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Research article

Role of bacterivorous organisms on fungal-based systems for natural tannin degradation

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ABSTRACT

Tannery wastewater presents high concentrations of organic load and pollutant recalcitrant molecules (e.g. tannins), which reduce the efficiency of biological treatment processes. Recent studies showed that several fungal species and strains are effective in the degradation of tannins. However, high bacterial load can negatively affect fungal growth, reducing system stability and degradation performances.

The aim of the present study was to evaluate the effects of the introduction of bacterivorous grazers (ciliates and/or rotifers) in batch scale experiments using fungi to remove Tara tannin, i.e. to check the potential synergistic effect between fungi and bacterivorous grazers in the degradation of recalcitrant compounds. In this context, the ciliated grazers *Paramecium calkinsi*, *Tetrahymena* sp., *Pseudovorticella* sp., and the rotifer *Lecane inermis*, preliminary selected according to their ability to grow in a solution prepared with Tara tannin, were separately tested. Activated sludge, including a complex mixture of native grazers, was used as experimental control. The following parameters were monitored: bacterial load, number of grazers/mL and Soluble Chemical Oxygen Demand (SCOD). Colony Forming Unit (CFU)/grazers ratio was also calculated. Particular attention was paid to: i) bacterial load reduction and ii) enhancement of recalcitrant compounds degradation, and we observed that in all experimental conditions where grazers occurred bacterial load was significantly reduced and the system achieved a higher SCOD removal in a shorter time. Our findings provide useful insights for the stabilization of fungal-based systems in non-sterile conditions.

1. Introduction

Several types of wastewater contain a varying concentration of compounds that are recalcitrant to conventional activated sludge processes (Munz et al., 2009; Schofield et al., 2001). In particular, tannery wastewaters can present high concentrations of tannins, defined as water-soluble polyphenolic compounds able to precipitate proteins in the solution, leading to stable complex products (Aguilar and Gutiérrez-Sánchez, 2001). Considering the recalcitrance of tannins, such wastewaters pose a threat to the environment and require a tertiary treatment before discharge (Kumari et al., 2018).

Biological treatment processes are well-known systems used to improve the quality of wastewater, before discarding it into receiving bodies. Among them, activated sludge processes represent the most common technology applied for wastewater treatment. They are based on the use of microbial consortia where bacteria play a dominant role in pollutants degradation (Foissner, 2016; Sehar and Naz, 2016).

Recent studies have shown that the use of fungi could be promising in the degradation of recalcitrant compounds like tannins (Prigione et al., 2018a; Aguilar et al., 2001; Anastasi et al., 2012). Within fungi, several groups have been reported as effective in degradation of tannins (León-Galván et al., 2010; Banerjee and Mahapatra, 2012). In particular,

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White Rot Fungi (WRF) produce several extracellular enzymes, including laccases, lignin peroxidases and manganese peroxidases, which are able to degrade tannins (Husain, 2006). Among them, several Ascomycetes including *Aspergillus* sp. and *Penicillum* sp., are particularly effective in degrading tannins, due to their ability to produce also the enzyme tannase (Prigione et al., 2018b; Dashtban et al., 2010).

Starting the depolymerization of several recalcitrant compounds, fungi could transform them into compounds degradable by bacteria (Kim et al., 2003; Huang et al., 2013). For this reason, it was suggested that fungi and bacteria could operate synergistically in the degradation of some pollutants (Spina et al., 2012). However, it has been demonstrated that the positive effect of their synergistic action is generally difficult to maintain in non-sterile conditions (Gullotto et al., 2015). Indeed, bacteria and fungi compete for available organic substrates, and this interferes negatively with fungal metabolism (Spina et al., 2014). As an example, in a recent study Spennati et al. employed the fungal strain *Aspergillus tubingensis* MUT 990, embedded on PolyUrethane Foam (PUF) cubes, in the treatment of Tara natural tannin through bioreactors. A Soluble Chemical Oxygen Demand (SCOD) removal of 90% was achieved after twenty days of operation. However, such result was associated with fungal biomass outcompetition by bacteria (Spennati et al., 2019).

The majority, if not all, of these studies evaluating the suitability of fungi in the degradation of recalcitrant compounds has been performed up to now in batch or lab scale reactors, in which bacterivorous organisms (protists and metazoans) were not present or monitored. On the other side, it is well known that, in activated sludge microbial communities, heterotrophic protists and micro-metazoans affect the bacterial community (Madoni, 2011). In this context, ciliates are one of the key taxonomic groups involved in bacterial load control (Decamp and Warren, 1998). According to their trophic role, ciliates of wastewater sludge are divided in carnivorous and bacterivorous. Bacterivorous ciliates can be subdivided into three functional categories based on their behavior: free-swimming ciliates, which swim in the water column (e.g. peniculids and hymenostomes); crawling ciliates (e.g. hypotrichs), which move on the surface of the sludge flocs; and sessile ciliates (e.g. peritrichs), which are firmly attached to the sludge floc. The last two categories represent the dominant components in a well-functioning community of activated sludge (Madoni, 1994, 2011) and affect sludge production by reducing it (Madoni, 1983, 2011). Metazoans can play a critical role as well; indeed, recently, it has been shown that the artificial introduction of *Lecane* sp. rotifers into activated sludge prevented the over-proliferation of filamentous bacteria (Kocerba-Soroka et al., 2013; Pajdak-Stós et al., 2017; Drzewicki et al., 2015; Fiałkowska et al., 2016), whose excessive growth lead to bulking phenomena (Martins et al., 2004). In addition, the presence of the hypotrich bacterivorous ciliate *Aspidisca cicada* can positively affect flocs quality, the proliferation of *Lecane inermis* population and the plant oxygenation (Walczyńska et al., 2018).

Assuming that in not sterile condition bacteria and fungi can compete for nutrients, in this study we aimed to evaluate the effect of the addition of bacterivorous protists and Metazoa on batch-scale in: i) reducing the bacterial load and ii) enhancing the degradation of the selected recalcitrant compound (Tara tannin) by the fungus *Aspergillus tubingensis* in attached form. The rationale of this study was to verify whether the presence of bacterial grazers can actually reduce bacterial load and indirectly favor both the fungal process and the "fungus-bacteria" system stabilization.

Two batch experiments were designed and performed on this purpose: the first was aimed at determining the effect of several selected grazers on bacterial load and recalcitrant removal; the second at determining the short-term effect of each selected grazer culture on bacterial load and recalcitrant removal. In both the experiments the following parameters were measured: a) the number of Colony Forming Unit (CFU), to quantify possible bacterial reduction in the experimental conditions where bacterivorous organisms were present; b) the count of grazers, to disclose whether different categories of bacterivorous organisms were able to grow and contributed to the process in the tested conditions. Additionally the CFU/grazers ratio was also calculated, in order to investigate possible associations between grazers concentration and bacterial load; c) the SCOD (Soluble Chemical Oxygen Demand), to address whether the presence of grazers could promote the degradation of Tara tannin; d) the pH, to observe possible fluctuations along the experiment, which can affect the fungal growth; e) the PUFs Dry Weight, to record fungal biomass increase/decrease in the tested conditions.

