BIOREMEDIATION OF ESTRONE FROM WATER MATRICES USING THE ENZYME LACCASE COMBINED WITH MATHEMATICAL MODELLING

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ABSTRACT

The presence and impact of steroid estrogens in natural water matrices has driven development and evaluation of wastewater treatment technologies that may reduce the steroid load entering water environments. This work was undertaken to assess and predict the ability of *Trametes versicolor* laccase to degrade estrone (E1) in water matrices under realistic conditions to wastewater treatment plants (WWTPs) and with consideration of the complex and variable nature of the wastewater matrix. A robust experimental procedure was developed to ensure the efficiency of the enzyme laccase to degrade E1 in water matrices was not overestimated due to errors arising from poor experimental design. These experiments demonstrated that commercially-obtained laccase in concentrations above >1 mg/ml are inhomogeneous requiring centrifugation prior to use to reduce error and provide more accurate evaluation of laccase capability. Sample filtration, which is necessary for chromatographic analysis, identified regenerated cellulose (RC) membrane filters as the optimum filters for particulates removal from E1 solutions due to their low affinity toward E1 (3.2 ±1.72 %). An optimum enzyme inactivation procedure using hydrochloric acid was also developed to ensure that the enzyme laccase was instantly inactivated without affecting the target steroid E1 itself.

Using the established experimental procedure, bench-scale studies evaluating the efficiency of laccase-based treatment in a 'clean' water matrix were investigated. Experiments in deionised water provided a proof of concept of laccase ability to degrade E1 in water under realistic ranges of temperature [6˚C - 25˚C] and contact time [0.5 hr – 8 hrs] to the WWTP and evaluate the use of models to fit experimental data and predict within that system. Box Behnken Design (BBD) was applied to determine the number and the conditions of the performed experiments. The experimental data was then utilised to build two different models to predict E1 removal efficiency under any set of conditions and optimise the performance of laccase-based treatment system. The goodness of the fit for each model was tested using statistical indices such as coefficient of determination (R^2), mean squared error (MSE)

and absolute average deviation (AAD). The artificial neural network (ANN) model showed a better fit to the experimental data than the response surface methodology (RSM) model (RSM and ANN of R^2 = 0.9908 and R^2 = 0.9992 respectively. In addition, the predictive capabilities of RSM and ANN were tested using a set of statistically designed unseen data that was not previously used in models' training. Both models showed limited predictive capabilities.

The ability of laccase-based treatment to remove E1 in real-world wastewater was studied at bench scale. To account for the complexity and variability of the wastewater matrix, effluent samples during the period December 2014 - June 2015 were characterised for standard water quality parameters, where the temporal variation in wastewater chemical oxygen demand (COD), total suspended solids (TSS) and pH, were observed. A new water quality parameter, "Benchmark" was also developed and applied to quantify the impact of wastewater variability on laccase performance for E1 removal. The average benchmark value in the period between December 2014 and June 2015 was 79.8±3.7%. In addition, the impact of laccase inhibitors, which are likely to be present within the wastewater matrix, such as chloride, copper, iron and zinc, on laccase activity was investigated. The inhibitory effect of chloride ions increased with increasing chloride concentration above 200 mg/l. Copper and zinc ions exhibited negative effects on the enzymatic degradation of E1 at concentrations equal or above 10 mg/l and 200 mg/l.

The impact of water matrix temperature, contact time and laccase concentration were studied in wastewater effluent and the experimental data was used to build RSM and ANN models. The predictive capability of the generated RSM model was relatively poor (R^2 = 0.863) and even lower than the achieved predictive capability in clean matrix when tested using unseen data, this was partially attributed to the variability of wastewater matrix that could have not been addressed in this type of models. Whilst the improved ANN model showed a better predictive capability than RSM (R^2=0.991) An advantage of the ANN model compared to the RSM model and reported for the first time, was the ability to include the impact of matrix complexity and variability on laccase performance, assessed via the benchmark data added as a

forth factor in the ANN model. The final ANN model incorporating the matrix variability observed temporally during the sampling period had extremely high predictive capabilities ($R^2 > 0.99$). This model approach holds the potential to help researchers evaluate and optimise laccase-based treatment (as well as other treatment technologies) and predict the removal efficiency of various bioactive chemicals under a wide range of conditions. Performing laccase-based treatment in a continuous reactor, utilising actual wastewater effluent and under realistic conditions to WWTPs, is the next stage that should be investigated in detail.

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LIST OF ABBREVIATIONS

AAD	Absolute Average Deviation
ABTS	2,2′-Azino-Bis (3-Ethylbenzothiazoline-6-Sulfonic Acid)
AC	Ammonium Acetate
ACN	Acetonitrile
ANN	Artificial Neural Network
AS	Activated Sludge
BBD	Box Behnken Design
BOD	Biochemical Oxygen Demand
BPA	Bisphenol A
CCD	Central Composite Design
CIP	Chemical Investigation Programme
COD	Chemical Oxygen Demand
E1	Estrone
E1-3G	Estrone-3β-D-Glucuronide
E1-3S	Estrone-3-Sulfate
E1-3S	Estrone-3-Sulfate
E2	17 B - Estradiol
E2-17G	17 B - Estradiol-17-Glucuronide
E2-3G	17 B - Estradiol-3-Glucuronide
E2-3S	17 B - Estradiol-3-Sulfate
E3	Estriol
E3-16G	Estriol-16-Glucuronide
E3-3S	Estriol-3-Sulfate
EDCs	Endocrine Disrupting Chemicals
EE2	17 A - Ethinylestradiol
EE2	17 A - Ethynylestradiol
EE2-3G	17 A - Ethynylestradiol 3β -D-Glucuronide
EE2-3S	17 A - Ethynylestradiol 3-Sulfate
EQS	Environmental Quality Standards
GAC	Granular Activated Carbon

GMF	Glass Micro Fibres
HBT	1-Hydroxybenzotriazole
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
HRT*	Hormone Replacement Therapy
LiP	Lignin Peroxidase
LMS	Laccase-Mediator System
LOD	Limit of Detection
LOQ	Limit of Quantification
MeOH	Methanol
MnP	Manganese Peroxidase
MSE	Mean Squared Error
MWD	Multiple Wavelength Detector
Na_2HPO_4	Sodium Phosphate Dibasic
NaCl	Sodium Chloride
NaH_2PO_4	Sodium Phosphate Monobasic
NSAID	Non-Steroidal Anti-Inflammatory Drug
PAHs	Polycyclic Aromatic Hydrocarbons
PES	Polyethersulphone
PNEC	Predicted No Effect Concentration
PTFE	Polytetra-Fluoroethylene
R^2	Coefficient of Determination
RC	Regenerated Cellulose
RMSE	Root Mean Squared Error
SA	Specific Activity
SRT	Solids Retention Time
TSS	Total Suspended Solids
UV	Ultraviolet
WFD	Water Framework Directive
WWTP	Wastewater Treatment Plant
$ZnSO_4.7H_2O$	Zinc Sulfate

1 INTRODUCTION

1.1 THESIS OVERVIEW

This work investigates the ability of laccase-based treatment to remove estrone (E1) from water matrices under realistic conditions to wastewater treatment plants (WWTPs), and the feasibility of optimising laccase-based treatment using models with high predictive capabilities. Chapter 2 reviews the literature to explain the various sources of bioactive chemicals such as steroids in the aquatic environment, their adverse effects on the living organisms and the efficiency of the current wastewater treatment technologies in removing these pollutants. It also provides an introduction to the enzyme laccase, its potential as a treatment technology and its substrates, mediators and inhibitors. Wastewater variability (both spatial and temporal) and its impact on laccase-based treatment have been highlighted as well. An overview about experimental designs, response surface methodology (RSM) and artificial neural network (ANN) models and their applications are discussed as well in Chapter 2 leading to identifying issues arising from the literature review that informed the scope of this work.

Following a detailed description of the methods and materials used for this work in Chapter 3, Chapter 4 contains a comprehensive assessment of the required controls and preliminary experiments during the degradation of E1 by laccase in water matrices. Utilising laccase-based treatment at bench-scale to remove E1 from deionised water matrix and the application of RSM and ANN models to fit experimental data and predict the system is investigated in Chapter 5. Chapter 6 focuses on understanding the complexity and variability of real-world wastewater matrices sampled from one WWTP through characterising water quality indicators, and developing a new ‘benchmark’ water quality parameter to better understand the impact of that variability on laccase-based treatment. This chapter also demonstrates the impact of laccase inhibitors common to the wastewater environment on laccase activity. The removal of E1 using laccase enzyme in actual wastewater effluent under realistic conditions to WWTPs is undertaken in Chapter 7 and incorporates

model approaches from Chapter 5 to evaluate RSM and ANN predictive capabilities and the possible approaches to improve these capabilities. Chapter 8 contains the overall summary and the main findings of this thesis.

1.2 RATIONALE FOR RESEARCH

Nowadays bioactive chemicals such as steroids and pharmaceuticals are receiving global attention in the scientific community, with public concern also growing[1]. Although bioactive chemicals in aquatic systems are usually present at low concentrations (ng/l- µg/l), their adverse effects on living organisms has led to Governmental and legislative drivers to assess and remediate their presence in water matrices[2]. Urban wastewater is the main source of bioactive chemicals in the aquatic environment. However wastewater treatment plants (WWTPs) were never designed to remove bioactive chemicals from wastewater, resulting in these pollutants reaching the receiving water courses and the organisms living within. Therefore, there is a need to develop new technologies to remediate these pollutants during.

Steroids estrogens such are commonly detected in effluents from WWTPs, the majority of the estrogenic activity in wastewater comes from free natural steroids: estrone (E1) and 17β-estradiol (E2) and synthetic steroid 17α-ethynylestradiol (EE2)[3]. In their free forms these steroids can cause abnormal sexual development in animals and feminisation in male fish [4, 5]. The presence of these pollutants in water has also been tentatively linked to increasing cases of cancer and decreased male fertility in human [6, 7]. Although there are several possible treatment technologies to remove bioactive chemicals from water matrices, bio-catalytic approaches, such as laccase-based treatment, offer environmentally friendly alternatives that capture the interest of many research groups around the world [8-13]. Laccase has been shown to directly degrade a wide range of bioactive chemicals such as steroid estrogens from water matrices, with the pollutant range can also be expanded to other pollutants by implementing various mediators[14, 15]. Benefits to utilising *Trametes versicolor* laccase are its ability to efficiently operate at relevant pH and temperatures to WWTPs, it is commercially available and it can be extracted from cheap and available media (white rot fungi).

To evaluate the potential of laccase as a treatment technology for remediating bioactive chemicals and move up the Technology Readiness Levels to full scale, there is a need to assess the impact that the WWTP conditions and wastewater matrix have on laccase performance. The performance of laccase-based treatment is influenced by many factors such as matrix temperature, contact time, laccase concentration, the composition and variability of the wastewater matrix, and will inform on reactor design. Robust experimental procedures for batch experiments and quantifying the variability of wastewater are both essential for collecting meaningful and accurate data. Experimental data determines the optimum factors for reactor design, however limitations in time and resource mean a system can only be partially mapped by actual experiments. In addition, the factors under consideration for reactor design (e.g. contact time of water matrix with laccase enzyme) or WWTP environment (e.g. water temperature) needs to be considered as interactive rather than independent factors. For these reasons, experimental factorial designs and mathematical models can help to not only describe and visualise this complex investigated system, but also to predict and optimise its performance.

1.3 AIM AND OBJECTIVES

The aim of this work is to investigate the ability of laccase-based treatment at bench scale to remove estrone (E1) from clean water and wastewater matrices under realistic conditions to wastewater treatment plants, utilising response surface methodology (RSM) and artificial neural network (ANN) models to predict and optimise laccase treatment performance.

The specific objectives of this research are:

1. Develop a robust bench-scale experimental procedure to ensure the efficiency of laccase to remove E1 from water matrices is not overestimated due to errors arising from experimental design.
2. Provide a "proof of concept" by demonstrating the feasibility of laccase-based treatment of E1 in a clean water matrix and the capability of models to predict and optimise the investigated system.
3. Characterise wastewater effluent and assess its variability using standard water quality parameters.
4. Quantify the impact of the variability of the wastewater effluent on the efficiency of laccase-based treatment by developing a new "Benchmark" parameter.
5. Investigate the impact of common laccase inhibitors within the wastewater environment on the activity of laccase.
6. Develop a model that is able to accurately predict E1 removal efficiency by laccase under realistic conditions of a WWTP.

2 LITERATURE REVIEW

2.1 THE SCOPE OF THE PROBLEM: BIOACTIVE CHEMICALS IN AQUATIC ENVIRONMENTS

Bioactive chemicals such as steroids and pharmaceuticals have been commonly detected in various environmental matrices. Effluents from wastewater treatment plants (WWTPs) are the main source of these pollutants in the aquatic environments. Unlike many other contaminants, bioactive chemicals cannot be reduced at source because they are either naturally produced by the human body e.g. steroids, or necessary for human health e.g. pharmaceuticals. In the human body, all bioactive chemicals undergo metabolism prior their excretion with urine or faeces. The excreted bioactive chemicals are either free or conjugated with glucuronide or sulfate. The conjugation process increases the hydrophilicity of the bioactive chemicals and thus, ease their urinary excretion from the body [16]. The urban water cycle and role of the WWTP as a gatekeeper to water quality is illustrated in Figure 2.1

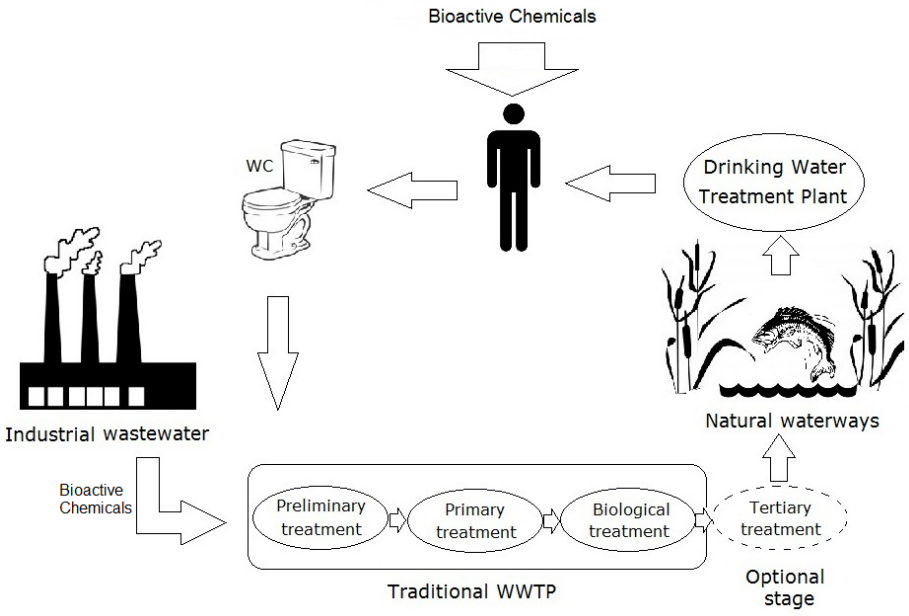


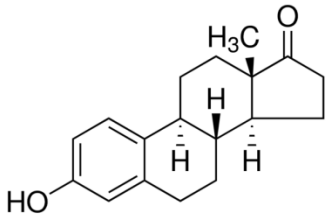
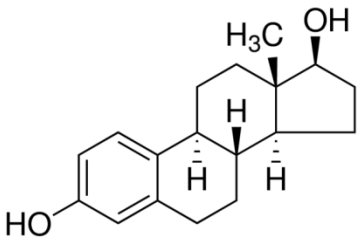
Figure 2.1 The urban water cycle, bioactive chemicals and the role of the wastewater treatment plant (WWTP).

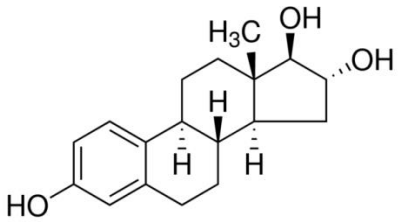
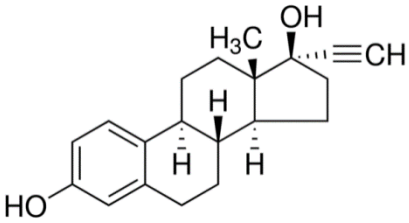
Before reaching the WWTP, the wastewater has to pass through the sewerage system where the transformation, deconjugation or even degradation of some bioactive chemicals can take place. However, some bioactive chemicals are more recalcitrant and show limited degradation during current treatment stages. These compounds are detected in the final effluents of WWTPs and eventually in the surface water. The biological activity of these pollutants can cause abnormal sexual development in animals, impair reproductive function in adults of either sex and evolve intersexuality in fish [4, 5]. To date, there is no evidence on adverse effects caused by bioactive chemicals in humans, in part due to the issues arising with environmental epidemiology and linking environmental pollutants to impacts on human health. However, increasing cases of testicular, prostate, breast and ovarian cancer and decreasing sperm counts have been tentatively linked to the presence of these pollutants in water [6, 7].

2.1.1 Metabolism of Bioactive Chemicals in the Human Body and Their Excretion

Human urine, via the WWTP is the main source of natural and synthetic estrogens in the aquatic environment [17, 18]. There are three steroid estrogens that are naturally excreted from human body: Estrone (E1), 17β-Estradiol (E2), and Estriol (E3)[19]. The chemical structures of common steroid estrogens are shown in Table 2.1.

Table 2.1The chemical structures of common steroid estrogens.

Steroid estrogen	Chemical structure
Estrone (E1)	
17β - Estradiol (E2)	

Estriol (E3)	
17α- Ethynyl Estradiol (EE2)	

Free estrogens are rarely detected in urine, apart from some E3 in pregnancy urine [20] and are instead principally excreted as inactive polar conjugates. For instance, E2 was predominantly found as 17β-estradiol-3-glucuronide (E2-3G), E1 as estrone-3-sulfate (E1-3S) and E3 as estriol-16-glucuronide (E3-16G) [21]. However and under suitable environmental conditions which are usually met in sewerage systems or within WWTPs, several deconjugation and transformation processes may occur and the free E2 may metabolise into E1. Although the estrogenic activity of E1 is lower than the estrogenic activity of E2, the concentration of E1 in the final wastewater effluent is usually higher than E2 and it is still able to cause endocrine-disrupting effects in living organisms [22]. Table 2.2 shows commonly detected steroids in human urine.

Table 2.2 Commonly detected steroids in human urine.

Steroid estrogens in human urine	**Reference**
Estriol-3-Glucuronide (E3-3G)	
Estriol-16-Glucuronide (E3-16G)	
Estriol-3-Sulfate (E3-3S)	
17 β-Estradiol-3-Glucuronide (E2-3G)	
17 β-Estradiol-17-Glucuronide (E2-17G)	[23]
17 β-Estradiol-3-Sulfate (E2-3S)	
Estrone-3β-D-Glucuronide (E1-3G)	
Estrone-3-Sulfate (E1-3S)	
17α-Ethynylestradiol-3β-D-glucuronide (EE2-3G)	

2-Methoxy-17α-Ethynylestradiol 17α-Ethynylestradiol-3-Sulfate (EE2-3S) 17α-Ethynylestradiol	[19, 24]

It is worth noting that the concentration of excreted bioactive chemicals from the human body can be much higher in the case of using oral contraceptives or hormone replacement therapy (HRT*). Pharmaceuticals are also excreted from human body either in the free or conjugated forms and have the potential to be biologically active [25].

2.1.2 Sources of Bioactive Chemicals in the Aquatic Environment

Although effluents from WWTPs are the main sources of bioactive chemicals in the aquatic environment, there are several other sources that contribute to the concentration of these pollutants in water environments.

2.1.2.1 Anthropogenic Sources

Wastewater from pharmaceutical industries can present a real challenge to the municipal WWTP. It has been found that discharging industrial wastewater into the sewage system can significantly (10-1000 times) increase the pharmaceutical concentrations in the final WWTP effluent in comparison with the WWTPs that do not receive any industrial wastewater [26]. Both manufacturing and packaging facilities of pharmaceuticals discharge active pharmaceutical compounds into their wastewater [27], discharges from hospitals, inappropriate household disposal also contribute to the total pharmaceutical load in the WWTP [28]. Unused pharmaceuticals are commonly disposed either via the toilet, thereby entering the WWTP or to the bin. The latter being sent to landfill, of which the leachate is then treated by municipal WWTPs providing a further route for anthropogenic sources to enter [29].

2.1.2.2 Non-Anthropogenic Sources

Livestock waste is another source of bioactive chemicals in the aquatic environment [30]. Untreated discharges of animal wastes into surface water or

by applying waste as a fertiliser to agricultural lands can cause contamination of soil and water with bioactive chemicals [31]. Studies from several dairy manures confirmed the presence of bioactive chemicals such as steroids in the produced animal wastes and highlighted the adverse effects of these wastes on the surrounding environment [32-34].

2.1.3 Bioactive Chemicals in the Wastewater Treatment Environment

After excretion from the human body, bioactive chemicals travel with the municipal wastewater via the sewage system which ends up in the WWTP. It was reported that the glucuronide conjugates of estrogens are largely deconjugated in the sewerage system, while the sulfate conjugates are more recalcitrant and for that reason can be detected in the effluent of WWTPs and rivers [35, 36]. Another study showed that endocrinologically inactive conjugates of natural steroids and contraceptives can become active again upon their deconjugation in the raw sewage or in the WWTP [29]. The deconjugation process in the WWTP is achieved by a mixture of various microorganism populations that can cleave the glucuronic acid and sulfate moieties with varying success and release the free steroids. The removal efficiencies of some pharmaceuticals e.g. carbamazepine, atenolol, metoprolol, trimethoprim are less than 10% in conventional WWTP [37]. In addition, some studies found that pharmaceutical conjugates can also deconjugate during the treatment process and as a result the concentration of some pharmaceuticals in the effluent maybe higher than their concentration in the influent [38].

2.1.4 Bioactive Chemicals in Environmental Matrices

Bioactive chemicals from different therapeutic classes e.g. antibiotics, analgesic, anti-inflammatory, β-blockers, anti-epileptics, contraceptives and steroids, can enter the aquatic environment through various routes such as industrial wastewater, improper disposal of unused drugs and metabolic excretion. Municipal wastewater is the main point of entrance for bioactive chemicals into the aquatic environment [39]. Due to the limited removal efficiency of these pollutants during the wastewater treatment process, many

bioactive chemicals end up in the aquatic environment. Steroids, pharmaceuticals and their metabolites are usually detected at trace levels (ng/l or μg/l) in water bodies. Despite their low concentrations their effects can be severe, especially in case of antibiotics and steroids that may lead to a development of resistance in natural bacterial populations or a disruption in the endocrine system of the living organisms [40]. Bioactive chemicals can be unintentionally consumed by humans through drinking water as the majority of drinking water treatment plants are not designed to remove this type of pollutants. For instant, carbamazepine, an anti-epileptic drug, and diclofenac, non-steroidal anti-inflammatory drug (NSAID), have been detected in drinking water [41]. Table 10.1 and Table 10.2 in Appendix A summarise the pharmaceutical residues that were detected in various water matrices all around the world.

2.2 LEGISLATIVE DRIVERS FOR REMEDIATING BIOACTIVE CHEMICALS FROM WASTEWATER

In UK and the rest of Europe the target concentrations of chemicals in water sources are based on the "non-harmful" concentration of that chemical in water. Keeping the concentration of any chemical below its target concentration, ensures that the sensitive species within the aquatic environment is not adversely affected by its presence. Despite the extremely low concentrations of steroids estrogens in the aquatic environment, their estrogenic activity have made them of interest to the legislative bodies in Europe[42]. In 2013 the European Commission added E2 and EE2 to the "Watch List" of priority substances that are controlled under the Water Framework Directive (WFD - Directive 2000/60/EC) where further studies and evidence will be required before these pollutants can become priority substances. Once a pollutant is identified as a priority hazardous substance, all the member states of the European committee are required to decrease their discharges of that pollutant into the aquatic environment within a 20 year period [2]. As a result, the Water Industry that discharges priority hazardous substances may face significant pressure to achieve the set standards by the environmental regulators. With time, additional substances such as E1 may be

added to the “Watch list”. Though E1 is not listed on the Watch List, it is well understood that E1 is excreted in higher proportions that E2 and also that E2 in the water environment when subjected to microbial activity, is removed to a higher degree than E1[43].

2.3 TREATMENT TECHNOLOGIES TO REMOVE BIOACTIVE CHEMICALS FROM WASTEWATER

Numerous studies from all around the world have reported the presence of bioactive chemicals not only in influents and effluents of WWTPs[44-48] but also in surface[25, 49, 50] and drinking waters[51]. The majority of these substances are biologically active even at minute concentrations as low as ng/l[29] and can pose serious risks to the aquatic living organisms[30]. Therefore, removing bioactive chemicals during the wastewater treatment process is essential to protect both humans and aquatic environments from potential adverse effects of steroids and pharmaceuticals residues. WWTPs were never designed to remove these pollutants. Therefore research has focused on two aspects:

i. Evaluating current treatment technologies in WWTPs in order to modify and improve their ability to remediate bioactive chemicals, .
ii. Develop new treatment technologies that can be added, usually as a tertiary option to existing WWTPs in order to improve the process for reducing the bioactive chemical load entering receiving water courses.

The body of work evaluating current and new treatment technologies is extensive but focuses on non-bio-based treatment technologies. Ozonation is a non-biological technology[52] that has been used to disinfect and improve the quality of drinking water and -in some cases- wastewater. The utilised ozone can either directly attack the organic pollutants or form hydroxyl radicals that can degrade these pollutants (indirect oxidation). In 2003, Ternes et al. investigated the ability of ozonation to remove specific bioactive chemicals (e.g. estrone, carbamazepine) from a municipal WWTP effluent [53]. The results showed that 10-15 mg/l ozone was able to remove a wide range of

pharmaceuticals and estrone below their limits of quantification (LOQ) (LOQ for Estrone= 0.003 mg/l) after contact time of 18 mins. Another study reported that more than 90% of diclofenac, naproxen and estrogens ,which were present in WWTP effluent, were oxidised by ozone's dose of ≈ 2 mg/l [54]. However, the main drawback of this technology is that, in some cases, the generated by-products can be as toxic and/or active as their parent compounds [55, 56]. In addition, ozonation is considered an expensive and energy-consuming treatment option [57].

Activated carbon – both granular activated carbon (GAC) and powder activated carbon (PAC) - can be effectively utilised to remove many non-polar bioactive chemicals[29]. One study reported that the removal efficiency of estrogens by GAC was greater than 90% whereas the initial concentrations of the removed estrogens were between 100-200 ng/l [58]. Another research group found that GAC can decrease the concentration of carbamazepine and a wide range of endocrine disrupting compounds (EDCs)[59]. Effluent from WWTP usually contains other pollutants such as humic acids, surfactants and dissolved organic materials which compete with adsorption sites of GAC and block it pores. Thus, using GAC to treat complicated matrices can be challenging and associated with frequent replacement or an energy-consuming regeneration of the used GAC. Both scenarios will lead to a considerable increase in treatment expenses. In addition, this technology does not degrade the problematic compounds, it just transfers them from one medium (liquid) to another (solid) and further disposing options of the used GAC must be considered [60].

In March 2015 *Dutch Foundation for Applied Water Research (STOWA)* took the initiative to undertake the first overall study to understand the current achieved removal of micropollutants from effluents of municipal wastewater treatment plants (WWTPs) in Germany and Switzerland that utilise advanced tertiary treatments[61]. The aim of this work was to translate the current experience in full scale applications from Germany and Switzerland to Dutch conditions, especially on costs involved. Tertiary treatment technologies such as ozonation, PAC and GAC were extensively studied on a large scale in

effluents from WWTPs. Table 2.3 shows the estimated cost of three tertiary treatments in WWTPs with different population equivalents (p.e.) in order to achieve a “high removal” efficiency of the micropollutants.

Table 2.3 Cost per one meter cubic of tertiary treated effluent from a municipal WWTP for micropollutants removal in the Netherland (adapted from Mulder at al., 2015[61]).

WWTP capacity (p.e.)	**20,000 p.e.**	**100,000 p.e.**	**300,000 p.e.**
Ozonation + Sand filtration	€ 0.22 ± € 0.04	€ 0.18 ± € 0.03	€ 0.16 ± € 0.03
PAC + Sand filtration	€ 0.26 ± € 0.04	€ 0.20 ± € 0.03	€ 0.18 ± € 0.03
GAC	€ 0.29 ± € 0.04	€ 0.27 ± € 0.04	€ 0.26 ± € 0.04

The removal efficiency of any micropollutant depends on the implemented tertiary treatment, the characteristics of wastewater influent and the nature of the micropollutant itself. Therefore different substances will have different removal rates. STOWA has found that many micropollutants were removed in the rage of 30%-50% to more than 80%, while other were not removed at all during the used tertiary treatment[61].

In Germany and Switzerland it is advised to implement a biological sand filtration step after ozonation, to remove any biodegradable toxic transformation products formed during ozonation. However it is still unknown if this implemented sand filtration is sufficient to remove all the harmful transformation products. Sand filtration is also required post PAC treatment to remove the small PAC particles from the final effluent.

The cost figures in Table 2.3 show that tertiary treatment of micropollutants is more cost effective when implemented in large WWTPs. It also shows that ozonation combined with sand filtration is noticeably cheaper than GAC treatment where the cost of regenerating/ replacing the old GAC can be significantly high[61].

In conventional WWTP the wastewater passes through various treatment stages to remove total suspended solids (TSS) and decrease the biological oxygen demand (BOD). Activated sludge (AS) is one of the popular secondary

treatment options, the typical design of a WWTP that uses this option is depicts in Figure 2.2

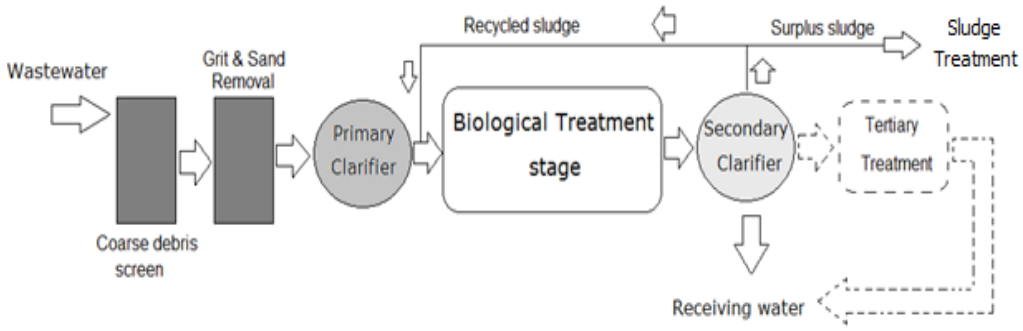


Figure 2.2 Treatment stages in wastewater treatment plant with activated sludge process as a secondary treatment stage.

The sequence of preliminary, primary and secondary treatment stages is insufficient in fully removing recalcitrant bioactive chemicals [62] and as a result many of these pollutants reach and affect the aquatic environment. The removal efficiency of bioactive chemicals in AS WWTP depends on various parameters such as seasonal variables (e.g. ambient temperature, rainfall, pH) and operational parameters of the biological treatment process (e.g. solids retention time (SRT) and hydraulic retention time (HRT))[63]. One study showed that a high removal efficiency of three steroids was achieved in a municipal AS WWTP (98% for E1 and E2 and 90% for EE2)[64]. Another study found that some pollutants such as E1 and EE2 cannot be fully removed during the typical treatment times in AS process. The data field showed that WWTP with AS process can consistently remove around 85% of E2, E3 and EE2. However, the removal of E1 is usually less and more variable[65]. According to another research, the removal efficiency of E1 and EE2 was less than 10% in one the AS WWTP in Germany[66]. The above studies demonstrate that the removal of many bioactive chemicals in WWTPs is incomplete and there is a need to improve the current treatment processes in WWTPs to reduce the amount of bioactive chemicals entering and polluting the aquatic environment[63].

2.4 ENZYMES AS A POTENTIAL TREATMENT PROCESS OF BIOACTIVE CHEMICALS IN WASTEWATER

Utilising laccase to remove various bioactive chemicals for water matrices has been investigated by many research groups. Studies were performed using different types of laccases, different experimental conditions and several water matrices. This is a developing technology that has the potential to remove a wide range of bioactive chemicals from wastewater matrix. This new treatment approach will be explained in detail in the following sections.

2.4.1 Introduction into Enzymes

Enzymes are highly specialised proteins with remarkable catalytic power. The catalytic ability of enzyme usually exceeds the catalytic ability of synthetic or inorganic catalysts. The majority of enzymes can function in aqueous solutions under mild conditions of temperature and pH [67]. Laccases (EC 1.10.3.2) are one of the earliest discovered enzymes (Mot, 2012). In the nineteenth century, laccase was discovered in the Japanese tree *Rhus vernicifera*[68] and since then it has been found in various plants, fungi, insects and even bacteria. The oxidising ability of laccase depend on its source [69]. In plants, laccases are participating in the synthesis of lignin, a complex insoluble biopolymer of phenolic compounds [69]. The ligninolytic system of white-rot fungi consist of three types of enzymes: lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. Laccase, which is a multi-copper enzyme that belongs to the blue oxidases family, catalyses the single-electron oxidation of substrates such as phenols, polyphenols and aromatic amines, simultaneously it reduces oxygen into water through four-electron transfer process. Although, the catalytic mechanism of laccase has been investigated for many years, it is not completely understood, especially in terms of the reduction of dioxygen to water[70, 71]. For many years studies focussed just on LiP and MnP due to their relatively high redox potentials, as a result laccases were neglected and were not considered an important part of the ligninolytic system[72]. Although laccases cannot degrade the non-phenolic part of lignin, they are secreted, by

fungi, in big quantities and their oxidising ability can be extended by the use of various mediators[73].

2.4.2 The Enzymatic Mechanism

Enzymatically catalysed reaction takes place inside the enzymatic pocket of the enzyme or what also called "The active site". The surface of the enzymatic pocket is lined with amino acid residues that bind the substrate and transform it chemically. During the chemical transformation, the bounded substrate is surrounded by the active site and isolated from solution (Figure 2.3).

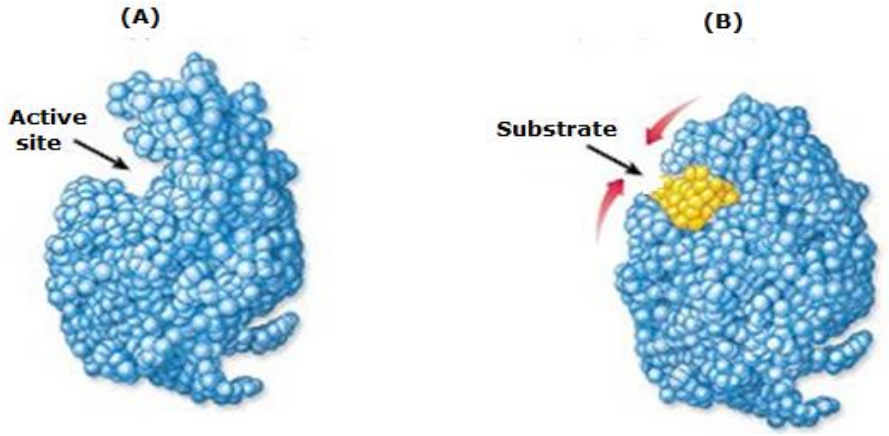


Figure 2.3 An illustration of the active site of an enzyme and the formation of "enzyme-substrate" complex.

The enzymatic reaction can be simply displayed as follows in Figure 2.4 where the substrate binds to the active site of the enzyme and creates a transient complex, which is then transformed into "enzyme-product" complex before releasing the final product(s) of the reaction into the reaction mixture.

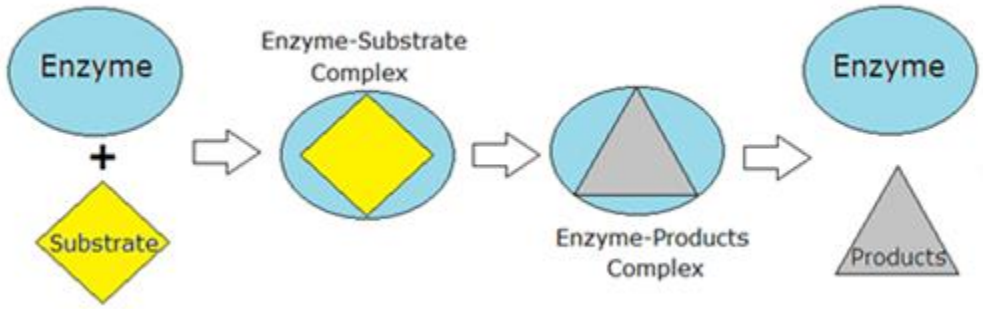


Figure 2.4 the stages of the enzymatic reaction.

Multi copper enzymes such as *Trametes versicolor* laccase contain three copper (Cu) sites; T1, T2 and T3. The T1 Cu-site is responsible for oxidising the substrate and transferring the generated electrons to the T2 and T3 Cu-sites. Laccase catalysis involves (i) binding the substrate to the T1 site and subsequent reduction of the Cu^{2+} to Cu^{1+} within the same site, followed by (ii) internal electron transfer from T1 to T2/T3 Cu cluster, and finally (iii) the binding and subsequent reduction of the molecular oxygen (O_2) to water (H_2O) at T2/T3 cluster[74]

2.4.3 Laccase Substrates

Phenolic compounds are the main laccase's substrate. Substrate's oxidation usually involves losing one electron (from the substrate) and generation of free radicals. The formed radical are not stable and may participate in further laccase-catalysed or non-enzymatic reactions [75]. The difference in redox (oxidation) potentials between laccase and substrate plays a big role in the electron transfer from substrate to the active site of laccase. To achieve a high oxidation percentage of the substrate it is important that the oxidation potential of the laccase is much higher than the substrate's oxidation potential [69].

2.4.4 Laccase-Mediator System

In nature, laccase plays a role in lignin biodegradation. However, due to its low redox potential, it can only degrade the phenolic substances [72]. Application of suitable mediators during the enzymatic treatment can increase the oxidation capability of laccase and oxidise non-phenolic compounds. Many studies showed that there is much potential for the laccase–mediator system (LMS) and that it can be used in various applications e.g. detoxification of industrial effluents such as pulp, paper and textile industries, bioremediation of urban wastewater and polymer synthesis.

This system was first developed for the paper and pulp industry, to improve the bio-bleaching of wood pulps[76]. According to that study, the ability of laccase to degrade lignin in kraft pulp can be improved by a number of synthetic compounds that have low molecular weight and can act as hydrogen

donors. The reaction mechanism of the laccase-mediator system passes through a number of stages: first, oxygen reacts with laccase and activates it, the activated laccase oxidise the mediator. The oxidised mediator diffuses out of the laccase, oxidises the substrate (e.g. lignin) and transforms/ degrades it (Figure 2.5).

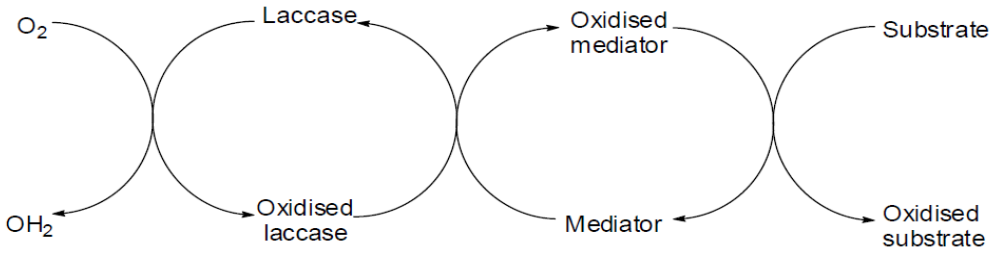


Figure 2.5 laccase-mediator system: Substrate oxidation [48].

With the assistance of mediators, laccase can be also used to degrade a wider range of pharmaceutical classes. For example, a partial degradation (22%) of carbamazepine, an antiepileptic drug, was achieved after 24 hours treatment by laccase-mediator system. The system consisted of *Trametes versicolor* laccase and synthetic mediator 1-hydroxybenzotriazole (HBT)[77]. A study within the same laccase-mediator system was conducted to remove tetracycline antibiotics, the results showed a complete elimination of problematic compounds after one hour treatment [78].

2.4.5 Current Applications of Laccases

Fungal Laccases are ideal green catalyses due to their wide substrate range and low energy consumption. These properties have encouraged the industrial community to implement laccase in various industrial and biotechnological processes in order to develop sustainable, efficient and environmentally-friendly applications.

2.4.5.1 Pulp and paper industry

The biological degradation of lignin can overcome the disadvantages of chemical and mechanical pulpings. For instant, in kraft pulping (a common type of chemical pulping), treating aspen chips with laccase from *Phanerochaete chrysosporium* can increase the yield and improve the

brightness and tensile strength. While using *Phelebia brevispora* laccase reduces the refining energy of mechanical pulping by 47%. During the pulping process around 90% of lignin is removed. However, the remaining lignin can affect the colour of the produced paper and thus, it is necessary to remove the remaining 10% as well. The conventional methods to degrade the remaining lignin are very effective. However, they require the use of toxic and harmful chemicals e.g. chlorine-based oxidants [79]. The enzymatic delignification is an extremely attractive alternative. It overcomes the disadvantages of conventional delignification process and can be easily implemented in the already existing technology.

2.4.5.2 Textile Dye Decolourisation

The non-specific extracellular system of laccases allows them to degrade compounds such as textile dyes that resist microbial degradation. Blanque, et al. studied the continuous treatment process of textile dye (*Grey Lanaset*) in a bioreactor with pellets of *Trametes versicolor* fungus. As a result a high efficiency (>80%) of decolourisation was achieved after contact time > 18 hours. The treated effluent met the environmental standards in relation to colour and it can be discharged into the sewerage system[80].

2.4.5.3 Bioactive Chemicals Removal

Although laccase-based treatment of bioactive chemicals in wastewater is still under study and has not been implemented on a full scale, there is much evidence of its ability to remove the problematic pollutants. One research group investigated the ability of *Trametes versicolor* laccase to degrade two common steroid estrogens (17β-estradiol (E2) and 17α-ethynylestradiol (EE2)) in deionised water matrix[12]. High removal efficiency (above 97%) of E2 and EE2 was achieved after 1 hour. In some cases laccase can form a part of enzymatic treatment system that consists of a mixture of different enzymes. For instance, a study combined between a fungal laccase from *Trametes versicolor* and β-D-glucuronidase in order to degrade a conjugated steroid (17β-estradiol 3-(β-D-glucuronide)). The enzymatic system was able to

effectively deconjugate the conjugated steroid and then degrade it with its by-product[13].

The majority of bench-scale studies were conducted in synthetic matrix (deionised water, synthetic wastewater). However, a number of research groups investigated the efficiency of laccase-based system in more complicated matrices. For example, Lloret, et al. (2013) developed an enzymatic membrane reactor that can provide a continuous removal of E1, E2 and EE2 from filtered secondary effluent with minimal laccase concentration [8]. Figure 2.6 depicts the designed reactor. Experiments were performed in both buffer solution and wastewater, with high (4 mg/l) and low (0.1 mg/l) concentrations of the target pollutants. In both cases, high removal efficiencies (80-100%) and reduction in the estrogenic activities (84-95%) were achieved. When the designed reactor was used to treat wastewater effluent with real concentrations of bioactive chemicals (0.29-1.52 ng/l) promising results were also achieved.

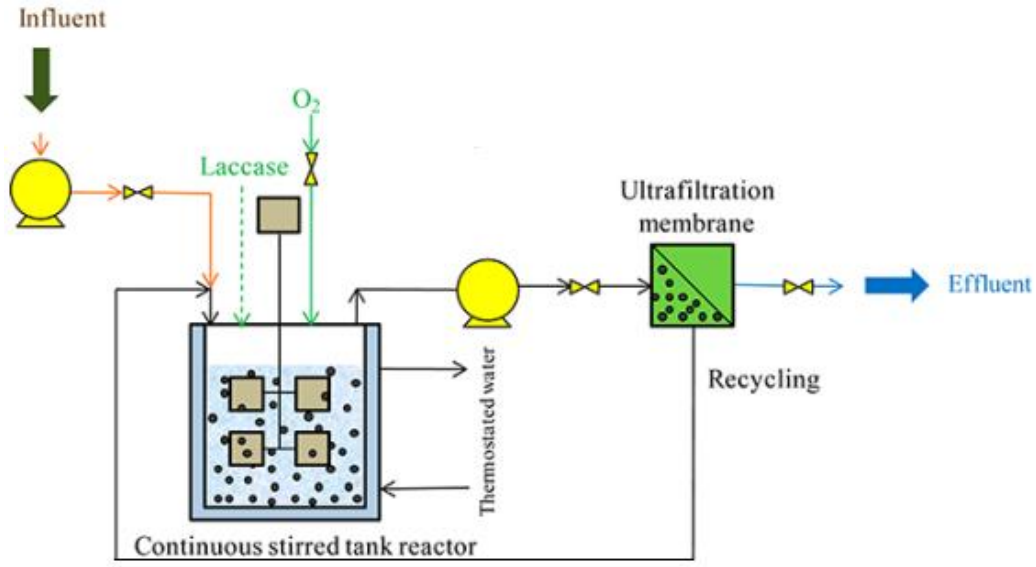


Figure 2.6 Continuous enzymatic membrane reactor for estrogens removal[8].

The by-products of the enzymatic treatment of steroid estrogens were studied by a number of research papers. Suzuki et al. reported high removal efficiencies of E2 using the laccase-mediator system. The authors also assumed

the cleavage of the aromatic rings of E2 during the enzymatic process. However no E2 metabolites were detected during the HPLC-UV analysis[81]. Suzuki's assumption was confirmed by another study using nuclear magnetic resonance (NMR) analysis[82].

Another explanation of steroid removal during laccase treatment process is the polymerisation of the phenolic compounds. Several studies suggested that laccase oxidises its substrate to free radicals which can then undertake further reactions and produce dimers and polymers that cannot be detected using HPLC-UV. As a result different analytical equipment are required to identify the formed by-products such as liquid chromatography mass spectrophotometry (LC-MS)[57, 83]. To ensure that the generated by-products during the enzymatic treatment of steroid were not more active/ toxic than the original compounds, the residual estrogenic activity of the treated effluent was measured by a number of research groups. Lloret et al. reported that their laccase-based membrane reactor managed to achieve a removal rate up to 95% for E1 and almost a complete degradation of E2 including a high reduction of the estrogenic activity (up to 97%) in the final effluent[84].

2.5 LACCASE-BASED TREATMENT FOR REMEDIATING BIOACTIVE CHEMICALS IN WATER MATRICES

Laccase-based treatment of bioactive chemicals has been investigated by several research groups around the world. Studies have been conducted in different matrices such as deionised water, buffers, synthetic and actual wastewater. The experimental studies have also been performed under different conditions of factors such as temperature, contact time, laccase concentration and pH. Various reactor designs for continuous treatment of bioactive chemicals have been also proposed. Table 2.5 demonstrates a number of laccase-based treatment studies focusing on steroids removal. The main points of interest in the previous laccase-based treatment studies are:

2.5.1 Experimental Factors and Their Ranges

In several cases the applied experimental conditions during laccase-based treatment studies, were often designed to match the optimum conditions for laccase activity rather than the actual conditions in WWTPs. For instance, utilising relatively high temperatures > 25˚C and pulses of pure oxygen showed very good removal efficiencies of target steroids[3, 84]. However, these experimental results do not provide a realistic evaluation of the efficiency of laccase-based treatment as their conditions are irrelevant to WWTP environment. Utilising pure oxygen for aeration will not be a feasible option in actual WWTP, which typically use air in its aeration tanks, due to the associated cost with that option. The temperature of wastewater is usually within the range of 10˚C - 25˚C and although laccase exhibit better activity at temperatures above that range[85], it is necessary to assess the performance of laccase-based treatment not at elevated temperatures such as 30˚C[13], but within the relevant temperature range to WWTPs as artificially increasing the temperature of the municipal wastewater would never be a cost effective/ realistic option. The pH is also one of the main factors influencing the activity of laccase. A significant change in laccase activity can be observed over a pH range of 2-7. According to one study, laccase activity dropped by 80% when the pH was increased from pH 3 to pH 7[9]. Therefore the efficiency of laccase-based treatment at acidic pHs such as pH 4.5-5[12, 13] does not provide a clear idea about the efficiency of that treatment at WWTP relevant pH (6.5 - 8.5).

The influence of contact time, temperature and laccase concentration on the enzymatic removal efficiency of a substrate can be estimated using the first principles such as Michaelis-Menten equation. For a reaction with a single substrate, Michaelis-Menten equation can be expressed as follows:

$$\nu = (V_{max} * [S]) / (K_M + [S]) \qquad \text{[Equation 2.1]}$$

Where;
ν is the reaction rate of the enzyme under the studied conditions, V_{max} is the maximum reaction rate of the enzyme, [S] is substrate concentration and K_M is Michaelis constant.

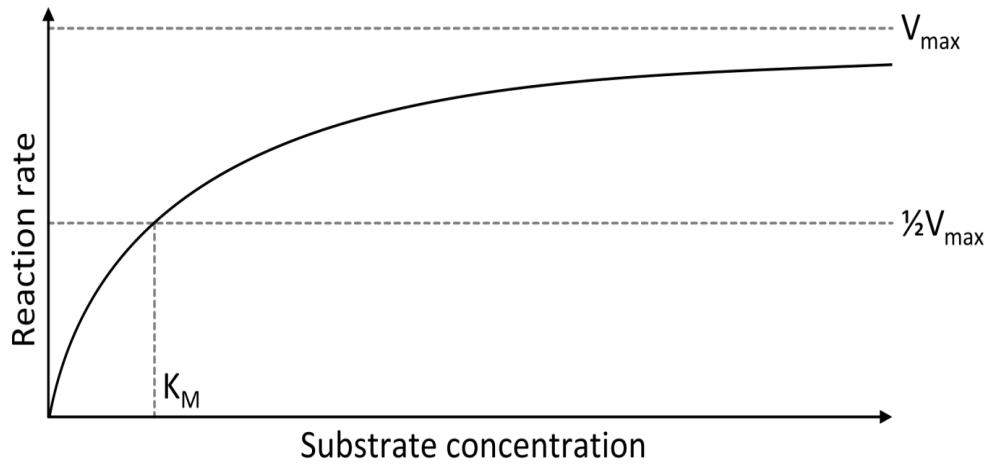


Figure 2.7 Typical Michaelis-Menten graph to estimate the kinetic parameters of an enzyme.

The Equation 2.1 is expressed using two parameters: (i) K_M: a Michaelis constant which is not dependent on either enzyme concentration or substrate concentration; (ii) V_{max}: a parameter containing the enzyme concentration ([E0]) and the catalytic rate constant (k_{cat})[86]. Based on the above it is possible to write Michaelis-Menten equation as follows:

$$v= (k_{cat}. [E].[S])/(K_M+[S]) \qquad \text{[Equation 2.2]}$$

k_{cat} is the catalytic constant that defines the capacity of the enzyme-substrate complex, once formed, to form a product. It's also called the "turnover number" as it represents the number of the catalytic cycles (or " turnovers") the enzyme can undergo in a unit of time, or the number of molecules of substrate that one molecule of enzyme can convert into products in one unit of time[86].

For an enzyme that obeys Michaelis-Menten equation, the kinetic parameters such as K_M and V_{max} can be determined by experimentally constructing a Michaelis–Menten graph (Figure 2.7) over a wide range of substrate concentrations in which the reaction rate varies noticeably. A suitable substrate range should be typically extended from about 0.1 K_M to about 10 K_M so the value of the determined parameter K_M is in the midpoint of that range.

These kinetic parameters can provide information about the suitable substrate range, the optimum enzyme concentration to achieve certain removal efficiency and the required contact time to achieve it[87].

The below equation [Equation 2.3] was derived from the standard Michaelis Menten equation [Equation 2.2] to determine the required contact time between the enzyme and the substrate to achieve a specific removal efficiency (X) in a batch reactor using kinetic parameters: K_M and V_{max}.

$$t = \frac{K_M}{V_{max}} \times \ln\left(\frac{1}{1-X}\right) + \frac{X.[S]_0}{V_{\max}} \qquad \text{[Equation 2.3]}$$

Where;
t is batch reaction time (hr)
X is the required removal efficiency of the pollutant/ substrate: $X = ([S]_0 - [S])/[S]$
$[S]_0$, [S] Initial and final substrate concentrations, respectively (M).
K_M Michaelis constant (M)
V_{max} Maximum reaction rate of an enzyme $M.hr^{-1}$

A study was conducted by Auriol et al. to determine the kinetic parameters of laccase-catalysed reaction with several steroid estrogens including estrone (E1)[11]. Experiments were performed in 0.1 M phosphate buffer at pH 7, 25±1 ºC and laccase concentration of 0.8 U/ml. The obtained kinetic parameters for estrone (E1) are shown in Table 2.4.

Table 2.4 Experimental kinetic parameters of laccase-catalysed reaction with estrone at pH 7.0, 0.8 U/ml laccase and 25 ± ˚C. (Adapted from Auriol at al.[11]).

Compound	Michaelis-Menten Model	
	K_M (µM)	**V_{max} (µM/min)***
E1	3.4	1.08

* The source of this table i.e. Auriol et al. has a typo in its table (Table 2) which shows the unit of V_{max} as µM/s instead of µM/min.

