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Removal of total phosphorus, ammonia nitrogen and organic carbon from non-sterile municipal wastewater with *Trametes versicolor* and *Aspergillus luchuensis*

Brigita Dalecka[a,b,*], Martins Strods[b], Talis Juhna[b], Gunaratna Kuttuva Rajarao[a]

[a] *Department of Industrial Biotechnology, School of Engineering Science in Chemistry, Biotechnology and Health, Albanova University Centre, KTH Royal Institute of Technology, Roslagstullcbacken 21, Stockholm, Sweden*
[b] *Water Research Laboratory, Riga Technical University, Kipsala, 6A-263, Riga, Latvia*

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ABSTRACT

Discharge of organic load from treated wastewater may cause environmental eutrophication. Recently, fungi have gained much attention due to their removal of pharmaceutical substances by enzymatic degradation and adsorption. However, the fungal effect in removing nutrients is less investigated. Therefore, two fungal species, the white-rot fungus *T. versicolor* as a laboratory strain and the mold *A. luchuensis* as an environmental isolate from the municipal wastewater treatment plant, were studied to determine the fungal potential for phosphorus, nitrogen, and the total organic carbon removal from municipal wastewater, carrying out a batch scale experiment to a fluidized bed pelleted bioreactor. During the batch scale experiment, the total removal (99.9 %) of phosphorus by *T. versicolor* was attained after a 6 h-long incubation period while the maximal removal efficiency (99.9 %) for phosphorus from *A. luchuensis* was gained after an incubation period of 24 h. Furthermore, both fungi showed that the pH adjustment to 5.5 kept the concentration of nitrogen constant and stabilized the total organic carbon reduction process for the entire incubation period. The results from the fluidized bed bioreactor demonstrated opposite tendencies on a nutrient removal comparing to a batch experiment where no significant effect on phosphorus, nitrogen, and total organics carbon reduction was observed. The obtained results from this study of batch and fluidized bed bioreactor experiments are a promising starting point for a successful fungal treatment optimization and application to wastewater treatment.

1. Introduction

White-rot fungi mainly have been studied for the removal of micropollutants as emerging concerns from wastewater throughout the last decade (Bulkan et al., 2020; Mir-Tutusaus et al., 2018). However, the removal of organic load and nutrients by fungi have been less investigated under non-sterile wastewater (Vasiliadou et al., 2016). Thus far, Shoun et al. (1992) have shown that a wide variety of fungi can perform denitrification (Shoun et al., 1992); Sankaran et al. (2010) have demonstrated the nitrogen source for fungi can be nitrates, nitrites, ammonium or organic nitrogen substances such as a yeast extract and peptone, depending on the type of fungi (Sankaran et al., 2010). Chemical precipitation, e.g., struvite precipitation, and biological assimilation by microorganisms, including fungi, are two major technologies used to remove P from municipal wastewater (Ye et al., 2015). In contrast to chemical precipitation, the fungal removal of phosphorus is considered as a more environmentally favorable and less expensive technology to remove and recover P from wastewater (He et al., 2019). Compared with bacteria, filamentous fungi have the advantage of being easy to harvest due to the mycelium growth and greater resistance to toxic and inhibitory compounds (Guest and Smith, 2007; Ye et al., 2015). Thus, filamentous fungi might be a promising candidates not only for micropollutant removal, but also to improve the classical biological treatment for wastewater to reduce the concentration of nutrients (Millan et al., 2000). However, several questions should be investigated in order to use fungi at full-scale

* Corresponding author at: School of Engineering Science in Chemistry, Biotechnology and Health, Albanova University Centre, KTH Royal Institute, Roslagstullcbacken 21, Stockholm, Sweden.
E-mail addresses: brigita@kth.se, brigita.dalecka_1@rtu.lv (B. Dalecka), martins.strods_4@rtu.lv (M. Strods), talis.juhna@rtu.lv (T. Juhna), gkr@kth.se (G.K. Rajarao).

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bioreactor, e.g., the pH effect on fungal growth; the need of fungal bioaugmentation in order to find optimal conditions of natural microorganisms interaction with fungi.

Trametes versicolor from the white rot fungi class has been shown as a promising candidate for removing micropollutants in wastewater treatment due fungal ability to use multiple biochemical and physical reactions to breakdown intermolecular bonds, demethylation, hydroxylation, dichlorinations, and the opening the aromatic rings (Freitas et al., 2009). Furthermore, all of these transformations are developed together to be combined with the enzyme system and the ability of adsorption, deposition and ion exchange (Dalecka et al., 2020a,b; Pedroza-Rodríguez and Rodríguez-Vázquez, 2013). However, most of the studies investigated the removal of pharmaceutical substances from municipal wastewater using laboratory strains from culture collections, including *T. versicolor* (He et al., 2019; Hultberg and Bodin, 2017; Spina et al., 2012). Therefore, Guest and Smith (2007) have suggested using fungi that are naturally available in a municipal wastewater treatment plant due to their adaptation to the environmental and operation conditions. For instance, recently *Aspergillus luchuensis,* - as an environmental isolate from a municipal wastewater treatment plant recently - has been showed as a promising candidate for a pharmaceutical substances removal from non-sterile wastewater (Dalecka et al., 2020a,b). Moreover, the experiments on both fungal strains have been performed in a batch-scale while only few works have examined the *T. versicolor's* potential of wastewater treatment under non-sterile conditions, using a bioreactor (Dalecka et al., 2020a,b; Pezzella et al., 2017). However, the removal efficiency can be affected by interaction with microbial community from the non-sterile wastewater (Dalecka et al., 2020a,b). Therefore, in terms of a fungi application to full scale wastewater treatment systems, more research is encouraged in this field (Mook et al., 2012).

In this paper, 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 a laboratory strain and *A. luchuensis* as an environmental isolate, 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, an observation of results were done under a batch-scale experiment with *T. versicolor* and *A. luchuensis*. During this phase, the removal of P, NH_4-N, and TOC was 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. Furthermore, 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. To the best of our knowledge, this is the first study where the nutrient removal of P, NH_4-N and TOC has been tested in a fluidized bed pelleted bioreactor, using *T. versicolor* and *A. luchuensis*.

