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TITLE: Tannery mixed liquors from an ecotoxicological and mycological point of view: risks vs potential biodegradation application

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Abstract

Fungi are known to be present in the activated sludge of wastewater treatment plants (WWTP). Their study should be at the base of an overall vision of the plant effectiveness and of effluents sanitary impact. Moreover, it could be fundamental for the implementation of successful bioaugmentation strategies aimed at the removal of recalcitrant or toxic compounds. This is one of the first studies on the cultivable autochthonous mycoflora present in the mixed liquors of two WWTP treating either vegetable or chromium tannery effluents. All samples showed a risk associated with potential pathogens or toxigenic species and high ecotoxicity (*Lepidium sativum* and *Raphidocelis subcapitata* were the most sensitive organisms). Diverse fungal populations developed, depending on the origin of the samples (63% of the 102 identified taxa were sample-specific). The use of a fungistatic was determinant for the isolation and, thus, for the identification of sample-specific species with a lower growth rate. The incubation temperature also affected the mycoflora composition, even though at lower extent. A selective medium, consisting of agarised wastewater, allowed isolating fungi with a biodegradation potential. *Pseudallescheria boydii*/*Scedosporium apiospermum* species complex was ubiquitously dominant, indicating a possible role in the degradation of pollutants in both WWTP. Other species, i.e. *Trichoderma spp., Trematosphaeria grisea, Geotrichum candidum*, *Lichtheimia corymbifera, Acremonium furcatum*, *Penicillium simplicissimum*, *Penicillium dangeardii*, *Fusarium solani, Scopulariopsis brevicaulis* potentially could be involved in the degradation of specific pollutants of vegetable or chromium tannery

wastewaters. However, several of these fungi are potential pathogens and their application, for an *in situ* treatment, must be carfully evaluated.

Key Words: Tannery wastewater, activated sludge, fungi, autochthonous mycoflora, ecotoxicity.

1 Introduction

The secondary treatments, which consist of biological processes operated with either suspended or attached biomasses, are generally the core of wastewater treatment plants (WWTPs) (Leyva-Diaz et al., 2017). The knowledge on composition and dynamics of microbial community has been fundamental for the development of biological treatment technologies (Orhon, 2015). However, studies were mainly focused on the bacterial fraction of these complex ecosystems, as well as mathematical models applied to describe the processes (Orhon, 2015). Recently, the literature has pointed out a consistent presence of autochthonous mycoflora in the activated sludge of WWTPs treating paper mill, vinery or municipal wastewaters (Grabinskaloniewska et al., 1993; Awad and Kraume, 2011; Evans and Seviour, 2012). Its characterisation should be included in an overall vision of the effectiveness and impact of a treatment plant, considering functional, ecological and sanitary aspects (More et al., 2010; Awad and Kraume, 2011; Korzeniewska, 2011).

The role of fungal organisms in the depuration process has been recently demonstrated. In particular, fungi seem to perform a complementary action with respect to bacteria in the removal of pollutants from wastewaters and can actively interact with them (Liu et al., 2017; Svobodová et al., 2016; Anastasi et al., 2012).

The study of autochthonous fungi in WWTPs may acquire particular importance when recalcitrant or toxic pollutants are present in wastewaters, in order to improve depuration performance by bioaugmentation or biostimulation of naturally present fungi (More et al., 2010; Djelal and Amrane, 2013; Herrero and Stukey, 2015).

Tannery wastewaters, regardless of the type of industrial process (chromium or vegetable), are among the most difficult to treat, basically on account of their recalcitrance and/or their toxicity towards bacteria (Lofrano et al., 2013). On the contrary, the ability of fungi in the degradation of tannery pollutants has been already demonstrated (Sharma and Malaviya, 2016; Zhang et al., 2015).

Besides the study of diversity and ecological function of the microbial community, a successful bioaugmentation strategy should contemplate the assessment of possible toxicity effects towards the biota (Herrero and Stukey, 2015). Ecotoxicity tests, associated with chemical and biological characterisations, can be a informative tool for the efficiency evaluation and the management of the secondary treatment in WWTPs (Chapman, 2000). Nevertheless, these procedures are not so diffused at industrial level. This is mainly due to the lack of knowledge about the methods for data interpretation and to the complexity of the information that these analyses can provide (Chapman, 2000).

The present study is focused on the analysis of cultivable autochthonous mycobiota present in mixed liquors of the oxidation tanks of two wastewater treatment plants, which collect vegetable and chromium tannery effluents, respectively. Selective media were exploited for the isolation of fungi with biodegradation potential and/or the ability to compete with other microorganisms. Moreover, the samples were incubated at both 25 °C and 15 °C, in order to acquire information about the effect of seasonal temperature fluctuations on mycoflora development. Finally ecotoxicological aspects of the samples were assessed by means of four bioassays.

2 Materials and Methods

2.1 Wastewaters

The samples (three in total) were collected from two WWTPs located in Tuscany (Italy):

Co – mixed liquor from the aerobic tanks of Cuoiodepur vegetal tannery WWTP (San Miniato, Pisa, Italy).

F1 – effluent from the settler of the first aerobic stage of the chromium tannery WWTP of Consorzio Aquarno SpA (Santa Croce sull'Arno, Pisa, Italy).

F2 - mixed liquor from the second biological stage of chromium tannery wastewater treatment plant of Consorzio Aquarno SpA (Santa Croce sull'Arno, Pisa, Italy).

Cuoiodepur manages a consortial WWTP, where the effluents of about 100 tanneries, operating vegetable tanning process, are treated. The influent wastewaters are characterized by a high organic and nitrogen load (COD > 15000 mg L^{-1}), high salinity and by the presence of natural and synthetic tannins. The Cuoiodepur WWTP treats about 5000 m^3 d^{-1} of tannery wastewaters and the treatment train is composed of: pretreatments, simultaneous equalization and sulphide oxidation with pure oxygen, primary settling, conventional suspended activated sludge (denitrification nitrification) biological stage and coagulation flocculation as tertiary treatment. The temperature of the biological reactors ranges between 19 °C and 35 °C, the hydraulic retention time (HRT) is about 3 days and the solids retention time (SRT) is usually between 50 and 70 d.

Aquarno is a joint-stock company by large majority private operating in the Tuscany tannery district for the treatment of chromium tannery wastewaters. The Aquarno WWTP treats about 12000 m^3 d^{-1} of chromium tannery wastewaters. The treatment train is composed of: pretratments, aerobic activated sludge system (at low SRT to remove sulphide), second conventional activated sludge system for nitrogen and carbon removal, and Fenton tertiary treatment. The average temperature of the influent is in the range 25-30 °C, the HRT of biological sections is about 7 days and the SRT of the second biological stage is close to 30 days.

2.2 Chemical analyses

The pH was determined with a Hach-Lange's probe. The ammonium and metals were measured by means of a nitrogen analyser (TOC-L, Shimazdu) and an inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer), respectively. The Chemical Oxygen Demand (COD) and soluble Chemical Oxygen Demand (sCOD) were determined using Hach-Lange's cuvettes after filtration (at 0.45 μm). The Total Suspended Solids (TSS) were measured as the dry weight (1 hour at 105 °C) of the residue of the filtered sample. The Volatile Suspended Solids (VSS) were measured as the dry weight (30 min at 570 °C) of the residue of the filtered sample.

2.3 Ecotoxicity tests

A battery of four bioassays was performed in order to evaluate the toxicity of the samples. The target organisms were: the unicellular green alga (I) *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, J.Kristiansen & O.M. Skulberg (UNI EN ISO 8692:2005); the dicotyledonous plants (II) *Cucumis sativus* L. and (III) *Lepidium sativum* L. (UNICHIM N. 1651, 2003); the monocotyledonous plant (IV) *minor* L. (ISO SO/WD 20079). The samples were filtered (Whatman type 1) for the execution of algal test only. In fact, filtration was needed to avoid interference with the spectrophotometric lectures performed at the end of this test.

Each dose–response curve consisted of six dilutions (in triplicate for plant test and in quadruplicate for algal test) and the control was performed with four and six repetitions for plant and algal tests, respectively.

Significant differences between dose-effect regression lines were analysed using T test ($p<0.05$ for line slope, $p<0.001$ for translation), for all the possible pairs.

2.4 Isolation and identification of autochthonous mycoflora

Aliquots of 1 mL of each sample were placed in Petri dishes (16 cm diam.) containing 30 mL of culture medium. Three different media were used: a modified Malt Extract Agar (MEAp) (agar 20 g, glucose 2 g, malt extract 2 g, peptone 0.2 g, water up to 1 L); Dichloran Rose Bengal Agar (DRBC 31.5 g, water up to 1 L);

Wastewater-Agar (WA; agar 20 g, glucose 2 g, sample supernatant after 5 min at 10000 rpm up to 1 L). A set of three antibiotics was added to all media: streptomycin 0.015 g L^{-1}, chloramphenicol 0.05 g L^{-1}, and ampicillin 0.05 g L^{-1}. The trial was performed with 20 replicates for each medium and sample. Ten replicates were incubated at 25 °C and ten at 15 °C, in the dark.

