Application of White-Rot Fungi for the Biodegradation of Natural Organic Matter from Potable Water

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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“Be less curious about people and more curious about ideas”

Marie Curie

Declaration

This is to certify that:

i. The thesis comprises only my original work towards the PhD except where indicated;
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iii. This work has not been submitted previously, in whole or in part, to qualify for any other academic award
iv. The content of this thesis is the result of work which has been carried out since the official commencement date of the approved research program
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Signed ……..On……../……../……..

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Abstract

Natural organic matter (NOM) originates from the degradation of plant and animal materials and represents the largest fraction of organic substances in terrestrial and aquatic ecosystems. NOM is heterogeneous and composed of yellowish-brown organic compounds that affect the aesthetic characteristics of colour, taste and odour of water. NOM impacts on water quality by increasing disinfectant and coagulant demand, providing precursor material for the formation of disinfection by-products (DBPs) and by enhancing bacterial regrowth in distribution systems. Most conventional processes for the removal of NOM involve the use of chemicals and the generation of sludge, thus leading to disposal problems. Consequently there is interest in the development of alternative biological NOM treatments, such as those utilising fungi, that by-pass the current problems. Ligninolytic fungi (i.e., white rot fungi-WRF) secrete non-specific oxidative enzymes that can oxidise a wide range of recalcitrant organic compounds including lignin and humic substances (HS). Previous work by Rojek *et al.* (2004) and Lee (2005) has shown the potential of WRF to degrade MIEX NOM concentrate using cultured laboratory strains of *Phanerochaete chrysosporium* and *Trametes versicolor* with nutrient supplementation. This research investigated the degradation of MIEX NOM concentrate obtained from the regeneration process of the magnetic ion exchange (MIEX) resin and NOM from Beaconsfield Reservoir by WRF without the addition of nutrients.

Twenty-one isolates from diverse fungal genera were obtained from various sources and screened for their ability to degrade NOM and cellulose using plate assays. All fungi grew on the NOM agar and exhibited sparse mycelial coverage, but only the WRF, in particular *Bjerkandera adusta,* decolourised it. Fungi that grew but did not decolourise NOM were mostly non-WRF isolated from Beaconsfield Reservoir and enrichment cultures. NOM decolourisation was evident after the first day of incubation and varied in clarity, degree and pattern. When the fungi were assessed for their cellulolytic ability, most WRF expressed greater cellulase activity with a more clearly defined zone of hydrolysis than the micromycetous fungi.

Based on preliminary screening, *B. adusta, Trametes* sp.*, Polyporus* sp., *Trametes versicolor* ATCC 7731 and *Pycnoporus cinnabarinus* VIC demonstrated good clarity and degree of NOM decolourisation on solid medium and so were further investigated in shake flask culture. The selected WRF decolourised the MIEX NOM concentrate (100 mg C L^{-1}) in distilled and tap water under nutrient limited conditions. Higher levels of colour removal (by 5-25%) were obtained in the NOM-tap water preparation by *Trametes* sp., *T. versicolor* ATCC 7731, and *Polyporus* sp., and this was attributed to the increased concentrations of mineral nutrients, particularly of Ca^{2+} and Mg^{2+}. Of the WRF, the newly isolated *B. adusta* attained the greatest decolourisation of 65%. NOM decolourisation by the five WRF coincided with a drop in culture pH, suggesting metabolic activity. Following maximal colour removal, the phenomenon of ‘‘negative decolourisation”, i.e., increase in colour after initial reduction, was observed for most WRF, but was particularly marked for *P. cinnabarinus*

and the two *Trametes* strains. The decolourisation of NOM in tap water correlated well with the moderate reduction in UV-absorbing compounds (A_{254}), whereas little change in A_{254} was observed for distilled water.

Decolourisation of NOM in both tap and distilled water coincided with ligninolytic enzyme activity, particularly that of Laccase (Lac) and Manganese peroxidase (MnP). Lac predominated for the *Trametes* strains, *Polyporus* sp., and *P. cinnabarinus*, whereas the activity of MnP was greatest for *B. adusta* in NOM-tap water. LiP activity was detected only for *T. versicolor*, *B. adusta* and *P. cinnabarinus.* The activities of Lac and MnP were greater in tap water and correlated with the greater NOM decolourisation and reduction in A_{254}. Enzyme activity patterns varied between the two NOM solutions. For most fungi, NOM decolourisation in tap water occurred in conjunction with increasing activity of Lac and MnP, whilst in distilled water the onset of Lac and MnP activity was evident after an initial stage of decolourisation (10-15%) due predominantly to adsorption. Adsorption was suggested by the slight discolouration of the pellets after the experiment and further confirmed with autoclaved controls. The simultaneous secretion of Lac and MnP activity suggests that they had a dual or synergistic action in the decolourisation of NOM. Although *B. adusta* had markedly lower Lac and MnP activity compared with the other fungi, it gave the greatest reduction in colour and this may be due to the action of a ‘‘hybrid” manganese-lignin or versatile peroxidase (VP).

The decolourisation of NOM concentrate was accompanied by the removal of higher molecular weight (MW) compounds (>1 kDa) and formation of lower MW intermediates (<1 kDa), the latter being most marked for *B. adusta,* which gave the greatest NOM decolourisation. Greater reductions in the average MW of the UV-absorbing species were observed in the NOM-tap water preparation and these correlated well with the increased decolourisation, greater reduction in A_{254} and corresponding enzyme activities for *Trametes* sp., *T. versicolor* and *Polyporus* sp. Fractionation of the treated NOM-tap water preparation demonstrated the extensive breakdown of the very hydrophobic acids (VHA) which contributed most of the colour of NOM, and less of the slightly hydrophobic acids (SHA). It was considered that the breakdown of the hydrophobic acids contributed to the increase in the charged and neutral fractions (CHA and NEU).

When NOM decolourisation was investigated at various concentrations (13-500 mg C L^{-1}) by *Trametes* sp., maximal decolourisation (50%) occurred at 100 mg C L^{-1}, with the minimum (21%) at 500 mg C L^{-1}. Although NOM decolourisation increased and then decreased with NOM concentration, the equivalent amount of NOM transformed (mg C L^{-1}) measured as colour increased. For most NOM contents (13-300 mg C L^{-1}) the initial mechanism of NOM decolourisation was attributed to adsorption followed by enzymatic breakdown, whereas for 500 mg C L^{-1} the decolourisation was almost all due to adsorption. At maximal NOM decolourisation the activity of Lac was greater than that of MnP, suggesting its predominant role in the decolourisation of NOM. Lac and MnP activity increased with increasing NOM content, but declined at NOM concentrations greater than 30 and 50 mg C L^{-1}, respectively, suggesting possible enzyme inhibition. Fungal treatment led to increased A_{254} and DOC, the proportional increases which were attributed to cell lysis, being

greater for lower NOM concentrations. When the fungi-treated NOM samples were subjected to a consortium of microbes (as biologically active sand) for a period of 51 days, considerable reduction in the colour, UV absorbance and fungal-generated DOC was observed, particularly for NOM contents of 30-50 mg C L^{-1}. This correlated to reductions in the higher MW fractions of the coloured and UV-absorbing compounds and the removal of most of the lower MW fungal products.

Decolourisation of NOM in Beaconsfield Reservoir water by *Trametes* sp., *Polyporus* sp. and *B. adusta* yielded high colour removals (70-80%), followed shortly after by a high degree of negative decolourisation. Decolourisation was accompanied by Lac and MnP activity, as well as LiP activity for *B. adusta*. The late onset of enzyme activities for *Trametes* sp. and *Polyporus* sp. indicated the initial removal of NOM to be predominantly via adsorption, whereas for *B. adusta* it was by a combination of adsorption and enzymatic breakdown. This is further supported by the high initial rate of colour removal and by the high degree of adsorption onto heat-killed biomass for the three WRF. Decolourisation of Beaconsfield water by *Trametes* sp. at 15ºC attained a greater reduction of colour (by 10%) with a lower degree of negative decolourisation than at 30ºC, but over a much longer time. When a small volume of culture was periodically replaced with fresh Beaconsfield water, decolourisation initially fluctuated but later stabilised and negative decolourisation did not occur. This was attributed to the nutrients from the periodic addition of Beaconsfield water. The rate and extent of NOM decolourisation by *Trametes* sp. and *B. adusta* was significantly greater in Beaconsfield water than NOM concentrate at the same concentration of 13 mg C L^{-1}. When both treated NOM samples were subjected to bacterial treatment, 55 and 70% of the DOC released by *Trametes* sp. and *B. adusta*, respectively, was biodegradable. As observed for NOM concentrate, fungal treatment of Beaconsfield water resulted in the breakdown of higher MW fractions and the concomitant formation of lower MW intermediates which subsequent bacterial treatment preferentially removed.

In vitro decolourisation of NOM concentrate (100 mg C L^{-1}) by commercial Lac, MnP and LiP preparations attained colour removals of 24%, 9.5% and 8.3% respectively. NOM decolourisation by the three enzymes was accompanied by reduction in UV absorbance, which was related to the removal of colour for each enzyme. In the presence of H_2O_2 (and $MnSO_4$ for MnP only), both MnP and LiP attained a greater rate and degree of colour removal. Increased NOM content (>100 mg C L^{-1}) inhibited the initial rate of NOM decolourisation for all three enzymes, as observed for the *in vivo* studies. The decolourisation of NOM by Lac, MnP and LiP was accompanied by a decrease in the average MW of both the coloured and UV-absorbing species. Lac gave the greatest reduction of the coloured and UV-absorbing material across a broad range of MWs, whereas MnP and LiP preferentially removed the higher MW fractions of the coloured compounds. Resin fractionation of the Lac-treated NOM showed a similar degree of reduction of the SHA and VHA fractions, the products of which probably contributed to the pool of the CHA and NEU fractions.

The major outcomes of this research included the isolation and characterisation of a range of NOM-degrading fungi from several sources, of which the newly isolated *B. adusta* was the most actively decolourising fungus. To our knowledge this is the first demonstration of the potential of *B. adusta* for the decolourisation of NOM concentrate under conditions of limiting nutrients. Furthermore, this is the first report of trends in extracellular enzyme activity, in particular of MnP, during NOM decolourisation by *B. adusta*. Although these findings demonstrated the ability of the WRF to decolourise and depolymerise NOM by the action of their ligninolytic enzymes, they were limited in their decolourisation capacity due to cell lysis caused by the low nutrient availability. Thus their potential to be used in the treatment of NOM in drinking water is questionable, unless however, a biological treatment technology based on the combined utilisation of fungi and bacteria is employed. This is a possible alternative for the removal of NOM, particularly at lower NOM concentrations (30-50 mg C L^{-1}) where a high percentage of the released DOC is biodegraded by bacteria. The *in vitro* application of extracellular enzymes, especially Lac, clearly has the potential for NOM biodegradation, however, their application on a commercial scale is uncertain due to their high cost.

List of publications and presentations arising from this work

Solarska, S., May, T., Roddick, F.A. & Lawrie, A.C. (2009). Isolation and screening of natural organic matter-degrading fungi. *Chemosphere,* 75**,** pp. 751-758.

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Abbreviations

A_{254}	Absorbance measured at 254 nm
A_{446}	Absorbance measured at 446 nm
ABTS	2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
AOC	Assimilable Organic Carbon
AOP	Advanced Oxidation Process
AMW	Apparent Molecular Weight
AWQC	Australian Water Quality Centre (located in Adelaide, South Australia)
BDOC	Biodegradable Dissolved Organic Carbon
BLAST	Basic Local Alignment Search Tool
BRP	Bacterial Regrowth Potential
BV	Bed Volume
CF	Correction Factor
CHA	Hydrophilic Charged Fraction of NOM
CMC	Carboxymethylcellulose
C:N	Elemental Ratio of Carbon to Nitrogen
Da	Dalton
DAX-8	Non-ionic macroporous resin used to adsorb the VHA fraction
DBPs	Disinfection By-Products
DDT	Dichlorodiphenyltrichloroethane
DMP	2, 6-dimethoxyphenol
DNA	Deoxyribonucleic acid
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
ε	Molar absorptivity (M^{-1} cm^{-1})
EEMs	Excitation-Emission Matrices
EPRS	Electron paramagnetic resonance spectroscopy
FA	Fulvic Acids
HA	Humic Acids
HAAs	Haloacetic acids

HAAFP	Haloacetic Acid Formation Potential
HPSEC	High Performance Size Exclusion Chromatography
HS	Humic Substances
ICP-MS	Inductively Coupled Plasma Mass Spectroscopy
IHSS	International Humic Substances Society
IRA-958	Resin used to adsorb the CHA fraction, effluent from this resin constitutes the NEU fraction
ITS	Internal Transcribed Spacer
Lac	Laccase
LiP	Lignin Peroxidase
LMEs	Lignin Modifying Enzymes
MEA	Malt Extract Agar
MEB	Malt Extract Broth
MeOH	Methanol
MIEX™	Magnetic Ion Exchange Resin
Milli-Q	Milli-Q water (organic free and deionised water)
Mi	The molecular weight of each ith fraction eluted at the ith volume in the chromatogram
MnP	Manganese-dependent Peroxidase
M_n	Number average molecular weight (Da) where $M_n = \Sigma_i n_i.M_i / \Sigma n_i$
MW	Molecular Weight
M_w	The weight average molecular weight (Da) where $M_w = \Sigma_i n_i.M_i^2 / \Sigma_i n_i.M_i$
n	Number of replicate experiments
NEU	Neutral Hydrophilic Fraction of NOM
Ni	Nickel
NOM	Natural Organic Matter
ρ	Polydispersity where $\rho = M_w/M_n$
PAH	Polyaromatic Hydrocarbons
PCBs	Polychlorinated Biphenyls
PCP	Pentachlorophenol
PCR	Polymerase Chain Reaction
POC	Particulate Organic Carbon
POM	Particulate Organic Matter
PVDF	Polyvinylidene Fluoride (PVDF) Membrane (Hydrophobic)

R	Correlation coefficient
rpm	Revolutions Per Minute
SD	Standard Deviation
SHA	Slightly Hydrophobic Acids
SOM	Soil Organic Matter
SUVA	Specific UV Absorbance measured at 254 nm
THMs	Trihalomethanes
THMFP	Trihalomethane Formation Potential
t	Time (days, hours or minutes)
TOC	Total Organic Carbon
U	International Enzyme Unit
VA	Veratryl Alcohol
VHA	Very Hydrophobic Acids
VP	Versatile Peroxidase
WRF	White Rot Fungi
XAD-4	Non-ionic macroporous resin used to adsorb the SHA fraction
λ	Wavelength (nm)

List of Organisms

Organism	Abbreviation used in text
Aspergillus niger	*A. niger*
Bjerkandera adusta	*B. adusta*
Fusarium oxysporum	*F. oxysporum*
Lopharia crassa 10644	*L. crassa*
Mucor racemosus	*M. racemosus*
Penicillium citrinum	*P. citrinum*
Perenniporia tephropora 7904	*P. tephropora*
Phanerochaete chrysosporium ATCC 24725	*P. chrysosporium* ATCC 24725
Phoma sp.	*Phoma* sp.
Polyporus sp.	*Polyporus* sp.
Punctularia strigosozonata	*P. strigosozonata*
Pycnoporus cinnabarinus VIC	*P. cinnabarinus* VIC
Pycnoporus cinnabarinus SQ	*P. cinnabarinus* SQ
Pycnoporus coccineus 1096	*P. coccineus* 1096
Pycnoporus coccineus 6004B	*P. coccineus* 6004B
Pycnoporus sanguineus 2256	*P. sanguineus* 2256
Trametes sp.	*Trametes* sp.
Trametes versicolor	*T. versicolor*
Trametes versicolor ATCC 7731	*T. versicolor* ATCC 7731
Trametes versicolor CV 5691	*T. versicolor* CV 5691
Trichoderma atroviride	*T. atroviride*

Chapter 1 Introduction

Growing concern for the supply and quality of drinking water has prompted the water industry to investigate alternative and preferably environmentally friendly treatment technologies. Natural water sources contain complex and coloured natural organic matter (NOM) which poses problems in the storage, treatment and distribution of drinking water. The presence of NOM is undesirable as it leads to unacceptable taste, odour and turbidity in water. In addition to the aesthetic problem, it interferes with most water treatment processes (Morran *et al.*, 1996). Furthermore, a major concern among water utilities is the formation of disinfection by-products (DBPs), some of which may be carcinogens (Suffet and MacCarthy, 1989), when NOM reacts with chlorine-based disinfectants. It may also lead to the formation of biofilms within the distribution system and so pose a microbial health risk to consumers (Gottschalk *et al.*, 2000). As disinfection is an integral part of maintaining pathogen-free water, it cannot be compromised in attempting to control by-product formation, as the health risks from DBPs at the levels at which they occur in drinking water are small in comparison with the risks associated with inadequate disinfection. Alternative treatments that maximise the removal of NOM and minimise the formation of DBPs, whilst being environmentally sound, are of interest.

Depending on the nature of NOM, it is removed from raw water directly or indirectly and to varying degrees by several treatment processes (Matilainen *et al.*, 2002). Conventional methods of NOM removal include membrane filtration, activated carbon adsorption, ozonation, enhanced coagulation and the use of magnetic ion exchange resin (MIEX™). These processes are effective for removing NOM but have high capital costs and can produce residues which can be problematic in disposal (Carlson and Silverstein, 1997). Alternative treatments such as those based on bioremediation offer a more environmentally benign technology for the removal of NOM that is potentially more cost-effective and limits by-product formation. Biological processes, particularly those utilizing microorganisms, offer the prospect of a more natural solution without the disadvantages of current problems.

However, the complex nature of NOM means that not all NOM is biologically labile. Although some biological treatments reduce NOM concentration in treated water (Bouwer and Crow, 1988), most water-soluble NOM exists in the form of humic substances that are of high molecular weight (MW) and refractory to microbial degradation (Marhaba, 2000). Despite these properties, the decomposition of humic substances by saprotrophic fungi has been well documented by Grinhut *et al*. (2007). The most active humic acid (HA) degraders are the basidiomycetous white rot fungi (WRF) (Gramss *et al.*, 1999). Unique to this group is the efficient degradation of lignin (Hatakka, 1994), a highly recalcitrant polymer that contributes to the formation of humic substances.

The efficiency of WRF in modifying lignin and humic substances is associated with their extracellular enzyme system, which is generally composed of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) (Hatakka, 1994). These lignin-degrading enzymes participate in different oxidative reactions that break the aromatic ring structure and bonds binding together the basic units of lignin, and could potentially break down similar structures in NOM to lower MW compounds that are more easily degraded by a wide range of organisms.

Considerable research on the degradation of natural and synthetic HAs by WRF has been undertaken (Dehorter and Blondeau, 1992; Hofrichter et al., 1997; Willmann and Fakoussa, 1997). The ability to decolourise and depolymerise humic substances *in vivo* has been shown for a number of WRF. Cohen and Gabriele (1982) showed that *Trametes versicolor* (a WRF) and *Poria monticola* (a brown-rot fungus) solubilised and degraded lignite coal. Studies by Gramss *et al*. (1999) and Steffen *et al.* (2002) showed that WRF degraded HAs extracted from soil. Despite these and other studies, there appear to be no reports in the literature on the degradation of concentrated aquatic NOM by WRF without the addition of nutrients. Research on the degradation of HAs has mainly focused on a few organisms, namely *Phanerochaete chrysosporium* and *Trametes versicolor* (Dehorter and Blondeau, 1992). As NOM reduces water quality and increases the cost of treatment, there may be a role for WRF and their enzymes in removing NOM from water with lower environmental impact than conventional processes.

This research investigated the application of WRF for the removal of NOM from drinking water and aquatic NOM concentrate from water without the addition of nutrients. The objectives of this work were to:

- Isolate, screen and select fungi capable of decolourising aquatic NOM concentrate
- Evaluate the efficiency of the selected fungi to decolourise NOM concentrate
- Identify and evaluate the mechanisms implicated in the removal of NOM and characterise the changes in NOM
- Assess the feasibility of using WRF as an alternative biological treatment for the removal of NOM from a sample of drinking water, as exemplified by water from Beaconsfield Reservoir
- Investigate the application of Lac, MnP and LiP in the *in vitro* decolourisation of NOM concentrate

In Chapter 2 the current literature on NOM, its impact on water treatment, its formation, and structural characteristics is reviewed. The literature on WRF, their lignin modifying enzymes (LMEs) and the potential application of WRF for the bioremediation of NOM are discussed.

Chapter 3 details the methods employed in the isolation, screening and application of WRF for the treatment of NOM and the techniques employed in the characterisation of NOM.

Chapter 4 describes the preliminary isolation, identification and screening of fungi for their potential to decolourise concentrated aquatic NOM using NOM plate assays. The relative tolerance of these organisms to a range of NOM concentrations is evaluated, along with their ability to degrade cellulose using cellulose plate assays.

Chapter 5 further investigates the decolourisation of NOM in shake flask cultures by the fungi that displayed greatest NOM decolourisation on solid medium. These WRF were examined for their ability to decolourise NOM concentrate in distilled and tap water without the addition of nutrients.

Chapter 6 investigates the effect of initial NOM concentration on the decolourisation of NOM concentrate by *Trametes* sp., and examines the biodegradability of the treated NOM using a consortium of bacteria.

Chapter 7 compares the decolourisation of NOM in a sample of drinking water and NOM concentrate at the same DOC level by several WRF, and its subsequent biodegradability.

Chapter 8 evaluates the application of extracellular enzymes as a method of NOM removal.

Chapter 9 discusses the major findings derived from this research.

Chapter 10 makes recommendations for further work.

Chapter 2 Literature Review

2.1 Natural Organic Matter

The term 'natural organic matter' is generally used to describe a group of naturally occurring organic compounds present in all terrestrial and aquatic (fresh) ecosystems including natural waters, marine and lake sediments. Due to its abundance, the production and decomposition of NOM plays a significant role in the global carbon cycle. In the environment, NOM represents a large part of soil organic matter (SOM) and consequently influences soil structure, water holding capacity, soil fertility, nutrient availability and binding of contaminants (Wershaw, 2004).

2.1.1 Impacts of NOM on water treatment

Natural organic matter is composed of yellowish-brown organic compounds that directly affect the aesthetic characteristics of water by influencing its colour, taste and odour. Some of these tastes and odours may be removed through chlorination, but in some cases, it may also intensify them. Furthermore, NOM acts as a food source for bacterial regrowth and biofilm formation in potable water distribution systems. To date the removal of NOM is accomplished by various processes such as coagulation-sedimentation, adsorption on activated carbon and membrane filtration. Conventional treatments utilise inorganic coagulants to remove turbidity and colour and so involve chemical addition, production of sludge and residuals, and operate over a limited applicable concentration range (Vickers *et al.*, 1995). Consequently, a novel treatment technology with a low requirement for chemical and energy use, along with reduced sludge production and treatment costs, is desirable. Greater interest in and awareness of water quality, and increasingly stringent standards, mean there is a need for the development of a more effective NOM treatment process.

The removal of NOM from potable water has become more challenging as the natural concentrations of NOM in source waters have increased over time (Sharp *et al.*, 2006). Natural organic matter has been associated with the production of potentially harmful DBPs following chlorination (Gottschalk *et al.*, 2000). Of these DBPs, the halogenated organic by-products classified as trihalomethanes (THMs) (Rook, 1974) are the most prevalent. Another class of DBPs, the haloacetic acids (HAAs), have been reported to have a greater degree of carcinogenic risk than THMs (Xiaojian, 2000). As disinfection is required for maintaining pathogen-free water, and chlorine residual protects against microbial growth in the distribution network, its use in drinking water treatment is unlikely to be eliminated in the short term. Consequently, there has been much effort focused on reducing NOM levels prior to chemical disinfection.

2.1.2 Structure of NOM

The structure of NOM differs with source and this consequently affects its role in the aquatic ecosystem. Natural organic matter can either be formed in place from the biota growing in water bodies (autochthonous) or it can be formed externally in a different ecosystem and transported into a water body (allochthonous) (Wershaw *et al.*, 2005). Terrestrial plant litter and microbial biomass, are the major parent materials involved in the formation of SOM (Kaiser *et al.*, 2002). The leaching of soluble organic compounds derived from SOM in the surrounding catchment will eventually contribute to the nature of allochthonous NOM. Autochthonous NOM is generally formed from exudates and decomposition products of aquatic and littoral organisms (Wershaw *et al.*, 2005). These may include algae, benthic organisms, bacteria, plankton and macrophytes, which will inevitably contribute carbohydrates, amino acids, proteins, lipids etc. (Hayakawa, 2004). The contribution of autochthonous NOM is relatively small compared with allochthonous NOM; however it becomes relatively important when primary production accelerates in summer (Lehtonen, 2005).

The composition of NOM reflects its biological and chemical production and decomposition, chemical and physical adsorption, and transport processes in the soil (Huang *et al.*, 1998). Natural organic matter can be represented as a macromolecule composed of interacting compartments of partially degraded plant tissue, biomass from organisms, organic precipitates, organic coating on mineral grains, pyrolytic carbon and dissolved organic matter in interstitial soil water (Wershaw, 2004).

The dissolved fraction of NOM in natural waters is commonly referred to as dissolved organic matter (DOM), or dissolved organic carbon (DOC), and is able to pass through a 0.45 µm membrane filter (Thurman, 1985). The residual fraction remaining on the filter following filtration is particulate and is commonly referred to as particulate organic matter (POM). Natural DOM comprises two fractions, one that is readily biodegradable and the other that is recalcitrant. Not all biodegradable DOM can be metabolised, and the fraction that is biodegradable consists of components that range from very labile to near refractory (Kaplan and Newbold, 1995). Although DOM provides energy and nutrients for heterotrophic bacteria and other microorganisms, dissolved humic substances, which make up most of the coloured constituents of DOM, tend to be resistant to decomposition.

Dissolved organic matter is generally separated into two categories: aquatic humic substances and non-humic substances (hydrophilic-polar molecules with an affinity for water) (Hood *et al.*, 2003). The non-humic fractions are of low MW and include organic compounds such as carbohydrates, amino acids, fatty acids, phenols, sterols, natural sugars, hydrocarbons, urea and porphyrins (Boggs *et al.*, 1985). The non-humic materials have relatively larger nitrogen and carboxyl compounds and lower aromatic carbon and phenolic contents (Croue´ *et al.*, 1999). Despite its solubility in water, DOM is composed of high MW compounds derived from the decomposition of lignin. Lignin, an amorphic three-dimensional substance, is a constituent

of cell walls providing plant strength as well as contributing to the formation of humic substances (HS). Lignin is highly resistant to microbial degradation due to its size and irregular structure, and is therefore one of the main precursors of NOM (Kirk and Farrell, 1987).

2.1.3 Humic substances

Humic substances in soils are fully decomposed (humified) remains of plant and animal organic matter, which make up a high percentage of total DOC, 50-80% in natural waters (Alborzfar *et al.*, 1998), or up to 80-90% in most brown-coloured lakes (Thurman, 1985). Humic substances are major organic constituents of soils and sediments, and their formation is a direct result of both chemical and bacterial action on plant tissue, coupled with secondary processes that occur in the soil. These secondary processes include the leaching of polyphenols from the surrounding leaf litter by the action of rain, condensation and polymerisation of polyphenols, quinones and proteins, and the action of microorganisms to produce proteins (Martin *et al.*, 1971).

Humic substances have long been recognised for their beneficial effects on soils and plant growth. Some of the benefits of HS include: improved nutrient uptake (particularly of phosphorus, sulphur and nitrogen), improved soil structure, chelation of soil nutrients, solubilisation of minerals, stimulation of the biological activity of soil, reduction of the need for nitrogen fertilisation, and improved water holding capacity of soil for better drought resistance (Mayhew, 2004).

2.1.4 Structure of humic substances

Humic substances are brown-coloured well-defined organic compounds, predominantly composed of carbon, hydrogen, nitrogen, oxygen and small amounts of sulphur. Humic substances comprise a heterogeneous mixture of relatively high molecular mass compounds (MW 0.5 kDa - 20 kDa) with mixed aliphatic and aromatic (containing one or more benzene rings) characteristics (Stevenson, 1994). The principal functional groups commonly identified in HS include carboxyl, phenolic-OH groups, methoxy, quinone, hydroxyquinone, ether and alcoholic-OH (Table 2-1) (Stevenson, 1994). The properties of HS are unique to a specific environment and changes in solution pH, concentration or the presence of metal ions significantly alter the molecular structure of the macromolecules (Zimmer, 2004). Humic substances are made up of hundreds of different molecules bound together by weak dispersive forces (Piccolo *et al.*, 2000). Slight changes in solution pH cause the humic polymer to fracture, resulting in disassociated molecules that can subsequently affiliate with free radicals, metals and impurities (Piccolo *et al.*, 2000). An alkaline environment opens up the long chains of humic polymers, whilst low pH makes them close up (Piccolo *et al.*, 2000). At high pH, HS tend to disperse and their molecular arrangement becomes smaller, whilst at low pH,

protonation of the dissociated functional groups may occur, thereby leading to the formation of intermolecular H-bonds and increase in molecular sizes (Kucerik *et al.*, 2003).

Humic substances tend to aggregate into long fibres. Based on their solubility, HS can be separated into three main fractions: HA, fulvic acid (FA) and humin, each consisting of highly complex heterogeneous mixtures. Humic acid and FA can be distinguished and isolated on the basis of properties such as hydrophobicity, pH-dependent solubility and other characteristics of chemical structure (Aiken *et al.*, 1992). Some of these characteristics are summarised in Table 2-1. The structure of humic and FAs differ depending upon whether they are derived from marine or terrestrial organic matter. Stuermer and Payne (1976) showed that marine FA are less aromatic and more aliphatic in character and have a lower molecular weight than terrestrial FA.

Humic acids are complex macromolecules that are considered to be resistant to microbial degradation. A study of the biodegradability of HS and other fractions of decomposing leaf litter by Qualls (2004) found HA to be the least mineralized (12.7%) followed by FA (29.2%) and ground litter (38%). The humin fraction in soil has a mean residence time comparable to that of HA, and is composed of bound HA and alkyl components formed over long periods of decomposition in soil (Rice, 2001). Despite the recalcitrance of HS, organotrophic microorganisms, particularly lignin-degrading fungi, brown rot fungi, ectomycorrhizal fungi, bacteria and soil-borne micro-fungi have been able to decolourise it (Gramss *et al.*, 1999).

Fulvic acids contain aliphatic and aromatic components with mainly carboxylic and phenolic functional groups, and represent 80-90% of typical humic substances (Shin and Lim, 1996). Fulvic acids are typically held together by hydrogen and ionic bonds, and the charge on both FA and HA is caused by the deprotonation of the carboxylic and phenolic groups (Van Zomeren, 2008). The resultant charged groups can consequently complex heavy metals.

Table 2-1. Characteristics of HS

Fraction of HS	Chemical compounds	MW range (Da)	Solubility	Colour	Consistency	References
Humic Acid (HA)	Aromatic rings, aliphatic chains bearing O-, N-, S- containing functional groups	500-5000	Soluble in alkali and precipitated in acid (pH 1-2)	Dark brown (dry black)	Crystals as humates	Stevenson (1994) Thurman (1985)
Fulvic Acid (FA)	Aromatic, aliphatic structures, functional groups containing –COOH, -OH and C=O	300-2000	Soluble in both acid and alkali	Yellow to golden brown	Amorphous	McKnight *et al*. (1994) Kordel *et al*. (1997)
Humin	Predominantly aliphatic, 1 and 2 (hydro)-aromatic ring units, may consist of HA in association with mineral water, fungal melanins, paraffinic substances and condensed insoluble humic matter	Three-dimensional macromolecule 100,000	Insoluble in acid and alkali	Black	Solid	Stevenson (1994) Fakoussa and Frost (1999)

2.1.5 Formation of humic substances

Humic substances are formed during the humification process when high and low MW molecules derived from decomposing biomass are coupled. This process involves the oxidative decomposition, solubilisation and polymerisation of precursor molecules (generally derived from lignin and its transformation products including NOM and polyphenols) resulting in the production of HS, which vary considerably in their nature. Nevertheless, typical structures of the original lignin polymer are still preserved in HS (Stefanova *et al.*, 2004).

Two hypotheses exist for the formation of HS. The first hypothesis involves the depolymerisation of refractory plant biopolymers, namely lignin, during the humification process to form the central core of HS. The second involves the formation of HS by condensation or re-polymerisation of small reactive organic molecules which were generated from the breakdown of the original biopolymer (Koivula, 2004). These HS are gradually able to form aggregates, resulting in the formation of higher MW super-molecules (Hosse and Wilkinson, 2001). Lignin and its transformation products such as polyphenols are important starting materials in the humification process, with the lignin content of the organic matter, along with its heterogeneity, ultimately affecting the composition of HS (Miikki *et al.*, 1994).

The resultant HS comprise heterogeneous macromolecules that are chemically reactive in aquatic environments, yet refractory to microbial decomposition. The recalcitrance of HS may be linked to the interactions of a diverse range of HS derived from several sources (Hayes and Clapp, 2001). As lignin is a major parent material in the formation of HS by providing aromatic building blocks, the fungi responsible for its mineralisation have also been implicated in the degradation of HS. Although different microorganisms are able to degrade HS to some extent, the filamentous WRF and litter-decomposing fungi are the most efficient at doing so (Willmann and Fakoussa, 1997).

2.2 White Rot Fungi

White rot fungi constitute a physiological group comprising mostly of basidiomycetous, and to a lesser extent, litter-decomposing fungi (Wesenberg *et al.*, 2003). WRF are the most abundant wood degraders in nature, and are so named because they leave a bleached appearance of the wood fibres following their attack. These fungi are distinctive among eukaryotes in having evolved non-specific mechanisms for the complete degradation of lignin and lignin-like substances. WRF and related litter-decomposing fungi are the most efficient degraders of lignin, although mixed cultures of fungi, mycorrhizal fungi, brown rot fungi, actinomycetes, and bacteria in soil and compost have also been implicated in the degradation process. Litter-decomposing fungi such as *Agaricus bisporus* and *Mycena inclinata* reduce lignin content by 35-60% (Durrant *et al.*, 1991; Steffen *et al.*, 2007). Numerous fungi have been implicated in the biodegradation of lignin, but the most extensive research has been on the WRF *Phanerochaete chrysosporium* (Kirk *et al.*, 1978; Tien and Kirk, 1988; Gold *et al.*, 1989). The bioremediation capability of *P. chrysosporium* has been demonstrated for a wide variety of environmentally persistent xenobiotics and chlorinated hydrocarbons (Thomas *et al.*, 1992). Recently, however, there has been growing interest in studying the lignin-modifying enzymes of other WRF, including: *Trametes* sp. (Johansson and Nyman, 1993), *Bjerkandera* sp. (Moreira *et al.*, 2007), *Pleurotus* sp. (Ardon *et al.*, 1998), *Phlebia radiata* (Niku-Paavola *et al.*, 1988) and *Pycnoporus cinnabarinus* (Schliephake *et al.*, 1993). Most of these species have shown potential for bioremediation (Hestbjerg *et al.*, 2003) and are also characterised by their ability to colonise lignocellulose substrates, which constitute cellulose, hemicellulose and lignin. The degradation of these substrates requires the secretion of a complex set of lignolytic enzymes and the presence of corresponding metabolites. This process entails an oxidative and non-specific process of decreasing the methoxy, phenolic, and aliphatic content of lignin, cleaving aromatic rings, and creating new carbonyl groups (Hatakka, 2001).

WRF vary considerably in the way they attack lignin, with some fungi preferentially removing lignin without a substantial loss of cellulose. In selective decay, as found with *P. chrysosporium*, lignin and hemicellulose are degraded significantly more than cellulose, whilst in non-selective decay involving *T. versicolor,* equal amounts of all lignocellulose components are degraded (Hatakka, 2001). The physiological conditions associated with lignin degradation and the combined enzyme systems involved in its attack are fungus-specific. Differences in lignin removal may be connected to the taxonomic position and ecology of the fungi (substrate specialisation-hardwood or softwood). A second group of basidiomycetes belonging to the brown rot fungi has also been implicated in the decomposition of lignin. The extracellular enzymes secreted by the brown rot fungi alter lignin minimally via hydroxylation and demethylation of the methyl groups, thus leading to the formation of hydroquinone structures (Essington, 2003).

2.3 Lignin – modifying enzymes

WRF employ a distinctive mechanism to degrade lignin. This comprises a hydrolytic system that produces hydrolases responsible for polysaccharide degradation, and in most, a ligninolytic system composed of lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase (Lac). Different combinations of lignin-modifying enzymes (LMEs) are produced by WRF, with some producing all three classes of the LMEs, while others only one or two of these enzymes (Hatakka, 1994). More than one isoform of the LMEs are expressed by different taxa and under different culture conditions (Wesenberg *et al.*, 2003). These LMEs are highly oxidative in nature and act synergistically to mineralise an array of organic pollutants structurally similar to lignin (Lonergan, 1992). They do this by catalysing a one-electron oxidation, which results in the formation of radicals that undergo further spontaneous reactions (Kluczek-Turpeinen, 2007). Due to the highly branched and irregular structure of lignin, combined with its large molecular size, the LMEs are extracellular and have lower substrate specificity than typical biological catalysts. Low MW mediators are also secreted with the LMEs; these aid the penetration of wood, which otherwise would not be possible due to the large molecular size of the enzymes (Evas *et al.*, 1994). These low molecular mass compounds are secreted during fungal metabolism and include veratryl alcohol (VA), oxalate, malate and fumarate (Leonowicz *et al.*, 1999). In addition to these, H_2O_2-utilising enzymes such as glyoxal oxidase (a copper-radical protein) and aryl alcohol oxidase (a flavoprotein) are also excreted. Another isolated enzyme known as versatile or hybrid peroxidase has also been reported in lignin degradation (Mester and Field, 1998). Some of the characteristics of the LMEs are listed in Table 2-2.

LMEs are produced during secondary metabolism as lignin oxidation provides no net energy to the fungus (Wesenberg *et al.*, 2003). The synthesis and secretion of LMEs is stimulated by limited nutrient availability (C:N ratio), with nitrogen being the limiting nutrient for fungal growth in most wood and soils (Kirk and Farrell, 1987). The depolymerisation of lignin enables the fungi to gain access to cellulose and hemicellulose, which they utilise as carbon and energy sources (Kirk and Farrell, 1987). If lignin is not modified or removed by lignolytic fungi, then cellulose is not available as a carbon source for other microbial decomposers. To date no organism is known to use lignin as a sole source of carbon (Kirk and Farrell, 1987). Lignin degradation leads to the formation of water-soluble compounds and, where mineralisation occurs, the formation of CO_2.

The non-specific nature of the LMEs enables the mineralisation of a diverse range of recalcitrant pollutants that are similar in structure to lignin (Mansur *et al.*, 2003). The synthesis of the enzymes is independent of pollutant concentrations, allowing the fungi to tolerate highly toxic compounds without the need to be pre-conditioned (Karapinar and Kargi, 2002). In addition, the extracellular nature of the enzymes allows the fungi to access non-polar and insoluble compounds that intracellular processes such as those that occur in the cytochrome P450 system and single cell organisms cannot (Levin *et al.*, 2003).

Table 2-2. Properties of LMEs

Enzyme	MW (kDa)	Optimal pH	Cofactor	Enzyme Assay	Reaction	Reference
Lac	50-110	3.5-7	O_2	Oxidation of guaiacol to tetraguaiacone Reaction monitored at 465 nm	Phenols are oxidised to phenoxy radicals-mediator radicals	Fakoussa and Frost (1999) Thurston (1994) Fakoussa and Hofrichter (1999)
MnP	38-50	4-4.5	H_2O_2	Oxidation of 2,6-dimethoxyphenol (DMP) to coerulignone Reaction monitored at 469 nm	Mn^{2+} are oxidised to Mn^{3+}, Mn^{3+} oxidises aromatic substrates, oxidation of phenolic compounds to phenoxyl radicals that cleave C_α-C_β and alkyl aryl bonds	Fakoussa and Frost (1999) Fakoussa and Hofrichter (1999) Gold and Alic (1993) Hofrichter (2002)
LiP	38-47	2.5-3	H_2O_2	Oxidation of VA to veratraldehyde Reaction monitored at 310 nm	Abstracts an electron from the substrate aromatic ring, generating an aryl cation radical which decomposes by enzymatic and non enzymatic processes	Gold and Alic (1993) Kirk and Farrell (1987) Fakoussa and Hofrichter (1999)
Versatile Peroxidase (VP)	-	pH 5 (for Mn^{2+}) pH 3 (for aromatic substrates)	H_2O_2	Oxidation of VA and DMP	Same effect on aromatic compounds as MnP and LiP	Camarero *et al.* (1999)

2.3.1 Peroxidases

Both MnP and LiP are heme-containing glycoproteins. These peroxidases require hydrogen peroxide as an oxidant to catalyse the oxidation of substrates using the H_2O_2 as a final electron acceptor (Table 2-2). There are three steps involved in this catalytic process (Dunford, 1999). The initial stage involves the ground state ferric peroxidase reacting with hydrogen peroxide to yield a two-electron oxidized intermediate, known as compound I. This compound is then reduced by a substrate in a one-electron reaction to compound II, which is then reduced back to the ground state through a one-electron reaction (Dunford, 1999). The process of lignin mineralisation is initiated by the synthesis and secretion of substrates, including VA, Mn^{2+} and hydrogen peroxide, which aid the oxidative activity of the extracellular enzymes. Veratryl alcohol and Mn^{2+} are redox mediators for LiP and MnP, respectively.

2.3.2 Manganese-dependent peroxidase (MnP)

Manganese peroxidase is the most common LME produced by most WRF and litter-decomposing species (Wesenberg *et al.*, 2003). Apart from *P. chrysosporium* (Vijay *et al.*, 2003), MnP has also been found in many other basidiomycetes including: *Bjerkandera adusta* (Heinfling *et al.*, 1998), *Trametes versicolor* (Dehorter and Blondeau, 1993), *Phlebia radiata* (Hofrichter *et al.*, 2001), *Irpex flavus* (Gill and Arora, 2003), and *Dichomitus squalens* (Gill and Arora, 2003).

Manganese peroxidase is extracellular, glycosylated and contains heme as the prosthetic group (Glenn and Gold, 1983). Like LiP, MnP is also expressed in multiple forms with MWs from 38 to 50 kDa (Table 2-2) (Fakoussa and Hofrichter, 1999). The catalytic cycle of MnP is similar to that of LiP and other peroxidases, including horseradish peroxidase, but requires the presence of Mn^{2+} to complete the cycle. The cycle is initiated by the binding of H_2O_2 to the native ferric enzyme and the formation of an iron-peroxide complex (1) (Figure 2-1) (Hofrichter, 2002). A subsequent two-electron transfer from the heme (2) is required to cleave the peroxide dioxygen bond and form MnP compound I. Subsequent reduction proceeds through MnP compound II. Mn^{2+} ion is oxidised to Mn^{3+} and acts as a one-electron donor to make compound II (3). The reduction of compound II occurs with the formation of another Mn^{3+} from Mn^{2+}, consequently leading to the generation of native enzyme (4) (Hofrichter, 2002). High concentrations of H_2O_2 cause reversible inactivation of MnP and the formation of compound III (5) (Wariishi *et al.*, 1988).

The presence of ligands such as malonate and oxalate assists in the stabilisation of Mn^{3+} ions and promotes their release from the enzyme into the surrounding environment (Hofrichter *et al.*, 1999). The highly reactive Mn^{3+} successively oxidises phenolic rings of lignin to unstable phenoxy radicals which further undergo spontaneous disintegration (Hofrichter, 2002). The highly reactive phenoxy radicals are also involved in the cleavage of C_α-C_β bonds, and similarly alkyl-phenyl bonds, resulting in the formation of smaller intermediates

including quinones and hydroxyquinones. Oxalic acid is another stabilising chelator secreted by the fungi and acts as a diffusible redox-mediator, allowing MnP to oxidise and depolymerise the natural substrate lignin as well as recalcitrant xenobiotics and textile dyes (Heinfling *et al.*, 1998). In the absence of radical mediators, MnP mainly oxidises phenolic lignin substructures, whilst in the presence of mediators, MnP oxidises non-phenolic lignin substructures (Wariishi *et al.*, 1989). MnP also catalyses the oxidation of several mono-aromatic phenols, including aromatic dyes.

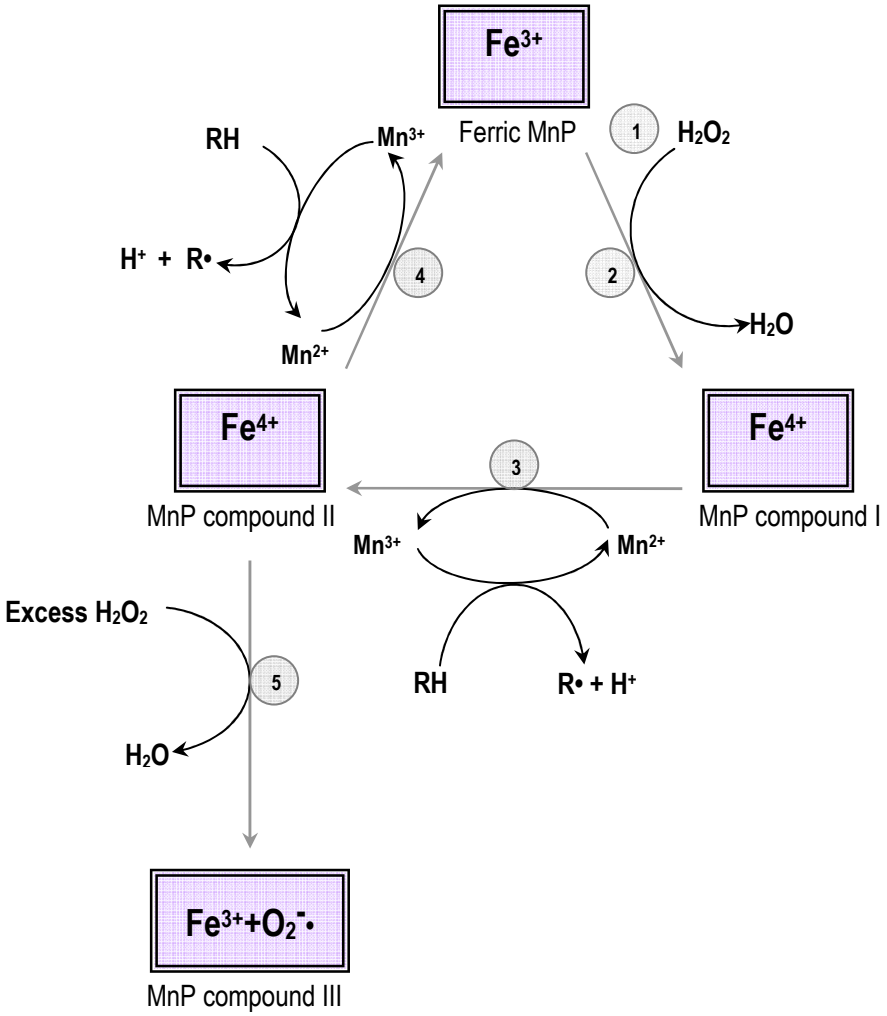

Figure 2-1. Catalytic cycle of MnP. Redrawn from Hofrichter *et al.* (2002) and Gold *et al.* (1989).

2.3.3 Lignin peroxidase (LiP)

Lignin peroxidase was one of the first enzymes to be implicated in the depolymerisation of lignin by the WRF *Phanerochaete chrysosporium* (Glenn *et al.*, 1983; Tien and Kirk, 1983). Other species which produce LiP include: *Trametes versicolor*, *Pleurotus ostreatus*, *Phlebia radiata* (Niku-Paavola *et al.*, 1988) and *Bjerkandera adusta* (Nakamura *et al.*, 1999). As only some fungi are able to excrete LiP, it is thought not to be essential in the degradation of lignin (Hatakka, 1994).

Like MnP, LiP is a glycoprotein that contains the iron protoporphyrin IX (heme) group that is dependent on H_2O_2 for catalytic activity (Table 2-2). LiP is expressed in multiple forms (isozymes) with MWs of 38-47 kDa (Table 2-2) (Fakoussa and Hofrichter, 1999). Lignin peroxidase has a high redox potential which permits the enzyme to catalyse one-electron oxidation of a wide range of aromatic substrates, including phenolic and non-phenolic structures. The oxidation of non-phenolic substrates yields the formation of aryl cation radicals which undergo further spontaneous degradation (Sayadi and Odier, 1995). The oxidation of non-phenolic compounds requires the presence of oxygen. Phenolic substrates, on the other hand, are oxidised to products that are similar to those produced by peroxidases (Kluczek-Turpeinen, 2007).

The catalytic cycle of LiP initially involves the oxidation of native Fe (III) enzyme by H_2O_2 to LiP compound I (1) (Figure 2-2). A one-electron reduction of compound I with an aromatic compound like VA results in the formation of compound II and a substrate radical (2). Compound II then undergoes another oxidation via a second aromatic substrate (3), whilst the free radical undergoes spontaneous reactions (Table 2-2). In the presence of excess H_2O_2 compound II can be converted back to an inactive form of the enzyme LiP (compound III) (4) (Gold *et al.*, 1989; Wariishi *et al.*, 1991).

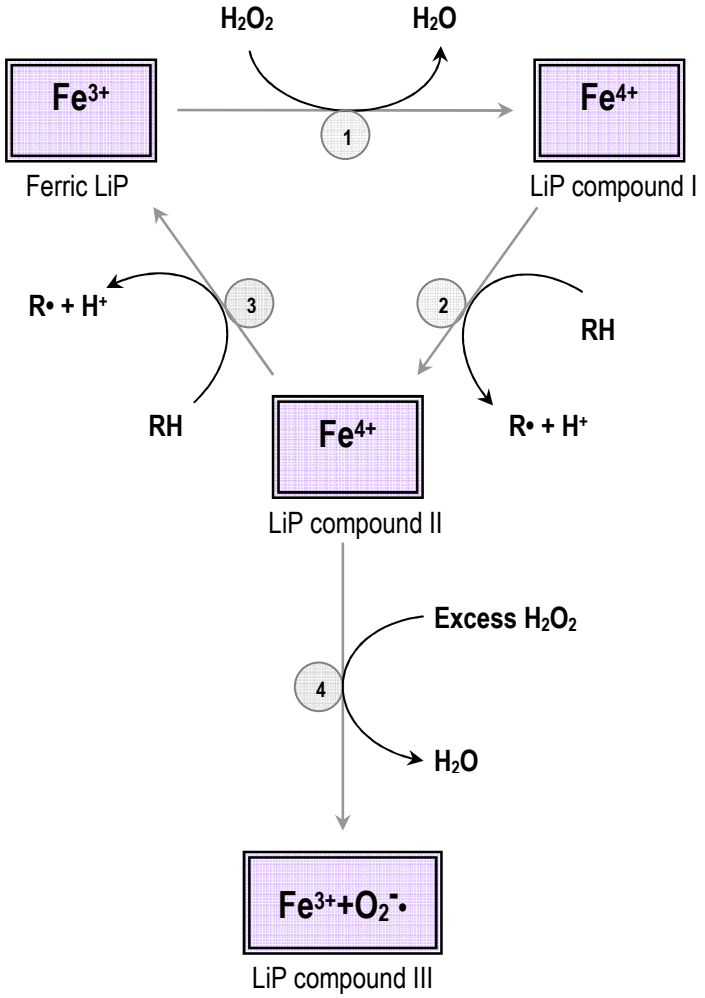

Figure 2-2. Catalytic cycle of LiP. Redrawn from Gold *et al.* (1989).

Among the oxidation reactions catalysed by LiP are the cleavage of the C_α-C_β and aryl C_α bonds, ring cleavage in β-O-4 compounds, aromatic ring opening, demethylation and phenolic oxidation (Kirk and Farrell, 1987). Aitken and Irvine (1989) reported that the stability of LiP is greatly enhanced by increasing culture pH (from 3-4.5) and the concentration of the enzyme. They also noted that controlled addition of H_2O_2, along with the presence of VA, is required for the enzyme to be activated. The presence of VA is also reported to protect against inactivation of LiP by excess H_2O_2 (Wariishi *et al.*, 1989). Lignin peroxidase can also lead to the mineralisation of a variety of recalcitrant aromatic compounds, such as polyaromatic hydrocarbons (PAH) and polychlorinated biphenyls (Shim and Kawamoto, 2002).

2.3.4 Laccase (Lac)

Laccases are widespread in nature and can be found in plants, insects, bacteria, and above all in fungi (Galhaup *et al.*, 2002a). Fungal laccases are produced by almost all litter- and wood-transforming basidiomycetes. Some producers of Lac include *Coriolus versicolor* (Moroshi, 1991), *Panus tigrinus* (Maltseva *et al.*, 1991) and *Pycnoporus cinnabarinus* (Eggert *et al.*, 1996). These copper-containing proteins have been implicated in a number of processes, which include not only lignin degradation, but also humification processes (Bourbonnais and Paice, 1990), pigment formation in mycelia and fruiting bodies (Clutterbuck, 1990), differentiation of sporulation and resting structures in basidiomycetes, as well as the formation of polyphenolic glue that binds hyphae together (Thurston, 1994).

Laccase is a member of the 'blue copper oxidase' family (Thurston, 1994) and is capable of catalysing the oxidation of phenolic and non-phenolic compounds (Bourbonnais and Paice, 1990). Lac is a glycosylated protein that is expressed in multiple forms and shows a high variability in MW of 50-110 kDa (Table 2-2) (Fakoussa and Hofrichter, 1999). The catalytic site on the Lac molecule includes four copper ions (Cu^{2+}) that are involved in the four-electron reduction of molecular oxygen to water without the formation of hydrogen peroxide. Most Lac molecules can be classified into three groups using UV/visible and electron paramagnetic resonance spectroscopy (EPR) (Leontievsky *et al.*, 1997). Type 1 copper is detectable with EPR and is responsible for the intense blue colour of the enzymes at 600 nm, type 2 copper is colourless and detectable with EPR, whilst type 3 copper consists of a pair of copper atoms that give a weak absorbance near the UV spectrum, but no EPR signal. The type 2 and 3 copper sites are close together and form a trinuclear centre (Leontievsky *et al.*, 1997).

The catalytic reaction of Lac initially involves the oxidation of substrate molecules with type 1 copper by one-electron transfers. Four electrons are required for the full reduction of the Lac enzyme. The re-oxidation of Lac is brought about by type 3 copper pair, which transfers four electrons in two-electron steps to oxygen (Claus, 2003). The oxidation of substrates creates reactive radicals that can undergo several non-enzymatic

coupling reactions such as: oxidative coupling of monomers including phenolic compounds or aromatic anilines, degradation of polymers such as lignin, and ring cleavage of aromatic compounds (Claus, 2003).

In addition, fungal Lac works synergistically with MnP in oxidising phenols and phenolic substructures of lignin through decarboxylation and subsequent demethylation of methoxy groups (Schlosser and Hofer, 2002). These pathways proceed via the formation of oxygen-mediated phenoxy radicals, like a carbon-centred cation radical formed in a MnP reaction (Kersten *et al.*, 1990). In the presence of low MW mediators (including natural phenols), Lac oxidises non-phenolic substrates with a high oxidation-reduction potential (Bourbonnais and Paice, 1990).

2.3.5 Versatile Peroxidase (VP)

In addition to Lac, an enzyme expressing the catalytic mechanisms of classical peroxidases has been isolated from the genera *Bjerkandera* and *Pleurotus* (Camarero *et al.*, 1999). The novel hybrid, otherwise known as a versatile peroxidase (VP), shares the catalytic properties of MnP and LiP (oxidation of lignin model dimers). These hybrid properties are due to the coexistence of different catalytic sites on a single protein (Camarero *et al.*, 2000) which allows for the oxidation of a broad range of substrates including the oxidation of Mn^{2+} (the substrate of MnP) to Mn^{3+}, the oxidation of 2,6-dimethoxyphenol (DMP) and VA (the typical LiP substrate). In addition to its high affinity for substituted hydroquinones (Mester and Field, 1998), VP also oxidises phenolic compounds and dyes, which are the substrates of other peroxidases such as horseradish peroxidase (Veitch, 2004).