2. Materials and methods

2.1. Target Tara tannin medium

The synthetic solution used to simulate a tannery effluent was prepared with Tara tannin according to Spennati et al. (2019). Tara Medium (TM) is composed according to the following formula: 1 g/L Tara powder, 0.1 g 99.8% NH_4Cl and 0.01 g 99.5% KH_2PO_4, dissolved in 1L of tap water. Tara powder, extracted from the leguminous tree *Caesalpinia spinosa,* was purchased from Chimont International S.p.A. (Montopoli V/Arno, PI, Italy).

2.2. Fungal strain cultivation and its immobilization on PUFs

The fungal strain used in this study, *Aspergillus tubingensis* MUT 990, previously isolated from commercial tannin powder, was obtained from *Mycotheca Universitatis Taurinensis* (MUT) collection, University of Turin, Department of Life Science and System Biology. The strain was cultivated on Malt Extract Agar (MEA: glucose 20 g/L, malt extract 20 g/L, peptone 2 g/L and agar 20 g/L) and preserved at 4 °C. The culture was maintained by inoculating a plug (approximately 1 cm^3) on fresh MEA plate and incubating it at 25 °C in the dark for fourteen days (Anastasi et al., 2010). *A. tubingensis* was selected due to its ability to produce tannases in presence of tannins and to grow using tannin as sole carbon source (Prigione et al., 2018b; Tigini et al., 2018; Banerjee and Mahapatra, 2012).

After cultivation on MEA, the strain was homogenized in a sterile saline solution (9 g/L NaCl). The homogenate was added to GLucose and Yeast medium (GLY) (glucose 5 g/L and yeast extract 1.9 g/L), with a ratio of 1.5 mL of homogenate for each PUF (Bardi et al., 2017). Flasks were kept in agitation at 23 ± 2 °C for ten days.

2.3. Grazers culture establishment and laboratory maintenance

The cultures of ciliates and rotifers employed in the present study were obtained from individuals isolated under a stereoscopic microscope (Wild Heerbrugg, Switzerland) at a magnification of × 10 by means of a micropipette from two activated sludge samples collected from Consorzio Cuoiodepur S.p.A. in San Romano – San Miniato (Pisa, Italy) Wastewater Treatment Plant (WWTP) and Central Aeration Station on Bely Island (CASBI) in Saint Petersburg (Russia). Samples from Cuoiodepur were collected in January 2016, while those from CASBI were collected in April 2016 (details in Supplementary Materials, Table S1). Sample temperature and pH were measured with the probe Crison BasiC 20 (Crison-Hach, Loveland, CO, USA). In the activated sludge sample from Cuoiodepur pH was 6.97 and temperature was 22.8 °C, while in the activated sludge sample from CASBI pH was 7.01 and temperature was 24.2 °C. Grazers, i.e. ciliates and rotifers, were briefly *in vivo* investigated under the stereoscopic microscope at a magnification of × 10–40 and a Differential Interference Contrast (DIC) microscope (Leitz, Germany) at a magnification of × 300–1250 to obtain a rough taxonomic classification based on morphology (organism size and general shape) and movement (Fokin et al., 2019). In order to provide a more reliable identification, a molecular analysis was later performed (see below). The detailed list of the grazers (monoclonal or polyclonal) cultures is provided in Supplementary Materials, Table S1.

Except for the ciliate *Euplotes* sp., which was fed on *Dunaliella tertiolecta* 5‰ diluted 1:10 in San Benedetto water (San Benedetto S.p.A), all the other cultures were weekly fed on bacterized (overnight incubation) Cerophyll Medium (CM) (Castelli et al., 2019; Nitla et al., 2019). Each time, about 10% of culture volume was discharged and replaced with an equivalent volume of bacterized medium. The latter consisted of: 1) bacterized CM (i.e. Standard Culture Condition, SCC) for *Paramecium* sp. and rotifers from Cuoiodepur; 2) bacterized CM diluted in San Benedetto water 1:1 (DwCC) for *Tetrahymena* sp. and rotifers from CASBI; 3) bacterized CM diluted in autoclaved original wastewater medium 1:1 (DomCC) for peritrichs (i.e. *Vorticella*-like and *Vaginicola*-like organisms).

2.4. Ciliate and rotifer selection for batch tests

As a first step, the ability of ciliate and rotifer laboratory monoclonal/polyclonal cultures to grow in Tara tannin was tested so to obtain a selection of cultivated grazers suitable for performing the next two batch tests. Tara tannin was preliminary filtered with filter paper (size: 500 × 500 mm). Then, 200 mL aliquots of Tara tannin were poured in different flasks, together with grazer culture and bacterized CM with a 40:40:20 (v/v) ratio of grazers culture: Tara tannin: bacterized CM. Cell/organism number, at each time-point, for each flask was estimated, after fixation in 2% (final concentration) Bouin's fluid (Sigma-Aldrich Chemie GmbH, Munich, Germany). According to the procedure by Castelli et al. (2015), the cell/organism number of each sample was estimated in three replicate sub-samples (100 μl each) in a clear-bottomed 24-well Krystal™ black microplate (Porvair Sciences Ltd., Wrexham, UK) with a self-made grid, under a dark field stereoscopic microscope (Wild Heerbrugg, Switzerland), at a magnification of × 10–40. The preliminary grazers selection test was carried out for two weeks. The cultures were selected according to: a) the organism ability to grow in the target solution, which was measured by evaluating the increase in cell/organism number; b) the grazers category, with the aim to include different functional categories of ciliates (such as free-swimming - e.g. *Euplotes*, *Paramecium*, *Tetrahymena*- and sessile- e.g. peritrichs) in the following batch tests; c) the difference in the growth rate, i.e. the increase of cell/organism-number/time unit.

Organism growth ability was tested using two different Tara tannin concentrations (always at pH 5), starting from 0.5 g/L up to harsher conditions, i.e. higher Tara tannin concentration up to 0.8 g/L. Cells/organisms were counted after the increase of tannin concentration (one week) and at the end of the experiment (two weeks); in case of rotifers, eggs were included in the count. From the grazer cultures Tara tannin selection test, a shorter list of organisms was obtained; these were subjected to molecular identification and batch experiments in a fungal-based system aimed at removing tannins.

