ASSESSMENT OF THE EVOLUTION OF MICROBIAL POPULATIONS DURING WASTEWATER TREATMENT PLANT PROCESSES

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This paper presents original results regarding the microbiological and physico-chemical characterization of urban wastewater treated at ECOAQUA Călărași Plant. Analyses in triplicate were performed using standardized methods and CHROMagar™ selective media. Significant reductions of ammonium, organic load and pathogenic bacteria were observed across treatment stages. However, residual Escherichia coli and Salmonella spp. persisted in the effluent, highlighting the need for improved disinfection. The study confirms the efficiency of the biological process and demonstrates the utility of chromogenic media for rapid microbial monitoring.

Keywords: wastewater treatment, microbial monitoring, fungi, mesophilic bacteria, pathogenic bacteria, CHROMagar™ selective media

1. Introduction

Urban wastewater results from domestic, industrial, commercial, and agricultural activities. It contains high concentrations of organic compounds of both natural and anthropogenic origin, along with heterotrophic allochthonous microorganisms entering the system with the influent, and autochthonous microorganisms present in the recirculated activated sludge [1,2]. The processes employed to remove these contaminants are complex and typically comprise several stages: an initial mechanical treatment phase for coarse fraction removal (large particles), primary sedimentation and degreasing to eliminate sand and fats, followed by secondary biological treatment aimed at reducing nitrogen (mainly in ammonium form), phosphorus, and carbon content, and concluding with secondary sedimentation to remove suspended solids. Mechanical-biological treatment technologies, first developed in the early 20th century and widely adopted in the latter half, have evolved to include so-called tertiary treatment steps (denitrification using external energy sources such as

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methanol) and more advanced treatment processes [3]. Recently, increased emphasis has been placed on disinfection practices and on controlling the microbial loads discharged into receiving water bodies. Although no strict regulatory thresholds are currently enforced, research increasingly indicates that microbial concentrations should be limited to reduce public health risks. Monitoring microorganisms in both water sources and treatment processes is essential for ensuring water quality and safeguarding public health by preventing disease transmission and protecting aquatic environments that receive treated effluents. Several disinfection solutions are available, including chlorination—a conventional method that may negatively impact indigenous aquatic populations—ultraviolet radiation (effective at low flow rates), and ozonation (characterized by high energy consumption and ozone losses into the atmosphere). However, these processes are not yet universally applied, have limited efficiency, and often require additional reagents and energy inputs [3,4]. The most relevant types of microorganisms commonly found in wastewater include fungi, which play essential roles in treatment processes [5], mesophilic bacteria and total coliform bacteria involved in lactose fermentation, as well as fecal indicator and pathogenic bacteria such as *Escherichia coli*, *Salmonella spp*., *Listeria spp*., and *Staphylococcus aureus*. These organisms serve as important indicators of fecal contamination, pathogenic potential, and overall treatment performance, reflecting risks related to the potential transmission of waterborne pathogens [6,7]. Coliform bacteria and *Escherichia coli* are typically found in the intestines of humans and warm-blooded animals. While many strains are harmless, some can cause serious illnesses, including urinary tract infections, gastroenteritis, and, in severe cases, hemolytic uremic syndrome [8]. Their presence in water is a common indicator of fecal contamination and suggests the potential presence of other pathogenic microorganisms. Fecal streptococci, part of the enterococci group, are also present in the normal intestinal flora of humans and animals. They are widely used as indicators of water quality, particularly for detecting fecal contamination in surface and marine waters. Certain strains can cause urinary tract infections, meningitis, and endocarditis, especially in immunocompromised individuals [9,10]. *Salmonella* is a genus of bacteria commonly found in the intestines of animals and humans and frequently associated with foodborne contamination. It can cause salmonellosis, an infection characterized by diarrhea, fever, and abdominal cramps. Its presence in water also indicates fecal contamination and poses a potential public health risk [11]. In addition to these, various other bacteria can be found in wastewater, originating from fecal inputs, industrial and agricultural discharges, and diverse pollution sources. For example, *Pseudomonas aeruginosa* is a ubiquitous bacterium notable for its resistance to many antibiotics. It acts as an opportunistic pathogen capable of causing infections in immunocompromised hosts [12]. *Legionella spp*. is known to cause Legionnaires' disease, a severe form of pneumonia, and is frequently found in standing water, including air conditioning systems and cooling towers [13]. *Staphylococcus aureus*, although more commonly associated with skin and soft tissue infections, can also persist in wastewater

