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Bioactivation of activated sludge microorganisms by Krebs cycle metabolites in wastewater treatment technologies

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ABSTRACT

The study addresses the challenge of intensifying biological wastewater treatment by regulating the metabolic activity of activated sludge. Insufficient stability in treatment plant performance, often caused by sludge bulking and poor sedimentation, necessitates innovative methods for microbiocenosis management. An ecobiotechnological approach was developed using tricarboxylic acid (TCA) cycle metabolites, specifically citrate and succinate, as biostimulation factors in ultra-low concentrations (0.1–10 mM). The study also examined the combined effect of these metabolites with malonate, a competitive inhibitor, on the Sludge Volume Index (SVI) and species diversity. For the first time, it was established that ultra-low concentrations of TCA metabolites effectively activate decomposer metabolism without disrupting cellular ionic balance. Citrate treatment led to a qualitative transformation of microbiocenosis, increasing the abundance of free-swimming ciliates 3.1-fold, attached ciliates 2.6-fold, and *Zoogloea ramigera* bacteria 1.8-fold. A significant novel finding is the 5-fold suppression of filamentous sulfur bacteria by citrate, which prevents sludge bulking. Succinate was found to stimulate higher trophic levels (*Rotifera, Tardigrada*), enhancing wastewater clarification through increased species diversity. Furthermore, the combined application of citrate, succinate, and malonate stabilized the population of each species within the biocenosis, successfully reducing the SVI from 235 to 195. The proposed activation technique ensures the sustainable development of modern wastewater systems by providing a controlled mechanism to improve sedimentation properties and treatment efficiency through metabolic regulation.

Keywords: biological activation, ecobiotechnology, citrate, succinate, malonate, sludge index.

INTRODUCTION

Biotechnological approaches to environmental protection remain highly relevant across all sectors, including the technologies for municipal and industrial wastewater treatment. In these systems, biological treatment methods operate effectively alongside physical and chemical processes. The search for ways to enhance the efficiency of all possible purification methods continues, increasingly involving innovative technologies (Crini and Lichtfouse, 2019; Koseoglu-Imer et al., 2022; Migdał et al., 2022). Among existing wastewater treatment methods, biological processes are the most economically viable and highly efficient for the degradation of pollutants. The high oxidative capacity of biological consortia allows for the effective decomposition of complex organic substances and the absorption of inorganic contaminants. Beyond its environmental safety, this technology is characterized by the high processing speed of large waste volumes and

a significant economic advantage over alternative treatment methods. Furthermore, it can serve as a valuable resource for biofuel production, contributing to a "green" future (Al-Gheethi et al., 2024; Darweesh et al., 2021; Paramonov et al., 2024; Zheng et al., 2026; Zoomi et al., 2021).

Microbial associations play a key role in the functioning of biological wastewater treatment plants. In addition to metabolizing organic matter, bacteria synthesize exopolysaccharides (EPS), which facilitate the formation of specific activated sludge flocs, biofilms, or granules. These structures are critically important for effective solid–liquid separation and overall water purification. Bacterial organisms are characterized by exceptional metabolic plasticity (Yang et al., 2020). Due to their wide spectrum of enzymatic reactions, they are capable of transforming a diverse range of primary substrates into components of their own biomass and essential cellular structures (Ahnert et al., 2026).

Traditional biological wastewater treatment is carried out by aerobic bacteria, which require dissolved oxygen and organic substances for respiration. The Krebs cycle (TCA cycle) serves as the primary enzymatic system, acting as a hydrogen generator for the respiratory chain. A specific feature of bacterial catabolism is the variability of respiratory pathways. At the electron transport stage, different taxonomic groups of microorganisms can engage alternative electron acceptors, depending on the species and environmental conditions. This adaptability ensures their survival across diverse ecological niches. Beyond its energetic function, the TCA cycle acts as a source of metabolic precursors. TCA cycle metabolites are utilized as substrates for the biosynthesis of glucose, amino acids, fatty acids, and other essential compounds (Kayser and Averesch, 2025). The absence of mitochondria in microorganisms does not halt the process; on the contrary, it necessitates greater metabolic flexibility. Microorganisms utilize their own cytoplasmic membrane for the functioning of the electron transport chain, where the periplasmic space acts as the site for the proton gradient. The diffusion of NADH and $FADH_2$ to the respiratory complexes occurs relatively rapidly.

A number of scientific studies have explored the multifaceted activation of activated sludge bacteria (Amirfakhri, 2023). Investigating the influence of bacterial metabolic byproducts and various external carbon sources – ranging from classical liquid sources (acetic acid, alcohols) to alternative ones (food industry waste, agricultural residues, etc.) – on their activity remains a promising direction for application in cutting – edge environmental protection technologies (Fu et al., 2022). One of the modern approaches to enhancing the efficiency of water purification by activated sludge microorganisms is the stimulation of their metabolic processes through the introduction of organic substrates that play a key role in energy metabolism (Ahmed et al., 2023; Mishra et al., 2025). Various carbon sources are most commonly used as metabolic activators, including glucose (Pang and Wang, 2021), sodium acetate (Fu et al., 2022), higher fatty acids (X. Li et al., 2023), glycerol (Schroeder et al., 2020), citrate (Mielcarek et al., 2017), and others.

