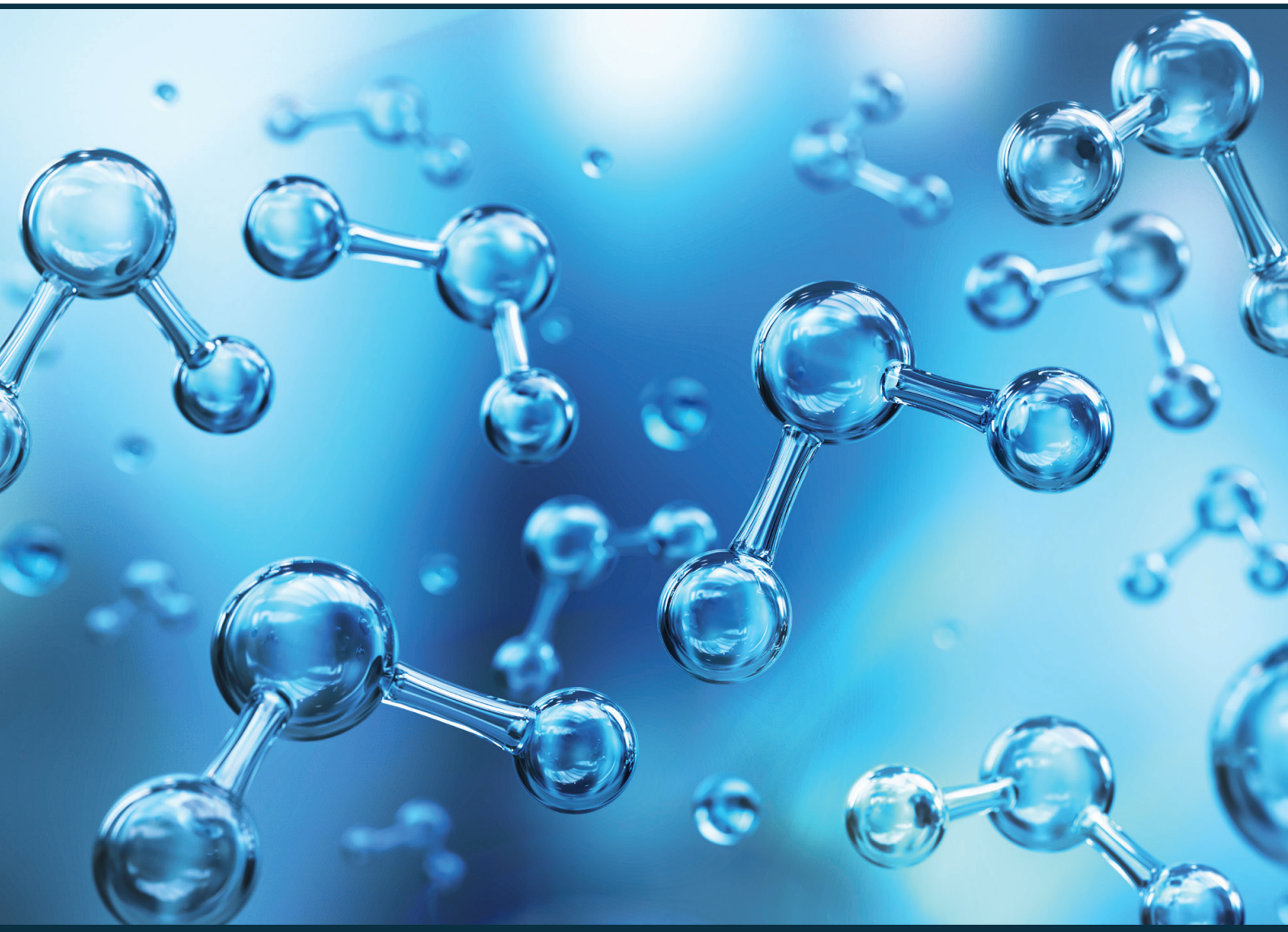
RIGA TECHNICAL UNIVERSITY

Elīna Strade

OPTIMIZATION OF THE BIOLOGICAL TREATMENT OF PHARMACEUTICAL PROCESSING WATERS UNDER MULTI-STRESS CONDITIONS

Summary of the Doctoral Thesis



RTU Press
Riga 2023

RIGA TECHNICAL UNIVERSITY
Faculty of Materials Science and Applied Chemistry
Institute of General Chemical Technology

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Doctoral Student of the Study Programme "Chemistry Technology"

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Summary of the Doctoral Thesis

Scientific supervisor
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RTU Press
Riga 2023

Strade, E. Optimization of the Biological Treatment of Pharmaceutical Processing Waters under Multi-stress Conditions. Summary of the Doctoral Thesis. Riga: RTU Press, 2023. 36 p.

Published in accordance with the decision of the Promotion Council "RTU P-01" of 9 August 2023, Minutes No. 04030-9.1/55.

ACKNOWLEDGMENTS

I thank my supervisor, Associate Professor Daina Kalniņa, for sharing knowledge and experience and for selfless help in the development of my Doctoral Thesis.

I thank my workplace JSC "Grindeks", for the opportunity to combine work with studies and conduct research in the wastewater treatment department. I thank the company's management and my colleagues for their supportive attitude in research performance.

My sincere thanks go to my family, who have always believed in my strength and have been a great support throughout my doctoral studies!

The Doctoral Thesis has been prepared using funding from the RTU Doctoral Student Grant for doctoral students of the Faculty of Materials Science and Applied Chemistry.

https://doi.org/10.7250/9789934229664
ISBN 978-9934-22-966-4 (pdf)

DOCTORAL THESIS PROPOSED TO RIGA TECHNICAL UNIVERSITY FOR THE PROMOTION TO THE SCIENTIFIC DEGREE OF DOCTOR OF SCIENCE

To be granted the scientific degree of Doctor of Science (Ph. D.), the present Doctoral Thesis has been submitted for the defence at the open meeting of RTU Promotion Council on 17 October 2023 14:00 at the Faculty of Materials Science and Applied Chemistry of Riga Technical University, 3/7 Paula Valdena Street, Room 272.

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DECLARATION OF ACADEMIC INTEGRITY

I hereby declare that the Doctoral Thesis submitted for review to Riga Technical University for promotion to the scientific degree of Doctor of Science (Ph. D.) is my own. I confirm that this Doctoral Thesis has not been submitted to any other university for promotion to a scientific degree.

Elīna Strade ……. (signature)
Date: ……

The Doctoral Thesis has been prepared as a collection of thematically related scientific publications complemented by summaries in Latvian and English. The Doctoral Thesis unites five scientific publications. The scientific publications have been written in English, with a total volume of 65 pages.

CONTENTS

ABBREVIATIONS

API	active pharmaceutical ingredient
BOD	biochemical oxygen demand
CFU	colony-forming unit
COD	chemical oxygen demand
EU	European Union
FDF	finished dosage form
GWWTP	wastewater treatment plant of JSC “Grindeks”
H	Shannon index
HPLC-MS	high-performance liquid chromatography-mass spectrometry
I. U.	International Unit
log K_{ow}	logarithm of the octanol-water partition coefficient
MP	skim milk powder
N_{tot}	total nitrogen
P_{tot}	total phosphorus
Q	flow, m^3/h
TOC	total organic carbon
WHO	World Health Organization
WWTP	wastewater treatment plant

GENERAL OVERVIEW OF THE THESIS

Topicality

According to the World Health Organization (WHO) data, water is the most used resource in the pharmaceutical industry [1]. It is used both as a raw material and solvent in the synthesis of active pharmaceutical ingredients (API) and in the production of finished dosage forms (FDF), as well as in the washing of equipment and for technical purposes-cooling and steam generation. The diverse use of water forces pharmaceutical factories to effectively manage both incoming and outgoing water flows in the production facility and to apply different treatment solutions in water preparation to guarantee that its quality meets regulatory requirements.

In the technological processes of pharmaceutical production, chemically polluted processing water flows occur, which differ significantly in terms of chemical composition, volume, and toxicity [2]–[4]. Depending on the specific API synthesis process, they may be polluted with various organic solvents, API residues, nitrogen-containing heterocyclic compounds, and various inorganic salts [2].

The high pollution concentration and flow fluctuations and the diverse composition of pollution of processing waters create multi-stress conditions. They can cause malfunctions in biological wastewater treatment plants (WWTP), negatively affecting their ability to provide a degree of wastewater treatment that meets the legislative requirements. Although biological treatment is one of the most commonly used technologies in pharmaceutical wastewater treatment [3], an increasing number of studies emphasize that biological WWTPs do not sufficiently treat wastewater from API residues and harmful compounds and, therefore, should be combined with physicochemical treatment technologies [4]–[6]. But this approach also has its limitations, as, for example, ozonation of processing water containing halogens can increase toxicity [7]. An alternative solution for increasing the efficiency of the biological treatment of processing waters may be the bioaugmentation of activated sludge systems with microorganism cultures [8], which, in turn, requires conducting a feasibility study, to determine the most suitable cultures for bioaugmentation. Additional challenges in the pharmaceutical industry are introduced by the changing demands for different APIs, which require a rapid reorientation of production and predicting how changes in production will affect the WWTP's ability to treat new types of pollution. Consequently, the development and implementation of new solutions that can be used to predict and preventively avoid or reduce the influence of various stress factors on the biological treatment process and improve the degree of wastewater treatment are highly topical.