2. Materials and methods

2.1. Fungal species

The fungal species – the white-rot fungus *Trametes versicolor* (L.) Lloyd strain DSM 6401 (Leibniz Institute DSMZ–-German Collection of Microorganisms and Cell Cultures, Brunswick, Germany) and the mold *Aspergillus luchuensis* (current name: *Aspergillus awamori* Nakaz.; an environmental isolate from a municipal wastewater treatment plant located in Stockholm, Sweden) were used in this study. The fungi were selected based on previous studies (Dalecka et al., 2020a,b) where *T. versicolor* and *A. luchuensis* have demonstrated a high potential to remove micropollutants.

2.2. Municipal wastewater samples

A municipal wastewater sample for a batch experiment was collected from the Henriksdal wastewater treatment plant (Stockholm, Sweden). The inlet wastewater was taken directly from an entry tank with the following composition: COD 500–700 mg/L, N_{tot} 40–50 mg/L, P_{tot} 4.0–5.0 mg/L, pH 7.6 – 7.7. For the pilot scale test the municipal wastewater sample was provided by the Daugavgriva wastewater treatment plant (Riga, Latvia). The inlet wastewater sample was directly taken from an entry tank with the following composition: COD 500–700 mg/L, N_{tot} 40–50 mg/L, P_{tot} 4.0–5.0 mg/L, pH 7.5 – 7.6.

2.3. Batch experiment

Fungal biomass was cultivated in the potato dextrose (PD) media (Oxoid, United Kingdom), incubating in a shaking incubator (50 rpm) for 5 days at 25 °C. To achieve a higher initial concentration of the selected fungi, the Kaldnes K1 carriers (AnoxKaldnes, France; diameter 9.1 mm) were used for a biofilm formation (one carrier unit per 1 mL) (Andersson et al., 2008). After growing, the fungal biomass was separated from the PD media and added to a non-sterile municipal wastewater sample without/with pH adjusting to 5.5 (1 M HCl acid). Additional samples were incubated in a shaker incubator (50 rpm) at 25 °C for a time period of 72 h. Further, an additional investigation of the P, NH_4-N, and TOC removal was done, and samples were taken every 3 for up to 72 h. All samples were filtered through a 0.22 μm membrane (Sartorius Stedim Biotech, Germany) and collected as culture filtrates for a further analysis of the P, NH_4-N, and TOC concentration, the laccase enzyme activity, and the pH level. Two additional negative controls – a control without carriers and a control without fungi – were prepared to compare the experimental results in order to be completely certain that the bioremoval of nutrients was induced by fungi. All experiments were carried out in duplicate or triplicate.

2.4. Bioreactor configuration and operating conditions

A fungal fluidized bed pelleted bioreactor was designed, consisting of a reactor, biomass tank for bioaugmentation, a feed peristaltic pump with flow-meter, air supply with flow regulator, and an effluent tank (Fig. 1). The reactor consisted of a 2 L cylindrical plastic column with working volume of 1.25 L. The up-flow velocity in the reactor was settled according to an experimental plan, i.e., approx. 1.08 mL/min or 0.11 mL/min where fungal biomass was maintained fluidized by air pulses generated by an air supply. At the beginning, the column was sterilized and filled with a 1.25 L synthetic wastewater medium (0.8 g/L KH_2PO_4; 0.2 g/L K_2HPO_4; 0.5 g/L $MgSO_4$; 0.2 g/L yeast extract (Oxoid, United Kingdom) and wet fungal biomass on Kaldnes K1 carriers (one carriers unit per 1 mL). After one week of adoption, the next amount of fungal biomass (25 g wet biomass per 100 mL) - harvested from 250 mL of a PD broth without carriers at least for 7 days cultivation period - was weighted and washed with deionized water. After washing, the wet biomass was homogenized with 250 mL non-sterile wastewater and added to the reactor. Before the fungal biomass adjustment, 250 mL of wastewater from the fungal fluidized bed pelleted bioreactor was removed through the effluent port and poured out in an effluent tank. Additionally, a negative control – a reactor with carriers without fungal biomass – was prepared to compare the experimental results in order to be completely establish a link to the nutrient removal induced by fungi. Samples were taken from the fungal fluidized bed pelleted bioreactor effluent port before (B) the adjustment of fresh non-sterile wastewater and after (A) the adjustment of