The biological activation of activated sludge microorganisms results in increased enzymatic activity and the intensification of water purification processes. Under the influence of metabolic activators, the species composition of the activated sludge changes, with aerobic bacteria serving as its primary components (Lee et al., 2025). The ability of bacteria to utilize pollutant molecules largely depends on the energy status of their cells.

The TCA cycle plays a decisive role in the energy supply of aerobic activated sludge microorganisms. Beyond its energetic function, it performs an integrative role by ensuring the oxidation of acetyl–CoA–a common metabolite in the metabolism of carbohydrates, lipids, and proteins. As a result of TCA cycle dehydrogenase reactions, reduced coenzymes $NADH+H^+$ and $FADH_2$ are formed, serving as substrates for the respiratory chain. The regulation of the TCA cycle is carried out through the activation and inhibition of key enzymes according to the energy needs of the cell. Under conditions of high ATP and $NADH+H^+$ concentrations in the cell, the rate of TCA cycle reactions decreases. The regulation of the TCA cycle is carried out through the activation and inhibition of key enzymes in accordance with the cell's energy requirements. Under conditions of high ATP and $NADH+H^+$ concentrations, the rate of TCA cycle reactions decreases (Akram, 2014). Inhibitors attract significant attention to understanding the regulatory mechanisms of cellular enzymatic activity. Based on their "enzyme–binding" characteristics, inhibitors are classified into reversible and irreversible (Blat, 2010). Competitive inhibition is a type of reversible enzyme inhibition in which

the inhibitor competes with the natural substrate for the enzyme's active site. Since the binding is competitive, an increase in substrate concentration can displace the inhibitor from the active site and restore enzymatic function. Malonic acid (malonate) acts as a competitive inhibitor of the enzyme succinate dehydrogenase (SDH) (Kim, 2002). Given the broad spectrum of biochemical reactions involving TCA cycle metabolites, their use as activators of cellular metabolic processes–alongside the application of a competitive inhibitor to regulate enzymatic activity–represents a promising research direction. In the study by (Murphy and Letter, 1986), the influence of succinate on the ability of activated sludge bacteria to degrade polyphosphates was investigated. More recently, (Chub et al., 2023) examined the effects of succinate and citrate on the efficiency of water purification by activated sludge microorganisms.

An analysis of the literature indicates the high efficiency of using TCA cycle metabolites as metabolic activators in microbial cells. However, the impact of these compounds on the species diversity of the activated sludge biocenosis remains insufficiently studied and requires further investigation. In this regard, the aim of this study is to determine the influence of citrate, succinate, and their combined effect with malonate on the abundance and species composition of activated sludge microorganisms, as well as the efficiency of water purification at municipal wastewater treatment plants.

MATERIALS AND METHODS

Research objects and materials

The study was aimed at studying the possibilities of intensifying wastewater treatment by regulating the metabolic activity of aerobic bacteria of activated sludge (AS). The main hypothesis is based on the fact that the use of ultra-low concentrations of Krebs cycle metabolites (0.1–10 mM) allows stimulating the energy metabolism of microorganisms without disturbing their ionic balance.

Citrate (citric acid) and succinate (succinic acid) were used as bioactivators, and for artificial regulation of enzymatic reactions – a competitive inhibitor malonate (malonic acid).

Reverse activated sludge (RAS) taken from sludge chambers was used for the study.

Experimental design

The experimental part was carried out in two stages: first in laboratory conditions, and then at the wastewater treatment plant in Sumy (Ukraine). In laboratory studies, samples with a volume of 1.0 dm³ and specialized cultivators for 20 dm³ were used.

The experiment was built on the principle of gradual scaling – from laboratory samples to industrial application. Testing different concentrations (0.04% and 0.08%) and combinations of citrate and succinate (simultaneous or sequential introduction) in six parallel samples.

To stimulate the metabolism of aerobic bacteria, it is necessary to take into account the biochemical effect of each metabolite, as well as cellular energy metabolism (Table 1). Since high concentrations of citrate or succinate can disrupt the ionic balance of the cell, the concentrations of metabolites for sludge treatment should be ultra-low.

According to research protocols (Koenigsberg et al., 2020; Pengchao, 2022), to regulate TCA processes without complete inhibition, the concentration of TCA substrates should be in the range of 0.1–10 mM. It is also necessary to take into account external and internal factors influencing the metabolism of aerobic bacteria in the biocenosis of operating wastewater treatment plants, namely: insufficient BOD load requires a decrease in the concentration of the TCA substrate; increased acidity as a result of the combined action of citrate and succinate; malonate artificially regulates TCA, can be toxic in high doses.

To calculate the concentration of the TCA cycle substrate for the stimulation of aerobic biomass metabolism, the following formula is used

$$K_m = (\mathrm{C} \cdot \mathrm{M}_{mol})/V \quad (1)$$

where: K_m – mass concentration of the TCA cycle substrate (g/dm³); C – molar concentration of the TCA cycle substrate (mM); M_{mol} – molar mass of the TCA cycle substrate (g/mM); V – volume of the liquid (1 dm³).