At regular intervals of time, the colony forming units (CFU) were counted and the different fungal morphotypes were isolated in pure cultures. Fungi were identified conventionally, according to their macroscopic and microscopic features. After determination of their genera (Domsch et al., 1980; Kiffer and Morelet, 1997; von Arx 1981), they were transferred to the media recommended by the authors of selected genus monographs for species identification. Molecular identification was performed by amplification and sequencing of specific markers: actin for *Cladosporium* (Bensch et al., 2012); β-tubulin for *Penicillium* and *Aspergillus* (Glass e Donaldson 1995); D1/D2 region for yeasts (Fell et al., 2000); Internal Transcribed Spacer (ITS) for other genera (White et al., 1990). The resulting sequences were compared with reference sequences in online databases provided by the CBS-KNAW Collection (Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands) and the NCBI National Center for Biotechnology Information (USA).

The nonparametric Mann-Whitney test ($p \leq 0.05$) was run to assess the significance of the quantitative (fungal load) differences between all possible permutations of the trial. Differences within factors (tannery effluent, incubation temperature, and culture medium) were evaluated by applying a Permutational Multivariate Analysis of Variance (PERMANOVA; $p < 0.05$) and visualised by Non-Metric Multidimensional Scaling (NMDS). The contribution of individual species (in percentage) to the diversity observed was assessed by SIMilarity PERcentage (SIMPER) analysis.

3 Results and discussion

3.1 Chemical analyses

The results of chemical analyses are shown in Table 1. The pH of the samples ranged around neutral values, from 7.22 of F2 to 7.66 of Co. All tannery samples showed high values of COD (10000-24740 mg L^{-1}) and sCOD (362-518 mg L^{-1}). It is noteworthy that 50% of the sCOD was due to tannins in Co sample (Munz et al., 2009). Both in Co and F1, the TSS and VSS were very high (≥12.61 g L^{-1} and ≥9.61 g L^{-1}, respectively), while in F2 these values decreased (7.79 g L^{-1} and 5.77 g L^{-1}, respectively). The ammonium was 278 mg L^{-1} in F1 sample, whereas for Co and F2 it was 1.04 mg L^{-1} and 9.65 mg L^{-1}, respectively. Eventually, supernatant colour was dark brown for all the samples.

According to the literature, tannery wastewaters have very high COD, high nitrogen, and low phosphate concentrations (Prigione et al., 2009; Kim et al., 2013; Lofrano et al. 2013). On the whole, the three samples, Co, F2 e F1, were typical of tannery WWTPs, when compared to the data in the literature.

3.2 Sample ecotoxicity

In this work four ecotoxicity tests were applied in order to evaluate the effectiveness of tannery wastewater secondary treatment and the evaluation of potential impact of the samples on the environment.

The inhibition effects of samples on *Raphidocelis subcapitata* are shown in Figure 1. The maximum testable concentration was 70% for F2, 50% for Co and 40% for F1. At higher doses, the algal concentration was not detectable on account of the deep colour impact on the spectrophotometric analysis.

The recorded data were almost linearly distributed only for F2 and Co samples (see $R^2>0.9$ in Figure 1). On the contrary, F1 points seem to be distributed according to an S-shaped curve ($R^2<0.9$). The three tannery effluents showed no significant differences among angular coefficients of dose-effect regression lines, indicating a similar toxicity effect for the three samples. However, this elaboration is possible only for positive inhibition values, which are the minority of the recorded data. Thus, the scarcity of data exploitable for statistical analysis affected the reliability of this elaboration.

The results about the ecotoxicity test with *Lepidium sativum* and *Cucumis sativus* are shown in Figure 2.

The phytotoxicity tests towards terrestrial dicotyledons (Figure 2) indicated a clear linearity between doses and effects only for F2 ($R^2>0.9$). On the contrary, Co and F1 points are quite scattered indicating no linear relation between dose and effect, in particular towards *C. sativum*. Moreover, most of the tested dilutions resulted in negative inhibition effect (biostimulation), decreasing the number of effective data for statistical elaboration. No significant difference among angular coefficients of dose-effect regression lines was found. Also in this case, as for the algal test, the limited number of doses showing effects with positive value affected the strength of statistical elaboration.

L. minor, the tested aquatic monocotyledon, was not sensitive towards the three samples with the 0% inhibition of fronds number. Also the plant dry weight was not significantly affected by the exposition to the samples, with an average inhibition ranging from -17% to 8%.

By comparing the observed data, a first consideration on the sensitivity of the tested organisms towards this kind of wastewaters can be drawn. Among the three plants, *L. sativum* was the most sensitive, with inhibition of 65-90% at 80% sample dose. Its higher sensitivity with respect to *C. sativus* and *L. minor* was already signalled in a study on the landfill leachate (Tigini et al., 2014). On the contrary, *L. minor* was the least sensitive, pointing out its inadequacy for the ecotoxicity assessment of different kinds of wastewaters (Tigini et al., 2014; Tigini et al., 2016). Instead the alga showed high sensitivity to the sample, comparable to *L. sativum*. The alga has been previously signalled as particularly sensitive towards textile effluents (Bedoui et al., 2015, Novotný et al., 2006). However, a comparison with the other organisms is hard to define, on account of the limits due to the analytical interference caused by the samples colour. On the other hand, this acquatic organism can relieve the so-called physical toxicity, e.g. the growth inhibition due to the decrease of light penetration that turns in the reduction of photosynthesis. Moreover, the unicellular alga coud be more exposed to sample toxicity on account of its distinctive higher surface/volume ratio. Eventually, the presence of protozoa with dimension under 11 μm, not retained by the paper filter, could have preyed the alga, reducing its growth rate at the end of the experiment.

Another consideration can be drawn on the effectiveness of the second phase of biological treatment of chromium tannery wastewaters (comparing F1, sampled before the second aerobic stage treatment, and F2, sampled from the mixed liquor of the second aerobic stage treatment), in terms of toxicity removal. Despite the ammonium drastically decreased, the algal test did not relieve significant difference (T test) both in slope and translation of their regression lines. On the contrary, both the terrestrial plant tests underlined a difference in the translation of the two regression lines, with an increase in wastewater toxicity after the treatment between 35-38%. The reasons of this toxicity increase should be deeper investigated, since all the main chemical features decreased after the treatment, but the phosphorous (which should not reasonably be responsible for this toxicity increase).

Interestingly, no difference in chromium (F2) and vegetable (Co) tannery samples of mixed liquor was detected by all tested organisms. This indicates that chromium in treated tannery wastewater is not of concern. Chromium is generally present in low concentration and in trivalent form, the less toxic one (Prigione et al., 2009). Actually, hexavalent chromium is mostly adsorbed on the activated sludge flocks. Thus, the alga could not come in contact with this pollutant, since we tested the supernatant in order to avoid spectrophotometric interference. In the other tests, we analysed the entire samples, because in these cases sample turbidity did not represent an analytical limitation. However, pollutants adsorbed on activated sludge flocks could be not bioavailable to the test organisms. As a consequence, even in this case the toxicity due to these components could be not detectable.

Noteworthy, at the maximum concentration, all the samples were highly toxic, even exceeding the legal threshold value (i.e. 50% for the Italian law D.L. 152/06 and further modifications). This could be due to the Cl^- concentration, which exceeded 2900 mg L^{-1} in all the samples. As a consequence, a last comment is addressed to the use of dewater excess sludge and treated wastewaters in agriculture. This procedure is seen as a desirable option, in the light of Directive 2008/98/EC, which gives added value to wastes through their integrated management, moved by the aspiration to the reduction of mineral fertilizer use (Zhang et al., 2015). The hypothesized multiple benefits for agriculture and the environment are actually to be confirmed, by evaluating also the toxicity of these wastes. On the base of the obtained results, the tested samples are still heavily toxic for different terrestrial plants. The present results are in line with another study, which warns on the possible inappropriateness of direct application of activated sludge and treated effluents (Alvarenga et al., 2007). Moreover, the assessment of their impact on the human health should be evaluated.

3.3 Characterisation of cultivable autochthonous fungi

The total fungal load of the three wastewaters was particularly high, ranging from 2630 CFU 100 mL^{-1} (Co on WA 15 °C) to 11020 CFU 100 mL^{-1} (F2 on DRBC 25 °C). On the whole, a total of 102 fungi were isolated from the three samples (complete list in Table A of Supplementary materials). The most abundant species and their percentage contribution to intragroup (each column) similarity are reported in Table 2.