environments [14,15]. Other relevant microbial groups include nitrifying and denitrifying bacteria, which are essential components of the nitrogen cycle, converting ammonia to nitrates (nitrification) or reducing nitrates to nitrogen gas (denitrification). They play an important role in biological wastewater treatment processes [16]. A growing concern is the emergence of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs). Wastewater provides a favorable environment for their development and dissemination, representing an increasing threat to public health [17,18]. It is important to recognize that many of these bacteria are pathogenic and can pose significant risks to human health, particularly when wastewater is insufficiently treated prior to environmental discharge or reuse.

This paper presents experimental results obtained from quantitative microbiological analyses of urban wastewater collected from the ECOAQUA Călărași Wastewater Treatment Plant, Călărași County. Samples were taken from three distinct stages of the treatment process: the entry point (raw wastewater), after biological treatment (intermediate phase), and the exit point (treated effluent). Samples were characterized from a physicochemical perspective by measuring parameters such as pH, suspended solids, ammonium, chemical oxygen demand (using potassium dichromate), total nitrogen, ammonium, total phosphorus, and BOD_5. The presence and abundance of mesophilic fungi and bacteria at 37 °C were assessed, along with indicators of fecal contamination, including total coliforms, fecal coliforms, fecal streptococci, and pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Listeria spp*., and *Pseudomonas aeruginosa.* Chromogenic culture media were used as effective tools for rapid, selective detection and presumptive identification of these microorganisms. In response to the growing demand for affordable and rapid diagnostic methods, this work demonstrates how chromogenic media can be integrated into practical, accessible protocols for routine monitoring in municipal wastewater management [19].

2. Materials and Methods

The water samples were collected on 3 June 2025 from the Călărași Wastewater Treatment Plant, operated by ECOAQUA S.A. Călărași. Sampling was performed at three points along the treatment flow: the influent (raw wastewater), after the biological treatment stage (intermediate phase), and at the effluent (treated effluent). All samples were analyzed for both physicochemical and microbiological parameters. Water samples were transported to the Bucharest laboratory in sterile containers, under refrigerated conditions (4 °C), and analyzed within 24 hours to ensure sample integrity. The management of ECOAQUA S.A. Călărași provided their consent for the publication of the results obtained from the wastewater samples.

2.1 Physico-chemical analysis of water

From a physico-chemical perspective, the following parameters were determined: pH (pH meter, Model 370 Jenway, England), suspended solids (2100Q IS Portable

Turbidimeter, LED, 0–1000 FNU, Hach, Germany), chemical oxygen demand in the presence of potassium dichromate (CCO-Cr), biochemical oxygen demand over 5 days (BOD_5) measured with an Oxitop Control IS6 system (WTW, British-American company), as well as total phosphorus and ammonium concentrations analyzed using a DR3900 Laboratory Spectrophotometer (Hach, Germany).

2.2 Microbiological analysis

The water samples used for microbiological analyses were collected in accordance with SR ISO 5667-2. All plating procedures were performed in triplicate to ensure the reliability of the enumeration process. Sample preparation followed the McCrady method (Most Probable Number, MPN), which involves preparing a series of 9–16 decimal dilutions. For each dilution step, 1 mL of the previous dilution was transferred into 9 mL of sterile physiological saline solution. The analyses targeted fungi, mesophilic bacteria at 37 °C, total coliform bacteria, fecal coliform bacteria, and fecal streptococci, which were enumerated by seeding on solid and liquid culture media.

Table 1 summarizes the dilution series and the specific seeding methods applied to determine the different microbial populations included in this study.

2.2.1. Fungal analysis

The water samples intended for fungal enumeration were prepared using the method of successive decimal dilutions, ranging from 10^{-1} to 10^{-16}. Inoculation was performed on Potato Dextrose Agar (PDA), and the plates were incubated at 37 °C for 48 hours.