During the laboratory studies, four plug–flow aeration tanks were in operation with a total volume of:

$$W_a = 5255 + 5255 + 5245 + \\ +5245 = 21000 \text{ m}^3 \quad (2)$$

Table 1. Biochemical characteristics of the selected TCA cycle substrates

TCA cycle substrate	Biochemical impact	TCA substrate molar concentration, mM
Citrate	TCA cycle metabolite	2.0
Succinate	TCA cycle metabolite, electron donor for the respiratory chain	3.0
Malonate	Competitive inhibitor of the enzyme succinate dehydrogenase	0.5

Calculation of the return activated sludge (RAS) volume for the biological activation experiment under laboratory conditions

$$V_{RAS} = \frac{21000 \cdot 0.0001}{100} = \\ = 21.0\ dm^3 \quad (3)$$

Since the experimental volume V_{RAS} must not exceed the calculated volume $V_{RAScalc}$, we selected V_{RASexp} = 20 dm³ for the experiment (Zabara et al., 2023).

Calculation of the dosage of TCA cycle metabolites to be added to 20 dm³ of return activated sludge, accounting for external and internal factors influencing TCA metabolic processes (Table 2):

- Citrate mass: $M = 20 \cdot 0.4 = 8$ g
- Succinate mass: $M = 20 \cdot 0.4 = 8$ g
- Malonate mass: $M = 20 \cdot 0.05 = 1$ g

Conditions for the first stage of laboratory studies. A volume of 6.0 dm³ of RAS was collected from sludge chamber No. 6 (sludge concentration: 3.6 g/dm³, sludge pH: 7.12). 1.0 dm³ of the collected sludge was added to each of six glass laboratory containers (6 samples). Constant aeration was provided to each container. The addition of TCA metabolites was performed according to Table 2.

Second stage. Aeration of selected sludge (18–24 h) with the addition of CTC metabolites, B vitamins as cofactors and an inhibitor. Industrial stage: Introduction of activated sludge mixture (0.0001% of the total volume) into operating aeration tanks (Table 3).

Research parameters

For comprehensive monitoring of the state of the system, the following indicators were measured:

- biochemical and physicochemical: pH, temperature, salt content, dissolved oxygen concentration, redox potential (ROP) and BOD. These parameters allowed to control the stability of the environment and the efficiency of treatment;
- hydrobiological: sludge dose, SVI – to assess sedimentation properties;
- microbiological: number and species composition of indicator microorganisms, floc structure, presence of *Zoogloea ramigera* and filamentous bacteria - to assess the state of the biocenosis.

Equipment, methods and data analysis

Laboratory cultivators with a capacity of 20 l and Oxyboost APR-300 Plus microaerators (Aquael, Poland) were used for cultivation and aeration. Analysis of the aquatic environment was carried out using portable multiparameter devices HQ 2100 HACH and HQ 4300 HACH (Hach, USA).

Identification of AM microorganisms was carried out using a Motic BA310E light microscope (Germany) with a Moticam X5 Plus digital camera.

During microscopic analysis, visual control of the condition of cultures was carried out according to the methods (Eikelboom, 2000; Stier and Fischer, 1998). For quantitative assessment, at least 3 samples were analyzed with the calculation of the average value according to the Goryaev camera formula (Goltsev et al., 2025).

Statistical data processing and visualization were carried out using Microsoft Office tools (Microsoft Corporation, Redmond, WA, USA).

RESULTS AND DISCUSSION

In this study, we investigated the effectiveness of bioactivating activated sludge microorganisms using citrate and succinate (TCA cycle metabolites), as well as a mixture of citrate, succinate, and malonate.

At the initial stage of the research, the main attention was focused on monitoring the key physicochemical and technological parameters of the system, in particular the hydrogen value (pH) and the concentration of activated sludge. According to the data obtained, a steady trend

Table 2. Concentrations of TCA cycle metabolites for the first stage of the experiment

Sample №	TCA cycle metabolite and its concentration, %	Added solution volume, cm³	TCA metabolite concentration in the sludge mixture, %	Addition time interval, minutes
Sample 1	8% (w/v) citrate solution	10	0.08	–
Sample 2	8% (w/v) citrate solution	5	0.04	–
Sample 3	8% (w/v) Succinate solution	10	0.08	–
Sample 4	8% (w/v) Succinate solution	5	0.04	–
Sample 5	Mixture of 8% citrate and 8% succinate solutions	5 + 5	0.04 + 0.04	Simultaneously
Sample 6	Mixture of 8% citrate and 8% succinate solutions	5 + 5	0.04 + 0.04	After 30 minutes

Table 3. Biological activation of activated sludge microorganisms with TCA cycle substrates

Stages of biological activation	Biological activation procedure
Experimental (laboratory studies)	A 20.0 dm³ sample of return activated sludge is collected from the sludge chamber
	The collected activated sludge is aerated in a laboratory–scale cultivator
	A TCA substrate solution is added to the aerated activated sludge
	The treated sludge mixture is aerated for 18–24 hours
	The following components are added sequentially to the aerated mixed liquor: • clarified wastewater from the primary sedimentation tank; • B–group vitamin solutions as cofactors for enzymatic reactions; • an inhibitor solution (depending on the experimental requirements).
	The treated mixed liquor is aerated for 3 hours
	Microscopic analysis of the activated mixed liquor is performed
Industrial	Once positive microscopic results are obtained
	After 24 hours, microscopic analysis of the sludge in the aeration tanks is performed, and the hydrobiological parameters of the activated sludge are determined: sludge volume, sludge dosage, and sludge volume index (SVI)

towards an increase in the pH level was observed in each experimental sample. The most dynamic achievement of a neutral environment was recorded in Sample 6, where the pH value of 6.53 was achieved two days after the start of the experiment. In parallel, the highest concentration of activated sludge was recorded in this sample, which was 2.8 $g \cdot dm^{-3}$.

