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Application of traditional and modern microbiological methods to assess the effectiveness of the selected bio-preparation to reduce sanitary indicators in treated wastewater

Izabela Kiełb-Sotkiewicz[1*], Justyna Zamorska[1]

[1] Department of Water Treatment and Protection, Rzeszow University of Technology, al. Powstańców Warszawy, 35-959 Rzeszow, Poland
* Corresponding author's e-mail: kielb.izabela@gmail.com

ABSTRACT

Microbiological biopreparations have been successfully used for years in wastewater treatment plants for various purposes, including the removal of biogenic compounds such as nitrogen and phosphorus, acceleration of the biodegradation of organic substances, improvement of anaerobic processes, elimination of unpleasant odors, enhancement of activated sludge quality, and reduction of sludge volumes requiring disposal. Some biopreparation manufacturers also claim that these products can be effectively used for the revitalization of treated wastewater and for the "effective removal of pathogenic bacteria, pathogens, and parasites from wastewater and sewage sludge." This study evaluated the effectiveness of the biopreparation ACS ODO_1 in eliminating *coliform* bacteria from treated wastewater, analyzing the impact of different preparation dosages and oxygen conditions. Both traditional culturing methods (fermentation-tube, membrane filtration, and pour plate techniques) and modern techniques (ATP luminometry, flow cytometry [FCM], and molecular PCR analysis) were used for assessment. The results of classical culturing methods showed that the greatest reduction of *coliform* bacteria was achieved in samples with the addition of the preparation combined with aeration, and the effectiveness increased with higher biopreparation doses. However, not all conditions led to a reduction in indicator microorganisms; in some cases, an increase in the number of *coliform* bacteria was observed, possibly indicating selective proliferation of undesirable microflora or inappropriate environmental conditions. Compared to traditional culturing methods, modern detection techniques provided different insights. ATP values and the number of viable cells detected by FCM were high in all samples, regardless of culturing method results. This may indicate the presence of microorganisms undetectable by traditional techniques (e.g., bacteria in the VBNC state) or the presence of active microflora introduced with the biopreparation. The interpretive differences stem from the fact that classical methods identify only bacteria capable of growing on selective media, whereas ATP and FCM reflect the total biological activity of the sample. The results suggest that a comprehensive evaluation of hygienization effectiveness and actual microbiological risk requires a combined application of both classical and modern methods. Only their complementarity allows for a reliable analysis of microbiological threats and an accurate assessment of the real activity of microorganisms present in wastewater.

Keywords: microbial biopreparation, wastewater, microbiological monitoring, ATP luminometry, flow cytometry (FCM), molecular diagnostics.

INTRODUCTION

Sanitary safety of water and wastewater is one of the key challenges in modern environmental engineering, sanitary microbiology, and public health. The rapidly growing human population, intensification of industrial and agricultural activities, and climate change affecting the water resources are all contributing to an increased risk of microbiological contamination of aquatic environments. In addition to microbiological threats, treated wastewater may still contain micropollutants of anthropogenic origin – such as pharmaceuticals, personal care products, and industrial chemicals – which pose additional environmental and health risks [Rogowska et al., 2020] Effluents from wastewater treatment

plants, stormwater, and surface waters can contain significant quantities of pathogenic bacteria, including fecal microorganisms, which pose a serious threat to human and animal health, as well as the balance of aquatic ecosystems [Zamorska and Piech, 2014; Bergier and Włodyka-Bergier, 2017]. These risks are not limited to waterborne exposure – microbiological hazards can also be present in the air around wastewater treatment facilities, where bioaerosols may pose an additional threat to workers and nearby populations [Michałkiewicz, 2019].

