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Conference paper, prepared as extended version of the oral communication presented during SIDISA 2016, International Symposium on Sanitary and Environmental Engineering, 19-23 June 2016

Recalcitrant compounds removal in raw leachate and synthetic effluents using the white-rot fungus *Bjerkandera adusta*

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Received: date; Accepted: date; Published: date

Abstract: Recalcitrant compounds limit the efficiency of conventional biological processes for wastewater treatment, representing one of the major issues in the field. This study focused on the treatment of three effluents with the White-Rot-Fungus (WRF) *Bjerkandera adusta* MUT 2295 in batch tests, with biomass cultivated in attached form on polyurethane foam cubes (PUFs) to test its efficiency in the removal of target effluents' recalcitrant fraction. Treatment efficiency of *B. adusta* was evaluated on the landfill leachate (Canada) and the two solutions containing synthetic recalcitrant compounds, which were prepared with tannic and humic acid. Chemical Oxygen Demand (COD) and color removal, the production of manganese peroxidases and the consumption of a co-substrate (glucose) were monitored during the experiment. Biological Oxygen Demand (BOD_5) and fungal dry weight were measured at the beginning and at the end of the experiment. After co-substrate addition, effluents' COD were 2300 ± 85, 2545 ± 84 and 2580 ± 95 (mg/L) in raw leachate, tannic and humic acids, respectively. COD removal of 48%, 61%, and 48% was obtained in raw leachate and in the synthetic solutions containing tannic and humic acids, respectively. Color removal of 49 %, 25% and 42% was detected in raw leachate and in tannic and humic acid solutions, respectively. COD and color removals were associated with the increase of fungal dry weight, which was observed in all the trials. These results encourage the use of the selected fungal strain to remove tannic acid, while further investigations are required to optimize leachate and humic acid bioremediation.

Keywords: bioremediation, landfill leachate, recalcitrant compounds, wastewater treatment, white-rot fungi

1. Introduction

The concept of recalcitrant compounds was introduced to define structurally novel and naturally occurring compounds resistant to microbial degradation and persistent in the environment for extended periods [1]. The presence of these compounds have emerged as a major issue in wastewater

treatment processes since the state-of-the-art technologies for their removal are, in general, complex and costly [2]. The search for efficient and sustainable technologies led to an increasing interest in advanced biological processes.

In particular, White-Rot Fungi (WRF) and their extracellular enzymes have been investigated for the removal of hazardous and recalcitrant pollutants [3]. The efficiency of WRF in degrading recalcitrant molecules is related with their ability to secrete extracellular enzymes such as lignin peroxidases (LiP), manganese peroxidases (MnP) or laccases, which are involved in lignin and lignocellulosic substrates degradation [4]. Compared to the use of bacteria, treatments involving fungi could be advantageous as it offers an easier degradation of high molecular mass organic pollutants and a higher rate of COD reduction in several industrial wastewaters [4]. Thus, WRF potential to depolymerize several compounds, traditionally recalcitrant in conventional wastewater treatment processes, could be exploited as a preliminary step to allow subsequent bacterial degradation [5]. Up to date, the use of WRF was effective, at laboratory scale, toward several xenobiotic, including phenols, dyes, hydrocarbons, textile effluents, pharmaceuticals, waste products of pulp and paper mill industries, pesticides and insecticides [6].

Fungal-based approach showed promising results also on landfill leachate, a highly polluted wastewater, whose decontamination requires innovative and sustainable technologies [2]. Despite the high variability detectable in landfill leachate chemical composition, in the majority of landfill leachates a common basal composition can be observed [7]. In particular, according to Ghosh et al. [7], four main categories of pollutants can be grouped: i), dissolved organic matter, including volatile fatty acids and more refractory compounds like fulvic-like and humic-like compounds; ii) inorganic macro components; iii) heavy metals and iv) xenobiotic organic compounds like aromatic hydrocarbons, phenols and chlorinated aliphatics. Toxic effects of leachate have been reported on microbes, algae, invertebrates, plants as well as mammals, indicating the environmental risk posed by the landfill leachates to the ecosystem as a whole, including humans [8].

At the moment, only a few studies are available in the literature about the use of WRF in landfill leachate treatment and the majority of them have been performed through batch tests. Ellouze et al. [4,9] detected COD removal efficiencies of 68%, 79% and 90% for *Phanerochaete chrysosporium*, *Trametes trogii* and *Lentinus tigrinus*, respectively, with 50% diluted leachate (initial COD: 8000 mg/L), accompanied by a significant enzyme secretion and a high reduction in the toxicity expressed as percentage of *Vibrio fischeri* bioluminescence inhibition (% BI < 20%). Kalčikova et al.[3] reported a maximum COD removal of 60% using extracellular enzymes obtained from *Dichomitus squalens* on 50% v/v landfill leachate. Tigini et al. [10] provided evidence of the efficiency of autochthonous and allochthonous fungal strains in landfill leachate treatment, showing their ability to significantly reduce its toxicity. The same authors indicated 40% of color removal from landfill leachate, using *Lopharia spadicea* MUT 1585. Aswathi et al. [11] reported important COD reductions by inoculating the enzyme produced by *Trichoderma harzianum* in solid waste leachate. Furthermore, Ghosh and Thakur [12] reported considerable contaminant-level reductions achieved with *Phanaerochaete* sp. ISTL01 for biosorption of color from landfill leachate. Among the studies related to WRF in the bioremediation of landfill leachate, only a small percentage have been performed in continuous systems. Indeed, Saetang and Babel [13] reported 63% color removal, 52% reduction of 5-day-Biological Oxygen Demand (BOD_5), and 42% COD removals in continuous experiments with optimum conditions. Ghosh et al. [14] proposed a combined approach of fungal and bacterial treatment, using bioreactors. The combined treatment led to 76.9% of COD removal and 45.4% of decolorisation, under optimized conditions. Furthermore, Bardi et al reported a maximum COD removal of 63 % using WRF on old landfill leachate, which is characterized by low BOD_5/COD (<0.1) and high concentrations of high refractory humic and fulvic acids. These authors performed experiments on landfill leachate using bioreactors, in which two co-substrate dosages were applied: at the beginning of the test and when the performance, in terms of COD removal, was decreasing [15].

