RIGA TECHNICAL UNIVERSITY

Brigita Daļecka

WASTEWATER TREATMENT FROM PHARMACEUTICAL SUBSTANCES WITH FILAMENTOUS FUNGI

Summary of the Doctoral Thesis

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RIGA TECHNICAL UNIVERSITY
Faculty of Civil Engineering
Institute of Heat, Gas, and Water Technology

KTH ROYAL INSTITUTE OF TECHNOLOGY
School of Engineering Science in Chemistry, Biotechnology and Health

Brigita Daļecka
RTU Doctoral Student of the Study Programme "Heat, Gas, and Water Technology"
KTH Doctoral Student of the Study Programme "Biotechnology"

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Scientific Supervisors:

Professor Dr. sc. ing.
TĀLIS JUHNA

Docent Researcher
GUNARATNA KUTTAVA RAJARAO

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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 a remote defence at the open meeting of RTU Promotion Council on 26 March 2021, at 11.00 in the Conference Hall of Riga Technical University, 6 Azenes Street, and in the KTH Royal Institute of Technology, Room F3, 26 Lindstedtsvägen, Stockholm.

OFFICIAL REVIEWERS

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Universidade do Moinho, Portugal

Dr. habil. sc. ing. Paul Van der Wielen
KWR Water Research Institute, The Netherlands

DECLARATION OF ACADEMIC INTEGRITY

I hereby declare that the Doctoral Thesis submitted for the review to Riga Technical University and KTH Royal Institute of Technology for the promotion to the scientific degree of Doctor of Science is my own. I confirm that this Doctoral Thesis had not been submitted to any other university for the promotion to a scientific degree.

Brigita Daļecka ……………………………. (signature)

Date: ………………………

The Doctoral Thesis has been written in English. It consists of an Introduction; 4 chapters; Conclusions; 14 figures; 4 tables; 4 appendices; the total number of pages is 68, not including appendices. The Bibliography contains 133 titles.

TABLE OF CONTENTS

GENERAL OVERVIEW OF THE THESIS

Relevance of the Study

The ever-increasing concern about the widespread occurrence of pharmaceutical substances in the aquatic environment has been recognized as an emerging environmental issue, as it can cause undesirable effects on the ecosystem and human health (Besha *et al.*, 2017). The conventional wastewater treatment plant is typically designed to remove high concentrations, mostly biodegradable organic matter and nutrients (Margot *et al.*, 2015) (Fig. 1). However, the pharmaceuticals substances are not removed completely by convectional wastewater system (Cruz-Morató *et al.*, 2013). Therefore, new advanced treatment technologies should be developed and optimized (Yamashita and Yamamoto-Ikemoto, 2014).

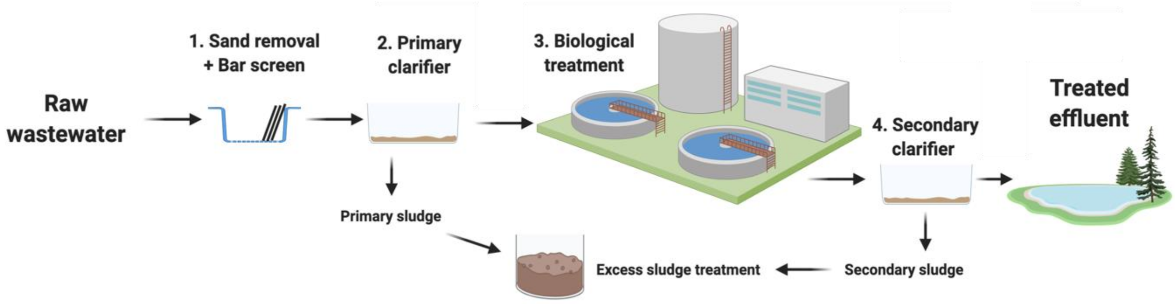

Fig. 1. Conceptual scheme of conventional wastewater treatment plant with biological treatment (created with BioRender.com).

For the last decade, the biological treatment by white-rot fungi has been shown to be a good candidate to remove pharmaceutical substances as a promising, cost-effective, and environmentally friendly method for removing the pharmaceutical substances from municipal wastewater (Mir-Tutusaus *et al.*, 2018). Furthermore, because of high adsorption capacity, enzyme systems, easy solid-liquid separation, relatively good adverse resistance, and broad degradation ability fungi are excellent candidates for wastewater treatment from pharmaceutical substances (Lu *et al.*, 2016). However, there are still many unanswered questions regarding a full-scale application of fungi for wastewater treatment (Espinosa-Ortiz *et al.*, 2016). One of the main scientific questions is how the use of different fungal strains could affect the removal efficiency of pharmaceutical substances, and how it would compete with other microorganisms under non-sterile conditions. The research needs to be done to determine if the fungal approach for wastewater treatment from pharmaceutical substances is efficient and flexible for municipal wastewater treatment applications.

Goal of the Study

The main goal of the study is to investigate the potential of filamentous fungi to remove pharmaceutical substances from municipal wastewater under non-sterile conditions without pH correction. Therefore, the main scientific question addressed in the study is: *Can filamentous fungi remove pharmaceutical substances from non-sterile municipal wastewater without pH correction?* According to this question, various tasks are set to accomplish this study:

- to identify the most common observed pharmaceutical substances in municipal wastewater;
- to investigate the efficiency of individual and mixed fungal cultures to remove pharmaceutical substances from municipal wastewater under non-sterile conditions;
- to examine the fungal removal mechanisms, biosorption, and biodegradation by laccase enzyme for pharmaceutical substances;
- to evaluate the effect on removal efficiency for pharmaceutical substances using carriers as a strategy to increase the fungal biomass;
- to isolate fungi from municipal wastewater and test their ability to remove pharmaceutical substances;
- to study the fungal removal efficiency of total phosphorus, ammonia nitrogen, and the total organic carbon from municipal wastewater;
- to design possible application and optimization of a fungal fluidized bed pelleted bioreactor for municipal wastewater treatment for the removal of pharmaceutical substances;
- to investigate the potential of bioaugmentation as a strategy for fungal treatment in fluidized bed pelleted bioreactor for municipal wastewater treatment for the removal of pharmaceutical substances;
- to evaluate the cost associated with a fungal treatment in a fluidized bed pelleted bioreactor and compare to classical and advanced treatment methods.