The different range of raw materials used in API syntheses and the specificity of technological processes determine that the degree of pollution of pharmaceutical processing waters, the content of biogenic elements and salts can also differ significantly between production plants. However, the different engineering solutions applied in biological WWTPs can have different effects on the resilience of the system to various stress factors and changes in the composition of wastewater [9]. This requires the use of an individual approach also in

evaluating the stress factors affecting the biological treatment and in developing the most suitable process optimization solutions.

The transition to a circular economy model in the field of wastewater treatment requires a focus not only on the reduction and treatment of industrial pollution but also on the recovery and reuse of water and chemicals [10]. Despite the fact that water reuse has been recognized as a key action to reduce water consumption in production plants and contribute to the achievement of sustainable development goals [11], [12], there is a lack of studies analyzing aspects of the implementation of the circular economy in a pharmaceutical plant using a holistic approach, evaluating them in the context of the specific water quality requirements set for the pharmaceutical industry; therefore it has been chosen as a topical research direction.

Aims and objectives

The aims of the Doctoral Thesis are:

1) develop solutions for increasing the efficiency of biological treatment of pharmaceutical processing waters under multi-stress conditions;

2) identify the possibilities and limitations of the reuse of processing waters in the pharmaceutical industry after evaluating the use of water in the pharmaceutical production plant and the quality requirements of the water used in pharmaceutical production according to Good Manufacturing Practice guidelines, Pharmacopoeia monographs, and the guidelines from European Medicines Agency and the World Health Organisation.

To achieve the aims, the following objectives have been set:

1) based on the analysis of the operation of WWTP of JSC "Grindeks" (GWWTP) over a period of 3 years, identify the main stress factors affecting the biological treatment of pharmaceutical processing waters;

2) develop a method for assessing the toxicity of chemically polluted processing water to activated sludge microorganisms;

3) in laboratory conditions, determine the most suitable microorganism cultures for bioaugmentation for increasing the efficiency of API and chemical oxygen demand (COD) degradation in biological WWTPs exposed to multi-stress conditions;

4) recommend alternative sources of P for ensuring the optimal level of nutrients in the process of biological treatment of pharmaceutical wastewater to reduce the dependence on commercial H_3PO_4.

Scientific novelty and main results

As part of the Doctoral Thesis, solutions for mitigating the effects of multi-stress in pharmaceutical WWTPs have been developed, which are based on the toxicity analysis of individual flows, the application of bioaugmentation strategy to increase the efficiency of API and COD degradation, and include proposals for the reuse of chemically polluted pharmaceutical processing water flows in accordance with the principles of the circular economy.

Within the framework of the Doctoral Thesis, a new method for evaluating the toxicity of chemically polluted wastewater to the biocenosis of activated sludge has been developed, which has been introduced at GWWTP and can also be adapted to the work of laboratories of other biological WWTPs treating chemically polluted industrial wastewater, especially in production plants that generate wastewater flows of variable chemical composition containing potentially toxic chemicals.

As a result of the conducted study, for the first time, the limitations of the reuse of pharmaceutical processing water in the pharmaceutical production plant were identified, highlighting the specific role of the pharmaceutical industry among the water-intensive industrial sectors. The solutions proposed and implemented in practice for the reuse of processing waters as chemicals in various stages of the biological treatment of pharmaceutical wastewater show a new approach to promote the circular flow of materials in the production plant, thus reducing the consumption of chemicals and the costs of wastewater treatment.

The selection of microorganism cultures suitable for bioaugmentation provides an opportunity for further optimization of WWTP operation through bioaugmentation in order to improve the efficiency of degradation of chemical pollution, especially API, which may be particularly relevant when introducing new regulatory requirements for the control of API emissions from pharmaceutical production plants based on the European Union (EU) strategic approach to pharmaceuticals in the environment [13], and increasing the responsibility of pharmaceutical manufacturers for the treatment of API pollution arising from the use of their products, in the context of the planned changes in the Urban Wastewater Treatment Directive 91/271/EEC [14].

Structure and volume of the Thesis

The Doctoral Thesis was prepared as a collection of thematically related scientific publications on a) the development of the method for assessing the toxicity of chemically polluted pharmaceutical processing waters on activated sludge biocenosis (**Publication 1**); b) the application of bioaugmentation strategy for increasing API treatment efficiency in biological WWTPs (**Publication 2**); c) possibilities of increasing COD removal efficiency using bioaugmentation with selective microorganism cultures (**Publication 3**); (d) circular economy-based phosphorus management (**Publication 4**); e) possibilities and limitations of reuse of processing waters in the pharmaceutical industry (**Publication 5**).

Publications and approbation of the Thesis

The main results of the Doctoral Thesis are presented in 5 scientific publications. The research results were presented at 4 scientific conferences.

Scientific publications:

1. **Strade, E.,** Kalnina, D. Cost Effective Method for Toxicity Screening of Pharmaceutical Wastewater Containing Inorganic Salts and Harmful Organic Compounds. *Environ. Clim. Technol.* **2019**, *23*, 52–63.

2. Neibergs, M., **Strade, E.**, Nikolajeva, V., Susinskis, I., Rozitis, Dz., Kalnina, D. Application of bioaugmentation to improve pharmaceutical wastewater treatment efficiency. *Key Eng. Mater.* **2019**, *800*, 122–131.

3. Rozitis, D., **Strade, E.** COD Reduction Ability of Microorganisms Isolated from Highly Loaded Pharmaceutical Wastewater Pre-Treatment Process. *J. Mater. Environ. Sci.* **2015**, 6, 507–512.

4. Smol, M., Preisner, M., Bianchini, A., Rossi, J., Hermann, L., Schaaf, T., Kruopienė, J., Pamakštys, K., Klavins, M., Ozola-Davidane, R., Kalnina, D., **Strade, E.**, Voronova, V., Pachel, K., Yang, X., Steenari, B.-M., Svanström, M. Strategies for Sustainable and Circular Management of Phosphorus in the Baltic Sea Region: The Holistic Approach of the InPhos Project. *Sustainability* **2020**, *12*, 2567.

5. **Strade, E.**, Kalnina, D., Kulczycka, J. Water efficiency and safe re-use of different grades of water-Topical issues for the pharmaceutical industry. *Water Resour. Ind.* **2020**, *24*, 100132.

Publication in a collective monograph:

Olsson, L. E., **Strade, E**., Ekenberg, E., Torresi, E, Quadri, L., Morgan-Sagastume F. Il Sistema MBBR per il trattamento degli scarichi da industrie farmaceutiche: aspetti tecnici ed esperienze gestionali. In: *La gestione degli impianti di depurazione MBBR*; Vaccari, M., Favali, G., Eds.; Maggioli Editore: Santarcangelo di Romagna, **2021**, pp. 172–183 (Italian).

Participation in scientific conferences:

1. Neibergs, M., **Strade, E.**, Nikolajeva, V., Susinskis, I., Rozitis, Dz., Kalnina, D. Application of bioaugmentation to improve pharmaceutical wastewater treatment efficiency. *59th International Scientific Conference of Riga Technical University Section of Materials Science and Applied Chemistry*. Riga, Latvia, 26 October **2018**.

2. **Strade, E**. The pollution of water with pharmaceutical residues: a growing environmental concern. *60th International Scientific Conference of Riga Technical University Section of Materials Science and Applied Chemistry.* Riga, Latvia, 24 October **2019**.

3. **Strade, E**. Biological nitrogen removal from pharmaceutical wastewater. *61st International Scientific Conference of Riga Technical University Section of Materials Science and Applied Chemistry*. Riga, Latvia, 23 October **2020**.

4. **Strade, E**., Kalnina, D., Kulczycka, J. Water Diversity and Problems in Water Re-use in Pharmaceutical Enterprises. *12th Eastern European Young Water Professionals Conference*. Riga, Latvia, 1–2 April, **2021**.

MAIN RESULTS OF THE THESIS

1. Identification of stress factors affecting the biological treatment of pharmaceutical processing waters

By conducting a study of the technological process of GWWTP and evaluating the monitoring data on the characteristics of the chemical and physical parameters of wastewater and the microbiological parameters of activated sludge, five main factors influencing the biological treatment of pharmaceutical wastewater were identified: toxicity, unbalanced nutrient content, flow and concentration fluctuations, elevated wastewater temperature, and pH fluctuations (Fig. 1). Each of these factors cause stress to activated sludge microorganisms and negatively affects the operation of the biological treatment process, but most often the effects of these factors occur simultaneously, creating multi-stress conditions.