Modern wastewater treatment plants are increasingly using microbiological biopreparations as tools to support the efficiency of treatment processes, including the reduction of pathogenic microorganisms. The microorganisms contained in these products – including aerobic bacteria, lactic acid bacteria, yeasts, and photoautotrophic bacteria – exhibit antagonistic effects against indicator bacteria such as *Escherichia coli*. Their mechanisms include competition for nutrients, acidification of the environment, production of antimicrobial substances, and creation of conditions unfavorable to pathogen growth [Elshafai and Elmoteleb, 2017, Cydzik-Kwiatkowska and Zielińska, 2016] These interactions are well-documented in microbiological literature and reflect fundamental ecological principles governing microbial communities [Madigan et al., 2018].

The hygienic effectiveness of biopreparations has been confirmed in locations such as Starachowice, Gdańsk, and numerous municipal treatment plants, where significant reductions in *coliform* bacteria counts have been observed [Mazur et al., 2020; Mazur et al., 2022; ACS Poland]. A particularly important factor influencing the activity of biopreparations is the presence of oxygen – many bacterial strains contained in these products are most effective under aerobic conditions, which promote their proliferation and enhance their ability to outcompete fecal microorganisms. According to literature and manufacturers, a reduction in *E. coli* levels can occur within several to several dozen hours after application, provided that appropriate aeration and temperature conditions are maintained [ACS Poland; Bitton, 2011].

However, the actual effectiveness of biopreparations in eliminating indicator bacteria requires a reliable microbiological assessment, taking into account not only quantitative changes but also the biological activity of the microbial community. Traditional culturing techniques – such as the fermentation-tube method, pour plate method, and membrane filtration – are basic diagnostic tools used in wastewater quality monitoring. Nonetheless, these methods are prone to errors, such as failure to detect bacteria in a VBNC (viable but non-culturable) state [Oliver, 2005; Fricker et al., 2013], or misidentification of lactose-fermenting bacteria, such as *Klebsiella* or *Citrobacter* [Pławińska-Czarnak et al., 2021].

In this context, modern and rapid microbial detection techniques are gaining increasing importance, as they allow for assessment of microbial viability, metabolic activity, and the presence of genetic material. Their effectiveness over traditional culture-based methods – particularly in environmental samples – has been well documented [Garcia-Muñoz et al., 2023, Jasson et al., 2010, Martínez-Pascual and Zornoza, 2022]. ATP luminometry enables rapid determination of biological activity through measurement of adenosine triphosphate levels – a universal marker of living cells [Chrzanowski and Ławniczak, 2016; Liu et al., 2023]. Flow cytometry (FCM) allows for direct analysis of the microbial population structure in a sample, including differentiation of live, dead, and damaged cells [Sun et al., 2023; Thompson and Ward, 2025, Van Nevel et al., 2013]. Recent advances have integrated these techniques with automated systems to enable real-time monitoring of wastewater microbiology [Lee and Park, 2023, Santos and Ferreira, 2024]. In turn, molecular biology techniques enable to identification or quantification of even relatively small amounts of specific DNA copies, e.g. from indicator bacteria, such as those in the *Enterobacteriaceae* family [Ritalahti et al., 2006].

The development of these techniques opens up new possibilities for real-time monitoring and dynamic assessment of water sanitary status. They are particularly valuable in situations where a rapid response is critical – such as in water supply systems, wastewater treatment plants, or during emergency management in the event of a sanitary failure – and are also important in the context of epidemiological risks [UNICEF, 2023].

METHODOLOGY OF RESEARCH

Study material

The material for the study consisted of treated wastewater samples collected from the municipal wastewater treatment plant in Tarnobrzeg (50°37’02.5”N 21°42’14.6”E). This

facility serves the city of Tarnobrzeg and the surrounding areas. The plant has a hydraulic capacity of approximately 12,000 m³ per day, with a maximum pollutant load capacity equivalent to 75,000 PE (population equivalents). The treatment plant operates using a mechanical–biological–chemical system, employing activated sludge technology with sludge recirculation and aeration in continuous-flow biological reactors. After treatment, the wastewater is discharged into the Vistula River in accordance with its water permit. Samples for the study were collected during the summer months – June to August – under conditions that ensured repeatability in terms of temperature and microbial activity [Suchy et al., 2003; Żmuda, 2011].