Scientific Novelty and Application

To the best of the author's knowledge, this is the first study where the pharmaceutical and nutrient removal from non-sterile municipal wastewater in a fluidized bed pelleted bioreactor without pH level adjustment has been tested using bioaugmentation as a strategy for operating the fungal bioreactor treatment system. The author sees this work as a valuable result which can be used as a first step to improve the fungal technology for municipal wastewater treatment and gain strong inside knowledge of fungi application in municipal wastewater treatment system.

Description of the Main Chapters and Methodology

This Thesis is based on four separate journal papers which are referred to by Roman numbers. The main aim of **Paper I** was to investigate the potential of five worldwidedistributed fungal strains – *Trametes versicolor*, *Irpex lacteus*, *Pleurotus ostreatus*, *Trichoderma reesei*, and *Fusarium solani* – to remove pharmaceutical substances from municipal wastewater under sterile/non-sterile batch-scale experiments. In this paper, the effect of pH level and carriers on the removal efficiency and the enzymatic laccase activity were examined for each strain separately and in mixed cultures in batch-scale experiments for a certain period of time (Fig. 2).

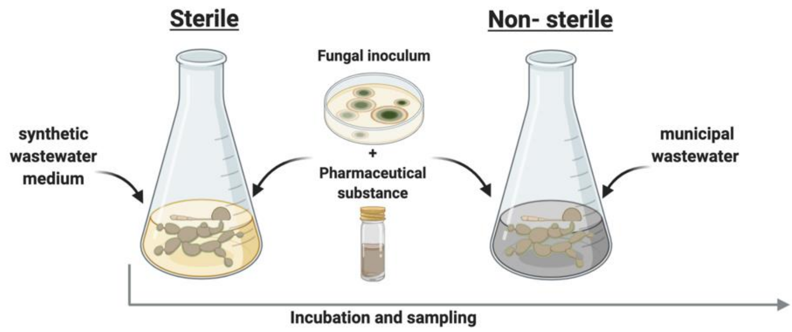

Fig. 2. Conceptual scheme of batch experiment with fungi (created with BioRender.com).

The effect of non-sterile municipal wastewater using fungal biofilm carriers K1 with the most promising strain *T. versicolor* was tested to assess the potential of fungal treatment to remove pharmaceutical substances. Additionally, the difference in initial inoculum for fungal cultures was analyzed in order to determine the removal efficiency in non-sterile municipal wastewater. Finally, the biosorption experiment was done to better understand fungal removal mechanisms for pharmaceutical substances.

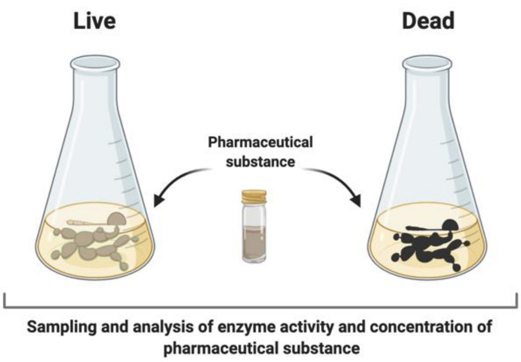

Fig. 3. Conceptual scheme of biosorption test with fungi (created with BioRender.com).

In **Paper II**, the main focus was to investigate the isolation of fungal strains from municipal wastewater and to test their ability to remove pharmaceutical substances. To achieve this, fungal isolates were isolated and cultivated on a synthetic wastewater media in the presence of selected pharmaceuticals. In this paper, the effect of the pH level on removal efficiency was studied. The most promising isolate was further identified as *Aspergillus luchuensis* and analyzed in non-sterile municipal wastewater for pharmaceutical substances removal. Finally, a biosorption experiment was conducted with the isolate, and enzyme activity was measured to better understand the removal mechanisms of pharmaceutical substances (Fig. 3). All results of the fungal isolate were compared to *T. versicolor* to evaluate the potential of an isolated fungal strain and the advantages of its application in wastewater treatment to remove pharmaceutical substances.

In **Paper III**, the investigation of the total phosphorus (P), ammonia nitrogen (NH_4-N) and the total organic carbon (TOC) removal from non-sterile municipal wastewater of two fungal species, *T. versicolor* as the most promising strain from **Paper I** and *A. luchuensis* as wastewater isolate from **Paper II**, was done. The removal efficiency of P, NH_4-N, and TOC was studied and compared taking into consideration the aspect of process design possible application and optimization of a fungal fluidized bed pelleted bioreactor. The investigation consisted of two phases. First, observation of results was done under a batch-scale experiment with *T. versicolor* and *A. luchuensis*. During this phase, the removal of P, NH_4-N, and TOC were analyzed. In the second phase, the fungal fluidized bed pelleted bioreactor was designed, and both fungal cultures were incubated in reactors allowing collecting the data of P, NH_4-N, and TOC removal in order to compare the nutrient removal efficiency from the batch-scale to the bioreactor (Fig. 4).

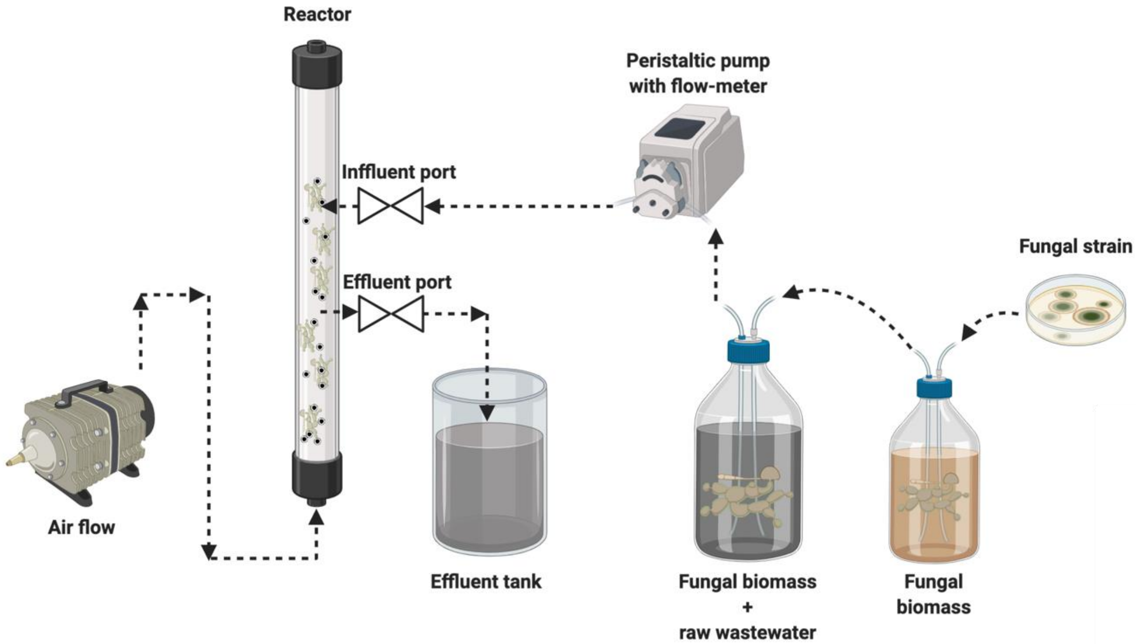

Fig. 4. A scheme of a fungal fluidized bed pelleted bioreactor (created with BioRender.com).