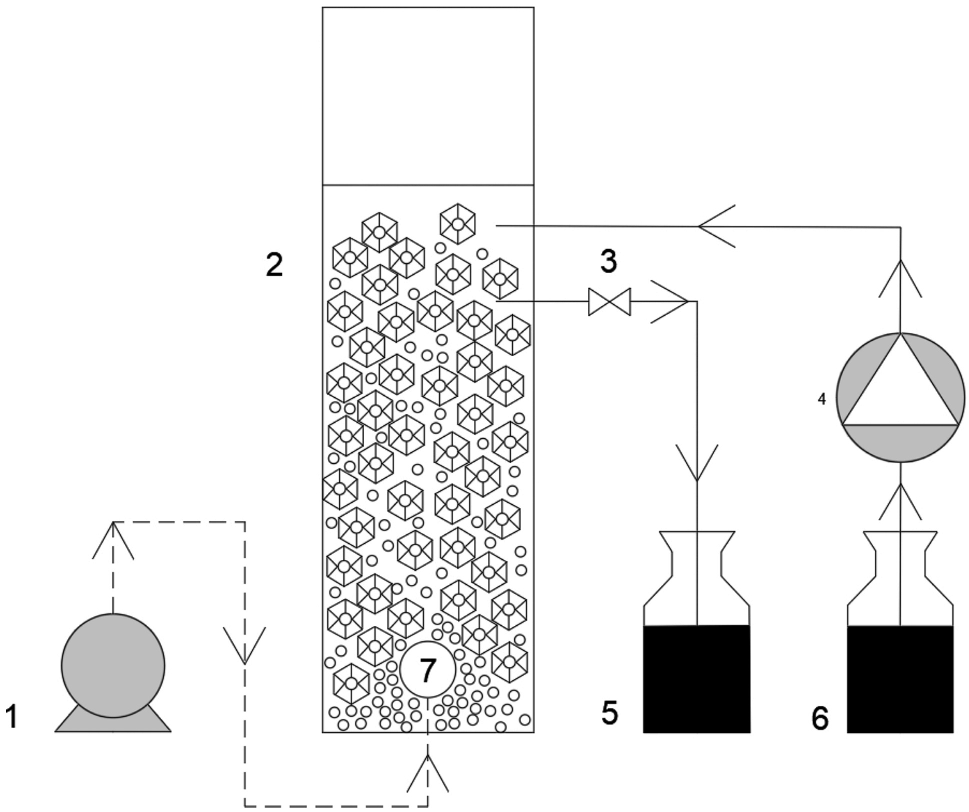

Fig. 1. A scheme of a fungal fluidized bed pelleted bioreactor. (1) Air flow; (2) Reactor; (3) Effluent port; (4) Peristaltic pump and flow-meter; (5) Effluent tank; (6) Fungal biomass tank; (7) Reactor with carriers.

250 mL fresh non-sterile municipal wastewater. All experiments were carried out in duplicate.

2.5. Analytical methods

2.5.1. P, NH_4-N, and TOC analysis

The Hach-Lange (Germany) spectrophotometric system and kits were used to determine the standardized procedure of PO_4^--P (total phosphorus; LCK 349; 0.05–1.5 mg/L), NH_4^--N (total ammoniacal nitrogen; LCK 304,; 0.015–2 mg/L), and TOC (total organic carbon; LCK 385; 3–30 mg/L). The determination of total P in a wastewater sample, using cuvette tests, is based on the reaction between phosphate ions and molybdate ions, as well as, subsequent reduction by ascorbic acid. The determination of total ammonical N is based on the ammonium ions reaction with hypochlorite ions and salicylate ions in the presence of sodium nitroprusside as a catalyst to form indophenol blue at the pH 12.6. The determination of the total TOC consists of a two-stage process. First, the total inorganic carbon is expelled with the help of the TOC-X5 shaker. Thus, the TOC is oxidized to carbon dioxide (CO_2). The CO_2 passes through a membrane into the indicator cuvette, where it causes a color change to occur, which is evaluated with a spectrophotometric system.

2.5.2. Quantification of bacteria

A direct counting method, using DAPI (4′,6-diamidino-2-phenylindole), was applied to obtain the total bacterial count in the wastewater sample (Zafiriou and Farrington, 1980). Shortly, a respective volume of sample was filtered onto a 25-mm-diameter filter (a pore size: 0.2 μm; Whatman, Germany). The sample was fixed with 3–4 % (v/v) formaldehyde and stained with 10 μg/mL DAPI for 10 min. A cell number was obtained by counting 20 random fields of view with an epifluorescence microscope (Leica DMLP, Germany), combined with a 50-W power supply, mercury lamp, and filter sets for DAPI (Ex.: 340/380 nm; Em: > 425 nm nm).

2.5.3. Enzymatic activity and pH measurements

The laccase activity was measured spectrophotometrically, using the standardized procedure of the enzymatic assay by Sigma-Aldrich (Germany). In brief, the test reaction contained 2.2 mL of a 100 mM potassium phosphate buffer (KH_2PO_2, pH 6.0), 0.5 mL of laccase from *T. versicolor* (crude powder, ≥50 units/mg solids, Sigma-Aldrich) and 0.3 mL of a 0.216 mM syringaldazine solution ($C_{18}H_{20}N_2O_6$, Sigma-Aldrich). The absorbance changes were measured for 10 min at 530 nm. The measurements were carried out in triplicate.

The pH level was measured during the batch and reactor experiments in every sampling by using the universal pH-indicator strips (pH 0–14; Merck KGaA, Germany).

3. Results and discussion

3.1. Nutrient removal in a batch experiment and the effect on pH

Over the last decades, it is stated that fungi, which have been isolated from wastewater treatment plants are more likely to be adopted to the natural environment, minimizing the operating conditions and costs (Guest and Smith, 2007). Therefore, during the initial study, the nutrient removal efficiency and the effect of pH on nutrient removal by two fungi - *T. versicolor* a laboratory strain and *A. luchuensis* an environmental isolate from a municipal wastewater treatment plant - were studied and compared to nutrient reduction in non-sterile municipal wastewater under a batch and pilot-scale experiment. The fungi were selected based on previous studies (Dalecka et al., 2020a,b) where *T. versicolor* and *A. luchuensis* have demonstrated a high potential to remove micropollutants as diclofenac and carbamazepine, during the wastewater treatment. However, there is still limited research about these fungi and their ability to remove nutrients from municipal wastewater under non-sterile conditions. The insight in the fungal potential to remove not only pharmaceuticals but also nutrients from municipal wastewater, could also help to better understand the fungal long-term goal of developing incorporation of fungal treatment technology (Mook et al., 2012).