2.4 Degradation of humic substances by WRF

Microbial degradation of HS is an important part of humus turnover and is therefore essential in maintaining the global carbon cycle (Haider, 1998). The production and degradation of HS is highly complex and still not well understood, with different microorganisms playing various roles in its synthesis and degradation. Amongst these the white rot and brown rot fungi and litter-decomposing basidiomycetes are the most important (Table 2-3). These fungi are actively involved in the degradation and mineralisation of refractory organic matter (Grinhut *et al.*, 2007) and the transport of nutrients. Other microorganisms including ectomycorrhizal fungi, soil-borne fungi and bacteria are also involved in the degradation process (Table 2-3) (Gramss *et al.*, 1999). Although bacteria participate in the turnover of HS, their direct involvement in the degradation of HS and lignin is only minimal (Gramss *et al.*, 1999) and is probably limited to the utilisation of lower MW species following fungal treatment.

The mechanisms of HS degradation probably entail an array of enzymes, of which the non-specific and oxidative types would be the most important in its breakdown. Supporting enzymes including H_2O_2-generating enzymes, and low MW organic acids (oxalate, malate and malonate) also play a role. Grinhut *et al.* (2007) proposed that the oxidation of HS by the LMEs combined with the formation of unstable radicals could lead to two distinct pathways: (i) degradation and some mineralisation of HS and or (ii) the transformation and polymerisation of HS. The type of pathway taken is likely to vary between fungal species, and depend upon the substrates involved and the surrounding conditions such as pH, temperature, humidity, ratio of C:N, and the presence of oxygen and other compounds (Grinhut *et al.*, 2007). The unique ability of LMEs to mineralise lignin, which is a main parent material for the formation of HS, should also permit the WRF to be effective in degrading HS (Hofrichter and Fritsche, 1996).

Considerable research has been undertaken on the degradation of natural and synthetic HA by WRF (Dehorter and Blondeau, 1992; Hofrichter *et al.*, 1997; Willmann and Fakoussa, 1997). Research undertaken with well-known basidiomycetes such as *Trametes versicolor* and *Phanerochaete chrysosporium* has demonstrated their ability to degrade high molecular mass HA to low MW FA and carbon dioxide (Steffen *et al.*, 2002). A comparison between *P. chrysosporium* and *T. versicolor* confirmed the latter to be more effective at degrading HAs (Dehorter and Blondeau, 1992). Dehorter and Blondeau found a correlation between HA degradation and the expression of lignin-degrading enzymes. They suggested that MnP played a more important role in the depolymerisation and mineralisation of different HAs *in vitro* than LiP. Similar findings were obtained for a soil-inhabiting fungus, *Collybia dryophila,* where extracellular MnP played a key role in the conversion of natural HA to lower molecular mass FA and carbon dioxide (Steffen *et al.*, 2002). The biodegradation of natural and synthetic HS by different basidiomycetes involves the secretion of different combinations of LMEs, with the predominant enzymes involved being MnP and Lac (Table 2-3).

The secretion of different combinations of enzymes may reflect the different mechanisms involved in the breakdown of recalcitrant compounds such as HA (Grinhut *et al.*, 2007).

The role of LiP in the transformation of HS remains questionable. Nevertheless, studies carried out with isolated LiP have shown its potential for degrading high MW coal substances (Fakoussa and Frost, 1999). Its involvement in the decolourisation and depolymerisation of HS has only been reported for *P. chrysosporium* and *T. versicolor* (Table 2-3). Blondeau (1989) reported that the LiP system was at least partly responsible for the degradation of humus by *P. chrysosporium*. Dehorter and Blondeau (1992) found that no extracellular LiP could be detected without the incorporation of soil HAs in the medium, and that LiP production was only important in the presence of VA. Studies by Ralph and Catcheside (1994) also reported the strong requirement of VA during decolourisation studies by LiP. The presence of an adequate mediator is an important factor in degrading HA; Fakoussa and Frost (1999) showed that *T. versicolor* degraded coal-derived HA (Table 2-3) and the fungus produced its own mediator from the components of the complex medium. Apart from the three ligninolytic enzymes other enzymes, such as glyoxal oxidase, cellobiose dehydrogenate, aryl alcohol oxidase, VP and cytochrome P450 may also be involved in the degradation of HS (Grinhut *et al.*, 2007).

Although much attention has been given to the degradation of HS by the basidiomycetes, the true extent of their degradation of HA is uncertain, as these WRF are mainly restricted to woody environments. Microfungi, including ascomycetes, are common inhabitants of soil, forests, grasslands and compost, and their utilisation of HS has been studied to a lesser extent than that of the WRF. Nonetheless, they modify HS via decolourisation, polymerisation and mineralisation with phenoloxidase enzymes (Table 2-3). Rezacova *et al.* (2006) found the extent of FA utilisation by soil microfungi to be comparable to that with *P. chrysosporium*. Similar findings were also reported by Koukol *et al.* (2004) who noted greater decolourisation of HA by the litter-inhabiting ascomycete *Chalara longipes* than by certain basidiomycetes.

Like lignin biodegradation, the depolymerisation of HA is a co-metabolic event. Temp *et al*. (1999) found that the depolymerisation of low rank coal by *Pycnoporus cinnabarinus* and *Polyporus ciliatus* required the presence of glucose as the primary carbon source to support fungal growth. On the other hand, Rezacova *et al.* (2006) found no significant effect of glucose on the utilization of either HA or FA by microfungi. They also found that adding mineral nutrients decreased the degradation rate, measured as the removal of FA aromaticity.

Table 2-3. Microorganisms that degrade HS

Species	Type of HS	Enzymes involved	Transformation of HS	References
Wood rotting Basidiomycetes				
Phanerochaete chrysosporium	^{14}C-labelled HA		Mineralisation of HA	Haider and Martin (1988)
Phanerochaete chrysosporium BKM-F 1767	Natural and synthetic melanoidin HA	LiP	Decolourisation of HA, reduction in MW and production of CO_2	Blondeau (1989)
Trametes versicolor	Lignite brown coal-derived HA	Lac, Peroxidase	Decolourisation of HA (80%), decrease in HA and increase in FA concentration	Fakoussa and Frost (1999)
Trametes versicolor	HA and FA from forest soil	MnP, LiP	Mineralisation of HA (80% bleaching)	Dehorter and Blondeau (1992)
Panus tigrinus 8/18	Chernozem-derived HA HA from peat and HA from soddy-podzol soil	Lac	Depolymerisation and polymerization of HA	Zavarzina *et al.* (2004)
Clitocybula dusenii	Lignite HA	MnP	Depolymerisation of HA to FA	Ziegenhagen and Hofrichter (1998)
Clitocybula dusenii	Forest soil HA	MnP, Lac	Decolourisation (56%)	Gramss *et al.* (1999)
Nematoloma frowardii	Synthetic fluorinated HA	MnP, Lac	Decolourisation (45-60%), degradation and partial defluorination of HA, with formation of lower MW FA-like substances	Wunderwald *et al.* (2000)
Hypholoma frowardii	Forest soil HA	Lac, MnP, Peroxidase	Decolourisation (73%)	Gramss *et al.* (1999)
Pycnoporus cinnabarinus *Polyporus ciliatus*	Coal HA	Lac, MnP Lac, MnP	Depolymerisation and decolourisation reduction in high MW HA fraction and increase in FA fraction	Temp *et al.* (1999)
Bjerkandera adusta	Soil humic extract- humic mull soil	MnP	Decolourisation of HA (56%)	Gramss *et al.* (1999)
Pleurotus ostreatus	Soil humic extract	Lac, MnP, Peroxidase	Decolourisation of HA (41.3%)	Gramss *et al.* (1999)
Fomitopsis pinicola	Soil humic extract	-	Decolourisation of HA (72%)	Gramss *et al.* (1999)
Forest litter Basidiomycetes				
Collybia dryophila	Natural HA isolated from pine forest litter and synthetic ^{14}C-labelled HA	MnP, Lac	Mineralisation of HA and formation of FA and CO_2	Steffen *et al*. (2002)
Agaricus bisporus	Soil humic extract	Lac, MnP	Decolourisation of HA (26%)	Gramss *et al*. (1999)
Ectomycorrhizal Fungi				
Glomus claroideum BEG-23	HA	-	Stimulated root colonization and production of mycelium	Gryndler *et al*. (2005)
Amanita muscaria *Hebeloma crustuliniforme* *Lactarius deliciosus* *Lactarius deterrimus*	Soil humic extract (black humic mull soil)	Lac MnP Lac, Peroxidase Lac, Peroxidase	Decolourisation of HA (13%) “ “ (19%) “ “ (18%) “ “ (14%)	Gramss *et al.* (1999)

Lactarius torminosus *Morchella conica* *Morchella elata* *Paxillus involutus* *Suillus granulatus* *Tricholoma lascivum*		Lac, Peroxidase Lac, Peroxidase, MnP Lac, Peroxidase, MnP Lac, Peroxidase Lac, Peroxidase	" " (8%) " " (32%) " " (35%) " " (12%) " " (3%) " " (18%)	
Soil Microfungi				
Alternaria alternata *Phoma* sp. *Paecilomyces lilacinus* *Clonostachys rosea*	Soil HA	MnP, Lac-neglible MnP, Lac-neglible MnP, Lac-neglible MnP, Lac-neglible	Decolourisation of HA (15%) " " (18%) " " (22%) " " (27%) Reduction in MW of HA, some reduced aromaticity of HA	Rezacova *et al*. (2006)
Paecilomyces inflatus	Synthetic and natural HA	Purified Lac	Partial mineralisation, modification and decolourisation of HA in compost environment plus moderate changes in MW distribution	Kluczek-Turpeinen *et al*. (2005)
Cladosporium cladosporioides	HA from groundwater and bog lake	Lac	Degradation of HA (60%)	Claus and Filip (1998)
Streptomyces aureofaciens	Andosol HA from soil	-	Decolourisation of HA (86%)	Yanagi *et al*. (2002)
Acremonium murorum *Botrytis cinerea* *Chaetomium globosum* *Cunninghamella elegans* *Rhizoctonia solani* *Scytalidium lignicola* *Trichoderma* sp.	Soil humic extract (black humic mull soil)	- Lac - - - Lac -	Decolourisation of HA (26%) " " (37%) " " (3%) " " (17%) " " (21%) " " (77%) " " (25%)	Gramss *et al*. (1999)
Bacteria				
Pseudomonas fluorescens	Aquatic HS from a bog lake water	-	Decrease in particle size, loss in aromaticity and aliphatic carbon	Hertkom *et al*. (2002)
Alcaligenes eutrophus *Alcaligenes faecalis* *Bacillus brevis* *Bacillus cereus* *Pseudomonas fluorescens* *Pseudomonas putida* *Xanthomonas campestris*	Soil humic extract (black humic mull soil)	- - - - - - -	Decolourisation of HA (2.5%) " " (3.4%) " " (7.3%) " " (8%) " " (2.5%) " " (3%) " " (3.7%)	Gramss *et al.* (1999)

2.5 Application of WRF in bioremediation

Among their industrial applications, WRF have shown great potential in the bioremediation of effluents from the pharmaceutical, textile, dyeing and printing industries. Their decolourisation and detoxification potential is attributed to the mechanisms of biodegradation and biosorption, with the extent of colour removal being dependent on the dye complexity (Shah and Nerud, 2002). Their use has also been documented in the pulp and paper processing industry for both the pulping process and treatment of effluents, the latter containing high concentrations of phenol, chlorinated lignin compounds and dyes. Fungal pre-treatment of pulp has led to decreased lignin content, a reduction in pulping time, and reduced consumption of bleaching chemicals (Wolfaardt *et al.*, 2004).

There is great potential for using lignin-modifying enzymes in bioremediation as they are able to break down recalcitrant compounds showing partial structural similarities to lignin. Some of the xenobiotic compounds that have already been degraded by whole cultures of WRF under laboratory conditions include: chlorinated phenols, polychlorinated biphenyls (PCBs), DDT, dioxins, PAHs, alkyl halides, nitrotoluenes, chloroanilines and dyes (Field *et al.*, 1993).

2.6 Application of WRF for the removal of NOM

One approach to the removal of DOM from water is the application of WRF and their extracellular enzyme system. As noted earlier, the LMEs are independent of pollutant type, allowing the fungi to tolerate highly toxic compounds without the need to be pre-conditioned (Karapinar and Kargi, 2002). Moreover, their extracellular nature allows the enzymes to access non-polar and non-soluble compounds that other intracellular processes cannot (Levin *et al.*, 2003). The application of WRF is a potentially 'environmentally friendly' approach to the removal of NOM from concentrated NOM wastes and possibly from water. The implementation of a biological treatment for NOM removal would overcome some problems associated with conventional treatment processes, including chemical usage and sludge management. This approach is further supported by Carraro *et al.* (2000) who demonstrated that biological treatment of drinking water avoids the need for break-point chlorination and limits by-product formation and associated mutagenic generation. Carraro *et al.* (2000) also reported that biological treatment transformed hazardous compounds, including xenobiotics, to oxidised assimilable organic carbon and reduced ammonia, iron and manganese levels to form biologically stable water, which would then reduce biofilm formation in the water distribution system. Biological treatment may also be used in association with conventional treatment for the removal of NOM from chemical sludges and membrane reject streams.

WRF and their LMEs have been extensively studied due to their ability to oxidise a variety of recalcitrant organic compounds with structural similarities to lignin. Of these, the HS, which comprise a major portion of NOM, have been extensively investigated. The study of the biodegradation of HS has mainly focused on each of the

individual fractions, predominantly HA and FA, using a few organisms. Most of the studies have focused on the removal of natural and synthetic HS in the presence of an easily available carbon source. Although the degradation of HS by saprotrophic fungi has been well documented, there appear to be no reports in the literature on the degradation of NOM in drinking water concentrated aquatic NOM without the addition of nutrients.

Previous work by Rojek *et al.* (2004) and Lee (2005) has shown the potential of WRF to degrade concentrated NOM using laboratory strains of *P. chrysosporium* and *T. versicolor* with nutrient supplementation. Rojek *et al*. showed that *P. chrysosporium* ATCC 34541 decolourised 40-50% of NOM in solution, the decolourisation mechanism was mainly via adsorption and only partly metabolically linked. Lee (2005), on the other hand, found NOM decolourisation by *T. versicolor* (up to 73%) to be associated with high oxidative extracellular enzyme activity, particularly of Lac. Lee found that lower decolourisation of NOM by *T. versicolor* occurred with higher glucose concentrations. The decolourisation was attributed to the breakdown of higher MW compounds to lower MW materials. This approach clearly has the potential for biodegradation of Beaconsfield NOM and MIEX NOM concentrate under conditions of limited nutrients with new species of fungi, as investigated in this thesis.

Chapter 3 Materials and Methods

3.1 Microorganisms

Fungi were isolated using three methods: trapping from NOM-containing water, isolation from fruiting bodies on dead wood and using enrichment culture.

3.1.1 Trapping and isolation from water

Potential NOM-degrading fungi were isolated from Beaconsfield Reservoir, Victoria, Australia, over three months from September to November (spring) 2004. Part of the Cardinia/Tarago catchment, the reservoir is rich in NOM (Table 3-1) and is not presently in use.

Wood from different species of both dead and live trees was used as bait in capturing the fungi. Baits included peat, and wood blocks of eucalypts (*Eucalyptus* sp. and *Corymbia ficifolia),* flowering ash (*Fraxinus ornus)* and pine (*Pinus* sp.*).* The wood was initially soaked in sterile distilled water for several days before suspension in the reservoir. Fly-fishing meshes were used to house five wood pieces (1-2 cm in length, width and depth) of each species and were suspended from a fishing line at 1 m and 2.5 m depths. Wood samples at each depth were collected from the reservoir weekly and incubated at room temperature in sterile Petri dishes containing 5 mL of Beaconsfield Reservoir water until mycelial growth was seen. Repeated subculturing of each sample on malt extract agar (MEA) (Oxoid) was required to obtain pure fungal isolates.

3.1.2 Isolation from fruiting bodies on dead wood

Several fruiting bodies of white and brown rot fungi were collected from dead tree stumps and rotting twigs in the Dandenong Ranges, Victoria, Australia, over autumn (March-May) in 2005. The fruiting bodies were placed in sterile plastic bags, transported to the laboratory and identified using morphological and molecular techniques. Samples of the internal tissue were excised aseptically and inoculated onto MEA and incubated at 30°C for 3 days until hyphal growth was seen, and repeatedly subcultured on MEA until pure fungal isolates were obtained.

3.1.3 Enrichment cultures

Enrichment culture was used to isolate fungi capable of utilising NOM as a sole carbon source by incubating 500 mL of 300 mg C L^{-1} NOM concentrate (Table 3-1) with 400 g of biologically active sand obtained from Big Pats Creek, Warburton, Victoria. The culture was aerated for 2 weeks and plated out (100 µL) onto plates

containing concentrated NOM (300 mg C L^{-1}) in 17 g L^{-1} agar (Oxoid) pH 6.8. NOM plates were incubated at 25°C for up to two weeks until fungal growth was visible, with further subculturing onto MEA to obtain pure fungal isolates.

3.2 Molecular identification of fungal isolates

Unidentified fungal species were pre-grown in 100 mL of malt extract broth (MEB), under continuous agitation. Mycelia from 7-day cultures were harvested and ground to a powder in a mortar containing liquid nitrogen. DNA was isolated with a DNeasy Mini kit (Qiagen) following the manufacturer's protocol. Isolated genomic DNA was used as a template in PCR reactions using primers (ITS1 and ITS4) to amplify the internal transcribed spacer (ITS) region of ribosomal DNA (White *et al.*, 1990).

PCR templates for sequencing were column-purified using a QIAquick PCR purification kit (Qiagen) before proceeding with sequencing. Sequencing was performed with an ABI BigDye Terminator Mix version 3.1 (Applied Biosystems, CA) in a GeneAmp 2400 (PerkinElmer, CA) following the manufacturer's instructions. Capillary electrophoresis and detection were carried out at the Micromon DNA Sequencing Facilities, Monash University (Victoria, Australia) using a 3730S Genetic Analyser (Applied Biosystems, CA). Identification of sequences was performed with a BLAST algorithm (Altschul *et al.*, 1997) running against the nr database in GenBank (National Centre for Biotechnology Information).

3.3 Culture maintenance

MEA at 2% (w/v) was employed as the growth medium for all species of fungi isolated. The medium was sterilised by autoclaving at 121ºC for 20 minutes and poured into plates in a laminar flow cabinet prior to being inoculated with fungi. All fungi were maintained by subculturing on MEA plates every 2 months. For prolonged storage of micro-organisms, MEA slants were inoculated with fresh mycelium and then grown at 30°C prior to being stored at 4ºC.

3.4 Preparation of fungal inoculum

Fungi were inoculated as pellets (10 g wet weight) (Section 3.4.2) prepared from mycelial suspensions (Section 3.4.1).

3.4.1 Production of mycelial suspension

Conidial suspensions were prepared from strains previously cultured on 2% (w/v) MEA plates at 30°C. Fresh mycelium was aseptically scraped off, suspended in Milli-Q water and then filtered through glass wool. Conidial suspensions were stored in sterile Schott bottles (100 mL) at 4°C for a maximum of 7 days prior to pellet production.

3.4.2 Pellet cultivation of WRF

Fungal pellets were cultivated from conidial suspensions (Section 3.4.1). Erlenmeyer flasks (500 mL) containing 200 mL of MEB (10 g L^{-1}) were inoculated with an appropriate volume of mycelial suspension to reach a desired spore concentration of 2×10^5 spores mL^{-1}. The spore concentration was determined by measuring the absorbance at 650 nm and calculated on the basis that A_{650} = 1.0 cm^{-1} corresponded to 5 × 10^6 spores per mL (Kirk *et al.*, 1978). The cultures were incubated at 30°C for 8-10 days in an orbital shaker at 125 rpm. After cultivation, the fungal pellets were rinsed and stored in sterile Milli-Q water at 4°C for up to 10 days.

3.5 NOM samples

Several sources of NOM, including NOM from a drinking water reservoir (Beaconsfield NOM), NOM concentrate and treated NOM obtained from Hallam tap water, were utilised as sources of organic matter for the experimental work. Reservoir NOM was obtained from Beaconsfield Reservoir, Victoria, Australia, over 3 months from September to November (spring) 2004. A highly concentrated and coloured NOM known as MIEX™ NOM concentrate was obtained from Hope Valley Reservoir, South Australia. The NOM concentrate was obtained from the regeneration of the magnetic anion exchange (MIEX™) resin. All NOM samples were filtered (0.45 µm hydrophilic PVDF, Millipore Millex-HV) and stored at 4°C prior to treatment and analysis. The characteristics of the NOM samples are listed in Table 3-1.

Table 3-1. Characteristics of NOM samples

NOM Sources	Beaconsfield NOM	MIEX NOM concentrate	Treated NOM
Source	Beaconsfield Reservoir	Hope Valley Reservoir	Hallam tap water
Collection Date	Sep-Nov 2004	August 2003	2006-2008
DOC (mg C L^{-1})	13	13	1.3
Absorbance at 446 nm (A_{446}) cm^{-1}	0.023	0.024*	0.00
Absorbance at 254 nm (A_{254}) cm^{-1}	0.290	0.380*	0.023
SUVA UV Abs. (cm^{-1})× 100/ DOC (mg L^{-1})) (L mg^{-1} cm^{-1})	2.23	2.92	1.8
pH	6.51	6.50	6.4

* Dilution factor of 2385 of NOM concentrate stock (31 g L^{-1})

3.6 Plate studies

Preliminary screening for NOM and cellulose degradation was conducted using NOM and cellulose plate assays, respectively.

3.6.1 Screening for cellulose degradation

Cellulase activity was assayed using carboxymethylcellulose medium (CMC-Agar), consisting of 5 g L^{-1} of CMC and 17 g L^{-1} plain agar (Oxoid) as described by Hankin and Anagnostakis (1977). Fungal isolates were point-inoculated onto the CMC-Agar and incubated for 5 days at their respective temperatures (Table 4-1). For observation of cellulolytic activity, the plates were flooded with 5 mL of 1% Congo Red dye for 15 min and de-stained with 3 M NaCl for 10-15 min. Relative cellulase activity was recorded as the ratio of the cleared zone diameter to the colony diameter (Teather and Wood, 1982). Fungi that produced clear zones, which are indicative of cellulose breakdown, were further tested for their ability to degrade NOM.

3.6.2 Screening for NOM degradation on solid medium

NOM degradation was screened on agar plates containing concentrated NOM (300 mg C L^{-1}). Point inoculations of each fungus were made on duplicate NOM plates and incubated as appropriate (Table 4-1). Growth rates were determined by measuring the increase in diameter of the mycelial mat every 24 h. Controls were set up by point-inoculating each fungus onto plain agar (Oxoid) without NOM. NOM degradation was determined by measuring the ratio of the diameter of the cleared zone to the diameter of colony growth after 7 days of incubation.

3.6.3 Screening for inhibition/tolerance tests on NOM agar

The relative tolerance of *Fusarium oxysporum*, *Mucor racemosus*, *Trichoderma atroviride* and *Trametes versicolor* ATCC 7731 was tested on agar containing varying NOM concentrations (100-600 mg C L^{-1}). Single plug (1 cm^2) inoculations of each fungus were made on duplicate NOM plates at each concentration and incubated as appropriate (Table 4-1). Growth rates were determined by measuring the increase in diameter of the mycelial mat every 24 h for 8 days. Controls were set up by point-inoculating each fungus onto plain agar without NOM.

3.6.4 Growth studies in liquid NOM plates

To ascertain if NOM alone would sustain fungal growth, the biomass of a single plug inoculum of *T. versicolor* ATCC 7731, *F. oxysporum*, *T. atroviride* and *M. racemosus* was monitored in liquid NOM plates. A single plug (1 cm^2) of actively growing culture, pre-incubated for one week on MEA, was excised and placed in the centre of a duplicate plate containing 25 mL of concentrated NOM (200 mg C L^{-1}). Controls were set up by plug-inoculating each fungus onto plates containing sterile distilled water without NOM. The inoculated plates were incubated as appropriate (Table 4-1) and monitored for growth daily. Dry weight of the fungal biomass was determined every two days by filtering the contents of the culture fluid through a pre-weighed filter paper (Whatman No. 1, 1.2 µm pore size) and drying at 60°C up to 4 h to constant weight. The filtered NOM was further filtered (0.45 µm hydrophilic PVDF, Millipore Millex–HV) and analysed for pH, colour (A_{446}) and the presence of conjugated and aromatic bonds (A_{254}) as described in Section 3.10.3.

3.7 NOM degradation *in vitro*

Fungi displaying good degradation of NOM in plate assays were further investigated in liquid cultures.

3.7.1 Shake-flask cultures

Degradation experiments were conducted on all three NOM sources by selected strains of WRF. For comparison NOM concentrate was diluted in both distilled and tap water to assess if the limited nutrients present in distilled water compared with tap water reduced the ability of fungi to decolourise NOM. To determine if NOM had any inhibitory effect on the decolourisation ability of the fungi, the degradation experiments were conducted at varying concentrations of NOM (13, 20, 30, 50, 100, 300 and 500 mg C L^{-1}) diluted with tap water and distilled water.

For all shake-flask experiments, unless otherwise stated, Erlenmeyer flasks (500 mL) containing 200 mL of filter-sterilised (0.45 µm hydrophilic PVDF, Millipore Millex-HV) NOM at pH 6.5 were inoculated with 10 g (wet

weight) pre-grown fungal pellets. The flasks were incubated at 100 rpm at either 15°C or 30°C for a period of 10–30 days, depending on the fungus and NOM source used. Experiments were conducted in triplicate or quadruplicate and sampled (5-7 mL) at time intervals appropriate to the fungal species.

3.7.2 Experimental controls

Several controls were set up and run in parallel with the test samples. Controls containing NOM solution without fungal biomass were used to see whether any external processes apart from fungi altered the state of the NOM solution. To determine if there was any adsorption of NOM to the fungal biomass, 10 g (wet weight) of fungi was autoclaved at 121°C for 20 minutes and incubated with the NOM solution. Controls of live fungi in tap and distilled water were also run in parallel to the test cultures.

3.7.3 Biomass determinations

The dry weight of the fungal biomass was determined at the end of each experiment by filtering the contents of the culture fluid through a pre-weighed filter paper (Whatman No. 1, 1.2 µm pore size) and drying at 60°C to constant weight. Biomass was expressed as the dry weight of fungal mycelium per 200 mL of culture fluid.

3.8 Enzyme assays

All enzyme assays were performed spectrophotometrically (Unicam, Model UV2) at 50°C, and the reaction mixtures were buffered with 0.2 M Na_2HPO_4-citric acid buffer at the pH of the culture at the specific time of sampling. Prior to enzyme analysis, the extracellular culture fluid was filtered (0.45 µm hydrophilic PVDF, Millipore Millex–HV).

3.8.1 Lac activity

Lac activity was determined as described by Coll *et al*. (1993) by monitoring the oxidation of 1 mM guaiacol (Sigma) at 465 nm (ε = 12,100 M^{-1} cm^{-1}) to the polymer tetraguaiacone. Guaiacol solution was prepared fresh for each day of assay. The following assay mixture was used in determining Lac activity (Table 3-2).

Table 3-2. Assay mixture for Lac activity

Assay components	Volume (µL)
1.0 mM Guaiacol	300
0.2 M Na_2HPO_4 - citric acid buffer	1800
Milli-Q water	600
Extracellular culture fluid	300
Total volume	3000

The reaction was initiated by the addition of guaiacol. The enzyme activity was expressed in U L^{-1}, where one unit (U) of Lac activity was defined as the amount of enzyme catalysing the oxidation of 1 µmole of guaiacol to form tetraguaiacone per minute.

3.8.2 MnP activity

Manganese-dependent peroxidase activity was determined as described by Wariishi *et al.* (1992) by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) (Aldrich, 99%) to coerulignone at 469 nm (ε = 49,600 M^{-1} cm^{-1}). DMP and hydrogen peroxide solutions were prepared fresh on the day of the assay. The following assay mixture was used in determining MnP activity (Table 3-3).

Table 3-3. Assay mixture for MnP activity

Assay components	Volume (µL)
0.5 mM DMP	300
0.2 M Na_2HPO_4 - citric acid buffer	1800
1.0 mM $MnSO_4$	300
0.5 mM H_2O_2	300
Extracellular culture fluid	300
Total volume	3000

The reaction mixture was initiated by the addition of DMP and hydrogen peroxide. One unit of activity (U) was defined as the amount of enzyme catalysing the oxidation of 1 µmole of DMP to form coerulignone per minute in the presence of H_2O_2.

3.8.3 LiP activity

LiP activity was measured according to Tien and Kirk (1884) by monitoring the oxidation of VA (Aldrich, 96%) to veratraldehyde at 310 nm (ε = 9300 M^{-1} cm^{-1}). VA and hydrogen peroxide solutions were prepared fresh on the day of the assay. The following assay mixture was used in determining MnP activity (Table 3-4).

Table 3-4. Assay mixture for LiP activity

Assay components	Volume (µL)
20 mM VA	300
0.2 M Na_2HPO_4 - citric acid buffer	840
4.0 mM H_2O_2	300
Extracellular culture fluid	1560
Total volume	3000

One unit (U) of activity was defined as the amount of enzyme catalysing the oxidation of 1 µmole of VA to form veratraldehyde per minute in the presence of H_2O_2.

3.8.4 Optimal enzyme pH and temperature determinations

Enzyme assays for optimal pH and temperature determinations were performed on NOM samples taken during the period of maximal NOM decolourisation. For determination of pH optimum, assays were performed at 50°C at pH 2.2, 4, 5, 6, and 8. To determine the optimum temperature, the enzyme activities were determined at 18, 30, 40, 50, 60, 70, and 80°C at the previously determined optimal pH for the enzyme.

3.9 *In vitro* decolourisation of NOM by Lac, MnP and LiP

The enzymes were purchased from Sigma Aldrich. The characteristics of the enzymes are listed in Table 3-5.

Table 3-5. Characteristics of purchased enzymes

Enzyme	EC number	Organism	Specific activity (Stock) U mg^{-1}
Lac	1.10.3.2	*Trametes versicolor*	≥ 20
MnP	1.11.1.13	Not specified	0.027
LiP	1.11.1.14	Not specified	0.52

3.9.1 Determining the activities of enzymes

Prior to use in the decolourisation experiments, Lac was dissolved in 0.2 M Na_2HPO_4 - citric acid buffer, whilst MnP and LiP were dissolved in sterile Milli-Q water. The reconstituted enzymes were filtered (0.45 µm hydrophilic PVDF, Millipore Millex-HV), dispensed into sterile Eppendorf tubes and stored at -18ºC prior to use.

The activity of Lac, MnP and LiP was measured according to the methods described in Sections 3.8.1-3.8.3, with the following amendments:

- Lac and MnP assays were buffered to pH 4.5, and LiP to pH 3

- Extracellular culture fluid was replaced with filter-sterilised enzyme solution (diluted if necessary with sterile Milli-Q water)

The activity of reconstituted enzymes was calculated by using the Beer-Lambert law (Appendix 1). The determined concentrations (U mL^{-1}) are reported in Table 3-6.

Table 3-6. Enzyme activities

Enzyme	Amount of reconstituted enzyme	Enzyme activity (U mL^{-1})	Total activity units (U)
Lac	100 mg in 20 mL	117	2320
MnP	10 mg in 10 mL	0.101	1.01
LiP	10 mg in 10 mL	0.52	5.20

3.9.2 Decolourisation of NOM by Lac

Decolourisation of NOM concentrate by Lac was monitored at 30ºC over 6 days by measuring A_{446} and A_{254}. Lac at varying concentrations (11.7 U mL^{-1}, 5.8 U mL^{-1} and 0.12 U mL^{-1}) was incubated with NOM concentrate (50 and 100 mg C L^{-1}) and Na_2HPO_4 - citric acid buffer (pH 4.5) in a 3 mL reaction mixture (Table 3-7). The decolourisation of NOM concentrate was initiated by the addition of freshly thawed enzyme. Controls using heat-denatured Lac (100°C for 10 min) were run in parallel with the decolourisation experiments. All enzyme assays were carried out in duplicate.

Table 3-7. Assay mixture used in the decolourisation of NOM by Lac

Assay components	Volume (µL)
200 mg C L^{-1} NOM concentrate	1500
0.2 M Na_2HPO_4 - citric acid buffer	1200
Lac solution	300
Total volume	3000

3.9.3 Decolourisation of NOM by MnP

Decolourisation of NOM concentrate by MnP was monitored at 30ºC over 4 days by measuring A_{446} and A_{254}. MnP at 0.0101 U mL^{-1} was incubated with NOM concentrate (50 and 100 mg C L^{-1}) and Na_2HPO_4 - citric acid buffer (pH 4.5) in a 3 mL reaction mixture (Table 3-8). The decolourisation of NOM concentrate was initiated by the addition of freshly thawed enzyme. Controls using heat-denatured MnP (100°C for 10 min) were run in parallel with the decolourisation experiments. All enzyme assays were carried out in duplicate. The

decolourisation of NOM concentrate (100 mg C L^{-1}) was also conducted in the presence of 0.5 mM H_2O_2 (300 µL) and 1 mM $MnSO_4$ (300 µL) with a reduced buffer (600-900 µL) volume to reach a final volume of 3000 µL.

Table 3-8. Assay mixture used in the decolourisation of NOM by MnP

Assay components	Volume (µL)
200 mg C L^{-1} NOM concentrate	1500
0.2 M Na_2HPO_4 - citric acid buffer	1200
MnP solution	300
Total volume	3000

3.9.4 Decolourisation of NOM by LiP

Decolourisation of NOM concentrate by LiP was monitored at 30ºC over four days measuring A_{446} and A_{254}. LiP at 0.052 U mL^{-1} was incubated with NOM concentrate (50 and 100 mg C L^{-1}) and Na_2HPO_4 - citric acid buffer (pH 3 and pH 4.5) in a 3 mL reaction mixture (Table 3-9). The decolourisation of NOM concentrate was initiated by the addition of freshly thawed LiP. Controls using heat-denatured LiP (100°C for 10 min) were run in parallel with the decolourisation experiments. All enzyme assays were carried out in duplicate. The decolourisation of NOM concentrate (100 mg C L^{-1}) was also conducted in the presence of 4 mM H_2O_2 (300 µL) at pH 3 with a reduced buffer volume (900 µL) to reach a final volume of 3000 µL.

Table 3-9. Assay mixture used in the decolourisation of NOM by LiP

Assay components	Volume (µL)
200 mg C L^{-1} NOM concentrate	1500
0.2 M Na_2HPO_4 - citric acid buffer	1200
LiP solution	300
Total volume	3000

3.10 Analytical methods

3.10.1 pH

A Mettler Toledo pH meter was used to measure the pH of the medium directly after sampling of the culture. Adjustments of pH were made with NaOH and HCl as appropriate.

3.10.2 Dissolved organic carbon (DOC)

A total organic carbon (TOC) analyser (Sievers, Model 820) was utilised to measure the DOC concentration. The device uses a combination of UV/persulfate oxidation of organics to form CO_2, and a permeation/conductivity detection method. Prior to DOC analysis all samples were filtered (0.45 µm hydrophilic PVDF, Millipore Millex–HV) and if necessary diluted appropriately with Milli-Q water.

3.10.3 Absorbance

A double beam scanning UV/vis spectrophotometer (Unicam, Model UV2) was used to measure absorbance. Prior to analysis, samples were filtered (0.45 µm hydrophilic PVDF, Millipore Millex–HV) and diluted where appropriate with Milli-Q water. The absorbance of the NOM solution was measured using a quartz cuvette of 1 cm path length for decolourisation (colour measured as absorbance at 446 nm), and for the presence of conjugated and aromatic bonds (at 254 nm). Decolourisation was calculated by means of the formula:

$$\text{Decolourisation}(\%) = \frac{A_i - A_f}{A_i} \times 100 \qquad \text{(Equation 3.1)}$$

Where:

D = Decolourisation (%)

A_i = Initial absorbance at 446 nm

A_f = Final absorbance at 446 nm

3.10.4 Correlation between NOM concentration and A_{446}

The correlation between the NOM concentration and the absorbance at 446 nm was determined by measuring A_{446} for NOM concentrations of 13-500 mg C L^{-1}. For NOM concentrations >300 mg C L^{-1} dilution was made with Milli-Q water prior to A_{446} measurements. The relationship between the NOM concentration and colour absorbance is provided in Appendix 2.

3.10.5 Absorbance correction factor (CF)

The absorbance of MIEX NOM concentrate at both 446 nm and 254 nm varied with pH, and as a result corrections were made where necessary according to the method of Lee (2005). NOM solutions at varying concentrations (50, 100 and 200 mg C L^{-1}) and at different pH (2-6), were prepared in duplicate and the absorbance at 446 nm and 254 nm was measured. An almost linear relationship (R^2=0.987) was established between the change in pH (2-6) and the absorbance correction factor (%) (Figure A-3) using the least squares

method. Plots illustrating the influence of pH on absorbance at 446 nm and 254 nm for MIEX NOM concentrate and description of calculations can be referred to in Appendix 3.

3.11 High performance size exclusion chromatography (HPSEC)

The MW distribution of NOM was determined by high performance size exclusion chromatography (HPSEC) at the Australian Water Quality Centre (AWQC), Adelaide, South Australia.

Samples were analysed with a Waters 2690 Alliance system comprising a temperature-controlled oven at 30°C and a Shodex KW 802.5 glycol functionalised silica gel column and a Waters 996 Photo Diode Array detector set at 260 and 446 nm. Samples (0.22 µL filtered) were injected into a carrier solvent consisting of 0.002 M phosphate buffer at pH 6.8, with an ionic strength of 0.1 M. An isocratic flow rate of 1 mL min^{-1} was used.

Calibration standards using polystyrene sulphonate were used to obtain the apparent molecular weight (AMW) of NOM which was calculated from the regression of the relationship between the retention time (t, minutes) and the logarithm of MW of the standards (log (M_w)): $\log_{10}(M_w) = -(0.4028)\,t + 7.1755$. Thus an AMW of 1000 corresponds to a retention time of 10.36 min. The MW distribution of NOM is represented by several parameters including the weight average MW (M_w), the number average MW (M_n) and polydispersity (ρ). M_w can be defined as the second moment of the curve and it provides an accurate evaluation of the proportion of the higher MW material. M_w is the weight of the molecule to which the “average” atom belongs. M_n is the first moment of the curve and is related to the proportion of low MW material. M_n is the weight of the “average” molecule. For a pure substance with a single MW, M_n will be equal to M_w, whereas for a mixture of molecules, M_n will be less than M_w and ρ will be greater than 1 (Zhou *et al.*, 2000). M_w, M_n and ρ were calculated from Equations 3.2, 3.3 and 3.4, respectively, according to Conte and Piccolo (1999).

$$M_w = \frac{\sum n_i M_i^2}{\sum n_i M_i} \qquad \text{Equation 3.2}$$

$$M_n = \frac{\sum n_i M_i}{\sum n_i} \qquad \text{Equation 3.3}$$

$$\rho = \frac{M_w}{M_n} \qquad \text{Equation 3.4}$$

Where:

n_i = The molecular height of each ith fraction eluted at the ith volume in the chromatogram

M_i = The MW of each ith fraction eluted at the ith volume in the HPSE chromatogram

3.12 Biodegradable dissolved organic carbon (BDOC)

The biodegradability of treated NOM was assessed by change in DOC concentration after bacterial degradation according to Volk *et al*. (1994). Biologically active sand (sand colonised by bacteria) obtained from Big Pats Creek, Warburton, Victoria, Australia was used for the BDOC experiments. Prior to experimentation the sand was washed vigorously with 10 L of distilled water to remove any particulate matter and debris that might affect the validity of the experiment.

A NOM sample (300 mL) was exposed to the biologically active sand (125 g moist weight) for 10 days under aerobic conditions. For concentrated NOM samples, the BDOC test was measured over 2 months. Samples were extracted daily or as appropriate, filtered (0.45 µm hydrophilic PVDF, Millipore Millex–HV), and the DOC and the absorbance at 254 nm and 446 nm measured (Sections 3.10.2 and 3.10.3 respectively). The BDOC was calculated according to equation 3.5:

$$\text{BDOC}\,(\%) = \frac{\text{DOC}_i - \text{DOC}_f}{\text{DOC}_i} \times 100 \qquad \text{Equation 3.5}$$

Where:

DOC_i = Initial DOC recorded (mg L^{-1}) on Day 0

DOC_f = Lowest DOC recorded over the duration of the experiment (mg L^{-1})

To determine whether the sand was active, a solution of 10 mg C L^{-1} sodium acetate was used as a positive control. To illustrate whether the age of the sand would impact on the BDOC test, the assay was conducted on old sand that was aerated for 24 months, and on fresh sand recently obtained from Big Pats Creek. A greater reduction in DOC (by 17% (1.74 mg C L^{-1})) was obtained using the fresh sand in comparison with the old sand. To demonstrate that the DOC removal was due to the bacterial inoculum present in the sand and not any other processes, a sample of sand was autoclaved at 121°C for 20 minutes to inactivate all bacteria and then exposed to sodium acetate as a negative control (Figure 3-1). A negative control of NOM solution exposed to autoclaved sand was similarly run in parallel with the BDOC tests. There was no DOC reduction for both the negative controls. In between experiments the biological activity of the sand was maintained by constantly aerating the sand in raw water obtained from the Beaconsfield Reservoir.

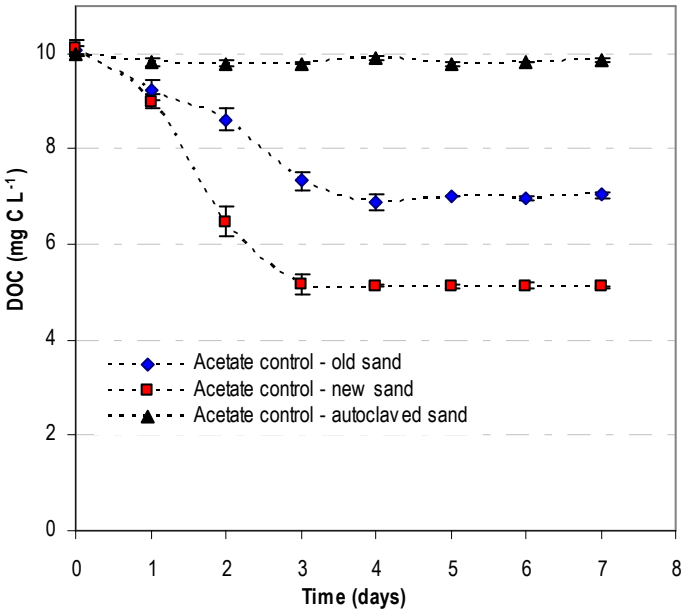

Figure 3-1. Validation of the BDOC assay, using old sand (2 yrs) and new sand from Big Pats Creek.

3.13 Fractionation of NOM

The rapid resin fractionation method (Chow *et al.*, 2004) used for the characterisation of NOM was based upon the full-scale fractionation scheme reported by Croué *et al*. (1994) and Bolto *et al*. (1999). The method uses three resins: DAX-8, on which the very hydrophobic acids (VHA) are adsorbed; XAD-4 on which the slightly hydrophobic acids (SHA) are adsorbed; and IRA-958, on which the charged (CHA) molecules are adsorbed. For this work, rapid resin fractionation was conducted only on the DAX-8 and XAD-4 resins, where the effluent from the XAD-4 resin contained the hydrophilic neutral (NEU) and charged (CHA) fractions. The fractionation unit consisted of two 20 cm glass columns of 1.3 cm diameter, containing pre-cleaned DAX-8 and XAD-4 resins to a bed depth of 10 cm, as illustrated in Figure 3.2.

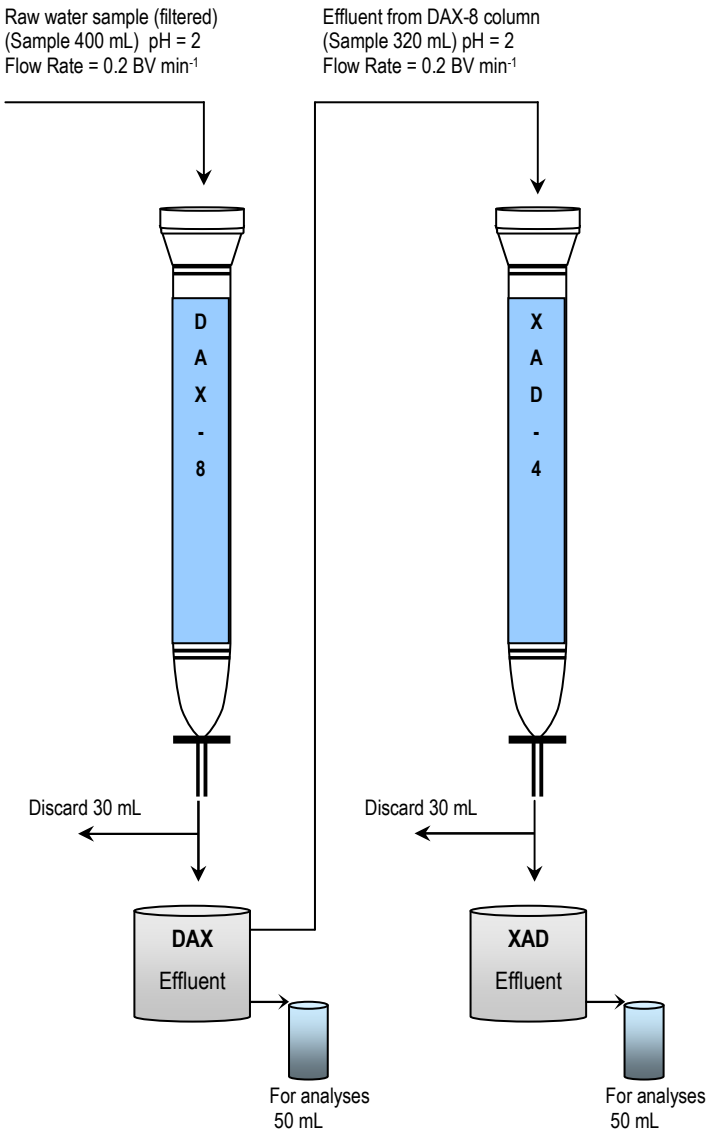

Figure 3-2. Schematic diagram of the fractionation unit used in this study (Chow *et al.*, 2004).

The two resins were filled to a bed volume of approximately 15 mL and flushed with 2-4 L of Milli-Q water prior to and after sample separation. The sample (400 mL) was filtered (0.45 µm hydrophilic PVDF, Millipore Millex-HV), acidified to pH 2 (HCl, AR grade) and passed through the DAX-8 column at a rate of 0.2 bed volumes (BV) per min using a peristaltic pump (Gilson Minipuls3, 8 channel). The first two bed volumes (30 mL) were discarded and the remaining effluent (370 mL) was collected. A sub-sample of 50 mL was collected for DOC, A_{254} and A_{446} analysis and the remaining effluent (320 mL) passed through the XAD-4 column. The effluent from the XAD-4 column was collected for DOC, A_{254} and A_{446} analysis. The DOC of each fraction was calculated by measuring the difference in DOC (at the respective pH of each column) before and after contact with the resin as shown below;

$$VHA = Raw - (DAX\text{-}8\,effluent) \quad \text{Equation 3.6}$$

$$SHA = (DAX\text{-}8\,effluent) - (XAD\text{-}4\,effluent) \quad \text{Equation 3.7}$$

$$CHA + NEU = XAD\text{-}4\,effluent \quad \text{Equation 3.8}$$

3.14 Excitation - emission matrix (EEM) spectra

Fluorescence spectroscopy was used to characterise NOM components. EEM fluorescence analysis of NOM samples was conducted using a Perkin Elmer Luminescence Spectrometer LS 50B at an excitation and emission wavelength range of 200-600 nm. FL WinLab 3D Viewer Version 1(c) Perkin-Elmer (1995-1996) Software was used to view and process the EEM spectra of the NOM samples.

3.15 ICP- MS analysis

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis was used to determine the concentrations of trace elements (Na, Mg, Mn, Al, P, K, Ca, Fe, Ni, Cu, and Zn) present in both NOM in tap and distilled water and Beaconsfield NOM. ICP-MS analysis was conducted by the School of Applied Sciences, RMIT University, using a Hewlett-Packard 4500 ICP-MS Instrument. The parameters used in the detection of trace elements are listed in Table 3-10.

Table 3-10. ICP-MS operating conditions for the analysis of NOM in tap and distilled water

Parameter	Setting
RF Power	1350 W
Sampling depth	8 mm
Carrier gas flow	1.17 L min^{-1}
Peri-pump speed	0.1 rps

Chapter 4 Isolation and preliminary screening of NOM-degrading fungi

Preface

The production and degradation of NOM is highly complex and still not well understood. Different micro-organisms play various roles in its synthesis and degradation. Amongst these, fungi play an important role in the decomposition of recalcitrant organic matter and the transport of nutrients (Frey *et al.*, 2000). This chapter describes the isolation and characterisation of a range of NOM-degrading fungi from several sources, and their potential to use concentrated aquatic NOM as a sole source of nutrients.

4.1 Isolation of fungi

Twenty-one isolates from a diverse genera of fungi were obtained from various sources, including the Dandenong Ranges, Beaconsfield Reservoir, lab strains, and from enrichment cultures of biologically active sand. A summary of identified fungi and their respective sources is presented in Table 4-1. No particular trend was found between wood species and genus of fungus isolated from Beaconsfield Reservoir. The predominant fungus isolated from most wood varieties was *Trichoderma atroviride* followed by *Mucor racemosus* and *Fusarium oxysporum* (Figure 4-1), whilst *Aspergillus niger* was the least frequent isolate.

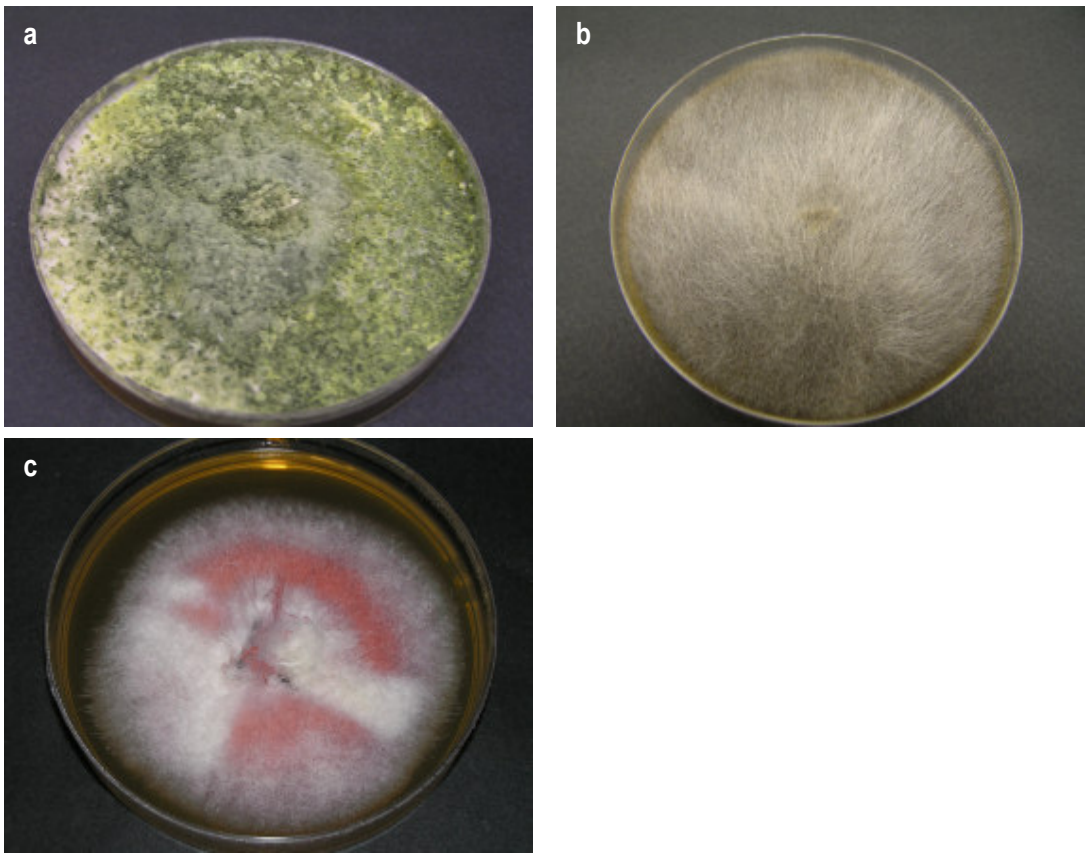

Figure 4-1. Morphological appearance of fungi isolated from Beaconsfield Reservoir on MEA. (a) *Trichoderma atroviride* (b) *Mucor racemosus* and (c) *Fusarium oxysporum*

Fruiting bodies (Figure 4-2) collected from dead tree stumps and rotting twigs were of the white-rot type and belonged to the Basidiomycetes.

Figure 4-2. Collected fruiting bodies of (a) *Trametes versicolor* (b) *Trametes* sp. (c and d) *Bjerkandera adusta* (e) *Pycnoporus cinnabarinus* (VIC) and (f) *Punctularia strigosozonata*

Table 4-1. Fungal strains investigated in this study

Fungal species	Type	Type of Rot	Source	Original Source	Optimal Growth Temperature (ºC)*
Aspergillus niger Tiegh.	Deuteromycete	Soft rot	Water	Beaconsfield Reservoir, Australia	30
Fusarium oxysporum Schltdl.	" "	" "	" "	" "	25
Mucor racemosus Bull.	Zygomycete	" "	" "	" "	25
Penicillium citrinum Thom.	Deuteromycete	" "	Enrichment culture	NOM concentrate	25
Phoma sp. Sacc.	Zygomycete	" "	" "	" "	25
Trichoderma atroviride P. Karst	Ascomycete	" "	Water	Beaconsfield Reservoir, Australia	25
Bjerkandera adusta (Willd.) P. Karst.	Basidiomycete	White-rot	Dead wood	Toorak, Victoria, Australia	30
Lopharia crassa (Lév.) Boidin 10644	" "	" "	Lab. strain	CSIRO Forest Products, Melbourne, Australia	30
Perenniporia tephropora (Mont.) Ryvarden 7904	" "	" "	" "	" "	37
Phanerochaete chrysosporium Burds. ATCC 24725	" "	" "	" "	The American Type Culture Collection, Rockville MD, USA	37
Polyporus sp. P. Micheli ex Adans. or Fr.	" "	" "	" "	Melbourne, Victoria, Australia (in-house strain)	30
Punctularia strigosozonata (Schwein.) P.H.B. Talbot	" "	" "	Dead wood	Dandenong Ranges, Victoria Australia	25
Pycnoporus cinnabarinus (Jacq.) Fr. VIC	" "	" "	" "	Warburton, Victoria, Australia	35
Pycnoporus cinnabarinus (Jacq.) Fr. SQ	" "	" "	Lab. strain	Coolum, Queensland, Australia (in-house strain)	37
Pycnoporus coccineus (Fr.) Bondartsev & Singer 1096	" "	" "	" "	CSIRO Forest Products, Melbourne, Australia	37
Pycnoporus coccineus (Fr.) Bondartsev & Singer 6004B	" "	" "	" "	Dept. of Natural Resources, Forestry Dept. Brisbane, Queensland, Australia	37
Pycnoporus sanguineus (L.) Murrill 2256	" "	" "	" "	" "	37
Trametes sp. Fr.	" "	" "	Dead wood	Dandenong Ranges, Victoria, Australia	30
Trametes versicolor (L.) Lloyd	" "	" "	" "	" "	30
Trametes versicolor (L.) Lloyd ATCC 7731	" "	" "	Lab. strain	" "	30
Trametes versicolor (L.) Lloyd CV 5691	" "	" "	" "	Dept. of Natural Resources, Forestry Dept. Brisbane, Queensland, Australia	30

*Optimum growth temperature was determined by growth on MEA and from optimum temperature data provided by Dr. Kirsten Schleiphake.

4.2 Identification of fungi

The fungi isolated from Beaconsfield Reservoir, enrichment culture and dead wood, and one laboratory strain were first identified on the basis of morphology using standard mycological texts. They were further identified by using molecular methods (Section 3.2) due to difficulties with their morphology, particularly with the WRF. The closest fungal matches of the GenBank database are shown in Table 4-2.

