USE OF FUNGI AND BACTERIA FOR THE REMOVAL OF RECALCITRANT COMPOUNDS FROM TANNERY WASTEWATER

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SUMMARY

Tannins are polyphenolic compounds produced by plants that are used in the vegetable tanning of leather at an industrial scale. Tannins differ from most other natural phenols since they precipitate proteins and are used in the tanning process to bind to the collagen proteins of the animal skin to make leather more durable and not putrescible. Tannins represent one of the low-biodegradability substances in tannery wastewaters with a highly recalcitrant soluble chemical oxygen demand; moreover, at high concentrations they can inhibit biological treatment. This soluble recalcitrant fraction of tannery effluents is usually removed by means of chemical processes. Therefore, a biological treatment that could remove effectively this fraction would have both environmental and economic advantages. Despite the antimicrobial properties of tannins, there are organisms that are able to grow on them; in fact their biodegradation in the environment is mainly associated with fungi rather than bacteria. Fungi could thus be exploited for the bioremediation of wastewater streams of the tanning industry. However, in environmental biotechnology applications, fungi tend to be outcompeted by bacteria. The application of a fungal-based bioreactor, that has a similar performance under sterile and non-sterile conditions in long-term operations, is still a challenging task. The present thesis was aimed at developing and testing technologies to remove tannins with an engineered ecosystem based on fungi and bacteria for application in typical tannery wastewater treatment trains (non-sterile conditions). *Aspergillus tubingensis* MUT 990 was selected as a suitable fungal strain. Literature research and previous experiences were used to design and build lab-scale (4 litres volume) and pilot-scale (1.5 m^3 volume) reactors. The selected fungal strain was immobilised in polyurethane foam cubes carriers and inoculated in a novel rotating, submerged, packed bed reactor. The first test in bioreactors was carried out to evaluate the effect of rotation and the co-substrate addition on tannin removal. The second test in bioreactors was carried out in two submerged packed bed reactors run in parallel, the first one was fed with a condensed tannin (Quebracho tannin), and the second with hydrolysable tannin (Tara tannin), both were inoculated with *Aspergillus tubingensis* in attached form. The hydraulic retention time and the concentration of tannins in the medium were varied to maximise the removal capacity and to analyse the substrate inhibition. A stable biofilm was maintained in the first reactor during the 180 days of operation. On the other hand, in the second reactor there was a biomass detachment during the start-up and then grown as a suspended culture throughout the operational period (226 days). Soluble chemical oxygen demand removal up to 53% and 90% were achieved in the first and second bioreactor, respectively, without the addition of co-substrates. The microbial communities of the reactors, made up of fungi and bacteria, were characterised with a metagenomic analysis. In addition, an innovative technique, the heterogeneous respirometry, was applied to assess the biological activity of immobilised cells (biofilm). The data obtained were used to develop a mathematical model and to perform a kinetic and stoichiometric characterisation of the biomass. Since fungal biomass is poorly characterised with modelling and respirometry, dedicated experiments were set-up along with a respirometric procedure. The lab-scale results were used to design a pilot-scale reactor. Finally, after a start-up phase the pilot-scale reactor was fed with real tannin-rich wastewater collected from tanneries. At the end of the experimentation the biofilm was stable and an encouraging performance had been achieved. We believe the results obtained represent the first step for a future real-scale application to reach an efficient biological removal of tannins from tannery wastewater.

RIASSUNTO

I tannini sono composti polifenolici prodotti dalle piante e sono utilizzati nella concia al vegetale a scala industriale. I tannini si differenziano dalla maggior parte degli altri composti fenolici naturali per la loro capacità di far precipitare le proteine e sono utilizzati nel processo di concia per legarsi al collagene delle pelli animali, al fine di renderle più resistenti e non putrescibili. I tannini sono una delle sostanze solubili a bassa biodegradabilità presenti nelle acque di scarico della concia e presentano un'elevata domanda chimica di ossigeno recalcitrante. Inoltre, ad elevate concentrazioni possono inibire i trattamenti biologici. Questa frazione recalcitrante e solubile delle acque di scarico della concia è solitamente rimossa tramite processi chimici. Pertanto, un trattamento biologico in grado di rimuovere efficacemente questa frazione presenterebbe vantaggi sia ambientali che economici. Nonostante le proprietà antimicrobiche dei tannini, esistono organismi in grado di usarli come substrato di crescita, la loro biodegradazione nell'ambiente è principalmente associata ai funghi piuttosto che ai batteri. I funghi quindi possono essere sfruttati per il biorisanamento delle acque reflue dell'industria conciaria. Tuttavia, in questa tipologia di biotecnologie ambientali, i funghi non riescono a competere con batteri. L'utilizzo di un bioreattore fungino, con simili prestazioni in condizioni sterili e non sterili in prove a lungo termine, rappresenta ancora un compito impegnativo. La presente tesi si è focalizzata nello sviluppo e sperimentazione di tecnologie atte a rimuovere i tannini sfruttando un ecosistema di funghi e batteri, il fine ultimo è stato quello di sviluppare un trattamento da integrare a quelli convenzionali per le acque reflue (condizioni non sterili). Il ceppo fungino *Aspergillus tubingensis* MUT 990 è stato selezionato come il più adatto per il presente studio. La letteratura di settore e le esperienze precedenti hanno fornito un importante contributo per la progettazione e costruzione dei reattori a scala di laboratorio (4 litri) e un reattore a scala pilota (1.5 m^3). Il ceppo fungino selezionato è stato immobilizzato in supporti costituiti da cubi di schiuma poliuretanica ed è stato inoculato in un innovativo reattore a letto rotante e sommerso. La prima prova con i bioreattori ha avuto il fine di valutare l'effetto della rotazione e dell'aggiunta del co-substrato sulla rimozione del tannino. La seconda prova è stata effettuata in parallelo in due reattori, il primo è stato alimentato con un tannino condensato (Quebracho) e il secondo con tannino idrolizzabile (Tara), entrambi sono stati inoculati con *Aspergillus tubingensis* immobilizzato sul supporto sopradescritto. Il tempo di ritenzione idraulico e la concentrazione di tannino sono stati i principali parametri studiati per massimizzare la capacità di rimozione e per analizzare l'inibizione del substrato. Nel primo reattore si è mantenuto un biofilm stabile durante i 180 giorni di funzionamento. Nel secondo reattore invece si è verificato un distacco del biofilm e lo sviluppo di una biomassa in sospensione per tutto il periodo sperimentale (226 giorni). Nel primo reattore è stata raggiunta una rimozione della domanda chimica di ossigeno solubile del 53% e nel secondo reattore del 90%, in entrambi senza l'aggiunta di co-substrati. Le comunità microbiche dei reattori, costituite da funghi e batteri, sono state caratterizzate da un'analisi metagenomica. Inoltre, è stata applicata una tecnica innovativa, la respirometria eterogenea, per valutare l'attività biologica del biofilm. I dati ottenuti sono stati utilizzati per sviluppare un modello matematico e per eseguire una caratterizzazione dei parametri cinetici e stechiometrici della biomassa. Poiché in letteratura la respirometria e la modellistica sono scarsamente applicate alle biomasse fungine è stata messa a punto una procedura respirometrica ed esperimenti dedicati. I risultati delle prove di laboratorio sono stati impiegati per la progettazione del reattore a scala pilota. Infine, dopo una fase di avviamento, il reattore a scala pilota è stato alimentato con refluo conciario contenente un'elevata concentrazione di tannini. Al termine della sperimentazione il biofilm è rimasto stabile mostrando una buona capacità di rimozione. I risultati ottenuti rappresentano il primo passo per una futura applicazione, su scala reale, di efficace trattamento biologico delle acque reflue conciarie.

RESUMEN

Los taninos son compuestos polifenólicos producidos por plantas que se utilizan en el curtido vegetal. Los taninos se diferencian de la mayoría de los fenoles naturales porque precipitan las proteínas y se usan en el proceso del curtido para unirse a las proteínas del colágeno de la piel del animal para hacer que el cuero sea más duradero. Representan una de las sustancias de baja biodegradabilidad en las aguas residuales de la curtiduría con una alta y recalcitrante demanda química de oxígeno soluble. Además en grandes concentraciones, dichos taninos pueden inhibir el tratamiento biológico. Este fragmento soluble y inflexible de los efluentes de las curtiduría generalmente se elimina mediante procesos químicos. Por tanto, un tratamiento biológico que pudiera eliminarlos eficazmente, tendría ventajas tanto ambientales como económicas. Los hongos podrían ser utilizados para el tratamiento de aguas residuales de la industria del curtido. La aplicación de un bioreactor a base de hongos que tenga un rendimiento similar en condiciones estériles y no estériles en operaciones de largo plazo sigue siendo una tarea desafiante. La presente tesis se ha centrado en desarrollar y probar técnicas para eliminar los taninos con un ecosistema diseñado a base de hongos y bacterias para su aplicación en trenes típicos de tratamiento de aguas residuales en las curtidurías. El *Aspergillus tubingensis* MUT 990 fue seleccionado como la cepa fúngica adecuada. La investigación bibliográfica y las experiencia previa se utilizó para diseñar y construir reactores a escala de laboratorio (4 litros) y piloto (1.5 m^3). La cepa fúngica se inmovilizó en portadores cúbicos y se inoculó en un nuevo reactor giratorio de lecho sumergido. La primera prueba en bioreactores se llevó a cabo para evaluar el efecto de la rotación y la adición del co-sustrato en la eliminación de los taninos. La segunda prueba se realizó de forma paralela en dos reactores de lecho sumergido fijo; el primero se alimentó con un tanino condensado (Quebracho) y el segundo con un tanino hidrolizable (Tara). Ambos se inocularon con el *Aspergillus tubingensis*. El tiempo de retención hidráulico y la concentración de taninos en el medio se fueron variando para maximizar la capacidad de eliminación y para analizar la inhibición del sustrato. Se mantuvo estable una biopelícula en el primer reactor durante 180 días de operación. Por otro lado, en el segundo reactor hubo un desprendimiento de biomasa durante la puesta en marcha y luego creció como un cultivo suspendido durante el período operativo (226 días). La eliminación de la demanda de oxígeno soluble fue hasta del 53% y del 90% y se logró en el primer y en el segundo bioreactor respectivamente sin la adición de co-sustratos. Las comunidades microbianas de los reactores formadas por hongos y bacterias se caracterizaron con un análisis de ADN. Además se aplicó la respirometría heterogénea, para evaluar la actividad biológica de la biopelícula. Los datos obtenidos se utilizaron para desarrollar un modelo matemático y realizar una caracterización cinética y estequiométrica de la biomasa. Se establecieron experimentos específicos junto con un procedimiento respirométrico para la biomasa fúngica. Los resultados a escala de laboratorio se utilizaron para diseñar un reactor a escala piloto. Finalmente, después de una fase de puesta en marcha, el reactor a escala piloto se alimentó con aguas residuales ricas en taninos recolectadas en las curtiembres. Al final del experimento, la biopelícula era estable y se había logrado un desempeño alentador. Creemos que los resultados obtenidos representan el primer paso para una futura aplicación a escala real y lograr eficientemente la eliminación biológica de los taninos en las aguas residuales de las curtidurías.

RESUM

Els tanins són polifenols produïts per plantes que s'utilitzen en l'adobat vegetal. Aquests es diferencien de la majoria dels fenols naturals perquè fan precipitar les proteïnes i s'utilitzen en el procés d'adobament per unir-se a les proteïnes del col·lagen de la pell de l'animal, fent que el cuir sigui més durador. Es tracta d'una substància de baixa biodegradabilitat, amb una elevada demanda química d'oxigen soluble. A més, la presència d'aquests tanins en grans concentracions poden inhibir el tractament biològic. Generalment aquest fragment soluble i inflexible dels efluents de les adoberies s'elimina mitjançant processos químics. Es per això que un tractament biològic que pogués eliminar-los eficaçment tindria avantatges tant ambientals com econòmics. D'aquesta manera els fongs podrien ser utilitzats al tractament d'aigües residuals d'aquest tipus d'indústria. Tot i així continua sent un desafiament important l'aplicació d'un bioreactor a base de fongs que, en operacions a llarg termini, tingui un rendiment similar en condicions estèrils i no estèrils. La present tesi s'ha centrat en desenvolupar i testejat diverses tècniques amb l'objectiu d'eliminar els tanins. Es fa mitjançant la creació d'un ecosistema dissenyat a base de fongs i bacteris. Aquest s'aplica a processos típics de tractament d'aigües residuals d'adoberies. *Aspergillus tubingensis* MUT 990 ha sigut la soca fúngica més adequada. S'han fet servir tant recerques bibliogràfiques com la pròpia experiència prèvia per dissenyar i construir reactors a escala laboratori (4 litres) i a escala pilot (1.5 m³). La soca fúngica es va immobilitzar en portadors cúbics i es va inocular en un nou reactor giratori de llit submergit. La primera prova en bioreactors es va dur a terme per avaluar l'efecte de la rotació i l'efecte de l'addició del co-substrat en l'eliminació dels tanins. La segona prova es va realitzar de forma paral·lela en dos reactors de llit submergit fix; el primer es va alimentar amb un taní condensat (Quebracho) i el segon amb un taní hidrolitzable (Tara). Tots dos es van inocular amb *Aspergillus tubingensis*. El temps de retenció hidràulic i la concentració de tanins en el medi es van anar modificant per maximitzar la capacitat d'eliminació i per analitzar la inhibició del substrat. Es va mantenir estable un biofilm en el primer reactor durant 180 dies d'operació. D'altra banda, en el segon reactor va haver un despreniment de biomassa durant la posada en marxa; després va créixer com un cultiu suspès durant el període operatiu (226 dies). L'eliminació de la demanda d'oxigen soluble va ser, sense l'addició de co-substrats, de fins el 53% i 90% en el primer i segon bioreactor respectivament. Les comunitats microbianes formades per fongs i bacteris dels reactors es van caracteritzar amb un anàlisi d'ADN. A més es va aplicar la respirometria heterogènia per avaluar l'activitat biològica del biofilm. Les dades obtingudes es van utilitzar per desenvolupar un model matemàtic i realitzar una caracterització cinètica i estequiomètrica de la biomassa. Es van establir experiments específics juntament amb un procediment respiromètric per a la biomassa fúngica. Els resultats a escala de laboratori es van utilitzar per dissenyar un reactor a escala pilot. Finalment, després d'una fase de posada en marxa, el reactor a escala pilot es va alimentar amb aigües residuals riques en tanins recollides d'adoberies. Al final de l'experiment, el biofilm era estable i s'havia aconseguit un exercici encoratjador. Creiem que els resultats obtinguts representen el primer pas per a una futura aplicació a escala real i aconseguir eficientment l'eliminació biològica de tanins en aigües residuals d'adoberies.

LIST OF ABBREVIATIONS

AcT - Tannic acid medium

ASM - Activated Sludge Models

AU – Tannase activity units

b_f - Decay coefficient of fungal biomass

b_h - Decay coefficient of heterotrophic biomass

BOD - Biological oxygen demand

BTF - Biotrickling filter

Cer2co - Research Centre for Tannery Wastewater laboratory

CFU - Colony forming units

COD - Chemical oxygen demand

CSTR - Continuous stirred tank reactor

D_f - Diffusion coefficient in the biofilm

D_w - Diffusion coefficient in water

DOC - Dissolved organic carbon

DNA - Deoxyribonucleic acid

DO - Dissolved oxygen

ECM - Extracellular matrix material

EPSs - Extracellular polymeric substances

f_{cv} - Conversion factor for VSS/COD

f_{dif} - Relative diffusivity

f_d - Fraction of inert COD generated in biomass decay

GENOCOV - Research Group on Biological Treatment and Valorisation of Liquid and Gas Effluents

GLY – Glucose and yeast medium

HRT - Hydraulic retention time

I.E. - Inhabitants equivalent

IWA - International Water Association

K_{la} - Oxygen mass transfer coefficient

K_i - half saturation constant for inhibition compound for heterotrophic biomass

K_{iq} - half saturation constant for inhibition compound for fungal biomass

K_s - Affinity constant of heterotrophic biomass

K_{sf} - Affinity constant of fungal biomass

MEA - Malt Extract Agar

MBfR – Membrane biofilm reactor

MIC - Minimum inhibitory concentration

MUT – Mycoteca Universitatis Taurinensis

MW - Molasses from wastewater distillery

OMW - Olive mill wastewater

OLR - Volumetric organic loading rate

ORR - Volumetric organic removal rate

OTUs - Operational taxonomic units

OUR - Oxygen uptake rate

OUR_{end} - Endogenous OUR

OUR_{ex} - Exogenous OUR

pCOD – Particulate COD

PLC - Programmable logic controller

PUF - Polyurethane foam

QQM - Quorum quenching molecule

QSM - Quorum sensing molecules

QT – Quebracho tannin

R1 – First bioreactor in preliminary tests with continuous operation

R2 - Second bioreactor in preliminary tests with continuous operation

R3 - Third bioreactor in preliminary tests with continuous operation

R4 - Fourth bioreactor in preliminary tests with continuous operation

RQ - Bioreactor fed with QT medium in long-term tests with continuous operation

RQ-28h - Sample collected from RQ corresponding to an HRT of 28 hours

RT - Bioreactor fed with TT medium in long-term tests with continuous operation

RT-9h – Sample collected from RT corresponding to an HRT of 9 hours

RT-28h - Sample collected from RT corresponding to an HRT of 28 hours

RE - Removal efficiency

RNA - Ribonucleic acid

sbCOD – Biodegradable sCOD

snbCOD – Non-biodegradable sCOD

sCOD – Soluble COD

SRT - Solid retention time

S/X - Substrate/biomass ratio

TA – Tannic acid

TOC - Total organic carbon

TSS - Total suspended solids

TT - Tara tannin

VFA -Volatile fatty acid

VSS - Volatile suspended solids

WA - Wastewater with agar

WWTP - Wastewater treatment plant

X_h - Heterotrophic biomass concentration

Y_f - Growth yield of fungal biomass

Y_h - Growth yield of heterotrophic biomass

Y_{obs} - Observed growth yield

μ_h - Maximum specific growth rate of heterotrophic biomass

μ_f - Maximum specific growth rate of fungal biomass

A modified version of some chapters of this thesis are in preparation or submitted or published in scientific journals as:

- STABILITY OF FUNGAL BIOFILMS AND REMOVAL EFFICIENCY OF QUEBRACHO AND TARA TANNINS IN BIOREACTORS CONTINUOUSLY FED UNDER NON-STERILE CONDITIONS
  F. Spennati, M. Mora, V. Tigini, S. La China, S. Di Gregorio, D. Gabriel, G. Munz.
  Journal of environmental management (submitted).

- THE ROLE OF CO-SUBSTRATE AND MIXING ON FUNGAL BIOFILM EFFICIENCY IN THE REMOVAL OF TANNINS
  F. Spennati, A. Ricotti, G. Mori, G. Siracusa, S. Becarelli, S. Di Gregorio, V. Tigini, G.C. Varese, G. Munz.
  Frontiers in environmental sciences (in preparation).

- KINETICS AND STOCHIOMETRIC CHARACTERISATION OF A LAB-SCALE FUNGAL BIOFILM BASED OF RESPIROMETRY AND MODELLING
  F. Spennati, M. Mora, V. Tigini, G. Munz, D. Gabriel.
  Environmental technology (in preparation).

- PILOT SCALE FUNGAL BIOREACTOR FOR TANNERY WASTEWATER TREATMENT: ANALYSIS OF THE PERFORMANCE AND MICROBIAL CONSORTIUM CHARACTERISATION
  F. Spennati, S. La China, G. Siracusa, S. Di Gregorio, V. Tigini, G. Mori, D. Gabriel, G. Munz.
  Water research (in preparation).

- TANNERY MIXED LIQUORS FROM AN ECOTOXICOLOGICAL AND MYCOLOGICAL POINT OF VIEW: RISKS VS POTENTIAL BIODEGRADATION APPLICATION
  V. Tigini, F. Bevione, V. Prigione, A. Poli, L. Ranieri, F. Spennati, G. Munz, G. C. Varese.
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XVIII

1   MOTIVATION, OBJECTIVES AND THESIS OVERVIEW

1.1   MOTIVATION AND OBJECTIVES

The research for the thesis was done in the Department of Civil and Environmental Engineering of the University of Florence, and in the Department of Chemical, Biological and Environmental Engineering of the Autonomous University of Barcelona. The thesis was jointly supervised by the University of Florence, the University of Pisa, the University of Perugia and the Autonomous University of Barcelona. The main activities were carried out in the laboratories of the Research Group on Biological Treatment and Valorisation of Liquid and Gas Effluents (GENOCOV) and the private/public joint Research Centre for Tannery Wastewater laboratory (Cer2co) inside the Consorzio Cuoiodepur wastewater treatment plant (WWTP) located in San Romano-Pisa (Tuscan tannery district), that treats vegetable tannery wastewater. The main partners of Cer2co were the University of Florence and Cuoiodepur. The research activity was within the research thread of the project MIUR FIR RBFR13V3CH financed by Italian Ministry of Education, Universities and Research and the project Lightan POR FESR 2014 – 2020 financed by the Region of Tuscany thanks to the European Regional Development Fund.

In municipal and industrial wastewater treatment plants, core treatments rely on engineered ecosystems: the activated sludge consists of microbial communities, predominantly bacteria, selected by environmental and operating conditions. Bacteria are effective in the degradation of pollutants and the removal of nutrients. In recent years, there have been growing concerns about recalcitrant compounds in industrial, but also municipal, wastewaters. Even though bacteria are effective in the removal of most of the organic compounds from municipal wastewater, industrial wastewater contains compounds, many chemicals, pharmaceuticals and, in tannery wastewater, tannins that are not biodegradable through the action of bacteria. Usually these compounds are removed through chemical treatments with high economic and environmental costs related to the consumption of reagents and energy. Many recalcitrant compounds, such as antibiotics and pesticides, however, could be

efficiently removed by fungi, which the scientific community has thoroughly investigated and whose potential has been demonstrated. Moreover, some fungal strains, such as *Aspergillus spp.* and *Penicillium spp.*, have been found in tannery wastewaters. However, the application of these biotechnologies has up to now been used very little in wastewater treatment, and the main issue is how to engineer an ecosystem composed of fungi and bacteria in the context of wastewater treatment. In long-term operation under non-sterile conditions fungi were usually outcompeted by bacteria. Nevertheless, a combined ecosystem of fungi and bacteria is interesting due to its potential synergies: fungi could reduce the general toxicity of the effluent and break the chemical bonds of recalcitrant compounds, while bacteria could bring these compounds to a final mineralisation.

The objective was the development and the test in long-term operation under non-sterile conditions of fungal bioreactors and the evaluation of recalcitrant compound degradation. Tannins (polyphenolic compounds produced by plants and used in the industrial vegetable tanning of leather) were chosen as target recalcitrant compounds, since they represent one of the low biodegradability components in tannery wastewaters with high recalcitrant soluble chemical oxygen demand (COD). Furthermore, high concentration of tannins can inhibit biological treatment. This soluble recalcitrant component of tannery effluents is usually removed by means of chemical processes, but the development of a biological treatment to effectively remove this component could lead to an environmental and economic advantage.

In this study, the reactor configurations and operating conditions were chosen and designed to create a favourable environment for fungi. Different operating conditions were tested and advanced molecular biology techniques, based on the sequencing of the genetic material present in the reactors, were used to identify and monitor the microbial populations (bacteria and fungi). Chemical and biological analyses were supported by respirometry. The experimental data were used to perform a kinetic and stoichiometric characterisation of the biomass. This work has allowed us to deepen our knowledge and optimise the biological removal of tannins (lab-scale and pilot-scale) that could be applied in a typical tannery wastewater treatment process, which is the main objective of many research projects.

1.2   THESIS OVERVIEW

In this first chapter of the thesis, the motivations, objectives and an overview of the thesis are presented. chapter 2 contains the general introduction with information about the leather tannery industry, tannery wastewater, tannins, fungi, respirometry and modelling. This information also provides the background needed to understand the requirements and objectives of the thesis and to facilitate understanding of the subsequent chapters by providing an explanation of many basic topics and concepts. Chapter 3 contains the Materials and Methods employed with a description of the equipment used (respirometers, batch set up, lab-scale bioreactors and pilot-scale bioreactors). Chapter 3 also describes the process operations of the lab-scale tests and pilot-scale tests performed to verify the hypothesis. Chapter 4 contains a discussion of the fungal strain selection and the test in continuous reactors. Lab-scale tests were designed and performed in the Cer2co and UAB laboratories. The first and second lab-scale tests serve to analyse the selective pressures (HRT, pH set point, load, co-substrate and rotation) applied in the novel reactor that were able to maintain the stability of the fungal engineered ecosystem and its removal capacity. In chapter 5, the results of the respirometric tests performed at the Cer2co and UAB laboratories are presented. The data obtained were used to perform a kinetic and stoichiometric characterisation of the biomass: this characterisation was performed with the Monod kinetics implemented in Aquasim software. The modelling results are presented in the same chapter. Chapter 6 describes the start-up of the pilot-scale reactor. The pilot reactor was created by scaling up continuous reactors, and the selective pressures were chosen via the results of the lab-scale tests. The pilot reactor successfully treated a real effluent, rich in natural tannins, from a tannery factory. Finally, chapter 7 summarises all of the results, presenting the conclusions and suggesting guidelines for future studies.

2   INTRODUCTION

2.1   LEATHER TANNING

2.1.1   Leather tanning process and the tannery industry

Leather tanning is one of the oldest crafts practiced by mankind. Primitive humans discovered that animal hides could be treated to become leather: a durable and flexible material suitable as protection against atmospheric agents and useful for a wide range of purposes. Tanning is the process used to obtain leather from animal skins, and vegetable tannins were the most used tanning agent for centuries (the etymology of both names is probably related to the Celtic name for "oak tree"). In the last century, tanning has been practiced on an industrial scale thanks to technological improvements such as the leather tanning drum. Leather production has been part of a wider sector. In fact, the raw hides are by-products of the meat industry. Despite the development of numerous synthetic fabrics, leather remains indispensable in many applications because of its distinctive properties: toughness, non-flammability, resistance to heat, low permeability to water, and permeability to air and water vapour. Nowadays leather is used to make various goods including clothing and furniture coverings. With a range of techniques, it is possible to obtain a wide variety of types and styles. The tanning process is composed of several sub-processes that could be summarised in three steps: pre-treatment of raw animal hides (beam house operation), treatment with a tanning agent, and finishing operations before transport to product manufacturers (Lofrano et al., 2013). In the slaughterhouses, the raw hides undergo a preliminary treatment: preservation by means of salting or drying to avoid deterioration during transport. The processing within the tannery industry is generally composed of six main operations, most of which are performed inside a rotating drum, as shown in Figure 2.1: soaking and liming, unhairing, fleshing, pre-tanning, tanning, and drying and finishing operations (Giaccherini, 2016).

1. Soaking and liming: in this stage, the raw hide regains its normal water content with a high discharge of COD, chloride and suspended solids load.
2. Unhairing: hair and epidermis are removed by chemical dissolution through alkaline medium and sulphide. The wastewater from this step is full of

sulphur compounds, proteins and fats.

3.   Fleshing and trimming: removal of non-structural components of the hides through mechanical operation, digestive enzymes and inorganic acids.

4.   Pre-tanning: this operation is commonly divided in two sub-processes:

Bating: pH neutralising, and removal of residual fat and hair using acid ammonium salts and enzymes.

Pickling: increasing the acidity of the hide to a pH of 3 to prepare it for the tanning phase. Addition of fungicides and bactericides.

5.   Tanning: tanning agents are added in this phase. The most common processes are chrome tanning or vegetable tanning.

6.   Drying and finishing operations: the leather is dried and treated with dye
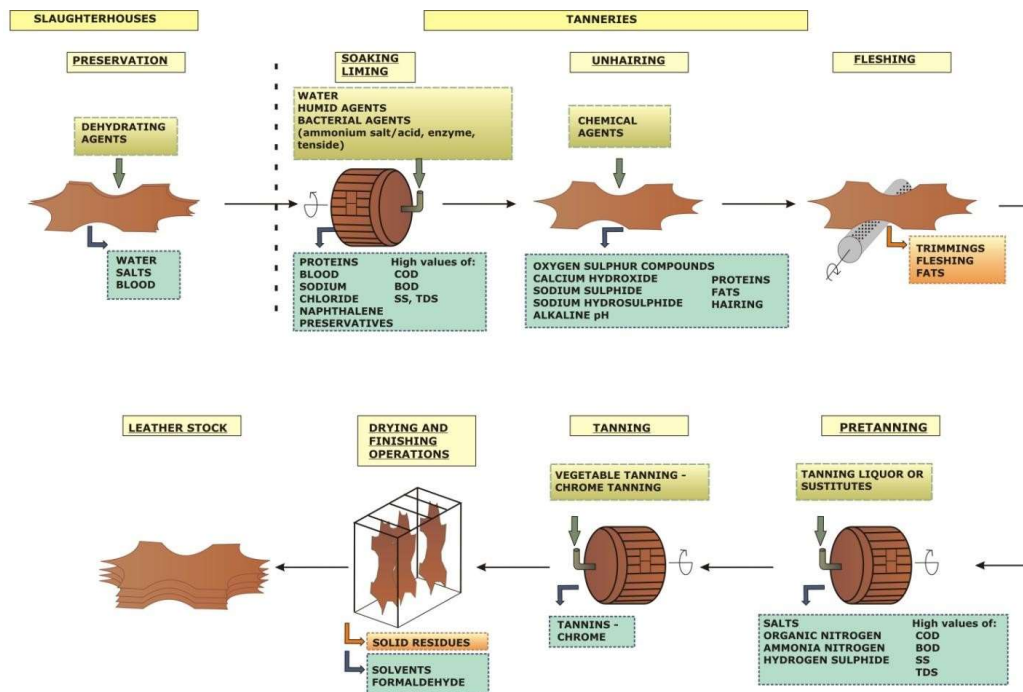
and fat to obtain the proper filling, smoothness and colour.

Figure 2.1: Tanning processes scheme (Giaccherini, 2016).

Historically leather was tanned with tannins extracted from wood, leaves, fruits, and roots. The phenolic groups of tannins create hydrogen bonding with collagen proteins, and the colour obtained depends also on the mix of tannins applied. Chrome tanning was invented in 1858. The process is based on the addition of chromium salts (chrome alum and chromium (III) sulphate) when the pH is low: the chromium ions bond with the free carboxyl groups in the collagen through a cross-linking reaction. For the last

hundred years, chrome-tanning has been the most widely used method due to the increased speed and the pliability and high quality of the leather obtained. It is currently the most used tanning process, and the product obtained is called wet-blue due to the colouration. Recently, efforts have been made into researching environmental friendly alternatives to chrome with the introduction of new products such as synthetic tannins. Vegetable tanning is one of the most interesting alternatives, and vegetable-tanned leather has increasingly been used in the footwear industry, by automobile manufacturers and for other products. The raw hides originate mostly as by-products of the food industry: bovine, sheep and goat are most commonly exploited for leathers, but other animal skins are also processed in the tanning industry. It has been estimated that the majority of all leather comes from bovine material and that 70% of the hides are produced in Latin America and the Far East. Table 2.1 reports the data sets on leather production in 2011 gathered from UN-FAO.

Table 2.1: Global leather production, 2011 (UN-FAO, 2013).

Country	Bovine Leather Production (10^3 tonnes per	Sheep and Goat Leather Production
Latin America	110	16
Africa	5	49
Near East	22	98
Far East	285	225
North America	21	6
Europe	71	73
Rest of Europe	0.4	1
Area Former	38	22
Oceania	3	6
Developed Other	6	0.3
World	562	496

In the near future, an increase in demand for meat has been estimated due to the World population and the general wealth growth (FAO, 2002); consequently the raw hide supply will continue to grow (about 3% each year) in step with the population, but the environmental costs related to raising and feeding the cattle at some point could change

this trend and favour other sources of raw hides. It should be noted that the main bovine breeder countries do not always correspond with the main leather producers: China, India, Italy, Brazil, Russia, Argentina, United States and Turkey (Figure 2.2). Nevertheless, the environmental and economic impact of leather production in each country could be better represented by comparing the production with the population and the land use. As represented in Figure 2.3, leather production in Italy is significant with an estimated 225 kg of leather produced per person and 146 tonnes of leather per km^2 (Giaccherini et al., 2017). It is important to underline that in the last century, following developments in materials science, leather has been substituted in some fields by synthetic materials able to perform better than leather in one or more selected properties. Ski boots are a clear example: in 1960 these were made with leather, while nowadays they are made with plastic materials. Even though in some cases leather could not compete with new materials developed during recent decades, it still represents an attractive and flexible product and presents the advantage of renewability because the hides are a by-product generated from the food industry. The uses of leather have changed and continue to change: nowadays designers in the fashion industry and other sectors appreciate the value of leather and are prone to adopt it in their products. In the global trade market, the value of leather and leather products has been estimated at approximately $100 billion USD per year. The leather sector plays an important role in the world economy, and further growth is expected in line with related markets such as the luxury market (UNIDO, 2010).
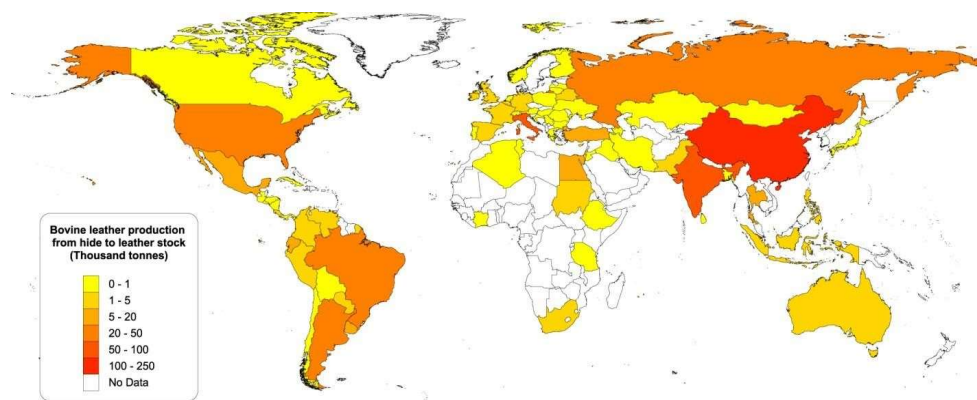
Figure 2.2: Global map of the bovine leather production (from hide to leather stock) in thousands of tonnes per year (Giaccherini et al., 2017).
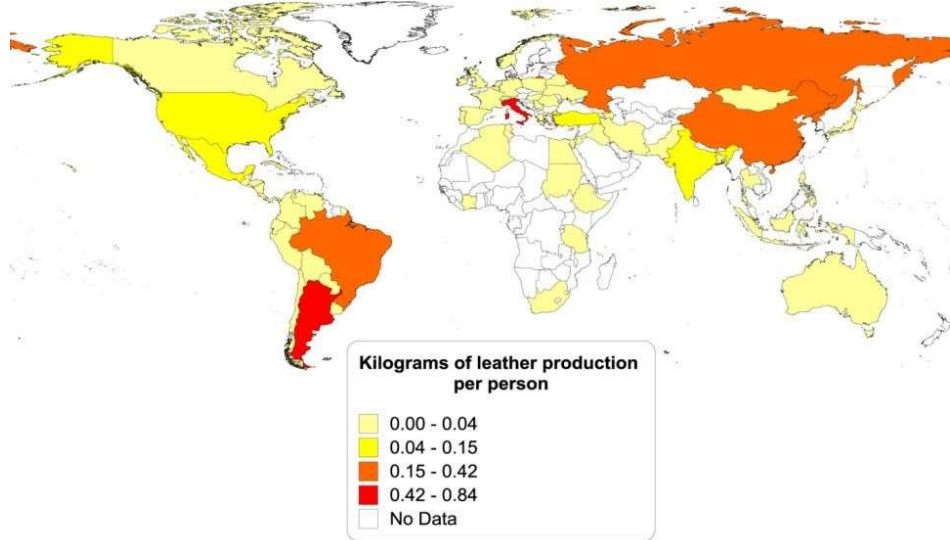
Figure 2.3: Map of the bovine leather production per capita for the 10 largest leather producers (Giaccherini et al., 2017).

2.1.2 Tannery wastewater and treatment

Although the leather tanning industry is known to be of prime economic importance in many countries, there has been increasing concern about the environmental impacts related to the production of leather, namely the related energy consumption, waste and emissions (UNIDO, 2012), and the release of various recalcitrant pollutants in tannery wastewaters (Lofrano et al., 2013; Romer et al., 2011). Tannins represent one of the refractory groups of chemicals in tannery effluents (G. Munz et al., 2009). Tannery wastewater is a complex mixture of organic and inorganic pollutants that is generally characterised by a high concentration of COD, Total suspended solids (TSS) and salt content (due to the sodium chloride from hide and skin preservation and sulphur compounds applied in the treatment). Tannery wastewater is rich in nitrogen (mainly organic nitrogen), but very poor in phosphorous. The raw hide origins, the chemicals used, and the process conditions, among other factors, including any mixing with municipality wastewater, strongly affect the tannery wastewater composition and characteristics as well as the temporal and spatial variability. Nevertheless, Table 2.2 reports an average mass balance of the tanning

process in which it has been estimated that the process generates 0.7-0.6 kg of solid waste (as fleshing) and 25-45 L of wastewater from 1 kg of raw hide. Typically the solid waste (fleshing and tannery sludge) is separated and sent to land management (Giaccherini et al., 2017) or subjected to lipid and protein recovery (in the Tuscany region, the fleshing is sent to S.G.S. Consortium).

Table 2.2: Leather tanning mass balance (UNIDO, 2000).

Component	Input (kg)	Output (kg)
Wet salted raw hide	1,000	0
Liquid-based		
Process water	16,700	16,000
Sodium chloride NaCl	-	200
Calcium hydroxide $Ca(OH)_2$	40	40
Sodium sulphide Na_2S	25	25
Ammonium salts	17	17
Enzyme	3	3
Solid-based		
Fleshing	-	300
Trimming	-	100
Unusable pelt	-	800
Finished product	-	300

In vegetable tanning, the tannins are dissolved in high concentrations in water and added in the rotating tanning drum to favour the reaction with the treated skins. The tannin-rich effluent of the drum is mixed with other effluents and so becomes a relevant fraction of the final tannery wastewater as shown in Table 2.3. The strategies to abate the pollution caused by leather production include the following (Lofrano et al., 2013; UNIDO, 2011):

- organisation of tanneries into industrial districts to exploit economies of scale of central treatment plants: this approach is widely adopted;
- segregation of waste streams with separated treatment in accordance with their specific characteristics: this approach is not widely applied;

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- evaluation of the products and leather technologies applied and replacement with less impactful solutions (salt-free preserved raw hides and skins, hair-save liming, low-ammonia or ammonia-free deliming and bating, advanced chrome management systems, etc.) in order to prevent pollution and reduce the overall cost of treatment: this approach is not widely applied;
- introduction of promising emerging technologies into conventional treatment (advanced oxidation processes, membrane technologies, water reuse, etc.): the literature demonstrates that efforts are ongoing, but that further are studies needed to reach an economically sustainable real-world application of these technologies.

Table 2.3: Vegetable tanning effluent composition (Mannucci et al., 2010).

Parameter	Units	Value	
		min	max
COD	mg L^{-1}	900	19,400
TSS	mg L^{-1}	660	16,144
pH	mg L^{-1}	7	8.5
N org	mg L^{-1}	360	-
NH$_4$	mg L^{-1}	102	400
Phosphorus	mg L^{-1}	8	40
Sulphate	mg L^{-1}	180	6,000
COD/SO$_4^{2-}$		1	33.8
Sulphide	mg L^{-1}	3	287
Alkalinity	mg CaCO$_3$ L^{-1}	350	5,000
Tannins	mg L^{-1}	600	2,900
Chloride	mg L^{-1}	353	8,400
VFA	mg L^{-1}	63	486

The conventional treatment train of tannery wastewater is a multistage process aimed at fulfilling the legal requirements. Generally, the core treatment of a tannery WWTP is a common activated sludge process, but the design of the plant is always tailored to

the requirements of a specific site. A general overview of tannery wastewater treatment is represented in Figure 2.4 and summarised below:

- Preliminary treatment focused on the removal of large particles, sand/grit and grease as well as chrome and sulphides;
- Physical-chemical treatment (primary) aimed at removing the settleable organic and inorganic solids;
- Biological treatment (secondary) aimed at removing nitrogen and organic matter using aerobic biological treatment processes. In some cases, phosphorus is added to the wastewater to avoid limitation in biological kinetics;
- Advanced (tertiary) treatment aimed at removing high COD due to the soluble recalcitrant compounds via the Fenton process or ozonation;
- Sludge handling and disposal, where the main process is dewatering. Sometimes sludges have a high concentration of chromium that hinders their application in agriculture as fertiliser;
- Odours and gaseous effluent treatment. Usually the main source of bad odours is the stripping of hydrogen sulphide (H_2S) in the first part of WWTP, which requires intensive aeration (sometimes it is necessary to add hydrogen peroxide or pure oxygen). Gas treatment facilities, such as chemical scrubbers, collect and treat the gaseous effluent from the tanks before release into the atmosphere.
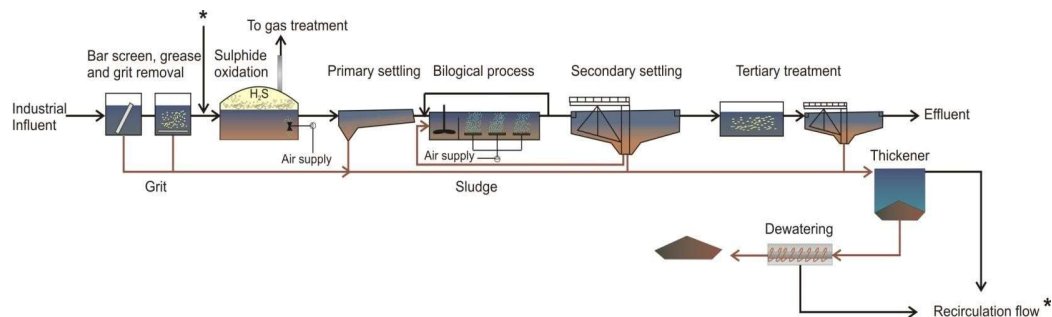
Figure 2.4: Tannery wastewater treatment process scheme (Giaccherini, 2016).