The above information can be utilised to estimate the required contact time (*t*) to achieve a desired conversion. For example, to achieve 90% conversion using 0.8 U/mL enzyme, assuming the enzyme activity is constant and equal to the initial activity and, by substituting the variables in Equation 2.3 with the known values, a total time of ~ 8 minutes is obtained, which is shorter compared to experimental values. This is due to probable enzyme deactivation.

$$t = \frac{3.4}{1.08} \times \ln\left(\frac{1}{1-0.9}\right) + \frac{0.9*0.4}{1.08} = 7.58\ mins$$

First principles can be also utilised to understand the potential impact of temperature on the activity and the stability of the studied enzyme. Figure 2.8 shows a typical "enzyme activity versus temperature" graph where the maximum enzymatic activity is achieved at what is called "the optimum temperature".

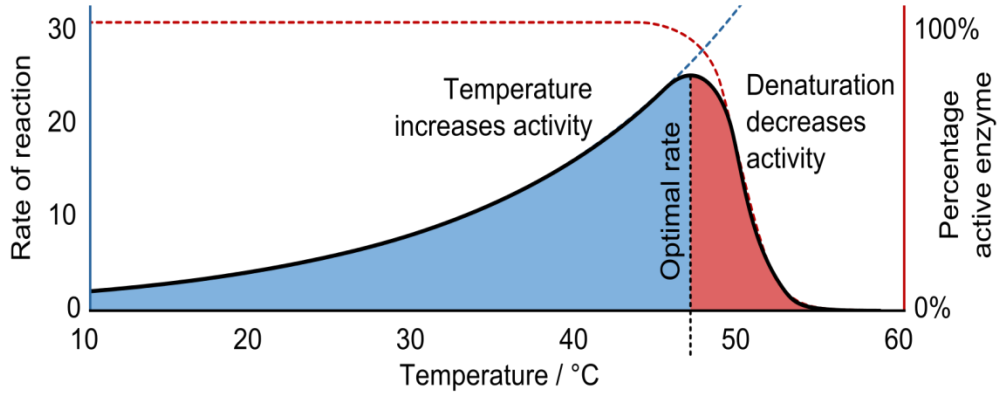


Figure 2.8 The impact of temperature on enzyme activity.

The enzymatic activity at different temperatures and the optimum operating temperature of any enzyme can be determined using Arrhenius law [88][Equation 2.4]:

$$k_{cat} = A.e^{-\frac{E_a}{RT}} \qquad \text{[Equation 2.4]}$$

Where;
A: the frequency or the pre-exponential factor (1/hr).
Ea: the activation energy ($J.mol^{-1}$)
R: the ideal gas constant (8.314 $J.K^{-1}.mol^{-1}$)
T: the absolute temperature measured in kelvin (K)

Arrhenius law describes the relationship between the rate of the reaction and its temperature, and can be applied to calculate k_{cat} of a specific enzyme at any given temperature. Once k_{cat} value (for a temperature T) is determined, both

V_{max} and K_M values can be calculated using the below equation and Equation 2.2:

$$Vmax = k_{cat}.[E]_0 \qquad \text{[Equation 2.5]}$$

As a result, either the contact time (*t*) in the reactor or the achieved removal efficiency (X) can be theoretically estimated at different temperatures by substituting V_{max} and K_M with their temperature-specific values and by solving Equation 2.3.

Some enzyme-based industrial processes are conducted at elevated temperatures (e.g. > 50˚C) where the enzyme may suffer from significant deactivation and lose part of its catalytic ability as the temperature raises. In this type of enzymatic reactions the thermal deactivation of the enzyme must be taken into account when calculating *t* or X values.

In the municipal wastewater environment, water temperature is typically between 6˚C and 25˚C and it is possible to assume that no significant thermal deactivation of an enzyme, such as the enzyme laccase, occurs within that range (Figure 2.8). This means that k_{cat} value can be calculated from Equation 2.5 without including any deactivation factors. Both *A* and E_a values can be experimentally determined from plotting ln (k_{cat}) vs 1/T, which should be related by equation (2.6) obtained from Arrhenius law:

$$\ln k_{cat} = \ln A - \frac{E_a}{R.T} \qquad \text{[Equation 2.6]}$$

For example, the enzymatic polymerization of phenol by laccase extracted from *Trametes versicol*or was investigated by Sengor et al. in 2009[89]. The activation energy of oxidative phenol polymerisation at pH 5 was determined as 21.175 kJ/mol

The above work shows that first principles can provide an estimation of the required amount of the enzyme, the suitable contact time and the optimum reaction temperature to achieve the maximum substrate conversion. However, this approach has its limitations and the accuracy of the estimated values using the first principles can be significantly compromised when the reaction is

performed in complex matrices. Performing kinetic studies in matrices such as wastewater may not be feasible due to the potential interferences between the various constituents within that matrix. In addition, the Michaelis-Menten model cannot account for the kinetic properties of many enzymes. *Allosteric* enzymes is an important group of enzymes that consist of multiple active sites and do not obey Michaelis-Menten kinetics[91].

As a result Artificial Neural Networks (ANN) and Response Surface Methodology (RSM) models are increasingly employed along with conventional first principle models to design and optimise the enzymatic processes and reactors[92]. The popularity of ANN models for chemical and enzymatic processes has been rapidly increasing due to their inherent ability to understand arbitrary complex functional relationships. ANN have been used to formulate approximate kinetic models for biological as well as conventional chemical reactors and to predict the efficiency of enzymatic treatments under various conditions using environmentally-relevant substrates and matrices[93]. ANN models have been also used to optimise the enzymatic system and identify the impact of each factor on substrate removal efficiency. Further details on ANN models are discussed in Section 2.10.2.

2.5.2 Evaluating the Impact of Each Factor

Studies in Table 2.6 investigated the impact of several factors (e.g. pH, contact time, temperature) on the efficiency of laccase-based treatment in removing bioactive chemicals. However these factors were investigated individually, by varying the value of one factor per time and maintaining the values of the others constant. This experimental approach is time consuming and does not address the interactions between the various factors. Utilising experimental designs such Box-Behnken Design (BBD) and Central Composite Design (CCD) (discussed in Section 2.8), is a more efficient way to study complicated systems such as laccase-based treatment[8, 94].

2.5.3 Water Matrix for Laccase-Based Treatment

Many experiments were performed in simple clean water matrices such as deionised water[12], buffers[84, 95] and synthetic wastewater, where the laccase is not subjected to the typical constituents within the wastewater matrix that may affect its activity. Some studies were also conducted in actual wastewater effluents [3, 11]. However the temporal variability of the wastewater was not accounted in any of them. The achieved removal efficiency of target pollutants in wastewater may change from day to day based on the composition of the utilised wastewater. Therefore studies of laccase-based treatment in wastewater should address the complexity and the variability of the used wastewater matrix that varies both temporally (during the day) and spatially (from WWTP to WWTP). In 2012, Gardner et al. studied 70 priority compounds in wastewater effluents from over 162 WWTPs in the United Kingdom, the results demonstrated the spatial variability of wastewater effluents as the concentration of each compounds varied among the WWTPs[42]. Table 2.5 shows the upper (97.5) and the lower (5) percentiles of the concentrations of several steroids and heavy metals. For example, the value of the upper percentile (97.5) of E1 was 0.1009 µg/l which means that 97.5% of the analysed wastewater samples had an E1 concentration that was less than/ equal to 0.1009 µg/l. While the value of the lower percentile (5) of E1 was 0.002 µg/l which means that only 5% of the analysed wastewater samples had an E1 concentration that was less than/ equal to 0.002 µg/l, as a result the majority of the analysed wastewater effluent samples had an E1 concentration in the following range [0.002 µg/l – 0.1009 µg/l]. Some of these heavy metals can act as inhibitors or mediators (as discussed in Section 2.6) of laccase. Therefore the efficiency of laccase-based treatment will vary from WWTP to WWTP. Heavy metals were used here just an example to demonstrate the spatial variability between the treatment plants, but many other factors may contribute to this variability. The temporal variability occurs in the WWTP as the wastewater influent varies throughout the day.

Table 2.5 Spatial variability of several compounds in wastewater effluents from 162 wastewater treatment plants in the UK.

Compounds in wastewater effluent	**Concentration percentile (µg/l)**	
	5	**97.5**
Iron	14	310
Copper	1.7	24
Lead	0.1	2
Nickel	1.6	14
Zinc	9.9	69
Estrone (E1)	0.002	0.1009
Estradiol (E2)	0.0002	0.0125
Ethinylestradiol (EE2)	0.0001	0.0016

At the moment there is a gap in the literature on quantifying the variability of wastewater and its impact on laccase-based treatment. Therefore, there is a need to develop a simple tool that can address this gap and provide a better understanding of the "wastewater variability" range in which laccase-based treatment has to operate if implemented in WWTPs.

Table 2.6 A summary of laccase-based treatment studies and some of their experimental conditions.

Source	Bioactive Chemical	Conc.	Matrix	pH	Temp. (˚C)	Contact time	Aeration/ mixing	Organism	Ref.
Auriol, *et al.* 2007	E1, E2, E3 and EE2	100 ng/l	Buffer	5-9	25	1 hr	magnetic stirring plate	*Trametes versicolor*	[11]
Auriol, et al. 2007	E1, E2, E3 and EE2	100 ng/l	Wastewater effluent	7	25	1 hr	magnetic stirring plate	*Trametes versicolor*	[11]
Xia et al. 2014	E2	1 mg/l	Buffer	4-8	Room temperature	2 hr	magnetic stirring plate	*Trametes versicolor*	[96]
Lloret et al. 2012	E1 and E2	50mg/l	Buffer	7	26	2-4	Pulses of oxygen	*Myceliophthora thermophila*	[84]
Lloret et al., 2013	E1, E2, E22	100 ug/l	Wastewater effluent	7	26	4	Pulses of oxygen	*Myceliophthora thermophila*	[3]
Tanaka, et al. 2009	E1, E2 and EE2	68 mg/l, 136 mg/l, 148 mg/l	Buffer + 20% ethanol	5	30	1 hr	Shaker 45 stroke/ min	*Trametes sp. Ha1*	[13]
Blanguez et al. 2008	E2 and EE2	10 mg/l	Deionised water	4.5	22	7 days	Agitation at 120 rpm	*Trametes versicolor*	[12]
Cardinal-Watkins et al. 2011	E2	2.7 mg/l	Buffer	4-7	21	40 mins	circulated using a pump	Immobilised *Trametes versicolor*	[95]

E1: Estrone; E2: 17β-estradiol, E3: Estriol, EE2: 17α-ethinylestradiol

2.5.4 Optimum Enzyme

Laccases from the blue oxidases family are one of the commercially available enzymes that are utilised in several fields of biotechnology[97]. The ability of one specific laccase from this family i.e. *Trametes versicolor* laccase to (i) catalyse a wide range of substrates such as phenolic and non-phenolic compounds as well as several environmental pollutants[68, 97] (ii) operate in a temperature range typical to the WWTPs e.g. [10˚C - 25˚C] and (iii) operate at near-neutral pH which is the typical pH range in municipal WWTPs, has made it an ideal candidate for micro-pollutants degradation in wastewater treatment plants[98]. *Trametes versicolor* laccase is also one of the most widely investigated enzymes to improve several industrial and biotechnological processes such as treating mill wastewater in pulp and paper industry[99] and degrading bioactive chemicals in municipal WWTPs[95, 100-102].

2.5.5 Optimum Location for Laccase-Based Treatment

Bench-scale studies performed in secondary wastewater effluent showed that there is a big potential to use laccase-based system as a tertiary treatment option following the secondary treatment stage in WWTPs[8, 98]. In some cases the secondary effluent was filtered through 0.45µm membrane filter in order to remove any particulates that may adsorb the target pollutant and thus provide incorrect results about the ability of laccase to degrade that pollutant.

Implementing laccase-based treatment as a tertiary treatment in conventional WWTPs has several advantages:

1) Full utilisation of the conventional treatment capacity

Conventional wastewater treatment plants were designed to remove suspended solids, organic matter, phosphorus and nitrogen from wastewater. However several studies have found that bioactive chemicals can be also reduced/ removed from wastewater during the primary and secondary treatment processes[103, 104] which may adversely impact on laccase activity, is

removed from wastewater during the primary and secondary treatment stages, leaving a much simpler wastewater matrix for laccase-based treatment.

2) Minimising the cost of laccase-based treatment

The secondary effluent will represent the influent to laccase-based treatment unit (Figure 2.9). This influent is a relatively clean wastewater matrix that contains the escaped bioactive chemicals from the conventional treatment. Applying laccase-based treatment into this matrix will allow the laccase to focus on the removal of these recalcitrant pollutants without being significantly consumed or inactivated by other substances. As a result the required amount of laccase to remove the target pollutants and subsequently its cost will be considerably smaller.

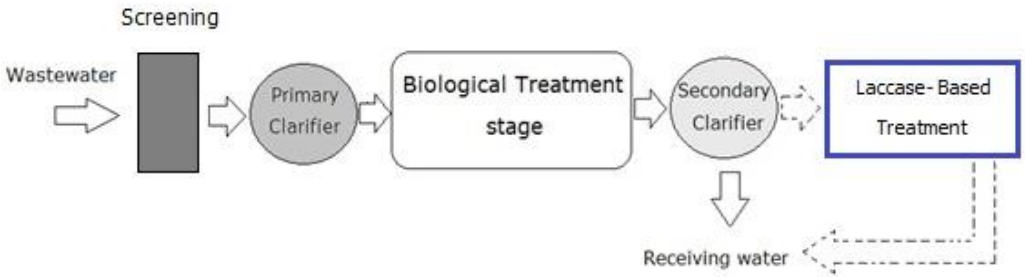


Figure 2.9 The potential location of laccase-based treatment in conventional wastewater treatment plant.

2.5.6 Target Pollutants

The ability of laccase to remove a wide range of pollutants has made it possible to consider laccase-based treatment to remove pollutants such as polycyclic aromatic hydrocarbons (PAHs)[105, 106], textile dyes[80, 107, 108], pharmaceuticals[109, 110] and steroids[3, 12, 96, 111-113] from water matrices. Removing biologically active steroids such as E1, E2 and EE2 from water matrices using laccase has attracted the interest of several research groups. The exposure to these chemicals, even at ng/l levels, in aquatic environments can cause adverse effects in animals and humans[4, 5, 114]. Three steroid estrogens: E1, E2 and EE2 have been included in the Chemicals Investigation Programme (CIP) performed by UK Water Industry. The study monitored more than 160 WWTPs for 70 compounds within the United

Kingdom. The concentration of E2 and EE2 in the final wastewater effluent exceeded the existing Environmental Quality Standard (EQS) in freshwater of $4x10^{-4}$ µg/l and $3.5x10^{-5}$ µg/l respectively in more than 50% of WWTPs, while the concentration of E1 was above the Predicted No Effect Concentration (PNEC) of 0.003 µg/l in 50% of the tested wastewater effluents. The compliance with the EQS or the PNEC could be achieved through dilution by the receiving water, however additional management/ treatment options may be required for some pollutants where the dilution alone may not be sufficient[42]. As a result several research groups around the world have been working on developing novel treatment technologies to remove these bioactive chemicals from water matrices and protect the aquatic environment.

2.6 LACCASE INHIBITORS

Wastewater is a complex matrix with an inherent variability. The feasibility of laccase-based treatment within this matrix depends on several factors such as temperature, pH and the presence of various constituents e.g. heavy metals and halides. The impact of metal ions and halides on laccase activity has been reported by several research papers[98, 108, 115-119].

2.6.1 Heavy Metals

Trace amounts of heavy metals such as Cadmium (Cd), Zinc (Zn) and Manganese (Mn) are essential for the growth of white-rot fungi. The fungal sensitivity toward heavy metals varies from species to species, however elevated levels of these metals can be toxic to fungi and adversely inhibit their growth and affect their catalytic activity. The toxicity of metals such as Copper (Cu) and Mercury (Hg) to some fungal strains has been utilised to develop antifungal wood preservatives[119]. Hg and Cd are considered the most toxic metals for all white-rot fungi[119], however, the concentrations of these two metals are generally very low/ below the detection limit in the majority of the municipal wastewater effluents (Table 2.5). Certain metal ions can act either as an inhibitor or an inducer of laccase activity at certain concentrations. Some studies reported that metal ions such as copper can act as inducers at low

concentration and as inhibitors at higher concentrations[117, 118]. The exact mechanisms of how these metallic ions interfere with the enzyme laccase are not fully understood. However, some studies reported that Cu and Fe ions could interrupt the internal electron transport system of laccase and lead to the inhibition of substrate conversion[116, 120, 121].The effect of metal ions on laccase activity may vary from laccase to laccase. However several studies agreed on the negative effects of heavy metals such as Mercury, Zinc, Cadmium, Iron, Silver, Manganese and Chromium, on the activity, stability or growth of laccases[115-118, 122]. Table 2.8 shows the impact of several metal ions on the activity of different laccases.

Although there are many potential inhibitors of laccase, the main focus should be on the constituents that are prevalence within municipal wastewater environment and exhibit an adverse impact on the efficiency of laccase-based treatment. The Chemicals Investigation Programme (see section 2.5.3 for details) included 10 metals in its study (Table 2.5). Samples of final wastewater effluent were analysed for both total and dissolved metals. The dissolved fraction of each metal represents its concentration in the discharged effluent and thus it can be directly used to determine the compliance with the EQS of the receiving water. The study found that the concentrations of dissolved Aluminium, Iron, Chromium, Mercury and Silver were all below the existing or the proposed EQS or PNEC values. However the concentration of Cadmium, Copper, Nickel, Lead and Zinc were above their PNEC/ EQS values [42].

Table 2.7 List of metals that have been included in Chemical investigation programme, their percentiles, maximum and minimum concentrations, proposed and existing consents [42].

Metal (dissolved)	Percentile (µg/l)		Conc. in wastewater effluent		EQS Annual Average (µg/l)	PNEC (µg/l)
	5	97.5	Minimum conc. (µg/l)	Maximum conc. (µg/l)		
Aluminium	4	122	0.1	125	N/A	N/A
Iron	14	310	14	310	N/A	1000^{DEFRA}
Cadmium	---	0.275	0.1	1.4	0.08	N/A
Chromium	0.5	2.1	0.5	2.1	N/A	3.4^{BLM}
Copper	1.7	24	0.1	50	N/A	11^{BLM}

Lead	0.1	2	0.1	10	1.2[bio]	6[BLM]
Mercury	---	0.08	0.001	0.013	0.07[MAC]	N/A
Nickel	1.6	14	0.5	50	4[bio]	6[BLM]
Silver	All results <0.5		<0.5	<0.5	N/A	N/A
Zinc	9.9	69	0.5	150	N/A	17[BLM]

BLM: "BLM adjusted PNEC" based on biotic ligand models available to Environmental Agency of England and Wales for pH 7.8, total hardness 125 mg CaCO3/l, 5 mg/l dissolved organic carbon (DOC).

N/A: No available information.

bio: Based on bioavailable fraction.

MAC: Maximum Admissible Concentration.

DEFRA:DEFRA Direction 2010:

http://archive.defra.gov.uk/environment/quality/water/wfd/documents/2010directions.pdf

Table 2.8 The impact of different metal ions on laccase activity based on various research papers

Metal	Laccase	conc. range	Impact	Ref
Iron	Trametes hirsuta	1-10 mM	Negative	[115]
Iron	*Trametes versicolor*	0.25-1 mM	Negative	[116]
Cadmium	Trametes hirsuta	1-10 mM	Negative	[115]
Cadmium	*Trametes versicolor*	0.5-80 mM	Negative	[117]
Cadmium	Peniophora sp.	0.5-15 mM	Positive	[118]
Chromium	Trametes hirsuta	1-10 mM	Negative	[115]
Copper	Trametes hirsuta	1-10 mM	Negative	[115]
Copper	*Trametes versicolor*	0.25-1 mM	Negative	[116]
Copper	*Trametes versicolor*	0.5-80 mM	Positive with metal conc. <1mM. Negative within metal conc. range= [2 mM -80 mM].	[117]
Copper	Peniophora sp.	0.5-15 mM	Positive with metal conc. ≤ 1 mM. Negative with metal with metal conc. > 1 mM.	[118]
Mercury	Trametes hirsuta	1-10 mM	Negative	[115]
Mercury	Peniophora sp.	0.5-15 mM	Negative	[118]
Nickel	*Trametes versicolor*	0.25-1 mM	No impact	[116]
Nickel	Peniophora sp.	0.5-15 mM	Positive within metal conc. range= [0.5 mM -1 mM]. No impact at 10 mM metal conc. Negative at metal conc. ≥15 mM	[118]
Nickel	*Trametes versicolor*	0.25-1 mM	Negative	[116]
Zinc	Trametes hirsuta	1-10 mM	Negative	[115]
Zinc	*Trametes versicolor*	0.25-1 mM	No impact	[116]
Zinc	*Trametes versicolor*	0.5-80 mM	No impact	[117]

The inhibition mechanism of laccase by metal ions has not been fully understood yet. However it has been reported that iron and copper ions can interrupt the electron transfer systems in laccase and inhibit the conversion of the substrate [120]. Both iron and copper ions are commonly detected in wastewater effluents and their presence in that matrix can potentially impact on the efficiency of laccase-based treatment [116].

2.6.2 Halides

Halides such as chloride (Cl^-) and fluoride (F^-) are common inhibitors of laccases, their ions can stop the molecular oxygen from being reduced at type T2/T3 trinuclear copper site and as a result break the terminal electron acceptance process. Some studies suggested that the extent of laccase inhibition depends on halides accessibility to the structural copper atoms of laccase and therefore larger halides such as bromine (Br) is a weaker laccase inhibitor that F^- or Cl^-. Chloride is essential for many aquatic habitats. However, high levels of chloride can adversely impact on the freshwater organisms and plants by altering their reproduction rates and changing the characteristics of the local ecosystem. In addition chloride can leach through the soil and affect the quality of the groundwater.

High levels of chloride can be found in areas where in-house water softening is applied. Water softeners commonly utilise sodium chloride salt (NaCl) to separate minerals from water. As a result the used salt breaks down into sodium (Na^+) and chloride (Cl^-) ions, reaches WWTP and eventually the aquatic environment.

Elevated levels of chloride (≈700 mg/l) were observed in the final wastewater effluent in Minnesota, USA, where in-house water softeners are heavily used. The final effluent is discharged to *Pomme De Terre* River and to protect the aquatic environment of the river, the Minnesota Pollution Control Agency (MPCA) issued a new regulation on chloride levels in sewage treatment plants that states that the permitted MPCA's chloride standard for sewage treatment ponds is 230 mg/l. As a result several WWTPs within that region had to implement chloride control measures to comply with the new standard. The

concentration of chloride in wastewater varies from site to site and it is influenced by the type of the sewerage system within the catchment, the existence and type of industrial wastewater inputs and even the presence of saline infiltration. One study reported that the typical concentration of chloride in municipal wastewater is around 177 mg/l[98]

Another research group reported that chloride ions exhibit both competitive and weaker uncompetitive inhibitory effects on laccase activity (Figure 2.10) and that there are interactive inhibitory roles of pH and chloride [123, 124]. Competitive inhibition occurs when the inhibitor (I) competes with the substrate (S) to bind to the active site of the enzyme (E), while in uncompetitive inhibition the inhibitor binds only to the enzyme-substrate (ES) complex. Raseda et al. proposed that the inhibition of laccase by chloride is similar to that by hydroxide ion, where both ions inhibit the electron transfer from the T1 site to T2/T3 trinuclear sites. The kinetic results of that study showed that the hydroxide anion and chloride share a common mechanism to inhibit the laccase activity [123].

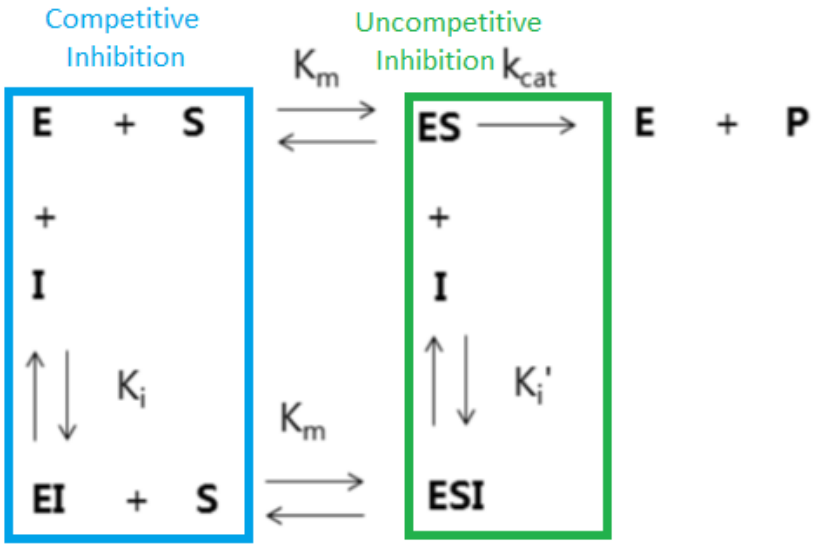


Figure 2.10 Schematic mechanism for the mixed inhibition of laccase by chloride (adapted from Raseda et al.)[123].

The impact of chloride ions on laccase activity was studied using ABTS as a substrate and varying the concentration of sodium chloride (NaCl) from 0 to 50 mM. Increasing NaCl concentration in the assay, significantly decreased laccase activity at pH 3, but had a limited effect on laccase activity at pH 6 [123]. Table 2.9 below shows the competitive and uncompetitive inhibition constants (expressed in mg of chloride per liter) for both pH 3 and pH 6. Inhibition constant (Ki) represents the potency of a certain inhibitor by measuring the required concentration of the inhibitor to produce half of the maximum inhibition and it's a measure of inhibitor's potency.

Table 2.9 Inhibition constant values of chloride ions at two different pHs during the oxidation of ABTS by *Trametes versicolor* laccase in citrate-phosphate buffers.

pH	**Chloride Inhibition Constant (mg/l as chloride ion)**	
	Competitive (K_{ic})	Uncompetitive (K_{iu})
3	12	642
6	840	11,000

Based on the Ki values in Table 2.9 it becomes clear that the inhibitory effects of chloride ions are significantly weakened at pH 6 in comparison with those at pH 3. The higher the Ki value, the weaker the inhibitor[123].

2.7 ENVIRONMENTAL ANALYSIS

2.7.1 High Performance Liquid Chromatography Analysis

High Performance Liquid Chromatography (HPLC) is a powerful analytical tool that have been utilised by research groups around the world to accurately quantify the concentration of various compounds in several matrices. The main components of the HPLC-UV system are shown in Figure 2.11. Once the sample is ready for analysis, it is injected and mixed with the used mobile phase in the HPLC. Manual sample injection is still utilised in some old HPLC units, however the majority of the modern HPLCs have a fully automated sample injection system that minimises human interference with the analytical process and the associated errors with that. The mixture of the sample and the mobile phase is then pumped under a high pressure through a chromatographic

column. HPLC column is typically packed with modified non-polar silica beads that are manufactured by attaching long hydrocarbon chains to its surface. This is a very common type of columns that is used in reverse phase chromatography. Polar solvents such as water and methanol are used with this type of columns and thus all the polar constituents within the mixture will be strongly attracted to and moving with the mobile phase rather than the modified silica beads (the stationary phase).

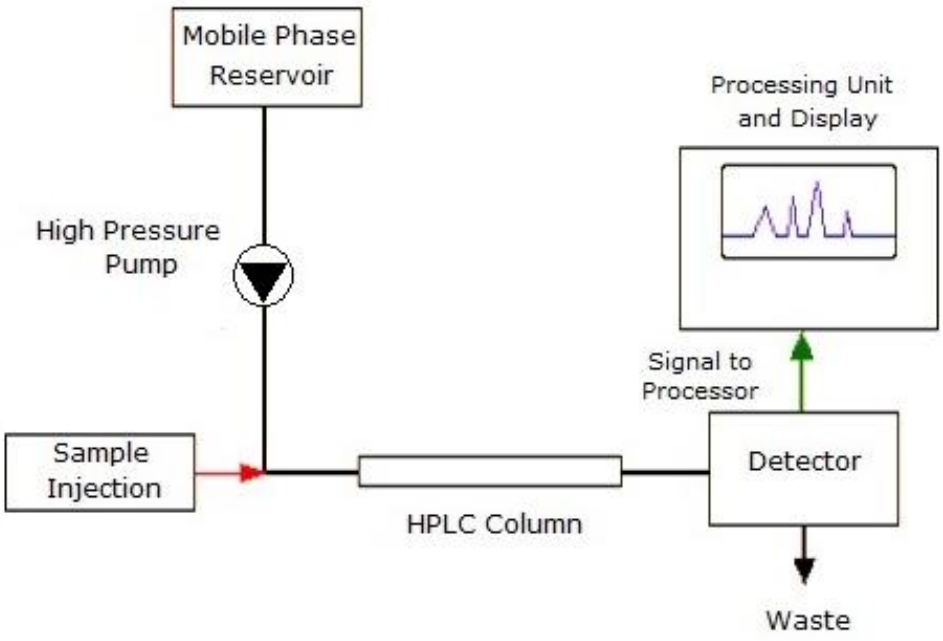


Figure 2.11 Typical components of the High Performance Liquid Chromatography unit coupled with UV detector.

Unlike the polar constituents, the non-polar ones will be attracted to the hydrocarbon groups of the stationary phase and less soluble in the moving solvent. As a result the non-polar constituents will move slower down the column, elute after the polar molecules and subsequently have a longer retention time. Retention time is the required time for a specific compound to pass through the column and reach the detector[125]. Retention time varies from compound to compound. Retention time of a specific compound depends on the applied pressure on the column, the type of the stationary phase e.g. particles size, the temperature of the column and the composition of the used mobile phase. After the separation of the mixture is completed, the mobile

phase reaches the detector. One of the commonly used detectors is the Ultraviolet (UV) detector which measures the ability of a sample to absorb light at one or more wavelengths (Figure 2.12). A wide range of organic compounds absorb various wavelengths of UV light.

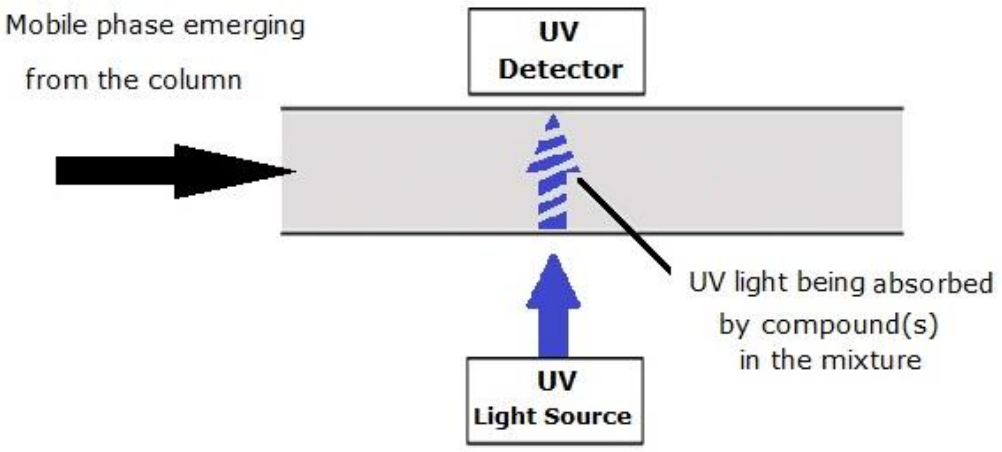


Figure 2.12 The principle of a UV detector.

The detector unit has a light source that emits a beam of UV light. The light then passes through the mobile phase emerging from the end of the column. On the opposite side of the UV lamp, there is a UV detector that measures the absorbed amount of light by the passing mobile phase. The amount of the absorbed light depends on the concentration of the specific compounds that is passing within the mobile phase at the time.

2.7.2 Ultraviolet-Visible Spectrophotometry Analysis

Ultraviolet-Visible (UV-Vis) spectrophotometry is one of the most frequently utilised techniques in enzymatic assays. It involves measuring the amount of ultraviolet or visible radiation absorbed by a compound in a solution. This technique is simple and a quick approach to measure enzyme activity under various conditions. Beer- Lambert law is the main law that governs the UV-vis spectrophotometric analysis and can be expressed using the following equation (Equation 2.7):

$$\Delta A_{min} = \text{Ɛ}^* \Delta C_{min}^* L \qquad \text{[Equation 2.7]}$$

Where;

ΔA_{min}: the change in the absorbance over time, Ɛ: the extinction coefficient of a substrate, L: path length of light through the sample, ΔC_{min}: the change in substrate's concentration over time.

Both "a" and "b" are constants, therefore the absorbance "A" is directly proportional to the concentration of the substrate[126]. The Absorption is usually measured at the wavelength that gives the maximum absorption (λmax) for a specific substrate. In laccase assays, ABTS is the most commonly used substrate, it can be rapidly oxidised by laccase into a green soluble by-product (ABTS+) and the increase in ABTS absorbance (at λmax = 420 nm) as a function of time can be easily observed using UV-vis spectrophotometer. In this type of assays the concentrations of ABTS and laccase have to be adjusted to give a good linear absorbance vs. time graph. The area where the absorbance values are below 1 considered to be the area of the maximum accuracy and precision and therefore absorbance values above 1 is not normally included in any further analysis.

2.8 EXPERIMENTAL DESIGNS

The removal efficiency of bioactive chemicals using laccase in water matrices depends on several factors including contact time, temperature, laccase concentration, pH and the presence of other compounds in the reaction mixture (inhibitors or mediators). The impact of these factors on the removal efficiency might prove to be either positive or negative, influenced by individual factors or by the interaction between them. As a result, evaluating this system by varying one factor per time is inefficient and time consuming approach. The use of statistically designed experiments is a popular way to deal with complicated systems that are influenced by multiple factors[8, 94]. The correct application of this approach can save both time and resources and provide a better understanding of the system as a whole.

Experimental designs have been incorporated in various fields including decolourisation of dyes[127], optimising the immobilisation of laccase[128] and the enzymatic treatment of steroid estrogens[8]. Each experimental design generates a matrix of conditions that identifies the required number of

experiments and their parameters. The size of this matrix depends on the number of factors and the type of the utilised design. There are two commonly used experimental designs; (i) Central Composite Design (CCD) with five levels per factor (-α, -1, 0, +1, +α) and (ii) Box-Behnken design (BBD) with three levels per factor (-1, 0, +1)[129],[130]. Both CCD and BBD were designed to cover the main points in the investigated system to give an overall understanding of system's response under different operating conditions. However, the points' locations in BBD and CCD are different (Figure 2.13). BBD was originally designed to reduce the number of the required experiments in quadratic model fitting. One of the characteristics of this design is that it does not have points where all the factors are at their extreme values. This feature may be quite useful in systems where undesired phenomena may appear at those specific points, however, this same feature could be considered a disadvantage due to the regions of poor prediction quality that it creates[131]. On the other hand, CCD can provide accurate predictions of the interactions effects between the factors influencing the studied process in any point within the system.

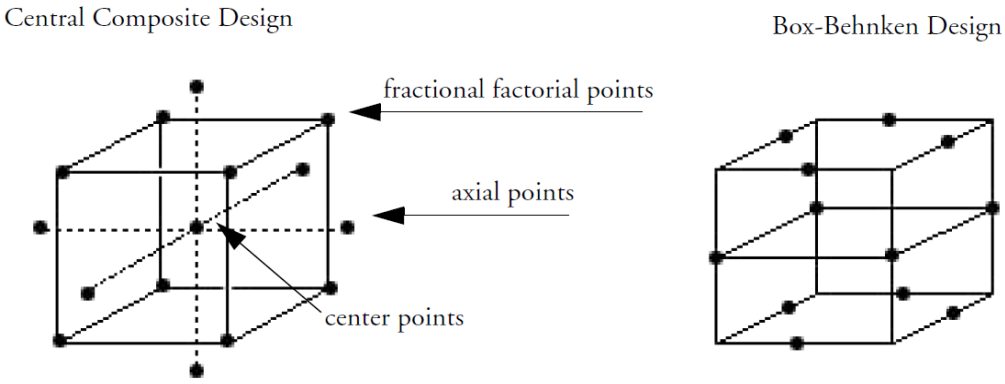


Figure 2.13 The locations of experimental points in the Central Composite Design (CCD) and Box-Behnken Design (BBD) [88].

2.9 THE IMPACT OF THE EXPERIMNETAL PROCEDURE ON THE VALIDITY OF LACCASE-BASED TREATMENT RESULTS

2.9.1 Estrone's Solubility in Water Matrices

Estrone is a common bioactive chemical that has been detected in many municipal wastewater effluents[42]. The feasibility of removing E1 from water matrices using laccase has been widely investigated. Due to the nature of free steroids, the aqueous solubility of E1, E2 and EE2 is low. However the main issue is that there is a large discrepancy in the reported aqueous solubility data of these steroids in the literature. For instance the solubility of E1 in aqueous solution varied from study to study within the range of [1.3 mg/l – 13 mg/l]. Table 2.10 summarises the reported solubility of three steroids: E1, E2 and EE2 in water. The variability of steroids solubility results could be attributed to the variation in the experimental conditions of each study, Yu, et al. (2004) obtained the solubility values after mixing studied solutions on a shaker for 7 days at 22±0.1˚C and pH of 6.8, while, Shareef, et al (2006) performed the experiments at 25±0.5˚C with only 4 days contact time and pH of 7 using purified nitrogen to mix the investigated solutions.

Table 2.10 Aqueous solubilities of selected free steroids from the literature.

Reference	**Solubility in water (mg/l)**		
	E1	E2	EE2
Shareef, et al., 2006 [22]	1.30	1.51	9.20
Kabasakalian, et al., 1966 [132]	----	5	10
Yamamoto, et al.,2004 [133]	1.53	3.85	19.1
Yu, et al., 2004[134]	2.09	3.1	3.1
Lai, et al 2000 [135]	13	13	4.8
Tanaka, et al., 2009 [13]	2.33	4.19	15.98
Han, et al., 2010 [136]	0.61	----	-----
E1: Estrone, **E2**: 17β-estradiol, **EE2**: 17α-ethynylestradiol			

As a result, a large number of laccase-based treatment research papers have utilised the solubility data from one of the above sources or from other solubility studies to prepare steroid solutions for their work. The majority of those research papers did not provide sufficient details about the followed preparation procedure of their steroid solutions and therefore it is impossible to

verify that the prepared solution for laccase-base treatment study (i) was prepared under the same conditions of the associated solubility value; (ii) was not over saturated. It is possible to increase the solubility of steroids by the addition of a solvent such as ethanol or methanol into the aqueous solution. However using this approach in laccase-based treatment studies means that the investigated matrix (solvent-water) is far from being a good representative of the actual environment where the laccase may end up being utilised i.e. wastewater effluent[13]. One paper studied the biodegradation of E2 and EE2 by *Trametes versicolor* using 10 mg/l solution for each steroid, the study reported achieving more than 97% steroids removal in both solutions after 24 hr contact time[12]. However and according to Table 2.10 the initial concentrations of E2 and EE2 solutions in that study were above the saturation point at least for one of these compounds. As a result the actual efficiency of laccase-based treatment might have been significantly over estimated as the initial concentrations of E2 and EE2 in the solutions were potentially lower than 10 mg/l. Similar study was performed using a mixture of three steroids E1, E2 and EE2 in a phosphate buffer pH 7, the individual concentration of each steroids was 4 mg/l which is much higher that the solubility values of E1 and E2 reported by Shareef, et al. in 2006[22]. The mixture was used again to study the ability of laccase to biodegrade these pollutants, high removal efficiencies of 80%, 87% and 85% were achieved for E1, E2 and EE2, respectively[3]. However and according to 3 previous solubility studies in Table 2.10, the used steroids mixture was oversaturated for E1 and E2 (and EE2 according to Yu, et al., 2004 [134]) which demonstrates again that the collected results from that study may not reflect the actual efficiency of laccase in degrading steroids. Many similar cases can be found in the literature which highlights the necessity of selecting suitable concentrations of steroid solutions that are high enough to be analytically quantified, but low enough to be below the aqueous saturation point of that steroid.

2.9.2 Adsorption of Bioactive Chemicals onto Membrane Filters

The hydrophobic nature of free steroids allows them to adsorb on various solid materials including membrane filters. Studies showed that the main routes of

steroids removal in conventional WWTPs are adsorption and biodegradation, adsorption into sludge in WWTPs is the main removal route for more hydrophobic steroids such as EE2 and E2[65, 137], while compounds with relatively weak hydrophobicity such as E1 and E3 could be removed from water matrix through biodegradation if suitable conditions are present. The hydrophobicity of a compound can be quantified using octanol-water partition coefficient (K_{OW}). This coefficient represents the ratio of compound's solubility in octanol, a non-polar solvent, to its solubility in water, a polar solvent. The higher the K_{OW}, the more hydrophobic (non-polar) the compound [138]. Table 2.11 shows the log K_{OW} and molecular weight (MW) values of the several steroid estrogens found in aquatic environments.

Table 2.11 Physicochemical properties of common steroids [30].

Bioactive chemical	**Molecular weight (g/mol)**	**Log K_{OW}**
Estrone (E1)	270.4	3.43
17 β-Estradiol (E2)	272.4	3.94
Estriol (E3)	288.4	2.81
17α-ethynylestradiol (EE2)	296.4	4.15

The nature of the used analytical equipments nowadays demands that the tested samples are particulate-free to avoid any blockages in the lines of the analytical systems such as HPLC-UV and LC-MS/MS.

Sample filtration is one of the most popular approaches for particulates removal, it is efficient, cost effective, does not require the use of expensive equipments and has been implemented in several laccase-based treatment studies to remove impurities from water matrices[139-141]. However there are very few studies that have investigated, acknowledged or highlighted the associated challenges with this step. One of the potential disadvantages of this method is the abiotic adsorption of some chemicals within the filtered solution onto the surface of the membrane filter itself. The percentage of the adsorbed amount depends on several factors such as the physicochemical properties of the adsorbed chemical, the acidity of the filtered sample and the membrane filter's material[136, 142, 143]. The adsorption of E1, a representative steroid

estrogen, into different membrane filters was investigated by Han, et al. (2010). The study demonstrated that nylon (NYL), polypropylene (PP) and some polytetrafluoroethylene (PTFE) filters have a high affinity toward E1 where more than 80% of E1 was adsorbed onto the surface of these membranes after a 0.4 mg/l E1 aqueous solution was passed through them. Unlike NYL, PP and PTFE filters, Regenerated cellulose (RC) and glass microfibers (GMF) filters showed a low affinity toward E1 and it adsorption on their surfaces was 8.1% and 2.3%, respectively. The study also showed that E1 adsorption was largely dependent on the material of the membrane and to a lesser extent on the pore size of the used membrane[136]. Selecting a suitable membrane filter during laccase-based treatment bench scale studies is essential to ensure that the obtained removal efficiency of E1, for example, is fully attributed to laccase and not to the abiotic adsorption onto the filter. Several studies in the literature could have used sample filtration as part of their experimental procedure without mentioning it in their final papers, other studies provided the pore size of the used filter without mentioning the actual material of that membrane filter [11] [116]. Table 2.12 highlights a number laccase-based treatment studies that focused on the biodegradation of steroids from water matrices and utilised sample filtration as part of their bench-scale experimental procedure.

Table 2.12 Sample filtration details of several enzyme-based treatment studies.

Ref.	Pore size (mm)	membrane material	Substrate	Matrix	Analytical equipment
[139]	0.45	U/S	E1, E2, E3	Water samples (Rain, River and Lake)	HPLC-UV
[141]	0.45	GMF	E1, E2, EE2, E3	Deionised water	GC-MS
[11]	0.45	U/S	E1, E2, E3, EE2	Phosphate buffer	LC-MS
[140]	0.45	U/S	E1, E2, E3, EE2	Wastewater effluent	LC-MS
[85]	0.2	PTFE	Phenol	Buffers	HPLC-UV

E1: Estrone, E2: 17 β-Estradiol, E3: Estriol, EE2: 17α-ethynylestradiol.
GMF: Glass microfiber, PTFE: Polytetra-fluoroethylene, U/S: Unspecified.

To identify the optimum filter for a specific chemical/ solution, several points must be considered;

- **Filter's compatibility with the used solvent** in the sample as some membrane filters can only be used with aqueous solutions e.g. RC filters.
- **The adsorption of the analyte onto the membrane filter** should be minimal and reproducible from filter to filter.
- **The filtered volume** is an important parameter that defines the suitable filter size (diameter). However the total capacity of the filter is also influenced by the characteristics of the filtered solution e.g. viscosity and pH.

2.9.3 Enzymatic Inactivation

During laccase bench-scale studies and in order to identify the residual concentration of the target bioactive chemical after a specific contact time, the enzymatic reaction must be stopped by inactivating the laccase instantly, permanently and without affecting the concentration of the target bioactive chemical within the solution. Several laccase inactivation options have been described in the current literature. In some studies laccase was inactivated by autoclaving it at 121˚C for 30 mins[144], this approach is suitable for experiments when the time is not a critical factor such as control experiments with inactivated laccase (to account for any abiotic removal of the target pollutant in the absence of the live laccase). However, when the biodegradation of the pollutant is studied as a function of contact time, there is a need to use faster inactivation approaches such as acidification. Several studies used strong acids such as sulfuric (H_2SO_4) and hydrochloric (HCl) acids to reduce the pH of laccase-active samples to pH 2 which –according to Lloret, et al. [9]- is the pH when the laccase becomes fully inactive. However, the same paper investigated the activity of laccase at different pHs in the range of [2-7] (Figure 2.14). Experiments were performed at 30˚C and the relative activity at each pH represented a ratio between the measured activity and the maximum activity of laccase. Figure 2.14 shows that at pH 2, the laccase still has 40% of its

maximum activity at pH 3, if the reported results in the below figure is correct that means that the followed inactivation approach is not efficient and the laccase may remain partially active even after the acid addition.

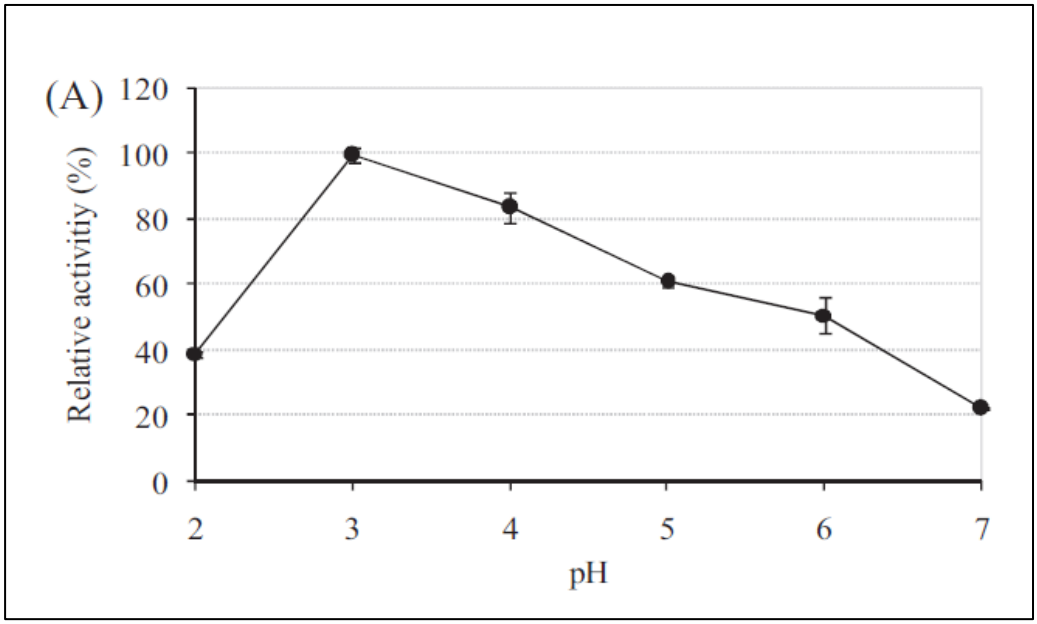


Figure 2.14 Effect of pH on the enzyme activity using ABTS as substrate (Adapted from [9]).

Another research paper utilised HCl to inactive laccase samples. However, no details were provided about the amount of the used acid, its strength or the achieved pH which make it impossible to assess the efficiency of that approach[140]. Cardinal-Watkins and Nicell (2011) used 100 µl of 1N H_2SO_4 per 1 ml of sample to reduce the pH to below 2.5 and inactivate any laccase that may have leached from the immobilised laccase in the reactor into the sampled effluent. The impact of pH 4, 5, 6 and 7 on the conversion of E2 was studied. However laccase activity at pH $\leq$ 2.5 was not included/ considered in this paper and it was not feasible for the reader to verify that full laccase inactivation was achieved at pH $\leq$ 2.5 in this immobilised laccase[95]. A summary of some of the utilised inactivation approaches is included in Table 2.13.

Table 2.13 Common enzyme inactivation approaches from the literature.

References	Inactivation approach	Enzyme type
Auriol *et al*., 2008 **[140]**	Acidification with Hydrochloric acid	*Trametes versicolor*
Cardinal-Watkins, *et al.*, 2011 **[95]**	Acidification with sulfuric acid	*Trametes versicolor*
Xia, *et al.*, 2014 **[145]**	1:1 sample dilution with methanol	*Trametes versicolor*
Kurniawati & Nicell, 2008**[85]**	1:9 sample dilution with 10% acetic acid	*Trametes versicolor*
Lloret, *et al.* 2013 **[9]**	Acidification with Hydrochloric acid	*Trametes versicolor*
Blanquez, *et al.* 2008 **[12]**	Autoclaving	*Trametes versicolor*

Acidification is a popular, simple and cost effective inactivation approach. However, the lack and –sometimes- the contradiction in the provided details in the literature may raise doubts in the reader mind about the actual efficiency of used inactivation approach and thus the reliability of the collected biodegradation results by laccase.

2.9.4 Steroids Stability in the Analysed Mixtures

The majority of laccase-based treatment studies utilise analytical equipment such as HPLC-UV and LC-MS to quantify the concentration of steroids before and after the enzymatic treatment. As it has been demonstrated previously in Table 2.12, samples with steroids can be prepared in various water matrices e.g. buffers, deionised water and wastewater. However, steroid estrogens are not stable in water matrices over long period of time and their concentration could slowly decrease when there is a long gap between the sample collection and the analysis[146]. The stability of steroids in those matrices can be affected by: the used inactivation approach (e.g. acidification, autoclaving), the storage conditions (e.g. temperature, duration), the original matrix of the sample (buffer, wastewater).Thus and aside from the investigated factor i.e. laccase activity, the concentration of steroids in water samples may decrease due to additional unmonitored factors. Therefore there is a need to check the stability of the studied steroid in the final sample by performing suitable

control studies. The details of the performed -if any- stability controls are usually dismissed in many research papers. One study investigated the degradation of E1, E2 and EE2 by laccase in a buffer at pH=5, however, no details were provided about the samples storage or the performed controls to ensure the efficiency of the inactivation procedure[13]. Another study with E1, E2, E3 and EE2 reported using H_2SO_4 to stop the enzymatic reaction by dropping the pH to 2 in the laccase-treated samples. The acidified samples were then refrigerated for 2 day prior further analysis. No details were provided about the storing temperate or about stability control is the acidified mixture[141].

Potentially some of these control experiments were performed by the research groups, but not included due to word count limits in some journals. However, it is important to at least briefly highlight that these controls were performed to give the reader a confidence in the presented results and remind other researchers to conduct this type of controls before initiating any new experimental studies.

2.10 MODELLING AND OPTIMISING LACCASE-BASED SYSTEM IN WATER AND WASTEWATER MATRICES

After selecting the suitable experimental design and performing the experiments in the lab, the experimental data can then be utilised to build a wide range of models that link system's response to the experimental conditions and predict the set of conditions for an optimum output. Response surface methodology (RSM) and artificial neural network (ANN) are some of the most popular models applied in laccase-based treatment studies [147]. Both models are able to represent complex nonlinear systems and the relationships between the independent factors[131].

2.10.1 Response Surface Methodology (RSM) Model

Response Surface Methodology (RSM) model is a collection of mathematical and statistical techniques that are used to describe the relation between a number of independent factors. The method was first developed in 1951 by

Box and Wilson and since then it was used to fit linear, square polynomial and other functions to experimental data generated using experimental designs such as BBD and CCD. Fitting the RSM model equation to the experimental data consists of two stages: (i) coding the experimental data, which involves transferring the input values of each factor into coded units such as +1, 0, -1 instead of their actual values. Experimental Design software such as Minitab can automatically transfer all the input values into coded units to ease the analysis and identify which factor has the largest impact on system's response. The next step involves (ii) fitting the coded experimental data to a suitable equation using the least square approach which is also incorporated in Minitab. Once the above two steps are completed, the response of the studied system can be depicted as a 3-dimensional graphs or as contour plots. Visualising the response is a fast way to understand the investigated system and identify the best routes to optimise its performance[131].