Table 4-2. Closest species matches in the GenBank database

Morphological Identification	Closest species from Blastn search*	GenBank Accession No.	Identity (%)	Base pairs
Fusarium sp.	*Fusarium oxysporum*	EU326216.1	99	547/551
Mucor sp.	*Mucor racemosus*	AY213662.1	98	616/626
Penicillium sp.	*Penicillium citrinum*	EU664458.1	99	548/552
Phoma sp.	-	AB303548.1	99	520/521
Trichoderma sp.	*Trichoderma atroviride*	EU280133	99	384/386
Bjerkandera adusta ‡	*Bjerkandera adusta* ‡	EF441742.1	100	376/376
Punctularia strigosozonata ‡	*Punctularia strigosozonata* ‡	-	-	-
Polyporus sp.	*Trametes ochracea*	AB158314.1	99	390/392
Pycnoporus cinnabarinus (VIC)	*Pycnoporus cinnabarinus*	AF363768.1	96	251/259
Trametes sp. ‡	*Trametes hirsuta* ‡	EU661882	99	360/363
T. versicolor ‡	*Trametes versicolor* ‡	EF524042.1	99	572/575

***** The expected (E) value for the closest Blast hit = 0.00
‡ The identity of *Punctularia strigosozonata*, *B. adusta* and *Trametes* sp. and *T. versicolor* was further confirmed by Tom May (Senior Mycologist) of the Royal Botanic Gardens, Melbourne, Victoria.

Sequences that shared over 99% identity were morphologically the same species, whilst sequences that shared 96-98% identity were mostly of the same genus. BLASTn searches were mostly consistent with the initial identification of fungi. Only one fungus, identified as *Phoma* sp., had no corresponding species match in the GenBank database. *P. strigosozonata* could not be identified using molecular techniques; this may have been the result of not obtaining a sufficiently pure DNA sample. The identities of *P. strigosozonata*, *B. adusta*, *Trametes* sp., and *T. versicolor* were further confirmed by Dr Tom May (Senior Mycologist) of the Royal Botanic Gardens, Melbourne, Victoria.

4.3 Degradation of NOM in solid medium

A preliminary screen based on the decolourisation of NOM-containing medium (Section 3.6.2) was used to differentiate fungi capable of degrading NOM. The plates were observed daily for fungal growth and clearing, with the colony diameter recorded daily. All fungi grew on the NOM agar, with most exhibiting sparse mycelial coverage over the 7-day incubation period. *P. strigosozonata* and *B. adusta* were the only WRF that displayed dense mycelial coverage (Table 4-3).

Mycelial mat diameter ranged from as little as 0.5 cm diameter for *A. niger* (no further growth was seen after day 1) to 8 cm for *F. oxysporum, T. atroviride* and *B. adusta* on the 7th day of incubation. Whilst variation in growth is expected between genera and species, variation also occurred within the same species, with *P. coccineus* 1096 growing to 3.7 cm and *P. coccineus* 6004B growing to 7.3 cm. Conversely the two *P. cinnabarinus* strains displayed comparable growth of 6.5 cm and 7.5 cm, as did the *T. versicolor* strains ATCC 7731 and CV 5691, which grew to 6.5 cm for the 7-day period.

The average mycelial extension on NOM agar ranged from as little as 0.3 ± 0.1 cm day^{-1} for *P. strigosozonata* to 1.1 ± 0.2 cm day^{-1} for *F. oxysporum*. The WRF had a lower average mycelial extension rate of 0.6 ± 0.2 cm day^{-1} than the other isolates, which averaged 0.7 ± 0.4 cm day^{-1} over the 7-day incubation period. A lower growth rate may allow sufficient time for nutrient exploitation, leading to the breakdown of the NOM structure and thus its decolourisation. A relatively high growth rate such as that exemplified by *F. oxysporum*, *M. racemosus* and *T. atroviride* of 1.0 cm day^{-1} is desirable in bioremediation processes as it can make the strains more competitive with native soil biota (Lamar *et al.*, 1990). Fungi that exhibited higher growth rates may either lack the capacity to break down the NOM molecule *in vitro*, or be starving due to limited nutrient availability and so maximising hyphal extension.

All fungi grew on plain agar, with the majority of strains exhibiting sparse mycelial coverage over the 7-day incubation period. Several fungi displayed mycelial clumping. All fungi exhibited higher extension rates on control plates of plain agar than on agar supplemented with NOM, except for *F. oxysporum*. More than half the isolates had an average mycelial extension rate of ≥1 cm day^{-1}. The WRF had an average growth rate of 1.1 ± 0.3 cm day^{-1} whilst the non-WRF averaged 0.7 ± 0.5 cm day^{-1} for the 7-day period. *P. chrysosporium* 24725 had the highest growth rate of 1.8 ± 0.7 cm day^{-1}, whereas *P. strigosozonata* had the lowest growth rate of 0.3 ± 0.1 cm day^{-1}. The higher growth rates of most fungal isolates on the plain agar compared with agar supplemented with NOM may indicate that the hyphal growth rate was stimulated by the limited nutrient availability and starvation. These fungi most likely sustained their growth by utilising the simple polysaccharides in the medium. Conversely the complex and heterogeneous nature of NOM probably contributed to the slower mycelial development of WRF, assisting them in the exploitation of a new carbon resource (Boddy, 1999).

Table 4-3. Characteristics of fungal growth on NOM agar

Fungal species	Growth characteristics	Average mycelial extension rate on NOM agar (cm day^{-1})	Average mycelial extension rate on plain agar (cm day^{-1})	NOM decolourisation coefficient (A)	Clarity of decolourisation zone
Aspergillus niger	+ (S)	0.1 ± 0.2	0.1 ± 0.2	0	-
Fusarium oxysporum	+	1.1 ± 0.2	1.0 ± 0.2	0	-
Mucor racemosus	+	1.0 ± 0.1	1.1 ± 0.1	0	-
Penicillium citrinum	+ (S)	0.4 ± 0.0	0.4 ± 0.0	0	-
Phoma sp.	+	0.5 ± 0.1	0.6 ± 0.0	0	-
Trichoderma atroviride	+ (S)	1.0 ± 0.3	1.3 ± 0.4	0	-
Bjerkandera adusta	+ + (Z)	0.8 ± 0.3	1.1 ± 0.3	1.21	1
Lopharia crassa 10644	+ (B) (C)	0.5 ± 0.1	0.9 ± 0.3	0.78	2-3
Perenniporia tephropora 7904	+ (Z)	0.7 ± 0.1	1.2 ± 0.2	1.03	2
Phanerochaete chrysosporium ATCC 24725	+ (Z)	0.4 ± 0.2	1.8 ± 0.7	0.28	-
Polyporus sp.	+ (Z)	0.7 ± 0.3	0.9 ± 0.3	1.09	1
Punctularia strigosozonata	++ (Z)	0.3 ± 0.1	0.3 ± 0.1	1.11	4
Pycnoporus cinnabarinus VIC	+ (S) (B) (M) (C)	0.6 ± 0.3	1.2 ± 0.2	0.96	1
Pycnoporus cinnabarinus SQ	+ (S) (B) (M) (C)	0.8 ± 0.2	1.3 ± 0.2	0.9	1
Pycnoporus coccineus 1096	+ (Z)	0.6 ± 0.1	1.2 ± 0.2	0.75	4
Pycnoporus coccineus 6004B	+ (S) (B) (M) (C)	0.8 ± 0.2	1.3 ± 0.3	0.78	2
Pycnoporus sanguineus 2256	+ (S) (B) (M) (C)	0.7 ± 0.2	1.2 ± 0.2	0.59	3
Trametes sp.	+ (Z)	0.7 ± 0.3	0.9 ± 0.3	1.15	1
Trametes versicolor	+ (M) (B) (C) (Z)	0.5 ± 0.1	0.9 ± 0.3	0.9	2
Trametes versicolor ATCC 7731	+ (M) (B) (C) (Z)	0.7 ± 0.2	1.0 ± 0.3	1	1
Trametes versicolor CV 5691	+ (Z)	0.7 ± 0.2	1.1 ± 0.3	0.96	1.5

Sparse mycelial growth (+), dense mycelial growth (++); Spore dispersal (S), alternating mycelial growth bands (B), mycelial clumping (M), alternating bands of clearing (C) and a single zone of clearing (Z); Clarity of NOM decolourisation: (1) very good, (2) good (3) moderate (4) minor.
A = coefficient of decolourisation, using *T. versicolor* ATCC 7731 as reference strain.
Average mycelial extension rate reported as mean ± standard deviation for two replicates.

Although all isolates grew on the NOM agar, only the WRF, in particular *B. adusta,* decolourised it. For most WRF, NOM decolourisation was evident after the first day of incubation and varied in its clarity, degree and pattern. Uninoculated controls showed no reduction in colour. Work undertaken by Hofrichter and Fritsche (1996) on the depolymerisation of low-rank coal by extracellular enzyme fungal systems also showed the basidiomycetes to be the only fungi capable of decolourising dark-brown HA agar.

The clarity of NOM decolourisation varied between the WRF. Seven organisms: *Trametes* sp., *T. versicolor* ATCC 7731, *T. versicolor* CV5691, *B. adusta, P. cinnabarinus* SQ, *P. cinnabarinus* VIC, and *Polyporus* sp. exhibited high clarity of NOM decolourisation, whilst *P. coccineus* 1096 and *P. strigosozonata* exhibited low clarity. Two distinct decolourisation patterns were observed for the WRF. Several fungi, including *P. cinnabarinus* SQ and *P. coccineus* 6004B, displayed alternating growth and decolourisation bands (Figure 4.3a), whilst others such as *Polyporus* sp. and *Trametes* sp. exhibited a single decolourised zone (Figure 4.3b). It was also observed that *T. versicolor* and *T. versicolor* ATCC 7731 displayed both decolourisation patterns.

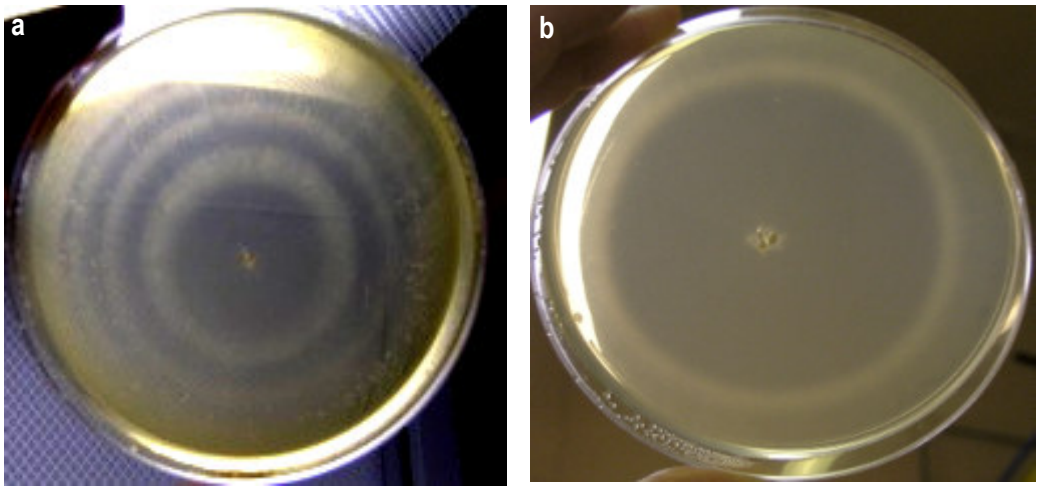

Figure 4-3. Decolourisation patterns on NOM medium by WRF. Alternating growth pattern for (a) *P. cinnabarinus* SQ and (b) a single decolourised zone for *Polyporus* sp.

The pattern of alternating cleared and growth bands as exemplified by *B. adusta*, *L. crassa* 10644, *P. cinnabarinus* VIC, *P. cinnabarinus* SQ, *P. coccineus* 6004B, *T. versicolor* ATCC 7731 and *Trametes* sp. may be associated with circadian rhythms which are driven by a central clock, and are likely to be linked with the metabolism and physiology of the organism (Watkinson *et al.*, 2005).

An indication of the NOM decolourisation activity of each species was determined by the decolourisation coefficient (A), which was calculated using *T. versicolor* ATCC 7731 as reference strain according to the formula of Machado (2005):

$$A = \frac{\left(\frac{H_t}{C_t} - \frac{H_{ref}}{C_{ref}}\right)}{H_{ref} - C_{ref}}$$

Equation 4.1

Where:

A	=	decolourisation coefficient
H	=	decolourisation disk diameter (cm)
C	=	growth disk diameter
t	=	test strain
ref	=	reference strain *Trametes versicolor* ATCC 7731

The test organism was classified as a good NOM degrader if A was greater than 1 and the clarity of decolourisation was very good (Table 4-3). Of the five WRF with A>1, *B. adusta* had the greatest value of 1.21, followed by *Trametes* sp. with 1.15 and *Polyporus* sp. with 1.09 (Table 4-3). These fungi also exhibited very good clarity of NOM decolourisation. *P. tephropora* 7904 had a large decolourisation coefficient of 1.03; however, the clarity of NOM decolourisation was only good. Although the decolourisation coefficients of *P. cinnabarinus* VIC, *P. cinnabarinus* SQ and *T. versicolor* CV 5691 were less than 1, they all gave very good clarity of NOM decolourisation. Both properties are highly desirable and thus the selection of a good NOM degrader could not be based merely on high clarity or a high decolourisation coefficient, but rather on the two properties which were combined to give the final rating in Table 4-3.

There was a close correlation between the NOM decolourisation coefficient and the growth rate (R^2=0.91) for strains expressing high NOM degradability. For example, *B. adusta* had the highest mycelial extension of 0.8 ± 0.3 cm day^{-1} and greatest decolourisation coefficient of 1.21, whereas *P. chrysosporium* ATCC 24725 had the second lowest mycelial extension of 0.4 ± 0.2 cm day^{-1} and the lowest decolourisation coefficient of 0.28. An exception was *P. strigosozonata,* which displayed a very low growth rate of 0.3 ± 0.1 cm day^{-1} but attained one of the highest decolourisation coefficients of 1.11 (Table 4-3). This may be associated with its characteristic dense mycelial coverage over the NOM medium (Figure 4.4b). Although there was no decolourisation zone apparent at the margin of growth as for the rest of the WRF, decolourisation was evident beneath the mycelium. A lower growth rate, as exemplified by *P. strigosozonata,* may allow sufficient time for nutrient exploitation, subsequently leading to the breakdown of the NOM structure and thus its decolourisation.

None of the WRF completely decolourised the entire NOM agar even if the mycelium reached the periphery of the plate. *B. adusta* came closest, with a decolourisation diameter of 7.5 cm. This suggests that the cessation of NOM decolourisation was probably due to nutrient depletion, or the depletion of easily available carbon from the NOM concentrate. For HA decomposition to take place, the presence of an easily assimilable carbon source is necessary. Very few fungi are capable of degrading HAs without nutrient supplementation and the need for an additional carbon and nitrogen resource is necessary to stimulate the degradation (Willmann and Fakoussa, 1997).

Because of their large size, lignin as well as coal HA cannot be taken up into the hyphae (Hofrichter and Fritsche, 1996), and thus the ability of the WRF to modify NOM by decolourisation was most likely associated with their extracellular system of non-specific lignolytic enzymes: LiP, MnP and Lac. This is further supported by the lack of visible adsorption of NOM onto the fungal hyphae, thus suggesting the release of extracellular enzymes into the medium and subsequent NOM decolourisation. Work by Hofrichter and Fritsche (1996) on the depolymerisation of low-rank coal, detected oxidase and peroxidase activities inside the bleached growth areas of various fungi on HA agar, thus indicating their role in the depolymerisation process. The activity of extracellular enzymes is dependent on the species in question and their secretion is triggered under conditions of limited nutrients (Mansur *et al.*, 2003), which may explain the varying capacity of WRF to decolourise NOM.

Fungi that grew, but did not decolourise NOM, were mostly isolated from the Beaconsfield Reservoir and the enrichment cultures. These micromycetous fungi (deuteromycetes, ascomycetes, zygomycetes) including *A. niger, F. oxysporum, M. racemosus, T. atroviride, Phoma* sp. and *P. citrinum*, lacked the capacity to break down the NOM molecule (Figure 4.4a) *in vitro* and may have utilised only the carbohydrate in the agar for growth, rather than the NOM. For the depolymerisation of low-rank coal by extracellular fungal enzyme systems, Hofrichter and Fritsche (1996) found that growth of micromycetes (e.g. strains of the genera *Trichoderma* sp., *Aspergillus* sp., *Penicillium* sp. and *Chaetomium* sp.) on HA agar produced large amounts of spores instead of hyphae, and no decolourisation. Similarly, in the present study *T. atroviride, Aspergillus* sp., and *P. citrinum* spored on the NOM agar with, however, only sparse mycelial growth (Figure 4.4a).

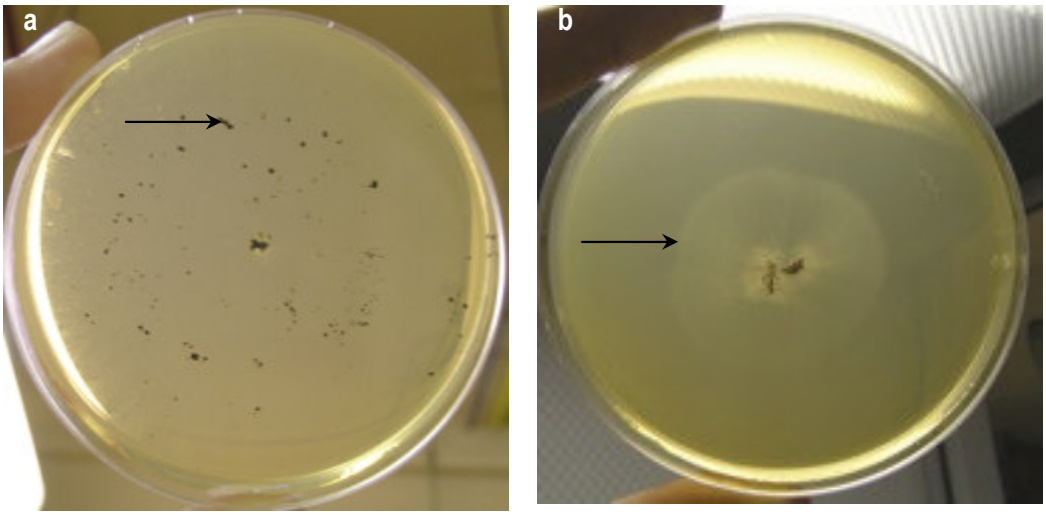

Figure 4-4. Growth on NOM agar. (a) Arrow indicates sporulation by *T. atroviride*, note absence of decolourisation, (b) Arrow indicates mycelial margin of *P. strigosozonata*

Related to these findings, *P. citrinum* (Polman *et al.*, 1994), *F. oxysporum* (Holker *et al.*, 1995), *T. atroviride* and *Phoma* sp. (Hofrichter *et al.*, 1997) were reported to solubilise (transform solid coal particles to dark brown coal droplets), although not depolymerise, coal. In most cases however, nitrogen-rich media and/or peroxidised or naturally oxidised coals were used, except for *Phoma* sp., which was screened on a nitrogen-reduced medium (Hofrichter *et al.*, 1997). The inability of these micromycetous fungi to decolourise NOM in the present study was probably associated with their lack of extracellular oxidative enzymes. Numerous studies have implicated these lignolytic enzymes in the depolymerisation of coal by several WRF (Hofrichter and Fritsche, 1996).

4.4 Inhibition/ tolerance tests on NOM agar

The structural complexity of NOM could have an inhibitory effect on microbial growth. To determine the relative tolerance of a range of the fungi to NOM, plug inocula (Section 3.6.3) of *F. oxysporum*, *M. racemosus*, *T. atroviride* and *T. versicolor* ATCC 7731 were grown on agar containing varying NOM concentrations (100-600 mg C L^{-1}) for 6 days (Figure 4.5).

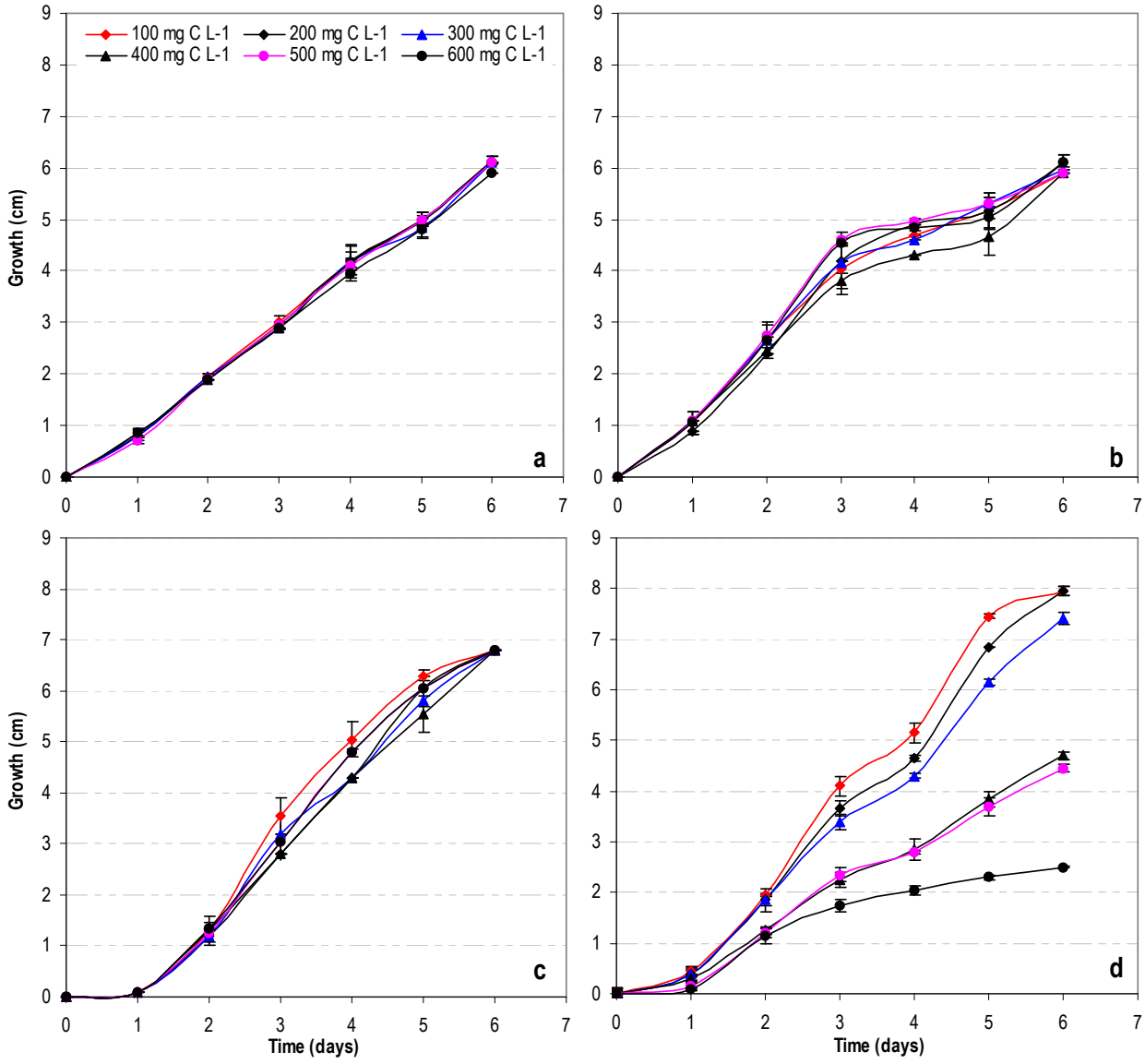

Figure 4-5. Mycelial growth of (a) *F. oxysporum* (b) *M. racemosus* (c) *T. atroviride* (d) and *T. versicolor* ATCC 7731 at various concentrations of NOM. Data points are means of two replicates ± 1SD.

There was little variation (1.0 ± 0.0, 1.2 ± 0.0 and 0.9 ± 0.0 cm day^{-1}, respectively) in the average mycelial extension of *F. oxysporum, M. racemosus* and *T. atroviride*, respectively, over the range of NOM concentrations. The low variation in mycelial extension rates of these three microfungi over the NOM concentrations tested suggests there was no inhibitory effect of NOM on their growth. These fungi grew sparsely on the NOM medium without decolourising it, and thus the primary nutrient source supporting their growth was probably the carbohydrates present in the agar.

However, increasing concentrations of NOM inhibited the growth rate of *T. versicolor* ATCC 7731. The highest growth rate of 1.2 ± 0.4 cm day^{-1} occurred at 100 mg C L^{-1} and decreased with increasing NOM concentration, with a sharp decline at greater than 300 mg C L^{-1} (Figure 4.5d). This is in agreement with the results of Meinelt *et al*. (2007) who reported that a range of humic substances reduced the growth of the water mould *Saprolegnia parasitica* in a concentration-dependent manner. The inhibitory effect of humic substances on the growth of *S. parasitica* was associated with the high aromaticity of the humic material, high C:H ratio and/or C:CH_2 ratio (Meinelt *et al.*, 2007).

When *T. versicolor* ATCC 7731 was subjected to a range of NOM concentrations, it displayed sparse mycelial growth with more scattered coverage at the lower NOM contents (Figure 4.6a). Although the growth rate of *T. versicolor* ATCC 7731 was greatly reduced at NOM concentrations greater than 300 mg C L^{-1}, the clarity and degree of NOM decolourisation remained unaltered.

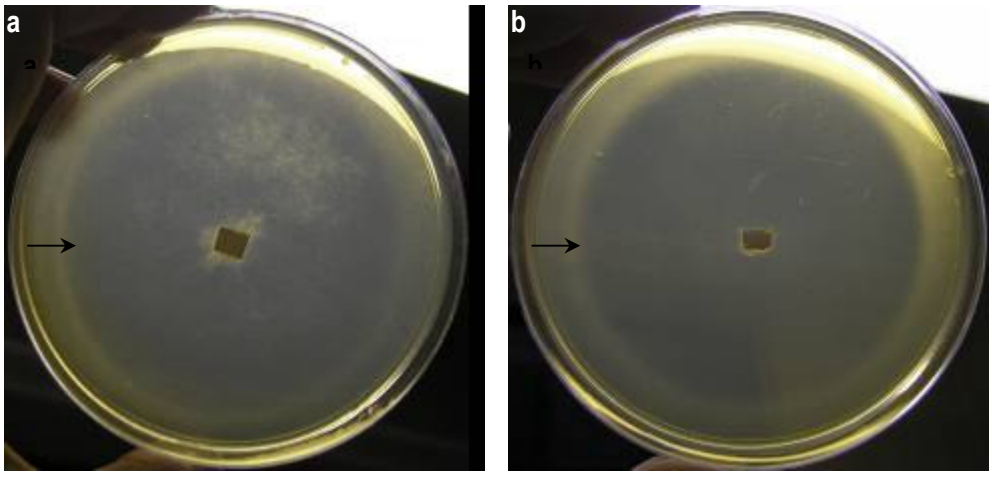

Figure 4-6. Morphological appearance of *T. versicolor* ATCC 7731 on 300 mg C L^{-1} (a) and 400 mg C L^{-1} (b) NOM-containing agar. Arrows indicate zone of decolourisation.

4.5 Growth studies in liquid NOM plates

To ascertain if NOM alone would sustain fungal growth, the biomass of a single plug inoculum of *T. versicolor* ATCC 7731, *F. oxysporum*, *T. atroviride* and *M. racemosus* was monitored in liquid NOM plates containing 200 mg C L^{-1} (Section 3.6.4). Figure 4.7 shows the data for biomass, pH, presence of conjugated and aromatic bonds (absorbance as 254 nm) (A_{254})) and decolourisation (colour measured as absorbance at 446 nm (A_{446})) over 8 days.

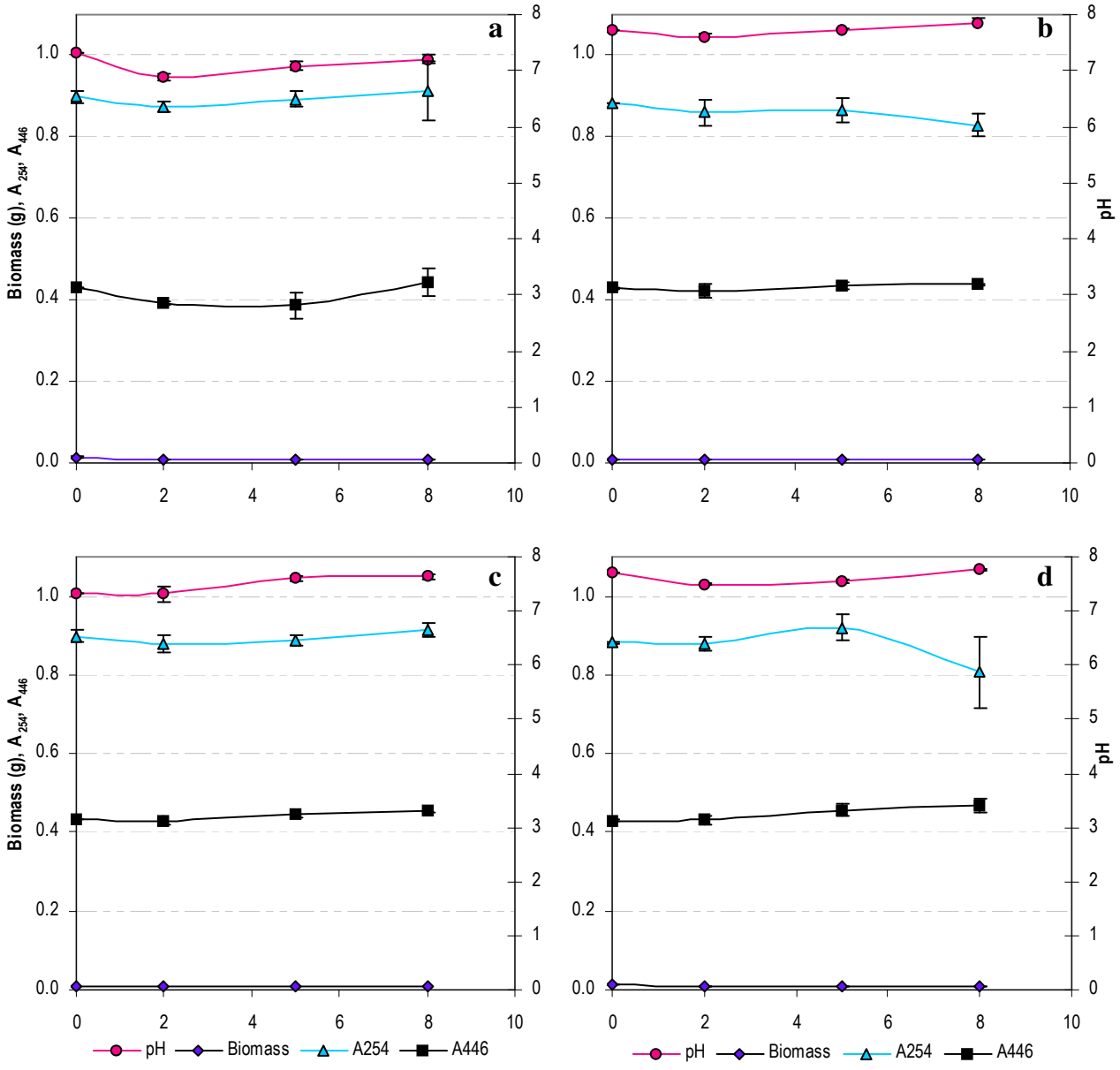

Figure 4-7. Growth in liquid NOM plates of (a) *T. versicolor* ATCC 7731 (b) *F. oxysporum* (c) *T. atroviride* and (d) *M. racemosus*. Data points are means of two replicates ± 1 SD.

Over the 8-day incubation period there was visible formation of a thin film of hyphae around the periphery of the plug inoculum for each fungus, as exemplified by *T. versicolor* (Figure 4.8).

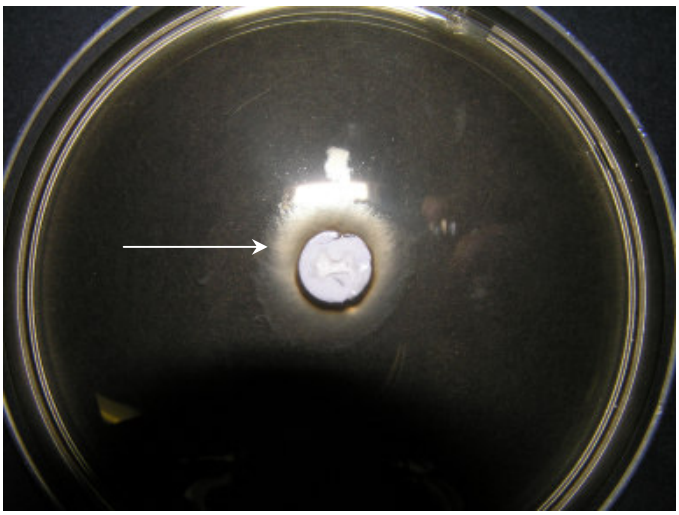

Figure 4-8. Thin film formation around the periphery of a *T. versicolor* plug. Arrow indicates film formation.

The presence of NOM in the plate cultures did not result in biomass production for any of the four fungi, but a very slight reduction instead (Figure 4.7). This suggests that the fungi were using the soluble carbohydrates present in the plug inoculum (containing MEA) to survive but not to grow. Similar results were also seen by Kluczek-Turpeinen (2007) for lignocellulose degradation and humus modification by *Paecilomyces inflatum*, where the presence of aromatic compounds led to reduced biomass formation. By contrast, work done by Dehorter and Blondeau (1992) on the production of extracellular enzymes during HA degradation showed stimulation of fungal growth for both *P. chrysosporium* and *T. versicolor* in the presence of HAs. The degradation of HA was, however, conducted in a basal medium containing nutrients, whereas in the current study there was no supplementation of the NOM and so results are not directly comparable.

Although there was no increase in the fungal biomass for the four fungi, several changes were observed for pH, A_{254} and A_{446} during the course of the experiment (Figure 4.7). The white rot *T. versicolor* ATCC 7731 was the only fungus that initially reduced the colour of NOM (as represented by the reduction in A_{446} by 9%), (Figure 4.7a), which later re-appeared. This decolourisation coincided with a drop in culture pH and reduction in A_{254}, i.e., breakage of aromatic and conjugated bonds. In comparison, *F. oxysporum*, *T. atroviride* and *M. racemosus* showed very small increases in A_{446}, and no significant changes in the culture pH or A_{254}. Control plates without NOM did not stimulate biomass formation; however, marginal increases in A_{254}, A_{446} and pH were apparent for the four fungi (results not shown) and were most likely due to cell lysis.

4.6 Cellulase activity

WRF are recognised for their ability to access and completely degrade all components of wood, of which cellulose makes up 40% and lignin 30% (Sjostrom, 1993). In wood, lignin is bound covalently to the side groups of different hemicelluloses by ester- or ether bonds and forms a matrix surrounding the cellulose microfibril (Nutt, 2006). As lignin oxidation provides no net energy, fungi have synergistically acting cellulases that exploit the energy and carbon available in cellulose (Knowles *et al.*, 1987). Due to the structural similarity of NOM to lignin, complete breakdown of NOM would necessitate both lignin and cellulose degradation, which can only be achieved by some strains of fungi. Lignolytic basidiomycetes, including *P. chrysosporium* and *T. versicolor* strains, give greater degradation of cellulose than lignin (Camarero *et al.*, 1999). A plate screening method (Section 3.6.1) based on the degradation of carboxymethylcellulose (CMC) was conducted to assess the cellulolytic ability of the fungal strains.

Table 4-4. Characteristics of fungal growth on CMC agar

Fungal species	Growth characteristics	Average mycelial extension rate on CMC agar (cm day^{-1})	Cellulase activity
Aspergillus niger	+ (S) (B)	0.5 ± 0.1	0.97
Fusarium oxysporum	+ (B)	0.8 ± 0.3	0.99
Mucor racemosus	+ (B)	1.3 ± 0.4	0.89
Penicillium citrinum	+ (S) (B)	0.6 ± 0.2	1.00
Phoma sp.	+ (B)	0.3 ± 0.1	1.29
Trichoderma atroviride	+ (S)	1.7 ± 0.3	0.40
Bjerkandera adusta	++ (B)	1.8 ± 0.2	0.99
Lopharia crassa 10644	+ (B)	0.9 ± 0.3	0.89
Perenniporia tephropora 7904	+ (B)	1.2 ± 0.2	0.98
Phanerochaete chrysosporium ATCC 24725	+ (B)	0.9 ± 0.3	0.89
Polyporus sp.	+ (B)	1.0 ± 0.3	0.99
Punctularia strigosozonata	++ (B)	0.3 ± 0.0	0.97
Pycnoporus cinnabarinus VIC	+ (S) (B)	1.0 ± 0.2	1.04
Pycnoporus cinnabarinus SQ	+ (S) (B)	1.0 ± 0.2	0.97
Pycnoporus coccineus 1096	+ (B)	0.8 ± 0.1	0.99
Pycnoporus coccineus 6004B	+ (B)	1.3 ± 0.1	0.98
Pycnoporus sanguineus 2256	+ (S) (B)	1.0 ± 0.2	0.88
Trametes sp.	+ (S) (B)	0.9 ± 0.3	0.85
Trametes versicolor	+ (B)	0.9 ± 0.2	0.91
Trametes versicolor ATCC 7731	+ (B)	1.2 ± 0.2	0.92
Trametes versicolor CV 5691	+ (B)	1.1 ± 0.3	0.83

Sparse mycelial growth (+), dense mycelial growth (++)
Spore dispersal (S), Blue halo formation (B)
Cellulase activity: ratio of cleared zone diameter to growth zone diameter.
Average mycelial extension rate reported as mean ± standard deviation for two replicates

All the fungi grew sparsely on the CMC medium and possessed cellulolytic ability (Table 4-4). The average mycelial extension rate on CMC agar ranged from as little as 0.30 ± 0.00 cm day^{-1} for *P. strigosozonata* to 1.8 ± 0.2 cm day^{-1} for *B. adusta* over the 5 day incubation period. Overall, the WRF had a greater growth rate than the micromycetous fungi.

The relative cellulase activity of each fungus was estimated from the diameter of the cleared zone (Figure 4.9) that resulted from CMC degradation. For most fungi, cellulase activity was strongly correlated (R^2=0.96) with the growth diameter of the fungus on the CMC agar, except for *T. atroviride* (Figure 4.9). Although *T. atroviride* did not display high cellulolytic activity in the present study, microfungi such as *T. reesei,* WRF such as *P. chrysosporium* and soft rot fungi have complete cellulolytic enzyme systems capable of breaking down crystalline cellulose to glucose (Nutt, 2006). The WRF are the only organisms known to degrade wood cellulose and lignin completely to CO_2 and H_2O (Kirk and Farrell, 1987).

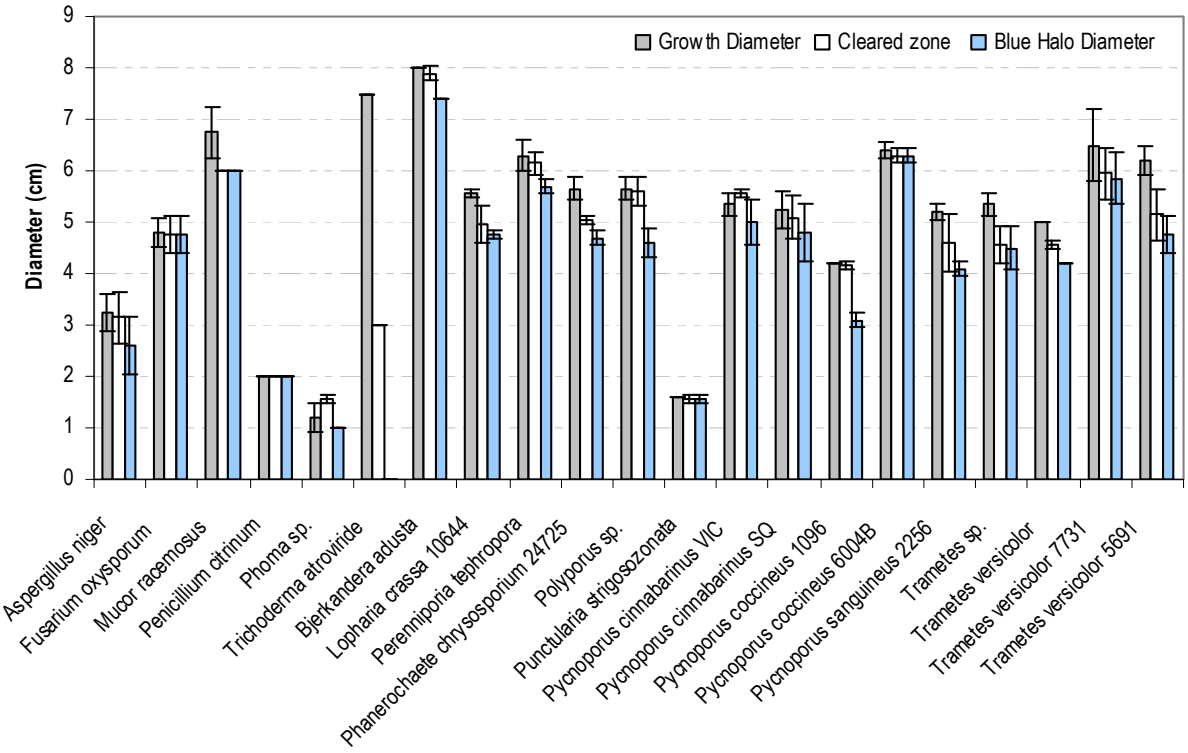

Figure 4-9. Cellulase activity on CMC agar (Day 5). Data points are means of two replicates ± 1 SD.

Most WRF expressed greater cellulase activity and a more clearly defined zone of hydrolysis than the micromycetous fungi, as exemplified in Figure 4.10. For most fungi the development of a blue halo was seen on staining the CMC agar (Figure 4.10b), indicating the presence of alkaline metabolic products. The region of cellulase activity exceeded the growth margin of the fungus for *Phoma* sp. and *P. cinnabarinus* VIC.

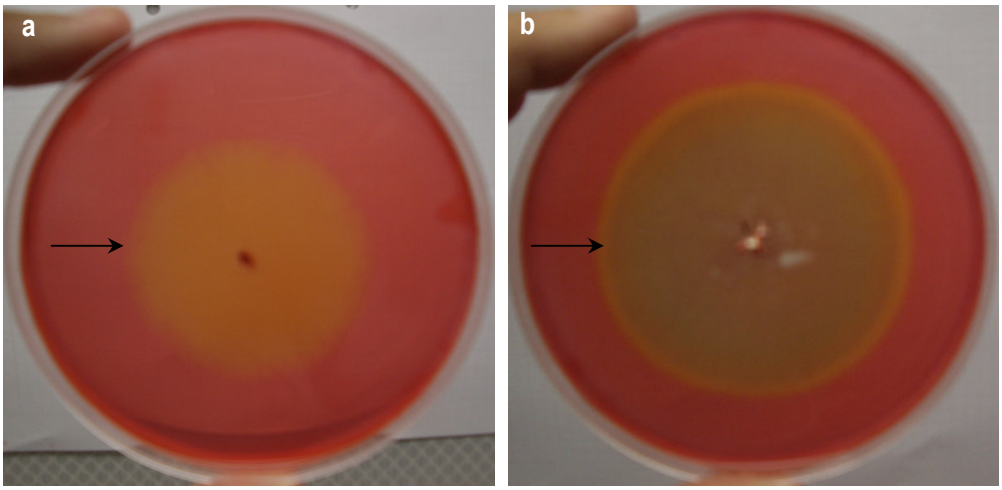

Figure 4-10. CMC-Agar plates inoculated with (a) *Fusarium* sp. (b) *Trametes* sp. after staining with Congo Red dye. Arrows indicate zone of CMC hydrolysis.

4.7 Conclusions for Chapter 4

Twenty-one isolates from diverse genera of fungi were obtained and screened for their ability to degrade concentrated aquatic NOM without the addition of nutrients. Potential NOM-degrading fungi were obtained from several sources, including the Dandenong Ranges, Beaconsfield Reservoir, lab strains, and from enrichment cultures of biologically active sand, using the NOM concentrate as substrate.

Two screening tests based on NOM and cellulase plate assays were utilised to indicate the degradation behaviour of the fungi. Of the 21 fungi investigated, the WRF, in particular *B. adusta,* were the only species capable of decolourising NOM in solid medium without the addition of further nutrients. The WRF had a lower average mycelial extension rate than the other isolates over the seven-day incubation period. Fungi that grew but did not decolourise NOM were mostly non-WRF and may have utilised the polysaccharides instead of the NOM in the agar for growth. NOM decolourisation varied in clarity, degree and pattern, with two distinct decolourisation patterns observed for the WRF. Several species, as exemplified by *P. cinnabarinus* SQ and *T. versicolor* displayed alternating growth and decolourisation bands, which may be associated with circadian rhythms. Others, such as *Trametes* sp. and *Polyporus* sp. exhibited a single decolourised zone. Over the range of NOM concentrations, micromycetous fungi displayed little variation in the average mycelial extension, whereas increasing concentrations of NOM inhibited the growth rate of *T. versicolor* ATCC 7731. The expression of greater cellulase activity, and the capacity to utilise NOM *in vitro* by the WRF, was most likely associated with the production of extracellular enzymes. Of the fungi screened, *T. versicolor* ATCC 7731, *Trametes* sp. *Polyporus* sp., *P. cinnabarinus* VIC and *B. adusta* displayed good degradation of NOM in plate assays and so were further investigated in shake–flask cultures.

Chapter 5 Degradation of MIEX^{TM} NOM concentrate in liquid culture by WRF

Preface

This chapter investigates the application of several WRF for the treatment of concentrated aquatic NOM in liquid culture. The treatment of NOM in distilled and tap water was investigated under limited nutrient conditions. WRF used in these studies were: *T. versicolor* ATCC 7731, *Trametes* sp. *Polyporus* sp., *P. cinnabarinus* VIC and *B. adusta*. These fungi were grown into pellet form and used in shake-flask cultures to promote enzyme production (Eggert *et al.*, 1996; Claus and Filip, 1998).

5.1 Selection of fungal inoculum

In order to use WRF effectively in the decolourisation of NOM, different types of inocula were trialled. Preliminary decolourisation studies involved the use of 1 cm^2 agar plugs excised from an active fungal colony of *T. versicolor* ATCC 7731 growing on a MEA plate (Figure 5.1a). The presence of glucose in the agar plug enhanced the decolourisation process by providing an easily available source of carbon. As the goal of the decolourisation work was to conduct the experiments without the addition of nutrients, the selection of an appropriate inoculum type was of great importance.

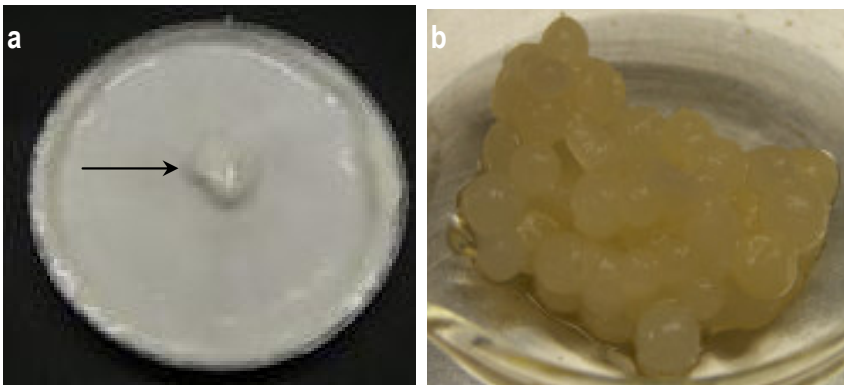

Figure 5-1. Single agar plug (a) (1 cm^2) of *T. versicolor* ATCC 7731 growing on MEA (b) cultivated pellets of *Trametes* sp.

The choice of fungal inoculum was narrowed down to using either mycelial suspensions or pellets. Spore suspensions were not selected for these studies, as fungal spores are metabolically dormant and may require stimulation with nutritional or environmental factors for active spore germination and vegetative growth to occur (Mitchell *et al.*, 1992). In addition, the concentration of spores in the inoculum has been shown to influence the morphology of fungi, with a high spore inoculum tending to produce a dispersed form of growth (Stanbury *et al.*, 1995), which could potentially (due to limited oxygen transfer) interfere with the decolourisation process. An advantage of using pellets in submerged culture is that this form tends to produce an essentially Newtonian system with much lower viscosity compared with dispersed filamentous growth, thus allowing improved oxygen transfer to take place within the culture medium (Stanbury *et al.*, 1995). In addition, the pellet form had the advantages that it was free from nutritional supplements, the morphological form remains unchanged, it was easy to use a consistent amount of inoculum, and the pellets retained their activity on storage in Milli-Q water at 4ºC for up to 3 weeks. Fungal pellets (Figure 5.1b) were therefore produced from spore suspensions of actively growing cultures of fungi on MEA plates (Section 3.4.2).

5.1.1 Morphology of fungal pellets

Pellet morphology may be influenced by the inoculum, medium, culture conditions and the species of fungus (Stanbury *et al.*, 1995). Pellets formed by *Polyporus* sp. (Figure 5.2a) and both the *Trametes* strains (Figure 5.1b, Figure 5.2b) illustrated tight wrapping of hyphae into compact spheres with defined edges. The pellets of *B. adusta*, however, were more loosely packed spheres with hyphae extending beyond the margin of the pellets (Figure 5.2c). The average pellet size differed between species, with *Trametes* spp. pellets being the largest (average of 5 mm diameter). The smallest pellets produced were from *P. cinnabarinus* (average 2-3 mm diameter), but these were difficult to produce as the fungus continuously spored in the shake-flask culture, leading to suspensions of combined hyphal fragments and pellets, thus making it difficult to isolate the pellets. This type of suspension was undesirable as the preferred type of inoculum comprised well-defined pellets. Nevertheless the pellets and hyphal fragments of *P. cinnabarinus* were used in the decolourisation experiments for comparison.

Figure 5-2. Pellets of (a) *Polyporus* sp. (~4 mm diameter), (b) *T. versicolor* ATCC 7731 (~ 5 mm diameter), (c) *B. adusta* (~3-4 mm diameter).

Although the pellet form tends to produce a low viscosity broth, which enhances oxygen transfer, large pellets may give rise to problems of oxygen availability. As the pellet habitat is dependent on simple oxygen diffusion, the core of a large compact pellet may be oxygen-deprived (Stanbury *et al.*, 1995).

5.2 Decolourisation studies of NOM in distilled water at 30ºC

A simple medium consisting of NOM (100 mg C L^{-1}) diluted in distilled water was chosen for the preliminary decolourisation studies to minimise the addition of nutrients which could influence the decolourisation of NOM. The rationale was to force the organism into a state of starvation, which should trigger the secretion of extracellular enzymes (Ruttimann-Johnson *et al.*, 1993). WRF used were: *T. versicolor* ATCC 7731, *Trametes* sp., *Polyporus* sp. and *P. cinnabarinus*. *B. adusta* was not used in this study as it was isolated during the later stages of this project.

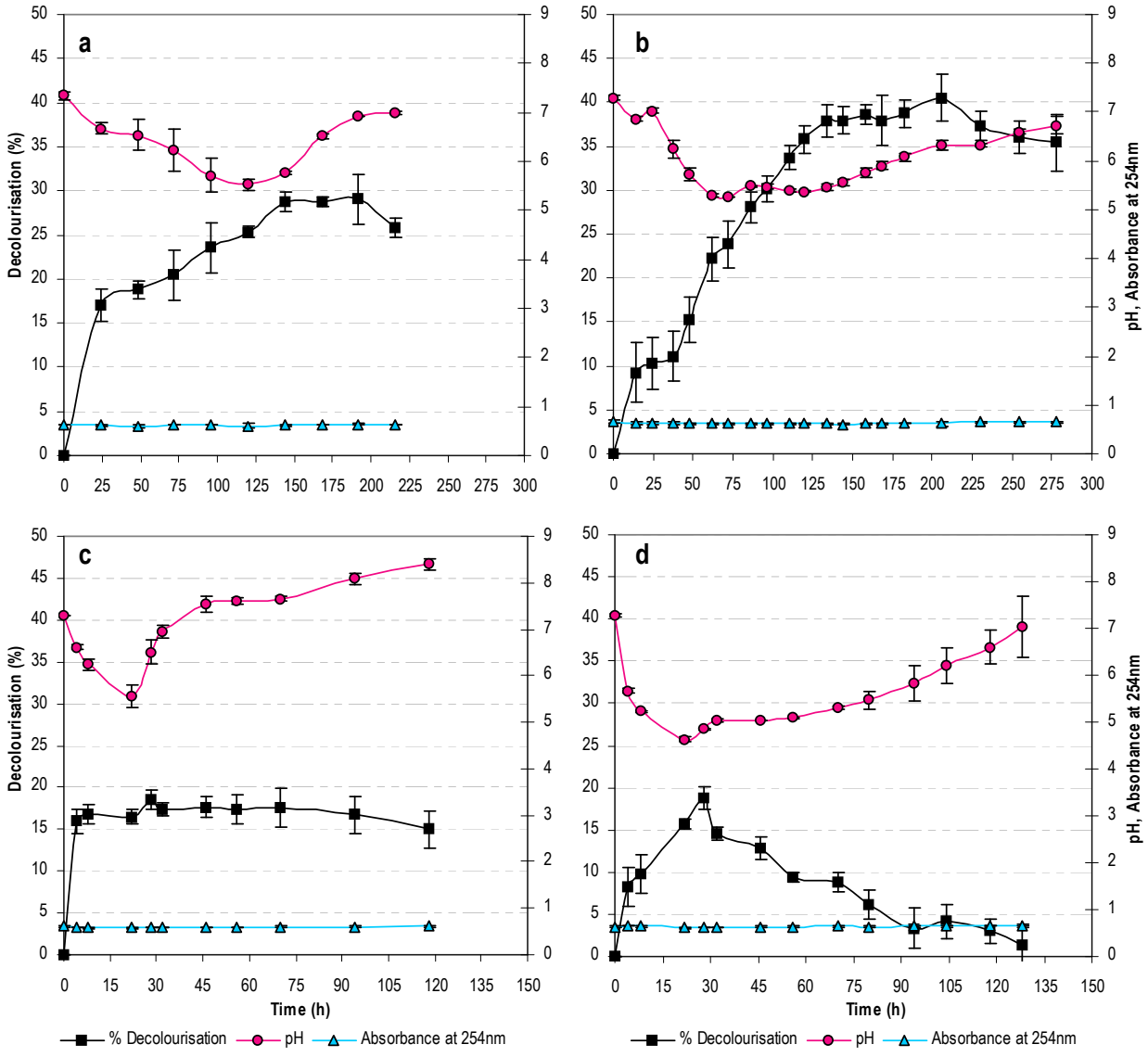

Figure 5-3. Decolourisation (in terms of reduction in A_{446}) of aquatic NOM concentrate by WRF using pellets as inoculum. (a) *T. versicolor* ATCC 7731, (b) *Trametes* sp., (c) *Polyporus* sp., (d) *P. cinnabarinus*. Data points are means of 4 replicates ± 1 SD.

All four WRF decolourised NOM initially as shown by the reduction in A_{446} (Figure 5.3). *Trametes* sp. attained the greatest reduction of 40%, followed by *T. versicolor* ATCC 7731 (29%) compared with 19% for *Polyporus* sp. and *P. cinnabarinus*. Decolourisation was reversed gradually after 30 h for *P. cinnabarinus* but not for the other fungi (Figure 5.3d).

Different decolourisation trends were observed for the four WRF (Figure 5.3). Maximal NOM decolourisation by *Polyporus* sp. and *P. cinnabarinus* (Figure 5.3c & d) was reached approximately four times faster than for both the *Trametes* species (Figure 5.3a & b). This high initial rate of colour removal by *Polyporus* sp. and *P. cinnabarinus* suggests that the mechanism for NOM decolourisation may initially be by adsorption to the fungal biomass. This was later confirmed by the slight discolouration of the pellets after the experiment. Fungi can remove colloidal particles of organic residues from water by adsorption to the hyphal surface and subsequently by incorporation into the hyphal network (Wainwright, 1992). By contrast, decolourisation of NOM by *Trametes* spp. occurred at a slower rate, which suggests a sustained metabolic activity. This was further confirmed with autoclaved controls in which only 2-3% of NOM was adsorbed onto dead biomass of *Trametes* spp., whilst for *Polyporus* sp. and *P. cinnabarinus* greater adsorption of 10-15% was obtained. Heat-treated pellets have modified surface binding sites from the denaturation of proteins on the cell wall structures (Bayramoglu *et al.*, 2009), which leads to increased surface area that facilitates more mass transfer.