2.5. DNA extraction and 18S rRNA gene characterization

DNA extraction and 18S rRNA gene sequencing were performed on selected grazers, i.e. those grazers which grow during the selection test in Tara tannin, in order to provide a more accurate identification and to better guarantee reproducibility of results. DNA extraction of ciliated grazers *Paramecium* sp., *Tetrahymena* sp. and *Vorticella*-like sp. used in this study, was performed through *NucleoSpin® Plant II DNA Kit (Macherey Nagel, Düren, Germany)*. The 18S rRNA codifying gene was amplified through the following amplification program: the primary denaturation was 94 °C for 180 s, then 94 °C for 30 s, the annealing temperature was 50 °C for 30 s and the elongation was 72 °C for 180 s, with a total of 35 cycles. The final elongation was 72 °C for 6 min. *Paramecium* sp. and *Tetrahymena* sp. 18S rRNA gene was amplified using the following primers: the forward universal primer F9 (5′-CTG GTT GAT CCT GCC AG-3′) (Medlin et al., 1988) and the universal primer reverse R1513 Hypo (5′-TGA TCC TTC YGC AGG TTC-3′) (Petroni et al., 2002). The PCR products were cleaned using the *Euro Gold Cycle Pure Kit* (EuroClone®, Milano, Italy) and sequenced. The sequencing primers employed for *Paramecium* sp. and *Tetrahymena* sp. were: 18S R536 (5′-CTG GAA TTA CCG CGG CTG-3′) (Modeo et al., 2013), 18S F300 (5′-AGG GTT CGA TTC CGG AGA-3′) and 18S F783 (5′-GAC GAT CAG ATA CCG TC-3′) (Modeo et al., 2013). *Vorticella*-like sp. 18S rRNA gene was amplified with the primers Peri 57F (5′-CAT GCA TGT GTA AGT ATA AGT A-3′) and R1513 Hypo, according to the PCR program above reported. As for *Vorticella*-like sp. rRNA gene sequence, four internal primers were employed: 18S R396 (5′-GAG AGT TGT TAT TTC TTG TC-3′), 18S R1052 (5′-AAC TAA GAA CGG CCA TGC A-3′) (Modeo et al., 2013), plus 18S R536 and 18S R783 above reported.

The rotifer 18S rRNA gene was obtained via Whole Genome Amplification (WGA) protocol from a single organism using *Repli G-Single Cell Kit* (QIAGEN, Hilden, Germany). The WGA amplicon was diluted 1:100 and amplified and sequenced with the same protocol used for *Paramecium* sp. and *Tetrahymena* sp. except for the annealing temperature that, in the case of the rotifer, was set at 44 °C in the first 5 cycles. All the obtained sequences were assembled using *Chromas Lite 2.1* (Technelysium, Brisbane, Australia) software and, afterwards, blasted on Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

2.6. Batch tests experimental design

Two different batch experiments were designed in order to investigate the effect of bacterivorous organism (protist and/or metazoans) introduction in a fungal-based system aimed at removing tannins. The experimental design of the two batch tests is showed in Figure 1. The experimental questions addressed by each parameter measured or calculated are listed in Table 1.

The first batch test was designed to evaluate the possible effect of the introduction of several selected bacterivorous organisms in a fungal-based system for the removal of Tara tannin. The second batch test was designed consequently to further evaluate the kinetic of bacterial load reduction and Tara tannin removal, using a single grazer culture.

In both batch tests each single experimental condition was performed in triplicate and each flask was prepared using the following final concentrations: Tara tannin medium (1 g/L), grazers (50 cells/mL) and Cerophyll Medium (CM) (0.5 g/L to sustain the growth of autochthonous bacteria). In the *first batch*, the flasks (500 mL), containing 260 mL of target solution, were kept in agitation at 23 ± 2 °C for eighteen days. In the *second batch*, the flasks, containing 280 mL of target solution, were kept in agitation at the same temperature for six days.

2.7. Parameters used to evaluate and to monitor the effect of ciliates and rotifers

During the experiments the following parameters were monitored: i) bacterial load measured as number of Colony Forming Unit (CFU) on LB plates; ii) number of grazers; iii) Soluble Chemical Oxygen Demand (SCOD); iv) pH; and v) PUFs Dry Weight.

In the *first batch* test, the bacteria count on plates was performed, in each trial, by plating 100 μL of properly diluted sample immediately after the beginning of the experiment (day zero), after six days, after twelve days, and after eighteen days. In the *second batch* test, the bacteria count on LB plates was performed at day zero, two, four, six. Bacterial colonies were counted after 48 h and 72 h. Different dilution rates were applied according to condition and time.

Count of grazers was performed using Bouin's fixative, as described above.

In both batch tests, SCOD was measured using *LCK 314 kit* (range 150–1000 mg/L) (Hach Lange, Düsseldorf, Germany) after filtering the samples with filter paper (Whatman) and 0.45 μm filter (Sartorius). The volumes removed during experiment to perform the analysis did not exceed 15% of flask total volume.

PUFs were weighted after drying in the oven at 105 °C overnight. From each measurement the dry weight of a single empty cube was subtracted.

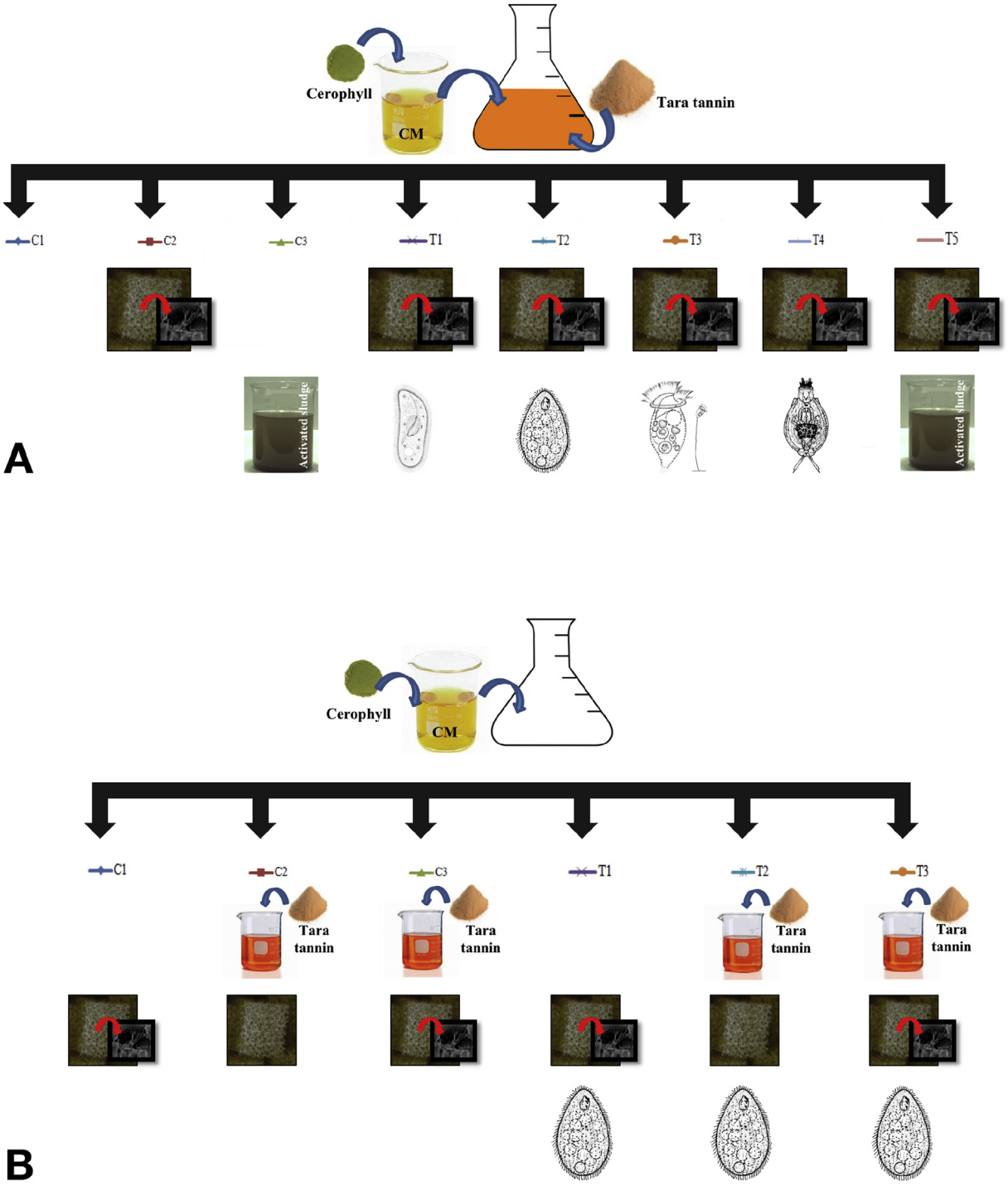

Figure 1. Batch tests experimental designs. A: First batch test, B: Second batch test.