The potential of WRF has been investigated also on humic acids, which are very complex and recalcitrant compounds, naturally occurring in landfill leachate. Thanks to their high adsorbing

abilities, humic acids can bind with other substances, leading to serious co-contamination problems. As a consequence, the presence of humic acids can negatively affect also the quality of public water supplies [16]. Batch tests performed on the removal of these compounds using *Bjearkandera adusta* showed a full decolorisation of 0.03% humic acids from brown coal and lessive soil, associated with the secretion of extracellular enzymes, suggesting the presence of a degradative process performed by the fungus [16]. Furthermore, about 80% of humic acid removal associated with extracellular enzymatic activity was reached using *Trametes versicolor* and *Phanerochaete chrysosporium* on a synthetic solution prepared with 0.8 % humic acid, through batch tests [17, 18]. The authors indicated that the observed bioremediation process was due to a combination of biodegradation and biosorption [17, 18]

Despite the increasing interest toward the use of WRF as a potential bioremediation tool, processes based on fungal biomass are still poorly applied. Indeed, long-term operations of fungal bioreactors under non-sterile conditions have been demonstrated to be difficult to maintain [5]. This is mainly due to bacterial contamination, which results in competition for available organic substrates, negatively affecting WRF metabolism [19]. At the moment very few is known about the optimal operating to enhance fungal bioremediation potential, such as fungal needs and responses to feeding and potential interferences on hyphal growth and fungal metabolism due to mixing or aeration [20]. This lack of knowledge may limit the scale-up of fungal-based processes. Indeed, several authors reported low yields or slow processes depending on the design and operation of the reactor [19].

In this study, the efficiency of *Bjerkandera adusta* MUT 2295, previously selected for its decolorisation capability on a sample of landfill leachate from Italy [20], was tested towards three effluent samples. This fungal strain, already reported as effective in the degradation and detoxification of textile, tannery and pharmaceutical wastewaters [21, 22], was evaluated, in the treatment of a raw landfill leachate (Canada) and, for the first time, on two synthetic wastewaters containing tannic and humic acids, which were selected as components of the recalcitrant fraction of landfill leachate [23, 24, 25]. In particular, several parameters, including COD and color removals and MnP activity, were measured in the three effluents to verify whether the selected strain could be exploited as a bioremediation tool for target effluents' recalcitrant fraction.

2. Materials and Methods

2.1 Fungal strain

Bjerkandera adusta MUT 2295 was obtained from the Mycotheca Universitatis Taurinensis Collection (MUT), University of Turin. This fungal strain was selected out of 12 strains due to its capability to treat raw leachates (Italy). Strain selection was performed through a biodegradation experiment in which decolorisation potential was used as main criteria, in which *B. adusta* MUT 2295 was able to remove the color up to 40% and produce MnP [20].

2.2 Target effluents

The efficiency of the treatment with *B. adusta* was tested on a raw leachate collected from Brady Road landfill (Winnipeg, Canada) and two synthetic recalcitrant compound solutions prepared with tannic acid (1.3 g/L) and humic acid (1.5 g/L). Both compounds were chosen as representatives of landfill leachate recalcitrant fraction [23, 24, 25]. The solutions were prepared by adding the chemicals directly into deionized water, without the addition of nutrients nor sterilization. Tannic and humic acids, used in this study, were of analytical grade and purchased from VWR Canada.

2.3 Fungal cultivation and experimental conditions

B. adusta was cultured on Malt Extract Agar (MEA, 20 g/L glucose, 20 g/L malt extract, 20 g/L agar, and 2 g/L peptone) at 25°C for one week (Figure 1a). After the cultivation, *B.adusta* was homogenized under aseptic conditions, with sterile saline (9 g/L NaCl) and inoculated into 1 L flasks containing glucose and yeast extract liquid broth (GLY, 5 g/L glucose, 1.9 g/L yeast extract) and 2 cm^3 polyurethane foam cubes (PUF); 1.5 mL of homogenate was added per cube. Flasks were incubated with agitation (150 rpm) and at room temperature (23 ± 2 C°) for one week, in order to enable the immobilization of the fungus onto the cubes (Figure 1c). After 7 days, the cubes were removed from the broth to start the experiment with raw leachate and the two synthetic effluents. In particular, two immobilized cubes were added to 500 mL flasks containing 160 mL of effluent. Flasks were kept in agitation (150 rpm) for ten days at room temperature (23 ± 2 C°). Trials were triplicated including the same number of controls, without fungal inoculum, for a total of 18 flasks. Glucose (1.0 g/L) was added to the three effluents as fungal co-substrate for growth. The pH was adjusted to 4.5 using 10% sulphuric acid in raw leachate and humic acid and using NaOH (1.0 N) for tannic acid. All the chemicals, used to prepare MEA and GLY and to adjust the solutions' pH, were of analytical grade and purchased from VWR, Canada. Grab samples for the evaluation of treatments' efficiency were collected after the few minutes and after 24, 48, 96, 168 and 240 h from the beginning of the test