Research by Willmann and Fakoussa (1997) suggested that the adsorption of HA molecules onto the fungal cell wall of the basidiomycete strain RBS 1K might be the first step in their biodegradation. A reasonably close correlation (R^2=0.75) was found between the rate of colour removal and the amount of NOM decolourised, the latter being greater at lower rates of NOM decolourisation. The lower rates of colour removal, as exemplified by the *Trametes* species, may allow for more exhaustive exploitation of nutrients and subsequent greater decolourisation of NOM. NOM decolourisation may be associated with metabolic activity, as illustrated by the drop in culture pH for the four WRF (Figure 5.3). Culture pH was found to be an important variable in the decolourisation process as it reduced the A_{446} by 5-10%. Consequently, corrections to NOM decolourisation have been applied where necessary to account for the contribution of colour removal by changes in pH (Section 3.10.5). The decolourisation of NOM probably ceased due to the depletion of essential nutrients and/or possibly by displacement from adsorption on cell walls by the change in pH.

The phenomenon of “negative decolourisation”, i.e., increase in A_{446} after initial reduction, was most marked for *P. cinnabarinus* (Figure 5.3d). This negative decolourisation, which occurred after 25 h incubation for *P. cinnabarinus*, coincided with a gradual increase in culture pH. Galhaup *et al.* (2002b) studied the production of Lac by *T. pubescens* in a medium containing glucose and peptone from meat, and similarly observed a sharp increase in culture pH shortly after glucose was exhausted from the medium. Solution pH

significantly affects the charge on both the carboxyl and phenolic functional groups of humic substances (Lin *et al.*, 2001) and the increase suggests the possible release of lytic products from fungal disintegration into the culture fluid, as suggested by Swamy and Ramsay (1999) for the decolourisation of Amaranth dye by *T. versicolor*. The breakdown of fungal pellets probably led to the release of adsorbed colour back into the culture solution, effectively contributing to negative decolourisation. An alternative explanation for this phenomenon of 'negative decolourisation' is that high pH leads to the dissociation of humic functional groups followed by their re-polymerisation (Stevenson, 1994). The possible re-polymerisation of dissociated humic constituents could have contributed to the subsequent increase in A_{446}. Cozzolino and Piccolo (2002) suggested that an observed increase in molecular size during a pH rise was due to a peroxidase-catalysed polymerisation, as dissociation of humic carboxyl groups disrupts the hydrophobic associations and humic constituents become available to react by a free radical mechanism.

There was little change in the UV absorbance (A_{254}) for the four WRF (Figure 5.3), which suggests that the UV-absorbing components of the NOM were neither broken nor greatly adsorbed by the fungal biomass. Alternatively, the degradation of UV-absorbing compounds could have been masked by the production or secretion of new UV-absorbing material.

5.2.1 Enzyme activities during NOM decolourisation in distilled water

Assays for phenoloxidases confirmed the presence of MnP, Lac and LiP in the fungal cultures. For all fungal strains, Lac predominated over MnP, whilst only *T. versicolor* ATCC 7731 and *P. cinnabarinus* showed a small amount of LiP activity under the culture conditions used (Figure 5.4).

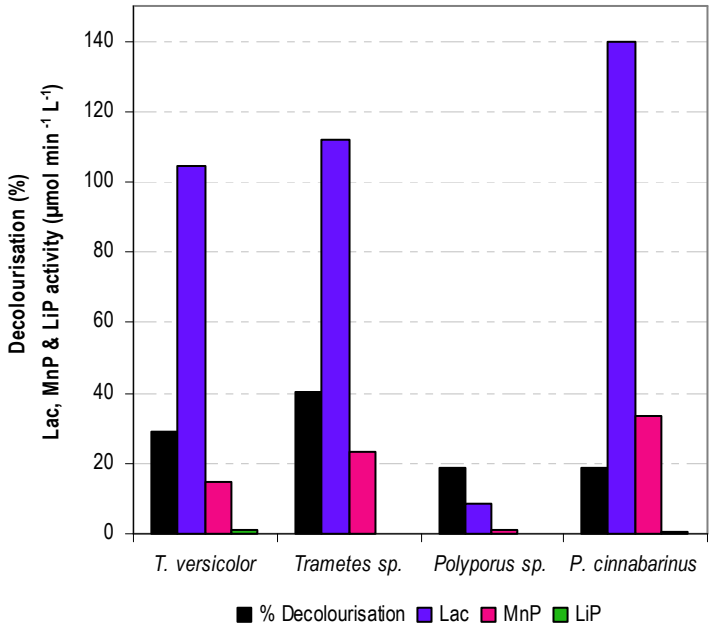

Figure 5-4. Maximum extracellular enzyme activities detected during NOM decolourisation by WRF at 30ºC.

As no LiP was detected for two of the fungal strains, different culture conditions may be required for its secretion. As noted previously, the secretion of extracellular enzymes occurs during secondary metabolism and under nutrient-limited conditions (Mansur *et al.*, 2003). NOM decolourisation occurred at different pH conditions for the different fungal strains, with the lowest pH occurring for *P. cinnabarinus,* which, although it demonstrated high MnP, the second highest Lac activity and the lowest LiP activity, gave one of the lowest reduction in A_{446}. This further indicates that the decolourisation of NOM by this fungus was partly by adsorption to the fungal biomass, as previously suggested by the brownish colouration of the pellets.

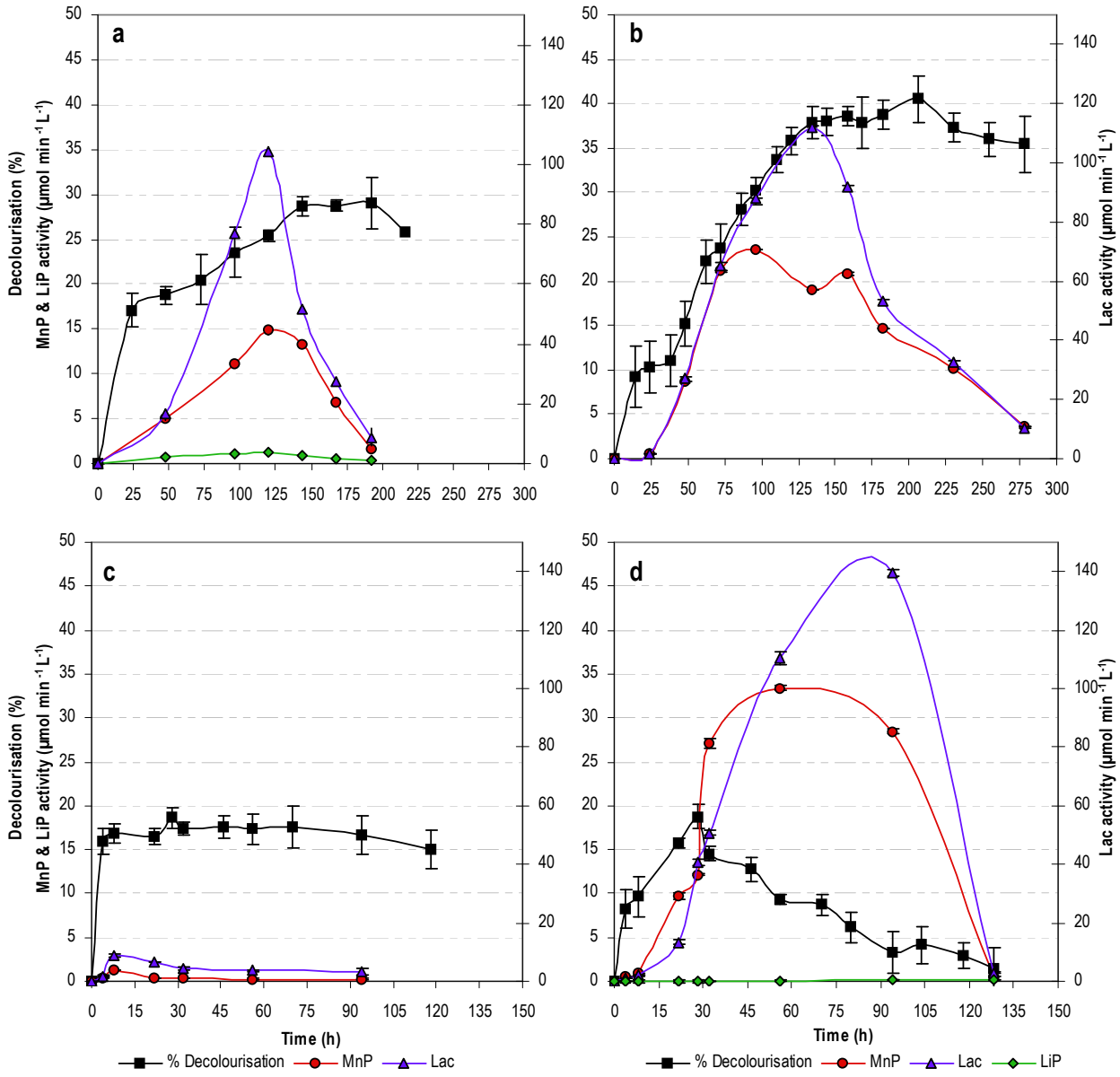

Figure 5-5. Enzyme activity profiles during NOM decolourisation at 30ºC by (a) *T. versicolor* ATCC 7731, (b) *Trametes* sp., (c) *Polyporus* sp., (d) *P. cinnabarinus.* Enzyme data points are means of 2 replicates ± 1 SD.

NOM decolourisation occurred simultaneously with increasing MnP and Lac activity for the two *Trametes* species (Figure 5.5), and enzyme activity patterns were similar. This finding agrees with that of Mansur *et al*. (2003) in that the activity of these extracellular oxidative enzymes is dependent on the species and strain of fungus. For the other two fungi, either there was little enzyme activity, suggesting adsorption (*Polyporus* sp.) or the enzyme activity was greatest after maximal decolourisation (*P. cinnabarinus*).

T. versicolor ATCC 7731 expressed all three enzymes, with MnP, LiP and in particular Lac, activity peaking at 120 h prior to maximal NOM decolourisation at 144 h, and shortly after the initial peak of NOM decolourisation (17%) at 25 h (Figure 5.5a). The activity levels of Lac and MnP were markedly greater than that of LiP, suggesting that these enzymes played a major role in the decolourisation process. The simultaneous secretion of Lac and MnP at 25 h, and increase in NOM decolourisation, was most pronounced for *Trametes* sp. (Figure 5.5b). Both enzymes emerged at 25 h during the initial peak of colour removal (10%) and peaked prior to maximal decolourisation at 206 h, with Lac peaking once at 134 h and MnP twice at 96 h and again at 158 h. Work by Stajic *et al*. (2004), on the screening of Lac, MnP and VP under submerged and solid state fermentation conditions, showed that differences in extracellular enzyme production exist among *Pleurotus* species, and even among strains of the same species.

Polyporus sp. exhibited the lowest enzyme activity levels of the four WRF (Figure 5.5c). Both MnP and Lac peaked within 8 h and during initial NOM decolourisation. The low enzyme activities for *Polyporus* sp. correlated well with the low degree of NOM decolourisation and further reaffirm the probable mechanism of NOM decolourisation to be almost all due to adsorption, with little due to enzymatic breakdown for this organism. In contrast, *P. cinnabarinus,* which gave the same degree of NOM decolourisation (19%), had the greatest MnP activity and the second greatest Lac activity of the four WRF. Both Lac and MnP were initially detected at 10 h, shortly after the initial stage of colour removal (10%), and peaked after maximal decolourisation, with MnP peaking prior to Lac and LiP (Figure 5.5d). The first peak of NOM decolourisation (10-17%) for *T. versicolor* ATCC 7731*, Trametes* sp., and *P. cinnabarinus,* prior to the onset of enzyme activities suggests that the initial mechanism of NOM decolourisation was largely due to adsorption. The subsequent rise in NOM decolourisation (>25 h), combined with the increase in enzyme activities, particularly for Lac and MnP, indicates that the later stages of colour removal by these three WRF were enzymatically driven. The high levels of MnP and Lac observed for *T. versicolor*, *Trametes* sp. and *P. cinnabarinus* are consistent with the findings of Golovleva *et al.* (1993), who reported that these enzymes worked synergistically in the degradation of lignin. The predominantly greater levels of Lac for the other three fungi, however, may be associated with the presence of aromatic compounds in NOM, which are said to induce Lac production (Maceiras *et al.*, 2001).

The onset of NOM decolourisation and increased MnP and Lac activities, coincided with the drop in culture pH (Figure 5.3) for the four WRF. For *T. versicolor*, *Trametes* sp. and *P. cinnabarinus*, a steep reduction in both enzyme activities (Figure 5.5) was seen shortly after maximal decolourisation; this may be attributed to the occurrence of secondary extracellular proteases on lysis of the cells (Moreira *et al.*, 1998) and subsequent increase in culture pH (Figure 5.3).

5.2.2 Molecular size distribution of NOM following decolourisation in distilled water

The molecular size distribution of the UV-absorbing components of NOM before and after fungal treatment was investigated. The decolourisation of NOM by the WRF was accompanied by a decrease in the average MW of the UV-absorbing species (Figure 5.6). All four WRF preferentially removed higher MW compounds (>1500 Da) and formed lower MW intermediates (<1500 Da), the latter being most marked for *Trametes* sp., which gave the greatest NOM decolourisation of 40% (Figure 5.3b).

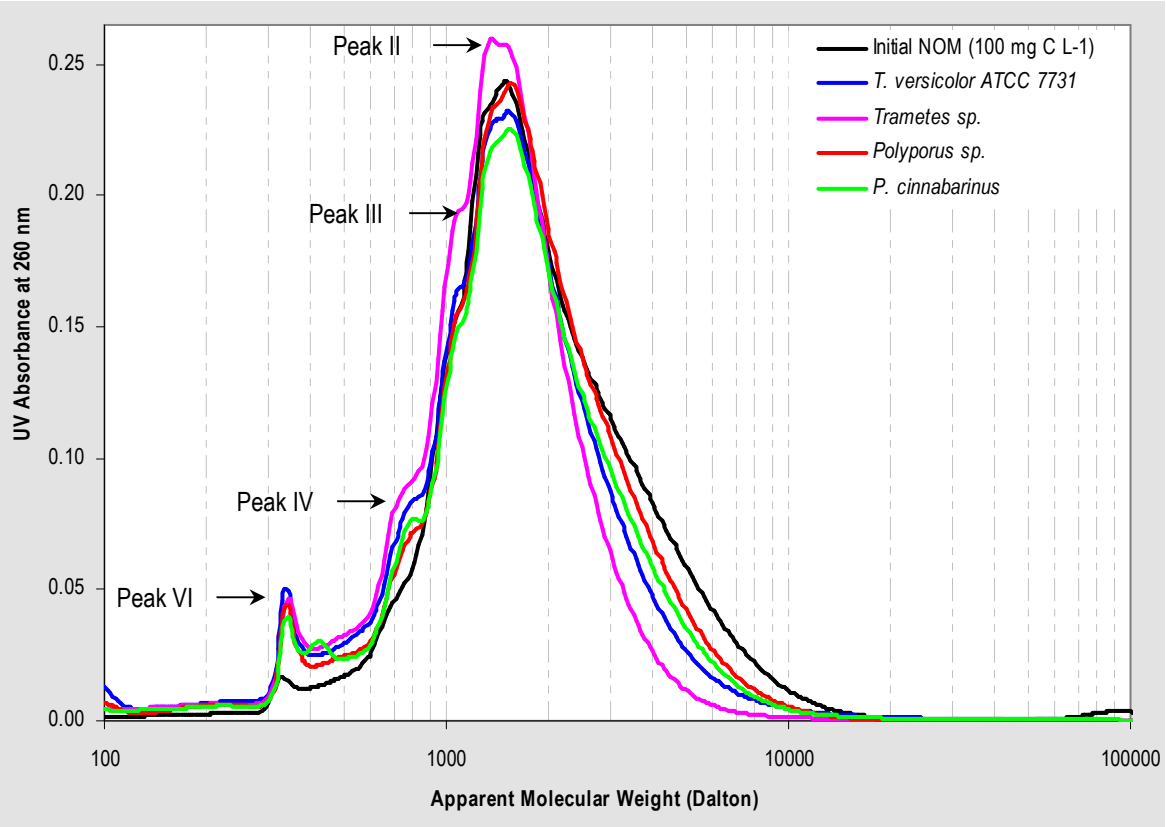

Figure 5-6. MW distribution of NOM in distilled water following treatment by WRF at 30ºC.

A peak-fitting approach based on the MW profile of DOM from surface water was developed by Chow *et al*. (2008) to characterise NOM from various sources. Six peaks were identified in the raw water NOM profile and are summarised in Table 5-1.

Table 5-1. Raw water peak fitting

Peak	NOM Peak	MW (Da)
I	Inorganic colloids and biological residues	50000
II	High MW humic substances (non-polar)	1900
III	Low MW humics 2	1200
IV	Low MW humics 1	800
V	Building blocks	500
VI	Low MW acids and N-containing aromatics	300

Following fungal treatment, four peaks (II, III, IV and VI) were identified in the MW distribution of the UV-absorbing components of NOM (Figure 5-6). The increased presence of lower MW compounds in the range of 350, 450, 700-800 and 1000-1500 Da for the four organisms indicates the likely formation of oxidation by-products such as organic acids (Swietlik *et al.*, 2004), potentially FAs (300 Da) and low MW humics (700-1200 Da)(Table 5-1) (Chow *et al.*, 2008; Huber, 2008), from the biodegradation of NOM. Fungal degradation of the predominant humic fraction (Peak II) of NOM may have liberated amino acids (Peak VI). A study by Thomson (2002), on the degradation of Hope Valley MIEX NOM by *P. chrysosporium*, similarly illustrated the preferential removal of higher MW chromophores with apparent increases in lower MW material.

To compare the variation in MW and the extent of polymerisation (p), the reduction in the proportion of the higher MW material (as represented by M_w) and lower MW material (as represented by M_n) was calculated after fungal treatment. The removal of higher MW compounds and formation of lower MW intermediates correlated well with the degree of NOM decolourisation by the four WRF (Figure 5.7).

Trametes sp. attained the greatest reduction (30%) in the proportion of the higher MW material and lower MW material, followed by *T. versicolor* ATCC 7731 (17%), *P. cinnabarinus* (13%), and *Polyporus* sp. with the lowest reduction of 10%. Although *P. cinnabarinus* had the same degree of NOM decolourisation (19%) as *Polyporus* sp., it led to a greater reduction in M_w and M_n (Figure 5.7), and this was most probably associated with the greater activities of Lac and MnP (Figure 5.5d). *Polyporus* sp. had the lowest reduction in M_w, which correlated well with the low activities of extracellular enzymes (Figure 5.5c), thus further supporting the mechanism of NOM removal to be mainly via adsorption for this organism. The reductions in the degree of polymerisation were consistent with the reductions in M_w and M_n for the four WRF.

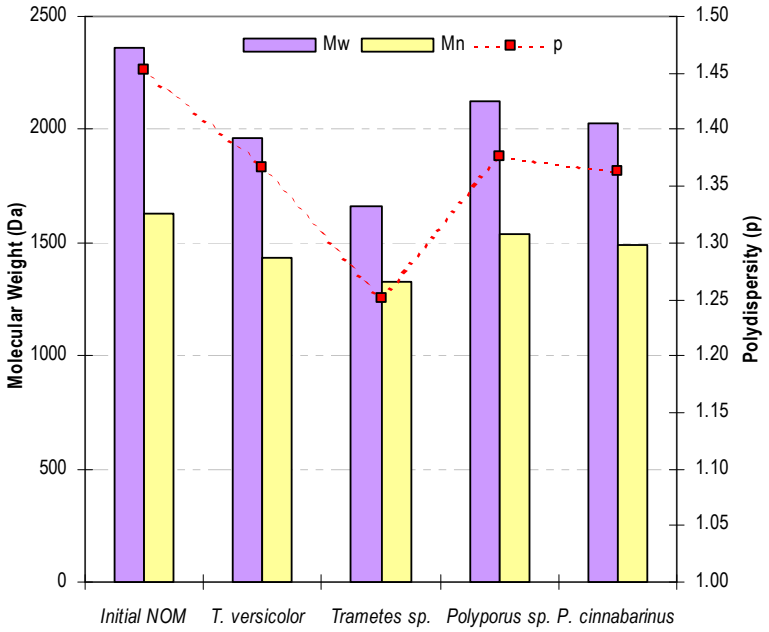

Figure 5-7. M_w, M_n and degree of polymerisation (ρ) of NOM in distilled water prior to and after treatment by WRF at 30ºC.

These findings demonstrate the ability of the extracellular enzymes to decolourise, depolymerise and perhaps even mineralise HS, as reported for other WRF such as *P. chrysosporium* and *T. versicolor* (Dehorter and Blondeau, 1992), *P. cinnabarinus* and *P. ciliatus* (Temp *et al.*, 1999). The enzymatic depolymerisation of the NOM molecule probably involved the cleavage of β-aryl ether (β-O-4) and β-1 bonds (Figure 5.8) and the formation of fulvic-like substances with lower molecular masses (Steffen *et al.*, 2002) that could ultimately be absorbed by the mycelium and metabolised.

These enzymes have been associated with the formation of unstable compounds which can then either undergo condensation and polymerisation (Zavarzina *et al.*, 2004) or even mineralisation (Steffen *et al.*, 2002). The lignolytic enzymes have also been found to contribute to the formation of humic substances as well as be involved in their cleavage and partial mineralisation (Steffen *et al.*, 2002). The degradation pathways taken by each enzyme would be dependent on the experimental conditions such as pH, temperature, humidity, oxygen, substrates and enzymes as well as the presence of other compounds such as mediators (Grinhut *et al.*, 2007).

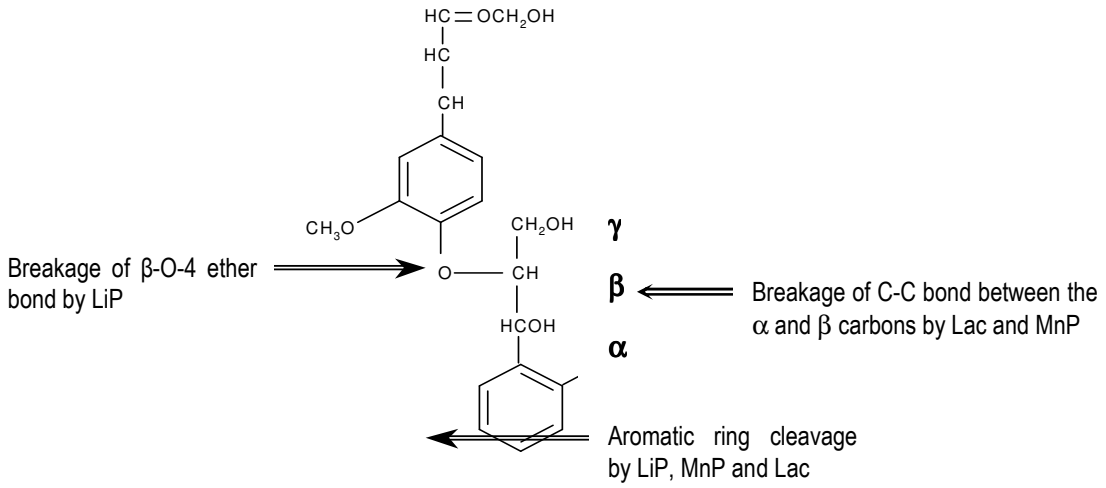

Figure 5-8. Proposed mechanism of NOM degradation by the extracellular enzyme system of WRF.

5.2.3 Decolourisation studies in distilled water at 15ºC

Preliminary studies on the decolourisation of NOM (100 mg C L^{-1}) were conducted at 30ºC, which was determined as the optimal growth temperature of the WRF. To assess their potential for the decolourisation of NOM in a reservoir where the temperature is much lower, decolourisation at 15ºC was investigated. Based on the different decolourisation trends and enzyme profiles observed at 30ºC, *Trametes* sp. and *P. cinnabarinus* were selected for this study.

Both *Trametes* sp. and *P. cinnabarinus* decolourised NOM at 15ºC, attaining colour reductions of 13% and 19%, respectively (Figure 5.9). The decolourisation of NOM attained by *Trametes* sp. was less than one third of that achieved at 30ºC (Figure 5.3b). Although maximal decolourisation occurred within 100 h, compared with 200 h at 30ºC, the rate of decolourisation halved at 15ºC. Negative decolourisation occurred shortly after maximal decolourisation for a further 170 h, whereas at 30ºC negative decolourisation was not as marked. There was no significant drop in culture pH for *Trametes* sp., and this combined with low decolourisation suggests that the mechanism of NOM decolourisation was predominantly via adsorption onto the fungal biomass. Zhou and Banks (1993) studied the mechanism of HA colour removal by fungal biomass and observed a greater uptake of HA by *Rhizopus arrhizus* at the low temperature of 16°C as opposed to 36°C. They implied that HA sorption by *R. arrhizus* was an exothermic process and the mechanism of removal was mainly via physical adsorption. By contrast, there was a decrease in culture pH for *P. cinnabarinus*, which indicates metabolic activity (Figure 5.9b).

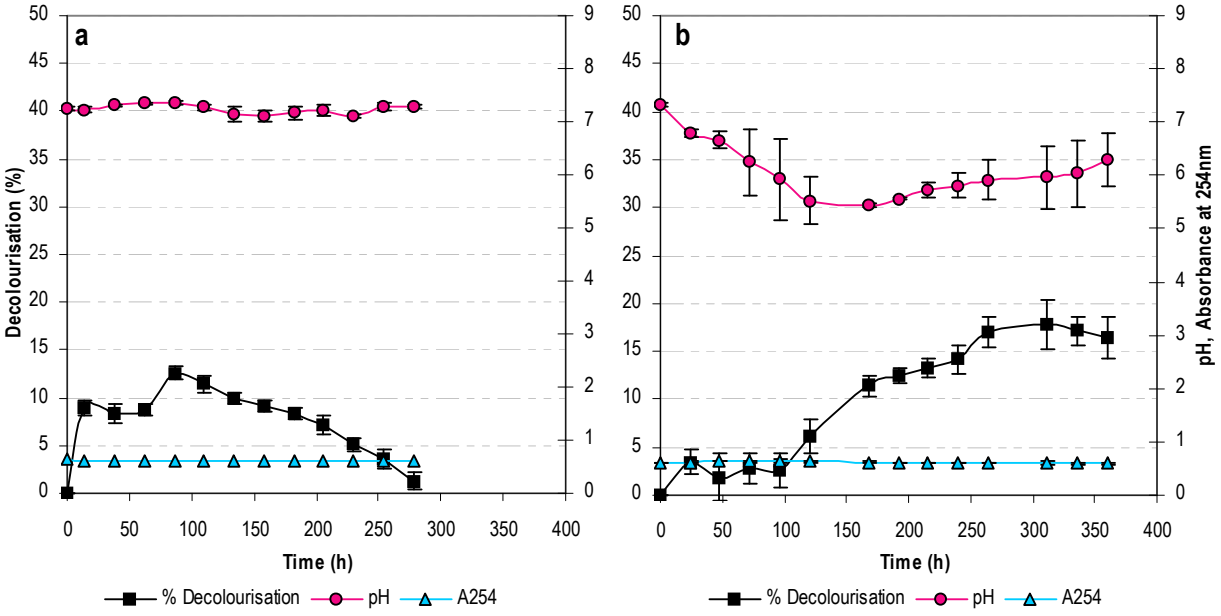

Figure 5-9. Decolourisation of NOM in distilled water at 15ºC by (a) *Trametes* sp., and (b) *P. cinnabarinus.* Data points are means of 4 replicates ± 1 SD.

Although the same degree of NOM decolourisation (19%) was achieved by *P. cinnabarinus* at both temperatures (Figure 5.9b), the rate of colour removal was significantly less at 15ºC. A lag phase was observed until 100 h, after which NOM decolourisation increased to the maximum value of 19% at 300 h (Figure 5.9b). The presence of a lag phase may indicate the need for adaptation to the colder conditions or enzyme induction. In contrast, NOM decolourisation at 30ºC peaked at 25 h, after which negative decolourisation occurred for the next 100 h (Figure 5.3d).

P. cinnabarinus pellets disintegrated more slowly at the lower temperature, which correlates well with the minimal negative decolourisation observed. At 30ºC, the pellets disintegrated shortly after 30 h, and consequently any colour adsorbed to the hyphal surface may have been released into the culture solution, thus resulting in a significant amount of negative decolourisation (Figure 5.3d). As for the experiments conducted at 30ºC, there was no change in A_{254} for both *Trametes* sp. and *P. cinnabarinus* at 15ºC (Figure 5.9).

5.2.4 Enzyme activities during NOM decolourisation at 15ºC

The combinations of phenoloxidase enzymes expressed by *Trametes* sp. and *P. cinnabarinus* at 15ºC were the same as at 30ºC, with Lac predominating (Figure 5.10).

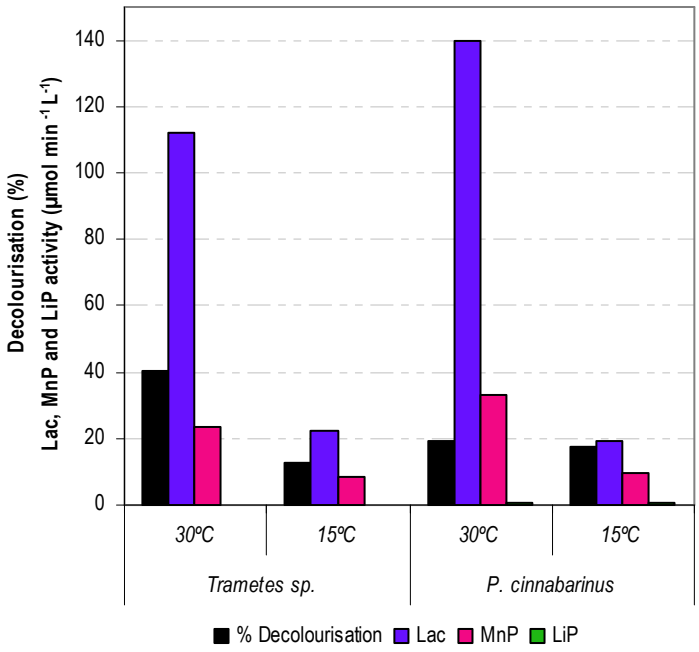

Figure 5-10. Maximum activities of extracellular enzymes during NOM decolourisation by *Trametes* sp. and *P. cinnabarinus* at 30ºC (from Figure 5.4) and 15ºC.

The maximum activities of Lac and MnP were markedly less at 15ºC for both species. The activity of MnP halved with the drop in temperature, whereas the activity of Lac was reduced to approximately 1/5th for *Trametes* sp., and 1/7th for *P. cinnabarinus*. The production of Lac is affected by factors such as medium composition, C:N ratio, pH and temperature (Maceiras *et al.*, 2001). The maximum activity of LiP for *P. cinnabarinus* was low at both the temperatures in comparison with Lac and MnP, and this suggests a negligible role in the decolourisation process. The reduction in enzyme activities was consistent with the lower decolourisation for *Trametes* sp., whereas the reduced enzyme activities for *P. cinnabarinus* did not relate to the amount of colour removed, as the latter remained the same at both temperatures.

The enzyme activity profiles for *Trametes* sp. at the lower temperature (Figure 5.11a) were somewhat similar to those obtained at 30ºC (Figure 5.5b and d). The initial peak of NOM decolourisation (~10%) at 14 h occurred prior to the onset of Lac and MnP activity (Figure 5.11a), as observed at 30ºC (Figure 5.5b). As most of the colour (10%) was removed prior to the secretion of enzymes, the mechanism of NOM removal by *Trametes* sp. at 15ºC was chiefly via adsorption. At the higher temperature, shortly after the initial peak of colour removal (>25 h), NOM decolourisation occurred simultaneously with increasing Lac and MnP activity (Figure 5.5b), indicating an enzymatically linked decolourisation process.

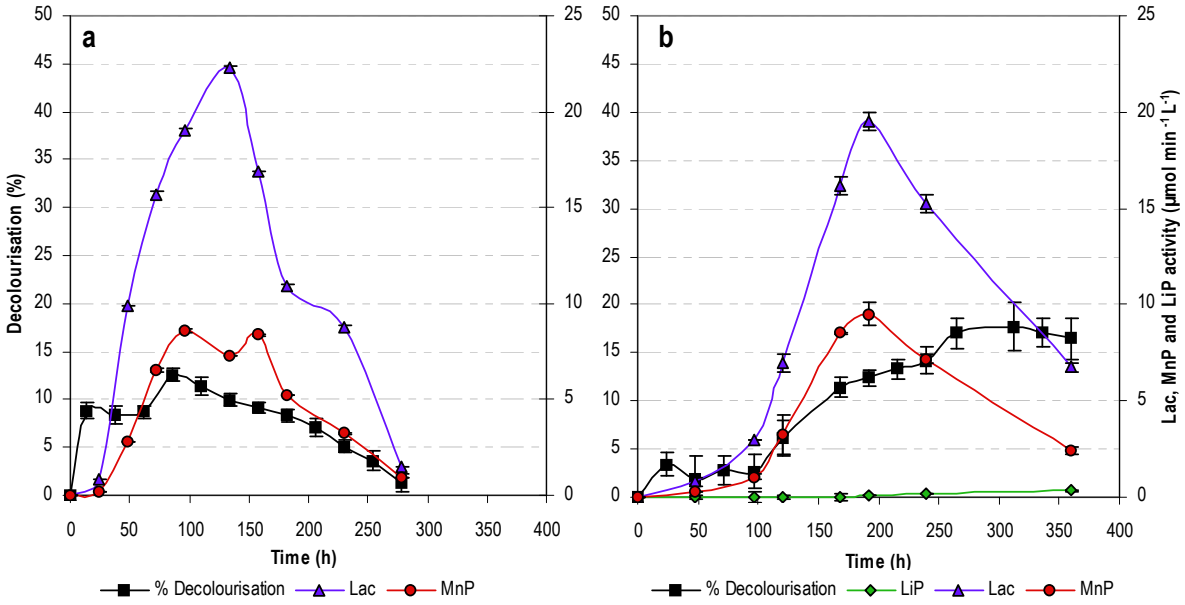

Figure 5-11. Enzyme activities during NOM (100 mg C L^{-1}) decolourisation at 15ºC by (a) *Trametes* sp., and (b) *P. cinnabarinus.*

The increase in NOM decolourisation at 15ºC by *P. cinnabarinus* coincided with the induction of Lac and MnP enzyme activity (>100 h), the activity of which increased with increasing NOM decolourisation up to 192 h (Figure 5.11b). Shortly after the peak of Lac and MnP activity at 192 h, LiP activity was detected and increased, coinciding with maximal NOM decolourisation at 300 h (Figure 5.11b). The simultaneous onset of Lac and MnP activity, together with increasing colour removal suggests enzymatic decolourisation up to 200 h. In contrast, the initial stage of NOM decolourisation (<10 h) at 30ºC was primarily due to adsorption, followed by enzymatic decolourisation (Figure 5.5d).

5.3 Decolourisation of NOM in tap water at 30ºC

Decolourisation studies were initially conducted in distilled water under strictly limited nutrient conditions. To further explore the decolourisation of NOM (100 mg C L^{-1}), but at the same time maintain low nutrient status, distilled water diluent was replaced with household tap water (Table 3-1). *T. versicolor* ATCC 7731, *Trametes* sp., *Polyporus* sp., and the newly isolated *B. adusta* strain were used in this study. As mentioned previously (Section 5.1.1) the production of *P. cinnabarinus* pellets was difficult to manage and only moderate decolourisation was obtained, and so this fungus was not selected for further study.

Two WRF gave greater NOM decolourisation in tap water (Figure 5.12a) than in distilled water (Figure 5.12b). *Polyporus* sp. had a two-fold increase and *T. versicolor* ATCC 7731 a 10% increase in NOM decolourisation. *Trametes* sp., on the other hand, had similar levels of colour removal for NOM in both

distilled and tap water. The newly isolated *B. adusta* strain attained the greatest reduction in A_{446} of 65% (Figure 5.12a) in tap water. The decolourisation of NOM in tap water by *T. versicolor* ATCC 7731 and *Trametes* sp. was twice as fast as in distilled water, with most of the colour being removed by the two species within the first 120 h of incubation. In contrast, *Polyporus* sp. decolourised NOM two and a half times faster in distilled water than it did in tap water; however this rapid rate of uptake was mainly attributed to adsorption, whereas for *T. versicolor* ATCC 7731 and *Trametes* sp., the mechanism of colour removal appeared to be a combination of initial uptake by adsorption followed by enzymatic action. NOM decolourisation by adsorption was further supported by the slight discoloration of the pellets for these organisms, and *B. adusta* and *Polyporus* sp., after the experiment.

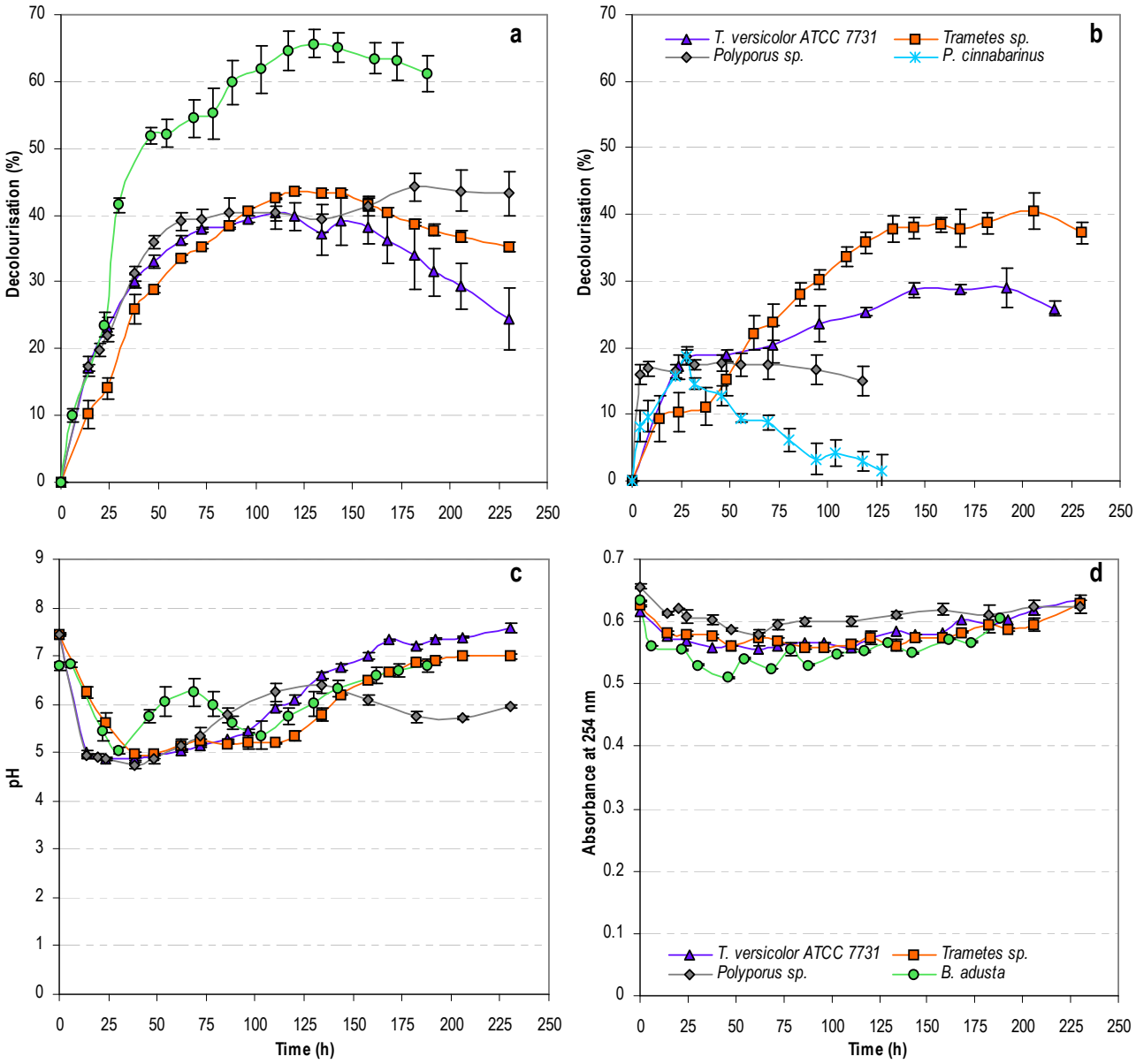

Figure 5-12. Decolourisation of NOM (100 mg C L^{-1}) in (a) tap water, (b) distilled water (included to aid comparison) by WRF at 30ºC. Profiles of (c) culture pH, (d) A_{254} during NOM decolourisation in tap water. Data points are means of 4 replicates ± 1 SD.

The autoclaved controls showed adsorption of NOM, with 15-16% of the colour being adsorbed by *Polyporu*s sp. and *B. adusta*, but only 4-6% by the *Trametes* strains. The adsorption of NOM in tap water was slightly greater for both the *Trametes* strains, but remained unchanged for *Polyporus* sp., even though the decolourisation of NOM increased two-fold for this organism. The variation in adsorption capacity between the four WRF may be due to several factors such as: culture pH, ionic strength of the medium, concentration of metal ions and temperature (Zhou and Banks, 1993).

An increase in A_{446} after the initial reduction was seen for the two *Trametes* strains shortly after 144 h incubation (Figure 5.12a), whereas in distilled water it was only slightly evident after 200 h (Figure 5.12b). The earlier onset of negative decolourisation by the two *Trametes* strains may be associated with the greater decolourisation rates (by 2-2.5 fold) in tap water. The subsequent depletion of essential nutrients probably led to the cessation of colour removal, followed by cell autolysis and the release of adsorbed colour back into the culture solution, effectively contributing to negative decolourisation. Negative decolourisation was not apparent for *B. adusta*, or for *Polyporus* sp. in both water types, although their decolourisation profiles in tap water fluctuated with culture pH (Figure 5.12c). As for distilled water, NOM decolourisation in tap water coincided with a drop of up to 2.5 units in culture pH, indicating that NOM removal is metabolically linked (Figure 5.12c). This drop in culture pH is in accordance with other studies, where the degradation of HAs resulted in the secretion of oxalic and other organic acids by various basidiomycetes (Takao, 1965).

The decolourisation of NOM in tap water by the four WRF correlated well (R^2=0.96) with the moderate drop in A_{254} (Figure 5.12d), the greatest being for *B. adusta* (20%) and the least for *T. versicolor* (10%). This decrease in A_{254} indicates that some UV-absorbing components of the NOM were cleaved and/or adsorbed by the fungal biomass, most notably by *Polyporus* sp. and *B. adusta.* This drop in A_{254} was not as great in distilled water, possibly owing to the lower levels of NOM decolourisation achieved by the WRF. The degradation of UV-absorbing compounds could have been masked by the production or secretion of new UV-absorbing material or cell lysis. The secretion of UV-absorbing material, possibly due to cell lysis, was further supported by the results for the controls of WRF in tap and distilled water without NOM. Significant increases in A_{254} and DOC were observed in tap and distilled water for the five fungi tested. Pellet disintegration was seen shortly after the first day of incubation, and this was attributed to the lack of nutrients provided by the NOM concentrate.

5.4 Elemental analysis of NOM in tap and distilled water

The difference in colour removal between the two water sources may be associated with the increased mineral content of the tap water. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis (Section 3.15) was used to determine the concentrations of trace elements present in both NOM solutions (Table 5-2).

Table 5-2. Elemental analyses of NOM (100 mg C L^{-1}) in tap and distilled water

Element	NOM in tap water (TWN) (mg C L^{-1})	NOM in distilled water (DWN) (mg C L^{-1})	Ratio of TWN:DWN
Na	280	172	1.6
Mg	30	2	15
Mn	0.15	0.054	3
Al	2.8	0.8	4
P	0.80	0.76	1
K	18.8	4.2	5
Ca	96	5.8	17
Fe	2.6	2.4	1.1
Ni	0.17	0.084	2
Cu	0.94	0.66	1.4
Zn	0.34	0.162	2.1

NOM-containing tap water contained greater concentrations of the divalent cations Mg^{2+} (15-fold higher), Mn^{2+} (3-fold higher) and Ca^{2+} (17-fold higher). A 4-fold and 5-fold increase in the Al and K^{+} concentrations was also observed. Both Mg^{2+} and Ca^{2+} have significant pH-buffering capacities and may have assisted in the decolourisation process and stabilisation of culture pH. Slightly greater concentrations of sodium, phosphorus, iron, nickel, copper and zinc were also present in the NOM-tap water preparation (Table 5-2) and could have aided the greater decolourisation by the WRF. Weinberg (1970) reported that the concentrations of manganese, iron and zinc are the most critical during secondary metabolism, which is when the secretion of extracellular enzymes occurs. Nutrient levels, mediator compounds and metal ions required by the enzymes (Mn^{2+} for MnP and Cu^{2+} for Lac) affect transcription of genes that code for multiple isoforms of the three LMEs (Wesenberg *et al.*, 2003). Galhaup *et al*. (2002a) showed that the addition of copper to the growth medium significantly increased Lac formation.

Magnesium, phosphorus, potassium, sulphur, calcium and chlorine are essential in media, as they are required by microorganisms for growth and metabolism. Other elements such as cobalt, copper, iron, manganese and zinc are also essential but are usually present as impurities in other major ingredients (Stanbury *et al.*, 1995). The greater concentration of mineral nutrients, in particular of Mg, Mn, Al, K, and Ca, present in the NOM-tap water preparation may have triggered a faster onset of biodegradation.

5.4.1 Enzyme activities during NOM decolourisation in tap water

Enzyme assays confirmed the presence of MnP and Lac in all WRF cultures, whilst LiP activity was found only for *T. versicolor* and *B. adusta* (Figure 5.13a). Lac predominated for both the *Trametes* strains and *Polyporus* sp., whereas the activity of MnP predominated for *B. adusta*, during NOM decolourisation. The absence of LiP from *Trametes* sp. and *Polyporus* sp. cultures suggests that different culture conditions could be required for its secretion.

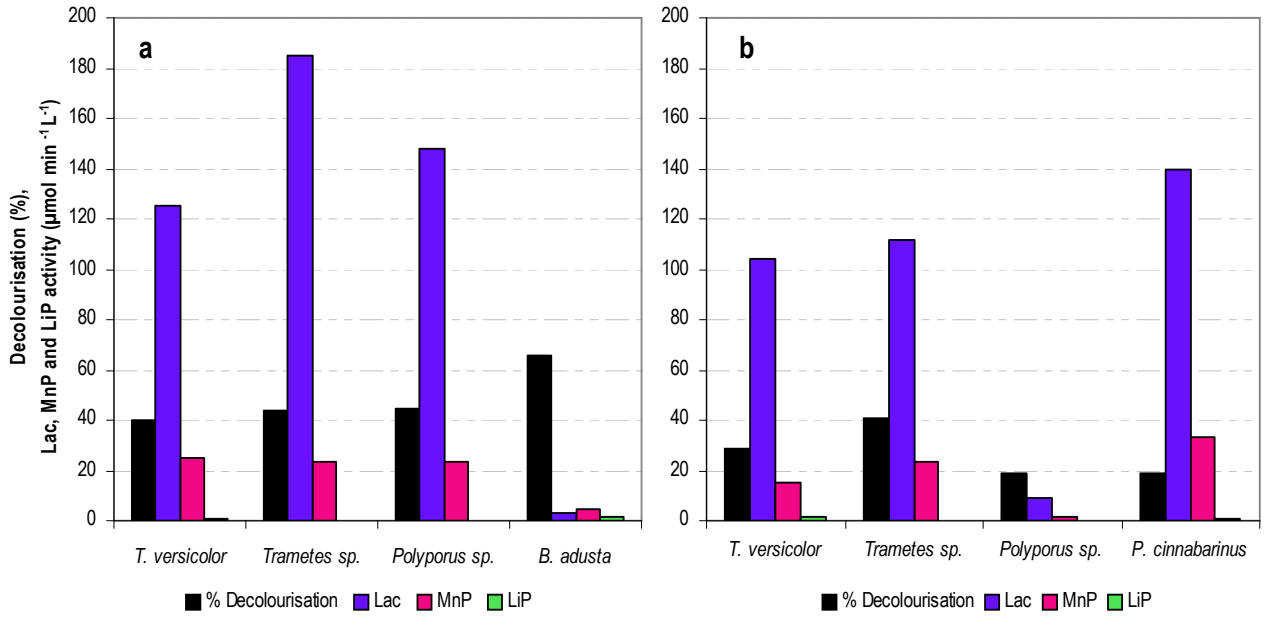

Figure 5-13. Maximum enzyme activities detected during NOM decolourisation by WRF at 30ºC in (a) tap water and (b) distilled water (from Figure 5.4).

The activity levels of Lac and MnP increased considerably (17- and 18-fold, respectively) for *Polyporus* sp. in tap water (Figure 5.13a) compared with distilled water (Figure 5.13b), while NOM decolourisation more than doubled. Greater enzyme activities, particularly for Lac, were also seen for *T. versicolor* (20%) and *Trametes* sp. (65%), whilst the increases for MnP were not so great. The greater enzyme activities for the three WRF may be due to the increased concentrations of some of the essential nutrients such iron and zinc present in the NOM-tap water solution, as the concentrations of these nutrients are most critical during secondary metabolism where the secretion of enzymes occurs after 2-3 days of the growth of the fungus (Pasti-Grigsby *et al.*, 1992). A study by Galhaup *et al.* (2002b) on the production of Lac by the wood-degrading basidiomycete *Trametes pubescens* showed Lac formation to be considerably stimulated by the addition of Cu(II).

The secretion of extracellular enzymes was not associated with the growth of WRF, as there was no increase in biomass in any of the fungi tested at the end of the experiment. Similar results were found with the growth studies conducted on NOM plates (Section 4.5), in which NOM did not trigger an increase in fungal biomass. In contrast to these results, Dehorter and Blondeau (1992) found that the presence of HAs (0.05%) in the culture medium always stimulated growth in *P. chrysosporium* and *T. versicolor*.

Maximal NOM decolourisation occurred at slightly different pH for the four organisms, although mostly at approximately pH 5.6. The highest pH (6.02) at maximal NOM decolourisation occurred for *B. adusta*, which gave the greatest reduction in A_{446} (65%). This decolourisation could be due to synergistic or cooperative action by the three phenoloxidase enzymes measured, but this seems unlikely, since *B. adusta* demonstrated markedly lower levels of MnP and Lac activity. Alternatively, the high decolourisation may be due to a ‘‘hybrid” manganese–lignin or versatile peroxidase (VP) (Davilla-Vazquez *et al.*, 2005), for which the enzyme assays were not optimized. In other WRF, VP oxidised Mn^{2+} to Mn^{3+} (as does MnP), degraded model dimer lignin and oxidised VA to veratraldehyde (as reported for LiP) (Ruiz-Duenas *et al.*, 2009).

Enzyme assays showed three different enzyme activity patterns, as exemplified by (1) the *Trametes* strains, (2) *Polyporus* sp., and by (3) *B. adusta* (Figure 5.14). For *T. versicolor* and *Trametes* sp., NOM decolourisation occurred in conjunction with increasing activity of Lac and MnP (Figure 5.14a and b). For *T. versicolor,* both Lac and MnP activity peaked simultaneously, just prior to maximal NOM decolourisation at 110 h. For *Trametes* sp., however, maximum decolourisation of NOM occurred at 120 h, shortly after the peak of MnP activity and during the peak of Lac activity. A steep reduction in both enzyme activities was seen shortly after maximal decolourisation, followed by an increase in culture pH (Figure 5.12c) for both *Trametes* spp. The reduction in enzyme activities may be related to several factors including: excess production of H_2O_2 by the fungi, decreased concentration of substrate, and increased protease activity which has been involved in the breakdown of ligninase-related protein (Wariishi *et al.*, 1988; Gold *et al.*, 1989; Dosoretz *et al.*, 1990; Maceiras *et al.*, 2001). Dosoretz *et al.* (1990), however, reported that the combined addition of glucose and veratryl alcohol to a growing culture of *P. chrysosporium*, repressed secondary proteolytic activity and stabilised MnP-peroxidase and ligninase activity.

The enzyme activity for *Polyporus* sp. fluctuated with NOM decolourisation (Figure 5.14c) and culture pH (Figure 5.12c). Lac activity peaked at close to maximum colour removal at 62 h, and again at 182 h during the time of maximal NOM decolourisation (44%). MnP activity, however, peaked prior to the initial peak of NOM decolourisation at 48 h (Figure 5.14c). The simultaneous secretion of Lac and MnP during the initial stages of NOM decolourisation by *T. versicolor*, *Trametes* sp. and *Polyporus* sp., suggests an enzymatically driven decolourisation process for the NOM-tap water preparation. By contrast, the initial decolourisation of the NOM-distilled water solution by the three WRF was primarily due to adsorption (Figure 5.5 and 5.12b).

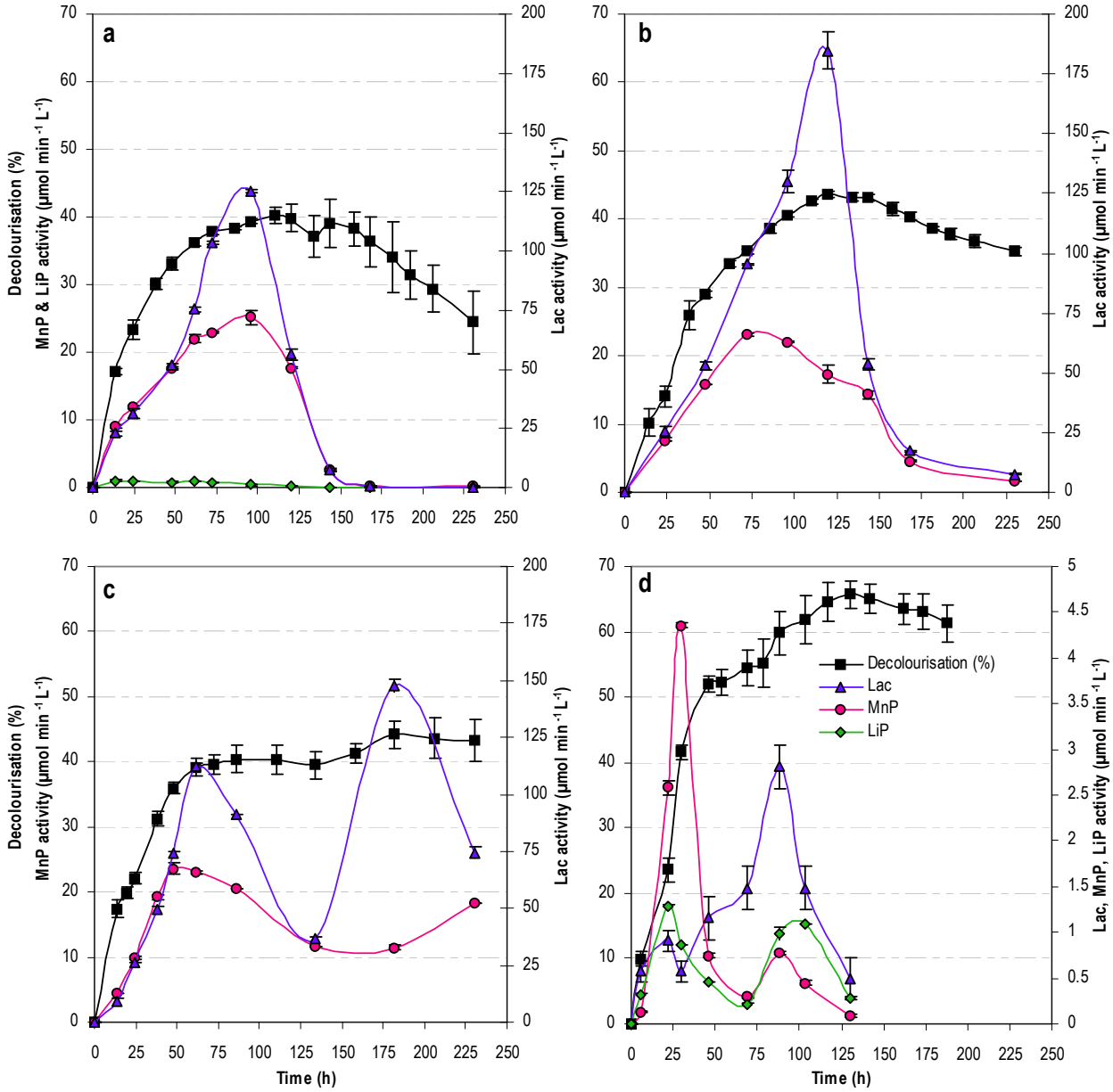

Figure 5-14. Enzyme activity profiles during NOM decolourisation in tap water at 30ºC by (a) *T. versicolor* ATCC 7731, (b) *Trametes* sp., (c) *Polyporus* sp., and (d) *B. adusta* (note: scales for Lac, MnP and LiP activities are markedly lower than for the rest of the WRF). Enzyme data points are means of 2 replicates ± 1 SD.