3.1.1. Phosphorus removal

Experimental results which are shown in the Fig. 2, reflect the effect on the pH level of the P removal under the batch-scale test which has undergone a 72 h-long incubation period, using *T. versicolor* and *A. luchuensis*. The control was prepared with carriers as a no-biomass addition sample to confirm that the P removal performance occurred by fungi only. The results indicated that both fungi were able to remove P in non-sterile municipal wastewater without/with a pH adjustment. For instance, *T. versicolor* was able to reduce P from 2.6 mg/mL to 0.7 mg/mL immediately after the incubation was started without a pH adjustment (Fig. 2 a). The total removal (99.9 %) of P by *T. versicolor* was reached after 6 and 12 h of incubation period without/with a pH level adjustment, respectively (Fig. 2 a and b). On the contrary, the total removal (99.9 %) of P with *A. luchuensis* was gained after a 24 h-long incubation period for both pH values (Fig. 2 a and b). At the same time, the P removal from the control was relatively slow compared to *T. versicolor* and *A. luchuensis.* For example, the P removal from the control without a pH level adjustment was reduced from 2.6 mg/mL to 0.5 mg/mL after a 48 h of incubation period (Fig. 2 a) while the control with a pH level adjustment could reduce P from 2.7 mg/mL to 0.1 mg/mL after a 36 h of incubation period (Fig. 2 b). Therefore, the removal efficiency with the fungal biomass adjustment showed a higher and faster removal efficiency compared with the control, i.e., a 6 h incubation period for *T. versicolor* and a 24 h of incubation period for *A. luchuensis*. Finally, the results indicated that there was no statistically significant effect ($p > 0.05$) on a pH adjustment to increase the P removal efficiency for selected fungi. Furthermore, the results did not indicate that the P was release back in municipal wastewater for the entire incubation period.

Similarly, researchers who have previously conducted research on the P removal from municipal wastewater under batch experiments, have also presented a fungal ability to reduce P where the removal efficiency has varied from 12 to 100 % (Hultberg and Bodin, 2017; Sankaran et al., 2010; Ye et al., 2015). For example, Hultberg and Bodin (2017) investigated the pH effect on the P removal by using fungi in synthetic brewery wastewater (Hultberg and Bodin, 2017). In addition, results showed there was no significant effect on the pH level. However, *T. versicolor* was able to reduce P by 28 % only. In the cited study the selected synthetic wastewater contained approx. a 20 times higher P concentration (60 mg/mL, sterile) compared to the present study (3 mg/mL, non-sterile). Ye et al. (2015) have claimed that possible mechanisms for P removal by fungi, including *T. versicolor*, might be adsorption. Therefore, the adsorption capacity of P for the selected fungus might be reached in the cited study. In this study, there is no release of P observed till 72 h. Thus, there might be involved other mechanism of P removal, e.g., cellular growth of fungi. Overall, in the future application, phosphorus reduction by fungi might play an important role in a wastewater treatment system and reduction mechanisms. Most of the conventional treatment process uses chemical precipitation to remove P which require addition of chemicals and sedimentation steps (Ye et al., 2015). The P reduction in this study showed a potential application of fungi in wastewater treatment process. Therefore, fungal capacity should be investigated (Hultberg and Bodin, 2017).

3.1.2. Removal of ammonia nitrogen

The Fig. 3 indicates the removal efficiency of NH_4-N by *T. versicolor* and *A. luchuensis* comparing the results from wastewater without/with an adjustment of pH value. Results from *T. versicolor* without the adjustment of pH value showed an increase of NH_4-N concentration from 0.25 mg/mL to 2.3 mg/mL immediately after the incubation was started (Fig. 3 a). The same tendency was observed from *A. luchuensis* where the NH_4-N concentration presented an increase from 0.2 mg/mL to 1.4 mg/mL (Fig. 3 a). 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, i.e., from 1.8 mg/mL to 2.7 mg/mL (Fig. 3 b). Similarly, the concentration of NH_4-N for the control stayed at a relatively low level (> 0.6 mg/mL) until the end of the incubation period of 72 h for both pH values (Fig. 3 a and b). In this study, the NH_4-N increment might be explained by the effect of the pH value in municipal wastewater.

According to Mook (2012) et al., there are two forms of NH_4-N in wastewater, free ammonia (NH_3) and ammonium ion (NH_4^+) - which are reversible (Mook et al., 2012). The composition ratio of NH_3 to NH_4^+ mainly depends on the pH value in wastewater (Luo et al., 2015). The higher the pH value, the higher proportion of NH_3 - conversely, the ammonium ion proportion is higher at a lower pH value (Luo et al., 2015; Rezagama et al., 2017). Furthermore, the previous studies have shown *T. versicolor* and *A. luchuensis* have the ability to decrease the pH level to 5 immediately after the fungal biomass incubation in wastewater (Dalecka et al., 2020a,b). Therefore, this might explains the increment of the ammonia nitrogen concentration in municipal wastewater without a pH adjustment for both fungi (Fig. 3 a). The same tendency has also been presented by Biplob et al. (2011) where the NH_4-N removal increased linearly with the raise of the pH level, indicating the importance of the pH for a system stability of wastewater treatment (Biplob et al., 2011).

On the contrary, the wastewater with the pH adjustment showed a

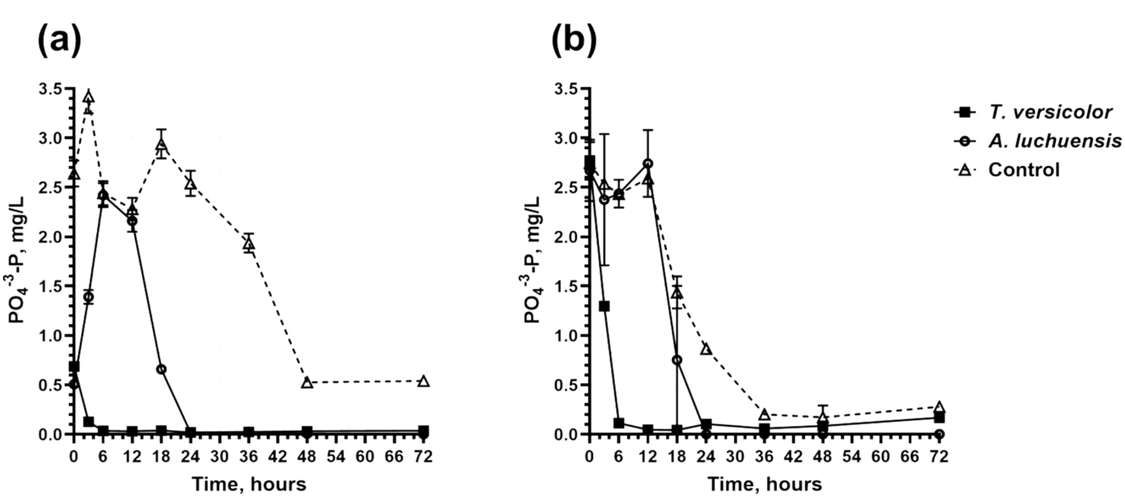

Fig. 2. P reduction from non-sterile wastewater by *T. versicolor* and A. *luchuensis* in a batch test (a) without a pH adjustment (b) with a pH adjustment to 5.5.