2.10.2 Artificial Neural Network (ANN) Models

Artificial neural network is an information processing paradigm that was inspired by the way biological nervous systems work. Unlike RSM, this model can utilise any set of experimental data even if it was not statistically designed with CCD, BBD or any other model. However, there is also no specific approach to determine the required number of data points to train this network. In addition to identifying the amount of the required/ available data, it is important to determine the architecture of the network itself. The feed-forward structure is the simplest network structure that is commonly used in the several research papers[148-150]and known for its great stability. A feedback network structure is a more complex structure that can handle more complicated calculations (Figure 2.15). However the feed-forward ANN considered suitable to model the relationship between 3 input data and one output[131].

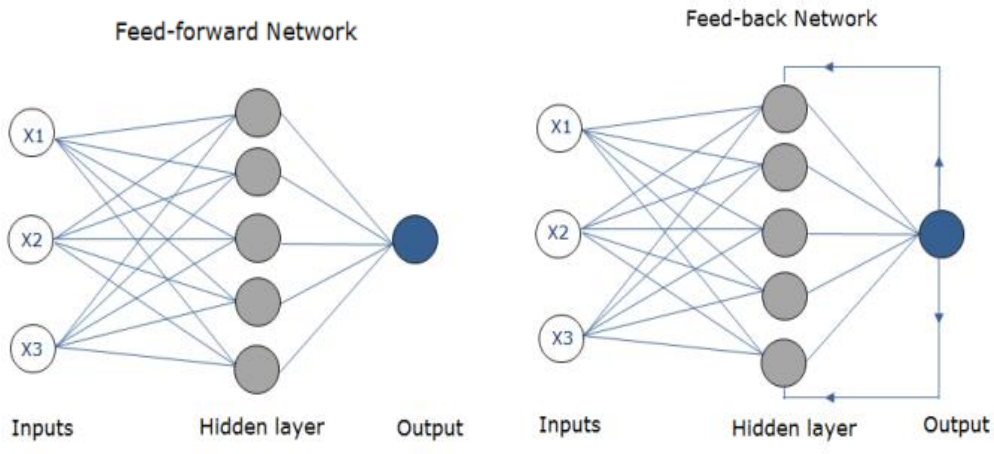


Figure 2.15 Common types of artificial neural network architecture.

The typical ANN model consists of three layers: inputs, hidden layer and outputs. The inputs layer contains the operational factors at their multiple levels (five levels for CCD, 3 levels for BBD). The hidden layer usually consists of 1-20 neurons that are closely interconnected with each other[151, 152]. A trial and error approach is usually utilised to identify the required number of neurons in the hidden layer, selecting too many neurons can significantly increase the training time in the network, while selecting too few may produce a network that is not sufficiently trained[131]. One of the best network training algorithms is the backpropagation method which is a commonly used algorithm in several research papers[118]. Levenberg & Marquardt (LM) is a more advanced training algorithm that can be used for training small-scaled cases. A comparison between BP and LM methods demonstrated that LM is much more suitable option for performance prediction. The last stage in designing an ANN is to verify and validate the produced model using a continuous error metric such as mean squared error (MSE) or the root of the mean squared error (RMSE) and ensure that their values are below the maximum acceptable error of the studied system. Validating the newly built model is an essential part of the ANN generating procedure. However, so far, there is no standard approach to validate a model and some researchers depend only on the value of the model performance function such as the previously calculated MSE and RMSE to demonstrate the quality of their model.

The ultimate objective of designing, training and efficiently validating an ANN model for a developing process is to create a model with good predictive capabilities that can be used to optimise the investigated process and report on the feasibility of scaling that process up.

2.11 ISSUES RISING FROM LITERATURE REVIEW

The current literature review has highlighted several issues associated with the performed laccase-based treatment studies so far. The main gaps and issues can be summarised below:

- The experimental procedure in several laccase-based treatment studies was relatively poor. Experiments were performed without conducting sufficient controls which then impacted on the validity and of the obtained results from those studies. Unquantified abiotic adsorption of target pollutants (e.g. E1) onto membrane filters, utilising oversaturated solutions of steroids and implementing inefficient laccase inactivation procedures, were some of the identified issues during this literature review.
- During some laccase-based treatment studies, the ranges of the investigated factors were not relevant to the actual ranges of those factors in WWTP environment. Relatively high temperatures, acidic pH values and pulses of pure oxygen were utilised during the degradation studies of bioactive chemicals and as a result the efficiency of laccase-based treatment under those conditions does not reflect the potential efficiency of that treatment in WWTPs.
- A number of laccase-based treatment studies were performed in actual wastewater matrix. However the spatial and temporal variability of wastewater was never quantified or addressed during these studies. As a result it was not feasible to understand the impact of wastewater variability on the performance of laccase-based treatment as well as the extent of that range.
- Many experiments investigated the impact of several factors on the efficiency of laccase-based treatment in removing bioactive chemicals,

individually. This experimental approach is time consuming and does not address the interactions between the various factors, which are common in complex systems.

This work is aiming to address the highlighted issues above and identify the optimum solution for each one of them to ensure that the efficiency of E1 removal by the enzyme laccase is accurately identified.

3 MATERIALS AND METHODS

This chapter describes all the utilised chemicals and reagents during this work and the details of the methodologies.

3.1 REAGENTS

The steroids, estrone (E1) (≥99%, CAS 53-16-7), 17β-estradiol (E2) (≥98%, CAS 50-28-2), and 17α-ethynylestradiol (EE2) (≥98%, CAS 57-63-6), were purchased from Sigma-Aldrich. For determining laccase activity, both ABTS ((2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) and laccase from *Trametes versicolor* (≥10 Units/mg) (CAS 80498-15-3) were purchased from Sigma-Aldrich (Poole, UK).

The laccase inhibitors, copper sulfate ($CuSO_4.5H_2O$) (CAS 7758-99-8), zinc sulfate ($ZnSO_4 \cdot 7H_2O$) (CAS 7446-20-0) and sodium chloride (NaCl) (CAS 7647-14-5) were all obtained from Sigma Aldrich, while ferric sulfate ($Fe_2(SO_4)_3$. 5H2O) (CAS 142906-29-4) was purchased from Fisher Scientific.

Methanol (MeOH), Acetonitrile (ACN) and water were HPLC grade and obtained from Fisher Scientific. Milli-Q ultrapure water (Milli-Q, 18.2 MΩ-cm resistivity, Millipore, Bedford, MA, USA) was provided in the lab and used for buffer preparation. Hydrochloric acid (CAS 7647-01-0) for laccase inactivation was purchased from Fisher Scientific. Sodium phosphate dibasic (Na_2HPO_4) (CAS 7558-79-4), sodium phosphate monobasic (NaH_2PO_4) (CAS 7558-80-7), glacial acetic acid (CH_3CO_2H) (CAS 64-19-7), ammonium acetate ($CH_3CO_2NH_4$) (CAS 631-61-8) were all purchased from Sigma-Aldrich.

For water quality analysis, Chemical Oxygen Demand (COD) cuvette test tubes LCI500 (0-150 mg/l O2) were purchased from HACH LANGE. Color-Coded buffer Solutions, pH 10, pH 7 and pH 4, for pH meter calibration were obtained from Fisher Scientific. Table 3.1 shows the different membrane filters used during this work and their suppliers.

Table 3.1 The utilised membrane filters used during this work and their suppliers.

Membrane material	**Pore size (μm)**	**Brand**	**Supplier**
Glass microfibres (GMF)	1.2	Fisherbrand	Fisher Scientific
Glass microfibres (GMF)	0.45	Whatman GD/X	Sigma-Aldrich
Regenerated cellulose (RC)	0.2	Minisart	Sigma-Aldrich
Polyethersulphone (PES)	0.2	Acrodisc	Pall corporation
Polytetra-fluoroethylene (PTFE)	0.2	Thermo Scientific Nalgene	Thermo Fisher Scientific

3.2 WASHING PROCEDURE

All glassware used in this work was cleaned with water and laboratory detergent. These were then rinsed with Milli-Q water 2–3 times and then three times with methanol to ensure no detergent remained, after which they were left to dry inside the fume hood prior to use.

3.3 HIGH PERFORMANCE CHROMATOGRAPHY WITH UV DETECTION

The concentrations of E1, E2 and EE2 at the beginning and at the end of the experiment were analysed by high performance liquid chromatography (HPLC) (Agilent 1260 HPLC system, Berkshire, UK) coupled to an Agilent multiple wavelength detector (MWD). The MWD set to ultraviolet (UV) detection for eight wavelengths: 192, 200, 206, 210, 220, 225, 254 and 280 nm, simultaneously. The lowest and the highest possible wavelengths on the detection system are 192 nm and 280 nm respectively.

The analyses were performed using SunFire C18 column (250 mm × 4.6 mm × 5 μm (Waters, Hertford-shire) at a mobile phase flow rate of 1 ml/min and column temperature of 27.5 ˚C. A binary mobile phase of water and ACN with a gradient elution was applied. The gradient was performed as follows: 60% water decreased to 40% in 12.5 min, then to 5% in 2 min, remained constant for 6 min, then increased to 60% again in 2.5 min and staying constant for 7 min. The total run was completed in 30 minutes (Figure 3.1). The limit of

quantification (LOQ) of E1, E2 and EE2 on this analytical equipment was 0.01 mg/l.

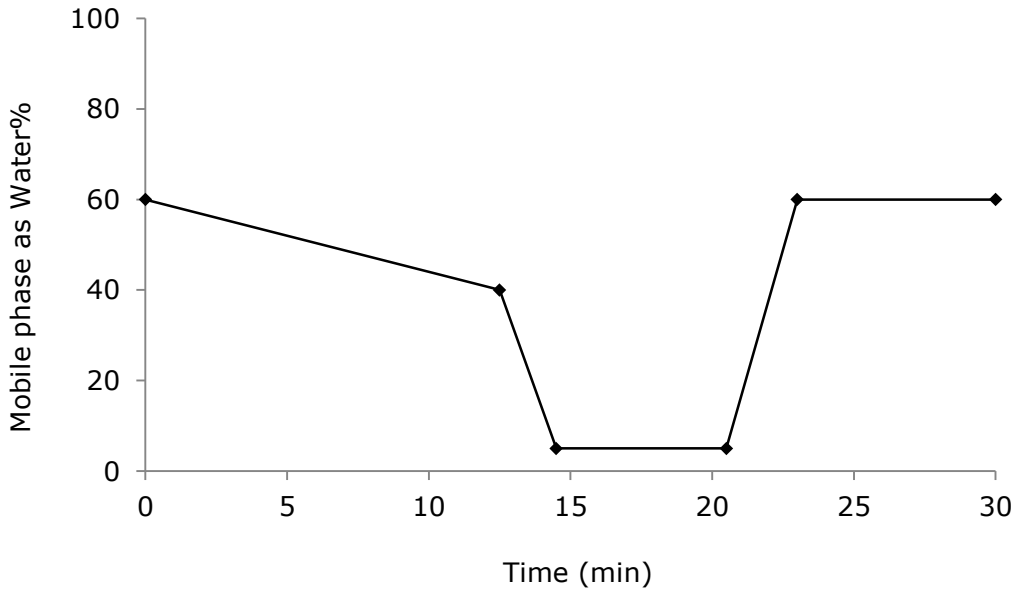


Figure 3.1 HPLC-UV chromatogram used to analyse individual solutions of Estrone (E1), 17 β-Estradiol (E2), 17α-ethynylestradiol (EE2) at 200nm, at column flow rate of 1 ml/ min and column temperature 27.5˚C.

3.4 STEROID CONCENTRATION AND SOLUBILITY

Due to the large discrepancy in the reported aqueous solubility data, highlighted in Section 2.9.1 of the literature review, the maximum used concentration of E1, E2 or EE2 in aqueous solutions in this work was 0.6 mg/l which is below the lowest reported solubility value for E1, E2 and EE2 in the literature (Table 2.4). All the performed laccase-based treatment studies in this work used 0.5 mg/l solution of E1 unless otherwise is specified. This concentration, 0.5 mg/l, is considered a suitable concentration as (i) it is low enough to ensure that the initial solution is not over saturated and (ii) high enough to track the degradation of E1 over a period of time. Using a much lower concentration of E1 means it will be harder to follow the removal of E1 by laccase as its concentration will drop below the LOQ within the first few minutes of the reactions.

3.5 SAMPLE PREPARATION BY FILTRATION FOR ANALYSIS

Prior to HPLC-UV analysis, all the samples were filtered through micro membrane filters to remove any impurities that may block the column or the instrument's lines. In order to identify the optimum membrane filter to be used with steroid solutions, the adsorption of E1, E2 and EE2 on four different types of membrane materials (Glass microfibres (GMF), Regenerated cellulose (RC), Polyethersulphone (PES) and Polytetra-fluoroethylene (PTFE)) were evaluated. For each steroid: E1, E2 or EE2, a 0.6 mg/l solution was prepared by diluting 0.06 ml of steroid's standard (500 mg/l in ACN) with deionised water using a 50 ml volumetric flask, which was then thoroughly mixed. The prepared solution was transferred into a clean beaker (Beaker A) and a 5 ml sample was withdrawn from it using a syringe filter. The withdrawn sample was then passed through one of the tested filter into another beaker (Beaker B). A 1 ml aliquot from Beaker B was transferred into a 2 ml HPLC vial to represent the "after filtration sample". The "before filtration sample" was prepared by taking a 1 ml aliquot from Beaker and transfer it into another HPLC vial. Both vials were analysed on HPLC-UV within 1 hour. Experiments were performed in triplicate for each filter. The obtained results from the HPLC-UV were used to calculate the adsorption percentage of the steroid onto the filter using the following equation:

$$R_{abiotic}\% = (C_0 - C_F / C_0) * 100 \qquad \text{Equation 3.1}$$

Where;

$R_{abiotic}$% is the abiotic removal percentage of a steroid on a specific filter (%)

C_0 is steroid's concentration before filtration in mg/l.

C_F is steroid concentration after filtration in mg/l.

Once the adsorption percentage was determined, the concentration of E1in the solution was corrected using the following equation:

$$E1_{After} = E1_{Before} \times ((100 - R_{abiotic}\%)/100) \quad \text{Equation 3.2}$$

Where;

$E1_{After:}$ E1 concentration after filtration (mg/l).

$E1_{Before}$: E1 concentration before filtration (0.5 mg/l).

3.6 STEROID STABILITY IN THE MATRIX

In deionised water with HCl, the stability of E1, E2 and EE2 in HCl acidified mixtures was evaluated over time. Standards of E1, E2 and EE2 were prepared in deionised water with initial concentration of 0.5 mg/l. A one ml aliquot was taken from each standard and mixed with 25µl of HCl. The pH value of the acidified samples was below pH=1.5. After the acid addition, the samples were then immediately analysed on HPLC-UV. The same samples were analysed again on HPLC-UV after the longest storage period (10 days). During the 10 day storage, the samples were kept on the bench in room temperature (20±1 ˚C). The difference between the initial and the final concentration was then calculated to check the impact of the acid on the concentration of the tested steroids.

In wastewater samples with laccase and HCl, filtered wastewater effluent was spiked with 0.5 mg/l of E1, the reaction started by adding a suitable amount of laccase into the reaction mixture at 20±1˚C. After 1 hr contact time a 1-ml aliquot of the reaction mixture was placed inside an HPLC-UV vial and mixed with 25 µl of HCl signifying the end of the reaction. This experiment was performed in triplicate. and the samples were analysed on HPLC-UV immediately after the inactivation and after 24 hours.

3.7 BUFFER PREPARATION

For phosphate buffers pH 7 (0.1M), Gomori buffers are the most commonly. They can be prepared by mixing the solutions of two salts: Sodium phosphate dibasic (Na_2HPO_4) and sodium phosphate monobasic (NaH_2PO_4) and by varying the amount of each salt, a range of buffers between pH 5.8 and pH 8.0 can be prepared. In this work, the above salt solutions were prepared in deionised water. A beaker with 0.1 M of Na_2HPO_4 was placed on a magnetic stirring plate and 0.1 M of NaH_2PO_4 was used to adjust its pH into pH 7. The change in the pH was continuously monitored using a calibrated pH meter. The prepared phosphate buffer was stored in the fridge at 4˚C ready for use.

Ammonium acetate (AC) buffer pH 4.5 (0.1M) was prepared using two salt solutions: 0.1 M of acetic acid solution and 0.1 M of AC solution. After preparing both solutions, a beaker with AC solution was placed onto a magnetic stirring plate and the acetic acid solution was used to adjust its pH into pH 4.5. The change in the pH was continuously monitored using a calibrated pH meter. The prepared AC buffer was stored in the fridge at 4˚C ready for use.

3.8 WASTEWATER SAMPLING AND CHARACTERISATION

Wastewater samples were collected from Stoke Bardolph WWTP, a nearby municipal WWTP operated by Severn Trent Water. The plant serves about 650,000 population equivalent (P.E.), discharging 148,000 m^3/day of secondary treated effluent into the River Trent. Stoke Bardolph WWTP has a conventional activated sludge process and no tertiary treatment, the final effluent is directed into an open channel that is then discharged into River Trent. The final effluent samples were taken from the open channel using a stainless-steel container. Sampling campaigns were carried in the mornings in the period between December 2014 and June 2015. Grab samples of the final effluent were collected in 2.5L clean amber glass winchesters and transported to the University's laboratory. The temperature of the final effluent, its pH and dissolved oxygen (DO) were measured on site using DO meter (Jenway 970,

Staffordshire, UK) and thermocouple thermometer (Digi-Sense, Cole-Parmer Instrument Ltd., UK) (Figure 3.2)

Figure 3.2 Final wastewater effluent sampling point and the used equipments to collect and characterise the sample on site e.g. dissolved oxygen meter (on the right) and thermometer.

In the laboratory, part of the sample was used for Total Suspended Solids (TSS) analysis, the remaining part was filtered through 1.2 µm glass microfiber membrane (GMF) (Fisherbrand) and passed for further analysis: chloride content and chemical oxygen demand (COD) analysis. The TSS and COD wastewater quality analysis were performed following the American Public Health Association's *Standard methods for the examination of water and wastewater*[153]:

3.8.1 Total Suspended Solids (TSS)

For total suspended solids (TSS) analysis, half a litre (0.5 L) of well-mixed final effluent sample was measured using a clean volumetric flask. The content of the flask was filtered through 1.2µm GMF filter paper using a vacuum filter kit. The filter paper retained on its surface all the suspended solids larger than 1.2µm within the sample. The filter paper was then placed into a preheated oven at 105˚C till complete dryness (3 hrs). The dried filter paper was weighed out, giving a value of W_T. The weight of the filter paper before the filtration (W_0) was recorded at the start of the experiment. The concentration of the TSS within the sample was calculated using the following equation:

$$TSS_{(mg/l)} = ((W_T - W_0)/V_{sample})$$

Where;
TSS: Total Suspended Solids (mg/l) in the final wastewater effluent.

W_0 : the weight of the filter paper before filtration (mg).

W_T: the weight of the filter paper after filtration and oven drying at 105 ˚C (mg).

V_{sample}: The volume of the filtered sample (L).

3.8.2 Chloride Concentration

The concentration of chloride ions in wastewater effluent were determined by titrating it against a standard silver nitrate ($AgNO_3$) solution. A standard silver nitrate solution of 0.0141M was prepared by dissolving 1.1976 g of $AgNO_3$ in 500 ml of deionised water. To test the accuracy of the titration process and identify the colour of its end point, a standard sodium chloride (NaCl) solution of 200 mg/l was titrated against the prepared $AgNO_3$. A burette (25 ml) was filled with $AgNO_3$ and a conical flask with 50 ml of NaCl solution and 10 drops of potassium chromate indicator was placed under the burette. The flask was placed on a magnetic stirring plate to ensure the homogeneity of the solution during the titration process. The titration process started by adding $AgNO_3$ solution into the conical flask and the end point of the titration was

reached when the mixture changed its colour from yellow into orange. The experiment was performed in triplicate and the used volume of $AgNO_3$ was recorded after each titration. The average used volume of $AgNO_3$ was 12.3 ±0.1 ml. The below equation was used to test the accuracy of this titration:

$$C_A \times V_A = C_B \times V_B \qquad \text{[Equation 3.3]}$$

Where; C_A and C_B are the concentrations of silver nitrate and sodium chloride (mol/l), respectively. V_A and V_B are the used volumes of silver nitrate and sodium chloride (ml), respectively.

$0.0141\ M \times 12.3\ ml = C_B \times 50\ ml \rightarrow C_B = 3.4686 \times 10^{-3}\ M$

1 mole of NaCl is 58.44 g →

$C_B = 3.4686 \times 10^{-3} \times 58.44 = 0.2027$ g/l = 203 mg/l which is very close to the actual value of NaCl solution.

The concentration of chloride ions only can be calculated using the molecular weight of chloride ions in NaCl solution, which is 35.45 g/mol.

Chloride concentration$= 3.4686 \times 10^{-3} \times 35.45 = 0.123$ g/l = 123 mg/l

After checking the accuracy of the titration process using a standard NaCl solution, similar process was used to determine the concentration of chloride ions within the filtered wastewater effluent samples. Figure 3.3 shows the colours of the initial solution, end point solution and beyond the end point solution.

In the wastewater matrix, a titration of the final effluent of nearby conventional WWTP against sliver nitrate was performed. Ten wastewater effluent samples were collected over a period of 40 days. Each sample was filtered through 1.2 µM GMF paper and titrated within 2 hrs after collection.

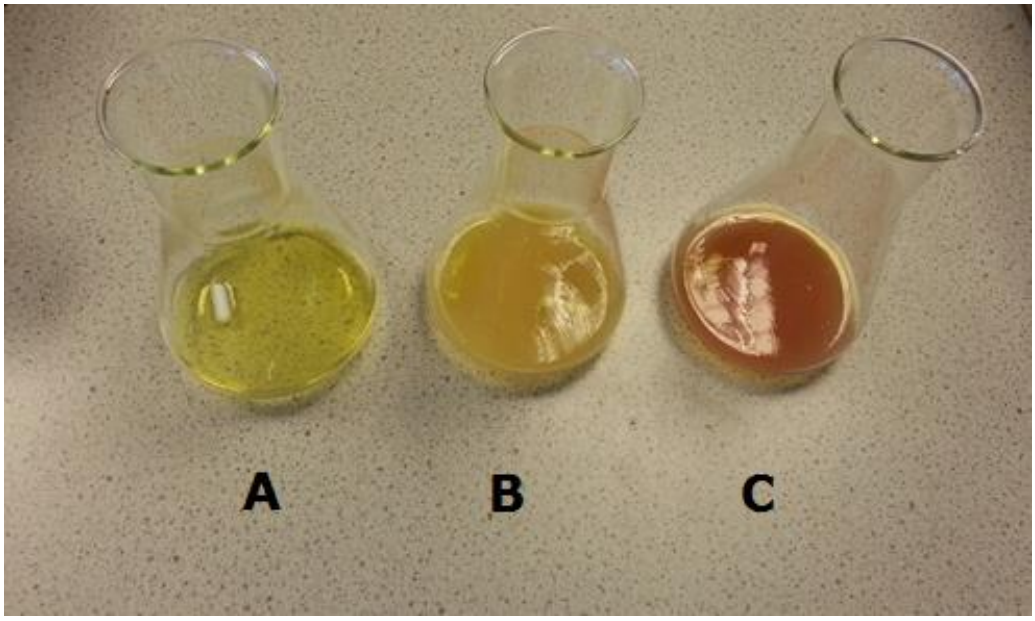


Figure 3.3 The change in colour during the titration of filtered wastewater effluent against silver nitrate solution to determine the concentration in chloride ions. A: the initial colour of the filtered wastewater solution mixed with 10 drops of potassium chromate indicator; B: the end point colour after adding silver nitrate into flask "A"; C: the colour of the solution when an excessive amount of silver nitrate is added.

3.8.3 Chemical Oxygen Demand (COD)

Chemical oxygen demand (COD) is the water quality parameter used to identify the oxidising capability and biodegradability of organic pollutants in wastewater. It represents a parameter that is frequently mentioned in various wastewater legislations and acts as a design basis and performance indicator in many WWTPs around the world. Utilising pre-dosed COD test tubes, makes this test simple, accurate and environmentally friendly. The COD test was performed by adding 2 ml of the tested matrix e.g. filtered wastewater effluent into the purchased COD test tube (0 -150 mg/l) which was then placed inside a LT200 thermostat (Hach Lange, UK) for 2 hours at 148˚C. By the end of the contact time, the sample was allowed to cool down to room temperature. The COD of the cool sample was measured using DR 2800 spectrophotometer (Hach Lange, UK). A control sample with deionised water was performed

simultaneously with the wastewater sample and used to represent COD=0 mg/l and calibrate the used spectrophotometer.

3.9 LACCASE PREPARATION

To ensure the commercially obtained laccase for this work was homogeneous and particulate free, the standard laccase solution of 1mg/ml was centrifuged for 5 min at 6000 rpm. The activity of laccase in the uncentrifuged and centrifuged laccase solutions were measured following the same procedure as in Section 3.10.

To retain activity and decrease error through poor experimental design, the supernatant was transferred by pasteur pipette into a clean beaker stored on ice and then aliquoted into 1ml plastic tubes and frozen for future use, with each tube being defrosted only once and the remaining laccase in the tube disposed if unused. With laccase stable at or near neutral pH values [122], all laccase stock solutions were prepared in deionized water and stored in these 1ml plastic tubes at-20±0.5 ˚C. Each time a new batch of centrifuged laccase was prepared, one of the 1 ml plastic tubes was used to determine the activity of the centrifuged laccase. A control experiment was conducted on one of the defrosted tubes to ensure that laccase storage procedure was appropriate and did not affect laccase activity. The details of measuring laccase activity are provided in Section (3.10). Tubes with laccase stock solution of 1mg/ml were used for all of the performed experiments in clean water matrices such as buffers and deionised water, unless otherwise was specified.

The main disadvantage of using laccase solution is the associated matrix dilution with it, which may affect the concentration of the target pollutant in the matrix. However, since this approach is utilised only with relatively low laccase concentrations (<1 U/ml), the dilution percentage will be always less than 10%. For example, if a 50 ml of 0.5 mg/l E1 solution was spiked with 0.25 ml of laccase solution, then the actual concentration of E1 in the reaction mixture can be calculated using the below equation:

$E1_D = E1_{T=0} \times ((V_0/(V_0+V_{lac}))$ [Equation 3.4]

Where;

$E1_{D:}$ E1 concentration after matrix dilution (laccase addition) (mg/l).
$E1_{T=0}$: E1 concentration before matrix dilution (0.5 mg/l).
V_0: The volume of E1 solution before laccase addition (50 ml)
V_{lac}: The volume of the added laccase solution (0.25 ml)

Based on the values in this example, the concentration of E1 after dilution will drop from 0.5 mg/l to 0.498 mg/l which is less than 0.4% difference. This correction was applied to all of the performed experiments with laccase solutions.

For experiments that required higher concentrations (>1 U/ml) of laccase e.g. experiments in wastewater matrix, using laccase solution of 1 mg/ml (≈10 U/ml) to achieve the required laccase concentration can lead to significant (>10%) matrix dilution. For example, to achieve laccase concentration of 5 U/ml in 50 ml of E1 solution in wastewater sample, 25 ml of laccase solution has to be added into the 50 ml of wastewater. According to Equation 3.4, the concentration of E1 in this new solution is going to be 0.33 mg/l instead of the original 0.5 mg/l which equates to 34% dilution of the original matrix. This high dilution percentage with deionised water (as laccase solution was prepared in deionised water) can make this matrix not an ideal representative of wastewater effluent matrix.

Therefore and to avoid this undesirable outcome, all wastewater experiments in this work were performed using powder laccase. The activity of the powder laccase was determined by preparing a low concentration (0.025 mg/ml) of laccase solution in deionised water. The solution was not centrifuged due to its low concertation and the activity was measured according to the explained procedure in Section 3.10. The activity of the powder laccase was measured for every new batch of laccase. Based on the measured laccase activity, the required amount of laccase powder for each experiment was determined on a 4-decimal point balance.

3.10 DETERMINATION OF LACCASE ACTIVITY

The activity of *Trametes versicolor* laccase was measured by the oxidation of ABTS. The process was performed according to the standard Fukuda method [154]. The reaction mixture consisted of 5.0 mM ABTS prepared in 0.1M oxygen-saturated AC buffer (see Section 3.7 for details), pH 4.5, and a suitable amount of laccase solution in a total volume of 1.0 ml was incubated at 37˚C. One unit of enzyme activity (U) was defined as the amount of laccase that catalyses the oxidation of 1 μmol of ABTS per min at 37 ˚C. Oxidation of ABTS was followed by increase in absorbance at 420nm with an extinction coefficient (e) of 3.6×10^4 $M^{-1}.cm^{-1}$. The specific activity (SA) of laccase, the number of laccase activity units per gram, was calculated using Beer – Lambert law.

ABTS concentration (M/min) = $\frac{A/min}{\varepsilon} = \frac{S\times60}{36000}$

The specific activity of the laccase (U/mg) = $\frac{ABTS_{Oxi}}{E}$

Where;

The molar absorptivity (Ɛ) of ABTS is 36000 L/(cm.mol); S is the slope of the line in the "Absorbance vs Time" graph.

$ABTS_{Oxi}$ is the amount of ABTS oxidised in the cuvette per minute (μmol/ min);

E is the amount of laccase in the cuvette (mg).

To obtain robust results, the activity assay experiments were performed in triplicate. Each sample was placed in 1 ml cuvettes and analysed in Ultraviolet-Visible (UV-Vis) Spectrophotometer (Agilent 8453, Waldbronn, Germany) for a contact time of 5 mins. An attached water bath (SubAqua 12, Grant) provided a constant temperature during the experiments. The collected results from the UV-vis spectrophotometer was utilised to prepare an Absorbance vs. Time graph to calculate the actual activity of laccase.

The impact of matrix pH on laccase activity was evaluated by UV-vis spectrophotometer at 2 pHs: pH 4.5 (AC buffer) and pH 7 (phosphate buffer). Laccase activity in both buffers was determined using the same approach

described in section 3.10, but under different conditions: The assay consisted of 850 µl of buffer (either at pH 4.5 or pH 7), 50 µl of 0.2 mg/ml laccase solution and 100 µl of 5mM ABTS solution, experiments were conducted in triplicate at 20˚C. The initial rate of laccase reaction was calculated using Beer - Lambert's law, during the first minute of the reaction (Equation 3.5):

$$\nu = \Delta C_{min} = (\Delta A_{min})/(L * \text{Ɛ}) \qquad \text{[Equation 3.5]}$$

Where;

ν: initial reaction rate (Mol/min); ΔC_{min}: Change in the ABTS concentration during the first 2 mins of the reaction; ΔA_{min}: Change in absorbance during the first two minutes of the reaction; Ɛ: the extinction coefficient of ABTS at 420 nm (Ɛ =36000 $M^{-1}.cm^{-1}$); L: the path length of the used cuvette.

Once the reaction rate was determined, it was divided by the actual amount of laccase in the reaction mixture to determine the specific activity (SA) of laccase in each buffer. The SA at pH 4.5 was then compared with the SA at pH 7. The experiments were performed in triplicate.

To evaluate the performance of HCl in inactivating an oxidative enzyme, *Trametes versicolor* laccase, the ability of laccase to oxidise ABTS was determined under the following conditions: laccase concentration=0.5 U/ml, contact time= 1 hr, temperature= 20±1˚C, reaction matrix= phosphate buffer at pH 7. During the contact time the absorbance of ABTS was automatically measured at 420 nm every 20 seconds. A trial and error approach was utilised to determine the suitable amount of the HCl acid to achieve a complete inactivation of laccase, which was found to be 25µl of HCl per 1 ml of sample, regardless of the used matrix. Laccase activity was measured under the specified conditions described above.

3.11 EVALUATING IMPACT OF INHIBITORS ON LACCASE ACTIVITY

The impact of four laccase inhibitors: chloride, copper, iron and zinc on laccase was investigated from two perspectives, 1) the impact of inhibitors on laccase activity alone using the standard substrate, ABTS measured by UV-vis spectrophotometer 2) the impact of inhibitors on laccase ability to degrade an environmentally relevant substrate, E1 measured by HPLC-UV.

For evaluating laccase activity by the UV-vis spectrophotometer, each experiment was performed in 1 ml cuvette that contained: suitable amount of AC buffer (pH 4.5), 20 µl of 0.1 mg/ml laccase solution, 100 µl of 5 mM of ABTS and suitable amount of inhibitor's solution in AC buffer. All experiments were performed in triplicate at 20˚C. The absorbance was measured at 420 nm and the obtained "Absorbance" versus "Time" graphs from the UV-Vis spectrophotometer were utilised to calculate the initial rate of the reaction (ν) for each experiment using Beer-Lambert's law (Equation 3.4.).

The percentage of the caused inhibition by a specific ion was determined by calculating the difference between the reaction rate of the control ($\nu_{control}$) (in the absence of the inhibitor) and the reaction rate in the presence of certain inhibitor concentration (ν_I). The standard deviation between the triplicates was also determined.

For all of the inhibition studies (whether with ABTS or E1), controls in the absence of inhibitor were undertaken to represent 100% of laccase activity. Control experiments in the absence of laccase were also performed to demonstrate that the oxidation of ABTS was fully attributed to laccase activity.

3.11.1 Selected Concentrations

The investigated concentrations of chloride, copper, iron and zinc, and the used analytical equipment are shown in Table 3.2

Table 3.2 The tested concentrations of the selected inhibitors.

Inhibitor	Tested concentrations (mg/l)	
	ABTS as a substrate	**Estrone as a substrate**
Chloride (Cl^-)	100, 200, 500,1000	100, 200, 500,1000
Copper ($\mathbf{Cu^{2+}}$)	0.05, 0.1, 10, 50, 500	0.05, 0.1, 10, 50
Iron ($\mathbf{Fe^{3+}}$)	0.1, 0.3, 10, 50, 100	-----
Zinc ($\mathbf{Zn^{2+}}$)	0.05, 0.15, 10, 50, 100, 200	0.05, 0.15, 10, 50,

The obtained "Absorbance versus Time" graphs were used to calculate the initial rate of the reaction (ν) for each experiment. The percentage of the caused inhibition by copper was determined by calculating the difference between the reaction rate of the control ($\nu_{control}$) (in the absence of copper) and the reaction rate in the presence of certain copper concentration (ν_{Cu}). The standard deviation between the triplicates was less than 1 for all of the performed experiments

To study the impact of inhibitors on laccase activity in the presence of environmentally relevant substrate such as E1, the use of HPLC-UV experiments were designed in AC buffer (pH 4.5). The impact of each inhibitor was tested at different concentrations (Table 3.2) in the presence of 0.5 mg/l of E1 and 0.5 U/ml of laccase solution, at 20˚C in AC buffer. The concentration of E1 was quantified by HPLC-UV before laccase addition and after 1 hour contact time. The percentage of the caused inhibition by a specific ion was determined by calculating the difference between E1 removal efficiency in the control experiment ($R\%_{control}$) (in the absence of the inhibitor) and E1 removal efficiency in the presence of certain inhibitor concentration ($R\%_I$). All the HPLC-UV experiments were performed in duplicate.

3.12 EVALUATING IMPACT OF THE WASTEWATER MATRIX ON LACCASE ACTIVITY

To study the impact of wastewater matrix on laccase activity, the degradation of E1 by laccase was studied in two matrices: (i) phosphate buffer (pH 7) and (ii) filtered wastewater effluent (pH 7). The pH of the final effluent was adjusted-when needed- to pH 7 using acetic acid. Experiments were performed in both matrices under the following conditions: contact time=1 hour,

temperature= 20˚C, initial E1 concentration = 0.5 mg/l, laccase concentrations= 0.5 U/ml, 2 U/ml and 3 U/ml. The experimental procedure was identical for both matrices. A specific volume of each matrix was placed in a flask and spiked with suitable amount of E1 standard solution (prepared in ACN) to give an initial E1 concentration of 0.5 mg/l. A 5 ml sample was taken from the flask, filtered through 0.2 µm of RC filter, mixed with 25 µl of hydrochloric acid (HCl) per 1 ml of sample and placed into HPLC vial ready for analysis. The reaction was started by the addition of the powder laccase into the flask and placing it onto a magnetic stirring plate inside the incubator for 1 hour at 20 ˚C. By the end of the contact time, a 5 ml sample was withdrawn, filtered through 0.2 µm of RC filter and mixed with 25 µl of hydrochloric acid (HCl) per 1 ml of sample ready for analysis. All experiments were performed in duplicate.

In order to quantify the impact of wastewater effluent on laccase activity, a benchmark experiment was designed. This experiment was performed in filtered wastewater effluent – unless otherwise was specified- under a set of standard conditions: 5 U/ml laccase conc., temperature=20˚C, contact time=1 hr and 0.5 mg/l of E1. The benchmark was performed after each sampling trip and the removal efficiency of E1 was calculated at the end of the contact time. The designed benchmark uses E1 as environmentally relevant substrate as it is the target pollutant in this work and a good representative of other steroid estrogens found in wastewater effluents. Benchmarks with other substrates can be similarly performed for studies that focus on different groups of bioactive chemicals such as antibiotics.

The collected wastewater effluent was filtered through 1.2µm GMF and spiked with suitable amount of E1 standard solution (prepared in ACN) to give an initial E1 concentration of 0.5 mg/l. A 5 ml sample was taken from (E1+filtered wastewater) solution, filtered through 0.2 µm of RC filter, mixed with 25 µl of hydrochloric acid (HCl) per 1 ml of sample and placed into HPLC vial ready for analysis. The reaction was started by the addition of the powder laccase into (E1+filtered wastewater) solution, then the flask with the reaction mixture was placed onto a magnetic stirring plate inside the incubator

for 1 hour at 20 ˚C. By the end of the contact time, a 5 ml sample was withdrawn, filtered through 0.2 µm of RC filter and mixed with 25 µl of hydrochloric acid (HCl) per 1 ml of sample ready for analysis. All benchmarks were performed in duplicate.

3.13 FACTORIAL EXPERIMENTAL DESIGN

Two factorial experimental designs, Central Composite Design (CCD) and Box-Behnken Design (BBD) were chosen to study the influence of three independent factors: temperature (X1), contact time (X2) and laccase concentration (X3), on the removal efficiency of E1 in deionised water and wastewater effluent. The CCD and BBD matrices of factors were generated in Minitab (V.16.2.2). The range for each factor was 6 – 26 ˚C, 0.5 – 8 hours and laccase concentrations in the range of 0.01 U/ml - 0.1 U/ml for clean water matrix and 0.5 U/ml – 6 U/ml for wastewater effluent matrix. Once the number of the main factors and their ranges were defined, Minitab was used to generate either a BBD or a CCD matrix of conditions which specified the number of the required experiments and their conditions.

3.14 ESTRONE DEGRADATION STUDIES

The initial concentration of E1 in both clean and wastewater effluent matrices was 0.5 mg/l for both BBD and CCD experiments. Experiments were performed in an open reactor to allow the natural diffusion of oxygen from the atmosphere into the solution. Continuous mixing was achieved through magnetic stirring. Experiments were performed in 500 ml Erlenmeyer flask containing 200 ml of reaction mixture, the required temperature was achieved and maintained using an incubator (LMS cooled incubator, Kent, UK). The experimental procedure for each sample was as follows: a 5 ml sample was withdrawn from each flask containing the studied matrix before laccase addition, filtered through 0.2 µm Regenerated Cellulose (RC) filters, transferred into a vial and mixed with 25 µl of hydrochloric acid (HCl) to ensure that the C_0 and C_t samples were subjected to the same conditions and could be compared with each other directly. This sample was labelled as (C_0).

Laccase was then spiked into the flask either as solution or powder, signifying the starting point of the reaction. After a specific contact time (t), a 5 ml sample was withdrawn, filtered through an RC filter, transferred into a vial and mixed with 25 µl of hydrochloric acid (HCl) to inactivate laccase and stop the enzymatic reaction, the final sample was labelled as (Ct). The followed experimental procedure was summarised in Figure (3.4).

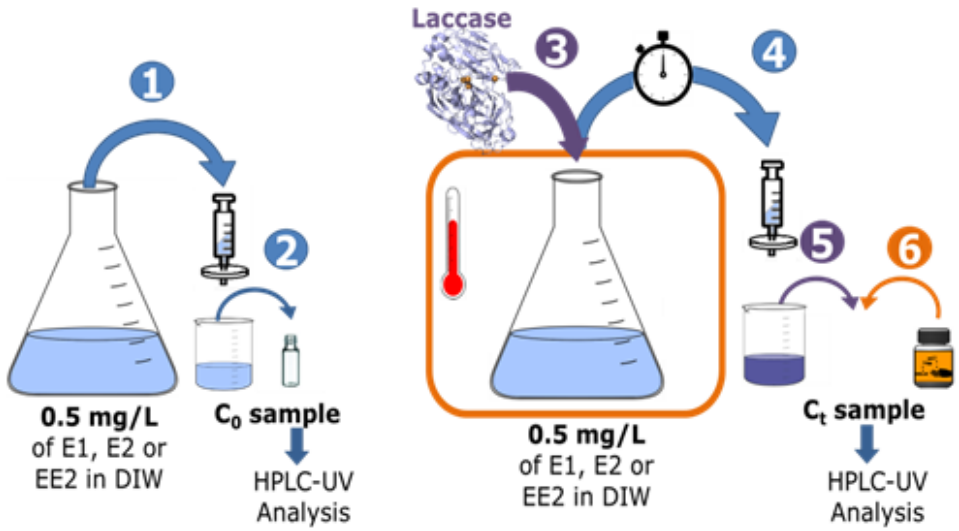


Figure 3.4 Summary of the followed experimental procedure during laccase-based treatment of estrone in both clean and wastewater matrices.

Control studies were performed to identify the required amount of HCl to achieve instant and permanent inactivation of laccase without affecting E1 concentration. In addition, the adsorption of E1 onto various membrane filters was assessed, with RC filters showing the lowest E1 adsorption. Samples were then analysed by HPLC-UV and the actual removal efficiency (R_{Act}%) was calculated using the following equation:

$$R_{Exp}\% = \frac{Co-Ct}{Co}.100 \qquad \text{Equation 3.6}$$

Where,

R_{Exp}%: the experimental removal efficiency of E1 as a percentage, C_0 and Ct are E1 concentrations at time=0 and time=t, respectively.

3.15 MODELLING THE LACCASE-BASED TREATMENT SYSTEM

3.15.1 Design of the RSM and ANN models

The obtained experimental data from BBD and CCD was utilised to build two mathematical models - response surface methodology (RSM) and artificial neural metwork (ANN) models.

The RSM model was generated in Minitab (V.16.2.2) to connect the system response with the CCD and BBD experimental conditions. The same software was used to perform the analysis of variance (ANOVA) and determine the statistical significance of each factor. Experimental removal efficiency was related to the experimental conditions through second order polynomial equation (Equation 3.7):

$$R_{pred}\ (\%) = \beta_0 + \sum_{i=1}^{n} \beta_i \,.\, X_i + \sum_{i=1}^{n} \beta_{ii} \,.\, {X_i}^2 + \sum_{i<j} \sum \beta_{ij} \,.\, X_i . X_j + \varepsilon$$

[Equation 3.7]

Where;

R_{pred} is the predicted removal efficiency of E1; Xi , Xj are the independent factors' levels; β0 is the intercept term; βi represents the coefficients for linear terms; βij represents the interaction coefficients; and βii stands for the quadratic coefficients; Ɛ is the error.

For the ANN model, a typical feed-forward ANN with three layers of neurons was built in MATLAB R2011a. The inputs layer contained the independent factors at their multiple levels, followed by the hidden layer. A trial and error approach was applied to identify the number of neurons in the hidden layer to generate an ANN model with the best fit to the experimental data. The output layer represented the predicted removal efficiency of E1, as a percentage, under specific conditions. The hidden layer in the final ANN model consisted of 6 neurons with 70% of the experimental results were utilised in ANN training, 15% for ANN validation and 15% for ANN testing. The used iterative scripts to generate ANN models for this work have been included in Appendix

B at the end of this thesis. The network performance function for all ANN models was MSE<2.

3.15.2 Evaluating the performance of RSM and ANN models

The performance of the built RSM and ANN models was assessed using two different approaches, statistical indices and with unseen data (not used in generating the model).

Statistical indices such as the coefficient of determination (R^2) (a non-adjusted R^2 value was used throughout this work, see Equation 3.8), the mean squared error (MSE), the root mean squared error (RMSE) and the absolute average deviation (AAD), were used to evaluate the goodness of fit of the build RSM and ANN models. This approach has been implemented by several research groups to assess the quality of nonlinear models [92, 152, 155, 156]. The best model was identified by the lowest AAD, MSE and RMSE values and the highest R2 value.

$$R^2 = 1 - \frac{\sum y_i - y_{EXPi}}{\sum y_i - y_m} \quad \text{[Equation 3.8]}$$

$$MSE = \frac{1}{n}\sum_{i=1}^{n}(y_i - y_{EXPi})^2 \quad \text{[Equation 3.9]}$$

$$RMSE = (MSE)^{\wedge 0.5} \quad \text{[Equation 3.10]}$$

$$AAD = \{[\sum_{i=1}^{n}(|y_i - y_{EXPi}|/y_{EXPi})]/n\} \times 100 \quad \text{[Equation 3.11]}$$

Where y_i and y_{EXPi} are the predicted and the experimental removal rates of E1, respectively; y_m the mean of the response values; n is the number of the conducted experiments.

Unseen data was also utilised to assess the predictive capabilities of ANN and RSM models. Unseen date being a set of data that was not used to generate the corresponding model [157]. A model with good predictive capability can

determine the system's response to any given set of conditions within that system. The conditions of the unseen data were either statistically designed using a different experimental design to the original model or was selected at random points within the studied system. Once the conditions of the unseen data were determined, these new experiments were performed in the lab and the actual E1 removal efficiency under those conditions was experimentally determined. The difference between the predicted and the experimental values was calculated and the obtained values were inversely proportional to model's accuracy.

4 : RESULTS AND DISCUSSION A COMPREHENSIVE ASSESSMENT OF THE REQUIRED CONTROLS AND PRELIMINARY EXPERIMENTS WHEN REMOVING ESTRONE USING LACCASE

4.1 INTRODUCTION

Bench-scale studies are very important stage in any experimental research. The obtained data from these studies can be utilised to demonstrate the ability of laccase to remove the target pollutant in different water matrices and assess the feasibility of moving this new technology up to the next Technology Readiness Level (TRL). However such studies require a robust experimental setup to ensure that the obtained results represent the actual efficiency of laccase-based treatment and the impact of the evaluated conditions without being affected by other factors. To accurately assess the ability of the enzyme laccase to degrade estrone in water matrices, several control and preliminary experiments have been undertaken, these experiments ensure that the final E1 removal efficiency is solely attributed to laccase activity and the evaluated conditions and not to poor experimental design and various unassessed abiotic processes.

Utilising over-saturated solutions of bioactive chemicals (see section 2.9.1), filtering the solutions through unsuitable membrane filters (see 2.9.2) and using an inefficient laccase-inactivation procedure (see section 2.9.3), are all common issues that can abiotically contribute to the removal of bioactive chemicals and have been detected in a number of research papers. All the above issues – if left unassessed- may deliver conflicting and inaccurate results and lead to an overestimation of the efficiency of laccase-based treatment. The potential implications of dismissing the above issue were discussed in section 2.11 from the literature review.

This chapter investigates three main points to ensure that the performed bench-scale experiments in the following chapters have addressed the 3 issues that have been omitted by several previous studies:

(1) The adsorption of E1 onto 4 membrane filters to identify its abiotic removal on each filter and account for it during the bench-scale studies.

(2) Developing a fast and an effective inactivation procedure to stop the enzymatic reaction exactly at the end of the tested contact time.

(3) Assess the stability of E1 in the reaction mixture, before and after the inactivation to ensure that this procedure does not abiotically degrade E1.

4.2 HIGHLIGHTS

- Centrifuging laccase solutions can increase the robustness of laccase bench-scale studies.
- The affinity of E1 toward four membrane filters was assessed by measuring its concentration on HPLC-UV before and after filtration.
- The RC filters have the lowest affinity toward E1 of 3.2 ± 1.7 %.
- The adsorption of other natural and synthetic steroids onto RC filters was assessed.
- The filter-to-filter reproducibility was determined for all of the tested membrane filters.
- An efficient acidification procedure was developed to instantly stop the enzymatic reaction.
- The stability of E1 in the acidified mixture was evaluated over time to ensure that the acid did not abiotically degrade E1 in the sample.

4.3 LACCASE ACTIVITY IN CENTRIFUGED AND UNCENTRIFUGED SOLUTIONS

The centrifugation of laccase solution (see section 3.9) before measuring its activity is one of the possible approaches to homogenise the solution and increase the robustness of the experiment procedure in general. Using inhomogeneous laccase solution during a set of experiments can produce

incorrect experimental data as the activity of the first aliquot from the vial with laccase solution may be quite different from the activity of the last aliquot in that vial. The impact of centrifugation on laccase activity was studied by measuring laccase activity in 1mg/ml laccase solution before centrifugation and after it. The used laccase in this work is a commercial unpurified enzyme and as a result its powder may contain several impurities that are insoluble in water and may precipitate out of laccase solution. Preliminary studies with this commercial laccase showed that laccase stock solutions higher than 1mg/ml (1 mg laccase powder/ 1 ml deionised water) exhibit solubility issues by forming a layer of precipitates at the bottom of laccase solution vial if left undisturbed. Lower concentrations of laccase solution may also contain various insoluble materials. However, it may be harder to spot with the naked eye. To ensure that the used laccase solution in this work was homogeneous and particulate free, the standard laccase solution of 1mg/ml was centrifuged. After the centrifugation a visible amount of precipitates was accumulated at the bottom of the tube (Figure 4.2). The activity of laccase in uncentrifuged and centrifuged laccase solutions were measured following the same procedure as in section 3.10.

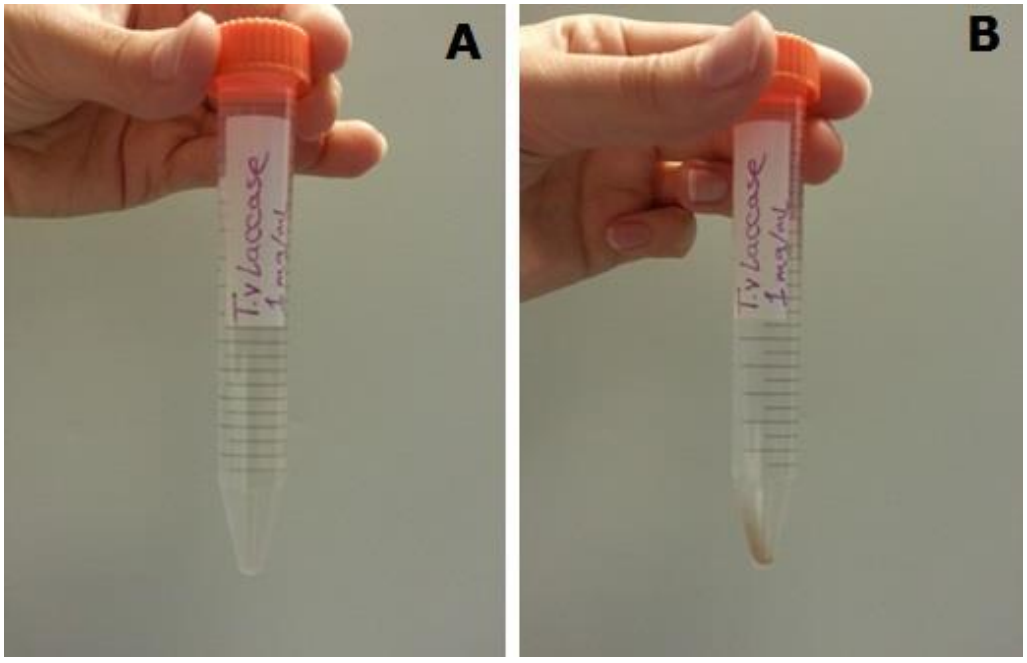


Figure 4.1 Comparison between un-centrifuged (A) and centrifuged (B) 1 mg/ml laccase solution.

One of the 1 ml plastic tubes was used to determine the activity of the centrifuged laccase every time a new batch of centrifuged laccase was prepared. The main disadvantage of using laccase solution is the associated matrix dilution with it, which may affect the concentration of the target pollutant in the matrix. However, since this approach is utilised only with relatively low laccase concentrations (<1 U/ml), the dilution percentage was always less than 10%.

On the other hand, experiments in wastewater effluent were performed with relatively high laccase concentrations (>1 U/ml) and to avoid significant (>10%) matrix dilution which may make the wastewater matrix not an ideal representative of actual wastewater effluent, all wastewater experiments in this work were performed using powder laccase. The activity of the powder laccase was measured for every new batch of laccase (as described in Section 3.10) The main disadvantages of utilising a powder laccase:

- The variability between the samples as the same weight of laccase does not always equal to the same activity (some particulates of the unpurified laccase are inert).
- Cannot be applied to experiments with low laccase concentrations as a very small amount of powder will have to be accurately weighed out (unless a big reaction volume is utilised).

However, unlike laccase solution approach, this method eliminates the issue with matrix dilution after laccase addition.

The graph in Figure 4.2 was used to calculate the specific activity of laccase (SA) following the described procedure in section 3.10. The SA of the uncentrifuged laccase was 12 U/mg, while SA of the centrifuged laccase was only 10.7 U/mg. This shows that a small part of laccase activity can be lost during the centrifugation process and therefore it is necessary to measure laccase activity for every new centrifuged laccase solution.