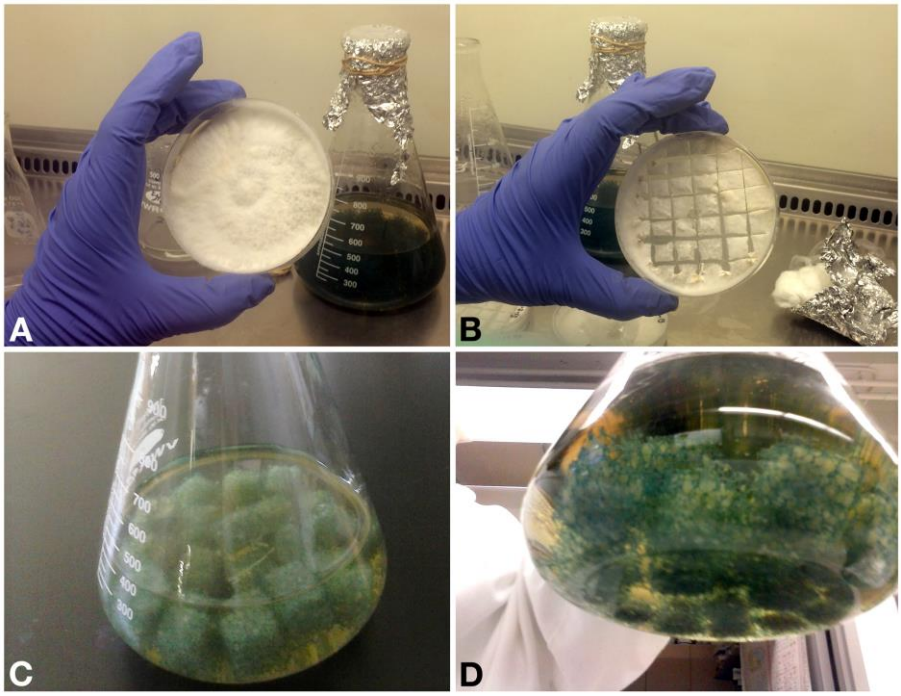

Figure 1. Fungal cultivation (A) and immobilization on PUF (B, C, D).

2.4. Parameters for the evaluation of treatments' efficiency

Effluent pH was measured using Eutech pH 5^+ pH-meter. Glucose was used as co-substrate for fungal growth and its concentration was measured during the first 3 days of treatment, according to the reducing sugars protocol [26]. COD removal, decolorisation, MnP activity and BOD_5 were used to evaluate the efficiency of the treatment. All these parameters, except BOD_5, were measured after the few minutes and after 24, 48, 96, 168 and 240 h from the beginning of the test. COD was measured according to the Standard Methods for Examination of Water and Wastewater 20^{th} edition Section

5220, Hach Spectrophotometric procedure (spectrophotometer DR2800) [27]. COD removal % was calculated as follows:

$$\%\ \ COD\ \ \ removal = \left(\frac{COD0h - COD240h}{COD0h}\right) * 100 \quad (1)$$

BOD_5 was measured at the beginning of the test, after glucose addition, and at the end of the treatment, according to Standard Methods for Examination of Water and Wastewater (SMEW, 20th Edition) [27].

The decolorisation percentage (DP) in raw leachate and the humic acid solution was determined spectrophotometrically as the decrease of the spectrum area (the integral of the absorbance spectrum) in the visible range (380-760 nm) with respect to the unseeded control [21]. DP calculation is showed in equation 2, in which the term At is the spectrum area of the trials inoculated with the fungus and the term Ac refers to the spectrum area of the unseeded trials .

$$DP = 100 - \left(\frac{Atreat * 100}{\text{Acontrol}}\right) \quad (2)$$

The removal of tannic acid solution was monitored spectrophotometrically as the decrease of the absorbance spectrum area in UV range (200-380 nm) with respect to the unseeded control, with particular attention to the peaks corresponding to tannic acid (274 nm) and gallic acid (310 nm).

The MnP activity was measured at 25 °C, monitoring the oxidation at 590 nm of dimethylaminobenzoic acid/3-methyl-2-benzothiazoline hydrazone hydrocloride (DMAB/MBTH), in 0.1 M succinate lactate buffer pH 4.5 [28]. The enzymatic activity was calculated in international Units (U), where 1 unit is defined as the amount of enzyme that oxidases 1μmole of substrate per minute. The calculation of MnP activity is showed in equation 3, whose terms can be explained as follows:

- Δ is the difference between the absorbance at 590 nm of the sample and the one of respective blank, which was performed without substrate addition;
- $Vsample$ is the volume (μL) of the sample in the reaction mixture (20 μL), Vmixture is the total volume of the mixture(100 μL);
- $\varepsilon 590$ is the molar extinction coefficient for DMAB and MBTH.(32900 M^{-1} cm^{-1})

$$MnP\ \ U/L = \left(\frac{\Delta * 1000 * Vsample}{\varepsilon 590 * Vmixture}\right) \quad (3)$$

Samples for ammonium nitrogen (NH_4^+-N), nitrites (NO_2^--N) and nitrates (NO_3^--N) were previously filtered, using Whatman Filter Papers Grade 1, and their concentrations were measured via a flow injection analyzer (Quick Chem 8500, LACHAT Instruments).

The dry weight of fungal biomass was measured before starting the experiment, at the end of immobilization in GLY, and at the end of the experiment. To discriminate the weight of fungal biomass inside a cube and the weight of the cube, from each measurement the dry weight of a single empty cube was subtracted. Empty cube dry weight was calculated as the average among three replicates. For each flask, two PUFs were used, for a total of two PUFs after the immobilization phase and 6 PUFs/condition at the end of the experiment. The dry weight was measured after overnight standing at 65 °C. Dry weight increase was calculated as the difference between the dry weight of fungal biomass/flask at the end of the experiment and at the end of the immobilization phase.

At the end of the experiment, Total Suspended Solids (TSS) were measured in each flask, according to Standard Methods for Examination of Water and Wastewater (SMEW, 20th Edition) [27]. The total biomass was calculated at the end of the experiment as the sum of dry weight increase, expressed in mg/L, and TSS (mg/L).

2.5 Statistical analysis

The data of COD and color removals were analyzed with the aid of one-way ANOVA, using STATISTICA 7.0 (StatSoft, Inc., Tulsa, OK). Variance homogeneity was tested using Levene's Test. In case of $p<0.05$ (Levene Test), ANOVA was performed through Welch's Test.