Although there was markedly lower MnP and Lac activity (by 82 and 98%, respectively) for *B. adusta* compared with the other three fungi, it gave the greatest decolourisation of NOM, and this may be because of the action of a VP that was not detected in the assays used. All three enzymes were active for *B. adusta*, and peaked prior to maximal NOM decolourisation at 130 h (Figure 5.14d). The initial drop in culture pH at 25 h (Figure 5.12c) coincided with the initial peak of Lac, MnP and LiP activity (Figure 5.14d). A subsequent drop in pH at 100 h coincided with the second peak of Lac, MnP and LiP activity. A steep reduction in all enzyme activities occurred prior to maximal colour removal, and was particularly evident for the peroxidases,

for which the activities fell to below 0.5 µmol min^{-1} L^{-1} within 75 h of incubation. This rather rapid inactivation of MnP and LiP compared with the LMEs of the other WRF (Figure 5.14a, b and c) could be due to the depletion of Ca^{2+} ions, as observed by Verdin *et al*. (2006) during their studies on the mechanism of VP inactivation by Ca^{2+} depletion in *B. adusta*. In addition, they observed a decrease in the redox potential and the formation of an active intermediate compound I. The decolourisation of NOM, coinciding with the early secretion of enzymes by *B. adusta*, suggests that the mechanism of NOM decolourisation may be a combination of enzymatic breakdown and adsorption. As, however, the activity of VP was not assayed in the cultures of *B. adusta*, it is difficult to elucidate the role of Lac, MnP and LiP in the decolourisation process, especially with such low activities.

In previous studies, *B. adusta* degraded daunomycin and HA (Balcarz *et al.*, 2005). Furthermore, it completely decolourised HA from brown coal and lessive soil in the presence of glucose whilst showing high Lac and lipase activity (Balcarz *et al.*, 2005). The present study is the first demonstration of the potential of *B. adusta* for the decolourisation of aquatic NOM concentrate under conditions of limiting nutrients. Furthermore, this is the first report of trends in extracellular enzyme activity, in particular of MnP, during NOM decolourisation by *B. adusta*.

5.4.2 Molecular size distribution of NOM following decolourisation in tap water

The AMW distribution of the UV-absorbing species for the NOM-tap water preparation was determined following treatment by WRF. A greater shift from higher MW (>2000 Da) to lower MW (<1000 Da) species was observed (Figure 5.15) compared with the NOM-distilled water preparation (Figure 5.6). The preferential removal of these higher MW fractions (Peak II) suggests a depolymerisation type mechanism based on radical reactions, where the extracellular enzymes targeted specific bonds (Figure 5.8) within the larger humic fractions, thus resulting in the concomitant formation of smaller MW intermediates that could be easily absorbed by the fungus. As for the NOM-distilled water system, the removal of these higher MW compounds was accompanied by the formation of lower MW material with AMWs of 300 (Peak VI), 360, 700 (Peak IV), 1000 (Peak III) and 1500 Da by the four WRF (Figure 5.15). The increase in Peak VI (300 Da) indicates the formation of small organic acids, amino acids or simple sugars derived from the biological process (Jarusutthirak and Amy, 2007) (Table 5-1). The lower MW acids could exist either as moieties within the humic substances or as sorbed components (Keil and Kirchman, 1993). Contrary to these results, Blondeau (1989) found no accumulation of lower MW species after the reduction of higher MW humic fractions by *P. chrysosporium*.

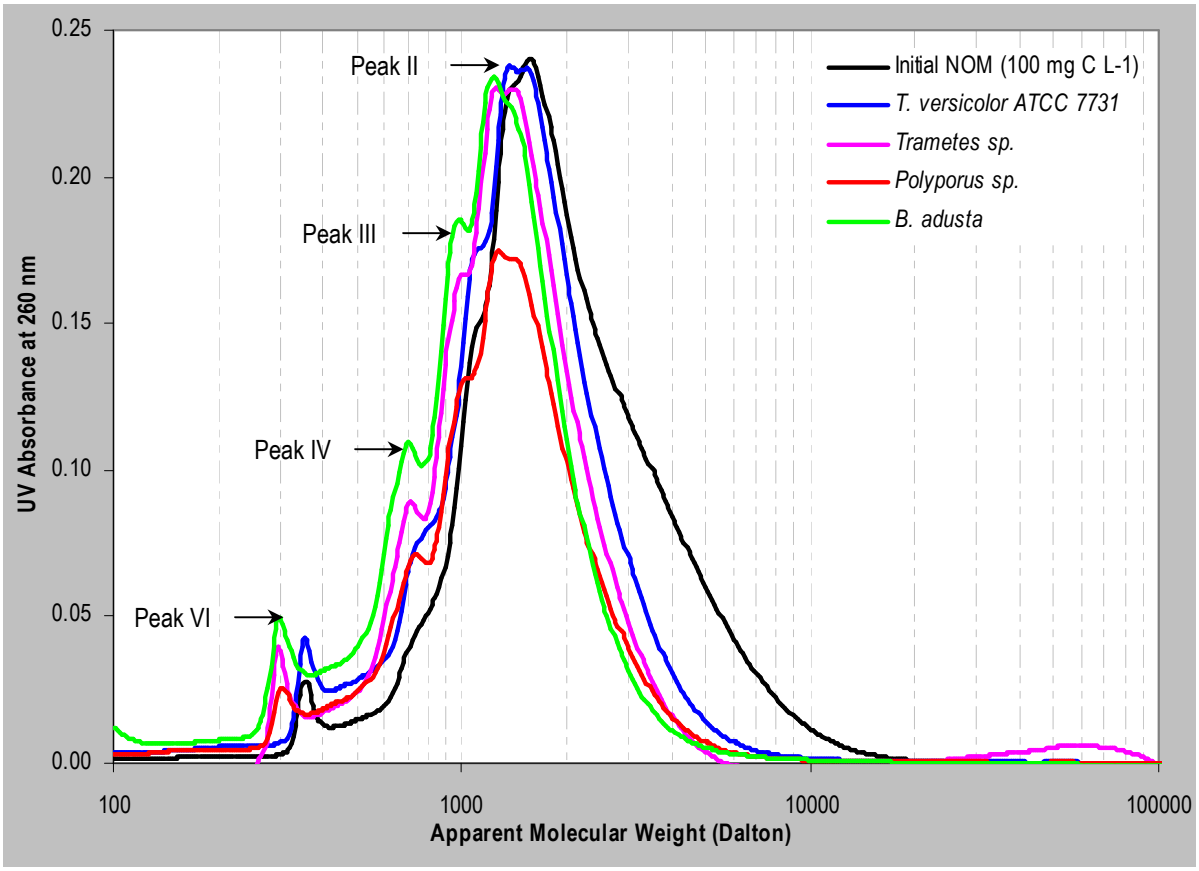

Figure 5-15. HPSE chromatograms for NOM-tap water preparation following treatment by WRF at 30ºC.

The proportions of the higher MW material (M_w) and lower MW material (M_n) were calculated for the four WRF following treatment of NOM-containing tap water (Figure 5.16a). Reductions in M_w and M_n for each fungus following treatment were determined by subtraction from the initial NOM values of 2389 Da and 1602 Da, respectively. Greater reductions in M_w and M_n were observed for *Trametes* sp. (33% and 37%), *T. versicolor* (44% and 45%) and *Polyporus* sp. (70% and 75%) in tap water (Figure 5.16a) than in distilled water (Figure 5.16b). These greater reductions in M_w and M_n correlate well with the increased decolourisation and corresponding enzyme activities observed for the three species in tap water. The greatest reduction in M_w (44%) and M_n (39%) was obtained for *B. adusta*, which also had the greatest formation of lower MW intermediates and greatest removal of colour (65%). Similar findings were reported by Moreira *et al*. (2007) on lignin transformation by a versatile peroxidase from *Bjerkandera* sp. strain. They observed a strong reduction of the high- and intermediate-molecular mass fractions of the soluble lignin fraction and an increase of the lower molecular mass fraction of lignin in solution. They suggested that the degradation products (i.e., lower molecular mass fractions) were probably produced by an enzymatically-catalysed oxidation. The large reduction in M_w by *B. adusta* and *Trametes* sp. indicates that these fungi target and break down the higher MW components of the NOM sample, whilst *Polyporus* sp. appears to

reduce UV-absorbance across a wider range of molecular sizes of NOM; hence the average MW does not change significantly.

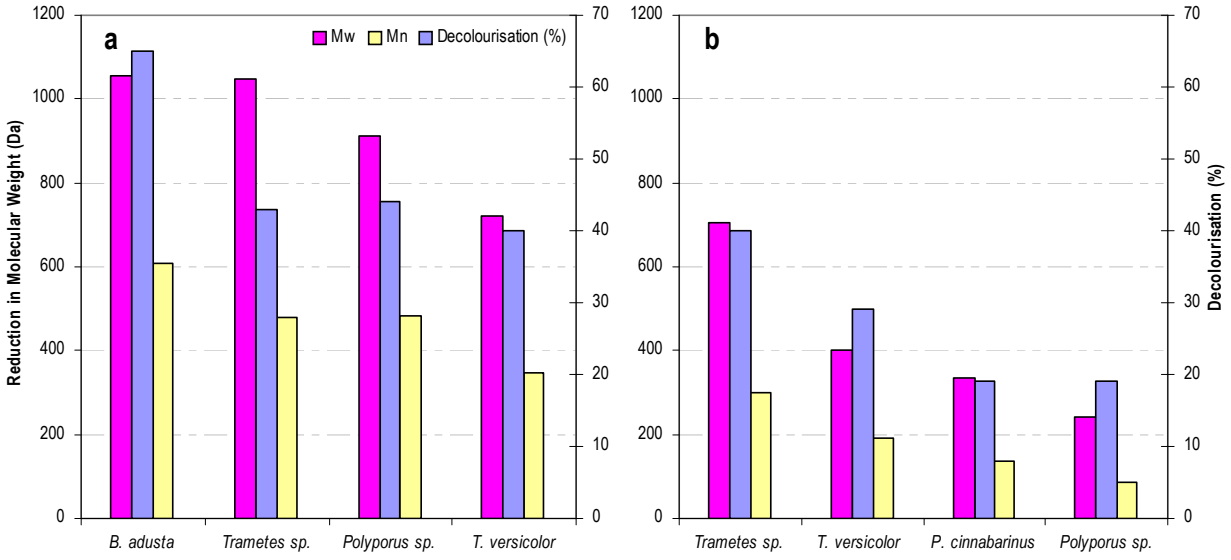

Figure 5-16. Reduction in M_w and M_n after treatment of (a) NOM- tap water and (b) NOM-distilled water preparation by WRF (redrawn from Figure 5.7).

The formation of lower MW intermediates did not directly correlate with the degree of NOM decolourisation achieved by the WRF. This is illustrated by *Polyporus* sp., which had the second greatest decolourisation of NOM (44%), but the least formation of lower MW intermediates and the third least reduction in M_w (Figure 5.16a). *Polyporus* sp. did, however, show a decrease in the UV-absorbing species at 1500 Da. This suggests that the mechanism for NOM decolourisation by *Polyporus* sp. was partly adsorption to the fungal biomass and enzymatic conversion to non UV-absorbing and/or smaller UV-absorbing molecules, some of which may have been metabolised by the fungus.

5.5 Fractionation of raw NOM concentrate

To further characterise the changes in NOM after fungal treatment, raw and treated NOM samples were fractionated (Section 3.13) according to the method of Chow *et al.* (2004) and analysed for DOC, A_{254}, A_{446} and MW distribution. Rapid resin fractionation of the raw NOM concentrate in tap water (10 mg C L^{-1}) showed that the very hydrophobic acid (VHA), slightly hydrophobic acid (SHA) and the hydrophilic charged and neutral fractions (CHA + NEU) accounted for 69%, 16%, and 15% of the NOM preparation, respectively (Figure 5.17).

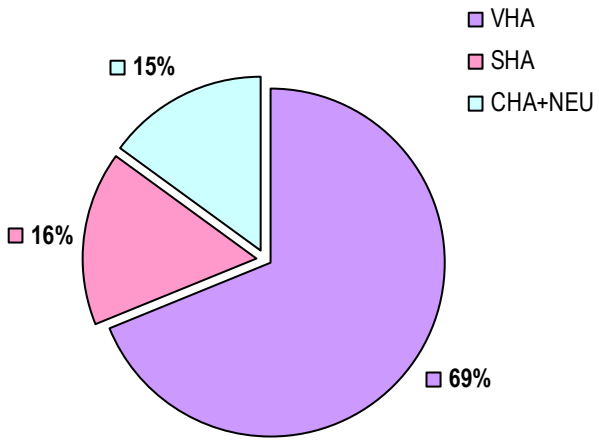

Figure 5-17. Proportions of individual fractions of raw NOM concentrate following fractionation (n=2).

The predominant components of the NOM preparation were the hydrophobic acids, as similarly found by Lee (2005) in the fractionation of the MIEX™ NOM concentrate (VHA - 69%, SHA - 17%). The typical classes of compounds observed for the VHA and SHA fractions are listed below (Leenheer, 1981; Aiken *et al.*, 1992; Marhaba, 2000):

- Hydrophobic acid: soil FAs; 1- and 2-ring aromatic carboxylic acids; 1- and 2-ring phenols
- Hydrophobic base: proportion of the HS retained by the XAD-8 resin at normal pH 7 that can be eluted by HCl
- Hydrophobic neutral: a mix of hydrocarbon and carbonyl compounds

The least abundant components of the NOM preparation were the hydrophilic fractions (CHA + NEU), which accounted for 15%. These constitute the non-humic substances of NOM, and comprise (Leenheer, 1981):

- Hydrophilic acid: organic compound containing hydroxyl acid group
- Hydrophilic base: amphoteric proteinaceous materials containing amino acids, amino sugars, peptides and proteins
- Hydrophilic neutral: organic compounds made up of polysaccharides

The relatively low proportion of the hydrophilic fractions can be explained by the method used to obtain the NOM concentrate. The MIEX™ resin is an anionic exchanger and so cannot remove the neutral components of NOM (Slunjski *et al.*, 2000); thus the NOM concentrate accounts for approximately 80% of the NOM present in natural waters. NOM fractions constitute a mixture of different compounds, and the character of compounds in each fraction will depend on the water source (Wong *et al.*, 2002). Hydrophobic fractions tend to possess a greater aromaticity and a greater average MW than the hydrophilic fractions.

To determine the relative contribution of colour and UV-absorbing components of each fraction in the NOM preparation, A_{446} and A_{254} were measured and normalised against the DOC concentration of each fraction (Figure 5.18).

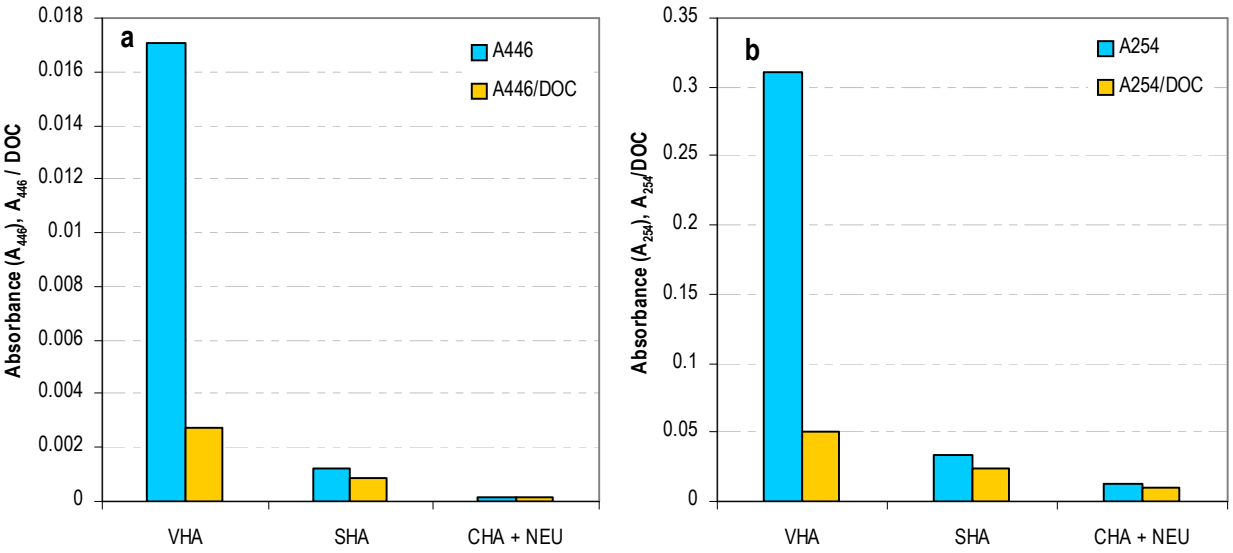

Figure 5-18. Relative colour (A_{446}, A_{446}/DOC) and UV absorbance (A_{254}, A_{254}/DOC) of individual NOM fractions.

The hydrophobic fractions, predominantly the VHA fraction, contributed the bulk of the NOM colour (as A_{446}), whereas the hydrophilic fractions (CHA and NEU) made little contribution to the colour of the NOM preparation (Figure 5.18a). Thus the decolourisation of NOM by the four WRF was associated with the removal/breakdown of the hydrophobic fractions, which account for a large proportion of HS (Swietlik *et al.*, 2004). The normalised values of A_{446} against the DOC concentration of each fraction (calculated as A_{446}/DOC) further illustrate the relatively high contribution of VHA and SHA fractions to the colour of NOM in comparison with the CHA and NEU fractions.

The hydrophobic fractions, particularly the VHA fraction, were the most intensely UV absorbing, with a low contribution by the hydrophilic fractions (CHA and NEU) (Figure 5.18b). The normalised A_{254} values, which provide an estimate of the relative abundance of the UV-absorbing components per unit DOC, show a large

contribution by the hydrophobic fractions. Thus the reduction in A_{254} would correspond to the breakdown or removal of the VHA and SHA fractions, less so of the CHA and NEU fractions.

5.6 Fractionation of NOM treated by WRF

Following treatment by *Trametes* sp., *Polyporus* sp. and *B. adusta*, the NOM-tap water preparation was fractionated to identify the changes in the fractions (Figure 5.19).

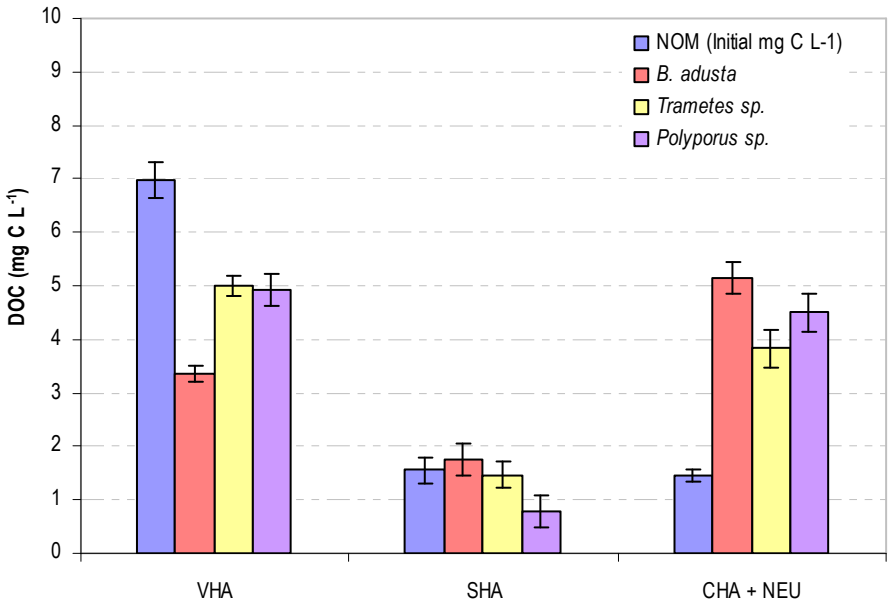

Figure 5-19. Proportion of individual NOM fractions after treatment by WRF (Section 5.3).

Fungal treatment led to the preferential removal of the VHAs (Figure 5.19). Of the WRF, *B. adusta* gave the greatest reduction (by 36%) of the VHA fraction, which correlated well with the highest decolourisation (65%) of NOM attained by this fungus (Figure 5.12a). *Trametes* sp. and *Polyporus* sp. had comparable reductions of 20-21% respectively, which corresponded well with the similar degrees of colour removal (43-44%) by these fungi. The SHA fraction remained relatively constant after treatment by *B. adusta* and *Trametes* sp., whilst for *Polyporus* sp. it halved. The combined reduction of the hydrophobic fractions (VHA and SHA) by *Polyporus* sp. might be associated with the ability of the fungus to reduce the UV absorbance across a range of the NOM molecular sizes, which was not observed for *B. adusta* or *Trametes* sp. (Figure 5.15). In contrast to the decrease in hydrophobic acids, the CHA and NEU fractions increased for the three WRF. This would be due to the production of organic acids, various charged and neutral organic compounds and proteinaceous material by the WRF, thus contributing to an overall increase in the hydrophilic fractions. This increase may also be associated with cell lysis upon nutrient depletion.

5.6.1 MW distribution of the NOM fractions following treatment by WRF

The AMW distribution of the UV-absorbing species for the individual NOM fractions prior to and following fungal treatment was investigated by HPSEC (Figure 5.20). The HPSE chromatograms of the NOM fractions were obtained by difference; the AMW data for the raw NOM preparation, DAX-8 and XAD-4 resin effluents were subtracted according to Equations 3.6 and 3.7 to obtain the plots of the respective fractions. The effluent from the XAD-4 column contained the CHA and NEU fractions.

The untreated VHA fraction was predominantly composed of high MW humic material, with the main UV-absorbing peak at 1700 Da and a lower peak at 370 Da, which may indicate some conjugated moieties (Figure 5.20a). Fungal treatment led to a considerable reduction in the VHA fraction of the NOM preparation, with a shift towards the lower MW compounds (Figure 5.20a). *B. adusta* gave the greatest reduction of the chromophoric (UV-absorbing) material across the range of MWs of 350-10000 Da. By contrast, *Trametes* sp. and *Polyporus* sp. preferentially degraded the higher MW chromophores in the VHA fraction, leading to marginal increases in the lower MW species (700–1300 Da) (Figure 5.20a). *B. adusta*, *Trametes* sp., and *Polyporus* sp. reduced the height of the VHA peak by approximately 65%, 27%, and 8% respectively.

The SHA fraction of the NOM sample contained fewer high MW compounds than the VHA fraction, with four peaks at 1450 Da, 1190 Da, 800 Da and 370 Da (Figure 5.20b). Fungal treatment led to a lower decrease of the higher MW material in the SHA fraction than in the VHA fraction. As observed for the VHA fraction, *B. adusta* reduced the UV-absorbing chromophores of the SHA fraction across a wide range of MWs (390-3000 Da), whereas *Trametes* sp. and *Polyporus* sp. reduced the SHA fraction across a narrower range of MWs (800-3000 Da) than in the VHA fraction (Figure 5.20b). *B. adusta*, *Trametes* sp., and *Polyporus* sp. reduced the height of the SHA peak by approximately 68%, 48%, and 39% respectively.

All three WRF increased the lower MW moieties (100-800 Da) of the hydrophilic (CHA + NEU) fractions, with *B. adusta* and *Trametes* sp. also showing increases between 800-960 Da (Figure 5.20c). A reduction of the high MW peak at 1430 Da was, however, observed for all three WRF, and may have resulted in the concomitant formation of the lower MW intermediates, with greater absorptions. The breakdown of the VHAs and/or the SHAs led to the formation of CHA and NEU components, as similarly observed for the VUV and UVC photooxidation of NOM as reported by Buchanan *et al.* (2005). However, contribution to this pool from lysis of the fungal cells is also likely.

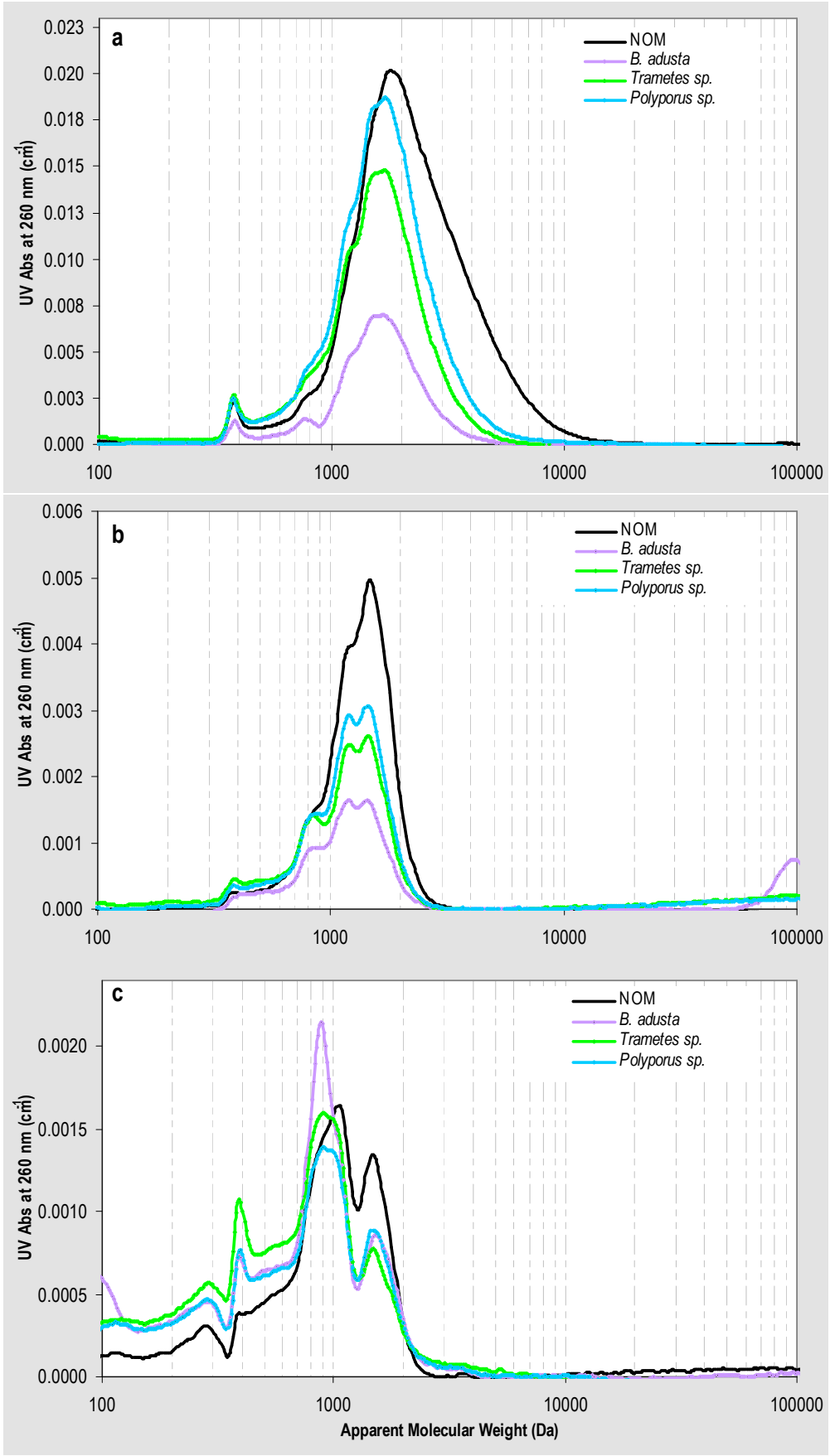

Figure 5-20. HPSEC profiles of the UV-absorbing species in (a) VHA, (b) SHA, (c) CHA+NEU fractions of raw and treated NOM-tap water preparation by WRF.

5.6.2 M_w and M_n of NOM fractions following treatment by WRF

The proportions of M_w and M_n were calculated for each of the NOM fractions following treatment by the WRF (Figure 5.21). The values of M_w and M_n for the individual NOM fractions were determined by subtraction, as previously conducted for the chromatograms of each NOM fraction.

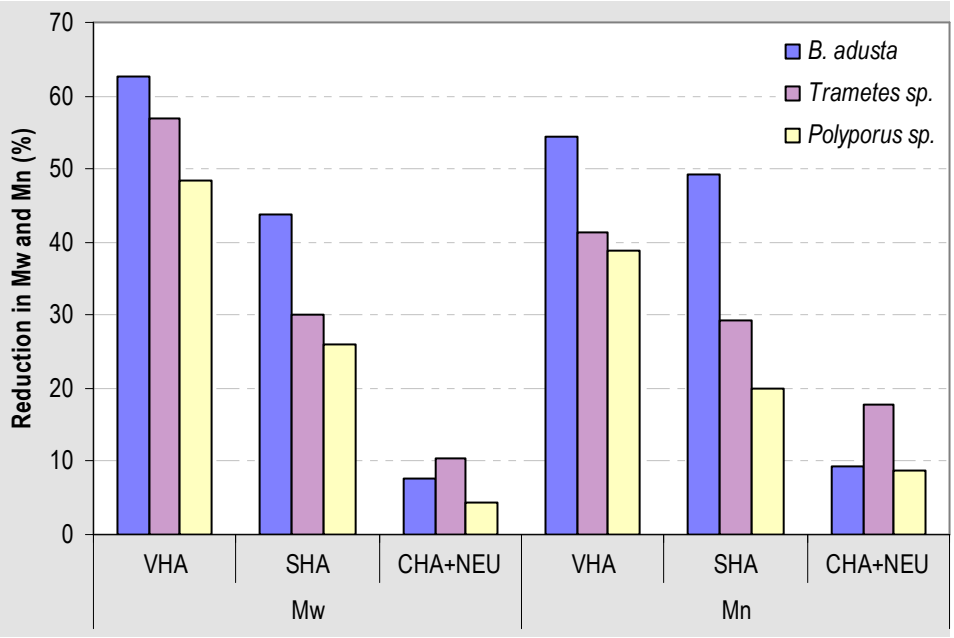

Figure 5-21. Reduction (%) in M_w and M_n of individual NOM fractions following treatment by WRF.

The reduction in M_w and M_n by the WRF for the individual NOM fractions was greatest for the hydrophobic components, in particular for the VHA fraction. *B. adusta* gave the greatest reduction in M_w (63%, 44%) and M_n (54%, 49%) for both the VHA and SHA fractions, and this correlated well with the greater degree of NOM decolourisation attained by this fungus (Figure 5.14). *Trametes* sp. and *Polyporus* sp. had lower reductions in M_w and M_n for the VHA (57%, 48%) and SHA (30%, 26%) fractions respectively, and this correlated well with the lesser decolourisation by these fungi. The predominant removal of the hydrophobic fractions suggests that the decolourisation of NOM involves oxidative reactions that target specific bonds within the VHA and SHA fractions, resulting in their depolymerisation. Thus the predominant reduction of the hydrophobic fractions would yield a less aromatic and less coloured NOM, with a lower MW. This is further supported by the degree of polymerisation (ratio of M_w:M_n) for the individual NOM fractions, which declined in the order of VHA>SHA.

The lowest reduction in M_w and M_n by the three WRF was observed for the hydrophilic fractions. Difficulty in removing these fractions from water has been reported for other treatments, including coagulation (Bolto *et al.*, 2002). As fungal treatment led to an increase in the lower molecular weight compounds of the CHA and

NEU fractions (Figure 5.20 and 5.21), a sequential treatment involving bacteria may be applicable in utilising these lower molecular MW species. Thomson (2002) studied the effect of UV and subsequent bio-treatment on the molecular size distribution of NOM, and observed that bacteria preferentially utilised the lower molecular weight compounds of the irradiated NOM.

5.7 Conclusions for Chapter 5

This chapter investigated the application of *T. versicolor* ATCC 7731, *Trametes* sp. *Polyporus* sp., *P. cinnabarinus* and *B. adusta* for the treatment of aquatic NOM concentrate. The potential of these fungi to decolourise NOM without the addition of nutrients was demonstrated in both tap and distilled water. Different trends and degrees of NOM decolourisation were observed for the WRF in the two NOM preparations. Greater levels of colour removal were obtained in the NOM-tap water preparation for three of the WRF and this was attributed to the increased presence of mineral nutrients.

The decolourisation of aquatic NOM concentrate by the five strains of WRF coincided with the onset of extracellular enzyme activity, in particular that of Lac and MnP. The magnitude and timing of the activity of these extracellular enzymes varied with the strains of fungi and culture conditions used. For most WRF, the activities of Lac and MnP increased in tap water, correlating well with the greater NOM decolourisation, compared with distilled water. The enzyme activities of the newly isolated *B. adusta* were markedly less, with MnP activity predominating. Despite this, the greatest decolourisation of NOM (65%) occurred for *B. adusta*, and this was attributed to the action of a VP. To our knowledge this is the first demonstration of the potential of *B. adusta* for the decolourisation of NOM concentrate under conditions of limiting nutrients. Furthermore, this is the first report of trends in extracellular enzyme activity, in particular of MnP, during NOM decolourisation by *B. adusta*.

Different rates and mechanisms of initial colour removal were observed for the different WRF and the two NOM solutions. NOM decolourisation in distilled water involved a preliminary adsorption step before the onset of enzyme activity, whilst in the tap water preparation, initial NOM decolourisation occurred almost simultaneously with the rise in enzyme activities. The simultaneous secretion of enzymes (mainly Lac and MnP) suggests that they played a dual or synergistic action in the decolourisation of NOM.

The decolourisation of NOM in both distilled and tap water was accompanied by a decrease in the average MW, the concomitant formation of lower MW intermediates being most marked for *B. adusta*. Fractionation of the treated NOM-tap water preparation demonstrated the breakdown of the hydrophobic fractions by the WRF, in particular of the VHA fraction. The breakdown of the hydrophobic fractions may have contributed to

the increase in the hydrophilic fractions (CHA and NEU). Thus the decolourisation of NOM by WRF was accompanied by the predominant removal of the higher MW humic fractions, which accounted for most of the colour and aromaticity of NOM.

Chapter 6 Effect of NOM concentration and bacterial supplementation on NOM degradation by *Trametes* sp.

Preface

The decolourisation of aquatic NOM concentrate was further investigated by studying its removal at various NOM concentrations in liquid culture. *Trametes* sp. was selected for these studies as it was one of the most effective for the decolourisation of NOM in both tap and distilled water. In addition, the selection of this fungus was based on the reproducibility of its results and ease of culture. Although *B. adusta* attained the greatest decolourisation of NOM in tap water, it was not used in the following work as it was difficult to obtain reproducible results between experimental runs but not within a run, during the period of this study. The effect of initial NOM concentration on the biodegradation of NOM was investigated to determine the maximum concentration tolerated by *Trametes* sp. in liquid culture under nutrient limited conditions. Following fungal treatment, the biodegradability of the treated NOM at various concentrations was assessed with a consortium of bacteria (BDOC determination).

6.1 Decolourisation studies at various NOM concentrations

The effect of initial NOM concentration (13-500 mg C L^{-1}) on the decolourisation of NOM-tap water by *Trametes* sp. was investigated under nutrient-limited conditions. A NOM concentration range of 13-500 mg C L^{-1} was selected for this study to cover the potential concentrations used in drinking water and concentrated NOM wastes.

6.1.1 Effect of initial NOM concentration on the removal of NOM

Maximal decolourisation (50%) by *Trametes* sp. occurred at 100 mg C L^{-1}, with the lowest (21%) at 500 mg C L^{-1} (Figure 6.1a). Although the proportion of NOM decolourised increased and then decreased with NOM concentration, the equivalent amount of NOM converted to mg C L^{-1} from A_{446} at maximum colour removal (Section 3.10.4) increased linearly up to 300 mg C L^{-1} (Figure 6.1b).

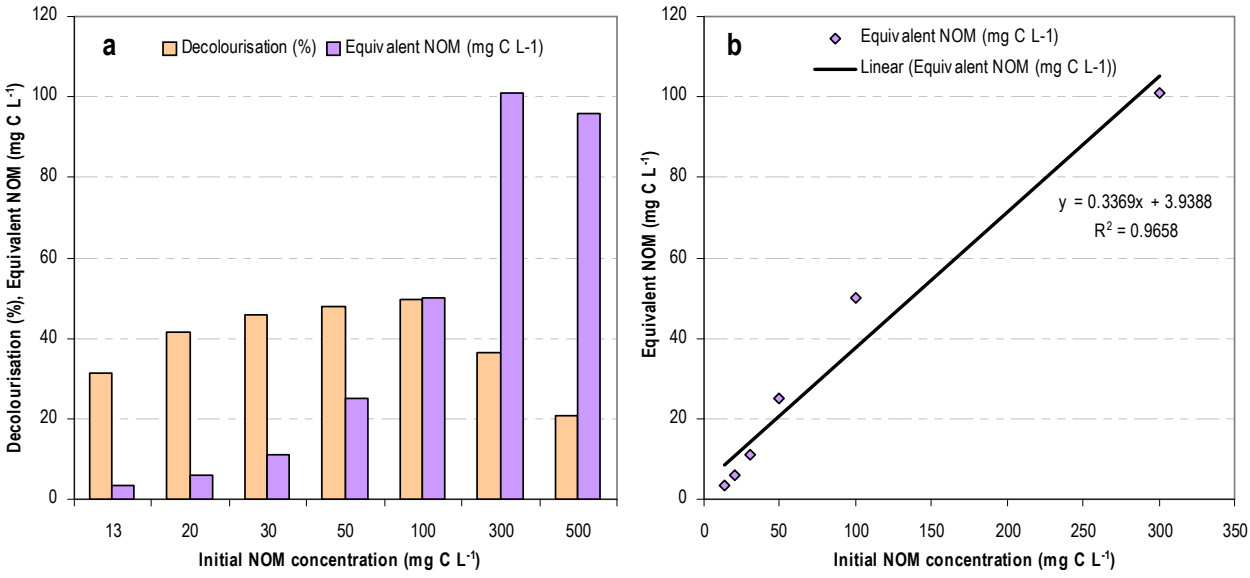

Figure 6-1. Effect of initial concentration on NOM decolourisation by *Trametes* sp. (a) and the equivalent amount of NOM converted to mg C L^{-1} from A_{446}, (b) relationship between initial NOM concentration and NOM removal (converted from A_{446}).

The linear relationship (R^2=0.97) between the initial NOM concentration (13-300 mg C L^{-1}) and the equivalent amount of NOM converted in terms of mg C L^{-1} (Figure 6.1b) is consistent with a similar study conducted by Lee (2005) on the treatment of MIEX™ NOM concentrate in Waksman medium (a simple growth medium) by *T. versicolor* ATCC 7731. Lee determined a closer relationship for the biodegraded NOM by *T. versicolor* ATCC 7731 as y = 0.0952x + 6.6653 (R^2=0.99). A comparison of NOM removals in terms of A_{446} by the two strains is illustrated in Figure 6.2.

NOM biodegradation by *Trametes* sp. was 2-3 times greater than that by *T. versicolor* ATCC 7731 for NOM concentrations of 30-500 mg C L^{-1}. The culture of *Trametes* sp. here was under limited nutrient conditions (Table 5.2), whereas the culture of *T. versicolor* ATCC 7731 (Lee, 2005) was in Waksman medium containing 2 g L^{-1} glucose. The absence of an easily available carbon source in the cultures of *Trametes* sp. may have triggered a stronger biodegradation response, particularly with greater NOM concentrations (with up to 100 mg C L^{-1} NOM converted). Lee (2005) observed that increasing the initial glucose concentration from 2 to 5 g L^{-1} did not improve the biodegradation of NOM by *T. versicolor* ATCC 7731. Similar observations were made by Rezacova *et al*. (2006), who did not find any significant effect of glucose on the utilisation of either HA or FA by microfungi.

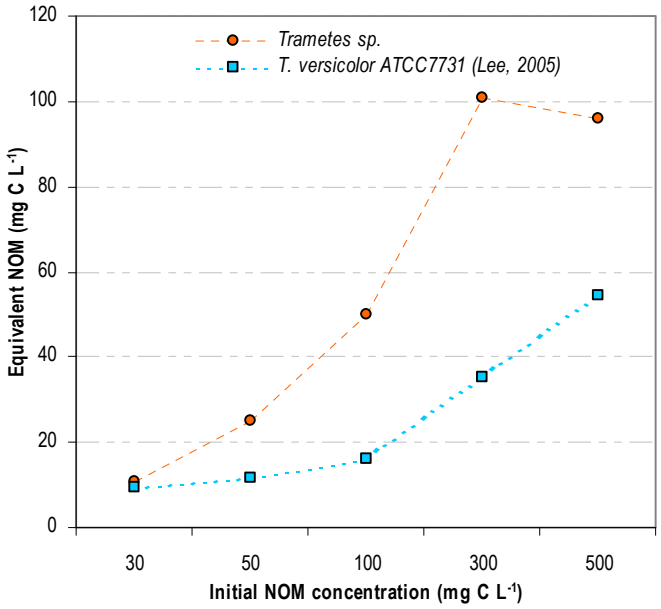

Figure 6-2. NOM biodegradation by *Trametes* sp. (this study) and *T. versicolor* ATCC 7731 (Lee, 2005).

Variation in nitrogen and carbon sources, C:N ratio, microelements and mediators all affect the biodegradation process of humic substances (Fakoussa and Frost, 1999). NOM includes fulvic and humic moieties with a C:N ratio of 10:3; the low C:N ratio suggests low aromaticity due to long ageing or microbial origin and is the likely explanation for only WRF being able to access its N content (Hood *et al.*, 2005). In another study conducted by Lee (2005), *P. chrysosporium* cultures grown in media with different C:N ratios achieved the greatest decolourisation with the lowest C:N ratio. This is also in agreement with work conducted by Rojek *et al*. (2004) with the same organism who also demonstrated that the biodegradation of NOM increased with decreased C:N ratio for a different NOM preparation. The difference in NOM biodegradation may be attributed to the strain of fungus used and media composition.

No linear correlation existed between the initial NOM concentration and the breakdown of UV-absorbing species (A_{254}). In contrast, Lee (2005) obtained a strong relationship (R^2=0.92) between the initial NOM concentration and the decrease in UV-absorbing NOM.

6.1.2 Profiles of NOM decolourisation at various NOM concentrations

For most NOM concentrations there was a high initial rate of NOM decolourisation that occurred within the first 15 h of incubation (Figure 6.3a and b). This high initial rate of colour removal suggests preliminary adsorption onto *Trametes* sp. pellets, prior to enzymatic breakdown. The rate of NOM decolourisation (up to maximum colour removal) increased linearly (R^2=0.98) with increasing NOM concentration up to 30 mg C L^{-1}, and decreased linearly (R^2=0.98) for NOM concentrations of 100-500 mg C L^{-1}.

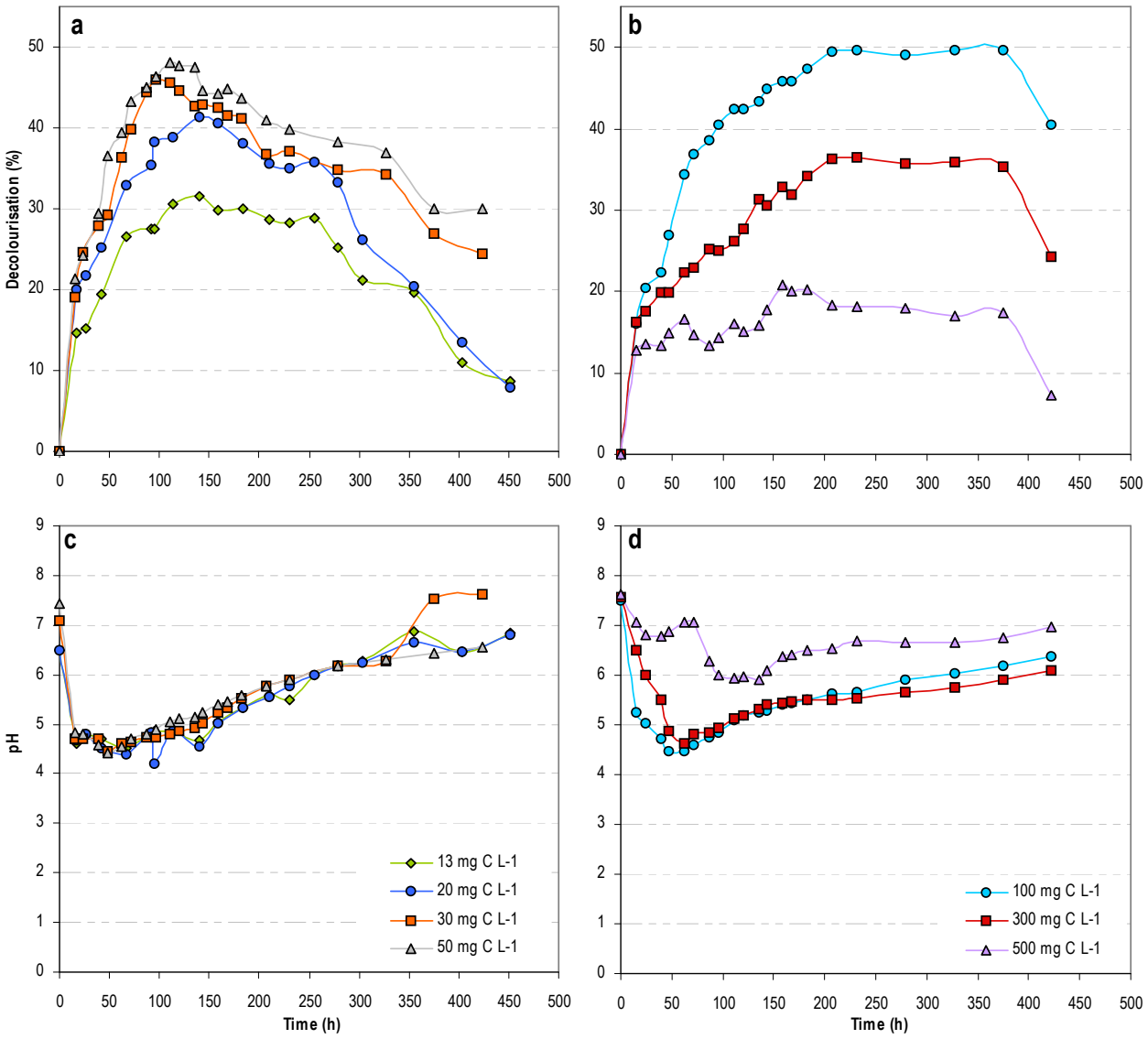

Figure 6-3. Decolourisation profiles at various NOM concentrations (a) 13–50 mg C L^{-1}, (b) 100-300 mg C L^{-1} and variation in pH at various NOM concentrations (c) 13–50 mg C L^{-1}, (d) 100-300 mg C L^{-1} for *Trametes* sp. at 30ºC. Data points are means of 4 replicates.

The reduced rate of colour removal at ≥100 mg C L^{-1} could indicate the onset of inhibition, whilst the greater rate of NOM decolourisation at the lower NOM contents (≤50 mg C L^{-1}) may be due to lower concentration of DOC and lesser nutrient availability. NOM decolourisation peaked between 96 and 140 h for NOM contents of 13-50 mg C L^{-1} (Figure 6.3a), and between 160 and 206 h for NOM contents of 100-500 mg C L^{-1} (Figure 6.3b). Shortly after reaching maximum decolourisation at the lower NOM contents, the onset of negative decolourisation was seen at approximately 140 h (Figure 6.3a). In contrast, maximum NOM decolourisation remained constant for approximately 170 h at the higher NOM contents, prior to negative decolourisation at 375 h (Figure 6.3b). This sustained period of maximum decolourisation coincided well with the increased NOM biodegradation (as converted from A_{446}) (Figure 6.1a). Although the biodegradation of NOM increased, NOM decolourisation increased then decreased with increasing NOM concentration, suggesting inhibition at NOM contents higher than 100 mg C L^{-1}. Work by Ziegenhagen and Hofrichter (1998) on the degradation of HA by MnP from the white rot fungus *Clitocybula dusenii*, similarly demonstrated a reduced decolourisation at the higher HA contents of 300-500 mg C L^{-1}.

As shown previously in section 5.2 and 5.3, the decolourisation of NOM appeared to be associated with metabolic activity, as indicated by the drop in culture pH for all NOM concentrations (Figure 6.3c and d). There was a general trend of rapid decrease in pH from 7.5 to 4.5 in the first 60 h, after which pH gradually increased to approximately pH 6-7. With increasing NOM concentrations (>300 mg C L^{-1}), however, the decrease in culture pH was not so great and correlated well with the reduced decolourisation. This drop in culture pH probably resulted from the release of organic acids such as malonate and oxalate and from the production of CO_2. The high initial rate of NOM decolourisation being due to adsorption was confirmed by the increasing colouration of the pellets with increasing NOM concentration (Figure 6.4).

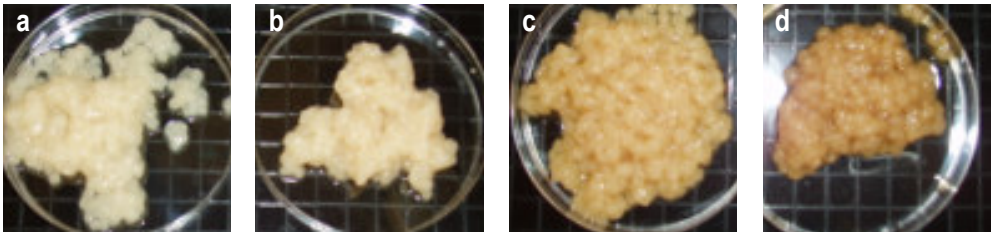

Figure 6-4. Adsorption of NOM to fungal biomass after treatment with *Trametes* sp. (Day 19) (a) 30 mg C L^{-1}, (b) 50 mg C L^{-1}, (c) 300 mg C L^{-1}, (d) 500 mg C L^{-1}.

Adsorption of NOM onto the fungal biomass was further confirmed with the autoclaved controls, where the amount of colour adsorbed by *Trametes* sp. increased with NOM concentration, the greatest being 9% for 500 mg C L^{-1}.

6.2 Effect of initial NOM concentration on enzyme activities

As noted previously, assays for phenoloxidases confirmed the presence of Lac and MnP in the culture filtrates, with no LiP being detected. As previously noted, the enzyme system of WRF exhibits different characteristics depending on the species, strains and culture conditions used (Rogalski *et al.*, 1991), with some fungi such as *T. versicolor* producing all three enzymes, while others produce one or two (Hatakka, 1994).

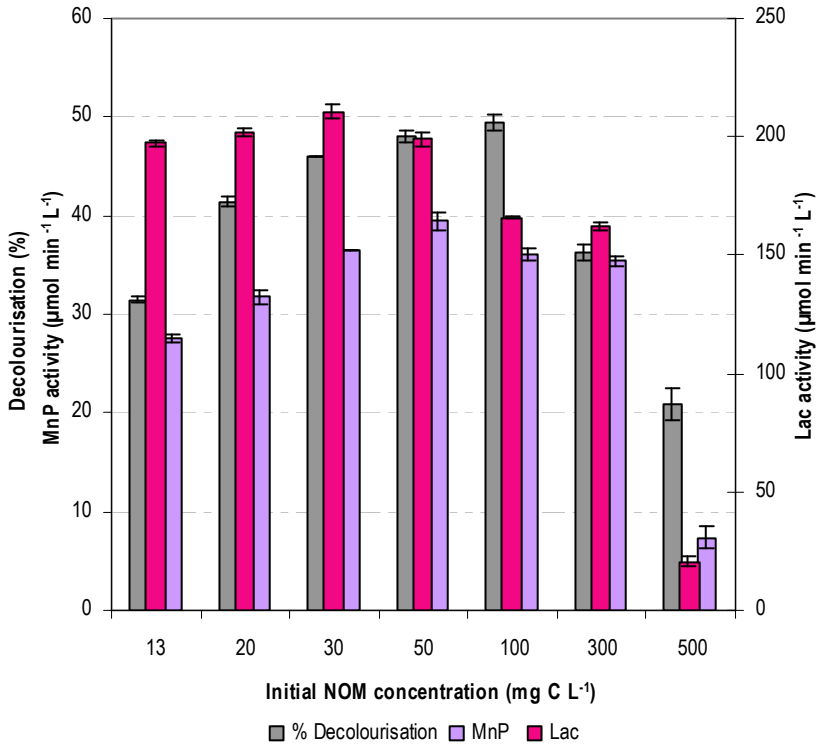

Figure 6-5. Effect of initial NOM concentration on the activities of Lac and MnP during NOM decolourisation by *Trametes* sp. Note: The activities of Lac and MnP are the maximum values recorded over the incubation period of 19 days.

For NOM concentrations of 13-500 mg C L^{-1}, the activity of Lac was greater (3-7 fold) than that of MnP, suggesting its predominant role in the decolourisation of NOM (Figure 6.5). With increasing NOM concentration the ratio of MnP:Lac (based on enzyme activity) increased, with maximal decolourisation occurring at a MnP:Lac ratio of 0.2:1. Lac and MnP activity increased with increasing NOM content up to 30-50 mg C L^{-1}. This finding is in accordance with that of Dehorter and Blondeau (1992) who found increasing production of MnP and LiP with increasing concentration of HAs. They suggested that the surfactant properties of HAs were responsible for the increased enzymatic activity seen for *P. chrysosporium* and *T. versicolor*. The activity of Lac and MnP gradually declined at NOM concentrations greater than 30-50 mg C L^{-1}, but were significantly reduced (5-fold for MnP and 8-fold for Lac) above 300 mg C L^{-1} (Figure 6.5).

These trends indicate enzyme inhibition at NOM contents greater than 300 mg C L^{-1}. Lee (2005) found inhibition of LiP and MnP activities of *T. versicolor* for NOM concentrations of 300 to 600 mg C L^{-1}, and Dehorter and Blondeau (1992) found that HA concentrations of greater than 1% (equivalent to 300 mg C L^{-1}) were inhibitory to enzyme production and the first stage of growth in *T. versicolor* and *P. chrysosporium*. The studies by Dehorter and Blondeau and by Lee, however, were conducted with different fungi and in the presence of glucose.

For NOM concentrations of 100-300 mg C L^{-1}, the maximum activities of Lac and MnP for *Trametes* sp. were 1-5 times higher than those of *T. versicolor* ATCC 7731 (Figure 6.6) and corresponded with the greater biodegradation of NOM (Figure 6.2). The lower activities, in particular of Lac for *T. versicolor* ATCC 7731, may be attributed to the presence of glucose in the culture medium, which has been reported to repress Lac synthesis in fungi (Eggert *et al.*, 1996; Galhaup *et al.*, 2002b).