Biopreparation

The biopreparation ACS ODO_1 was used to assess hygienization potential. A key criterion for selecting this biopreparation was its high content of lactic acid bacteria and their associated production of active antagonistic metabolites [ACS Poland; Mazur et al., 2022; Mazur et al., 2020]. The biopreparation ACS ODO_1 is composed of lactic acid bacteria at a concentration of 8.1 × 10^7 CFU per gram of dry matter, photoautotrophic bacteria, and yeasts. This microbial consortium is supplemented with fermented wheat bran and minerals, while sugar cane molasses serves as the substrate.

Treated wastewater (1 dm³) was placed in sterile laboratory containers, to which a specified amount of biopreparation was then added. After thorough mixing, the containers were covered with sterile cotton gauze. This method of covering ensured a free flow of oxygen – necessary for the development of microorganisms – while simultaneously protecting the sample contents from secondary contamination from the environment. The samples were incubated in a shaking incubator at 20 °C under natural daylight conditions, aiming to closely replicate the environmental conditions in which such preparations might be used in practice. The study was conducted under various experimental conditions, taking into account, among others, variations in the dose of the biopreparation and the presence or absence of additional aeration (Table 1).

Methodology of determinations

In each of the prepared test samples, the effect of a selected variable (in accordance with the experimental design shown in Table 2) on the effectiveness of eliminating *coliform* bacteria from treated wastewater was assessed.

Simultaneously, changes in ATP levels (expressed as RLU) were monitored, and the abundance and structure of the microbial population were analyzed using flow cytometry (FCM). A separate set of tests was conducted for molecular analysis. For this, 5 cm³ of the ACS ODO_1 biopreparation per 1 dm³ of treated wastewater was added to the sample. The sample was shaken for five consecutive days at a temperature of 20 °C. Then, a defined volume of the sample was collected, immediately cooled and transported to the laboratory of the Department of Agricultural Microbiology (IUNG-PIB, Puławy), where DNA was extracted and genetic analyses, including 16S rRNA gene amplification and sequencing, were performed [Nowak et al., 2024]

The abundance of microorganisms was determined using both classical and modern detection methods, in accordance with applicable procedures (Table 2).

Total ATP concentration was determined using a luminometer, following the manufacturer's

Table 1. The configurations in which each research series was conducted, depending on the determining factor

Series	Determinant factor	Characteristics
1.	Dose of biopreparation	Different doses of biopreparation were used: 1 cm³, 5 cm³ and 10 cm³ of biopreparation/dm³ of treated wastewater.
		Analyses were performed on the day of wastewater collection and 24h after dosing with biopreparations.
2.	Additional oxygenation	The study was carried out in two variants: with supplemental oxygenation and with limited oxygen. For this purpose, an oxygen generator from Hailea (with parameters: 220–240 V; 50 Hz; 2.5 W; 0.2 L/min; ≥ 0.018 MPa) was used.
		The biopreparation dose used was: 5 cm³ of biopreparation/dm³ of treated wastewater.
		Analyses were performed on the day the wastewater was collected and 24 hours after the test samples were set.

Table 2. Microbiological quality indicators of tested wastewater and biopreparations [University of Warmia and Mazury in Olsztyn, n.d.]

1.	Number of *coli* bacteria	MPN - Most Probable Number (fermentation-probe) method according to PN-ISO 4831:2007
2.	Number of *coli* and *Escherichia coli* bacteria	- Culture method according to PN-EN ISO 9308-2:2014-06 - Koch plate method - chromogenic medium for the determination of bacteria in an environment with a high content of organic compounds
3.	Luminometry determination of ATP concentration *	PROMEGA protocol for 20/20 luminometer
4.	FCM	Sysmex – Partec protocol for CytFlow Cube 6
5.	PCR	Sequencing method; results are quantitative

Note: * at work given as the RLU value – general.

instructions. To the tested sample, 100 µl of the sample and an equal volume of BacTiter-Glo™ Substrate combined with BacTiter-Glo™ Buffer were added. The mixture was incubated for 30 seconds at 37 °C. After incubation, the sample was mixed thoroughly using a vortex motion and then placed in a luminometer to measure light intensity, expressed in relative light units (RLU).