The impact of certain stress factors, such as pH, flow, and concentration fluctuations, on the efficiency of the biological wastewater treatment process can be reduced, technologically regulated, or completely eliminated by providing appropriate technological solutions already during the design of WWTPs [15], [16]. However, wastewater characteristics such as toxicity and nutrient composition cannot be predicted in the long term, as the composition of processing water can change dramatically depending on changes in the range of manufactured products and global demand for specific pharmaceutical products.

The solutions developed in the Doctoral Thesis for optimizing the biological treatment process of pharmaceutical processing waters under multi-stress conditions are reflected in the scientific publications, the connection of which with the set aims and objectives of the Doctoral Thesis is shown in Fig. 1.

Data on the technological process of GWWTP, characteristics of the chemical and physical parameters of influent wastewater of GWWTP, as well as activated sludge microbiological parameters used for the identification of stress factors, are summarized in an appendix available under request.

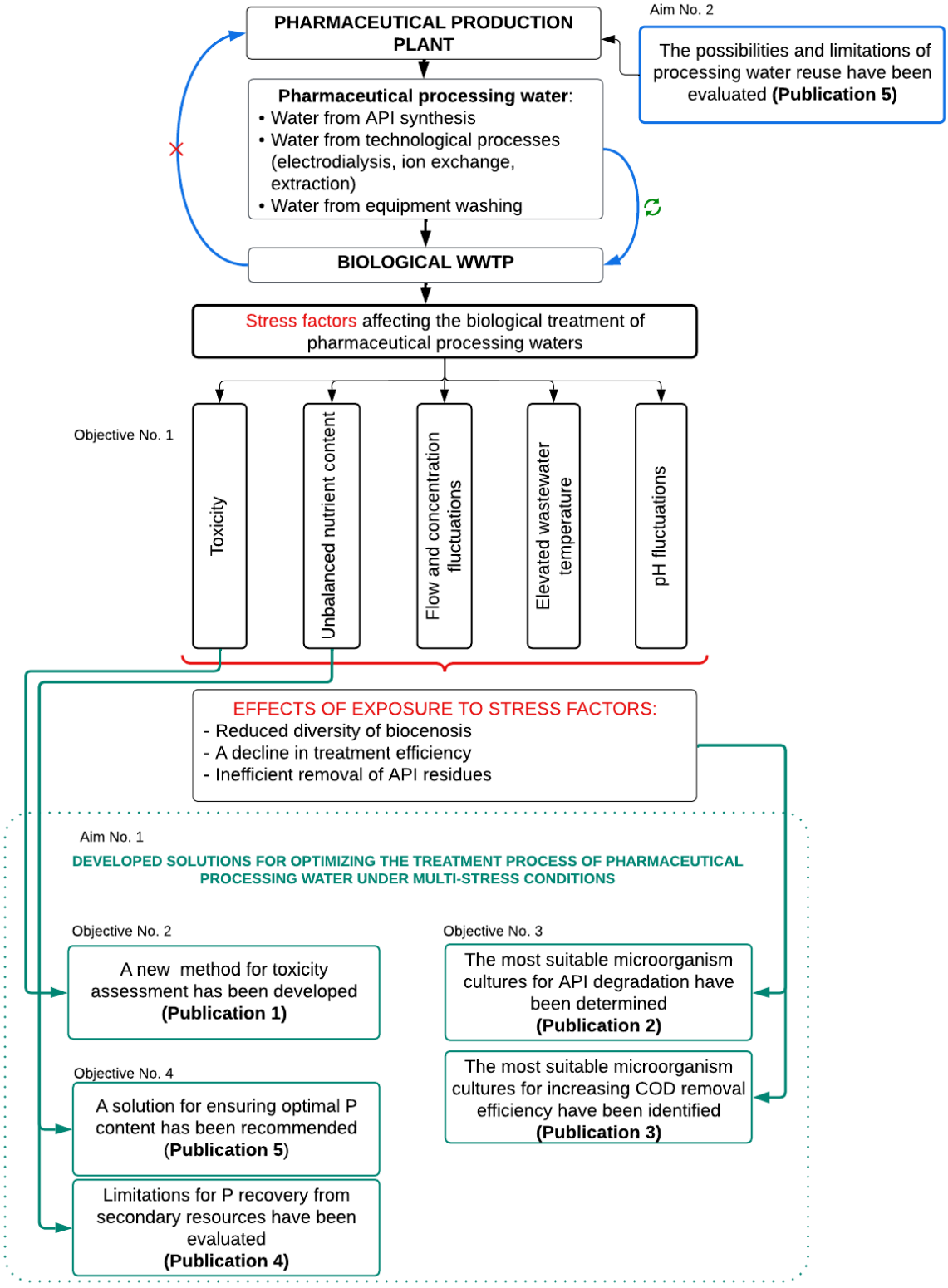


Fig. 1. Stress factors affecting the biological treatment of pharmaceutical processing waters and the structure of the Doctoral Thesis.

2. Assessment of the toxicity of pharmaceutical processing waters

The chemical characteristics of wastewater do not provide direct information about the toxicity of wastewater. Therefore, to prevent the death of activated sludge biocenosis under the influence of toxic substances and to avoid cases when the level of wastewater treatment does not comply with the requirements of the legislation, it is important, in addition to chemical analyses, to carry out toxicity tests and take preventive measures if toxicity is detected.

To objectively assess the toxicity of the influent water of WWTP, it is important to use activated sludge from a given treatment plant as test organisms since the result of the toxicity test depends on the test organism chosen and the reference function [17]. Studies have shown that test organisms not specific to the WWTP system, such as the luminescent bacteria *Vibrio qinghaiensis* and *Vibrio fischeri*, may show increased sensitivity, not reflecting the actual toxicity of the wastewater to the WWTP biocenosis [18]–[20], while in other cases, the short incubation time may lead to reduced toxicity results [21], resulting in an inadequate assessment of potential harm and toxicity risk.

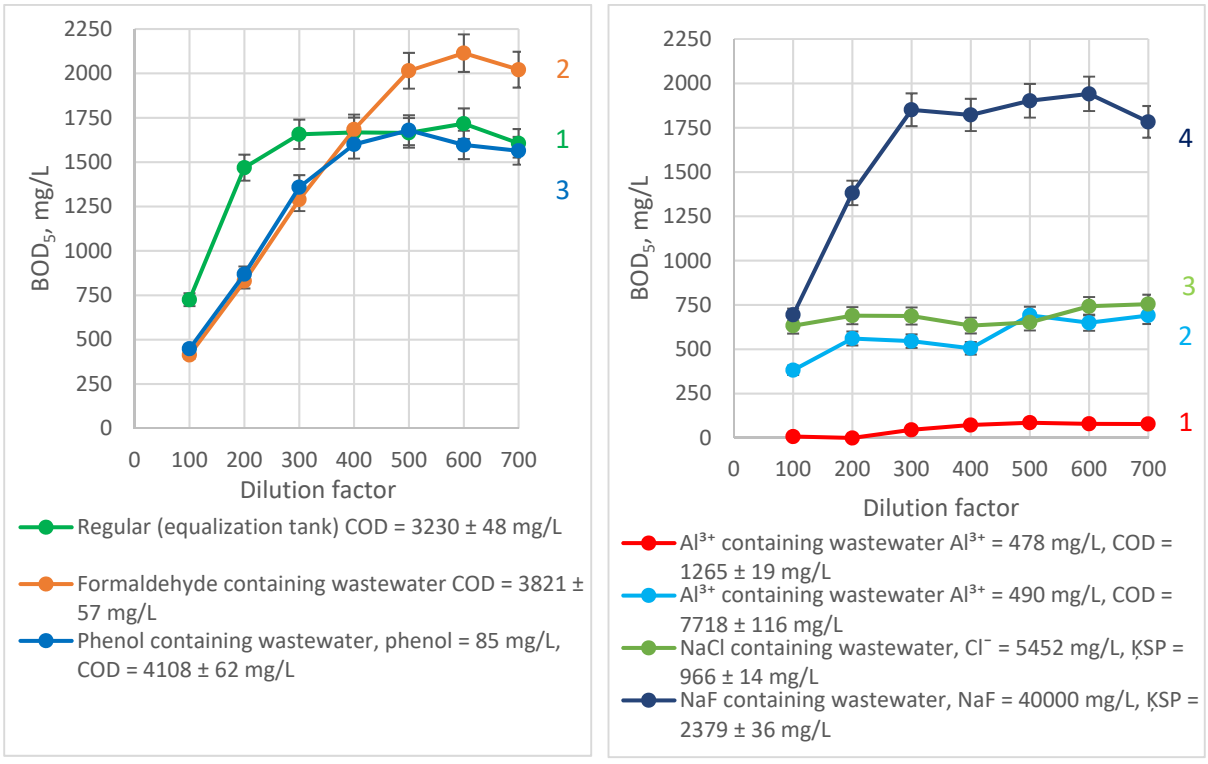
Publication 1 [22] presents the developed method for the systematic assessment of the toxicity and biodegradation potential of chemically polluted processing waters based on BOD measurements made in an extended range of initial concentrations. By applying this method, experimental characteristic curves are obtained that allow us to evaluate how the biodegradability and toxicity of wastewater to activated sludge microorganisms changes with dilution. If the BOD values increase markedly with increasing dilution, it indicates that the water contains substances that, in high concentrations, have an inhibitory effect on the activated sludge biocenosis and may cause disturbances in the treatment process. If the curve quickly reaches a plateau and the ratio BOD/COD is high (≥50 %), this indicates good biodegradability in the activated sludge system and a low level of toxicity [23]. Whereas, if the ratio BOD/COD remains low (<10 %) and does not change with dilution, then this indicates that the tested wastewater is toxic and the organic compounds in its composition are not biodegradable [24]; therefore, they cannot be discharged to biological WWTPs to avoid the toxic shock of microorganisms.