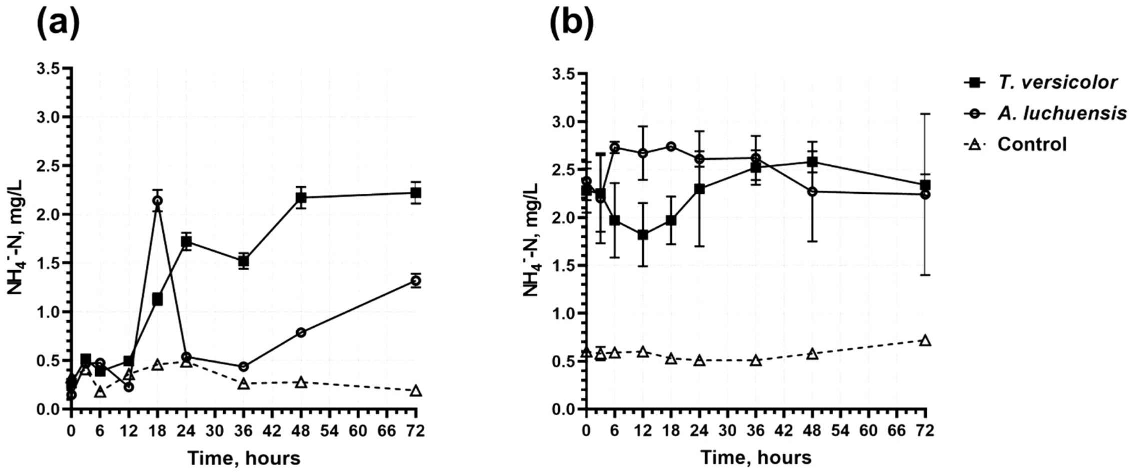

Fig. 3. The ammonia nitrogen reduction from non-sterile wastewater by *T. versicolor* and A. *luchuensis* in a batch test (a) without a pH adjustment (b) with a pH adjustment to 5.5.

more stable concentration of NH_4-N throughout the entire incubation time due of the pH adjustment to 5.5. Thus, the fungal biomass 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.

3.1.3. Removal of total organic carbon

Results of the Fig. 4 demonstrate the removal of TOC by *T. versicolor* and *A. luchuensis* from non-sterile municipal wastewater, compared to a control without fungal biomass as a negative control. When evaluating the TOC removal efficiency after a fungal treatment, it can be stated that *T. versicolor* and *A. luchuensis* can reduce TOC from > 3000 mg/mL to <2100 mg/mL after a 72 h of incubation period with a pH level adjustment for wastewater (Fig. 4 b). 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 (Fig. 4 a). For instance, both fungi showed the TOC reduction until 12 h of incubation and started to decrease after 18 h of incubation time. In the meantime, the control demonstrated relatively small changes in the TOC concentration reduction throughout the entire incubation time of 72 h for both pH values (Fig. 4 a and b). 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. The same tendency of TOC reduction in wastewater was observed by Kim et al. (2004). The cited research investigated *T. versicolor* and a membrane filtration potential of TOC removal from dye wastewater. Results showed that the TOC reduction by *T. versicolor* was relatively low (< 5 % from the starting concentration of TOC). Furthermore, the TOC removal was mainly caused by membrane filtration (Kim et al., 2004). Thus, the results of this study showed that selected fungi might have a less significant effect on the TOC reduction from municipal wastewater compared to the control. The inconsistency of the TOC reduction may require more time to adapt the fungi within the wastewater microbial community. However, the adjustment of the pH was able to keep the TOC removal more stable and constant for the entire incubation time.

Finally, to better understand the fungal mechanisms behind the nutrient removal of P, NH_4-N, and TOC by selected fungi, the pH and laccase activity were also monitored in this study. Fig. 5 demonstrates the pH changes and laccase enzyme activity derived from the batch experiment for the entire incubation time. The results showed that *T. versicolor* and *A. luchuensis* decrease the pH value immediately when the incubation was started and kept the pH value around 5 for the entire incubation time (Fig. 5 a and b) while the control without a fungal biomass adjustment presented a pH value at 6.5−7.5. Furthermore, the laccase activity was observed for white-rot fungus *T. versicolor* only. Laccases occur in many white-rot fungi together with lignin-peroxidases, manganese-peroxidases and further degrading agents

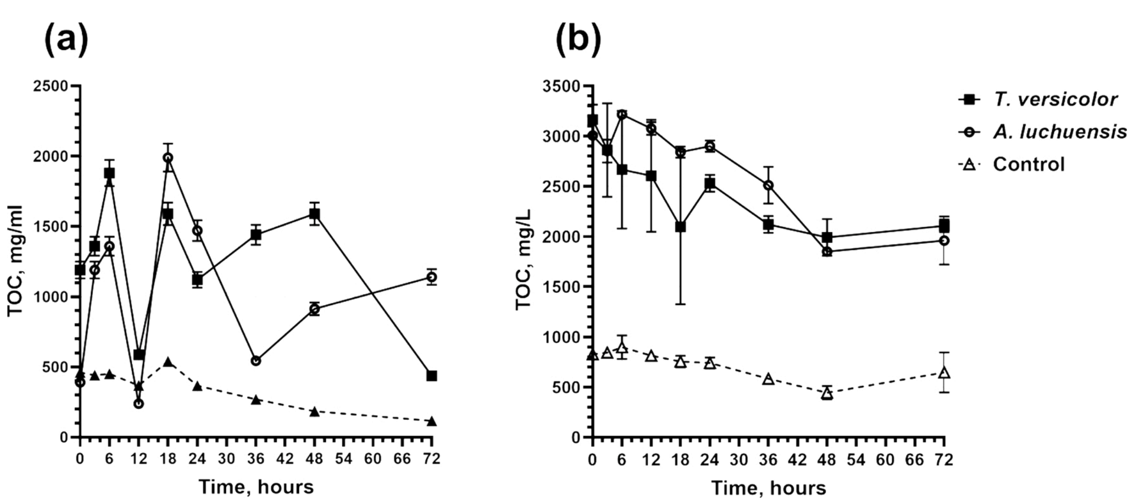

Fig. 4. TOC reduction from non-sterile wastewater by *T. versicolor* and A. *luchuensis* in a batch test (a) without a pH adjustment (b) with a pH adjustment to 5.5.