Finally, to better understand the removal mechanism of nutrients and fungal interaction with natural microorganisms in municipal wastewater, the pH value, laccase enzyme activity, and quantification of total bacteria were determined.

In **Paper IV**, the bioaugmentation as a strategy for the fungal approach for wastewater treatment from pharmaceuticals was tested in the fungal fluidized bed pelleted bioreactor. The fungal fluidized bed pelleted bioreactor was optimized from **Paper III,** and both fungal cultures, *T. versicolor* and *A. luchuensis*, were incubated in reactors allowing collecting the data of total P, N, nitrate and nitrite, and TOC removal. To better understand the removal mechanism of nutrients and fungal interaction and adaption with the microbial community in municipal wastewater, the pH value, laccase enzyme activity, and quantification of total bacteria were determined. Additionally, the removal efficiency of pharmaceutical substances such as diclofenac, ketoprofen, carbamazepine, ibuprofen, sulfamethoxazole, and metoprolol were tested and analyzed (Table 1).

Table 1

Physio-Chemical Properties of the Selected Pharmaceutical Substances

Pharmaceutical	Molecular formula	Functional group	log K_d	Classification	Reference	Paper
Diclofenac	$C_{14}H_{11}C_{12}NO_2$	Amine, chlorine, carboxylic (–COOH)	2.7	Nonsteroidal anti-inflammatory drug (nsaids)	Baresel *et al.*, 2015; Carballa *et al.*, 2004; Kramer *et al.*, 2018; Martín *et al.*, 2012; Maurer *et al.*, 2007; Taheran *et al.*, 2016; Ternes, 1998; Wang and Wang, 2016; Zahmatkesh *et al.*, 2017	Paper I, II and IV
Ketoprofen	$C_{16}H_{14}O_3$	Carboxylic acids and salts, ketones and ketals	2.4	Nonsteroidal anti-inflammatory drug (nsaids)		Paper I and IV
Carbamazepine	$C_{15}H_{12}N_2O$	Heterocycles	1.15	Anticonvulsants		Paper II and IV
Ibuprofen	$C_{13}H_{18}O_2$	Alkane substituents and a carboxylic acid	2.1	Nonsteroidal anti-inflammatory drug (nsaids)		Paper II and IV
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	Amines and amine salts, heterocycles, sulfur containing groups	1.04	Nonsteroidal antibacterial-inflammatory drug (nsaids)		Paper II and IV
Metoprolol	$C_{15}H_{25}NO_3$	Alcohols and phenols, amine salts, ethers and oxides	2.4	Antihyper-tensives		Paper IV

In this Thesis, diclofenac, ketoprofen, carbamazepine, ibuprofen, sulfamethoxazole, and metoprolol were selected as model compounds due to the high level of consumption in Latvia and Sweden and as the most reported and detected compounds in the wastewater treatment plant influent and effluents with relevant low removal efficiency by conventional wastewater treatment. Furthermore, these compounds contain different functional groups that might provide new insight into the removal efficiency by fungi.

RESULTS AND DISCUSSION

Removal of Pharmaceuticals by Fungi in Municipal Wastewater

The results from **Paper I** in batch-scale experiment demonstrated that the initial concentration of a fungal inoculum was relatively low. Therefore, the fungi might be outcompeted by *Bacteria, Archaea,* and other fungal species from wastewater. Thus, the initial fungal concentration of biomass was increased in further experiment by K1 carriers (Fig. 5).

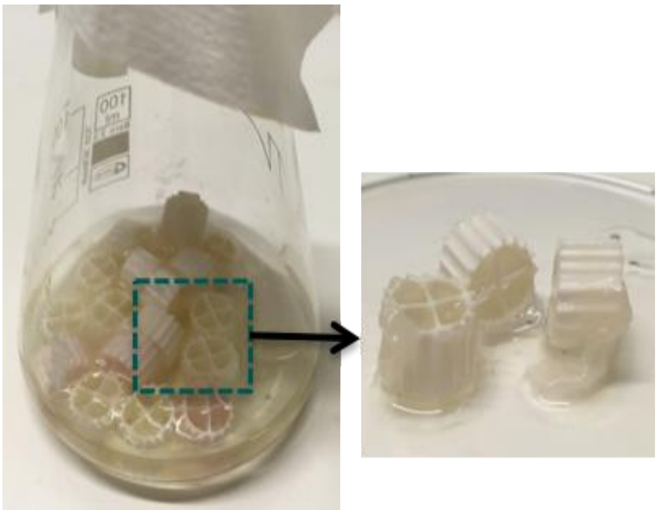

Fig. 5. Fungal growth on K1 carriers.

The results demonstrated that all fungal cultures were able to completely remove (>99.9 %) diclofenac after 3 days of incubation (Fig. 6). However, a longer incubation time demonstrated a release of the diclofenac from all fungal strains back to wastewater, except for *T. versicolor* (Fig. 6 (B)). The obtained results might be explained by the use of different removal mechanisms of tested fungi. Therefore, the removal mechanisms by *T. versicolor* were further investigated and analyzed by biosorption test.

The results of the biosorption test in **Paper I** indicated that *T. versicolor* used both mechanisms – enzyme activity and biosorption – to remove diclofenac. The observation of biosorption test showed that laccase was responsible for removing ~20 % of diclofenac while the *T. versicolor* live biomass could remove ~80 % of diclofenac.