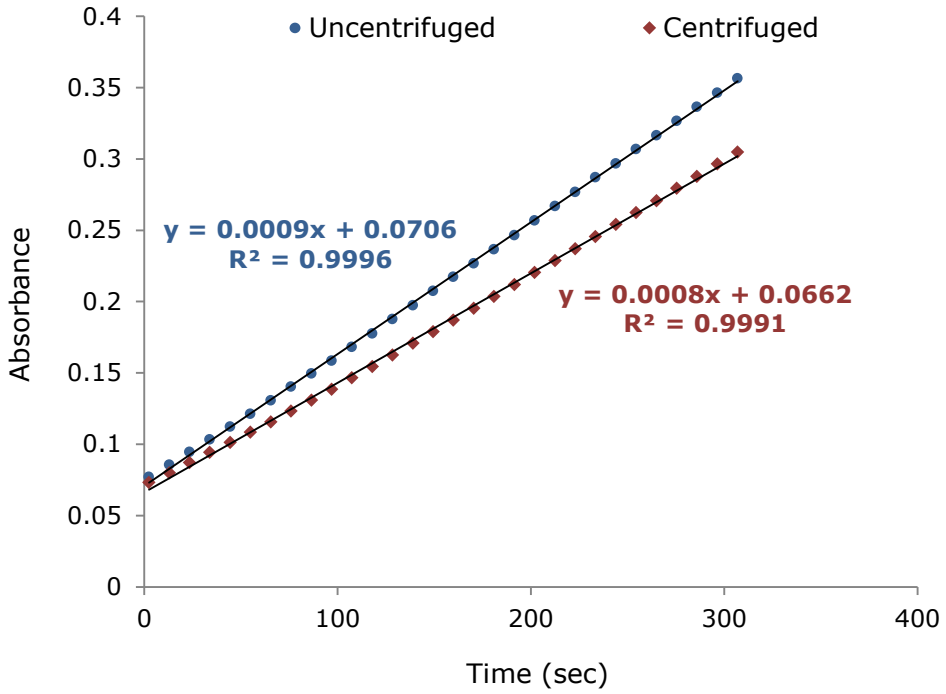


Figure 4.2 The oxidation of ABTS by laccase using 1 mg/ml of either un-centrifuged laccase solution or centrifuged laccase solution under the following conditions: contact time= 5 mins, temperature= 37±0.5˚C, reaction matrix= ammonium acetate buffer at pH 4.5. The coefficient of variance (CV%) between the triplicates was 1.0% and 0.85% for centrifuged and uncentrifuged samples, respectively.

The main advantage of centrifuging laccase solutions during bench-scale studies is the homogeneity of the produced laccase solution and the even distribution of laccase activity units in it. Subsequently this will ensure the accuracy and robustness of the performed experiments. Figure 4.2 shows the "Absorbance vs Time" graphs of ABTS oxidation using uncentrifuged and centrifuged laccase solution.

4.4 SAMPLE FILTRATION AS AN ADDITIONAL ROUTE FOR STEROIDS REMOVAL BY ADSORPTION

Filtering samples before analysis is essential to maintain the analytical equipment in a good state, especially when working with environmental matrices[158]. However a percentage of the target steroid may be removed by adsorption during the filtration process. For HPLC analysis it is advised to

filter the samples using filters in the range of 0.2-2µm nominal porosity[158]. One study identified that certain membrane filters could adsorb up to 100% of E1 (Initial E1 concentration of 0.4 mg/l) while other filters have an extremely low affinity and may adsorb as little as 2.3% of the same compound. These findings highlight the importance of selecting the suitable membrane filter for each planned experiments [136]. The abiotic adsorption of three steroid estrogens; E1, E2 and EE2 onto four different types of membrane filters; glass microfibers (GMF), regenerated cellulose (RC), polyethersulphone (PES) and polytetrafluoroethylene (PTFE) was investigated. The four filters were selected to demonstrate steroid affinity toward different membrane filters, some of these filters have been frequently used in several studies which are shown in Table 2.12. The characteristics of the utilised filters are listed in Table 4.1

Table 4.1Characteristics of the used membrane filters.

Membrane material	**Pore size (µm)**	**D (mm)**	**Brand**	**Wettability**
Glass microfibres (GMF)	0.45	25	Whatman GD/X	Hydrophilic
Regenerated cellulose (RC)	0.2	15	Minisart	Hydrophilic
Polyethersulphone (PES)	0.2	15	Acrodisc	Hydrophobic
Polytetra-fluoroethylene (PTFE)	0.2	15	Thermo Scientific Nalgene	Hydrophobic

In this work E1 (intial E1 concentration of 0.6 mg/l) was selected as a representative steroid estrogen to study its adsorption onto GMF, RC, PES and PTFE membrane filters.

For each experiment a total volume of 5 ml was passed through a new filter. The selected volume was sufficient for further analysis as only 1 ml of the filtrate was analysed on the HPLC-UV, the 5 ml volume was also below the breakthrough volume (>10 ml) and above the hold-up volume (10-30 µl) of filters with 15 mm diameter[158].

The adsorption of E1 from clean water matrix onto four membrane filters at 20 ± 0.5 ˚C and at pH 7 was performed to identify the optimum filter for this work that has a minimal affinity to E1. The optimum filter should have a minimal affinity toward the filtered compound. The obtained results show that the

adsorption of E1 onto PES filters is extremely high (98%) which makes it unsuitable option for this type of studies (Table 4.2). PTFE filters demonstrated a better performance with adsorbing only 33.11% of E1. However this value is still relatively high and using PTFE filters may prove to be problematic when working with solutions with low E1 concentration as quantifying E1 in the filtered sample can be an analytically challenging when the concentration of E1 in the filtrate drops below the limit of quantification (LOQ) due to abiotic adsorption onto PTFE filter.

Table 4.2 Estrone adsorption (%) onto selected membrane filters using 0.6 mg/l estrone aqueous solution (n=3).

Membrane material	$A_{ave}\%^{*}$	STDEV**	CV%***
Glass microfibres (GMF)	4.25	0.0142	2.52
Regenerated cellulose (RC)	3.20	0.0095	1.72
Polyethersulphone (PES)	98.00	0	0.00
Polytetra-fluoroethylene (PTFE)	33.11	0.0301	10.24

* Average adsorption of estrone to the filter (%)
** Standard Deviation based on the post-filtration concentration of the triplicates
*** CV is the coefficient of variance based on the post-filtration concentration of the triplicates.

Both RC and GMF filters demonstrated a low affinity toward E1 where the adsorption values of E1 onto these two filters were 3.20% and 4.25% respectively. These results come in line with findings from a previous study where both GMF and RC filters showed low affinity toward E1[136]. However, according to Han, et al. the GMF had a lower affinity to E1 than the RC. This can be related to the different experimental conditions e.g. initial concentration, pore size, temperature, filtration speed. In order to accurately identify the concentration of the bioactive chemical in the filtered sample, it is essential to utilise the right membrane material during the experimental work. Several studies reported filtering their steroid solutions using membrane filters without specifying the actual material of the used membrane[11, 139, 140]. Wang et al, (2011) worked on the simultaneous determination of E1, E2 and E3 in environmental waters such as rivers and lakes. Before the analysis, all the samples were filtered through 0.45 μm membrane filters of unspecified material. This could mean that the reported concentrations of steroids in the studied samples may have been underestimated if PTFE or PES filters were

utilised in that work[139]. Similar situation was observed in another research paper that studied the enzymatic degradation of E1, E2 and E3 by laccase in wastewater. After 1 hour of enzymatic treatment, steroids solution in wastewater was filtered through 0.45 µm of unspecified membrane filter. In this case the efficiency of the enzymatic treatment was potentially overestimated as some of the steroids may have been removed by adsorption onto the membrane filter[11]. One of the important points that should be considered when using membrane filters is the filter-to-filter reproducibility. This means that the adsorption of the target bioactive chemical under the same experimental conditions onto a specific type of filters is constant. The filter-to-filter reproducibility is usually guaranteed by the manufacturer. However it should be checked by the researcher as well to ensure that the followed filtration approach is reproducible. The filter-to-filter reproducibility was tested by calculating the coefficient of variance (CV) between the triplicates after the filtration process. The difference between triplicates is mainly attributed to the filter-to-filter variation as all the other experimental factors were maintained the same. The CV values of GMF and RC filters were less than 3% (2.52% and 1.72% respectively) (Table 4.3). The reproducibility of these two filters makes it possible to account for the abiotic removal of the target compound by these two materials. On the other hand, the CV values of PTFE filters were relatively high (>10%), which means that E1 adsorption on PTFE filters may significantly vary from filter to filter and therefore it is not feasible to use a constant value to account for abiotic E1 adsorption onto the PTFE filter. Based on the above results RC filters are the most suitable filters to work with aqueous E1 solutions. To account for the adsorbed E1 concentration, a simple correction (described in section 3.5 of Methods and Materials) was applied to all of the filtered E1 solutions.

E2 and EE2 are another two common steroid estrogens that are detected in aquatic environments with E1. The affinity of RC filters toward these two steroid estrogens was studied and compared to the affinity of RC filters toward E1. Experiments were undertaken under the same experimental conditions as E1 adsorption study and the obtained results showed that the affinity of RC filters toward E2 is very similar to its affinity toward E1, this could be

attributed to the fact that both these natural steroids have very close values of octanol-water partition coefficient (K_{OW})(Table 4.3).

Table 4.3 Adsorption (%) of the selected steroid estrogens onto regenerated cellulose (RC) membrane filters using 0.6 mg/l aqueous solution of each steroid.

Target bioactive chemical	**Adsorption %**	**Log K_{OW}**	**CV%***
Estrone (E1)	3.20	3.43	1.72
Estradiol (E2)	3.81	3.94	0.57
Ethinylestradiol (EE2)	6.68	4.15	2.17
* CV is the coefficient of variance based on the post-filtration concentration.			

The affinity of RC filters toward the synthetic steroid, EE2, was higher than its affinity toward the natural steroids (i.e. E1 and E2). However, the adsorption of EE2 onto RC filter was still relatively low (6.68%) which makes RC a suitable membrane material even when working with aqueous solutions of synthetic steroids. The Filter-to filter reproducibility was also checked for E2 and EE2 and the filtration results were highly reproducible with CV values less than 3%.

4.5 ENZYMATIC INACTIVATION BY HYDROCHLORIC (HCL) ACID

Enzymatic treatment studies (including laccase-based treatment studies) usually contain an inactivation step to stop the treatment process at preselected time intervals[11, 85, 95, 145]. Having a robust and efficient inactivation procedure is essential to evaluate treatment's efficiency over time. There are several inactivation approaches highlighted in the literature e.g. acidification[9] autoclaving[12, 144] and dilution with methanol[145]. However, acidification remains one of the most practical inactivation approaches - if applied correctly. The efficiency of one acidification approach in inactivating a representative enzyme, *Trametes versicolor* laccase, was investigated in this section. Acidification with hydrochloric acid (HCl) has been used by several research groups to inactivate laccase[9, 11]. However the efficiency of this inactivation procedure has not been investigated in detail so far.

Regardless of the selected inactivation method, a successful inactivation procedure must:

i. instantly and permanently stop the enzymatic reaction; and
ii. not impact on the concentration of the target compound.

To evaluate the performance of HCl in inactivating an oxidative enzyme, *Trametes versicolor* laccase, three sets of control experiments were performed in duplicate under the specified conditions in Section (3.10) of Chapter 3: (A) Standard laccase activity assay without any inhibitors; (B) Standard laccase activity assay with 25 µl HCl per ml of reaction mixture; (C) Standard laccase activity assay with 25 µl HCl per ml of reaction mixture but without any laccase (Figure 4.3). The ABTS oxidation in the assays was monitored for 1 hour. However Figure 4.3 depicts only the first 5 minutes of that reaction as the area where the absorbance values are above 1 considered to be the area of lower accuracy and should not be included in any further analysis. A trial and error approach was utilised to determine the suitable amount of the HCl acid to achieve a complete inactivation of laccase. The preliminary experiments showed that 25 µl of HCl per 1 ml of solution is sufficient to fully inactivate the enzyme laccase regardless of laccase concentration.

Graph "A" demonstrates that the used laccase was active and able to oxidise the chosen substrate (ABTS) under the tested conditions. Graph "B" shows that the addition of 25 µl of HCl into 1 ml of the reaction mixture can fully and instantly inhibit the laccase and stop its enzymatic activity, while graph "C" confirms that the oxidation of ABTS was solely attributed to laccase and not to HCl or any other factors. The complete absence of laccase activity in experiment "B" was evidenced by the similarity between "B" and "C" absorbance graphs as experiment "C" was performed without any laccase addition. The absorbance curve of experiment "B" also shows that laccase inactivation by HCl was permanent as no increase in absorbance was observed during the 1 hr contact time (Figure 4.3 shows only the first 5 minutes of the reaction where the absorbance value is below 2).

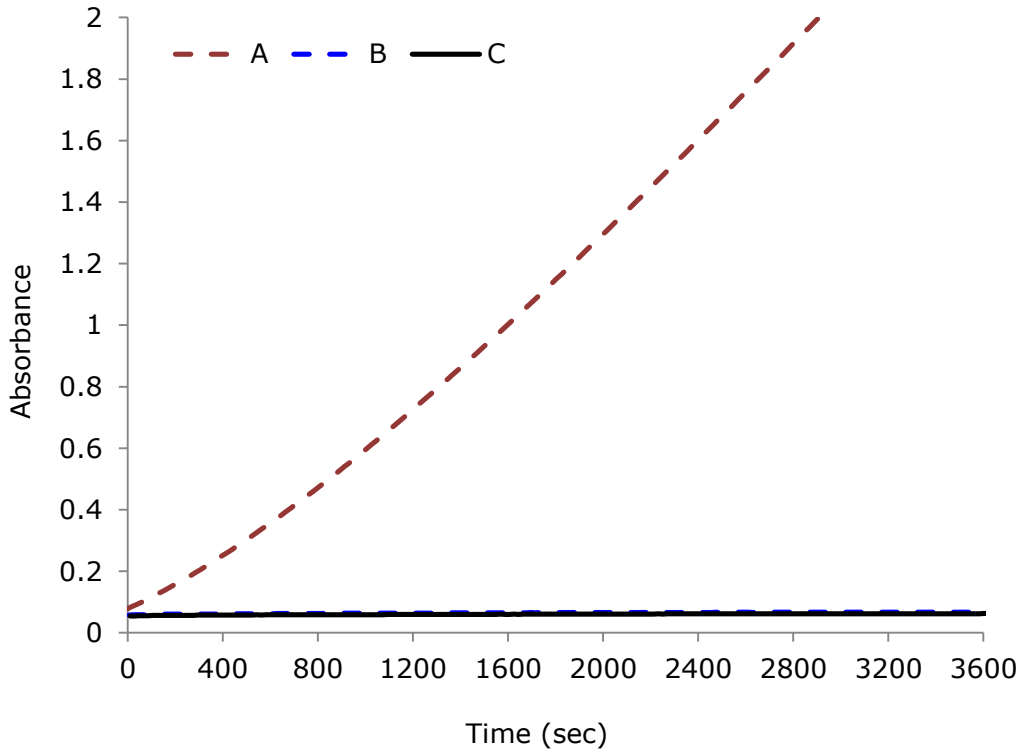


Figure 4.3 The oxidation of ABTS by laccase under the following conditions: laccase concentration=0.5 U/ml, contact time= 1 hour (only the first 5 mins are shown in this graph), temperature= 20±1˚C, reaction matrix= phosphate buffer at pH 7. (A) laccase activity assay under the above conditions, (B) laccase activity assay under the above conditions with 25 µl of HCl (C) laccase activity assay under the above conditions with 25 µl of HCl but without laccase. The coefficient of variance (CV%) between the triplicates was less than 2.0%

4.6 STEROIDS STABILITY IN THE ACIDIFIED MIXTURE

After the acidification, the enzyme becomes inactive and unable to degrade the target pollutant, E1, any more. However, the added acid may abiotically degrade E1 within the solution over time leading to an overestimation of the actual efficiency of the enzymatic treatment. The stability of E1, E2 and EE2 in HCl acidified mixtures was evaluated over time. Standards of E1, E2 and EE2 were prepared in deionised water with initial concentration of 0.5 mg/l. A 1 ml aliquot was taken from each standard and mixed with 25µl of HCl. The pH value of the acidified samples was below pH 2 which should guarantee a complete inhibition of enzyme's activity [159]. The samples were then analysed on HPLC-UV (i) immediately after acid addition and (ii) after a 10 day period which is the longest storage period before analysis. During the 10

day period, samples were stored on the bench in room temperature (20±1 ˚C). The difference between the initial and the final concentration was then calculated to check the acid's impact on steroid concentration overtime (Table 4.4).

Table 4.4 The stability results of E1, E2 and EE2 in deionised water (DIW) acidified with 25µl hydrochloric acid/ml DIW. The initial concentration of each steroid is ≈0.5 mg/l.

Target bioactive chemical	Concentration (mg/l)			Difference* (%)	
	Initial	After 24 hrs	After 10 days	After 24 hrs	After 10 days
E1	0.526	0.520	0.516	1.1%	1.8%
E2	0.478	0.479	0.472	-0.2%	1.1%
EE2	0.491	0.491	0.482	-0.1%	1.9%

E1: Estrone; E2: 17 β-Estradiol; EE2: 17α-ethynylestradiol.

***** Difference between the initial concentration of the steroid and its concentration by the end of the storage period.

Table 4.4 shows that even after 10-day contact time the difference between the initial and final steroid concentration was insignificant (less than 2%). Part of that difference can be also attributed to the analytical variability of HPLC-UV.

An additional set of experiments were performed with E1, a representative steroid estrogen, using an actual wastewater effluent to demonstrate that 25 µl of HCl is able to effectively inactivate the enzyme laccase even in complex matrices. A 0.5 mg/l standard of E1 was prepared in filtered wastewater effluent with initial concentration of ≈0.5 mg/l. A 1 ml aliquot was taken from the standard and mixed with 25µl of HCl. The experiment was performed in triplicate and the samples were analysed on HPLC-UV immediately after the acid addition and after 24 hrs, the longest storage period of the wastewater samples. The results in Table 4.5 show that the change in E1 concentration during the 24 hr contact time with the acid and the wastewater was less than 3% and no significant abiotic degradation of E1 in wastewater and HCl, was observed.

Table 4.5 The concentration of estrone (E1) in wastewater effluent (in the absence of the enzyme laccase) immediately and after 24 hrs of inactivation by 25µl hydrochloric acid/1ml solution. The coefficient of variance between the triplicates was less than 2%.

Estrone concentration		Difference (%)
immediately after the acid addition	After 24 hrs	
0.518	0.524	-1.07
0.501	0.501	0.00
0.497	0.486	2.28

The previous set of experiments did not contain laccase within its reaction mixture and only the impact of HCl acid and wastewater matrix on E1 concentration was evaluated over time. However during laccase-based treatment experiments, HCl acid is used to inactivate the enzyme laccase and stop the enzymatic reaction. Therefore there is a need to evaluate the stability of E1 in such matrix to obtain a more realistic idea about the impact of 24 hr storage time on E1 concentration. Experiments with E1 in the presence of laccase were performed in filtered wastewater effluent in a similar manner to the previously performed experiments in deionised water (details are provided in section 3.14 of Chapter 3). The vials were analysed immediately after the inactivation on HPLC-UV to determine the remaining E1 concentration, the same vials were re-analysed after storing them for 24hr at 20±1˚C (Table 4.6). The results demonstrate that even in the presence of laccase and wastewater, the inactivation procedure by HCl acid is still able to instantly and permanently inactivate the laccase without affecting the concentration of E1 even after 24 hr contact time.

Table 4.6 The concentration of estrone in wastewater effluent inactivated laccase by 25µl hydrochloric acid/ 1ml solution.

Estrone concentration (mg/l)		Difference (%)
immediately after the inactivation	After 24 hrs	
0.118	0.120	-0.94
0.114	0.119	-4.30
0.135	0.134	0.46

The acidification approach can be a reliable and cost effective inactivation procedure. However it is important to evaluate the suitability of this approach for each experimental scenario.

The suitability of the inactivation procedure is affected by multiple factors:

- The type and the amount of the used acid.
- The type of the used enzyme.
- The type of the studied bioactive chemical
- The presence of other constituents in the reaction mixture.

A re-assessment of the inactivation procedure should be carried out if any of the above factors are altered.

4.7 CONCLUSIONS

- Centrifuging laccase solution can potentially decrease its activity, but at the same time it can improve its homogeneity and increase the overall robustness of laccase bench-scale studies.
- The analysed samples on HPLC-UV must be particulates-free to avoid any blockages in the lines of the analytical equipment. Filtering the samples through suitable membranes can be a simple and effective procedure as long as the abiotic removal of the target compound by the membrane assessed and accounted for.
- The adsorption of E1 onto membrane filters depends on the type of the used membrane. PES filters had the highest affinity toward E1, while RC filters had the lowest one which made RC filters the optimum membrane material when working with E1 solutions.
- The filter-to-filter reproducibility in the RC filters was assessed and the results showed that the adsorption of the target steroid under the same experimental conditions onto these filters was relatively constant.
- Acidification with 25 µl of concentrated HCl acid per 1 ml of sample was able to instantly and permanently stop the enzymatic reaction without impacting on the concentration of E1 in the reaction mixture.
- The stability of E1, a representative steroid estrogen, in the presence of inactive laccase, HCl acid and wastewater was assessed. The results showed that E1 concentration remained constant during the tested periods.
- The efficiency of the acidification procedure to inactivate the enzyme laccase has to be re-assessed if any of the following factors are modified: the type and the amount of the used acid, the type of the used enzyme, the studied matrix and the studied bioactive chemical.

5 : RESULTS AND DISCUSSION ENZYMATIC TREATMENT OF FREE STEROID ESTROGENS IN CLEAN WATER MATRIX – ESTRONE AS A CASE STUDY

5.1 INTRODUCTION

The experimental considerations and the essential controls to evaluate the capability of laccase to remove the target steroid, E1, were discussed in detail in Chapter 4. The obtained results in Chapter 4 were applied in this chapter to study E1 removal by laccase from deionised water. A simple matrix such as deionised water was selected as a starting point to investigate the feasibility of generating predictive models of laccase-based system. This chapter investigates the individual and interrelated effects of three independent factors: temperature, contact time and laccase concentration on the removal efficiency of E1, a representative bioactive chemical, in deionised water. Deionised water matrix was selected as simple to test the feasibility of conducting and modelling this treatment. The ranges for the environmental factor i.e. temperature and the reactor design factor i.e. contact time were based on the WWTP environment. Experiments were statistically designed using Box-Behnken Design (BBD) and the experimental data was utilised to build two models namely response surface methodology (RSM) and artificial neural network (ANN). Both models were evaluated for their capability to determine the effectiveness of laccase enzyme at removing estrone from water. The Models' performance was initially evaluated using popular statistical indices. Afterward, the predictive capabilities of RSM and ANN models were assessed -for the first time- using statistically designed unseen data based on the Central Composite Design (CCD).

5.2 HIGHLIGHTS

- Unlike many other laccase-based treatment studies, this chapter utilised realistic temperature range to WWTP in its experiments

- A good agreement was achieved between the built RSM and ANN models (R^2=0.995).
- For the first time, a set of statistically designed unseen data was used to test the predictive capabilities of RSM and ANN models.
- The ability of RSM and ANN models to become predictive models and not only descriptive ones, were assessed.

5.3 MODELLING LACCASE-BASED TREATMENT PROCESS

5.3.1 Box Behnken Design (BBD)

BBD is the most efficient and popular experimental design used with RSM models. Compared to other experimental designs such as Central Composite Design (CCD), BBD is more labour efficient as it requires fewer experiments to complete its matrix of conditions (only 3 levels per factor) and it has been successfully implemented to investigate and optimise the performance of several laccase-based treatment studies [8, 127] and actual wastewater treatment processes[160]. Therefore in this work BBD was selected as the main experimental design to study the impact of three factors: water temperature, contact time and laccase concentration of E1 removal efficiency in deionised water matrix. The BBD matrix of conditions for the investigated 3 factors consisted of 12 unique experiments and 3 replicates at the middle of each continuous factor's range (experiments 1, 5 and 14 in Table 5.3). The replicates, also known as centre points, act as detection mechanism to determine whether the performed experiments are reproducible or not. However, due to various random errors and the nature of laccase itself, the system response will always vary. To ensure the reproducibility of the performed experiments, the coefficient of variance (CV) was calculated for the centre points, the obtained value (CV=1.84%) showed that the system's response for specific set of conditions was constant and the system's variability from experiment to experiment was extremely low. Table 5.1 demonstrates the studied factors and their coded and uncoded ranges.

Table 5.1 The studied factors, their levels and ranges using Box Behnken Design

Factor	Factor code	Factor levels and range		
		-1	0	+1
Temperature (˚C)	X_1	6	15.5	25
Contact time (hour)	X_2	0.5	4.25	8
Laccase conc. (U/ml)	X_3	0.01	0.055	0.1

5.3.2 Selecting the Range for Each Factor

Temperature has a significant impact on laccase activity. However laccase-based treatment should be able to operate within the range of the typical temperatures in WWTPs. The mean annual temperature of wastewater depends on the geographical location e.g. the annual wastewater temperature range in the United States varies from 3 to 27˚C[161]. One study reported that the temperature range in wastewater varies between 15˚C -25˚C[162], and similar range [10˚C - 25˚C] was also mentioned in another study[163]. As a result a similar realistic range was selected for this work where the temperature was confined between +6˚C and +25˚C. The contact time is another factor that has a strong impact not only of the efficiency of laccase-based treatment but also on laccase activity[163]. The contact time will have a direct impact on the size of the required reactor if this treatment is implemented in WWTPs, very long contact time will require extremely large treatment unit, while very short contact time may not be sufficient to achieve the required removal efficiency of the target pollutant. Therefore a suitable range of the contact was considered between 0.5 and 8 hours. Preliminary studies were performed to identify the range of laccase concentration for both clean and wastewater matrices, a brief example of these scoping studies in clean water was included in Appendix D. Suitable laccase range should provide a good breadth of steroids' removal in the selected matrix and ensure that all the obtained removal percentages are above 0% but less than 98% (which is the maximum possible removal efficiency in this study as the limit of quantification (LOQ) of E1 on the used HPLC-UV is 0.01 mg/l). Maintaining the removal values within that range was

essential to ensure that the collected data from the experimental studies could be utilised to build models with high predictive capabilities. In case the E1 concentration at the end of the contact time was less than the LOQ (0.01 mg/l), the achieved removal efficiency was reported as ≥ 98%. Based on the above requirements and on several scoping studies (Appendix D), laccase concentration was confined between 0.01 U/ml and 0.1 U/ml. The investigated factors and their ranges are shown in Table 5.2.

Table 5.2 The ranges of the investigated factors in deionised water.

Parameters	Range
Temperature (˚C)	6 - 25
Contact time (hr)	0.5 - 8
Laccase concentration (U/ml)	0.01 – 0.1

The initial concentration of E1 in this matrix (as well as in wastewater effluent matrix) was 0.5 mg/l. Although this concentration is relatively higher than the environmentally relevant level of E1 in the aquatic environment, it is still lower than the used concentration in the majority of similar studies [12, 95]. Performing experiments at environmentally relevant levels (ng/l - µg/l) is very challenging from the analytical point of view, especially when it comes to detect the concentration of the targeted steroid at the end of the contact time. Therefore a higher concentration of E1 was selected to easily track the change in E1 concentration over time.

The impact of temperature, contact time and laccase concentration on the removal efficiency of E1 could have been also estimated using the first principles as described in Section 2.5.1. In this work a standard Michaelis Menten graph was constructed using ABTS as a substrate. GraphPad Prism software was used to estimate the main kinetic parameters: K_M and V_{max} of that reaction and the results of those experiments are included in Appendix C. It is worth noting that in complex matrices, such as wastewater, K_M and V_{max} values may change in the presence of various compounds that can act as enzyme inhibitors/ mediators which makes it difficult to utilise them to calculate the contact time in the batch reactor.

For these reasons, this work focused on using batch experiments and models such as RSM and ANN instead of the first principles to determine the impact of the selected parameters on the enzymatic removal efficiency of E1.

5.3.3 Response Surface Methodology (RSM) Model

The RSM model was generated in Minitab (V.16.2.2) to connect the system response with the CCD and BBD experimental conditions. The polynomial equation of the built RSM model is presented in the Equation 5.1 below:

$$R\% = -93.87 + 5.99X_1 + 21.35X_2 + 1583.18X_3 - 0.11{X_1}^2 - 1.52{X_2}^2 - 8752.05{X_3}^2 - 0.09X_1X_2 - 11.38X_1X_3 + 21.21X_2X_3 \quad \text{[Equation 5.1]}$$

Where;

$R_{\%}$ is the predicted removal efficiency of E1; X_1: temperature (˚C); X_2: contact time (hour); X_3: laccase concentration (U/ml).

The predicted removal efficiency of E1 by RSM (R%) was calculated by replacing X_1, X_2 and X_3 from the Equation 5.1 with their level values from the BBD matrix of conditions (Table 5.3). The absolute error represented the percentage difference between the actual and predicted removal efficiency and was calculated using the below equation (Equation 5.2):

$$\text{Absolute Error (\%)} = \left|\left(\frac{R_{ACT} - R_{RSM}}{RACT}\right) \times 100\right| \quad \text{[Equation 5.2]}$$

Table 5.3 The actual and predicted removal efficiencies of estrone by laccase using Box Behnken Design (BBD) and response surface methodology (RSM) model.

Run	Factors			Removal %		
	Temperature (˚C)	Duration (Hrs)	Laccase conc. (U/ml)	Actual	RSM prediction	Absolute Error (%)
1	15.5	4.25	0.055	86.74	85.80	1.08
2	6	8	0.055	77.89	70.40	9.62
3	25	0.5	0.055	39.78	38.59	2.99
4	6	0.5	0.055	5.09	8.68	70.53
5	15.5	4.25	0.055	84.93	85.80	1.02
6	15.5	8	0.01	52.32	53.52	2.29

7	25	4.25	0.01	54.09	53.93	0.30
8	6	4.25	0.01	8.90	14.29	60.56
9	6	4.25	0.1	74.27	72.10	2.92
10	25	8	0.055	≥ 98.00	100	2.36
11	25	4.25	0.1	≥ 98.00	92.28	5.84
12	15.5	0.5	0.1	35.70	39.88	11.71
13	15.5	0.5	0.01	2.34	0.00	----
14	15.5	4.25	0.055	88.31	85.80	2.84
15	15.5	8	0.1	≥ 98.00	100	3.68

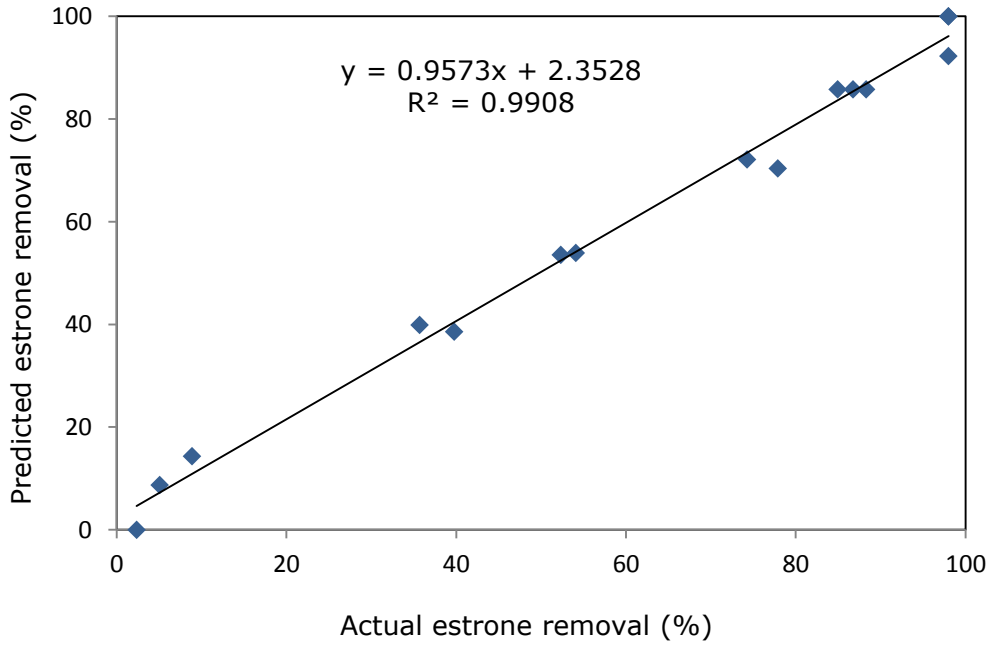


Figure 5.1 Comparison between the experimental and the predicted values of estrone removal efficiency using response surface methodology (RSM) model.

The statistical significance of the RSM model and its linear terms (X_1, X_2, X_3), quadratic terms (X_1^2, X_2^2, X_3^2) and cross-factor interactions ($X_1.X_2$, $X_1.X_3$, $X_2.X_3$) where X_1: temperature (˚C), X_2: contact time (hours) and X_3: laccase concentration (U/ml), were evaluated by ANOVA which is a reliable approach to assess the lack-of- fit of the RSM model to the experimental data[164]. The results of ANOVA analysis are presented in Table 5.4.

Table 5.4 Analysis of variance (ANOVA) of the BBD-RSM model using uncoded units.

Source	Coefficient value	Sum of squares	Mean square	F-value	P-value
X_1	5.99	840.3	873.46	23.13	0.005
X_2	21.35	4146.7	2507.96	66.41	0.000
X_3	1583.18	2353.9	1859.22	49.23	0.001
X_1^2	-0.11	341.9	341.86	9.05	0.030
X_2^2	-1.52	1682.6	1682.61	44.56	0.001
X_3^2	-8752.05	1159.8	1159.76	30.71	0.003
$X_1.X_3$	-0.09	94.7	94.67	2.37	0.353
$X_1.X_2$	-11.38	39.6	39.58	1.05	0.174
$X_2.X_3$	21.21	51.3	51.27	1.36	0.297
Residual Error	7	188.8	37.76	-	-
Lack-of-Fit	5	183.1	61.03	21.33	0.045
Pure Error	2	5.7	2.86	-	-

The term or the interaction was considered statistically significant when its probability (P) value was ≤0.05. Subsequently terms with P value > 0.05 were considered insignificant. The lack-of-fit of the built RSM model has a P value of 0.045 which is lower than 0.05 and as a result the lack-of-fit of the RSM model is significant and its ability to represent the investigated system is poor and inaccurate.

All the linear and quadratic terms from Equation 5.1 were found to be significant. While the P values of the interaction terms were statistically insignificant with all P values above 0.05. This shows that there are no cross-factor interactions that influence the removal of E1 by laccase. The coefficient value of each linear term (X_1, X_2 and X_3) can be also used as an indicator of the intensity of its impact on E1 removal efficiency. However, it is important to compare the coefficients using the coded factors' levels (-1, 0, +1) rather than the uncoded ones (the actual levels of each factor). Analysing a model using in uncoded units may mean that the model is not orthogonal any more. Orthogonality allows the users to estimate model terms independently and remove any insignificant terms without impacting on the remaining terms within the model. The default analysis in Minitab is typically performed using coded units[165]. The coefficients of the polynomial equation of the RSM

model are given in Table 5.5 for both coded and un-coded units. The positive value of an individual coefficient implies that it has a positive impact on E1 removal efficiency, while the negative value shows that the factor has a negative impact on E1 removal.

The larger the coded coefficient of a factor, the bigger its impact on E1 removal efficiency. As a result contact time was identified as the main factor influencing this system with a coefficient of 30.91, followed by laccase concentration with a coefficient of 24.04 and then temperature with a coefficient of 15.97. All of the investigated factors had positively impacted on E1 removal percentage. The contribution of each individual factor to E1 removal percentage can be displayed using Figure 5.2

Table 5.5 Estimated regression coefficients of the response surface methodology model using both coded and un-coded units.

Term	**Coefficient**	
	Un-coded	Coded
Temperature (˚C) X_1	5.99	15.97
Contact time (hours) X_2	21.35	30.91
laccase concentration (U/ml) X_3	1583.18	24.04
X_1^2	-0.11	-9.62
X_2^2	-1.52	-21.35
X_3^2	-8752.05	-17.72
$X_1.X_2$	-0.09	-3.15
$X_1.X_3$	-11.38	-4.87
$X_2.X_3$	21.21	3.58
Constant	-93.87	86.66

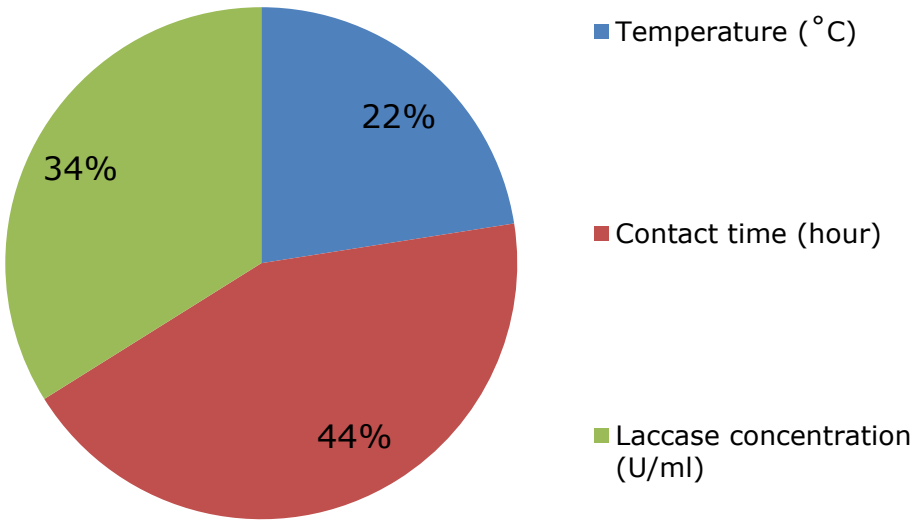


Figure 5.2 The contribution of each individual factor to E1 removal percentage based of the coefficients values of the coded units.

5.3.4 Artificial Neural Network (ANN) Model

Due to the lack of fit of the RSM model, a different type of models was built to represent this system. ANN model was built in MATLAB using the same experimental data as the RSM model. During the generation of the ANN model, 70% of the experimental results were utilised in ANN training, 15% in ANN validation and 15% in ANN testing. The built ANN model was used to predict the achieved E1 removal efficiency under the specified conditions in BBD matrix of conditions. The conditions of the performed experiments, the experimental E1 removal percentages and the ANN predicted E1 removal percentages were included in Table 5.6. The ANN model was able to predict the exact experimental value in 12 out of 15 experiments and as a result the absolute error value for each of those 12 experiments was zero.

Table 5.6 Actual and predicted removal efficiencies of estrone by laccase using artificial neural network (ANN) model.

Run	Factors			Removal %		
	Temperature (˚C)	Duration (Hrs)	Laccase conc. (U/ml)	Actual	ANN prediction	Absolute Error (%)
1	15.5	4.25	0.055	86.74	86.74	0.00
2	6	8	0.055	77.89	77.89	0.00
3	25	0.5	0.055	39.78	39.78	0.00
4	6	0.5	0.055	5.09	5.09	0.00
5	15.5	4.25	0.055	84.93	86.74	2.13
6	15.5	8	0.01	52.32	52.32	0.00
7	25	4.25	0.01	54.09	54.09	0.00
8	6	4.25	0.01	8.90	8.90	0.00
9	6	4.25	0.1	74.27	74.27	0.00
10	25	8	0.055	$\geq$ 98.00	100.00	2.04
11	25	4.25	0.1	$\geq$ 98.00	100.00	2.04
12	15.5	0.5	0.1	35.70	35.70	0.00
13	15.5	0.5	0.01	2.34	0.42	82.05
14	15.5	4.25	0.055	88.31	86.74	1.78
15	15.5	8	0.1	$\geq$ 98.00	100.00	2.04

The predicted values were compared against the experimental values and the high value of the coefficient of determination (R^2 =0.9992) indicated the good fit between the ANN predictions and the experimental data (Figure 5.3). A biosorption study also utilised an experimental design (Central Composite Design) to generate the optimal architecture of ANN model. The good correlation between the experimental and the ANN predicted data was demonstrated by R^2 of 0.967[131].

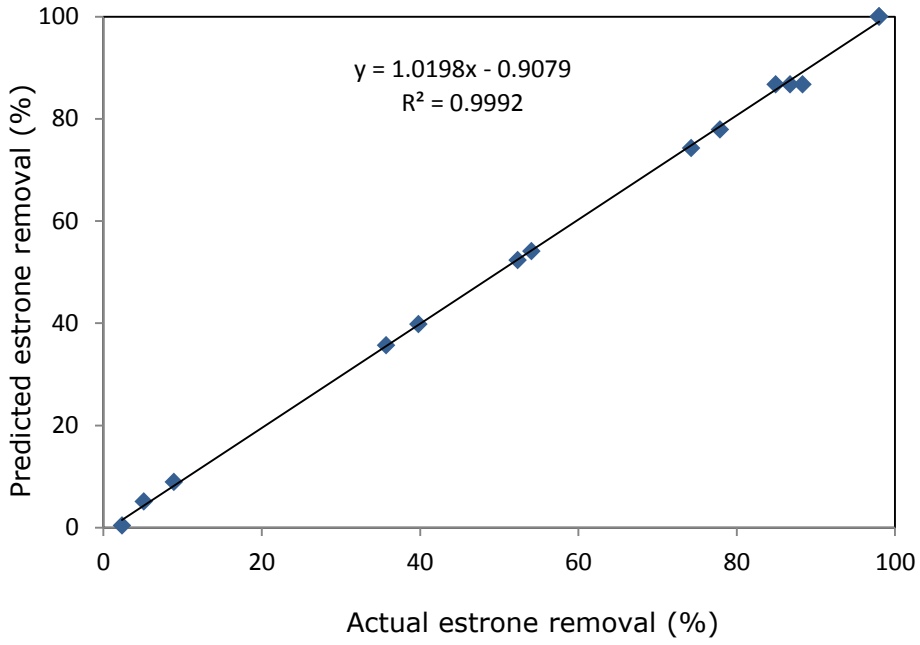


Figure 5.3 Comparison between the experimental and predicted values of estrone removal efficiency using artificial neural network (ANN).

5.4 EVALUATING THE GOODNESS-OF-FIT OF RSM AND ANN MODELS USING STATISTICAL INDICES

One of the applied approaches to evaluate the quality of the RSM and ANN models is to plot the difference between the actual and the predicted values (the residual) against the number of the conducted experiments (15 experiments)[166]. Table 5.7 shows the values of the residuals for both RSM and ANN models.

Utilising the values from Table 5.7, the distribution of residuals for both ANN and RSM models was depicted in Figure 5.4. In a good model, the residuals occur near the centre line in a random pattern without trending or clustering[166]. The fluctuation of the ANN residuals was very small which demonstrates a model with good fit to the experimental data. While the poor fit of the RSM model was demonstrated by a much higher deviation of the residuals from the centre line.

Table 5.7 The values of ANN and RSM residuals.

Run	Estrone Removal (%)			ANN Residual	RSM Residual
	Actual	ANN Predicted	RSM predicted		
1	86.74	86.74	85.8	0	0.94
2	77.89	77.89	70.4	0	7.49
3	39.78	39.78	38.59	0	1.19
4	5.09	5.09	8.68	0	-3.59
5	84.93	86.74	85.8	-1.81	-0.87
6	52.32	52.32	53.52	0	-1.2
7	54.09	54.09	53.93	0	0.16
8	8.9	8.9	14.29	0	-5.39
9	74.27	74.27	72.1	0	2.17
10	≥98.00	100	100	-2.00	-2.00
11	≥98.00	100	92.28	-2.00	5.72
12	35.7	35.7	39.88	0	-4.18
13	2.34	0.42	0	1.92	2.34
14	88.31	86.74	85.8	1.57	2.51
15	≥98.00	100	100	-2	-2

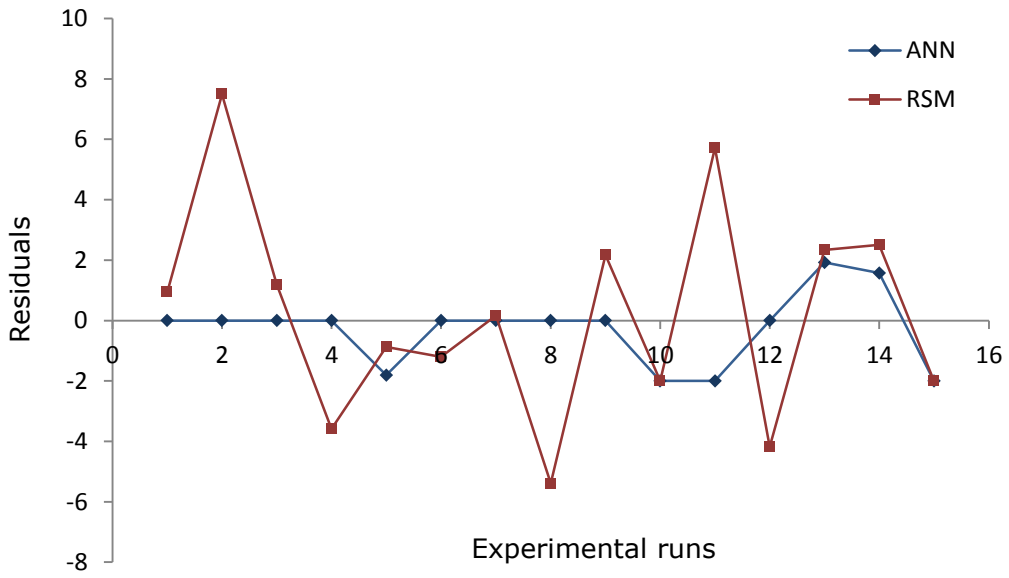


Figure 5.4 The residuals of RSM and ANN models in deionised water.

The performance of the generated RSM and ANN models can be also assessed using popular statistical indices such as the mean squared error (MSE), the root

mean squared error (RMSE) and the absolute average deviation (AAD) (see section 3.15.2 for details). These indices have been used by several research papers to evaluate the goodness of fit of the nonlinear models [92, 152, 155, 156]. The best model was identified by the lowest AAD, MSE and RMSE values. The values of these indices for both RSM and ANN models are presented in Table 5.8.

Table 5.8 The statistical indices of the built models.

Model	**Index**	**Value**
RSM model	R^2	0.9908
	MSE	11.81
	RMSE	3.44
	AAD	18.38
ANN model	R^2	0.9995
	MSE	1.43
	RMSE	1.19
	AAD	6.13

The results clearly show that the ANN model is superior to the RSM model where the MSE, RMSE and AAD values of the ANN model are noticeably smaller than their values in the RSM model. This comes in line with the R^2 values of both models, where the R^2 of ANN model (0.9995) is higher than the R^2 of RSM model (0.9908). However, the agreement between the two models themselves is still very high with R^2=0.9926 (Figure 5.5) which shows that both models can provide a good estimation of E1 removal efficiency by laccase. The impact of the three independent factors on E1 removal efficiency is visualised as 3D graphs for both RSM and ANN models in Figure 5.6.

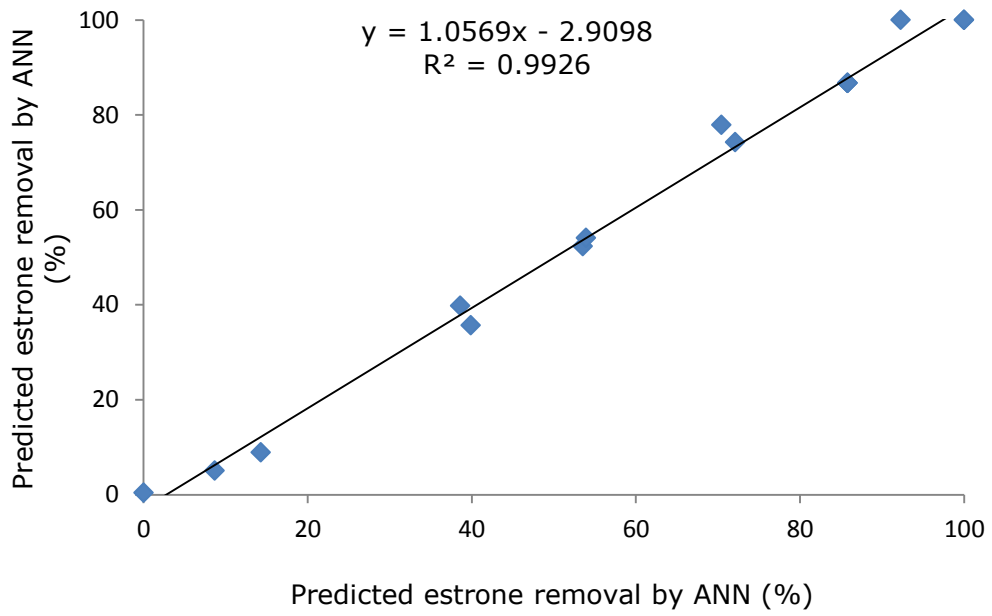


Figure 5.5 Comparison between the predicted removal efficiency of estrone by laccase using RSM and ANN models.

Although the ANN and RSM graphs are noticeably different when representing the whole system, the depicted impact of each factor on E1 removal efficiency in both models' graphs is in agreement. The contact time versus temperature graphs show that higher removal efficiency can be achieved with higher temperature and longer contact time. In addition to this intuitive outcome, both RSM and ANN graphs show that the gradient of the contact time is higher than the temperature one which means that the contact time has a higher impact on the removal efficiency of E1 than the temperature. This observation comes in line with the values of the coded RSM coefficients in Table 5.5 where the coefficient value of the temperature (X_1=15.97) is lower than the contact time one (X_2=30.91) and, as it has been mentioned previously, factors with higher coefficients have a higher influence on the system response. The laccase concentration versus contact time graphs also correspond to the coefficient values of these two factors (24.04 and 30.91 respectively) where the contact time shows a higher impact on E1 removal efficiency. The final two graphs illustrate the influence of temperature and laccase concentration on E1 removal efficiency where the later factor has a

higher impact on the system's response. Thus, despite the different nature of ANN and RSM models, both of them have similarly ranked the influence of the investigated factors on E1 removal efficiency where the highest impact was attributed to the contact time, followed by laccase concentration and then temperature.

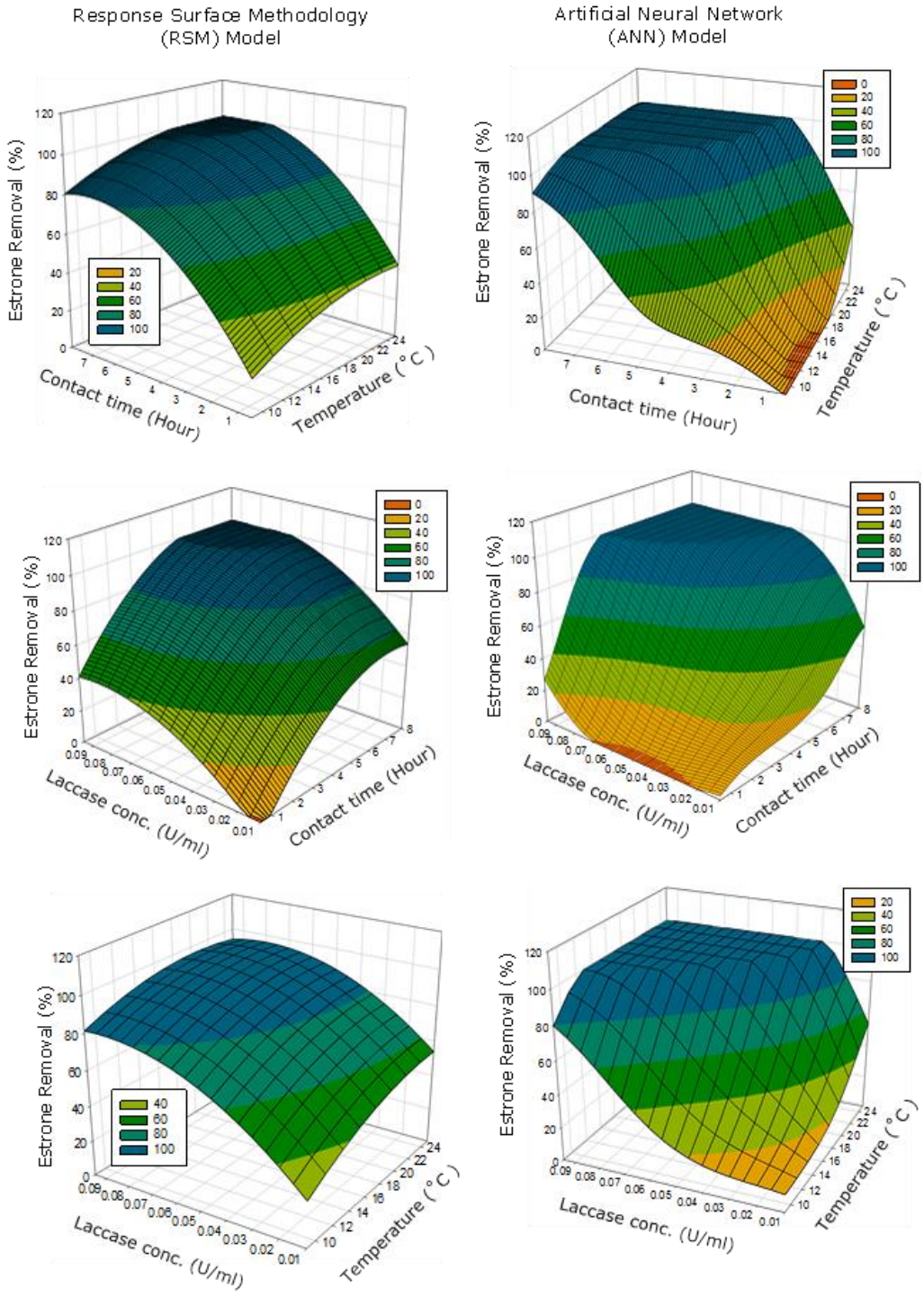


Figure 5.6 The predicted removal efficiencies of estrone by laccase using RSM and ANN models, under the influence of three independent factors: temperature, contact time and laccase concentration.