2.1.3 Tuscany tannery district and Cuoiodepur WWTP

There are about 1,400 tanneries in Italy distributed throughout four regions: Campania, Lombardy, Tuscany, and Veneto. Their output made up 17% of total world trade and 62% of the European Union total (Lofrano et al., 2013). Italian tanneries import raw materials from foreign counties, but they are close to the leather processing industries, such as the European fashion houses. Italy's leather sector is a dominant player in the international leather market thanks to tradition and outstanding skill as well as operational synergies in specialised clusters for fashion markets. However, in recent years the Italian tanneries have suffered a decline due to emerging international competitors. To overcome this challenging situation, Italy's leather sector must invest in research efforts to improve the efficiency of the process and offer high quality at affordable prices, while at the same time solving the emerging environmental issues related to leather production. (The environmental regulations in Europe became stricter with the adoption of EU Water Framework Directive EC 32/2008 and the Registration, Evaluation, Authorisation and Restriction of Chemical substances REACH Act, EC 1907/2006). Among the chemicals applied as tanning agents, vegetable tanning is likely to gain ground on non-chrome tanning methods, due to the environmental concerns of the latter and certain advantages of the former (UNIDO, 2010). Vegetable tanning has become quite popular with footwear, automobile manufacturers and other products. Italy is one of the three largest traditional footwear producers (in terms of product value), and Italian leather is widely used in luxury market products such as automotive upholstery. However, as previously noted, the sector is under pressure, and Italy's footwear output declined to 242 million pairs in 2007 (UNIDO, 2010). The Tuscan tannery district is the second largest in Europe, and its production consists of 35% of Italian leather production. The district is located in an area between Pisa and Florence (San Miniato, San Romano, Santa Croce, Montopoli Val d'Arno, San Donato and Ponte a Egola) as shown in Figure 2.5. The peculiarity of the district is its division into two different sectors or sub-districts where chrome and vegetable tanning processes are operated separately. The Cuoiodepur WWTP (San Miniato, PI, Italy) treats vegetable tanning wastewater from the tanneries located in the southern part of the district (mainly located near San Miniato and Montopoli Val d'Arno), but also treats the municipal wastewater of the area

surrounding San Romano, San Donato, San Miniato Basso and Ponte a Egola. The size of the plant is significant from the point of view of both organic load and the influent flow (850,000 inhabitants equivalent (I.E.), 130 g COD I.E. d^{-1}). In 2016, Cuoiodepur treated 1.6 million m^3 year^{-1} of tannery wastewater and 1.3 million m^3 year^{-1} of municipality wastewater. The treatment scheme of Cuoiodepur WWTP is reported in Figure 2.6. The plant has a complex chain of treatment: biological and chemical treatments in the water line as well as scrubbers to remove the hydrogen sulphide from gaseous effluents. The treatment train of Cuoiodepur WWTP for industrial wastewater was composed of conventional pre-treatment, an equalisation in two circular tanks to attenuate fluctuation and promote sulphide oxidation with pure oxygen, followed by a primary settling. A biological treatment with a denitrification process is the first step, which is followed by an oxidation-nitrification reactor, a secondary settling and final a tertiary treatment that includes flocculation with ferrous chloride (FeCl$_2$) and polyelectrolytes at basic pH (the pH of the final effluent is neutralised before discharge). The average temperature is 20-23°C (range 19°C to 35°C), and the hydraulic retention time (HRT) of biological section is about 3 days, while the solid retention time (SRT) is usually between 50 and 70 days.
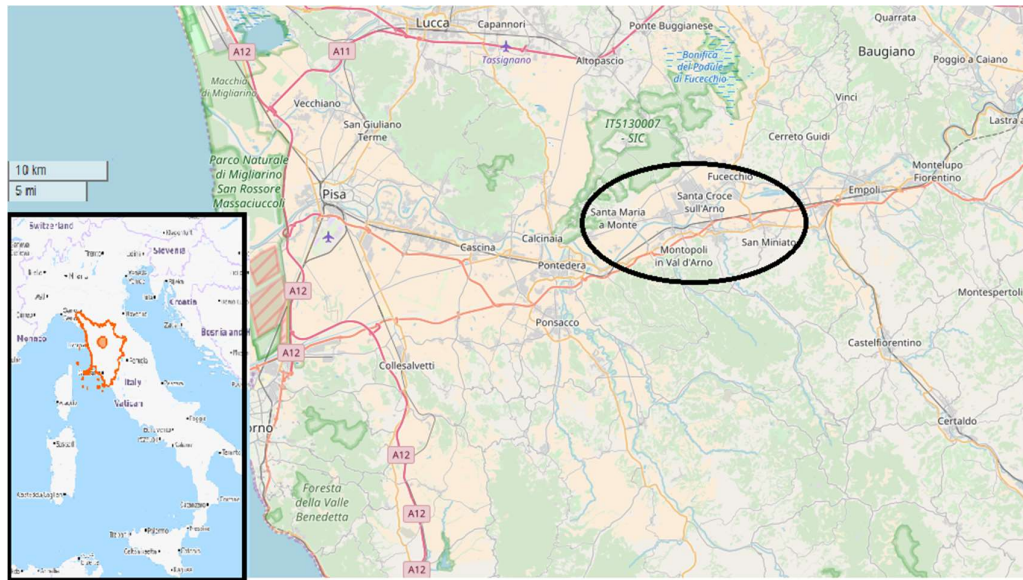
Figure 2.5: Tuscany tannery district georeferencing (from www.openstreetmap.org).
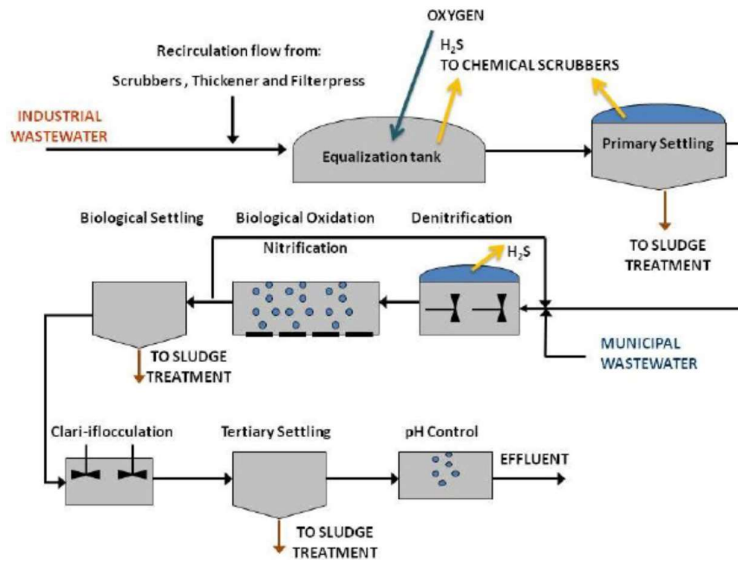
Figure 2.6: Cuoiodepur WWTP plant treatment scheme (Giaccherini, 2016).

The reactors of the primary line are covered, and the gaseous effluents are treated to avoid odour emissions due to the sulphide stripping as H_2S. The main aim of Cuoiodepur WWTP is the removal of nutrients and organic carbon from the complex influent wastewater (Table 2.4), where the tannins contribute to a high soluble recalcitrant COD.

Table 2.4: Industrial wastewater influent in Cuoiodepur WWTP (year 2016 – data kindly provided by Cuoiodepur).

Parameter	Average	Max	Min	STD
pH	**7.8**	12.6	6.2	1.2
COD_{total} (mg L^{-1})	**12,639**	19,560	3,480	4,836
sCOD (mg L^{-1})	**6,435**	12,360	1,926	2,628
TSS (mg L^{-1})	**5,388**	10,370	940	2,312
$N-NH_4^+$ (mg L^{-1})	**378**	582	83	109
N-total (mg L^{-1})	**831**	1,648	568	302
P-total (mg L^{-1})	**45.1**	62.3	20.4	23.1
Sulphides (mg L^{-1})	**295**	525	44	136
Sulphates (mg L^{-1})	**2,581**	4,111	888	692
Chlorides (mg L^{-1})	**5,860**	13,125	2,210	1,841
Cr-total (mg L^{-1})	**45**	101	19	21

The Cuoiodepur WWTP configuration is closest to the typical one described in the previous paragraph and provides an effective treatment of the tannery wastewater produced by the district. Nevertheless, the actual process presents some drawbacks that may justify the need for changes in the future:

- the production of pure oxygen has an important influence in the overall environmental and economic cost of the WWTP. This requires research into sustainable alternatives for H_2S removal with respect to the currently employed expensive chemical scrubbers;

- the sludge is used to produce fertilisers, but strengthened legal requirements could prompt the need for alternative sludge management in the near future;

- the tertiary treatment is focused on the removal of the high recalcitrant soluble chemical oxygen demand (sCOD) (approximately 500 mg L^{-1}, mainly related to tannin content), with a substantial consumption of chemicals and consequent environmental impact. An innovative and effective biological process could reduce this final section of the WWTP.

2.2   TANNINS

2.2.1   Introduction to tannins

Tannins are the most abundant and widely distributed polyphenolic compounds in nature. They are produced by plants (pteridophytes, gymnosperms and angiosperms) and are accumulated in leaves, fruit, bark, roots, seeds and wood. They usually account for the dry weight of tree leaves in the range of 5% to 10%. Tannins provide advantages to plants as deterrents from predation, aiding pathogen resistance and protecting tissues against decay (Bhat et al., 1998). These compounds have a range of effects on various organisms, ranging from toxic effects on animals to growth inhibition of microorganisms (Ayres et al., 1997; Barbehenn and Peter Constabel, 2011). Tannins differ from other natural phenols in their ability to precipitate proteins (Lorenz et al., 2014), and they are used in the tanning process to bind to the collagen

matrix of animal skin and make the leather more durable and resistant to decay. Plants produce tannins as a defence mechanism, with this defence involve different processes:

- protein binding and consequent enzyme inhibition and/or substrate deprivation;

- formation of metal ion complexes and consequent reduction of the bioavailability of vitamins and minerals;

- Reaction with and creation of reactive oxygen species;

- Interference with biochemical processes at cell membranes.

Herbivores show a different sensitivity to each type of tannin, which can have different effects on different herbivores (Ayres et al., 1997). Tannin toxicity in insects is related to the formation of free-radicals in the high-pH environment of insect guts, while tannins affect the digestion of proteins in vertebrate herbivores (Barbehenn and Peter Constabel, 2011). In general, the biochemical activity is the result of the interaction between the herbivore gut physiology and the tannin structures. Natural tannins can be subdivided into hydrolysable (gallotannins and ellagitannins), condensed (or proanthocyanidins), and complex tannins (Khanbabaee and Ree, 2001) as shown in Figure 2.7. Hydrolysable tannins are polyesters of gallic acid and various sugars (Figure 2.8). Condensed tannins are polymers of flavonoids (like catechin) connected by carbon linkages and are the most resistant to microbial attack (Figure 2.9). There are also tannins consisting of combinations of these two structures (McSweeney et al., 2001). However, many microorganisms are able to overcome this defence through different detoxification mechanisms, such as the production of tannin-resistant enzymes or tannin-binding polymers. Some microorganism are also able to oxidise tannins or perform a degradation of tannins for use as a sole carbon source. The degradation of hydrolysable tannins, particularly gallotannins, is well understood (Mingshu et al., 2006), but the degradation pathways of the other tannins are less well defined, requiring further study. Tannins are usually defined as water soluble polyphenolic compounds with high molecular mass characterised by the ability to precipitate proteins; however, their identification and measurement is rather complex (Pasch et al., 2001; Schofield et al., 2001). In recent years, tannins have been studied

not only to develop a better understanding of their ecological role (Barbehenn and Peter Constabel, 2011), but also with regard to their physical and chemical proprieties (Pizzi, 2006; Tondi et al., 2009) and biological activity. There are still more 1,000 natural products, however, that have not been adequately defined and classified (Khanbabaee and Ree, 2001).
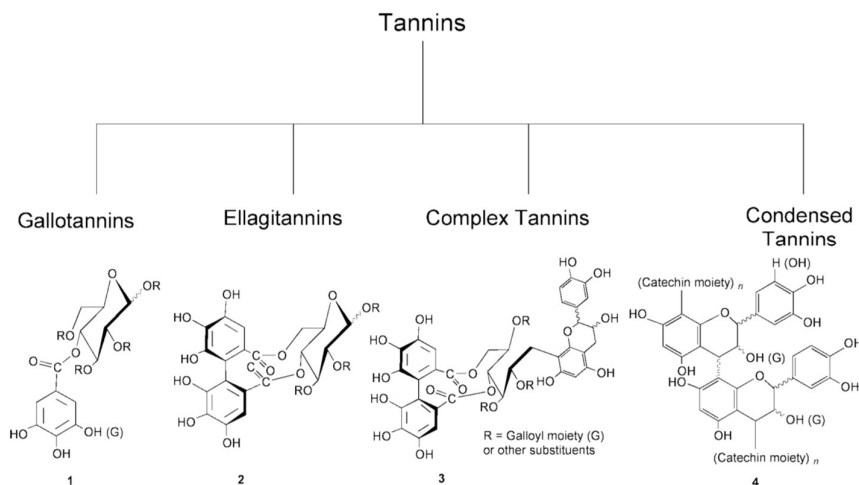
Figure 2.7: Classification of tannins (Khanbabaee and Ree, 2001).
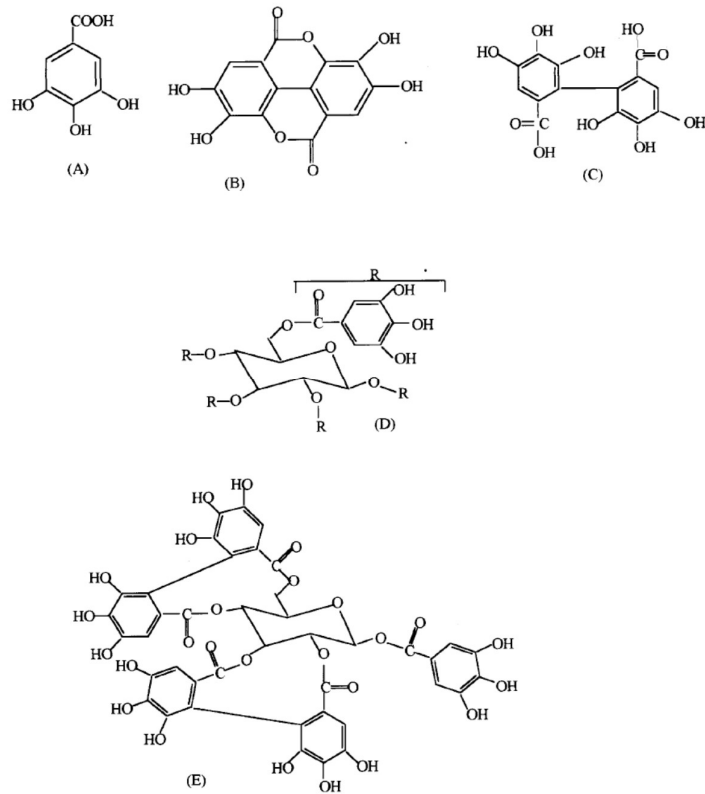
Figure 2.8: Structure of (A) gallic acid, (B) ellagic acid, (C) hexahydroxydiphenic acid, (D) gallotannin and (E) ellagitannin (Bhat et al., 1998).
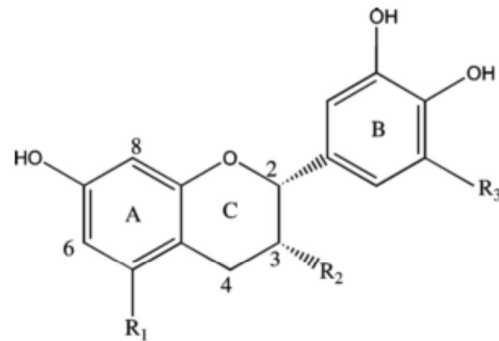
R_1	R_3	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Figure 2.9: The basic repeating unit in condensed tannins (Schofield et al., 2001).

2.2.2 Tannins in the leather tannery industry

Tanning is an essential phase in one of civilisation's oldest processes, the transformation of skins into leather, and vegetable tannins were probably the earliest used reagents. Tannins are applied in the leather tanning industry to transform the animal skin, making leather more durable and resistant to decay. Tannins used in the tanning processes are polyphenolic compounds with elevated molecular weight (from 500 to 4000 Da). The most used tannins in the leather industry are Quebracho (*Schinopsis spp*.), Wattle (*Mimosa spp.*), Chestnut (*Castanea spp.*) and Tara (*Cæsalpinia spp.*). Tannins represent one of the low biodegradability substances present in tannery wastewater, and a high concentration of tannins can inhibit biological treatment (Mannucci et al., 2010; G. Munz et al., 2009; Tilli et al., 2010). Moreover, the toxicity on Sea urchin embryogenesis of one of the vegetable tannins used (*Mimosa spp.*) was shown have effects at levels ≥ 1 mg L^{-1} (De Nicola et al., 2007). Tara tannin (TT) is obtained from the fruit pods of *Cæsalpinia spp*., and the principal components are hydrolysable tannins based on a galloylated quinic acid structure (Sciences et al., 2007). Quebracho tannin (QT) is obtained from the wood of *Schinopsis spp*. and is composed mainly of condensed tannins; it produces a more pronounced inhibition than TT (Nelson et al., 1997). In fact, QT has also been suggested as a potential antifouling agent (Bellotti et al., 2012). In the present study, as described in further detail in the sections below, TT has been used as a representative of hydrolysable tannins and QT as a representative of condensed tannins, among the natural tannins applied as tanning agents. Hydrolysable tannins are more easily biodegraded than condensed tannins (He et al., 2008). Tannins are part of the soluble recalcitrant fraction of tannery effluents with a high soluble COD, which could also interfere with nitrification and with anaerobic digestion (Mannucci et al., 2010; G. Munz et al., 2009). This is usually removed by means of chemical processes (Lofrano et al., 2013), since conventional activated sludge systems (Li et al., 2009) and anaerobic digestion (Mannucci et al., 2010) are not effective. Alternative biological treatment processes able to effectively remove this recalcitrant organic fraction could lead to environmental and economic advantages (Giaccherini et al., 2017).

2.3   FUNGI

2.3.1   Introduction to fungi

The universal phylogenetic tree of life is divided into three domains: Bacteria (prokaryotic), Archaea (prokaryotic) and Eukarya (eukaryotic). Plants, animals and fungi are the major multicellular organisms of Eukarya, and these three kingdoms diverged from one another roughly half a billion years ago (Woese, 2000). Fungi are abundant worldwide, because they can exist and survive in a wide range of habitats. Within their ecosystems, fungi might be symbionts, parasites or saprobes of plants, animals and other fungi. They play an important ecological role in the decomposition of organic matter and in the cycle of nutrients. The fungi kingdom is divided into five phyla (Chytridiomycota, Zygomycota, Glomeromycota, Ascomycota and Basidiomycota) and includes moulds, mushrooms and yeasts. Even though less than 75,000 species have been classified, there are estimated to be at least 1.5 million different species of fungi (Hawksworth, 2001). All fungi have a range of features that separate them from other organisms (Deacon, 2006):

- All fungi are eukaryotic. In fact, they have a membrane-bound nucleus containing chromosomes, and they also have cytoplasmic organelles.
- Fungi are heterotrophs (chemo-organotrophs). In other words, they need organic compounds as energy and a carbon source for cellular synthesis. They are specialised in the production of enzymes and exoenzymes able to depolymerise complex organic structures for the uptake of released nutrients through the membrane.
- Generally, fungi grow as filaments called hyphae (singular: hypha), but they do not grow like other filamentous organisms. Instead, they grow by continuously extending their extreme tips as a chain of cells (apical growth). The hyphae may grow linearly or by branching. The hyphae network is called mycelium; these structures allow fungi to explore and reach into fresh zones of substrate. Protoplasm and other substances are continuously moving into the hyphae from the older regions to the newest one. Some fungi, such as yeast,

can grow as two shapes (hyphal growth or budding); these are referred to as dimorphic fungi.

- A distinguishing feature of the fungal cell wall is the inclusion of chitin and glucans;

- Fungi typically have haploid nuclei. However, fungal hyphae often have several nuclei within each hyphal compartment. Many budding yeasts are diploid.

- Fungi can generally reproduce by asexual and sexual means and produce spores. Spores allow dispersion in the environment and could also aid dormant survival.

- Fungi have a characteristic range of soluble carbohydrates and storage compounds such as mannitol, trehalose and glycogen.

Fungi make an essential contribution not only to the biosphere, but also to human industry, medicine and research (Deacon, 2006; Stajich et al., 2009). They have, historically and to the present day, represented both an important source of food (yeast in bread-making, mushroom production, brewing process, etc.) and one of the leading crop diseases. Fungi have various biochemical activities that can be exploited for the production of compounds useful in many fields, such as antibiotics, steroids and cyclosporins. Nevertheless, fungi are also responsible for synthesising carcinogenic aflatoxins or other mycotoxins in human foods and animal feedstuffs. Fungi, thanks to their enzymatic systems, are one of the main players in the biosphere in the recycling of organic matter (in fact, fungi are the main decomposer of complex natural compounds such as lignin). Unfortunately, fungal diseases are the most significant cause of death in immunocompromised or immunosuppressed patients (AIDS, transplant surgery, etc.). Recently, fungi have garnered attention for their involvement in biotechnological applications aimed at producing heterologous gene products, for their use as biological control agents, in investigations of cell biological processes, and for their potential use in the remediation of wastes and wastewaters.

2.3.2   Fungal wastewater treatment

Organic carbon sources, nutrients and pathogens contained in civil and industrial wastewaters are usually removed with a combination of treatments based on physical-chemical technologies and engineered biological ecosystems. Environmental biotechnology, however, represents the core of WWTPs and allows an economical and sustainable treatment of wastewater (Metcalf & Eddy et al., 2002). Nowadays, WWTPs represent a filter that allow the closure of the anthropic water cycle and the preservation of the water sources' quality. This protects not only human health, but also the integrity of the ecosystem. In recent years, new emerging organic compounds (Bolong et al., 2009; Meffe and de Bustamante, 2014; Pal et al., 2014) such as endocrines disrupters (Liu et al., 2009), pharmaceuticals (Li, 2014), and micropollutants (Luo et al., 2014) have become relevant. These compounds belongs to a group of recalcitrant pollutants, as they can easily escape current treatment processes, remaining in the discharged effluent (Bui et al., 2016). The removal of recalcitrant pollutants represents a challenging task. Although some recalcitrant compounds are effectively eliminated via conventional treatment methods, the need for better technologies for the treatment of wastewater to fulfil water quality requests in an economical and sustainable way is growing. The lack of knowledge about the effects of recalcitrant compounds underlies increasing concerns about their impacts on the population. Consequently, more stringent limits on the discharge of WWTPs are expected in the near future (Barbosa et al., 2016). Current biological wastewater treatments are engineered biological ecosystems based on bacteria, which are able to tolerate diverse conditions, grow faster and efficiently degrade a rather broad range of pollutants. However, bacteria are ineffective in the removal of several compounds. Although fungi represent a promising biological resource in environmental biotechnology (Harms et al., 2011), they have rarely been applied in wastewater treatment due to a lack of knowledge regarding the optimal process conditions and due to their lack of stability in non-sterile conditions (Palli, 2016; Svobodová and Novotný, 2017). Most research on fungi in wastewater treatment is focused on the degradation of pharmaceuticals or complex pollutants (e.g., dyes and pesticides) at lab-scale (flasks), with important limitations and, above all, mostly in sterile conditions. Fungi also play a role in the biodegradation of phenols, chlorinated phenolic compounds,

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chlorinated alkanes and alkenes, polycyclic aromatic hydrocarbons, petroleum hydrocarbons and other emerging contaminants. Fungal exoenzymes and enzymes, such as peroxidases, laccases and enzyme mediators, are relevant to the biodegradation of toxic chemicals given their ability to break a wide range of strong chemical bonds. Generally, biomasses can be thought to interact with contaminants via three kinds of interactions: induced precipitation, adsorption and metabolism. However, they could all be present and acting at the same time or be sequential, for example, as adsorbed compounds could be later degraded (Cerboneschi et al., 2015). Fungi have been studied not only for their degradation capacity, but also as biosorbent material that could also be applicable to heavy metals uptake (Singh, 2006). In experiments, the biosorption mechanism occurred mainly within 24 hours. The selectivity, capacity and efficiency of the process is related to the nature of the biosorbent material and the pollutant (Fu and Viraraghavan, 2001; Srinivasan and Viraraghavan, 2010). Ascomycetes, in particular *Aspergillus spp.*, could remove contaminants through adsorption onto the cell surface as well as through enzymatic degradation (Anastasi et al., 2009; Öngen et al., 2007). Nevertheless, if adsorption is the main process, a change in the process conditions or long term operation could cause backward transformation or desorption (Badia-Fabregat et al., 2015). Fungi have successfully been used in the detoxification of contaminated sites and other solid matrices (Biiatt et al., 2002; Di Gregorio et al., 2016; Siracusa et al., 2017) and as biofilters for gas treatment (Van Groenestijn et al., 2001). However, fungi are usually outcompeted in wastewater treatment by detrimental bacteria. Autochthonous microorganisms of real wastewaters could be a source of microbial stress to fungi, negatively affecting the fungal degradation capacity in non-sterile conditions (Espinosa-Ortiz et al., 2016; Svobodová and Novotný, 2017). Bacterial contamination in fungal-based reactors causes, particularly during the start-up period, damage to the fungal mycelium (Rene et al., 2010) and the reduction or inhibition of fungal enzymes and in general their activity (Gao et al., 2008; Libra et al., 2003). Bacteria outcompete fungi in a bioreactor for several reasons. Fungi are eukaryotes and their grow rate is therefore intrinsically much slower than bacteria. Fungi prefer an acidic environment as opposed to most bacteria. They are able to brake complex bonds, but sometimes they require the addition of substrates for co-metabolism (co-substrates) (Palli et al., 2014). The majority of fungi grow in temperatures ranging from 5 to 35°C, but optimal growth is

usually around 25°C. Filamentous fungi are able to grow submerged, but they naturally grow at the water-air interface (Deacon, 2006; Singh, 2006). For these reasons, it is clear that the typical conditions of conventional WWTPs are unfavourable to fungi. In the literature, multiple strategies have been suggested (Anastasi et al., 2010; Badia-Fabregat et al., 2017; Espinosa-Ortiz et al., 2016; Gao et al., 2008; Mir-Tutusaus et al., 2017; Palli et al., 2016; Torán et al., 2017) to enhance the resistance of fungi in non-sterile conditions:

- immobilisation of fungal cultures;
- reduction of medium pH;
- limitation of nitrogen in the medium;
- elevated HRT (1-3 days);
- selective disinfection or microscreens;
- addition of co-substrates;
- fungal inoculation;
- bioreactor configurations;
- starting with mature and well-developed cultures of fungi.

There are two main possible configurations of the treatment train: enzyme dosage (produced in a dedicated side reactor) or processes based on fungal biomass. Since enzyme dosage presents some limitations (such as inactivation) and because enzyme degradation pathways for tannins are not completely defined, the latter approach was chosen in the present study. Long-term experiments in continuous mode bioreactors allow a better understanding of the process, which facilitates the upscaling and highlights possible unexpected effects, like an ecotoxicity increase in the final effluent that could sometimes occur (Chanda et al., 2016; Cruz-Morató et al., 2013). A preliminary classification of fungal bioreactors could be characterised by immobilised or suspended biomass (there are some other solutions like mycelium encapsulation in alginate, gelatine, agarose etc. (Perullini et al., 2010)) as briefly summarised in Table 2.5. Immobilised mycelia reactors present several advantages. They are more resistant to mechanical stresses and pH changes, have a higher degradation capacity, and minimal clogging is usually observed (Anastasi et al., 2012; Ramsay et al., 2005; Xin et al., 2012). Moreover, immobilisation of the fungal biomass is preferable when the

effluent includes a toxic pollutant with high concentrations (Kaushik and Malik, 2009; Prigione et al., 2008). Packed bed long-term stability and efficiency is significant higher than stirred tank reactors (Rodarte-Morales et al., 2012). The most widely used reactors with immobilised fungal biomass in non-sterile conditions are moving bed biofilm reactors (Park et al., 2011), packed beds (Palli et al., 2016) and biotrickling filters (Ehlers and Rose, 2005; Novotný et al., 2011). Mixing in a bioreactor plays an important role in oxygen and mass transfer in biofilms and reduces non-uniformity and gradients of relevant parameters such as temperature, pH, gases, and solids. However, mixing also influences the biofilm shape and thickness (Picioreanu et al., 2001). Rotating biological contactors with fungal biofilms have been investigated for their removal performance and resistance to microbial stress (Malachova et al., 2013; Pakshirajan and Kheria, 2012; Svobodová et al., 2012; Tanaka et al., 2003). Rotation could also control biomass growth in immobilised systems (Spennati et al., 2017). Fungi, due to their structure, are sensitive to shear stress, and excessive agitation could damage biofilms or pellets (Espinosa-Ortiz et al., 2016; Palli, 2016). Based on the considerations described above, a novel packed bed reactor type, with the ability to rotate, was designed and built as described in the Materials and Methods chapter. Table 2.6 lists the operational conditions employed in a typical non-sterile fungal reactor used for the treatment of phenol rich wastewaters as olive mill wastewater (OMW) and molasses from wastewater distillery (MW). It is important to design the process conditions by also taking in account the characteristics of the target compounds.

Table 2.5: Typical fungal bioreactors for removal of pollutants (Espinosa-Ortiz et al., 2016; Singh, 2006).

Biofilm or immobilised systems	Suspended growth
Trickling filter	Stirred tank
Packed bed	Bubble column
Rotating biological contractor	Airlift tower loop
Upflow fixed film	Fluidised bed
Slurry	
Packed bed	
Immobilised (various types)	

Table 2.6: Non-sterile fungal bioreactor conditions used for phenol rich wastewaters.

Fungal strains	Target compound	Reactor	Carriers	Temp. °C	pH	Co-substrate	Removal	Ref.
N. crassa	phenols	MBfR	Membrane	-	-	Sucrose	92-100%	(Luke and Burton, 2001)
Fungal consortium	MW	Novel reactor (14 L)	Straw	24 ± 2°C	8.2	Straw	65%	(Pant and Adholeya, 2010)
A. tubingensis	MW	Novel reactor (6 L)	No carriers	20-30°C	4.45	-	90%	(Watanabe et al., 2009)
Fungal consortium	phenols	BTF (6.3 L)	chips Pinewood	19-25°C	-	Pinewood	81%	(Ehlers and Rose, 2005)
P. ostreatus	OMW	airlift (5 L)	no carriers	28°C	5	Potato dextrose	95%	(Olivieri et al., 2006)

2.3.3   Tannin-degrading fungal strains

Despite the antimicrobial properties of tannins, many fungi, bacteria and yeast are quite resistant to tannins and can use tannins as carbon and energy sources. The biodegradation of natural tannins in the environment is mainly associated with fungi rather than bacteria. The role of fungi in tannin degradation was reported as early as 1900, when Fernbach and Pottevin independently reported the hydrolysis of tannins using a cell-free preparation of *Aspergillus niger*. Fungi express a large number of degradative enzymes including peroxidases, laccases and tannases. Tannin acyl hydrolase (E.C.3.1.1.20), commonly referred to as tannase, is an enzyme accidentally discovered by Tieghem in 1867 (in *Aspergillus Niger*, Van Tieghem).

Tannase catalyses the hydrolysis of ester bonds, releasing glucose, gallic acid and galloyl esters. The enzyme finds application in many industrial sectors, including the pharmaceutical, food, chemical, and beverages industries (Aguilar et al., 2007; Belmares et al., 2004). Tannase is considered one of the most expensive enzymes, and an economically feasible process needs to be developed for tannase production (Belur and Mugeraya, 2011). Some authors suggest a correlation between tannase activity from fungi in phenol-rich wastewater and the reduction of COD, Total Organic Carbon (TOC) and phenol content (Hanafi et al., 2013; Kachouri et al., 2005; Kanagaraj and Mandal, 2012; Salgado et al., 2016). However, the effect of tannase on condensed tannins has not yet been adequately defined. In this context, the potential of fungi for the bioremediation of wastewater streams from the tanning industry (Tilli et al., 2010) cannot be exploited until suitable technologies for their selection in a typical wastewater treatment train can be developed. In fact, the main operational concerns are related to bacterial contamination and to the robustness of the bioprocess in the long-term, since fungi are easily outcompeted by bacteria. Currently, a bioreactor able to maintain the stable growth and performance of fungi under sterile and non-sterile conditions is still a challenging task. Furthermore, the fungal degradation of most of the recalcitrant compounds frequently require the supply of an external carbon source (co-substrate), since recalcitrant compounds may not be a suitable growth substrate (Espinosa-Ortiz et al., 2016; Palli et al., 2016). Nevertheless, among recalcitrant compounds present in tannery wastewaters (G. Munz et al., 2009), tannins represent a potential carbon source for fungi, despite their antimicrobial properties. In particular, *Aspergillus spp.* and *Penicillium spp.* have been isolated from tannery wastewaters and were reportedly able to biodegrade tannins (Batra and Saxena, 2005; Bhat et al., 1998; Mingshu et al., 2006; Murugan et al., 2007; Ramos et al., 2011). In particular, tannin-degrading strains of *Aspergillus* (section Nigri) were found in tannery wastewater rich in QT (León-Galván et al., 2010). They were also able to grow on tannic acid (TA) (Van Diepeningen et al., 2004) and express degradative enzymes such as tannase (Batra and Saxena, 2005; Renovato et al., 2011; Sharma et al., 2007). Based on this, it can be hypothesised that fungi will grow in the recalcitrant fraction of wastewater containing a high concentration of tannins, such as tannery wastewater. Most research on the biodegradation of natural tannins and polyphenolic compounds by fungi has been performed in petri dishes or flasks: using tea by-products (Ni et al., 2015), tannin

extracts (Belmares et al., 2009) or on TA (Van Diepeningen et al., 2004). There have been no previous experiments using continuously fed bioreactors (under sterile or non-sterile conditions) for the removal of QT, TT or other natural tannins with fungi. It is important to highlight that in a co-culture, microbial interaction could promote alternative intermediate compounds or metabolites and therefore different degradation pathways of the target pollutant (Bertrand et al., 2014).

2.4   RESPIROMETERS AND MODELLING

2.4.1   Respirometry and titrimetry

Respirometry is a technique that measures the oxygen consumption from a biomass in the presence of a substrate. Metabolic respiration is a metabolic process that produces adenosine triphosphate due to the electron transfer from an electron donor (substrate) to an electron acceptor (O_2, NO_2, NO_3, SO_2, etc.). Respirometry is the measurement and interpretation of the biological electron acceptor consumption (usually oxygen) under well-defined experimental conditions (Andreottola and Esperia, 2001). Respirometry is a powerful tool that can be combined with other techniques to provide information relative to biological wastewater treatment (and in general environmental biotechnologies), including COD fractionation and the characterisation of wastewater, the estimation of kinetic and stoichiometric parameters, the monitoring of bioprocesses, and the evaluation of potential toxicity and inhibition effects over different suspended cultures, mainly in aerobic processes (Cokgor et al., 2009; Gernaey et al., 2001; Guisasola et al., 2007; Spanjers et al., 1996; Vanrolleghem et al., 1999). Respirometric tests are performed in a respirometer. A respirometer is a vessel equipped with probes to measure various elements (temperature, pH, oxygen concentration etc.) and actuators that control the conditions (mixing device, thermostatic water bath, pH dosage solutions, etc.). The first respirometers were created to measure biological oxygen demand (BOD), and more recent models are connected to a computer or a programmable logic controller (PLC). Oxygen can be measured as a difference of pressure (manometric respirometer) or the

oxygen production necessary to balance the pressure (electrolytic respirometer). In recent years, commercial oxygen sensors (such as electrochemical and optical sensors) have been largely adopted. The classification of respirometers is based on a three-letter configuration as reported in Table 2.7. The first letter indicates whether the oxygen measurement is performed in the gas (G) or liquid (L) phase. The second and third letters indicate whether the gas and liquid phases are static (S) or flowing (F), respectively.

Table 2.7: Summary of respirometer configurations (Mora, 2014).

			Measurement in liquid phase				Measurement in gas phase			
	Process	Coefficient	LSS	LFS	LSF	LFF	GSS	GFS	GSF	GFF
Liquid-phase balance	Respiration	$V_L r_o$	-1	-1	-1	-1	-1	-1	-1	-1
	Dissolved Oxygen accumulation	$d\,(V_L S_o)\,dt^{-1}$	-1	-1	-1	-1	-1	-1	-1	-1
	Liquid flow	$Q_{in}S_{o,in} - Q_{out}S_o$			1	1			1	1
	Gas exchange	$V_L K_L a\,(S_o^* - S_o)$		1		1	1	1	1	1
Gas-phase balance	Gaseous Oxygen accumulation	$d(V_G C_o)\,dt^{-1}$					-1	-1	-1	-1
	Gas flow	$F_{in}C_{o,in} - F_{out}C_o$						1		1
	Gas exchange	$V_L K_L a\,(S_o^* - S_o)$					-1	-1	-1	-1

The oxygen uptake rate (OUR) represents the consumption of dissolved oxygen (DO) per unit of time. This value is related to the catabolic and anabolic functions of the biological system. It is possible to distinguish between the endogenous OUR (OUR_{end}), in other words, the consumption of oxygen observed without a substrate, and the exogenous OUR (OUR_{ex}), the consumption of oxygen observed in the presence of a substrate and related to its oxidation. During endogenous respiration, or decay, the cells exploit material stored internally as an electron acceptor, and the biomass of the system decreases due to the internal storage depletion, lysis, predation, etc. (Andreottola and Esperia, 2001). A respirometric test, thus allows for the study of how the whole process evolves and the different biodegradability of the components of the substrate used. The OUR can be calculated with continued aeration or discontinued

aeration. In the first case, the OUR calculation is based on the DO balance in the respirometer, which is different depending on its configuration. An equilibrium of the DO is reached in the respirometer, thus the OUR_{end} as well as the overall oxygen mass transfer coefficient (K_{la}) can be calculated thanks to a short interruption of aeration as represented in Figure 2.10. The mass balance applied to obtain the OUR_{ex} (mg O_2 L^{-1} h^{-1}) is calculated through Eq. 2.1 for LFS respirometric tests performed at a constant volume.

$$OUR_{ex} = K_{la} \cdot (S_o^* - S_o) - \frac{dS_o}{dt} - OUR_{end} \qquad (2.1)$$

Where S_0^* is the DO saturation (mg O_2 L^{-1}) and S_0 the DO concentration (mg O_2 L^{-1}).
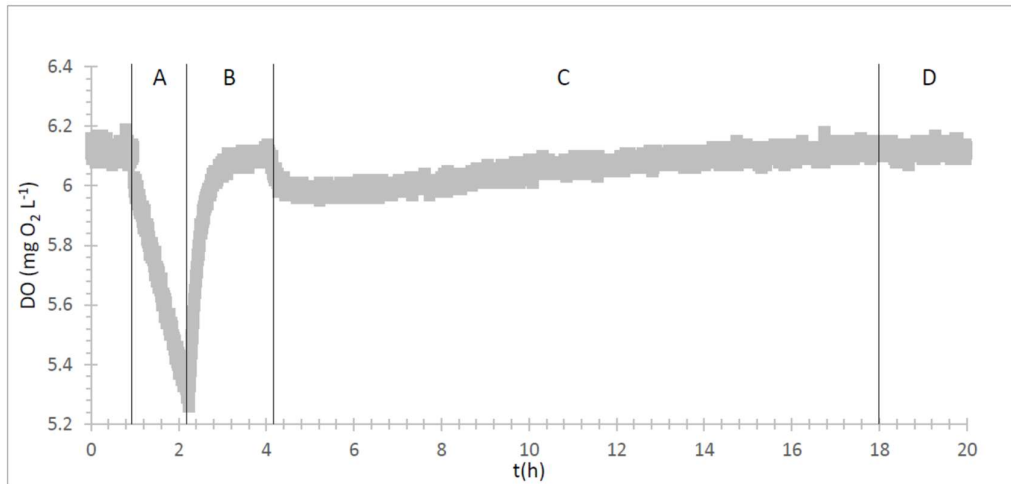
Figure 2.10: Respirogram of *Aspergillus t.* immobilised with TT pulse. A) Aeration is cut off to calculate the OUR_{end}; B) Aeration is re-started to evaluate the K_{la}; C) Pulse of substrate and calculation of OUR_{ex}; D) The biomass returns to endogenous conditions.

In the second case, the DO in the respirometer is maintained between a lower and an upper DO set point by switching on and off the aeration (the lower set point is chosen to avoid the oxygen limitation of the biological process). The increasing lines represent the aeration, while the pendent of the descending lines is the OUR. An example of a respirogram is shown in Figure 2.11. The OUR_{end} is the OUR calculated in absence of substrate.
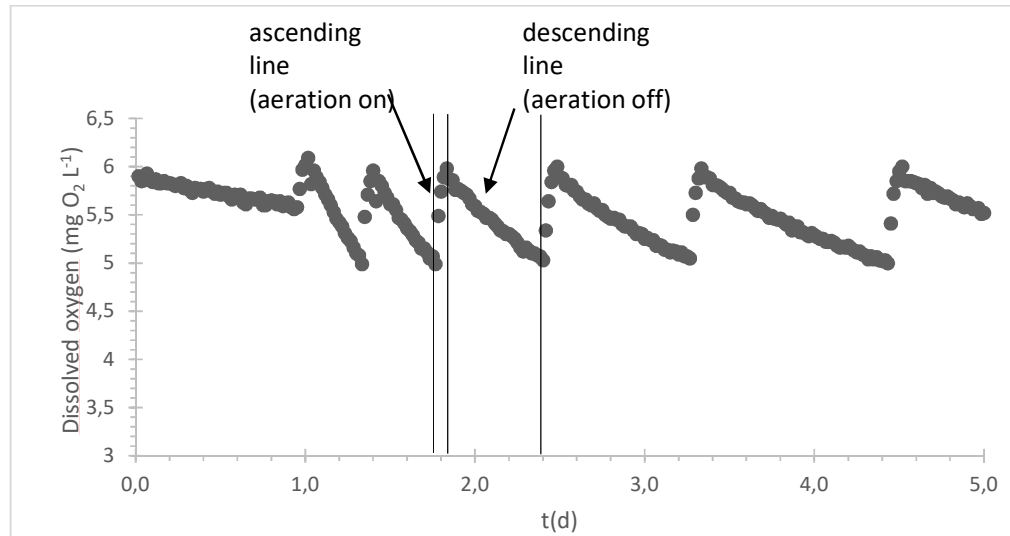
Figure 2.11: Respirogram of activated sludge with TT pulse.

Two respirometric procedures were proposed and developed in the present work: homogeneous and heterogeneous respirometry. The main differences between the two techniques are described below.

- Homogeneous respirometry

Homogeneous respirometry is usually applied with suspended biomass, but could also be applied to immobilised biomass. This technique and the mathematical models used are well known. The mixing in the vessel allows for the maintenance of homogeneous conditions. The main drawback of this system is that the hydrodynamic conditions and diffusion phenomenon of biofilm are not considered because biofilms are destroyed and instead tested as a suspended culture (Mora, 2014).