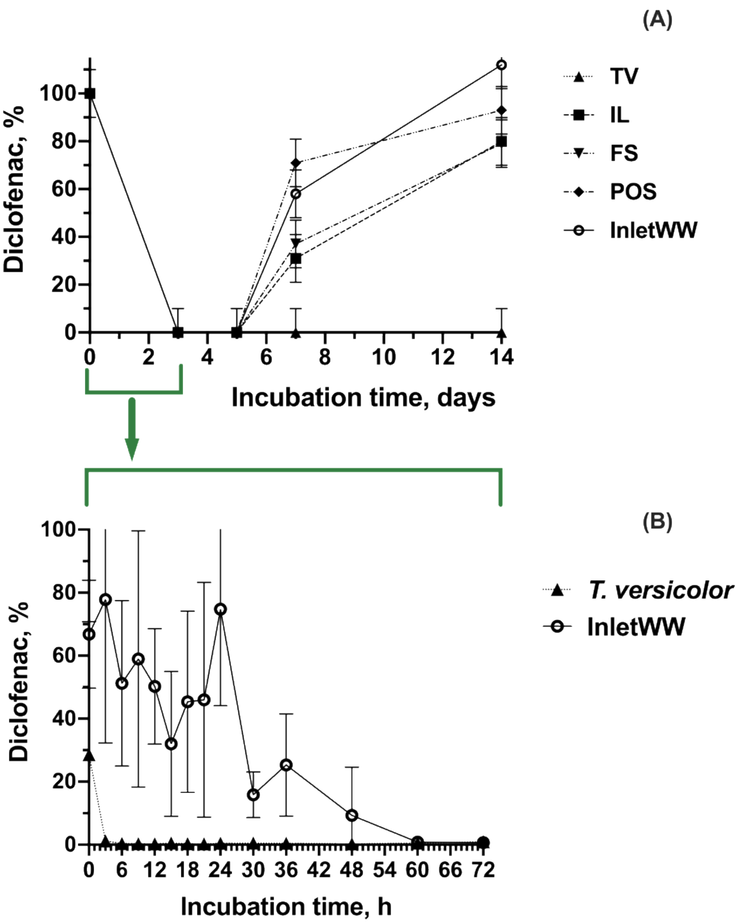

Fig. 6. (A) The removal efficiency (%) of diclofenac from non-sterile municipal wastewater with fungal biofilm on K1 carriers of *T. versicolor* (TV), *I. lacteus* (IL), *F. solani* (FS) and *P. ostreatus* (POS); **(B)** Removal efficiency (%) derived from the additional research of diclofenac removal within 3 days with *T. versicolor* in non-sterile municipal wastewater with fungal biofilm on K1 carriers.

Isolation of Fungal Strains From Municipal Wastewater

In **Paper II**, the municipal wastewater was used for fungi isolation. Isolated fungi were grown on agar plates in the presence of all selected pharmaceutical compounds, such as carbamazepine, diclofenac, ibuprofen, and sulfamethoxazole. Based on growth efficiency, a total of seven fungal isolates were used for further study to investigate their ability to remove pharmaceutical substances in synthetic wastewater media, and the results were compared to *T. versicolor* in order to see the differences in removal efficiency. Furthermore, the pH effect on removal efficiency was also investigated.

The results from **Paper II** indicated difficulties to obtain relatively high removal efficiency (>80 %) from carbamazepine by tested isolates. The results from diclofenac removal showed that one of the fungal isolates could completely (>99.9 %) remove this pharmaceutical after 6 days of incubation at pH 5.5, while complete reduction of diclofenac at pH 6.3 was obtained after 10 days of incubation. The same result was observed for *T. versicolor*. Therefore, further in **Paper II**, the removal efficiency for diclofenac in municipal wastewater with the most promising isolate was examined and compared with *T. versicolor*. The most promising isolate was identified as *Aspergillus luchuensis*.

When evaluating the diclofenac removal efficiency after fungal treatments at various pH values for a non-sterile municipal wastewater sample, it can be stated that the fungal isolate of *A. luchuensis* can remove >95% of diclofenac for both pH values for the entire incubation time (Fig. 7 A, B). In contrast, *T. versicolor* demonstrated a relatively slower diclofenac removal efficiency at the pH 7.8 after 24 h of the incubation time, compared to the pH 5.5 when diclofenac was completely removed (>99.9%) after 3 h of an incubation period. In order to better understand the removal mechanisms for *A. luchuensis*, the enzyme activity of laccase was measured and the biosorption test was done to better understand the relationship between the enzyme activity, fungal biomass, and the removal efficiency for diclofenac (Fig. 7 C, D).