5.5 EVALUATING THE PREDICTIVE CAPABILITY OF RSM AND ANN MODELS USING UNSEEN DATA

Although statistical indices such as AAD, MSE, RMSE and R^2, are commonly used to assess models' performance, it is important to remember that these indices are usually affected by size of the sample, especially when it is smaller than 100 data points[167]. Determining the predictive capability of a model based only on its AAD, RMSE and R^2 values may lead to inaccurate conclusions about its ability to predict the removal efficiency of E1 under other conditions within the same system. To evaluate the model in more efficient ways, the predictive capabilities of any model can be assessed using a set of data that have not been used to generate the corresponding model, also referred to as "unseen data"[157]. A model with good predictive capability should be able to determine the system response for any given set of conditions within that system. The difference between the predicted and the experimental values is inversely proportional to model's accuracy.

5.5.1 Preparing the Unseen Data Set

The unseen data represents a new set of data for model testing that has not been used to train or build the tested model. The set is usually prepared by performing few additional experiments which represent some randomly selected points within the studied system. This approach ensures that the built model is not only a descriptive, but also a predictive one and is able to accurately predict the output of any point within the system. In this work the unseen data set was statistically designed (rather than randomly selected) to provide a better coverage of the investigated system. CCD is another popular experimental design known for its high quality predictions of linear and quadratic interaction effects of factors affecting the studied system[131]. The CCD and BBD are covering different sets of points in the investigated system (as described in section 2.8). Therefore CCD was used to generate a set of unseen data that were located within the studied system but was not previously used in RSM and ANN models' training. The CCD matrix of conditions consisted of twenty experiments studying the impact of the same 3 factors

within the same ranges as the BBD (Table 5.2). Six of these experiments represented the centre points and the reproducibility of CCD experiments was evidenced by the extremely low coefficient of variance (CV=0.71%). Since the centre points in both BBD and CCD had the same conditions (temperature= 15.5˚C, Contact time-= 4.25 hours, Laccase concentration=0.055 U/ml), it was decided to exclude them from the unseen data set during the assessment of the models. After removing the centre points, the CCD was left with 14 data points that were fed into the previously built ANN and RSM models. The experimental and the predicted E1 removal efficiencies of the unseen data for both RSM and ANN models, are presented in Table 5.9.

Table 5.9 The predicted removal efficiency of RSM and ANN using unseen data

Run	**Factors**			**Removal %**			**Residual**	
	Temp (˚C)	Time (Hour)	Laccase conc. (U/ml)	Actual	RSM	ANN	RSM residual	ANN residual
1	21.3	6.55	0.0274	92.86	79.91	74.77	12.95	18.09
2	9.7	6.55	0.0826	93.6	96.15	97.22	-2.55	-3.62
3	9.7	1.95	0.0274	29.6	22.99	20.13	6.61	9.47
4	21.3	1.95	0.0826	90.37	70.44	72.81	19.93	17.56
5	15.5	4.25	0.1	94.61	92.07	97.14	2.54	-2.53
6	15.5	4.25	0.01	34.37	44	20.56	-9.63	13.81
7	6	4.25	0.055	63.49	60.9	55.62	2.59	7.87
8	15.5	0.5	0.055	12.29	33.63	7.41	-21.34	4.88
9	15.5	8	0.055	95.59	95.14	92.64	0.45	2.95
10	25	4.25	0.055	96.85	90.76	100	6.09	-3.15
11	21.3	6.55	0.0826	≥98.00	100	100	-2.00	-2.00
12	21.3	1.95	0.0274	63.34	47.33	23.08	16.01	40.26
13	9.7	1.95	0.0826	61.76	53.4	39.63	8.36	22.13
14	9.7	6.55	0.0274	64.56	60.38	40.61	4.18	23.95

The results showed that the overall agreement between the experimental and

predicted E1 removal values dropped significantly for both RSM and ANN models when they were tested using unseen data. Figure 5.7 compares between the experimental and the predicted removal efficiencies of RSM and ANN models using unseen data.

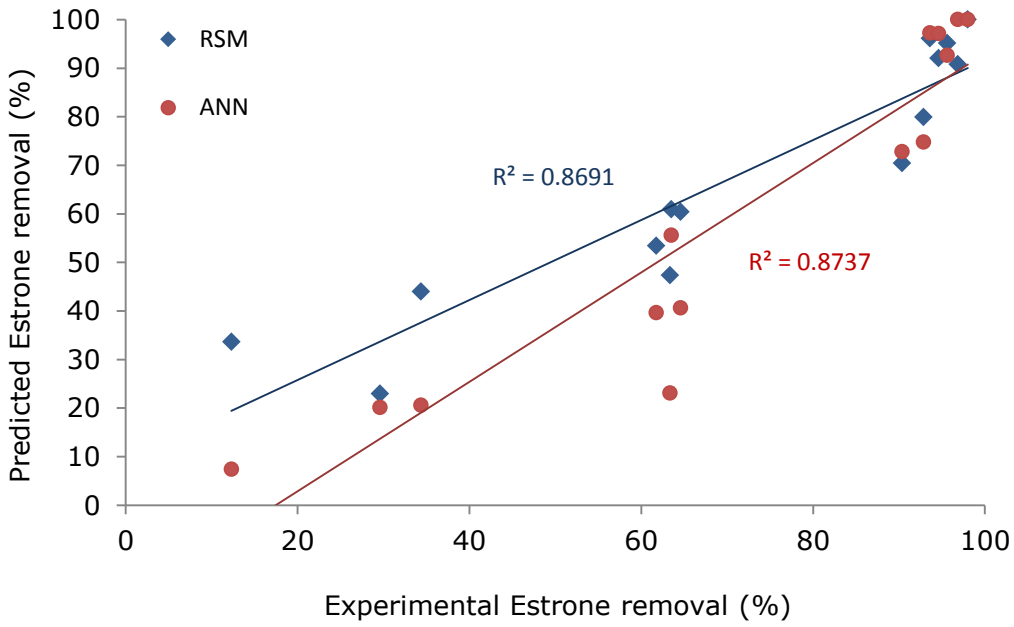


Figure 5.7 Comparison between the actual and predicted values of estrone removal efficiency using unseen data.

The RSM model was able to provide a relatively accurate prediction (±10%) when both of the contact time and laccase concentration (the main two factors) were at their higher coded levels (0,+1,+α) (the corresponding un-coded units for CCD for each factor are presented in Table 5.10), while the temperature value seemed less significant and does not have a clear impact on the accuracy of the prediction.

Table 5.10 The coded units of the Central Composite Design (CCD) and their corresponding un-coded units.

Factors	**Coded Units**				
	-α	-1	0	+1	+α
Temperature (˚C)	6	9.7	15.5	21.3	25
Contact time (Hour)	0.5	1.95	4.25	6.55	8
Laccase conc. (U/ml)	0.01	0.0274	0.055	0.0826	0.1

Unlike the RSM model, there was no clear connection between the points that were accurately predicted (±10%) by the ANN model. This can be justified by the nature of the ANN model itself. Before generating any ANN model, the neural network passes a training process where it learns to associate specific input patterns with corresponding outputs. When the fully built ANN model is used, it detects the input pattern and associates it with suitable output. If the entered input pattern has no associated output with it, then the ANN model provides an output that corresponds to a learnt input pattern that is most similar to the entered pattern. Since the training process cannot be externally controlled there is always a potential to over fit the model to its original data or produce inaccurate predictions during the testing process [111]. Table 5.11 summarises the achieved statistical indices for each model, for both standard data (15 BBD experiments that were used to build the models) and unseen data (14 CCD experiments that were not used for training/ building the models).

Table 5.11 The statistical indices of the built models using standard and unseen data sets.

Model	Index	Standard data	Unseen data
RSM model	R^2	0.9908	0.8691
	MSE	11.81	119.81
	RMSE	3.44	10.95
	AAD	18.38	22.96
ANN model	R^2	0.9995	0.8737
	MSE	1.43	286.48
	RMSE	1.19	16.93
	AAD	6.13	22.32

The AAD value increased from 18.38 to 22.96 for RSM model and from 6.13 to 22.32 for ANN model. While R^2 decreased from 0.9908 to 0.8691 form RSM model and from 0.9995 to 0.8737. Although both models showed a relatively good fit to the standard experimental data, they were unable to accurately predict E1 removal efficiency for points outside the standard data set. The poor predictive capabilities of the built ANN model could be improved by increasing the number of the used data points and their distribution within the system. The observed results demonstrate the

importance of utilising unseen data to evaluate the predictive capabilities of the built models and not relying only on the statistical indices.

Further studies on how to improve the predictive capabilities of a model and the removal efficiency of E1 in actual wastewater matrix are discussed in Chapter 7.

5.6 CONCLUSIONS

- This work provides a proof-of-concept of laccase ability to degrade E1 in clean water under realistic ranges of temperature and contact time.

- The centre points of the BBD and the CCD were used as a detection mechanism of experiments reproducibility. The result showed that the system's response for specific set of conditions is constant and the system's variability from experiment to experiment in deionised water is very small with CV less than 2%.

- The goodness of the fit of the RSM and ANN models to the experimental data were evaluated using residuals graphs and statistical indices for nonlinear models such as MSE, RMSE and AAD. The results showed that the ANN model had better predictive capabilities than the RSM model.

- Assessing the quality of RSM and ANN models using unseen data demonstrated that both models had poor predictive capabilities in several areas of the system. Adding experiments from those areas into the training data may improve the quality of the built models.

- The RSM model was able to provide a relatively accurate prediction (±10%) only when both of the contact time and laccase concentration were at their higher coded levels (0, +1,+α). No specific pattern or trend was observed with the ANN model.

6 : RESULTS AND DISCUSSION UNDERSTANDING AND CHARACTERISING COMPLEX ENVIRONMENTAL MATRICES

6.1 INTRODUCTION

The efficiency of laccase-based treatment in clean water matrix was fully investigated in Chapter 5. The performed experiments in Section 5.3 demonstrated the feasibility of laccase to degrade an environmentally relevant pollutant such as E1 under a temperature and a contact time relevant to the wastewater treatment environment. While the generated RSM and ANN models were able to describe the studied system and predict its performance under specific set of conditions.

As it was mentioned previously in Section 2.5.5, the optimum location for laccase-based treatment is at the end of the municipal wastewater treatment plant (WWTP) which typically consists of preliminary, primary and secondary treatment stages. The effluent from the secondary wastewater treatment stage will potentially form the influent into the laccase-based treatment stage.

Even after passing the conventional treatment stages, the secondary wastewater effluent is still a complex and a variable matrix that contains a wide range of constituents that may impact of the performance of laccase-based treatment. Before assessing laccase performance in this complex matrix, it is essential to understand the potential impact of effluent's complexity and its variability on laccase-based treatment.

This chapter demonstrates the temporal variability of wastewater effluent using several water quality parameters; it also depicts the direct impact of this variability on E1 removal efficiency by laccase and develops a new approach to account for wastewater temporal variability. Following that, the chapter investigates the impact of 4 potential inhibitors: chloride (Cl^-), copper (Cu^{2+}), iron (Fe^{3+}) and zinc (Zn^{2+}) on laccase activity and determine the impact of different concentrations of each individual ion on laccase-based treatment.

6.2 HIGHLIGHTS

- Unlike other laccase-based treatment studies, this work assesses the temporal variability of the wastewater effluent and evaluates its impact on laccase-based treatment.
- A new parameter "Benchmark" was developed to quantify the amenability of the wastewater effluent to be treated by laccase.
- The inhibitory effect of chloride ions on laccase-based treatment is much stronger at pH 4.5 than at wastewater-relevant pH.

6.3 QUALITY PARAMETERS OF WASTEWATER EFFLUENT

Municipal wastewater is a complex matrix which is influenced by a wide range of factors[161]. A wide range of parameters are commonly used to characterise the wastewater matrix and these include the concentration of: biological oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), dissolved oxygen (DO), ammonia, metals, pH and temperature[153]. Some of these parameters e.g. BOD, TSS and ammonia form the main part of the discharge consent in the majority of WWTPs[168]. In the UK, consents usually reflect the objectives of local water quality, the requirements of national legislation or the requirements of the European Urban Waste Water Treatment Directive (UWWTD). The location of the final discharge point, the maximum permitted flow rate of the treated effluent and its quality are all specified within the associated consent to minimise the negative impact on the receiving water [169]. These water quality parameters can inform not only on the compliance of the WWTP with the relevant legislations but can also indicate the temporal variability of the wastewater which can potentially impact of the efficiency of laccase-based treatment.

Therefore developing a new treatment technology for complex and variable matrices such as wastewater, it is essential to establish the impact of matrix variability on the performance efficiency of the new technology. Some treatment technologies can be very sensitive to the changes in the characteristics of their influent, while others have a higher tolerance toward matrix variability. The sensitivity of laccase-based treatment will have a direct

impact on the feasibility of implementing this technology in a real wastewater matrix.

To understand the variability of wastewater, the final effluent of a WWTP with a population equivalent on 650,000 P.E. and operating biological treatment as activated sludge, was monitored between December 2014 and June 2015. The following water quality parameters were measured for each collected final effluent sample: temperature (˚C), COD (mg/l), TSS (mg/l), pH and DO (mg/l). These parameters are not necessarily independent of each other and due to the presence of various constituents within the watewater and its daily variability, the relationship between two parameters may vary. Hence it may be unfeasible to predict the value of one of these parameters based on the value of another.

To demonstrate the impact of the TSS on the COD value in wastewater, a set of experimnets was performed on seven different sampling dates. The COD values were measured for both filtered (without TSS) and unfiltered (with TSS) wastewater effluent samples. Effluents with high TSS are expected to have high COD values as the TSS may act as sites for microbial attachment and may also comprise of organic material themselves. The TSS were separated from the wastewater sample by the 1.2 µm glass microfiber (GMF) filter. The results in Figure 6.1 show that the COD of the unfiltered sample (COD_{unfilt}) was always higher than the COD of the filtered same wastewater sample (COD_{filt}) as the TSS and any associated microorganisms in the unfiltered sample would consume additional oxygen which will subsequently increase the COD value. However the relationship between COD_{unfilt} and COD_{filt} data points varied from day to day depending on the composition of the wastewater effluent.

In Figure 6.1 both sample (1) and sample (7) had the same TSS value of 5.2 mg/l, but their measured COD values (both COD_{unfilt} and COD_{filt}) were noticably different (Table 6.1).

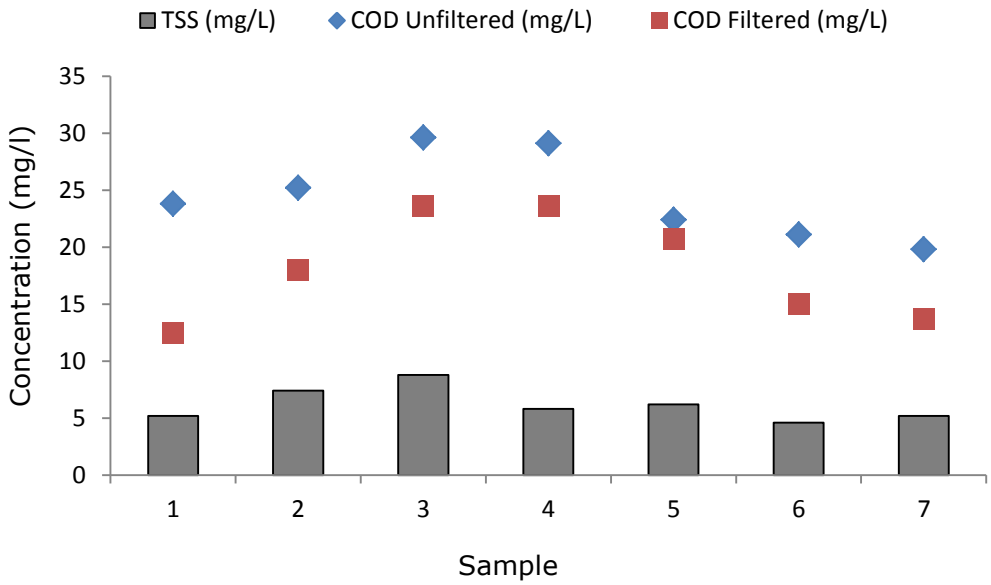


Figure 6.1 Average chemical oxygen demand (COD) values of the wastewater effluent samples that were either unfiltered or filtered through 1.2 µm of glass microfibers filter (GMF). The associated Total Suspended Solids (TSS) values of the unfiltered samples are presented as well. The coefficient of variance between the COD readings was less than 2%.

Table 6.1 Comparison between the filtered and unfiltered Chemical Oxygen Demand (COD) values of two wastewater effluent samples with the same Total Suspended Solids (TSS).

Sample ID	COD Unfiltered (mg/l)	COD filtered (mg/l)	TSS (mg/l)
1	23.8	12.5	5.2
7	19.8	13.7	5.2

The filtration process of sample (1) reduced its COD by 47%, while the filtration process of sample (7) reduced the relevant COD only by 31%. There are several consitituents within the final effluent such as dissolved inorganic salts (e.g. sodium chloride and sodium sulfate) that may impact on the COD value of a sample without affecting its TSS which makes the relationship between the COD_{unfilt} and COD_{filt} variable from day to day[170, 171]

Measuring the values of the TSS and the COD_{filt} of the wastewater effluent duirng a 6-month period demonstrated the continuous variability and fluctuation of this matrix (Figure 6.2).

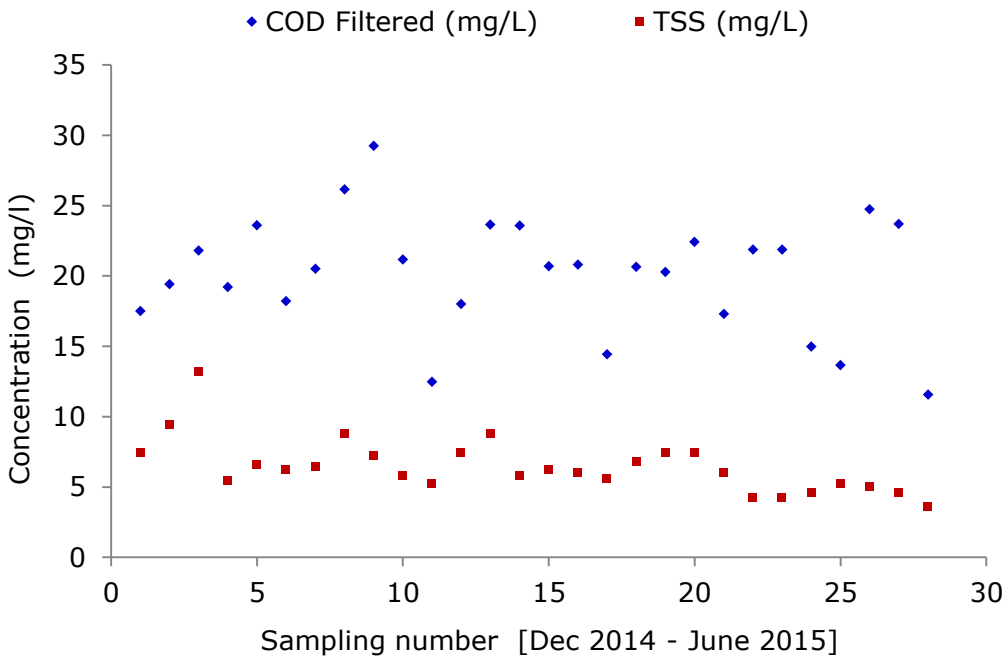


Figure 6.2 The values of the total suspended solids (TSS) and the chemical oxygen demand (COD_{filt}) of the filtered wastewater effluent during a 6-month period (Dec 2014 - June 2015).

In water matrices, the solubility of the oxygen and its concentration are mainly controlled by the temperature of water, its salinity and the partial pressure of the oxygen in the atmosphere[171, 172]. In wastewater effluents, the DO is consumed by the biodegradation of carbonaceous materials and endogenous respiration of the microorganisms as a result the DO concentration in the final effluent continuously varies[173]. The temperature of the final effluent and its DO concentration were measured on site immediately after collecting each sample. The increase in water temperature was generally associated with a decrease in DO value. However this observation did not hold true for all the samples as there are several other factors that may impact on the DO concentration such as effluent's salinity, the presence of organic matter and the turbulence of the discharged effluent[173] (Figure 6.3).

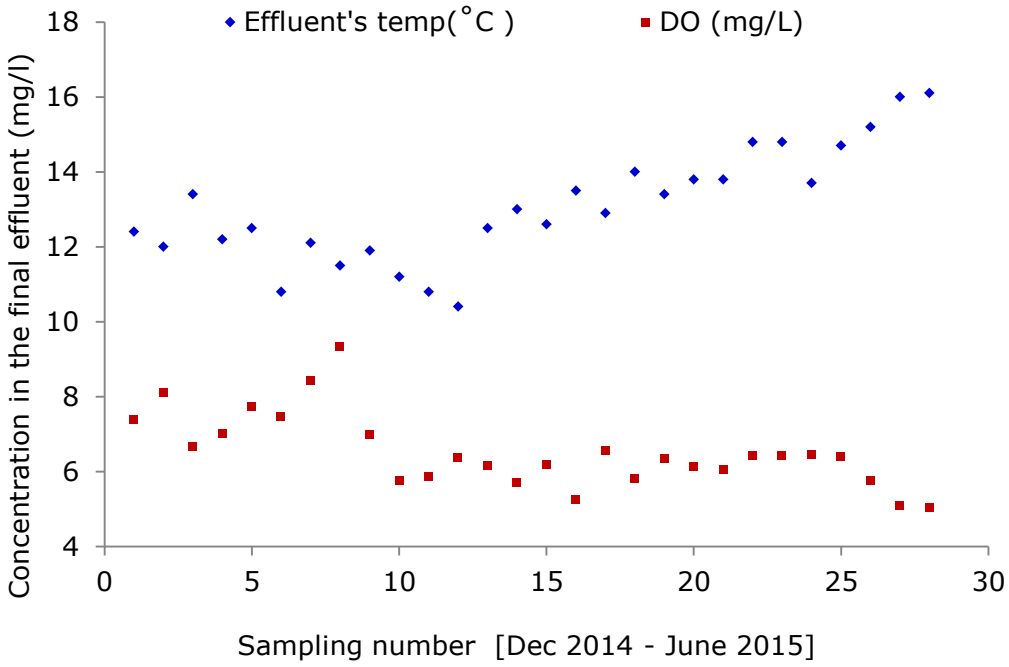


Figure 6.3 The values of the dissolved oxygen (DO) and the temperature of the final effluent during a 6-month period (Dec 2014- June 2015).

During the sampling period, Dec 2014 – June 2015, at the studied WWTP, the pH value of the final effluent remained relatively constant and within the neutral range: pH_{Ave}=7.4±0.2 (Figure 6.4). The permitted pH range for treated wastewater effluents discharged into the aquatic environment is usually between pH=6.5 to pH=8.5 [171] which is the typical pH range for municipal wastewater with limited –if any- industrial inputs. The majority of the microorganisms of the biological processes live and thrive within a narrow pH range (typically 6 to 9), acidic wastewater with low pH (less than 6) is difficult to treat through biological processes and the pH of its final effluent should be neutralised to avoid any negative impacts on the surrounding environment post the discharge[171]. As mentioned previously in Section 2.5.5, this work investigates the feasibility of adding another biological treatment unit (enzyme -based) at the end of the secondary treatment process of a WWTP. Figure 6.4 demonstrates that the pH of the currently produced final (secondary) effluent is within the pH range of the majority of the municipal WWTPs and that the effluent itself is suitable to be treated biologically.

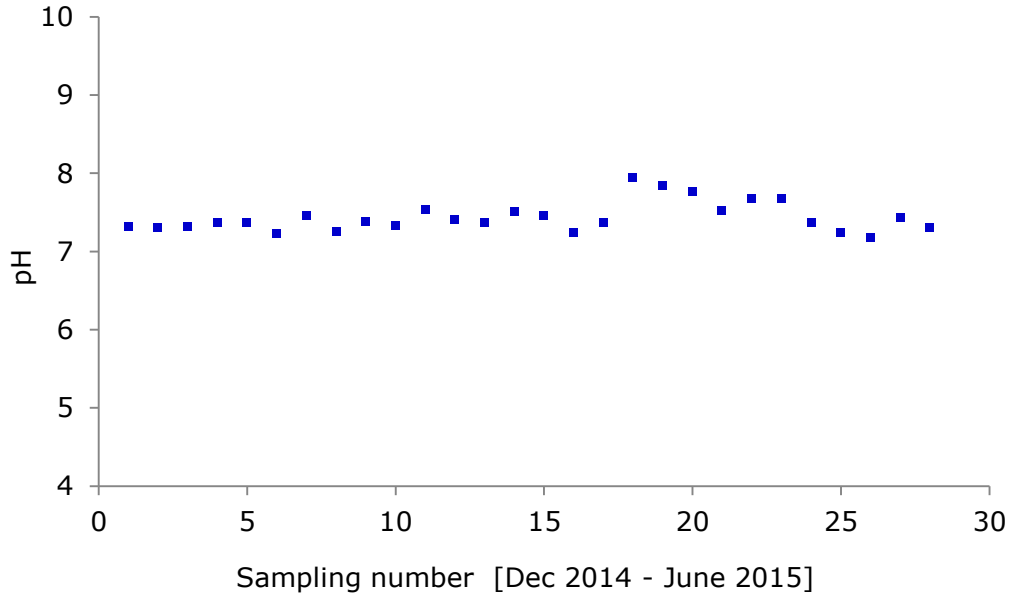


Figure 6.4 The pH values of the treated wastewater effluent during Dec 2014-June 2015.

Another study acknowledged the complexity of the relationship between various wastewater parameters in activated sludge WWTP of a hospital, and worked on developing a model to predict the TSS and the COD of the effluent based on four wastewater influent characteristics: pH, temperature, TSS and COD. The results showed that the pH of the influent had a significant impact on both effluent TSS and effluent COD. However, no clear relationship was identified between the COD and TSS of the final effluent themselves[174]

6.4 EVALUATING THE IMPACT OF WASTEWATER VARIABILITY ON LACCASE-BASED TREATMENT

6.4.1 Benchmarking Wastewater Effluents

The maturity of any developing technology can be assessed using a technology management tool called Technology Readiness Levels (TRLs) (Figure 6.5). Each evolving technology has to pass through 9 different levels starting from Basic principles (TRL1) and finishing at actual technology qualified through successful mission operations (TRL9) [175].

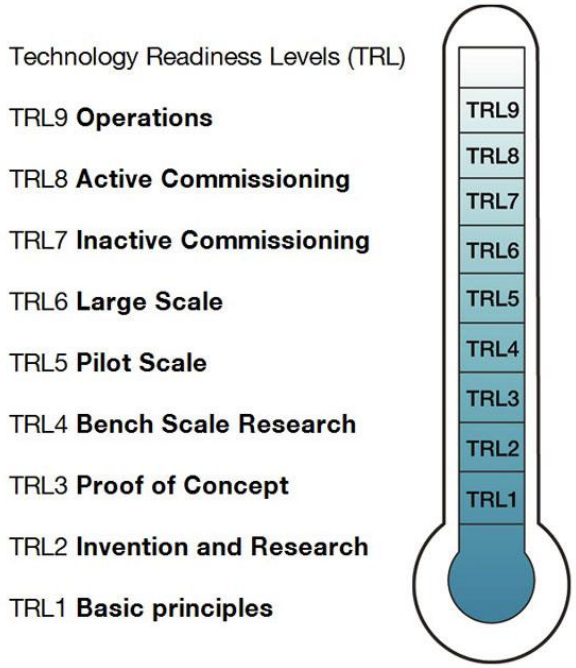


Figure 6.5 Technology Readiness Levels (TRLs)[176].

To ensure that a treatment technology utilising the enzyme laccase is able to progress up the TRLs, it is essential to assess its performance in environmentally relevant matrix such as wastewater effluent. Contrary to many other process industries, wastewater is inherently complex and its characteristics vary both temporally (throughout the day and seasonally) and spatially (within a site at the different treatment stages, and from site to site) [42]. Factors such as rainfall, temperature, the implemented secondary treatment (e.g. trickling filter, conventional activated sludge, advanced activated sludge designs) and influent characteristics (e.g. the presence of trade inputs, the size and the population equivalent of the serviced catchments), will directly impact on the performance and efficiency of laccase-based treatment in removing bioactive chemicals from the wastewater matrix.

The efficiency of laccase-based treatment in a specific wastewater effluent is influenced by multiple factors such as the investigated water quality parameters in Section 6.3. However none of those parameters represents the amenability of wastewater effluent to be treated by the enzyme laccase efficiently. With this in mind, a different parameter was needed to assess the potential performance of laccase-based treatment for a specific WWTP. As a

result, a new water quality parameter (termed ‘benchmark’) was designed using an environmentally relevant substrate (estrone (E1)) to quantify the efficiency of laccase-based treatment with respect to wastewater temporal variability. The conditions for the new water quality parameter are described in Methods and Materials Section 3.12. The laccase concentration of the benchmark was selected by scoping studies to ensure that the achieved removal efficiency of E1 in the benchmark was above 0% and below 100% in wastewater effluent under 20˚C and after 1 hour of contact time. The studies showed that 5 U/ml was a suitable laccase concentration to achieve E1 removal efficiency between 70% -80%, depending on quality of the wastewater effluent. The selected contact time of the benchmark was relatively short (1 hr) to ensure that the benchmark was not a highly time consuming parameter.

The results for the benchmark water quality parameter over a 6-month period between Dec 2014 and June 2015 showed that the variation in the wastewater effluent has a direct impact on the efficiency of laccase-based treatment. To identify the impact of the effluent’s variability on laccase performance, benchmark experiments were performed (in duplicate) over a 6 month period. Estrone removal percentage of the benchmarks varied between 71.74% and 87.65% throughout the sampling period with an average across the sampling period of 79.80 ± 3.74% (Figure 6.6).

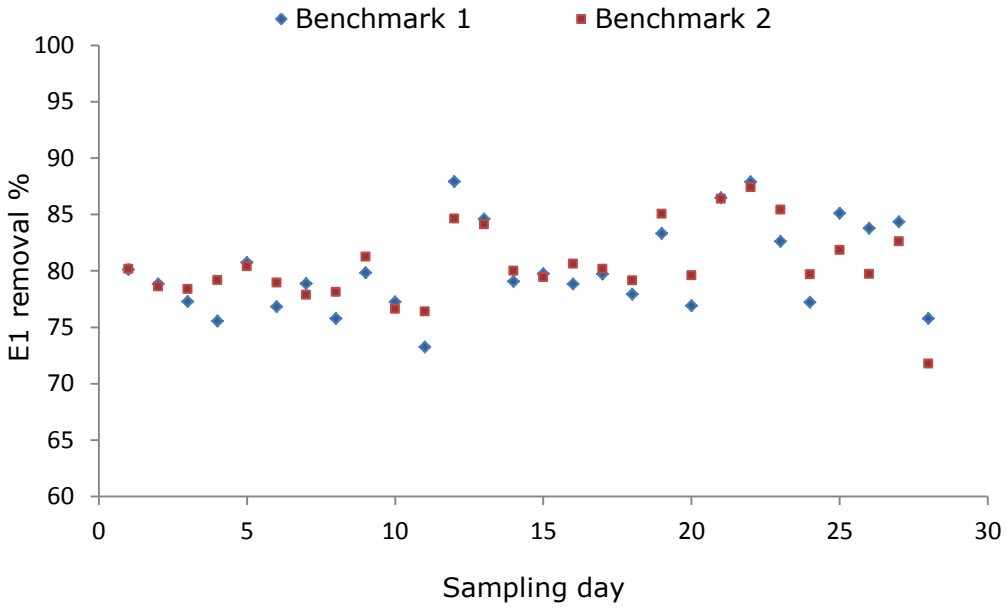


Figure 6.6 The achieved estrone (E1) removal percentage for each performed benchmark in filtered wastewater effluent under the following conditions: 5 U/ml laccase conc., Temp=20˚C, contact time=1 hr and initial E1 conc. =0.5 mg/l, during Dec 2014- June 2015. Bench mark 1 and benchmark 2 represent the results of the duplicates.

The difference within a duplicate demonstrates the variability observed due to the biological component (matrix and enzyme) even when all other conditions are the same. The data in Figure 6.6 shows that the efficiency of laccase-based treatment varies temporally with the wastewater effluent. This new water quality parameter is also called a benchmark because benchmarking is particularly important when studying the impact of a specific factor e.g. temperature or contact time, on laccase performance using effluent samples collected on different days. Omitting the variability of the effluent during such studies can mask the actual impact of the studied factor on laccase performance and lead to incorrect conclusions. Performing a benchmark water quality parameter to quantify laccase ability to degrade E1 in that sampled wastewater matrix (effluent) allows a meaningful comparison between laccase experiments that are conducted on different dates.

Studies evaluating laccase-based treatment for remediating bioactive chemicals commonly use ‘clean’ or non-environmental samples [13, 96]. However in

recognition of needing to understand how a laccase-based treatment technology may operate in reality, several studies have started evaluating laccase performance in environmental matrices[3, 11, 177]. The complexity of wastewater matrix (filtered wastewater effluent) was highlighted in some of those studies by demonstrating a decrease in laccase activity in wastewater in comparison with its activity in clean matrix[3]. However, the temporal variability of the used wastewater and its potential impact on the performance of laccase-based treatment was never acknowledge or quantified in any of them. Lloret et al. (2013) mentioned that the wastewater effluent was collected from the outlet of a municipal WWTP, however it was unclear if all the utilised wastewater samples were collected from a single trip or multiple sampling trips. Similar ambiguity about the number of wastewater sampling trips was observed in another laccase-based treatment study[11]. Utilising wastewater that was sampled on different days, in laccase-based treatment study without addressing its temporal variability can affect the validity of the obtained results as the compositions of wastewater matrix varies with time (as discussed in Section 6.3). In addition, the concentration of various constituents and potential laccase inhibitors in wastewater matrix also varies temporally, this was thoroughly demonstrated during the UKWIR Chemical Investigation Programmes where the wastewater effluents from 162 WWTPs were sampled during a one year period with sampling frequency of 14-28 samples per site[178].

The designed benchmark above allows the researcher to (i) realistically assess laccase-based treatment in a complex and variable matrix such as wastewater, and (ii) understand the actual range of the temporal wastewater variability that may influence this potential treatment technology in real WWTP.

6.4.2 Benchmarks: Filtered vs Unfiltered Wastewater Effluents

As demonstrated in Section 6.4.1, performing a benchmark after each sampling trip is essential to account for the variability of the wastewater matrix and the impact on the treatment efficiency. It is worth noting that all wastewater experiments were performed in filtered wastewater effluent (through 1.2 µm GMF filter), unless otherwise specified (see Section 3.14). This was to ensure the effluent did not contain any coarse materials e.g. fibres or insects that may cause additional variability between the duplicates[3]. However, by filtering the wastewater samples, the TSS was removed from the effluent and potentially simplified the wastewater matrix. Therefore there was a need to compare between filtered and unfiltered benchmark values to gain a better understanding of the degree of the variability between these two sets of experiments (filtered versus unfiltered) as in the real scenario laccase will be subjected to the presence of the TSS in wastewater effluent i.e. the influent into laccase-based treatment unit.

To achieve that, six benchmark experiments were performed in filtered and unfiltered effluents on 6 different sampling dates. The obtained results showed that the difference between the two sets of samples (filtered vs unfiltered) for the 6 days sampled was an average of 1.6 ± 0.6% (Figure 6.7) and part of that difference can be attributed to the analytical variability. The very limited impact of the filtration process on the efficiency of laccase-based treatment may be attributed to the fact that the constituents that affect laccase-based treatment are dissolved within the wastewater and thus unaffected by filtration. The results also demonstrate that filtering the wastewater effluent during the bench scale studies does not reduce the environmental relevance of these experiments to the WWTP environment.

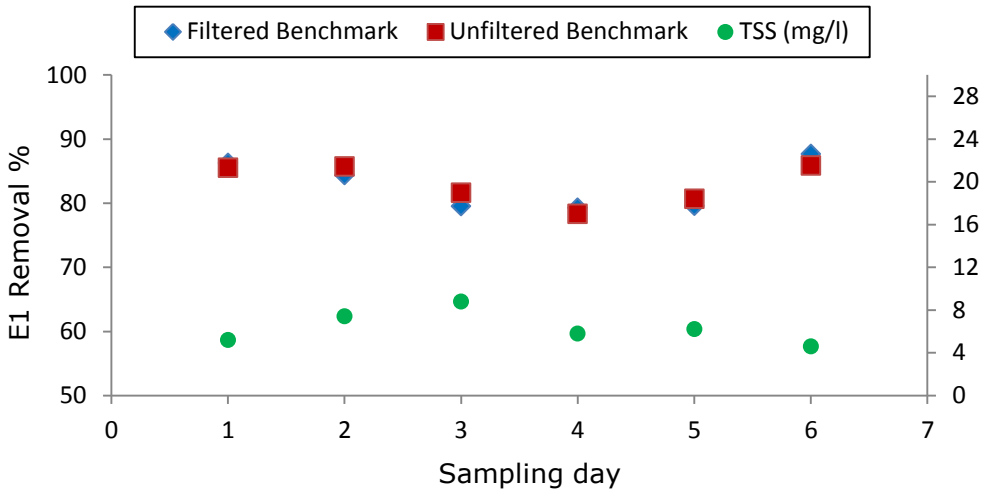


Figure 6.7 Estrone (E1) removal percentage by laccase under the following conditions: 5 U/ml laccase conc., Temp=20˚C, contact time=1 hr and initial E1 conc. =0.5 mg/l, using both filtered and unfiltered wastewater effluents, duirng Dec 2014- June 2015.

The majority of current studies have no appreciation of the impact of matrix variability on the performance of the investigated technology, this partially attributed to the fact that many experiments are performed in clean or synthetic wastewater matrices rather in actual wastewater matrix. The new parameter is performed in actual wastewater matrix and can quantify the change in wastewater characteristics and relate it back to the efficiency of laccase-based treatment which is essential when studying the actual impact of any factor on the efficiency of laccase-based treatment. Benchmark experiment can be used to identify the range of wastewater variability and subsequently the window in which the laccase-based treatment should be able to operate.

6.5 INFLUENCE OF MATRIX pH ON LACCASE ACTIVITY

The enzyme laccase exhibits a higher catalytic activity at acidic pHs such as pH 5[116]. As a result, many studies have chosen acidic pHs to evaluate laccase performance for removing bioactive chemicals such as the case study, estrone. However, the wastewater matrix has a higher pH, for example effluent from a municipal WWTP is typically within the 6.5-8.5 pH range[171] and as the studied WWTP in this work had an effluent with pH of 7.4 ± 0.2 (Figure

6.4). Although it is possible to modify the pH of the wastewater to make it more acidic and therefore more suitable for laccase, this is unfeasible at industrial scale due to the associated costs and the inclination of Water companies to minimise the use of chemicals during the treatment process. Several laccase-based treatment studies have performed their experiments at the optimum pH for laccase activity (acidic pH), rather than at relevant pH to WWTPs (pH 6.5 – 8.5). Therefore this work compared the impact of two different pHs relating to laccase optimal performance (pH 4.5) and the wastewater environment (pH7), respectively, on the efficiency of laccase-based treatment to degrade E1.

Table 6.2 combined with Figure 6.8 demonstrate the significance of the pH value on the specific activity (SA) of laccase where the SA at pH 4.5 is about 300 times higher than the SA at pH 7. This shows that at pH 4.5 laccase was significantly more active and therefore more efficient in oxidising/ degrading the substrate (ABTS). Selecting a relevant pH to WWTP during laccase-based treatment studies is essential to obtain relevant results to the WWTP scenario. Otherwise the efficiency of laccase-based treatment in removing the target pollutants will be extremely over estimated. According to Lloret et al. (2013), laccase activity at pH 4 was 60% higher than laccase activity at pH 7 [9]. Therefore the efficiency of laccase-based treatment at acidic pHs such as pH 4.5-5[12, 13]does not provide a clear idea about the efficiency of that treatment at WWTP relevant pH (6.5 -8.5). Similar experiments but in wastewater effluent were discussed later in Section 6.6.

Table 6.2 The impact of the pH on laccase activity using ABTS as a substrate. The slope values correspond to mean values of triplicate with a standard deviation less than 0.5%.

pH	Buffer	Slope	Laccase conc. (mg/ml)	Specific activity (U/mg)
4.5	Ammonium Acetate	0.0247	0.01	4.1188
7.0	Phosphate	0.0001	0.01	0.0134

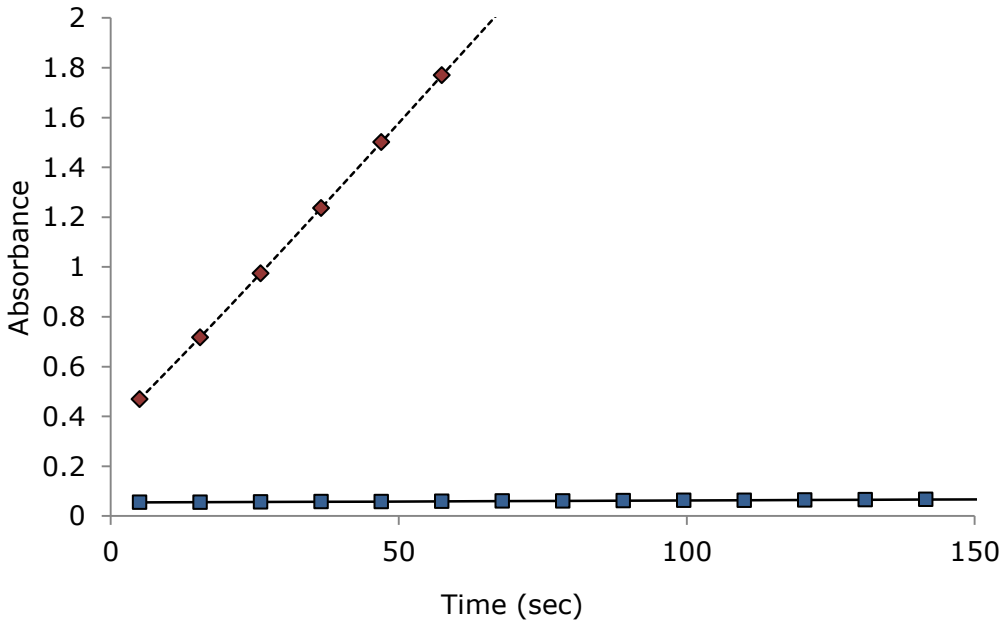


Figure 6.8 Comparison between laccase activity at pH 4.5 (red) and pH 7 (blue) at 20˚C using 5mM ABTS as a substrate. The points represent an average of three readings with coefficient of variance less than 2%.

The final aim of laccase-based treatment is to degrade a wide range of bioactive chemicals such as steroids and pharmaceuticals in wastewater under environmentally relevant conditions. The impact of pH on laccase activity using a standard substrate such as ABTS, is different from its impact in the presences of environmentally relevant substrate. This point was demonstrated by Kurniawati et al. (2008) where pH 6 was determined as the optimum pH for phenol conversion[85], while the optimum pH for laccase activity using ABTS was reported by one study to be pH 3[9]. Another laccase-based treatment study reported that the maximum conversion of bisphenol A was achieved at pH 5[116]. The above points demonstrate that the optimum pH for the enzymatic treatment of a certain pollutant depends not only of the type of the used enzyme, but also of the type of the target pollutant. In this work, the efficiency of laccase-based treatment at removing E1 at a relevant pH to WWTP (pH 7) was investigated without focusing on the optimum pH for the enzymatic degradation of steroids, which was reported to be pH 6[11].

Scoping studies were conducted to determine a suitable range for laccase concentration that could be applied to both buffers (pH 4.5 and pH 7) and provide a range of E1 removal efficiencies above 0% and below 100%. Using the same laccase concentration in both buffers allows a direct comparison between the impacts of two pHs on E1 removal by laccase. The results showed that laccase efficiency for removing E1 from this matrix (buffer) at pH 4.5 was always higher than the removal efficiency at pH 7 (Figure 6.9). However, the difference between the percentage of E1 removed for pH 4.5 and pH 7 decreased as laccase concentration increased. This result confirmed literature observations, and could be explained by several factors:

(a) Higher pH values have higher concentrations of hydroxide ions. These ions bind to the type 2/ type 3 (T2/T3) trinuclear copper sites (see Section 2.4.2 for details) and inhibit laccase activity by interrupting the internal electron transfer from the T1 to the T2/T3 centres resulting in lower laccase catalytic activity at higher pHs [123, 179].
(b) Previous studies show that the pH value affects the catalytic activity of laccase and its stability. Low pH such as pH 4.5 provides a relatively high catalytic activity, which is normally associated with high removal rates of the target pollutant and almost a full deactivation of laccase by the end of the contact time. Contrarily, almost no loss in laccase stability was observed at neutral pH (pH 7), but lower removal efficiencies were achieved [62].
(c) Laccase is less stable at lower concentrations [180] therefore the difference between E1 removal efficiency at pH 4.5 and at pH 7 decreases as the concentration of laccase increases.

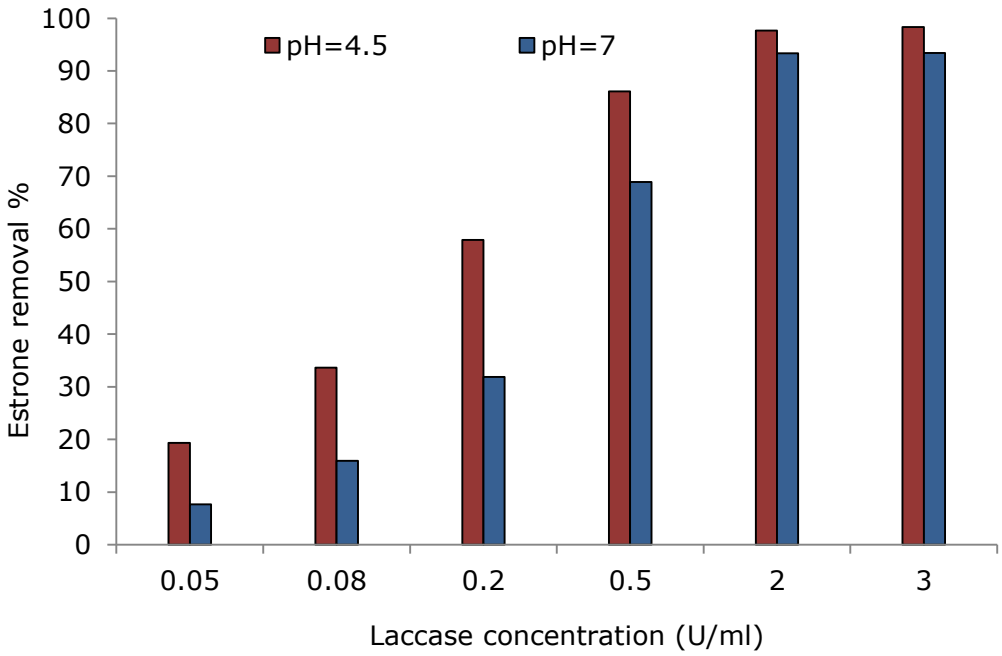


Figure 6.9 The impact of the optimum pH (pH 4.5) and the environmentally relevant pH (pH 7) on the removal efficiency of estrone (E1) by laccase. Experiments were performed either in ammonium acetate buffer (pH 4.5) or in phosphate buffer (pH 7) under the following conditions: temperature=20˚C, contact time=1hr, estrone conc.=0.5 mg/l. The variability between the duplicates was less than 2%.

6.6 IMPACT OF WASTEWATER MATRIX ON LACCASE ACTIVITY

Wastewater contains a wide range of constituents that may impact on laccase performance and affect its activity, potential laccase inhibitors and mediators were discussed in detail in Sections 2.6 and 2.4.4, respectively. The temporal variability of wastewater was also demonstrated in Section 6.4.1, where the achieved E1 removal efficiency varied from day to day based on the composition of the wastewater at the time of sampling. Municipal WWTPs remove a large percentage of laccase-consuming compounds and inhibitors which make the final effluent a more suitable matrix for laccase-based treatment that may potentially be implemented at the end of conventional treatment process (as discussed in Section 2.5.5). Lloret, et al. (2013) also studied the matrix effect on laccase activity by comparing the degradation of E1, E2 and EE2 by laccase in buffer and filtered wastewater effluent. The results showed a 20% reduction in laccase activity and a loss of E1, E2 and EE2 removal efficiencies in wastewater matrix[3]. These observations were

explained by the presence of other constituents in wastewater that could compete with the target compounds for the enzyme. To evaluate the impact of the used wastewater in this work on laccase activity, experiments were performed in two matrices:

a) Phosphate buffer at pH 7 (see Section 3.7 for details)
b) Filtered wastewater effluent at pH 7

The phosphate buffer was used as a control representing a clean water matrix, it is also one of most used buffers in the literature within the range of pH 5.8 and pH 8.0 [3, 96]. The performed experiments showed that the degradation of E1 by laccase was strongly influenced by the type of the used matrix (Figure 6.10). The low removal efficiency of E1 in wastewater matrix (70% less than the achieved E1 removal efficiency in phosphate buffer at 2 U/ml of laccase concentration) can be attributed to a wide range of constituents that either competes for the active site of laccase or inhibits it[3]. Different result was reported by Auriol et al. (2007) when they studied the wastewater matrix effect on the efficiency of laccase-based treatment in removing E1, E2, E3 and EE2. The study found that at pH 7, 25±1˚C laccase-based treatment was not significantly affected by the municipal wastewater matrix in comparison with phosphate buffer matrix. However it is worth noting that a much higher laccase concentration (20 U/ml) was utilised during their study[11].

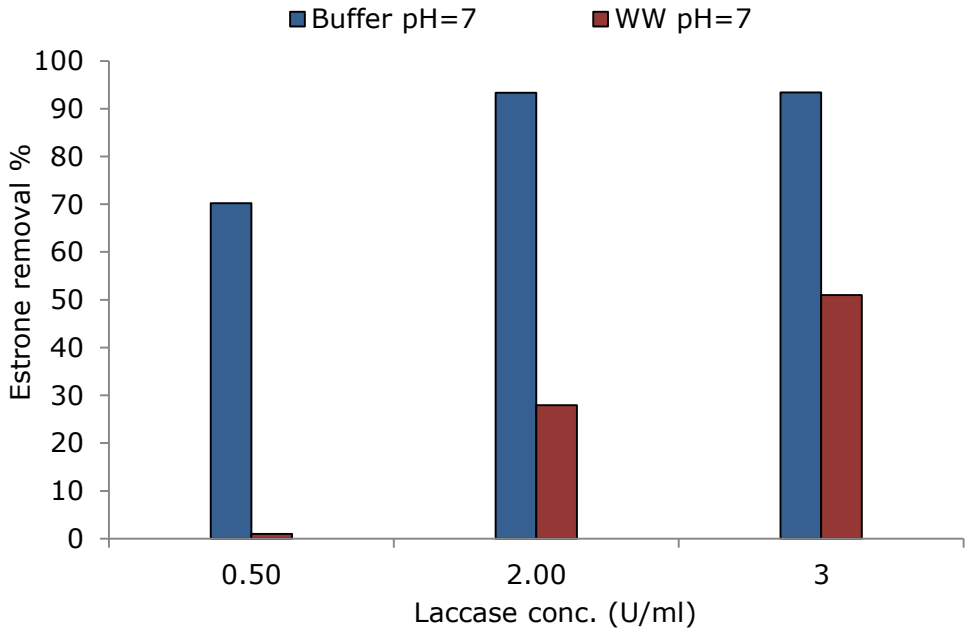


Figure 6.10 The average (n=2) removal efficiency of estrone (E1) by laccase in phosphate buffer at pH 7 and in filtered wastewater effluent at pH 7 under the following conditions: temp=20˚C, pH 7, contact time=1 hr, E1 conc.=0.5 mg/l, Laccase concentrations: 0.5 U/ml, 2 U/ml and 3 U/ml. The difference in E1 removal was less than 3% between wastewater duplicates and less than 2% between the buffer duplicates.

The performance of laccase-based treatment in wastewater was evaluated at pH 4.5 (the most used pH in laccase activity assays[117, 181]) and at pH 7 (a relevant pH to WWTP). The removal of E1 by laccase in wastewater effluent under three different laccase concentrations: 0.5 U/ml, 2 U/ml and 3 U/ml, and 1 hr contact time, was investigated (Figure 6.11).