There are few studies focusing on the characterisation of the cultivable mycobiota associated to tannery industries. Most of them are referred to the contamination of work environments, i.e. indoor/outdoor air, superficies, raw materials, etc. (Skóra et al., 2014; Gutarowska et al., 2014; Castellanos-Arevalo et al., 2016). In others, the mycoflora associated to samples of the final effluent was characterised (Prigione et al., 2009). Nevertheless, a common key aspect emerged about tanneries as high-risk work environments, due to the massive presence of fungal propagules in bioaerosol, up to 2800 CFU m^{-3} (Skóra et al., 2014).

As far as we know, this work is one of the first reported studies focusing on a detailed analysis of the cultivable fungi present in influents and mixed liquors of a tannery WWTP.

In this study, several opportunistic pathogenic fungi, such as *Aspergillus fumigatus*, *Candida tropicalis*, *Geotrichum candidum*, *Lichtheimia corymbifera*, *Rhodotorula* sp., and *Pseudallescheria boydii*/*Scedosporium apiospermum* species complex were isolated. These fungi are emerging pathogens with an incredibly high ecological success in the last decades. They are often associated with polluted sites and their rapid spread is particularly alarming on account of their increasing impact on humans (Rougeron et al., 2015). Moreover, several potential toxigenic species have been identified in this study, in particular *Aspergillus flavus* (see Table A, in Supplementary material).

Besides potential pathogens and toxicogenic species, even the presence of other fungi, characterised by high sporulating rate, can be considered of concern. Actually, they consistently contribute to the bioaerosol with 0.65-2.1 μm diameter (classified as PM 2.5), which is one of the main professional risk factors in this field. In particular, five filamentous fungi have been determined as indicator of high health risk in tannery work environments according to their prevalence, source of isolation and health implications (Sokora et al., 2014). Noteworthy, two of them, *Cladosporium cladosporioides* and *Penicilliun crustosum*, were found in this work, too.

3.3.1 Effect of sample origin

Quantitative (fungal load) differences among the three samples were significant in most cases (Figure 3).

Co had a lower fungal load with respect of F2 and F1. It showed a similar fungal load to F2 only in MEA 15 °C and to F1 only in DRBC 15 °C. As regards the comparison between the two samples coming from chromium tannery, F2 and F1, they had different fungal loads (with the sole exception of WA 25 °C). However, which of them achieved the highest fungal load depended on the trial (F2>F1 in MEAp 25 °C, DRBC 25 °C, and DRBC 15°C; whereas F2<F1 in MEAp 15 °C, and WA 15 °C).

The three samples were qualitatively different, too. Among the 102 taxa, only 20 were common to all the samples. Their origin can be likely ascribable to air or working surfaces and they accidentally can be grown in WWTPs tanks. Actually, some of them are common air contaminant (e.g.. *Cladosporium* spp., *Penicillium* spp., and *Trichoderma* spp.), and can be easily sampled from outdoor environments (Skóra et al., 2014). Others are well known agents of deterioration of tanned leathers or chemicals used in this kind of industries, e.g. *Chaetomium globosum*, *Aspergillus* spp., *Fusarium* spp. (Orlita, 2004).

On the contrary, most of the isolated taxa were exclusive of a single sampling site. In particular, 30 were exclusive of Co, 11 were exclusive of F2, and 26 were exclusive of F1.

The differences recorded in the fungal composition of the three tanks indicate the development of three separated ecological niches. Actually, activated sludge in WWTPs tends to form a characteristic community mainly in relation to the influent composition (Evans and Seviour, 2012). This was particularly evident with the medium DRBC, on which the fungi of the three samples showed a similarity lower than 26% (Figure 4).

Probably, this result was due to the inhibition of fast growing fungi caused by DRBC medium that turned in the development of sample-specific fungi.

3.3.2 Effect of medium

Selective media with low glucose concentration (MEAp) or fungistatic (DRBC) were adopted, in order to limit the rapid growing fungi. Actually in preliminary exploring trials they were particularly abundant in all the samples. Nevertheless, these fast growing fungi still caused the failure of isolation of some fungal colonies with a slower growth rate (indicated as "unidentified" and with taxon name ending with sp. in Table 2 and in Table A of Supplementary material). Moreover, a medium containing wastewater (WA) as principal source of carbon was used in order to select fungi with a biodegradation potential toward the main organic compounds present in the samples. The use of different media significantly affected both fungal load (Figure 3) and biodiversity (Figure 5). Intergroup dissimilarity percentages (PERANOVA test) were in the ranges of 77-100% in Co, 51-77% in F2 and 61-96% in F1.

In four out of six cases, samples inoculated on DRBC showed the highest fungal load. The two exceptions were Co 25 °C and F1 15 °C, in which fungal load detected on DRBC was not significantly higher with respect to other media. The higher fungal load retrieved on DRBC is probably due to its higher nutrient's concentration (up to 25 fold for N source and five fold for C source) with respect to the other media, in which the low nutrient concentration could represent a limiting factor for fungal growth. Besides, higher peptone concentration could activate fungal propagules of dormant species (Thanh and Nout, 2004).

In two cases of particular interest (F2 15 °C and F1 25 °C), WA showed a significantly higher fungal load with respect to MEAp. Actually WA has the same glucose concentration, but lacks of malt and peptone. Moreover, it definitively exercised a selection due to the toxicity of the wastewater used to prepare the culture medium.

Among the most frequent taxa, some highly sporulating fungi are present. Thus, the fungal load of these fungi could be overestimated, due to the culture dependent method used for the isolation and identification of fungi (Evans and Seviour, 2012). However, the massive presence of these fungi in such selective medium indicates at least a great tolerance to pollutants in WWTPs tanks.

DRBC was the richest medium in terms of taxa diversity (17-25 taxa), whereas, MEAp showed the lowest fungal biodiversity (9-21 taxa). Surprisingly, WA showed also a good biodiversity (11-21 taxa). The presence of toxic or recalcitrant substrates generally leads to the reduction of microbial biodiversity in favour of the development of few species capable of tolerating such limiting factors (Cudowski et al., 2015). However, the relative abundance changed drastically, since the dominant species on WA (*Scedosporium apiospermum* up to 62% in Co, *Pseudallescheria boydii* up to 51% in F1 and 52% in F2) were sporadically counted in other media (0-20%). These species, which are an anamorphic and teleomorphic forms, respectively, belonging to the *Pseudallescheria boydii* species complex, have been already signalled to degrade several xenobiotics (Claussen and Schmidt, 1998; Ishii et al., 2009; Santos and Linardi, 2004) and are typically present in human-impacted areas (Rougeron et al., 2015).

Even other species, present in lower percentages (8-20%), have interesting degradation capability, i.e. *Trematosphaeria* (Mabrouk et al., 2012), *Scopulariopsis* (Verma et al., 2017) and *Geotrichum* (Ayed et al., 2016).

The use of WA culture medium, thus, decreased the species stocastically present, enhancing the development of a few species probably active in the degradation of pollutants in the WWTPs. These fungal species could potentially belong to the theoretical 20% of determinative species in the community that cause the 80% of the WWTP effectiveness, according the 20-80% ratio among cause-effect relationship (Pareto law). The knowledge of this determinative 20% species is at the base of a successful bioaugmentation strategy, since not all the species retrieved in a certain environment can effectively degrade of pollutans (Herrero and Stuckey, 2015). However, since most of them imply a concrete risk for human health, their massive exploitation in open WWPTs should be accurately evaluated.

Intuitively, the fungi isolated form the WA media consisting of chromium tannery samples, which are subsequent in the same plant (F1 and F2) showed the highest similarity (48.6%). Whereas Co was significantly different, with similarity to both F1 and F2 lower than 29% (Figure 4). The species in Co that mostly contributed to Co dissimilarity towards the other two samples were: *T. virens*, *S. apiospermum, T. asperellum*, *T. grisea* and *T. capillare.* These species could be potentially involved in tannins degradation.

On the other hand, in chromium tannery samples, the species that mostly contributed to F1/F2 dissimilarity towards Co were: *G. candidum*, *P. boydii*, *L. corymbifera A. furcatum*, *T. chromospermum*, P. *simplicissimum*, *P. dangeardii*, *F solani, S. brevicaulis.* These species, instead, could be rather involved in the degradation of pollutants present in chromium tannery wastewaters. None of these species have been reported for bioremediation of tannery wastewaters. However, several *Trichoderma* species have been signalled in the literature for biosorption of hexavalent chromium from tannery wastewaters (Shukla and Vankar, 2014). Moreover, other species of Penicillium and Fusarium, isolated from contaminated soil, have been used for bioremediation experiment of tannery wastewater (Sharma and Malaviya, 2016). The role of the fungi isolated in the present study should be investigated with specific bioremediation experiments, in order to validate this hypothesis.

3.4 Cultivable autochthonous fungi – effect of incubation temperature

The effect of temperature on bacterial composition of activated sludge was recently assessed (Niu et al., 2015). However, no study about the effect of temperature on fungal community in WWTPs has been performed.