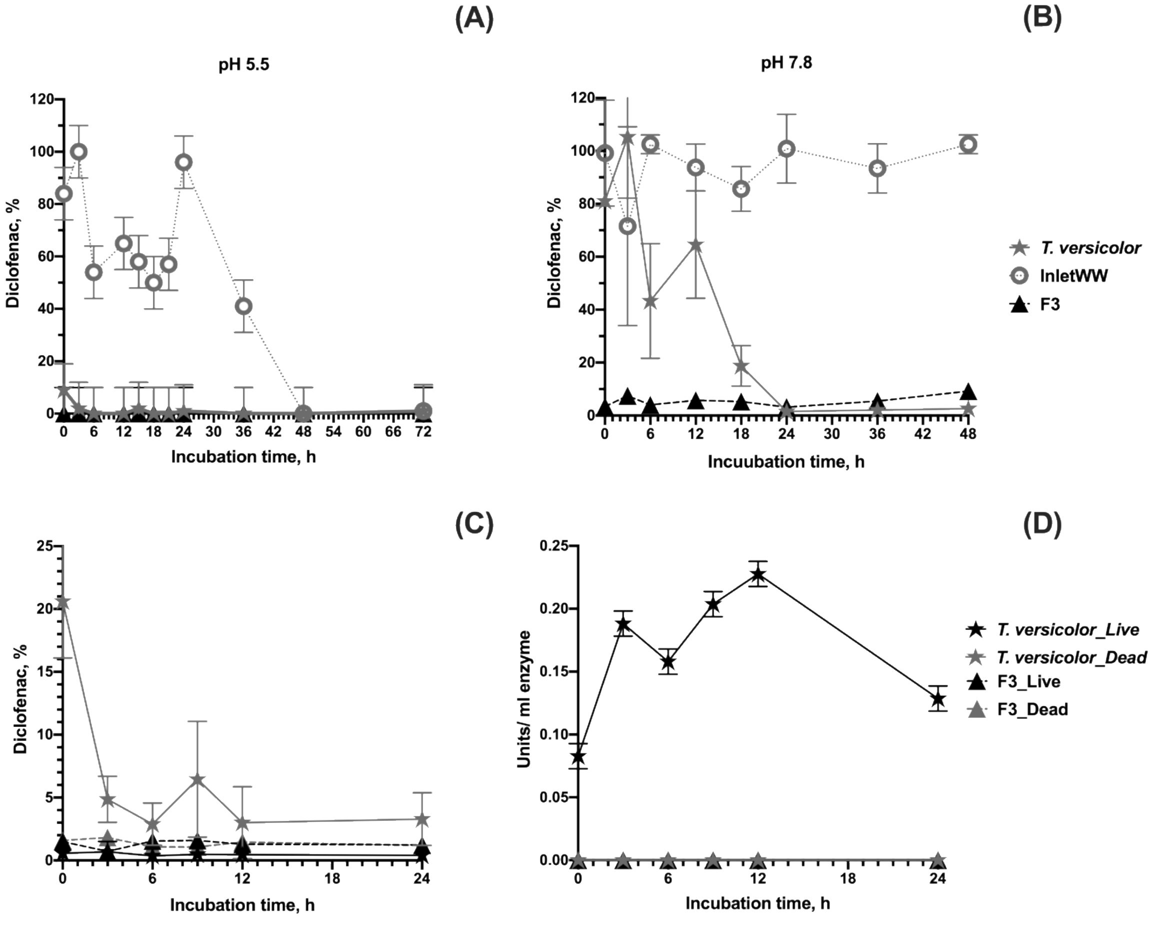

Fig. 7. The removal efficiency by the isolate *A. luchuensis* and *T. versicolor* (%) of diclofenac at **(A)** pH 5.5; **(B)** pH 7.8 from non-sterile municipal wastewater; **(C)** the removal efficiency (%) of diclofenac from the biosorption test of live and dead fungal biomass by isolates *A. luchuensis* and *T. versicolor*; **(D)** enzyme activity of laccase from the biosorption test of live and dead fungal biomass by isolate *A. luchuensis* compared to *T. versicolor.*

The results indicated that there was no enzyme activity detected for *A. luchuensis* and wastewater without a fungal inoculum, while *T. versicolor* produced laccase for the entire incubation period for both non-sterile municipal wastewater samples with different pH values. Furthermore, the live biomass of *A. luchuensis* did not present any laccase enzyme activity throughout the incubation period. Moreover*, A. luchuensis* showed complete removal (>99.9 %) of diclofenac for both live and dead fungal biomass immediately after the biomass adjustment indicating that the removal of diclofenac could be due to the biosorption mechanism.

Removal of Total P, NH_4-N and TOC Under Batch Scale Experiment

In **Paper III**, the nutrient removal efficiency and the effect of pH on nutrient removal by two fungi – *T. versicolor* as a laboratory strain from **Paper I** and *A. luchuensis* isolate from a municipal wastewater treatment plant from **Paper II** – were studied and compared to nutrient reduction in non-sterile municipal wastewater under a batch and pilot-scale experiment.

The results in **Paper III** from the batch scale experiment indicated that both fungi were able to remove phosphorus (P) in non-sterile municipal wastewater without/with a pH adjustment. Results of ammonia nitrogen (NH_4-N) removal by *T. versicolor* without the adjustment of pH value showed an increase of NH_4-N concentration immediately after the incubation was started. The same tendency was observed by *A. luchuensis*. On the contrary, the results of *T. versicolor* and *A. luchuensis* with adjustment of pH value showed relatively small changes in NH_4-N concentration throughout the incubation time. Therefore, both fungi had no direct effect on NH_4-N reduction in municipal wastewater, i.e., it is believed that both fungi did not use NH_4-N in their metabolic pathway to reduce the nitrogen concentration in wastewater. However, a further investigation is required to better understand the fungal role in the NH_4- N reduction in municipal wastewater. When evaluating the total organic carbon (TOC) removal efficiency after both fungal treatments, it can be stated that *T. versicolor* and *A. luchuensis* can reduce TOC concertation after a 72 h of incubation period with a pH level adjustment for wastewater. In contrast, the results of *T. versicolor* and *A. luchuensis* without a pH level adjustment showed diverse changes in the TOC concentration throughout the incubation period of 72 h. Therefore, the pH value adjustment might stabilize the TOC removal process for fungi, while wastewater without a pH value adjustment showed an unsteady reduction of TOC for the entire incubation time of 72 h.

Removal of P, NH_4-N and TOC in Fluidized Pelleted Bioreactor

Once the results from the batch experiments achieved a relatively good success in the P reduction by fungal treatment and showed that the pH adjustment to 5.5 helped to stabilize the N and TOC reduction process, the removal analysis was further tested in a fluidized bed pelleted bioreactor.

The results in **Paper III** from fluidized pelleted bioreactor showed that both fungi were able to reduce more than 80 % of P until the end of the incubation period. The result of the NH_4-N concertation did not show any changes for both fungi until the end of incubation period. Finally, the results of TOC demonstrated that the TOC has been reduced by 35 % compared to the starting concentration of TOC after 15 days of the incubation period. Overall, the results of a fluidized bed bioreactor demonstrated different tendencies on nutrient removal using *T. versicolor* and *A. luchuensis* compared to a batch experiment. Therefore, in

Paper IV, the fluidized pelleted bioreactor was optimized and the bioaugmentation as a strategy to add fresh fungal biomass was used.

Removal of Pharmaceuticals in Fluidized Pelleted Bioreactor by Bioaugmentation

In order to better understand the bioaugmentation effect on the removal efficiency, two biomass, 10 g and 50 g of wet biomass for *T. versicolor* and *A. luchunesis*, were tested in **Paper IV**. The results in Fig. 8 indicate the efficiency of removal by *T. versicolor* and *A. luchunesis* for diclofenac, ketoprofen, carbamazepine, ibuprofen, sulfamethoxazole, and metoprolol.