The application of the method is clearly demonstrated by analyzing the influent wastewater of GWWTP (from the buffer tank) and specific pharmaceutical processing water streams polluted with organic compounds and inorganic salts. The samples and the information on the main chemical pollutant and its concentration in the wastewater were received from the production department, and it was used to identify samples. Therefore, the COD analysis was used as a suitable indicator for the characterization of the total organic content of the wastewater.

Dilutions in the range from 100–700 were tested, which corresponds to the actual amount of wastewater generated per day in the manufacturing processes and their possible dilution in the buffer tank (equalization tank) of GWWTP. Seeded dilution water was used as a blank sample. The oxygen consumed over a 5-day incubation period of the blank sample shall not exceed 1.5 mg/L of oxygen. Tests were performed in duplicate.

Figure 2 a) shows that the BOD values of the analyzed buffer tank wastewater sample change little with dilution. An inhibitory effect on activated sludge microorganisms is observed at the dilution factor of 100, however the curve reaches the plateau phase already at the dilution factor of 200. The character of the curve shows that the wastewater is relatively well biodegradable, and no strong toxic effect on activated sludge microorganisms is expected. Depending on the degree of dilution, the ratio of BOD/COD varies from 22–53 % (Table 1).

The obtained experimental curves show that activated sludge microorganisms are strongly inhibited by pharmaceutical processing waters containing phenol and formaldehyde. As can be seen in Fig. 2 a), the character of the toxicity curves obtained during the testing of both wastewater flows is similar – the biodegradability strongly depends on the degree of dilution of the sample, and the curve reaches the plateau phase only at the dilution degree of 500 that corresponds to the degree of dilution of the wastewater sample in the test solution. To prevent the risks of intoxication of microorganisms, this type of wastewater, which is biodegradable but in high concentrations toxic to the activated sludge biocenosis, can be collected in separate reservoirs and dosed in a controlled manner in the total wastewater flow.



a) b)

Fig. 2. Toxicity characteristic curves for a) buffer tank wastewater, processing waters containing phenol and formaldehyde; b) processing waters containing inorganic salts.

When evaluating the toxicity of pharmaceutical wastewater containing inorganic salts, it was concluded that aqueous solutions containing Al^{3+} ions, which are produced in the synthesis of API milnacipran hydrochloride, where $AlCl_3$ is used as a Lewis acid, have a highly toxic effect on the biocenosis of activated sludge. Toxicity tests were performed on wastewater samples with different organic matter content (COD concentration) and practically the same

Al^{3+} concentration. As can be seen in Table 1, the ratio BOD/COD for both samples remains low even at a high degree of dilution of the sample and does not exceed 10 %, indicating that these wastewaters are practically non-degradable. Moreover, the toxicity does not change depending on the COD, which confirms that the toxic effect is caused by Al^{3+}. The obtained results allow us to conclude that wastewater containing Al^{3+} should not be let into biological treatment plants, as it can cause a toxic shock to microorganisms and destroy the activated sludge biocenosis. Such toxic and non-biodegradable wastewater streams must be separately collected at production sites and pre-treated prior to entering biological WWTPs or disposed of as hazardous waste.

Analysing NaCl-containing wastewater flow, no toxic effect on activated sludge microorganisms was detected when comparing at equal dilutions (Fig. 2 b)). This can be explained by the fact that the NaCl content in the tested wastewater sample corresponds to the salt concentration in the physiological solution and the BOD measurements were performed in a high dilution. The experimental results allow us to conclude that the organic matter in the wastewater has a good biodegradability – the BOD/COD ratio is greater than 65 % (Table 1). When performing a toxicity test for NaF containing wastewater flow, the inhibitory effect on activated sludge microorganisms was observed only at the dilution factor of 100 (Fig. 2 b)), but at the dilution factor of 200, the BOD/COD ratio already reached 58 % (Table 1), indicating that at this dilution, fluorides no longer affect the biodegradation of organic matter present in wastewater.

Table 1

BOD/COD ratio (%) depending on the degree of dilution of wastewater

Type of wastewater	COD, mg/L	BOD/COD ratio, %						
		Dilution factor						
		100	200	300	400	500	600	700
Regular wastewater (equalization tank)	3230 ± 48	22.4	45.4	51.3	51.6	51.5	53.2	49.7
Formaldehyde containing wastewater	3821 ± 57	10.8	21.7	33.7	44.1	52.7	55.3	52.9
Phenol containing wastewater Phenol = 85 mg/L	4108 ± 62	10.9	21.2	33.1	38.9	40.9	38.9	38.1
Al^{3+} containing wastewater Al^{3+} = 478 mg/L	1265 ± 19	0.6	0.5	3.6	5.8	6.8	6.3	6.3
Al^{3+} containing wastewater Al^{3+} = 490 mg/L	7718 ± 116	5.0	7.3	7.1	6.6	9.0	8.4	9.0
NaCl containing wastewater Cl^{-} = 5452 mg/L	966 ± 14	65.5	71.4	71.3	65.6	67.5	76.9	78.2
NaF containing wastewater NaF = 40000 mg/L	2379 ± 36	29.2	58.1	77.8	76.6	79.9	81.6	75.0

The data obtained during the experiment demonstrate that the ratio of BOD/COD for pharmaceutical processing waters can vary up to ten times at the same dilutions (Table 1). This confirms that waters from various technological processes differ significantly in their biodegradability and toxicity to activated sludge microorganisms, which is directly influenced by the different compositions of pollutants and the ecotoxicity of compounds.

The results show that the developed toxicity method is sufficiently sensitive and allows comparing different processing water flows to each other and identifying compounds toxic to WWTP biocenosis. The extended range of initial concentrations used in the testing shows at what dilution the toxicity of the wastewater changes, which can be used to predict the impact on the treatment process of increasing product production volumes. A significant advantage of the method is the relatively low cost and the ability to determine the effect of toxic substances on the biocenosis of specific treatment plants, using it as seed material.

3. Selection of microorganism cultures suitable for bioaugmentation to increase target API and COD degradation in pharmaceutical WWTPs

API residues that enter the environment with insufficiently treated pharmaceutical wastewater have received increased attention in recent decades, as studies have shown that pharmaceutical production plants can be an important point source of API emissions [25], [26]., Still these substances are biologically active even at very low concentrations. Several studies confirm that their release into the aquatic ecosystem leads to negative consequences for aquatic organisms and human health [27].

Bioaugmentation of activated sludge systems with selective cultures of microorganisms is one of the technologies that can be applied to increase the efficiency of API degradation in biological WWTPs of both municipal and pharmaceutical wastewaters [28], [29]. The selection of suitable cultures for bioaugmentation is one of the key aspects that determines the effectiveness of a bioaugmentation strategy. Cultures must not only provide a high ability to degrade target compounds but also be resistant to wastewater environmental conditions, toxicity, and be able to survive in competition with other activated sludge microorganisms [30].

The range of pollutants in pharmaceutical wastewater is very wide. Therefore, in order to ensure that bioaugmentation is effective and that the total water pollution indicators in the effluent of WWTP comply with the requirements of the legislation, the microorganism cultures used should not only provide a high degree of API treatment but also be able to effectively degrade other chemical compounds present in water. Studies by other authors confirm that the bioaugmentation strategy allows for intensifying the degree of treatment of pharmaceutical wastewater and improving the efficiency of COD degradation [31], therefore, within the framework of the Doctoral Thesis, the most suitable microorganisms for bioaugmentation were searched, which can provide both a high degree of degradation of the target API and effectively reduce COD, and would be suitable candidates for increasing the efficiency of wastewater treatment in GWWTP.

Publication 2 [32] of the Doctoral Thesis presents the screening results obtained by testing the ability of ten bacteria, ten yeasts and three filamentous fungi isolated from the GWWTP-

activated sludge to degrade three APIs with different chemical structures and therapeutic effects – the cyclic nonapeptide oxytocin, the heterocyclic nitrogen compound zopiclone and a hydrazine derivative meldonium dihydrate (Table 2). These APIs were chosen as model compounds for the study because their microbiological degradation has not been studied so far, but according to the volume of production, they occupy a significant place in the product range of JSC "Grindeks", and during their production, a large amount of chemically polluted processing water is generated. Table 2 clearly shows that the selected compounds differ significantly in terms of their chemical properties, which determine their behavior in water, i.e. water solubility and logarithmic values of octanol-water partition coefficient log K_{ow}.