A comprehensive analysis of these indicators allowed us to draw a conclusion about the effectiveness of the selected stimulation strategy. Thus, the optimal activation conditions were determined: the best results were provided by the citrate and succinate addition regime, where the working concentration of each metabolite in the sludge mixture was 0.04% (Table 4).

After establishing the optimal dosages and confirming the positive impact of the selected concentrations on the overall condition of the system, we moved on to the next stage - detailed identification of activated sludge microorganisms. With the help of regular microscopic studies, a deep analysis of the transformation of the biocenosis was carried out, which allowed us to assess the qualitative changes in the species composition and structure of the microbiocenosis under the influence of the established doses of bioactivators.

Microscopic studies of activated sludge were aimed at a detailed analysis of the structural and quantitative indicators of the microbiocenosis (Figures 1–4), which allows for a prompt assessment of the condition of the treatment system. The study of the structure of the biocenosis was based on the identification of indicator microorganisms that reflect the efficiency of metabolic processes.

Particular attention was paid to the presence of the bacteria *Zoogloea ramigera*, which are responsible for the formation of dense flocs and the effective destruction of organic pollutants.

The quantitative content of free-floating and attached ciliates, as well as representatives of higher trophic levels, such as rotifers (Rotifera) and tardigrades (Tardigrada), was assessed.

Citrate treatment of activated sludge improves floc formation. This is attributed to the activating

Table 4. Results of the study on the parameters of biological sludge activation by TCA metabolites

Sample №	pH after addition of TCA metabolites	pH after 4 hours	Hafter 48 h with 25 cm³ clarified wastewater	Microbial species count after 48 h	Microbial species count after 48 h	Sludge concentration, g·dm⁻³ after 2 days
Sample 1	4.57	4.84	5.26	10	$20 \cdot 10^3$	2.4
Sample 2	5.60	6.96	8.36	18	$41 \cdot 10^3$	2.6
Sample 3	4.69	4.84	5.03	8	$11 \cdot 10^3$	2.5
Sample 4	5.55	7.46	8.30	24	$50 \cdot 10^3$	2.7
Sample 5	4.71	4.84	5.42	6	$8 \cdot 10^3$	2.4
Sample 6	4.75	5.83	6.53	18	$14 \cdot 10^3$	2.8

effect of citrate on the synthesis of extracellular polymeric substances (Sun et al., 2026), which participate in the aggregation of activated sludge components (An et al., 2023).

The ability of certain bacteria to uptake citrate is utilized in the development of modern methods for measuring its concentration (Kanlaya et al., 2024). The application of citrate as a metabolic activator significantly enhances the structural and functional characteristics of activated sludge. The metabolic integration of citrate into microbiocenosis not only supports cellular energy needs but also facilitates a profound qualitative transformation of the biocenosis. According to the research findings, citrate treatment acts as a potent biological activation factor, leading to a substantial increase in the population of decomposer microorganisms. Specifically, a marked expansion in the ciliate community was observed (Table 5): Free-swimming ciliates increased 3.1-fold; Attached (sessile) ciliates increased 2.6-fold; Suctorial ciliates (Suctoria) showed the most significant growth, increasing 4.3-fold. This shift toward higher trophic levels indicates a more mature and efficiently functioning activated sludge biocenosis. Beyond population growth, citrate treatment addresses a critical operational challenge: sludge bulking. The study revealed that citrate effectively improves the sedimentation properties of the sludge by reducing the abundance of filamentous sulfur bacteria fivefold (Table 5). Since an overgrowth of filamentous organisms is a primary cause of high SVI (W.-M. Li et al., 2020; Sam et al., 2022) and the subsequent carryover of solids from secondary clarifiers (S. Li et al., 2024), this inhibitory effect is vital for maintaining effluent quality. The suppression of filamentous bacteria, combined with the stimulated growth of *Zoogloea ramigera* ensures a dense, well-settling sludge