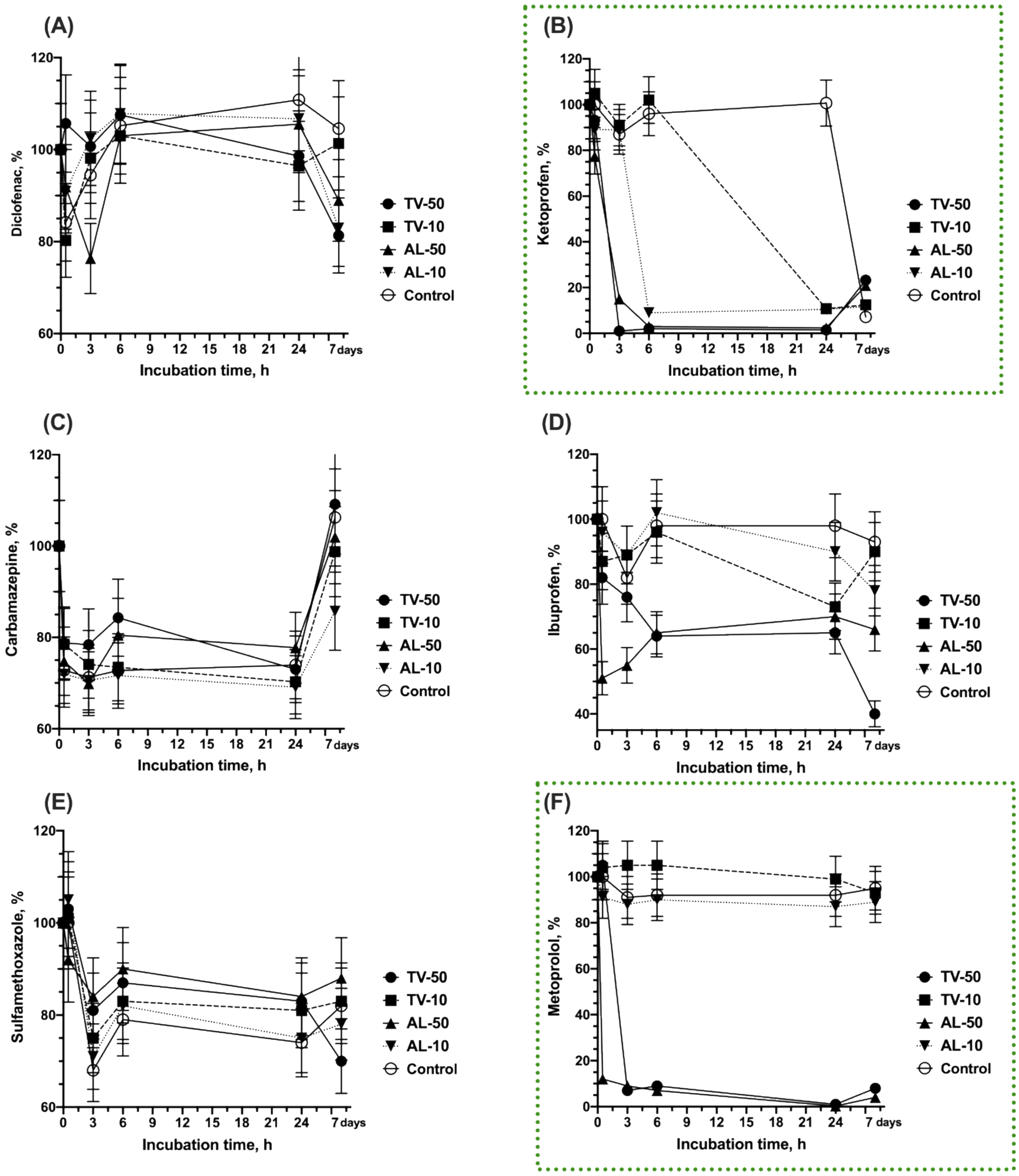

Fig. 8. The bioaugmentation effect on removal efficiency of pharmaceutical substances (%) **(A)** diclofenac, **(B)** ketoprofen, **(C)** carbamazepine, **(D)** ibuprofen, (**E)** sulfamethoxazole, and **(F)** metoprolol by fungi *T. versicolor* (TV) and *A. luchuensis* (AL) with the adjustment of 10 g and 50 g wet biomass.

Results from both fungi showed that adjustment of 50 g of wet biomass demonstrated relatively high removal efficiency (>90 %) for ketoprofen and metoprolol (Fig. 8, B and F) compare to the adjustment of 10 g of wet biomass after 3 hours of incubation time. At the same time, relatively low removal efficiency for both bioaugmentation strategies (>40 %) was detected for diclofenac, carbamazepine, ibuprofen, and sulfamethoxazole.

Cost Evaluation of Fungal Treatment

In **Papers III** and **IV**, the results showed that with bioaugmentation it is possible to maintain domination of fungi over bacteria without a pH adjustment and effectively remove nutrients and pharmaceutical substances such as metoprolol and ketoprofen. However, it does require an additional cost, including an extra source of the organic substrate, to cultivate fungi. In **Paper III**, the author has estimated costs based on the current average market prices in Europe. All estimated fungal treatment costs (EUR/m^3) include the cost of fungal growth and operation in a fluidized bed pelleted bioreactor (Table 2).

Table 2

The Average Price of Different Wastewater Treatment Technologies and the Cost of the Studied Fungal Treatment by *T. versicolor* and *A. luchuensis*

Wastewater Treatment Technology	Cost, EUR/m^3	Reference
Fungal Treatment	From 200 to 2000	This study
Coagulant-Flocculant	From 0.35 to 8.5	Pelendridou *et al.*, 2014; Yoo, 2018
Membrane-Based Treatment	From 2	Rongwong *et al.*, 2018
Conventional Biological Treatment	From 0.036 to 1	Hansen *et al.*, 2007

According to the literature (Hansen *et al.*, 2007; Pelendridou *et al.*, 2014; Rongwong *et al.*, 2018; Yoo, 2018) the cost of typical treatment technologies such as a coagulation-flocculation process for wastewater treatment, is in the range from 0.35–8.5 EUR/m^3; for membrane-based technologies – from 2 EUR/m^3; for conventional biological treatment – from 0.035 EUR/m^3 to 1 EUR/m^3 while the fungal treatment growth and operation costs may vary from 200 EUR/m^3 to 2000 EUR/m^3.

CONCLUSIONS

The objective of this Thesis was to investigate the potential of filamentous fungi to remove pharmaceutical substances from municipal wastewater under non-sterile conditions without pH level correction. To achieve the goal of this Thesis, the literature study was done and filamentous fungi potential for wastewater treatment from pharmaceutical substances were examined.