The total number of microorganisms present in the tested wastewater was determined using flow cytometry. The analysis was performed with a Partec Cube 6 (Sysmex-Partec) flow cytometer equipped with a blue laser (488 nm), forward scatter (FSC) and side scatter (SSC) detectors, and three fluorescence detectors (FL1: 536 ± 20 nm, FL2: 590 ± 25 nm, FL3N: 615 nm). The fluorescent dye SYBR Green and the TM BacCount Viable reagent set were used for the analysis. SYBR Green I, diluted 10.000-fold in DMSO, is activated by argon laser light at 488 nm, enabling staining and detection of cells.

During analysis, two main bacterial population regions were distinguished:

- LNA (Low Nucleic Acid, Reg. 1): dead cells with low nucleic acid content, which may also include viruses or fragments of genetic material,
- HNA (High Nucleic Acid, Reg. 2): live cells with high nucleic acid content, mainly bacteria.

Due to the high particulate content in wastewater, samples were diluted to concentrations of 10^{-1} to 10^{-2} cm³. For each measurement, 50 µl of the sample was used; the flow lasted 60 seconds at a rate of 0.5 µl/s, and thanks to dilution, the load measured in kPart/s was maintained in the range of 1 to 3.

Data were analyzed according to the manufacturer's recommendations, using a defined gating strategy, where two logarithmic-scale histograms were created: H1 (SSC) and H4 (FL1 – green fluorescence) two logarithmic-scale scatter plots were prepared: P1 (FL1 vs. FL3 – green vs. red fluorescence) and P3 (FL1 vs. SSC), and on each bacterial population, three polygonal gating areas were designated:

- "PG1" (FL1 vs. FL3) for live bacteria,
- "PG2" (FL1 vs. SSC) for LNA bacteria,
- "PG3" (FL1 vs. SSC) for HNA bacteria.

RESULTS

Below are the results of the microbiological analyses conducted to assess the effectiveness of the ACS ODO_1 biopreparation in eliminating *coliform* bacteria from treated wastewater (Table 3).

The reduction in the number of *coliform* bacteria was dependent on the presence of oxygen. In most cases, the use of the biopreparation without aeration did not lead to a reduction in the number of these bacteria; in fact, in some trials, an increase in MPN/100 cm³ was observed compared to the control sample. This may indicate low effectiveness of the preparation under anaerobic conditions or a possible stimulation of undesirable microbial growth (Figure 1).

The results of *coliform* bacteria counts obtained using other culture-based methods are presented in Table 4.

The application of a higher dose of the biopreparation (10 cm³/dm³) resulted in a decrease in bacterial counts; however, the reduction was more pronounced for *E. coli* than for the entire *coliform* group. In three series, a value of 0 CFU/100 cm³ was achieved for *E. coli* with the highest dose of the applied biopreparation. The analysis conducted using the membrane filtration method showed that a high dose of the ACS ODO_1 biopreparation

Table 3. *Coliform* count – MPN method

Series	Treated wastewater (control)	Biopreparation ACS ODO_1	ACS ODO_1 + supplemental oxygenation
	MPN/100cm^3		
1	1.1×10^4	1.1×10^4	1.1×10^3
2	4.6×10^2	9.2×10^2	1.1×10^2
3	1.1×10^4	6.1×10^4	9.3×10^3
4	4.1×10^1	6.1×10^1	1.1×10^1

Table 4. *Coliform* counts as a function of biopreparation dose, averaged results from tests performed in successive batches (1–3)