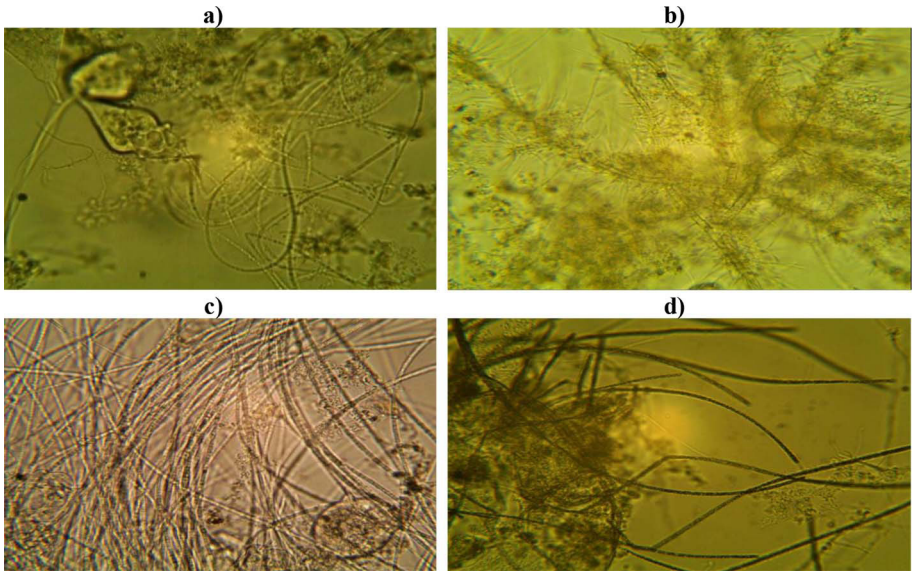

Figure 1. Micrographs of filamentous sulfur bacteria in activated sludge prior to biological activation (40 × 10 magnification): a) – Type 1863; b) – Type 1701; c) – Type 021N; d) – *Thiothrix*

Table 5. Changes in the abundance of activated sludge microorganisms during biological activation with citrate (citric acid)

Indicator groups of activated sludge microorganisms	Average abundance of microorganisms prior to biological activation (ind · 10^3·см$^{-3}$) *	Average abundance of microorganisms after biological activation (ind · 10^3·см$^{-3}$) *
Filamentous sulfur bacteria (filamentous index)**	5	0
Zoogloea ramigera ***	+	++
Sarcodines	59	250
Flagellates	7	3
Free–swimming ciliates	182	579
Sessile ciliates	471	1231
Suctorial ciliates	3	13
Rotifers	46	130
Total number of species	15	17

Note: * – determined using the Goryaev chamber formula (Goltsev et al., 2025). ** – determined by the filamentous index (D. H. Eikelboom, 1975; D. H. Eikelboom and Geurkink, 2002; H. D. Eikelboom, 2000; Yurchenko and Tkachenko, 2024). *** – determined by the degree of colony presence *Zoogloea* (D. H. Eikelboom and Buijsen, 1981).

structure (Figure 2). Thus, the use of citrate in ultra-low concentrations (Table 2) represents an effective ecobiotechnological strategy for the stable and intensified performance of wastewater treatment facilities.

Succinate plays a vital role in energy metabolism, serving as both a TCA cycle metabolite and a potent electron donor for the respiratory chain. The supplementary intake of succinate into cells positively affects their energy balance by activating oxidative phosphorylation. The application of succinate as a bioactivation factor leads to an increase in the species diversity of the activated sludge biocenosis, rising from 17 to 24 species (Table 6).

Treatment of activated sludge with succinate leads to a significant 6.3–fold increase in the number of rotifers, as well as the appearance of a substantial population of tardigrades. Furthermore, under the influence of succinate, the abundance of suctorial ciliates significantly increases by 3.8 times. Meanwhile, the numbers of *Zoogloea ramigera* and filamentous bacteria remain stable (Table 6).

The growth in the number of species and individuals of rotifers indicates that a sufficient food supply has emerged in the treatment system–specifically, a well–developed population of other microorganisms. A complex food chain is formed: bacteria – ciliates – rotifers. Additionally, the presence of many rotifers leads to further clarification and improvement in the quality of the treated wastewater (Figure 3).

The combined effect of citrate and succinate on the abundance of activated sludge microorganisms is stabilizing compared to the individual effects of these compounds (Table 7).

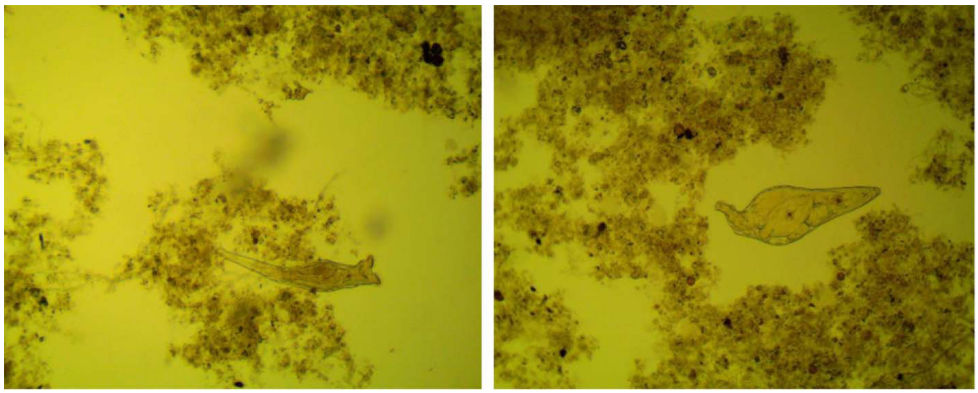

Figure 2. Micrograph demonstrating the absence of filamentous bacteria in the activated sludge biocenosis following the application of citrate as a bioactivator (40 × 10 magnification)