Paper I showed the potential of five fungal strains to remove ketoprofen and diclofenac individually and in mixed cultures. Experiments with synthetic wastewater media showed that fungi compete with each other, since higher removal efficiency was observed if the fungi were grown individually. The capability of selected fungal strains to remove diclofenac in a non-sterile municipal wastewater sample using carriers showed a promising approach for the wastewater treatment process. The results show that *T. versicolor* could fully (> 99.9 %) remove diclofenac after a 3 h long incubation period in non-sterile municipal wastewater. Moreover, *T. versicolor* demonstrated an ability to use both mechanisms – enzyme activity and biosorption – to remove diclofenac. Therefore, *T. versicolor* could be a promising candidate to remove pharmaceutical substances by wastewater treatment process and was further used in **Papers II**, **III**, and **IV**.

The results from **Paper II** have shown that *A. luchuensis* has a higher removal efficiency in a non-sterile wastewater sample without a pH correction than *T. versicolor*. Thus, *A. luchuensis* has a high potential for use in industrial wastewater treatment due to minimized specific pH requirements. Therefore, in further studies in **Papers III** and **IV**, these two strains were selected and tested in a fluidized bed pelleted bioreactor for removal of nutrients and pharmaceutical substances.

In **Paper III**, the fluidized bed bioreactor was designed and the removal efficiency of nutrients was tested. During this study, fungi have demonstrated a high potential to remove phosphorus from municipal wastewater efficiently and successfully under a batch scale experiment, while the results from the fluidized bed bioreactor did not demonstrate a significant decrease of nutrients. Therefore, in **Paper IV**, the fluidized pelleted bioreactor was optimized and the bioaugmentation as a strategy to add fresh fungal biomass was used.

The results from **Paper IV** showed that bioaugmentation can be a promising strategy to optimize and operate the fungal bioreactor. However, the results indicated that fungi have a variety of strategies on how to counteract pharmaceutical substances.

Finally, the fungal treatment costs highly depend on fungal growth requirements (temperature, incubation time, electricity of shaking, composition of media). Thus, the cost of the fungal treatment presented here is among the highest reported in the literature, declaiming the hypothesis that the fungal treatment can be a cost-effective treatment technology. However, the fungal treatment still has a high potential to be an environmentally friendly and sustainable treatment method for wastewater treatment not only considering the nutrient load perspective but also for pharmaceutical substances removal and complement to the conventional biological step (Mir-Tutusaus *et al.*, 2018). Furthermore, fungi might give valuable benefit in a long term by recovering value-added products from fungal biomass and fine-tuning of the wastewater treatment process thereby reducing the cost of the operation process (Sankaran *et al*., 2010).

Recommendations and Future Studies

The main benefit of pharmaceutical treatment by fungi is that it is a biological treatment and does not require the specific use of chemicals (Pointing, 2001). The author of this study believes that there might be two possible practical applications for this method:

- fungal pre-treatment for industrial wastewater where specific pharmaceuticals need to be removed;
- fungal post-treatment for pharmaceutical substances removal at the end of the municipal wastewater treatment system (Fig. 9).

However, the results derived from this study showed that fungal growth in non-sterile conditions in the bioreactor is limited and the removal efficiency decreases compared to the results from batch-scale experiments. Sterilization of wastewater is not a cost-efficient and suitable option for wastewater treatment by fungi (Espinosa-Ortiz *et al*., 2016; Ferreira *et al*., 2020; Mir-Tutusaus *et al*., 2019). Therefore, in future studies development of a symbiotic fungal-bacterial/fungi-algae consortium for the removal of pharmaceuticals from non-sterile wastewater can be investigated (Muradov *et al*., 2015; Wei *et al*., 2018). Further research is needed to better understand the operational challenges and requirements for full-scale applications (Mir-Tutusaus *et al*., 2018). In **Paper IV**, the fungal bioreactor has a relatively high concentration of added fungal biomass inhibiting the fresh biomass adaption and growth in the bioreactor. Therefore, the operation of how to efficiently remove the used biomass has to be implemented in the bioreactor. Furthermore, *T. versicolor* and *A. luchuensis* are fungi that are able to make spores (Benson *et al*., 2019; Hong *et al*., 2013). Thus, the strategy of what to do with fungal biomass after wastewater treatment needs to be discussed and developed (Sankaran *et al*., 2010). Furthermore, this study showed that fungi can use two strategies for removing pharmaceutical substances – enzyme activity and biosorption. As fungi can use biosorption for pharmaceutical substance removal, the biomass can be relatively rich in concertation of removed pharmaceutical substances (Jureczko, 2018). Additionally, the strategy of what to do with this biomass needs to be discussed and the possible impact on the environment needs to be investigated.

Finally, full-scale application of fungal treatment for wastewater does not exist at the moment. According to researcher Mir-Tutusaus, developments in fungal treatment direction depend on overcoming several shortcomings, namely: (1) maintaining a stable activity of the fungal pellets over prolonged periods of time, and (2) preserving good performance in non-sterile conditions (Mir-Tutusaus *et al*., 2019). Already, the fungal treatment costs highly depend on fungal growth requirements (temperature, incubation time, the electricity of shaking, the composition of media) (Mir-Tutusaus *et al*., 2018; Sankaran *et al*., 2010). Thus, the cost of the fungal treatment presented in **Paper III** is among the highest reported in the literature, declaiming the hypothesis that the fungal treatment can be a cost-effective treatment technology (Arikan *et al*., 2019; Lu *et al*., 2016; Wang and Wang, 2016). However, the author of this study believes that fungal treatment still has a high potential to be an environmentally friendly and sustainable treatment method for wastewater treatment not only considering the nutrient load perspective but also for pharmaceutical substance removal. Furthermore, the fungal biomass after treatment can be used as a source for valuable byproducts therefore covering the incurred costs of growth (Sankaran *et al*., 2010).