The method of determination used	Sample	*Coliform* [cfu/100 cm^3]	*E.coli* [cfu/100 cm^3]
Series 1			
Membrane filtration method	Treated wastewater	4.5×10^2	1.3×10^1
	Dose of 1 cm^3 Biopreparation ACS ODO_1	1.98×10^4	1.3×10^1
	Dose of 5 cm^3 Biopreparation ACS ODO_1	2.71×10^3	5
	Dose of 10 cm^3 Biopreparation ACS ODO_1	9.5×10^2	0
Series 2			
Membrane filtration method	Treated wastewater	2.55×10^3	1.25×10^1
	Dose of 1 cm^3 Biopreparation ACS ODO_1	2.325×10^4	1.35×10^1
	Dose of 5 cm^3 Biopreparation ACS ODO_1	1.45×10^3	1×10^1
	Dose of 10 cm^3 Biopreparation ACS ODO_1	1.985×10^3	1
Series 3			
Depth culture method	Treated wastewater	6×10^2	4
	Dose of 1 cm^3 Biopreparation ACS ODO_1	1.375×10^4	5
	Dose of 5 cm^3 Biopreparation ACS ODO_1	1.345×10^3	2
	Dose of 10 cm^3 Biopreparation ACS ODO_1	7.7×10^2	0

leads to a reduction in *E. coli*, although other *coliform* bacteria remain present (Table 5, Chart 1).

The culture-based methods used in the study targeted *coliform* bacteria, whereas the ATP and flow cytometry (FCM) analyses reflect the total number of live microorganisms in the sample, regardless of species. The ATP analysis confirmed a very high microbial count, and FCM confirmed the presence of thousands of live bacterial cells. This may indicate the presence of microorganisms in the VBNC state – alive but undetectable by classical culture methods – or the dominance of other metabolically active microflora that do not ferment lactose. The observed high ATP levels and increased number of live cells (FCM) in samples treated with the biopreparation, especially in the variant with additional aeration, may indicate not only the survival of the existing wastewater microflora but also the assimilation and proliferation of microorganisms introduced with the biopreparation. These microorganisms are metabolically active and contribute to the increase in total biomass, which raises the ATP and FCM values (Table 3, Table 5, Figure 2, Table 6, Figure 3).

In the control sample, a decrease in RLU values was observed after 24 hours, which may indicate the natural die-off of microorganisms without external stimulation. In the case of applying the biopreparation ACS ODO_1, a sharp increase in metabolic activity was recorded – the RLU value exceeded 88 million just 24 hours after application. This suggests intense microbial proliferation or significant stimulation of the metabolic activity of the microflora present in the wastewater.

The variant with the addition of oxygen also showed an increase in RLU – though lower (up to about 48 million) – which suggests that aeration partially limits the rate of microbial growth but does not stop it entirely.

Interpreting these results in relation to those obtained by culture methods (MPN and

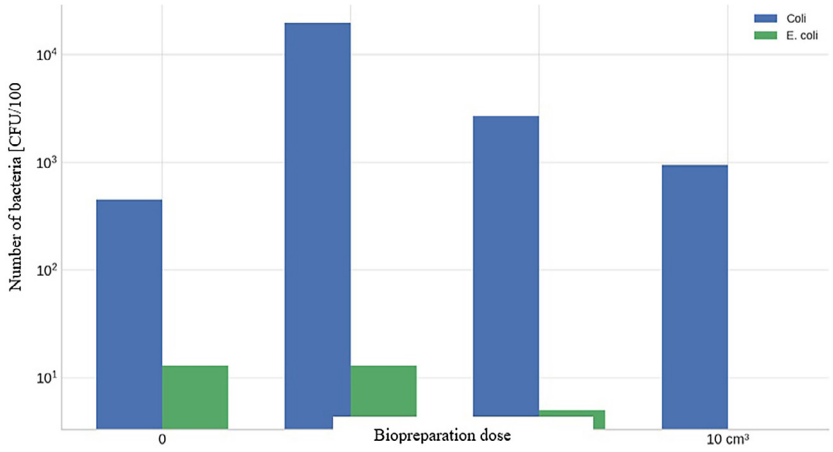

Figure 1. *Coliform* and *E.coli* counts as a function of ACS ODO_1 biopreparation dose