3. Results

3.1 Target effluents characterization

Details of the raw leachate chemical characterization were as follows: pH 7.6, COD 1636 ± 84 (mg/L), ammonium nitrogen 704 ± 202 (mg/L), phosphorus 3.36 ± 0.98 (mg/L), BOD_5 150 (mg/L), BOD_5/COD ratio 0.091. These characteristics suggest that the landfill leachate used in this study was old, with high refractory carbon load and high ammonium nitrogen concentration. After glucose addition, raw leachate COD was 2300 ± 85 (mg/L). Initial COD values of the two synthetic solutions that simulate raw leachate in terms of organic load, were 1630 ± 9 and 1740 ± 37 (mg/L) before glucose addition and 2545 ± 84 and 2580 ± 95 (mg/L) after glucose addition in tannic acid and humic acid solution, respectively.

3.2 pH values

In the trials inoculated with *B. adusta*, effluents' pH average value was 5.07 ± 1.03 with a minimum value of 4.17 and a maximum of 7.78. In the unseeded controls, the average pH value was 4.57 ± 0.45 with minimum value of 4.07 and maximum of 5.34 (Figure 2). These results indicated higher pH stability in unseeded controls, compared to the trials inoculated with the selected fungal strain, in which a gradual pH increase could be observed starting from 96 h from the beginning of the experiment. In particular, in raw leachate trials pH increase from 4.75 ± 0.03 at 48 h to 5.79 ± 0.015 at 96 h, reaching a final value of 7.78 ± 0.04 at the end of the experiment (240 h). In those flasks containing humic acid, pH rise could be observed only in the last measurement ranging from 4.88 ± 0.20 at 168 h and 6.26 ± 0.54 at 240 h.

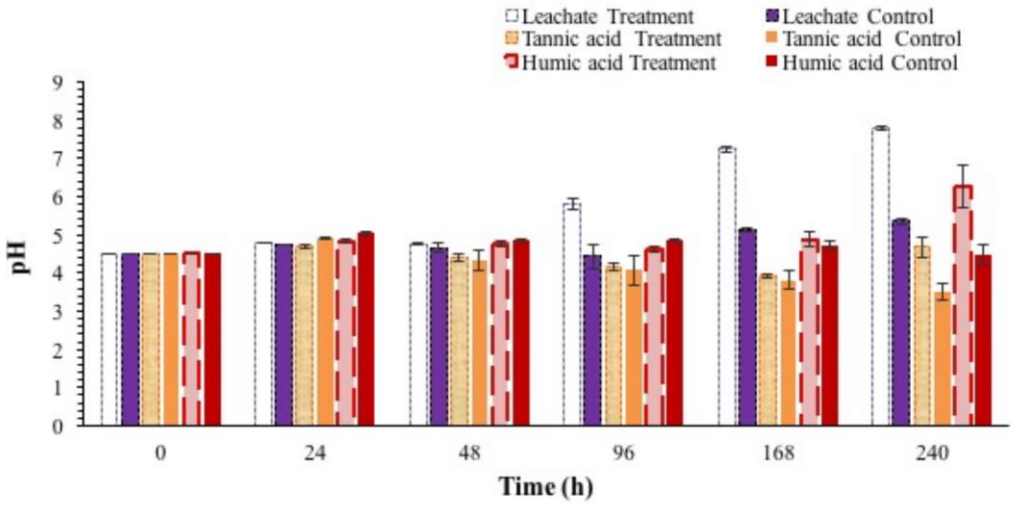

Figure 2. Effluents pH values during the treatment given as the average among trials; error bars indicate the standard deviations (SD) of the three replicates.

3.3 Glucose consumption and BOD_5 removal

Consumption of glucose, co-substrate for fungal growth, was measured after 3 days from the beginning of the experiment. As shown in Table 1, in the trials inoculated with *B. adusta*, average glucose consumption percentage was 60 ± 5.63, with a minimal consumption of 54 % in tannic acid trials and a maximum one of 65 % in raw leachate. The results showed 5 % and 12% of glucose consumption in the controls of humic acid and tannic acid solutions, respectively. On the contrary, 66% of glucose consumption was detected in raw leachate control, suggesting the presence of autochthonous microorganisms capable of using glucose as carbon source.

In table 1, the results of BOD_5 removals in the three effluents at the end of the treatment are shown. BOD_5 removal was complete in raw leachate and 89% and 75% in tannic acid and humic acid solutions, respectively. In the case of raw leachate, the initial BOD_5 value of 150 mg/L was fully removed, implying the complete removal of leachate biodegradable organic matter and total glucose consumption. In the two synthetic solutions, 11% and 25% of BOD_5 was detected at the end of the treatment, which is attributable to residual glucose or an eventual increase in the biodegradability (as BOD_5/COD) of the effluents, due to the fungal treatment. Further investigations, such as respirometric tests, could clarify this point.

Table 1. BOD_5 concentration in the three effluents at the beginning of the test (after glucose addition), at the end of the treatment (240 h) and BOD_5 removal (%) between 0 and 240 h. Glucose concentration at the beginning of the test, at 96 h and glucose consumption (%) measured against the different effluents during the test. Values are given as the average among triplicates. Bars indicated standard deviations (SD) T: trials inoculated with *B.adusta*; C; controls. RL: Raw Leachate; TA: Tannic Acid and HU: Humic acid.

		Beginning (0 h)	**End of the experiment (240 h)**		**Beginning (0 h)**		**96 h**		
	Effluent	**BOD_5 mg/L**	**BOD_5 mg/L**	**BOD_5 removal (%)**	**Glucose mg/L**	**SD**	**Glucose mg/L**	**SD**	**Glucose removal (%)**
T	RL	840	< 40	100	933	293.79	329	6.053	65
	TA	720	80	89	1140	201.09	527	7.176	54
	HA	770	190	75	1286	193.78	494	106.85	62
C	RL	n.a	n.a	n.a	974	154.49	336	10.53	66
	TA	n.a	n.a	n.a	1444	56.28	1265	233.62	12
	HA	n.a	n.a	n.a	1483	n.a.	1406	66.67	5

n.a: data not available.

3.4 COD removal

COD removal in the three effluents after 10 days from the inoculation of *B. adusta* MUT 2295 is reported in Table 2. The results showed COD removals of 48%, in the trials with raw leachate and humic acid and 61% in those containing tannic acid.