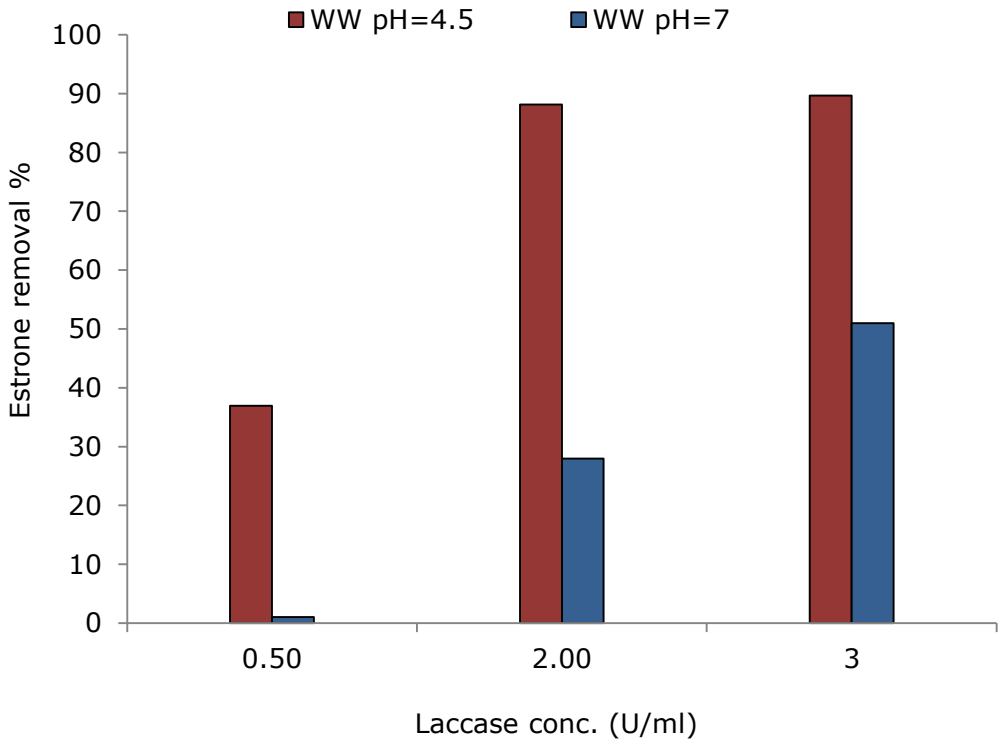


Figure 6.11 The average (n=2) removal efficiency of estrone (E1) by laccase in wastewater effluent at pH 7 and pH 4.5 under the following conditions: temp=20˚C, contact time=1 hr, E1 conc.=0.5 mg/l , Laccase concentrations: 0.5 U/ml, 2 U/ml and 3 U/ml. The difference in E1 removal between the duplicates was less than 3%.

The results from Figure 6.11 shows that modifying the pH of the wastewater from pH 7 to pH 4.5 can improve the removal efficiency of E1 during the 1 hr contact time by 68% using 2 U/ml of laccase concentration. Modifying the pH of wastewater effluent can significantly increase the treatment cost and lead to several environmental implications if the pH of the discharged effluent is not neutralised before being discharged into the aquatic environment. In addition, the stability of the enzyme laccase during the contact time drops rapidly at acid pHs. According to one study the stability of laccase decreased by 80% after 24 hrs contact time at pH 4.5, while at pH 7 and under the same conditions the activity decreased only by 10%[9]. The achieved removal efficiency of E1 at pH 7 can be improved by increasing laccase concentration and/or the contact time. During the contact time, the concentration of E1 decreases and the chances of binding E1 into the active site of laccase become smaller as the

concentration of E1 drops. Therefore increasing the contact time or laccase concentration increases the binding chance between E1 and laccase which will subsequently improves the achieved E1 removal efficiency.

Adjusting the pH of the wastewater can be a cost-effective option only when the full cost of pH adjusting facilities and the used chemicals during that process is equal to/ less than the cost of the additional laccase that has to be used at the natural pH (pH 7) of the wastewater rather than at the adjusted pH 4.5.

There is a large number of available chemicals that can be used to adjust the pH of the wastewater, the final choice will depend on the suitability of a certain chemical for a specific application and the associated cost. The secondary ions of each acid or alkaline must be considered as well, for example the secondary ion of hydrochloric acid (i.e. Chloride) acts as laccase inhibitor which makes this acid unsuitable candidate for pH adjustment in laccase-based treatment. The below points are provided just as an example of potential chemicals that can be implemented within the pH adjustment process. The secondary ions (i.e. Sulfate and Sodium) of the selected chemicals below have no negative impact on the activity of the enzyme laccase.

It is worth noting that the final effluent from each WWTP has different composition and therefore different buffering abilities which means that the required amount of acid/ alkaline to adjust its pH will change greatly between sites. The capital cost of such facilities will also vary between sites.

1) **Reducing the pH of the wastewater to pH 4.5 using acids**

Sulfuric acid (H_2SO_4) is the most commonly used and the least expensive chemical for pH adjustment and neutralization reactions. It is easier and safer to handle than hydrochloric acid and it is more potent than all of the other acids except of the phosphoric acid. However this acid may not be the best option for pH adjustment in calcium-rich wastewater due to the formation of calcium sulfate ($CaSO_4$), also known as gypsum, which can precipitate in the reactor. Sulfuric acid is readily available in concentrations ranging from 25% to 96% from various suppliers. For example, the cost of 96% sulfuric acid

from APC Pure is around £2.8 per litre (when purchasing 25 L container). The required amount of sulphuric acid to adjust the pH from 7 to 4.5 depends on wastewater characteristics and its buffering ability. However a rough theoretical estimation can be provided for a 96% sulfuric acid as below:

The molarity of 96% sulfuric acid is 18 M, and every mole of H_2SO_4 contains 2 moles of hydrogen, as a result the concentration of hydrogen ions $[H^+]$ within this acid is 18*2= 36 M.

The required pH of the adjusted wastewater is pH 4.5 and since $pH=-\log[H^+]$, this equates to $[H^+] = 10^{-4.5}$ M. The required amount of acid to achieve pH 4.5 in 1 m^3 of wastewater can be calculated from the below equation: $C_W \times V_W = C_A \times V_A$

Where ;

C_W : the required concentration of hydrogen ions in wastewater (M).
C_A : the concentration of hydrogen ions in the acid (M).
V_w, V_A: the volumes of wastewater and acid, respectively (liter).

Solving the above equation shows that the required acid volume for each 1 m^3 of wastewater is (at least) 0.88 ml of 96% sulfuric acid which costs less than £2.5×10^{-3} per m^3.

2) Increasing wastewater pH back to pH 7 using basic chemicals

After the enzymatic reaction is completed, wastewater pH has to be raised back to pH 7 before discharging the final effluent into the environment. This procedure is necessary to prevent any negative impact of the acidic wastewater on the aquatic environment and avoid breaching the discharge consent for pH. Sodium Hydroxide (NaOH) is one of the most common alkaline neutralising chemicals in several industries. It is easy to handle, very effective, highly soluble and relatively cheap (£5.75 per litre of 50% (w/w) NaOH).

The molarity of 50% NaOH is 19.4 M, and every mole of NaOH contains 1 mole of hydroxide (OH^-), as a result the concentration of hydroxide ions $[OH^-]$ in NaOH is 19.4 M as well.

The amount of [OH^-] in wastewater at pH 4.5 can be calculated using the following equation:

$$[OH^-] = \frac{1\times10^{-14}}{[H^+]} \qquad \text{[Equation 6.1]}$$

At pH 4.5 the concentration of hydrogen ions is: $[H^+] = 10^{-4.5}$ M, by solving Equation 6.1 we find that at pH 4 the concentration of hydroxide ions is:

$[OH^-]= 3.16 \times 10^{-10}$ M.

This means that the available amount of hydroxide ions is 3.16×10^{-10} M, while the required amount of these ions to achieve pH 7= pOH 7 is 10^{-7} M.

The difference between these two concentrations will be covered using hydroxide ions from NaOH,

Additional required $[OH^-] = (10^{-7}) – (3.16 \times 10^{-10}) = 9.97 \times 10^{-8}$ mol/l.

For 1 m^3 we need: 9.97×10^{-5} mole of hydroxide

For 50% NaOH, there are 19.4 moles of hydroxide in 1 litre → 5.14×10^{-6} litre contain 9.97×10^{-5} moles of hydroxide.

Thus: 5.14×10^{-3} ml of 50% NaOH will be required to raise the pH from pH 4.5 to pH 7 for 1 m^3 of wastewater. The cost per 1 m^3 is less than £0.03.

The above points provide a very rough estimation of the volume of the required chemicals and their cost without accounting for wastewater buffering ability as it varies from site to site. They also show that the cost of the pH-adjusting chemicals is inexpensive and unlikely to act as a limiting factor. However, the capital cost of such facilities may be relatively high. In addition, the cost of removing the secondary ions from the final effluent after laccase-based treatment may have to be considered in some cases.

At the moment, extracellular laccase is mainly produced on a small scale and purchased by research groups investigating the possible applications of this enzyme. The limited demand for laccase makes its production cost relatively

high (≈ £50 per 1 gram of *Trametes versicolour* laccase of a specific activity ≥10 U/mg) and as a result the price of laccase-based treatment technology appears to be quite expensive. However many studies have been investigating how to make the production process of laccase as cost-efficient as possible [182, 183]. One study established that reducing the cost of the culture medium, which accounts for over than 95% of the total production cost, is the most effective way to decrease the overall cost. The study developed a cheaper medium and unlike the conventional one, the new medium does not contain the expensive malt extract in it, laccase pellets were then produced in a 10 litres air-pulsed bioreactor. In addition to the very good performance of these pellets, the new production process reduced the total production cost up to 94.4% per unit volume of wastewater treated. the 95% of the production process cost [182]. This type of studies demonstrates the feasibility of producing the enzyme laccase on a large scale and in a cost-efficient manner.

6.7 EVALUATION OF THE IMPACT OF WASTEWATER INHIBITORS ON LACCASE ACTIVITY

As demonstrated in Section 6.6 wastewater effluent has a strong inhibitory effect on E1 enzymatic removal compared to phosphate buffer. By its nature, wastewater effluent contains a wide range of constituents that may act either as inhibitors or inducers of laccase activity (see Section 2.6). The current literature highlights a wide range of compounds that may impact on laccase activity such as heavy metal and halides[121]. This work focuses on inhibitors that have been detected in several effluents from municipal WWTPs and that are known to have an impact on laccase activity. Based on the above criteria and Table 2.4 and Table 2.5 from Chapter 2, four ions were selected for this study: chloride (Cl^-), copper (Cu^{2+}), iron (Fe^{3+}) and zinc (Zn^{2+}). The impact of the maximum ion concentration in wastewater effluent (according to the findings of the Chemical investigating programme that screened 162 WWTP in the UK) was tested (Table 2.5) as well as a number of other concentrations that were tested by other research papers (Table 2.4).

Four different salts were used to prepare the solutions of the selected ions. A literature review was conducted to identify the suitable secondary ion for each solution to ensure that only the impact of the primary ion i.e. Cl^-, Cu^{2+}, Fe^{3+} and Zn^{2+} was being evaluated. According to Kim et al., sodium (Na^+) and sulfate ions (SO_4^-) did not inhibit laccase catalysed conversion of pollutants[116]. Sodium chloride (NaCl), copper sulfate ($CuSO_4.5H_2O$), ferric sulfate ($Fe_2O_{12}S_3.5H_2O$) and zinc sulfate ($ZnSO_4.7H_2O$) salts were selected as as sources of the studied compounds.

Sulfate solutions of Cu^{2+}, Fe^{3+} and Zn^{2+} in deionised water are all acidic (Table 6.3.) and therefore cannot be used as such to study the ions' impact on laccase activity at neutral/ alkaline pH. This is because the metal hydroxides formed at high pH are not soluble[184]. Therefore, experiments were performed under acidic conditions in ammonium acetate buffer at pH 4.5. Another research group also investigated the impact of several metal ions including Zn^{2+} and Cu^{2+} on the enzymatic degradation of bisphenol A at acidic pH 5 [116].

Table 6.3 pH values of the studied solutions (0.5 g/l of the primary ion) during the inhibition studies. All solutions were prepared in deionised water.

Solution	Solution's pH (temp=20˚C)
Copper Sulfate ($CuSO_4$)	4.7
Zinc Sulfate ($ZnSO_4$)	6.1
Ferric Sulfate ($Fe_2(SO_4)_3$)	2.7
Sodium Chloride (NaCl)	7

This study was designed to assess the impact of several inhibitors on the enzymatic activity of laccase as well as on its removal efficiency of E1. The experiments were performed using two sets of approaches:

1) Laccase activity with ABTS as substrate, determined by UV-vis spectrophotometer

ABTS is the standard substrate that is commonly used to determine laccase activity[154]. The spectrophotometer can be used as a quick diagnostic tool to evaluate the potential impact of certain ions/ concentrations on laccase activity under specific pH and temperature. However one study in the literature has highlighted that this standard laccase activity assay was not suitable to monitor

laccase activity during batch experiments with pollutants such as phenol due to interferences of reaction species with assay colour formation [85]. Similar issue may occur when working with complex matrices such as wastewater effluent that has a residual colour typically attributed to the presence of coloured minerals and dyes, humic breakdown substances and iron.

2) Estrone removal determined by HPLC-UV

Whilst the UV-vis spectrophotometer can be utilised to quickly evaluate the impact of certain ions on laccase activity in the presence of an alternative substrate such as ABTS, evaluating laccase performance in the presence of metal ions and an environmentally relevant substrate such as E1 requires LC-based capability to measure the substrate removal from the matrix. Experiments using HPLC-UV approach were designed to indirectly measure laccase activity based on E1 removal percentage under different scenarios. The initial concentration of both E1 and laccase was constant during the experiments. The impact of each ion on laccase activity was tested individually and under several ion concentrations. Estrone concentration was measured at the start of the reaction and after 1 hr contact time. The removal percentage of E1 during the reaction was calculated and linked to laccase activity.

6.7.1 Influence of Chloride Ions (Cl^-) on Laccase Activity and Estrone Removal Efficiency

As discussed in Section 2.6.2, halide ions such as Chloride (Cl^-), Fluoride (F^-) and Bromine (Br^-) are some of the common inhibitors of laccases, with several research papers reporting a reduction in laccase activity and laccase-based treatment efficiency in the presence of these ions [179, 185, 186]. However the impact of chloride ions on the enzymatic degradation of E1 in actual wastewater matrix has not been yet evaluated. In this work chloride was selected as a representative of the halide group due to its common presence in wastewater matrix. Chloride is essential for many aquatic habitats. However, high levels of chloride can adversely impact on the freshwater organisms and plants by altering their reproduction rates and changing the characteristics of

the local ecosystem[187]. In addition chloride can leach through the soil and affect the quality of the groundwater.

The concentration of chloride in wastewater varies from site to site, it is influenced by the type of the sewerage system within the catchment, the existence and type of industrial wastewater inputs and even the presence of saline infiltration. High chloride concentration can be found in areas where in-house water softening is applied. Water softeners commonly utilise sodium chloride salt (NaCl) to separate minerals from water. As a result the used salt breaks down into sodium (Na^+) and chloride (Cl^-) ions, reaches WWTP and eventually the aquatic environment[188].

Before undertaking the planned experiments on the UV-vis and HPLC-UV it was necessary to identify the realistic chloride concentration in the wastewater of the studied WWTP. To achieve that, chloride concentrations in wastewater effluent from 10 sampling trips were measured to determine the range of chloride concentrations in wastewater effluent that will represent the influent into laccase-based treatment. Figure 6.12 shows the measured chloride content in each wastewater effluent sample and the associated E1 removal % during the benchmark experiment (as described in Section 6.4.1)

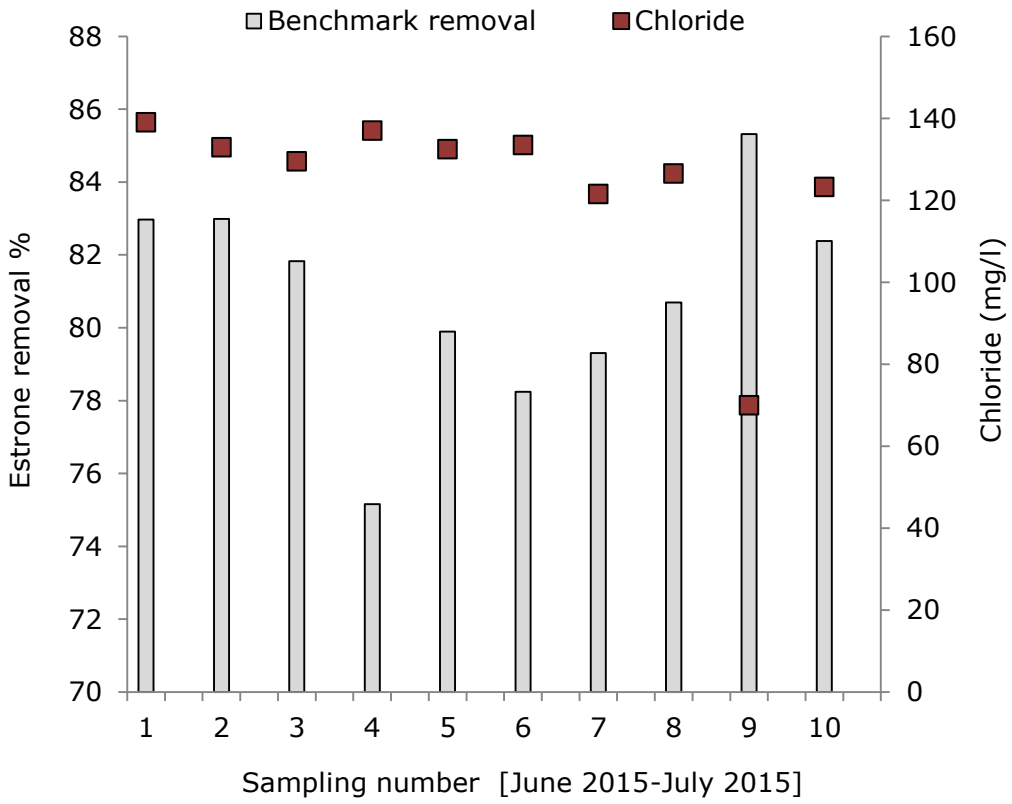


Figure 6.12 The achieved estrone (E1) removal percentages under the following benchmark conditions: 5 U/ml laccase conc., Temp=20˚C, contact time=1 hr and initial E1 conc. =0.5 mg/l, in filtered wastewater effluent during June 2015- July 2015.

Chloride concentration in wastewater effluent varied between 70 mg/l and 139 mg/l (average 125±20 mg/l). However no clear correlation between chloride levels and E1 removal in the benchmark was observed. Figure 6.12 shows that the highest E1 removal percentage (under the benchmark conditions) was 85.3%, this value was achieved when the chloride concentration was at its lowest value of 70 mg/l, but on the other sampling days the relation between E1 removal and chloride concentration was not that clear, this was potentially attributed to the narrow chloride range [121.5 mg/l - 139 mg/l] within the sampled effluent and the presence of other factors that may mask chloride's impact on E1 removal efficiency.

The sampled WWTP in this work had an average chloride concentration in it effluent around 100 mg/l. However, effluents from some other WWTPs may contain a much higher chloride concentration (around 700 mg/l)[188].

Therefore this work has investigated the impact of the average relevant chloride concentration (Cl^- =100 mg/l) to the current WWTP and three higher chloride concentrations: 200 mg/l, 500 mg/l and 1000 mg/l on E1 removal efficiency by laccase. The inhibition percentage for each tested chloride concentration is shown in Table 6.4. The obtained results showed that chloride ions have a clear negative impact on laccase activity, with the inhibition of laccase activity directly proportional to the increasing concentration of chloride ions. The inhibition in laccase activity increased from 13.9% (for 100 mg/l of chloride ions) up to 57.9% (for 1000 mg/l of chloride ions) (Figure 6.13), 50% inhibition in laccase activity at pH 4.5 was achieved in the presence of ≈ 620 mg/l of chloride ions, this concentration comes in line with the previously reported data in the literature where 50% competitive inhibition (K_{ic})of *Trametes versicolor* laccase was achieved using 840 mg/l of chloride ions at pH 6 [123], and since the impact of chloride ions on laccase activity decreases with the raise of pH value, it is expected that the K_{ic} at pH 6 will be higher than the K_{ic} at pH 4.5.Control experiments in the absence of laccase were also performed at identical conditions to the shown experiments in Table 6.4, no increase in ABTS absorption was observed during any of those experiments. This demonstrates that the oxidation of ABTS was fully attributed to laccase activity.

Table 6.4 The average (n=3) inhibition of laccase by different concentrations of chloride ions: 0 mg/l, 100 mg/l, 200 mg/l, 500 mg/l and 1000 mg/l using ABTS as a substrate (0.5 mM). Experiments were performed in ammonium acetate buffer at pH 4.5 at 20˚C.

Chloride conc. (mg/l)	Inhibition %	STDEV	Standard Error*
0	0.00	0.00	0.00
100	13.92	0.88	0.51
200	26.67	0.07	0.04
500	43.90	1.08	0.63
1000	57.90	0.42	0.24

* The standard error is calculated by dividing the standard deviation (STDEV) by the square root of number of measurements (N=3).

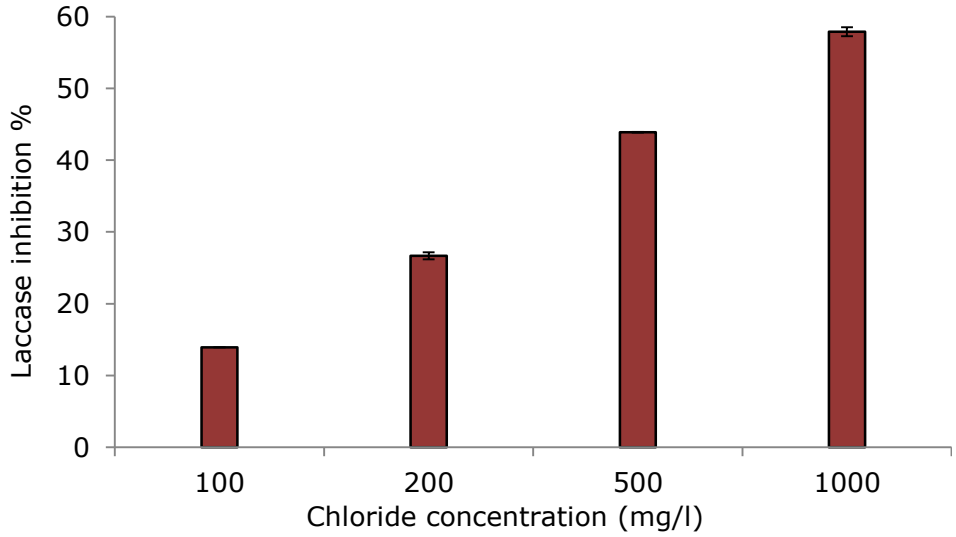


Figure 6.13 The average (n=3) inhibition of laccase by different chloride concentrations: 0 mg/l, 100 mg/l, 200 mg/l, 500 mg/l and 1000 mg/l using ABTS as a substrate (0.5 mM). Experiments were performed in ammonium acetate buffer at pH 4.5 at 20°C.

Table 6.5 shows the reduction in E1 removal under the influence of the previously selected chloride concentrations: 100 mg/l, 200 mg/l, 500 mg/l and 1000 mg/l. Unlike the performed experiments using ABTS as a substrate, chloride concentrations of 100 mg/l have no impact on E1 removal. However, higher chloride concentrations (≥200 mg/l) cause a reduction in E1 removal percentage when compared to the achieved E1 removal percentage in the control sample. The reduction in E1 removal percentage can be as high as 52% for samples with 1000 mg/l chloride ions. These results come in line with obtained data from the UV-vis spectrophotometer.

Table 6.5 The removal efficiency of estrone (E1) by laccase in the presence of different concentrations of chloride ions: 0 mg/l, 100 mg/l, 200 mg/l, 500 mg/l and 1000 mg/l using E1 as a substrate under the following conditions: pH 4.5 (ammonium acetate buffer), laccase concentration = 0.5 U/ml, temperature=20˚C, contact time=1 hr and initial E1 conc. =0.5 mg/l.

Chloride conc. (mg/l)	E1 Removal %	Reduction in E1 removal %
0	85.15	---
100	85.15	0.00
200	74.33	12.46
500	59.24	30.24
1000	40.92	51.81

The previous experiments were performed under the optimum pH value for laccase activity (pH 4.5). To study the impact of chloride ions on E1 removal under wastewater effluent-relevant pH, experiments with pH 7 were performed under the exact same conditions as the experiments with pH 4.5.

The achieved E1 removal efficiency during the control experiment (Cl^-=0 mg/l) demonstrated once again that laccase has a higher activity at pH 4.5. The results showed that mixtures with less than 100 mg/l of chloride ions had no significant impact on E1 removal. Kim et al. (2006) studied the impact of chloride ions (≈886 mg/l) on bisphenol A (BPA) conversion at pH 5 and the results showed that the presence of chloride ions at that elevated concentration caused a 14% decrease in BPA conversion in comparison with chloride-free control[116].

Similar to pH 4.5, higher concentrations of chloride (≥ 200 mg/l) had a noticeable negative impact on E1 removal at pH 7. Figure 6.14 depicts that chloride has a stronger inhibitory effect on laccase at pH 4.5 rather than at pH 7, 1000 mg/l of chloride decreased E1 removal efficiency by 52% at pH 4.5 and only by 15% at pH 7. Similar observation was made by Raseda, Hong [123] who reported that the inhibitory effects of chloride get weaker as the pH of the solution increases. At higher pHs (e.g. pH 7) the inhibition of laccase by hydroxide ions takes effect, while the inhibition of laccase by chloride ions significantly decreases. Both hydroxide and chloride anions share a common inhibition mechanism and the interaction between these two anions reduces the inhibition strength of chloride ions to a great extent. These results demonstrate that decreasing the pH of the wastewater to pH 4.5 (optimum pH for laccase activity) is unlikely to bring the desired increase in treatment efficiency due to the potential presence of halides within that matrix. It also shows that the inhibitory effect of chloride can be 'softened' by retaining a pH of ≥7.

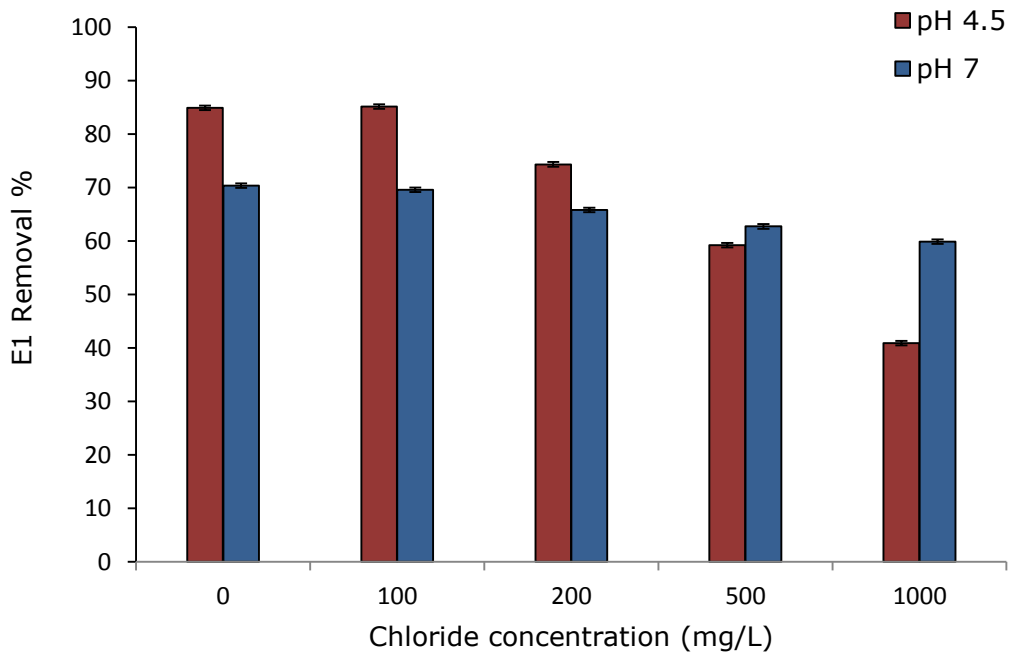


Figure 6.14 Comparison between estrone (E1) removal efficiency by laccase at pH 4.5 and pH 7 in the presence of 4 different concentrations of chloride ions: 100, 200, 500 and 1000 mg/l. Contact time=1 hour, laccase concentration=0.5 U/ml, initial E1 concentration=0.5 mg/l, temperature=20˚C.

Control experiments in the absence of laccase and in the presence of various concentrations of chloride ions showed that E1 removal was fully attributed to laccase activity.

6.7.2 Influence of Copper Ions (Cu^{2+}) On Laccase Activity and Estrone Removal Efficiency

Copper sulfate (CuSO4. 5H2O) was used to prepare 0.5 g/l Cu^{2+} ions standard in AC buffer (pH 4.5). Table 6.6 shows the inhibition percentage for each tested copper concentration.

Table 6.6 The inhibition of laccase by different concentrations of copper ions (Cu^{2+}): 0.05 mg/l, 0.1 mg/l, 10 mg/l, 50 mg/l and 500 mg/l using ABTS as a substrate (0.5 mM). Experiments were performed in ammonium acetate buffer (pH 4.5) at 20˚C.

Copper ions concentration (mg/l)	Inhibition* %	STDEV	Standard error**
0.05	-3.29	1.38	0.80
0.1	-3.92	0.64	0.37
10	-5.68	0.92	0.53
50	-12.51	0.57	0.33
500	2.33	0.68	0.40

* Negative inhibition values mean that the associated concentration has a positive effect on laccase activity.

** The standard error is calculated by dividing the standard deviation (STDEV) by the square root of number of measurements (n=3).

It was highlighted previously in the literature that low concentrations of Cu^{2+} may induce laccase activity[118, 189]. The obtained results demonstrate that Cu^{2+} ions have an inhibitory effect on laccase activity only when their concentration is above 500 mg/l. The lower concentration of Cu^{2+} ions can increase laccase activity up to 12.5% (Figure 6.15). Control experiments in the absence of laccase and in the presence of various concentrations of Cu^{2+} ions, demonstrated that the oxidation of ABTS was fully attributed to laccase activity.

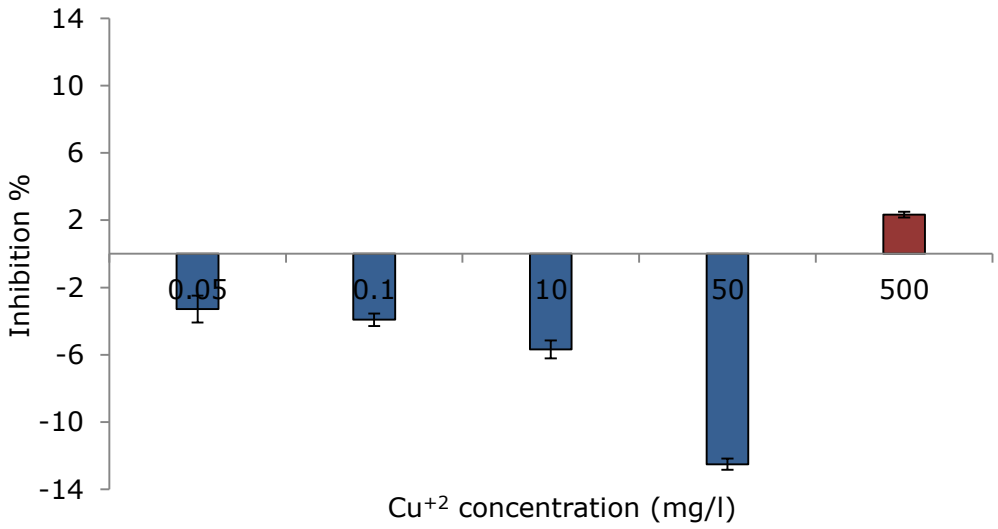


Figure 6.15 The inhibition of laccase by different concentrations of copper ions (Cu^{2+}): 0.05 mg/l, 0.1 mg/l, 10 mg/l, 50 mg/l and 500 mg/l using ABTS as a substrate (0.5 mM). Experiments were performed in ammonium acetate buffer (pH 4.5) at 20°C.

To study the impact of copper ions on laccase activity in the presence of E1, several batch experiments were performed under standard conditions where the initial and final E1 concentrations were determined by HPLC-UV.

The impact of copper ions on E1 removal in the presence of laccase was tested. Four relevant concentrations to the wastewater effluent and industrial effluent were selected: 0.05mg/l, 0.1 mg/l, 10 mg/l and 50 mg/l. The obtained results were presented as reduction in E1 removal % rather than E1 removal % in order to ease their comparison with the previously obtained results from UV-vis. HPLC-UV results showed that copper has a positive impact on E1 removal when its concentration in the reaction mixture is equal or less than 0.1 mg/l. Copper concentrations of 10 mg/l and above have adverse effect on E1 removal % (Figure 6.16).

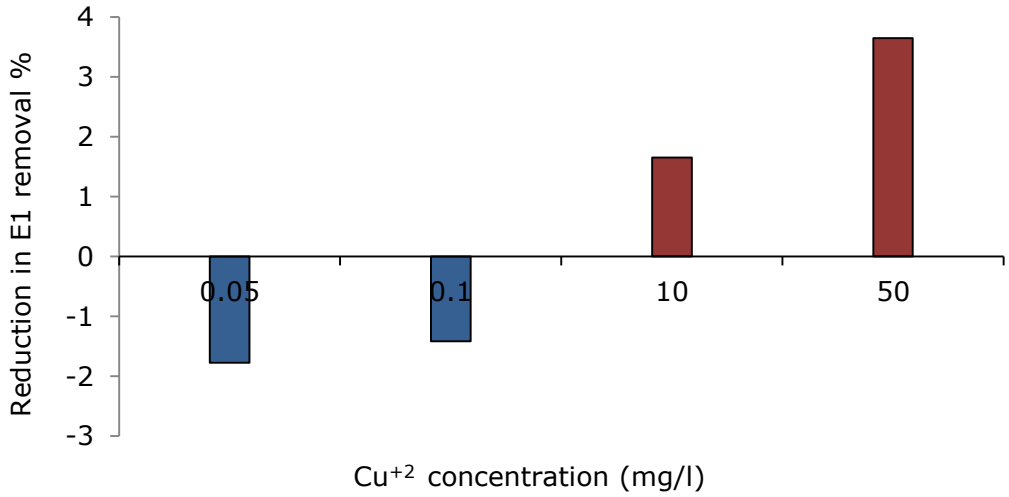


Figure 6.16 A reduction in estrone (E1) removal percentage at 4 different concentrations of copper ions (Cu^{2+}): 100, 200, 500 and 1000 mg/l. Contact time=1 hour, laccase concentration=0.5 U/ml, initial E1 concentration=0.5 mg/l, temperature=20˚C, pH 4.5.The difference between the duplicates was less than 2%.

The behavior and the impact of copper ions on laccase performance change in the presence of E1, low Cu^{2+}concentrations such as 0.05 mg/l and 0.1 mg/l slightly improved E1 removal efficiency. However, Cu^{2+} ions ≥ 10 mg/l exhibited a negative impact on that removal efficiency. Control experiments in the absence of laccase and in the presence of various concentrations of Cu^{2+} ions, demonstrated that E1 removal was fully attributed to laccase activity.

Both analytical approaches (i.e. UV-vis and HPLC-UV) showed that copper ions have a positive impact on laccase activity when their concentration is equal to/ lower than 0.1 mg/l. However for Cu^{2+} concentrations of 10 mg/l and above, there was a discrepancy between the two approaches.

The inducing effect of the low concentrations of Cu^{2+} on laccase activity has been previously reported in the literature [117, 190]. One study found that copper regulates laccase transcription in *T. versicolor*. Increasing Cu^{2+} concentration (maximum tested concentration was 0.16 mg/l) in the growth medium, increased laccase activity. No increase in laccase activity was detected in Cu-free cultures. The author suggested that the enzyme laccase remained inactive in Cu-free cultures because Cu ions were not available for

incorporation to produce functional laccase protein [191]. Considering the structure of *T. versicolor* laccase which contains four Cu atoms essential for its activity, this finding is not surprising. However the exact mechanism of this stimulation process has not been fully understood yet. The inhibitory effect of the high concentrations of Cu^{2+} has been also demonstrated in the literature. Kim, et al. reported that Cu ions in concentrations above 6 mg/l reduced bisphenol A (BPA) conversion by interrupting the electron transport system of laccase [116]. Measuring laccase activity in the absence of environmentally relevant substrate such as E1 may not provide the right idea about the impact of certain ions on E1 removal efficiency. As a result HPLC-UV experiments are a better representative of laccase ability to remove E1 in presence of other chemicals.

6.7.3 Influence of Iron (Fe^{3+}) on Laccase Activity

The impact of Fe^{3+} on E1 removal efficiency was investigated. Ferric sulfate solution of 0.5 g/l was prepared in AC buffer and used as a source of Fe^{3+} ions (see Section 3.11 for details).

A range of Fe^{3+} concentrations were studied 0.1 mg/l, 0.3 mg/l (wastewater effluent relevant concentrations), 10 mg/l, 50 mg/l and 100 mg/l. Control experiments in the absence of laccase and in the presence of various concentrations of Fe^{3+} ions, demonstrated that the oxidation of ABTS was fully attributed to laccase activity.

The collected data from the UV-vis spectrophotometer showed that laccase activity was negatively affected by Fe^{3+} ions at concentrations equal to/ above 0.3 mg/l. The maximum inhibition in laccase activity was achieved using 10 mg/l of Fe^{3+} ions. Increasing the concentration of Fe^{3+} ions above 10 mg/l did not increase laccase inhibition (Figure 6.17). Several research papers reported the inhibitory effect of Fe^{3+} on laccase activity [115, 192] and on the enzymatic substrate conversion [116]. The inhibition mechanism of Fe^{3+} is suggested to be very similar to Cu^{2+}, where the metal ions interrupt the electron transfer system of the laccase [121].

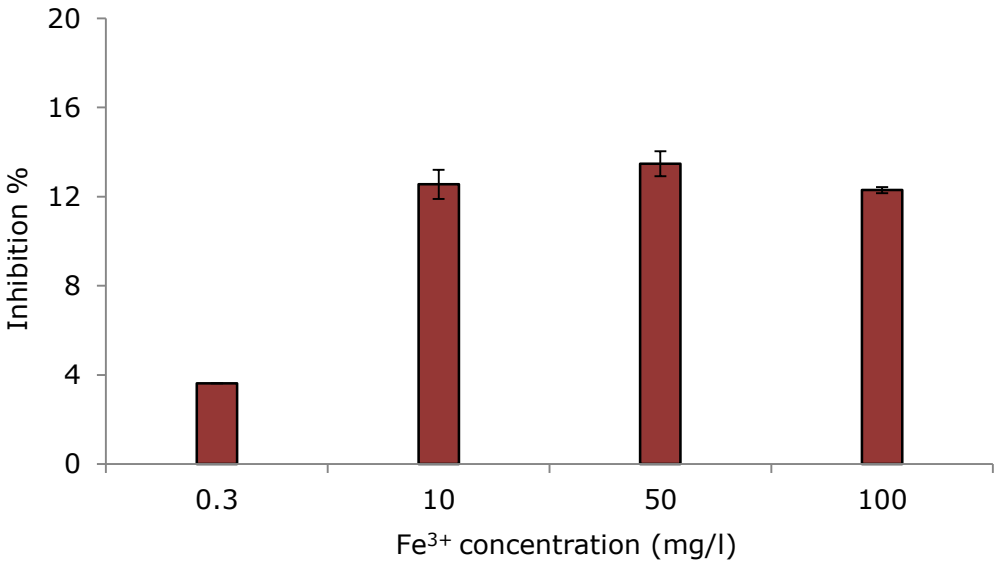


Figure 6.17 The inhibition of laccase by different concentrations of iron ions (Fe^{3+}): 0.3 mg/l, 10 mg/l, 50 mg/l and 100 mg/l using ABTS as a substrate (0.5 mM). Experiments were performed in ammonium acetate buffer (pH 4.5) at 20°C.

The impact of Fe^{3+} on laccase activity in the presence of E1 was studied using HPLC UV. Control experiments with: E1 and Fe^{3+} in the absence of laccase, showed that there is a potential reaction between Fe^{3+} ions, HCl and E1 which affects E1 concentration even in the absence of laccase (Table 6.7).

The concentration of E1 at T=0 was measured in two sets of control experiments:

a) One ml of 0.5 mg/l of E1 solution prepared in AC buffer and mixed with 25 µl of HCl after being placed in HPLC-UV cuvette ready for analysis.
b) One ml of 0.5 mg/l of E1 solution prepared in AC buffer that contains 50 mg/l of Fe^{3+} ions, mixed with 25 µl of HCl after being placed in HPLC-UV cuvette ready for analysis.

Table 6.7 The impact of iron ions (Fe^{3+}) on estrone's (E1) concentration in the presence of hydrochloric acid (HCl) in ammonium acetate buffer (pH 4.5): (a) E1 solution mixed with 25 µl of HCl, (b) E1 solution with 50 mg/l of Fe^{3+} ions mixed with 25 µl of HCl.

Experiment	E1 conc. at T=0 (mg/l)
a	0.47
b	0.01

Experiment (a) demonstrates the actual E1 concentration in the reaction mixture, E1 concentration of experiment (b) should be similar to experiment (a) unless Fe^{3+} ions have a direct or indirect impact on E1 concentration.

Table 6.7 shows that 50 mg/l of Fe^{3+} ions or its mixture with HCl significantly reduces E1 concentration (98% reduction) in the reaction mixture which makes it impossible to utilise this approach to evaluate Fe^{3+} impact on E1 removal efficiency by laccase.

The exact mechanism of E1 removal in the presence of Fe^{3+} ions and HCl is unknown. However it could be associated with Fenton reaction that has been successfully used to degrade a wide range of pollutants such as pesticides, phenols and steroids [193]. Fenton reaction is based on the fact that some metals such as iron have a strong catalytic power to generate highly reactive hydroxyl radicals ($^{\bullet}OH$). This reaction utilises a solution of hydrogen peroxide with ferrous (Fe^{2+}) ions as a catalyst that is used to oxidise steroids or any other pollutants [194]. Fe^{2+} is oxidized by hydrogen peroxide (H_2O_2) to Fe^{3+} forming a hydroxyl radical, Fe^{3+} is then reduced to Fe^{2+} by another H_2O_2, forming water as byproduct and 2 oxygen radical species that oxidise the target pollutant e.g. E1.

6.7.4 Influence of Zinc (Zn^{2+}) on Laccase Activity and Estrone Removal Efficiency

Zinc sulfate standard of 0.5 g/l as Zn^{2+} ions was prepared in AC buffer. The standard was used to make several sub-solutions with different Zn^{2+} concentrations: 0.05 mg/l, 0.15 mg/l (concentrations in wastewater effluent), 10 mg/l, 50 mg/l, 100 mg/l and 200 mg/l. The UV-vis experiments showed that

the lower concentrations of Zn^{2+} ions (≤100 mg/l) did not have a significant impact on laccase activity while higher concentrations such as 200 mg/l inhibited laccase activity by ≈ 9% (Figure 6.18). The negative impact of elevated Zn^{2+} concentrations (> 65 mg/l) on *T. hirsute* laccase activity has been reported by Couto in 2005 [115]. Another study with *T.versicolor* laccase found that Zn^{2+} concentrations less than 65 mg/l had no significant impact on BPA conversion by laccase [116].

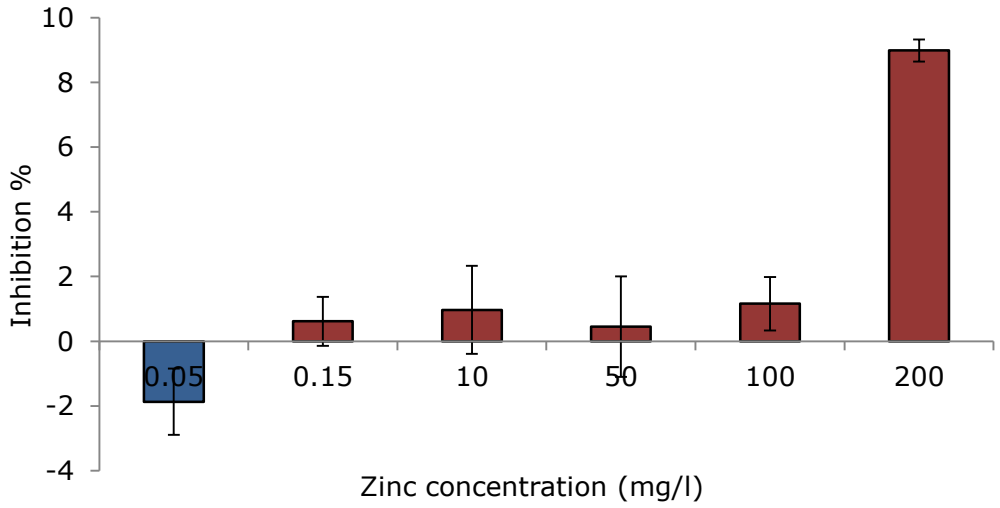


Figure 6.18 The inhibition of laccase by different concentrations of zinc ions (Zn^{2+}): 0.05 mg/l, 0.15 mg/l, 10 mg/l, 10 mg/l, 100 mg/l and 200 mg/l using ABTS as a substrate (0.5 mM). Experiments were performed in ammonium acetate buffer (pH 4.5) at 20°C.

Control experiments in the absence of laccase and in the presence of various concentrations of Fe^{3+} ions, demonstrated that the oxidation of ABTS was fully attributed to laccase activity.

Experiments in the presence of E1 were performed only on the lowest 4 concentrations of Zn^{2+} ions (0.05 mg/l, 0.15 mg/l, 10 mg/l and 50 mg/l) as they are considered the most relevant to the municipal wastewater environment (see Table 2.4 Section 2.6) (Figure 6.19). Control experiments in the absence of

laccase and in the presence of various concentrations of Fe^{3+} ions, demonstrated that E1 removal was fully attributed to laccase activity.

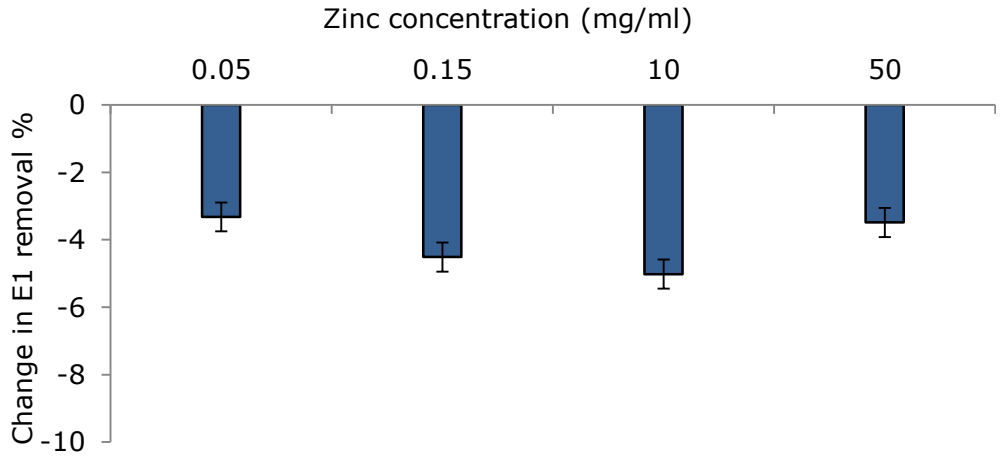


Figure 6.19 A reduction in estrone (E1) removal percentage at 4 different concentrations of zinc ions (Zn^{2+}): 0.05, 0.15, 10 and 50 mg/l. Contact time=1 hour, laccase concentration=0.5 U/ml, initial E1 concentration=0.5 mg/l, temperature=20˚C.

All the tested concentrations seemed to have a slight positive impact (< 5%) on E1 removal efficiency (Figure 6.19). However, increasing Zn^{2+} concentration did not subsequently improve E1 removal efficiency. These findings come in line with the recent literature where a limited positive impact or no impact of Zn^{2+} ions on laccase activity was reported[116, 117].

6.8 CONCLUSIONS

- Wastewater is a complex and inherently variable matrix, the standard water quality parameters such as COD, DO, TSS and pH are used to characterise this matrix in the Water Industry and these parameters demonstrate matrix variability temporally. Understanding the variability of this matrix is essential to develop new treatment technologies that can efficiently operate in this challenging environment that varies both temporally and spatially.
- Benchmark wastewater quality parameter was developed for this work to quantify the temporal variability of wastewater effluent and understand its impact on the performance of laccase-based treatment.
- Unlike other water quality parameters, the wastewater benchmark represents the amenability of wastewater to be treated by laccase using an environmentally relevant substrate.
- The potential location of laccase-based treatment in this work is at the end of the conventional WWTP (at the end of the secondary treatment stage). The pH of the secondary wastewater effluent is typically around pH 7, which is far from the optimum pH (pH 4.5) for laccase activity. The specific activity (SA) of laccase at pH 4.5 is about 300 times higher than its SA at pH 7. However, previous studies showed that the catalytic activity of laccase is inversely proportional to its stability and that laccase is more stable at pH 7 than at pH 4.5. Having relatively high laccase stability near neutral pHs can be a desired property when operating laccase-based treatment as a continuous process.
- Halides and heavy metals are common pollutants to the wastewater environment and are also known to inhibit laccase activity. The impact of 4 ions on laccase-based treatment was studied using two different substrates: ABTS (standard substrate) and E1 (wastewater relevant substrate), the result showed that:
    - Chloride exhibits a strong inhibitory effect on laccase at pH 4.5, where 200 mg/l of chloride ions can reduce laccase activity by 27%. This effect is significantly weaker at pH 7. This shows

that decreasing the pH of the wastewater to pH 4.5 which is optimum pH for laccase activity may not to bring the desired increase in treatment efficiency due to the potential presence of halides ions within that matrix. It also shows that the inhibitory effect of chloride can be reduced by keeping the pH around pH 7.

- The influence of copper ions on laccase-based treatment is concentration dependent. Copper ions (Cu^{2+}) has a slight positive impact (less than 2%) on E1 removal efficiency when its concentration in the reaction mixture is equal to or less than 0.1 mg/l and a slight negative impact when its concentration is equal to or above 10 mg/l e.g. 50 mg/l of Cu^{2+} solution can reduce E1 removal efficiency by less than 4%. As a result, the relevant Cu^{2+} concentrations to the municipal wastewater environment (less than 50 mg/l) will not have a significant impact on laccase-based treatment.
- Fe^{3+} ions have a negative impact on laccase activity at concentrations equal to/ above 0.3 mg/l. The maximum inhibition in laccase activity (≈13%) was achieved using 10 mg/l of Fe^{3+} ions. Increasing the concentration of Fe^{3+} ions above 10 mg/l did not increase the inhibition percentage.
- The presence of Zn^{2+} ions across the concentration range from 0.05–50 mg/l did not significantly impact on laccase ability to remove E1 from the water matrix. All the tested Zn^{2+} concentrations have a slight positive impact (less than 5%) on E1 removal efficiency.

- The impact of any inhibitor on laccase-based treatment can be better investigated using wastewater - relevant substrates. Unlike the UV-vis experiments with generic substrate e.g. ABTS, the HPLC-UV experiments can utilise environmentally relevant substrates such as E1 and provide a clearer idea about the effect of certain inhibitors on the removal efficiency of that substrate/ pollutant by laccase.

7 : RESULTS AND DISCUSSION ENZYMATIC TREATMENT OF FREE STEROID ESTROGENS IN WASTEWATER WATER MATRIX

7.1 INTRODUCTION

The performed experiments in Chapter 5 showed that E1 removal efficiency by laccase can be described in clean matrix using response surface methodology (RSM) and artificial neural networks (ANN) models, the results also demonstrated the limited predictive capabilities of the generated RSM and ANN models in that matrix. However predicting steroids removal efficiency in wastewater matrix is more challenging and environmentally relevant at the same time. This chapter utilises the obtained conclusions from Chapter 5 and Chapter 6 to study the removal efficiency of E1 in wastewater effluent under the influence of three factors: temperature, contact time and laccase concentration. The ranges of the selected factors were relevant to the actual conditions in wastewater treatment plants (WWTPs). The experimental data were used to build RSM and ANN models to predict E1 removal efficiency under different conditions in wastewater matrix. Similar to Chapter 5, the goodness of fit and the predictive capabilities of two models RSM and ANN were evaluated. To minimise the amount of the required experiments, BBD with three factors was utilised. The ranges of temperature and contact time remained the same as in Chapter 5. However the range of laccase concentration was increased to overcome the inhibitory effect of various wastewater constituents that were discussed in Chapter 6. Benchmark experiments were also employed in this chapter to quantify wastewater variability and optimise the performance of the ANN model.

7.2 HIGHLIGHTS

- The ability of laccase to degrade estrone in actual wastewater effluent and under realistic conditions to WWTP, was demonstrated.
- RSM model had a significant lack-of-fit to the experimental data when used in wastewater matrix.
- For the first time, wastewater variability was included in an ANN model in a form of benchmark data, to improve its predictive capability.
- Enzymatic degradation of estrone was modelled in wastewater matrix and the agreement between the experimental and the predicted data was very high with R^2 of 0.991.
- The predictive capability of the ANN model noticeably decreases for points outside the investigated system.