- Heterogeneous respirometry

The heterogeneous respirometry concept is a respirometric technique applied directly to a biofilm or part of a biotrickling filter without any pre-treatment (Mora, 2014). The respirometer vessel contains liquid and the biofilm undisturbed.

It is worth mentioning that a different electron acceptor could be used in a respirometric test (NO_2, NO_3, SO_2, etc.), referred to as anoxic respirometry, depending on the species

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monitored. Titrimetry is a pH-stat test with an acid or base dosage in which pH is perfectly controlled. This technique is useful to monitor biological processes where the reactions affect the pH and usually have a well-known stoichiometric relation such as nitrification. In fact, this test provides an indirect measurement of proton production due to the dosage of an acid or base solution with a defined molarity (Spanjers et al., 1996). In some cases it is also possible to couple respirometry and titrimetry to obtain further information on the biological process being tested (Mora et al., 2014; Giulio Munz et al., 2009).

In the literature, heterogeneous respirometry has been applied mainly to characterise the kinetics of biological filters for gas treatment (Bonilla-Blancas et al., 2015; González-Sánchez et al., 2014). Respirometry has rarely been applied to fungal biomasses. The majority of studies focus on homogeneous respirometry in sterile conditions (Fuentes et al., 2015; Michel et al., 1992; Schinagl et al., 2016), on sterile solid matrices (Willcock and Magan, 2001), on soil matrices to analyse some behaviours of fungi and bacteria (Boening et al., 1995), or with OMW treatment (Caffaz et al., 2007). Sometimes oxygen consumption is exploited to model fungal growth. However, respirometric assays for heterogeneous respirometry of a fungal and bacterial biofilm in non-sterile conditions are still lacking. It has been hypothesised that it is possible to effectively remove the target recalcitrant in non-sterile conditions with a reactor based on fungi. It may also be possible to use respirometry and modelling as tools for the monitoring and design of fungal growth. In this thesis, a procedure to apply heterogeneous respirometry of a fungal and bacterial biofilm in non-sterile conditions has been developed, which has also been used as a modelling tool for the fungal bioprocess.

2.4.2   Biofilms

Microorganisms can be observed in the environment as planktonic (free-swimming) organisms or as aggregated communities called biofilms. The transition between these two forms is a complex and highly regulated process characterised by a strong interaction between cells and various environmental signals (O'Toole et al., 2000). Biofilms may be adhered to a surface or in the form of granules.

In nature, biofilms are usually composed of heterogeneous and dynamic communities and structures (Gonzalez-Martinez et al., 2015; Rodriguez-Sanchez et al., 2016; Stoodley et al., 1999). Moreover, various interactions between the microorganisms, including cooperation and competition phenomena, are present inside the biofilm (Allen and Nowak, 2013; Pérez et al., 2014). Multiple drives influence biofilm formation and structure, and biofilm detachment generally occurs when external forces overpower the internal strength of the matrix holding the biofilm together. For instance, the substrate and nutrient availability and the liquid shear are influencing parameters as shown in Figure 2.12.
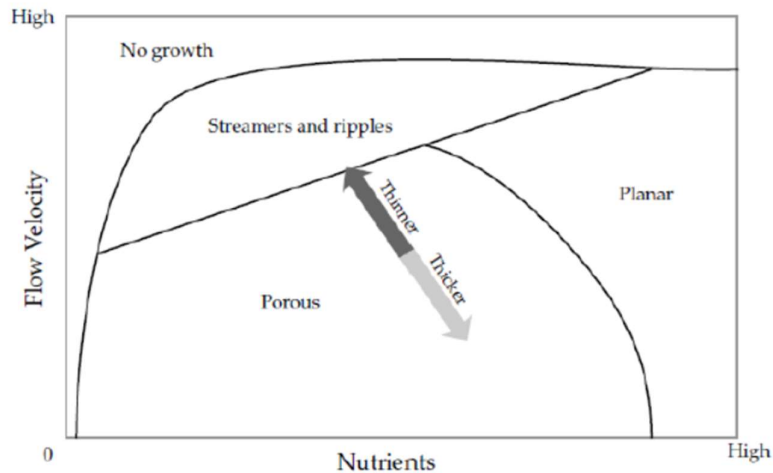
Figure 2.12: Influence of nutrients and liquid share on biofilm structure (Stoodley et al., 1998)**.**

Usually biofilms grow in phases: adhesion at the substratum, colonisation, maturation and dispersal (Ramage et al., 2009). The biofilm allows for greater resistance to external stress (such as antibiotics) compared to the planktonic form (O'Toole et al., 2000). Biofilms promote diffusion in the environment or, in the case of pathogens, act as persistent sources of infection (Hentzer and Givskov, 2003). The different transport phenomena of compounds due to the biofilm structure is a crucial feature with multiple implications. Moreover, the biofilm includes the formation of extracellular matrix material (ECM) with functions as diverse as cell-cell aggregation, nutrient storage (Lagree and Mitchell, 2017) and cell-substrate adherence (Blankenship and Mitchell, 2006). Inside the biofilm, as an additional feature of resistance, some cells became less

active, but more resistant to the external stresses. These are called persistent cells (Ramage et al., 2012). Finally, cell-cell communication allows the population to coordinate behaviours. This is achieved through the secretion of signalling molecules called quorum sensing molecules (QSM) (Lee et al., 2016). Biofilms are widely studied since they could be detrimental (biofouling, infections) or positive (biofilm able to remove pollutants) (Henze et al., 2008). The interest in fungal biofilms is not well developed and relates mainly to the pathogens dimorphic fungi *Candida albicans* and other pathogens such as *Aspergillus fumigatus*. Even so, fungal biofilms have received less attention than bacterial biofilms (Blankenship and Mitchell, 2006; Lagree and Mitchell, 2017; Ramage et al., 2009). In wastewater treatment, fungal biofilms have been studied due to their occurrence within municipal water distribution systems (Doggett, 2000; Siqueira et al., 2011). Quorum sensing is a typical prokaryotic mechanism, but it has also been discovered in some fungi. For example, the dimorphic *C. albicans* produce QSM to inhibit/promote filamentation (Albuquerque and Casadevall, 2012). Interestingly, the first fungal QSM discovered, Farnesol, also acts as a bacterial quorum quenching molecule (QQM) that significantly interferes with the production of the extracellular polymeric substances (EPSs), reducing, for example, membrane biofouling (Lee et al., 2016). Microbial networks undergo time-space evolution, and the performance of the system is strictly dependent on the inner interactions. Though the dominant organisms could be outcompeted by others via several mechanisms, the interactions between bacteria and fungi in biofilms are not well understood and require further study (Jeong et al., 2016).

2.4.3   Activated sludge models and biofilm modelling

A mathematical model defines a purposeful description of a system and provides a simplification of reality that may help to understand or design the system. Models are also used to deal with metabolic processes in systems biology. However, an inadequate knowledge of complex metabolic networks could severely hamper the application of models in ecological systems. Despite important recent developments, a wider understanding of processes in systems biology is needed to estimate their associated parameters. The first necessary step to build a model is the analysis of the system and the definition of the goal of the model. In other words, one must build a

conceptual model as the basis for the mathematical one. Each model has assumptions and limitations that must be highlighted for a correct application. The second step is the choice of the processes that will be implemented and the type of model. Processes could besteady state, dynamic state or frozen state. Furthermore, the resolution required and the level of detail in the time and space scales must be clarified. A model may follow the black box, grey box or glass box approach. The black box approach is an empirical type of model, while the glass box is a mechanical type of model. In the former, the mechanisms occurring inside the model are not known, while in the latter, the mechanisms are described with equations. Thus, for a biological system, a deep understanding of biochemical and microbiological processes and the transport phenomena are required. Models are applied in systems biology to fulfil multiple objectives. They can be used in wastewater treatment to get insight into the processes and the performance, to simulate different scenarios (for a plant design or upgrade and to support management decisions), and to develop new monitoring and control schemes. At the end of the modelling it is necessary to "come back to reality" and validate the results obtained.

The Activated Sludge Models (ASM) of the International Water Association (IWA) are the reference models for activated sludge processes. The activated sludge model development were biokinetic models based on multi-substrate Monod-type equations and aimed to describe oxygen and substrate consumption and sludge production. ASM1 is considered the reference model to describe the growth and death dynamics of heterotrophs in activated sludge where COD is used for the measurement of the organic matter. ASM2 is used for phosporus removal, and ASM3 integrates the substrate storage inside the cells. Through the years, different integrations and other derivative models such as TUDP (Henze et al., 2008) have been proposed. Figure 2.13 contains a summary of the most important models.
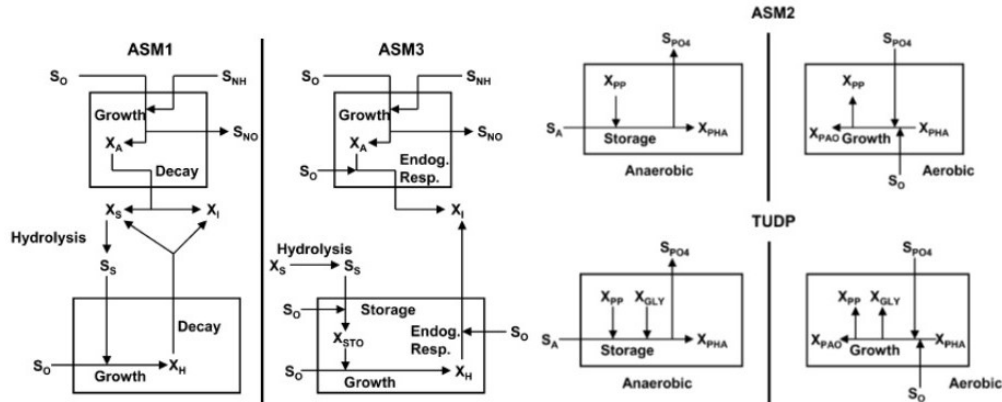
Figure 2.13: Simplified schematics of ASM1, ASM2, ASM3 and TUDP (Gernaey et al., 2004)**.**

Below is a list of the most widely used simulator environments in this field:

AQUASIM (www.aquasim.eawag.ch)

BioWin (www.envirosim.com)

EFOR (www.dhisoftware.com/efor)

GPS-X (www.hydromantis.com)

MATLAB/SIMULINK (www.mathworks.com; a general purpose simulator)

PetWin (www.envirosim.com/products/petwin)

SIMBA (www.ifak-system.com)

STOAT (www.wrcplc.co.uk/software)

SUMO (www.dynamita.com/the-sumo/)

WEST (www.hemmis.com)

Among the simulator environments, Aquasim is particularly suitable for the simulation of biofilms thanks to a "compartment" that integrates the biofilm dynamics and transport phenomena (Wanner and Morgenroth, 2004). For this reason, Aquasim was chosen as the simulating environment adapted for the present study. Biofilm modelling is research area in developing (Boltz et al., 2017; Rauch et al., 1999).

2.4.4   Stoichiometry and kinetics of fungi in environmental biotechnologies

The kinetics of fungal growth are generally studied in industrial processes for continuous culture systems in chemostat for the production of antibiotics or other relevant commercial products. However, the industrial processes are traditionally based on batch cultures. The main aim of the industrial processes is the production of relevant metabolites, so the substrate is usually glucose and fungal growth is in an axenic culture broth (Deacon, 2006). The conditions adopted in an industrial context are generally different from those in environmental biotechnologies. For bacterial biomasses, Monod kinetics and death regeneration IWA concepts are commonly applied, but there are also other options for fungi (Sole-Mauri et al., 2007). However, Monod-kinetics could also describe growth in filamentous fungi (Kelly et al., 2004; Villena and Gutiérrez-Correa, 2011), and this model allows comparisons between both microorganisms. Even though the enzymatic pathways were not clear, this approach was chosen to model the fungal biomass. There are few examples in the literature of kinetic models of tannin (tannic acid derived from vegetable tannins) degradation by activated sludge in industrial WWTP (Lu et al., 2009; Tramšek et al., 2006) and even fewer for fungal biomass (Wang et al., 2008). Fungi are rarely used in environmental biotechnologies related to wastewater due to their instability in non-sterile conditions. Few studies in the literature have performed a stoichiometric and kinetic characterisation. Moreover, the parameter estimation did not include COD. Of note is the fact that fungi used in the removal of phenols from vinasses (as a sole carbon source) showed a maximum specific growth rate of 0.06-0.047 h^{-1}, a growth yield of 0.38-0.39 g cells g COD^{-1} and a saturation constant of 13,525-4558 mg COD L^{-1} (García García et al., 1997). In the present work, we have hypothesised and demostrated that fungi biomass developed in a bioreactor under non-sterile conditions can be described using Monod kinetics with inhibition. In this study, we determined not only that a fungal base bioreactor is stable in non-sterile conditions, but that it was also able to be modelled.

3 GENERAL MATERIALS AND METHODS

3.1 SELECTION OF FUNGAL STRAINS

3.1.1 Preliminary isolation of suitable fungal strains from tannery wastewater

The first phase of this project was focused on the isolation, identification and selection of fungi able to tolerate and degrade target recalcitrant compounds. This first step was performed in collaboration with the Mycotheca Universitatis Taurinensis (MUT - Department of Life Sciences and Systems Biology of the University of Turin). MUT is affiliated with the World Federation Culture Collections and European Culture and oversees the acquisition, identification, characterisation, preservation and distribution of more than 5,000 fungi belonging to the collection. The collaboration with the MUT research team allowed for the identification and isolation of fungi to be further tested in bioreactors. The evaluation of the most suitable strains included both those belonging to the MUT collection and a group of strains isolated from samples collected from WWTPs treating tannery and municipal wastewaters. Three samples of approximately of 15 L each were collected from the mixed liquor of Cuoiodepur vegetal tannery wastewater treatment plant (C), from the mixed liquor of the WWTP for the treatment of chromium tannery wastewaters of Consorzio Aquarno SpA (Santa Croce sull'Arno, Pisa, Italy) (A1) and from the effluent of the settler of the first aerobic step of Aquarno (A2). The Cuoiodepur WWTP was described in the previous chapter. Aquarno is a joint-stock company run by a large private majority operating in the Tuscany tannery district for the treatment of chromium tannery wastewater. The WWTP was designed to treat 2 million I.E. and currently treats 1.2 million I.E. The plant is divided into two lines, with municipality wastewater accounting for approximately 40-45% of the total annual flow. The treatment of municipality wastewater is carried out in three main steps: primary sedimentation, organic oxidation and secondary sedimentation. The treatment of industrial wastewater is composed of a pre-treatment section, followed by conventional active sludges and a post-denitrification tank. Afterwards, the degradation of the more slowly biodegradable compounds occurs in a second oxidation

section coupled with a secondary sedimentation. The tertiary treatment is based on the Fenton process with a subsequent clarification with polyelectrolyte dosage. The average temperature of the wastewater is in the range 25-30°C, and the HRT for the biological section is approximately 7 days. Table 3.1 reports the chemical characterisation as measured from the samples collected.

Table 3.1: Chemical characterisation of the three samples (C, A1, A2) collected in the selected WWTPs.

	C	A1	A2
pH	7.66	7.22	7.33
Ammonium (mg L^{-1})	1.04	9.65	278
Total phosphorus (mg L^{-1})	5.92	3.46	2.43
Chlorides (mg L^{-1})	2,906	4,218	4,100
COD (mg L^{-1})	14,280	10,000	24,740
sCOD (mg L^{-1})	362	518	760
TSS (g L^{-1})	12.93	7.49	12.61
VSS (g L^{-1})	10.78	5.77	9.61

Aliquots of 1 millilitre of each sample were placed in Petri dishes (16 cm diameter) containing 30 millilitres of culture medium. Three different (generic and selective) culture media were used for the isolation and identification of the autochthonous mycoflora: Malt Extract Agar (MEA) (agar 20 g, glucose 2 g, malt extract 2 g, peptone 0.2 g, water up to litre); DRBC (Dichloran Rose Bengal Agar 31.5 g, water up to 1 L); Wastewater with agar (WA) (agar 20 g, glucose 2 g, samples supernatant after 5 min at 10,000 rpm up to 1 L). A set of three antibiotics was added to each medium, and two different incubation temperatures were chosen (15°C and 25°C). At regular time intervals, the colony forming units (CFU) were counted and the different fungal morphotypes were isolated in pure culture. Fungi were identified conventionally according to their macroscopic and microscopic features. Several hundreds of fungi were isolated from WWTPs samples and various strains were determined to be active towards target compounds in accordance with the parameters considered. Finally, among the potentially suitable fungi, the safest (Generally Recognised As Safe, GRAS) and most adaptable strains were chosen. The selected strains were also tested for their ability to grow in attached form on the selected support media as described in the following paragraphs. At the end of the procedure described above, 15 autochthonous fungal strains were isolated from tannery effluents. Finally, 4

allochthonous strains that are well described in the literature for their ability to degrade tannins were added, provided by the fungal collection of MUT. The selected strains were tested, as described in the following section, to identify the optimal strain that could be exploited in the subsequent tests in bioreactors (Table 3.2).

Table 3.2: Allochthonous and Autochthonous fungal strains selected.

Allochthonous fungal strains	Autochthonous fungal strains
Aspergillus niger MUT 983	*Scedosporium apiospermum* n. 1
Aspergillus niger MUT 918	*Scedosporium apiospermum* n. 3
Aspergillus tubingensis MUT 990	*Graphium putredinis* n. 1
Paecilomyces varioti MUT 1125	*Scedosporium apiospermum* n. 8
	Scedosporium apiospermum n. 9
	Cladosporium cladosporioides
	Pseudallescheria ellipsoidea n. 3
	Chaetomium sp.
	Trematosphaeria grisea n. 1
	Myrotecium sp.
	Trematosphaeria grisea n. 2
	Chaetomium cochliodes
	Chaetomium tarraconensis
	Trematosphaeria grisea n. 3
	Sterile mycelium

3.1.2   Fungal strain selection

The 19 preliminarily selected fungal strains were tested to identify a suitable strain for experimentation in bioreactors as described below. In order to reduce the variability in subsequent tests in bioreactors due to biological interactions between different fungal strains, a single fungal strain was chosen among the preliminarily selected strains. The fungal strains were evaluated for their capacity to treat a tannin-rich sample collected from a WWTP. The test sample was collected from the effluent of the secondary clarifier of the biological treatment of Cuoiodepur WWTP. A composite sample (made up of samples collected three times per day during an entire week) was used to perform the selection via flask tests with the support of MUT's researchers. The sample was characterised as follows: pH 7.8, COD 608 mg L^{-1}, sCOD 496 mg L^{-1}, TOC 259 mg L^{-1}, and volatile suspended solids (VSS) 0.11 g L^{-1}. The

sample also presented a concentration of Ammonium 0.54 mg L^{-1}, Phosphorus 1 mg L^{-1}, Chloride 2009 mg L^{-1}, Sulphates 1160 mg L^{-1}, and Iron 2.44 mg L^{-1}. The aim of the flask test was to evaluate the removal capacity in terms of COD in non-sterile conditions and also to evaluate ecotoxicity and tannase production. The fungal strains were cultivated in 9 cm diameter Petri dishes containing 10 mL of a generic solid medium of MEA. Fungi were incubated in the dark at 24°C for two weeks. Ten agar plugs of each fungal strain (5 mm diameter), collected from the margin of colonies in active growth, were put in 100 mL Erlenmeyer flasks containing 40 mL of GLY sterile medium (5 g L^{-1} glucose, 1.9 g L^{-1} yeast extract). The experiment was performed in four replicates. The flasks were incubated in the dark at 25°C and 110 rpm in a INFORS™ shaker. In the other 3 flasks, the culture broth was replaced with 40 mL of modified sample (adjusted to pH 6.4 with HCl, with 1 g L^{-1} of glucose added in order to provide the fungi with a supplementary carbon source). Additionally, an abiotic control (without any biological inoculum) was set up. The flasks were incubated at 25°C and 110 rpm for 7 days (168 h). The COD was measured using Hach cuvettes. The ecotoxicological analyses were carried out before and after the fungal treatment to verify the ecotoxicity of the samples. The samples were tested following the UNI EN ISO 8692:2005 protocol, using the green alga *Raphidocelis subcapitata*. An amount equal to 104 cell mL^{-1} was inoculated in 24 multi-well plates containing 2.5 mL of diluted sample. Each dose-response curve consisted of 6 dilutions. The test was performed in triplicate, and a fourth repetition without the algal inoculum was used as the abiotic control. Moreover, a biotic control was set up in 6 replicates. The samples were incubated for 72 h at 23°C and 8000 lux. The cell concentration was then evaluated spectrophotometrically. Absorbance at 663 nm (which is conventionally used for the chlorophyll concentration of culture) was measured and converted into cell concentration by means of a previously plotted regression line developed via analyses of cell suspensions at known concentrations. The inhibition percentage was calculated according to the following adimensional equation:

$$I_{ui} = \frac{(\mu_c - \mu_i)}{\mu_c \cdot 100} \qquad (3.1)$$

where:

$$\mu_c = \frac{(\ln(abiotic\ control) - \ln(10000))}{3} \qquad (3.2)$$

$$\mu_i = \frac{(\ln(number\ of\ cells\ in\ the\ sample - abiotic\ control) - \ln(10000))}{3} \qquad (3.3)$$

The glucose concentration in the culture was determined using the 2,4-dinitrosalicyclic acid (DNS) assay for reducing sugars according to the Miller method (Miller, 1959). A further test of the enzymatic tannase activity was conducted in order to assess the enzymatic activities involved in the biodegradation. The fungal strains were inoculated, in three replicates, onto Petri dishes (60 mm diameter) containing 10 mL of MEA solid medium. The plates were incubated in the dark at 24°C for 15 days. For each fungal strain, a mycelium disk (5 mm diameter) was cut from the colony in active growth and inoculated in sterile polystyrene flat-bottom 48-well microtiter plates, containing 1 mL of one of the following media:

- AcT (tannic acid 1 g, peptone 0.02 g, K_2HPO_4 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.03 g, $CaCl_2 \cdot 2H_2O$ 0.01 g, H_2O 1 L, mineral stock* 1 mL);
- AcT + MEA (tannic acid 1 g, glucose 0.2 g, malt extract 0.2 g, peptone 0.02 g, K_2HPO_4 0.2 g, $MgSO_4 \cdot 7\ H_2O$ 0.03 g, $CaCl_2 \cdot 2H_2O$ 0.01 g, H_2O 1 L, mineral stock* 1 mL);

*mineral stock, composition for 100 mL distilled water: 0.05 g $MnSO_4 \cdot 5\ H_2O$, 0.1 g NaCl, 0.01g $FeSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 6H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$, 0.001 g, $CuSO_4 \cdot 5H_2O$, 0.001 g $AlK(SO_4)2$, 0.001 g H_3BO_3, 0.001 g $NaMoO_4 \cdot 2H_2O$.

Three triplicates per medium were performed, with plates incubated in the dark at 24°C and monitored daily for 7 days to evaluate tannase activity (activity units, AU). Aliquots of 20 µL were placed in Eppendorf tubes, diluted with distilled water (480 µL), and centrifuged at 24,000 rpm for 5 min. A further dilution was performed by taking 20 µL of the supernatant and adding 180 µL of distilled water. The final dilution was transferred into UV-transparent 96-well flat-bottom polystyrene microtiter plates. The spectrophotometric reading was conducted at the maximum absorption peak for TA of 274 nm and 310 nm for gallic acid. TA variation was estimated according to the following formula (adimensional):

$$VT = 100 \cdot \frac{(Ab_{S0} - A_{st})}{Ab_{S0}} \qquad (3.4)$$

Where Abs_0 is the absorbance at time 0 and Ab_{st} is the absorbance at time t, measured at the maximum visible wavelength (λ_{max}) of TA. Tannase activity can be estimated by decreased absorbance at 274 nm.

3.2   PREPARATION OF FUNGAL CULTURES

3.2.1   Immobilisation of fungi on supports

Based on the results of the previous selection described in detail in chapter 3.1 *Aspergillus tubingensis* MUT 990, a black Aspergillus belonging to the section Nigri, was chosen as the inoculum for the present study. This fungal strain was originally isolated from commercial TT powder and is preserved at MUT on MEA at 4°C. The selected fungal strain was inoculated on 20 generic MEA plates (150 mm diameter) and incubated at 25°C in the dark for seven days. After incubation, the fungal colonies were cut to obtain a homogenate: for each cm^2 of mycelium, 1 mL autoclaved distilled water was added (Anastasi et al., 2012). Polyurethane foam (PUF) cubes were chosen as suitable carriers (Spina et al., 2012). PUF carriers were cubes of about 2 cm per side (density 25 kg m^{-3}, Pores/Volume ratio: 0.97 and specific surface 600 m^2 m^{-3}), which were inserted in six 1000 mL Erlenmeyer flasks (35 cubes each) containing 500 mL of GLY medium and autoclaved at 121°C for 30 min. Following this, 10 mL of fungal homogenate was added to each flask. Flasks were cultured under stirring conditions at 110 rpm with a shaking table at 25°C in the dark for seven days. This procedure allowed the immobilisation of the selected fungal strain onto the PUF. The dry mass in each PUF was determined after biomass drying in an oven at 105°C. The collected PUFs were left drying in the oven to remove humidity, after which they were weighed to obtain the weight sum of the dry mass and the mass of the PUF support. Subsequently, the PUFs was submerged into a commercial solution of sodium hypochlorite to remove the dry mass. The clean PUFs were dried again in an oven and

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weighed to find the mass of the PUF support. The dry mass weights were determined by subtraction.

3.2.2   Inoculum preparation for pilot-scale reactor

The strain tested in this study, *Aspergillus tubingensis* MUT 990, was inoculated onto generic MEA plates (150 mm diameter) and incubated at 25°C in the dark for seven days. After the incubation period, 10 plates were homogenised with 200 mL of physiological solution in sterile conditions. Six L of GLY medium were prepared in six bottles (1 L effective volume and about 1.1 L total volume), which were autoclaved at 121°C for 30 min. At the same time, 2 air stone diffusers and related plastic pipettes and 16 PUF cubes (2 cm per side) were autoclaved, while two glass vessels of 5 L total volume with covers were sterilised under a UV lamp for 1 h. The following was inserted into each glass vessel: 1 stone diffuser, 8 PUF cubes, 3 L of GLY solution and 100 mL of homogenised fungi. The previous procedure was performed under a biological hood, with the outlet and inlet air pipe linked to 0.2 μm air filters. Finally, the reactors were connected with the air blower and incubated at room temperature for 64 days. Figure 3.1 includes pictures of the set-up described above and the fungal biomass. Fresh GLY solution was added to achieve 4 L volume in each reactor. Before the inoculation in the pilot reactor, the biomass was weighed, the medium was removed and the fungi was homogenised and poured into the reactor as described in the pilot-scale start-up.

Figure 3.1: One of the two vessel for inoculum growth, from left to right at day 0, after 3 weeks and after 6 weeks.

3.3 PRELIMINARY TESTS WITH CONTINUOUS BIOREACTOR OPERATION

3.3.1 Experimental set-up

The reactor design was based on the scientific literature and the outcomes of previous work. Firstly, an immobilised biomass was chosen due to the expected increased resistance as described in the Introduction. PUF cubes were adopted as carriers since they have already been demonstrated as the most suitable based on previous experience (Spina et al., 2012). The packed bed reactor type was chosen based on the outcomes of previous work with different substrates (Anastasi et al., 2010; Palli et al., 2016). The reactor was built with a rotating cage where the immobilised cubes could be inserted. The cage rotation was a process parameter able to improve the mixing inside the reactor and the biomass growth control due to the shear stress (Spennati et al., 2017). The submerged configuration was also chosen for safety reasons due to sporulation. The final rotating, submerged, packed bed reactor scheme is represented in Figure 3.2 and described below.
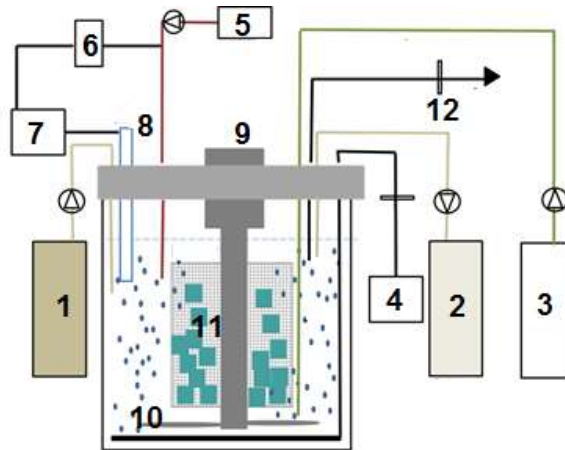
Figure 3.2: Rotating, submerged, packed bed reactor scheme: 1) medium tank; 2) discharge tank; 3) co-substrate tank; 4) air blower; 5) acid tank; 6) valve; 7) pH control panel; 8) pH probe; 9) engine to allow cage rotation; 10) air diffuser; 11) the metal cage and the PUF cubes; 12) air filter 0.2 μm.

The experimental set-up consisted of four aerated and completely mixed reactors with a volume of 5 L each (total volume, leaving 4 L of effective volume). The vessel had

a cylindrical shape with a diameter of 18 cm and a height of 27.5 cm. The four rotating, submerged, packed bed reactors were designed, built and installed in the Cer2co laboratory inside the Cuoiodepur WWTP as shown in Figure 3.3. The reactors had a cylindrical cage filled with 60 PUF cubes immobilised with the procedure described above. The cage was completely submerged at a liquid level of 4 L, and, consequently, the immobilised fungi were submerged. The cage had a cylindrical shape with a diameter of 5 cm and a height of 18 cm, assembled with a wide open stainless steel grid and perforated stainless steel plate as shown in Figure 3.4. Each cage was connected with an engine through a stainless steel rod in the cylindrical axis and was able to rotate. The engines were brushless (BL018.240 P42/3B, Intecno, Italy), electrically powered by Intecno card BLD07, and were modified to reach a maximum rotation velocity of 6 rpm. Each vessel was covered with a plastic device with nine circular openings along the border for probes and pipes and a central opening for the metal rod that connected the packed bed with the engine. The inlet and outlet flow was controlled by four peristaltic pumps (TEC-R, Aqua, Italy) and controlled by a PLC (LOGO! 230RC, Siemens, Italy). The co-substrate flow rate and the acid solution were controlled by peristaltic pumps (300 series, Watson and Marlow, USA) equipped with a multichannel head. In each reactor, pH was measured with Hamilton pH probes and automatically controlled with a pH controller (M20R-pH) by dosing sulphuric acid at 1 mol L^{-1}. An oxygen concentration close to saturation was ensured by air diffusers located at the bottom of the reactors. The reactors were located in a temperature-controlled room (20 ± 2°C). The DO concentration was measured regularly with a portable galvanic DO sensor (Oxi 340i with CellOx 325, WTW, Germany). Outlet air was filtered at 0.2 µm (for safety reasons).

Figure 3.3: The Cuoiodepur experimental set-up consisted of four rotating, submerged, packed bed reactors.

Figure 3.4: The packed bed composed of stainless steel cylindrical cages. On the left, an external view and on the right, an internal view with some PUF cubes.

The immobilised fungal biomass obtained was weighed: the initial average dry weight of solids in a PUF cube was 0.2 g and, consequently, the fungal biomass concentration in the reactors was about 3 g L^{-1} (60 PUF cubes were added in each reactor). The dry weight was calculated by subtraction after 1 h in an oven at 105°C, measuring the PUF weight in triplicate in the presence and absence of fungal biomass. The immobilisation of fungi on the PUF cubes and their positioning inside the cage are shown in Figure 3.5.
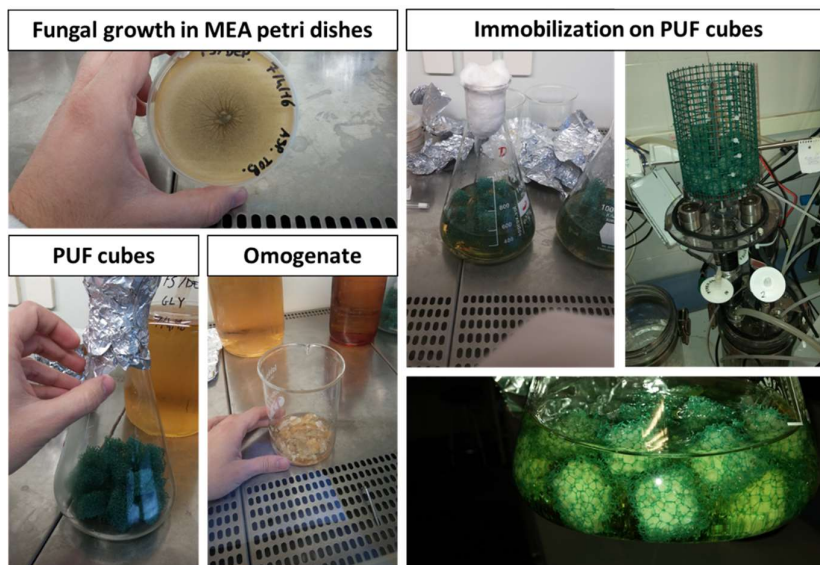
Figure 3.5: Pictures of the biomass preparation process.

3.3.2   Process operation

The first experiment was designed to evaluate the role of specific process parameters: initial inoculum, co-substrate addition, and rotation. The evaluation was aimed at assessing the stability of the bioprocess and performance in the removal of the target recalcitrant compounds (tannins) in non-sterile conditions. Non-sterile conditions are simulated in lab-scale studies by using non-sterilised tap water, non-filtered air for aeration, industrial grade chemicals without sterilisation and by allowing direct air-liquid contact inside the reactor. The reactors were fed with synthetic wastewater, and a solution of malt extract (analytic grade) at 1 g L^{-1} was dosed as a co-substrate. The experiment was also aimed at providing an evaluation of the degradation performance in the presence of a co-substrate for fungal growth (Palli et al., 2014). Malt extract as a co-substrate could allow the fungi to inhibit the growth of competitive bacteria (Svobodová et al., 2016). The synthetic wastewater was a mixture of four tannins for a total concentration of 1 g L^{-1} (0.25 g L^{-1} each) in tap water. Chimont International Spa, Montopoli (Italy) provided the industrial tannins (Quebracho, Wattle, Chestnut and Tara powders). These tannins are the most commonly used in the tannery factories in the Tuscany tannery district. All other reagents used in the present study were of analytic grade (Sigma-Aldrich). The

operating conditions were designed to enhance the development and degradation capacity of the selected fungal strain: the acidic pH (5-6) of the medium (Mohan et al., 2013), the HRT equal or higher than 24 h (More et al., 2010), and the immobilisation on carriers (Hai et al., 2013). The process operation was designed to achieve a pH set point at 5.5 ± 0.2 and HRT of 24 h to enhance the exoenzyme tannase production (Ramos et al., 2011; Rodriguez-Duran et al., 2011; Trevino et al., 2007). The inlet flow of co-substrate medium and synthetic wastewater was equal. Reactor R1 was inoculated with the immobilised PUF cubes, and it was the only reactor without co-substrate dosing. In this way, Reactor R1 served as the control reactor to evaluate the effect of the co-substrate compared to the other reactors. Reactor R2 was filled with empty PUF cubes and an inoculum of 2 mL of activated sludge from the Cuoiodepur WWTP (Pisa, Italy). Even though the activated sludge of tannery WWTP is acclimated to natural tannins, their degradation is poor at the conventionally used WWTP operational conditions. The objective of the test was to evaluate the tannin removal capacity of a WWTP autochthonous biomass in the steady-state conditions of the new reactor configuration (pH set point, co-substrate feeding, etc.) and to compare the performance of reactors inoculated with the selected fungal strain. Reactor R3 was inoculated with the immobilised PUF cubes and was fed with co-substrate. R4 was identical to R3, except that the cage of reactor R4 rotated at 3 rpm. The R4 was used to evaluate the effect of rotation, in terms of tannase production and diffusion in the reactor and natural tannin degradation. The test was conducted for 50 days and is schematically summarised in Figure 3.6.
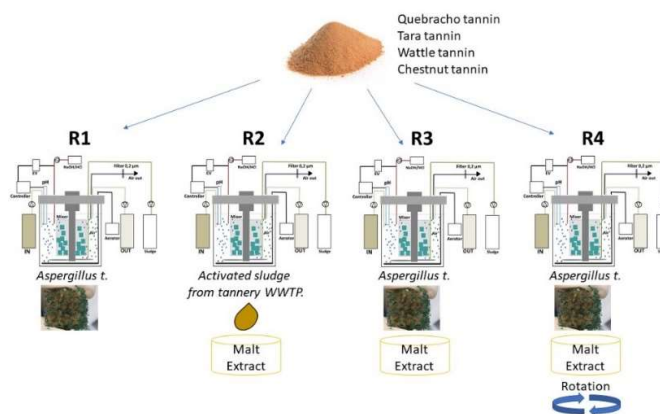
Figure 3.6: Conceptual model of the experiment performed with the four bioreactors installed at Cuoiodepur.

3.4 LONG-TERM TESTS WITH CONTINUOUS BIOREACTOR OPERATION

3.4.1 Experimental set-up

Two similar additional submerged, packed bed reactors were designed, built and installed at UAB (GENOCOV laboratories). In the setup developed for this project, each reactor consisted of a 5 L vessel (4 L of effective volume and 1 L of headspace), equipped with pH stabilisation at 5.8 ± 0.2, achieved by dosing 1 M solutions of NaOH and HCl. Air was injected through a stone diffuser and manually controlled with a rotameter (100 NL h^{-1}). Outlet air was filtered at 0.2 μm (for safety reasons). The air flow allowed for complete mixing inside the reactor. Inside the vessel, a submerged plastic cylindrical cage was inserted containing 100 immobilised PUF cubes. The cage had a radius of 5 cm, and the grid was square with 1.25 cm sides. Given the outcomes of the previous experiment, which are described in the results section, the cage was not rotating in this set-up. The probes for continuous pH, DO and temperature measurements were located inside the vessels. DO and temperature were measured with a galvanic DO sensor (Oxi 340i with CellOx 325, WTW, Germany) and pH electrodes (53 33, Crison, Spain), respectively. Temperature was maintained at $23 \pm 2°C$ (room temperature). A computer and a SCADA system developed in-house were used for data acquisition and pH regulation. A schematic representation and picture of the reactor is shown in Figure 3.7 and in Figure 3.8.
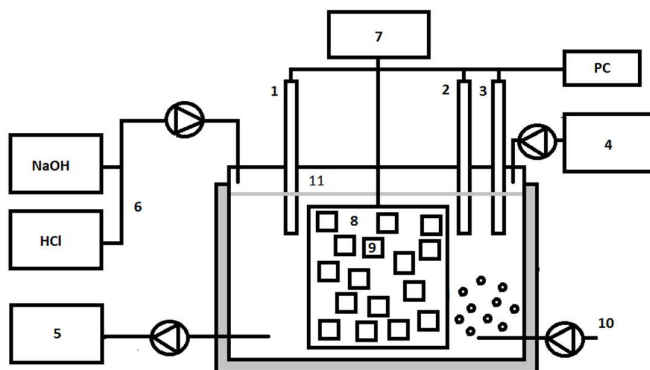
Figure 3.7: Schematic of the cage reactor design: 1) DO probe; 2) temperature probe; 3) pH probe; 4) feeding tank and pump; 5) discharge tank and pump; 6) acid tank and caustic tank; 7) engine for cage rotation (not used in this case); 8) plastic cage equipped with PUF carriers; 9) PUF cubes, 10) aerator and air sparger; 11) reactor and water level.

Figure 3.8: The UAB experimental set-up consisted of two rotating, submerged, packed bed reactors.

3.4.2   Process operation

Tests were carried out in two submerged, packed bed reactors run in parallel. The HRT and tannin concentration in the medium were varied to maximise the removal capacity. QT and TT are widely used and were selected as model recalcitrant compounds for condensed (QT) and hydrolysable (TT) tannins in tannery effluents (Romer et al., 2011; Tilli et al., 2010). RQ was fed with QT while RT was fed with TT as carbon sources. The feed of RQ was composed of 1 g L^{-1} QT, 0.1 g L^{-1} NH$_4$Cl and 0.01 g L^{-1} KH$_2$PO$_4$ dissolved in tap water, while the feed of RT was composed of 1 g L^{-1} of TT, 0.1 g L^{-1} NH$_4$Cl and 0.01 g L^{-1} KH$_2$PO$_4$ dissolved in tap water. The COD of the QT medium solution prepared to feed RQ at a concentration of 1 g L^{-1} QT was 1,433 $\pm$ 56 mg L^{-1}, which corresponded to the sCOD due to the lack of pCOD. The sCOD of the TT medium solution prepared to feed RT at a concentration of 1 g L^{-1} of TT was 786 $\pm$ 67 mg L^{-1}, which also corresponded to the total COD due to the lack of pCOD. The inlet and outlet flows were controlled with peristaltic pumps (300 series, Watson and Marlow, USA), and HRT ranged from 52 to 9 h (see Table 3.3 and Table 3.4). The tannin concentrations in the feed ranged from 0.1 to 8 g L^{-1} as reported in Table 3.3 and Table 3.4. Despite the fact that the two reactors were run in parallel, the operating conditions tested were different due to the completely different

behaviour of the bioreactors. As described in results section, different QT concentrations were tested to optimise the removal efficiency (RE) in RQ (Table 3.3). As described in the results section, the biofilm in RT was disrupted and a second start-up was performed with similar results. Consequently, the cage was removed and different HRT and TT concentrations were tested to evaluate the reactor performance and to assess the inhibition on the suspended biomass (Table 3.4). In RQ, the simultaneous treatment of QT and TT was tested from day 154 onward. RQ was fed with a medium composed of TT (1 g L^{-1}) and QT (0.1 g L^{-1}) corresponding to a total inlet sCOD of 879 ± 63 mg L^{-1}.

Table 3.3: Operating conditions of RQ fed with QT. (*) From day 154 onward, the feeding medium was composed of TT (1 g TT L^{-1}) and QT (0.1 g QT L^{-1}).

Time (days)	0	56	84	100	106	113	141	154
HRT (hours)	28	52	52	52	28	28	28	28
QT concentration (g QT L^{-1})	1	1	0.5	1	0.5	0.2	0.1	0.1*
OLR (g COD m^{-3} h^{-1})	51.1	27.5	12.9	27.5	24.0	7.2	3.6	31.0
COD (mg O$_2$ L^{-1})	1,433	1,433	672	1,433	672	202	104	869

Table 3.4: Operating conditions of RT fed with TT.

Time (days)	0	150	155	159	164	169	174	179	187	201	211
HRT (hours)	28	52	28	16	12	9	52	28	28	28	28
TT concentration (g TT L^{-1})	1	1	1	1	1	1	1	1	2	8	1
OLR (g COD m^{-3} h^{-1})	27.9	15.0	27.9	48.9	65.2	87	15.0	27.9	55.9	223.9	15.0
COD (mg O$_2$ L^{-1})	786	786	786	786	786	786	786	786	1,570	6,270	786

3.4.3   Adsorption of tannins on mycelium

The mycelium adsorption of QT and TT was determined (as sCOD) in one-week tests with flasks. The flasks were filled with 100 mL of tannins (QT and TT separately) plus two immobilised and autoclaved PUF cubes (with the same liquid/PUF ratio as the bioreactors) and stored with agitation in an orbital shaker for one week. sCOD was measured in triplicate after 24 h, 72 h and 168 h (less than 15% of the total volume of the liquid was sampled). Flasks without PUF were used as control.