First batch test

Conditions	Descriptive labels
C1	TM, CM, and empty PUFs
C2	TM, CM, and fungus embedded on PUFs
C3	TM, CM, empty PUFs, and activated sludge including a complex mixture of native grazers
T1	TM, CM, fungus embedded on PUFs, and *Paramecium* sp.
T2	TM, CM, fungus embedded on PUFs, and *Tetrahymena* sp.
T3	TM, CM, fungus embedded on PUFs, and Vorticella-like sp.
T4	TM, CM, fungus embedded on PUFs, and rotifer
T5	TM, CM, fungus embedded on PUFs, and activated sludge including a complex mixture of native grazers

Second batch test

Conditions	Descriptive labels
C1	CM and fungus embedded on PUFs
C2	TM, CM, and empty PUFs
C3	TM, CM, and fungus embedded on PUFs
T1	CM, fungus embedded on PUFs, and *Tetrahymena* sp.
T2	TM, CM, empty PUFs and *Tetrahymena* sp.
T3	TM, CM, fungus embedded on PUFs, and *Tetrahymena* sp.

Figure 1. (*continued*).

The dry weight of fungal biomass was measured before starting the experiment (three PUFs), after the immobilization in GLY, and at the end of the experiment (two PUFs/condition). Empty cube dry weight was calculated as the average among three replicates. 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.

2.8. Statistical analyses

Statistical analyses were performed to analyze the significant differences concerning bacterial load, grazers' growth, Colony Forming Unit (CFU)/grazers ratio and Soluble Chemical Oxygen Demand (SCOD) patterns.

In particular, significant differences during the experiment were assayed using two-ways ANOVA, followed by Tukey multiple comparison. When ANOVA's assumptions were not met, non-parametric analyses were performed using Steel-Dwass test. Two-ways ANOVA was applied for the following variables of *first batch*: bacterial load, CFU/grazers ratio and SCOD. In the *second batch* it was applied solely for CFU/grazers ratio. Instead, Steel-Dwass test was applied for the following variables of *second batch*: bacterial load, grazers growth and SCOD. In the *first batch* it was applied solely for grazers growth.

Analyses were carried out using JMP (statistical software), produced by JMP business unit of SAS Institute.

3. Results

3.1. Culturing, selection, and 18S ribosomal DNA-based identification of bacterivorous organisms

Several monoclonal strains and polyclonal cultures of bacterivorous eukaryotic organisms were obtained from the two wastewater treatment plants. Data on these ciliates/organisms are reported in Supplementary Materials, Table S1. The preliminary experiment concerning organism ability to grow in Tara tannin disclosed the following result: *Tetrahymena* sp., *Paramecium* sp., *Vorticella*-like sp., and the rotifer could grow along the experiment. The growth curves of these organisms are reported in

Table 1. Experimental questions addressed by each parameter measured or calculated.

Measured parameters	Questions to address
Number of Colony Forming Unit (CFU)	to quantify a possible bacterial reduction in the experimental conditions where bacterivorous organisms were present.
Count of grazers	to disclose whether different categories of bacterivorous organisms were able to grow and contributed to the process.
CFU/grazers ratio	to investigate possible associations between grazers concentration and bacterial load.
Soluble Chemical Oxigen Demand (SCOD)	to address whether the presence of grazers could promote the degradation of Tara tannin.
pH	to observe possible fluctuations along the experiment, which can affect the fungal growth.
PUFs Dry Weight	to record fungal biomass increase/decrease in the tested conditions.

Supplementary Materials, Figure S1; only the organisms able to grow during the whole time are indicated.

As for the molecular identification of the organisms through 18S rRNA gene sequencing, results are reported in Table 2. In detail, *Tetrahymena* sp. was confirmed as a species of the genus, *Paramecium* sp., was identified as *Paramecium calkinsi*, and the rotifer was identified as *Lecane inermis*. As for *Vorticella*-like sp., the 18S rDNA sequence was identical to that previously attributed to *Pseudovorticella* sp. (accession number KM222116.1) (Gao et al., 2016) although an intron of 919 bp has been found within the sequence herein provided.

Henceforth the grazers cultivated and employed in the two batch experiments will be referred to, according to molecular identification, as follows: *Tetrahymena* sp., *Paramecium calkinsi*, *Pseudovorticella* sp., and *Lecane inermis*.

3.2. First batch experiment with Tara tannin: effect of different grazers on bacterial load and recalcitrant removal

3.2.1. 1 Count of number of Colony Forming Unit (CFU) on LB plate

Results of count of bacteria on LB plates are shown in Figure 2A. ANOVA analysis showed significant differences among different conditions, along experimental time, and considering the interaction time/conditions (Table 3). Multiple comparison results are reported in Supplementary Materials, Table S2a.

At day zero (D0), Colony Forming Units (CFU) count was not uniform across the conditions. In particular, bacterial load was extremely low in sample C2 and uncountable in C1. Control 1 and 2 significantly differed from the other conditions (details in Supplementary Materials, Table S2d) in which CFU number was initially negligible and increased up to 2.33E+05.

As shown in Figure 2A, three main patterns could be observed: 1) the trials in which grazers were not added (C1 and C2); 2) the trials in which one single grazer was tested (T1, T2, T3, T4) and 3) the trials including activated sludge (C3 and T5). C1 and C2, in which bacterial load was not present or irrelevant at the beginning of the experiment, after six (C2) or twelve (C1) days were characterized by a bacterial load significantly higher than trials with grazers (detail in Supplementary Materials, Table S2d). After day six (D6), the value of C2 slightly decrease probably due to senescence (i.e. loss of cultivability and membrane integrity) of bacterial population; this phenomenon was not visible for C1 due to its lag in the growth curve. The trials in which grazers were individually tested (i.e. T1-T4), presented a similar pattern, characterized by a decrease of bacterial load up to D6 followed by somehow stable conditions. At D6, bacterial load was significantly lower than in C2 and, in most cases, was also significantly lower at day twelve (D12) with respect to C2 and C1. Such differences decreased at day eighteen (D18), probably due to bacteria senescence in sample C2.

In the trials containing activated sludge, i.e. C3 and T5, the highest reduction of bacterial load was recorded among all the trials after D6 and was further reduced in the following checkpoints. In terms of removal percentage, at the end of the experiment the reduction of bacterial load could be quantified as follows: 99% in C3, 56% in T2, 75% in T4, 92% in T1, 94% in T3 and 99% in T5.