7.3 MODELLING LACCASE-BASED TREATMENT PROCESS

The matrix of conditions in wastewater effluent was prepared using Box Behnken Design (BBD) that was also implemented in Chapter 5 to study the enzyme degradation of E1 in clean water matrix. Similar to Chapter 5, the impact of three individual factors (temperature, contact time and laccase concentration) on E1 removal by laccase was investigated. The ranges of the temperature and the contact time remained the same as described in Section 5.3.2, as both ranges reflected the relevant values to wastewater treatment plants (WWTPs). However the range of laccase concentration was increased to overcome the complexity of wastewater effluent and the inhibitory effects of various wastewater constituents that were discussed in Chapter 6. Trial and error approach was utilised to select the boundaries of the new range of laccase. This was typically performed by conducting enzymatic degradation experiments of E1 at the least favourable conditions i.e. the lowest temperature (6˚C) and the shortest contact time (0.5 hour), and the most favourable conditions i.e. the highest temperature (25˚C) and the longest contact time (8 hr), and at different laccase concentrations to determine the lower and the upper values of laccase concentration range and ensure that the achieved E1

removal efficiency was less than 100% at the most favourable conditions and above 0% at the least favourable ones. As a result, laccase range was confined between 0.5 U/ml and 6 U/ml. Table 7.1 shows the investigated factors and their ranges for the performed experiments in wastewater effluent.

Table 7.1 The ranges of the investigated factors in wastewater effluent.

Parameters	Range
Temperature (˚C)	6 - 25
Contact time (hr)	0.5 - 8
Laccase concentration (U/ml)	0.5 - 6

7.3.1 Response Surface Methodology (RSM) Models

Similar to the clean water matrix in Chapter 5, BBD was utilised to build the matrix of conditions in wastewater effluent (12 unique experiments and 3 replicates). The statistically designed experiments were performed in the lab, and the collected results were fed into Minitab to build a RSM model. Experiments at the centre points were conducted on different sampling dates but under identical experimental conditions and were used to check system's reproducibility. The results in Table 7.2 show that the variability in E1 removal efficiency on those three sampling dates was relatively high with a coefficient of variance (CV) of 4.5% in comparison with the previously calculated CV% value in the clean water matrix of 1.8%. This variability and lack of reproducibility can be attributed to the variable and complex nature of the wastewater matrix (as described in Section 6.4). This means that E1 removal efficiency by laccase under the same conditions (temperature, contact time and laccase concentration) may vary with the quality of the wastewater effluent from day to day.

Table 7.2 Actual and RSM predicted removal efficiencies of E1 in wastewater matrix under the conditions of the Box Behnken Design (BBD) centre points.

Run	Factors			Removal (%)		
	Temperature (˚C)	Contact time (Hrs)	Laccase conc. (U/ml)	Actual	Predicted by RSM	Absolute Error (%)
6	15.5	4.25	3.25	72.02	73.06	1.44
9	15.5	4.25	3.25	70.4	73.06	3.78
11	15.5	4.25	3.25	76.72	73.06	4.77

The predicted results by the built RSM model for the 15 BBD experiments (Table 7.3) demonstrate that the quality of the wastewater effluent forms an additional factor that may significantly affect E1 removal efficiency. Due to the nature of the RSM model it was not feasible to include wastewater variability within its design. Not including this factor within the model design will adversely impact on its fit to the actual experimental data and reduce its predictive capabilities.

Table 7.3 Actual and predicted removal efficiencies of estrone (E1) using Box-Behnken Design (BBD) and Response Surface Methodology (RSM) model.

Run	**Factors**			**Removal (%)**		
	Temperature (˚C)	Duration (Hrs)	Laccase conc. (U/ml)	Actual	Predicted by RSM	Residuals
1	6	4.25	0.5	0.49	0.00	0.49
2	15.5	8	6	91.95	98.58	-6.63
3	15.5	8	0.5	0	3.95	-3.95
4	6	4.25	6	75.22	65.07	10.15
5	25	4.25	6	95.23	102.71	-7.48
6	15.5	4.25	3.25	72.02	73.06	-1.04
7	25	8	3.25	93.53	79.47	14.06
8	15.5	0.5	6	64.42	60.50	3.92
9	15.5	4.25	3.25	70.4	73.06	-2.66
10	6	8	3.25	47.4	50.93	-3.53
11	15.5	4.25	3.25	76.72	73.06	3.66
12	6	0.5	3.25	12.25	26.34	-14.09
13	25	4.25	0.5	4.34	14.52	-10.18
14	15.5	0.5	0.5	1.52	0.00	1.52
15	25	0.5	3.25	60.43	56.93	3.50

The coefficient of determination (R^2) was calculated to determine the degree of correlation between the predicted removal efficiency by RSM and the actual (experimental) one. In the clean water matrix (section 5.3.3) a higher R^2 (R^2=0.9908) was achieved using the same model, which means that the lack of fit of the current RSM model is mainly attributed to the inherent variability of the wastewater that was not included in its design. (Figure 7.1).

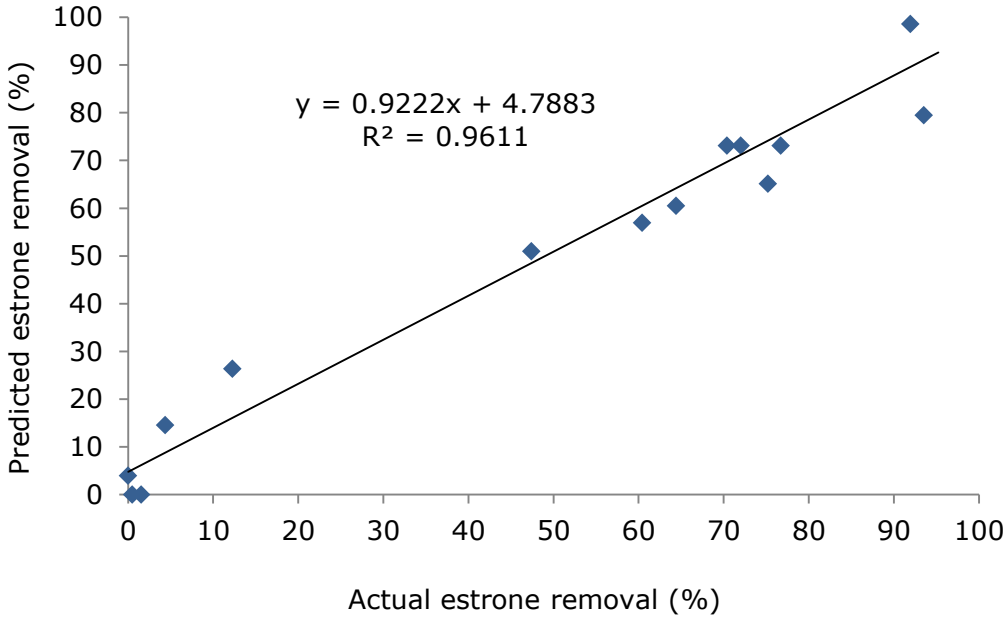


Figure 7.1 Comparison between the actual removal efficiency of estrone (E1) and the predicted one by response surface methodology (RSM) in wastewater effluent.

To illustrate the negative impact of dismissing the wastewater variability during the model building, the residuals of the RSM model in clean water matrix (Table 5.7 in Chapter 5) were compared to the residuals of the RSM model in wastewater effluent. Figure 7.2 shows that the residuals in wastewater matrix are further from the centre line than the ones in clean water matrix and therefore, RSM model in wastewater has a poor fit to the actual experimental data.

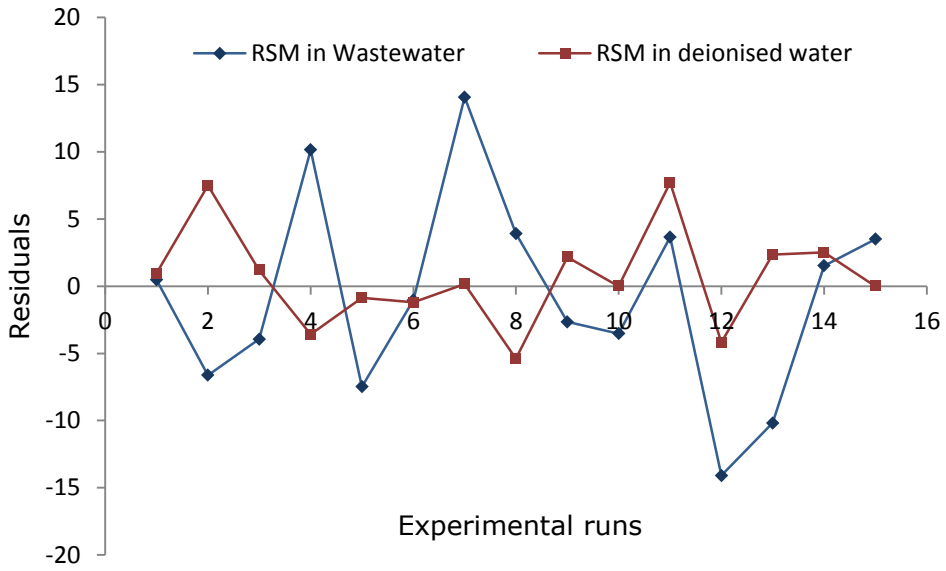


Figure 7.2 The residuals of RSM models in both deionised water and wastewater matrices.

Similar to section 5.3.3, the Analysis of Variance (ANOVA) for E1 removal was performed on this model and the results showed that the lack-of-fit of RSM model in wastewater was significant with P value of 0.037.

7.3.2 Artificial Neural Networks (ANN) Model

Similar to the built ANN model in Section 5.3.4, the experimental data from BBD in wastewater matrix was utilised to build ANN model in MATLAB. The actual and the ANN predicted values in wastewater matrix are presented in Table 7.4.

Table 7.4 Actual and predicted removal efficiencies of estrone (E1) using Artificial Neural Network (ANN) model in wastewater matrix.

Run	**Factors**			**Removal%**		
	Temperature (˚C)	Duration (Hrs)	Laccase conc. (U/ml)	Actual	Predicted by ANN	Residual
1	6	4.25	0.5	0.49	0.49	0.00
2	15.5	8	6	91.95	92.15	-0.20
3	15.5	8	0.5	0	0.00	0.00
4	6	4.25	6	75.22	75.22	0.00
5	25	4.25	6	95.23	95.23	0.00
6	15.5	4.25	3.25	72.02	72.02	0.00
7	25	8	3.25	93.53	93.53	0.00
8	15.5	0.5	6	64.42	64.42	0.00

9	15.5	4.25	3.25	70.4	72.02	-1.62
10	6	8	3.25	47.4	47.40	0.00
11	15.5	4.25	3.25	76.72	72.02	4.70
12	6	0.5	3.25	12.25	12.25	0.00
13	25	4.25	0.5	4.34	4.34	0.00
14	15.5	0.5	0.5	1.52	1.52	0.00
15	25	0.5	3.25	60.43	58.25	2.18

The agreement between the predicted and the actual removal efficiencies of E1 was demonstrated by the R^2 value (Figure 7.3). The relatively high R^2 value (R^2=0.9986) indicates that this ANN model fits the experimental data much better than the RSM model with R^2 of 0.9611.

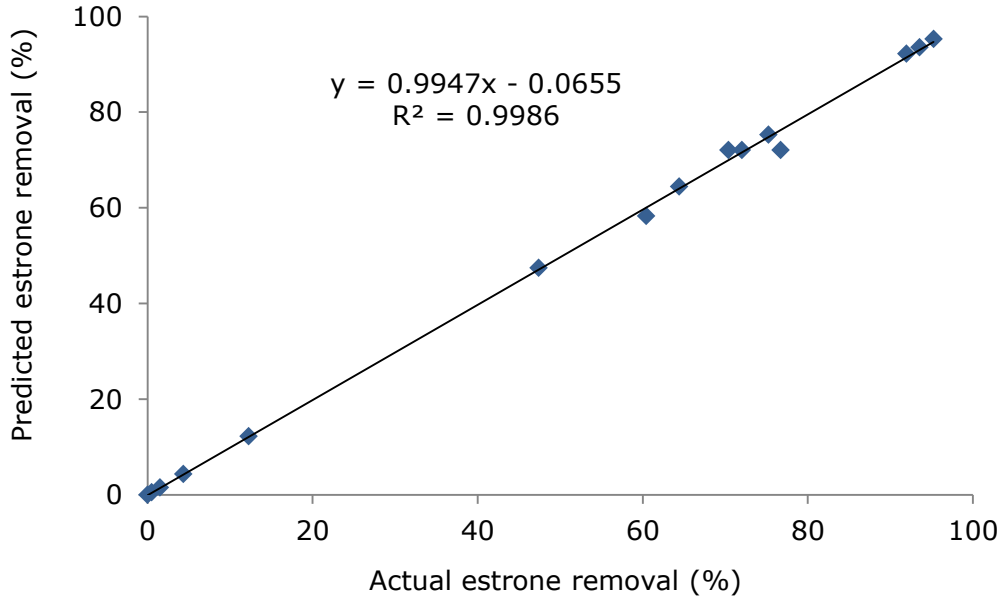


Figure 7.3 Comparison between the actual and the predicted removal efficiency of estrone (E1) by the ANN model.

7.4 EVALUATING THE GOODNESS-OF-FIT OF RSM AND ANN MODELS USING STATISTICAL INDICES

The residuals of RSM and ANN models in wastewater matrix (Table 7.3 and Table 7.4, respectively) were compared against each other to visualise the fit of the models to the actual data. Figure 7.4 shows clearly that the residuals of the RSM model have a much higher deviation from the centre line than the residuals of the ANN model, and therefore ANN shows a better fit to the actual data than the RSM in wastewater.

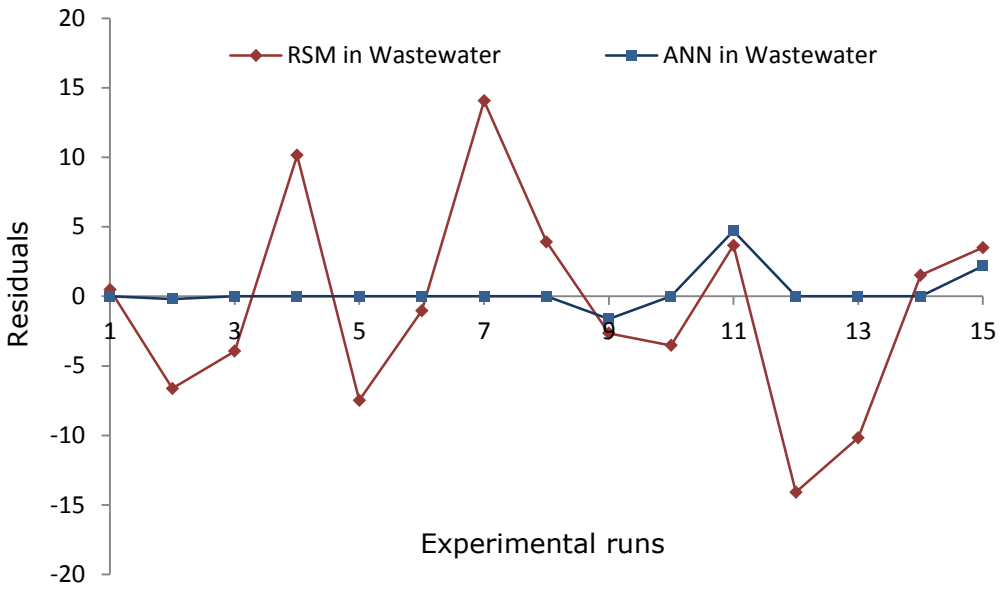


Figure 7.4 The residuals of RSM and ANN models in wastewater effluent.

The statistical indices of both RSM and ANN models were also calculated and compared against each other. Table 7.5 demonstrates that all the statistical indices of the ANN model were significantly better than the statistical indices of the RSM model. The variability of wastewater adds additional complexity to the investigated system and RSM with its second-order polynomial equation may not be the most suitable model to represent such a system.

Table 7.5 The statistical indices of the built models in wastewater.

Model	Index	Value
RSM model	R^2	0.961
	MSE	49.96
	RMSE	7.07
	AAD	41.31
ANN model	R^2	0.999
	MSE	1.97
	RMSE	1.40
	AAD	0.83

7.5 EVALUATING THE PREDICTIVE CAPABILITY OF RSM AND ANN MODELS USING UNSEEN DATA

A set of 6 unseen data points, randomly selected and located within the ranges of the investigated factors, was prepared in wastewater effluent. This set of data was used to test the predictive capability of both RSM and ANN models in wastewater matrix. The experimental conditions, the actual removal and the predicted E1 removal efficiencies for both models are included in Table 7.6.

Table 7.6 The experimental conditions of the unseen experiments in wastewater.

Run	**Factors**			**Removal%**		
	Temperature (˚C)	Duration (Hrs)	Laccase conc. (U/ml)	Actual	Predicted by RSM	Predicted by ANN
1	20	3	2.5	52.22	60.77	56.38
2	15	2	2	19.52	40.26	17.06
3	10	1	6	66.61	52.30	49.78
4	20	1	6	77.92	73.43	79.97
5	25	1.5	3	69.59	61.78	62.78
6	12	3	1.5	6.03	29.51	5.91

The agreement between the actual and the predicted results of the unseen data was shown in (Figure 7.5). The ANN model had an R^2 value of 0.9324, while the R^2 of the RSM model was relatively lower (R^2 = 0.8630). This comes in line with the obtained results from section 7.4 that showed that the RSM had a poor fit even to that standard data set that was used to build it.

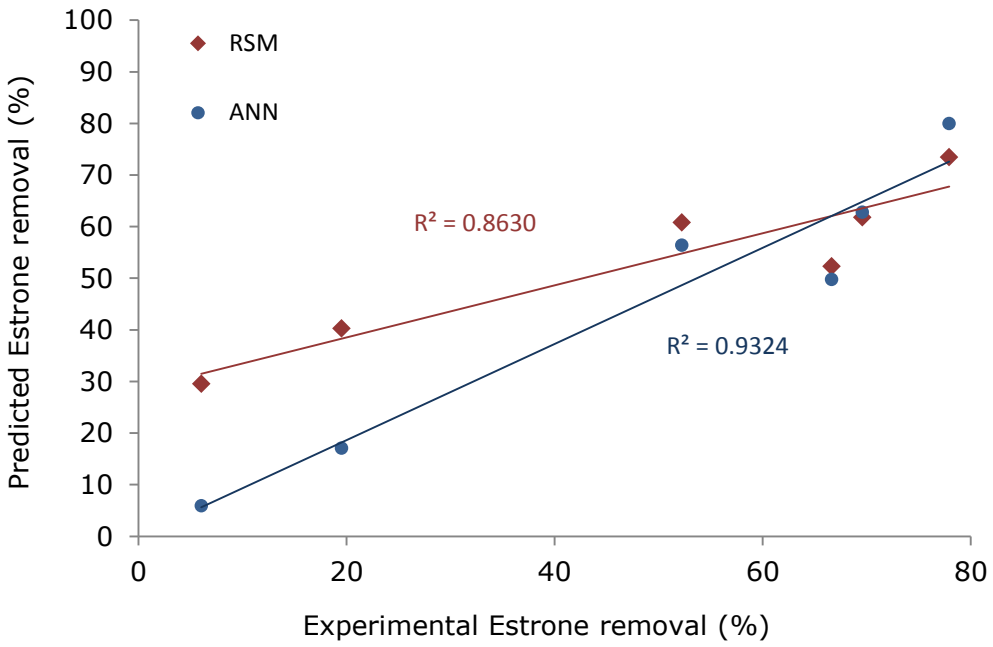


Figure 7.5 Comparison between the actual and predicted values of estrone removal efficiency in wastewater effluent using unseen data.

To demonstrate the impact of wastewater variability on the performance of RSM and ANN models, the obtained R^2 value for each matrix (clean and wastewater), model (RSM and ANN) and data set (standard and unseen), was presented in Table 7.7. RSM model showed a better performance in clean water matrix for both standard and unseen data. ANN had the same high R^2 value of 0.999 in both clean and wastewater matrices, demonstrating its ability to describe both complex and simple systems. However, the predictive capabilities (using unseen data) of all models, in all matrices, were very limited with $R^2 \leq 0.932$. It was also observed that R^2 value of the unseen experiments was always lower than R^2 of the standard data.

Table 7.7 Coefficient of determination values for the seen and unseen experiments both in clean and wastewater matrices.

Matrix	**Model**	**R^2**	
		Standard data	Unseen data
Clean	RSM	0.991	0.869
	ANN	0.999	0.874
Wastewater effluent	RSM	0.961	0.863
	ANN	0.999	0.932

Therefore there is a need to improve the predictive capabilities of the used models, so they can act not only as a descriptive tool of a system, but also as a predictive one. Unlike the RSM model, new factors such as wastewater variability can be simply added to the existing matrix of conditions to build and enhance ANN model. The next section investigates a number of options to improve the predictive capability of ANN model in a variable matrix such as wastewater effluent.

7.6 IMPROVING THE PREDICTIVE CAPABILITY OF THE ANN MODEL

7.6.1 Including Wastewater Variability in the ANN Model

Modelling the enzymatic treatment in wastewater is challenged by the complexity and the variability of this matrix. To obtain a model of this system with good predictive capability, the variability of the wastewater should be included within the model. To achieve that a fourth factor was introduced into the previous 3-factor ANN model. The additional parameter represented the percentage of E1 removal in wastewater during the benchmark experiment (as described in section 6.4.1). Each benchmark was then added to the relevant experiments (i.e. the experiments that were performed in the same wastewater as the benchmark) in the matrix of conditions. The input factors of the new ANN model were: temperature, contact time, laccase concentration and the benchmark value of the used wastewater in that experiment. The number of input experiments remained 15 and the output represented the predicted E1 removal percentage in wastewater. Table 7.8 shows the modified matrix of conditions and the obtained removal using the new ANN model.

Table 7.8 The actual and the predicted removal efficiencies of estrone (E1) using ANN model with 4 factors in wastewater matrix (standard data set).

Run	Factors				Removal%	
	Temperature (˚C)	Duration (Hrs)	Laccase conc. (U/ml)	Benchmark %	Actual	Predicted by ANN
1	6	4.25	0.5	84.18	0.49	0.00
2	15.5	8	6	78.25	91.95	91.95
3	15.5	8	0.5	78.25	0	0.00
4	6	4.25	6	79.39	75.22	74.16
5	25	4.25	6	79.72	95.23	92.49
6	15.5	4.25	3.25	81.83	72.02	71.42
7	25	8	3.25	78.54	93.53	93.53
8	15.5	0.5	6	79.28	64.42	64.42
9	15.5	4.25	3.25	79.59	70.4	70.40
10	6	8	3.25	79.94	47.4	47.40
11	15.5	4.25	3.25	84.02	76.72	76.72
12	6	0.5	3.25	79.39	12.25	12.25
13	25	4.25	0.5	84.36	4.34	4.34
14	15.5	0.5	0.5	81.83	1.52	1.52
15	25	0.5	3.25	84.02	60.43	60.43

The correlation between the actual and the predicted E1 removal efficiency was quantified using R^2. A very good R^2 value of 0.9991 was obtained for the 15 input experiments (Figure 7.6). However and despite including wastewater variability within the new ANN model, the R^2 value of the unseen experiments (the same unseen experiments as in Table 7.6) was lower (R^2=0.883) than the R^2 of the previous ANN model (R^2=0.932) that did not account for wastewater variability. This could be attributed to the small data set (15 experiments) that was used to build the ANN model. Figure 7.6 and Figure 7.7 show the correlation between the actual and the predicted E1 removal efficiency using ANN model with 4 factors, with both standard and unseen data sets, respectively.

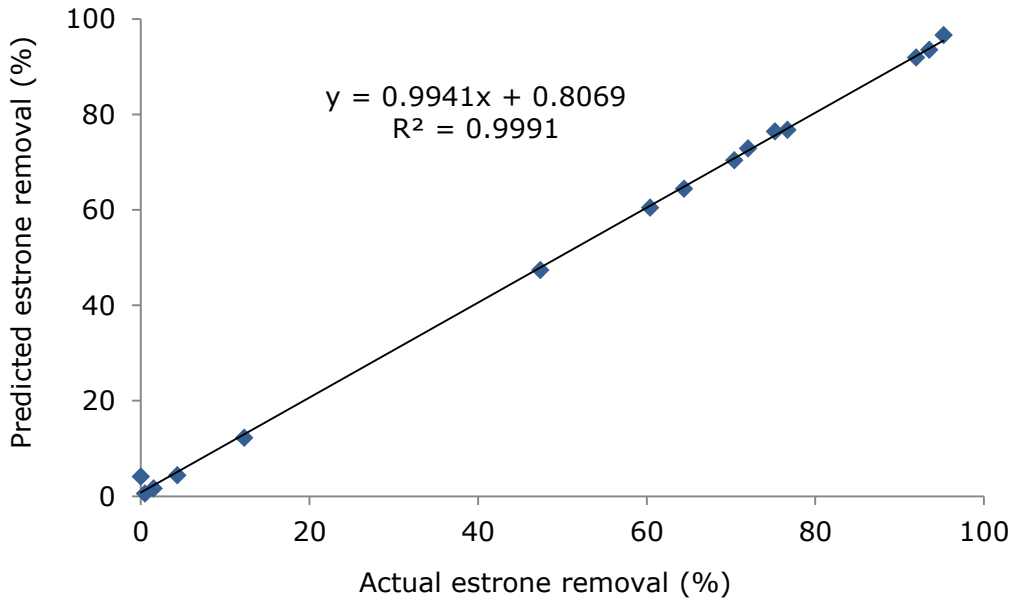


Figure 7.6 Comparison between the actual and the predicted removal efficiency of estrone (E1) by the ANN model (4 factors) for the standard data.

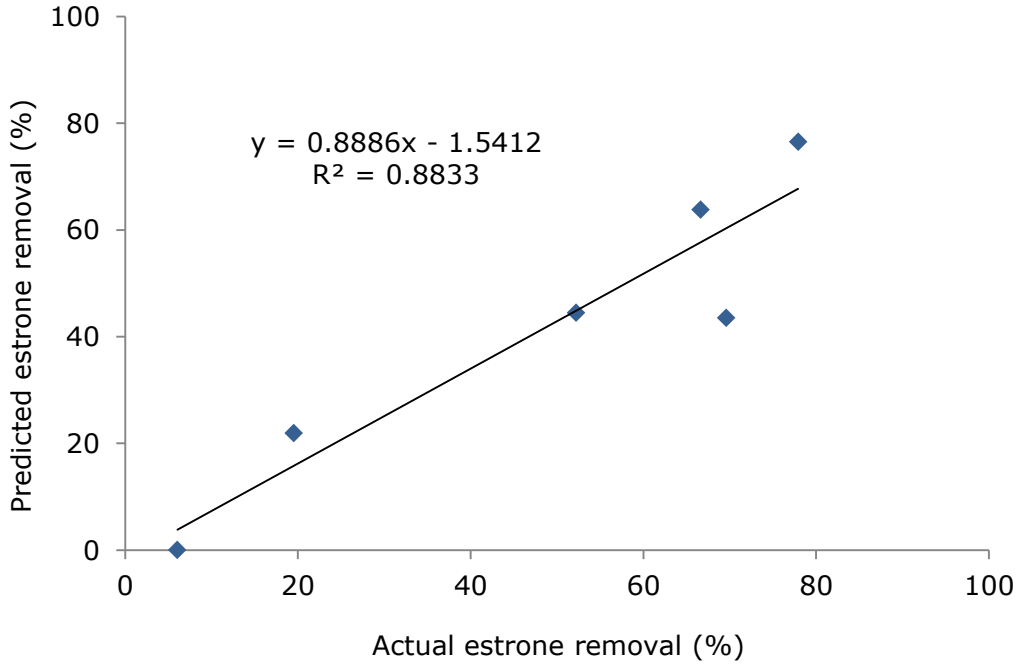


Figure 7.7 Comparison between the actual and the predicted removal efficiency of estrone (E1) by the ANN model (4 factors) for the unseen data.

In similar manner to wastewater benchmark, standard wastewater quality parameters such as TSS, COD and pH, could be also included in the structure of the ANN model in order to improve its predictive capabilities. However,

increasing the number of factors (inputs) requires a substantial increase in the number of data points (experiments). Once a sufficiently large data set is available, ANN model could then utilise it to learn and understand the complex relationships and patterns between these inputs and their effects on E1 removal efficiency by laccase (output). As a result, the predictive capability of that generated model will be significantly improved.

7.6.2 Utilising a Larger Data Set to Build the ANN Model

During the generation of the ANN model, 70% of the experimental results were utilised in ANN training, 15% in ANN validation and 15% in ANN testing. Increasing the number of the experiments will provide a larger set of data to train, test and validate the model which should subsequently improve its accuracy.

To increase the number of system's data points, 28 benchmark experiments were added to the input data in the ANN model. The additional benchmark experiments were separately performed on various dates to monitor the seasonal variability of the final effluent. All the benchmarks were performed under a standard set of conditions within the ranges of the BBD matrix: temperature=20˚C, contact time=1 hr, laccase conc.=5 U/ml, therefore each benchmark experiment can be considered as another data point and its actual E1 removal is equivalent to the benchmark removal. Table 7.9 shows all the used data points, the actual and the predicted E1 removal efficiencies by this new ANN model.

Table 7.9 The actual and the predicted removal efficiencies of estrone (E1) using ANN model with 4 factors and a larger data set.

Run	**Factors**				**Removal%**	
	Temperature (˚C)	Contact time (hour)	Laccase conc. (U/ml)	Benchmark %	Actual	Predicted by ANN
1	6	4.25	0.5	84.18	0.49	0.00
2	15.5	8	6	78.25	91.95	91.95
3	15.5	8	0.5	78.25	0	0.00
4	6	4.25	6	79.39	75.22	74.16
5	25	4.25	6	79.72	95.23	92.49
6	15.5	4.25	3.25	81.83	72.02	71.42

7	25	8	3.25	78.54	93.53	93.53
8	15.5	0.5	6	79.28	64.42	64.42
9	15.5	4.25	3.25	79.59	70.4	70.40
10	6	8	3.25	79.94	47.4	47.40
11	15.5	4.25	3.25	84.02	76.72	76.72
12	6	0.5	3.25	79.39	12.25	12.25
13	25	4.25	0.5	84.36	4.34	4.34
14	15.5	0.5	0.5	81.83	1.52	1.52
15	25	0.5	3.25	84.02	60.43	60.43
16	20	1	5	80.15	80.15	80.14
17	20	1	5	78.71	78.71	78.71
18	20	1	5	71.74	71.74	71.80
19	20	1	5	77.83	77.83	77.84
20	20	1	5	77.35	77.35	77.36
21	20	1	5	80.57	80.57	80.57
22	20	1	5	77.9	77.9	77.91
23	20	1	5	73.91	73.91	73.84
24	20	1	5	78.36	78.36	78.37
25	20	1	5	76.94	76.94	76.93
26	20	1	5	73.52	73.52	73.46
27	20	1	5	80.54	80.54	80.54
28	20	1	5	75.94	75.94	75.90
29	20	1	5	86.28	86.28	86.30
30	20	1	5	84.36	84.36	84.38
31	20	1	5	79.53	79.53	79.52
32	20	1	5	79.28	79.28	79.27
33	20	1	5	79.59	79.59	79.58
34	20	1	5	79.39	79.39	79.38
35	20	1	5	81.83	81.83	81.86
36	20	1	5	79.72	79.72	79.71
37	20	1	5	79.94	79.94	79.93
38	20	1	5	78.54	78.54	78.55
39	20	1	5	84.18	84.18	84.20
40	20	1	5	78.25	78.25	78.26
41	20	1	5	86.44	86.44	86.48
42	20	1	5	87.65	87.65	87.93
43	20	1	5	84.02	84.02	84.05

The correlation between the actual and the predicted data was featured using the R^2 value. The results showed that almost a perfect correlation between the two sets was achieved with R^2 of 0.9999 (Figure 7.8). The new ANN model (43 data points) managed to achieve a better R^2 value than the previous ANN

model (15 data points_ Section 7.6.1) even with the unseen data (R^2 0.9006) (Figure 7.9).

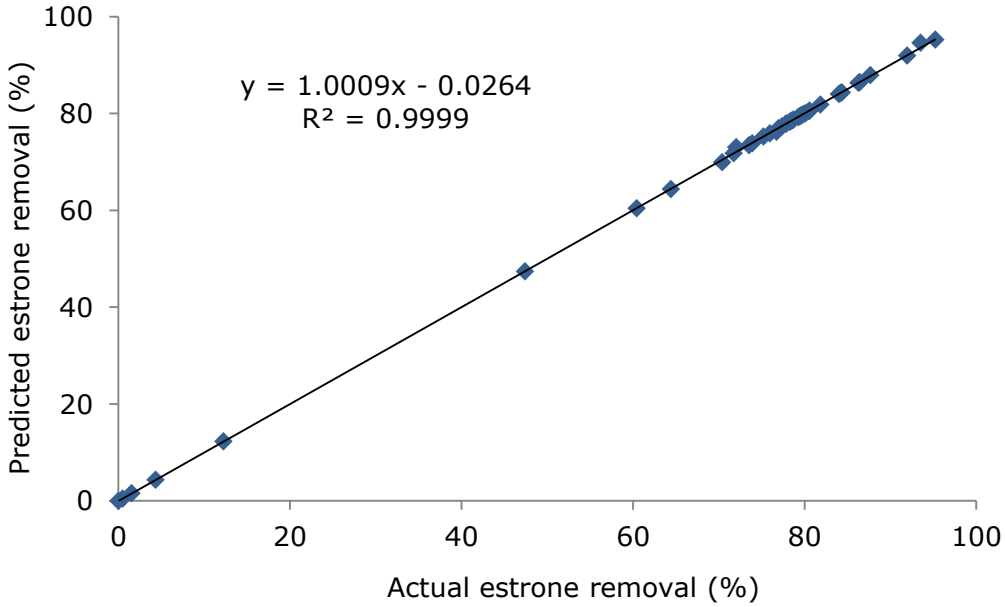


Figure 7.8 Comparison between the actual and the predicted removal efficiency of estrone (E1) by the ANN model (4 factors, 43 data points) for the standard data.

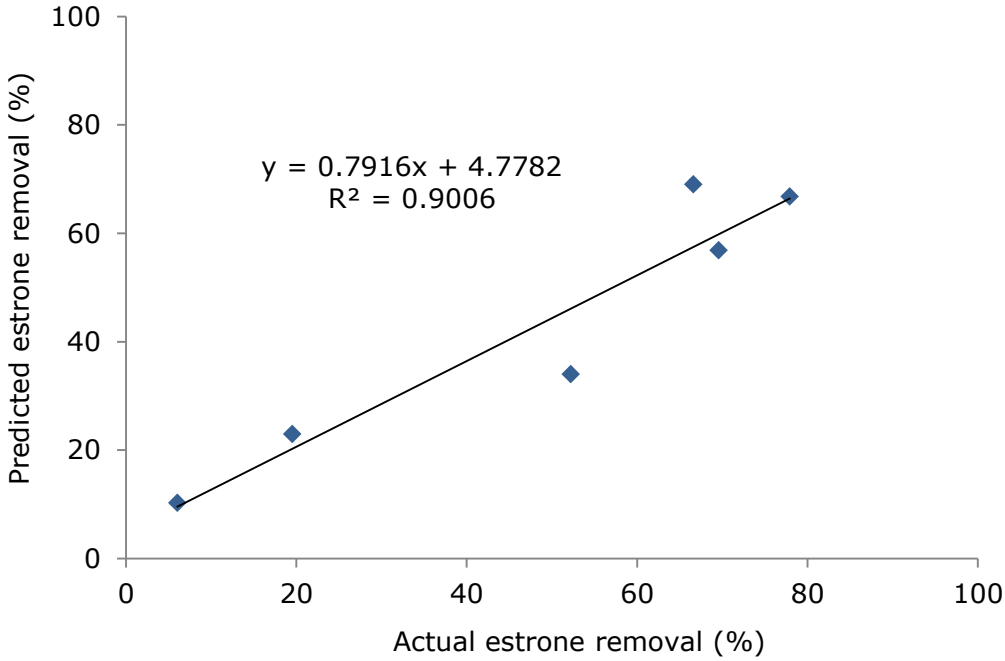


Figure 7.9 Comparison between the actual and the predicted removal efficiency of estrone (E1) by the ANN model (4 factors, 43 data points) for the unseen data.

7.6.3 Changing the Type of the Network Training Function

In all the previous ANN models in this work, Levenberg-Marquardt (trainlm) was used as the network training function. This function updates weight and bias values according to Levenberg-Marquardt optimisation, trainlm is often the fastest backpropagation algorithm in MATLAB toolbox and commonly used as a first-choice supervised algorithm [195]. However trainlm requires more memory than the other algorithms and the validation vectors are used to stop the training early if the network performance on the validation vectors fails to improve or remains the same for max_fail epochs in a row [195]. As a result, this early stop in training may affect the accuracy of the produced model.

Bayesian regularisation (trainbr) is another network training function that updates the weight and bias values according to Levenberg-Marquardt optimisation too [196]. It minimises a combination of squared errors and weights, and then determines the correct combination so as to produce a network that generalises well. The training can continue until an optimal combination of errors and weights is found as the stops in the validation process are disabled by default in this function (max_fail=0) [196].

In order to improve the predictive capabilities of the ANN model, trainlm function was replaced by trainbr in the iterative script of ANN model (APPENDIX B). The used set of data points was the same as in Table 7.9. Table 7.10 shows the actual and the predicted E1 removal % values. The correlation between the actual and predicted values was optimum with R^2=1.00 (Figure 7.10) and an extremely high R^2 of 0.9914 was achieved between the actual and the unseen data points (Figure 7.11).

Table 7.10 The actual and the predicted removal efficiencies of estrone (E1) using ANN model (4 factors, MSE <2 and 43 data points (trainbr)).

Run	Factors			Removal%		
	Temperature (˚C)	Duration (Hrs)	Laccase conc. (U/ml)	Benchmark %	Actual	Predicted by ANN
1	6	4.25	0.5	84.18	0.49	0.49
2	15.5	8	6	78.25	91.95	91.95
3	15.5	8	0.5	78.25	0	0.00
4	6	4.25	6	79.39	75.22	75.22
5	25	4.25	6	79.72	95.23	95.23
6	15.5	4.25	3.25	81.83	72.02	72.02
7	25	8	3.25	78.54	93.53	93.53
8	15.5	0.5	6	79.28	64.42	64.42
9	15.5	4.25	3.25	79.59	70.4	70.40
10	6	8	3.25	79.94	47.4	47.40
11	15.5	4.25	3.25	84.02	76.72	76.72
12	6	0.5	3.25	79.39	12.25	12.25
13	25	4.25	0.5	84.36	4.34	4.34
14	15.5	0.5	0.5	81.83	1.52	1.52
15	25	0.5	3.25	84.02	60.43	60.43
16	20	1	5	80.15	80.15	80.15
17	20	1	5	78.71	78.71	78.71
18	20	1	5	71.74	71.74	71.74
19	20	1	5	77.83	77.83	77.83
20	20	1	5	77.35	77.35	77.35
21	20	1	5	80.57	80.57	80.57
22	20	1	5	77.9	77.9	77.90
23	20	1	5	73.91	73.91	73.91
24	20	1	5	78.36	78.36	78.36
25	20	1	5	76.94	76.94	76.94
26	20	1	5	73.52	73.52	73.52
27	20	1	5	80.54	80.54	80.54
28	20	1	5	75.94	75.94	75.94
29	20	1	5	86.28	86.28	86.28
30	20	1	5	84.36	84.36	84.36
31	20	1	5	79.53	79.53	79.53
32	20	1	5	79.28	79.28	79.28
33	20	1	5	79.59	79.59	79.59
34	20	1	5	79.39	79.39	79.39
35	20	1	5	81.83	81.83	81.83
36	20	1	5	79.72	79.72	79.72
37	20	1	5	79.94	79.94	79.94
38	20	1	5	78.54	78.54	78.54
39	20	1	5	84.18	84.18	84.18
40	20	1	5	78.25	78.25	78.25
41	20	1	5	86.44	86.44	86.44
42	20	1	5	87.65	87.65	87.65
43	20	1	5	84.02	84.02	84.02

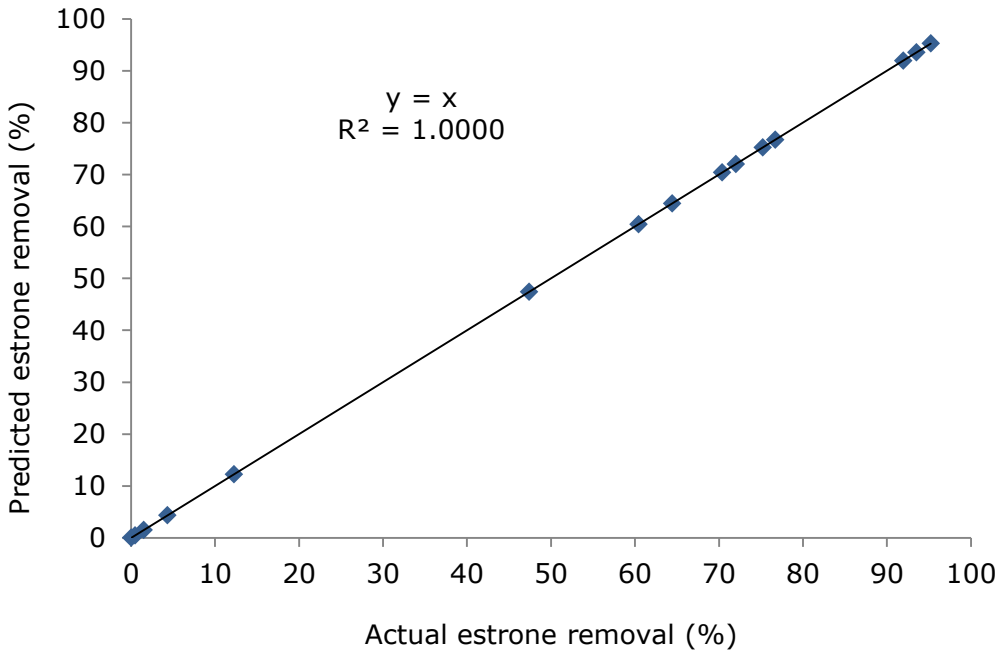


Figure 7.10 Comparison between the actual and the predicted removal efficiency of estrone (E1) by the ANN model (4 factors, 43 points (trainbr)).

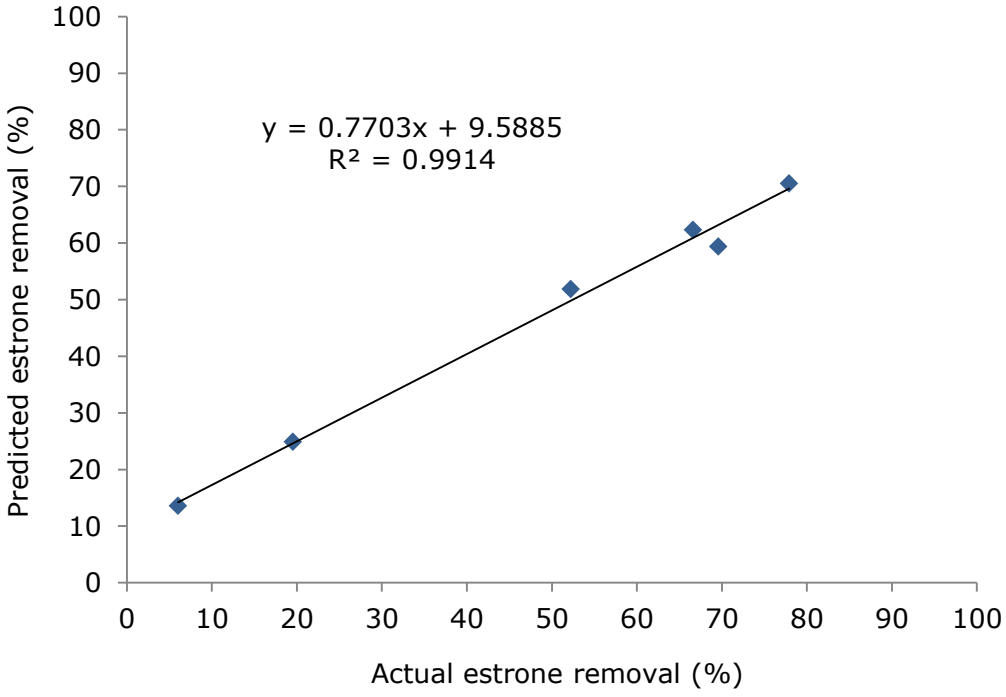


Figure 7.11 Comparison between the actual and the predicted removal efficiency of estrone (E1) by the ANN model (4 factors, 43 data points (trainbr)) for the unseen data.

Seven additional unseen experiments were performed to test the performance of this final model (Table 7.11), six of them were located within the same system as the BBD matrix of conditions while the seventh experiment was selected from outside that system.

Table 7.11 The conditions of the 2nd set of the unseen data and the achieved removal efficiencies inside and outside the investigated system.

Run	Inside/ Outside the System	Factors				Estrone removal (%)		
		Temp. (˚C)	Contact time (hr)	Laccase conc. (U/ml)	B* (%)	Actual	ANN Predicted	Abs. error (%)
1	Inside	20	1	0.5	79.9	1.03	0.98	4.60
2	Inside	20	1	1	79.9	4.97	5.43	9.27
3	Inside	20	1	2	80	21.98	22.90	4.23
4	Inside	20	1	3	82.4	50.99	51.64	1.28
5	Inside	20	1	4	80	70.91	72.15	1.75
6	Inside	20	1	5	80.7	82.38	80.70	2.04
7	Outside	20	1	8	79.9	90.65	62.55	31.0

The point was considered inside the system if all its conditions fall within the specified range of: temperature [6˚C, 25 ˚C], contact time [0.5 hr, 8 hrs] and laccase concentration [0.5 U/ml, 6 U/ml]. Laccase concentration in experiment 7 was 8 U/ml which is outside the investigated range above. The absolute error values (calculated using Equation 5.2) showed that the predictive capability of ANN model outside the investigated system (Exp. 7) was noticeably lower than its predictive capability inside the system (Experiments 1-6). Figure 7.12 demonstrates the absolute error for each unseen experiment in Table 7.11. The absolute error was less than 10% for the experiments inside the system and above 30% for the only experiment outside the system. This highlights the fact that the predictive capability of any model may significantly drop outside the investigated system and it important to understand the boundaries of the built model and utilise it only for points within its system.

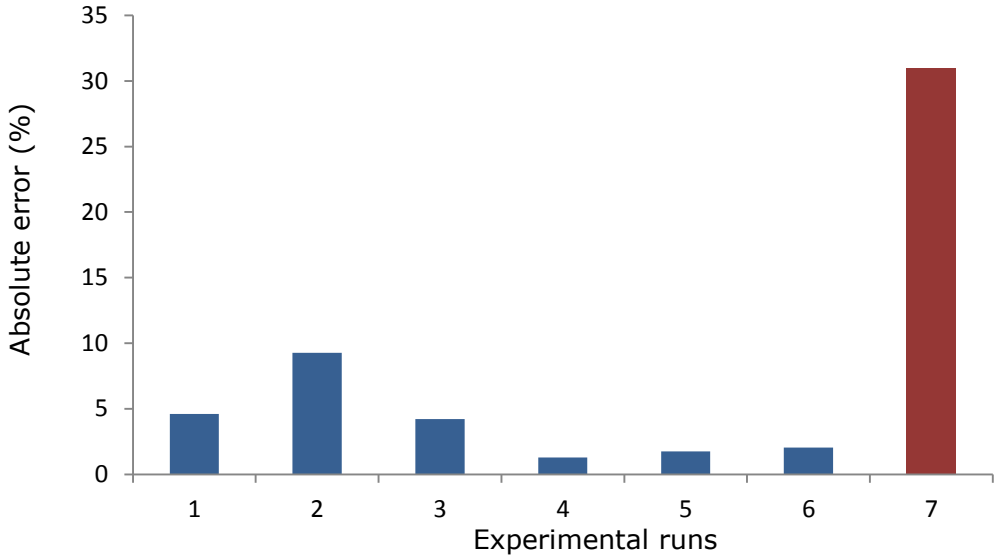


Figure 7.12 Comparison between the actual and the predicted estrone removal efficiency using the improved ANN model with the 2nd set of unseen data from inside (blue) and outside (red) the tested system. The coefficient of variance between the duplicates was less than 2%.

7.7 VISUALISING THE IMPROVED ANN MODEL USING 3D GRAPHS

The improved ANN model (Table 7.10) was visualised using 3D graphs generated in SigmaPlot similar to the used visualisation in Chapter 5 Figure 5.6. Unlike the ANN model in clean water matrix, laccase concentration has the biggest impact on the removal efficiency of E1 in wastewater. The relation between the E1 removal efficiency and laccase concentration was not linear and the graph approached a plateau when laccase concentration was increased above 4.5 U/ml (Figure 7.13). This observation may be explained by the fact that increasing laccase concentration reduces the required contact time to achieve a specific removal percentage. However as the reaction continuous, the concentration of the target pollutant (E1) decreases, as well as the interaction between active laccase and the free pollutant. Table 7.11 showed that increasing laccase concentration from 1 U/ml to 4 U/ml (additional 3 U/ml), improves the removal efficiency by 65.9%. However, increasing laccase concentration from 5 U/ml to 8 U/ml (additional 3 U/ml), improves the efficiency only by 8.3% as the experiment approaches the 100% E1 removal. Increasing laccase concentration beyond a certain point may not be cost effective and cost benefit analysis should be undertaken to decide the target

removal efficiency within the treatment unit. According to Figure 7.14, the temperature had the second biggest impact on E1 removal from wastewater, followed by the contact time, which is different from the reported order of significance in clean water matrix in Chapter 5. Figure 7.15 shows the impact of laccase concentration and temperature on E1 removal efficiency in wastewater. The impact of laccase concentration on E1 removal is again noticeably higher than the impact of the temperature.

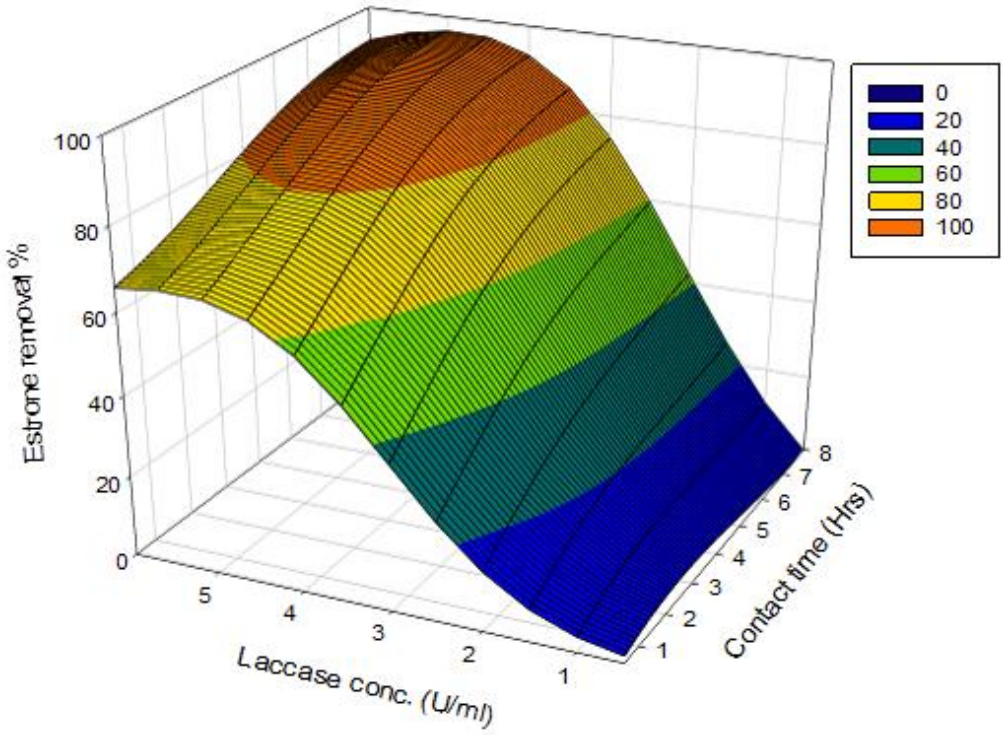


Figure 7.13 3D graph of the final ANN model. The graph represents the predicted impact of laccase concentration and contact time on estrone removal efficiency, the temperature was held constant at the median condition =15.5˚C.