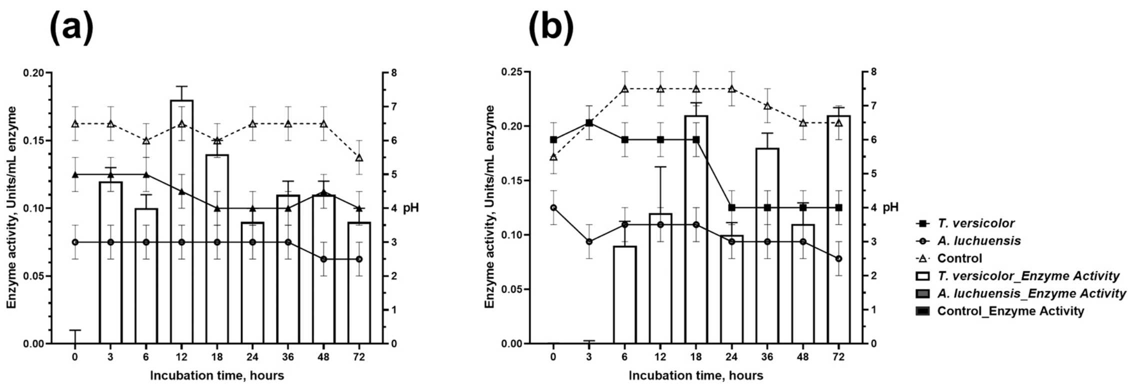

Fig. 5. A laccase enzyme activity (U/mL enzyme) and pH changes derived from a batch experiment (a) without a pH adjustment (b) with a pH adjustment to 5.5.

(Naghdi et al., 2018). However, laccases were also found in some other fungi like molds such as Aspergillus spp. which only discolor wood on its surface (Ramos et al., 2011). Therefore, it is believed that the mold *A. luchuensis* for the P removal used biosorption while *T. versicolor* - both mechanisms, i.e., biosorption and metabolism mechanisms. Moreover, the obtained results of the pH demonstrated that the pH value had a significant effect on N and TOC concentrations. However, results demonstrated that there is no need to adjust the pH value to 5.5 in non-sterile municipal wastewater because of the ability of fungi to decrease and keep stable the pH value to 5 naturally.

3.2. Nutrient removal in a fluidized bed pelleted bioreactor and an effect on flow

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 fluidized bed bioreactor is one of the most commonly used reactors for fungal treatment of wastewater (Andrews, 1988; Espinosa-Ortiz et al., 2016). The use of a fluidized bed bioreactor for wastewater treatment offers many advantages such as a compact bioreactor size due to a short hydraulic retention time, long biomass retention on the carriers, a high conversion rate due to fully mixed conditions, and consequently high mass transfer rates, no channeling of flow, dilution on an influent concentration due to a recycle flow (Moreira et al., 1996; Özkaya et al., 2019). Therefore, the fungal bioreactor is widely applied in the environmental engineering field for many purposes, including to minimize the organic compound load for the treatment process of different wastewater types (Özkaya et al., 2019). However, when a process is scaled up to a bioreactor, aeration and agitation may change when compared to a batch experiment. Thus, fungal biomass may responds differently to the mechanical and oxidative stress and fungal metabolic activity may change in a fluidized bed pelleted bioreactor (Spina et al., 2014). In this study, the removal efficiency of P, NH_4-N, and TOC, using *T. versicolor* and *A. luchuensis* was studied and analyzed. Furthermore, two up-flow velocity rates −1.08 L/min as maximal permissible flow for peristaltic pump and 0.11 L/min as 10 times lower flow compare to maximal permissible flow - were selected and tested in order to find the best optimal conditions for fungal adaption and growth in a fluidized bed pelleted bioreactor. The optimization of the flow can result in a continuously high density production of enzymes and a biomass formation in a bioreactor (Musoni et al., 2015).

3.2.1. Removal of P, NH_4-N, and TOC

Initially, the fluidized bed pelleted bioreactor was designed of three identical bioreactors (Fig. 1). Two of the bioreactors were used for each fungal species separately while the third bioreactor was used as a negative control without an addition of fungal biomass in order to compare the reduction of P, NH_4-N, and TOC between the selected fungi and exclude the interference on the nutrient reduction of any other microorganisms present in municipal wastewater. A sampling was done before (B) the adjustment of fresh non-sterile wastewater and after (A) the adjustment of 250 mL fresh non-sterile municipal wastewater in order to compare the changes in the nutrient load and total bacteria count throughout the entire incubation period.

The P removal profiles for both up-flow velocity rates, using fluidized bed bioreactors with *T. versicolor* and *A. luchuensis* are shown in the Fig. 6 (a and b). The results presented that both fungi were able to reduce more than 80 % of P until the end of the incubation period for both up-flow velocity rates. However, there was no statistically significant difference on the P reduction efficiency between fungi and the negative control ($p > 0.05$). Therefore, the results showed that there was no effect on the fungal adjustment on the P reduction, using a fluidized bed bioreactor, compared to results of batch experiments (Fig. 2).