The incubation temperature significantly affected the fungal load in five cases out of nine (CFU in greater number at 15 °C for Co on DRBC and for F1 on MEAp; CFU in greater number at 25 °C for F2 on MEAp and DRBC and for F1 on DRBC) (Figure 3). The temperature affected the biodiversity too, since the PERANOVA test pointed out significant differences in all cases. However, the intragroup similarity was 60-81% for both the temperatures. This turned in a low intergroup dissimilarity percentage 29-60%, with the exception of F1 cultured on MEAp and DRBC, which achieved respectively 85% and 74% dissimilarity between the two incubation temperatures. This is visible in Figure 4, in which the two incubation temperature of F1 on MEAp and DRBC are clearly distinct (intergroup similarity lower than 40%). Temperature does not seem to determine differences in mycoflora development as medium does, which is graphically evident in Figure 5.

Seasonal variations in bacterial composition in activated sludge treating tannery wastewater have been already observed, in particular during the summer. However, this phenomenon seems to be more related to the different influent composition and load during that period than to the high temperature (Giordano et al., 2016). Actually, starvation period can definitively modify the bacterial composition of the activated sludge (Cabezas et al., 2009). This could be true also for fungi, whose cellular structure (with a wall cell) protects them from environmental stress.

4 Conclusions

This is one of the first detailed studies on the cultivable fungi present in mixed liquors in chromium and vegetable tannery WWTPs aimed at developing novel bioremediation applications. Strong differences in mycoflora indicated the development of specific fungal population in each treatment tank. This study raised some questions about the risk associated with both potential pathogens / toxigenic species and high ecotoxicity, regardless the origin or the stage of depuration. In such kind of samples, the identification of sample-specific species with lower growth rate is strongly linked to the use of fungistatic. Contrarily, incubation temperature, in the range of seasonal fluctuations, does not substantially contribute to the development of different fungal species.

In further studies, the use of culture-independent methods based on next-gen sequencing (e.g. 454, SOLiD, and illumina, etc.) is needed, in order to achieve the complete identification of the fungi present in these kinds of samples, which are considerably rich in microorganisms.

From the applicative point of view, instead, a selective medium, consisting of agarised wastewater as principal source of carbon, allowed to select fungal strains that can be potentially exploited for bioaugmentation or biostimulation, with different suitability for vegetable or chromium tannery wastewaters. However, several of these species are potential pathogens and, thus, their application for *in situ* treatment must be evaluated with caution.

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References

1. Alvarenga, P., Palma, P., Gonçalves, A.P., Fernandes, R.M., Cunha-Queda, A.C., Duarte, E., Vallini, G., 2007. Evaluation of chemical and ecotoxicological characteristics of biodegradable organic residues for application to agricultural land. Environ. Int. 33, 505–513.
2. Anastasi, A., Spina, F., Romagnolo, A., Tigini, V., Prigione, V., Varese, G.C., 2012. Integrated fungal biomass and activated sludge treatment for textile wastewaters bioremediation. Bioresour. Technol. 123, 106-111.
3. Awad, M.F., Kraume, M., 2011. Mycological survey of activated sludge in MBRs. Mycoses. 54, e493–e498.
4. Ayed, L., Asses, N., Chammem, N., Hamdi, M., 2016. Improvement of green table olive processing wastewater decolorization by *Geotrichum candidum*. Desalin. Water Treat. 57, 17322–17332.

5. Bedoui, A., Tigini, V., Ghedira, K., Varese, G.C., Ghedira, L.C., 2015. Evaluation of an eventual ecotoxicity induced by textile effluents using a battery of biotests. Environ. Sci. Pollut. Res. 22, 16700–16708.

6. Bensch, K., Braun, U., Groenewald, J.Z., Crous, P.W., 2012. The genus *Cladosporium*. Stud. Mycol. 72, 1–401.

7. Cabezas, A., Draper, P., Etchebehere, C., 2009. Fluctuation of microbial activities after influent load variations in a full-scale SBR: recovery of the biomass after starvation. Appl. Microbiol. Biotechnol. 84, 1191-1202.

8. Castellanos-Arevalo, A.P., Camarena-Pozos, D.A., Castellanos-Arevalo, D.C., Rangel-Cordova, A.A., Pena-Cabriales, J.J., Arevalo-Rivas, B., de Pena, D.G., Maldonado-Vega, M., 2016. Microbial contamination in the indoor environment of tanneries in Leon, Mexico. Indoor Built Environ. 25, 524–540.

9. Chapman, P.M., 2000. Whole effluent toxicity testing-usefulness, level of protection, and risk assessment. Environ. Toxicol. Chem. 1, 3–13.

10. Claussen, M., Schmidt, S., 1998. Biodegradation of phenol and p-cresol by the hyphomycete *Scedosporium apiospermum*. Res. Microbiol. 149, 399–406.

11. Cudowski, A., Pietryczuk, A., Hauschild, T., 2015. Aquatic fungi in relation to the physical and chemical parameters of water quality in the Augustów Canal. Fungal Ecol. 13, 193–204.

12. Djelal, H., Amrane, A., 2013. Biodegradation by bioaugmentation of dairy wastewater by fungal consortium on a bioreactor lab-scale and on a pilot-scale. J. Environ. Sci. 25, 1906–1912.

13. Domsch, K.H., Gams, W., Anderson, T.H., 1980. Compendium of soil fungi, first ed. Academic Press, New York.

14. Evans, T.N., Seviour, R.J. 2012. Estimating biodiversity of fungi in activated sludge communities using culture-independent methods. Microb. Ecol. 63, 773–786.

15. Fell, J.W., Boekhout, T., Fonseca, A., Scorzetti, G., Statzell-Tallman, A., 2000. Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. Int. J. Syst. Evol. Microbiol. 50, 1351-1371.

16. Giordano, C., Boscaro, V., Munz, G., Mori, G., Vannini, C., 2016. Summer holidays as break-point in shaping a tannery sludge microbial community around a stable core microbiota. Sci. Rep. 6, 30376.

17. Glass, N.L., Donaldson, G.C., 1995. Development of prime sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Appl. Environ. Microbiol. 61, 1323–1330.

18. Grabinskaloniewska, A., Slavikova, E., Furmanska, M., Slomczynski, T., 1993. Fungi in activated-sludge biocenosis. Acta Microbiol. Pol. 42, 303–313.

19. Gutarowska, B., Skóra, J., Stępień, Ł., Twarużek, M., Błajet-Kosicka, A., Otlewska, A., Grajewski, J., 2014. Estimation of fungal contamination and mycotoxin production at workplaces in composting plants, tanneries, archives and libraries. World Mycotoxin J. 7, 345–355.

20. Herrero, M., Stuckey, D.C., 2015. Bioaugmentation and its application in wastewater treatment: A review. Chemosphere 140, 119–28.

21. Ishii, K., Furuichi, T., Tanikawa, N., Kuboshima, M., 2009. Estimation of the biodegradation rate of 2,3,7,8-tetrachlorodibenzo-p-dioxin by using dioxin-degrading fungus, *Pseudallescheria boydii*. J. Hazard. Mater. 162, 328–332.

22. Kiffer, E., Morelet, M., 1997. Les deutéromycètes. Classification et clés d'identification générique, first ed. INRA, Paris.

23. Kim, I.S., Ekpeghere, K., Ha, S.Y., Kim, S.H., Kim, B.S., 2013. An eco-friendly treatment of tannery wastewater using bioaugmentation with a novel microbial consortium. J. Environ. Sci. Health Part A. 48, 1732–1739.

24. Korzeniewska, E., 2011. Emission of bacteria and fungi in the air from wastewater treatment plants – a review. Front. Biosci. 3, 393–407.

25. Leyva-Diaz, J., Martin-Pascual, J., Poyatos, J.M., 2017. Moving bed biofilm reactor to treat wastewater. Int. J. Environ. Sci. Technol. 14, 881–910.

26. Liu, J., Li, J., Tao, Y., Sellamuthu, B., Walsh, R., 2017. Analysis of bacterial, fungal and archaeal populations from a municipal wastewater treatment plant developing an innovative aerobic granular sludge process. World J. Microbiol. Biotechnol. 33:14.

27. Lofrano, G., Meric, S., Zengin, G.E., Orhon, D., 2013. Chemical and biological treatment technologies for leather tannery chemicals and wastewaters: A review. Sci. Total Environ. 461: 265–281.

28. Mabrouk, A.M., Kheiralla, Z.H., Hamed, E.R., Youssry, A.A., Abd El Aty, A.A**.,** 2012. Characterization and purification of the crude *Trematosphaeria mangrovei* laccase enzyme. Egypt. Pharmaceut. J. 11, 93–98.

29. More, T.T., Yan, S., Tyagi, R.D., Surampalli, R.Y., 2010. Potential use of filamentous fungi for wastewater sludge treatment. Bioresour. Technol. 101, 7691–7700.

30. Munz, G., De Angelis, D., Gori, R., Mori, G., Casarci, M., Lubello, C., 2009. The role of tannins in conventional and membrane treatment of tannery wastewater. J Hazard. Mater. 164, 733–739.