Grazers growth and non-parametric pairwise comparison for grazers growth (Steel-Dwass) are reported in Supplementary Materials, Table S2b. In general, growth curves from higher to lower were: *Tetrahymena* sp. (T2) > Activated Sludge without fungi (C3) = Activated Sludge (T5) > *L. inermis* (T4) > *P. calkinsi* (T1) = *Pseudovorticella* sp. (T3). Consequently, it is rather clear that both, the bacterial load and the number of grazers were substantially stable from D6 to D12 suggesting that, in this time, stable condition were reached and grown bacteria were removed by the population of present grazers.

3.2.2. 2 CFU/grazers ratio

In order to compare the different "removal capability" of each grazer group, the ratio bacterial load (CFU)/grazer was calculated and plotted for the different time points (Figure 2B; Table 4). In all treatments the ratio CFU/grazers decreased from D0 to D6 (significantly in C3, T1, and T3), remaining stable between D6, D12, and D18 (in *Tetrahymena* sp. at D18 a modification of the ratio was evident but not statistically significant). Considering grazers ability to control bacterial load, it is possible to observe two different groups of organisms: the first is represented by *P. calkinsi*, *Pseudovorticella* sp. and, possibly, *L. inermis* (high CFU/grazers ratio); the second one is represented by heterotrophic protists of the activated sludge plus *Tetrahymena* sp., with the latter showing the lower CFU/grazer ratio. Nevertheless, differences between the two groups were not statistically significant in multiple comparison Tukey test (detail in Supplementary Materials, Table S2c).

3.2.3. 3 Soluble Chemical Oxygen Demand (SCOD)

The results of Soluble Chemical Oxygen Demand trend, along the experiment, are represented in Figure 3. ANOVA and Tukey multiple comparison results for SCOD values along the experiment are reported in Table 5 and in Supplementary Materials Table S2e, respectively. Significant differences have been detected among conditions, over time and considering the interactions of time and condition (Table 5). CM SCOD (0.5 g/L) value was approximately 200 mg/L, while Tara tannin SCOD (1 g/L) value was approximately 700 mg/L. Controls and treatments show the same SCOD value at D0 with the exception of trials T1 and T2 that are significantly higher if compared to the others (Figure 3). Such differing

Table 2. BLAST results of 18S ribosomal RNA coding genes and the accession numbers in Genbank.

Strain/name	Accession number	Higher blast score	Accession number	Identity (%)	Molecular identification
Paramecium sp.	MT012297	*Paramecium calkinsi*	MH819561.1	99.53	*Paramecium calkinsi*
Tetrahymena-sp.	MT012299	*Tetrahymena setosa*	AF364041.1	99.94	*Tetrahymena* sp.
Vorticella-like sp.	MT025819	*Pseudovorticella* sp.	KM222116.1	100	*Pseudovorticella* sp.
Rotifer	MT025820	*Lecane inermis*	KY859765.1	100	*Lecane inermis*

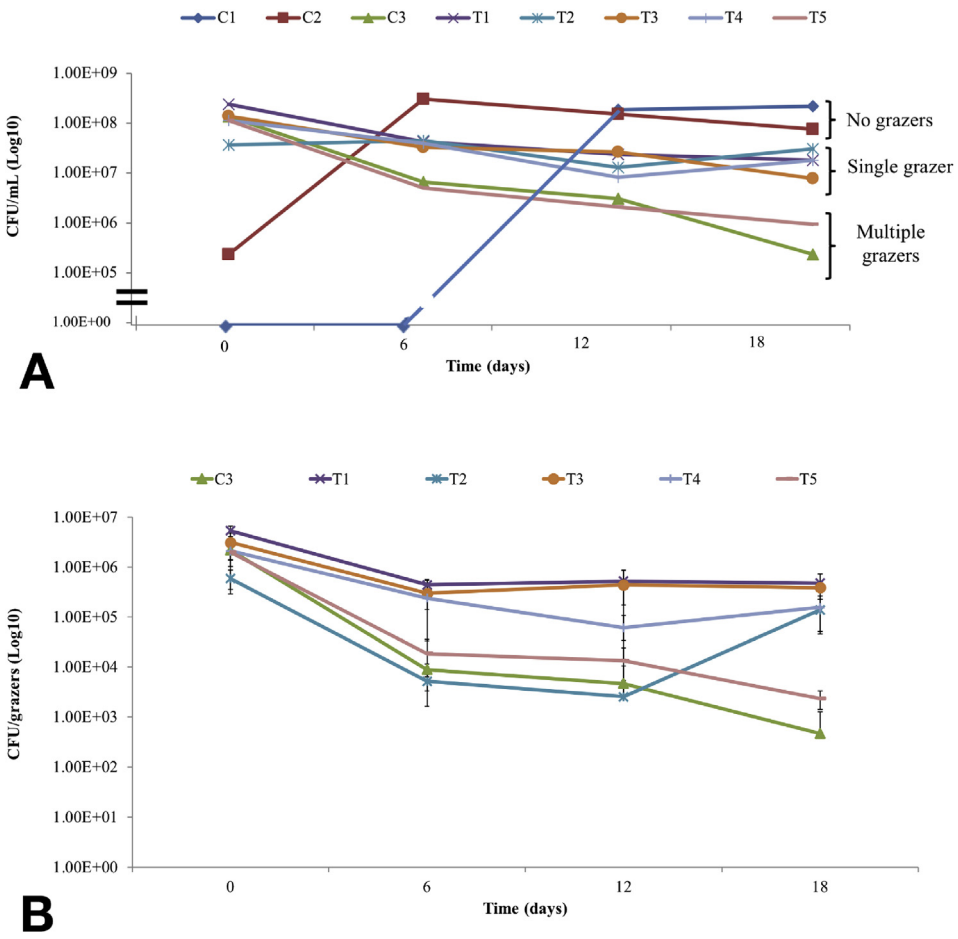

Figure 2. (A): Bacterial load: Colony Forming Unit (CFU)/mL. As for the first two measurements of C1, the initial bacterial load was below the resolution power of the dilution applied. (B): ratio between number of CFU and number of grazers at the different time points. All values are given as average among three biological replicates ± Standard Deviation (SD), except for (A), in which the values of SD were not added to make clearer the graph reading (full data are shown in Supplementary Materials, Table S2d).

pre-experiment conditions could be attributed to the presence of a higher SCOD associated with cultures of *Tetrahymena* sp. (T2) and *P. calikinsi* (T1) used as inoculum. As shown in Figure 2, a faster and significant removal of SCOD occurred in all conditions where fungi or grazers were added and could be observed already at D6 of the experiment (comparison between C1 and any of other trials). At D6, samples containing grazers showed a lower SCOD respect to the sample containing only the selected fungal strain (C2) but this difference was statistically significant only in pairwise comparison with T5. At the end of experiment (D18), the lowest final SCOD value was obtained in presence of activated sludge, in both cases, with the selected fungal strain (T5) and without fungi (C3).