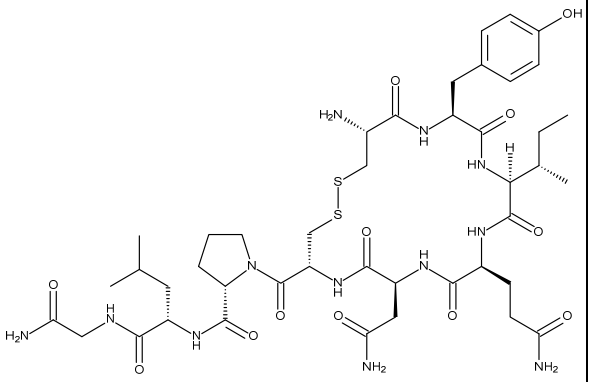
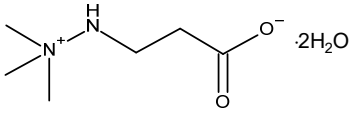
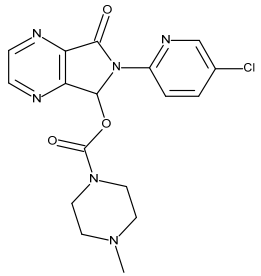
The research was conducted in laboratory-scale experiments, testing the ability of microorganisms to eliminate API as a sole carbon source and by cometabolism with the addition of skim milk powder (MP) as an additional nutrient. MP was chosen as an additional nutrient source based on the results published by Quintana et al., which demonstrate that the addition of powdered milk allows co-metabolic degradation of those APIs that could not be utilized as the sole source of carbon and energy [33]. API removal efficiency % during the 168 h incubation period determined by HPLC-MS analysis is shown in Tables 3 and 4. The chosen incubation period corresponds to the longest possible hydraulic retention time in GWWTP. The elimination of API from the solution is a total result of sorption and biodegradation. In our experiments these two processes were not differentiated; however, logarithmic values of octanol-water partition coefficient log K_{ow} for selected API are low (Table 2), indicating that sorption onto activated sludge is of minor importance in the removal process [34].

Most of the tested microorganism cultures, incl. bacteria – *Acinetobacter schindleri, Bacillus cereus, Chryseobacterium balustinum, Myroides odoratus, Sphingobacterium thalpophilum*; yeasts – *Apiotrichum montevideense, Cutaneotrichosporon arboriforme, Trichosporon asahii* and the filamentous fungus *Fusarium udum* within 168 h completely (>99 %) utilized all oxytocin in a solution where API was the sole carbon source. The addition of skim milk powder in a ratio of 1:10 to the starting concentration of API significantly improved the removal efficiency of oxytocin in biodegradation tests with the yeasts *Candida inconspicua 1, Cutaneotrichosporon cutaneum, Farysia acheniorum,* and the filamentous fungus *Talaromyces radicus.*

Studies show that the oxytocin molecule is not stable in aqueous solutions, and at elevated temperatures its degradation occurs, and biological activity is lost [35], [36]. During the bioaugmentation experiments, oxytocin degradation in the control sample without the addition of microorganisms was 12 % within 168 h, which confirms that microbiological degradation was still the primary form of oxytocin degradation in the conducted studies.

Table 2

APIs used in bioaugmentation studies

API	Chemical formula and structure	Molecular weight, g/mol	CAS No.	Solubility in water, g/L	log K_{ow}	Therapeutic class
Oxytocin	$C_{43}H_{66}N_{12}O_{12}S_2$ 	1007.19	50-56-6	12	–6.27	Exogenous hormone
Meldonium dihydrate	$C_6H_{14}O_2N_2 \cdot 2\ H_2O$ 	182.26	86426-17-7	20.2	0.45	Cardiovascular
Zopiclone	$C_{17}H_{17}ClN_6O_3$ 	388.81	43200-80-2	0.151	1.54	Sedative/ hypnotic nonbarbiturate

Limited information is available in the literature on the biodegradability of the heterocyclic nitrogen compound zopiclone. When treating hospital wastewater in a membrane bioreactor, its removal efficiency was less than 10 % [37]. Zopiclone removal efficiency varied significantly between different groups of microorganisms in our studies. The yeast *Apiotrichum domesticum* removed >99 % of zopiclone from the test solution in the presence of skim milk powder, while the other yeasts showed a limited ability to degrade zopiclone. *Pseudomonas putida* and *Moraxella osloensis* were identified as the most effective bacterial cultures for the selective degradation of zopiclone and showed 85 % and 89 % removal efficiency, respectively, over a 168 h incubation period. Whereas the filamentous fungi *Fusarium solani* and *Fusarium udum* removed more than 90 % of zopiclone both in tests where this API was the sole carbon source and in the presence of skim milk powder, indicating the high potential for further application of these cultures for the purpose of API degradation.

Meldonium dihydrate was identified as the most persistent compound against biodegradation. Most of the microorganism cultures tested could not degrade meldonium dihydrate as the sole carbon source. Yeast *Apiotrichum domesticum* degraded 65 % of meldonium dihydrate in 168 h in the presence of MP, while other yeast cultures showed low removal efficiency (<20 %). Out of all ten tested bacterial species, only *Sphingobacterium thalpophilum* showed a high removal efficiency of meldonium dihydrate in the presence of skim milk powder – 91 % within 168 h, while the other bacteria utilized meldonium dihydrate in the range of 1.3–40 % during the 168 h incubation period, both in test solutions where meldonium dihydrate was the sole carbon source, both in the presence of MP. Filamentous fungi *Fusarium solani* and *Fusarium udum* in test solutions, where meldonium dihydrate was the sole carbon source, showed 21 % and 46 % removal efficiency, respectively, but in the presence of skim milk powder, the removal efficiency increased significantly, reaching 91 % and 94 %, respectively.

Table 3

API removal efficiency (%) after 168 h incubation period in test solutions where API is the sole carbon source (initial concentration of API 20 mg/L) and in the presence of MP (initial concentration of API 5 mg/L) using bioaugmentation with bacterial cultures

	Oxy-tocin	Oxy-tocin + MP	Zopi-clone	Zopi-clone + MP	Meldonium dihydrate	Meldonium dihydrate + MP
Acinetobacter schindleri	>99	>99	54	82	4.7	35
Aeromonas caviae	99	99	59	71	1.3	1.8
Bacillus cereus	>99	>99	63	60	3.0	12
Chryseobacterium balustinum	>99	>99	68	81	5.2	13
Comamonas testosteroni	99	>99	63	67	12	23
Moraxella osloensis	69	57	89	91	14	40
Myroides odoratus	>99	>99	64	60	24	27
Pseudomonas aeruginosa	58	57	50	79	10	18
Pseudomonas putida	73	68	85	87	19	34
Sphingobacterium thalpophilum	>99	>99	75	84	17	91
Control sample without microorganisms	12	12	25	17	17	10

Although the degradation processes and metabolites of meldonium in the human and animal body have been relatively extensively studied [38]–[41], there are no studies in the literature that analyze the mechanisms of microbial degradation of this API in activated sludge systems or with selective microbial cultures and evaluate the role of cometabolism in the degradation process. The low results of meldonium elimination show that most of the studied microorganisms do not have the appropriate enzymes to degrade this API, but the microorganisms *Sphingobacterium thalpophilum, Fusarium solani,* and *Fusarium udum* can efficiently degrade meldonium only in the presence of an additional carbon source.

Table 4

API removal efficiency (%) after 168 h incubation period in test solutions where API is the sole carbon source (initial concentration of API 20 mg/L) and in the presence of MP (initial concentration of API 5 mg/L) using bioaugmentation with yeast and filamentous fungal cultures

	Oxy-tocin	Oxy-tocin + MP	Zopi-clone	Zopi-clone + MP	Meldonium dihydrate	Meldonium dihydrate + MP
Yeasts						
Apiotrichum domesticum	98	>99	42	>99	17	65
Apiotrichum montevideense	>99	>99	21	44	14	11
Candida inconspicua 1	13	51	29	35	6.7	8.5
Candida inconspicua 2	98	>99	56	26	12	3.5
Cutaneotrichosporon arboriforme	>99	>99	0.7	32	6.0	8.0
Cutaneotrichosporon cutaneum	1.0	7.1	16	29	1.6	15
Farysia acheniorum	11	>99	14	28	13	25
Rhodotorula mucilaginosa	91	61	16	47	13	14
Saprochaete gigas	45	81	64	59	7.6	16
Trichosporon asahii	>99	>99	7.7	24	4.4	2.8
Filamentous fungi						
Fusarium solani	99	>99	98	>99	21	91
Fusarium udum	>99	>99	>99	91	46	94
Talaromyces radicus	12	98	2.3	26	5.8	11
Control sample without microorganisms	12	12	25	17	17	10

Summarizing the obtained API degradation screening results, it was concluded that the most effective cultures for target API degradation are the bacterium *Sphingobacterium thalpophilum* and the filamentous fungi *Fusarium solani* and *Fusarium udum*. These microorganism cultures were able to eliminate all three tested APIs with an efficiency above 84 % over a 168 h incubation period.