The result of the NH_4-N concentration with up-flow velocity rates 1.08 L/min did not show any changes for both fungi until the 15th days of incubation period while the results of the negative control demonstrated an increase of the NH_4-N concentration for the entire incubation period (Fig. 6 c). On the contrary, the results from fluidized bed bioreactors with up-flow velocity rate of 0.11 L/min, indicated relatively small changes in the NH_4-N concentration for the entire incubation time (including the negative control) (Fig. 6 d).

Finally, the Fig. 6 (e and f) presents the TOC reduction results of *T. versicolor* and *A. luchuensi,* comparing to the negative control without an adjustment of fungal biomass. The results demonstrated that TOC has been reduced from 700 mg/L to > 250 mg/L after 15 days of the incubation period for both up-flow velocity rates. Furthermore, there was no statistically significant difference among both fungi and the negative control ($p > 0.05$) when the TOC concentration for both up-flow velocity rates after 15 days of the incubation time, were compared. Overall, the results of a fluidized bed bioreactor demonstrated different tendencies on the nutrient removal, using *T. versicolor* and *A. luchuensis* compared to a batch experiment. For example, the batch experiment showed a significant effect on the P reduction by *T. versicolor* and *A. luchuensis* compared to a negative control while there was no significant effect on P reduction by fungi in a fluidized bed bioreactor. One of the main problem to achieve a successful bioreactor performance in stable conditions with fungi is related to limiting hyphal growth, as well as, avoiding diffusional restrictions (Moreira et al., 1996). In bioreactor, the excessive growth of fungi provokes operational problems, i.e., growth back along the nutrient feed and sampling lines, decrease in the treatment efficiency due to increase of viscosity and mass transfer limitations (Moreira et al., 1996). The previously mentioned factors cause practical and technical difficulties in culturing fungi. Therefore in this study, the ability to control the fungal growth and regulate hyphal extension,

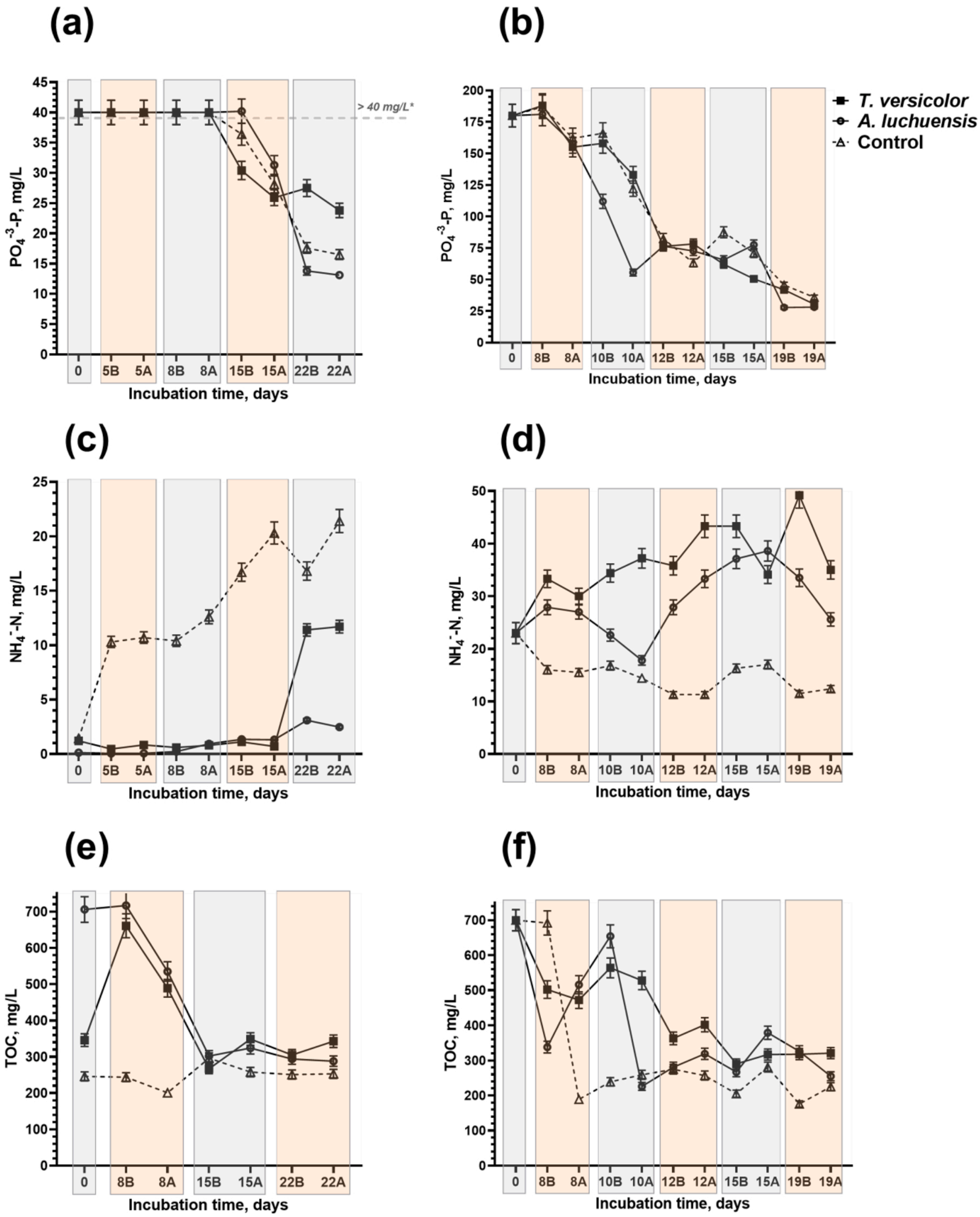

Fig. 6. A nutrient removal from non-sterile wastewater by *T. versicolor* and A. *luchuensis* in a fluidized bed pelleted bioreactor before (B) the adjustment of fresh non-sterile wastewater and after (A) the adjustment of 250 mL fresh non-sterile municipal wastewater. (a) P removal with a flow of 1.08 L/min (b) P removal with a flow of 0.11 L/min; (c) NH_4-N removal with a flow of 1.08 L/min; (d) NH_4-N removal with a flow of 0.11 L/min; (e) TOC removal with a flow of 1.08 L/min; (f) TOC removal with a flow of 0.11 L/min.