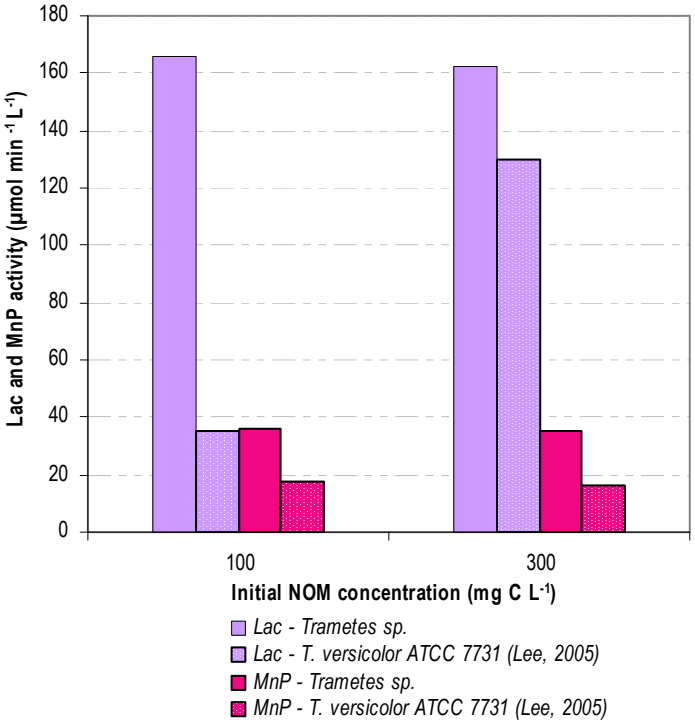

Figure 6-6. Maximum activities of Lac and MnP of *Trametes* sp. and *T. versicolor* ATCC 7731 in cultures containing varying NOM concentrations at 30ºC.

6.2.1 Profiles of enzyme activities at various NOM concentrations

Different enzyme activity profiles of Lac and MnP were observed during the decolourisation of NOM at various concentrations (Figure 6.7). The slow onset of Lac and MnP activity at approximately ≥ 15 h for most NOM concentrations is consistent with the mechanism of initial NOM decolourisation (≤ 15 h) being chiefly via adsorption (Figure 6.3a and b). With increasing NOM content (>100 mg C L^{-1}) the onset of enzyme activity occurred more slowly. This was more apparent at the highest NOM concentration, where the induction of Lac and MnP was seen only after 90 h of incubation. The later appearance of the extracellular enzymes, combined with their lower activities, reaffirm the probable mechanism of NOM decolourisation at 500 mg C L^{-1} being almost entirely due to adsorption, with little due to enzymatic breakdown. The rise in enzyme activities, combined with the increase in NOM decolourisation for NOM contents of 13-300 mg C L^{-1}, strongly indicate an enzymatically driven decolourisation process after the initial adsorption stage (Figure 6.3a and b).

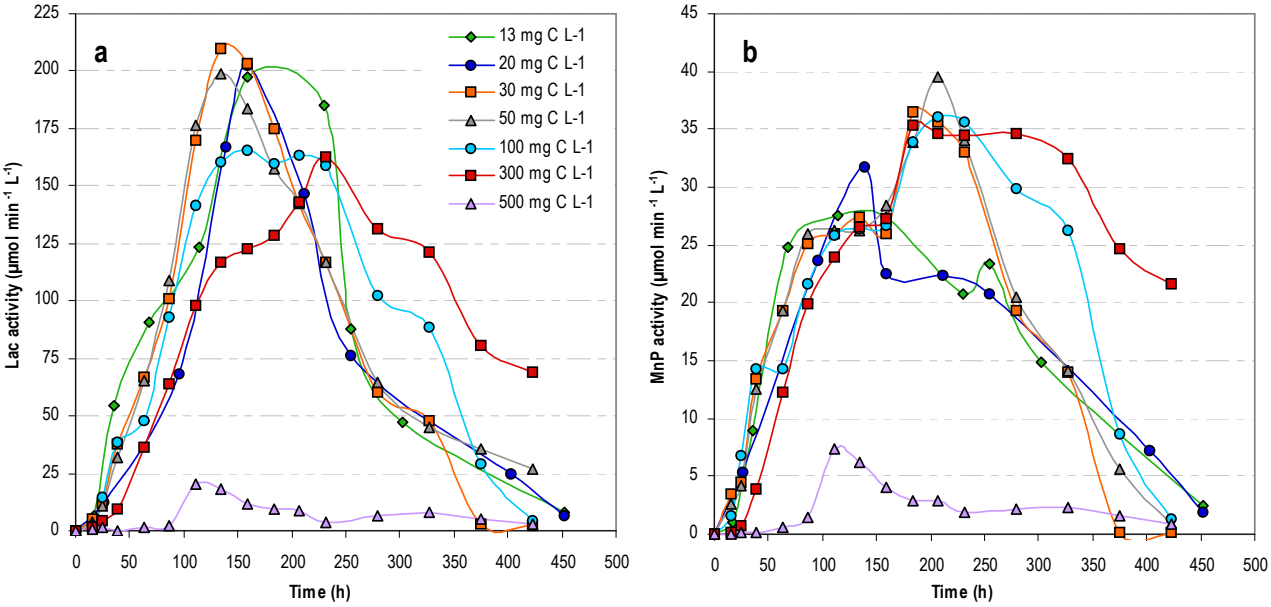

Figure 6-7. Enzyme activity profiles for (a) Lac and (b) MnP during NOM decolourisation by *Trametes* sp. at 30ºC.

Lac activity peaked at approximately 150 h, coincident with maximum NOM decolourisation (100-200 h) (Figure 6.7a), whereas MnP activity initially peaked at 100 h during the onset of NOM decolourisation and again at a greater level shortly after maximum decolourisation at 200 h for most NOM concentrations (Figure 6.7b). A steep reduction in both enzyme activities was seen shortly after reaching maximum decolourisation (100-200 h), the decline being steeper for lower NOM concentrations. The reduction in enzyme activities correlated well with the increase in culture pH for all NOM contents (Figure 6.3c and d). The profiles of enzyme activity at 100 mg C L^{-1} (Figure 6-7) are similar to those for an earlier experimental run with *Trametes* sp. (Figure 5-14b), where maximum decolourisation occurred during the peak of Lac activity and shortly after the initial peak of MnP activity.

A positive relationship was derived for the simultaneous activity of Lac and MnP during the decolourisation of NOM at varying concentrations by *Trametes* sp. (Table 6.1).

Table 6-1. Relationship between cooperative enzyme activity and initial NOM concentration

Initial NOM concentration (mg C L^{-1})	13	20	30	50	100	300	500
Correlation of Lac and MnP activity (R^2)	0.72	0.74	0.76	0.77	0.88	0.95	0.99

With increasing NOM content a stronger correlation (R^2) between the activity of Lac and MnP was observed, and this correlated well with the greater removal of NOM at higher concentrations (Figure 6.2). This indicates that the two enzymes work in a cooperative, complementary or even synergistic manner. These ligninolytic enzymes can either function separately or cooperatively for the degradation of complex organic compounds (Shah and Nerud, 2002).

6.3 Effect of initial NOM concentration on A_{254} and DOC

Fungal treatment led to increased A_{254} (for NOM concentrations of 13-50, and 500 mg C L^{-1}) and DOC, the increases being greater for lower NOM concentrations (Figure 6.8). Absorbance at 254 nm is often used as a surrogate measure for DOM (Edzwald, 1993). A good correlation (R^2 = 0.903) was found for the increase in DOC and increase in A_{254} for NOM content of 13-50 mg C L^{-1}.

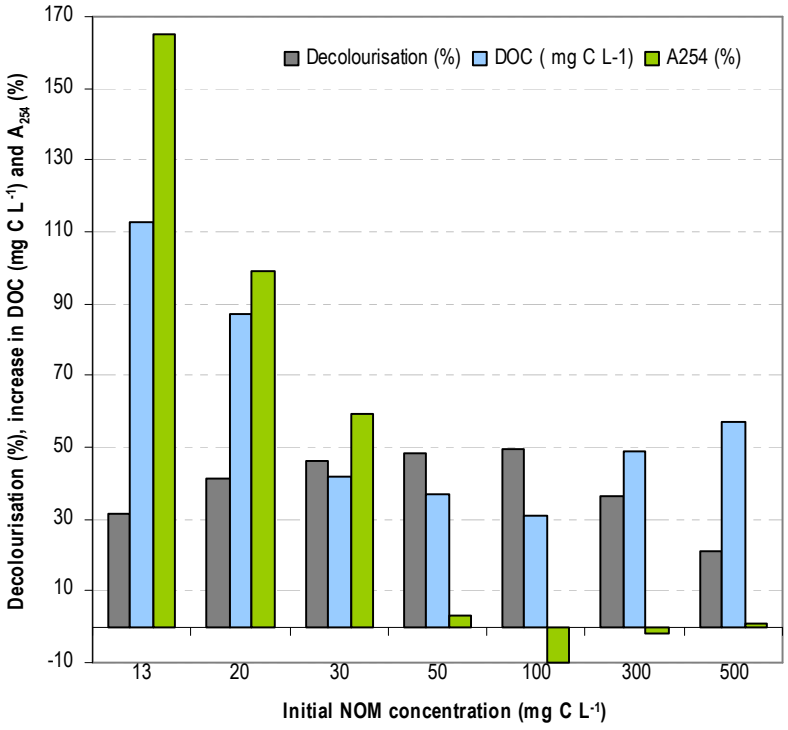

Figure 6-8. Changes in A_{254} and DOC following NOM decolourisation by *Trametes* sp. at 30ºC.

The overall increase in DOC and A_{254} was attributed to the release of soluble organic compounds into the culture from (Sigee, 2005):

- The passive diffusion of polysaccharides, amino and organic acids from active cells
- The active production and secretion of the extracellular enzymes
- Soluble breakdown products from cell lysis
- Release of NOM from fungal cell walls following adsorption

Work by Reid (1998) on the fate of residual lignin during delignification of kraft pulp by *T. versicolor* similarly showed an increase in the UV-absorbing material (A_{280}). The greater increase in A_{254} and DOC at the lower NOM contents of 13-30 mg C L^{-1} may be linked to the greater rate of NOM decolourisation and the early onset of 'negative decolourisation', which lasted for approximately 310 h (Figure 6.3a). This extended period of 'negative decolourisation' may have contributed to a greater release of NOM from fungal cell walls and from cell disintegration upon the utilisation of nutrients. At greater NOM concentration (100-500 mg C L^{-1}), negative decolourisation occurred much later, after approximately 360 h, thus possibly contributing to the lesser quantities of DOC and A_{254} (Figure 6.8). With increasing NOM concentration, the increase in DOC declined and was at its least at 100 mg C L^{-1}, coinciding with maximum colour removal (of 50%) and reduction in A_{254} (by 10%). Above 100 mg C L^{-1}, the generation of DOC increased, coinciding with a reduction in colour removal. The considerable increase in new UV-absorbing material at the lower NOM contents may have masked the degradation of the UV-absorbing NOM compounds, thus resulting in an overall increase in A_{254} (Figure 6.8). Additionally, the re-polymerisation of dissociated humic constituents at high pH could have contributed to the overall increase in A_{254}. For NOM concentrations greater than 100 mg C L^{-1}, A_{254} decreased.

6.4 The effect of fungal treatment on the biodegradability of treated NOM

As fungal treatment led to increases in DOC levels (41-57 mg C L^{-1}) for NOM contents of 30-500 mg C L^{-1} respectively (Figure 6.9), the biodegradability of the treated NOM was determined using the biodegradable dissolved organic carbon (BDOC) method (Volk *et al.*, 1994). The BDOC test utilises heterotrophic microorganisms to ascertain the fraction of DOC that can be mineralised by bacteria.

When the treated NOM samples were subjected to the biologically active sand for 51 days, 51-167 mg C L^{-1} of the increased DOC was assimilated by the bacteria to levels below the original NOM concentration (Figure 6.9). At lower NOM concentrations (30-50 mg C L^{-1}) a large fraction of the DOC released from fungi was biodegraded (61-70%), whereas at higher NOM concentrations (300-500 mg C L^{-1}), only 30-35% of the DOC was biodegraded.

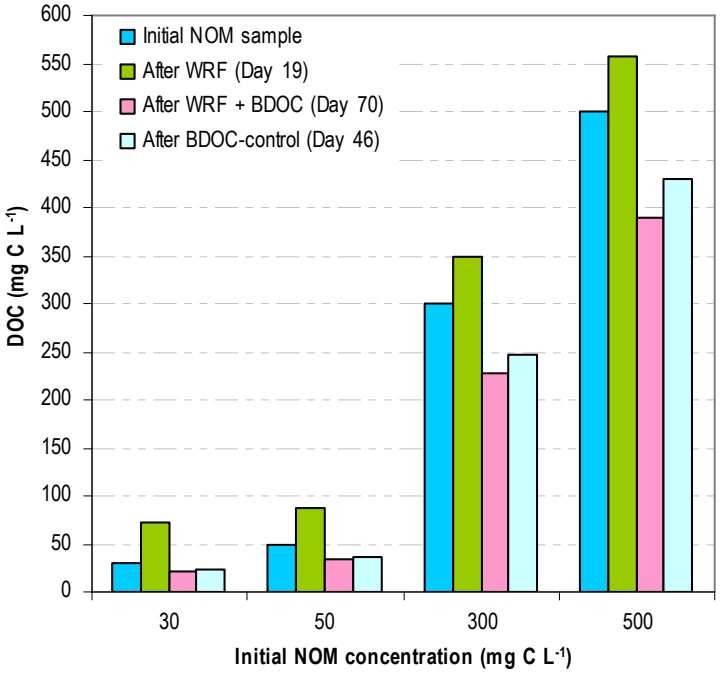

Figure 6-9. DOC concentrations after fungal (Day 19), sequential fungal and bacterial treatment (BDOC, Day 70) and bacterial treatment alone (control) (Day 46).

Although a considerable amount of the increased DOC was assimilated by the bacteria, *Trametes* sp. only slightly enhanced the biodegradability of the treated NOM (5-10 %), as evidenced by the BDOC controls of NOM without fungal pre-treatment (Figure 6.9). This was most apparent at the lower NOM contents (30-50 mg C L^{-1}) where the overall reduction in DOC of the WRF-treated NOM was only slightly greater (by 2-2.4 mg C L^{-1}) than that by BDOC alone. For higher NOM concentrations (300-500 mg C L^{-1}), however, sequential fungal and bacterial treatment removed more DOC (by 18-40 mg C L^{-1}) than BDOC alone, which suggests that the WRF could be suited more for the pre-treatment of higher NOM concentrations (e.g. membrane reject streams). Thus it seems that the fungus aided in the removal of the more refractory material by the formation of a mixed substrate from the addition of biologically labile compounds, as observed by Tranvik (1998).

Fungi contributed to the increased biodegradability of NOM through several possible mechanisms:

- By forming lower MW substrates such as organic acids (Makela *et al.*, 2002).
- By increasing the pool of smaller MW intermediates, through the depolymerisation of higher MW fractions (Hofrichter and Fritsche, 1996), as is discussed in Section 6.5.

In addition to the considerable reduction in DOC of the WRF-treated NOM, subsequent bacterial treatment led to reductions in A_{446} and A_{254} (Table 6-2).

Table 6-2. Reduction in A_{446} and A_{254} after sequential fungal and bacterial treatment

Initial NOM concentration (mg C L^{-1})	Maximum decolourisation (%)		Reduction in A_{254} (%)	
	WRF + BDOC	BDOC*	WRF + BDOC	BDOC*
30	75	40	59	20
50	80	51	76	29
300	44	40	27	26
500	31	25	24	12

***** Controls of NOM concentrate subjected to BDOC without prior fungal treatment

NOM decolourisation (%) initially increased then decreased with NOM content (≥ 300 mg C L^{-1}), attaining a maximum colour removal of 80% at 50 mg C L^{-1}. For NOM contents of 30-50 mg C L^{-1}, sequential fungal and bacterial treatment removed 29-32% more colour from solution than fungal treatment alone. Although *Trametes* sp. led to the generation of new-UV absorbing material, particularly at lower NOM concentrations (Figure 6.8), subsequent BDOC treatment significantly reduced the increased A_{254} (Table 6-2).

The lesser levels of A_{446}, A_{254} and DOC removed by BDOC alone indicate that fungal pre-treatment facilitated the removal of more refractory compounds, whilst the bacteria were responsible for the mineralisation of intermediate products released through fungal activity (Ruttimann *et al.*, 1991). Presumably the most biologically labile molecules were metabolised first, with the more recalcitrant molecules needing longer residence times (Volk *et al.*, 1997).

6.5 MW distribution of NOM after fungal and sequential fungal and bacterial treatment

HPSEC with UV (260 nm) and colour (446 nm) detection was conducted to examine the changes in molecular size distribution of the organic matter after fungal (Day 19), and sequential fungal and bacterial treatment (Day 70).

The decolourisation of NOM by *Trametes* sp. resulted in a shift from higher MW hydrophobic fractions to lower MW hydrophilic fractions for all NOM concentrations (Figure 6.10). The greatest reductions in average AMW of the coloured species occurred for NOM contents of 30-50 mg C L^{-1}, which also coincided with the greatest NOM decolourisation of 46-48% (equivalent to the decolourisation of 11-25 mg C L^{-1} NOM) by *Trametes* sp. (Figure 6.8). With increasing NOM content the shift from higher MW towards lower MW compounds declined, suggesting inhibition at NOM contents greater than ≥ 50 mg C L^{-1}. Coinciding with the shift in AMW was also a drop in the absorbance of the initial peak at 2500 Da for all NOM contents (Figure 6.10). *Trametes* sp. reduced the peak height by approximately 41%, 43%, 19% and 3% for NOM concentrations of 30, 50, 300 and 500 mg C L^{-1} respectively. These reductions correlated to the reductions

in colour of the NOM solution. For NOM concentrations of 30-50 mg C L^{-1}, an increase in absorbance over the AMW range of 300-1500 Da was observed (Figure 6.10) and coincided well with the greater degree of 'negative decolourisation' at these low NOM contents (Figure 6.3a). With increasing NOM concentration this increase in absorbance declined and matched well with the low degree of negative decolourisation at higher NOM concentrations (Figure 6.3b). The formation of a small peak at an AMW of 700 Da for 30 mg C L^{-1} and at 900 Da for 50 mg C L^{-1}, indicates the formation of lower MW humic material (Huber, 2008) from the breakdown of higher MW humic fractions.

When the treated NOM was subjected to the BDOC test for 51 days, further reductions in the average MW of the coloured species were observed (Figure 6.10). For most NOM contents, bacteria assimilated the coloured material across the MW range of 350-10000 Da with greater removal at lower NOM concentrations of 30-50 mg C L^{-1}. The most apparent reduction was for 50 mg C L^{-1} and correlated well with the greatest removal of colour (80%) from the fungal treated NOM solution (Table 6-2). Following fungal treatment, the bacteria further reduced the absorbance of the peak at 2500 Da, leading to combined fungal and bacterial reductions of 64, 81, 35 and 21% for NOM concentrations of 30, 50, 300 and 500 mg C L^{-1}, respectively. These reductions were correlated to the reductions in colour of the NOM solution (Table 6-2).

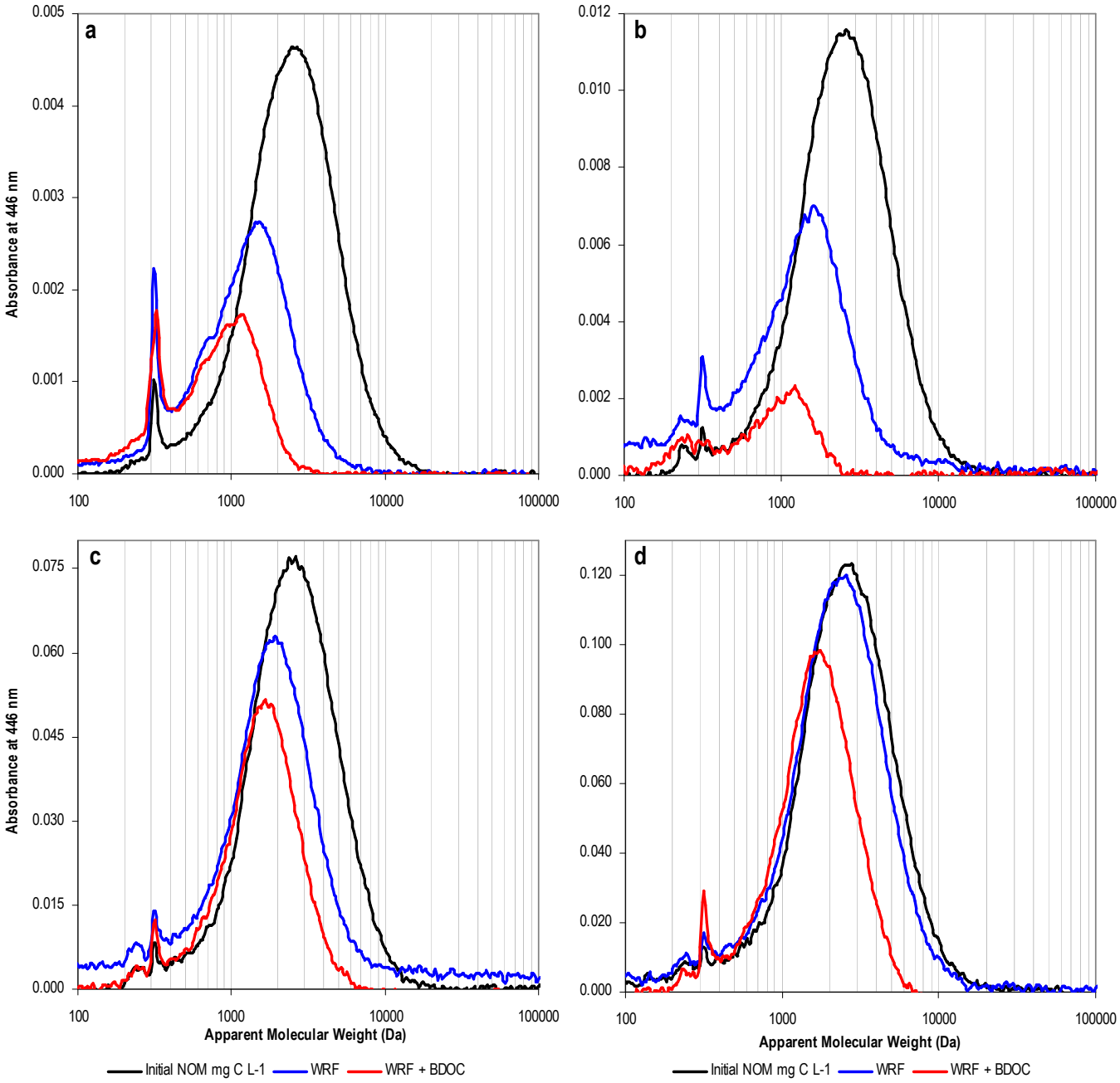

Figure 6-10. HPSEC-A_{446} chromatograms following fungal treatment (Day 19) and sequential fungal and bacterial treatment (Day 70) of NOM concentrate for (a) 30 mg C L^{-1} (b) 50 mg C L^{-1} (c) 300 mg C L^{-1} and (d) 500 mg C L^{-1}. Note: different scales for A_{446}.

HPSEC with detection at 254 nm further illustrated the preferential breakdown of higher MW compounds (>1500 Da) by *Trametes* sp. for all NOM concentrations (Figure 6.11). The shift from higher MW fractions was accompanied by the concomitant formation of lower MW UV-absorbing peaks, which decreased in their intensity with increasing NOM concentration. The increased presence of lower MW intermediates in the range of 300, 700 and 970 Da, particularly apparent for NOM contents of 30-50 mg C L^{-1}, represent the soluble organic compounds released by cell lysis and enzyme production as well as lower MW humics. The depolymerisation of NOM by extracellular enzymes would also have contributed to the formation of smaller MW humic fractions with lower UV absorbance; however, the active production of enzymes and release of fungal breakdown products led to the overall increase in A_{254} and DOC, thus masking the reduction in A_{254}. With increasing NOM content (≥300 mg C L^{-1}) the formation of lower MW compounds decreased, which correlated with the reduction in A_{254} (Figure 6.8).

Sequential fungal and bacterial treatment led to further reductions in the higher MW material (>1000 Da) and most of the lower MW fungal products in the range of ≥300 Da and ≤1000 Da (Figure 6.11). For lower NOM concentrations of 30-50 mg C L^{-1}, UV-absorbing material across the AMW range of 300-3000 Da was reduced. The marginal reduction in the higher MW humic material at NOM concentrations greater ≥300 mg C L^{-1} suggests that fungal treatment did not modify these higher fractions to a more biodegradable form, and thus a proportion of this material remains refractory to microbial assimilation under the conditions used.

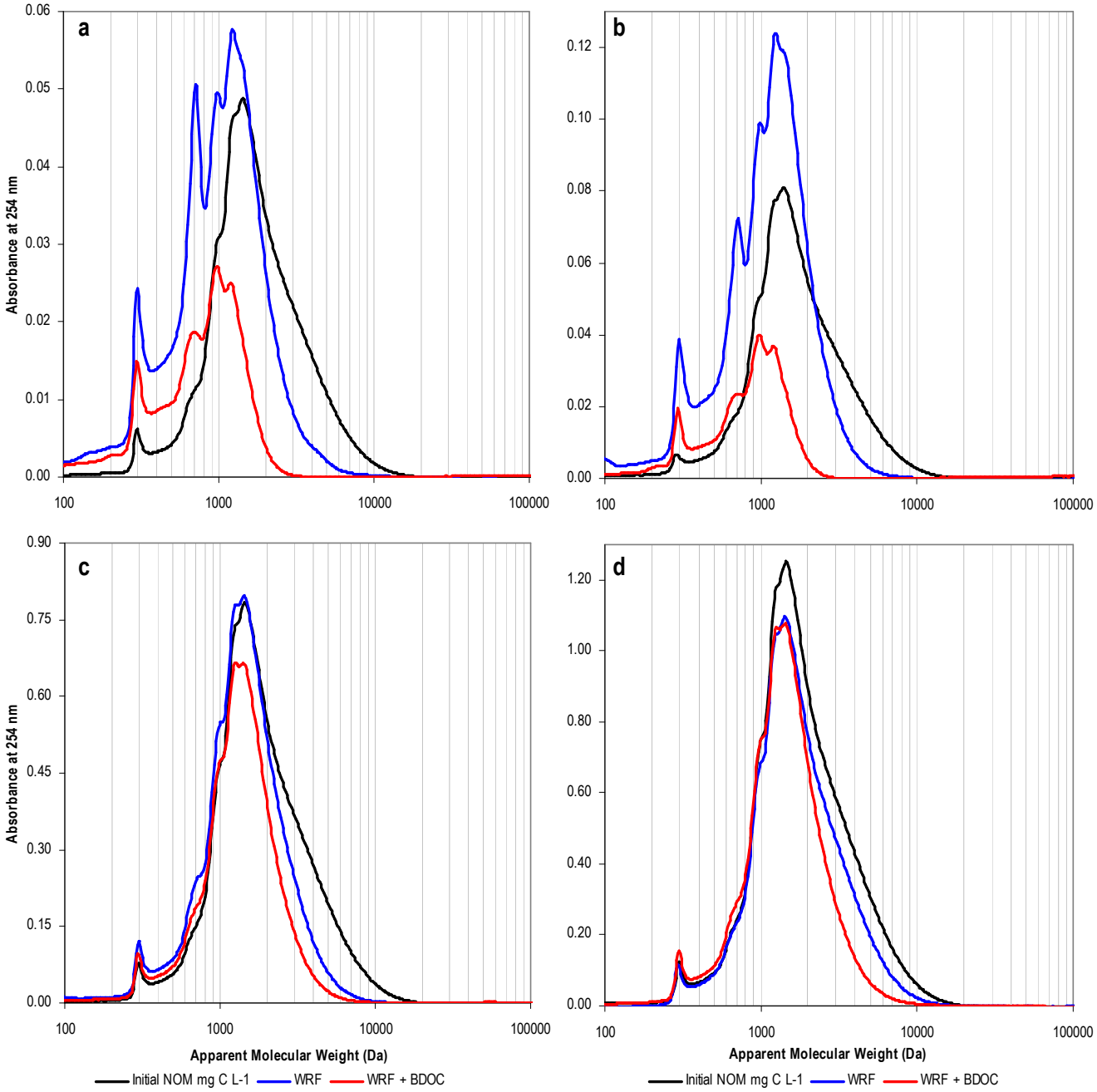

Figure 6-11. HPSEC-UV chromatograms following fungal treatment (Day 19) and sequential fungal plus bacterial treatment (Day 70) of NOM concentrate for (a) 30 mg C L^{-1} (b) 50 mg C L^{-1} (c) 300 mg C L^{-1} and (d) 500 mg C L^{-1}.

To compare the variation in MW and the extent of polymerisation, the reduction in the proportion of the higher MW material (Mw) was calculated after fungal and sequential fungal and bacterial treatment of NOM at various contents (Figure 6.12).

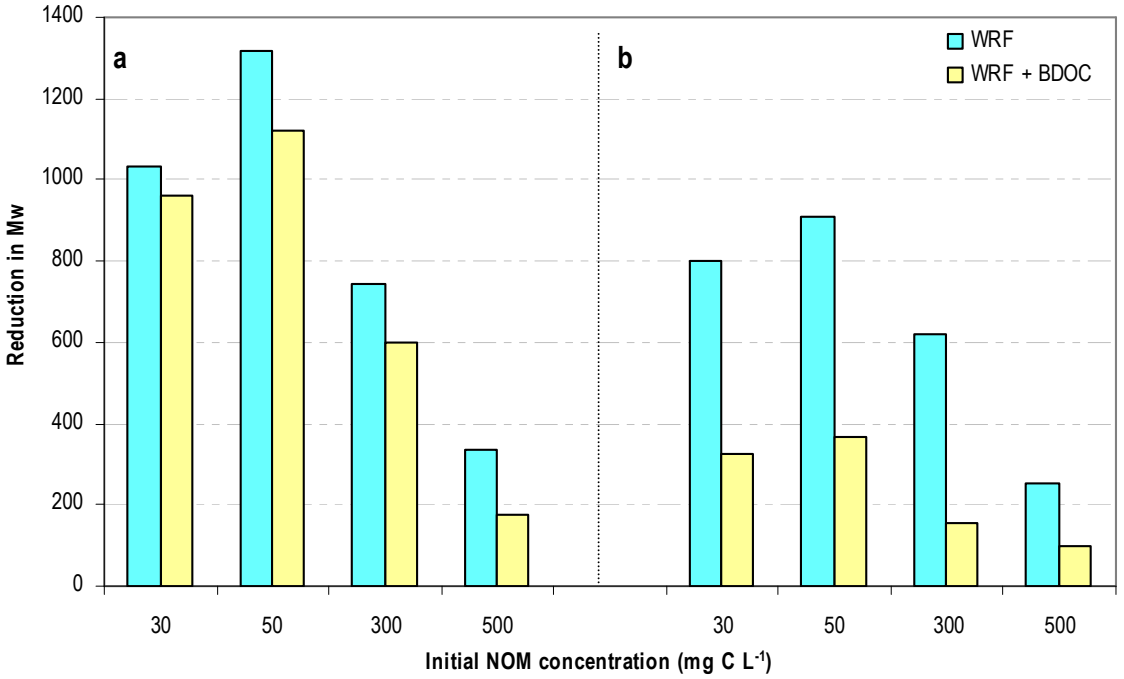

Figure 6-12. Reduction in M_w for NOM after fungal (Day 19) and subsequent fungal and bacterial treatment (Day 70) for (a) coloured molecules (A_{446}) and (b) UV-absorbing molecules (A_{254}).

NOM decolourisation by *Trametes* sp. was accompanied by considerable reductions in the weight average MW of the coloured (1317-335 Da) and UV-absorbing (911-255 Da) compounds (Figure 6.12a and b). With increasing NOM content the reduction in Mw declined at 446 and 254 nm, suggesting inhibition above 50 mg C L^{-1}. This is in accordance with previous results, where the decolourisation of NOM by *Trametes* sp. was inhibited at higher NOM concentrations. Subsequent bacterial treatment led to further reductions in Mw of the coloured (1119-174 Da) and UV-absorbing species (368-97 Da), which declined similarly with increasing NOM content.

For all NOM contents, NOM decolourisation by *Trametes* sp. was associated with the breakdown of the hydrophobic fractions of both the coloured and UV-absorbing species with the concomitant formation of lower MW hydrophilic compounds of reduced colour and UV absorbance (Figure 5.19, 6.12). This is further supported by the degree of polymerisation, which declined for both UV-absorbing and coloured species after fungal treatment. The ability of *Trametes* sp. to degrade NOM over a range of concentrations was attributed to the activities of Lac and MnP, which may have acted in a cooperative and possibly synergistic manner (Table 6-1). The enhanced biodegradability of NOM was most apparent for NOM contents of 30-50 mg C L^{-1}

and can be attributed to the greater accumulation of biodegradable compounds (present in the CHA and NEU fractions) from the subsequent depolymerisation of the higher MW hydrophobic fractions after fungal treatment and cell lysis. Oxidative techniques such as ozonation have similarly led to the generation of more bioavailable hydrophilic compounds for microbial assimilation from the decomposition of higher molecular mass structures (Hesse *et al.*, 1999). The production of lower MW compounds from NOM by photochemical processes has also been correlated with increased biodegradability (Frimmel, 1998).

6.6 Fluorescence spectroscopy of NOM after fungal and sequential fungal and bacterial treatment

After fungal, and sequential fungal and bacterial treatment, the NOM was further characterised by fluorescence spectroscopy (Figure 6.13). The excitation-emission matrix (EEM) spectrum of NOM was divided into five regions, with the peak location being defined by the excitation and emission boundaries according to Chen and colleagues (2003) (Table 6.3).

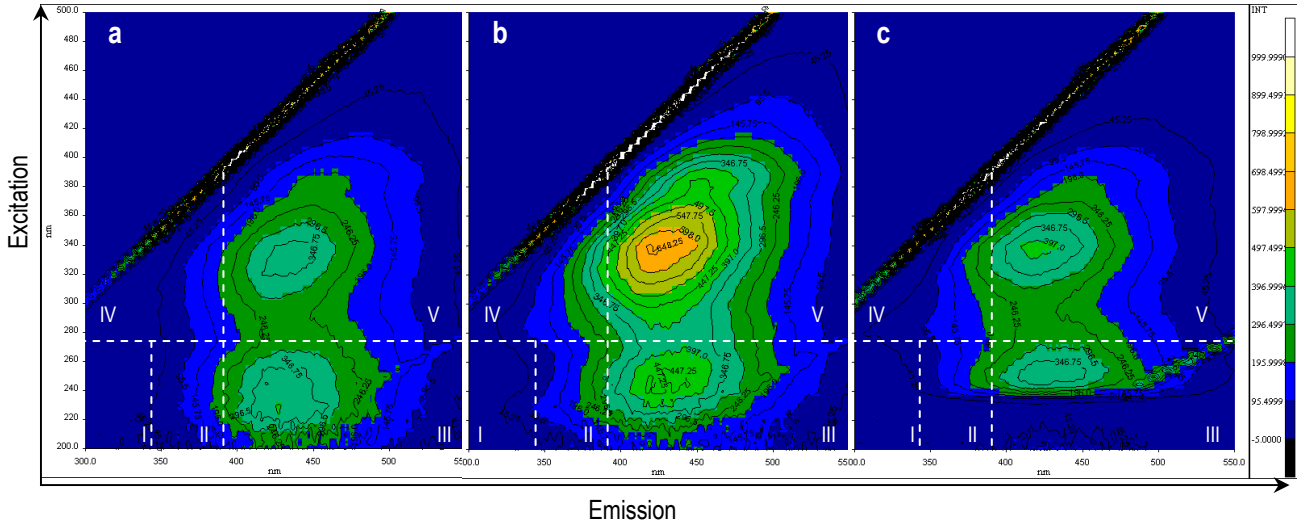

Figure 6-13. EEMs for NOM at (a) initial concentration at 30 mg C L^{-1} (b) after fungal treatment (Day 19) (c) after fungal and bacterial treatment (Day 70).

Table 6-3. Major peak locations of NOM

Region	NOM Peak	Excitation Peak (E_x)	Emission Peak (E_m)
I	Aromatic protein	200-270	300-330
II	Aromatic protein II	200-270	330-380
III	FA-like	200-270	380-540
IV	Soluble microbial product-like	270-340	300-380
V	HA-like	270-400	380-540

New fluorophores were formed after fungal treatment of NOM (30 mg C L^{-1}) as indicated by the EEM (Figure 6.13b). The decolourisation of NOM by *Trametes* sp. led to an increase in the humic-like (region V) and fulvic-like (region III) material with peaks at Ex_{max}/Em_{max} = 330/430 and Ex_{max}/Em_{max} = 240/430 respectively (Figure 6.13b).

These increases indicate polymeric products arising from enzyme-catalysed oxidative reactions (Piccolo *et al.*, 2000). These changes are consistent with the breakdown of some of the larger NOM components and the subsequent increase in both lower and medium MW moieties (Figure 6.11a and b). The polymerisation reactions would involve the formation of carbon-carbon or carbon-oxygen bonds (Piccolo *et al.*, 2000), which is consistent with the greater increase in A_{254} and DOC at lower NOM contents (Figure 6.8). Although the specific physiological functions of Lac are not completely understood, there are several indications that it is involved in the formation of humic matter (Claus, 2003).

The synthesis of extracellular enzymes, along with the breakdown of the fungal pellets which led to cell lysis, was most likely responsible for the increase in DOC, increase in the shoulders of soluble microbial product peak (Figure 6.13b, Region IV) and proteinaceous material (Figure 6.13b, Region II). Subsequent bacterial treatment led to an overall decrease in the fulvic-like (region III), humic-like (region V) and soluble microbial product-like (region IV) fluorescence (Figure 6.13c). The decrease in fluorescing material was attributed to the bacterial assimilation of some of the lower MW fungal products, as seen by the reduction in DOC (Figure 6.9), A_{446} and A_{254} (Table 6.2).

6.7 Conclusion for Chapter 6

Chapter 6 investigated the effects of initial NOM concentration on the decolourisation of aquatic NOM concentrate by *Trametes* sp. without nutrient addition. NOM decolourisation decreased with increasing NOM content; however, the degradation of the equivalent amount of NOM (mg C L^{-1}) measured as colour increased. For most NOM concentrations (13-300 mg C L^{-1}) the initial mechanism of NOM decolourisation was attributed to adsorption followed by enzymatic breakdown, whereas for 500 mg C L^{-1} the decolourisation was almost all due to adsorption. Following the initial adsorption stage, NOM decolourisation coincided with the onset of extracellular enzyme activities, in particular of Lac and MnP. Increasing activity of Lac and MnP was seen with increasing NOM content, with the activity of both enzymes declining at NOM concentrations greater than 30 and 50 mg C L^{-1}.

Fungal treatment led to increased A_{254} and DOC, the proportional increases being greater for lower NOM concentrations. For lower NOM concentrations (30-50 mg C L^{-1}), 70-61% of the released DOC was biodegraded, whereas for higher NOM concentrations (300-500 mg C L^{-1}) only 35-30% of the DOC was

assimilated by bacteria. Bacterial treatment after fungal treatment led to further reduction in the colour of NOM. Thus it seems that sequential fungal and bacterial treatment worked best for a NOM content of around 50 mg C L^{-1}, from which it preferentially removed the higher MW fractions of NOM. For all NOM concentrations, decolourisation of NOM by *Trametes* sp. resulted in the breakdown of the higher MW colour and UV-absorbing species, followed by formation of lower MW intermediates. Subsequent bacterial treatment led to further reductions of the higher MW fractions and most of the lower MW fungal products. EEMs indicated the formation of new fluorophores in the humic- and fulvic-like regions after fungal treatment, indicating possible polymeric products from enzyme-catalysed oxidative reactions.

Chapter 7 Degradation of Beaconsfield Reservoir water

Preface

The decolourisation of aquatic NOM concentrate by *Trametes* sp., *Polyporus* sp. and *B. adusta* was investigated in Chapter 4. To ascertain if these WRF are capable of decolourising NOM from a fresh water body, decolourisation experiments were conducted on Beaconsfield Reservoir water. The results were compared with the decolourisation of MIEX NOM concentrate at the same DOC concentration (13 mg C L^{-1}). Following fungal treatment, the biodegradability of the treated NOM (Beaconsfield and concentrate) was assessed with a consortium of bacteria via the BDOC test.

7.1 Decolourisation studies on Beaconsfield NOM at 30ºC

Decolourisation of Beaconsfield NOM by *Trametes* sp., *Polyporus* sp., and *B. adusta* was conducted in shake flask cultures at 30°C under nutrient-limited conditions. Controls using heat-killed fungi were run in parallel to the test cultures to determine passive adsorption.

The decolourisation of Beaconsfield NOM at 30ºC by *Trametes* sp., *Polyporus* sp., and *B. adusta* and the corresponding enzyme activities are illustrated in Figure 7.1. A high initial rate of colour removal was observed within the first 17 h of incubation by the three WRF (Figure 7.1, 1-3a). *B. adusta* had the greatest decolourisation of 80% with the highest rate of colour removal of 11% per hour, followed by *Polyporus* sp. with 75% decolourisation at 5% per hour, and lastly with *Trametes* sp. with 70% decolourisation at a rate of 4% per hour. These high rates of NOM decolourisation suggest that the underlying mechanism of removal was mainly via adsorption. This is further supported by the results for heat-killed biomass, which accounted for 67% of removal by adsorption in *Polyporus* sp. and 46-48% in *B. adusta* and *Trametes* sp. (Figure 7.1). Taking into consideration the removal of NOM by passive adsorption, the maximum enzyme-mediated decolourisation of NOM by the pellets of *B. adusta*, *Polyporus* sp. and *Trametes* sp. accounted for 34%, 22% and 8% respectively. Adsorption of NOM to autoclaved pellets is a physicochemical process which involves van der Waals interactions between non-ionic moieties present on the NOM molecule (Tan and Kilduff, 2007). Rojek *et al.* (2004) observed that approximately 60-70% of the removal of NOM (MIEX concentrate) by *P. chrysosporium* was predominantly due to sorption, 50% of which was attributed to physicochemical sorption.

The time-course of NOM adsorption by heat-killed pellets of *Polyporus* sp. most closely followed the profile of NOM decolourisation for live pellets, whereas for *Trametes* sp. and *B. adusta* NOM adsorption peaked within the first 25 h and was shortly followed by desorption (Figure 7.1, 1-3a). Desorption of colour from heat-killed biomass was attributed to pellet breakdown. The high rate of decolourisation was followed by a high degree of negative decolourisation for the three WRF. An inverse proportional relationship (R^2=0.99) was found for the rate of NOM decolourisation and the rate of negative decolourisation for the three WRF.

The initial rate of colour removal was observed to be 11-22 times greater for Beaconsfield NOM than for the NOM-tap water preparation (100 mg C L^{-1}) for the three WRF (Figure 5.12a). The greater rate of Beaconsfield NOM decolourisation was attributed to the lower concentration of DOC and lower nutrient availability, which probably triggered a faster degradation response. A comparative study of the decolourisation of Beaconsfield NOM and the NOM concentrate at the same DOC level was undertaken and is discussed in section 7.5.

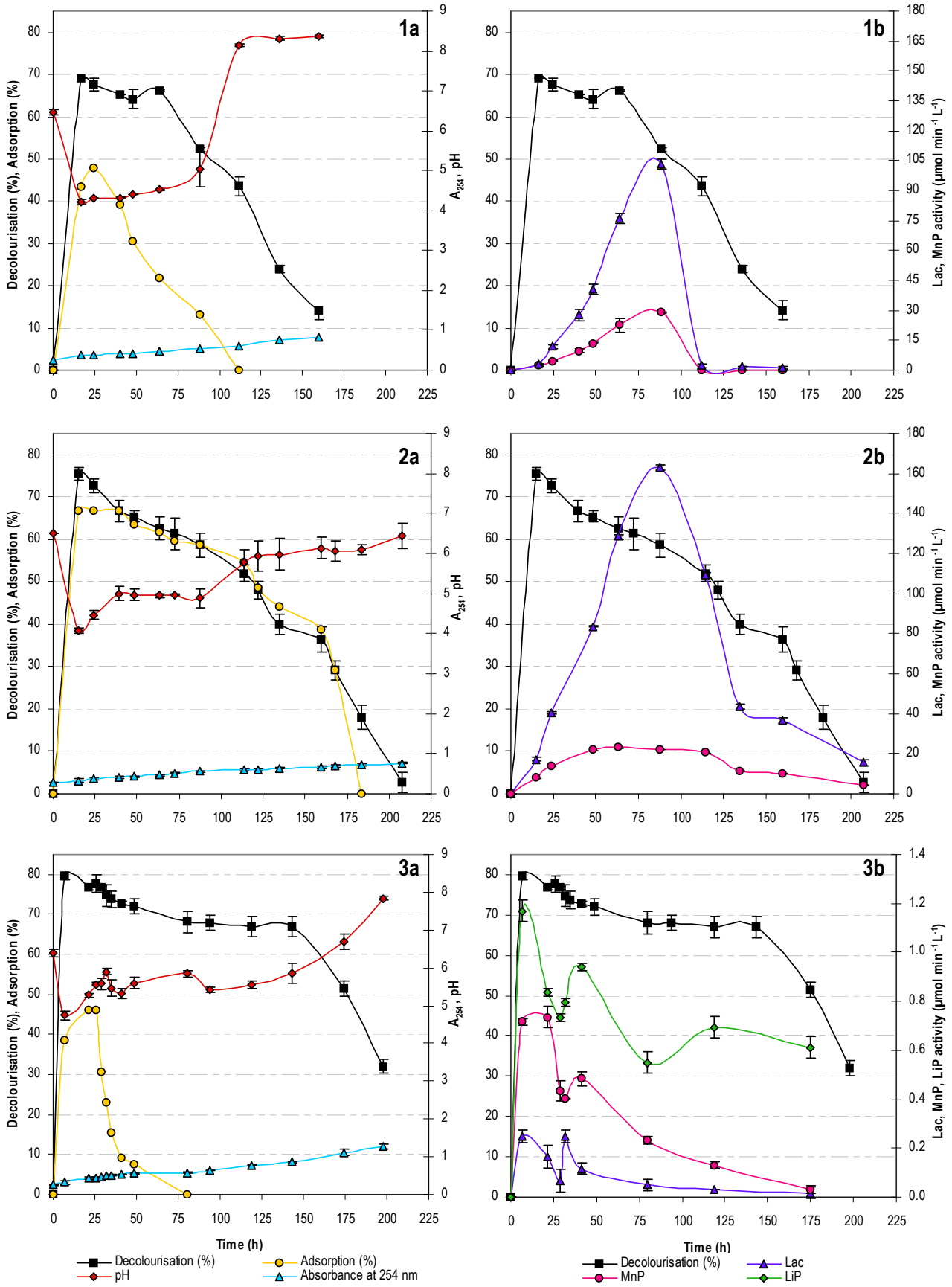

Figure 7-1. Decolourisation of (a) Beaconsfield Reservoir water and (b) associated enzyme activities by (1) *Trametes* sp. (2) *Polyporus* sp. and (3) *B. adusta* at 30ºC (note: scales for Lac, MnP and LiP activities are markedly lower than for the rest of the WRF). Plots of adsorption (%) refer to the amount of NOM removed (%) by heat-killed pellets. Data points are means of 3 replicates ± 1 SD.

7.2 Enzyme activities during decolourisation of Beaconsfield NOM

Decolourisation of Beaconsfield NOM was accompanied by extracellular enzyme activity, in particular of Lac and MnP (Figure 7.1, 1-3b). The later onset of Lac and MnP activity shortly after maximal NOM decolourisation at ≥ 17 h and ≥ 15 h for *Trametes* sp. and *Polyporus* sp., respectively, indicates the mechanism of colour removal to be chiefly via adsorption for these organisms (Figure 7.1, 1-2b). For *Trametes* sp. the subsequent rise in enzyme activities ≥ 25 h coincided with a small second peak of NOM decolourisation, which suggests that this may have been enzymatically driven. In contrast, the increase in enzyme activities for *Polyporus* sp. coincided with the onset of 'negative decolourisation'. This is further evidence for the mechanism of colour removal to be largely due to adsorption and to a lesser extent to enzymatic breakdown, as evidenced by 67% of colour removal by adsorption for this fungus (Figure 7.1, 2a & b). For both fungi the maximum activities of Lac and MnP occurred at approximately 88 h, during 'negative decolourisation'.

B. adusta expressed all three enzymes, which peaked during the time of maximum NOM decolourisation at 7 h and again at 40 h during negative decolourisation (Figure 7.1, 3b). The profiles of NOM decolourisation and passive adsorption, combined with the trends in enzyme activity, suggest that the mechanism of NOM decolourisation by *B. adusta* was by a combination of adsorption and enzyme breakdown. Although *B. adusta* had the lowest enzyme activities of the three fungi, it gave the greatest reduction in colour, as also seen for NOM concentrate, and this was probably associated with the action of a versatile peroxidase (VP).

The onset of NOM decolourisation and enzyme activities coincided with the drop in culture pH for the three WRF (Figure 7.1). For *Trametes* sp. and *Polyporus* sp. a reduction in Lac and MnP activities was seen shortly after 90 h, whereas the enzyme profile for *B. adusta* initially fluctuated with culture pH and then decreased at 41 h. For the three WRF, the rise in culture pH coincided with negative decolourisation and was attributed to fungal breakdown, given the low nutrient status and low concentration of NOM. This is consistent with previous findings in the decolourisation of NOM concentrate by *Trametes* sp. (section 6.3) where greater fungal disintegration was seen with lower NOM concentrations.

7.3 MW distribution of Beaconsfield NOM after treatment by WRF

The molecular size distribution of the UV-absorbing species was determined for Beaconsfield Reservoir water (13 mg C L^{-1}) following treatment by *Trametes* sp., *Polyporus* sp. and *B. adusta* at 30ºC. The removal of Beaconsfield NOM was accompanied by a shift of high MW hydrophobic species, predominantly humics >1500 Da, to lower MW hydrophilic species <1500 Da for the three WRF (Figure 7.2).

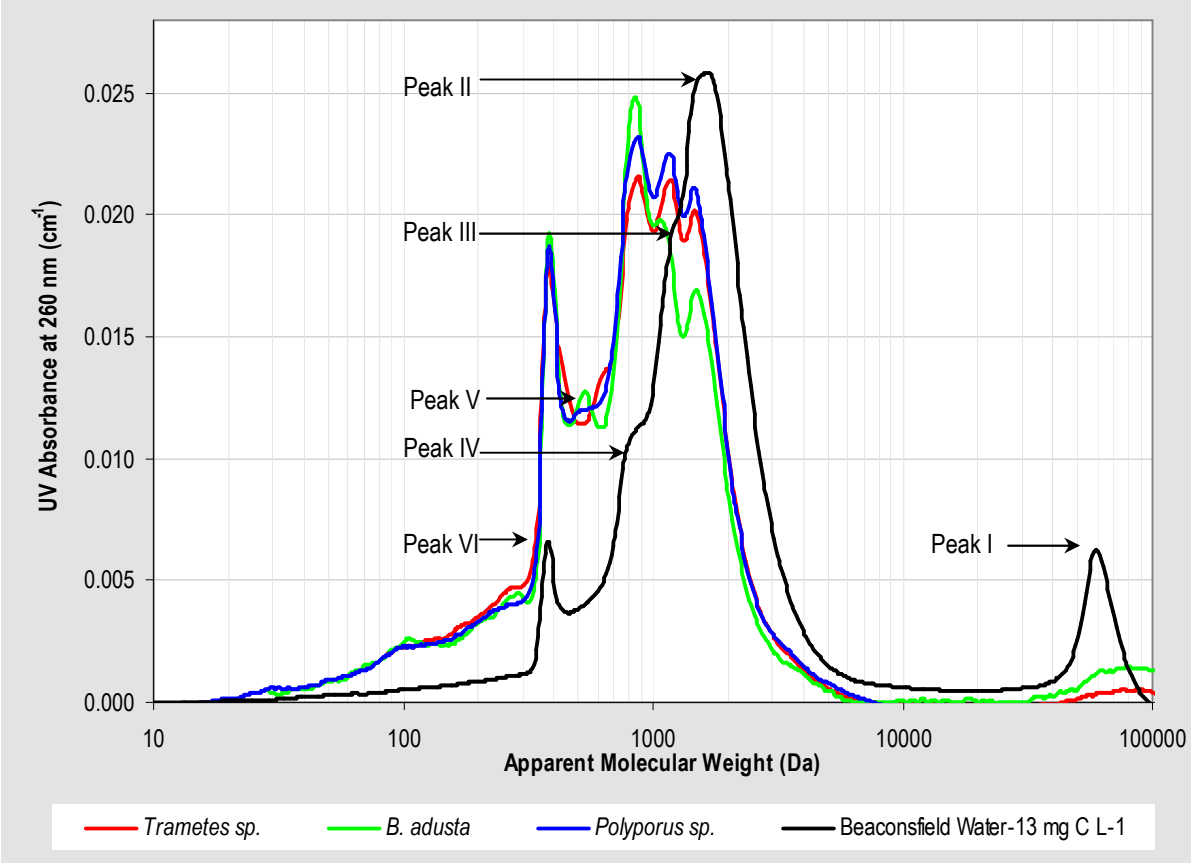

Figure 7-2. HPSE-UV chromatogram for Beaconsfield Reservoir water before and after treatment by WRF at 30ºC.

Biologically derived colloidal material (Peak I), which most probably contained some NOM-metal complexes (Allpike *et al.*, 2005), and had an AMW of 60,000 Da was largely removed by all fungi (83%, 95% and 100% for *B. adusta, Trametes* sp. and *Polyporus* sp., respectively). The high MW humic peak (Peak II) at 1540 Da was correspondingly reduced by 35%, 22% and 18% respectively for the three species, with the concomitant formation of lower MW humic intermediates at 840 and 1110 Da (Peak III and IV). For *B. adusta* there was also the formation of an intermediate peak at 509 Da (Peak V) corresponding to building blocks.

The increase in UV-absorbing material shown in these chromatograms correlated well with the increase in A_{254} and DOC for all WRF, and was particularly high for *B. adusta*. As suggested by Frimmel (1998), the increases were most likely ascribable to the break-up of larger chromophoric material and the subsequent

formation of products with higher molar absorptivity. The greater increase, especially at 800 Da, for *B. adusta* may be related to the action of LiP which converts dimethoxybenzene to a more highly absorbing p-benzoquinone (Kersten *et al.*, 1985). Furthermore, the HPSE chromatogram exhibited a very sharp increase in Peak VI at an AMW of 380 Da. This increase was most prominent for *B. adusta* (190%), followed by *Polyporus* sp. (183%) and with *Trametes* sp. (169%).

The molecular size distribution of the coloured species was also determined following treatment by the three fungi (Figure 7.3). The decolourisation of Beaconsfield NOM by the three WRF resulted in the shift from higher MW hydrophobic fractions to lower MW hydrophilic fractions. The greatest reduction in the average MW of the coloured species occurred for *B. adusta*, and also coincided with the greatest NOM decolourisation of 80%. Coinciding with the shift in AMW was also a drop in the absorbance of the predominant humic peak at 1600 Da by 4%, 5% and 15% for *Polyporus* sp., *Trametes* sp. and *B. adusta* respectively.