In other API degradation studies, the fungus *Fusarium solani* has been identified as a suitable culture for the removal of API diclofenac and ketoprofen from wastewater [42], while the bacterium *Sphingobacterium thalpophilum* has shown the ability to degrade the antibiotic nitrofurantoin [43]. To the best of our knowledge, the role of *Fusarium udum* in increasing the efficiency of API degradation has not been investigated so far.

The experimental data summarized in **Publication 3** [44] show that among the 65 microorganism isolates tested, the bacterium *Sphingobacterium thalpophilum* and the filamentous fungi *Fusarium solani* and *Fusarium udum* are among those cultures that have also shown the highest COD reduction when treating pharmaceutical wastewater. Pharmaceutical wastewater samples for the tests were taken from the buffer tank of the GWWTP, into which processing waters from all production sites enter and mix before being discharged for treatment in the first bioreactor. Using such a complex sample, it is possible to investigate the ability of microorganisms to degrade a wide range of chemical compounds, which is an essential condition for bioaugmentation to be effective under changing production conditions.

As can be seen in the results summarized in Figs. 3 and 4, during 120 h of incubation, the bacterium *Sphingobacterium thalpophilum* and the filamentous fungi *Fusarium solani* and *Fusarium udum* reduced COD by 75.9 %, 89.4 %, and 88.7 % respectively. Since COD is a combined indicator of the level of organic pollution in wastewater and is also included in the regulatory requirements governing the maximum allowable pollutant concentrations in the discharge of WWTP, the high COD reduction rates obtained in microorganism screening tests confirm that the cultures of *Sphingobacterium thalpophilum, Fusarium solani* and *Fusarium udum* could be suitable candidates for increasing the treatment efficiency of pharmaceutical wastewater by bioaugmentation.

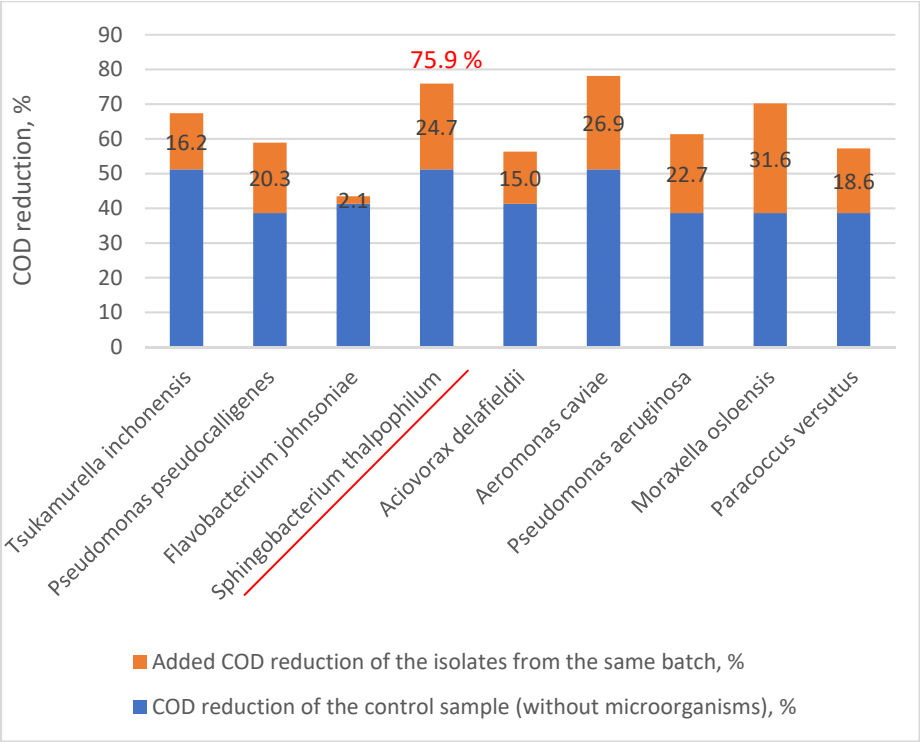


Fig. 3. COD reduction efficiency using bioaugmentation with bacterial cultures.

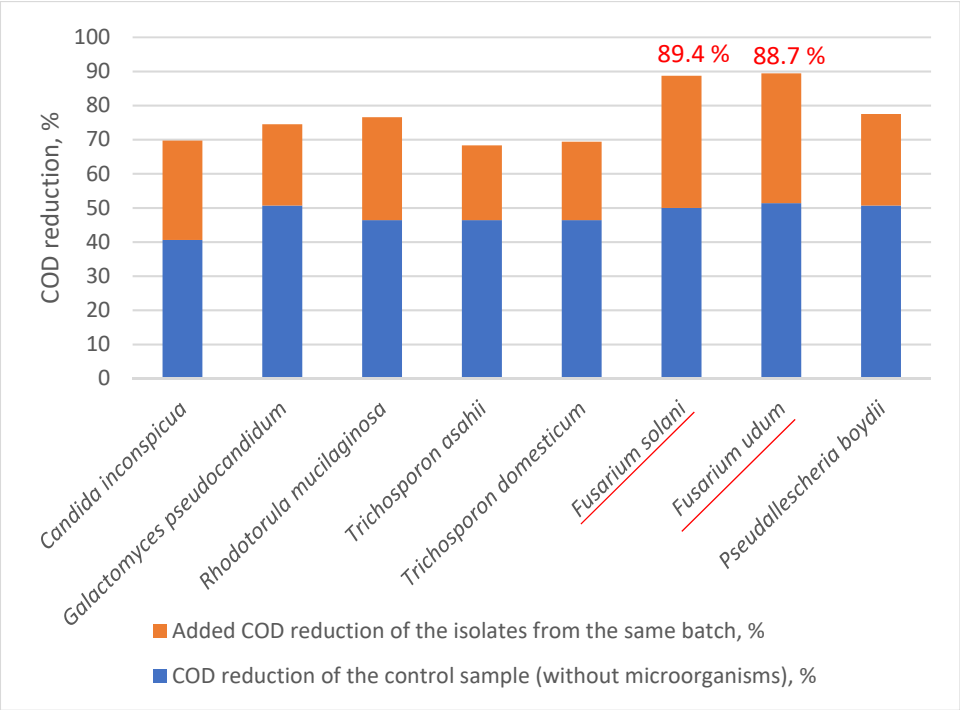


Fig. 4. Efficiency of COD reduction using bioaugmentation with yeast and filamentous fungal cultures.

4. Solutions to the problem of phosphorus deficiency in pharmaceutical wastewater biological treatment systems

According to the wastewater pollution monitoring data from GWWTP, the median COD and N_{tot} concentration of the influent water is 5500 mg/L and 160 mg/L, respectively, while the average PO_4-P content is only 3 mg/L, which is insufficient to ensure the optimum ratio COD : N : P of 100 : 5 : 1 for the treatment process. The lack of phosphorus in wastewater impairs the efficiency of biological treatment, promotes the development of filamentous bacteria (Fig. 5), and leads to an increase in the volume and structural change of sludge, which negatively affects its settleability and dewaterability [45]–[48]. To ensure an optimal P level for the biological treatment process, H_3PO_4 is usually used as an additional P source.

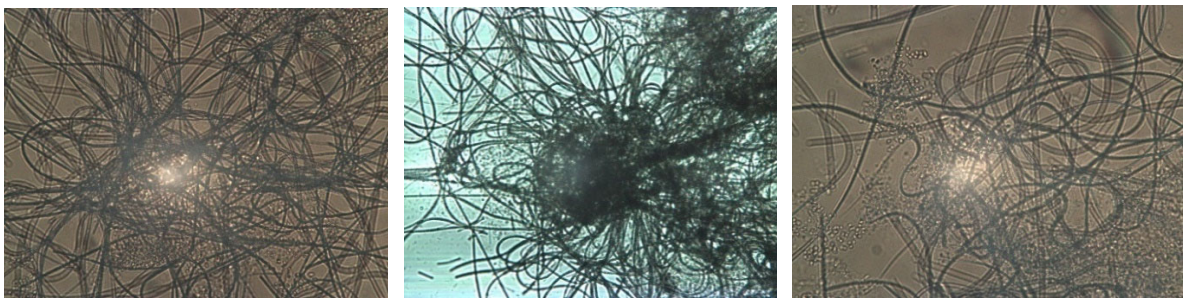


Fig. 5. P deficiency-induced dominance of filamentous bacteria in GWWTP activated sludge (magnification 100 x, light microscope Optika B-600 Tiph).