31. Novotný, C., Dias, N., Kapanen, A., Malachová, K., Vándrovcová, M., Itävaara, M., Lima, N., 2006. Comparative use of bacterial, algal and protozoan tests to study toxicity of azo- and anthraquinone dyes. Chemosphere 63, 1436–1442.

32. Niu, L., Li, Y., Wang, P., Zhang, W., Wang, C., Wang, Q., 2015. Understanding the linkage between elevation and the activated-sludge bacterial community along a 3,600-meter elevation gradient in China. Appl. Environ. Microbiol. 81, 6567–76.

33. Orhon, D., 2015. Evolution of the activated sludge process: the first 50 years. J. Chem. Technol. Biotechnol. 90, 608–640.

34. Orlita, A., 2004. Microbial biodeterioration of leather and its control. Int. Biodeterior. Biodegrad. 53, 157–163.

35. Prigione, V., Zerlottin, M., Refosco, D., Tigini, V., Anastasi, A., Varese, G.C., 2009. Chromium removal from a real tanning effluent by autochthonous and allochthonous fungi. Bioresour. Technol. 100, 2770–2776.

36. Rougeron, A., Schuliar, G., Leto, J., Sitterle, E., Landry, D., Bougnoux, M-E., Kobi, A., Bouchara, J-P., Giraud, S., 2015. Human-impacted areas of France are environmental reservoirs of the *Pseudallescheria boydii*/ *Scedosporium apiospermum* species complex. Environmental Microbiology; 17: 1039–1048.

37. Santos, V.L., Linardi, V.R., 2004. Biodegradation of phenol by a filamentous fungus isolated from industrial effluents-identification and degradation potential. Process biochem. 39, 1001–1006.

38. Sharma, S., Malaviya, P., 2016. Bioremediation of tannery wastewater by chromium resistant novel fungal consortium. Ecol. Eng. 91, 419-425.

39. Shukla, D., Vankar, P.S., 2014. Role of Trichoderma Species in Bioremediation Process: Biosorption Studies on Hexavalent Chromium, in: Gupta, V.K., Schmoll, M., HerreraEstrella, A., Upadhyay, R.S., Druzhinina, I., Tuohy, M.G. (Eds), Biotechnology and biology of trichoderma, Elsevier Science BV, The Netherlands, pp: 405-412.

40. Skóra, J., Gutarowska, B., Stępień, L., Otlewska, A., Pielech-Przybylska K., 2014. The evaluation of microbial contamination in the working environment of tanneries. Medycyna Pracy 2014; 65:15–32.
41. Svobodová, K., Petráčková, D., Kozická, B., Halada, P., Novotný, C., 2016. Mutual interactions of *Pleurotus ostreatus* with bacteria of activated sludge in solid-bed bioreactors. World J. Microbiol. Biotechnol. 32:94.
42. Thanh, N.V., Nout, M.J.R., 2004. Dormancy, activation and viability of *Rhizopus oligosporus* sporangiospores. Int. J. Food Microbiol. 92, 171–179.
43. Tigini, V., Franchino, M., Bona, F., Varese, G.C., 2016. Is digestate safe? A study on its ecotoxicity and environmental risk on a pig manure. Sci. Total Environ. 551, 127–132.
44. Tigini, V., Prigione, V., Varese, G.C., 2014. Mycological and ecotoxicological characterisation of landfill leachate before and after traditional treatments. Sci. Total Environ. 487, 335–341.
45. von Arx, J.A., 1981. The genera of fungi sporulating in pure culture, second ed. Port Jervis Lubrecht & Cramer Ltd, New York.
46. Verma, A., Singh, H., Anwar, S., Chattopadhyay, A., Tiwari, K.K., Kaur, S., Dhilon, G.S., 2017. Microbial keratinases: industrial enzymes with waste management potential. Crit. Rev. Biotechnol. 37, 476–491.
47. White, T.J., Brun, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenesis, in: Innis, M.A., Gelfand D.H., Sninsky, J.J., White, T.J. (Eds.) PCR Protocols: A Guide to Methods and Applications, Academic Press Inc., New York, pp. 315–322.
48. Zhang, S., Gao, X., He, L., Qiu, Y., Zhu, H., Cao, Y, 2015. Novel trends for use of microbial tannases. Prep. Biochem. Biotech. 45, 221–232.

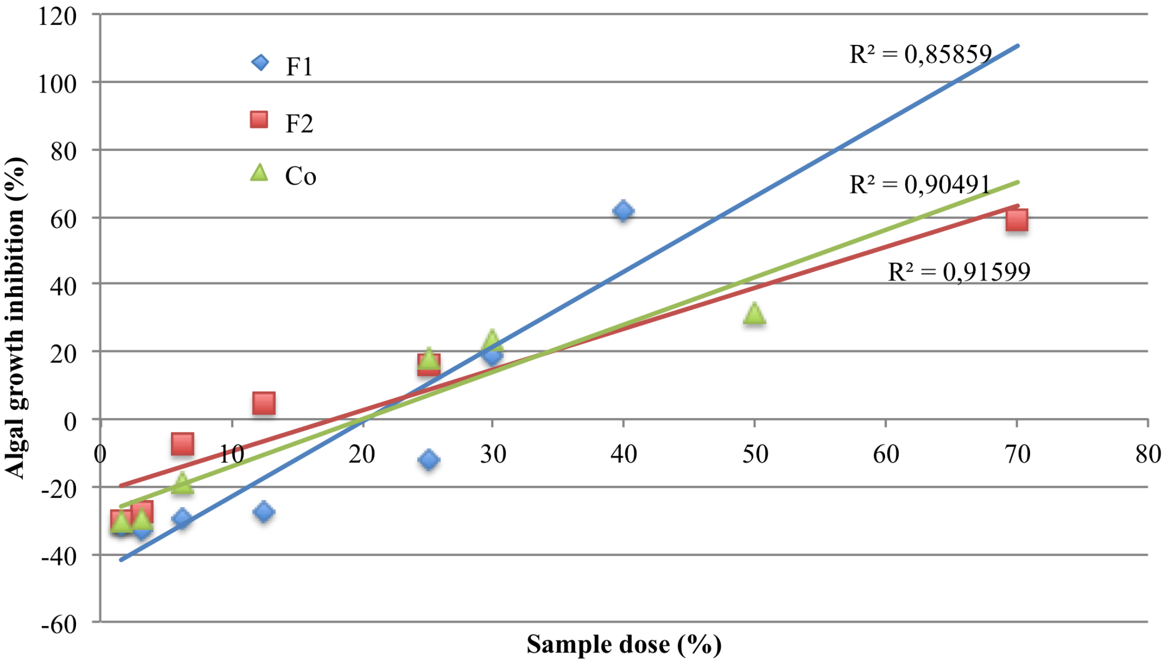


Figure 1. Algal growth inhibition caused by the three tannery effluents (Co, F1, and F2).

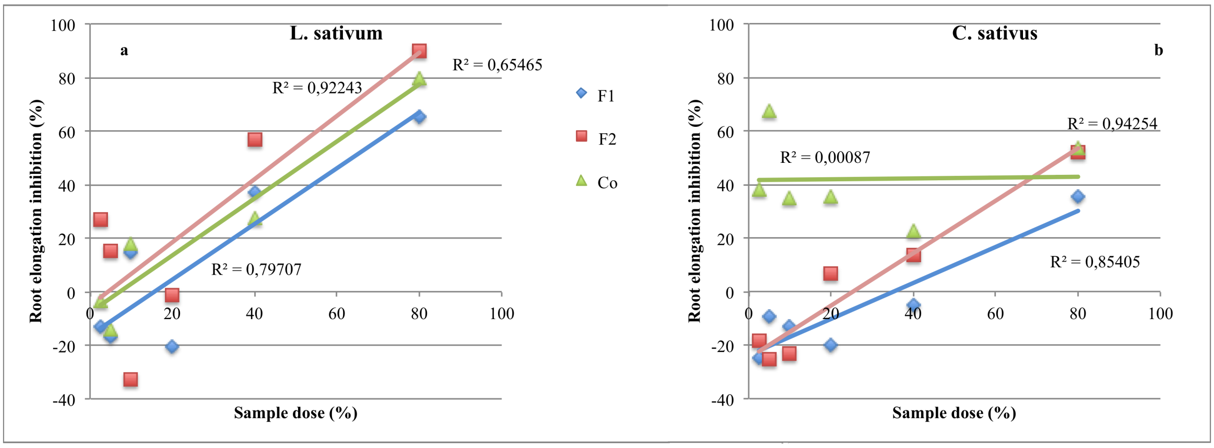


Figure 2. Phytotoxicity caused by the three tannery effluents (Co, F1, and F2).