2.2.2. Bacteria analysis

The water samples intended for the enumeration of mesophilic bacteria growing at 37 °C were prepared using the method of successive decimal dilutions, ranging from 10^{-1} to 10^{-12}. Inoculation was performed on Nutrient Agar, and the plates were incubated at 37 °C for 48 hours. After incubation, colonies were counted on plates containing fewer than 300 colonies

The number of colonies was calculated using the following formula:

$$\text{Number of mesophilic bacteria} = \frac{\sum(nd)}{NV};\ [\text{CFU/cm}^3] \quad (1)$$

where:

n = number of colonies counted per Petri dish
d = reciprocal of the dilution factor of the inoculated sample
N = number of Petri dishes considered
V = volume of the sample plated (cm^3)
CFU = colony forming units

Table 1

Microbiological analyses performed and reference methods

No.	Microbiological analysis	Dilution range	Determination method	Reference / Standard
1	Fungi	$10^{-1} – 10^{-16}$	Incorporation method using solid and liquid media	SR EN ISO 6222:2004
2	Mesophilic bacteria at 37 °C	$10^{-1} – 10^{-12}$	Incorporation method using solid and liquid media	STAS 3001-91
3	Total coliform bacteria	$10^{-1} – 10^{-10}$	Membrane filtration method	STAS 3001-91
4	Fecal coliform bacteria	$10^{-1} – 10^{-10}$	Membrane filtration method	STAS 3001-91
5	Fecal streptococci	$10^{-1} – 10^{-10}$	Membrane filtration method	STAS 3001-91
6	*Escherichia coli*	$10^{-1} – 10^{-10}$	CHROMagar™ ECC	[20]
7	*Staphylococcus aureus*	$10^{-1} – 10^{-10}$	CHROMagar™ Staph aureus	[21]
8	*Salmonella spp.*	$10^{-1} – 10^{-10}$	CHROMagar™ Salmonella	[22]
9	*Listeria monocytogenes*	$10^{-1} – 10^{-10}$	CHROMagar™ Listeria	[23]

The determination of total coliform bacteria and fecal streptococci was performed by filtering the water samples through a membrane filter with a diameter of 30–45 mm and a pore size of 0.45 μm.

The culture media used for inoculation were as follows: Endo agar (containing sodium sulfite and basic fuchsin) for total coliforms, and Slanetz–Bartley agar (sodium azide–triphenyltetrazolium chloride glucose medium) for fecal streptococci.

Fecal coliforms were enumerated using the Multiple Tube Fermentation Method, consisting of two stages: presumptive testing in Lauryl sulfate broth and confirmation in MacConkey medium (lactose bile broth with bromcresol purple indicator).

Sample filtration was carried out using a MultiVac 300–MB filtration system (AC 220 V, 50 Hz; manufacturer: Rocker, Taiwan). Incubation of the samples was performed in a natural circulation incubator (Memmert, Germany).

Determination of pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella spp.*, and *Listeria spp.*) was performed by aseptically plating 0.1 mL from each dilution step onto Petri dishes containing the following CHROMagar™ selective media: CHROMagar™ ECC for *Escherichia coli*, CHROMagar™ *Staph aureus*, CHROMagar™ *Salmonella*, and CHROMagar™ *Listeria*. Samples were spread evenly using a Drigalski spatula. The plates were incubated aerobically at 37 °C for 24–48 hours using a LABOSHAKE Gerhardt orbital incubator. After incubation, colony-forming units (CFUs) were counted on plates presenting between 30 and 300 colonies. Presumptive identification was based on colony morphology and chromogenic response according to the manufacturer's instructions [20-23]. Although CHROMagar™ enabled fast presumptive identification, culture-based detection may underestimate viable but non-culturable (VBNC) cells, potentially present in effluents.

All plating procedures were performed in triplicate to ensure reliability of the enumeration process. Statistical analysis was performed using one-way ANOVA to assess differences in microbial counts across sampling points. Post-hoc comparisons were conducted using Tukey's test, and results were considered statistically significant at $p < 0.05$. Data were expressed as mean ± standard deviation (SD) based on triplicate plating. For visualization purposes, CFU/mL values were $\log_{10}$ transformed in figures.

3. Results and Discussion

3.1. Physico-chemical characterization of the water

The physico-chemical characteristics of the water samples collected from the three sampling points are summarized in Table 2. Analysis of the data presented in Table 2 shows that the influent water exceeded the maximum permissible limit only for the ammonium parameter, which recorded a concentration of 62.3 mg/L, compared to the regulatory limit of 30 mg/L established by NTPA 002/2002.