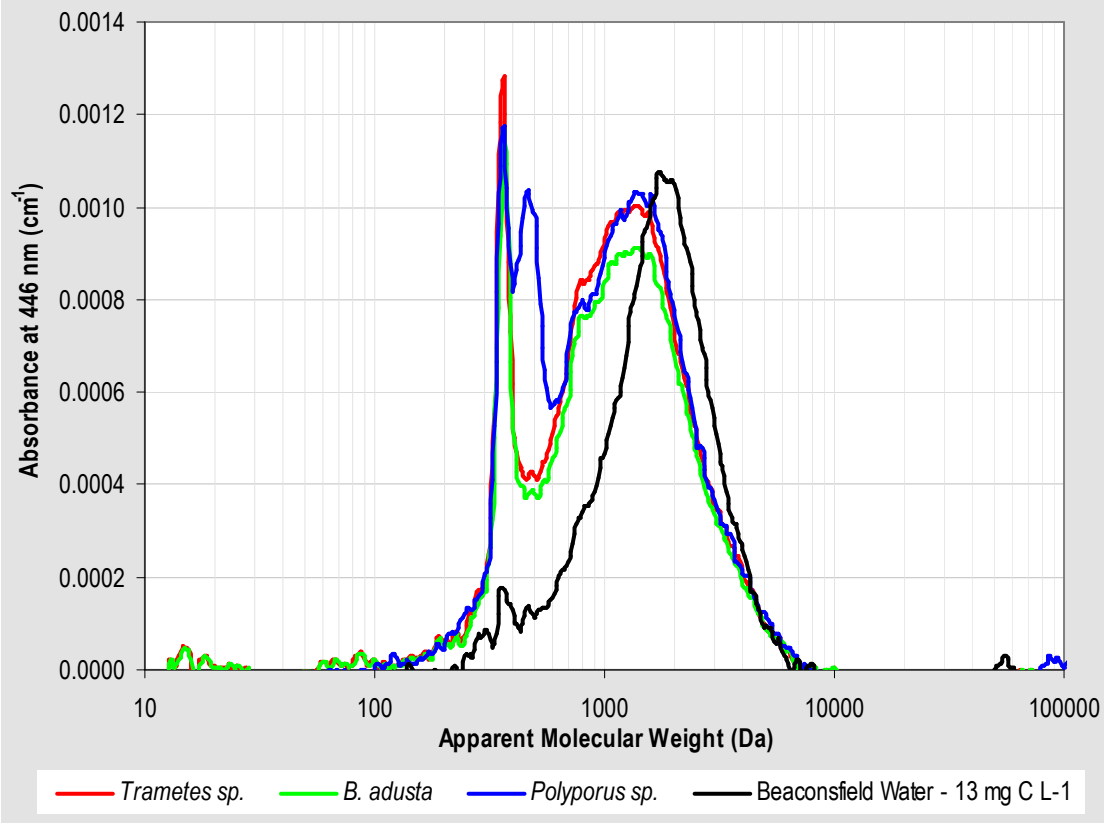

Figure 7-3. HPSE-A_{446} chromatogram for Beaconsfield Reservoir water before and after treatment by WRF at 30ºC.

Increases in absorbance over the AMW range of 100-1500 Da were observed and coincided with the high degree of 'negative decolourisation' for the three fungi (Figure 7.1, 1a-3a). The formation of an intermediate peak at 455 Da by *Polyporus* sp. was also observed.

The proportion of higher MW fractions (M_w) and lower MW fractions (M_n) of the coloured and UV-absorbing species was calculated for the three WRF (Figure 7.4). Decolourisation of the NOM by the three WRF was accompanied by considerable reductions in the weight average MW (M_w) of the coloured (516-572 Da) and UV-absorbing (614-683 Da) compounds for the three WRF (Figure 7.4a and b). Reduction in the proportion of the smaller MW material (M_n) was lower for the UV-absorbing compounds (381-418 Da) but higher for the coloured material (558-632 Da) in comparison to the reductions in M_w. As for the NOM-tap water preparation (Figure 5.17), *B. adusta* gave the largest reduction in M_w and M_n (30% and 44%, respectively) of the coloured species, which was consistent with the greatest decolourisation (77%). Although *Polyporus* sp. gave the lowest reduction in M_w and M_n (27% and 38%, respectively) of the coloured material, it gave the second greatest decolourisation of NOM (75%). These findings are similar to those obtained for the NOM-tap water preparation (100 mg C L^{-1}), where *Polyporus* sp. also had the second greatest colour removal (44%) accompanied by one of the lowest reductions in M_w and M_n (38% and 31%, respectively) (Figure 5.17). The two-fold increase in decolourisation of Beaconsfield NOM, combined with a similar reduction in M_w and M_n for both NOM samples, strongly indicates that the mechanism of Beaconsfield NOM decolourisation by *Polyporus* sp. was largely due to adsorption. This is further supported by the autoclaved controls, where 67% of Beaconsfield NOM was adsorbed onto the *Polyporus* sp. pellets. However, *Trametes* sp. gave the lowest decolourisation of Beaconsfield NOM (64%) and the second greatest removal of M_w and M_n (27%, 40%), as also observed for the NOM–tap water preparation.

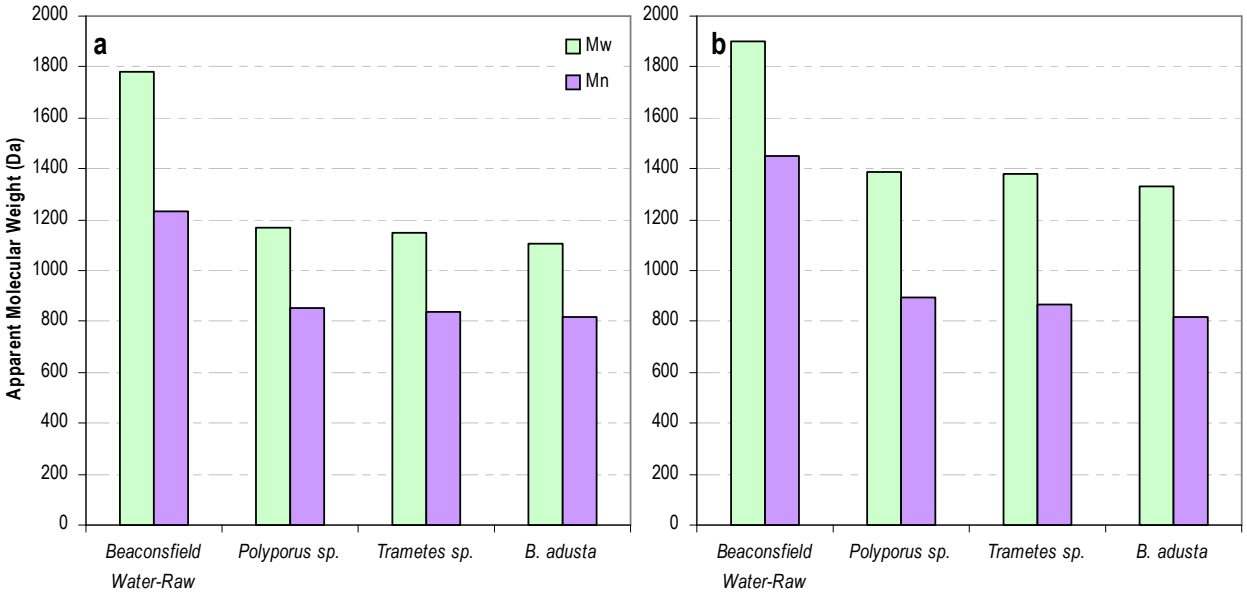

Figure 7-4. M_w and M_n of (a) the coloured and (b) UV-absorbing species after treatment of Beaconsfield NOM by WRF.

7.4 Decolourisation of Beaconsfield NOM at 15ºC by *Trametes* sp. with nutrient replacement

Trametes sp. was examined for its potential to decolourise Beaconsfield NOM at 15ºC, i.e., the approximate temperature of the water in Beaconsfield Reservoir (Figure 7.5, 1a and 1b). The effect of adding nutrients on the decolourisation of NOM was investigated by replacing 5 mL of the 200 mL culture with an equal volume of Beaconsfield water at the time of sampling (Figure 7.5, 2a and 2b).

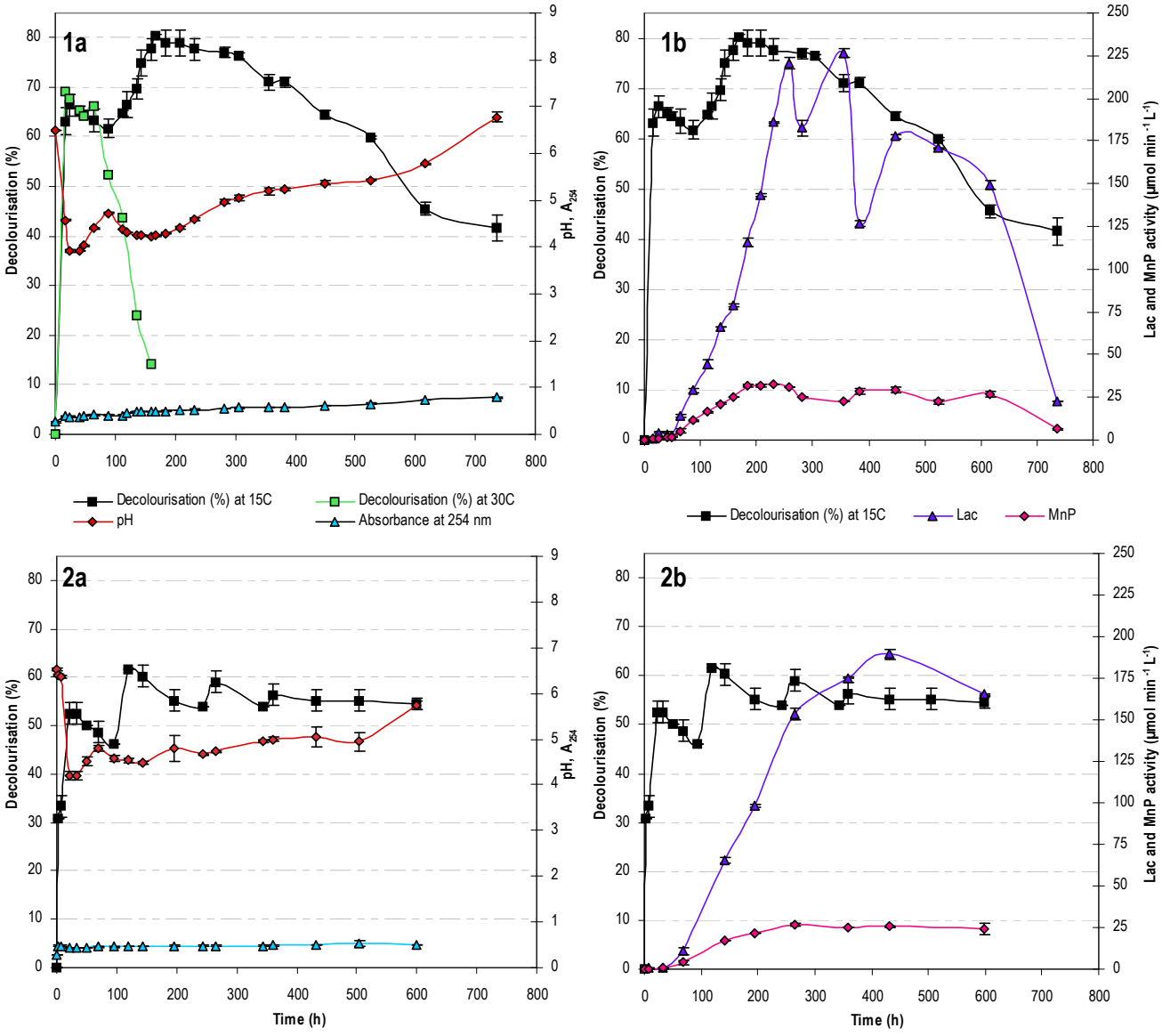

Figure 7-5. Decolourisation of (a) Beaconsfield NOM and (b) associated enzyme activity at 15ºC by *Trametes* sp. without Beaconsfield NOM replacement (1) and with Beaconsfield NOM replacement (2). Data points are means of 3 replicates ± 1 SD.

Trametes sp. gave greater decolourisation of Beaconsfield NOM (by 10%) at 15ºC than at 30ºC; however, it took nine times longer to reach maximum decolourisation. The initial peak of colour removal (70%) at 15ºC followed closely the profile of NOM decolourisation at 30ºC, and then it increased to a maximum value of 80% at 170 h, whereas at 30°C it declined to approximately 14% at 160 h (Figure 7.5, 1a). The initial mechanism of colour removal at the lower temperature was attributed to adsorption as for 30ºC, because of similar kinetics. This mechanism is further supported by the slow onset of Lac and MnP activity (>50 h) which coincided with the second peak of colour removal at 170 h (Figure 7.5, 1b). Both enzymes peaked shortly after maximum NOM decolourisation, as similarly observed at 30ºC. The maximum activity of Lac doubled to a value of 226 U L^{-1} at the lower temperature and this correlated well with the increased colour removal, whereas the activity of MnP only marginally increased (5%). Initial adsorption of NOM was further confirmed with heat–killed biomass, which accounted for 50% decolourisation. At the lower temperature there was less breakdown of the pellets and thus a correspondingly lesser increase in UV-absorbing compounds (by 12%), which correlated well with the lower rate (by 6-fold) of negative decolourisation relative to the experiment at 30ºC (Figure 7.5, 1a).

When Beaconsfield water was decolourised without the periodic replacement of NOM, the decolourisation of NOM ceased soon after reaching maximum colour removal at both 15ºC and 30ºC. The depletion of essential nutrients probably led to the cessation of colour removal, followed by cell autolysis and the release of adsorbed colour back into the culture solution, effectively contributing to negative decolourisation. When 5 mL of the culture was replaced with an equal volume of Beaconsfield water at the time of sampling, a considerable difference in the decolourisation profile was observed (Figure 7.5, 2a). Following the initial peak of colour removal (53%) at 23 h, the decolourisation of NOM fluctuated between 46 and 62% for approximately 340 h, after which it remained constant at 55%. This period of sustained fluctuation was probably due to the nutrients from the periodic addition of Beaconsfield water. Although negative decolourisation did not occur when the culture was supplemented with fresh NOM, maximal colour removal reached only 62% compared with 80% for unsupplemented NOM (Figure 1a and 2a). The periodic addition of NOM to the shake-flask culture or the use of a recycled flow-through system could be a way of minimising or even eliminating the phenomenon of ‘negative decolourisation’ and thus maintaining a constant state of colour removal, as observed here.

The enzyme activity profile was also affected by the addition of Beaconsfield NOM (Figure 7.5, 2b). Both Lac and MnP were initially detected at approximately 70 h, shortly after the initial stage of colour removal, and peaked after maximal decolourisation (62%), with MnP peaking prior to Lac. Whilst the activity of Lac declined soon after peaking, the activity of MnP remained constant and coincided with the stable level of NOM decolourisation of 55% at >360 h. With the periodic addition of nutrients there was less breakdown of the pellets and thus lower generation of UV-absorbing compounds (by 125%) compared with the unsupplemented NOM.

7.5 Comparison of Beaconsfield NOM and MIEX NOM concentrate after treatment by WRF at 30ºC

The decolourisation of Beaconsfield NOM and MIEX NOM concentrate at the same DOC level of 13 mg C L^{-1} by *Trametes* sp. and *B. adusta* at 30ºC was compared. Controls of heat-killed fungi were run in parallel to the test cultures to determine passive adsorption.

7.5.1 Profiles of Beaconsfield NOM and NOM concentrate decolourisation

The profiles of Beaconsfield NOM and NOM concentrate decolourisation were markedly different for *Trametes* sp., and to a lesser extent for *B. adusta* (Figure 7.6). *Trametes* sp. gave more than a two-fold increase (37%) in the removal of colour from Beaconsfield NOM and at a significantly greater rate (21-fold) than for NOM concentrate (Figure 7.6, 1a). Similar trends were observed for *B. adusta*, which gave a 23% increase in the decolourisation of Beaconsfield NOM at a greater rate (4-fold) (Figure 7.6, 2a) compared with *Trametes* sp. This greater rate and higher degree of Beaconsfield NOM decolourisation by both fungi was predominantly due to adsorption, whereas for NOM concentrate the initial stage of colour removal of 13 and 25% for *Trametes* sp. and *B. adusta,* respectively, was attributed to adsorption. The greater decolourisation and adsorption of Beaconsfield NOM compared with NOM concentrate may be attributed to the structure of NOM and ionic strength of the NOM solution. Zhou and Banks (1993) studied the mechanism of HA colour removal from natural waters by *R. arrhizus* and argued that the variation in biosorption between the two HAs was due to their difference in elemental composition and structure. Changes in ionic strength can also alter the function of enzymes and ligands and activate particular sites for catalysis or binding.

Solution pH may too play a role in the adsorption of NOM to fungal biomass. During maximum decolourisation, *Trametes* sp. and *B. adusta* reduced the solution pH for Beaconsfield NOM to approximately 4.5, whereas for NOM concentrate it was reduced only to pH 5.5. The lower pH of Beaconsfield NOM may have provided more favourable conditions for sorption to take place by increasing the binding capacity of HA onto fungal mycelia. At low pH the overall surface of the fungal biomass becomes positively charged, which may result in an electrostatic attraction between the negatively charged NOM and positively charged cell wall constituents such as chitin, lipids and amino acids (Aksu and Tezer, 2000). Rojek (2003) observed that a low pH (pH 3) promoted NOM binding to the fungal mycelium.

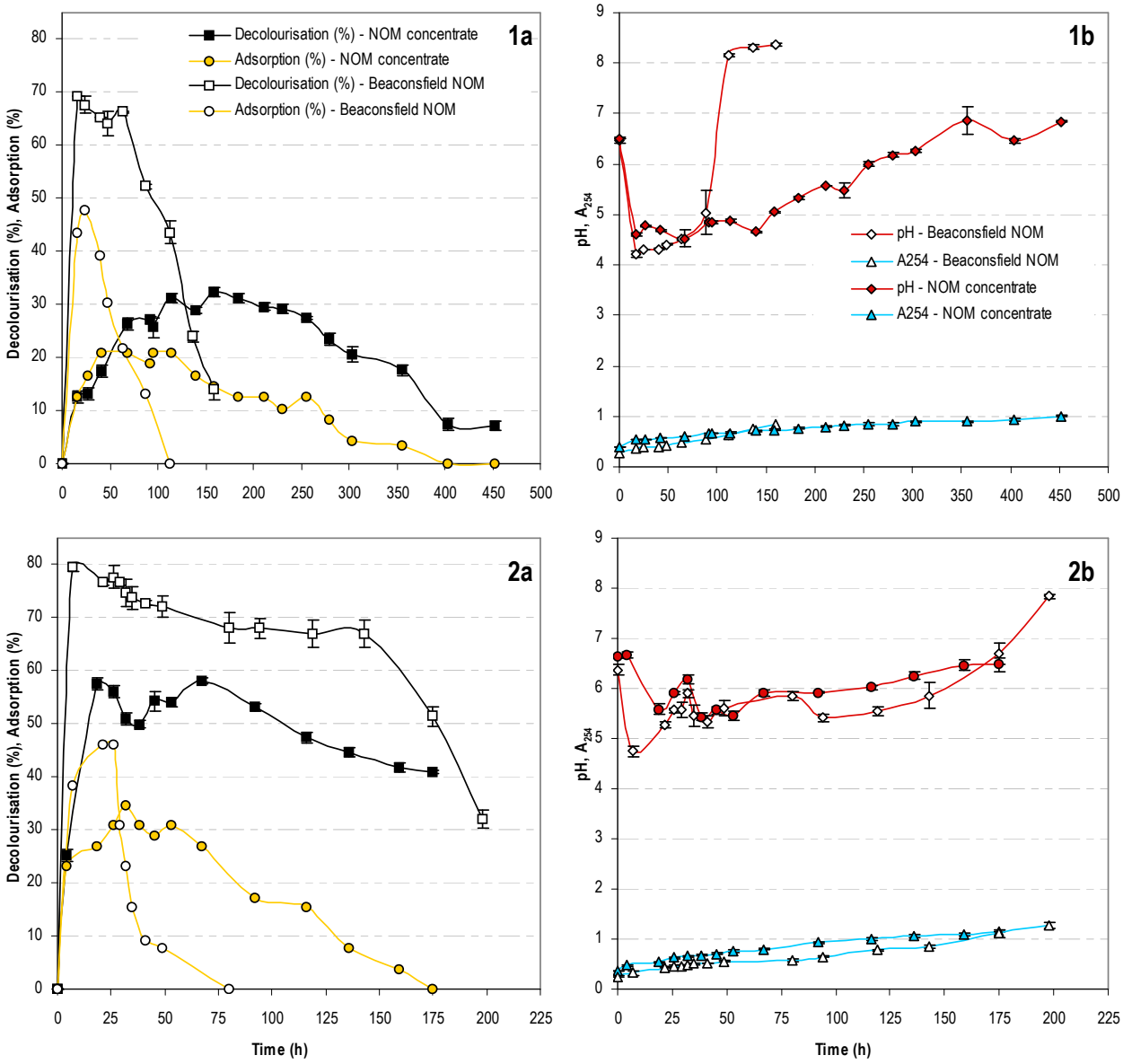

Figure 7-6. Profiles of (a) Beaconsfield NOM and NOM concentrate decolourisation and adsorption, (b) with associated A_{254} and pH during decolourisation by *Trametes* sp. (1) (from Figure 6-3a) and *B. adusta* (2) at 30ºC. Data points are means of 3 replicates ± 1 SD.

The rate of negative decolourisation was also greater in Beaconsfield NOM by 3 and 7-fold for *Trametes* sp. and *B. adusta*, respectively, and this correlated well with the greater increase in A_{254} (6%, 12%) and culture pH (Figure 7.6, 1b and 2b). ICP-MS analysis (Section 3.15) was used to determine the concentrations of trace elements present in Beaconsfield NOM (Table 7-1), as previously conducted for MIEX NOM concentrate (at 100 mg C L^{-1}) in Chapter 5, Section 5.4.

Table 7-1. Elemental analysis of NOM-tap water and Beaconsfield NOM at 13 mg C L^{-1}

Element	Beaconsfield NOM (BN) (mg C L^{-1})	NOM in tap water (TWN) (mg C L^{-1})	Ratio of BN:TWN
Na	78	36	2.1
Mg	11	4	2.8
K	4.2	2.4	1.7
			Ratio of TWN:BN
Mn	0.0014	0.007	4.9
Al	0.14	0.36	2.5
Ca	6.0	12.5	2.1
Fe	0.18	0.34	1.9
Ni	0.0026	0.011	4.2
Cu	0.031	0.12	3.9
Zn	0.014	0.04	3.1

*Elemental analysis for MIEX NOM concentrate was taken from Table 5-2. Note: Elemental concentrations for TWN were corrected to account for dilution.

In addition to NOM structure the difference in the degree and rate of colour removal between the two NOM sources may also be associated with the increased concentrations of some of the trace elements present in Beaconsfield NOM (Table 5-2). The greater concentrations of mineral nutrients, in particular of Mg^{2+} (3-fold), Na^{+} (2-fold) and K^{+} (2-fold), present in Beaconsfield NOM may have assisted in the greater degree of colour removal. Beaconsfield NOM, did however, contain much lower concentrations of the divalent cations Mn^{2+} (5-fold), Cu^{2+} (4-fold), and Fe^{2+} (2-fold). Apart from these elements, a 3-fold and 4-fold decrease in Al^{3+} and Ni^{+} concentrations was also observed, in comparison with NOM concentrate. Overall, the lower concentrations of trace elements present in Beaconsfield NOM may have triggered a faster biodegradation response upon the utilisation of available nutrients. This may explain the accelerated disintegration of *Trametes* sp. and *B. adusta* pellets and thus the more rapid release of fungal products and adsorbed colour back into the NOM solution.

7.5.2 Profiles of enzyme activity during Beaconsfield NOM and NOM concentrate decolourisation

Following the initial stage of adsorption (≤17 h and ≤4 h for *Trametes* sp. and *B. adusta* respectively), decolourisation of NOM concentrate occurred simultaneously with the onset of enzyme activity for these organisms (Figure 7.7a and b). For *Trametes* sp. Lac and MnP were active for much of the decolourisation period (290 h) and peaked during the time of greatest colour removal (Figure 7.7a), whilst for Beaconsfield NOM the enzymes were active for a shorter period (90 h) and peaked shortly after maximum NOM decolourisation (Figure 7.1,1b). This further supports the mechanism of NOM concentrate decolourisation by *Trametes* sp. being primarily due to adsorption followed by enzymatic breakdown, whereas for Beaconsfield NOM it was mostly due to adsorption with limited enzyme involvement. The higher concentrations of divalent cations Cu^{2+} and Mn^{2+} present in the NOM concentrate may have assisted in the greater involvement of enzymes in the decolourisation process. Levin *et al.* (2002) studied copper induction of lignin-modifying

enzymes in *Trametes trogii* and observed that addition of copper (up to 1 mM) strongly stimulated lignolytic enzyme production.

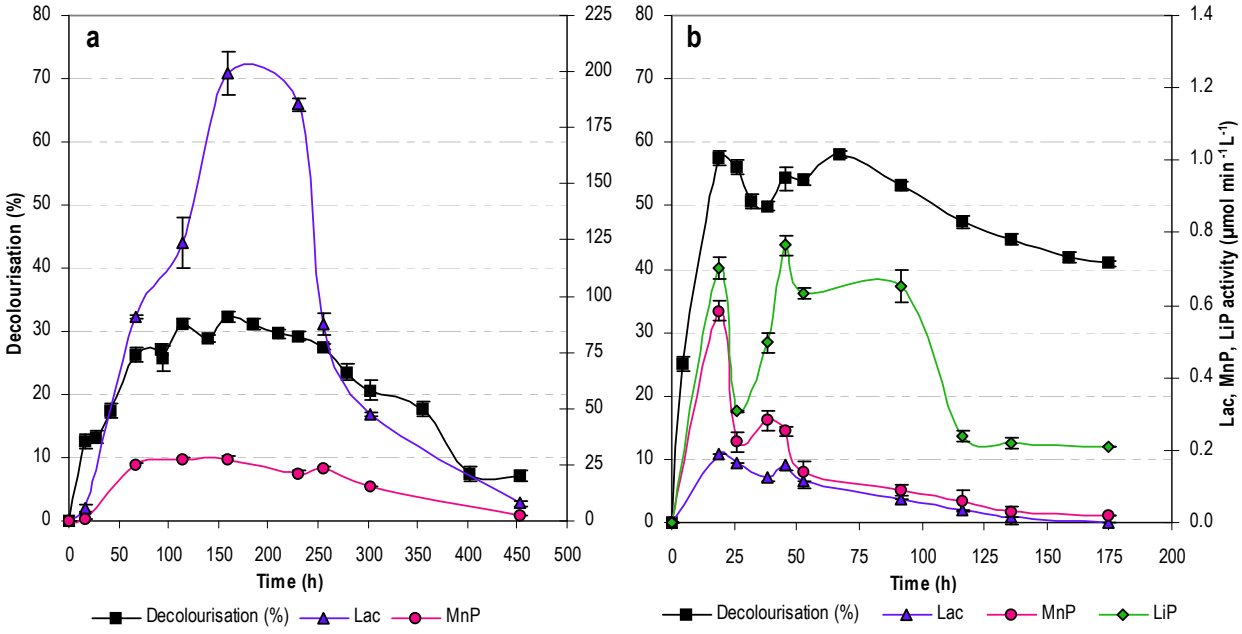

Figure 7-7. Decolourisation of MIEX NOM concentrate and associated enzyme activities by (a) *Trametes* sp. (from Figure 6-7) and (b) *B. adusta* at 30ºC (note: scales for Lac, MnP and LiP activities are markedly lower than for *Trametes* sp.) Data points are means of 2 replicates ± 1 SD.

The enzyme activity profiles during the decolourisation of NOM concentrate by *B. adusta* (Figure 7.7b) were not much different from those of Beaconsfield NOM (Figure 7.1, 3b). Although the activities of Lac, MnP and LiP were marginally lower for the decolourisation of NOM concentrate, they peaked during maximum colour removal at 19 h and again during the second peak of colour removal (38-45 h), as observed for Beaconsfield NOM. The similar profiles of NOM decolourisation and enzyme activities for both NOM samples by *B. adusta* in comparison to *Trametes* sp., could be attributed to the action of versatile peroxidase, which is known to have a very broad substrate range compared with MnP and LiP (Camarero *et al.*, 2000).

7.5.3 BDOC of Beaconsfield and NOM concentrate following treatment by WRF

Decolourisation of Beaconsfield NOM and NOM concentrate by *Trametes* sp. and *B. adusta* led to substantial release of DOC (65 and 139 mg C L^{-1}, respectively) (Figure 7.8) and increase in A_{254} (Figure 7.6, 1b and 2b). The release of DOC by *Trametes* sp. and *B. adusta* was 1.6-fold greater for NOM concentrate compared with Beaconsfield NOM, even though the latter was decolourised to a greater extent. *B. adusta* generated more DOC (13 and 26 mg C L^{-1}, respectively) from Beaconsfield NOM and NOM concentrate than *Trametes* sp., and this correlated with the greatest reduction in colour for both NOM samples (Figure 7.6,

2a). The increase in DOC levels was probably associated with the production of extracellular enzymes and the disintegration of fungal pellets upon depletion of available nutrients as discussed in Chapter 6 (Section 6.3). When the fungal-treated NOM samples were subjected to biologically active sand in the BDOC test for a period of 8 days, 55-70% of the DOC was removed by the bacteria (Figure 7.8). Work by Lonborg *et al*. (2009) on the production of bioavailable and refractory DOM by coastal heterotrophic microbial populations similarly observed a large proportion (34%) of the produced DOC to be more bioavailable than that in natural systems.

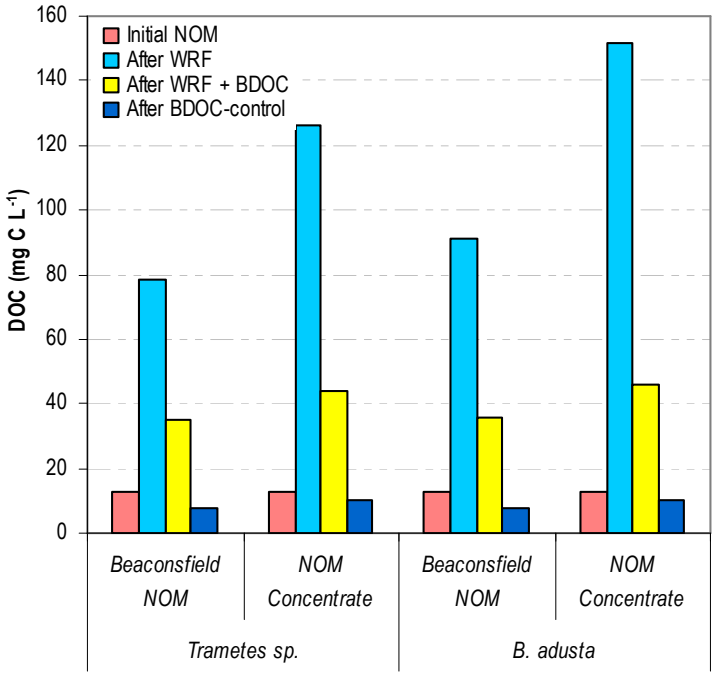

Figure 7-8. DOC concentrations after fungal and sequential fungal and bacterial treatment.

The variation in BDOC concentration is influenced by the quality and quantity of the carbon source. Although a large proportion of the released DOC (43-106 mg C L^{-1}) was assimilated by bacteria, the residual DOC content of 35 and 46 mg C L^{-1} for Beaconsfield NOM and NOM concentrate, respectively, following BDOC treatment was greater than the original NOM content of 13 mg C L^{-1} (Figure 7.8). This pool of residual DOC was refractory to further bacterial transformation under the experimental conditions used and suggests that the bacteria preferentially removed the lower MW fungal products and less of the more refractory and higher MW components of NOM. This is further supported by the marginal reduction of the higher MW fractions of NOM following BDOC treatment (Section 7.5.4).

Although a considerable amount of the increased DOC was assimilated by the bacteria, both *Trametes* sp. and *B. adusta* did not enhance the biodegradability of the treated NOM as evidenced by the BDOC controls of NOM without fungal pre-treatment (Figure 7.8). The biodegradability of Beaconsfield NOM (40%) without fungal pre-treatment is comparable to that in previous work by Tranvik and Jorgensen (1995), who reported

a biodegradability of 5-53% of DOC from fresh water. The lower biodegradability of NOM concentrate (20%) compared with Beaconsfield NOM (40%) may be attributed to the difference in NOM structure.

7.5.4 MW distribution of Beaconsfield NOM and MIEX NOM concentrate after fungal and sequential fungal and bacterial treatment

HPSE chromatograms of Beaconsfield NOM and MIEX NOM concentrate were initially compared to evaluate the differences in the molecular size distribution of the NOM (Figure 7.9). Table 5-1 was used to aid in the identification of peaks present in the raw water NOM samples according to Chow *et al*. (2008). Although both NOM sources were examined at the same DOC content, some important variations were observed in their AMW distribution (Figure 7.9).

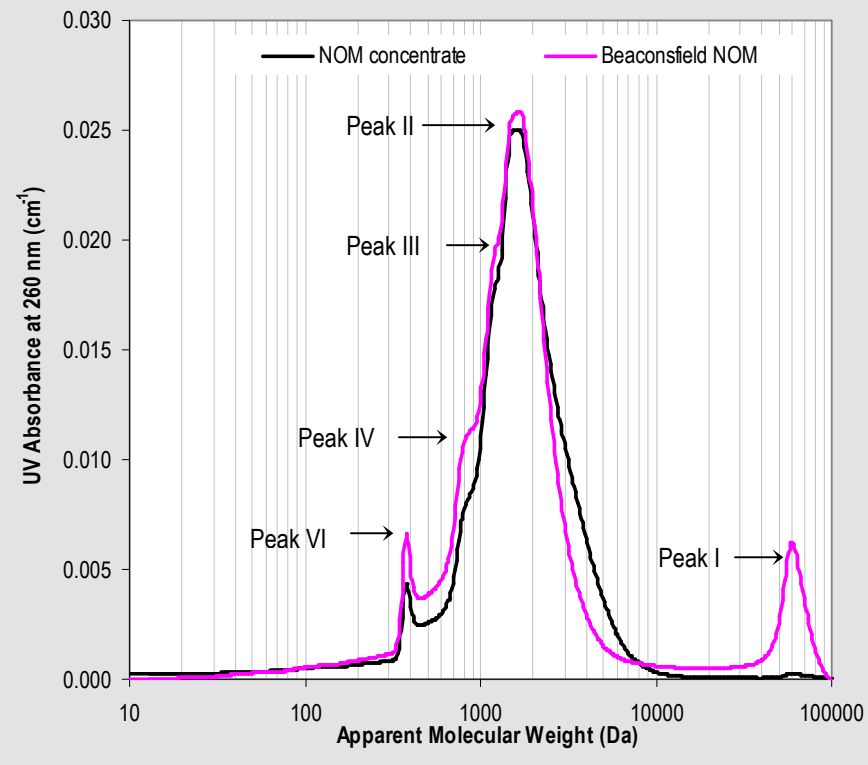

Figure 7-9. HPSE chromatograms of Beaconsfield NOM and MIEX NOM concentrate at 13 mg C L^{-1}.

The chromatogram for Beaconsfield NOM exhibited more defined peaks, particularly for the lower MW humics-1 (800 Da) and humics–2 (1200 Da) (Peaks IV and III) (Table 5-1), and a larger peak at 380 Da for lower MW acids and aromatics (Peak VI). Inorganic colloids and biological residues (Peak I) at 60000 Da, were present only in the Beaconsfield NOM, and have been associated with newly introduced organic matter having a short residence time in the water body (Fabris *et al.*, 2008). The variation in NOM characteristics can be explained by the nature of NOM concentrate, which represents approximately 80% of the NOM present in natural waters and does not contain the neutral fractions (Lee, 2005). The absence of Peak I in

the NOM concentrate is expected, as the MIEX resin does not entrap this fraction. NOM concentrate had a greater content (by 11.4%) of higher MW material (M_w) and a 13% greater weight average of the lower MW material (M_n) (Figure 7.11). The greater content of higher MW humic material present in NOM concentrate is further supported by the higher SUVA value of 2.92 L mg^{-1} cm^{-1}, compared with 2.23 L mg^{-1} cm^{-1} for Beaconsfield NOM (Table 3-1). The polydispersity (M_w:M_n) was slightly lower (1.42) for NOM concentrate compared with Beaconsfield NOM (1.45), indicating a slightly smaller degree of polymerisation.

The effect of fungal and sequential fungal and bacterial treatment on the apparent molecular size distribution of Beaconsfield NOM and NOM concentrate is illustrated in Figure 7.10. The apparent molecular size distribution of Beaconsfield NOM after treatment by *Trametes* sp and *B. adusta* was previously discussed in Section 7.3, and so the following discussion will focus on the comparison between the two NOM samples after fungal treatment. The decolourisation of NOM concentrate by *Trametes* sp. was accompanied by the breakdown of higher MW humic material >1600 Da (Peak II) with the formation of lower MW organic acids and humics with peaks at 380, 860 and 1090 Da (Figure 7.10, 1b). These intermediate fractions were defined by sharper and larger peaks than for Beaconsfield NOM (Figure 7.10, 1a) and correlated well with the greater increase in DOC (Figure 7.8). The higher MW humic peak at 1500 Da was increased in height, whereas for Beaconsfield NOM it was reduced.

Decolourisation of NOM concentrate by *B. adusta* was similarly accompanied by the reduction of the higher MW humic peak at 1500 Da (Peak II) with the formation of low MW humic fractions with peaks at 825 and 1090 Da (Figure 7.10, 2b), the former being smaller than the same peak in Beaconsfield NOM and the latter being larger than the observed peak at 970 Da for Beaconsfield NOM (Figure 7.10, 2a). Furthermore there were two intermediate peaks, at 82 and 107 Da, and the absence of the building block peak at 500 Da in the treated NOM concentrate solution. The higher MW humic peak at 1500 Da was marginally reduced, whereas for Beaconsfield NOM it was markedly reduced.

Sequential fungal and bacterial treatment of the two NOM samples led to significant reductions in the lower MW humics and possibly lower MW acids and N-containing aromatics (Table 5-2) in the range of ≥100 Da and ≤1600 Da (Figure 7.10) for both fungi. These intermediate and lower MW fractions are generally the most difficult to remove by most treatment methods (Pidou *et al.*, 2008). Greater reductions in the smaller MW species generated by *Trametes* sp. and *B adusta* occurred for the NOM concentrate preparation and this correlated well with the greater reduction in DOC than from Beaconsfield NOM (Figure 7.8). The preferential reduction of the lower MW fractions by subsequent bacterial treatment indicates their greater biodegradability relative to the larger NOM material at >1500 Da. The marginal reduction in the higher MW humics ≥1500 Da for the two NOM samples indicates that not all of these fractions were able to be converted to a more biodegradable form, and thus remain refractory to bacteria.

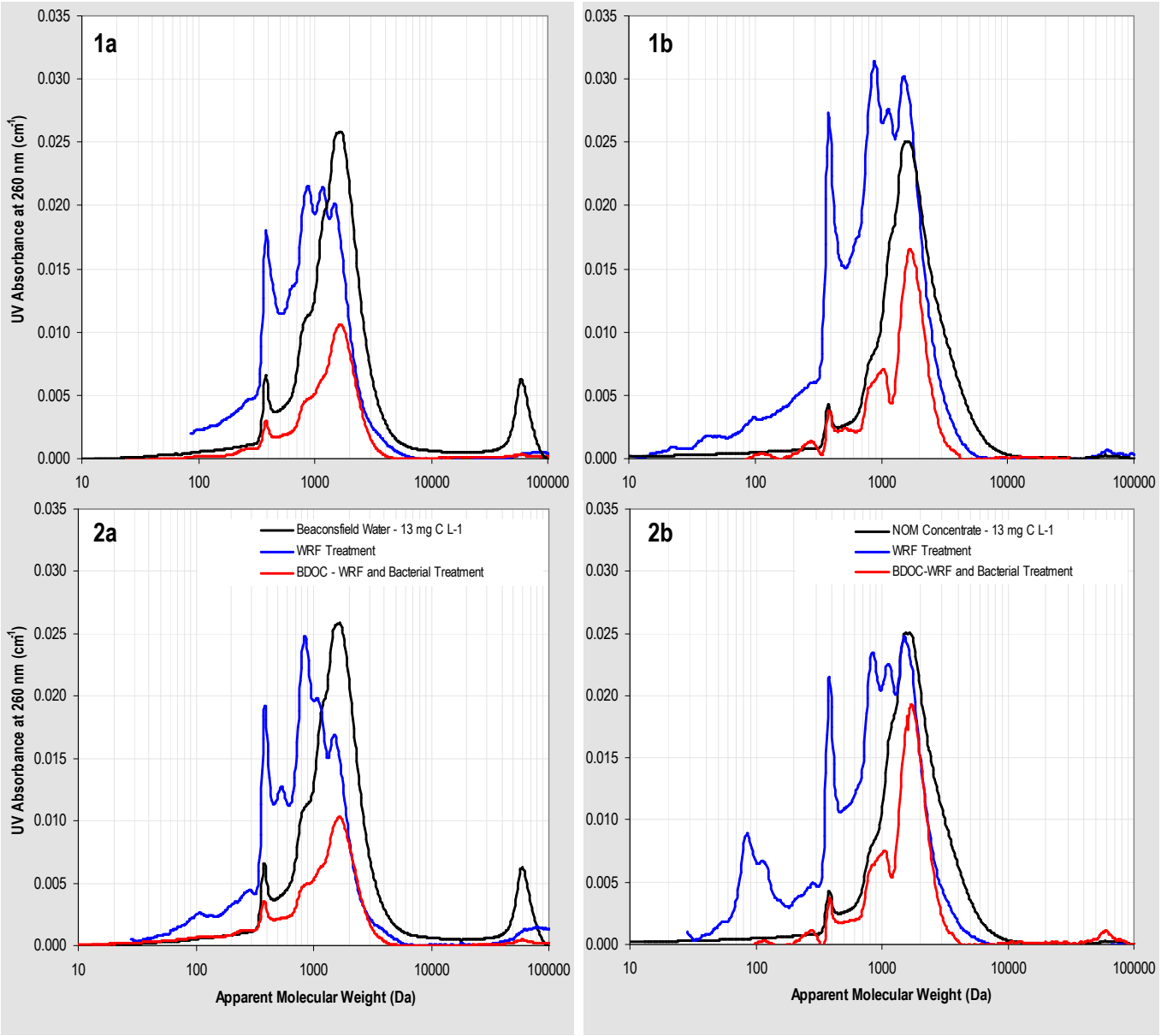

Figure 7-10. HPSE chromatograms of (a) Beaconsfield water and (b) NOM concentrate at 13 mg C L^{-1} after treatment by (1) *Trametes* sp. and (2) *B. adusta* Note: HPSE chromatograms for treated Beaconsfield NOM by *Trametes* sp. and *B. adusta* are repeated from Figure 7.2 for comparison.

The calculated weight average of the higher MW material (M_w) and lower MW material (M_n) of the Beaconsfield NOM and NOM concentrate after fungal and sequential fungal and bacterial treatment is summarised in Figure 7.11. *Trametes* sp. removed a greater proportion of the higher and lower MW material from NOM concentrate (40% and 38%, respectively) than from Beaconsfield NOM (36% and 32%, respectively), despite only achieving half the decolourisation of Beaconsfield NOM. The greater reduction in the higher MW fractions suggests the involvement of enzymes, rather than adsorption, as indicated for Beaconsfield NOM (Section 7.1). This was further confirmed by the greater activities of MnP, and particularly of Lac, which doubled during the decolourisation of NOM concentrate (Figure 7.6, 1a). In comparison with

Trametes sp., *B. adusta* reduced an equivalent amount (38%) of the higher MW fractions from both Beaconsfield NOM and NOM concentrate, even though it removed 22% more colour from Beaconsfield NOM (Figure 7.6, 2a). The equal reduction of M_w for both NOM samples by *B. adusta* may be attributed to the action of lignolytic enzymes, which for both NOM samples peaked during maximum colour removal. Overall, *B. adusta* had a marginally greater reduction in M_w and M_n for the decolourisation of Beaconsfield NOM, whereas *Trametes* sp. had a greater reduction in M_w and M_n for the decolourisation of NOM concentrate.

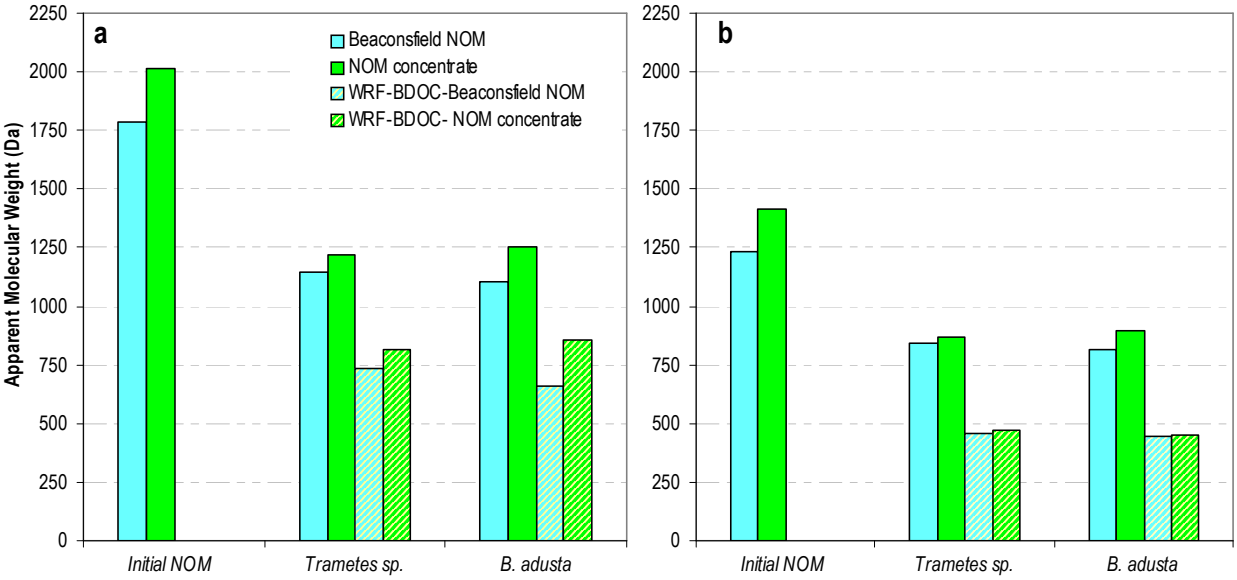

Figure 7-11. M_w (a) and M_n (b) of untreated Beaconsfield NOM and MIEX NOM concentrate at 13 mg C L^{-1} and following fungal (*Trametes* sp. and *B. adusta*) and sequential fungal and bacterial treatment at 30ºC.

Sequential fungal and bacterial treatment led to further reductions in M_w and M_n of the two NOM samples (Figure 7.11). Greater reductions in the proportion of the smaller MW material (M_n) in comparison to the reductions in higher MW material (M_w) were observed when the NOM samples were subjected to bacterial treatment. Reduction in M_n was lower for Beaconsfield NOM, and this correlated well with the lower reduction in DOC (Figure 7.8). In contrast to Beaconsfield NOM, however, NOM concentrate had a lower reduction in the proportion of higher molecular weight material. Contrary to these findings, when NOM concentrate at ≥ 30 mg C L^{-1} was subjected to BDOC treatment following initial decolourisation by *Trametes* sp., a substantially greater reduction of the higher MW humics in the range of >1500 Da was observed (Figure 6.11). With increasing NOM content (>50 mg C L^{-1}), however, the bacterial assimilation of these higher MW moieties declined. Thus, it seems that sequential fungal and bacterial treatment works best for a NOM content of around 50 mg C L^{-1}, from which it preferentially removes the higher MW fractions.

7.6 Conclusions for Chapter 7

The potential of WRF to decolourise NOM from Beaconsfield Reservoir was investigated. Treatment at 30ºC by cultures of *Trametes* sp., *Polyporus* sp. and *B. adusta* yielded high colour removals of 70%, 75% and 80%, respectively, followed shortly after by a high degree of negative decolourisation. Decolourisation was accompanied by extracellular enzyme activity, in particular of Lac and MnP as well as LiP for *B. adusta*; however, the late onset of enzyme activities for *Trametes* sp. and *Polyporus* sp. indicates the initial removal of NOM to be predominantly via adsorption, whereas for *B. adusta* it was by a combination of adsorption and enzymatic breakdown. Decolourisation of Beaconsfield NOM at 15ºC by *Trametes* sp. attained a greater degree of colour removal (by 10%) than at 30°C, but over a much longer time. When a small volume of the culture was periodically replaced with Beaconsfield NOM, the removal of colour initially fluctuated but later stabilised, and negative decolourisation did not occur. The rate and extent of NOM decolourisation by *Trametes* sp. and *B. adusta* was significantly greater in Beaconsfield NOM than NOM concentrate at the same concentration of 13 mg C L^{-1}. The variation in colour removal between the two water sources was attributed to different NOM structures and the lower concentrations of trace elements (in particular of cations Mn^{2+} and Cu^{2+}), present in the Beaconsfield NOM. This low nutrient status may have triggered a faster biodegradation response upon the utilisation of available nutrients, consequently leading to the breakdown of fungi. Decolourisation of Beaconsfield NOM and NOM concentrate by the WRF led to substantial release of DOC (65-139 mg C L^{-1}) of which 55% and 70%, respectively, was biodegradable. The breakdown of the two NOM samples was accompanied by the reduction of higher MW humic fractions and the formation of lower molecular intermediates, which subsequent BDOC treatment preferentially removed.

Chapter 8 Degradation of NOM by Lac, MnP and LiP

Preface

Chapters 5-7 focused on the decolourisation of NOM concentrate and reservoir NOM in shake-flask cultures. Several WRF have shown the ability to decolourise NOM, which was accompanied by extracellular Lac, MnP and LiP activity, of which the former two predominated. To date there appears to be no report on the decolourisation of aquatic NOM by purified Lac, MnP and LiP. In batch cultures, the concentrations of enzymes are constantly changing and it is difficult to ascertain whether one or more enzymes plays a role in decolourisation *in vivo* (Champagne and Ramsay, 2005). The *in vitro* action of these enzymes often show different results compared with *in vivo* studies. To further ascertain the involvement of each of Lac, MnP and LiP in the breakdown of NOM, *in vitro* decolourisation studies were conducted on the NOM-tap water preparation.

8.1 Decolourisation studies of NOM by Lac, MnP and LiP

Enzymes were purchased from Sigma Aldrich and tested for their ability to decolourise NOM concentrate at 30ºC. Lac was isolated from *T. versicolor*, but the source of MnP and LiP was unknown. Concentrations of Lac, MnP and LiP were determined as the total enzyme activity units (U mL^{-1}) with respect to substrate oxidation (Section 3.9.1) (Table 3-6).

8.1.1 Decolourisation of NOM by Lac

The decolourisation of NOM concentrate (50 and 100 mg C L^{-1}) by Lac of varying activity was monitored by the decrease in A_{446} at 30ºC at pH 4.5 (Figure 8.1). A control experiment with heat-denatured (100°C for 10 min) Lac at 11.7 U mL^{-1} showed no reduction in the colour absorbance.

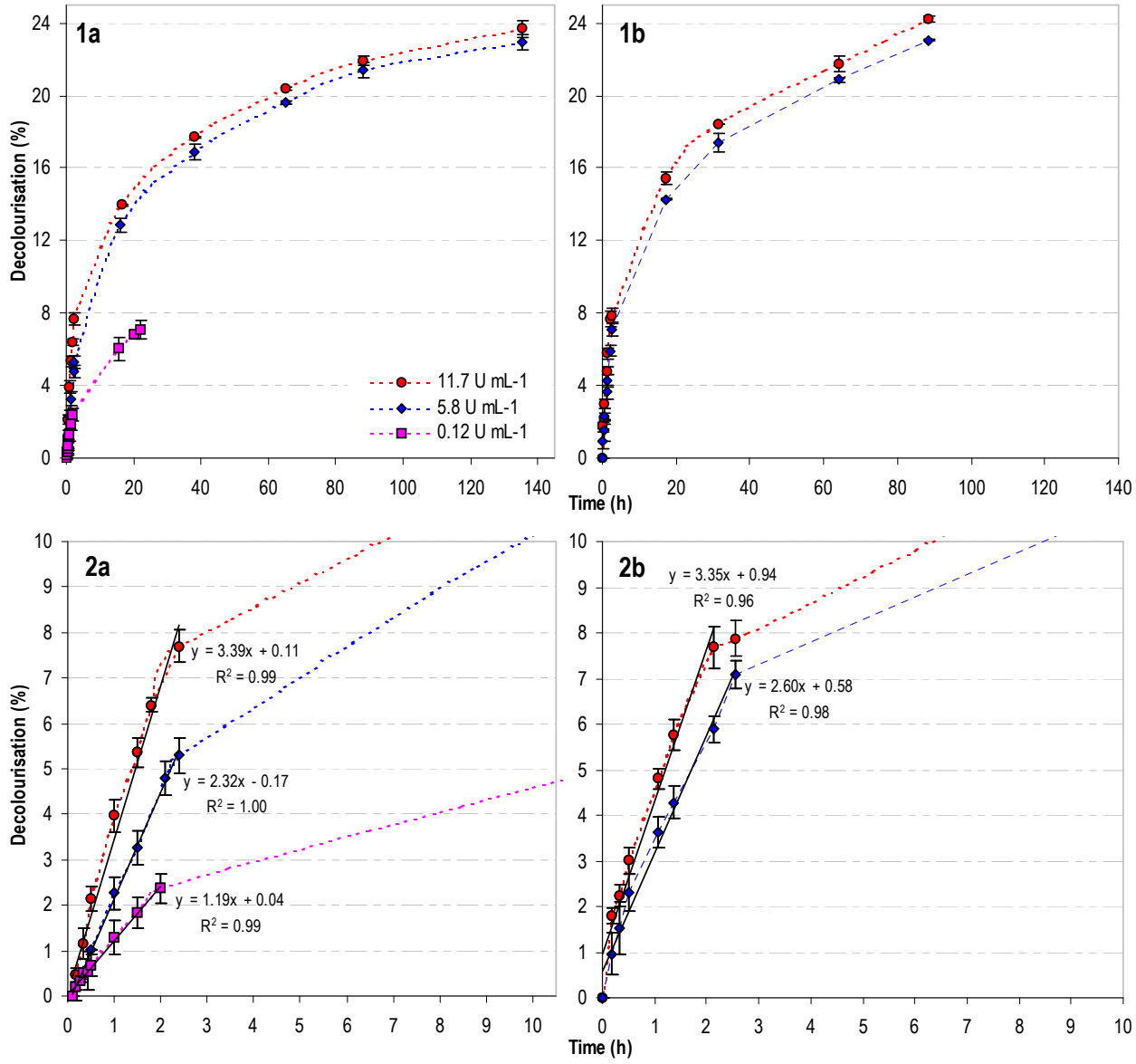

Figure 8-1. Decolourisation of the NOM - tap water preparation at 100 mg C L^{-1} (1a) and 50 mg C L^{-1} (1b) by Lac. Plots 2a and 2b show inset sections of plots 1a and 1b, respectively. Data points are means of 2 replicates ± 1 SD.

At the stock concentration of 11.7 U mL^{-1}, Lac decolourised 24% of the NOM concentrate (100 mg C L^{-1}) in just over 135 h (Figure 8.1, 1a). An equivalent amount of colour was removed by Lac at the lower concentration of 50 mg C L^{-1}, although at a faster rate (1.5 times) than observed at 100 mg C L^{-1} (Figure 8.1, 1b). The reduced rate of colour removal at the higher NOM concentration may indicate that the activity of Lac was inhibited. Zavarzina *et al*. (2004) reported that the inhibitory effect of HA was competitive and increased with increasing HA concentration (0.002-0.2 mg mL^{-1}).

The decolourisation of NOM by *Trametes* sp. showed similar findings to that of Lac alone, with a greater rate of NOM decolourisation (1.8 times) at 50 mg C L^{-1} and reductions in colour of 48-50% for NOM contents of 50 and 100 mg C L^{-1} respectively (Figure 6.5). Although *Trametes* sp. had twice the decolourisation of NOM in contrast with Lac alone, the probable mechanism responsible for the initial NOM decolourisation of NOM was adsorption. This is further supported by the low activities of Lac (0.17-0.2 U mL^{-1}) detected in the culture filtrates of *Trametes* sp., for NOM contents of 50 and 100 mg C L^{-1}. The decolourisation of NOM by Lac *in vitro* occurred in the absence of a redox mediator. Zavarzina *et al.* (2004) similarly observed that Lac alone without any mediators performed depolymerisation (as well as polymerisation) reactions of HA *in vitro.* In contrast, Claus *et al*. (2002) found that the decolourisation of a mixture of eight dyes by Lac from a strain of *T. versicolor* required the presence of mediators.