3.5   RESPIROMETER SETUP

3.5.1   Respirometer at Cuoiodepur

The MARTINA respirometer/titrimeter (Spes Srl, Italy) can operate as an open static liquid-flowing gas/static gas (LFS/LFF) respirometer. A schematic layout of the respirometer and a picture are shown in Figure 3.9 and Figure 3.10, respectively. The control unit was able to perform two tests in two (2 L) plexiglass vessels simultaneously. The two jacketed vessels were each connected to a thermostatic bath (SBF 7, Falc, Italy), continuously mixed with magnetic agitators, and monitored with a temperature probe, a Hamilton pH probe and a DO probe (CellOx 325, WTW, Germany). The control unit was able to stabilise the pH set point by dosing with HCl and NaOH solutions (0.1 M) through a total isolation micro solenoid valve (D301V51, Sirai, Italy). The control unit allows the switching on and off of the aeration in order to calculate OUR and maintain the DO in the reactor within two selected DO thresholds. MARTINA also allowed the performance of tests in pH-stat, DO-stat (thorough the dosage of a diluted solution of H_2O_2) and other titrimetric tests.
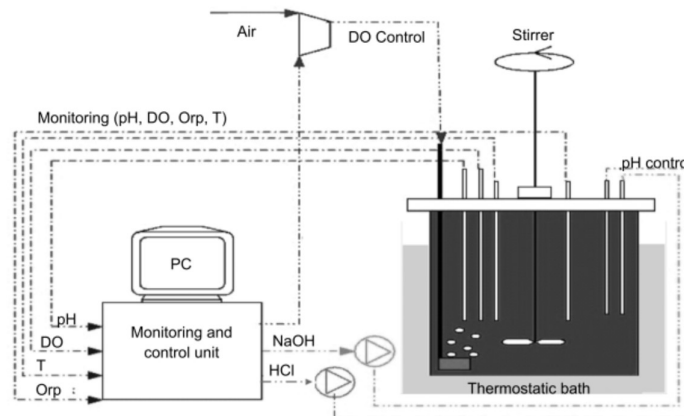
Figure 3.9: The schematic layout of the MARTINA respirometer (Munz et al., 2010).

Figure 3.10: A picture of the MARTINA respirometer.

3.5.2   Respirometer at UAB

The respirometer/titrimeter at UAB laboratories is an LFS controlled by a computer with dedicated control software in Visual Basic. Two different volumes of glass vessels were used: 0.3 L and 1 L. The 0.3 L vessels had a stainless steel cover and a stainless steel air diffuser. The respirometer was jacketed with temperature control via the recirculation of a thermostatic water batch (Polystat24, Fisher Scientific, Spain). The 1 L vessel had a stone air diffuser and was immerged in a thermostatic bath. Vessel mixing was ensured by magnetic stirrers. Temperature and pH probes (SenTix82, WTW, Germany) and a DO probe (CellOx 325, WTW,

Germany) were connected to a control station (Inolab Multi 740, WTW, Germany) and the computer for data monitoring. The pH set point was maintained by dispensed burette and the dosage of diluted solutions of NaOH and HCl (Multi-Burette 2-SD, Crison Instruments, Spain). A schematic layout of the respirometer and a picture of the set-up and the small vessel are reported in Figure 3.11 and Figure 3.12, respectively.
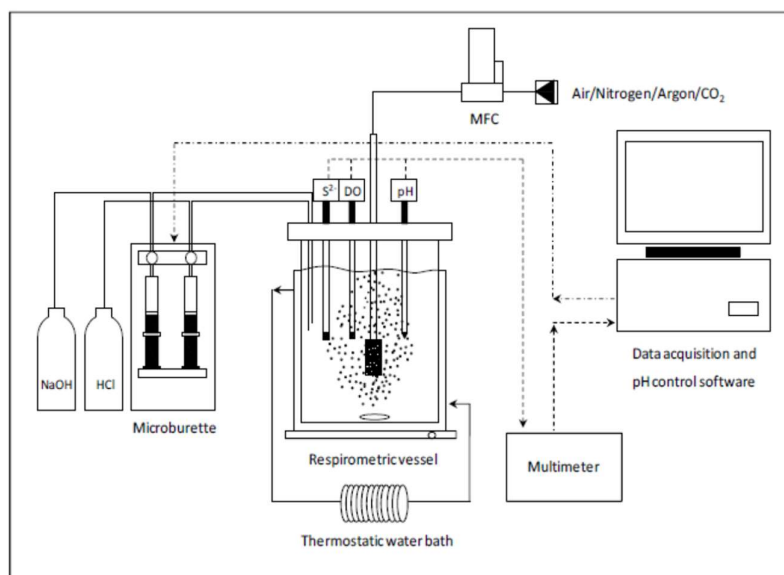
Figure 3.11: A schematic layout of the respirometer at UAB laboratories (Mora, 2014).

Figure 3.12: A picture of the respirometer at UAB laboratories and detail of the 0.3 L vessel filled with immobilised PUF cubes.

3.5.3   Tannin degradation tests by respirometry

The biodegradable sCOD fractions (sbCOD) of QT and TT (medium solution containing 10 g L^{-1} of tannins filtered at 0.45 µm) were estimated with a respirometer operating LFF (MARTINA, Spes Srl, Italy). The conventional procedure for COD fractioning was adopted, as described elsewhere (Andreottola and Esperia, 2001). The activated sludge used was obtained from Cuoiodepur tannery WWTP to ensure its acclimation to tannins. Respirometric tests were performed at pH 7.8 with a concentration of 250 mg sCOD L^{-1} of QT and a concentration of 120 mg sCOD L^{-1} of TT. The biodegradability of QT and TT were also estimated with a suspended culture of RT and immobilised PUF cubes of RQ with respirometric tests in a 1 L vessel and 0.3 L vessel, respectively. The same pH set point (pH 5.8 ± 0.2) and temperature set point (25°C) were used. A concentration of 3 g L^{-1} of suspended biomass from RT and a concentration range of 7.8 mg L^{-1} to 78 mg L^{-1} of sCOD of TT and QT were chosen for respirometric tests in the 1 L vessel. The biomass was first maintained for 24 hours in endogenous conditions and, subsequently, the samples to be tested (TT or QT) were spiked with two/three pulses to "wake up" the microbial consortia (Mora et al., 2015). The use of respirometric tests with immobilised biomass is a new technique without a defined conventional procedure, thus requiring several months of attempts to identify a feasible procedure (Mora, 2014). Fungal pellets, fresh immobilised biomass and immobilised biomass sampled from RQ were used to test and define the respirometric procedure. Since the dry mass was assessed with 3 PUF cubes, the respirometric test procedure (using a 0.3 L vessel) required the removal of 10 + 3 PUF cubes from RQ. The PUF cubes were placed in sterile water for 24 hours in endogenous conditions before the test water was replaced. The pH set point was controlled during the tests by dosing with 0.05 M NaOH and HCl solutions. The air flow was regulated at 10 N mL min^{-1} with a mass flow controller (TecFluid, USA), and mixing was guaranteed with a magnetic stirrer. The PUF cubes were fixed in the middle of the vessel, far from the surface and the bottom of the vessel to avoid direct contact with the final part of the probe and to minimise fluctuations. The resulting profile was accepted as repeatable after multiple pulses. Before each pulse, the endogenous conditions and K_{la} were evaluated with an air cut (Mora, 2014). The concentrations tested were within the

range of 47 mg L^{-1} to 476 mg L^{-1} of sCOD for QT, while the biodegradability of TT was also evaluated. The results of the respirometric tests on biomass samples from RT and RQ were coupled to the continuous operation performance and parameters estimation of Monod kinetics with Aquasim.

3.6   LONG-TERM TESTS WITH PILOT-SCALE BIOREACTOR

3.6.1   Experimental set-up

The results obtained from the experiments in bioreactors were used to design a scale-up test to evaluate performance at pilot scale. The pilot reactor was built by Italprogetti Spa (San Romano, Pisa) and installed at the tannery WWTP of Cuoiodepur (San Miniato, Pisa) in the area of Cer2co where the start-up and test phases were performed. A schematic layout of the piping and diagram of the instruments is shown in Figure 3.13. Figure 3.14 contains an overview of the pilot reactor. The pilot reactor was a cylindrical polypropylene vessel with a volume of 1.44 cubic metres with a 600-litre rotating cage inside. The cage could rotate from 1 rpm to 3 rpm and included four removable sectors filled with carriers. Each sector of the cage was filled with carriers with different sizes to evaluate the optimal size for immobilisation. The first sector was filled with 2.5 cm PUF cubes, the second one with 5 cm PUF cubes, the third one with 7.5 cm PUF cubes, and the last one with PUF cut-to-shape. The last sector was substituted with 2.5 cm PUF cubes after two weeks, however, because the sector was not stable due to aeration (Figure 3.15 and Table 3.5). In the bottom part of the reactor, an air diffuser was connected to an air blower and a peristaltic recirculation pump to avoid sedimentation inside the reactor. The feeding tank was an external, completely mixed 1 m^3 vessel. The pilot reactor was connected to a 500-litre mixed tank with co-substrate solution and a 500-litre unmixed tank with a diluted HCl solution (0.1 M during the start-up and 0.05 M during the treatment phase). Two membrane pumps (500 series, Watson and Marlow, USA) allowed the dosing of both reagents inside the reactor. A pH probe, temperature probe, redox probe and dissolved oxygen probe were installed inside the vessel. Table 3.6 reports the list of the main actuators and probes. The pH, temperature, DO, and ORP were measured with Hach probes connected to two Hach Sc200 controllers

and a dedicated PLC made by Italprogetti. Actuators and probes were connected to the control panel in order to record the data (acquisition time every 5 minutes in csv file automatically saved onto a USB drive), control the flow, maintain the pH set point, maintain the temperature set point and report malfunctions. The embedded programme of the control panel could maintain the chosen pH set point and also the temperature thanks to the recirculation of hot water (70°C) in the internal pipes of the jacketed vessel. The temperature control was a low bound control, while the pH control was a high bound control, both designed to avoid unfavourable conditions for the fungi biomass at low temperature and basic pH.
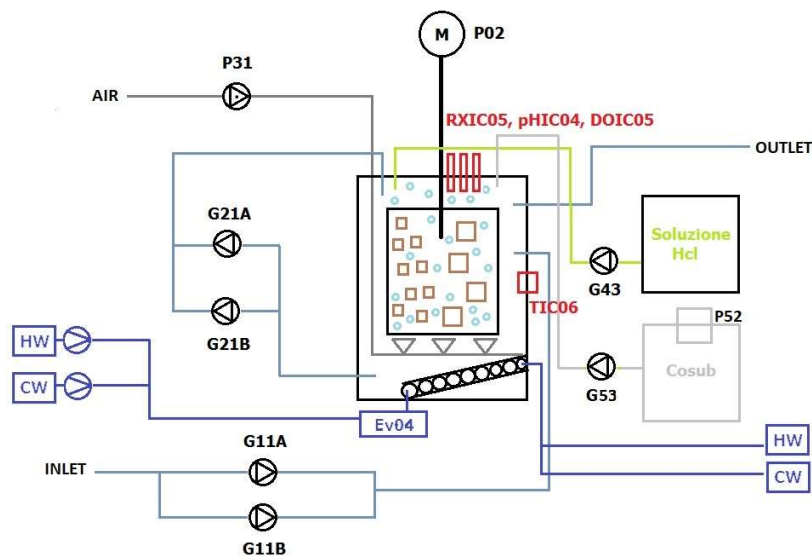
Figure 3.13: Schematic layout of the pilot-scale reactor (HW and CW: Hot water and cold water pipes; Cosub: Co-substrate tank; HCl: Acid tank).

Figure 3.14: Lateral view of the pilot reactor (left) and front view of the pilot reactor (right).

Table 3.5: Pilot-scale reactor: sector composition.

Sector	Number of PUF cubes	Type of PUF cubes
Sector 1	6,000	2.5 cm PUF cubes
Sector 2	620	5 cm PUF cubes
Sector 3	170	7.5 cm PUF cubes
Sector 4	6,000	2.5 cm PUF cubes

Figure 3.15: Internal view of the rotating cage (left) and one sector full of PUF cubes (right).

Table 3.6: Pilot-scale reactor: main sensors and actuators installed.

Actuators				
P&I	**Description**	**Supplier**	**Model**	**n**
P02	Vertical mixer	Italprogetti	Engine 63	1
G11A/B	Peristaltic pump	Watson marlow	521F/R2C	2
G21A/B	Peristaltic pump	Watson marlow	521F/R2C	2
P31	Air compressor	Techma gp	LAM200	1
G43	Dosing pump	Obl	RBB 30 P 95	1
P52	Vertical mixer	Italprogetti	Engine 71	1
G53	Dosing pump	Obl	RBB 30 AC 95	1
EV04	Valve	Omal	GF3/4"	1
Sensors				
DOIC03	DO probe	Hach	LXV416.99.20001	1
PHIC04	pH probe	Hach	DPD1R1.99	1
RXIC05	Redox probe	Hach	DRD1R5.99	1
TIC06	Temperature probe	Seico	PT 100	1

3.6.2   Process operation: start-up

The two main critical issues of the start-up were to avoid that the selected fungal strain was outcompeted by bacteria and to promote mycelium growth inside the PUF. Each sector of the cage was filled with carriers. The homogenised inoculum was equally distributed into each sector together with 2 additional L of sterile GLY (8 L in total). Every 48 h for the entire start-up, the 1 cubic metre mixed feeding tank was cleaned and renewed with a mineral medium containing 1 g L^{-1} QT, 0.1 g L^{-1} N-NH_4Cl, 0.01 g L^{-1} P-H_3PO_4, and 3 mL of commercial antifoam dissolved in tap water (COD 1,645 $\pm$ 339 mg L^{-1}). The conditions chosen to promote immobilisation in non-sterile conditions were:

- A pH set point of 5 $\pm$ 0.2 pH;
- A temperature set point of 25 $\pm$ 2ºC;
- HRT of 3.4 days;
- Cage rotation at 3 rpm for 1 minute followed by 30 minutes of pause;
- Recirculation flow activated;
- Aeration activated with low-bound control at DO 7 mg O_2 L^{-1}.

3.6.3   Process operation: treatment phase

The start-up achieved biofilm development in the pilot reactor. The purpose of the subsequent treatment phase was to test the performance of the fungal biomass on the degradation of a tannin-rich effluent collected from a Tuscany district tannery factory after the vegetable tanning phase (tanning bath). In this second phase, the same process conditions used during the start-up were maintained. The supernatant of the effluent (characterised in detail in Table 3.7) was diluted 40 times with tap water in order to obtain a COD value comparable to the start-up phase (COD 1,579 $\pm$ 331 mg L^{-1}). Only phosphorus was added as a nutrient (0.01 g L^{-1} P-H_3PO_4) since nitrogen was already not limiting for growth (the ammonium concentration was approximately 20 mg N L^{-1}). The start-up lasted 55 days and the treatment phase lasted 66 days, thus the entire experiment lasted 121 days in total.

Table 3.7: Characterisation of tannin-rich effluent collected from a tannery factory (analysed in the Cuoiodepur laboratories).

Parameter	Unit	Value
pH	pH	3.6
sCOD	mg O L^{-1}	64,331
DOC	mg C$_{org}$ L^{-1}	14,546
Total N.	mg N L^{-1}	1,071
TSS	mg TSS L^{-1}	12,540
VSS	mg VSS L^{-1}	9,050
Chlorides	mg Cl L^{-1}	13,983
Sulphates	mg S L^{-1}	5,369
Sulphides	mg S L^{-1}	120
Nitrites	mg N L^{-1}	4.7
Nitrates	mg N L^{-1}	0.5
Ammonium	mg N L^{-1}	834.7
Conductivity	mS cm^{-1}	45.3
Metals		
Chromium	mg L^{-1}	0.4
Iron	mg L^{-1}	15.8
Cadmium	mg L^{-1}	< 0.2
Lead	mg L^{-1}	< 0.2
Zinc	mg L^{-1}	3.2
Aluminium	mg L^{-1}	32.2
Copper	mg L^{-1}	0.6
Boron	mg L^{-1}	0.4

3.7 ANALYTICAL PROCEDURES

3.7.1 Analytical methods for preliminary tests with continuous bioreactor operation

The medium and co-substrate solutions were prepared three times per week, and the pH and temperature measured via the pH reader (M20R-pH) were recorded. The inlet solutions were prepared without suspended particles. Dissolved Organic Carbon (DOC) and sCOD were measured from composite samples (filtered at 0.45 µm) twice a week using a DOC and nitrogen analyser (TOC-L series, Shimazdu Analyser, Japan) and Hach cuvettes. The DOC was measured after 20 days of activity. The dissolved oxygen was measured with a Hach LDO probe. Laccase, manganese peroxidase and tannase activity were measured. The measurements of enzyme activity

were based on enzymatic oxidation with DMP (2,6-dimethoxyphenol) for laccases and manganese peroxidases (Liew et al., 2011). Tannase activity was measured by incubating the samples with TA and measuring the production of glucose. Blank controls and tannin suspension/solution controls were performed. The sCOD of the malt solution at 1 g L^{-1} was 690 mg L^{-1}, and the sCOD of the tannin solution (the medium) at 1 g L^{-1} was 720 mg L^{-1}. The average DOC of the malt solution at 1 g L^{-1} was 470 mg L^{-1} and the average DOC of the tannin solution (the medium) at 1 g L^{-1} was 540 mg L^{-1}. The standard deviation of these solutions and the outlet samples was less than 10%.

3.7.2 Analytical methods for long-term tests with continuous bioreactor operation

Samples were collected from the reactors and feeding tanks three times per week. The concentrations of DOC, COD and sCOD in the collected samples were determined using a TOC-TN analyser (TOC-L series, Shimazdu Analyser, Japan) and Lovibond COD cuvettes, respectively. The COD and sCOD measurements were performed immediately after sampling, while samples were filtered and frozen at -40°C to subsequently measure DOC. DOC and sCOD were measured after the samples were filtered with 0.45 μm acetate filters. Periodically, PUF cubes were removed to evaluate the dry mass as follows: the sampled PUF cube was dried at 105°C until constant weight. Then the PUF cube was submerged in 100 mL of water with 1 mL of commercial bleach until the biomass was completely detached. Finally, the PUF cube was cleaned with deionised water and dried again at 105°C until constant weight. TSS in the liquid influents and effluents from RQ and RT were measured according to standard methods (APHA-AWWA-WPCF, 2005).

3.7.3 Analytical methods for pilot reactor tests

A sample of inoculum (pure culture of *Aspergillus tubingensis* for the pilot reactor) was subjected to elemental analysis (C, H, N and S) through combustion at 1,200°C and an elemental analyser (CHNS Flash 2000, Thermo Fisher Scientific, Spain). Analyses were performed at the laboratories of the WWTP managing company.

During the start-up and treatment phase, the system was sampled to analyse the COD, sCOD, and DOC with the same procedures described in section 3.7.1. Phosphorus, Sulphate, Nitrite, Nitrate and Ammonium were measured with Hach cuvettes and a Hach spectrophotometer. The dry mass of PUF cubes (2.5 cm size), TSS and VSS were measured in triplicate according to APAT CNR IRSA 2003. All analyses were performed for inlet and reactor samples with the frequency described in Table 3.8. Furthermore, a liquid sample and a biofilm sample obtained from PUF cubes were frozen every week. Some samples were later selected to perform DNA extractions with FastDNA™ spin kit for soil (MpBio, US), and the corresponding water samples were used for ecotoxicology analysis (data not shown, extraction process ongoing).

Table 3.8: Measurement of the pilot reactor during the start-up and the treatment phase. 3w and 1w: the parameter was measured three times per week or once per week, respectively.

Parameter	Sampling Point	Frequency	Methods
COD	Inlet	1w	Hach cuvettes
sCOD	Inlet	1w	Hach cuvettes
COD	Reactor	3w	Hach cuvettes
sCOD	Reactor	3w	Hach cuvettes
DOC	Inlet	3w	TOC analyser
DOC	Reactor	3w	TOC analyser
TN	Inlet	3w	TOC analyser
TN	Reactor	3w	TOC analyser
NH_4^+	Inlet	1w	Hach cuvettes
NH_4^+	Reactor	1w	Hach cuvettes
NO_2^-	Inlet	1w	Hach cuvettes
NO_2^-	Reactor	1w	Hach cuvettes
NO_3^-	Inlet	1w	Hach cuvettes
NO_3^-	Reactor	1w	Hach cuvettes
PO_4^{3-}	Inlet	1w	Hach cuvettes
PO_4^{3-}	Reactor	1w	Hach cuvettes
TSS, VSS	Inlet	3w	Standard methods
TSS, VSS	Reactor	3w	Standard methods
Dry mass	Reactor	1w	Standard methods (modified)

3.7.4   Metagenomic DNA analysis in long-term tests with continuous bioreactor operation

Fungi and bacteria communities were analysed in the biofilm collected from PUF cubes in RQ after 70 days of operation (RQ-28h), while suspended fungi and bacteria communities were analysed in RT under steady-state conditions on day 159 (RT-28h) and 174 (RT-9h), corresponding to an HRT of 28 hours and 9 hours, respectively. The biofilm sample from a PUF cube (RQ) and suspended biomass samples (RT) were collected and washed with a phosphate buffered saline solution (0.01 M P-PO$_4^{3-}$). The biofilm sample of a PUF cube removed from the cage was obtained by removing the biomass with a sterile spatula. The suspended biomass from RT was collected in a sterile Eppendorf. Pellets were obtained by centrifugation (5,000 rpm) followed by removal of the supernatant (Thermo Scientific Hareus Pico17, USA). DNA was extracted using a MoBio PowerBiofilm® DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer instructions. DNA quality and quantity were measured using a NanoDrop® spectrophotometer (Thermo-Scientific, USA). A total of 50 ng of DNA was used for the production paired-end libraries and for the sequencing of the 16S and ITS2 fragments. The primers used are reported in Table 3.9. The resulting paired-end libraries were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Bio-tek) and pooled in equimolar amounts according to the Qubit dsDNA high-sensitivity (HS) assay. The pools were sequenced using Illumina MiSeq PE300 run. The raw data obtained from sequencing was de-multiplexed, and the sequences of the primers were removed. The fastq files were assembled using a PEAR assembler (Zhang et al., 2014), followed by a quality check using BBmap tools and FastQC (Bushnell, 2014). Sequences with a length of less than 300 bp and bases with a Phred quality score lower than 30 were removed. Chimera sequences were removed using UCHIME (Edgar, 2016). The assembly was processed using the QIIME (Caporaso et al., 2011) pipeline to determine the Operational Taxonomic Units (OTUs) and to analyse the alpha diversity. The Ribosomal Database Project (RDP) 16S and the UNITE ITS database were used as reference databases in the selection step of the OTUs, performed by the SortMeRNA methods with a 97% threshold. The OTUs identified with low-confidence, related to less than 0.1% of the reads (0.1% is the estimate index miss-assignment on Illumina

Miseq), were removed. The numbers of high-confidence sequences were normalised based on the smallest library by using the Deseq2 method, except for rarefaction curve calculation. The Shannon, Chao and Simpson alpha-diversity indexes were calculated by using QIIME and Mothur methods to characterise the microbial community.

Table 3.9: Primers used to amplify 16S and ITS2 regions to sequence bacterial and fungal rRNA**.**

16 S	515F:
	5'- GTG CCA GCM GCC GCG GTA A - 3'
	909R:
	5' - CCG TCA ATT YHT TTR AGT - 3'
ITS2	ITS4:
	5' - TCCTCCGCTTATTGATATGC - 3'
	ITS3:
	5' - GCATCGATGAAGAACGCAGC - 3'

4 FUNGAL STRAIN SELECTION AND LAB-SCALE CONTINUOUS REACTOR OPERATION

Chapter abstract

This chapter describes the results of the lab-scale tests performed. The chapter is divided in three sub-sections: the fungal strain selection, the preliminary tests with continuous bioreactor operation and the long-term tests with continuous bioreactor operation.

In the first sub-section describing the fungal strain selection, the results of the tests in flasks are described. These tests allowed for the selection of one fungal strain that was used in subsequent operations with continuous bioreactors. The selection tests were carried on preliminarily isolated and identified suitable allochthonous and autochthonous fungal strains. The medium used was a sample collected after the clarifier of Cuoiodepur WWTP. COD removal, glucose consumption, ecotoxicology results and tannase production were assessed.

In the second sub-section on preliminary tests with continuous bioreactor operation, the first experiment in bioreactors is presented. Bioreactors labelled as R1, R2, R3 and R4 operated with continuous feeding for two months in non-sterile conditions. The experiment was designed to evaluate the role of specific process parameters such as the initial inoculum (R1-R2), co-substrate addition (R3) and rotation (R4). The evaluation focused on the stability of the bioprocess and performance in the removal of the target recalcitrant compounds (tannins) in non-sterile conditions. The reactors were fed with a mix of tannins, while malt extract was used as a co-substrate. DOC, sCOD removal and laccase, manganese peroxidase and tannase production were measured.

The last sub-section describes the long-term tests with continuous bioreactor operation, in which two lab-scale reactors, RQ and RT, were fed continuously under

non-sterile conditions with QT and TT that are widely used and were selected as model recalcitrant compounds for condensed (QT) and hydrolysable (TT) tannins in tannery effluents, respectively. The tests were carried out in two submerged, packed bed reactors run in parallel. The reactors were inoculated with *Aspergillus tubingensis* attached to polyurethane foam cubes. The reactors were operated for approximately 200 days under different operational conditions with respect to HRT and the organic loading rate. Moreover, RQ was fed with both tannins simultaneously during its final operation stage. No co-substrate was added. The removal efficiency, volumetric COD elimination rate, specific maximum growth rate and yield coefficient were measured. Microbial characterisation was carried out on samples from both reactors using biomolecular analysis techniques. A stable fungal biofilm was maintained in RQ by efficiently removing DOC and sCOD, while fungal biofilm detachment and proliferation of bacteria were observed in RT. Since the biomass was in suspended form, the support medium was removed and the reactor became a standard continuous-flow stirred-tank reactor. Despite the detachment, the performance of the reactor was promising (90% RE of sCOD).

4.1   FUNGAL STRAIN SELECTION

The composite sample (collected after the clarifier of Cuoiodepur WWTP) used in the test was modified to a glucose concentration of 1 g L^{-1}, corresponding to a COD of 1,245 mg L^{-1} in the abiotic sample at the end of the treatment. As shown in Table 4.1, CODs obtained at the end of the test were inconclusive since many species showed an important reduction in COD (calculated with respect to the abiotic sample), further complicated by the difficultly in distinguishing between the effect of adsorption and effective degradation.

Table 4.1: COD values measured after the test. RE COD calculated with respect to the abiotic sample.

n	Fungal strains	COD (mg L^{-1})	RE COD (%)
1	*Aspergillus niger* MUT 983	400	68%
2	*Aspergillus niger* MUT 918	574	54%
3	*Aspergillus tubingensis* MUT 990	318	74%
4	*Paecilomyces varioti* MUT 1125	353	72%
5	*Scedosporium apiospermum* n. 1	261	79%
6	*Scedosporium apiospermum* n. 3	279	78%
7	*Graphium putredinis* n.1	305	76%
8	*Scedosporium apiospermum* n. 8	278	78%
9	*Scedosporium apiospermum* n. 9	312	75%
10	*Cladosporiu cladosporioides*	228	82%
11	*Pseudallescheria ellipsoidea* n. 3	340	73%
12	*Chaetomium sp.*	440	65%
13	*Trematosphaeria grisea* n. 1	296	76%
14	*Myrotecium sp.*	308	75%
15	*Trematosphaeria grisea* n. 2	329	74%
16	*Chaetomium cochliodes*	278	78%
17	*Chaetomium tarraconensis*	282	77%
18	*Trematosphaeria grisea* n. 3	287	77%
19	Sterile mycelium	282	77%

Glucose consumption was monitored, with results indicating that all the allochthonous fungi and five autochthonous strains completely consumed the available glucose after 24 hours of treatment (with residual glucose concentration between 0.097 g L^{-1} and 0.105 g L^{-1}). It is important to note that low glucose content sustains the fungal biomass

during the first growth phases, which later generally switch to the consumption of secondary carbon sources (Yang et al., 2009). The glucose was consumed within 72 hours of treatment, but this later consumption could also be related to the bacteria naturally present in the sample. The residual glucose concentration ranged between the 0.052 AU L^{-1} for *T. grisea* n. 1 to 0.098 AU L^{-1} for *T. grisea* n. 2 as shown in Figure 4.1.
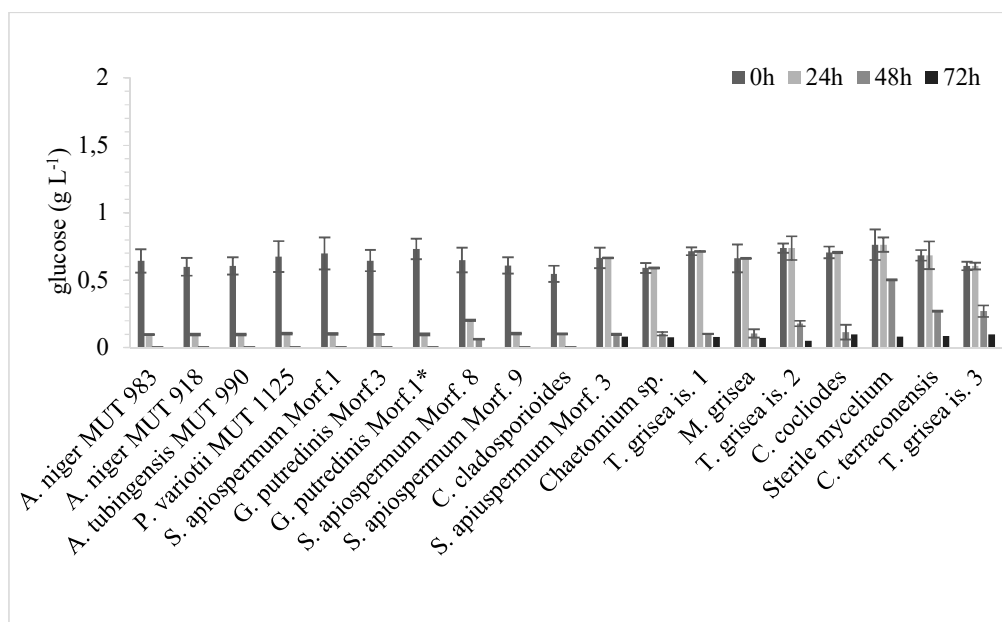
Figure 4.1: Glucose concentration measured during the experiment.

Ecotoxicity was expressed as inhibition of algal growth in a dose-effect chart (Figure 4.2). The inhibition effect was very well correlated to the dose of the sample, achieving 31% of inhibition in the presence of a dose of 50%. Modification of sample pH did not modify the ecotoxic effect over 25% of dosing, whereas below this value the sample no longer demonstrated the biostimulation effect. The results of the ecotoxicity test performed after the fungal treatment are reported in Table 4.2. Ecotoxicity variation is expressed with respect to both the untreated sample and the abiotic control at a dose of 12.5%. After treatment, almost all fungi caused an algal growth that was inhibited in the range of 5-50%, determined by *S. apiospermum* 9 and *M. grisea*, respectively. The results correspond to an increase of the sample ecotoxicity of 148-623% with respect to the untreated tannery effluent. In three cases (*A. niger* MUT 918, *A. tubingensis* MUT 990 and *G. putredinis* 3), the sample still created a biostimulation effect (from -4% to -19%) with an ecotoxicity decrease of 63-97%. The elaboration of

ecotoxicity variations with respect to the abiotic control amplified this effect even more: the ecotoxicity increase was between 313% and 2,438%. On the contrary, the ecotoxicity reduction was between -62% and -782%. Another relevant parameter was the variation of the tannin concentration present in the Act as an indirect measure of tannase enzymatic activity, that was related to the degradation of TA into glucose and gallic acid in absence of other carbon source, as shown by spectrophotometric analyses. At the end of the experiment, all strains tested achieved a decrease in TA. The highest tannin decrease was obtained by *A. niger* MUT 918 (88%), followed by *A. tubingensis* MUT 990 (86%), *A. niger* MUT 983 (80%) and *M. grisea* (59%).

Table 4.2: Results of ecotoxicity tests performed after fungal treatment, expressed as ecotoxicity variation percentage (%) with respect the untreated sample and the abiotic control (*the value of algal growth inhibition refers to the 12.5% sample dilution).

Tannery wastewater	Fungal strain	Inhibition (%)*	st. dev	ecotoxicity Δ (%) with respect to the untreated sample	ecotoxicity Δ (%) with respect to the abiotic control
1	*A. niger* MUT 983	9.16	± 4.04	196	530
2	*A. niger* MUT 918	-3.56	± 3.39	63	-67
3	*A. tubingensis* MUT 990	-18.78	± 2.99	-97	-782
4	*P. variotii* MUT 1125	21.44	± 4.32	325	1,107
5	*S. apiospermum* n.1	7.42	± 14.37	178	448
6	*G. putredinis n.3*	-7.53	± 12.06	21	-253
7	*G. putredinis n.1*	5.80	± 9.47	161	372
8	*S. apiospermum n. 8*	6.93	± 3.20	173	425
9	*S. apiospermum n. 9*	4.54	± 1.31	148	313
10	*C. cladosporioides*	16.81	± 6.40	277	889
11	*S. apiospermum n. 3*	12.72	± 5.06	234	697
12	*Chaetomium* sp.	17.88	± 7.96	288	939
13	*T. grisea* n. 1	17.35	± 1.67	282	915
14	*M. grisea*	49.80	± 34.34	623	2,438
15	*T. grisea* n. 2	11.59	± 3.89	222	644
16	*C. cochliodes*	17.33	± 3.95	282	914
17	*C. tarraconensis*	47.40	± 2.42	598	2,325
18	*T. grisea* n. 3	23.46	± 1.67	346	1,201
19	Sterile mycelium	15.37	± 9.45	261	822

Among the allochthonous fungi, on the other hand, the results indicated that all strains were suitable as potential candidates for the tannery treatment. Of particular interest

was the *A. tubingensis* MUT 990 due to the encouraging ecotoxicology results, COD removal and glucose consumption in the first 24 hours, as well as the high tannase production. This strain is widely diffused among black Aspergillus (Mirhendi et al., 2016). Moreover, the isolation substrate of the strain (pure tannin powders) was an indication of its ability to exploit tannins as a carbon source. In general, *Aspergillus spp*. have shorter cultivation periods (2–7 days) than white-rot fungi (Öngen et al., 2007). However, there are some constraints on the use of *Aspergillus spp*.: some strains of *Aspergillus* section *Nigri* members have the ability to produce ochratoxin (Varga et al., 2003). As a consequence, this study has focused on the use of non-ochratoxin-producing *Aspergillus spp.*, such as *A. tubingensis* MUT 990.
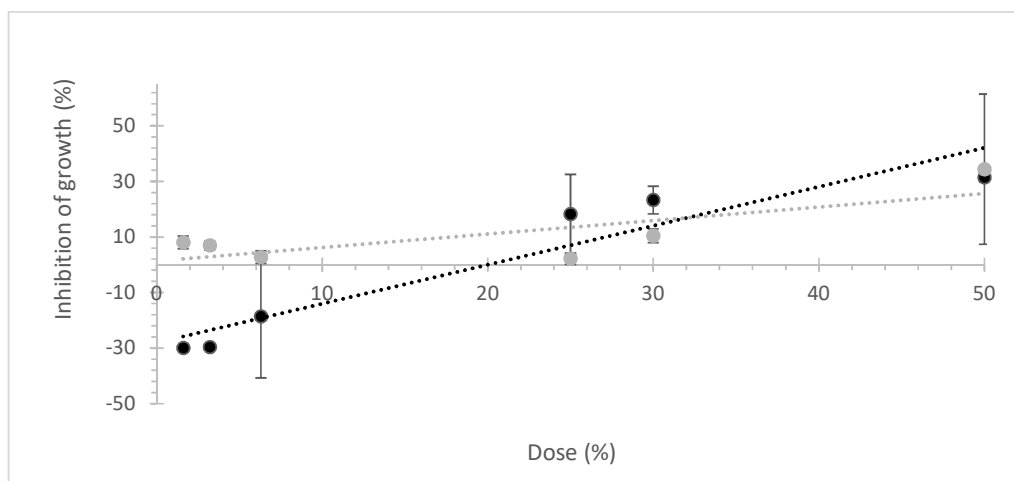
Figure 4.2: Initial ecotoxicity of *R. subcapitata* before fungal treatment, comparing the Dose/Inhibitory effect of untreated composite sample (black) at different dilutions with the abiotic control (pH and glucose modified) at different dilution (grey).

4.2 PRELIMINARY TESTS WITH CONTINUOUS BIOREACTOR OPERATION

4.2.1 Bioreactor performance

Bioreactors R1, R2, R3 and R4 operated with continuous feeding for two months under non-sterile conditions. They were stable and able to reach a high RE for DOC and sCOD. R1, working in the absence of malt extract as the co-substrate

for fungal biomass growth, initially showed good performance with sCOD removal of up to 70-80% (Figure 4.3).
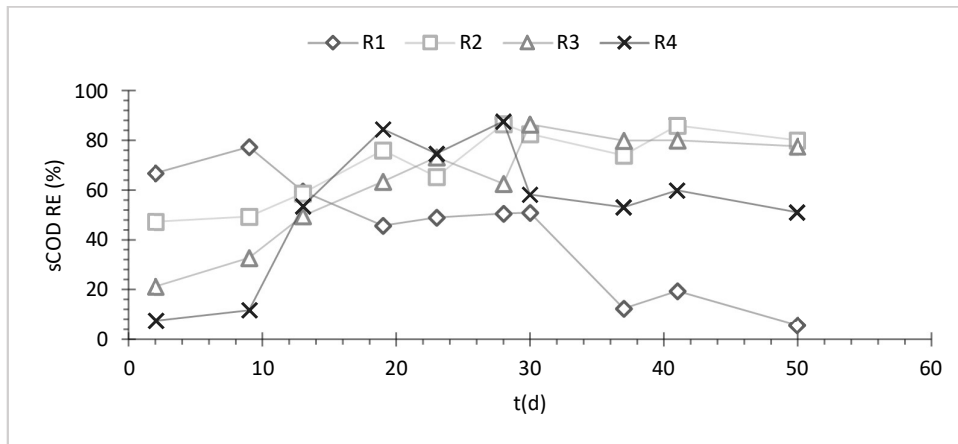
Figure 4.3: The sCOD RE during preliminary tests with continuous bioreactor operation.

However, after the first ten days of operation, tannin removal was reduced to lower values and the sCOD RE dropped to about 20% in the final period. As shown in the comparison between R1 and R3 (with co-substrate addition), the malt extract dosage was able to support the biomass and the RE. In fact, during the first 10 days of incubation, reactor R3 showed decreased performance with respect to R1. However, within 30 days, R3 achieved a high level of removal performance that was stable (Figure 4.3 and Figure 4.4), with 80-90% RE of DOC and sCOD recorded. The observed trend can be explained by an initial fungal growth on malt extract (the co-substrate), with a shift to tannins as the carbon source when the fungal biomass reaches a critical point allowing it to overcome the inhibition of tannins and maintain competitiveness within the microbial community found in the non-sterile environment.
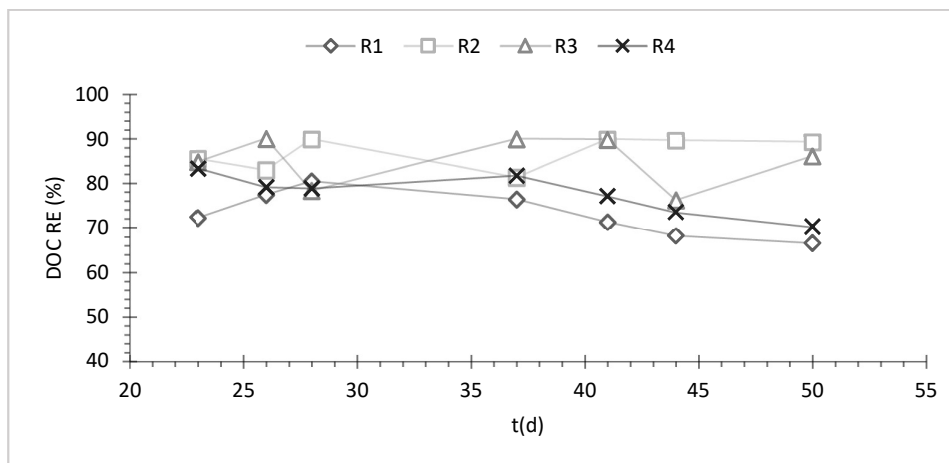
Figure 4.4: The DOC RE during preliminary tests with continuous bioreactor operation.

At the same time, it was evident that the fungal biomass in R1 showed a significant sCOD RE, suggesting that the fungal strain was a candidate for tannin removal in non-sterile conditions once the growth conditions were optimised. Reactor R4 operated under the same conditions of R3, except for the addition of cage rotation at 3 rpm with respect to R3, which lacked rotation. Reactor R4 showed lower performance at the beginning and at the end of the experiment. However, after 30 days of operation, it showed encouraging sCOD RE percentages (up to 90%) with a similar trend in RE compared to R3. Generally, mechanical stirring devices impact the system by both improving the contact between phases (i.e., air bubbles and microorganisms) and causing damage to cells at high turbulent flow due to shear stress (Serrano-Carreón et al., 2015). However, in this case the rotation rate was very low and the fungal mycelium was protected by both the PUF and the metal cage. Thus, the negative stress due to shear forces was negligible and an important decrease in RE was not recorded. Reactor R2 was inoculated with activated sludge and represents the theoretical performance of WWTP autochthonous biomass developed in the operational conditions used in the fungal reactors. As shown in Figure 4.3, the sCOD RE was stable at approximately 80% after 30 days and, as shown in Figure 4.4, the DOC RE was at approximately 90%. The performances obtained were similar to those recorded in reactor R3 inoculated with the selected fungal strain. An inhibitory effect of the tannins on the biomass was evident during the first 10 days of incubation when the RE in R2 was significantly lower than that observed in R1. Results obtained suggested

that the fungal strain was more resilient to the eventual inhibition of tannins than WWTP activated sludge, confirming the possibility of exploiting the fungal candidate's resilience.