Following biological treatment, the ammonium concentration decreased significantly to 12.5 mg/L, and after secondary sedimentation it was further reduced to 1.12 mg/L, well below the maximum allowed threshold. These results indicate that domestic wastewater from this facility exceeded the permissible limits exclusively in terms of ammonium content, which required targeted removal processes. In this context, a biological treatment stage combining aerobic, anoxic, and anaerobic phases was implemented.

Table 2

Physico-chemical characteristics of the water samples collected from the three sampling points

No.	Parameter name	Unit	Sampling point – Influent	After biological treatment	Sampling point – Effluent	Maximum allowable limit (NTPA 002/2002)	Analytical method (standard)
1	pH (25 °C)	–	7.65	7.15	7.32	6.5–8.5	SR EN ISO 10523:2012
2	Total suspended solids	mg/L	196	6270	30	350	SR EN ISO 872:2009
3	Ammonium	mg/L	62.3	12.5	1.12	30	SR ISO 7150-1:2001
4	CCO-Cr	mg O_2/L	402.5	230.4	46.4	500	SR ISO 6060:1996
5	Total nitrogen	mg/L	51.2	13.06	4.1	–	SR EN ISO 11905-1:2003
6	Total phosphorus	mg/L	5.1	1.34	0.6	5	SR EN ISO 6878:2008
7	BOD_5	mg O_2/L	197.87	112.4	9.3	300	SR EN 1899-1:2003

The process proved highly effective, as the final ammonium concentration at the plant effluent reached 1.12 mg/L, demonstrating compliance with the regulatory limit of 30 mg/L. Suspended solids, although within the permissible limit of the influent, were reduced to 27 mg/L at the effluent, demonstrating that the installation is highly efficient, particularly during the secondary settling stage. The organic load, initially measured at 402.5 mg/L, decreased substantially to 46.4 mg/L in the final effluent, a value well below the permissible limit of 500 mg/L. These results confirm the high effectiveness of the biological treatment processes implemented at the facility. Total phosphorus, present in the wastewater primarily in the form of orthophosphates, had a concentration of 4.8 mg/L at the plant influent, which decreased to 0.7 mg/L at the effluent—well below the maximum allowable limit of 5 mg/L. Biochemical oxygen demand was initially very high due to the elevated concentrations of nutrients and microorganisms. It decreased from 197.87 mg/L in the influent to 112.4 mg/L after biological treatment and further to 9.3 mg/L in the final effluent, remaining significantly below the permissible limit of 300 mg/L. During the biological treatment stage, microorganisms played an essential role in the mineralization of organic matter and the reduction of nitrogen, phosphorus, and carbon compounds.

3.2. Microbiological analyses

Based on microbiological analyses, it was observed that domestic wastewater contains high loads of various types of microorganisms (Fig. 1). The concentration of these organisms progressively decreased throughout the technological treatment stages. In the samples collected at the plant influent, the highest concentrations of fungi, mesophilic bacteria, coliforms, and pathogenic bacteria (*E. coli*, *Salmonella spp*., etc.) were recorded, reflecting the expected microbial load in raw wastewater prior to treatment. Fungi represented the most abundant group present in the analyzed wastewater, forming irregular white colonies on PDA at initial concentrations on the order of 2.8×10^{16} CFU/mL. Their abundance decreased to 10^{16} CFU/mL after biological treatment and further to 3×10^{9} CFU/mL in the final effluent.

Mesophilic bacteria cultivated at 37 °C developed as white and yellow colonies with various shades of brick and brown, round with uniform edges, or as white, acicular colonies. Their concentrations ranged from 3.9×10^{13} CFU/mL in the influent to 2.9×10^{7} CFU/mL in the effluent. Total coliform bacteria were identified by the formation of metallic red-violet, round colonies. Their concentration decreased from 2.6×10^{6} CFU/mL in the influent to 4.5×10^{4} CFU/mL in the effluent. Fecal coliform bacteria were detected by the production of gas resulting from lactose fermentation in Durham tubes and the discoloration of the culture medium. Their concentrations declined from 1.4×10^{5} CFU/mL in the influent to 2×10^{2} CFU/mL in the treated effluent. Fecal streptococci were found in the highest concentrations among the bacterial groups analyzed, decreasing from 3.2×10^{9} CFU/mL at the influent to 2×10^{8} CFU/mL at the effluent.