Table 6. Changes in the abundance of activated sludge microorganisms during biological activation with succinate (succinic acid)

Indicator groups of activated sludge microorganisms	Average abundance of microorganisms prior to biological activation (ind. · 10^3·cm^{-3}) *	Average abundance of microorganisms after biological activation (ind. · 10^3·cm^{-3}) *
Filamentous sulfur bacteria (filamentous index)**	3	2
Zoogloea ramigera ***	+	+
Sarcodines	250	517
Flagellates	3	49
Free–swimming ciliates	579	455
Sessile ciliates	1231	839
Suctorial ciliates	13	49
Rotifers	130	819
Tardigrades	0	33
Total number of species	17	24

Note: * – determined using the Goryaev chamber formula (Goltsev et al., 2025), ** – determined by the filamentous index (D. H. Eikelboom, 1975; D. H. Eikelboom and Geurkink, 2002; H. D. Eikelboom, 2000; Yurchenko and Tkachenko, 2024), *** – determined by the degree of colony presence *Zoogloea* (D. H. Eikelboom and Buijsen, 1981).

Under the influence of both succinate and citrate, the number of sessile (attached) ciliates increases 4.4–fold (Figure 4), which has a positive impact on the sedimentation processes of the activated sludge and promotes water clarification. Meanwhile, the population of *Zoogloea ramigera* and filamentous bacteria remains stable.

Identification of peritrichid ciliate species was carried out according to the following sources: (Fiałkowska et al., 2005; Pajdak-Stós et al., 2017). The species diversity of the activated sludge biocenosis varies depending on the presence of specific substrates in the wastewater. The use of succinic acid as a biostimulator of cellular metabolic processes may lead to hyperactivation of succinate dehydrogenase, potentially resulting in excessive generation of reactive oxygen species (ROS) and the development of oxidative stress. In this regard, to mitigate the imbalance within the free radical oxidation – antioxidant defense system, it is expedient to use malonic acid, which acts as a competitive inhibitor of succinate dehydrogenase (Chouchani et al., 2014; Dimroth and Hilbi, 1997). Therefore, in combination with Krebs cycle metabolites, malonate can be applied to regulate the intensity of oxidation processes in microbial cells.

According to the results of the study, the combined application of citrate, succinate, and malonate does not have a pronounced effect on the abundance or species composition of the activated sludge biocenosis (Table 8).

Apparently, under these conditions, the effect of citrate and succinate as activators of energy metabolism is neutralized by the inhibitory effect of malonate on the TCA cycle. Thus, under the combined action of citrate, succinate, and malonate, the composition of the activated sludge biocenosis does not undergo significant changes and remains stable.

Sludge concentration and index were controlled throughout the experiment. Biological activation with citrate, succinate, and malonate resulted in a significant reduction in the sludge index. The post–activation index value of 195 demonstrates effective prevention of sludge carryover in secondary clarifiers (Table 9).

The results obtained fully confirmed our working hypothesis that the regulation of metabolic activity through substrates of the tricarboxylic acid cycle (TCA) allows to intensify the processes of biological wastewater treatment. The established effect of improving the sedimentation properties of sludge due to a decrease in the content of filamentous bacteria and an increase in the number of ciliates and rotifers (Rotifera) is consistent with classical works on the hydrobiology of activated sludge (Minnie et al., 2022; Wei et al., 2023). However, our study demonstrates higher efficiency when using ultra-low concentrations of metabolites (0.1–10 mM), which avoids the disruption of the ionic balance of cells that some authors have warned about. (Koenigsberg et al., 2020). In particular,