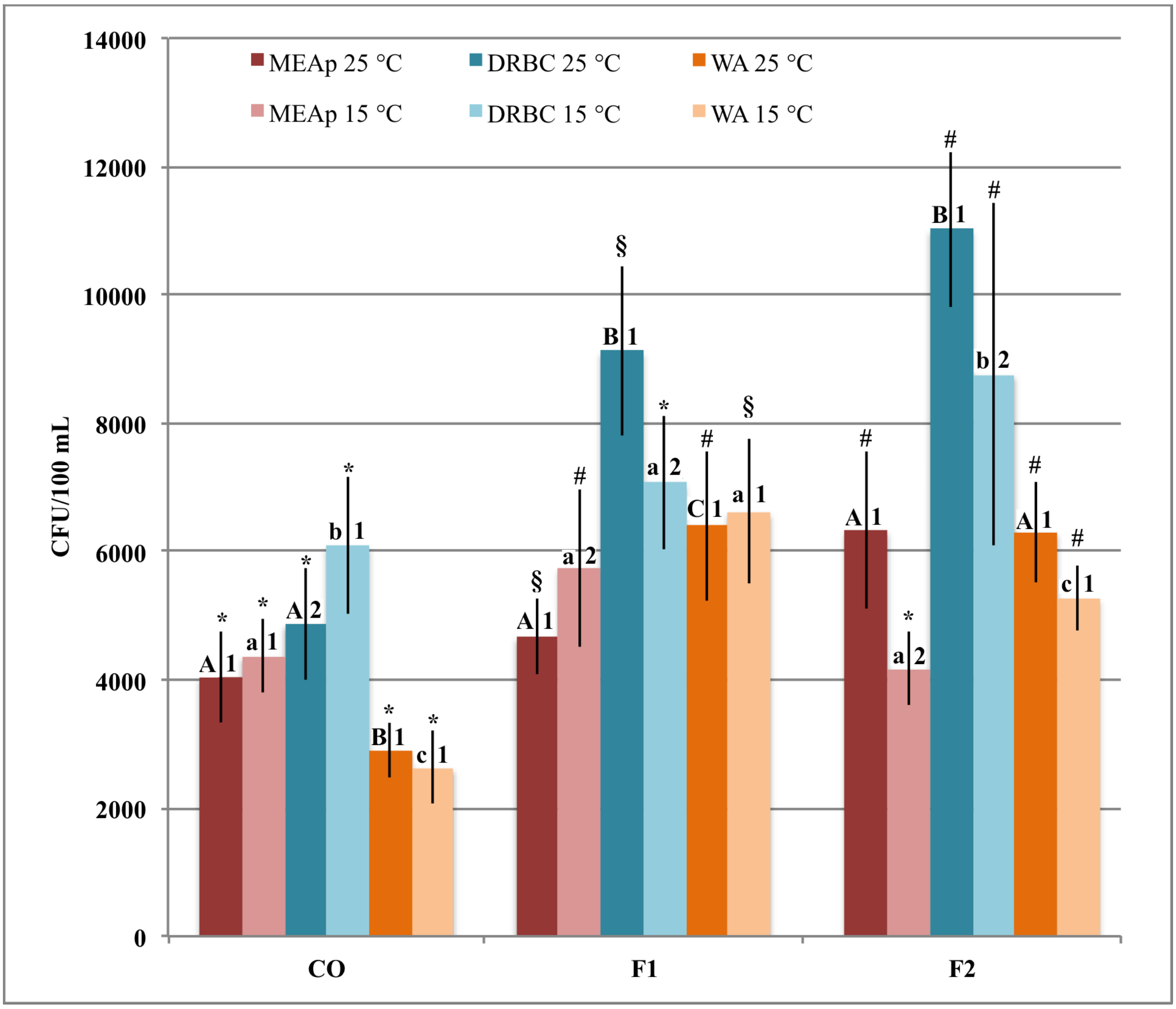


Figure 3. Total fungal load of the three samples (Co, F1, and F2) inoculated on three different media (MEAp, DRBC, and WA) and incubated at two temperatures (25 °C and 15 °C). Capital letters indicate significant differences among fungal loads observed on different culture media at 25 °C incubation; small letters indicate significant differences among fungal loads observed on different culture media at 15 °C incubation; numbers indicate significant differences between incubation temperature; symbols indicates significant differences among sample origins (Mann Whitney, P≤0.05).

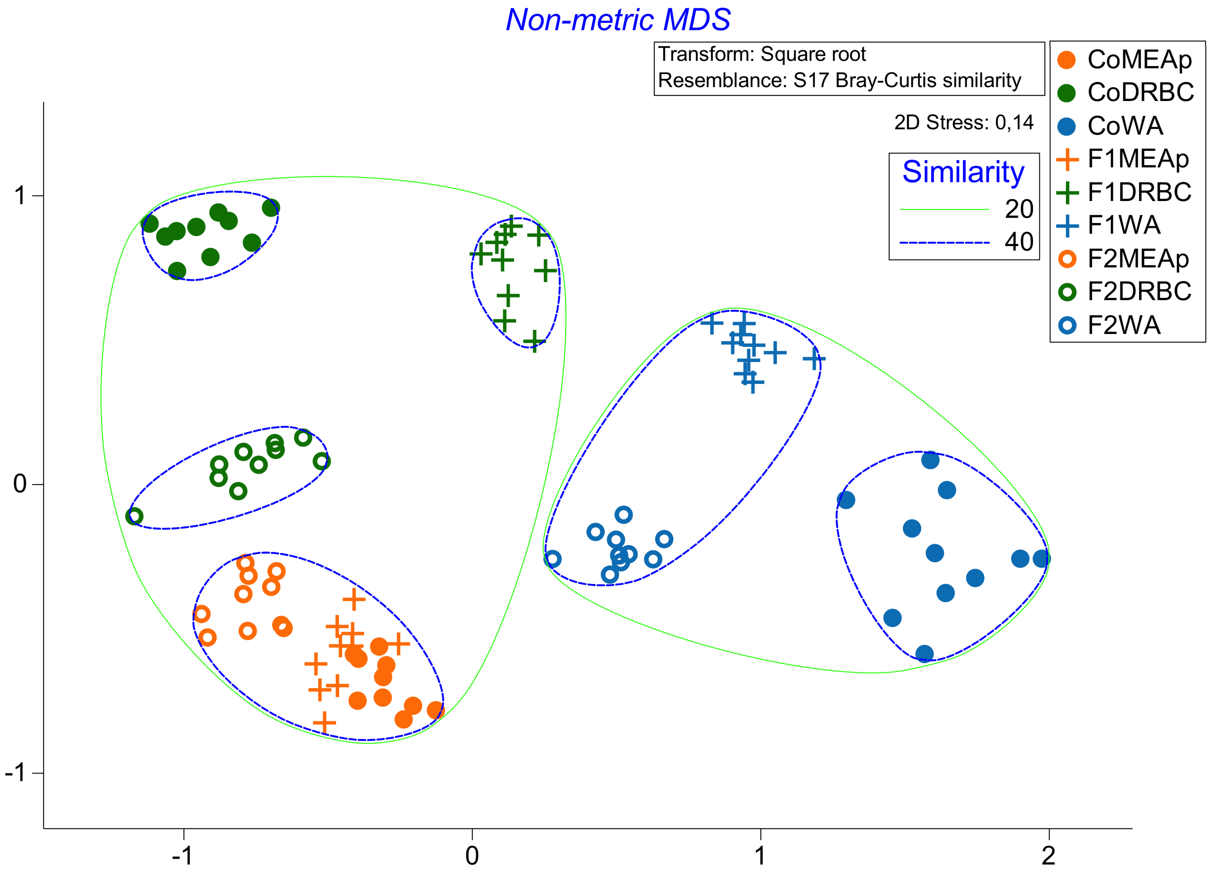
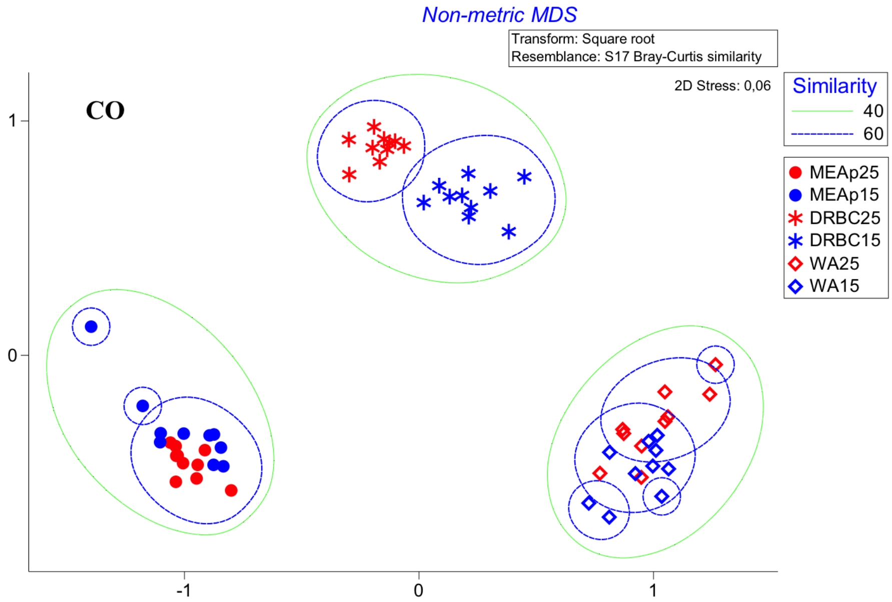
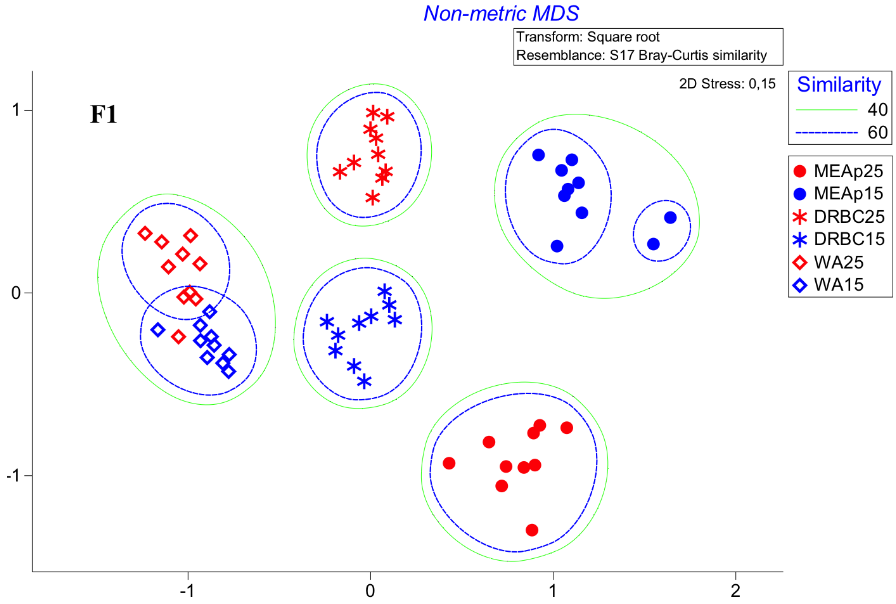