Chromogenic media facilitated the rapid and visually distinctive identification of specific bacterial species at different stages of the treatment process (Fig. 2). *Salmonella spp.* colonies appeared as dark purple to bluish black with a metallic sheen on CHROMagar™ Salmonella, being especially numerous in the influent sample, consistent with the highest CFU/mL values observed in the bar graph (~300,000 CFU/mL). *E. coli* colonies developed a characteristic deep blue coloration on CHROMagar™ ECC, while total coliforms were pale pink to slightly beige. On CHROMagar™ Staph aureus, the expected mauve pigmentation was rarely observed; most colonies remained small and unpigmented, indicating low presence. *Listeria monocytogenes* formed turquoise to blue-green colonies with subtle opaque halos on CHROMagar™ *Listeria* and were clearly present in the influent but significantly reduced or absent in post-treatment samples.

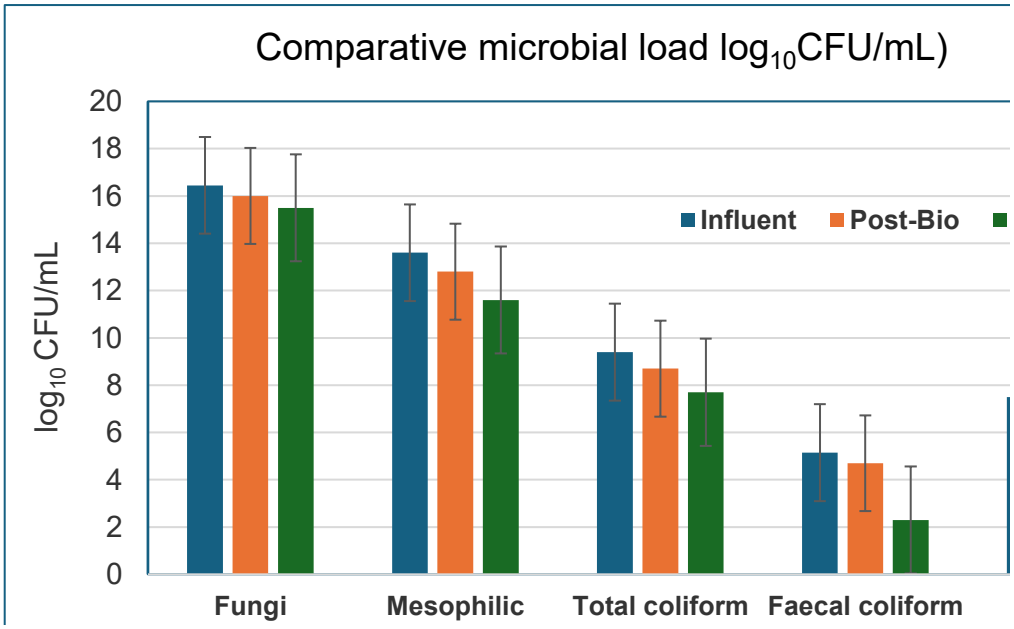

Fig. 1. Comparative microbial load (log_{10} CFU/mL) for major microbial indicators during the wastewater treatment process at the three sampling points: influent, post-biological treatment, and effluent. Error bars represent standard deviations.

The comparative microbial load profiles, expressed as log_{10} CFU/mL, clearly illustrate the substantial reduction in bacterial counts achieved across the treatment stages (Fig. 3). In the influent samples, *E. coli* and *Salmonella spp.* exhibited the highest loads, exceeding 5 log units, while *Listeria spp.* and *Staphylococcus aureus* showed initially lower counts. Following the biological treatment, *Salmonella spp.* declined markedly by over 2 log units, and *E. coli* levels decreased significantly, although remaining relatively elevated, suggesting that additional disinfection steps might be necessary to ensure further reduction. In contrast, *Listeria spp.* and *Staphylococcus aureus* were effectively diminished to near or below the detection limit in the effluent, reflecting the efficacy of the applied treatment train against these pathogens. The low standard deviations observed in most measurements indicate good reproducibility, with slightly higher variability recorded for *Staphylococcus aureus* after the biological stage, possibly due to counts approaching the detection threshold. Overall, these findings underscore the importance and efficiency of multi-stage treatment processes in mitigating microbial contamination and ensuring the microbiological safety of the final effluent. To evaluate the statistical relevance of the observed differences, a one-way ANOVA was performed on microbial counts obtained from the three sampling points.