4.2.2   Tannase activity

Tannase activity was measured in all reactors as shown in Table 4.3. On the contrary, neither laccases nor manganese peroxidases were detected in the reactors. Similar results have been described in the literature. Hanafi (2013) and Öngen (2007) found 70% sCOD removal from OMW and 370-650 AU L^{-1} of tannase activity, with negligible laccase and peroxidase activity. Enzyme measurements revealed a maximum tannase activity of up to 360 AU L^{-1} achieved in R4 at day 9. This high tannase activity recorded in R4 could be partially related to the rotation that improved the tannase diffusion in the liquid. However, shaking conditions have also been demonstrated to improve tannase production by *Aspergillus spp*. (Aboubakr et al., 2013). The mechanisms regulating microbial tannase production are currently not well understood. Some authors suggest that gallic acid (a degradation product of tannin degradation) could act as both an inducer and inhibitor of tannase production, depending on its concentration (C. N. Aguilar et al., 2001). This could partially explain the behaviour observed in R4, which was inversely proportional to the sCOD RE, with an initial high tannase activity in the first week followed by a decrease. In reactor R2, which was not inoculated with fungi, measurable tannase activity was recorded (ranging from a maximum of 62 to a minimum of 9 AU L^{-1}). However, the presence of tannase activity cannot be considered the only variable in evaluating fungi activity, since some bacteria can also produce tannase (Vaquero et al., 2004). Tannase activity is, nevertheless, an index of the natural presence of organisms with the ability to biotransform tannins via tannase in activated sludge from tannery wastewater treatment plants. Actually, each wastewater treatment plant selects a typical community of activated sludge able to degrade the pollutants that are present in the plant (Shchegolkova et al., 2016). The autochthonous microorganisms in R2, on the whole, achieved similar sCOD RE performance, but the enzymatic activity of the selected fungus in R3 is substantially higher. A comparison of the tannase activity in

R1 and R3 provides information about the effect of malt extract as a co-substrate in the treatment. The results indicated that the additional source of carbon promoted tannase production, contrary to that observed by other authors (Aboubakr et al., 2013). Alternatively, the co-substrate may have supported the growth of the fungal biomass and, potentially, tannase production. Finally, the low enzymatic activity recorded in the first 10 days in R1 and R2 confirms the hypothesis that biosorption was the main process involved in the pollution removal during that initial period.

Table 4.3: Maximum tannase activity measured in each reactor during preliminary tests with continuous bioreactor operation.

Tannase (AU L^{-1})	R1	R2	R3	R4
Day 9	56	49	163	360
Day 23	63	62	109	205
Day 41	119	46	162	145
Day 50	88	9	94	186

4.2.3 Biomass degradation capacity

Even though *Aspergillus tubingensis* has been successfully used in a biotrickling filter reactor to treat gas with monoethylene glycol (Esmaeili and Loghmani, 2016), to the best of our knowledge no fungal-based bioreactors have been used for the biological degradation on natural tannins (either non-sterile or sterile). A stable system based on a fungal consortium was reached in R3 thanks to the selection of the operating conditions. It is possible to hypothesise that the conditions chosen for R2 allowed for the development of a tannin-degrading microbial consortium, including both fungi and bacteria. Nevertheless, further studies are required to better characterise the organisms developed. It was observed that the fungal biomass changed to a darker colour, probably due to the adsorption of tannins onto the biomass as previously suggested by other authors (Öngen et al., 2007). Adsorption is a relatively fast phenomenon, and it played a role in removal within the first few days of operation. The recorded decrease of sCOD and DOC at the later sampling times could be

plausibly ascribed to biotransformation processes. The ratio between sCOD and DOC allowed insight into whether the biotransformation of tannins occurred. The typical ratios COD/TOC in municipal wastewater is in the range 3.5-2 (Henze et al., 2008). The average ratio between sCOD and DOC was 1.48 for the inlet malt extract solution and 1.35 for the inlet tannin solution. However, the ratio between sCOD and DOC in the outlet changed with a higher value (as represented in Table 4.4), and this behaviour could be related to a tannin depolymerisation.

Table 4.4: Average ratio between sCOD and DOC in the outlet during the whole testing period of preliminary tests with continuous bioreactor operation.

	R1	R2	R3	R4
sCOD/DOC	2.8 ± 0.6	2.3 ± 0.6	2.5 ± 0.2	2.7 ± 0.6

To summarise, it was possible to run a fungal reactor in non-sterile conditions for two months thanks to the appropriate choice of design and operating conditions. The selected fungal strain seemed able to cope with tannins, but it was hypothesised (and verified in the long-term tests described in section 4.3) that the degradation ability of the fungal strain may be different for different tannins. Rotation could be applied to control the biomass retention, but the frequency and duration of the rotation should be evaluated accurately to avoid excessive stress on the biofilm and obtain a fine tuning of the biomass retention time. Malt extract seemed to favour the fungal inoculum and also the development of a microbial consortium bioactive against one or more of the four tannins introduced (given the selected operational conditions and reactor design). It was hypothesised and confirmed (with the tests exposed in the next section) that different tannins may have a different effect on the systems and may influence the consortium.

4.3   LONG-TERM TESTS WITH CONTINUOUS BIOREACTOR OPERATION

The long-term tests with continuous bioreactors performed at UAB focused on two representative tannins. Hydrolysable (TT) and condensed (QT) tannins were

treated with similar process conditions without cage rotation (due to the apparent negative effects in the previously tested conditions) and without co-substrate (to have a better understanding of the effect of the tannins alone).

4.3.1 Performance of RQ in QT removal

In RQ, the biofilm was stable and effective for five months of operation in non-sterile conditions, leading to an average outlet concentration of TSS of 0.03 g L^{-1} during the experimental period. Thus, the outlet COD was mainly sCOD. The pCOD was less than 4% of total COD and likely composed of detached biomass. Consequently, sCOD was the parameter chosen to evaluate reactor performance. In addition, the ratio between sCOD and DOC at the reactor inlet was determined to be 3.02 ± 0.15. Unfortunately, DOC measurements in the effluent were not reliable due to a coagulation phenomenon that occurred during sample storage at -20°C. RQ was fed with a solution of 1,433 mg COD L^{-1} until day 56 of operation (Figure 4.5). Despite some oscillations, the sCOD RE was in the range of 4% to 34%. However, the average value (17%) was higher than the 8% RE determined via respirometric tests carried out with an activated sludge sample collected from a tannery WWTP. This difference was related to the fungal biomass inoculated, which was probably more effective in QT removal than the autochthonous species of the activated sludge from a tannery WWTP. It may also depend on the chosen operating conditions of the reactor (acidic pH vs neutral pH), which could have influenced the performance of the inoculated fungal strain and the microbial communities developed during reactor operation. As further discussed in the following section, the long-term operating conditions generated enough selective pressure to drive the evolution of the ecosystem by increasing the diversity of fungi and promoting the growth of bacteria. As described in Table 3.3, after a start-up phase of 56 days, the HRT was increased from 28 to 52 hours, maintaining the inlet COD concentration constant in order to verify if a longer contact time could improve the low RE due to the low processes kinetics. However, this strategy did not enhance the RE. The average RE during this period was 12%. Consequently, from day 84 to day 100, the inlet concentration was halved and the HRT was kept at 52 h to determine whether the high concentration of tannins was inhibiting

the biomass as suggested in the literature (Nelson et al., 1997). Since this strategy was not effective, the inlet concentration of QT was progressively reduced after day 106 from 1 to 0.1 g L^{-1} of QT (Table 3.3). As shown in Figure 4.5, as soon as the inlet concentration was reduced to 202 g COD m^{-3} (equivalent to 0.2 g QT L^{-1}), the RE started to increase, demonstrating the effectiveness of this strategy. A further inlet QT concentration decrease on day 141 day led to a sCOD RE increase to 53% once the system reached a new steady-state (which corresponded to a volumetric elimination capacity of 9 g sCOD m^{-3} h^{-1}). The biofilm showed a larger degradation capacity due to the reduced inhibition at lower inlet QT concentration, as also observed by other authors in lab-scale tests with fungi and bacteria and in continuous tests with antifouling coatings based on tannins (Bellotti et al., 2012; Nelson et al., 1997).
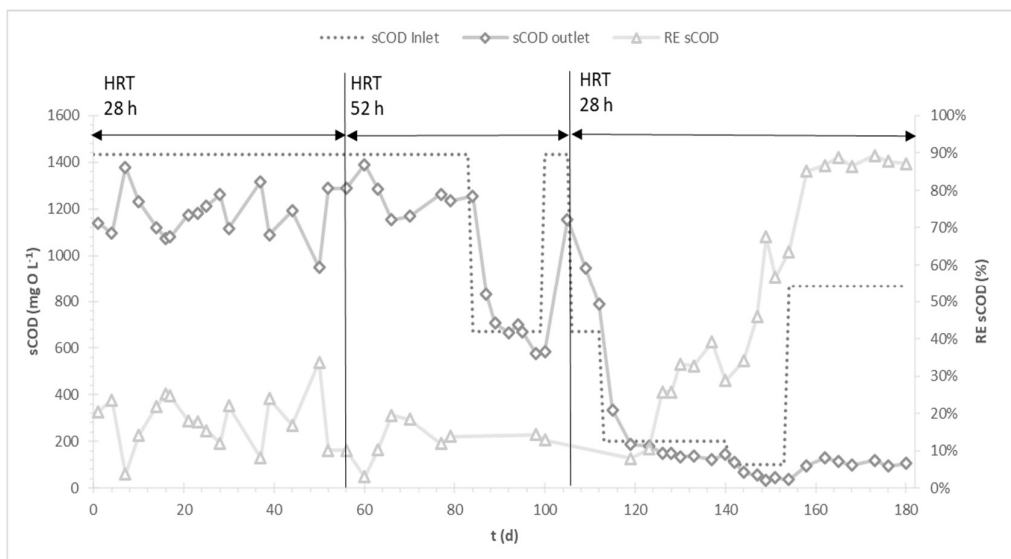
Figure 4.5: Inlet and outlet sCOD and sCOD removal percentage during continuous treatment in bioreactor RQ.

It is worth mentioning that some PUF cubes were collected from the reactor after 24 hours of operation and cut into two halves. The cross section showed that diffusion and adsorption of tannins occurred in the external layers of the PUF cube (Figure 4.6 and Figure 4.7). Thus, in order to assess the role of adsorption in COD removal, the adsorption of QT on mycelium was estimated with flask tests on autoclaved PUF cubes using the same ratio of fungi/liquid as used in the reactor. Adsorption reached equilibrium in approximately 72 h, while 31% of the initial sCOD concentration was

removed. Adsorption can thus be seen as a relevant phenomenon only at the beginning of the reactor operation as well as right after each change of influent load (either flow rate or inlet concentration changes). However, adsorption was considered negligible with respect to the observed COD removal once the system reached a new steady-state (at least 5-6 days after any stepwise inlet change performed at 28 hours HRT). The biomass content of the reactor was estimated on the basis of the dry mass inside PUF cubes (Figure 4.8). The dry mass inside PUF increased for 100 days from an initial value of 0.03 g to 0.1 g of dry mass per cube. Growth then slowed, and the dry mass reached 0.12 g per PUF at the end of the experiment, additionally due to the reduction of the inlet concentration of QT. The sCOD balance during the first 100 days of operation, considering the amount of biomass grown in that period, leads to an observed yield coefficient of 0.19 g COD g COD^{-1}, while the value for the biomass yield suggested in literature for *Aspergillus niger* (this strain belongs to section Nigri) with glucose was 0.37 g dry mass g glucose^{-1} (which would correspond to approximately 0.51 g COD g COD^{-1}).

Figure 4.6: Cross section of a PUF cube collected from the reactor RQ fed with QT after 24 h of operation (left) and after 1 week of operation (right). The tannin adsorption was clearly visible in the darker external ring of the cross-section of the PUF on the left.
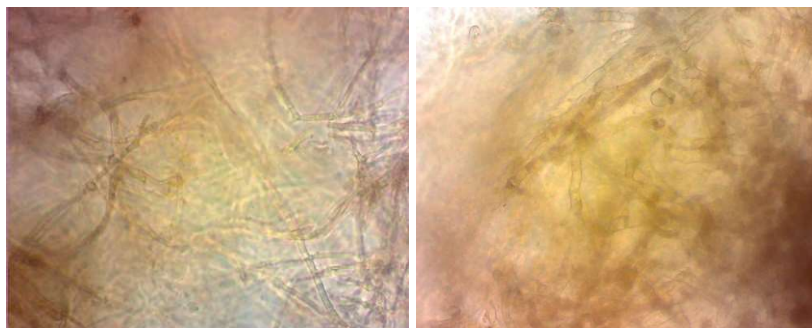
Figure 4.7: Photograph of the external border of a PUF cube after 24 hours (left) and after 7 days (right) taken at 100x magnification.
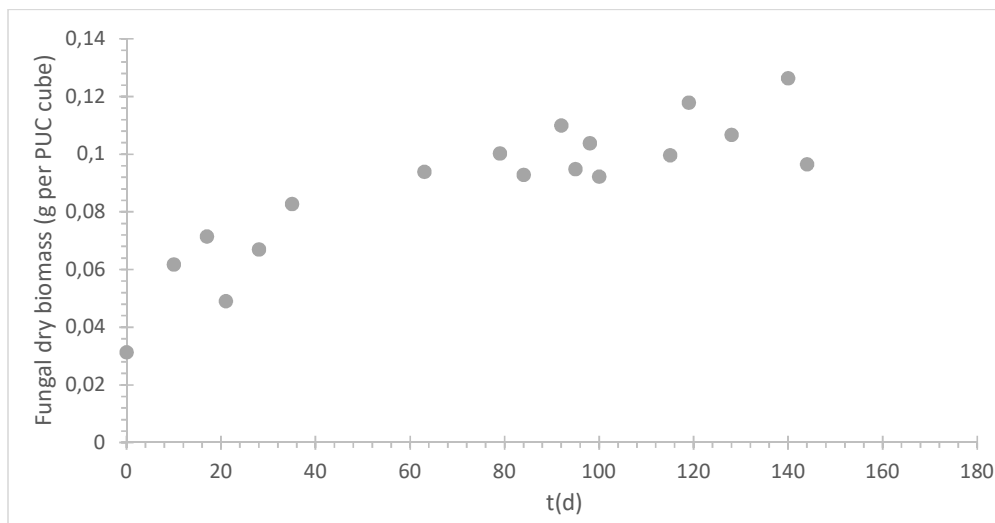
Figure 4.8: Average fungal dry biomass measured in PUF cubes during the experimental period in reactor RQ fed with QT.

4.3.2   Performance of RT in TT removal

Start-up of RT was performed as in RQ. However, detachment of the fungal biofilm and growth of the biomass in suspended form were observed during the first two weeks. Thus, a new start-up was performed with fresh *Aspergillus tubingensis* MUT 990 to verify the repeatability of the observed phenomenon. The same results were obtained (Figure 4.9): after two weeks the PUF cubes were almost devoid of biofilm (the dry mass inside the PUF cubes was below the detection limit of the measurement procedure). The fungal biofilm detachment may have been provoked by

bacterial proliferation, which could create competition for the TT substrate and also damage the mycelium (Espinosa-Ortiz et al., 2016).
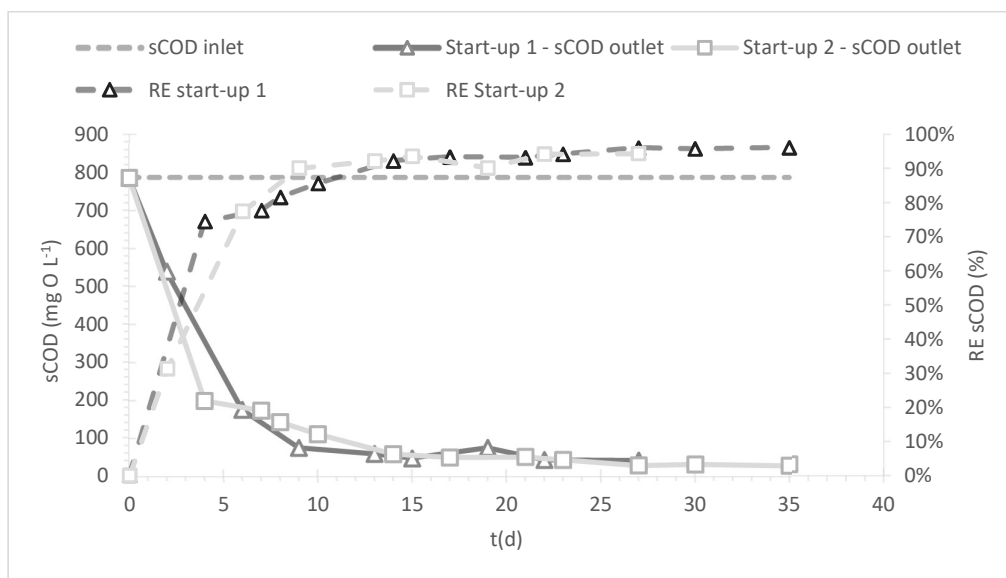
Figure 4.9: sCOD and sCOD removal (%) in two different start-up periods of RT fed with TT.

Since the biomass was in suspended form, the support medium was removed, and the reactor became a standard Continuous Stirred Tank Reactor (CSTR). Despite the detachment, the performance of the reactor was promising. After twenty days, a stable 90% sCOD RE was achieved (which corresponded to a volumetric elimination capacity of 40 g sCOD m^{-3} h^{-1}), much higher than the 19% RE found with respirometric tests with activated sludge from a tannery WWTP. Though TT, like other tannins, has toxic or inhibiting effects on various organisms, some bacteria, fungi and yeast have the ability to degrade tannins (Bhat et al., 1998). Since gallotannins, one of the main component of TT, are among the easiest tannins to be biodegraded, it is reasonable to think that there was a fast development of naturally selected suspended biomass in RT with an active role in TT biotransformation (Mingshu et al., 2006). Two experiments were carried out beginning on day 150 to evaluate the performance of the reactor at different inlet loads. Further tests were carried out after four months of operation under steady-state conditions. Firstly, the HRT was reduced stepwise from 52 hours to 9 hours while maintaining the inlet TT concentration. Secondly, the HRT was set at 28 hours, and the inlet concentration was increased stepwise up to 8 g L^{-1} of TT (Table

3.4). Performance was assessed once a steady-state was reached at each step (i.e. at least four times the HRT). At an HRT of 9 h, the biomass was almost washed out and the sCOD RE dropped to 43% (Figure 4.10). Chemostat conditions allowed the calculation of kinetic parameters and the observed growth yield of the process culture. The estimated maximum specific growth rate (μ_h) was 4.99 d^{-1}, while the growth yield (Y_h) was 0.68 g COD g COD^{-1} in chapter 5. These values were similar to other values reported in the literature with TA and activated sludge from a tannery WWTP (4.99 d^{-1} and 0.59 g COD g COD^{-1}) (Li et al., 2009). These similarities were reasonable given that commercial TA is usually extracted from plants such as *Caesalpinia spp.*, and TA is easier to degrade than TT. These results supported the hypothesis that bacteria were the main agent responsible for TT degradation, which was further confirmed in the microbial diversity analysis (section 4.3.4). In addition, the ratio between sCOD and DOC in the inlet and outlet were 2.80 ± 0.30 and 2.69 ± 0.44, respectively, which was another indicator of the large degree of mineralisation of TT. The DOC results are reported in Figure 4.11, showing a very similar profile to sCOD in Figure 4.12. The test with different concentrations was performed with 1, 2, and 8 g L^{-1} of TT. Inhibition was observed at just 2 g L^{-1} as RE decreased from 90% to 62%. The system was almost completely inhibited at 8 g L^{-1} (Figure 4.12). No toxicity was observed, since the RE recovered after day 210 to the initial RE of 90% when the inlet concentration was again reduced to 1 g L^{-1}. In fact, the reported minimum inhibitory concentrations (MICs) of TT for most bacteria is up to 1 g TT (Aguilar-galvez et al., 2014). Inhibition is further discussed in section 4.3.5.
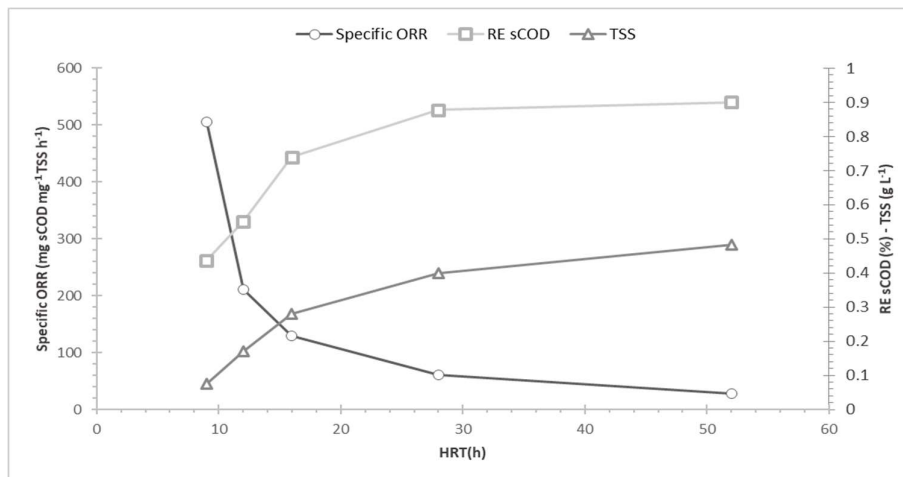
Figure 4.10: Influence of the HRT in RT over TSS, RE and specific ORR.
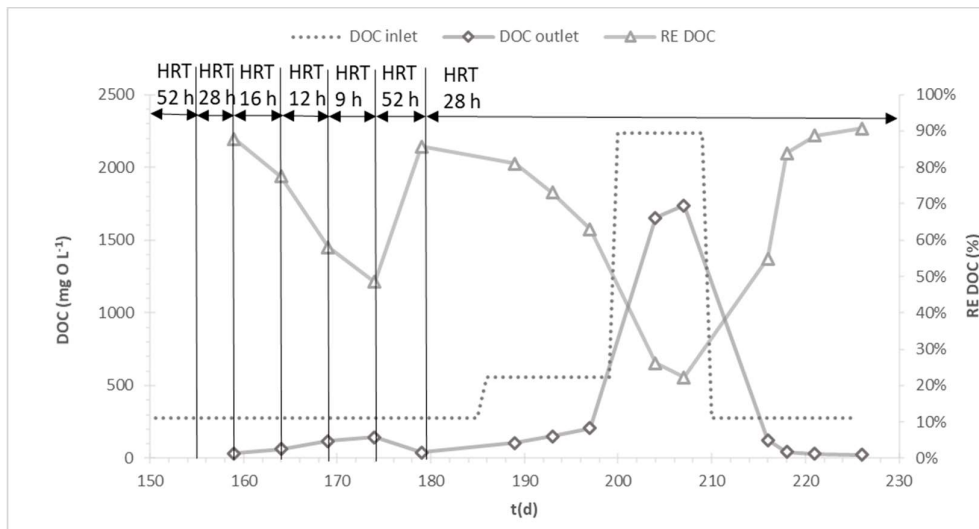
Figure 4.11: DOC and DOC removal (%) during specific experiments at different HRT in bioreactor RT fed with TT after day 150. Vertical lines show the different HRT used in the experiments.
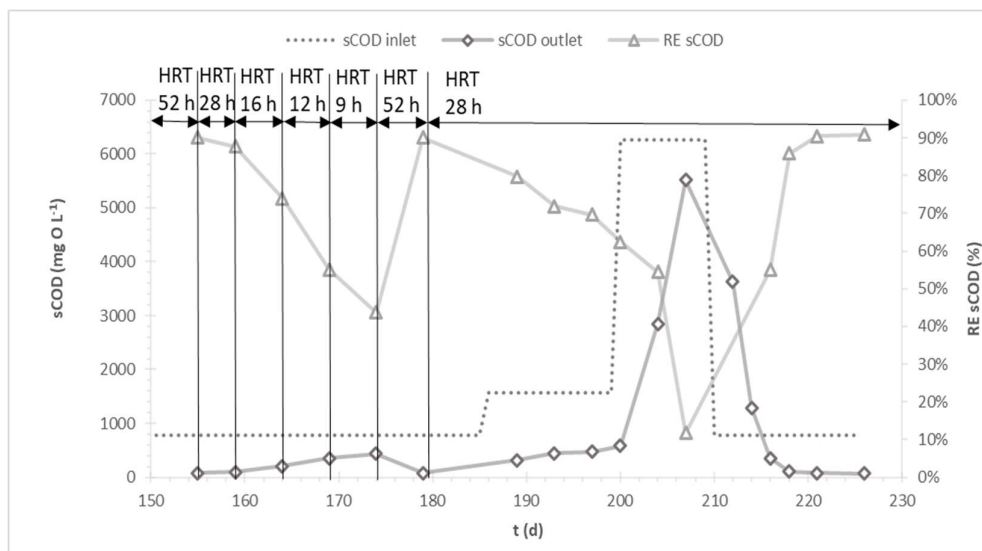
Figure 4.12: Inlet and outlet sCOD and sCOD removal percentage during continuous treatment in bioreactor RT in the test phase beginning on the 150. Vertical lines show the different HRT during reactor operation.

4.3.3 Simultaneous treatment of TT and QT

After the previous tests, RQ was fed with a medium composed of TT (1 g L^{-1}) and QT (0.1 g L^{-1}) (corresponding to a total inlet sCOD of 879 ± 63 mg L^{-1}) beginning on day 154. It was also inoculated with the bacteria developed in RT. Since the inhibition due to QT was strong even at a relatively low COD concentration, it was

hypothesised that QT contributed to biofilm preservation from bacterial growth. The RE of COD after 4 days of simultaneous QT and TT feeding was stable at approximately 88% on average (Figure 4.5). No synergistic effects were detected when comparing the weighted individual RE during simultaneous treatment with the RE for QT and TT obtained when fed individually during RQ and RT operation. The simultaneous feeding test lasted 26 days, and the biofilm did not detach from the support medium, maintaining a stable removal performance similar to the previous test phase with QT only. Fungi were shown to be more tolerant to condensed tannins (QT) compared to bacteria and, as previously observed (Mutabaruka et al., 2007), the presence of condensed tannins could increase the ratio between fungi and bacteria in a microbial ecosystem. In the literature, TA has been demonstrated to be an efficacy QQM (Patel et al., 2017), and others tannins also show quorum-sensing inhibitory activity (Coppo and Marchese, 2014; Yang et al., 2016). Moreover, QT is known for its antifouling features (Bellotti et al., 2014, 2012). Thus, it is possible that QT could also act as an efficacy QQM as one of its mechanisms of action against bacteria. These results, together with the selected operating conditions, could be used as a possible strategy to increase fungal competitiveness over bacteria and favour the development of a fungal-bacterial consortium that is symbiotically effective in recalcitrant compounds removal.

4.3.4   Microbial community analysis

The total number of high prokaryotic sequences obtained across the three samples was 229,296, with an average of 76,432 each. Regarding fungi, the amount of high-confidence sequences across the three samples was 85,259, with an average of 28,419 each. Rarefaction curves for both bacterial and fungal communities showed that sequencing results covered bacterial and fungal diversity, since the rarefaction curves tended toward a saturation plateau (Figure 4.13). The alpha diversity, Chao, Shannon and Simpson indexes showed that the bacterial and fungal communities in the bioreactor were well characterised (Table 4.5). Regarding the bacterial community, 97% of sequences were classified into 113 OTUs by using the 97% similarity threshold. At the phylum level, the OTUs were characterised by four phyla. The *Proteobacteria* phylum was the most abundant, with 88.1% representation

among the total sequences. The other phyla were: *Actinobacteria* (8.2%), *Bacterioides* (2.4%) and *Proteobacteria* (0.5%). At the genus level, most of the sequences were attributed to a taxonomical identity (Figure 4.14 and Figure 4.15). The most abundant genus was *Novosphingobium* (13.7%). Other representative genera were *Kaistia* (12.9%), *Comamonas* (11.9%), *Sphingobium* (7%) and *Microbacterium* (5.2%). The above-mentioned genera were distributed across the samples (from reactor RT, RT-9h and RT-28h, and reactor RQ, RQ-28h) in different ways (as shown in Figure 4.16, Figure 4.18, Figure 4.19, Figure 4.20): *Novosphingobium* was distributed with a percentage of 33.2% (RT-9h), 5.2% (RT-28h) and 2.7% (RQ-28h); *Kaistia* was very abundant in the RQ reactor (37.4 % into RQ-28h), while it reached only 0.7% (RT-9h) and 0.6% (RT-28h) of the total in the RT reactor. On the other hand, the *Comamonas* genus was most abundant in the RT reactor (with 5.6% in RT-9h and 28.5% in RT-28h) and less abundant in the RQ reactor (1.5% in RQ-28h). The genus *Sphingobium* was recorded at 15% in the RQ reactor, but less abundant in RT reactor (2.7% in RT-9h and 3.4% in RT-28h). The *Microbacterium* genus was abundant in the RT bioreactor (15.2% in RT-28h) and less abundant in the RQ bioreactor (less than 1% in RQ-28h). The remainder of the genera was represented with lower percentages than those reported above or not classified at the genus level. In the case of the fungal community, 93.1% of the total number of sequences represented 46 different OTUs. At the phylum level, the OTUs were divided into two phyla: *Ascomycota*, with an abundance of 65%, and *Basidiomycota*, with an abundance of 28.1%. The remaining 6.9% of sequences were uncharacterised. As shown in Figure 4.15 and Figure 4.16, the *Basidiomycota* phylum *Apiotrichum* was the most-represented genus (27.9% of total sequences). Within the phylum *Ascomycota*, the genera *Ophiostoma* (16.2%), *Myxocephala* (15.2%), *Exophiala* (11.1%) and *Aspergillus* (9.3%) were most common. The above-mentioned genera were distributed within the two bioreactors as follows: the *Apiotrichum* genus was predominant in RT, with an abundance of 24.4% in RT-9h, as compared to RQ where it accounted for just 15%. The *Ophiostoma* genus was most abundant in the RT bioreactor (23.4% in RT-28h) as compared to RQ where it accounted for just 14%. The *Myxocephala* genus accounted for 26% in the RT bioreactor (RT-9h), and for 4.4% in RQ. The *Exophiala* genus was more abundant in RQ (27.9%) compared to RT (2.3%). The *Aspergillus* genus accounted for 23% in RQ-28h and approximately 3% in RT (RT-9h and RT-28h). Since *Aspergillus tubingensis*

was the fungal strain inoculated (immobilised onto the PUF), it might be reasonable to assume that the strain constituted the majority of the total *Aspergillus ssp*. in the RQ (23% of the total fungi in the RQ) and RT (about 3% of the total fungi in the RT in both HRT). Furthermore, biomolecular analysis showed that there was important fungal consortium development at steady-state conditions in RQ. Starting from a single fungal strain inoculum, the *Aspergillus* relative abundance was reduced to a residual value in RT. It is worth mentioning that among the most abundant bacterial genera found in the reactors (*Kaistia* and *Sphingobium* in RQ; *Comamonas*, *Novosphingobium* and *Microbacterium* in RT), several species were identified that have not been previously reported in literature to be tannin degraders, but instead are reported to be related to the biotransformation of phenols. Likewise for the fungal genera *Exophiala*, *Aspergillus*, *Apiotrichum*, and *Ophiostoma* in RQ and *Apiotrichum*, *Myxocephala* and *Ophiostoma* in RT, several species were identified that were not previously reported as tannin degraders (Bhat et al., 1998; Mingshu et al., 2006). In our experimental context, the presence of a diversified microbial community in addition to the inoculated *Aspergillus* was reasonably linked to the non-sterile nature of the commercial tannins used and environmental contamination. The involvement of these communities in the tannin removal process under the experimental conditions adopted herein is reasonable. The relationship between organisms in a fungi and bacteria consortium is complex. A comparison between RQ and RT nevertheless provides some indications about the organisms more suitable to degrade TT or QT. Further studies are required to reach a better understanding about the interactions between microorganisms and the influence of the operating conditions of the reactor. As an example, the different HRT in RT was, as expected, a reactor operating condition able to strongly influence the microbial populations. Thus, *Basidiomycota* phylum was present with an abundance of 47.3% in RT-28h while decreasing sharply to 15.3% in RT-9h. In fact, the fungi of *Basidiomycota* phylum are characterised, in general, by a slower growth rate than *Ascomycota*.
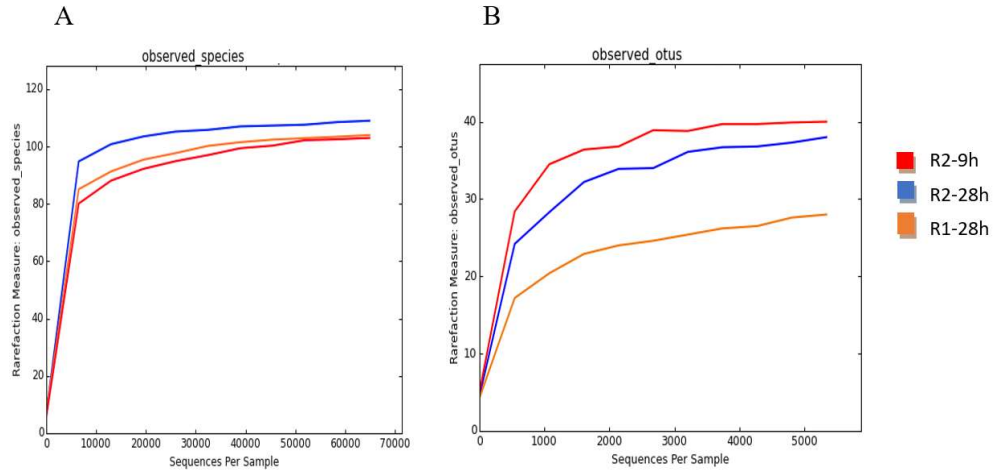
Figure 4.13: Rarefaction curves of observed OTUs per sample for bacterial (A) and fungal community (B).

Table 4.5: Alpha-diversity indexes (Shannon, Simpson, Chao) characterising both the bacterial and fungal communities in RQ and RT.

Fungal community			
Index	RT-9h	RT-28h	RQ-28h
Chao	40	39.5	31.33
Shannon	2.69	2.68	3.37
Simson	0.84	0.73	0.79

Bacterial community			
Index	RT-9h	RT-28h	RQ-28h
Chao	107.2	110.5	105.66
Shannon	3.77	4.42	4.33
Simpson	0.84	0.9	0.9

Figure 4.14: Legend of colours used for different fungal communities.
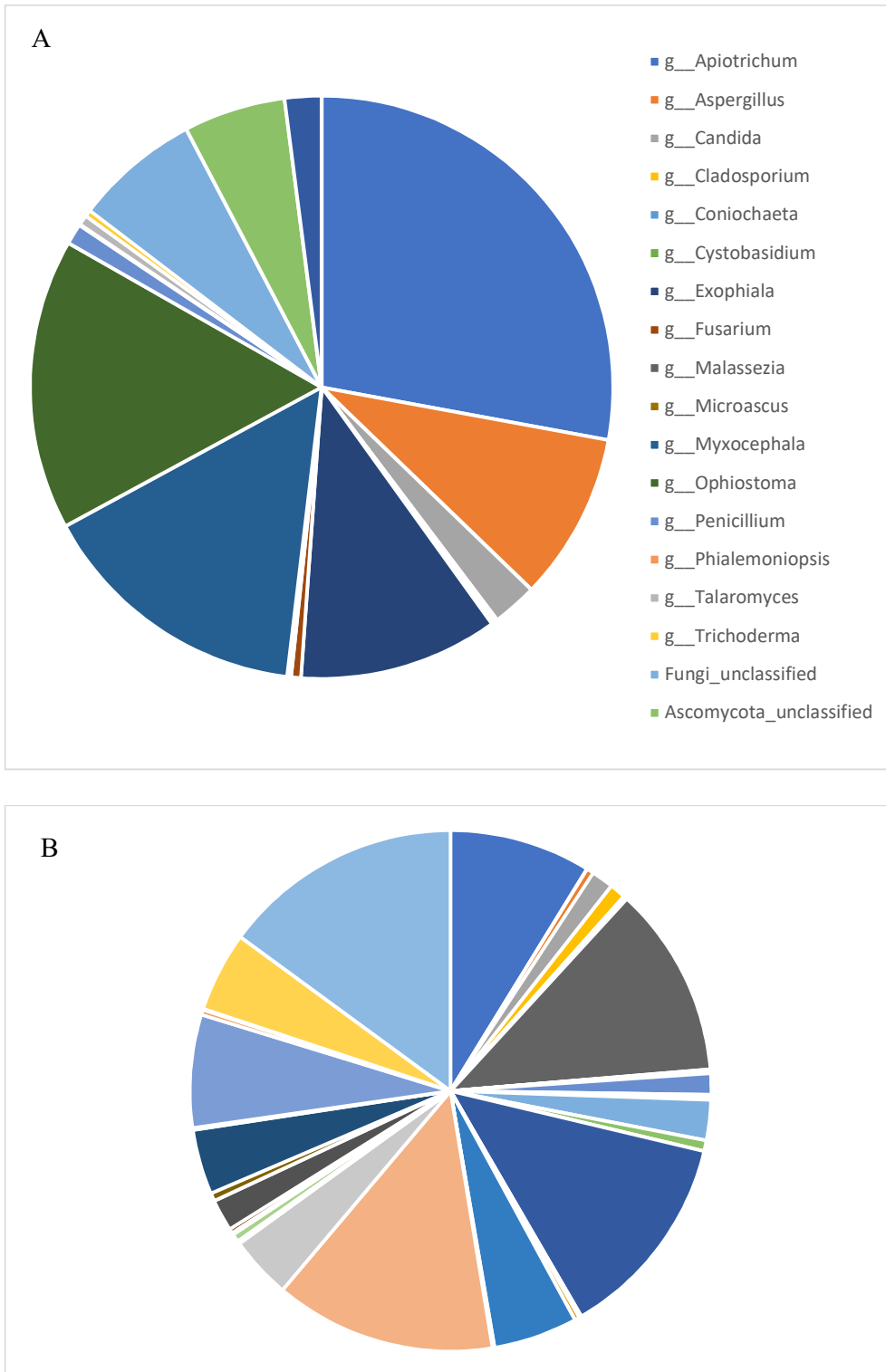
Figure 4.15: Total genus percentage for bacterial (A) and fungal (B) communities in both bioreactors.
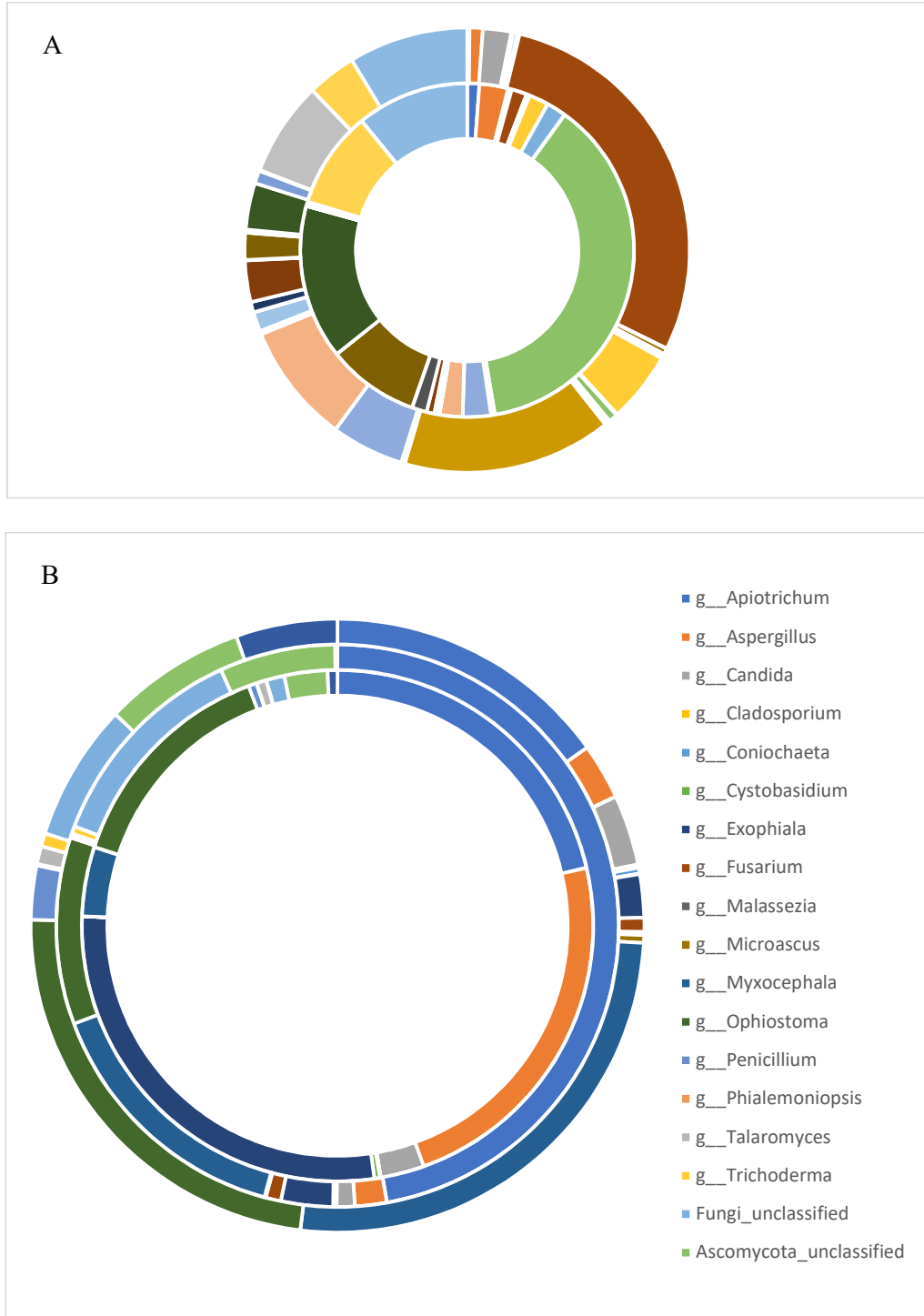
Figure 4.16: Genus percentage for bacterial (A) and fungal (B) communities in both bioreactors. From inside to outside: total percentage in RQ-28h, RT-28h and RT-9h.
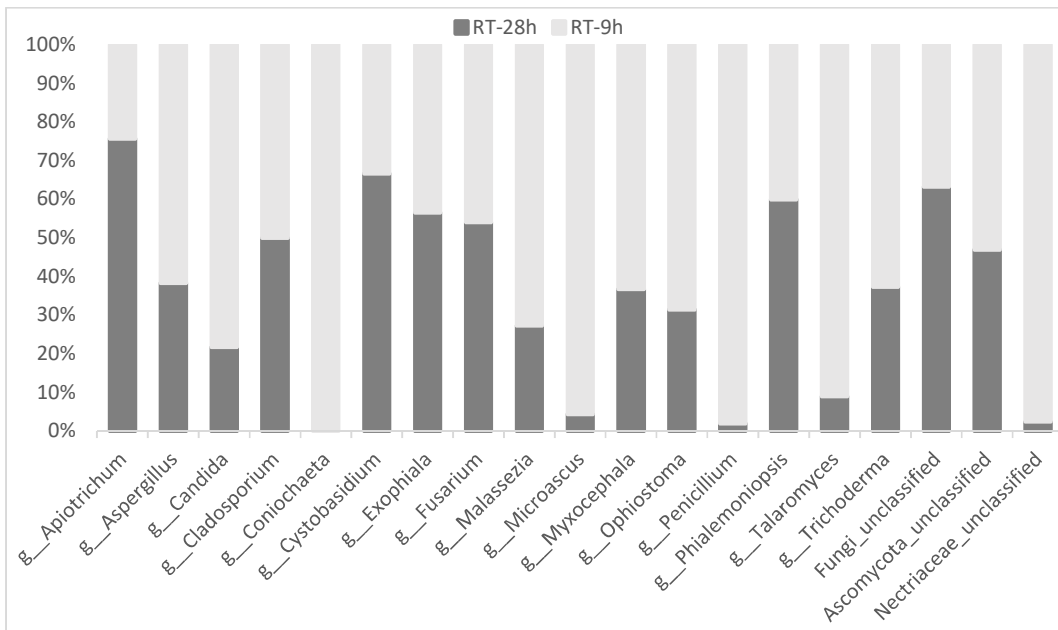
Figure 4.17: Bar chart showing the OTUS that characterised the fungal community in RT-28h and RT-9h.
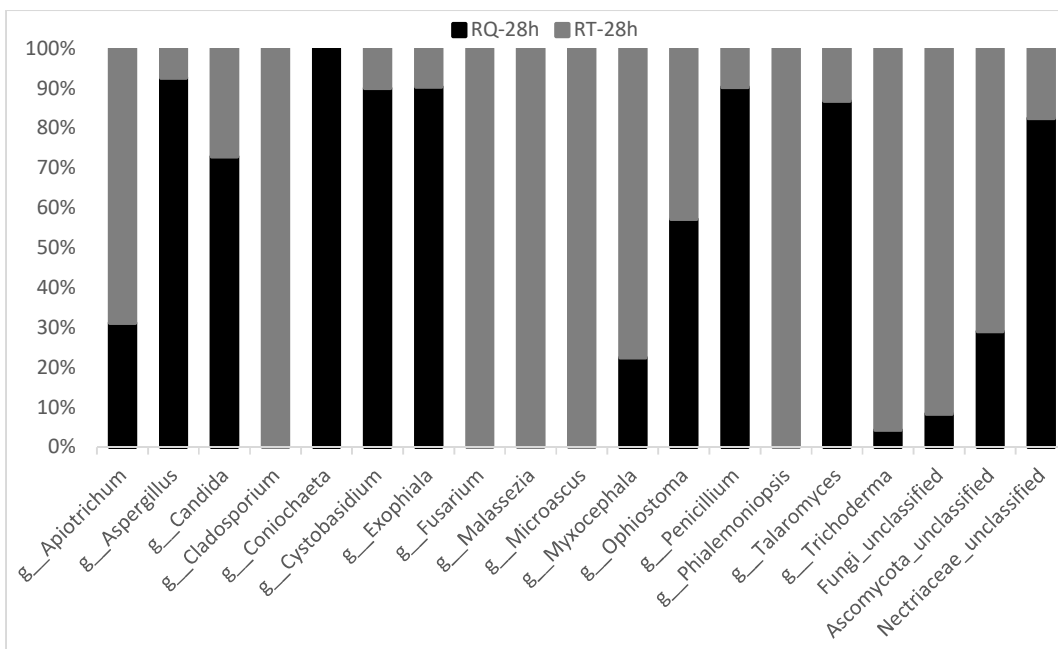
Figure 4.18: Bar chart showing the OTUS that characterised the fungal community in RQ-28h and RT-28h.
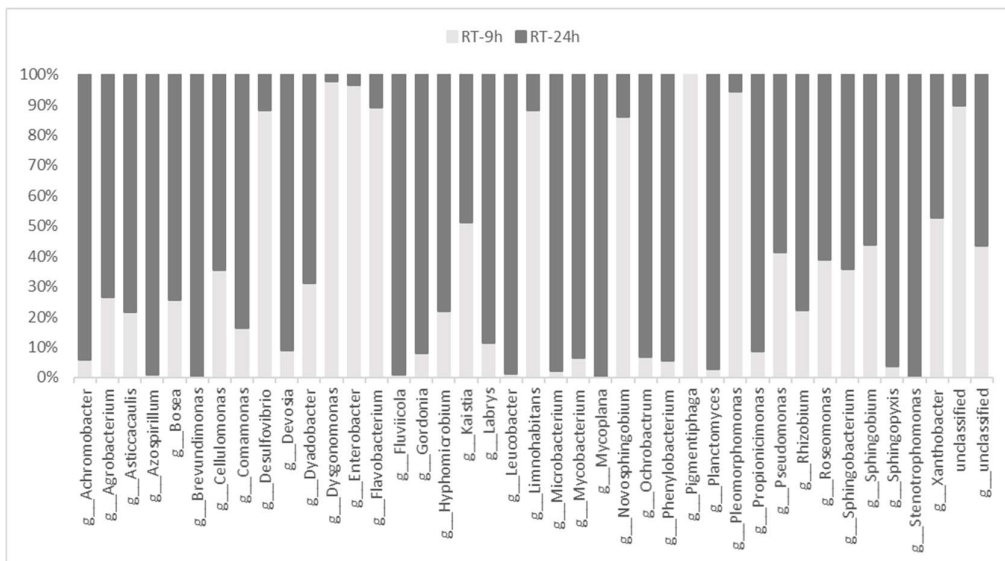
Figure 4.19: Bar chart showing the OTUS that characterised the bacterial community in RT-28h and RT-9h.
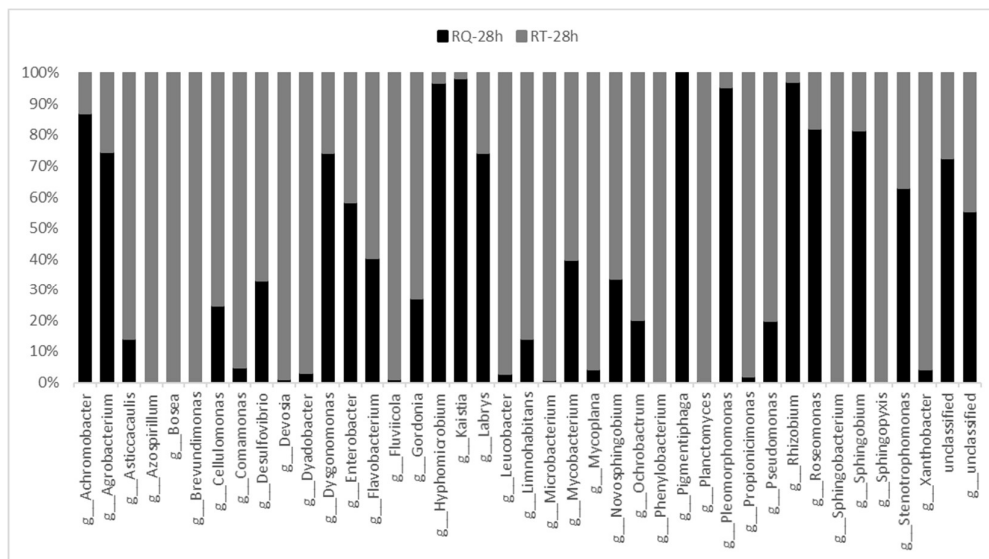
Figure 4.20: Bar chart showing the OTUS that characterised the bacterial community in RQ-28h and RT-28h.