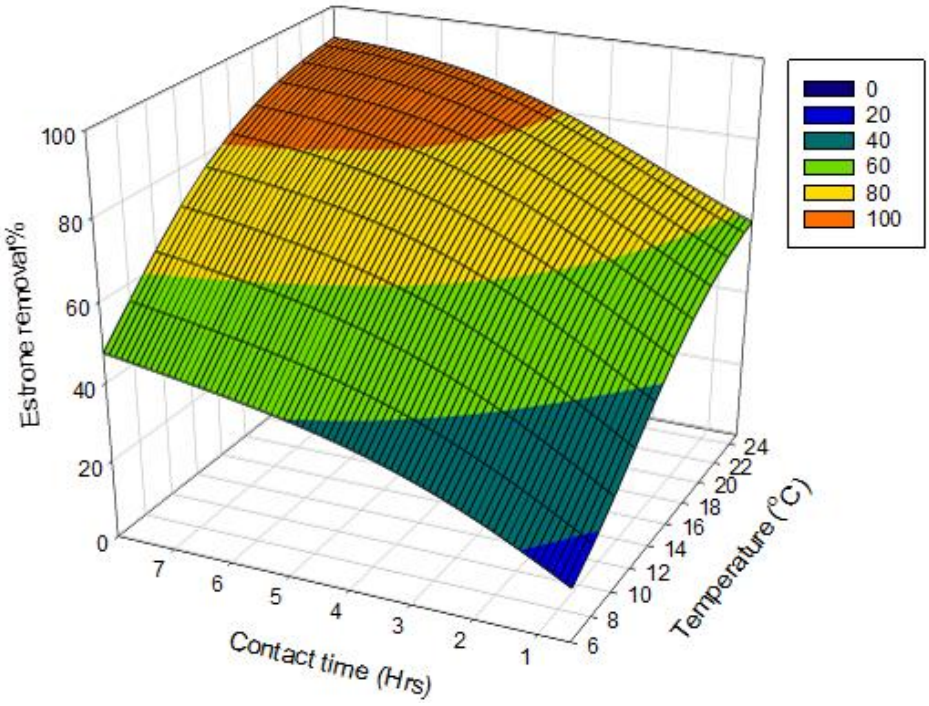


Figure 7.14 3D graph of the final ANN model. The graph represents the predicted impact of temperature and contact time on estrone removal efficiency, laccase concentration was held constant at the median condition =3.25 U/ml.

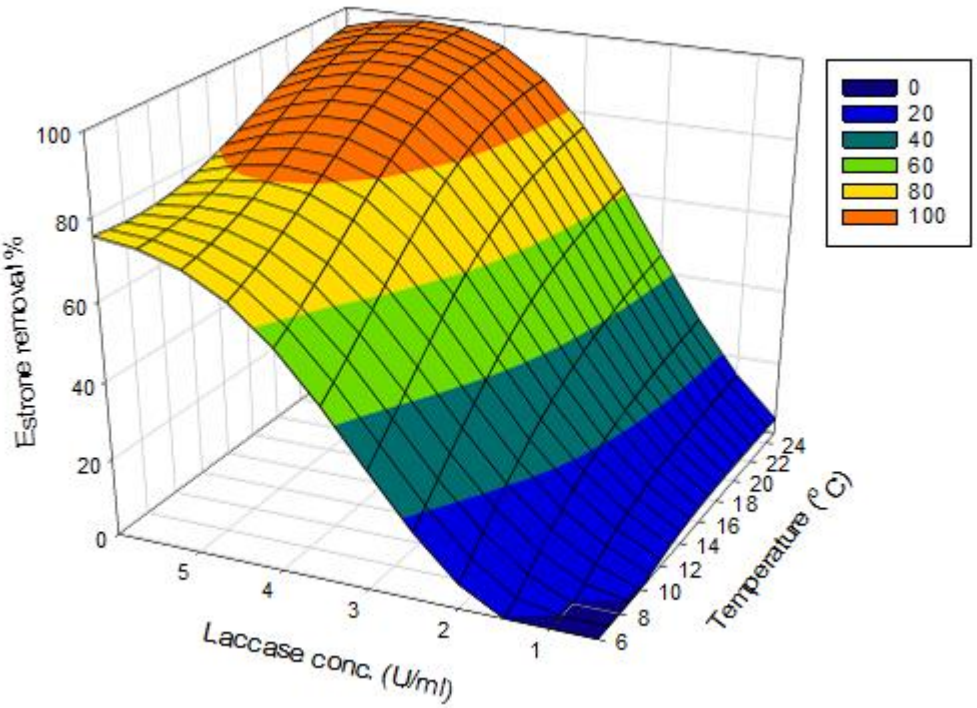


Figure 7.15 3D graph of the final ANN model. The graph represents the predicted impact of temperature and laccase concentration on estrone removal efficiency, the contact time was held constant at the median condition=4.25 hrs.

7.8 CONCLUSIONS

- Wastewater is a complex and variable matrix and a 2^{nd} order polynomial equation such as RSM model was not able to accurately represent this system. The ANN approach is more flexible as the generated model is not based on any particular equation that is used to fit the system.
- To improve ANN performance in complex matrices such as wastewater, it is necessary to include the variability of that matrix within the model itself and tie it up to the other investigated factors in the system. The variability in this chapter was represented by the benchmark experiment that was performed after each sampling trip.
- ANN model utilises the matrix of conditions (inputs) and the results of E1 removal efficiency (outputs) to train, test and validate itself. Increasing the number of experiments will create a model with better understanding of the tested system and more accurate prediction of the achieved removal efficiency under various conditions.
- The type of the used network training function plays a big role in defining the quality of the generated ANN model. Changing the network training function from trainlm to trainbr had significantly improved the prediction capabilities of the generated model for both standard and unseen experiments.
- The built ANN model can accurately predict the achieved E1 removal efficiency for any combination of conditions within the investigated system. The removal efficiency for a set of condition from outside that system cannot be accurately predicted using the same model.

8 CONCLUSIONS, RECOMMENDATIONS AND FUTURE WORK

8.1 CONCLUSIONS

The aim of this work was to investigate the ability of laccase-based treatment to remove estrone (E1) from water matrices under realistic conditions to wastewater treatment plants (WWTPs), utilising response surface methodology (RSM) and artificial neural network (ANN) models with high predictive capabilities to optimise laccase-based treatment system. This thesis contained four experimental chapters that were designed to achieve the above aim. The first experimental chapter (Chapter 4) identified the required controls and preliminary experiments to develop a robust experimental procedure to remove E1 from water matrices using laccase. The second experimental chapter (Chapter 5) demonstrated the ability of laccase-based treatment to degrade estrone (E1) in simple matrix such as deionised water. The experimental results from this chapter were utilised to build RSM and ANN models. The third chapter (Chapter 6) focused on characterising the final effluent from wastewater treatment plants (WWTPs), evaluating its variability and studying the impact of various inhibitors within that effluent on the efficiency of laccase-based treatment system. The final chapter (Chapter 7) was dedicated to study the performance of laccase-based treatment in actual wastewater effluent under relevant conditions to WWTPs. The variability of wastewater was included as an additional factor to the original Box Behnken Design (BBD) matrix of conditions to build a final ANN model with high predictive capabilities in wastewater matrix.

This chapter is dedicated to summaries the main findings of this work.

- **Developing a robust experimental procedure to study the degradation of estrone by laccase in water matrices**

This experimental procedure was developed by reviewing the impact of each step of the batch experiment on the activity of laccase or on the concentration of E1. The results showed that concentrated solutions (>1 mg/ml) of unpurified

Trametes versicolor laccase are inhomogeneous and may vary in their activity from aliquot to aliquot. Centrifuging such solution for 5 mins at 6000 rpm can improve its homogeneity, however the activity of laccase in the solution may decrease post centrifugation. Therefore, the actual activity of laccase in these solutions must be determined only after centrifugation.

Filtering samples through membrane filters is a route for abiotic removal of E1 that should be considered. The adsorption of E1 onto membrane filters largely depends on the type of the used membrane. Glass microfiber (GMF) and regenerated cellulose (RC) filters showed a minimal affinity toward E1 by adsorbing only 4.25% and 3.2% of E1 in the sample, respectively. RC filters can be successfully utilised in E1 studies where the adsorbed percentage can be accounted for and corrected during data analysis.

At the end of the enzymatic reaction, 25 µl of concentrated hydrochloric acid (HCl) per 1 ml of sample was able to instantly and permanently stop the enzymatic reaction without impacting on the concentration of E1 in the reaction mixture even after 10 day contact time with the acid.

- **Providing a "proof-of-concept" of laccase ability to degrade E1 in water matrices and modelling the system using RSM and ANN**

The ability of laccase to degrade E1 in deionised water, under realistic ranges of temperatures and contact times was demonstrated. The ANOVA results of the built RSM model showed that the most significant impact on E1 removal efficiency was caused by contact time followed by laccase concentration followed by temperature, where the impact of the temperature was around 50% of contact time's impact. The centre points of the Box Behnken Design (BBD) matrix of conditions showed a good reproducibility in clean water matrix with CV less than 2%. For the first time the predictive capabilities of the RSM and the ANN models were assessed not only with popular statistical indices, but also with statistically designed unseen data using Central Composite Design. Both models showed relatively poor predictive capabilities and further modifications to the models should be considered.

- **Quantifying the variability of the wastewater and its impact on laccase-based treatment**

Common water quality parameters such as chemical oxygen demand (COD), dissolved oxygen (DO), total suspended solids (TSS) and pH can be used to characterise wastewater effluent and demonstrate its variability. However, the majority of current studies have no appreciation of the impact of matrix variability on the performance of the investigated technology. "Wastewater Benchmark" is a new wastewater parameter that was specially designed to quantify the impact of the temporal wastewater variability on E1 removal efficiency by laccase, in situ. This parameter is performed in an actual wastewater effluent and represents the amenability of wastewater to be treated by laccase using a relevant substrate, E1. Thus, "Wastewater Benchmark" can be used to compare effluents from different WWTPs and assess the potential efficiency of laccase-based treatment in each plant.

- **Evaluating the impact of common laccase inhibitors on laccase activity and E1 removal efficiency**

The impact of 4 ions, namely chloride, copper, iron and zinc, on laccase-based treatment was studied using two different substrate: ABTS (a standard substrate) and E1 (wastewater relevant substrate), the result showed that chloride ions have a stronger inhibitory effect on laccase at pH 4.5, where 200 mg/l of chloride ions in the solution reduced E1 removal percentage by 12.5 %. This effect was significantly weaker at pH 7, where the same amount of chloride decreased E1 removal percentage only by 6.5%. No clear positive or negative impact of copper ions on E1 removal percentage was detected at Cu^{2+} concentrations below 50 mg/l. Experiments showed that even the presence of 50 mg/l of Cu^{2+} in the solution had a limited impact on laccase-based treatment, reducing E1 removal efficiency by less than 4%. The concentration of the Cu^{2+} in the majority of the municipal wastewater effluents is below 50 mg/l and therefore will not have a significant impact on laccase-based treatment. Experiments with Fe^{3+} and ABTS showed that laccase activity was reduced by around 12.5% in the presence of 10 mg/l, 50 mg/l and 100 mg/l of

Fe^{3+}. Solutions with 10mg/l or above of Fe^{3+} had the same inhibitory effect on laccase activity. Zinc ions within the following range [0.05–50] mg/l did not significantly impact on laccase ability to remove E1 from the water matrix. All the tested Zn^{2+}concentrations had a slight positive impact (less than 5%) on E1 removal efficiency, which could be partially attributed to analytical errors.

The potential location of laccase-based treatment in this work is at the end of the conventional WWTP (at the end of the secondary treatment stage). The pH of the secondary wastewater effluent is typically around pH 7, which is far from the optimum pH (pH 4.5) for laccase activity. The specific activity (SA) of laccase at pH 4.5 is about 300 times higher than its SA at pH 7. However, previous studies showed that the catalytic activity of laccase is inversely proportional to its stability and that laccase is more stable at pH 7 than at pH 4.5. Having relatively high laccase stability near neutral pHs can be a desired property when operating laccase-based treatment as a continuous process.

- **Modelling laccase-based treatment of E1 in wastewater effluent using ANN model with high predictive capabilities**

The degradation of E1 in wastewater effluent by laccase was performed in a similar manner to the previously conducted experiments in deionised water. However, the variability of this matrix affected the goodness of fit of the RSM model and its predictive capabilities. Unlike the RSM model, the ANN model was more flexible and able to easily include the variability of wastewater within its structure by introducing the benchmark as a fourth factor. In addition, the data from the benchmark experiments (the inputs and the output) were utilised to increase the number of data point within the ANN model and subsequently improve its predictive capabilities. Changing the ANN network training function from *trainlm* to *trainbr* had also significantly improved the predictive capabilities of ANN model, where R^2 between the actual and the predicted values for a set of unseen experiments was above 0.99. This means that the built ANN model can be used as to predict E1 removal efficiency for any set of conditions within that system. Optimising the performance of laccase-based treatment and maintaining the minimum required E1 removal efficiency by adjusting the other factors, can also be achieved using this model.

8.2 RECOMMENDATIONS AND FUTURE WORK

Laccase-based treatment is a promising developing technology that is steadily moving up the technology readiness levels (TRL). Several aspects of this treatment technology are still under study and require further work and research before this technology can be implemented in actual wastewater treatment plants.

- **Designing a reactor for continuous steroids removal in wastewater**

Designing an enzymatic reactor for continuous steroids removal from actual wastewater and under realistic conditions to WWTPs, is the next level for this technology and it must be thoroughly investigated. Several research groups have already presented possible designs for continuous enzymatic reactors using either suspended or immobilised laccase. However, the majority of these studies were performed either in clean water matrix or synthetic wastewater. A small number of studies were performed in actual wastewater effluent, but the applied conditions in the reactor were far from the actual conditions in wastewater treatment plants.

- **Study the enzymatic degradation of steroids using their environmentally relevant concentrations**

The concentrations of steroids in wastewater effluents vary from WWTP to WWTP, but in the majority of the cases their concentrations are around 0.1 µg/l or below. Understanding the efficiency of laccase-based treatment in removing extremely low concentrations of steroids is essential to evaluate the feasibility of implementing this treatment in actual WWTPs. This type of studies requires advanced analytical equipments to measure the concentration of steroid before the treatment and after it, and a robust experimental procedure.

- **Utilising a mixture of enzymes to degrade conjugated steroids in wastewater**

Laccase can effectively degrade free steroid estrogens without the help of mediators. However laccase on its own is unable to remove the conjugated forms of steroids that may later deconjugate in the aquatic environment and adversely impact of its living organisms. An enzyme such as β-D-glucuronidase can deconjugate the glucuronidase conjugates of steroids, releasing the free steroids into the water matrix, which can be then directly degraded by laccase. There are several aspects to be investigated in this system such as the efficiency of this treatment in actual wastewater.

- **Developing a methodology to produce laccase on a large scale and in a cost effective manner**

Implementing laccase-based treatment in WWTPs means that the utilised laccase should be commercially available in large quantities and at relatively low price. Therefore there is a need to consider the feasibility of producing *Trametes versicolor* laccase on a large scale including the availability of the feedstock (white rot fungi), the process of laccase extraction and the storage facilities of the produced laccase.

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10 APPENDICES

10.1 APPENDIX A

Literature screening of steroids and other bioactive chemicals present in environmental and non-environmental water matrices and their concentrations.

Table 10.1 The concentrations of detected steroid estrogens in water matrices around the world.

Steroid estrogens	concentration	water matrix	Country	Ref.
E1	14.5/8.3 ng/l	wastewater Influent/ wastewater Effluent	Japan	[36]
E2	19.8/1.6 ng/l	wastewater Influent/ wastewater Effluent	Japan	
E1-3S	6.8/ND ng/l	wastewater Influent/ wastewater Effluent	Japan	
E2-3S	5.6/0.2 ng/l	wastewater Influent/ wastewater Effluent	Japan	
E1-3G	ND/ND ng/l	wastewater Influent/ wastewater Effluent	Japan	
E2-3G	ND/ND ng/l	wastewater Influent/ wastewater Effluent	Japan	
17α-estradiol (α-E2)	ND/ND	wastewater Influent/ wastewater Effluent	Pennsylvania, USA	[47]
17β-estradiol (β-E2)	198.3/ND ng/l	wastewater Influent/ wastewater Effluent	Pennsylvania, USA	
17α-dihydroequilin	20.4/11.9 ng/l	wastewater Influent/ wastewater Effluent	Pennsylvania, USA	
17α-Ethinyl estradiol (EE2)	16.4/ND ng/l	wastewater Influent/ wastewater Effluent	Pennsylvania, USA	
Estriol (E3)	709.8/ ND ng/l	wastewater Influent/ wastewater Effluent	Pennsylvania, USA	
Estrone (E1)	49.2/ ND ng/l	wastewater Influent/ wastewater Effluent	Pennsylvania, USA	
E1	32.7/31.7 ng/l	wastewater Influent/ wastewater Effluent	Japan	[48]
E2	13/ND ng/l	wastewater Influent/ wastewater Effluent	Japan	
E3	140/ND ng/l	wastewater Influent/ wastewater Effluent	Japan	
E1-3S	7.7/0.7 ng/l	wastewater Influent/ wastewater Effluent	Japan	
E2-3S	ND/ND	wastewater Influent/ wastewater Effluent	Japan	
E3-3S	36.1/2.1 ng/l	wastewater Influent/ wastewater Effluent	Japan	
E1-3G	ND/ND	wastewater Influent/ wastewater Effluent	Japan	
E2-3G	ND/ND	wastewater Influent/ wastewater Effluent	Japan	
E3-3G	ND/ND	wastewater Influent/ wastewater Effluent	Japan	
E1	96/19 ng/l	wastewater Influent/ wastewater Effluent	Japan	[197]

E2	5/ ND ng/l	wastewater Influent/ wastewater Effluent	Japan	
E3	111/ND	wastewater Influent/ wastewater Effluent	Japan	
E1-3S	7/ ND	wastewater Influent/ wastewater Effluent	Japan	
E2-3S	ND/ND	wastewater Influent/ wastewater Effluent	Japan	
E3-3S	55/6	wastewater Influent/ wastewater Effluent	Japan	
EE2	154/- ng/l	wastewater Influent/ wastewater Effluent	Spain	[198]
E1-3S	160/35 ng/l	wastewater Influent/ wastewater Effluent	Spain	
E2-3S	76/Detected ng/l	wastewater Influent/ wastewater Effluent	Spain	
Estrone (E1)	15/3 ng/l	wastewater Influent/ wastewater Effluent	UK	[199]
E2	5/0.7 ng/l	wastewater Influent/ wastewater Effluent	UK	
Estriol (E3)	50/1 ng/l	wastewater Influent/ wastewater Effluent	UK	
17α-Ethinyl estradiol (EE2)	1.2/1 ng/l	wastewater Influent/ wastewater Effluent	UK	
E1-3S	10/12 ng/l	wastewater Influent/ wastewater Effluent	UK	
Estriol (E3)	1.81/2.18 mg/l	Male/Female urine (Max)	China	[200]
17β-Estradiol (β-E2)	<2.7 µg/l/10.64 mg/l	Male/Female urine (Max)	China	
17α-Estradiol (α-E2)	<4.6 µg/l /0.15 mg/l	Male/Female urine (Max)	China	
17α-Ethinylestradiol (α-EE2)	1.3/0.49 mg/l	Male/Female urine (Max)	China	
4-Nonylphenol (NP)	2.26/0.14 mg/l	Male/Female urine (Max)	China	
E1-3S	2.2 ng/l	Effluent from WWTP	Japan	[201]
E2-3S	1 ng/l	Effluent from WWTP	Japan	
E1	34 ng/l	Effluent from WWTP	Japan	
β-E2	2.5 ng/l	Effluent from WWTP	Japan	
E1-3S	0.67 ng/l	Tamagawa River	Japan	[201]
E2-3S	0.57 ng/l	Tamagawa River	Japan	
E1	4.6 ng/l	Tamagawa River	Japan	
β-E2	0.8 ng/l	Tamagawa River	Japan	

E1-3S	0.52 ng/l	Lake Kasumigaura	Japan	[201]
E2-3S	0.18 ng/l	Lake Kasumigaura	Japan	
E1	0.62 ng/l	Lake Kasumigaura	Japan	
E3-3G	Detected	wastewater collecting tank (16 h)	Rome, Italy	[20]
E3-16G	39 ng/l	wastewater collecting tank (16 h)	Rome, Italy	
E3-3S	47 ng/l	wastewater collecting tank (16 h)	Rome, Italy	
E2-3G	9 ng/l	wastewater collecting tank (16 h)	Rome, Italy	
E2-17G	Detected	wastewater collecting tank (16 h)	Rome, Italy	
E2-3S	9 ng/l	wastewater collecting tank (16 h)	Rome, Italy	
E1-3G	10 mg/l	wastewater collecting tank (16 h)	Rome, Italy	
E1-3S	27 ng/l	wastewater collecting tank (16 h)	Rome, Italy	
E3	62 ng/l	wastewater collecting tank (16 h)	Rome, Italy	
E2	9 ng/l	wastewater collecting tank (16 h)	Rome, Italy	
E1	58 ng/l	wastewater collecting tank (16 h)	Rome, Italy	
E3-3G	Detected/Detected	wastewater Influent/ wastewater Effluent	Rome, Italy	[20]
E3-16G	19/ Detected ng/l	wastewater Influent/ wastewater Effluent	Rome, Italy	
E3-3S	14/2.2 ng/l	wastewater Influent/ wastewater Effluent	Rome, Italy	
E2-3G	5.2/ Detected ng/l	wastewater Influent/ wastewater Effluent	Rome, Italy	
E2-17G	Detected/Detected	wastewater Influent/ wastewater Effluent	Rome, Italy	
E2-3S	3.3/Detected ng/l	wastewater Influent/ wastewater Effluent	Rome, Italy	
E1-3G	4.3/0.7 ng/l	wastewater Influent/ wastewater Effluent	Rome, Italy	
E1-3S	25/9 ng/l	wastewater Influent/ wastewater Effluent	Rome, Italy	
E3	72/2.3 ng/l	wastewater Influent/ wastewater Effluent	Rome, Italy	
E2	11/1.6 ng/l	wastewater Influent/ wastewater Effluent	Rome, Italy	
E1	44/17 ng/l	wastewater Influent/ wastewater Effluent	Rome, Italy	

Table 10.2 The concentrations of bioactive chemicals (pharmaceuticals) and other chemicals in water matrices from all around the world.

Pharmaceutical compound	Conc.	Water matrix	Country	Ref.
Paracetamol	11.96 µg/l	Surface water	England	[25]
Metformin hydrochloride	6.3 µg/l	Surface water	England	
Ibuprofen	4.96 µg/l	Surface water	England	
Amoxycillin	2.19 µg/l	Surface water	England	
Sodium valproate	1.45 µg/l	Surface water	England	
Sulphasalazine	1.42 µg/l	Surface water	England	
Mesalazine	1.24 µg/l	Surface water	England	
Carbamazepine	1.23 µg/l	Surface water	England	
Ferrous sulphate	1.15 µg/l	Surface water	England	
Ranitidine hydrochloride	1.11 µg/l	Surface water	England	
Cimetidine	1.09 µg/l	Surface water	England	
Naproxen	1.07 µg/l	Surface water	England	
Atenolol	0.89 µg/l	Surface water	England	
Oxytetracycline	0.83 µg/l	Surface water	England	

Erythromycin	0.81 µg/l	Surface water	England	
Diclofenac sodium	0.8 µg/l	Surface water	England	
Flucloxacillin sodium	0.72 µg/l	Surface water	England	
Phenoxymethylpenicillin	0.68 µg/l	Surface water	England	
Allopurinol	0.68 µg/l	Surface water	England	
Diltiazem hydrochloride	0.67 µg/l	Surface water	England	
Gliclazide	0.57 µg/l	Surface water	England	
Aspirin	0.55 µg/l	Surface water	England	
Quinine sulphate	0.51 µg/l	Surface water	England	
Mebeverine hydrochloride	0.47 µg/l	Surface water	England	
Mefenamic acid	0.44 µg/l	Surface water	England	
Benzoylecgonine (Cocaine metabolite)	2/0.5 µg/l	wastewater Influent/ wastewater Effluent	Castellon (Eastern Spain)	[202]
Cocaine	0.6/0.5 µg/l	wastewater Influent/ wastewater Effluent	Castellon (Eastern Spain)	
Codeine	N/A	wastewater Influent/ wastewater Effluent	Castellon (Eastern Spain)	
Cotinine (nicotine metabolite)	N/A	Influent	Castellon (Eastern Spain)	

Ketamine	N/A	Effluent from WWTP	Castellon (Eastern Spain)	
MDMA	3/0.5 μg/l	wastewater Influent/ wastewater Effluent	Castellon (Eastern Spain)	
Methamphetamine	0.5 μg/l	Effluent from WWTP	Castellon (Eastern Spain)	
norBenzoylecgonine	0.5 μg/l	Effluent from WWTP	Castellon (Eastern Spain)	
Oxazepam	N/A	Effluent from WWTP	Castellon (Eastern Spain)	
Temazepam	N/A	Effluent from WWTP	Castellon (Eastern Spain)	
Ibuprofen	N/A	Effluent from WWTP	UK	[203]
Paracetamol	N/A	Effluent from WWTP	UK	
Salbutamol	N/A	Effluent from WWTP	UK	
Mefenamic Acid	18.6/ 7.4 ng/l	wastewater Influent/ wastewater Effluent	UK	
Propranolol hydrochloride	196.6/157.4 ng/l	wastewater Influent/ wastewater Effluent	UK	
Bleomycin	13 ng/l	Drinking water	UK	[204]
Clofibric acid	Positive identification	Drinking water	UK	
Diazepam	10 ng/l	Drinking water	UK	
Trimethoprim	1879 /1004 ng/l	Influent/ Effluent from WWTP	UK, South Wales	[38]

Sulfamethoxazole	0 / 12 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Erythromycin	404 /830 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Metronidazole	2608/ 373 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Paracetamol	492340/ 1826 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Ibuprofen	3742/ 227 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Diclofenac	70/ 123 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Ketoprofen	102/ 23 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Naproxen	1082/ 400 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Aspirin	966/ 0 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Salicylic acid	17461/ 209 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Mefenamic acid	444/ 115 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Codeine	9766/ 3948 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Tramadol	44700 (59046) ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Carbamazepine	2593/ 3117 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Gabapentin	18474/ 21417 ng/l	Influent/ Effluent from WWTP	UK, South Wales	

Propranolol	542/ 388 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Metoprolol	110/ 68 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Atenolol	13874/ 2702 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Clofibric acid	52/ 17 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Bezafibrate	971/ 418 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Cimetidine	2494/ 2387 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Sulfasalazine	65/ 266 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Sulfapyridine	115/ 329 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
5-Aminosalicylic acid	4789/ 3072 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Furosemide	2197/ 1144 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Valsartan	676/ 344 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Diltiazem	920/ 95 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Salbutamol	130/ 66 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Amitriptyline	849/ 207 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Amphetamine	5236/ 127 ng/l	Influent/ Effluent from WWTP	UK, South Wales	

Cocaine	526/ 149 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
Benzoylecgonine	1229/1597 ng/l	Influent/ Effluent from WWTP	UK, South Wales	
paraxanthine	15001 ng/l	Effluent from WWTP	Spain	[37]
biphenylol	7662 ng/l	Effluent from WWTP	Spain	
4-AAA	7260 ng/l	Effluent from WWTP	Spain	
caffeine	5753 ng/l	Effluent from WWTP	Spain	
ofloxacin	4422 ng/l	Effluent from WWTP	Spain	
hydrochlorothiazide	3683 ng/l	Effluent from WWTP	Spain	
4-FAA	3386 ng/l	Effluent from WWTP	Spain	
ibuprofen	2567 ng/l	Effluent from WWTP	Spain	
gemfibrozil	2337 ng/l	Effluent from WWTP	Spain	
4-AA	2098 ng/l	Effluent from WWTP	Spain	
naproxen	1840 ng/l	Effluent from WWTP	Spain	
atenolol	1720 ng/l	Effluent from WWTP	Spain	
ketorolac	1289 ng/l	Effluent from WWTP	Spain	

chlorophene	1279 ng/l	Effluent from WWTP	Spain	
nicotine	1201 ng/l	Effluent from WWTP	Spain	
4-MAA	1051 ng/l	Effluent from WWTP	Spain	
furosemide	1050 ng/l	Effluent from WWTP	Spain	
codeine	1039 ng/l	Effluent from WWTP	Spain	
ciprofloxacin	923 ng/l	Effluent from WWTP	Spain	
diclofenac	826 ng/l	Effluent from WWTP	Spain	
ranitidine	684 ng/l	Effluent from WWTP	Spain	
ketoprofen	553 ng/l	Effluent from WWTP	Spain	
erythromycin	519 ng/l	Effluent from WWTP	Spain	
indomethacine	405 ng/l	Effluent from WWTP	Spain	
fluoxethine	398 ng/l	Effluent from WWTP	Spain	
trimethoprim	331 ng/l	Effluent from WWTP	Spain	
antipyrine	317 ng/l	Effluent from WWTP	Spain	
sulfamethoxazole	275 ng/l	Effluent from WWTP	Spain	

omeprazole	247 ng/l	Effluent from WWTP	Spain	
benzafibrate	233 ng/l	Effluent from WWTP	Spain	
fenofibric acid	186 ng/l	Effluent from WWTP	Spain	
chlorfenvinphos	163 ng/l	Effluent from WWTP	Spain	
metronidazole	160 ng/l	Effluent from WWTP	Spain	
diuron	138 ng/l	Effluent from WWTP	Spain	
mefenamic acid	138 ng/l	Effluent from WWTP	Spain	
carbamazepine	136 ng/l	Effluent from WWTP	Spain	
metoprolol	61 ng/l	Effluent from WWTP	Spain	
carbamazepine epoxide	52 ng/l	Effluent from WWTP	Spain	
Paracetamol (acetaminophen)	59 ng/l	Effluent from WWTP	Spain	
propanolol	44 ng/l	Effluent from WWTP	Spain	
sotalol	36 ng/l	Effluent from WWTP	Spain	
clofibric acid	27 ng/l	Effluent from WWTP	Spain	
simazine	16 ng/l	Effluent from WWTP	Spain	

diazepan	16 ng/l	Effluent from WWTP	Spain	
mepivacaine	15 ng/l	Effluent from WWTP	Spain	
salbutamol	15 ng/l	Effluent from WWTP	Spain	
terbutaline	15 ng/l	Effluent from WWTP	Spain	
salicylic acid	25-100 ng/l	Surface water	Netherlands	[49]
Bezafibrate	<25-100 ng/l	Surface water	Netherlands	
Bisoprolol	<25 ng/l	Surface water	Netherlands	
Carbamazepine	<25->100 ng/l	Surface water	Netherlands	
Clofibric acid	<25 ng/l	Surface water	Netherlands	
Dehydro erythromycin	<25-100 ng/l	Surface water	Netherlands	
Diclofenac	<25 ng/l	Surface water	Netherlands	
Metoprolol	<25-100 ng/l	Surface water	Netherlands	
Sulfamethoxazole	<25-100 ng/l	Surface water	Netherlands	
Ibuprofen	3086 ng/l	Effluent from WWTP	UK	[205]
Diclofenac	424 ng/l	Effluent from WWTP	UK	

Propranolol	76 ng/l	Effluent from WWTP	UK	
Dextropropoxyphene	195 ng/l	Effluent from WWTP	UK	
Mefenamic acid	133 ng/l	Effluent from WWTP	UK	
Erythromycin	<10 ng/l	Effluent from WWTP	UK	
Trimethoprim	70 ng/l	Effluent from WWTP	UK	
Acetyl-sulfamethoxazole	<50 ng/l	Effluent from WWTP	UK	
Sulfamethoxazole	<50 ng/l	Effluent from WWTP	UK	
Tamoxifen	<10 ng/l	Effluent from WWTP	UK	
Salicylic acid	330/3.6 µg/l	wastewater Influent/ wastewater Effluent	Canada	[206]
Ibuprofen	38.7/4 µg/l	wastewater Influent/ wastewater Effluent	Canada	
Fenoprofen	1.8/ND µg/l	wastewater Influent/ wastewater Effluent	Canada	
Ketoprofen	5.7/ND µg/l	wastewater Influent/ wastewater Effluent	Canada	
Diclofenac	1.3/ND µg/l	wastewater Influent/ wastewater Effluent	Canada	
Naproxen	40.7/12.5 µg/l	wastewater Influent/ wastewater Effluent	Canada	
Bezafibrate	0.6/0.2 µg/l	wastewater Influent/ wastewater Effluent	Canada	

Gemfibrozil	0.7/1.3 µg/l	wastewater Influent/ wastewater Effluent	Canada	
Clofibric acid	/	wastewater Influent/ wastewater Effluent	Canada	
Phenazone	/	wastewater Influent/ wastewater Effluent	Canada	
Pentoxifylline	0/0.5 µg/l	wastewater Influent/ wastewater Effluent	Canada	
Carbamazepine	0.7/0.7 µg/l	wastewater Influent/ wastewater Effluent	Canada	
Ifosfamide	/	wastewater Influent/ wastewater Effluent	Canada	
Cyclophosphamide	/	wastewater Influent/ wastewater Effluent	Canada	
Ibuprofen	545 µg/l	Urine	Berlin/ Germany	[207]
Bezafibrate	516 µg/l	Urine	Berlin/ Germany	
ß-Sitosterol	30.7 µg/l	Urine	Berlin/ Germany	
Diclofenac	22.5 µg/l	Urine	Berlin/ Germany	
Carbamazepine	9 µg/l	Urine	Berlin/ Germany	
Pentoxifylline	2.9 µg/l	Urine	Berlin/ Germany	
Phenazone	1.3 µg/l	Urine	Berlin/ Germany	
Paracetamol (acetaminophen)	1968/201000 ng/l	Surface water / Effluent from WWTP	Spain	[50]

4-aminoantipyrine (4-AA)	811/2770 ng/l	Surface water / Effluent from WWTP	Spain	
Atorvastatin	42/209 ng/l	Surface water / Effluent from WWTP	Spain	
Bezafibrate	49/312 ng/l	Surface water / Effluent from WWTP	Spain	
Ciprofloxacin	740/2292 ng/l	Surface water / Effluent from WWTP	Spain	
Clarithromycin	91/247 ng/l	Surface water / Effluent from WWTP	Spain	
Diclofenac	358/690 ng/l	Surface water / Effluent from WWTP	Spain	
Enalapril	88/236 ng/l	Surface water / Effluent from WWTP	Spain	
Enrofloxacin	70/220 ng/l	Surface water / Effluent from WWTP	Spain	
Erythromycin	78/82 ng/l	Surface water / Effluent from WWTP	Spain	
Flumequine	20/41 ng/l	Surface water / Effluent from WWTP	Spain	
Gemfibrozil	304/2008 ng/l	Surface water / Effluent from WWTP	Spain	
Ibuprofen	2850/15100 ng/l	Surface water / Effluent from WWTP	Spain	
Ketoprofen	70/583 ng/l	Surface water / Effluent from WWTP	Spain	
Lincomycin	47/142 ng/l	Surface water / Effluent from WWTP	Spain	
Lorazepam	0/81 ng/l	Surface water / Effluent from WWTP	Spain	

Moxifloxacin	205/540 ng/l	Surface water / Effluent from WWTP	Spain	
Nalidixic acid	14/60 ng/l	Surface water / Effluent from WWTP	Spain	
Naproxen	285/710 ng/l	Surface water/ Effluent from WWTP	Spain	
Norfloxacin	54/310 ng/l	Surface water/ Effluent from WWTP	Spain	
Ofloxacin	400/925 ng/l	Surface water/ Effluent from WWTP	Spain	
Pantoprazole	117/36 ng/l	Surface water / Effluent from WWTP	Spain	
Pefloxacin	64/112 ng/l	Surface water / Effluent from WWTP	Spain	
Pipedimic acid	245/430 ng/l	Surface water / Effluent from WWTP	Spain	
Pravastatin	0/69 ng/l	Surface water / Effluent from WWTP	Spain	
Roxithromycin	12/18 ng/l	Surface water / Effluent from WWTP	Spain	
Salicylic acid	1160/80000 ng/l	Surface water / Effluent from WWTP	Spain	[50]
Sarafloxacin	55/52 ng/l	Surface water / Effluent from WWTP	Spain	
Sulfamethoxazole	33/432 ng/l	Surface water / Effluent from WWTP	Spain	
Trimethoprim	151/232 ng/l	Surface water / Effluent from WWTP	Spain	
Venlafaxine	575/875 ng/l	Surface water / Effluent from WWTP	Spain	

Carbamazepine	356.1/251 ng/l	Influent/ Effluent from WWTP	Canada	[45]
CBZ-EP	39.2/19.1 ng/l	Influent/ Effluent from WWTP	Canada	
CBZ-2OH	59.0/70.4 ng/l	Influent/ Effluent from WWTP	Canada	
CBZ-3OH	55.4/69.2 ng/l	Influent/ Effluent from WWTP	Canada	
CBZ-10OH	22.2/32.5 ng/l	Influent/ Effluent from WWTP	Canada	
CBZ-DiOH	1001.2/1081.2 ng/l	Influent/ Effluent from WWTP	Canada	
Gemfibrozil	0.71/0.18 µg/l	Influent/ Effluent from WWTP	Sweden	[46]
Ibuprofen	3.59/0.15 µg/l	Influent/ Effluent from WWTP	Sweden	
Ketoprofen	0.94/0.33 µg/l	Influent/ Effluent from WWTP	Sweden	
Naproxen	3.65/0.25 µg/l	Influent/ Effluent from WWTP	Sweden	
Diclofenac	0.16/0.12 µg/l	Influent/ Effluent from WWTP	Sweden	
Carbamazepine	1.68/1.18 µg/l	Influent/ Effluent from WWTP	Sweden	
Atenolol	0.03/0.16 µg/l	Influent/ Effluent from WWTP	Sweden	
Metoprolol	0.16/0.19 µg/l	Influent/ Effluent from WWTP	Sweden	
Propanolol	0.05/0.03 µg/l	Influent/ Effluent from WWTP	Sweden	

Trimethoprim	0.08/0.04 µg/l	Influent/ Effluent from WWTP	Sweden	
Sulfamethoxazole	0.02/0.07 µg/l	Influent/ Effluent from WWTP	Sweden	
Hydroxy-ibuprofen	0.99/0.05 µg/l	Influent/ Effluent from WWTP	Sweden	
Carboxy-ibuprofen	10.75/0.43 µg/l	Influent/ Effluent from WWTP	Sweden	
Triclosan	0.38/0.16 µg/l	Influent/ Effluent from WWTP	Sweden	
Tris(2-chloro-isopropyl)phosphate	2.79/2.26 µg/l	Influent/ Effluent from WWTP	Sweden	
Tris(2-butoxyethyl)phosphate	9.44/1.89 µg/l	Influent/ Effluent from WWTP	Sweden	
BHT	2.53/0.61 µg/l	Influent/ Effluent from WWTP	Sweden	
BHT-aldehyde	0.56/0.49 µg/l	Influent/ Effluent from WWTP	Sweden	
Galaxolide(HHCB)	0.79/1.08 µg/l	Influent/ Effluent from WWTP	Sweden	
Nonyl phenol (NP)	1.14/0.34 µg/l	Influent/ Effluent from WWTP	Sweden	
Palmitic (hexadecanoic) acid	35.91/0.71 µg/l	Influent/ Effluent from WWTP	Sweden	
Stearic (octadecanoic) acid	41.00/0.80 µg/l	Influent/ Effluent from WWTP	Sweden	
Caffeine	3.69/0.22 µg/l	Influent/ Effluent from WWTP	Sweden	
Gemfibrozil	0.71 µg/l	Effluent from WWTP	Greece	[208]

Fenofibrate	0.16 µg/l	Effluent from WWTP	Greece	
Clofibrate	0.8 µg/l	Effluent from WWTP	Greece	
Ibuprofen	0.05 µg/l	Effluent from WWTP	Greece	
Diclofenac	0.89 µg/l	Effluent from WWTP	Greece	
Acebutolol	0.01 µg/l	Effluent from WWTP	Greece	
Metoprolol	0.1 µg/l	Effluent from WWTP	Greece	
Oxprenolol	0.01 µg/l	Effluent from WWTP	Greece	
Propranolol	0.01 µg/l	Effluent from WWTP	Greece	
Carbamazepine	1.03 µg/l	Effluent from WWTP	Greece	
Trimethoprim	0.08 µg/l	Effluent from WWTP	Greece	
Sulfamethoxazole	0.09 µg/l	Effluent from WWTP	Greece	
Ofloxacin	0.46 µg/l	Effluent from WWTP	Greece	
Lomefloxacin	0.29 µg/l	Effluent from WWTP	Greece	
Enoxacin	0.03 µg/l	Effluent from WWTP	Greece	
Norfloxacin	0.07 µg/l	Effluent from WWTP	Greece	

Ciprofloxacin	0.07 µg/l	Effluent from WWTP	Greece	
Gemfibrozil	4.76 µg/l	Effluent from WWTP	Italy	[208]
Fenofibrate	0.16 µg/l	Effluent from WWTP	Italy	
Bezafibrate	0.91 µg/l	Effluent from WWTP	Italy	
Clofibric acid	0.23 µg/l	Effluent from WWTP	Italy	
Ibuprofen	0.02 µg/l	Effluent from WWTP	Italy	
Flurbiprofen	0.34 µg/l	Effluent from WWTP	Italy	
Naproxen	5.22 µg/l	Effluent from WWTP	Italy	
Diclofenac	5.45 µg/l	Effluent from WWTP	Italy	
Acebutolol	0.11 µg/l	Effluent from WWTP	Italy	
Metoprolol	0.1 µg/l	Effluent from WWTP	Italy	
Oxprenolol	0.03 µg/l	Effluent from WWTP	Italy	
Propranolol	0.09 µg/l	Effluent from WWTP	Italy	
Carbamazepine	0.5 µg/l	Effluent from WWTP	Italy	
Trimethoprim	0.13 µg/l	Effluent from WWTP	Italy	

Sulfamethoxazole	0.03 µg/l	Effluent from WWTP	Italy	
Ofloxacin	0.31 µg/l	Effluent from WWTP	Italy	
Lomefloxacin	0.22 µg/l	Effluent from WWTP	Italy	
Enoxacin	0.03 µg/l	Effluent from WWTP	Italy	
Norfloxacin	0.06 µg/l	Effluent from WWTP	Italy	
Ciprofloxacin	0.04 µg/l	Effluent from WWTP	Italy	
Gemfibrozil	2.07 µg/l	Effluent from WWTP	Sweden	[208]
Clofibric acid	0.46 µg/l	Effluent from WWTP	Sweden	
Ibuprofen	7.11 µg/l	Effluent from WWTP	Sweden	
Naproxen	2.15 µg/l	Effluent from WWTP	Sweden	
Acebutolol	<0.01 µg/l	Effluent from WWTP	Sweden	
Metoprolol	0.39 µg/l	Effluent from WWTP	Sweden	
Propranolol	0.01 µg/l	Effluent from WWTP	Sweden	
Carbamazepine	0.87 µg/l	Effluent from WWTP	Sweden	
Trimethoprim	0.05 µg/l	Effluent from WWTP	Sweden	

Sulfamethoxazole	0.02 µg/l	Effluent from WWTP	Sweden	
Ofloxacin	0.12 µg/l	Effluent from WWTP	Sweden	
Lomefloxacin	0.13 µg/l	Effluent from WWTP	Sweden	
Enoxacin	0.01 µg/l	Effluent from WWTP	Sweden	
Norfloxacin	0.03 µg/l	Effluent from WWTP	Sweden	
Ciprofloxacin	0.03 µg/l	Effluent from WWTP	Sweden	
Triclosan	734 ng/l	Drinking water	USA	[51]
Carbamazepine	140-258 ng/l	Drinking water	USA	
Dilantin	1.3 ng/l	Drinking water	USA	
Primidone	40 ng/l	Drinking water	Germany	
Amitryptilline	1.4 ng/l	Drinking water	France	
Meprobamate	5.9 ng/l	Drinking water	USA	
Diatrizoate	1200 ng/l	Drinking water	Germany	
Iopromide	<50 ng/l	Drinking water	Germany	
Bezafibrate	27 ng/l	Drinking water	Germany	

Clofibric acid	50-270 ng/l	Drinking water	Germany	
Gemfibrozil	70 ng/l	Drinking water	Canada	
Paracetamol (acetaminophen)	210.1 ng/l	Drinking water	France	
Diclofenac	6-35 ng/l	Drinking water	Germany	
Ibuprofen	1350 ng/l	Drinking water	USA	
Ketoprofen	8 ng/l	Drinking water	Finland	
Phenazone	250-400 ng/l	Drinking water	Germany	
Propyphenazone	80-240 ng/l	Drinking water	Germany	
Codein	30 ng/l	Drinking water	USA	
Caffeine	60-119 ng/l	Drinking water	USA	
Carbamazepine	17 ng/l	Effluent from WWTP	Beijing, China	[209]
Clozapine	23 ng/l	Effluent from WWTP	Beijing, China	
Sulpiride	67 ng/l	Effluent from WWTP	Beijing, China	
Citalopram	5 ng/l	Effluent from WWTP	Beijing, China	
triclosan (TCS)	1.3/0.3 µg/l	wastewater Influent/ wastewater Effluent	Greece	

10.2 APPENDIX B

The iterative scrip of the final ANN model presented in Section 7.7:

```
% This script assumes these variables are defined:
%
%   INPUTS - input data.
%   AllOutputs - target data.
inputs = INPUTS';
targets = OUTPUTS';
mse=300
while mse > 2
% Create a Fitting Network
hiddenLayerSize =6;
net = fitnet(hiddenLayerSize);
% Choose Input and Output Pre/Post-Processing Functions
% For a list of all processing functions type: help nnprocess
net.inputs{1}.processFcns = {'removeconstantrows','mapminmax'};
net.outputs{2}.processFcns = {'removeconstantrows','mapminmax'};
% Setup Division of Data for Training, Validation, Testing
% For a list of all data division functions type: help nndivide
net.divideFcn = 'dividerand';  % Divide data randomly
net.divideMode = 'sample';  % Divide up every sample
net.divideParam.trainRatio = 70/100;
net.divideParam.valRatio = 15/100;
net.divideParam.testRatio = 15/100;
% For help on training function 'trainbr' type: help trainbr
% For a list of all training functions type: help nntrain
net.trainFcn = 'trainbr';  % Levenberg-Marquardt
% Choose a Performance Function
```

```
% For a list of all performance functions type: help nnperformance
net.performFcn = 'mse';  % Mean squared error
% Choose Plot Functions
% For a list of all plot functions type: help nnplot
% net.plotFcns = {'plotperform','plottrainstate','ploterrhist', ...
% 'plotregression', 'plotfit'};
% Train the Network
 [net,tr] = train(net,inputs,targets);
% Test the Network
outputs = net(inputs);
errors = gsubtract(targets,outputs);
performance = perform(net,targets,outputs)
% Recalculate Training, Validation and Test Performance
trainTargets = targets .* tr.trainMask{1};
valTargets = targets  .* tr.valMask{1};
testTargets = targets  .* tr.testMask{1};
trainPerformance = perform(net,trainTargets,outputs);
valPerformance = perform(net,valTargets,outputs);
testPerformance = perform(net,testTargets,outputs);
% View the Network
% view(net)
z=regress(targets',outputs');
a1=z*outputs;
a2=a1-targets;
a3=a2.^2;
a4=sum(a3);
 a5=a4/15
 mse=a5
 end
```

10.3 APPENDIX C

Constructing a Michaelis- Menten Graph

All the experiments were performed at 25 °C in a phosphate buffer (0.1 µM) at pH 7. Laccase concentration in the reaction mixture was 0.05 mg/ml. The volume of the reaction mixture was 1 ml. The increase in ABTS absorbance was measured at 420 nm using UV-vis spectrophotometer at 12 ABTS concentrations ranging between 2 µM and 1000 µM. Each experiment was performed in triplicate. Table 10.1 shows the average initial rate for each ABTS concentration. The data in this table was entered into GraphPad Prism software to construct a Michaelis-Menten graph and identify the kinetic parameters (K_M & V_{max}) for the used laccase.

Table 10.1 The average initial reaction rate of the enzyme laccase at 25 °C & pH 7 using several ABTS concentrations.

	ABTS conc. in the reaction mixture (µM)	Average **initial rate** (ν) **(µmol/min)**
1	2	0.203326
2	4	0.394253
3	6	0.580891
4	10	0.747994
5	20	0.977731
6	40	1.158599
7	60	1.246991
8	80	1.239011
9	100	1.245203
10	200	1.248168
11	500	1.238924
12	1000	1.221568

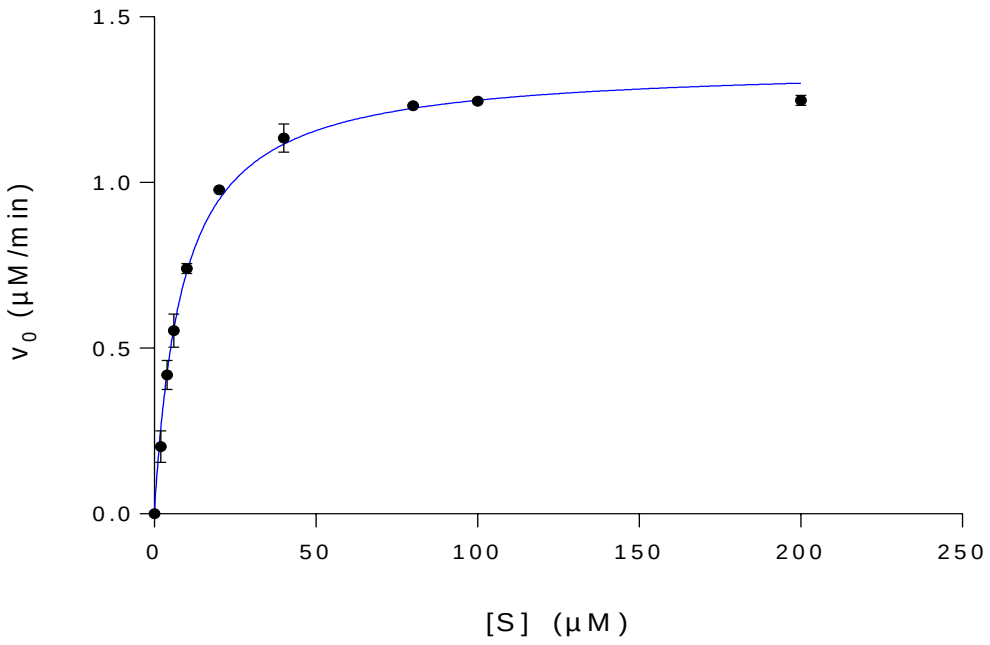

Figure 10.1 Michaelis Menten Graph generated in GraphPad Prism software.

The produced graph was analysed in GraphPad Prism and the main kinetic parameters were determined (Table 10.2).

Table 10.2 Kinetic parameters of *Trametes versicolor* calculated using GraphPad Prism software.

	K_M	**V_{max}**
Best-fit value	8.62 µM	1.36 µmol/min
Standard error	0.3991	0.0168
95% Confidence Intervals	7.798 to 9.438	1.321 to 1.391
R^2	0.9931	

The obtained K_M and V_{max} values above were relatively close to the previously reported values of *Trametes versicolor* laccase and ABTS by Raseda et al. at pH 6: K_M = 9.5 µM and V_{max} = 1.60 µmol/min[123]. Both Raseda and this work used buffers with same ionic strength (100 µM). However, Raseda et al. used laccase with specific activity of 15 U/mg, while we used *Trametes versicolor* laccase with specific activity ≥10 U/mg. Raseda et al. also determined Km and

Vmax values at pH 7 but in a buffer with lower ionic strength (20 mM): K_M = 4.1 μM and $V_{max} \approx 0.1$ μmol/min[123].

10.4 APPENDIX D

Scoping Studies to Determine the Range of Laccase Concentration in Deionised Water

The ranges of temperature [6 ºC - 25 ºC] and contact time [0.5 hr – 8 hrs] in this work were selected to be relevant to the wastewater treatment plant environment. However identifying the most suitable range for laccase concentration requires some scoping studies to ensure that there is a good breadth in estrone (E1) removal efficiency which is essential to generate a meaningful model. Scoping studies were performed using 3 different laccase concentrations: 0.01 U/ml, 0.05 U/ml and 0.1 U/ml. It has been assumed that the maximum E1 removal efficiency is achieved at 25 ºC and after 8 hr contact time, while the minimum removal efficiency is potentially achieved at 6 ºC and after 0.5 hr. The tested laccase concentrations represent the median of the potential range and the range is going to between x and 10 x, where x is the lower end of the range.

Table 10.3 Estrone removal efficiency during the scoping studies.

Laccase conc.(U/ml)	**Estrone removal efficiency (%) at:**	
	Temp: 6 ºC, Time: 0.5 hr	Temp: 6 ºC, Time: 0.5 hr
0.01	0	63.1
0.05	4.8	97.4
0.1	20.2	≥ 98.0

The above results show that 0.05 U/ml of laccase is a suitable concentration as the achieved removal efficiency of E1 at the favourable conditions was less than 98% i.e. E1 concentration at the end of the 8 hrs contact time was above the limit of quantification (LOQ) of E1 on HPLC-UV. It also shows that at the least favourable conditions, this laccase concentration is sufficient to achieve removal efficiency above 0%.

As a result the suitable range for laccase concentration in deionised water is 0.05 U/ml = (x+10x)/2 → X =0.0091 U/ml

To ease the calculation x was rounded up to 0.01 U/ml and the actual laccase range was [0.01 U/ml - 0.1U/ml].

Similar approach was utilised to determine the range of laccase concentration in wastewater matrix.