However, the availability of phosphoric acid has significantly decreased in recent years. One of the main reasons for this situation is the rapid depletion of natural P resources [49] and the fact that phosphate rock deposits in the world are located only in a few regions outside the European Union (EU), mainly in China, Morocco, the USA, and Russia, which makes the EU countries completely dependent on P compound imports and rising prices. Due to these circumstances, the phosphate rock and phosphorus used in the production of H_3PO_4 are included in the list of critical raw materials for the EU economy [50], and the need to recover P compounds from various waste streams and to search for the most optimal solutions for the transition to circular P management not only at the national but also at the regional level is particularly emphasized.

In **Publication 4** [51], analyzing various P flows and their current management practices in the countries of the Baltic Sea region, globally available technological solutions for the recovery of P compounds from secondary resources and the region-specific conditions, it was concluded that, despite the fact that several P-containing secondary resources have been identified, including wastewater, sewage sludge, and sewage sludge ash (Table 5), P recovery from them is not widely used in the countries of the Baltic Sea region, mainly due to the complex processing and high costs of installation and operation of P recovery technologies, as well as the lack of appropriate legislative framework [51].

Publication 5 [52] presents an alternative solution to the problem of P deficiency in GWWTP. It has been determined that the only technological process in which orthophosphate-rich production waters are generated (C = 10 g/L PO_4-P) in the production plant of JSC "Grindeks" is the synthesis of API ipidacrine. In order to avoid extreme peak loadings of phosphates and overdose of phosphorus during the production stage of this API, which lasts only several weeks a year, according to the recommendations provided within the work, these waters (~ 10 m^3 or ~ 50 % of the annual volume) are collected separately in the production site and reused as a P source during periods when the orthophosphate content in the GWWTP influent is low or insufficient. This practice allows to reduce the annual average consumption of H_3PO_4 by ~ 30 %. Currently, the use of the entire volume of ipidacrine synthesis waters is constrained by the limited area of the GWWTP, and the limited possibility of long-term storage of processing waters collected in separate containers for further use.

Table 5

Waste streams identified as possible secondary P sources

Sector	Secondary P sources
Industrial	Industrial wastewater, industrial sewage sludge, industrial sewage sludge ash, phosphogypsum, biomass ash
Agriculture	Manure, meat and bone meal, fish sludge
Municipal	Municipal wastewater, municipal sewage sludge, municipal sewage sludge ash, food waste

According to literature data, in the synthesis of APIs waters polluted with organic phosphorus compounds may also occur, which cannot be directly used as a bio-available source

of phosphorus for activated sludge microorganisms. However, the research conducted with the waters of the synthesis of the antibiotic Fosfomycin shows that a suitable solution for the recovery of P from such streams of pharmaceutical processing waters is wet air oxidation to transform organic phosphorus compounds into inorganic phosphates, followed by precipitation in the form of hydroxyapatite and struvite [53].

Despite this, it has been concluded that P deficiency cannot be attributed to all pharmaceutical WWTPs. In contrast to the insufficient P content in processing waters characteristic of GWWTP, other studies have shown that the opposite situation may also occur, and the orthophosphate content in pharmaceutical processing waters can reach up to 380 mg/L (at COD and N_{tot} concentrations of 4200 mg/L and 410 mg/L, respectively), which eliminates the need to dose an additional P source in biological treatment reactors [54]. But the use of phosphate-rich processing water from one production plant in the WWTP of another production plant is not economically justified due to the high water transportation costs and complicated logistics.

5. Possibilities and limitations of reuse of processing waters in the pharmaceutical industry

The transition to a circular economy model in the field of wastewater treatment requires evaluating the possibility of recovering not only P but also other chemicals from wastewater and at the same time obtaining reusable water to reduce the consumption of resources and the impact of production processes on the environment.

Multi-stage batch production processes, the diversity of processing water flows, and the fragmentation of technological processes do not allow the application of a universal solution for water treatment and reuse in the pharmaceutical industry. However, the application of individual solutions for each water flow is technically difficult and expensive, therefore limiting the implementation of circular economy-based technologies on a large scale.

The specificity of the pharmaceutical industry also demands that any changes in production processes must be validated and accepted by the relevant regulatory authorities, as well as approved by industrial customers [55], which is time-consuming and costly and does not motivate pharmaceutical production plants to introduce changes in the technological processes of manufacturing pharmaceutical products.

As a result of the research carried out in **Publication 5** it is concluded that due to the high chemical and microbiological purity requirements set for water, the reuse of treated processing water is not possible in production processes where the water comes into direct contact with the pharmaceutical product. Depending on the stage in the manufacturing process at which water is used, the formulation, and the route of administration, the water must meet the quality requirements of drinking or pharmaceutical-grade water defined in the Pharmacopoeia (Table 6). The available wastewater treatment technologies cannot guarantee such a high degree of water treatment, but the contamination of pharmaceutical products can pose serious risks to the health of patients and is not allowed according to the good manufacturing practice requirements set for the industry [56]. It should be considered that in multi-product

pharmaceutical factories, the residues of pharmaceutical substances also enter the processing water, which, when insufficiently treated water is reused, can pose risks of API cross-contamination. Since APIs are biologically active even at very low concentrations, product cross-contamination can critically impact the quality and safety of pharmaceutical products [57].

The study concluded that pharmaceutical-grade water preparation systems themselves cause significant water losses. According to the experience of JSC "Grindeks", due to the very high purity requirements of purified water, up to 50 % of the feed water in the reverse osmosis modules is discharged into the sewer as wastewater. However, this water is still clean enough to be reused in technological processes where water does not come into direct contact with pharmaceutical products, for example, in the cooling system, which is identified as the most water-intensive technological process in the production of pharmaceutical products. It was established that by implementing such an approach, the company could reduce the annual average water consumption by 7000 m^3, which corresponds to 12 % of the total volume of drinking water used in production.

Table 6

The quality requirements set for water used in pharmaceutical production and the relevant type of use [58]

Water grade	Quality requirements according to the European Pharmacopoeia	Applied treatment technologies	Usage in production
Potable water	Compliance with WHO guidelines for drinking water quality	Not specified	Chemical synthesis of intermediates of APIs; rinsing/cleaning of manufacturing equipment and facilities; production of pharmaceutical-grade water
Purified water	TOC < 500 µg/L Conductivity ≤ 4.3 µS/cm (20 ºC) Nitrate ≤ 0.2 mg/L Aerobic bacteria ≤ 100 CFU/mL	Ion exchange, ultrafiltration, reverse osmosis, distillation, activated carbon filtration	Final isolation and purification of APIs; preparation of nonsterile FDFs; a final rinse of equipment, containers, and closures in the manufacture of non-parenteral FDFs
Water for injections	TOC < 500 µg/L Conductivity ≤ 1.1 µS/cm (20 ºC) Nitrate ≤ 0.2 mg/L Aerobic bacteria ≤ 10 CFU/mL Bacterial endotoxins ≤ *I.U.*/mL	Reverse osmosis, electro-deionization, ultrafiltration, nanofiltration	Manufacturing of sterile pharmaceutical products

Evaluating the possibilities of reuse of biologically treated pharmaceutical wastewater for technological purposes, it was concluded that without additional tertiary treatment, their reuse, for example, in cooling systems, is not possible for the following reasons:

1) due to the high content of inorganic salts dissolved in water, the use of such wastewater can contribute to the scaling and corrosion of pipelines;

2) nutrients and activated sludge particles remaining in wastewater can contribute to the development of biofilm and the formation of sediments in water systems, thereby reducing the efficiency of the heat transfer process;

3) the temperature of wastewater after biological treatment can reach up to 42 °C, which is too high to be used for cooling purposes;

4) the chemical compounds remaining in the wastewater may not be compatible with the materials used in the cooling systems and may cause unpredictable risks of water leakage and chemical and microbiological contamination of pharmaceutical products.

The application of physico-chemical and thermal wastewater treatment technologies or their combinations allows to treat pharmaceutical wastewater to such a degree that it can be reused in technological processes where water does not come into contact with API or FDF, including cooling systems and steam generators [59], [60]. However, the feasibility of implementing such technologies should be evaluated in the context of local water availability and costs. Given that chemical and energy-intensive wastewater treatment processes need to be used to ensure that the quality of the treated wastewater meets the reuse specification, in some cases the reuse of wastewater can have a higher impact on the environment than the use of clean water [61].

Despite these limitations, the implemented and tested solutions presented in **Publication 5** show that elements of circular economy can be implemented in pharmaceutical wastewater management practice by reusing specific processing water streams and liquid wastes as resources in different stages of biological treatment of wastewater even without prior treatment (see Figure 6) if effective cooperation between the production department and the WWTP is ensured.