Figure 4. Graphical representation of similarity among the sample on the three culture media (MEAP, DRBC and WA for the three samples (Co, F1 and F2) incubated at 25 °C.


Non-metric MDS
Transform: Square root
Resemblance: S17 Bray-Curtis similarity
2D Stress: 0,06
CO
Similarity
40
60
MEAp25
MEAp15
DRBC25
DRBC15
WA25
WA15
1
0
-1
0
1


Non-metric MDS
Transform: Square root
Resemblance: S17 Bray-Curtis similarity
2D Stress: 0,15
F1
Similarity
40
60
MEAp25
MEAp15
DRBC25
DRBC15
WA25
WA15
1
0
-1
-1
0
1
2

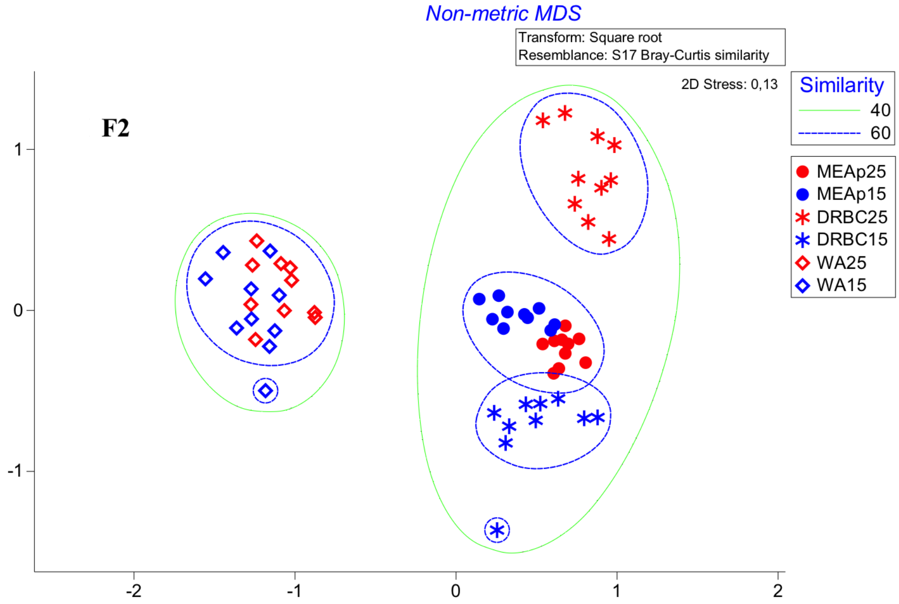

Non-metric MDS
Transform: Square root
Resemblance: S17 Bray-Curtis similarity
2D Stress: 0,13
F2
Similarity
40
60
MEAp25
MEAp15
DRBC25
DRBC15
WA25
WA15
1
0
-1
-2
-1
0
1
2

Figure 5. Graphical representation of similarity among all the trials (temperatures and culture media) for the three samples (Co, F1 and F2).

Table 1. Samples chemical features.

	Co	F1	F2
pH	7.66	7.33	7.22
Ammonium [mg L^{-1}]	1.04	278	9.65
Total phosphorus [mg L^{-1}]	5.92	2.43	3.46
Chlorides [mg L^{-1}]	2906	4100	4218
COD [mg L^{-1}]	14280	24740	10000
SCOD [mg L^{-1}]	362	760	518
TSS [g L^{-1}]	12.93	12.61	7.49
VSS [g L^{-1}]	10.78	9.61	5.77

Table 2: List of the most abundant species retrieved. For each sample (Co, F1, F2), medium (MEAp, DRBC, WA), and incubation temperature (25, 15 °C), contribution in percentage to the intragroup (each column) similarity and total number of taxa are reported.

	Sample	**Co**						**F1**						**F2**					
	Medium	**MEAp**		**DRBC**		**WA**		**MEAp**		**DRBC**		**WA**		**MEAp**		**DRBC**		**WA**	
	Incubation temperature	**25**	**15**	**25**	**15**	**25**	**15**	**25**	**15**	**25**	**15**	**25**	**15**	**25**	**15**	**25**	**15**	**25**	**15**
Contribution (%) to intragroup similarity	*Acremonium furcatum*															27			
	Aspergillus niveus																19		
	Candida tropicalis var. *tropicalis*								35										
	Cladosporium sp.											12							
	Fusarium solani				9														
	Geotrichum candidum	48	41					67						63	48	24	38	34	34
	Lichtheimia corymbifera									30									
	Penicillium simplicissimum	21	21	14					37	28	11			16		13			
	Pseudallescheria boydii						35			20	12	36	22					47	42
	Scedosporium apiospermum				22	60	40				42	12	19						
	Scopulariopsis brevicaulis											12	13						
	Trematosphaeria grisea					21													
	Trichoderma asperellum			29	33														
	Trichoderma capillare	17	13					12											
	Trichoderma chromospermum			12	8						15			17		12	18		
	Trichoderma virens			26											20				
	Undetermined												43						
	Total n° of taxa	12	18	21	19	16	20	13	21	23	17	19	21	10	9	25	17	11	20

Table A: List of the taxa retrieved. For each sample (Co, F1, F2), medium (MEAp, DRBC, WA), and incubation temperature (25, 15 °C), the abundance of single taxa and average of total load (both expressed as CFU/mL), and total number of taxa are reported.