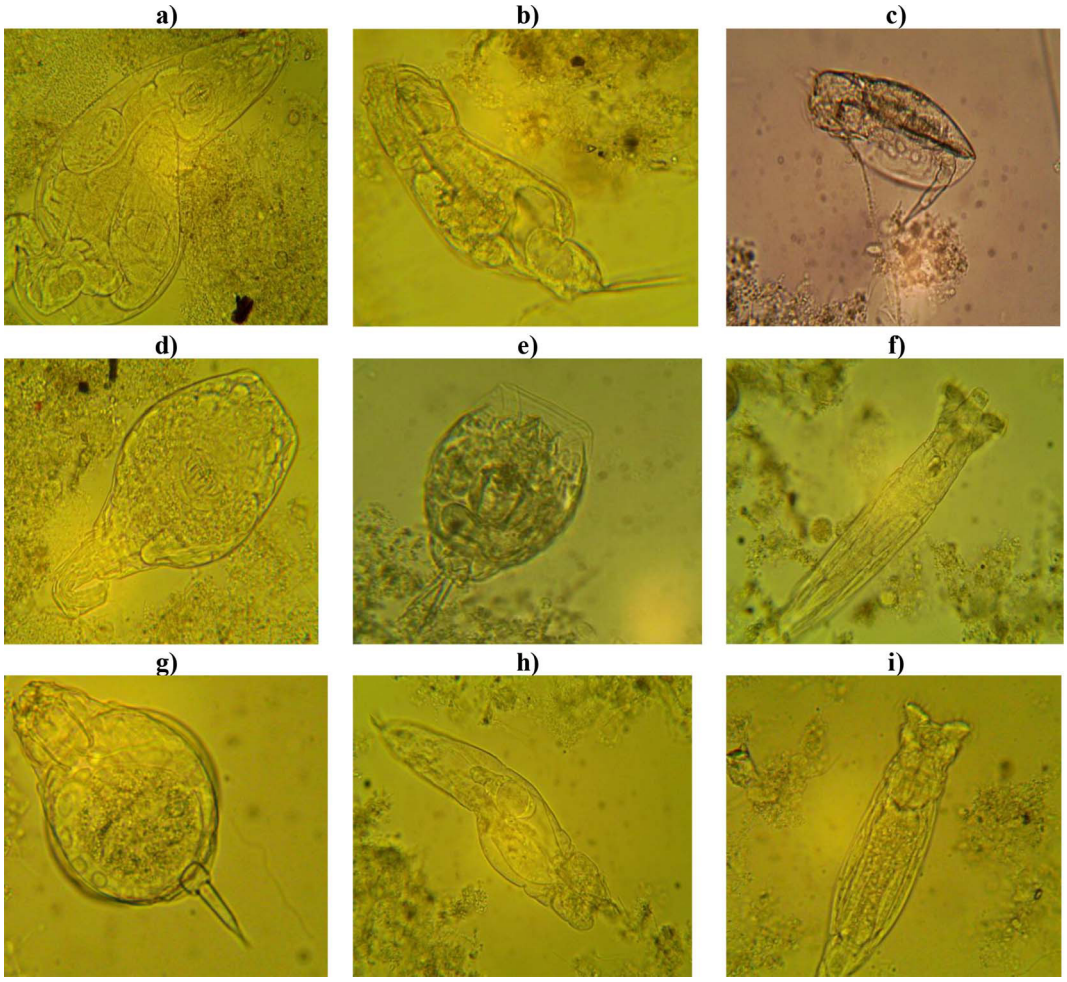

Figure 3. Micrograph showing the increase in rotifer diversity following biological activation with succinate (40 × 10 magnification): a), b), c) – rotifer species present prior to bioactivation. (a) – *Rotaria rotatoria*; b) – *Cephalodella incila*; c) – *Lepadella rhomboides*;) d), e), f), g), h), i) – additional rotifer species observed after bioactivation with succinate. (d) – *Eosphora ehrenbergi*; e) – *Lecane flexilis*; f) – *Rotaria tardigrada*; g) – *Lecane closterocerca Lepadella ovalis*; h) – *Encentrum lupus*; i) – *Philodina roseola*). Identification of rotifer species and individuals was performed according to (Fiałkowska et al., 2005; Pajdak-Stós et al., 2017)

Table 7. Changes in the abundance of activated sludge microorganisms during biological activation with citrate and succinate

Indicator groups of activated sludge microorganisms	Average abundance of microorganisms prior to biological activation (ind. · 10^3·cm^{-3}) *	Average abundance of microorganisms after biological activation (ind. · 10^3·cm^{-3}) *
Filamentous sulfur bacteria (filamentous index)**	2	2
Zoogloea ramigera ***	+	+
Sarcodines	60	70
Free–swimming ciliates	44	34
Sessile ciliates	111	489
Suctorial ciliates	10	8
Rotifers	294	276
Total number of species	26	26

Note: * – determined using the Goryaev chamber formula (Goltsev et al., 2025), ** – determined by the filamentous index (D. H. Eikelboom, 1975; D. H. Eikelboom and Geurkink, 2002; H. D. Eikelboom, 2000; Yurchenko and Tkachenko, 2024), *** – determined by the degree of colony presence *Zoogloea* (D. H. Eikelboom and Buijsen, 1981).