As glucose represented about 36-39% of effluent's COD, assuming its complete depletion at the end of the experiment, the detected COD removal percentages were higher than glucose consumption in all the samples inoculated with *B. adusta*. A significant difference in the COD values, between treatments inoculated with *B. adusta* and the respective unseeded controls was found in the trials with humic acid ($p=0.22$, $F=7.22$) and tannic acid ($p \leq 0.001$), respectively. In the case of raw leachate and humic acid, the data met the assumption of the homogeneity of the variance (Levene's Test), with $p=0.326$ and $p=0.148$. On the contrary, in the case of tannic acid the assumption of variance homogeneity was not fulfilled ($p=0.006$). For this reason the results of tannic acid removal were

analyzed using the Welch's Test and achieving $p<0.001$. The maximum COD removal was achieved in the trials containing tannic acid, with a final COD value of 992 ± 26.62 mg/L and 61 % of COD abatement with respect to the initial value, while only 6 % COD removal was found in the respective unseeded controls (Table 2). Although significant differences between treatments and controls were detected in both synthetic solutions, the COD removal observed in the trials containing humic acid and inoculated with *B. adusta* was presumably due to glucose consumption. The difference in the COD removal between treatments and controls was 33%, which was lower than the organic load represented by glucose (37 %). A similar pattern could be observed in raw leachate trials, in which the percentage of COD removal (48 %) was the same in the treatments, inoculated with *B. adusta*, and in the respective unseeded controls.

Table 2. COD values (mg/L) at the beginning of the experiment (0 h), at the end experiment (240 h) and COD removal percentage (0-240 h). Values were given as average among triplicates ± SD. *indicates $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$, one- way ANOVA, difference between the treatment and the respective unseeded controls. .

		Beginning (0 h)		End of the experiment (240 h)		
	Effluent	**COD mg/L**	**SD**	**COD mg/L**	**SD**	**COD removal (%)**
Treatments	Raw Leachate	2365	82.82	1185	80.11	48
	Tannic Acid***	2545	82.74	992	26.65	61
	Humic acid*	2580	94.76	1346	27.07	48
Controls	Raw Leachate	2747	451.55	1399	33.62	48
	Tannic Acid	2780	142.32	2610	185.33	6
	Humic acid	2758	47.38	2336	424.09	15

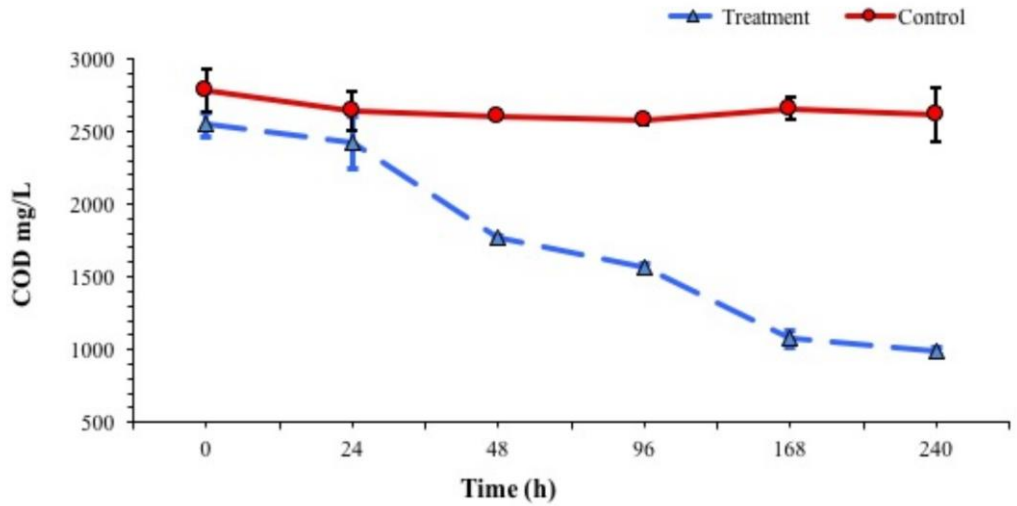

Figure 3. COD values as mg/L in tannic acid trials during the experiment. Values are given as the average among triplicates. Bars indicated standard deviations (SD).

3.5 Color removal

As shown in Figure 4a, *B. adusta* decolorized raw leachate, humic acid and tannic acid solutions up to 49%, 42% and 25% after one week of treatment, respectively. The results of raw leachate and humic acid met the assumption of homogeneity of the variance (Levene test), with $p=0.101$ and $p=0.074$ in raw leachate and humic acid, respectively. On the contrary, tannic acid results did not meet the homogeneity assumption ($p=0.006$) and were analyzed using Welch's test. In the case of

dark colored effluents (raw leachate and humic acid solution), differences in the spectrum area toward respective unseeded controls were significant after 96 h and highly significant after 168 h of treatment (Figure 5a, 5b). In particular, in raw leachate p=0.24 (F=8.30) after 96 h and p=0.009 (F=11.54) after 168h, while in humic acid p=0.04 (F=7.57) after 96 h and p= 0.008 (F=12,38) after 168 h.