The mechanism of interaction between fungi and bacteria (and between different types of bacteria and different types of fungi) are complex and require further study. Looking to the relative abundance, is it possible that the genera *Novosphingobium, Comamonas,* and *Microbacterium* could better tolerate/degrade TT with respect to QT and could be an invading species for most fungi. *Kaistia* and *Sphingobium*, on the other

hand, could have symbiotic interactions with fungi and could better tolerate/degrade QT. Species of *Novosphingobium* (Fu et al., 2017)*, Comamonas* (El-Banna and Qaddoumi, 2007; Shen et al., 2015)*,* and *Microbacterium* (Postma et al., 2005) are known have antagonistic effects on fungal growth and could be bioactive in the degradation of plant derivatives as reported in the literature: *Novosphingobium* (Chai et al., 2010)*, Comamonas* (Chai et al., 2010; Halecky et al., 2013) and *Microbacterium* (Zachow et al., 2008)*. Sphingobium spp*. have been found with fungal strains in aged, polluted soil (Lladó et al., 2013), and *Sphingobium chlorophenolicum* is reported to completely mineralise pentachlorophenol (Xun et al., 2010). *Sphingobium spp.* was able to increase its grow after a fungal bioaugmentation (Hong et al., 2017), but in other cases could exert an antagonistic effect on fungi (Fu et al., 2017). Nevertheless, it is also important to highlight that a community of non-fungal antagonist bacteria could express antagonistic behaviour (Fujiwara et al., 2016). Others have observed a consortium evolution in long-term phenol treatment operations with species such as *Comamonas testosteroni* and *Pseudomonas aeruginosa* (Halecky et al., 2013), which belong to genera also identified in this project. In general, a degradation activity drop of fungi could be related to unspecified damage by invading bacteria, where the little known invading bacteria population are absent or present only in marginal relative abundance (*Pseudomonas, Burkholderia*, etc.) (Jeong et al., 2016; Svobodová and Novotný, 2017). In recent years, the characterisation of the WWTP bacterial community is becoming more and more common (Hu et al., 2012; Muszyński et al., 2015), including for tannery WWTP (Giordano et al., 2016), while the fungal communities still are less well characterised (Maza-Márquez et al., 2016). These interactions are complex, and the studies are still in the early stages, but the characterisation of WWTP and the lab-scale tests have allowed insight into the microbial interactions and the influence of environmental parameters (Badia-Fabregat et al., 2017; Cortés-Lorenzo et al., 2016) and may later also allow for performance optimisation (Maza-Márquez et al., 2015).

4.3.5   Comparison between RQ and RT

The different performance of bioreactors RQ and RT can be explained on the basis of the different competition for substrates between fungi and bacteria. In fact, it has previously been observed that bacterial growth can lead to the disruption of the fungal mycelium (Rene et al., 2010). Moreover, TT was mainly composed of hydrolysable tannin structures based on gallic acid and quinic acid (Garro Galvez et al., 1997) and, in agreement with previous studies, the minimum inhibitory concentration (MIC) of TT for most bacteria was found to be in the range of 0.16 to 16 g TT L^{-1} (Aguilar-galvez et al., 2014). As a consequence, the selected fungal strain could be outcompeted by bacterial strains able to degrade TT (Mingshu et al., 2006), with a MIC higher than the concentration used in the medium solution (1 g L^{-1} of TT). The operating conditions in RT (such as acidic pH) allowed the development of a suspended biomass, with RE of the hydrolysable tannins present in TT for concentrations below 2 g L^{-1} of TT. On the other hand, in RQ the RE was satisfactory only in the range 0.2 to 0.1 g L^{-1} of QT. Furthermore, the high concentration of condensed tannins in QT allowed for the maintenance of the biofilm as verified during the simultaneous feeding of TT and QT in RQ. The volumetric organic loading rate (OLR) and the volumetric organic removal rate (ORR) in RQ and RT were compared in Figure 4.21 and demonstrated a higher substrate inhibition of QT comparted to TT. At an ORR of 28 mg sCOD L^{-1} h^{-1}, the specific OLR of RT was 61 mg sCOD g^{-1} h^{-1}, while that estimated for RQ was 80 mg sCOD g^{-1} h^{-1}. The OLR in RQ was calculated with an estimation of the total biomass of the reactor obtained from dry mass measurement in the PUF. Overall, the results obtained in the present study represent significant progress in the application of biological processes in tannin removal from wastewater. This is also the first report of the continuous removal of QT and TT, providing removal rates and operating conditions that could aid the design of industrial scale reactors. The reactor design and operating conditions demonstrated successful, stable tannin removal under non-sterile conditions, even without the addition of any co-substrate. Finally, in both RT and RQ, the development of a fungal and bacteria consortium was observed. It is reasonable to suppose that fungi were outcompeted by bacteria in RT for several evidences: biomolecular analysis results, microscope

observation of samples and visual comparison between RQ biofilm and RT biomass, the detachment of the fungal biofilm (and subsequent removal of cage), and the development of suspended biomass.
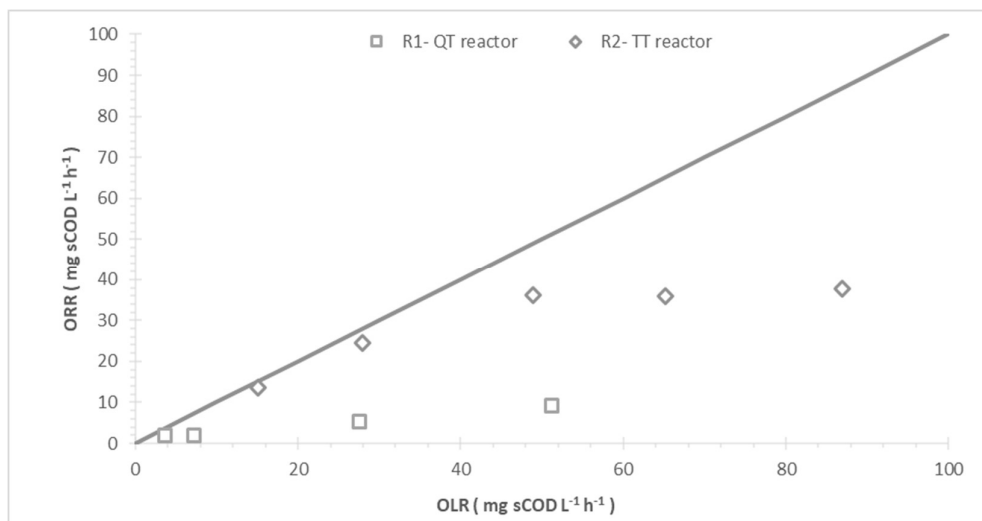
Figure 4.21: Overall performance rates of RQ and RT reactors.

The RQ, fed with QT, outlasted the colonisation of bacteria and allowed a stable fungal biofilm thanks to the operating conditions and QT concentration. A QT removal rate of 53% was obtained with 0.1 g L^{-1} concentration in the inlet solution. RT was fed with TT as a sole carbon source and caused the detachment of the inoculated fungal biofilm and formation of suspended biomass that reached 90% removal of hydrolysable tannins. *Aspergillus tubingensis* was inoculated and remained in long term operation, in both reactors, as a microbial consortium developed. Further discussion about the inoculum and long-term stability is included in the conclusion chapter. The results obtained in this section were coupled with respirometric tests to further develop a stoichiometric and kinetic characterisation of the biomass. The preliminary and long-term continuous bioreactor operation results confirmed the efficiency of the selected bioreactor design. Moreover, the conditions tested with RQ allowed the development of a stable, long-term fungal biomass in non-sterile conditions and a promising QT removal. Both of these aspects were supported by the design of the pilot reactor and its operating conditions during the start-up and treatment phase.

5 RESPIROMETRIC TECHNIQUES COUPLED WITH MODELLING FOR KINETIC AND STOICHIOMETRIC CHARACTERISATION OF BIOMASSES

Chapter abstract

The focus of this chapter is the kinetic and stoichiometric characterisation of biomasses continuously fed with TT (RT) and QT (RQ) in lab-scale bioreactors. The results described in chapter 4 and those of specifically designed respirometric tests were used to estimate the kinetic and stoichiometric parameters of the biomass. The chapter is divided into three sub-sections: respirometric tests design and application, modelling of RT and modelling of RQ.

In the first sub-section, the respirometric techniques were developed and applied to activated sludge of Cuoiodepur WWTP, suspended biomass from RT, immobilised PUF cubes of RQ and immobilised PUF cubes of a pure culture of *Aspergillus tubingensis*.

In the second sub-section, a Monod kinetic with substrate inhibition, biomass decay and hydrolysis was applied to simulate the behavior of the suspended biomass selected in RT. The model was designed and the parameters estimated with the software Aquasim. The results were compared with the literature values referred to tannins degradations by suspended microbial ecosystems.

In the last sub-section, the growth with a Monod kinetic with substrate inhibition, the decay and the hydrolysis process were used to represent the behavior of immobilised biomass of RQ. The model was designed and the parameter estimated with the software Aquasim by using the specific tool (biofilm compartment) to simulate the attached growth system. The biofilm features and transport parameters were measured or assumed from the literature.

5.1 RESPIROMETRIC TESTS

The various respirometric set-ups and the developed respirometric methodology for fungal attached biomass were described in detail in the Materials and the Methods and Introduction chapters. The activated sludge from a tannery WWTP, the QT degrading attached biomass from RQ and the TT degrading suspended biomass from RT were evaluated separately and with specific approaches as detailed in the following Table 5.1. The Activated sludge from Cuoiodepur tannery WWTP was tested in MARTINA respirometer with substrate concentration that allowed to have the ratio of substrate/biomass (S/X) (g COD g COD^{-1}) in the range 0.01 to 0.1. The *A. tubingensis* immobilised in PUF cubes and the immobilised biomass were tested in LFS respirometer with a vessel of 0.3 L of volume, the suspended biomass from RT was tested in LFS respirometer with a vessel of 1 L. The solutions dosed for all the tests were prepared at the concentration of 10 g tannin L^{-1} (TT or QT) and were filtered to remove eventual particles. The suspended biomass from RT was tested also with TA and Gallic acid solutions.

Table 5.1: Summary of the respirometric tests performed.

Biomass	Substrate	Respirometer	Range tested (mg COD L^{-1})
Activated sludge from tannery WWTP	QT	MARTINA	250
Activated sludge from tannery WWTP	TT	MARTINA	120
A. tubingensis immobilised	QT	LFS - UAB	47
A. tubingensis immobilised	TT	LFS - UAB	14-520
RT suspended biomass	QT	LFS - UAB	14-42
RT suspended biomass	TT	LFS - UAB	7.8-78
RT suspended biomass	TA	LFS - UAB	15
RT suspended biomass	Gallic Acid	LFS - UAB	41-51
RQ immobilised biomass	QT	LFS - UAB	47-476
RQ immobilised biomass	TT	LFS - UAB	26

5.1.1   Activated Sludge from tannery WWTP

The biodegradability of tannins was evaluated for acclimated activated sludge (in others words the conventional biological treatment adopted in a tannery WWTP) and were performed with the "MARTINA" respirometer at pH 7.8 with 3-4 g VSS L^{-1} of activated sludge sample collected from Cuoiodepur tannery WWTP. Two respirograms, with the OUR_{ex} and OUR_{end} after a TT and QT pulse, are reported in Figure 5.1 as example: the QT shown an average biodegradability of 8% ± 2%, while TT showed an average biodegradability of 19% ± 4%. The tests were performed in triplicate. The ΔCOD_b (mg COD L^{-1}) effectively biodegraded was calculated with the following formula (5.1) (Andreottola and Esperia, 2001) with the procedure described in Material and Methods and conventional growth yield Y_h for heterotrophic biomass of 0.45 (mg VSS mg COD^{-1}) and conversion factor (f_{cv}) for VSS/COD of 1.42 (mg COD mg SSV^{-1}) were adopted.

$$\Delta COD_b = \frac{1}{1-f_{cv}\cdot Y_h}\cdot \Delta O \cdot \frac{V_l+V_s}{V_l} \tag{5.1}$$

Where:

Y_h is the growth yield coefficient (mg VSS mg COD^{-1});

f_{cv} is the conversion factor for VSS/COD (mg COD mg SSV^{-1});

ΔO is the oxygen consumption calculated from the respirogram (mg O_2 L^{-1});

V_l is the volume of TT or QT samples dosed (L);

Vis the volume of the activated sludge in the vessel or the effective volume (L).

It is noteworthy to mention that the tests were slightly longer than the conventional 3-4 hours usually used for respirometry tests due to the slow biodegradable fraction that required longer time to be degraded: as shown in Figure 5.1 it was observed a fast peak of OUR and a following long tail till to the OUR_{end} level (around 2 mg O_2 L^{-1} min^{-1}). Moreover, sCOD measurements were performed at the beginning and at the end of the tests: lower values of sCOD than expected (on the basis of respirometry) were found at the end of the tests and thus higher removal efficiency (about 60% for both tannins).

This phenomenon was related to the adsorption of tannins on the activated sludge and it was verified with aerated batch tests: the adsorption occurred within 15 minutes from the tannins pulse. There is little literature about tannins biodegradability with activated sludge based on respirometric tests and mainly performed with one of the most biodegradable, i.e. TA: this tannin showed a biodegradability of 81.6% as COD (Li et al., 2009). From a mix of vegetable tannins (490 mg sCOD L^{-1}) 51.3% of sCOD was removed (as cumulated adsorption on activated sludge and effective degradation) during aerobic batch test lasting 6 days (He et al., 2007). Other authors obtained similar results with short term (5 days) and long term (30 days) tests with activated sludge in aerobic batch tests and a decreasing biodegradability at increasing concentration of tannins (He et al., 2008). It is important to note that, while the sole measurement of sCOD in aerated batch tests is affected by short-term effects such as adsorption, the respirometric tests and the long-term tests (at high SRT) conducted with continuous bioreactors operation provided the effective biodegradability of tannins by the selected biomasses.
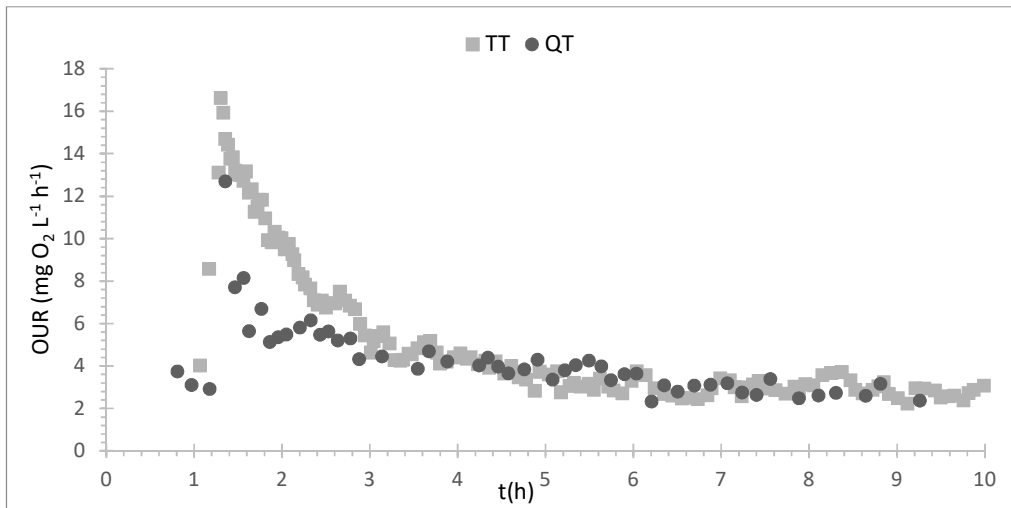
Figure 5.1: OUR profile of TT and QT pulses in a respirometer with activated sludge. In this picture is reported the OUR$_{ex}$ and OUR$_{end.}$

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5.1.2   Suspended Biomass from reactor RT

The suspended biomass from RT was tested in LFS UAB respirometer (1 L vessel) with the conventional procedure adopted for suspended biomass as described in Materials and Methods. Pulses of TT (within a concentration range from 7.8 mg L^{-1} to 78 mg L^{-1}) with an increasing concentration of COD were carried out with biomass collected from RT at steady-state conditions (during the period at 16 h HRT), as shown as example in Figure 5.2 (pulse TT at COD 7.8 mg L^{-1}). Numerous trials were performed, unfortunately it was not possible to remove the "noises" in the respirograms: in fact, the pulses with high concentration caused a pH fluctuation after the pulse that was difficult to control. However, as shown in Figure 5.3, respirometric tests also with Gallic Acid (one of the main components of TT) and TA were performed and were shown to be almost completely biodegradable. However, also QT pulses were tested, but the consumption of oxygen was very low and it was not possible to calculate the biodegradability of QT for the suspended biomass from RT.
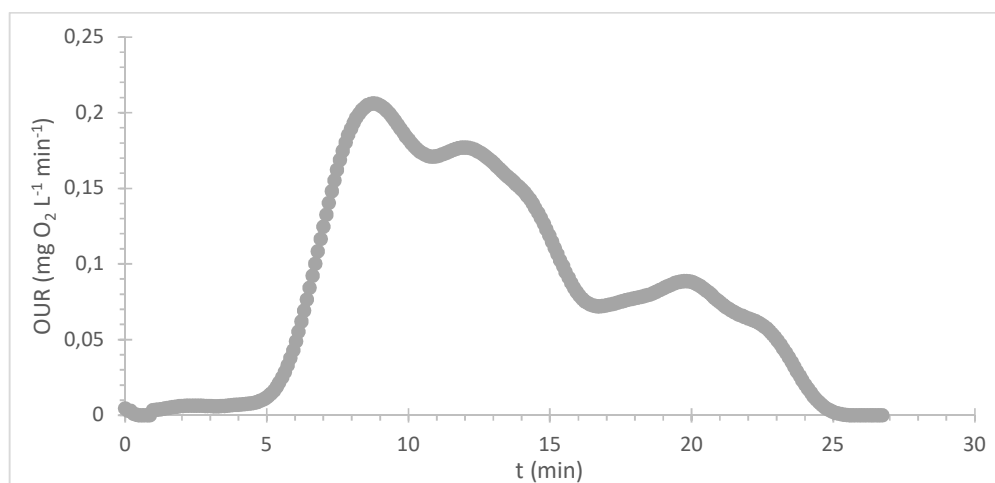
Figure 5.2: Respirogram of suspended biomass from RT with TT pulse (COD 7.8 mg L^{-1}). In this picture is reported the OUR_{ex}.
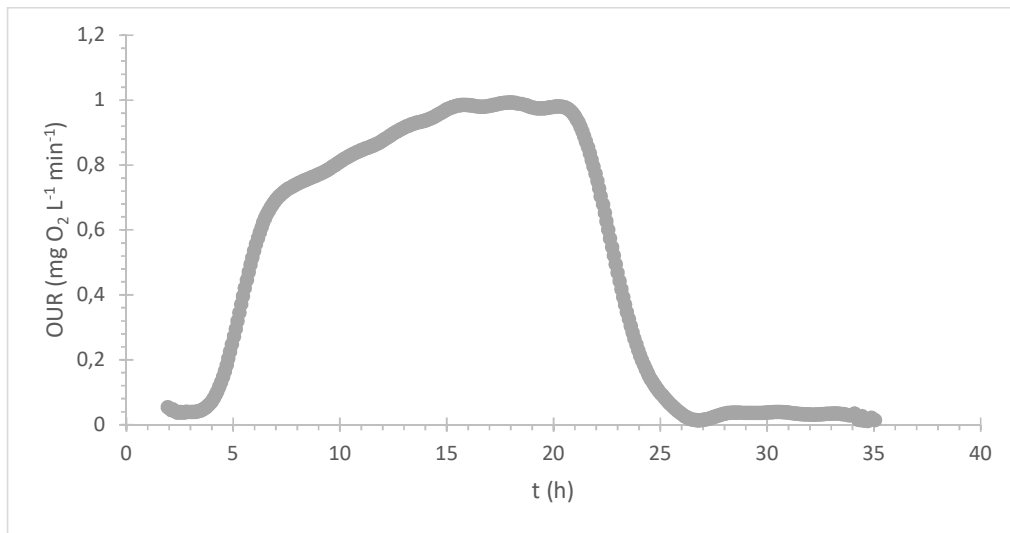
Figure 5.3: Respirogram of suspended biomass from RT with gallic acid pulse (COD 51 mg L^{-1}). In this picture is reported the OUR$_{ex}$.

5.1.3   Immobilised biomass from reactor RQ

The immobilised biomass from RQ was tested in UAB respirometer (0.3 L vessel) with a dedicated procedure described in Materials and Methods. The development of a procedure for respirometric tests with immobilised biomass required several trials also with pure fungi biomass (*Aspergillus tubingensis*). This pure fungal biomass required an endogenous phase and multiple wake up pulses. These tests were performed also to verify the tannins biodegradation capacity of selected fungal strain versus QT and TT. In Figure 5.4 the first three wake up pulses are reported and the pure fungal culture adaptation to the substrate. These tests were repeated until reaching an equal response after each pulse: approximately 3-4 pulses were needed. The immobilised biomass of RQ response on TT pulse was similar to the response of the acclimated pure culture of *Aspergillus tubingensis* and the suspended biomass collected from RT. The QT pulses were chosen, as shown in Table 5.2, in order to obtain a similar S/X ratio among the respirometer and the different condition tested in RQ. In Figure 5.5 four pulses of QT are represented, with an increasing concentration of COD, tested in the respirometer filled with the immobilised biomass sampled from RQ. The respirometric profiles were used, as described in the following section, for modelling and characterising of the biomasses.
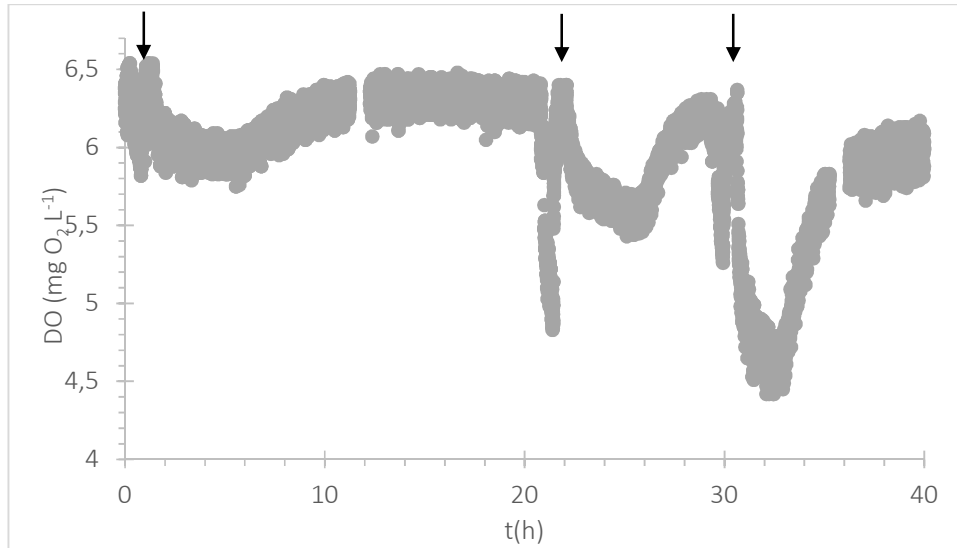
Figure 5.4: Three wake up pulses of 1 ml each (10 g TT L^{-1}) in a respirometry test with immobilised pure culture of *Aspergillus tubingensis.*
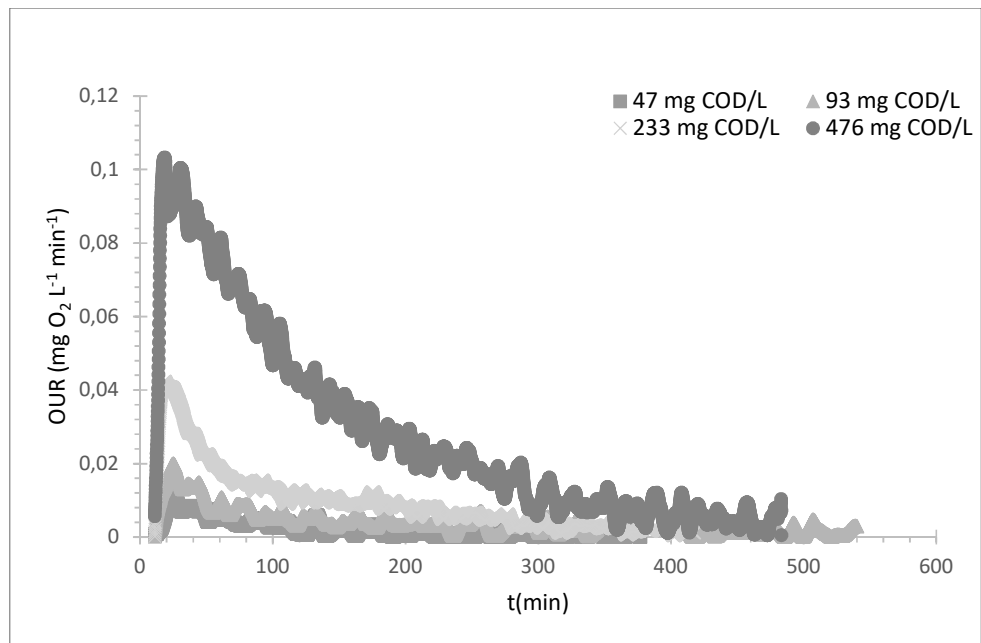
Figure 5.5: Respirograms with immobilised fungi from RQ and QT pulses. In this picture is reported the OUR$_{ex}$.

Table 5.2: Comparison between the different QT concentrations and S/X ratio in the bioreactors RQ and in the respirometer LFS UAB.

Vessel	QT (mg COD L^{-1})	S/X ratio (g COD g COD^{-1})
RQ	25	0.7
RQ	50	1.4
RQ	175	4.9
RQ	350	9.9
Respirometer LFS UAB	47	1.0
Respirometer LFS UAB	93	2.0
Respirometer LFS UAB	233	4.9
Respirometer LFS UAB	477	10.1

5.2   TARA TANNIN REACTOR MODELLING

The kinetic characterisation of TT degrading suspended biomass of RT was carried out by modelling the results of RT monitoring in combination with those of respirometric tests. Temperature was considered and kinetic parameters were corrected based on conventional Arrhenius coefficients for bacteria ($\theta_b = 1.04$, $\theta_\mu = 1.1$) and all the parameters were referred to 20 °C. The model was implemented in Aquasim (www.aquasim.eawag.ch), developed by Eawag for simulation of aquatic systems (Reichert, 1994). The software allowed to simulate dynamic processes; in particular, as described in the Introduction and Material and Methods, it was chosen to describe the growth and decay of the biomass according to Monod kinetics equations. The Gujer matrix of the IWA-ASM-like model is presented in the Table 5.3 and Table 5.4. The matrix represents the final model implemented to describe the suspended biomass from RT. The model incorporates the substrate inhibition and the Contois hydrolysis kinetic equation; conventional parameters were chosen to describe the decay (direct decay as in IWA ASM3) and the fate of the decay products (Barker and Dold, 1997; Kaelin et al., 2009).

Table 5.3: Matrix presentation of the model for RT suspended biomass.

Components (i)	S	X_S	X_P	X_H	S_o
Processes (j)					
Aerobic Growth of X_H	$-\dfrac{1}{Y_H}$			1	$1-\dfrac{1}{Y_H}$
Decay of X_H		$1-f_P$	f_P	-1	
Hydrolysis of X_S	1	-1			

Table 5.4: Process rates of the model for RT suspended biomass.

Processes (j)	Process Rate (ρ_j)
Aerobic Growth of X_H	$\mu_H \dfrac{S_O}{K_{OH}+S_O} \dfrac{S}{K_S+S+S^2/K_i} X_H$
Decay of X_H	$b_H X_H$
Contois hydrolysis of X_S	$K_H \dfrac{X_S/X_H}{K_X+X_S/X_H} X_H$

Where:

S Soluble concentration in bulk liquid of easily biodegradable COD (mg COD L^{-1});

X_s Slowly biodegradable COD (sbCOD) concentration in bulk liquid (mg COD L^{-1});

X_p Unbiodegradable particulate organics from biomass decay (mg COD L^{-1});

X_h Heterotrophic biomass concentration (mg COD L^{-1});

S_o DO concentration (mg O_2 L^{-1});

Y_h Growth yield of heterotrophic biomass (mg COD mg COD^{-1});

μ_h Maximum specific growth rate of heterotrophic biomass (d^{-1});

b_h Decay coefficient of heterotrophic biomass (d^{-1});

Ks Affinity constant (mg COD L^{-1});

f_d Fraction of inert COD generated in biomass decay (mg COD L^{-1});

K_i half saturation constant for inhibition compound (mg COD L^{-1});

K_{oh} half saturation constant for DO (mg O_2 L^{-1});

K_h Hydrolysis rate constant (d^{-1});

K_x Hydrolysis rate constant (g X_S g^{-1} X_H).

5.2.1 Microbial parameters estimation

The respirometer LFS UAB (1 L vessel volume) was filled with the suspended biomass collected from the CSTR bioreactor degrading TT (RT). The b_h estimation was performed with a respirometric test (pH and temperature-constant conditions as in RT) that lasted 70 h. The biomass was aerated and completely mixed in endogenous conditions (without any carbon source); the air supply (the flow was kept constant) was switched off four times and the calculated OURs (in the DO concentration range 7.8 to 7.6 mg O L^{-1}) were used to estimate b_h with an exponential curve as expressed in Equation 5.2 and shown in Figure 5.6 and Figure 5.7. The test was performed at T= 25°C, so the b_h was corrected at 20 °C. The b_h (20°C) estimated was 0.29 d^{-1}. The typical value of b_h (20°C) for heterotrophs was lower: 0.24 d^{-1} (Henze et al., 2000).
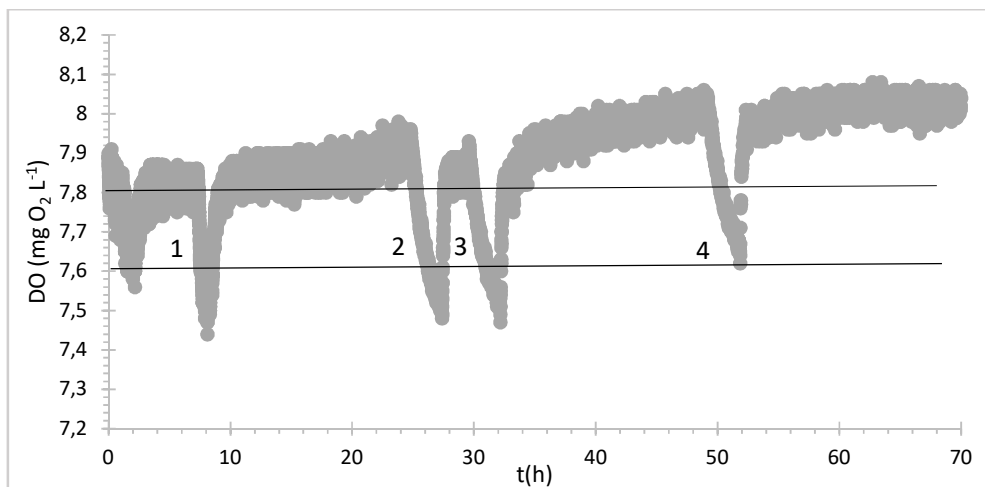
Figure 5.6: Oxygen profile from decay coefficient estimation for RT suspended biomass.
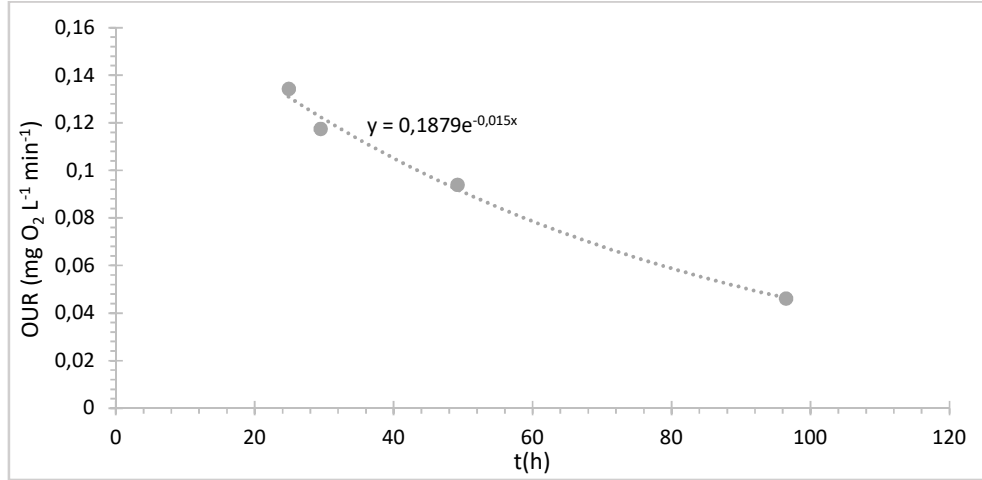
Figure 5.7: Endogenous OURs measurement for RT suspended biomass.

$$OUR(t) = -b_h \cdot X_{h,t=0} \cdot e^{(-b_h)t} \tag{5.2}$$

The estimation of the Y_h was carried out by applying Eq. 5.3 to four respirograms (within a range concentration of 7.8 mg L^{-1} to 78 mg L^{-1} of sCOD of TT). Since the maximum RE of the TA reactor (at HRT 52 h) was 90%, in order to estimate Y_h, the COD biodegraded during respirometric techniques was assumed as the 90% of the dosed COD. The Y_h estimation obtained was 0.68 ± 0.02 mg COD mg COD^{-1}. The conventional range of Y_h for heterotrophs is 0.38-0.75 and the typical value is 0.67 (Henze et al., 2000) and for TA was found 0.59 (Li et al., 2009).

$$Y_h = \frac{\Delta COD_{synthesi}}{\Delta COD_{bio}} = \frac{\Delta COD_{bio} - \int OUR_{es}(t)dt}{\Delta COD_{bio}} \tag{5.3}$$

The X_h in the reactor at steady-state was estimated at different HRT with the Eq. 5.4. In a CSTR the SRT is equal to the HRT and, as a consequence, Eq. 5.4 and Eq. 5.5 are equivalent. The estimated active biomass concentrations, calculated with the actual values, were reported in Table 5.5.

$$X_h = \frac{Y_h(S_{s0}-S_s)}{[1+(b_h SRT)]} \frac{SRT}{HRT} \qquad (5.4)$$

$$X_h = \frac{Y_h(S_{s0}-S_s)}{[1+(b_h HRT)]} \qquad (5.5)$$

Table 5.5: Active biomass estimation for RT suspended biomass.