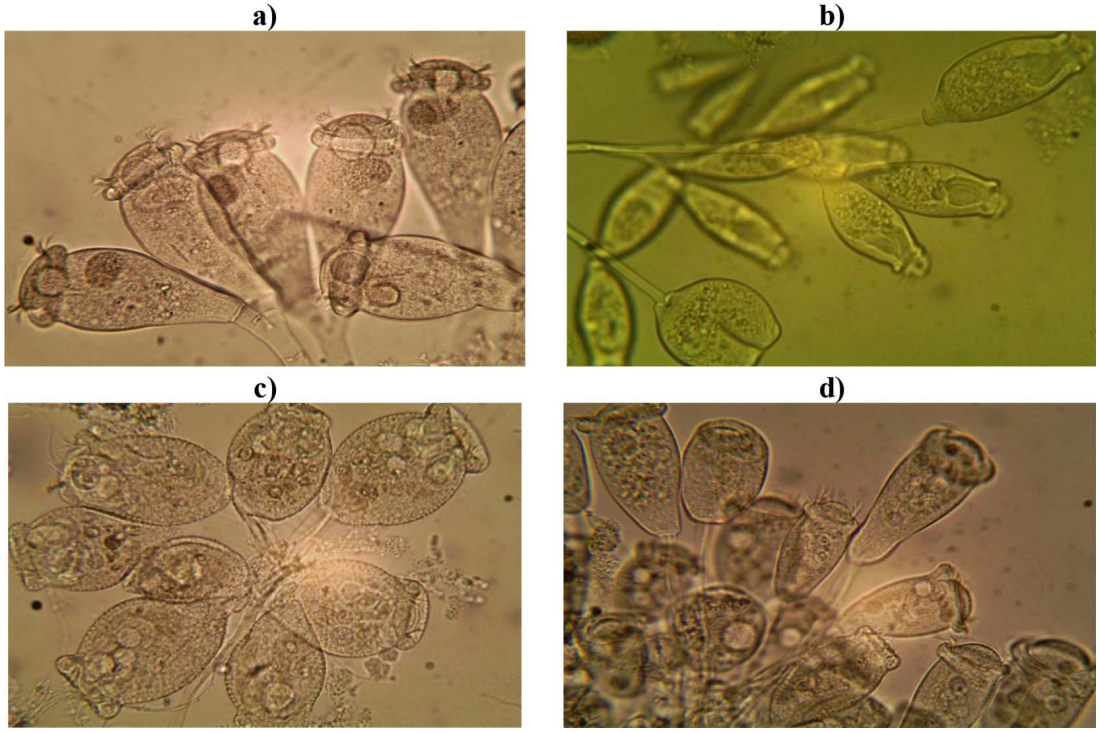

Figure 4. Micrograph showing the emergence of additional colonies of sessile ciliates following biological activation with citrate and succinate (40×10 magnification): a) – *Epistylis urceolata* colony; b) – *Opercularia coarctata* colony; c) – *Carchesium batorligetiense* colony; d) – *Vorticella convallaria* colony

Table 8. Changes in the abundance of activated sludge microorganisms during bioactivation with citrate, succinate, and malonate

Indicator groups of activated sludge microorganisms	Average abundance of microorganisms prior to biological activation (ind. · 10^3·cm^{-3}) *	Average abundance of microorganisms after biological activation (ind. · 10^3·cm^{-3}) *
Filamentous bacteria (filamentous index)**	2	1
Zoogloea ramigera ***	+	+
Sarcodina	103	103
Free-swimming ciliates	59	64
Sessile ciliates	323	282
Suctoria	5	5
Rotifers	64	43
Total number of species	27	27

Note: * – determined using the Goryaev chamber formula, ** – determined by the filamentous index (D. H. Eikelboom, 1975; D. H. Eikelboom and Geurkink, 2002; H. D. Eikelboom, 2000; Yurchenko and Tkachenko, 2024), *** – determined by the degree of colony presence *Zoogloea* (D. H. Eikelboom and Buijsen, 1981).

the stability of the biocenosis in the presence of malonate as an inhibitor may seem controversial, since the literature often emphasizes the toxicity of inhibitors. However, we have proven that it is the selective and dosed inhibition of succinate dehydrogenase that allows preventing "overheating" of metabolism and maintaining a stable species composition, which is a more rational approach for wastewater treatment plants with uneven loading. Thus, the obtained data open new horizons for the implementation of controlled biostimulation systems, which will allow not only to reduce the sludge index and prevent biomass removal, but also to ensure the stable operation of industrial aeration tanks in critical operating modes.

Table 9. Results of activated sludge dosage and sludge index determination under various biological activation conditions at the wastewater treatment plant over a one–year period

Month	Average sludge dosage, g·dm^{-3}	Sludge volume index	Sludge bioactivation with TCA cycle metabolites
January	1.8	444	–
February	2.0	380	–
March	2.4	363	citrate
April	2.6	333	citrate
May	2.9	276	succinate
June	3.3	235	succinate
July	3.1	258	citrate+succinate
August	3.3	235	citrate+succinate
September	3.4	232	citrate+succinate+malonate
October	4.1	195	citrate+succinate+malonate
November	3.7	210	–
December	2.7	289	–

CONCLUSIONS

The conducted study was aimed at substantiating an ecobiotechnological approach to intensifying biological wastewater treatment by regulating the metabolic pathways of the activated sludge microbiocenosis. The findings confirm that the strategic application of TCA cycle intermediates serves as a highly effective tool for directed biostimulation, ensuring both operational stability and enhanced purification.

The results of the study demonstrated that metabolic regulation via citrate and succinate fundamentally transforms the structural and functional state of the activated sludge. It was established that citrate acts as a selective regulator that suppresses the growth of filamentous sulfur bacteria, thereby eliminating the primary cause of sludge bulking. Simultaneously, the introduction of these metabolites shifts the biocenotic balance toward higher trophic levels – specifically increasing the abundance of diverse ciliate groups and rotifers – which directly correlates with improved effluent clarification and lower SVI. The synergy between activators and the competitive inhibitor malonate provides a mechanism for stabilizing the microbial population, preventing the carryover of solids from secondary sedimentation tanks even under fluctuating loads.

The practical significance of these results lies in the development of a low-dosage, energy-efficient technique for managing wastewater treatment plants. This method allows for the intensification of treatment processes without large-scale infrastructural changes, relying instead on the inherent metabolic plasticity of the existing biomass.

Further research should focus on investigating the long-term effects of metabolic biostimulation on the removal of specific persistent organic pollutants and nutrients (nitrogen and phosphorus). Additionally, the transition from municipal to complex industrial wastewater environments remains unexplored, representing a promising direction for future studies to test the versatility of this ecobiotechnological approach.

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