At D6 of the experiment, SCOD removal percentages could be quantified as follows: 12% in C1, 58% in C2, 72% in C3, 78% in T1, 81% in T2, 76% in T3, 75% T4, and 82% in T5. It is worth noting that, in the presence of grazers, the majority of SCOD reduction occurred in less days when compared with the control containing only fungi (C2), see Figure 3.

In the Figure, values of SCOD trend are given as the average among three replicates ± standard deviation (SD).

3.2.4. pH values and PUFs dry weight

During the experiment, the pH has been generally almost stable. Indeed, it only ranged between 5.5 ± 0.3 and 6.3 ± 0.3 However, in all the trials, pH increased in the last three days of experiment from 6.3 ± 0.3 to 7.5, with the exception of T5 in which the pH variation was lower.

At the end of the experiment, in T2 (with *Tetrahymena* sp.), in T3 (with *Pseudovorticella* sp.), and in T4 (with *L. inermis*) the fungal biomass had increased of 29 ± 13%, 26 ± 19% and 24 ± 4% respectively. On the contrary, at the end of the experiment the fungal biomass had decreased in C2 (fungi alone), in T1 (*P. calkinsi*) and in T5 (activated sludge). In particular, the highest decrease was observed in C2 (fungi alone) with a reduction percentage of 102 ± 29%. In this case, it was hypothesized a possible detachment of the fungal strain from the PUFs occurred without the addition of grazers.

3.3. Second batch experiment with Tara tannin: short term effect of a single grazer on bacterial load and recalcitrant removal

3.3.1. Count of number of Colony Forming Unit (CFU) on LB plate

Bacterial load along the experiment is shown in Figure 4A. Bacterial load at time zero evidenced a similar starting point for trials C1, C3, T1, and T3 (range 4.25E+07–5.56E+07); all these trials were containing fungi. Bacterial load of T2 (containing *Tetrahymena* sp. but not fungi) was relevant (Figure 4A) but "significantly" lower than C1, C2, T1, T3. Bacterial load of trial C2 (no fungi, no *Tetrahymena* sp.) at D0 was minimal

Table 3. Results of two-ways ANOVA (Bacterial load).

Source	SQ	DF	MQ	Ratio	p value
Condition	1.21E+15	7	1.74E+14	4.85	<0.0001
Time	3.88E+14	3	1.29E+14	2.83	<0.0002
Condition*Time	4.69E+15	21	2.23E+14	7.81	<0.0001

Table 4. Two-ways ANOVA for ratio CFU/grazers.

Source	N° parameters	DF	SQ	Ratio F	p value
Condition	5	5	1.67E+11	8.62	<0.0001
Time	3	3	7.45E+11	63.96	<0.0001
Condition*Time	15	15	2.23E+11	3 .83	0.0002

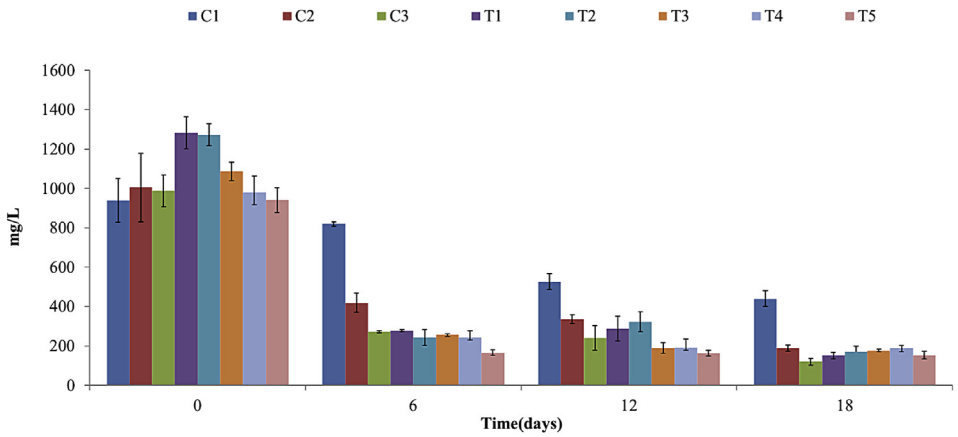

Figure 3. *First batch Tara.* Soluble Chemical Oxygen Demand (SCOD) trend.

(almost sterile condition, bacterial count = 0). These differences clearly indicate that initial bacterial load was associated to the fungal inoculum that was apparently carrying an associated, quantitatively relevant, microbial consortium. Bacteria load associated with *Tetrahymena* sp. was present (sample T2) but about two order of magnitude lower than the one associated to fungi (consequently the presence of *Tetrahymena* sp. in trials T1 and T3 did not significantly affect their bacterial load at D0 respect to samples without *Tetrahymena* sp.). Microbial consortium associated with Tara and CM was minimal and a derived bacterial load was measurable only at D6. Trend of bacterial load in the different trials resulted in two statistically significant differences: between trials T1 (fungi + *Tetrahymena* sp.) and C1 (fungi) and T3 (fungi + Tara + *Tetrahymena* sp.) and C3 (fungi + Tara), respectively. In both cases, the presence of *Tetrahymena* significantly reduced bacterial load ($p < 0.05$).

Although, in the presence of Tara, bacterial load was slightly higher after D2 (T3 > T1 and C3 > C1) these differences were not statistically relevant (detail in Supplementary Materials, Table S3a). In terms of percentage, the highest values of bacterial reduction were observed in T1 and T3, both characterized by the presence of *Tetrahymena* sp. and *A. tubingensis* MUT 990, achieving 67% and 33% of reduction respectively.

Grazer counts are shown in Supplementary Materials (Figure S3). Indeed, growth curves of trials T1 (*Tetrahymena* without Tara tannin) and T3 (*Tetrahymena* + Tara tannin + fungi) were not statistically different. On the contrary, these two curves were different from T2 (*Tetrahymena* without fungi) curve.

3.3.2. CFU/grazers ratio

In order to evaluate if the lower number of grazers in trial T2 was related to the lower bacterial load of the same sample, the ratio between bacterial load and grazers has been calculated for trials T1, T2 and T3 and values have been compared (Figure 4B; Table 6). It is evident that from D2, after conditions are stabilized, all the three ratios are comparable (differences statistically not significant, detail in Supplementary Materials, Table S3c), hence demonstrating that the lower number of grazers in T2 was proportionally determined by the lower bacterial load of the sample.

Table 5. Results of two ways ANOVA (SCOD).

Source	DF	SQ	Ratio F	p value
Condition	7	7.37E+05	28.35	<0.0001
Time	3	1.10E+07	967.57	<0.0001
Condition*Time	21	8.86E+05	11.36	<0.0001

3.3.3. Soluble Chemical Oxygen Demand (SCOD)

The results of SCOD values, over the experiment, are shown in Figure 5. Alike the *first batch*, CM SCOD (0.5 g/L) value was 200 mg/L, while Tara tannin SCOD (1 g/L) estimated value was 700 mg/L.

At D0, SCOD concentrations were approximately similar both among samples containing Tara (C2, C3, T2, T3) and between those without Tara (C1 and T1). As shown in the graph, it is possible to observe a trend of SCOD reduction from the beginning to the end of the experiment (D0) in all trials containing Tara.