HRT (h)	Active biomass (mg COD L^{-1})
52	293
28	286
16	240
12	179
9	142

A first estimation of the K_s and μ_h of the biomass was performed with a simple Monod kinetics without inhibition and used to simulate respirometric tests. The model was applied to a respirometric test with the biomass collected from the reactor at steady-state of HRT 16 h. The model was implemented in Aquasim as shown in Figure 5.8. Since the S/X ratio (0.032) was low, the inhibition was neglected in this preliminary estimation. The K_s and the μ_h estimation for RT suspended biomass were 9 mg COD L^{-1} and 4.47 d^{-1}, respectively.
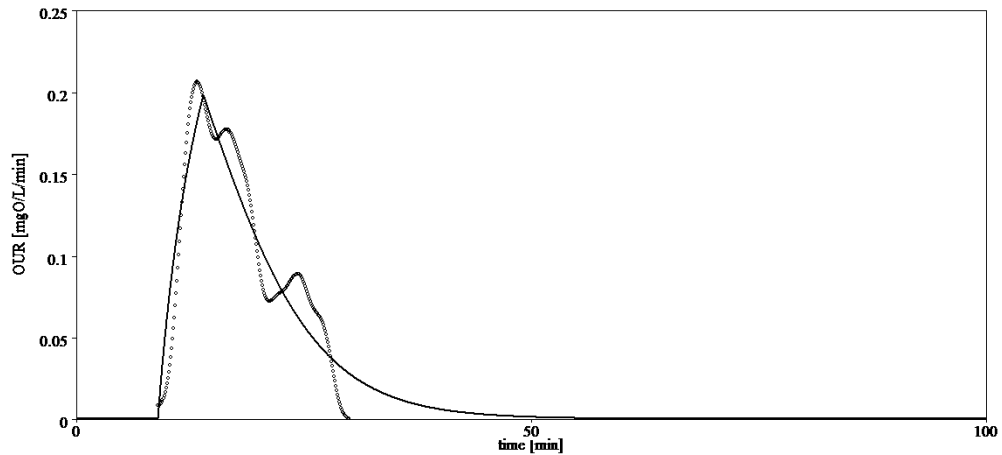
Figure 5.8: Measured OURs of respirometric tests with low S/X ratio and Monod kinetics implemented in Aquasim. K_s and μ_h estimation for RT suspended biomass. Points represents the OUR measured while the line represents the OUR modelled.

Moreover, a second estimation of μ_h and K_s was carried out with a Lineweaver-Burk plot: it was designed with the data of HRT test on RT (Figure 5.9). The K_s and the μ_h estimation for RT suspended biomass were 465 mg COD L^{-1} and 4.98 d^{-1}, respectively. The μ_h value was close to the one estimated with the respirometer test, while K_s was quite high compared to the previous estimated value. The sensitivity of $K_{s.}$ and μ_h to HRT revealed that 1 hour error in HRT measurements correspond to a change of 22% on K_s value and about 21% on μ_h value; an error of 20% on COD measurements correspond to a variability of 23% of K_s. A preliminary estimation of μ_h was performed with a Monod kinetics without inhibition applied to HRT test, but with a Contois hydrolysis kinetic to describe the fate of the decay products. The K_s and the μ_h estimation for RT suspended biomass were 240 mg COD L^{-1} and 4.99 d^{-1}, respectively. The simulation is represented in Figure 5.11.
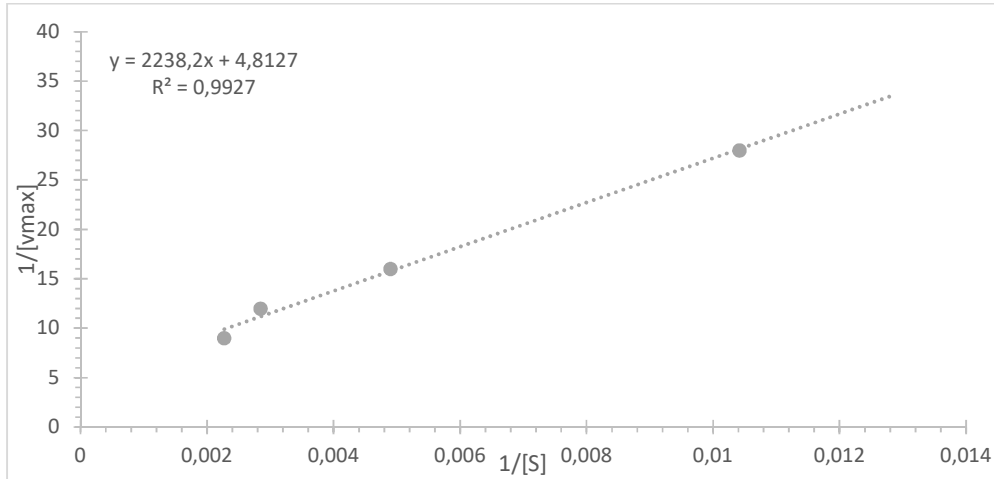
Figure 5.9: Lineweaver-Burk plot for RT suspended biomass.
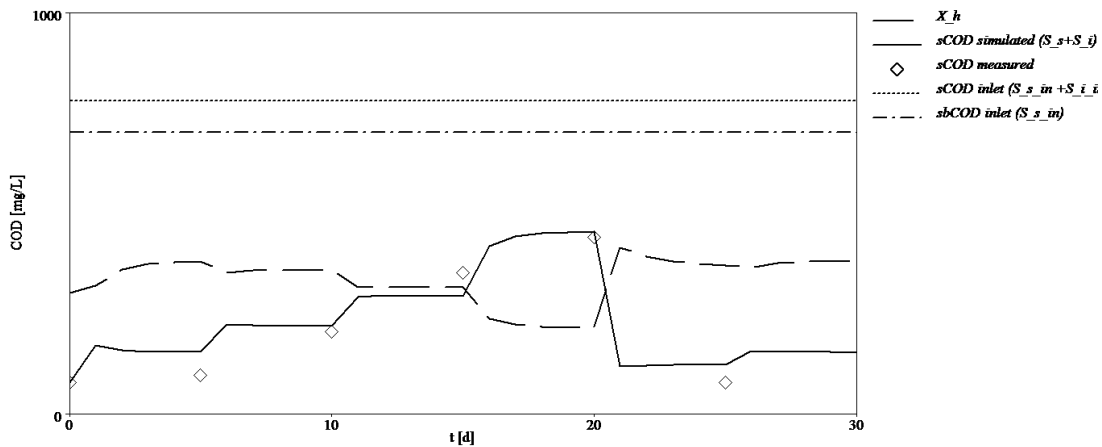
Figure 5.10: Simulated and experimental values of X_h and sCOD during the HRT test in RT used to estimate the maximum specific growth rate.

The μ_h was close to the value estimated with respirogram and Lineweaver-Burk plot. The K_s obtained was higher than one estimated with Lineweaver-Burk plot. In Figure 5.11 is reported the simulated curve with a K_s variation of 10% and 20%. Nevertheless, this estimation did not explain the observed behavior in the second part of the experiment (Figure 5.12), where inhibition occurred due to the high concentration of TT tested, thus substrate inhibition by substrate was introduced in the model.
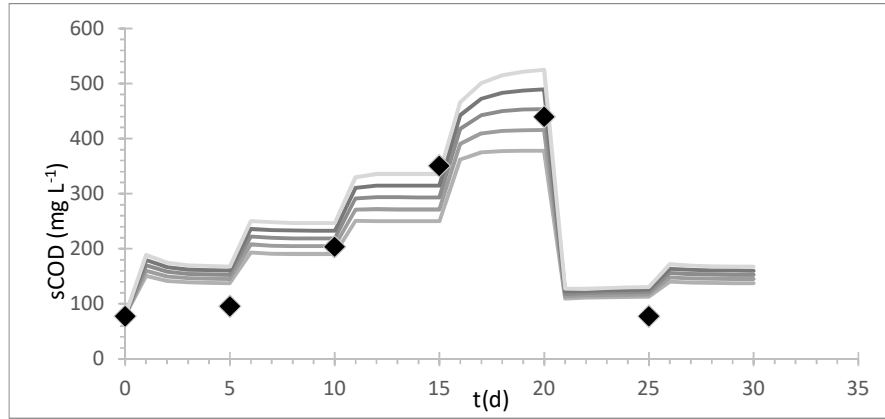
Figure 5.11: The picture shows the effect on the simulated curve with a K_s variation of 10% and 20%.
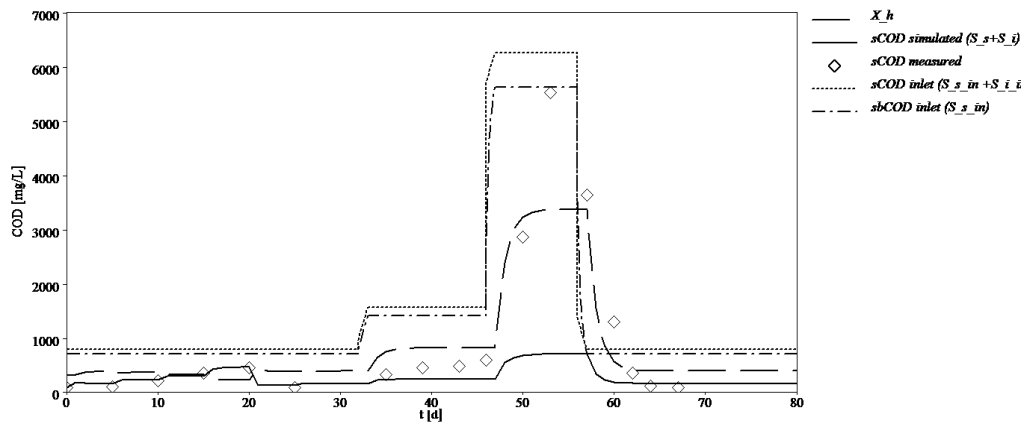
Figure 5.12: Modelling of the entire data set point of RT with the Monod kinetics without inhibition. The of K_s and μ_h were previously estimated from the results of HRT test in RT.

The estimation of K_i for substrate inhibition was performed using three calibration approaches: starting from the previous estimation of the K_s and μ_h (three pairs of values). The different K_i values estimated are shown in Table 5.6 and the optimal K_i was 637 mg COD L^{-1} with the last calibration approach. It was observed that the model slightly underestimates the outlet sCOD of the first part of the test as shown in Figure 5.13. Moreover, as reported in Figure 5.14, the model was able to approximate the solids concentration observed in the reactor (VSS measured was converted in COD with conventional 1.42 ratio for VSS/COD).

Table 5.6: Inhibition constant estimation for RT suspended biomass.

		K_s (mg COD L^{-1})	μ_h (d^{-1})	K_i (mg COD L^{-1})
1)	**Respirograms**	9	4.47	241
2)	**Lineweaver-Burk plot**	465	4.98	841
3)	**HRT test simulation**	240	4.99	637
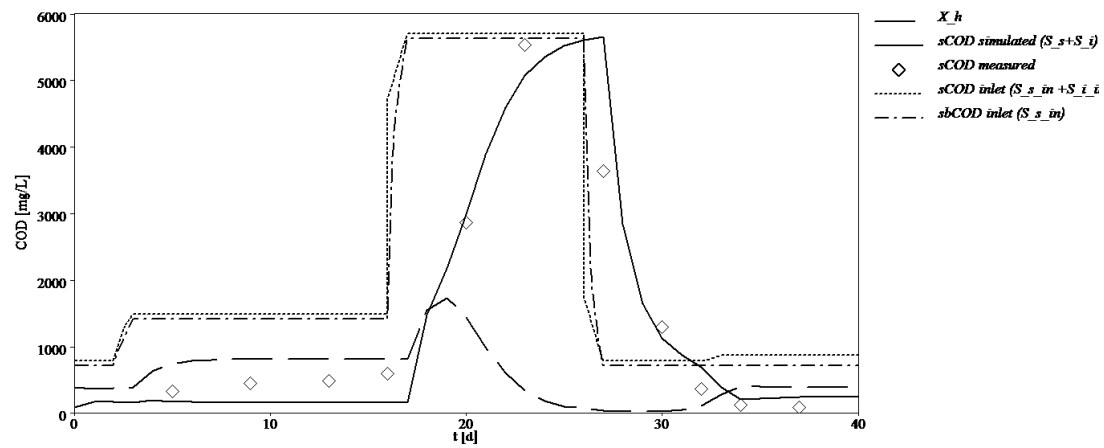
Figure 5.13: Estimation of K_i with the data of load test in RT. It was used the K_s and μ_h estimated from HRT test simulation (3° approach of Table 5.6).
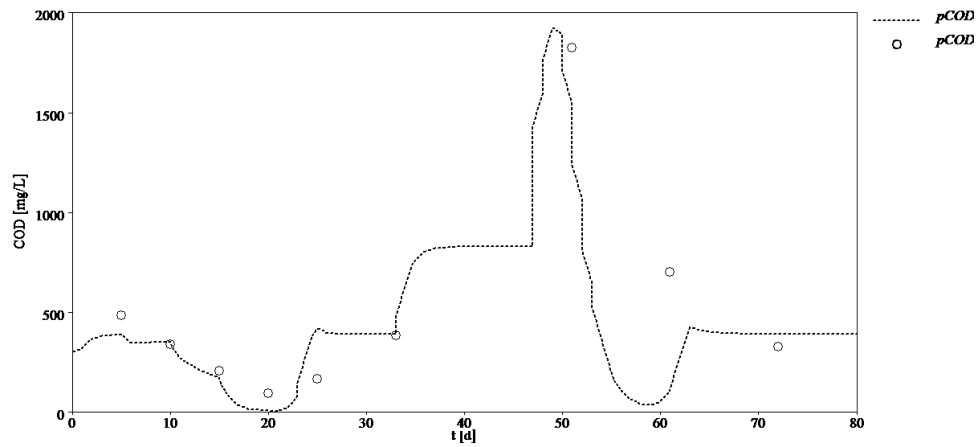
Figure 5.14: Biomass concentration (mg COD L^{-1}) measured and simulated in RT.

In Table 5.7 is reported a comparison between the results obtained and those of similar study in the literature about kinetic and stoichiometric characterisation of suspended biomass with TA and vegetable tannins (based on the same modelling approach used in the present study). It is possible to observe that in all cases high values of Ks were found when compared to the literature conventional range; this is possibly related to the complex pathway degradation of tannins. TT is an hydrolysable tannins that could be degraded by microorganism; however, as observed during the experiment in RT and RQ and as confirmed by the literature (He et al., 2007), the increasing concentration of tannins caused the inhibition of biomass activity (depending on the type of tannins).

Table 5.7: Literature comparison of hydrolysable tannins degrading biomass (*kg m^{-3} d^{-1}).

Reference	Substrate	K_s (mg COD L^{-1})		μ_h (d^{-1})	K_i (mg COD L^{-1})	Y_h (-)	b_h (d^{-1})
This Study	TT	240		4.99	637	0.68	0.29
(Li et al., 2009)	TA	226		4.99	522	0.59	0.22
(Tramšek et al., 2006)	Chestnut tannin	119		0.545*	234	n.d.	n.d.

5.2.2   Validation

The obtained inhibition model was validated with a wider data set (around 70 days). The model, with the values calculated with the HRT test, slightly overestimated the sCOD outlet concentration (Figure 5.15); nevertheless, it was more representative of the data set than the inhibition model with the K_i estimated from the μ_h and K_s values of Lineweaver-Burk plot and the respirogram. Moreover, different kind of inhibition kinetics (competitive inhibition, uncompetitive inhibition, non-competitive inhibition) depending on the snbCOD were tested, but the substrate inhibition allowed to better represent the experimental data (Figure 5.16, Figure 5.17 and Figure 5.18). The complete model is reported in Table 5.8. The mean squares error per approach showed that the substrate inhibition approssimate the measured data better than the other inhibitions types.
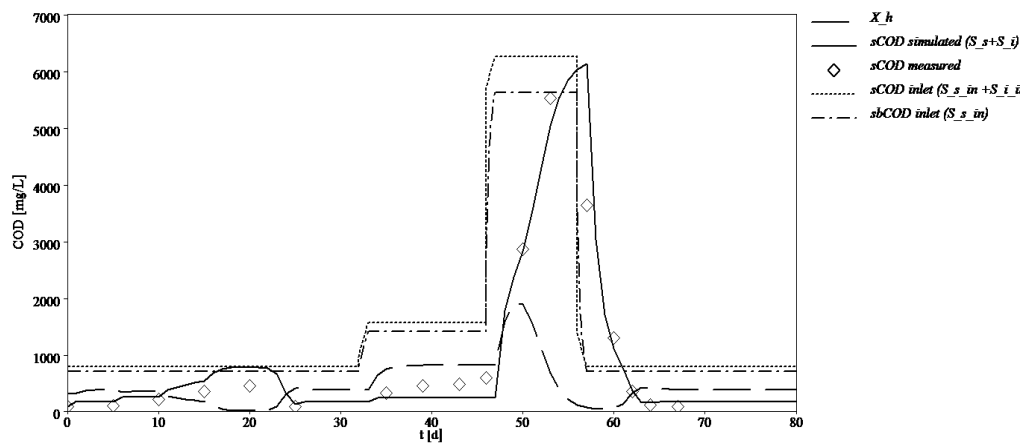
Figure 5.15: Validation of values obtained with HRT test results for RT suspended biomass.
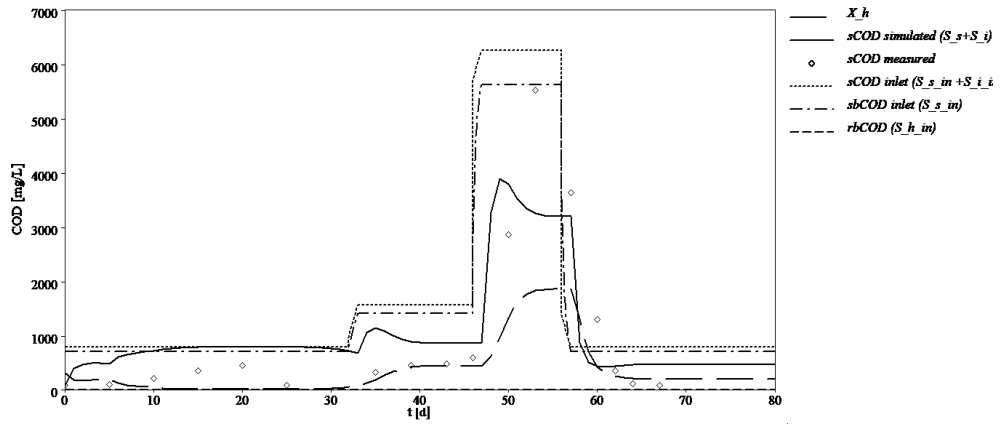
Figure 5.16: Inhibition constant estimation with competitive inhibition for RT suspended biomass.
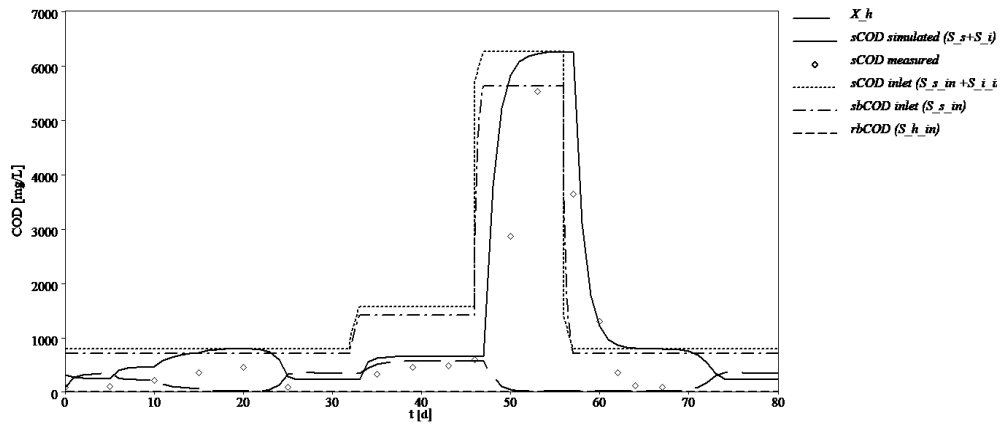
Figure 5.17: Inhibition constant estimation with non-competitive inhibition for RT suspended biomass.
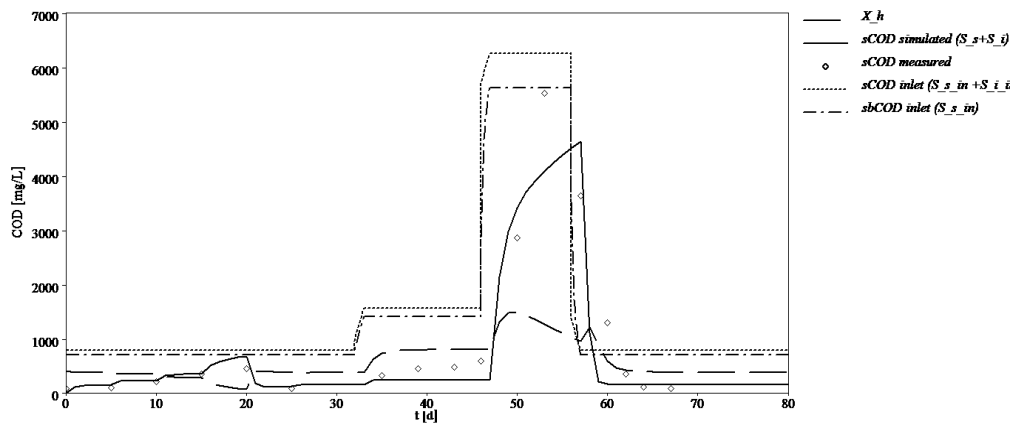
Figure 5.18: Inhibition constant estimation with uncompetitive inhibition for RT suspended biomass.

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Table 5.8: Summary of the estimated and assumed coefficients (substrate inhibition) for RT suspended biomass.

Symbol	Characterisation	Values	Units	Reference
K_{OH}	Saturation/inhibition coeff. for oxygen, het. growth	0.2	$g\ O_2\ m^{-3}$	(Henze et al., 2000)
K_H	Hydrolysis rate constant	3	d^{-1}	(Henze et al., 2000)
Y_H	Yield coeff. for heterotrophs in aerobic growth	0.68	$g\ COD$ $g\ COD^{-1}$	This study
b_H	Decay coefficient for heterotrophic biomass	0.29	d^{-1}	This study
f_P	Fraction of inert COD generated in biomass lysis	0.08	$g\ COD$ $g\ COD^{-1}$	(Andreottola and Esperia, 2001)
K_i	inhibition constant	240	$g\ COD\ m^{-3}$	This study
K_s	Half-saturation coefficient	637	$g\ COD\ m^{-3}$	This study
K_X	Hydrolysis rate constant	0.02	$g\ X_S\ g^{-1}\ X_H$	(Henze et al., 2000)
μ_H	Maximum growth rate on substrate	4.99	d^{-1}	This study

5.3 QUEBRACHO TANNIN REACTOR MODELLING

The modelling of QT degrading fungal biofilm was carried out by coupling the results of tests performed in bioreactor RQ with those of respirometric techniques applied to attached biomass. The tests in the respirometer, with the dedicated developed procedure explained in the Materials and Methods section, were used to perform a kinetic and stoichiometric characterisation. The model was designed with the software Aquasim and the dedicated compartment to simulate dynamic processes in biofilm bioreactors. The biofilm features and transport parameters were defined by

measurements or literature, while to describe the microbial kinetics it was selected a Monod kinetic equation with substrate inhibition where the parameters were calibrated with the data collected.

5.3.1 Biofilm model structure definition

The Aquasim software includes a biofilm compartment (consisting of a biofilm phase and a bulk fluid phase) that was used to simulate the behavior of biomass growing on the support media and the diffusion phenomena (Boltz et al., 2010). The Aquasim biofilm compartment supports the biofilm modelling in different type of biofilm bioreactors (Wanner and Morgenroth, 2004). In Figure 5.19 the dialog box of the software with all the features of this compartment was reported.
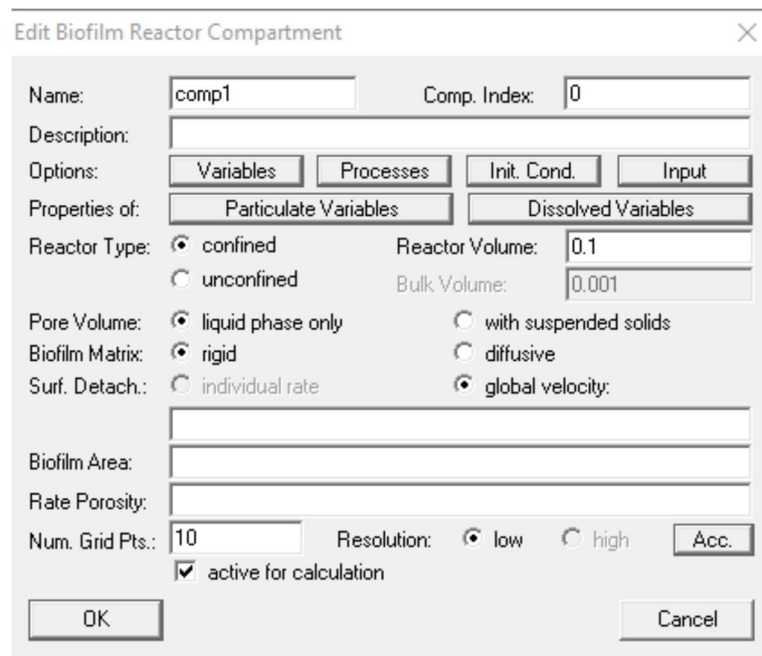
Figure 5.19: Dialog box for editing a biofilm reactor compartment in Aquasim.

The definition of the characteristics of the biofilm reactor was a necessary step before running the simulations. The first step was the definition of the boundary conditions of the reactor RQ required for the biofilm compartment. The reactor volume was imposed to be 4 L and the reactor type was "confined" with a constant total volume for biofilm

and bulk fluid (no biofilm growth out of the PUF). The pore volume was defined as "liquid phase only" and influent suspended solids were neglected, since the inlet COD was mainly sCOD. For the same reason, the biofilm matrix was modelled as "rigid" and the diffusive mass transport of solids was neglected. Among the main hypothesis of the model, the adsorption was neglected and the diffusion was considered only for tannins and oxygen. The surface area and the biofilm thickness are biofilm parameters described in the model as follow: the 100 immobilised PUF cubes (2x2x2 cm) were modeled as 100 spheres with 2 cm of diameter, in fact the observed diffusion of tannin shown an isotropic behavior and a radial gradient from outside to the center of the carriers. The plastic carriers were neglected due to the high porosity and the small volume occupied by the plastic support. The biofilm thickness was 1 cm (initial condition) and the surface area was described in Aquasim as described in the Eq. 5.6 as suggested in Aquasim manual.

$$A = 4\pi n_{sp}(r_{sp} + z)^2 \tag{5.6}$$

where:

A surface area (m^2);

n_{sp} number of spherical particles (100);

r_{sp} radius of the spherical particles (0.01 m);

z distance from the substratum (program variable).

The biofilm density was calculated from pure fungi samples. The samples were inserted in graduated volumetric vessel and the TSS and VSS of the biomass was measured with the standard procedure. The obtained pure fungal biofilm average density was 12.5 g L^{-1} as dry mass and this value was adopted in the model. Nevertheless, in the literature a high variability in fungal biofilm density (Spigno et al., 2003; Stewart, 1998) is reported. Subsequently, the transport phenomena and mass transfer related parameters were defined. The water diffusion coefficient of oxygen in water (D_w) was $8.96\cdot10^{-6}$ m^2 h^{-1} (Horn and Morgenroth, 2006) and the internal diffusion in the biofilm could be expressed with the Eq. 5.7.

$$D_f = f_{dif} D_w \tag{5.7}$$

Where:

D_f diffusion coefficient in the biofilm (m^2 h^{-1});

D_w diffusion coefficient in water (m^2 h^{-1});

f_{dif} relative diffusivity (adimensional).

The effective internal diffusion coefficient in biofilms can change with the biofilm density and the biofilm thickness; usually, the relative diffusivity (f_d) ranges from 40% to 90% (Hibiya et al., 2004). Some authors found a correlation between the oxygen profiles and the biomass distribution in biopellets of *Aspergillus niger* (Hille et al., 2005) and others proposed that the pellet density could allow to predict the steepness of oxygen concentration profiles (Fan LS1, Leyva-Ramos R, Wisecarver KD, 1990). The biofilm density reported in the previous paragraph, supports the adoption of a f_d value equal to 80% (Horn and Morgenroth, 2006). The diffusion of phenols in water was estimated to be $8.47 \cdot 10^{-10}$ m^2 s^{-1} ($2.35 \cdot 10^{-13}$ m^2 h^{-1}) (Fan LS1, Leyva-Ramos R, Wisecarver KD, 1990) and that of natural tannins to be $5 \cdot 10^{-11}$ m^2 s^{-1} ($1.38 \cdot 10^{-14}$ m^2 h^{-1}) (Tzibranska, 2000). The tannins diffusion was modelled with the same equation used for oxygen diffusion. However, the dissolved compounds (oxygen, tannins) should pass first through the mass transfer boundary layer (external mass transfer) via convection and, then, through the biofilm matrix (internal mass transfer) via diffusion (Khabibor Rahman et al., 2009), Nevertheless, in these simulations the external mass transfer was neglected due to the mixing in the reactor. The f_{dif} chosen was 80% (Stewart, 1998).

5.3.2   Microbial parameters estimation and model validation

The tests in the respirometer, with the dedicated procedure explained in Materials and Methods section, were used to preliminary characterise the kinetic and stoichiometric parameters of the fungal biomass. A simple Monod kinetics was used for a respirometric estimation of microbial parameters, while a biofilm model was used to simulate the reactor performance. For the decay coefficient (b_f) of fungal biomass it

was adopted the value suggested in literature 0.22 d^{-1} for *Aspergillus niger* (Wang et al., 2008). The estimation of the yield coefficient (Y_f) was done through the calibration of the parameters of Eq. 5.3 with the respirograms (Figure 5.5). The biodegradable COD was assumed as 17% of the total COD to estimate Y_h. This value was extrapolated from an average performance in the reactor at steady state, with similar conditions of the respirometric tests (concentration and S/X ratio). The obtained Y_f was 0.45 ± 0.01 mg COD mg COD^{-1} was quite close to the value suggested in literature for *Aspergillus niger* with glucose 0.37 mg dry mass mg glucose^{-1} (= 0.51 mg COD mg COD^{-1}) (C N Aguilar et al., 2001; Viniegra-González et al., 2003). The active biomass (X_f) in the reactor at steady-state was estimated at the two different HRT tested in the reactor with the Eq. 5.3. The SRT was calculated in the previous section. The estimated biomass concentrations were reported in Table 5.9.

Table 5.9: Active biomass estimation.

HRT (h)	Active biomass (mg COD L^{-1})
52	251
28	465

To estimate the affinity constant (K_{sf}) and the maximum specific growth rate (μ_f) of the biomass of RQ a simple Monod kinetics without diffusion in biofilm, adsorption neither inhibition was applied. The procedure was similar to that applied for the estimation of suspended biomass from RT. The model was applied to a respirometric test with the biomass collected from the reactor at steady state. The respirometric tests were performed with pulses of 1, 2, 5 and 10 millilitre of QT (10 g L^{-1}) in a vessel of 0.3 L with 10 PUF cubes (Figure 5.5). The best estimation of K_{sf} and μ_f were 800 mg COD L^{-1} and 2.88 d^{-1}, respectively, even though, the best fitting simulations overestimated the measured OURs, as represented in Figure 5.20 and Figure 5.21. Multiple simulations performed on respirometer tests results showed that the limiting step was the diffusion in the biofilm and it was not achieved a better fitting of the data since this model did not take in account diffusion in biofilm.
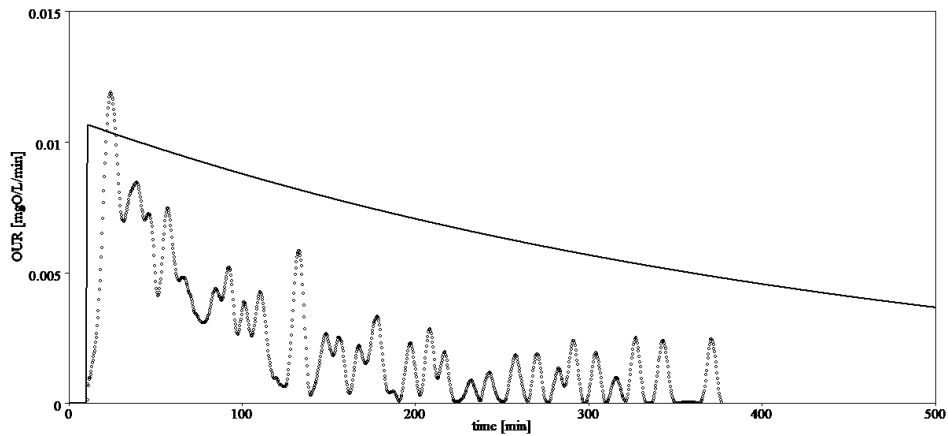
Figure 5.20: Measured OURs in a respirometric test with a pulse of 1 ml of QT (at 10 g L^{-1} concentration) and Monod kinetics implemented in Aquasim.
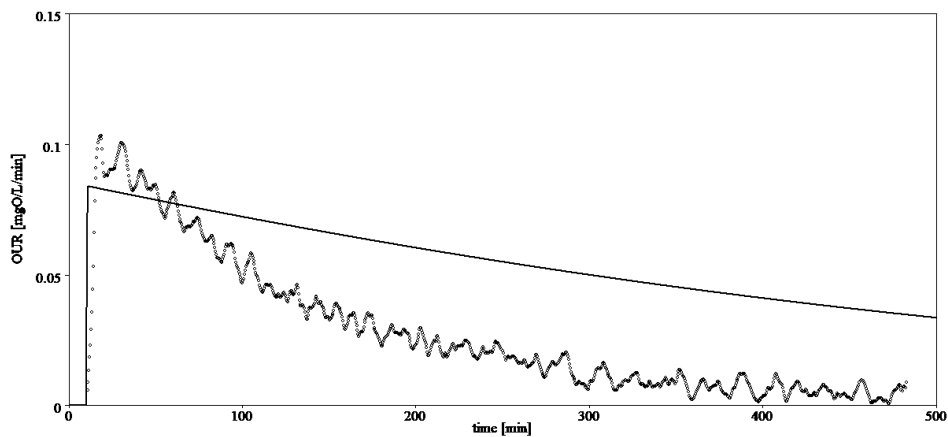
Figure 5.21: Measured OURs in a respirometric test with a pulse of 10 ml of QT (at 10 g L^{-1} concentration) and the Monod kinetics implemented in Aquasim.

The results of 154 days operation of the reactor RQ were used to obtain a better estimation of μ_{hf} and K_f estimate the inhibition due to the QT (K_{iq}). The inhibition kinetics were implemented in Aquasim with the biofilm compartment with all the equations and the parameters presented in the previous section. The best estimation obtained was represented in Figure 5.22 and the microbial kinetics coefficients of the biofilm were reported in Table 5.10. The applicative consequences of these μ_{hf} and K_f are that fungi can grow very quickly if the substrate (QT) is abundant, which is at this level more typical for solid matrices than for wastewater, even though the inhibition should be also considered.

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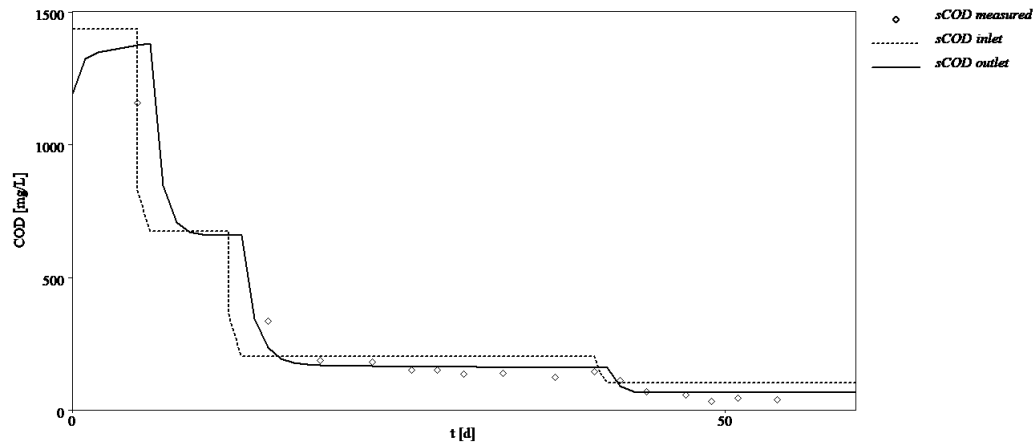
Figure 5.22: Measured and simulated values for outlet sCOD for RQ biofilm model.

Table 5.10: Summary table with estimated and chosen microbial kinetics coefficients.

Symbol	Characterisation	Value	Units	Reference
Y_f	Yield coeff. for fungi in aerobic growth	0.45	g COD g COD^{-1}	This study
b_f	Decay coefficient for heterotrophic biomass	0.22	d^{-1}	(Wang et al., 2008).
f_P	Fraction of inert COD generated in biomass decay	0.08	g COD g COD^{-1}	(Andreottola and Esperia, 2001)
K_{iq}	inhibition constant	13	g COD m^{-3}	This study
K_{sf}	Half-saturation coefficient	993	g COD m^{-3}	This study
μ_f	Maximum growth rate on substrate	5.39	d^{-1}	This study

6   PILOT SCALE TEST

Chapter abstract

The experiments described in the previous chapters proved that QT, the most recalcitrant tannin for leather tanning, have been used as carbon source by the fungal biomass and effectively removed. Moreover, QT showed an inhibition effect at increasing concentration that reduced its biodegradation efficiency; fungi has been revealed more resistant to this inhibition effect than bacteria and QT concentration allowed to maintain a stable fungal biofilm in non-sterile conditions. Other vegetable tannins, such as TT, had an inhibition effect at higher concentration. In this chapter, feeding with QT as a main carbon source and specific operating conditions have been verified as a good strategy to promote a fungal growth and immobilisation in a pilot-scale reactor in non-sterile conditions. Moreover, the pilot-scale reactor has been demonstrated effective in the removal of COD from an exhaust tanning bath collected from one of the tanneries of the Tuscany tannery district. The start-up phase lasted 55 days and the treatment phase lasted 66 days, thus the entire experiment lasted 121 days. The chapter is divided into two sub-sections dealing with the start-up phase and the treatment phase.

In the first sub-section describing the start-up phase, the main aim was to achieve the immobilisation of a fungal biofilm (able to remove tannins) in non-sterile conditions at pilot-scale. To reach the scope, as described in Materials and Methods, the results obtained in the bioreactors experiments have been exploited to define the optimal selective pressures. An inoculum of 8 L of pure culture of *Aspergillus tubingensis* was prepared and dosed inside the reactor.

In the second sub-section describing the treatment phase, a tannins bath with high content of QT and other tannins was collected from a tannery factory and stored in 1 cubic meter tank: that was treated with the pilot-scale reactor (after 40 times dilution). The fungal pilot-scale reactor was able to remove, in non-sterile condition and without any co-substrate, in average the 29% and 23% of COD and DOC of the tannins bath, respectively.

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6.1   START-UP PHASE

At the end of inoculum incubation, the GLY medium in vessels was removed under sterile conditions (the operation was performed under a biological hood). Subsequently, the fungal biomass, a pure culture of *Aspergillus tubingensis,* was homogenized and mixed with a new GLY solution (8 L in total). The first step of the start-up was the insertion of the inoculum inside the reactor. The inoculum was poured equally in all the sectors and after that the pilot-scale reactor was filled with the QT medium. At the beginning of the start-up the fungal biomass inoculated allowed to achieve a concentration of approximately 20 mg VSS L^{-1} in the reactor. The results obtained in the previous tests with continuous bioreactors operation were exploited to define the optimal selective pressures (acid pH, QT medium, PUF cubes, etc.) for the fungal biomass immobilisation and growth in non-sterile conditions as detailed described in Materials and Methods. The concentration of 1 g QT L^{-1} was chosen as feeding medium to avoid excessive inhibition effect and, at the same time, to provide sufficient substrate for fungal growth. The start-up lasted 55 days and the four sectors of the cage were filled with different PUF cubes size in order to identify the most suitable one for the fungal immobilisation (a sector was filled with PUF cut-to-shape, but after two weeks was substituted with PUF cubes with 2.5 cm size, because the sector was not stable due to the aeration). The elemental composition analysis of the inoculated fungus revealed a C/N ratio of about 13 and a C/H ratio of about 7, while sulphur was below the detection limit.   The inlet concentration of nitrogen and phosphorus in the medium solution was enough to avoid limitation effects. The measured COD concentration of the inlet solution was 1,645 ± 339 mg L^{-1}, while the DOC was 408 ± 79 mg L^{-1}. Even though the QT was completely soluble and a mixed vessel was used during the medium preparation, the formation of small aggregates of tannins occurred. The QT powder was slowly poured in the mixed feeding tank, however, due to the size of the tank (1 m^3) the QT powder was prone to create small aggregates that were slowly dissolved; in fact, an average value of pCOD 636 mg L^{-1} due to this aggregation was measured in the inlet; a consistent concentration of VSS (519 ± 325 mg VSS L^{-1}) was measured and an average ratio of pCOD/COD of 0.4 was estimated. The average ratio VSS/TSS was 0.84 ± 0.08. Noteworthy, it was observed a

released of nitrogen (part detected as ammonium) from the reactor in the first two weeks as reported in Figure 6.1. The release of nitrogen during the first two weeks could be related to the residuals of PUF production process possibly present on its surface, in fact, in the industrial PUF production process, isocyanate reacts with an amine leading to the synthesis of urea (double amide of the acid carbonate) as byproduct. During the start-up, biomass growth in all the supports was observed and at the end of the start-up the average dry mass concentration was (at 42 days) 0.078 ± 0.018 g of dry mass in each 2.5 cm cubes. It was observed that the PUF cubes of 2.5 cm size were the most effective (in terms of dry mass/PUF volume ratio) for biomass growth; in fact, the PUF cubes of 5 and of 7.5 cm had 2% and 32% less dry mass density than the smallest size. In the sampled PUF cubes of 7.5 cm size, it was visually observed that the mycelium did not grow more than 2 cm in depth approximately, probably due to transport limitation phenomena (representative pictures of PUF are reported in Figure 6.2 and Figure 6.3).
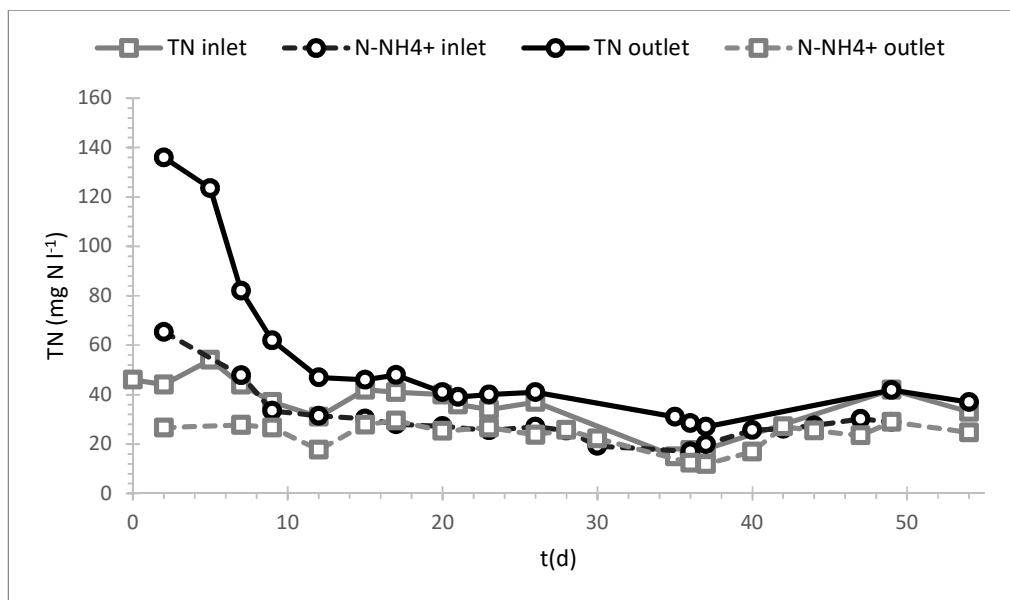
Figure 6.1: Total nitrogen and ammonium concentration inlet and outlet in the pilot reactor during the start-up phase.