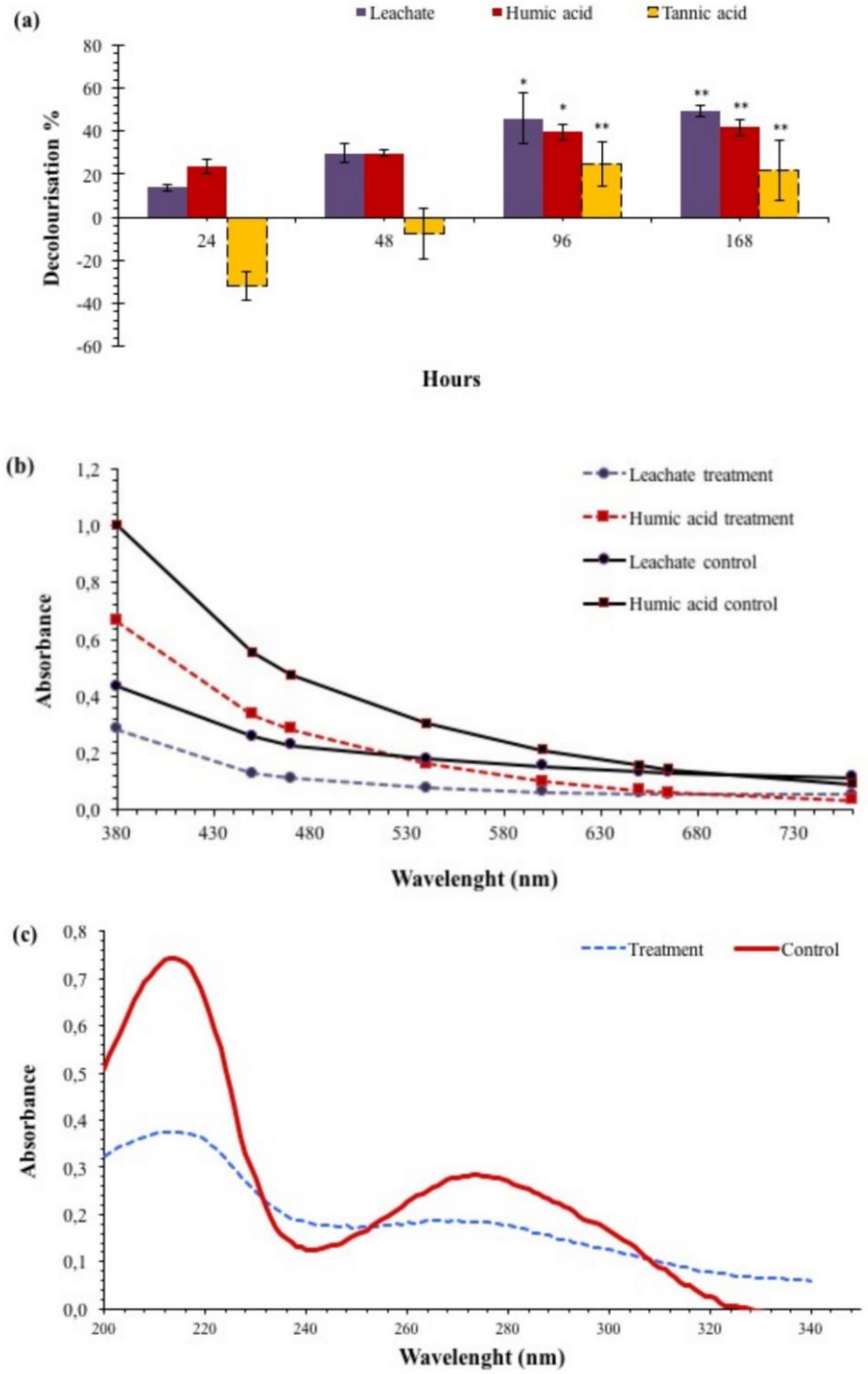

Figure 4. (a) Effluents decolorisation percentages, calculated toward respective unseeded controls, in one week of treatment. Error bars indicate Standard Deviation (SD). (b) Spectrum area reduction in raw leachate and humic acid after 168 h and (c) spectrum area reduction in tannic acid after 96 h. Decolorisation values are given as the average among triplicates with SD (+). Negative values should be considered as increases in the spectrum area. *indicates $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ (one-way ANOVA, difference between the treatments trials and the respective unseeded controls).

In the tannic acid solution, initially almost colorless, after a first increase in the spectrum area within the first 48 hours from the beginning of the experiment, a reduction up to 25% associated with highly significant differences toward respective unseeded controls, was observed in the trials inoculated with *B. adusta* (Figures 4a, c). Indeed, the spectra of these trials, after 96 h, were characterized by evident flattening of the shape, compared to the respective unseeded controls for the same duration. In addition, at 168 h, the spectra of treatment trials showed 33% and 38% reductions at 310 nm and 260 nm, respectively.

3.6 Enzymatic activities

As shown in Figure 5, MnP activity was detected in all the treatments, confirming that *B. adusta* was metabolically active during the experiment.

The maximum value was reached in the tannic acid solution after 72 hours with 8.9 ± 2.3 U/L, in correspondence to the beginning of spectrum area reduction. In the case of raw leachate, the maximum value of MnP was 1.92 ± 1.4 U/L, after 72 h from the beginning of the experiment. In the trials containing humic acid, a maximum MnP value of 5.6 ± 1.5 U/L was found after 120 h from the beginning of the test

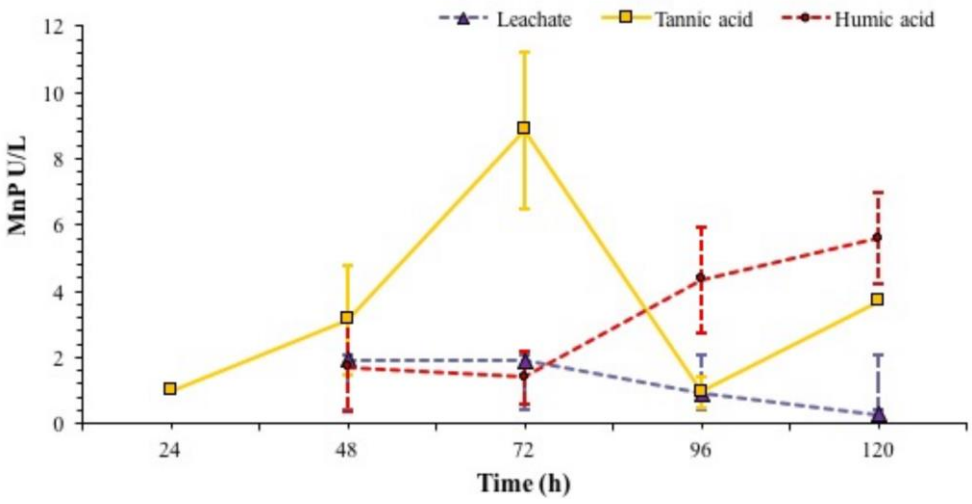

Figure 5. MnP production during the treatment. Values are given as the average among triplicates + Standard Deviation (SD).