In terms of removal percentage, the SCOD removal, from the beginning to the end of the experiment, could be quantified as follows: 17% in C1 (CM), 6% in C2 (CM, Tara), 26% in C3 (CM, Tara, fungi), 35% in T1 (CM, fungi and grazer), 47% in T2 (CM, Tara and grazer), and 67% in T3 (CM, Tara, fungi, and grazer), which represented the complete system.

From the pairwise comparison (details in Supplementary Materials, Table S3e) it is possible to observe several differences among experimental conditions. Tara tannin removal increases in the presence of fungi (C3 versus C2, removal increase of 20%) although this difference is not significant. Tara tannin removal was higher in the presence of *Tetrahymena* sp. (T2) than in the presence of fungi (C3) (T2 versus C3, removal increase of 21%) although this difference is not significant. A significant increase in Tara tannin removal was achieved in the presence of *Tetrahymena* sp. (T2 versus C2, removal increase of 41%); a significant increase in Tara tannin removal was also achieved in the presence of fungi when *Tetrahymena* sp. was added to the sample (T3 versus C3, removal increase of 41%). The combined presence of fungi and *Tetrahymena* sp. supported the higher Tara tannin removal. This removal increase was close to significance if compared to the one obtained with only *Tetrahymena* sp. (T3 versus T2, removal increase of 20%).

3.3.4. pH values and PUFs dry weight

In all the trials the pH was maintained stable, ranging between 6 and 7 from the beginning to the end of the experiment.

During the six days of the test, increases of the fungal biomass were not observed in any trials.

4. Discussion

Spennati et al. (2019) described the SCOD pattern obtained in bench-scale reactor, operating under non-sterile conditions, inoculated with *Aspergillus tubingensis* MUT 990 in attached form into PUFs. These authors observed a detachment of fungal biomass from the cubes after two weeks of operation. Molecular approach has shown that fungal inoculum was outcompeted by bacteria, which established a suspended biomass leading, in that system, to a stable removal of Tara tannin of 90% after twenty days of operation. In the *first batch* experiment of the present study we evaluated the effect of different bacterial grazers on a system

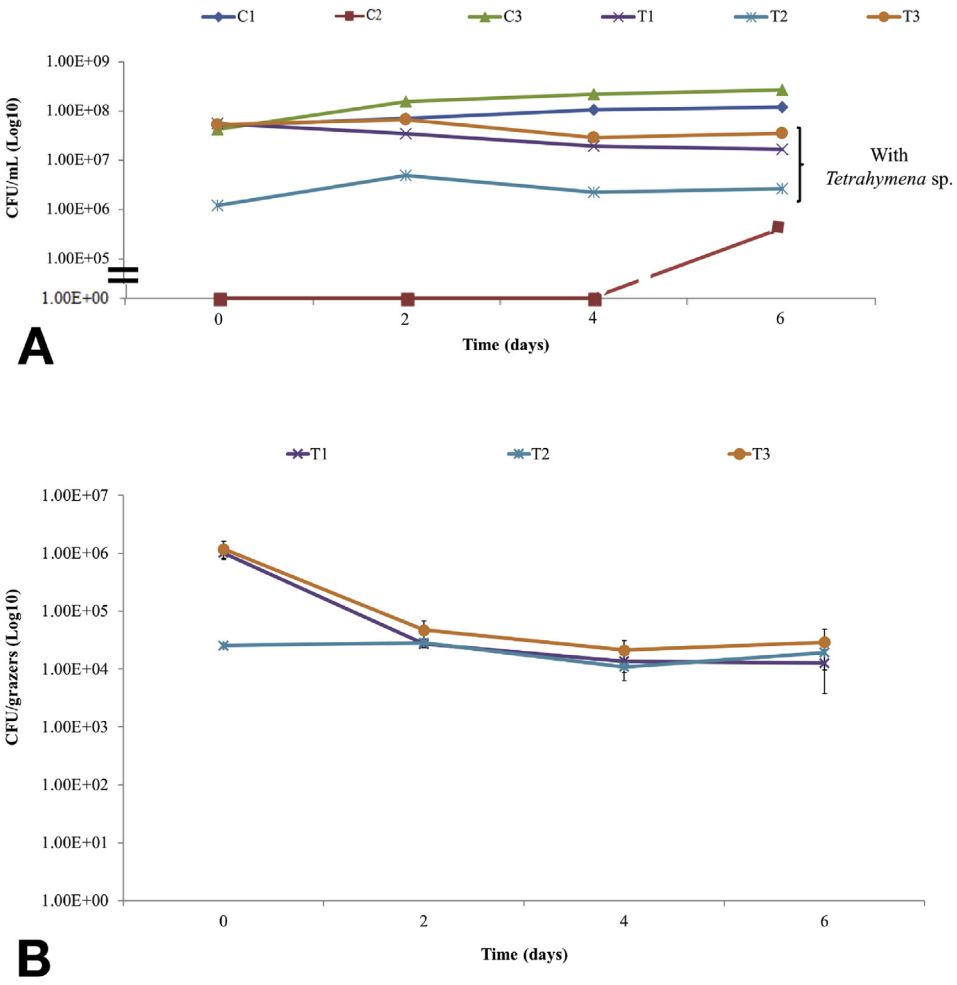

Figure 4. *Second batch Tara*. Legend: CM was added in all the trials. Additional components were as follows: C1, *A. tubingensis* MUT 990; C2, Tara tannin; C3, *A. tubingensis* MUT 990 and Tara tannin; T1, *A. tubingensis* MUT 990 and *Tetrahymena* sp.; T2, Tara tannin and *Tetrahymena* sp.; T3, *A. tubingensis* MUT 990, Tara tannin, and *Tetrahymena* sp. A) Count of bacteria on LB plate: Colony Forming Unit (CFU)/mL and (B) ratio between bacteria load and grazers. The values of standard deviation (SD) are reported in (B) but not in (A) to make clearer graph reading. SD are shown in Supplementary Materials, Table S3d.

similar to the one described in Spennati et al. (2019) (Tara tannin + fungi in attached form) with particular attention to: 1) the stability of the system and 2) possible increases in SCOD removal.

The results achieved in this experiment, suggested the capability of all employed grazers to keep bacterial load under control and stabilizing the system. Indeed, in the absence of grazers, bacterial load was reaching significantly higher values. Carrying capacity of grazers was different among different species with *Tetrahymena* sp. having a carrying capability about two orders of magnitude higher than *P. calkinsi*, *Pseudovorticella* sp., and *L. inermis* (Supplementary Materials, Figure S2). On the other side, despite different species of grazers were showing different growth curves (kinetics) reaching significantly different number of cells/mL, their efficiency in keeping reduced the bacterial load was comparable (Figure 2A). This suggests a higher efficiency of organisms such as *Paramecium*, *Pseudovorticella* and *Lecane* in controlling bacterial load, confirming previous observations (Madoni, 2011; Pajdak-Stós et al., 2017). These results are also in agreement with the current knowledge on the development of bacterial grazers' community in an activated sludge plant. In these plants, r-strategist species such as *Tetrahymena* develop in a first step, later followed by k-strategist species such as *Pseudovorticella* and rotifers, whose presence is considered as a good indicator of stability and efficiency of plant functioning (Martín-Cereceda et al., 1996; Salvado et al., 1995; Al-Shahwani and Horan, 1991; Madoni et al., 1993; Madoni, 2011; Moussa et al., 2005). Indeed, grazing activity was demonstrated to be essential for the clarification and treatment of sewage effluent (Curds, 1968; Dos Santos et al., 2014, 2015; Jiang et al., 2014; Zhong et al., 2017; Xu et al., 2018).