		Co						F1						F2					
	Taxa	**MEAp**		**DRBC**		**WA**		**MEAp**		**DRBC**		**WA**		**MEAp**		**DRBC**		**WA**	
		25	**15**	**25**	**15**	**25**	**15**	**25**	**15**	**25**	**15**	**25**	**15**	**25**	**15**	**25**	**15**	**25**	**15**
1	Absidia glauca Hagem								0.1										
2	*Acremonium berkeleyanum* (P. Karst.) W. Gams						0.1												
3	*Acremonium furcatum* Moreau & V. Moreau ex W. Gams	0.1														33.9			
4	*Acrostalagmus luteoalbus* (Link) Zare, W. Gams & Schroers											0.1							
5	*Ascodesmis macrospora* W. Obrist												0.1						
6	*Aspergillus caespitosus* Raper & Thom													0.3	0.4	0.2			
7	*Aspergillus candidus* Link							0.1	0.1	0.1	0.6	0.1							
8	*Aspergillus carneus* (Tiegh.)					0.1													
9	*Aspergillus clavatus* Desm.		0.3	0.2	0.1	0.1				0.1	0.1								
10	*Aspergillus flavus* Link									0.2	0.2			0.2					
11	*Aspergillus fumigatus* Fresen.		0.1	0.7	0.1							0.1				0.2			
12	*Aspergillus neoniveus* Samson	1.4	0.7																
13	*Aspergillus niger* Tiegh.										0.1								
14	*Aspergillus niveus* Blochwitz								0.1							0.2	11.7		
15	*Aspergillus ochraceus* K. Wilh.			2.4	1.5			0.2	0.1	1.3	0.3	0.6	0.3		0.1	6	0.2	0.4	
16	*Aspergillus ostianus* Wehmer																0.1		
17	*Aspergillus sojae* Sakag. & K.									0.1									
18	*Aspergillus terreus* Thom	0.1	3.4	0.1	0.2														
19	*Aspergillus versicolor* (Vuill.) Tirab.					0.1													
20	*Aureobasidium* sp.						0.1												
21	*Basifimbria* sp.					0.5										0.1			
22	*Bjerkandera adusta* (Willd.) P. Karst.				0.1														

23	*Botryotrichum piluliferum* Sacc. & Marchal									0.1									
24	*Byssochlamys laguncularia*e (C. Ram) Samson, Houbraken & Frisvad						0.2												
25	*Byssochlamys nivea* Westling															0.1			
26	*Candida tropicalis* var. *tropicalis*	1.6	0.1						14.2									0.2	
27	*Chaetomium bostrychodes* Zopf									0.1									
28	*Chaetomium elatum* Kunze									0.4									0.2
29	*Chaetomium globosum* Kunze ex Fr.					0.1	0.3		0.2		0.7	0.3	0.5						0.1
30	*Chaetomium homopilatum* Omvik		0.1				0.2					4.1	3.6					0.2	4.5
31	*Chaetomium pilosum* (C. Booth & Shipton) X.W. Wang & Crous											0.2	0.5						
32	*Chaetomium* sp.					0.4													
33	*Chaetomium tarraconense* Stchigel						0.3												
34	*Cladosporium cladosporioides* (Fresen.) G.A. de Vries						0.5		0.4										
35	*Cladosporium pseudocladosporioides* Bensch								1.1										
36	*Cladosporium* sp.				3.8	0.1						3.9					4.6	3.5	2.2
37	*Cladosporium xylophilum* Bensch, Shabunin, Crous & U. Braun,												3.3						
38	*Clonostachys solani* f. *nigrovirens* (J.F.H. Beyma) Schroers			0.2															
39	*Coelomycetes* sp.								0.3				0.1						
40	*Cryptococcus humicola* (Dasz.) Golubev					0.2													
41	*Emericellopsis terricola* J.F.H. Beyma,												0.1						0.1
42	*Eurotium* sp.												0.2						
43	*Fusarium keratoplasticum* D. Geiser, O'Donnell, Short et Zhang								7.2										
44	*Fusarium solani* (Mart.) Sacc.			0.1	4.2	0.2		1.9	0.2	0.1	0.2	1.3	3.3	0.1	0.2	0.8	2.8		1.8
45	*Geotrichum candidum* Link	24.2	22.2					38.3	0.3	0.3	1.4		0.3	50	26.4	28	53.1	19.2	14.6
46	*Gliocladium*	1.4	0.1																

	solani (Harting) Petch																		
47	*Gliomastix* sp.								0.2										
48	*Gymnascella hyalinospora* (Kuehn, G.F. Orr & G.R. Ghosh) Currah											0.1							
49	*Gymnaschella* sp.											0.6	0.1						
50	*Gymnoascus udagawae* Arx						0.5												
51	*Humicola fuscoatra* Traaen			0.2			0.7												
52	*Humicola grisea* Traaen												0.1						
53	*Isaria fumosorosea* Wize										0.1								
54	*Lichtheimia corymbifera* (Cohn) Vuill.		0.1	0.1						32.4		1.5	0.2			0.6		0.1	0.6
55	*Mammaria echinobotryoides* Ces.												0.1						0.1
56	*Monascus ruber* Tiegh.			0.1	0.1		0.2	0.2				0.1	0.1	0.1					
57	*Mucor circinelloides* var. circinelloides		0.1																
58	*Mucor luteus* Linnem. ex Wrzosek							0.6											
59	*Mucor plumbeus* Bonord.	1.5	3.0		0.3														
60	*Neosartorya fischeri* (Wehmer) Malloch & Cain	0.1																	
61	*Paecilomyces carneus* (Duché & R. Heim) A.H.S. Br. & G. Sm.									0.4									
62	*Paecilomyces variotii* Bainier									0.4						5.2			0.2
63	*Penicillium atramentosum* Thom		0.1	0.6					0.5								0.6		
64	*Penicillium camemberti* Thom													0.1					
65	*Penicillium crustosum* Thom		0.3																
66	*Penicillium dangeardii* Pitt															5.4			
67	*Penicillium expansum* Link						0.1												
68	*Penicillium funiculosum* Thom			0.1															
69	*Penicillium mexicanum* Visagie, Seifert & Samson									0.3						0.1			
70	*Penicillium paneum* Frisvad								0,7										
71	*Penicillium rugulosum*				0.5									0.3		0.2	1.2		

	Thom																		
72	*Penicillium simplicissimum* (Oudem.) Thom	5.1	5.4	3.8	2.5			2.3	26.4	25.6	4.6			3.2	3.5	16.1	1.8		
73	*Penicillium* sp.			0.8	0.1			0.1	0.7	0.1	0.4		0.6			0.1	0.2		
74	*Petriella guttulata* G.L. Barron & Cain											0.1							
75	*Phoma sorghina* (Sacc.) Boerema, Dorenb. & Kesteren							0.1											
76	*Phoma* sp.					0.4													
77	*Pseudallescheria boydii* (Shear) McGinnis, A.A. Padhye & Ajello				1.1	3.1	7.6			15.4	3.9	32.8	17.5					32.6	25
78	*Rhodotorula* sp.		1.6																
79	*Sagenomella diversispora* (J.F.H. Beyma) W. Gams	0.7	0.3																
80	*Sagenomella* sp.							0.1											
81	*Sarocladium strictum* (W. Gams) Summerbell																	0.2	
82	*Scedosporium apiospermum* (Sacc.) Sacc. ex Castell. & Chalm.				12	17.9	9.5				39.3	5.7	11.4		2.4	0.4	0.7	4.2	0.7
83	*Scedosporium aurantiacum* Gilgado					1.0	0.2										0.1	1.6	0.1
84	*Scolecobasidium* sp.					0.1	0.7												
85	*Scopulariopsis brevicaulis* (Sacc.) Bainier									0.1		4.4	5		0.1	0.4	0.5	0.7	1.3
86	*Sporormiella minima* (Auersw.) S.I. Ahmed & Cain															0.1	0.1		0.1
87	*Stachybotrys chartarum* (Ehrenb.) S. Hughes																		0.2
88	*Talaromyces flavus* (Klöcker) Stolk & Samson			0.7															
89	*Talaromyces* sp.															0.1			
90	*Talaromyces wortmannii* (Klöcker) C.R. Benj.															0.7			
91	*Thelebolus* sp.						0.1												0.2
92	*Trematosphaeria grisea* (J.E. Mackinnon, Ferrada & Montemartini) S.A. Ahmed, W.W.J. van de Sande, A. Fahal & de Hoog					4.4	4.3												0.5
93	*Trichoderma asperelloides* Samuels									0.1									
94	*Trichoderma asperellum*			17.3	23.6					1.8	0.2					0.5	0.1		

	Samuels, Lieckf. & Nirenberg																		
95	*Trichoderma capillare* Samuels & C.P. Kubicek	3.9	1.7	0.1				1.3	0.2	9.0									
96	*Trichoderma chromospermum* P. Chaverri & Samuels			4.0	3.6				3.0		6.8			4.3	1.8	9.8	8.7		
97	*Trichoderma harzianum* Rifai			0.7												0.2			
98	*Trichoderma virens* (J.H. Mill., Giddens & A.A. Foster) Arx			13.8	4.1			0.1	1.2	0.8	1.6	0.1		4.7	6.8	0.8	1.1		
99	*Trichosporon* sp.			0.1	0.4														
100	Undetermined	0.4	4.1	2.6	2.6	0.3	0.6	1.4	0.2	2	10.2	7.9	18.7						0.1
101	*Westerdykella dispersa* (Clum) Cejp & Milko						0.1												
102	*Westerdykella* sp.																		0.1
	Total n° of taxa	12	18	21	19	17	20	13	21	23	17	19	21	10	9	25	17	11	20
	Average of total CFU/mL	**40.5**	**43.7**	**48.7**	**60.9**	**29**	**26.3**	**46.7**	**57.4**	**91.2**	**70.7**	**64**	**66.1**	**63.3**	**41.7**	**110.2**	**87.6**	**62.9**	**52.7**

Species common to all samples are underlined in blue; species exclusively present in Co are underlined in red; species exclusively present in F1 are underlined in purple; species exclusively present in F2 are underlined in green.