As highlighted in the previous section, the PO_4^{3-} containing processing water from the API ipidacrine production is used as a P source in WWTP to ensure the optimal nutrient ratio for activated sludge microorganisms during periods when the PO_4^{3-} content of the influent wastewater is low. Aqueous solutions containing Al^{3+} from the synthesis of API milnacipran hydrochloride, where $AlCl_3$ is used as a Lewis acid, have been identified as highly toxic to the biocenosis of activated sludge (see section "Assessment of the toxicity of pharmaceutical processing waters"), and should therefore be disposed of as hazardous waste. A separate study determined that these waters can be reused as a coagulant for the precipitation of excess phosphorus and the separation of sludge in biological WWTP. At the current production capacity, the volume of processing waters containing Al^{3+} is 15 m^3/year, and they are used in full in the coagulation process, replacing the commercial $FeCl_3$ coagulant and thus reducing the consumption of chemicals and the costs of wastewater treatment. It has been verified in practice that organic solvents, which after several regeneration cycles are no longer suitable for use in pharmaceutical production processes, can be used as a carbon source in the denitrification

process to ensure the reduction of NO_3^- and NO_2^- to N_2 gas. During the year, JSC "Grindeks" uses ~ 9.5 t of organic solvents (mainly ethanol) for this purpose, which makes up ~ 3.5 % of the volume of solvents recovered at the production plant. The company's experience shows that concentrated alkaline solutions, resulting from production processes, can be collected separately and subsequently used for pH regulation in bioreactors of WWTP or for precipitation of heavy metals such as copper from other wastewater streams of the plant. In general, the presented examples allow us to conclude that the reuse of liquid waste from production in accordance with the principles of the circular economy is beneficial both for the reduction of environmental pollution and for economic reasons, as it allows to reduce both the volume and costs of hazardous waste to be disposed of, as well as to improve the efficiency of wastewater treatment and the management of chemically polluted water flows at the pharmaceutical production plant.

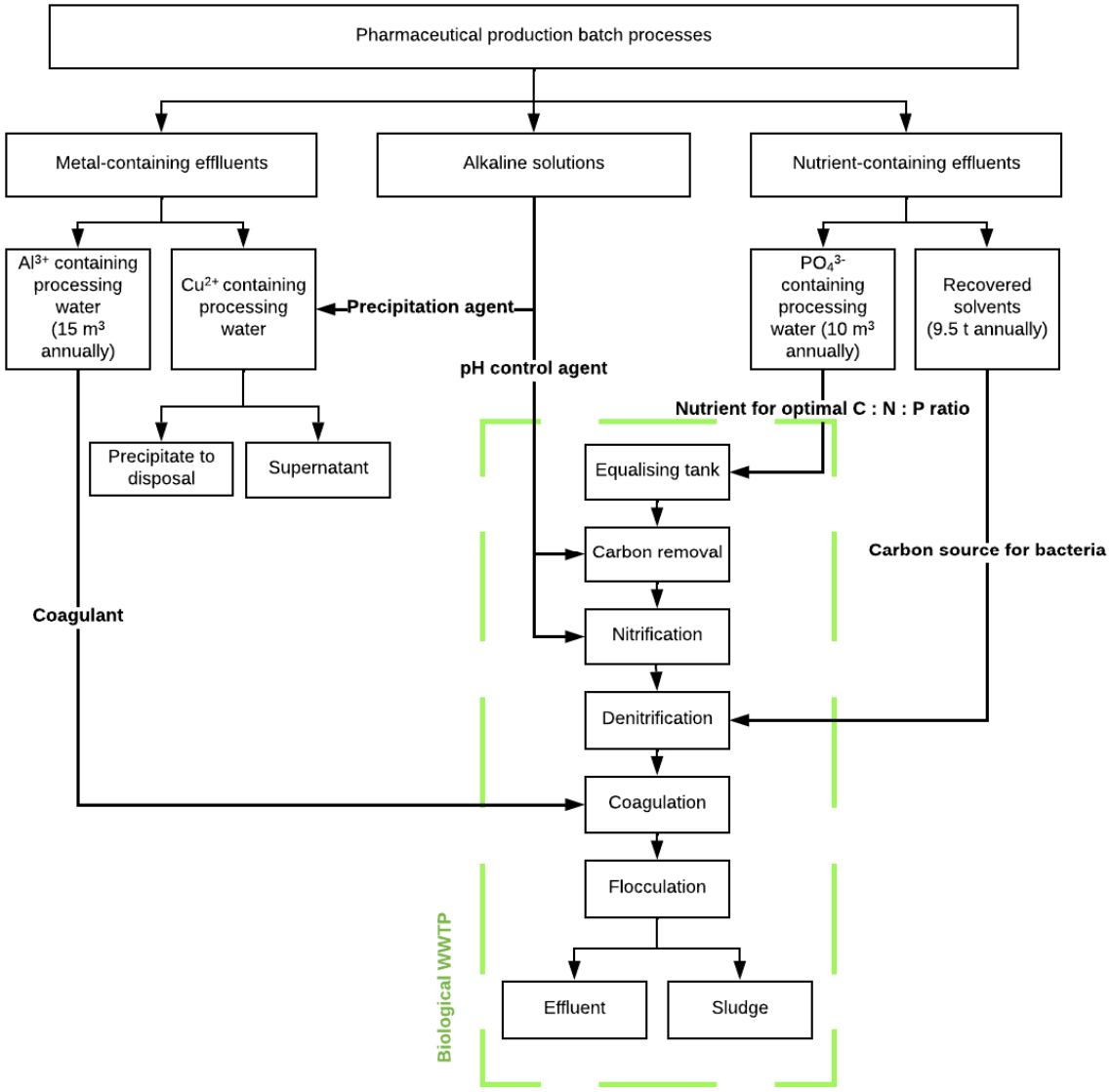


Fig. 6. Possibilities of reuse of specific pharmaceutical processing waters.

CONCLUSIONS

1. Analyzing the operation of GWWTP, the following main stress factors affecting the biological treatment of pharmaceutical production waters were identified:
   1) toxicity associated with the specifics of the chemical composition of processing waters;
   2) unbalanced nutrient content (>93 % of days per year the ratio COD : N_{tot} does not meet the optimal level of 100 : 5; 58–74 % of days per year PO_4-P concentration in the influent waters of WWTP < 5 mg/L in the analyzed 3-year period);
   3) elevated wastewater temperature in bioreactors (2–3 % of days per year t > 39 °C in the analyzed 3-year period);
   4) flow and concentration fluctuations (wastewater flow fluctuations: Q = 6–21 m^3/h, concentration fluctuations: COD concentration in GWWTP influent waters exceeds the designed load of GWWTP 15–21 % days per year, N_{tot} concentration 26–28 % of days per year);
   5) pH fluctuations in the influent waters of WWTP (pH = 4.3–12).
2. A new method for assessing the toxicity of chemically polluted processing waters to the biocenosis of activated sludge has been developed. The method provides that by performing BOD measurements of processing waters in an extended range of initial concentrations, experimental characteristic curves can be obtained, which allow:
   1) to identify toxic water flows to activated sludge biocenosis;
   2) to compare different chemically polluted processing waters in terms of toxicity;
   3) to predict the performance of the biological wastewater treatment process if production is intensified and the volume of wastewater or the concentration of specific pollutants increases.
3. It was found that among the microorganism cultures studied in the Doctoral Thesis, the bacterium *Sphingobacterium thalpophilum* and the filamentous fungi *Fusarium solani* and *Fusarium udum* are the most suitable candidates for increasing the efficiency of degradation of COD and API by bioaugmentation.
4. It was established that the addition of an additional carbon source (skim milk powder) significantly improves the degradation of API meldonium dihydrate by the bacteria *Sphingobacterium thalpophilum* and the filamentous fungi *Fusarium solani* and *Fusarium udum*.
5. The following circular economy elements have been developed and implemented in GWWTP:
   1) reuse of Al^{3+} containing waters from the production of API milnacipran hydrochloride (V = 15 m^3/year), which is toxic to activated sludge biocenosis in the process of activated sludge coagulation and chemical precipitation of excess P, replacing commercial $FeCl_3$;
   2) reuse of concentrated alkaline solutions from pharmaceutical production processes to ensure optimal pH in various stages of the wastewater treatment process, reducing the overall consumption of chemicals;

3) reuse of phosphate-containing waters from chemical synthesis of API ipidacrine (C = 10 g/L PO_4-P) to ensure optimal P content in bioreactors of GWWTP, which allows to reduce the consumption of commercial H_3PO_4 by 30 % annually;
4) reuse of the recovered solvents in the denitrification process as a carbon source, which allows for a reduction in the amount of organic solvents to be disposed of by ~ 3.5 % annually.

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APPENDICES

Characterization of pharmaceutical WWTP studied in the Thesis (available under request)

Elīna Strade (née Faltiņa) was born in 1986 in Ērgļi. She obtained a Bachelor's degree (2008) and a Master's degree in Chemical Technology (2010) from Riga Technical University. She started working at the Latvian Institute of Organic Synthesis in 2005. Since 2006, she has been working at JSC "Grindeks". Currently, she is Head of the Wastewater Treatment Department. From 2011 to 2015, she was a researcher at "Environment, Bioenergy and Biotechnology Competence Centre" Ltd. Her scientific interests are related to the biological treatment of industrial wastewater and the implementation of circular economy principles in wastewater management.