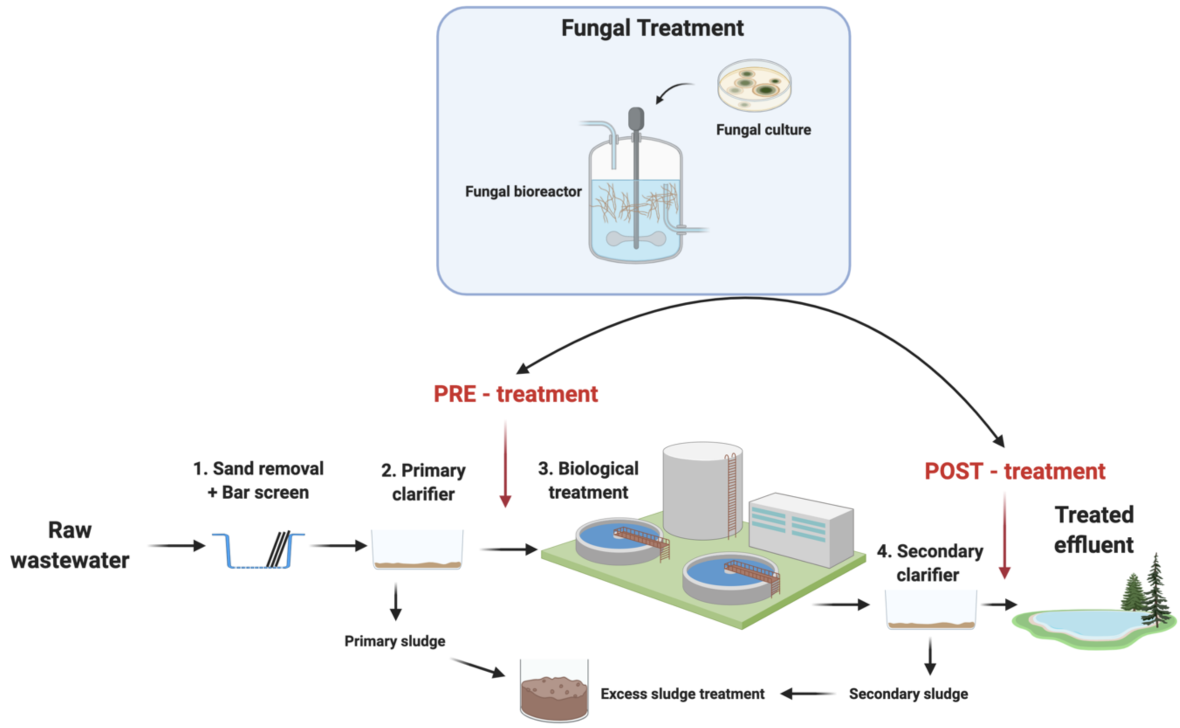

Fig. 9. Scheme of fungal application in conventional wastewater treatment system (created with BioRender.com).

Overall, the results from this Thesis approve the scientific question derived from this Thesis, i.e., filamentous fungi can remove pharmaceutical substances from non-sterile municipal wastewater. The observed results from this study give a relevant and useful knowledge for future investigations and help to improve the fungal application in real wastewater treatment system. For instance, the investigation of the synergetic study showed that fungi have relatively higher removal efficiency for pharmaceutical substances if they are incubated individually instead of mixed cultures of fungal strains. The results from this study also give better understanding of the effectiveness of fungal treatment and the mechanisms behind the removal of pharmaceuticals. However, there is still a need for finetuning of some of the aspects regarding a full-scale application of fungi in the wastewater treatment process, for example, the optimal operation conditions for fungal growth and adaption in fluidized bed bioreactor to efficiently remove nutrients and pharmaceutical substances from wastewater.

In the future, the researchers need to cooperate with stakeholders and the government to develop strategies and processes on how to implement innovations and optimize the existing methods for wastewater treatment. According to Global Goals for Sustainable Development, this study is mainly overlapping with Goal 6. The main aim of Goal 6 (called *Clean water* *and sanitation*) is to ensure the availability and sustainable management of water and sanitation for all people. One of the main objectives under Goal 6 is to improve water quality and wastewater treatment. Therefore, this study goes hand in hand with the objective of this goal. As this goal proposes that by 2030 the release of chemicals and hazardous substances needs to be minimized, the author thinks that the results derived from this Thesis can be used to improve the fungal treatment for pharmaceutical substances removal from wastewater. Furthermore, the improvement of wastewater treatment systems to remove pharmaceutical substances can also help to realize the objective to protect and restore water-related ecosystems such as rivers, lakes, and seas where relatively often the treated wastewater effluent is discharged.

APROBATION

List of Publications

1. Dalecka, B., Juhna, T., Rajarao, G. K. Constructive use of filamentous fungi to remove pharmaceutical substances from wastewater. Journal of Water Process Engineering, 33, 2020.
2. Dalecka, B., Oskarsson, C., Juhna, T., Rajarao, G. K. Isolation of fungal strains from municipal wastewater for the removal of pharmaceutical substances. Water, 12, 524, 2020.
3. Dalecka, B., Strods, M., Rajarao, G. K., Juhna, T. Removal of total phosphorus, ammonia nitrogen and organic carbon from non-sterile municipal wastewater with *Trametes versicolor* and *Aspergillus luchuensis*. Microbiological Research, 241, 2020.
4. Dalecka B., Strods M., Cacivkins P., Ziverte E., Rajarao G. K., Juhna T. Bioaugmentation with fungi: An emerging strategy for removing pharmaceutical substances in wastewater treatment process by fluidized bed pelleted bioreactor. Chemosphere, 2021 (under submission).

List of Conferences

1. Riga Technical University 61st International Scientific Conference, Latvia, 16 October 2020. Oral presentation "Filamentous fungi: a promising approach for nutrient removal from municipal wastewater".
2. Riga Technical University 60th International Scientific Conference, Latvia, 16 October 2019. Oral presentation "Wastewater treatment from hazardous substances by filamentous fungi".
3. IWA 11th Eastern European Young Water Professionals Conference, Prague, Czech Republic, 1–5 October 2019. Extended abstract and oral presentation "Potential use of filamentous fungi for diclofenac removal from municipal wastewater". Award for the best platform presentation.
4. IWA 9th International Young Water Professionals Conference, Toronto, Canada, 23–27 June 2019. Extended abstract and oral presentation "Isolation fungal strains from municipal wastewater for their use in pharmaceutical substances removal".
5. IWA 10th Eastern European Young Water Professionals Conference, Zagreb, Croatia, 7–15 May 2018. Extended abstract and oral presentation "Simple screening of fungal strains to determine potential application for the wastewater treatment process".
6. IWA 9th Eastern European Young Water Professionals Conference, Budapest, Hungary, 24–27 May 2017. Extended abstract and oral presentation "Study of potential PCR inhibitors in drinking water from Riga distribution system".
7. Riga Technical University 58th International Scientific Conference, Latvia, 16 October 2017. Oral presentation "Production and use of bioenergy in wastewater treatment plants: opportunities and perspectives".

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