biofilm formation, and interaction around the carriers became difficult and required improvements. Furthermore, the results from a fluidized bed bioreactor showed the P/N ratio 1:5 and 1:7 for a batch and fluidized bed bioreactor, respectively. The difference in the nutrient load might be explained by wastewater sampling from two different wastewater treatment plants and the use of synthetic wastewater in a fluidized bed bioreactor at the beginning of the incubation time. The synthetic wastewater was used to better adopt the fungal biomass in fluidized bed bioreactor conditions (Sankaran et al., 2010). Additionally, the changes in the nutrient load were caused by an adjustment of fresh non-sterile municipal wastewater. Therefore, the removal of P, NH_4-N, and TOC was relatively slower compared to the batch-scale experiment.

In this study, the results have also demonstrated that a sufficient and regular fungal biomass augmentation ($<$ 20 g wet biomass per 100 mL) to non-sterile wastewater in a bioreactor helped to adjust the pH level lower than $>$ 5. Therefore, the natural growth of microorganisms was limited and fungi were able to reduce P (Fig. 7 a). Moreover, the ability to decrease the pH level by *T. versicolor* and *A. luchuensis* may

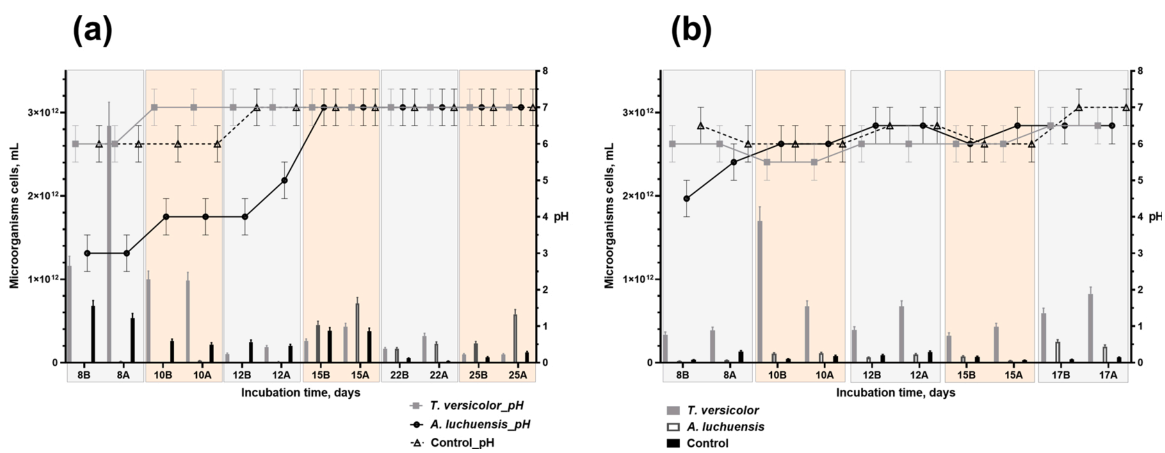

Fig. 7. A number of microorganism's cell and pH changes in a fluidized bed pelleted bioreactor with (a) a flow of 1.08 L/min; (b) a flow of 0.11 L/min. The total bacteria count was obtained before (B) the adjustment of fresh non-sterile wastewater and after (A) the adjustment of 250 mL fresh non-sterile municipal wastewater.

demonstrate an advantage to minimize the cost of the pH adjustment. Therefore, a preliminary analysis was performed to evaluate the cost associated with a fungal treatment in a fluidized bed pelleted bioreactor and compared to classical treatment methods.

3.2.2. *Cost evaluation of fungal treatment*

Due to the ability to apply the secretion of an extracellular non-specific enzymatic complex during their secondary metabolism, fungi have the unique ability to degrade the bulky, heterogeneous and recalcitrant polymers (Espinosa-Ortiz et al., 2016). This potential can be used to remove xenobiotics and micropollutants from wastewaters (Dalecka et al., 2020a,b; Naghdi et al., 2018). Thus, in the last decade there has been growing interest to integrate fungal bioreactors into the wastewater plants (Cruz del Álamo et al., 2020; Freitas et al., 2009; Mir-Tutusaus et al., 2019; Negi et al., 2020). The authors of this study believe that there are at least two possible ways to apply fungi at the WWTP: (i) to encourage fungi growth in situ on an organic substrate present in the wastewater, or (ii) to cultivate them separately and then dose in the process (bioaugmentation). In this study authors examined the second option. This study showed that with bioaugmentation is possible to maintain domination of fungi over bacteria without a pH adjustment and effectively remove P, NH_4-N, and TOC (Fig. 7). However, it does require an additional costs, including an extra source of the organic substrate to cultivate fungi. Here, the authors have 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 1).

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 to 1 EUR/m^3 while the fungal treatment growth and operation costs may vary from 200 to 2000 EUR/m^3. 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 micropollutant removal (Mir-Tutusaus et al., 2018). 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).

Table 1
The average price for 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)

4. Conclusions

In this study, a batch scale experiments using *T. versicolor* and *A. luchuensis* were performed for non-sterile municipal wastewater and the pH effect on the P, NH_4-N, and TOC reduction was analyzed. Additionally, the fluidized bed bioreactor was designed and removal efficiency was tested. Although, bacteria are still the preferred microorganisms to be used in bioreactors for the treatment of municipal wastewater, during this study, fungi have demonstrated a high potential to remove phosphorus from municipal wastewater efficiently and successfully under a batch scale experiment. In the further work, optimization and development of fluidized bed bioreactor operations, using fungi, should be investigated and evaluated.

Author contributions

T.J. and G.K.R. devised the project, its main conceptual idea, and proof outline. B.D. designed and carried out experiments. T.J. and M.S. designed the concept of the fluidized bed bioreactor. B.D. wrote the manuscript with support from M.S., T.J., and G.K.R. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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