Table 5. Changes in RLU values in samples with biopreparations: ACS ODO_1, ACS ODO_1 with supplemental oxygenation and in the control sample

Hours	Treated wastewater (control)	Biopreparation ACS ODO_1	ACS ODO_1 + supplemental oxygenation
0h	5 098 667	5 101 332	4 982 143
24h	1 862 252	88 160 586	48 149 692

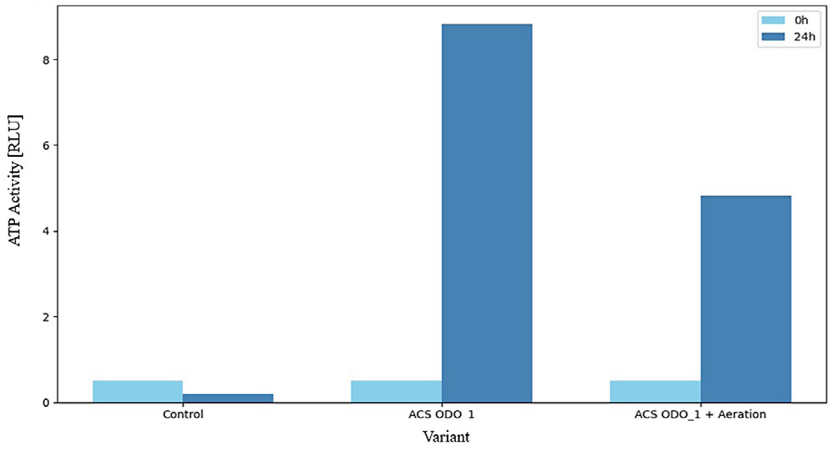

Figure 2. ATP Activity at 0h and 24h for different variants

membrane filtration), a significant discrepancy is noted: while some culture tests showed a decrease in the number of *coliform* bacteria or even no detection at high doses of the biopreparation, the RLU values in the same tests indicate strong biological activity. This means that although the number of bacteria capable of growing on selective media may have decreased, the wastewater environment remains microbiologically active – possibly due to the presence of VBNC bacteria or stimulation of other microbial groups.

Cytometric analyses (FCM) revealed clear changes in the abundance of both dead cells (Reg.1) and live cells (Reg.2) within the first 24 hours following the application of the biopreparation. In both variants – with limited oxygen access and with additional aeration – the number of live cells (Reg.2) significantly decreased, with the reduction being more pronounced under aerobic conditions. At the same time, the number of dead cells (Reg.1) increased, which may indicate the effectiveness of the biopreparation in eliminating active microflora. Nevertheless, even after 24 hours, considerable amounts of both live and dead cells were still present, confirming that the wastewater environment remains microbiologically active and that the hygienization effect is only partial.

Importantly, observations obtained by FCM contrast with the results of classical

Table 6. FCM analysis

Hours	Biopreparation ACS ODO_1		ACS ODO_1 + supplemental oxygenation	
	Reg. 1	Reg.2	Reg. 1	Reg.2
0h	48 934	48 647	23 521	20 003
24h	31 566	23 903	18 235	17 012

