of ozone in water decreases with increasing pH [16]. Secondly, the lack of complete removal of microorganisms from sewage may also result from the fact that the microorganisms occurred in clusters or were protected by other particles present in the sewage. The cell wall serves as a natural protective barrier for bacteria against the entry of foreign substances. When ozone is applied, it penetrates only after it has been previously damaged. As a result of ozone penetration, a reaction occurs between the cell wall and the substance in question, as a result of which proteins are released, fatty acid peroxides are formed and the permeability of the cell wall changes. Until the penetration of the outer wall is completed, the availability of ozone to the protoplasm of the cell is hindered, as well as the direct reaction of the oxidant with intracellular components. As a result, it can be deduced that the death of a bacterial cell is dependent on the result of damage to its outer layer [6].

Moreover, the literature shows that a positive effect of ozonation is the inactivation of only some optional pathogenic bacteria, including only some strains of *Escherichia coli* (in sewage from sewage treatment plants, reduction by several logarithmic units) [17,18,19]. The effectiveness of ozone as a disinfectant depends on several parameters, such as the reaction rate of ozone or its decay products with sewage sludge compounds, including bacteria [18]. Some species appear to be slightly more resistant to ozone than others and may therefore have been "positively selected" by ozonation, resulting in an increase in the proportion of dry varieties. Additionally, pigment and biofilm production may be factors influencing ozone sensitivity. Additionally, as Dodd [20] notes, antibiotic-resistant bacteria have an advantage in dealing with oxidative stressors such as ozone compared to bacteria that are not resistant to antibiotics.

Ozone has highly destructive properties against all microorganisms that may potentially occur in wastewater, including: virus particles or protozoan cysts/oocysts. The process of inactivation of microorganisms takes place at low (about 13 mg/dm^3) ozone concentrations. Even at residual concentrations (1 mg/dm^3 and lower), ozone inactivates microorganisms resistant to chlorination, such as poliovirus type 3 or protozoan cysts *Cryptosporidium* and *Giardia* [21, 22]. The inactivation of microorganisms associated with ozonation depends on several environmental factors: relative humidity, pH (the higher the pH, the more likely that ozonation will take place with an advanced oxidation process (AOP) due to the higher concentration of HO radicals), contact time, type of organism, dosage, turbidity, presence of organic substances, ozone demand or temperature (affects the solubility, stability and reactivity of ozone [23, 24].

During ozonation, bacteria are broken down due to cell wall degradation caused by protoplasm oxidation (cell lysis). The double bonds in lipids allow ozone to act as a disinfectant, causing increased permeability of cell walls and membranes through ozonolysis [25]. When the molecule breaks down at the site of the double bond between carbon atoms, it breaks into shorter chains, killing bacteria faster than chlorine would. Amazingly, there are approximately 106 double bonds in the lipids that form the cell membrane [26, 27]. Using O_3 for disinfection can disrupt the functioning of the bacterial cell membrane, enzymes, and cell-to-cell relationships within the bacterial cell. It has been shown that the effectiveness of bacterial inactivation is influenced by the composition (size, shape or composition of the cell membrane) and the physiological state of the bacterial community [28]. Despite the disinfectant's widespread effects on bacteria and other well-known organisms, the exact mechanism of action has yet to be determined. Ozone has a significant effect on cellular metabolism by oxidizing the coenzymes NADH and NADPH, which play an important role in a variety of synthesis and degradation reactions, such as respiration and fatty acid synthesis. The impact of this depends on the bacterial strain, as Gram-negative bacteria have weaker sensitivity to ozone than Gram-positive bacteria. As for viral disinfection, oxidative attack on ozone-coated protective proteins is considered; interactions with deoxyribonucleic acid or ribonucleic acid may also be an important factor to consider [29, 30, 31].

Ozone doses for biologically treated wastewater are estimated in the range of 15 to 30 mg O_3 /dm^3, and the contact time of wastewater with ozone is about 15÷20 minutes [3] Studies show that the ozone doses necessary to provide the recommended values for enterococci and *E. coli* bacteria, needed to inactivate 97-99% of pathogens, are more than 5.5 mg O_3 min/dm^3 and not less than 4.7 mg O_3 min/dm^3, respectively [32] .

The use of ozonation reduces the total number and biomass of bacterial cells. After applying a dose above 7 mgO_3/dm^3, the reduction was over 70%. It was found that ozone at a dose above 7 mgO_3/dm^3 causes particular destruction of smaller cells. Ozonation also effectively destroys viroplankton. Studies report a 99.9% reduction in viral particles [32].

Disinfection of treated wastewater using gaseous ozone, like chlorination, involves the difficulty of dissolving it in wastewater [33] . Disinfection with ozone contributes to the reduction of parameters such as pH, COD [34] , BOD_5, total suspended solids, and total phosphorus. In addition, it significantly affects color change. According to the literature, the exception is the concentration of total nitrogen - in the case of ozonation, the concentration of total nitrogen in wastewater after disinfection increases slightly.

In Poland, disinfection of municipal wastewater with ozone is not popular because it is too expensive. However with the technological progress it may change soon, and the use of ozone may become more cost-effective. Ozone is actually more than twice as effective as chlorine when it comes to eliminating microorganisms. In addition, ozone has the power to destroy potentially carcinogenic compounds, such as polycyclic aromatic hydrocarbons (PAHs), as well as eliminate offensive odors and refractive elements [35].

Unlike chlorine, ozone affects the *Cryptosporidium* family. Moreover, it decomposes quickly and completely. There are also no harmful residues. Ozonation is very effective to inactivate and decay microbial cells (Hammes et al. 2008, 2010; Ramseier et al. 2011b), but ozonation increases microbial regrowth potential by generating biodegradable organic matter (BOM), while it decreases microbial abundance. As changes in BOM and microbial community are complicated during the treatment process, little information is available on how microbial regrowth potential is shaped in the water [36].

What is more, ozone is produced on-site, so there are fewer safety issues related to transportation and handling. In addition, ozonation of wastewater has many advantages such as: an increase in dissolved oxygen, reduction in chemical consumption, and reduction in turbidity and color. Ozonation is a natural, ecological disinfectant to which no microorganisms show resistance (ozone has a short half-life and quickly reverts to oxygen atoms) [12].

5. CONCLUSIONS

1. Rapid microbiological techniques make it possible to access the microbiological quality of treated wastewater. The use of this assessment would allow to regulate the dose of ozon depending on the quality of disinfected wastewater.
2. The effectiveness of ozone in disinfection processes of treated wastewater was confirmed. As the contact time with ozone increased, lower abundance of mesophilic bacteria, psychrophilic bacteria and coliforms and *E. coli* were found.
3. The use of a 9-minute ozone contact time "with a 7 g/h generator" did not result in the complete elimination of microorganisms of treated wastewater.
4. The effect of ozonation time on the color of wastewater was observed. The longer the time, the lower the color determined in Pt mg/l.
5. The microbiological instability of wastewater after the biological treatment process was observed. Microflora recovery occurs both at 4 ˚C, 15 ˚C and 28 ˚C.

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