3.7 Fungal dry weight and TSS

In Figure 6, the results of fungal dry weight increase and TSS at the end of the experiment are shown.

At the end of the experiment, the average biomass increase detected in the three effluents was 60 mg/flask ± 3.7, with a maximum value of 64 mg detected in humic acid trials and a minimum value of 56 mg, detected in the ones containing tannic acid.

The lowest TSS concentration was detected in tannic acid trials with 2.25 ± 0.63 mg/L of TSS, while 2.35 ± 1.62 mg/L were measured in raw leachate and 10.95 ± 2.05 mg/L in humic acid trials.

The total biomass was 375 mg/L in raw leachate, 353 mg/L in tannic acid and 409 mg/L in humic acid trials.

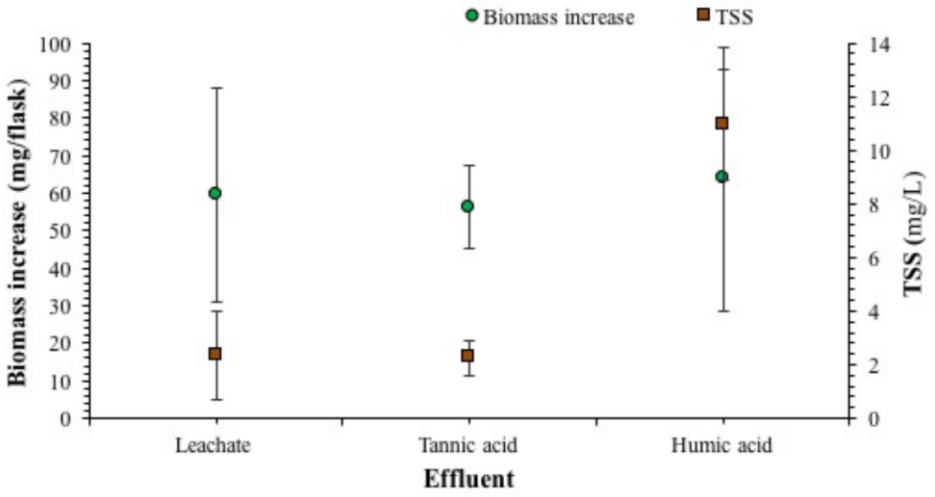

Figure 6 Circles represent biomass increase as mg/flask. Squares represent TSS as mg/L. All the values are given as average among triplicates. Bars indicated standard deviations (SD).

4. Discussion

In this study the efficiency of *B. adusta* MUT 2295, grown on PUFs, was tested toward raw leachate and two synthetic recalcitrant solutions prepared with tannic and humic acids. Although the high variability detectable in landfill leachate composition, the chemical characterization of the leachate used in this study is attributable to a sample of mature and high refractory landfill leachate [3].

The results suggested active fungal metabolism during the whole experiment. In particular, all the pH values, measured during the tests, were compatible with fungal active metabolism [29]. The increasing trend of the treatments pH values could indicate that *B. adusta* buffered the effluents at pH values as similar as possible to the optimum range for the enzymes involved in the process. It is known that many enzymes, including peroxidases, have an optimum pH between 5 and 6 [29]. In addition, Kaushik and Malik [31] have already described WRF buffering capacity. However, considering that pH increased, at 96 h, only in leachate treatment, a possible decline of *B. adusta* active metabolism in leachate trials could not be excluded. It is reasonable to hypothesize that the full co-substrate consumption could have negatively affected the metabolism of the fungus or its capability to compete with the autochthonous organisms. The same pattern could not be observed in the synthetic effluents. Indeed, an increase in the pH values of the humic acid treatment was detected only at the end of the treatment and no important increases were detected in pH values of the tannic acid treatment (Figure 2b).

Significant (61%) COD removals have been achieved in tannic acid trials. In particular, considering that COD removal in the trials containing tannic acid and inoculated with *B. adusta* was higher than glucose organic load (37 %) and the low COD removals in the respective controls, it is evident that a degradative process occurred in presence of the selected fungal strain. According to our knowledge, this is the first report about tannic acid COD removal, achieved using the selected fungal strain, *B. adusta* MUT 2295.

On the other hand, the same conclusions could not be drawn for the other two effluents tested in this study. The results achieved in raw leachate and humic acid suggest that the presence of fungal biomass could have led to possible degradative processes, which were not detectable through total COD measurements, such as increases in the biodegradable COD (bCOD) fraction, as previously reported by other authors for the fungal treatment of industrial effluents [32]. Besides the lack of evident COD decreases in raw leachate and humic acid trials that are attributable to the presence of the fungus, different rearrangements of the chemical structures of recalcitrant compounds and consequent enhancement of their bioavailability for other organisms, which was related to the treatment with *B. adusta*, could not be excluded. Since humic acid and tannic acid are considered part of leachate natural recalcitrant fraction [24, 25], the lack of evident COD decreases, due to a

degradative process in humic treatment, is in accordance with the pattern observed in raw leachate trials. The high removal detected in tannic acid trials could be explained considering that tannic acid is an hydrolysable tannin, which would start degrading in the early phase of leachate lifecycle where the biodegradable organic matter is also utilized by microorganisms [3]. Since the landfill leachate, used in this study presents the typical characterization of mature landfill leachate with the low BOD_5/COD ratio [2], it is reasonable to expect that the concentration of refractory condensed tannins was higher than that of hydrolysable ones.

Color removal was observed in all the effluents treated with *B. adusta*. However, only in the case of tannic acid trials, spectrum area reductions were positively related with significant COD removals Furthermore, in tannic acid trials inoculated with *B. adusta* MUT 2295 an evident flattening of tannic acid spectrum occurred, associated with the reduction of the absorbance at 310 and 260 nm, which indicates the removal of tannic and gallic acids [33]. All these results provided further evidence of the degradative process activated by *B. adusta* toward tannic acid.