Table 6. Two-ways ANOVA CFU/grazers ratio.

Source	N° parameters	DF	SQ	Ratio F	p value
Condition	2	2	6.08E+09	17.90	<0.0001
Time	3	3	3.46E+10	67.99	<0.0001
Condition*Time	6	6	1.73E+10	16.96	<0.0001

Considering SCOD removal, the results show that differences are present only within the first six days. The systems in which grazers were added showed a higher SCOD removal than control including only fungi. These results suggested that the presence of grazers was increasing the kinetic of Tara tannin removal.

In order to evaluate, in detail, the effect of bacterial grazers addition in implementing SCOD removal kinetics in the first six days of experiments, a *second batch* test has been designed and performed. In this experiment, an increase in the removal of SCOD was visible already after two days in samples with either fungi alone or *Tetrahymena* sp. alone. The combined presence of fungi and *Tetrahymena* sp. was producing an even higher SCOD removal, confirming the pattern observed in the first batch test.

In agreement with previous studies on the effect of bacterial grazers on bacterial metabolic activity (Biagini et al., 1998; Fenchel and Harrison, 1976) and degradation potential (Madoni, 2011; Pajdak-Stós et al., 2017), we hypothesize that the presence of *Tetrahymena* stimulates bacterial activity, providing a higher SCOD removal. Indeed, it is already known that ciliates can excrete compounds including vitamins, co-enzymes, amino acids and nucleotides, which stimulate the growth of bacteria (Ratsak et al., 1996). These compounds, together with protozoan lysis products, speed-up the production of cell material by bacteria and

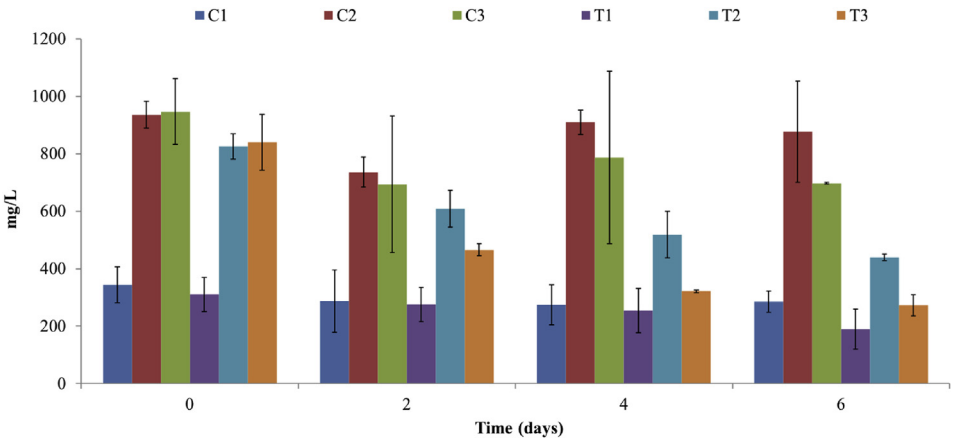

Figure 5. *Second batch Tara.* Soluble Chemical Oxygen Demand (SCOD). Legend: CM was added in all the trials. Additional components were as follows: C1, *A. tubingensis* MUT 990; C2, Tara tannin; C3, *A. tubingensis* MUT 990 and Tara tannin; T1, *A. tubingensis* MUT 990 and *Tetrahymena* sp.; T2, Tara tannin and *Tetrahymena* sp.; T3, *A. tubingensis* MUT 990, Tara tannin, and *Tetrahymena* sp. Values of SCOD over the experiment are reported as average among three biological replicates ± Standard Deviation (SD).

increase their consumption by grazers. Indeed, other sources, such as lysis products, including protozoan lysis products, may also be a suitable substrate for bacteria. This phenomenon has been described by several authors that reported increases of *E. coli* cells in the presence of filtrate obtained from a culture of *Tetrahymena pyriformis*, without additional substrate (Sambanis and Fredrickson, 1988; Ratsak et al., 1996). As a result, processes connected with assimilation, dissimilation and carbon mineralization in bacteria as well as in their predators, are accelerated.

Therefore, we hypothesize that the higher bacterial metabolic activity would have enhanced the degradative potential of bacteria on the tested recalcitrant compound. Our results also suggest a synergistic effect of fungi and bacterivorous grazers as evidenced by the higher SCOD removal of sample T3 (*Tetrahymena* and fungi). Indeed, grazers would keep under control bacterial growth stimulating bacterial metabolisms, and fungi would benefit from reduced competition with bacteria.

It is worth to mention that the experimental set-up applied in this study does not allow to unambiguously discriminate between the possible effect of fungi and that of the microbial consortium they are associated to. Seemingly, the same concern can be referred also to the effect of grazers and that of the microbial consortium they are associated to. To our best knowledge, this is the first report in which the effect of bacterial grazers on a fungal-based system has been investigated. Overall, results suggest a positive effect of grazers at two different levels: a) stabilizing fungi and their efficiency toward Tara tannin (possibly through bacterial load control) and b) stimulating bacterial degradative metabolism. These observations suggest that the faster kinetic in SCOD removal could be due to synergistic effect of the two levels.

Our experiments additionally support that the presence of grazers alone can increase and speed up recalcitrant compound degradation processes performed by bacteria as shown by the increase of SCOD removal in the presence of *Tetrahymena*. Indeed, such increase occurred also in the absence of fungi.

Finally, our results suggest that kinetics of recalcitrant compound removal could be significantly different in controlled lab experiments without bacterial grazers with respect to natural conditions where these grazers are present. It is worth to notice that the best removal results have been achieved in all controls where activated sludge (including grazers) was included in the inoculum.

To better understand the synergistic effect observed between fungi and grazers, the system could be studied on wider scale, using bioreactors.

5. Conclusions

In this study, the effects of bacterivorous organism introduction on batch-scale experiments for Tara tannin removal using fungi have been investigated. The presence of grazers led to significant reduction of bacterial load associated with a speed-up of Tara tannin Soluble Chemical Oxygen Demand (SCOD) reduction. Overall, these results suggest a synergistic role of bacterial grazers and fungi in recalcitrant compound removal.

As a final remark, the synergistic effect we observed between fungi and grazers could be relevant in the scaling up of fungal-based systems in not sterile condition. Further studies, using bioreactors, would be necessary to extend our knowledge on the system at a wider scale.

Declarations

Author contribution statement

Cristiana Sigona: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Alessandra Bardi: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Letizia Modeo: Analyzed and interpreted the data; Wrote the paper.

Gualtiero Mori: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Alexey Potekhin, Franco Verni: Contributed reagents, materials, analysis tools or data.

Giulio Munz: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Giulio Petroni: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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