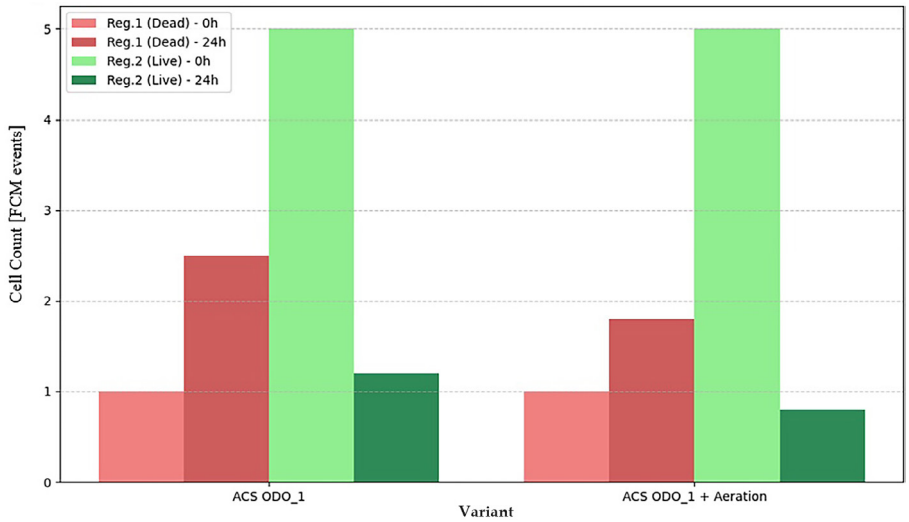

Figure 3. Comparison of Reg.1 (dead bacteria) and Reg. 2 (live bacteria) cells at 0h and 24h

culture-based assays, which in some samples – especially at higher biopreparation doses – suggested a significant reduction in *coliform* bacteria or even their complete absence. Flow cytometry, detecting live bacteria regardless of their ability to grow on media, revealed the presence of active microflora even in cases where culture methods yielded negative results. This indicates the presence of bacteria in a VBNC state or other microorganisms with intact cell membranes and physiological activity, which remain undetectable by conventional methods (Table 7).

The analysis of samples taken five days of treatment with the biopreparation ACS ODO_1 revealed the presence of DNA of the *Enterobacteriaceae* family, including genera such as *Raoultella*, *Klebsiella*, and *Citrobacter*, which together accounted for 2.49% of the total microbial community structure detected in the samples. Although the method did not explicitly identify the presence of *Escherichia coli*, it indicates the persistence of indicator microorganisms from the same family that have sanitary significance.

It is worth noting that in samples where membrane filtration did not detect *E. coli* (0 CFU/100 cm³), still specific DNA of related bacteria was presence. This may imply that the bacteria were not completely eliminated but existed in a cultivability-inactive state (e.g., VBNC) or in forms undetectable by classical microbiological methods. Moreover, PCR-based techniques, as a sensitive method, also come with several technical challenges, eg, the possibility of sample contamination, the selection of specific primers or potentially extracellular DNA amplification, which is particularly important for the analysis of small DNA copy numbers. Furthermore, the presence of genetic material from these bacteria after several days of treatment suggests that ACS ODO_1 did not effectively reduce the microflora with potential pathogenic activity.

Table 7. PCR results for *coliform* bacteria in treated wastewater with biopreparations

Biopreparation	Presence of *coliform* bacteria		Percentage of *coliforms* in samples after 5 days of biopreparation effect
ACS ODO_1	Current	*Raoultella* *Citrobacter* *Klebsiella*	2.4858%

DISCUSSION

The results of the conducted studies confirmed that the biopreparation ACS ODO_1 can contribute to reducing the number of *coliform* bacteria in treated wastewater; however, its effectiveness depends on environmental conditions – primarily the presence of oxygen and the applied dose. The presence of aerobic and facultative bacteria in the preparation is reflected in higher hygienic efficacy under aerated conditions, which aligns with mechanisms described in the literature [Mazur et al., 2020; Bitton, 2011].

The highest reduction of *coliform* bacteria – including *Escherichia coli* – was achieved at a dose of 10 cm^3 of the preparation per dm^3 of wastewater, under additional aeration. These effects were most apparent in results obtained by cultivation methods, indicating that the preparation effectively eliminates bacteria capable of growing on selective media.