The color removal observed in the three effluents was not positively associated with high enzymatic activity. Indeed, limited concentrations of MnP were detected during the experiment.

In the case of tannic acid, this result could be explained considering that other enzymes could be involved in the degradative process observed during the experiment. Although several enzymes, including tannases, laccases, and peroxidases, are capable of degrading tannins [33], the observation of the spectra suggests a possible involvement of tannase. This enzyme catalyze the depolymerisation of gallotannins producing gallic acid and glucose [33]. Hence, further analyses are needed to clarify the role of tannase in the observed degradation in tannic acid trials inoculated with *B. adusta* MUT 2295.

In the case of raw leachate, the maximum value of MnP of 1.92 ± 1.4 U/L was similar to the activity reported by Kalčikova et al. [3] in 100% mature landfill leachate. Although peroxidases have been described as the major enzymes involved in the decolorisation of leachate [10], the values observed were not positively associated with the spectra reduction of 49 %. Our results was in accordance to those achieved by Anastasi et al. [21], that have reported the lack of relation between enzymatic activity and color removal. The authors addressed the observed pattern to several possible reasons, including rapid enzymatic inactivation and presence of enzymatic isoforms with different substrate affinity. In this case, as suggested by the pH increase at 96 h, a possible decline of *B. adusta,* due to other microorganisms' competition or leachate high toxicity, could explain this result. On the other hand, further analyses would be necessary to explain the results achieved in raw leachate trials. Indeed, the low MnP activity observed could be due to the lack of a degradative process in those trials. According to this hypothesis, the detected COD removal could be explained because of biosorption of pigments to fungal biomass or, alternatively, by the presence of autochthonous microorganisms capable of removing a certain amount of recalcitrant compounds from the leachate itself [21].

The activity detected in humic acid trials was significantly lower compared to the results reported by others authors, which performed longer experiments with WRF on humic acid substances [17, 18 34]. However, considering the increasing trend in MnP activity observed at the end of our experiment in humic acid trials, further investigations are needed to assess a possible relation with the detected color removal.

The values of biomass increase were similar to those reported by other authors in similar conditions. In particular, Saetang and Babel [13] detected a biomass increase between 51 and 66 mg/PUF, using glucose as co-substrate and concentrated leachate. In our study, the increase was similar in the three effluents, suggesting that the fungus was able to grow in all the tested conditions, providing further evidence of the versatility of this strain, as reported by Anastasi et al. [21]. However, the lower values of Standard Deviation (SD) observed in tannic acid trials, compared to raw leachate and humic acid ones, suggested higher stability of biomass in tannic acid trials, which could be presumably associated with lower biomass loss from the cubes.

The TSS results provided further evidence of biomass stability in tannic acid trials in which the lowest TSS concentration was found, associated with a negligible SD. Although TSS could represent either biomass loss from the cubes or biomass growth that exceeded from the cube surface,

considering the full pattern of results achieved in tannic acid trials, our understanding is that the growth was homogeneous among replicates in tannic acid trials and only a negligible biomass loss occurred during the treatment. On the contrary, considering the full pattern of results in raw leachate and humic acid, we cannot exclude that, in several cubes, a partial biomass loss occurred.

All the results achieved at the end of the treatment, confirmed the presence of a degradative process in the tannic acid trials inoculated with *B. adusta*. In addition, several results, such as spectrum analyses and the low MnP activity observed, suggested the presence of tannase, which are continuously needed for diverse industrial purposes, including tannery effluents treatment [35]. Although other authors [33] described tannic acid degradation by fungi, according to our understanding, this is the first report about tannic acid degradation by *B. adusta* MUT 2295. These preliminary results could represent the first step for continuous experiments on wider scale aiming at optimizing process performances with the selected fungal strain.

5. Conclusions

Bjerkandera adusta MUT 2295 was metabolically active during the experiment. The treatment with the selected strain resulted in 49%, 25%, and 42% of color removal in raw leachate, tannic acid and humic acid, respectively. BOD_5 was completely removed in raw leachate, while 89% and 75% of removal occurred in tannic and humic acid solutions, respectively. In all the trials, biomass increase equal to 60 ± 3.7 mg/flask in the three effluents was detected at the end of the treatment. This indicates the ability of the fungus to grow in all the tested conditions while overcoming the recalcitrance of the chemicals used and its competitive nature against other microorganisms in the raw leachate trials.

In the case of tannic acid, the process is associated with 61% of COD removal and the quantification of MnP activity, indicating the presence of tannic acid biodegradation in the trials inoculated with *B. adusta*. This is the first published record of effective treatment with *Bjerkandera adusta* MUT 2295 towards tannic acid. These results encourage the use of the selected fungal strain toward effluents containing tannic acid, which could be exploited using larger volumes and for longer durations to discern a deeper understanding of the process. Further investigations could also assess possible degradative processes in raw leachate and humic acid.

Acknowledgments: This study was supported by MIUR with the FIR project (RBFR13V3CH_002) and by the EU Marie Curie Irses Program with the Carbala Project (PIRSES-GA-2011). Simone Gabrielli is gratefully acknowledged for his help in graphical artwork.

Author Contributions: Alessandra Bardi and Giulio Munz conceived and designed the experiments; Alessandra Bardi performed the experiments; Valeria Tigini, Federica Spina, Giovanna Cristina Varese, Alessandra Bardi and Francesco Spennati selected the fungal strain used during the study. Alessandra Bardi analyzed the data; Simone Becarelli and Federica Spina helped in enzymatic and spectra analyses. The experiment was performed in the laboratory of Qiuyan Yuan that contributed reagents/materials/analysis tools; Alessandra Bardi wrote the paper. Giulio Munz, Giulio Petroni, Qiuyan Yuan and Simona di Gregorio revised and improved the manuscript.

Conflicts of Interest: The authors declare no conflict of interest and no role of the funding sponsors in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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