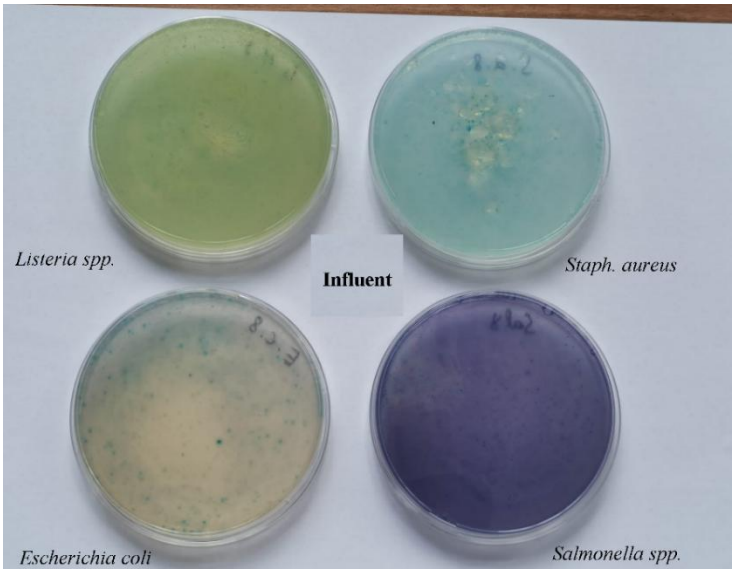

Fig. 2. Representative CHROMagar™ plates showing bacterial growth in influent wastewater sample.

The analysis indicated statistically significant variations in bacterial loads between stages of the treatment process ($p < 0.05$). Post-hoc Tukey's tests further confirmed that reductions between influent and effluent samples were particularly significant for *Escherichia coli* and *Salmonella spp.* The inclusion of standard deviations emphasized the reproducibility of the results and highlighted both biological and technical variability inherent to the chromogenic culture method. These findings support the consistency and reliability of the observed microbial decline during wastewater treatment. For clarity, $\log_{10}$ transformation was applied to CFU/mL counts in graphical representations.

These findings highlight the potential risk of pathogen release into natural waters and emphasize the importance of continued surveillance and upgrading disinfection stages.

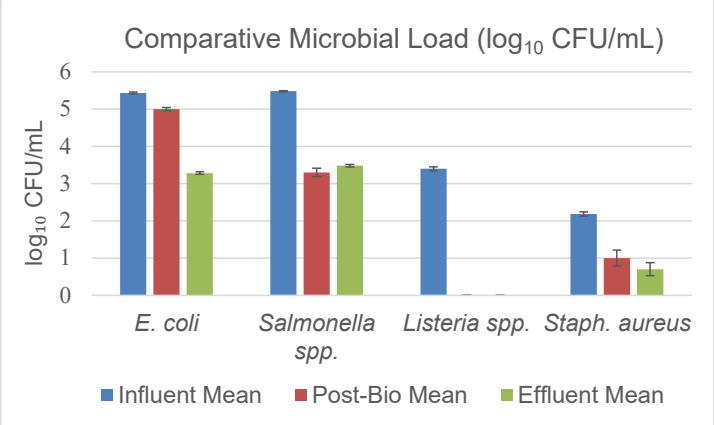

Fig. 3. Comparative microbial load (log_{10} CFU/mL) at the three sampling points: influent, post-biological treatment, and effluent. Error bars represent standard deviations. Note: Values below detection limit set to 1 CFU/mL (log10=1).

4. Conclusions

The ECOAQUA Călărași wastewater treatment plant demonstrated high efficiency in reducing both physico-chemical and microbiological contamination. Treatment processes significantly lowered ammonium, suspended solids, and organic loads to values compliant with regulations. Microbiological monitoring showed substantial decreases of fungi, mesophilic bacteria, and fecal indicators during treatment. Chromogenic culture media were effective tools for rapid detection and differentiation of pathogenic species such as *Escherichia coli*, *Salmonella spp.*, *Listeria monocytogenes*, and *Staphylococcus aureus*. However, the residual presence of *E. coli* and *Salmonella spp.* highlights the need to improve disinfection strategies. Overall, the results confirm the value of continuous microbiological surveillance and advanced treatment measures to protect public health and receiving water bodies.

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