However, evaluation based solely on classical methods may lead to a false conclusion of complete wastewater sanitization. In samples where fermentation-tube or membrane filtration methods did not detect bacteria, the presence of genetic material from E*nterobacteriaceae* family bacteria were confirmed, despite their lack of growth on culture media. It appears that enumeration of *coliform* bacteria in cultures with molasses-based biopreparations may be influenced by lactose content, as *coliform* detection mainly relies on lactose fermentation. Therefore, it is important to consider lactose content when interpreting *coliform* bacteria counts or to attempt to separate microorganisms present in the biopreparations from the medium (lactose). Membrane filtration may serve as one method to separate microorganisms from the growth substrate (lactose), enhancing the effectiveness of biopreparations in eliminating *coliform* bacteria.

In this context, it is worth emphasizing that the analytical methods used differ not only in sensitivity and assay time but primarily in the biological scope of the obtained data. Although biopreparations offer a promising biological approach to sanitization, physical methods such as membrane filtration also play a significant role in microbial removal from wastewater [Bodzek, 2013]. Cultivation methods provide information solely on microorganisms capable of growing on a given selective medium – in this study, mainly *coliform* bacteria. The results of these methods thus directly indicate the presence or elimination of specific indicator microorganisms relevant from a sanitary and water quality standards perspective.

Conversely, modern techniques are not exclusively targeted at specific bacteria such as *coliforms*. ATP luminometry measures the total number of viable cells in the sample, regardless of species affiliation. An increase in ATP after adding the biopreparation may therefore indicate intense proliferation of beneficial microflora (e.g., lactic acid bacteria), not necessarily fecal pathogens. Similarly, flow cytometry (FCM) allows assessment of the abundance and physiological state of all bacterial cells, without species distinction unless additional staining is applied.

This means that traditional cultivation methods and rapid detection techniques are not contradictory but complementary – they provide data on different aspects of the microbiological status of the sample. Classical methods are useful for assessing compliance with sanitary requirements (e.g., *E. coli* counts), while modern methods help determine whether the environment after biopreparation application is truly biologically "dormant" or remains active, albeit possibly dominated by a different – potentially non-pathogenic – microflora. Only an integrated approach can thus provide a comprehensive picture of sanitization effectiveness and sanitary safety.

Discrepancies between cultivation methods and modern techniques – including ATP luminometry, flow cytometry, and molecular techniques – highlight the need for an integrated approach. Only simultaneous analysis of specific pathogens and overall microbiological activity enables a complete evaluation of biopreparation efficacy and the actual microbiological risk in treated wastewater.

CONCLUSIONS

1. The conducted studies demonstrated that the effectiveness of wastewater sanitization using the biopreparation ACS ODO_1 is strongly dependent on environmental conditions (dose, aeration) and the method of microorganism detection. ACS ODO_1 shows limited sanitizing efficacy under anaerobic conditions – its effectiveness significantly increased with additional aeration. This indicates that the preparation may support biological treatment systems but does not guarantee complete elimination of microbiological hazards.

2. The application of ACS ODO_1 caused changes in the abundance of *coliform* bacteria, as confirmed by classical cultivation methods. The best effects were observed in the variant with additional aeration.
3. ATP measurements showed a clear increase in the abundance of microorganisms after applying ACS ODO_1, especially under aerated conditions; however, this does not necessarily indicate improved sanitization.
4. Flow cytometry (FCM) revealed a significant number of live bacterial cells in all samples. Despite the reduction of indicator bacteria (*coliforms*), the overall microflora remained active or was partially replaced by microorganisms originating from the biopreparation.
5. Genetic material from *Enterobacteriaceae* family bacteria was detected in all samples, including those where classical fermentation tests yielded negative results. In particular, *Citrobacter freundii* and *Raoultella planticola* were identified, confirming the risk of false results in lactose fermentation-based tests and highlighting the necessity of molecular techniques as a complement to classical analyses.
6. An integrated diagnostic approach – combining classical and modern methods – is essential for a reliable assessment of biopreparation efficacy and identification of the actual sanitary risk in wastewater. Only such an approach allows for proper microbiological risk estimation and accurate evaluation of sanitizing agents' effectiveness.

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