Figure 6.2: Representative pictures of the three sizes of PUF cubes (left) and one sector of the cage (right).
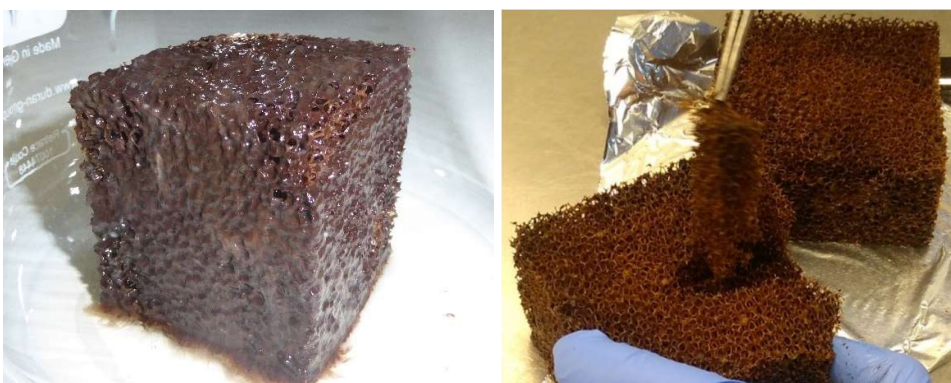
Figure 6.3: PUF cubes with 7.5 cm size: external view (left) and internal view (right).

The PUF cubes with different size occupied approximately a total volume of 337 L as detailed for each sector in Table 6.1. Therefore, the estimated dry mass at the end of the start-up (42 days) was roughly 1.55 kg of dry mass in total. Visual observations (Figure 6.4) and with a microscope (Figure 6.5) revealed a prevalently fungal biofilm development in the PUF and the ongoing metagenomic analysis will provide further information (and it will be possible to compare with RQ biomass). In conclusion, the PUF of 2.5 cm size seems the most suitable (compare to the other tested) to promote the fungal immobilization.

Table 6.1: Characteristic of the sectors of the pilot-scale reactor.

	Volume (m^3)	Surface (m^2)	dry mass (kg)	dry mass/volume (kg m^{-3})
Sector 1	0.094	56	0.468	4.99
Sector 2	0.078	47	0.377	4.86
Sector 3	0.072	43	0.243	3.39
Sector 4	0.094	56	0.468	4.99
Pilot bioreactor	0.337	202	1.556	4.56

Figure 6.4: Pictures of immobilised PUF cubes in the pilot at the end of the start-up phase. On the left a picture of PUF cubes located in the middle of the sector (less exposed to the share stress of rotation).
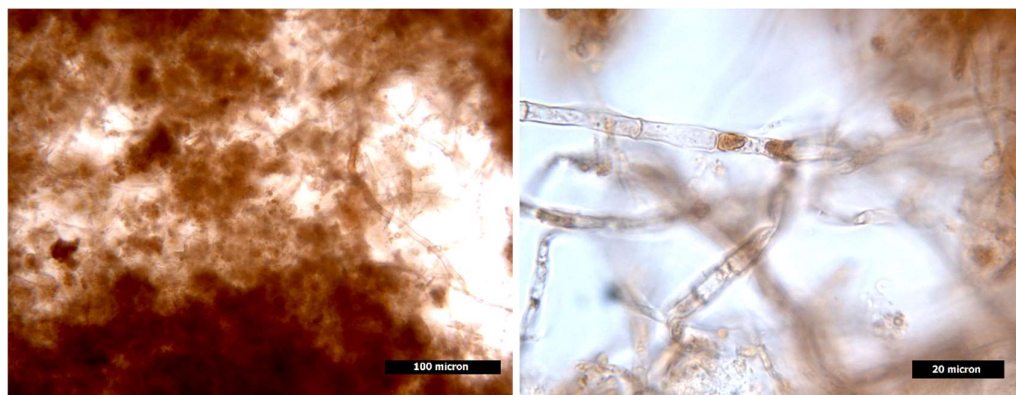
Figure 6.5: Microscope pictures of biofilm from immobilised PUF cubes in the pilot at the end of the start-up phase.

During the start-up, an average COD RE of 31% and a DOC RE of 21% were achieved, in addition a sCOD RE of 9% supported the hypothesis of tannins aggregates dissolution. In the inlet, due to the aggregation the pCOD concentration was high; however, in the reactor the pCOD dissolved into sCOD and thus the RE of sCOD was apparently lower than expected. The ratio among COD/DOC in the inlet and the outlet was 2.54 ± 0.35 and 3.05 ± 0.15, respectively. In the outlet the average ratio VSS/TSS was 0.77 ± 0.20 and the concentration of VSS was 30 ± 14 mg VSS L^{-1}. The estimated OLR was 19.99 mg COD L^{-1} h^{-1} and the ORR 6.30 ± 0.86 mg COD L^{-1} h^{-1}: this result was an improvement when compare to the test in the laboratory scale bioreactor RQ where the OLR was 27.6 mg COD L^{-1} h^{-1} and the correspondent ORR 5.3 mg COD L^{-1} h^{-1}. Moreover, the RE of the reactor RQ in similar conditions were lower, about 17%,

than what was found with the pilot-scale reactor. The μ'_f was estimated as described below from the dry mass measurements in the period between day 14 and day 28. During this period the biomass growth was approximated as exponential as showed in Eq. 6.1; in the following period the substrate concentration possibly decreased to limiting values in the reactor and in the previous period there was a lag phase; moreover, the substitution of the support media in a sector of the reactor, as well as the release of ammonium from PUF, could have interfered with the growth.

$$X(t) = X(t_0) \cdot e^{\mu_f t} \tag{6.1}$$

where:

$X(t)$ is the biomass concentration at time t (mg L^{-1});

$X(t_0)$ is the biomass concentration at time t = 0 (mg L^{-1}).

From the dry mass measurements between day 14 and day 28 it can be estimated an average doubling time of the biomass about 8.57 days. From Eq. 6.1 it is possible to estimate the specific growth rate μ'_f imposing the doubling time as showed in Eq 6.2. Even if μ'_f was estimated to be 0.08 d^{-1}, that is lower than the maximum specific growth rate μ_f of chapter 5 (5.39 d^{-1}).

$$\mu'_f = \frac{\ln}{T} \tag{6.2}$$

where:

T is the mean doubling time (d).

The trends of COD, DOC etc. of the start-up are reported in the next section with the results of the treatment phase for a better overview. However, it is worth mention that since the temperature control was a low bound control, while the pH control was a high bound control some fluctuations occurred. The average temperature in the start-up was 26.8°C (and it was probably advantageous for the fungi growth) and it was observed fluctuation of pH till 3. The pH occurred especially in the start-up as showed in Figure 6.6 . In the treatment phase those fluctuations were reduced with a fine tuning of acid solution dosing rate and modify the pH of feeding solution at 5. Moreover, thanks to that fluctuation it was possible to find a correlation among the redox potential and the pH (Figure 6.7).
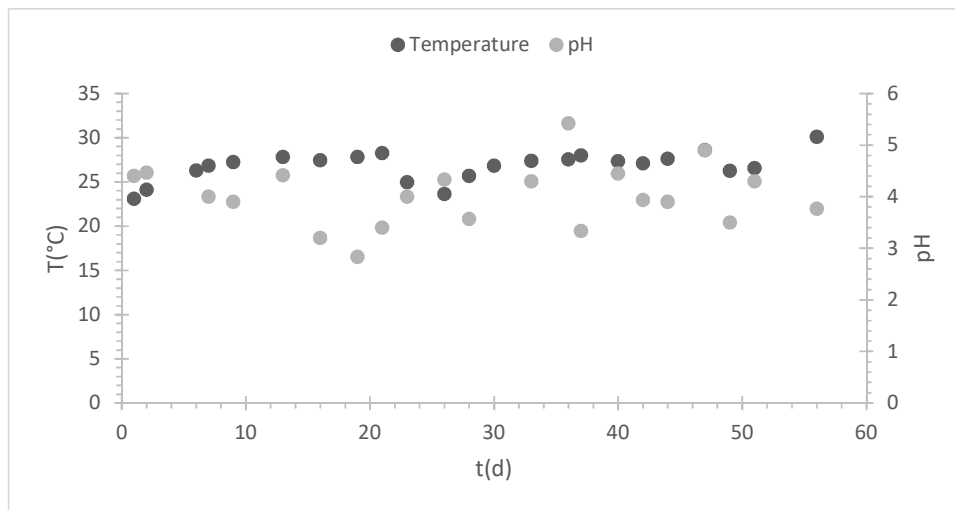
Figure 6.6: The average temperature and pH during the start-up in the pilot-scale reactor.
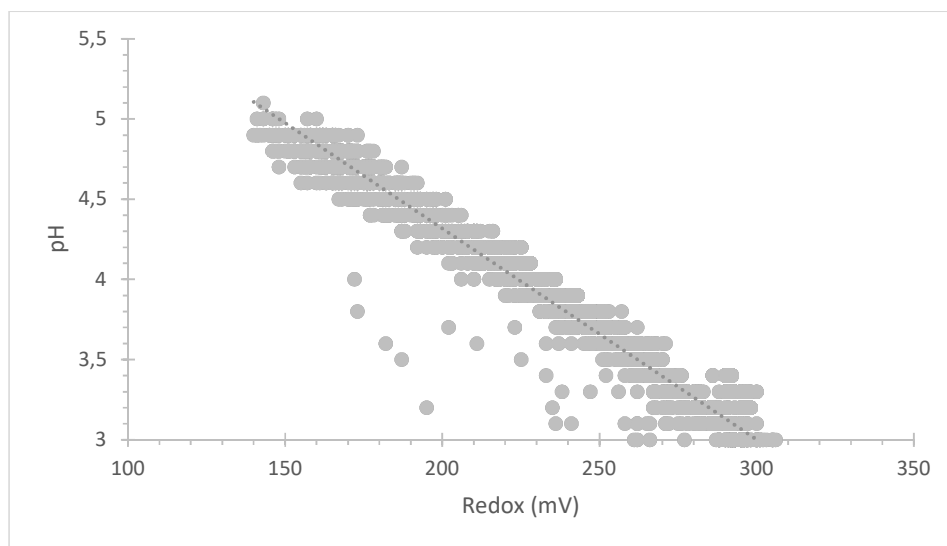
Figure 6.7: Correlation among pH and redox in the pilot-scale reactor.

6.2   TREATMENT PHASE

In the treatment phase a tanning bath with a high content of tannins was collected from a tannery factory and stored in a cubic meter tank. The tannins bath was treated with the pilot-scale reactor (after 40 times dilution) as described in Materials and Methods, the resulting average COD in the inlet solution was $1{,}579 \pm 331$ mg L^{-1}, while the DOC was 396 ± 40 mg L^{-1}. The pilot reactor, during the treatment phase, was

able to remove in average the 29% and 23% of COD and DOC, respectively. The trends of COD and DOC during the whole experimental period are shown in Figure 6.8 and Figure 6.9. The inlet concentration of COD and DOC was maintained very close to the value of the QT medium solution fed during the start-up phase also the removal performance of the reactor was similar to the previous start-up period. However, the sCOD RE was in average 21%, this value is higher respect to the start-up and more similar to COD RE (Figure 6.10). The pCOD and VSS in the inlet was much lower respect to the QT medium used during the start-up: pCOD was 138 ± 50 mg L^{-1} and the VSS about 159 ± 103 mg L^{-1}. The ratio among COD and DOC in the inlet and in the outlet were 4.11 ± 1.11 and 3.56 ± 0.30, respectively. The estimated OLR was 19.19 mg COD L^{-1} h^{-1} and the ORR 5.91 ± 0.98 mg COD L^{-1} h^{-1} similar to the previous phase.
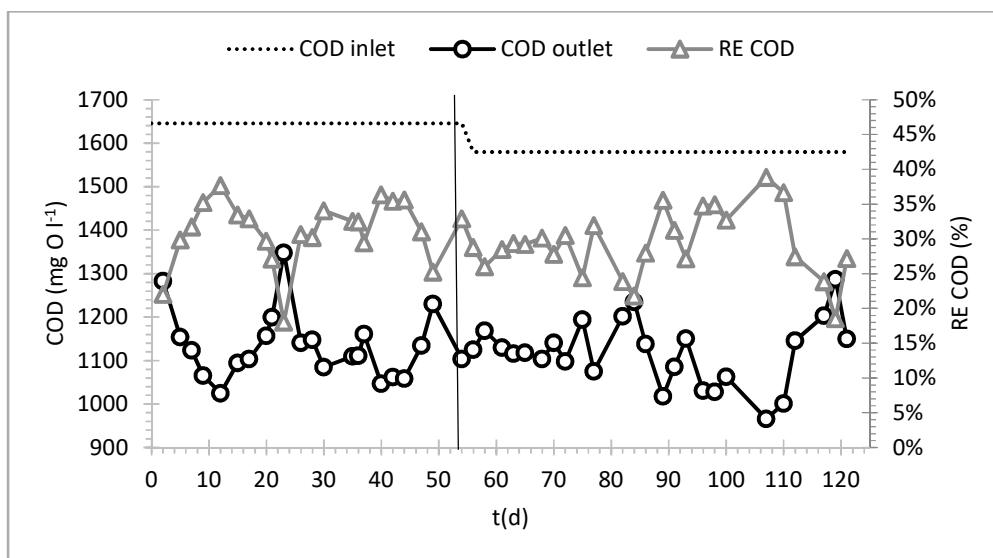
Figure 6.8: Inlet and outlet COD and COD removal percentage during continuous treatment in the pilot reactor. Vertical line divided the start-up phase from the treatment phase.

Figure 6.9: Inlet and outlet DOC and DOC removal percentage during continuous treatment in the pilot reactor. Vertical line divided the start-up phase from the treatment phase.

Figure 6.10: Inlet and outlet sCOD and sCOD removal percentage during continuous treatment in the pilot reactor. Vertical line divided the start-up phase from the treatment phase.

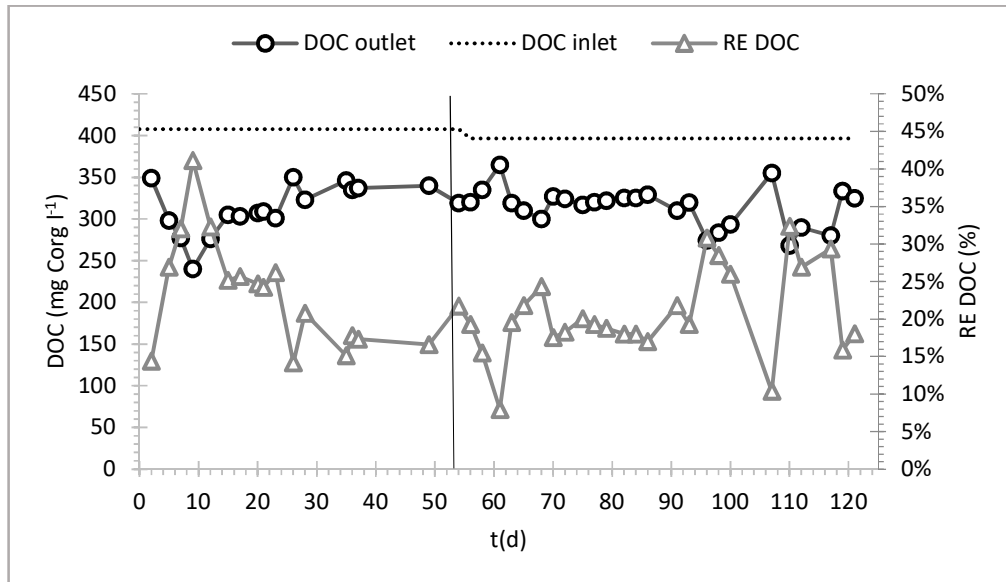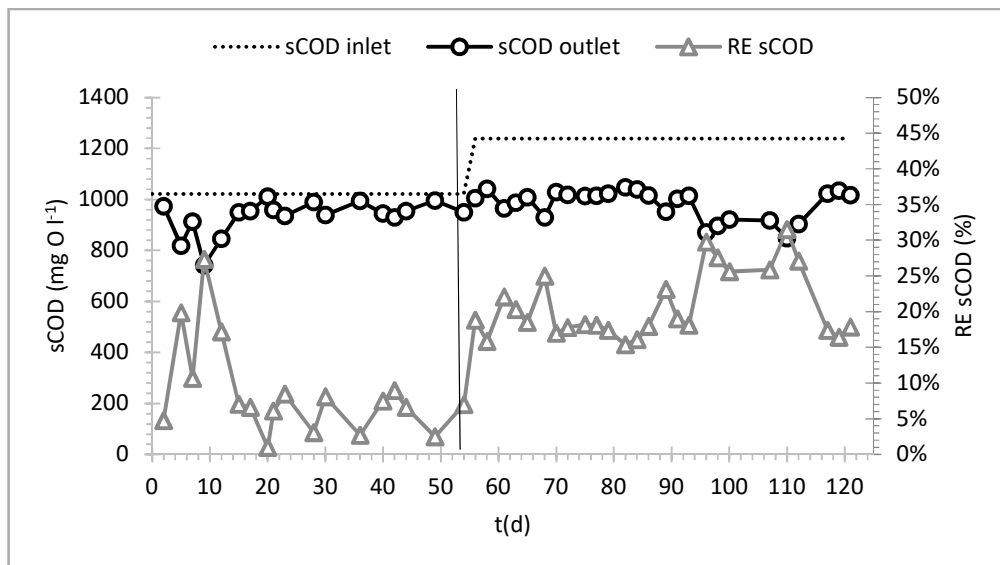The dry mass inside the PUF cubes of 2.5 cm increased during the experimentation, as shown in Figure 6.11, till 0.13 g for each PUF (day 110). The VSS are represented in Figure 6.12: in the outlet were about 28 ± 14 mg VSS L^{-1}. The dry mass of biofilm was

estimated about in 1.55 kg in the end of start-up period and 2.80 kg in the end of treatment phase. It is reasonable to estimate the COD of the biofilm with the conventional ratio 1.42 VSS/COD; thus, at the end of the start-up period the biomass growth until 2.20 kg of COD (1.55·1.42) while in the second period increased of further 1.77 kg COD. A COD mass balance during the entire period of the start-up revealed that the pilot reactor was fed with 37.99 kg of COD as QT, while in the outlet was measured 26.21 kg of COD: approximately were removed 11.78 kg of COD. During the entire period of treatment phase the inlet was 43.76 kg of COD, the outlet 31.07 Kg of COD and 12.69 kg of COD were removed. The biomass in the outlet was 0.69 kg as VSS in the start-up phase (approximate as 0.98 kg of COD) and in the treatment phase was 0.77 kg as VSS (approximate as 1.10 kg of COD). The observed growth yield (Y_{obs}) was calculated as for the start-up (Y_{obs_1}) and treatment phase (Y_{obs_2}) with the Eq. 6.3 and 6.4. The Y_{obs_1} was slightly higher than Y_{obs_2}:

$$Y_{obs\_1} = \frac{2.20 \; Kg \; COD + 0.98 \; Kg \; COD \; (Biomass)}{11.78 \; Kg \; COD \; (Substrate)} = 0.27 \; \frac{Kg \; COD}{Kg \; COD} \tag{6.3}$$

$$Y_{obs\_2} = \frac{1.77 \; Kg \; COD + 1.10 \; Kg \; COD \; (Biomass)}{12.69 \; Kg \; COD \; (Substrate)} = 0.23 \; \frac{Kg \; COD}{Kg \; COD} \tag{6.4}$$

The estimated SRT for the treatment phase, assuming an average value of 2.17 kg of dry mass for the treatment phase, was calculated with his definition (Eq. 6.5) as 184 d. It is teorically possible reduce this value increasing the rotation till to the biomass washout ($\mu'f^{-1} = 12$ days). The effect of the adsorption of tannins on mycelium, was not taken into account: it could play a role and it is difficult to estimate, but considering to the dry mass in the PUF and the degraded COD, biodegradation is the main process causing COD removal.

$$SRT = \frac{Average \; mass \; of \; biofilm}{average \; rate \; of \; biofilm \; detachmen} \tag{6.5}$$

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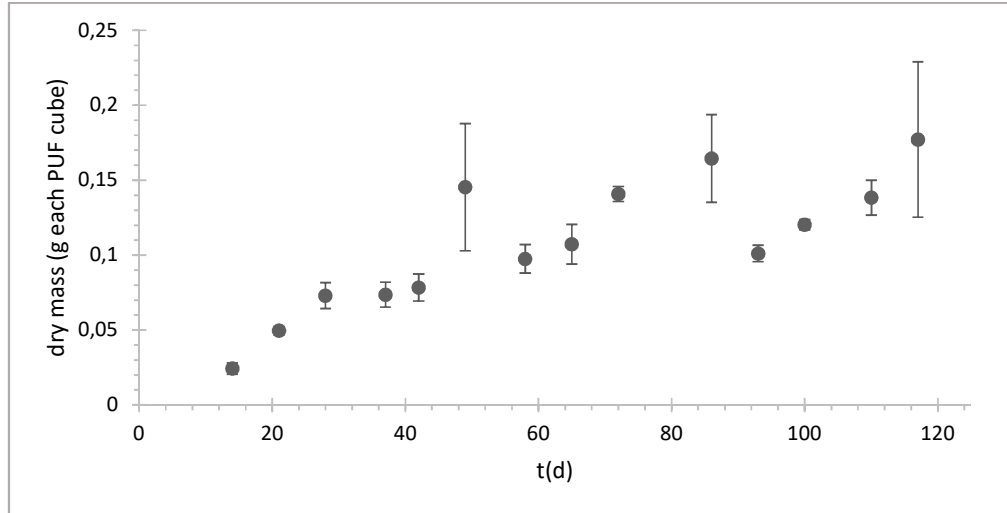**Figure 6.11:** Dry mass in PUF cubes collected from the pilot reactor.
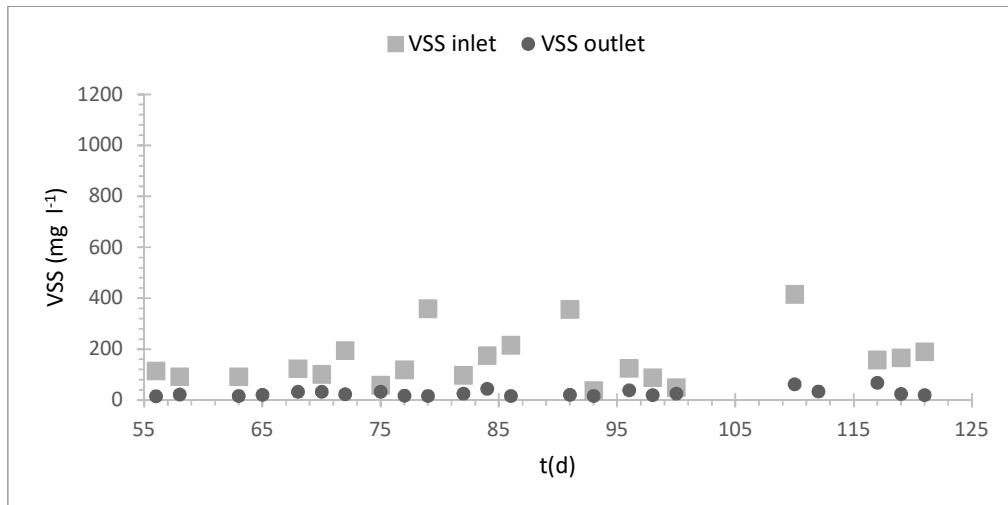
Figure 6.12: VSS inlet and outlet in the pilot reactor during the treatment phase.

Nitrogen and Phosphorus were measured in the inlet and outlet solution and, despite the high SRT and oxygen, Nitrification was not detected (probably due to low pH) and roughly 2/3 of the total Nitrogen was present as Ammonium (Figure 6.13).

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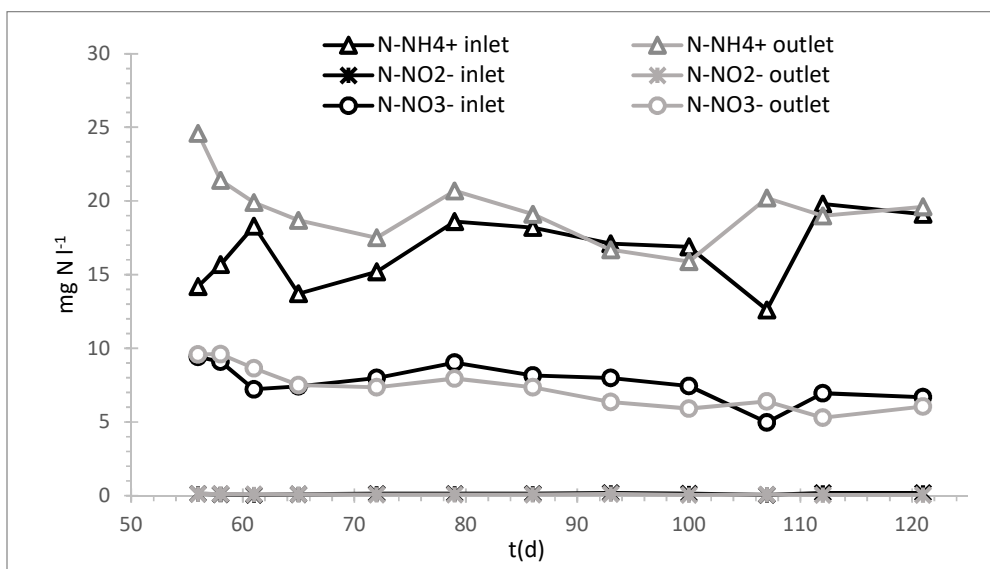
Figure 6.13: Ammonium, Nitrate and Nitrite concentration inlet and outlet in the pilot reactor during the treatment phase.

From elemental analysis of fungal biomass and the dry mass estimated in the end of the experiment, it was calculated a consumption of ammonium of about 3 mg N L^{-1} (estimated for the assimilation in the biomass). However, this consumption was not detected in the outlet. It was verified that tannins could interfere with the spectrophotometry measurements of ammonium with Hach cuvettes, thus could be a possible explanation of the missing ammonium in the mass balance. Moreover, fungal biomass could uptake nutrients (such as nitrogen) through the PUF support degradation (Khan et al., 2017; Loredo-Treviño, 2011) and this could be a second explanation, but the sampled PUF did not show visible damage or consumption.

The experiment demonstrated that it is possible to growth and immobilised a fungal biomass in non-sterile conditions and remove tannins effluents. Moreover, the SRT could be controlled with rotation. Future experiment, as suggested in the Conclusion chapter, will be focus on long-term testing of different rotation frequency and speed and correlate with the suspended solids concertation in the outlet. It is also possible to evaluate the use of continuous or intermittent rotation. Further implications of these findings are presented and discussed in the chapter 7. The pilot-scale reactor or an industrial-scale fungal reactor could be used in treatment of tanning baths. In some cases spent chrome tanning baths are treated separately to recover chrome (Gando-

Ferreira et al., 2015), while vegetable tannins bath are widely mixed with the others tannery effluents. However, a different management of tannery wastewaters could be possible: despite the high recalcitrant COD the volume of effluent of tanning bath is relative small compare to overall volume: a segregation of waste streams for treatment could be beneficial, but is little applied (Cassano et al., 2001; Lofrano et al., 2013). In literature some authors suggest a vegetable tanning bath treatment based on nanofiltration (Cassano et al., 2003; Molinari et al., 2004) ultrafiltration (Romero-Dondiz et al., 2015) for an eventually recycle (Romero-Dondiz et al., 2016; Teng et al., 2016), electrocoagulation process (Hassoune et al., 2017), treatment with ozone (Ramírez-Ramírez et al., 2016), adsorption and precipitation (Liu et al., 2015). Even though the possible alternatives, previous listed, to treats the separate flow of vegetable tanning batch the technology based on fungal biomass could provide advantage from environmental and economical point of view. Moreover, the tanning baths presents an acid pH. The treatment need be improved and further engineered, but the results presented in this study are promising.

The present study present reports one of the few fungal based reactor stable and effective in non-sterile conditions for wastewater treatment and recalcitrant removal at lab-scale (Espinosa-Ortiz et al., 2016; Svobodová and Novotný, 2017), but also at one the first at pilot-scale reactor stable and efficiency in long-term. In literature were found only three other fungal pilot-scale reactors: the first one was an agitated reactor (working volume 110 L.) with a fungal consortium for treatment of diary wastewater for 32 days and a co-substrate was added (2 g/L whey) (Djelal and Amrane, 2013); the second one a membrane reactor (170 L) focused on treatment of a synthetic textile wastewater by the mixed culture of *Aspergillus versicolor* and *Rhizopus arrhizus,* nevertheless this experiment was a short-term one since the entire test lasted only 15 days and long-term stability was not taken in account (Acikgoz et al., 2016); the third one a pilot-scale trickle-bed reactor (working volume 30 L) inoculated with *Pleurotus ostreatus* for the degradation of endocrine disrupters was installed in a wastewater treatment plant, but operated with some stability problem and for only 10 days (Křesinová et al., 2016).

7   CONCLUSIONS AND FUTURE RESEARCH

7.1   CONCLUSIONS

The starting point of the present study was the following research question: is it possible to exploit fungi in wastewater treatment? Or, more precisely, is it possible to develop a stable and effective process to remove recalcitrant compounds from wastewater based on fungal biomass in non-sterile conditions? Until now, operating a fungal reactor continuously and in non-sterile conditions with wastewater as influent has been the main bottleneck from an application perspective, since bacteria outcompete fungi. For decades, fungi have been investigated for their potential in the removal of recalcitrant compounds. Even though fungi have been exploited in the bioremediation of contaminated sites, they have found little application in wastewater treatments. The critical gap in scientific knowledge has been a lack of information about the environmental conditions and the reactor design needed to achieve a stable system able to operate long-term. Several attempts aimed at clarifying the operating conditions necessary to fulfil the previous scope have been reported in the literature, however no conclusive results have been reached. Since fungi are natural degraders of tannins, the present thesis was focused on the removal of these recalcitrant compounds. Tannins are polyphenolic compounds produced by plants that have been used for centuries in the vegetable tanning of leather. Tannins represent one of the least biodegradable substances in tannery wastewaters, with high recalcitrant soluble COD. A high concentration of tannins can additionally inhibit biological treatment. Moreover, tannins represent a complex class of compounds, and several studies are currently underway to improve our understanding of their physical and chemical properties, their effect on biological activity, their role in microbial ecosystems, and, finally, their potential applications in other fields (production of glues, pharmaceuticals, surfactants, etc.).

This study contributed to answering the above research questions and more specific sub-questions by demonstrating that an engineered fungal biofilm reactor can be applied as a stable and effective technology in the removal of recalcitrant compounds from wastewaters in the long-term and in non-sterile conditions both at

laboratory and pilot-scale. The research began with a definition of the experimental design. The first step was the selection of fungal strains, followed by the design and set up of six lab-scale reactors, where the fungal biomass was studied in preliminary and long-term tests while continuously fed with tannins. Modelling, respirometry and biomolecular analyses were combined to improve the understanding of the observed phenomena. The acquired knowledge allowed for the design and implementation of the final tests at pilot-scale, which provided promising results for a full-scale application of the new technology based on fungal biofilms. The use of an effective biological process for tannin removal is potentially advantageous from both an economic and environmental point of view. The treatment configuration, however, requires further modification: the tannin bath (effluent rich in vegetable tannins) should ideally be treated separately in a side stream reactor, as described in the next section. Finally, this study contributed to the validation of specific respirometric techniques and offered a modelling approach for fungal biofilm to characterise fungi from a kinetic and stoichiometric point of view, responding to the lack of knowledge in the literature on the application of fungi in environmental biotechnologies. The main findings of each chapter are summarised below.

In Chapter 4, preliminary laboratory tests allowed for the choice of a fungal strain suitable for the successful removal of tannins from tannery wastewater. Several strains were identified as potential candidates for tannery wastewater treatment. *A. tubingensis* MUT 990 fungi, a black *Aspergillus* belonging to section Nigri, was selected due to the encouraging ecotoxicology results, the COD removal, and the high tannase production during the first 24 hours of batch tests. In the first lab-scale tests, four novel rotating, submerged, packed bed reactors were inoculated with the selected fungal strain to achieve biodegradation of continuously fed tannins in non-sterile conditions. The reactor configurations were chosen to investigate the effects of the inoculum, the rotation and the co-substrate addition on the outcomes of tannin removal and biomass growth. The experiment ran actively for two months, resulting in the removal of up to 80% of COD and 90% of DOC. During this time, significant tannase activity was detected. These tests demonstrated that it was possible to grow the fungal biomass in non-sterile conditions for two months, thanks to the careful choice of the reactor design and operating conditions. The selected fungal strain appeared to cope

well with tannins. Rotation could be applied to control the biomass retention. However, the frequency and duration of the rotation required to avoid excessive stress on the biofilm and control the biomass retention time was not evaluated. Malt extract supported the growth of the fungal inoculum and contributed (within the chosen operating conditions and reactor design) to the development of a microbial consortium active against one or more of the four tannins dosed from a tannery activated sludge inoculum.

Because of questions raised regarding COD removal during the aforementioned tests, a second laboratory-scale test was performed. The effects of two different types of tannins, condensed and hydrolysable, on the fungal biomass were investigated. Condensed and hydrolysable tannins were separately tested in two reactors, termed RQ and RT, which were fed with QT and TT, respectively. The reactors were inoculated with the selected fungal strain in attached form. A stable biofilm was maintained in RQ during its 180 days of operation. On the other hand, the biomass detached during RT start-up and grew as a suspended culture during the operational period (226 days). RQ and RT achieved sCOD removal up to 53% and 90%, respectively, and a maximum elimination capacity of 9 and 40 g sCOD m^{-3} h^{-1}, respectively, without the addition of co-substrates. Furthermore, TT and QT were successfully co-treated in RQ during a final test phase. Biomolecular analyses showed that a fungal and bacterial consortium developed in RQ, while fungi were outcompeted by bacteria in RT. The RQ, fed with QT, outlasted the colonisation of bacteria to allow for the development of a stable fungal biofilm. A removal of 53% of QT (as COD) was obtained with 0.1 g L^{-1} concentration in the inlet solution (HRT 28 h and OLR 3.6 g COD m^{-3} h^{-1}). RT was fed with TT as a sole carbon source, which caused the detachment of the inoculated fungal biofilm and the formation of suspended biomass. However, the reactor went on to achieve 90% removal of COD (HRT 28 h and OLR 27.9 g COD m^{-3} h^{-1}). Finally, QT showed an inhibitory effect at increased concentrations that reduced its biodegradability. Fungi have been shown to be more resistant to this inhibitory effect than bacteria, and the increased QT concentration therefore allowed for the maintenance of a stable fungal biofilm in non-sterile conditions.

Chapter 5 described the validation of specifically designed respirometric techniques that were applied using activated sludge from Cuoiodepur WWTP, suspended biomass from RT, immobilised PUF cubes from RQ, and immobilised PUF cubes from a pure culture of *Aspergillus tubingensis*. The respirometric analyses confirmed the activities of the biomasses with respect to tannins. The RT consortium was not able to degrade QT, while the immobilised RQ PUF cubes were able to degrade QT and TT. Similar results were observed with a pure culture of *Aspergillus tubingensis.* The outcomes of the respirometric analyses were coupled with the results of the long-term operation of RT and RQ to construct the biomass activity model. Monod kinetics with substrate inhibition, biomass decay and hydrolysis were applied to simulate the behaviour of the suspended biomass sampled from RT and the immobilised biomass from RQ. The model was designed and the parameters estimated with Aquasim software by using the specific tool (biofilm compartment) to simulate the attached growth system. The biofilm features and transport parameters were either measured directly or estimated from the literature. Both reactors were modelled, and their kinetic and stoichiometric parameters were estimated. The chosen approach was able to provide a good description of RT performance in the different conditions tested. The results of the characterised biomass showed some similarities with the available literature on modelled tannin-degrading suspended biomasses. Nevertheless, the approach revealed important limitations to the characterisation of the immobilised RQ biomass, namely that diffusion in the biofilm played a crucial role. Further studies are required to achieve a better understanding of these and other mechanisms to improve the modelling of the immobilised RQ biomass.

The results of the pilot-scale test are described in Chapter 6. The pilot-scale reactor treated a real effluent: a tannin bath with a high concentration of QT and other tannins collected from one of the tanneries of the Tuscany tannery district. The first step of the start-up was the inoculation of the fungal strain inside the reactor, which lasted 55 days. The subsequent treatment phase lasted 66 days. A QT-based medium and the selected operating conditions (established during the previous lab-scale tests) were confirmed to promote fungal growth and immobilisation in the pilot-scale reactor in non-sterile conditions without any co-substrate dosage. During the start-up, an

average COD RE of 31% and a DOC RE of 21% were achieved with the QT medium. During the treatment phase, the pilot-scale reactor was able to remove, on average, 29% of COD and 23% of DOC from the tannin bath. The estimated OLR was 19.19 mg COD L^{-1} h^{-1}, and the ORR was 5.91 ± 0.98 mg COD L^{-1} h^{-1}. The dry mass of biofilm inside the reactor was estimated at approximately 1.55 kg at the end of the start-up period and at 2.80 kg at the end of the treatment phase. Given the promising results obtained, we have confirmed that technologies based on fungal biofilms can be used in real-world applications, although the technology would certainly benefit from further tests and improvements in engineering. These experiments have demonstrated that the results obtained from lab-scale tests can be scaled-up to pilot-scale, despite several differences observed between the two scales that need to be investigated further. The present study has described one of the first fungal-based, pilot-scale reactors that is stable and effective in non-sterile conditions in the treatment of wastewater and the removal of recalcitrant compounds. The operating conditions (pH set point, inoculum, QT dosage, etc.) played a fundamental role, but the range of application of each condition remains to be better clarified and understood (i.e., the relationship of RE with temperature). Finally, the limitations of this technology (i.e., the high HRT, which could potentially be reduced) need to be better defined, and a model of the pilot-scale reactor needs to be developed.

7.2   FUTURE RESEARCH

The outcomes of the present study represent a starting point for further research on application-based solutions and fungal bioreactor technology, requiring further investigation of some open questions related to the findings.

7.2.1   Future applications

Tannins in tannery wastewater are treated inefficiently by the activated sludge process and at high cost by physical-chemical treatment. One future application could be the full-scale use of a segregated flux of vegetable tannin bath, with an optimised process based on fungal bioreactors. Treatment in a side-stream reactor could reduce the overall cost of treatment, since the tertiary treatment applied for removing soluble recalcitrant COD from the tannery wastewater could be minimised.

The SRT of this first fungal biofilm reactor in non-sterile conditions could be fine-tuned with cage rotation, but this solution requires further study and engineering. Moreover, this reactor design could be used as a technology to produce fungal biomass for other purposes. For example, it could be exploited for bioaugmentation in activated sludge treatment (Djelal and Amrane, 2013), in other fields such as the clean-up of contaminated sites (Chiu et al., 2009), or as a bio-flocculant (to improve settling) for treatments based on microalgae (Nasir et al., 2015; Wrede et al., 2014).

The co-treatment of different streams of wastewaters to remove other recalcitrant compounds with fungi (e.g., tannery and pharmaceuticals (Cokgor et al., 2008)) could also be considered. However, because some fungi are potential pathogens, their use must be carefully evaluated.

7.2.2   Future investigations

A more in depth investigation is needed into the different degradation pathways of tannins and of the chemical compositions and structures of tannins. This research could be carried out, for example, with a LC-MS using specially-developed methods and procedures or, even better, with advanced equipment, i.e., MALDI-TOF (Pasch et al., 2001). The population monitoring involved in each degradation step, coupled with the enzymatic activity and mechanisms of action, could also improve the production of those enzymes at industrial scale, given that tannase is one of the most expensive enzymes to produce.

A second aspect that could be investigated is related to the different inhibitory mechanisms of QT and TT (and tannins in general). This would require an investigation of the different mechanisms of action of bacteria versus fungi, with an additional consideration of the possible interference of quorum sensing.

A third potential avenue of investigation would be the study of biofilm dynamics for modelling. The diffusion of tannins and oxygen inside the fungal biofilm and the associated biological reactions could be described with microsensors. A possible future application, which would also be applicable to other fields, is the coupling of respirometry/titrimetry of the biofilm with microsensors for the development of robust procedures and protocols. The combination of these two techniques could allow a better description and understanding of biofilm dynamics. This would improve the overall knowledge related to biofilms and would be beneficial for obtaining a more reliable model. Moreover, the microbial consortium composition, as revealed via biomolecular analysis, could be related to the overall reactor performance and respiratory activity (Boltz et al., 2017; Miłobędzka and Muszyński, 2016). Finally, a future application of the biofilm reactor could be related to the evaluation of online respirometry as a potentially useful tool to control the treatment. A deeper investigation into the compositions of microbial consortia in varying conditions and of the interaction with the reactor performance would allow a more rapid start-up and the adoption of optimal operational strategies.

One final aspect that could be investigated is the relationship between the inoculum and its influence on the long-term equilibrium of the microbial ecosystem.

Aspergillus tubingensis was inoculated in both reactors (RT and RQ), and microbial consortia developed. The inoculum *Aspergillus* had a relative abundance of approximately 23% after 70 days in RQ where the fungal biofilm was mantained. It is possible that the inoculum was the determining factor for the composition of the final microbial degradation consortium (Kanniah Goud et al., 2017). However, different inocula have been shown to produce distinctive microbial consortia with similar degradation capacities (Cortes-Tolalpa et al., 2016; Roeselers et al., 2006) and selective pressure has also been shown to be the determining factor for consortium composition (Gonzalez-Martinez et al., 2015; Park et al., 2010; Temudo et al., 2008). Additionally, the external species in the influent could have important effects (Cook et al., 2006). Different consortia, including both bacterial and fungal communities, could be composed of distinct populations with their own dynamic equilibria and unique enzyme activity patterns, while still achieving functionally similar substrate degradation. Given these uncertainties, the inoculum and its influence on long-term equilibrium require further investigation.

Acknowledgment

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