For NOM contents of 50 and 100 mg C L^{-1}, the initial rate of NOM decolourisation increased with increasing Lac concentration (Figure 8.1, 1a and 1b). Similarly, Champagne and Ramsay (2005) reported that the initial rate of Amaranth or RBBR decolourisation increased with increasing Lac concentration. For the first 2.5 h of incubation with 0.12-11.7 U mL^{-1} of Lac, NOM decolourisation was linear for both NOM concentrations used (Figure 8.1, 2a and 2b). During this time a marginal reduction in colour of 2.4-7.7% was observed for all Lac concentrations used. Following this initial linear period was a reduced rate of colour removal with the onset of a steady state period, which was particularly apparent at the highest NOM and Lac concentration (Figure 8.1, 1a). Studies by Schliephake (1997), on the decolourisation of Chicago Sky Blue dye by pure Lac similarly showed an initial linear rate of dye decolourisation that later reduced, with the onset of an apparent equilibrium.

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8.1.2 Decolourisation of NOM by MnP

The decolourisation of NOM concentrate (100 mg C L^{-1}) by MnP alone was minimal (4.6%) at the highest possible concentration of enzyme available of 0.01 U mL^{-1} (10 U L^{-1}) in the reaction mixture (Figure 8.2). A control experiment with heat-denatured MnP showed no reduction in the colour absorbance. MnP decolourised NOM to a lesser extent than Lac, possibly due, at least in part, to the lower concentration of MnP.

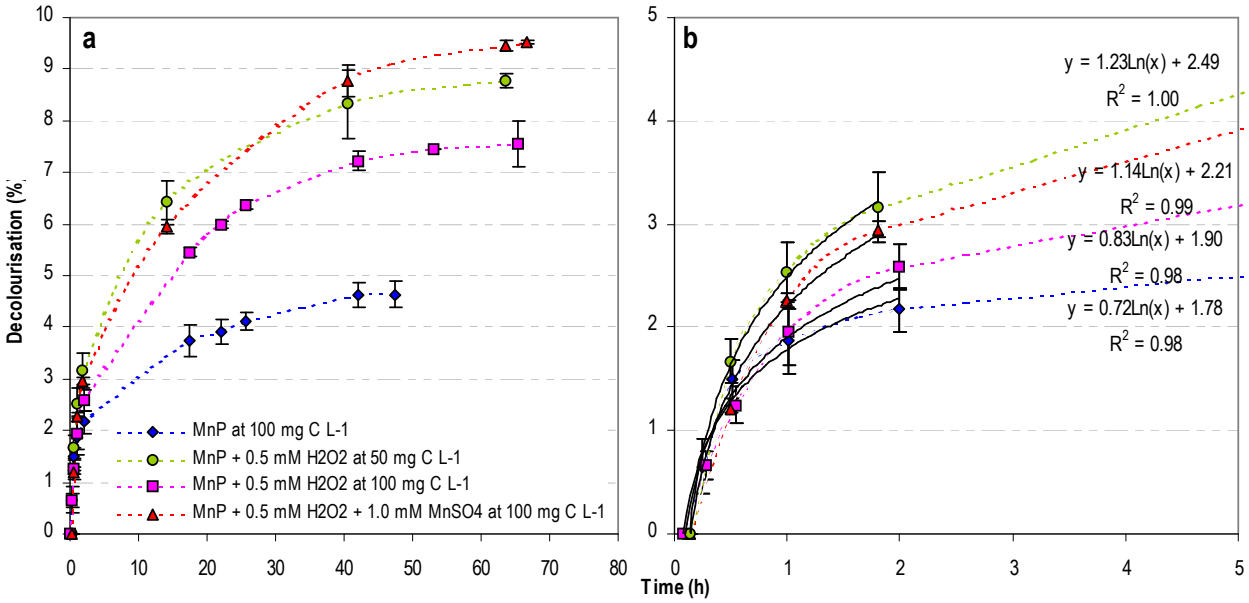

Figure 8-2. Decolourisation of the NOM-tap water preparation by MnP at 0.01 U mL^{-1} (a). Plot 8.2b shows inset sections of plot 8.2a. Data points are means of 2 replicates ± 1 SD.

The addition of 0.5 mM H_2O_2 to the reaction mixture enhanced the initial decolourisation rate and the degree of colour removed to 7.6% (Figure 8.2a and b). To exclude the possibility that NOM decolourisation was due to a non-enzymatic oxidation, NOM was incubated with H_2O_2 in the absence of MnP. The absorbance value did not change after a 20-hour incubation. The concentration of H_2O_2 used in the decolourisation study was not high enough to cause irreversible inhibition of MnP, which has been reported to occur at high H_2O_2 concentrations (Wolff *et al.*, 1986). By contrast, Ziegenhagen and Hofrichter (1998) reported a rapid decrease in MnP activity with H_2O_2 concentrations greater than 150 µM in the *in vitro* degradation of HA by MnP from *Clitocybula dusenii.* Decolourisation at the lower NOM concentration of 50 mg C L^{-1} by MnP and H_2O_2 increased the initial rate and extent of colour removal to 8.8%. The combined addition of H_2O_2 and Mn^{2+} (added as 1.0 mM $MnSO_4$) to the reaction mixture further increased the initial rate of NOM decolourisation at 100 mg C L^{-1} (Figure 8.2b), as similarly observed by Champagne and Ramsay (2005) in the decolourisation of Amaranth dye. The lower rate and extent of NOM decolourisation by MnP than by Lac could be due to a combination of factors including: the concentration of MnP, the concentration of H_2O_2, the concentration of $MnSO_4$, and the presence of organic acids in the reaction medium. Organic acids such as

malonate and oxalate, which are secreted by the WRF during secondary metabolism, stabilise Mn^{3+} (Wariishi *et al.*, 1992) and promote its (Mn^{3+}) release from the enzyme into the surrounding environment (Hofrichter *et al.*, 1999).

8.1.3 Decolourisation of NOM by LiP

LiP alone only marginally decolourised (~2%) NOM concentrate (100 mg C L^{-1}) at the greatest possible activity of 0.052 U mL^{-1} (Figure 8.3a). The limited involvement of LiP in the decolourisation of NOM was also observed in cultures of *T. versicolor* ATCC 7731, where the activity of LiP was found to be negligible (1.1 U L^{-1}) (Figure 5.15). Furthermore, as no LiP activity was detected for cultures of *Polyporus* sp. and *Trametes* sp., the decolourisation of NOM appears to be independent of LiP. This has previously been reported for lignin degradation, where some WRF degrade lignin without expressing LiP activity (Hatakka, 1994).

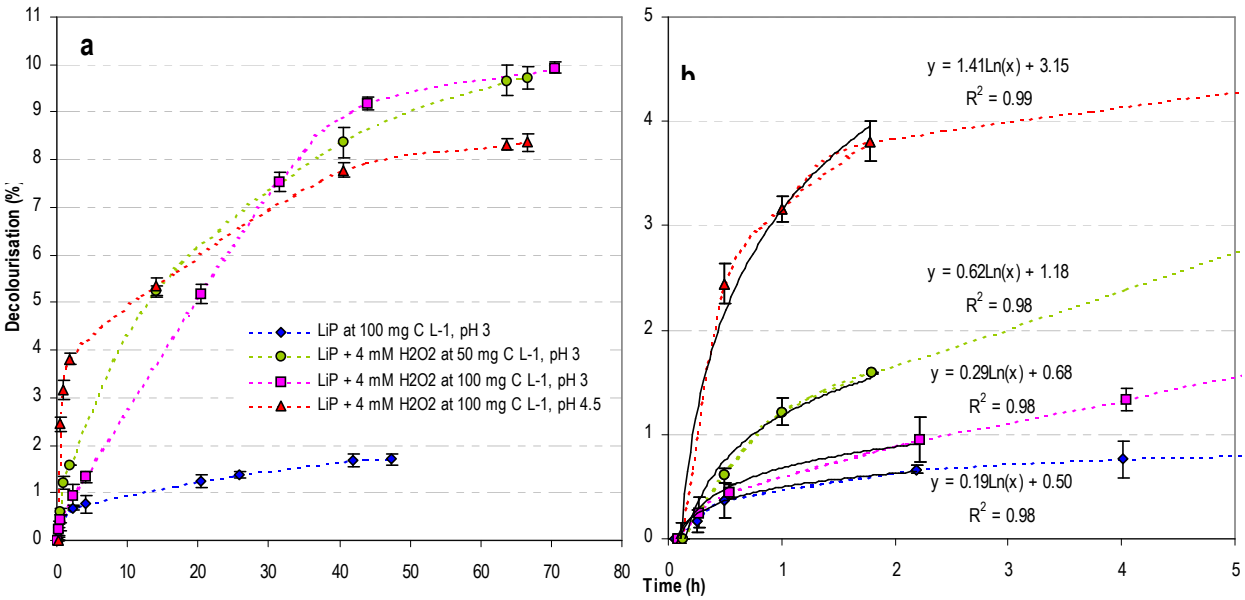

Figure 8-3. Decolourisation of the NOM-tap water preparation by LiP at 0.052 U mL^{-1} (a). Plot 8.3b shows inset sections of plot 8.3a. Data points are means of 2 replicates ± 1 SD.

Addition of H_2O_2 to the reaction mixture increased the initial rate and extent of NOM decolourisation to 10% (Figure 8.3a and b), as observed for Lac (Figure 8.2). This response was expected, as LiP requires hydrogen peroxide as a final electron acceptor to catalyse the oxidation of substrates. As illustrated for MnP (Section 8.1.2), H_2O_2 alone did not degrade NOM. Decolourisation of NOM by LiP was carried out at the enzyme's optimum pH of around 3 (Gold and Alic, 1993). When the reaction mixture was buffered to pH 4.5, as for Lac and MnP, an increase in the initial rate of NOM decolourisation and a lower degree of colour removal of 8.3% was observed (Figure 8.3a). Aitken and Irvine (1989) reported that the stability of LiP is greatly enhanced by increasing culture pH (from 3-4.5) and the concentration of the enzyme. As observed

for Lac and MnP, a greater rate (equivalent to that above with H_2O_2, for 100 mg C L^{-1}) of initial NOM decolourisation occurred at the lower concentration of 50 mg C L^{-1}. The lower rate of NOM decolourisation by Lac, MnP and LiP above NOM concentrations of 50 mg C L^{-1} suggests enzyme inhibition. In cultures of *Trametes* sp. the activities of Lac and MnP similarly declined at NOM concentrations greater than 30-50 mg C L^{-1} (Figure 6.5).

8.1.4 Decolourisation of NOM by Lac, MnP and LiP

All three enzymes decolourised NOM, with Lac attaining the greatest colour reduction of 24%, followed by MnP with 9.5% and LiP with 8.3% at the concentrations used (Figure 8.4). NOM decolourisation by Lac, MnP and LiP was also accompanied by a reduction in UV absorbance of 9.2%, 3.4% and 2.9% respectively (data not shown). The reduction in A_{254} was related to the removal of colour for the three enzymes and indicates that NOM containing conjugated bonds and aromatic rings were enzymatically broken. The lower decolourisation by MnP and LiP compared with Lac may be attributed to the lower specific activity of the enzymes (Table 3.5). Claus and Filip (1998) conducted an *in vitro* study on the degradation and transformation of aquatic HS, and suggested that the low decolourisation (5-10%) of HA by Lac could be due to the low specific activity of the enzyme (13 nkat mg^{-1}). They observed that a high activity Lac preparation (specific activity of 2500 nkat mg^{-1}) gave a greater (by 20%) decolourisation of lake water HA. The low decolourisation of NOM by MnP and LiP may also be attributed to their partial inhibition in the presence of NOM. HA inhibit peroxidase activity by competitive and non-competitive interactions, which may explain the lower rate and extent of NOM decolourisation by these enzymes (Blondeau, 1989).

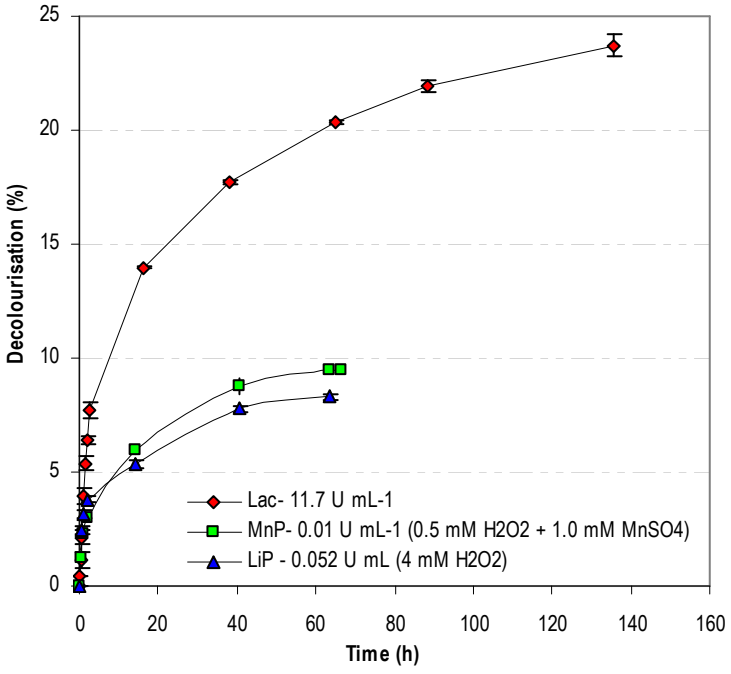

Figure 8-4. Decolourisation of NOM concentrate (100 mg C L^{-1}) by Lac, MnP and LiP at 30ºC.

Addition of H_2O_2 to the reaction mixture enhanced the rate and extent of NOM decolourisation for MnP and even more so for LiP. Similarly, addition of Mn^{2+} enhanced the decolourisation rate of MnP, but its addition was not required for NOM decolourisation to take place (Figure 8.2a). With time, the reduced rate of colour removal for both peroxidase enzymes was probably due to the consumption of H_2O_2 and Mn^{2+} as well as the consumption of readily degradable NOM. Moreira *et al*. (2000) studied the *in vitro* degradation of a polymeric dye by addition of H_2O_2, and noted that semicontinuous addition resulted in greater decolourisation and sustained decolourisation activity, whereas fed-batch additions caused rapid although limited decolourisation. Similar findings were obtained in cultures of *Trametes* sp. where the periodic addition of NOM minimised the phenomenon of 'negative decolourisation' and maintained a constant state of colour removal, combined with stable MnP activity (Figure 7-5, 2a and 2b).

Although the decolourisation studies were only carried out in the presence of one enzyme, it is highly probable that the presence of all three enzymes in the reaction mixture would result in a greater loss of colour. Champagne and Ramsay (2005) studied the decolourisation of Amaranth dye and showed that the rate of Amaranth decolourisation in the presence of both Lac and MnP was additive rather than synergistic. Based on this observation, NOM decolourisation in the presence of all three enzymes would yield a colour reduction of approximately 42% (Figure 8.4). This colour reduction is within the range of 40-45% attained by *T. versicolor* ATCC 7731, *Trametes* sp. and *Polyporus* sp. for the decolourisation of NOM–tap water (Figure 5.12a). The possibility of a cooperative interaction between the three-lignolytic enzymes cannot, however, be excluded. The complex nature of the system makes it difficult to ascertain the exact reactions involved in the decolourisation process; however the structural similarity of lignin and NOM suggests that similar mechanisms might be involved in its depolymerisation. The decolourisation of NOM may initially involve different hydrolytic enzymes that catalyse the cleavage of peptide, carbohydrate or other aliphatic side chains in HS (Claus and Filip, 1998). The aromatic nature of NOM, however, would necessitate the action of lignolytic enzymes, which would probably involve the cleavage of alkyl -aryl and C_α-C_β bonds of phenolic moieties by Lac (Kawai *et al.*, 1988), the oxidation of phenolic rings by the strong oxidant Mn^{3+}, alkyl phenyl cleavage by MnP (Gold *et al.*, 1989), and ring cleavage in β-O-4 compounds as well as aromatic ring opening by LiP (Kirk and Farrell, 1987).

8.2 MW distribution of NOM after *in vitro* decolourisation by Lac, MnP and LiP

The molecular size distribution of the UV-absorbing and coloured species following *in vitro* decolourisation of NOM by Lac, MnP and LiP is illustrated in Figure 8.5. Controls of Lac, MnP and LiP, reconstituted in Milli-Q water at the equivalent concentration added to the reaction mixture, showed little UV-absorbance for the AMW range of 300-10000 Da (Figure 8.5a). The three enzymes predominantly removed the higher MW fractions (≥1500 Da) of the UV-absorbing species, and to a lesser extent the lower MW fractions in the AMW range of ≥380 Da and ≤1600 Da. There was little variation in the reduction of the UV-absorbing material between the three enzymes, thus implying a similar degree of breakage of aromatic rings and double bonds for all three enzymes. The preferential removal of higher MW humics was also observed for cultures of *T. versicolor*, *Trametes* sp., *Polyporus* sp. and *B. adusta* (Figure 5.16). The enzyme-catalysed degradation of NOM was accompanied by the reduction of UV-absorbing material across a broad range of MWs of 300-10000 Da (Figure 8.5a), whereas fungal treatment led to increased generation of UV-absorbing species in the lower MW range (Figure 5.16). Lac, MnP and LiP preferentially reduced the higher MW humic peak (II) at 1620 Da by 54%, 60% and 61% respectively, with a smaller reduction of the lower MW humic peak (III) by 39%, 53% and 54%, respectively. Peaks IV and VI were also reduced but to a lesser extent than peaks II and III. The reduction in the average MW of the UV-absorbing species corresponded with the reduction in A_{254} for the three enzymes (Section 8.1.4), and indicates a depolymerisation-like structural transformation of the NOM.

The MW distribution of the coloured material following enzymatic decolourisation of NOM is illustrated in Figure 8.5b. Unlike for the HPSEC-UV traces, considerable variation in the removal of the coloured material was observed between the three enzymes, thus the extent of breakage of UV-absorbing bonds was not directly correlated to the reduction in colour. The reduction in the average MW of the coloured species was most marked for Lac, which also gave the greatest NOM decolourisation of 24%. Lac preferentially removed the coloured material across a broad range of MWs of 200-10000 Da, whereas MnP predominantly removed the higher MW species (>2000 Da) with little reduction below 2000 Da. The lowest reduction in the higher MW fractions was observed for LiP, which had an increase in the absorbance over the AMW range of 800-3000 Da. The slight reduction in high MW fractions and increase in absorbance by LiP, however, is questionable as the chromatogram of the initial NOM peak at 446 nm is slightly displaced to the right. Coinciding with the shift in AMW was a drop in the absorbance of the predominant humic peak at 2600 Da by 73% and 23% for Lac and MnP respectively, whereas for LiP the height of this peak increased by 7%. Overall, these reductions correlate well with the degree and rate of NOM decolourisation attained by the three individual enzymes (Figure 8.4).

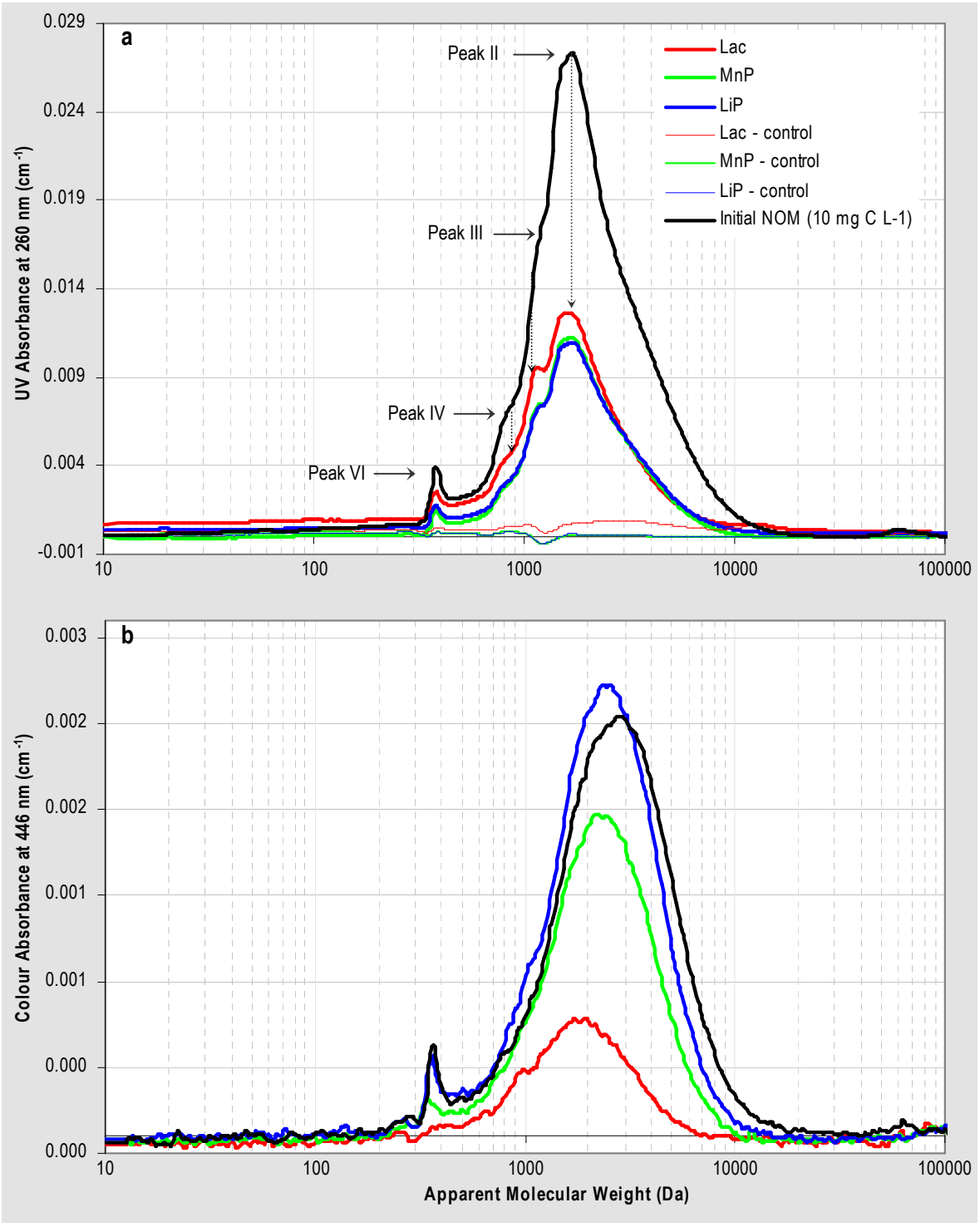

Figure 8-5. HPSEC with detection at 254 nm (a) and 446 nm (b) of NOM (diluted down to 10 mg C L^{-1}) after treatment by Lac (11.7 U mL^{-1}), MnP (0.01 U mL^{-1} + 0.5 mM H_2O_2 + 1.0 mM $MnSO_4$) and LiP (0.052 U mL^{-1} + 4 mM H_2O_2 at pH 4.5). Note: Chromatogram of Initial NOM peak at 446 nm is slightly displaced to the right (artefact).

Lac had the greatest reduction in the weight average MW (M_w) and the number average MW (M_n) of both the coloured (34%, 21%) and UV-absorbing compounds (37%, 30%). MnP had the second greatest reduction in M_w and M_n of the coloured (12%, 5%) and UV-absorbing compounds (19%, 12%) followed by LiP with (7%, 4%) and (14%, 9%) respectively (Figure 8.6). For all three enzymes a greater reduction in the proportion of the higher and lower MW material of the coloured compounds was observed relative to the UV-absorbing compounds.

The reduction in M_w (34%) of the coloured material by Lac was within the range attained by *Polyporus* sp. (38%) and *T. versicolor* (30%) following the decolourisation of NOM–tap water at 30ºC (Figure 5.17a). Although Lac and the two WRF caused comparable decreases in the quantities of the higher MW material, fungal treatment attained greater NOM decolourisation (by 16 and 20% for *Polyporus* sp. and *T. versicolor*, respectively), which was predominantly attributed to adsorption. The presence of organic acids including malonate and oxalate and a constant supply of oxygen would have also contributed to the enhanced decolourisation by the WRF.

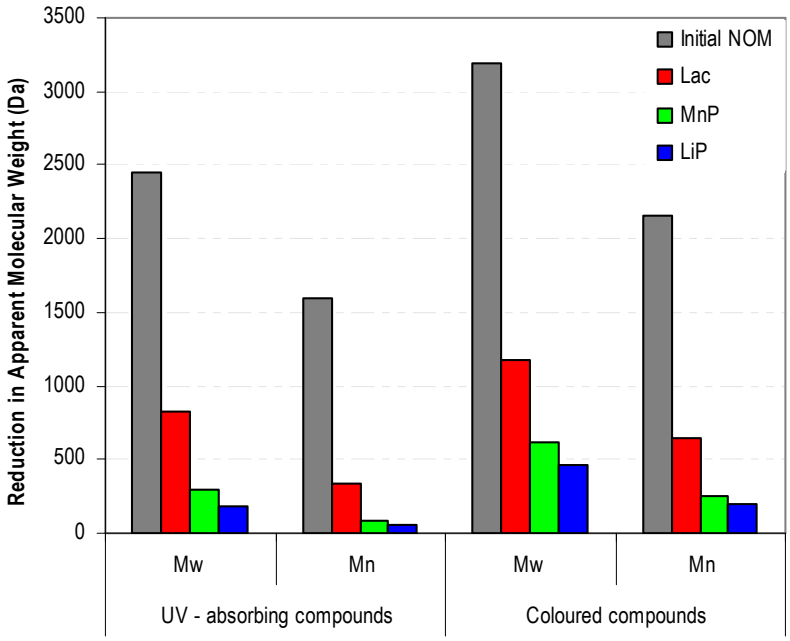

Figure 8-6. Reduction in M_w and M_n of the UV-absorbing and coloured compounds of NOM concentrate after treatment by Lac, MnP and LiP at 30ºC.

The greater reduction in colour, A_{254} and removal of the MW fractions of both the UV-absorbing and coloured compounds by Lac in comparison to MnP and LiP indicates its greater feasibility for the decolourisation of NOM–rich waters. The advantage of using Lac in the decolourisation of NOM is that it requires only molecular oxygen as a co-factor, whereas MnP and LiP require the addition of hydrogen peroxide and Mn^{2+}

(for MnP) to achieve greater colour removal, thus making them less economically viable. In addition, it is cheaper and easier to obtain commercial quantities of Lac as opposed to MnP and LiP.

8.3 Fractionation of NOM following treatment by Lac

Following treatment by Lac (11.7 U mL^{-1}), the NOM-tap water preparation (100 mg C L^{-1}) was diluted to 10 mg C L^{-1} and fractionated to identify the fractions susceptible to removal by Lac (Figure 8.7). A control of Lac alone in Milli-Q water at the equivalent concentration added to the reaction was also fractionated.

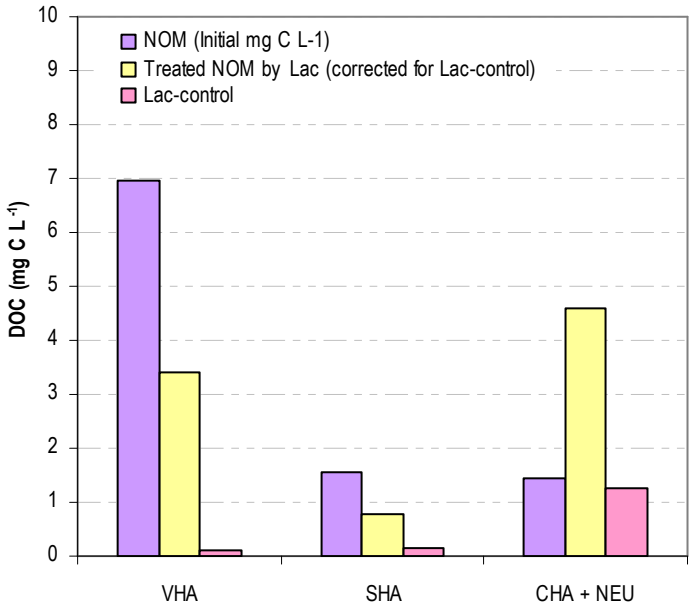

Figure 8-7. Proportion of individual NOM fractions prior to and after treatment by Lac with 11.7 U mL^{-1} at 30ºC. Note: Treated NOM fractions were corrected for Lac-control fractions.

Enzyme treatment led to an almost equal reduction of both the VHA (51%) and SHA (50%) fractions, whereas fungal treatment preferentially removed the VHA fraction (29-52%) from the initial NOM preparation (Figure 5.20). The removal of these fractions was most probably associated with the cleavage of aromatic and aliphatic rings by Lac (Claus, 2003) present within these hydrophobic fractions (Aiken *et al.*, 1992). The decrease in the VHA fraction by Lac alone was comparable to that attained by *B. adusta* of 52%, even though Lac gave a considerably lower decolourisation of NOM (24%) than *B. adusta* (65%). The combined reduction of the hydrophobic fractions (VHA and SHA), which account for a large proportion of colour and A_{254} (Figure 5.19a and b), may be associated with the ability of the enzyme to reduce the colour and UV absorbance across a wide range of the MWs of NOM (300-10000 Da) (Figure 8.5a and b), which was not observed for MnP or LiP. In contrast to the removal of hydrophobic acids, the charged and neutral (CHA + NEU) fractions increased by 3-fold (Figure 8.7). The breakdown of the VHAs and SHAs probably led to the formation of the CHA and NEU components, as observed by Buchanan *et al*. (2005) in the fractionation of

UV and VUV pre-treated (i.e., photo-oxidised) NOM from drinking water. By contrast with enzymatic treatment, the increase in the CHA+ NEU fractions after fungal treatment was predominantly due to the production of organic acids, organic compounds and proteinaceous material

8.4 MW distribution of fractionated NOM following treatment by Lac

The AMW distribution of the UV-absorbing species for the individual NOM fractions prior to and following Lac treatment is illustrated in Figure 8.8. The HPSE chromatograms of the NOM fractions were obtained by difference as similarly reported for fungal treatment. The controls for individual Lac fractions (Lac-control) were also plotted and subtracted accordingly from the uncorrected data (Lac-uncorrected) to give an accurate representation of the MW distribution of the individual NOM fractions following Lac treatment (Lac-corrected).

Enzyme treatment led to a considerable reduction of the VHA fraction across the range of MWs of 700-10000 Da (Figure 8.8a), without the increase in the lower MW species <1600 Da as observed for *Trametes* sp. and *Polyporus* sp. (Figure 5.21). Lac reduced the height of the predominant humic peak at 1700 Da by 55%, correlating to a similar reduction in the VHA fraction (51%) after fractionation (Figure 8.7). Lac preferentially removed the higher MW UV-absorbing material of the VHA fraction >1600 Da, which predominantly constitutes higher MW humic substances (Table 7-1). The chromatograms of the uncorrected and corrected VHA fractions are relatively similar for the entire range of MWs, as the control of Lac constituted only 3% of the VHA component.

For the SHA fraction, Lac reduced the chromophores across the range of AMWs of 600-2500 Da with the preferential decrease of the lower MW compounds <1400 Da (Figure 8.8b). The height of the humic peak at 1400 Da was reduced by 59% as similarly observed for the VHA fraction (55%). The profile of the uncorrected SHA fraction contained a greater proportion of the UV-absorbing compounds between 360-1400 Da than for the VHA fraction, and this is mainly due to the greater proportion of these lower MW compounds in the control sample of Lac (Figure 8.8b, Figure 8.7).

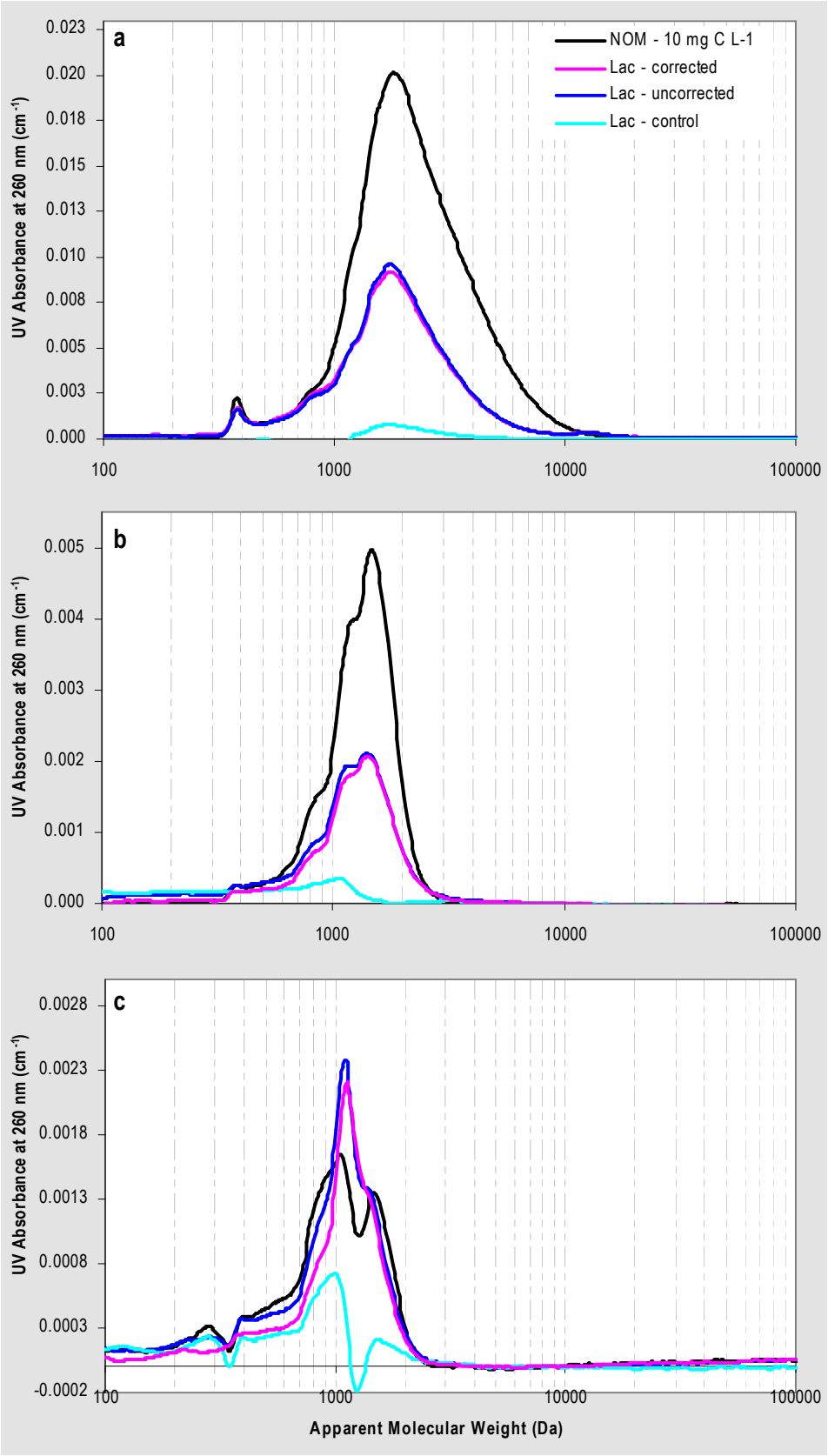

Figure 8-8. HPSEC profiles of the UV-absorbing species in the (a) VHA (b) SHA and (c) CHA + NEU fractions of the raw and treated NOM concentrate (10 mg C L^{-1}) by Lac at 11.7 U mL^{-1}. The controls for individual Lac fractions (Lac-control) were also plotted and subtracted accordingly from the uncorrected data (Lac-uncorrected) to give a representation of the MW distribution of the individual NOM fractions following Lac treatment (Lac-corrected).

Although Lac reduced the lower (100-960 Da) and higher (1400-2000 Da) MW moieties of the hydrophilic (CHA and NEU) fractions, it led to a considerable increase of the intermediate MW compounds between 960-1400 Da (Figure 8.8c). The reduction of the higher MW material >1400 Da may have contributed to the concomitant formation of the intermediate MW compounds, with a greater UV-absorbance. Alternatively the breakdown of the SHA and VHA fraction may have led to the formation of the CHA and NEU components of intermediate size. By contrast, fungal treatment led to an increase in the lower MW compounds (100-960 Da) and a decrease of the intermediate species between 1000-1700 Da (Figure 5.21c). The control of Lac accounted for a large proportion of the lower MW UV-absorbing components, as observed for the fractionation data (Figure 8.7).

The proportion of higher MW material (M_w) and lower MW material (M_n) was calculated for each of the NOM fractions following treatment by Lac (Figure 8.9). The values of M_w and M_n for the individual NOM fractions were obtained by difference as previously for the chromatograms of each NOM fraction.

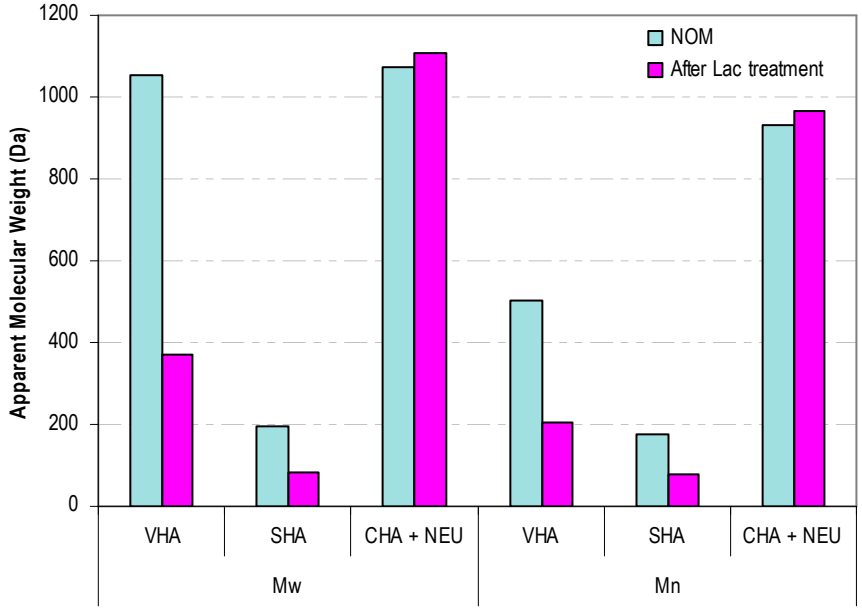

Figure 8-9. M_w and M_n of individual NOM fractions before and after treatment by Lac.

Lac treatment led to comparable reductions in the apparent MW of the higher (M_w) and lower MW (M_n) compounds for the hydrophobic fractions. Slightly greater reductions of M_w and M_n were observed for the VHA fraction (65%, 60%) than for the SHA fraction (57%, 54%) and correlate well to the reductions (DOC mg C L^{-1}) of these fractions after fractionation (Figure 8.7). The reduction in the AMW of the hydrophobic material suggests that Lac targets specific bonds such as the C_α-C_β bonds within the VHA and SHA fractions. The cleavage of these bonds would result in depolymerisation of material and so reduction in AMW, as illustrated in Figure 8.9. The depolymerisation of NOM by Lac is further evidenced by the reduction in the ratio of M_w:M_n from 1.28 to 1.18. The breakdown of these predominantly humic fractions would have

contributed to the formation of the CHA and NEU fractions with diverse MWs. In addition, Lac alone contributed to the overall pool of this fraction. Enzyme treatment resulted in a higher AMW of M_w (1108 Da) and M_n (964 Da) of the CHA + NEU fractions. This correlates well with the increase in intermediate species with AMWs of 960-1400 Da following Lac treatment. Alternatively the breakdown of higher and lower MW compounds could have resulted in re-polymerisation reactions of these broken fractions, thus leading to the formation of intermediate compounds.

8.5 Conclusions for Chapter 8

Chapter 8 investigated the *in vitro* decolourisation of NOM concentrate (100 mg C L^{-1}) by Lac, MnP and LiP. Lac attained the greatest colour removal of 24%, followed by MnP with 9.5% and LiP with 8.3% at the highest concentrations used (Figure 8.4). The greater extent of NOM decolourisation by Lac was probably due, at least in part, to the higher concentration of the enzyme. NOM decolourisation by the three enzymes was accompanied by a reduction in UV absorbance, which indicates that conjugated bonds and aromatic rings in the NOM were enzymatically broken. In the presence of H_2O_2, both MnP and LiP attained a greater rate and degree of colour removal, indicating that H_2O_2 facilitates the decolourisation process. A further increase in the initial rate of NOM decolourisation by MnP was observed following the addition of Mn^{2+}. The initial rates of NOM decolourisation by Lac, MnP and LiP decreased with increasing NOM content > 50 mg C L^{-1}, suggesting possible enzyme inhibition.

The decolourisation of NOM by Lac, MnP and LiP was accompanied by a decrease in the average MW of both the coloured and UV-absorbing species, which correlated well with the reductions in A_{446} and A_{254} attained by the three individual enzymes. Lac gave the greatest reduction of the coloured and UV-absorbing material across a broad range of MWs, whereas MnP and LiP preferentially targeted the higher MW fractions of the coloured compounds. Fractionation of the Lac-treated NOM solution gave an almost equal reduction of both the VHA and SHA fractions, with the removal of these hydrophobic fractions being attributed to the cleavage of aromatic and aliphatic rings by Lac present. The breakdown of the VHAs and SHAs probably contributed to the pool of the CHA and NEU fractions. The exact role of the individual enzymes in the decolourisation of NOM is still uncertain and further work is required to cover the gaps in knowledge. Although the *in vitro* decolourisation studies have illustrated the ability of the enzymes to degrade NOM, their potential application on a commercial scale is questionable due to their high cost.

Chapter 9 Conclusions

The application of WRF for the decolourisation of Beaconsfield water and aquatic NOM concentrate and NOM from Beaconsfield Reservoir without the addition of further nutrients was investigated. This research had several objectives:

1. To isolate, screen and select fungi capable of decolourising aquatic NOM concentrate
2. To evaluate the efficiency of the selected fungi to decolourise NOM concentrate
3. To identify and evaluate the mechanisms implicated in the removal of NOM and characterize the changes in NOM character
4. To assess the feasibility of using WRF as an alternative biological treatment for the removal of NOM from a sample of drinking water, as exemplified by water from Beaconsfield Reservoir
5. To investigate the application of Lac, MnP and LiP in the *in vitro* decolourisation of NOM concentrate.

Twenty-one isolates from diverse genera of fungi were obtained from various sources, including the Dandenong Ranges, Beaconsfield Reservoir, laboratory collections, and from enrichment cultures of biologically active sand. Fungi capable of degrading NOM (MIEX concentrate) were screened for by decolourisation of the NOM medium and assayed for cellulolytic ability. All fungi grew on the NOM agar and exhibited sparse mycelial coverage. Of the 21 isolates, the WRF were the only species that decolourised NOM. The WRF had a lower average mycelial extension rate than the other isolates, which may have allowed sufficient time for nutrient exploitation and the subsequent decolourisation of NOM. By contrast, the micromycetous fungi that grew but did not decolourise NOM were mostly non-WRF and may have utilised the polysaccharides in the agar for growth instead of the NOM. The decolourisation of NOM by WRF varied in clarity, degree and pattern, with good NOM degraders being chosen on the basis of having a high clarity and degree of NOM decolourisation. Of the WRF, *B. adusta* exhibited the highest NOM degradation coefficient, which for most fungi correlated well with the growth rate. When the fungi were assessed for their cellulolytic ability, all grew sparsely on the CMC agar, with the WRF exhibiting a greater mycelial extension rate than the other isolates. The ability of WRF to decolourise NOM, combined with their greater cellulolytic activity than the micromycetous fungi, was probably associated with the production of ligninolytic enzymes.

Based on preliminary screening, *B. adusta, Trametes* sp.*, Polyporus* sp., *T. versicolor* ATCC 7731 and *P. cinnabarinus* VIC demonstrated good NOM degradability and were further tested for their capacity to degrade NOM in liquid culture. The potential of these WRF to decolourise NOM without the addition of further nutrients was investigated in both tap and distilled water. Following an initial study into the selection of a suitable type of inoculum, the WRF were grown into pellet form and used in shake-flask cultures. The selected WRF decolourised NOM concentrate in tap and distilled water, with different trends and degrees of

decolourisation observed between the WRF in the two NOM solutions at 100 mg C L^{-1}. *Polyporus* sp. and *T. versicolor* ATCC 7731 had greater NOM decolourisation in tap water (by 26% and 10%, respectively), whilst *Trametes* sp. had similar levels of colour removal of 41-44%. Of the WRF, the newly isolated *B. adusta* strain attained the greatest decolourisation 65%. The variation in colour removal between the two water sources was attributed to the increased presence of mineral nutrients in the NOM-tap water preparation, which was confirmed with elevated concentrations of Ca^{2+}, Mg^{2+} and K^{+} by ICP-MS analysis. The initial rates of NOM decolourisation also varied for the WRF and the two NOM solutions. Both the *Trametes* spp. decolourised NOM in tap water twice as fast as in distilled water, whilst *Polyporus* sp. decolourised NOM faster (2.5 fold) in distilled water than in tap water. The initial high rates of NOM decolourisation were attributed to adsorption, which is considered to be the first step in the biodegradation of NOM. This was confirmed with autoclaved controls, where different degrees of adsorption to the fungal mycelium were observed for the five species. Greater adsorption was observed in the NOM-tap water preparation, with *Polyporus* sp. and *B. adusta* adsorbing 10-15% of the colour for both NOM solutions, whereas the *Trametes* spp. strains adsorbed only 4-6%.

NOM decolourisation by the WRF in both NOM solutions coincided with a drop in culture pH, suggesting metabolic activity. The drop in culture pH was attributed to the secretion of organic acids by the fungi during secondary metabolism. Following maximum colour removal, the phenomenon of 'negative decolourisation' was observed for most fungi, but was most marked for *P. cinnabarinus* and the two *Trametes* strains. Negative decolourisation probably occurred due to the depletion of available nutrients, which led to cell autolysis, and the release of adsorbed colour back into the culture solution. An alternative explanation is that the rise in culture pH shortly after maximal decolourisation led to the dissociation of humic functional groups, followed by their re-polymerisation, thus leading to an increase in colour and UV-absorbing compounds. Further research is required to optimize and control decolourisation and minimise negative decolourisation.

The decolourisation of NOM in tap water by the WRF correlated well with a moderate drop in A_{254}, the greatest being for *B. adusta* (20%) and least for *T. versicolor* (10%). This drop in A_{254} indicates that some UV-absorbing components of the NOM were cleaved to non-UV absorbing species and/or adsorbed by the fungal biomass as seen with *Polyporus* sp. and *B. adusta.* This drop in A_{254} was not as evident in distilled water, possibly owing to the lower levels of NOM decolourisation achieved by the WRF. Alternatively, the degradation of UV-absorbing compounds could have been masked by the production or secretion of new UV-absorbing material.

The decolourisation of aquatic NOM concentrate by the WRF coincided with the onset of extracellular enzyme activity, in particular that of Lac and MnP. In both NOM solutions, Lac activity predominated for both the *Trametes* strains and *Polyporus* sp., whereas MnP activity was greatest for *B. adusta* in NOM-tap water. LiP activity was detected only for *T. versicolor*, *B. adusta* and *P. cinnabarinus*, suggesting that specific

culture conditions may be required for its secretion. The magnitude and timing of the activity of these enzymes varied between the strains of fungi and culture conditions used. For most fungi, the activities of Lac and MnP were greater in tap water compared with distilled water, and correlated well with the greater NOM decolourisation achieved. These greater enzyme activities may be due to the increased concentrations of some of the essential nutrients and trace elements such as iron and zinc, present in the NOM-tap water solution. Enzyme activity patterns also varied between the WRF. For *T. versicolor* and *Trametes* sp., NOM decolourisation in tap water occurred in conjunction with increasing activity of MnP and Lac, whilst in distilled water the onset of Lac and MnP activity was evident after an initial stage of NOM decolourisation (of 10-15%), ascribed to adsorption. By contrast, *B. adusta* expressed all three enzymes, which peaked prior to maximal NOM decolourisation, suggesting that the mechanism of NOM decolourisation may be a combination of enzymatic breakdown and adsorption. Although the enzyme activities of the newly isolated *B. adusta* were markedly lower compared with the other fungi, it gave greater decolourisation of NOM and this may be due to the action of a versatile peroxidase. Although the degradation of coal-derived HA by various strains of WRF has been widely documented, this is the first report of the ability of the newly isolated *B. adusta* to decolourise aquatic NOM concentrate without the addition of nutrients. Furthermore, this is the first report of trends in extracellular enzyme activity, in particular of MnP, during NOM decolourisation by *B. adusta*. A steep reduction in all enzyme activities was seen shortly after maximal decolourisation; this may be attributed to the occurrence of secondary extracellular proteases on lysis of the cells and subsequent increase in culture pH. The simultaneous secretion of enzymes (mainly Lac and MnP) suggests that they played a dual or synergistic action in the decolourisation process of NOM.

The decolourisation of NOM in both distilled and tap water was accompanied by a decrease in its average MW, and the formation of lower MW intermediates. Greater reductions in the proportion of higher and lower MW material were observed in tap water than in distilled water for *Trametes* sp., *T. versicolor* and *Polyporus* sp. and these correlated well with the increased decolourisation and corresponding enzyme activities for the three species. *B. adusta* gave the greatest reduction in the higher MW fractions and the greatest formation of lower MW intermediates, which correlated with the greatest removal of colour (65%). Fractionation of the NOM-tap water preparation showed that the decolourisation of NOM concentrate by the WRF was predominantly associated with the removal of the hydrophobic fractions (in particular of the VHA fraction), which account for a large portion of the humic substances present in NOM. Thus the decolourisation of NOM by WRF was accompanied by the predominant removal of the higher MW humic fractions, which were found to account for most of the colour and aromaticity of NOM. The breakdown of the hydrophobic fractions (VHA and SHA) may have contributed to the increase in the hydrophilic fractions (CHA and NEU). These findings demonstrate the ability of the extracellular enzymes to decolourise and depolymerise humic substances, as reported for other fungi. The enzymatic depolymerisation of the NOM molecule probably coincided with the cleavage of β-aryl ether (β -O-4) and β -1 bonds within the NOM structure, and the subsequent formation of lower molecular masses with increased UV absorbance (CHA and NEU). In the presence of the oxidative

enzymes, these lower MW fractions may have re-polymerised, thus contributing to the increase in UV absorbance.

The decolourisation of aquatic NOM concentrate was further investigated by studying its removal at various NOM concentrations by *Trametes* sp. The effect of initial NOM concentration on the biodegradation of NOM was investigated to determine the maximum concentration tolerated by *Trametes* sp. under conditions of limited nutrients. Maximal decolourisation (50%) occurred at 100 mg C L^{-1}, with the lowest (21%) at 500 mg C L^{-1}. NOM decolourisation increased and then decreased with increasing NOM concentration, suggesting inhibition at NOM concentrations greater than 100 mg C L^{-1}. With increasing NOM concentration, the equivalent amount of NOM (converted to mg C L^{-1} from A_{446}) removed increased linearly up to 300 mg C L^{-1}. For most NOM concentrations (13-300 mg C L^{-1}) the initial mechanism of NOM decolourisation was attributed to adsorption followed by enzymatic breakdown, whereas for 500 mg C L^{-1} the decolourisation was almost all due to adsorption. At maximal NOM decolourisation, the activity of Lac was greater than that of MnP, suggesting its predominant role in the decolourisation of NOM. Lac and MnP activity increased with increasing NOM content, but declined at NOM concentrations greater than 30 and 50 mg C L^{-1}, respectively. These trends suggest enzyme inhibition, which was also reported during the degradation of HAs by other WRF. Fungal treatment led to increased A_{254} and DOC, the proportional increases being greater for lower NOM concentrations. The increase in DOC and A_{254} was attributed to the release of soluble organic compounds into the culture from the active production and secretion of extracellular enzymes as well as cell autolysis due to nutrient depletion. When the fungi-treated NOM samples were subjected to biologically active sand for a period of 51 days, a considerable reduction in the colour (31-75%), UV absorbance(24-59%) and fungal-generated DOC (51-167 mg C L^{-1}) was observed, particularly for NOM contents of 30-50 mg C L^{-1}. This was confirmed by the reductions in the higher MW fractions and by the removal of most of the lower MW fungal products following BDOC treatment. EEMs indicated the formation of new fluorephores in the humic- and fulvic-like regions, indicating possible polymeric products from enzyme-catalysed oxidative reactions.

To ascertain if WRF were capable of decolourising NOM from a fresh water body, experiments were conducted on Beaconsfield Reservoir water under conditions of limited nutrients. The decolourisation of Beaconsfield NOM by cultures of *Trametes* sp., *Polyporus* sp. and *B. adusta* occurred at a rate (of 4-11% per hour) with a much greater extent of colour removal (up to 70-80%) compared with that attained for NOM concentrate at 100 mg C L^{-1}. A high degree of negative decolourisation was observed for the three WRF following these high rates of colour removal. These high initial rates of NOM decolourisation were predominantly driven by passive adsorption, as demonstrated by the high degree of adsorption (46-67%) onto heat-killed biomass for the three WRF. The late onset of Lac and MnP activity (shortly after maximal NOM decolourisation) for *Trametes* sp. and *Polyporus* sp. further indicates the mechanism of colour removal to be chiefly via adsorption for these organisms, whereas for *B. adusta* it was by a combination of adsorption

and enzymatic breakdown. When the decolourisation of Beaconsfield NOM was conducted at 15°C by *Trametes* sp., a greater reduction of colour (by 10%) with a lower degree of negative decolourisation was attained, but over a much longer time than that at 30ºC. When a small volume of culture was periodically replaced with fresh Beaconsfield water, decolourisation initially fluctuated but later stabilised and negative decolourisation did not occur. The periodic addition of NOM to the culture could be a way of minimising or even eliminating the phenomenon of 'negative decolourisation' and thus maintaining a constant state of colour removal. When decolourisation of Beaconsfield water and NOM concentrate was conducted at the same concentration of 13 mg C L^{-1} by *Trametes* sp. and *B. adusta,* the rate and extent of colour removal by both fungi was significantly greater for Beaconsfield NOM. The variation in the rate and degree of colour removal between the two NOM samples may be attributed to the structure of NOM and ionic strength of the NOM solution. As observed for NOM concentrate, fungal treatment of the Beaconsfield NOM resulted in the breakdown of higher MW fractions and the concomitant formation of lower MW intermediates. When the fungal-treated NOM samples were subjected to biologically active sand, most of the lower MW fungal products were assimilated by the bacteria, but less so of the higher MW material. Although fungal treatment led to the generation of DOC, which is undesirable in distribution systems as it acts as a food source for bacterial regrowth, a high percentage (55-70%) of the produced DOC was assimilated by the bacteria. A biological treatment technology based on the combined utilisation of fungi and bacteria could be an attractive alternative for the removal of NOM.

To clarify the involvement of each ligninolytic enzyme in the decolourisation process, *in vitro* decolourisation studies were conducted on NOM concentrate with commercial preparations of Lac, MnP and LiP. All three enzymes decolourised NOM concentrate (100 mg C L^{-1}), with Lac attaining the greatest colour reduction of 24%, followed by MnP with 9.5% and LiP with 8.3% at the concentrations used. Lac was also the predominant enzyme involved in the decolourisation of NOM by the selected strains of WRF. The lower decolourisation by MnP and LiP compared with Lac may be attributed to the lower activity of the enzymes. Decolourisation by the three enzymes was accompanied by the reduction in UV absorbance, which was related to the removal of colour for each enzyme, and indicates that NOM containing conjugated bonds and aromatic rings was enzymatically broken. In the presence of H_2O_2 (and $MnSO_4$ for MnP only), both MnP and LiP attained a greater rate and degree of colour removal. Increased NOM content (>50 mg C L^{-1}) inhibited the initial rate of NOM decolourisation for all three enzymes, as similarly observed for the *in vivo* studies. Although the decolourisation studies were only carried out in the presence of one enzyme, it is highly probable that the presence of all three enzymes in the reaction mixture would result in a greater loss of colour unless H_2O_2 was limiting. The decolourisation of NOM by Lac, MnP and LiP was accompanied by a decrease in the average MW of both the coloured and UV-absorbing species. Lac gave the greatest reduction of the coloured and UV-absorbing material across a broad range of MWs, whereas MnP and LiP preferentially removed the higher MW fractions of the coloured compounds. Fractionation of the Lac-treated NOM solution gave equivalent reduction of the SHA and VHA fraction, and an increase in the CHA and NEU

fractions. The removal of these hydrophobic fractions was attributed to the cleavage of aromatic and aliphatic rings by Lac present. The complex nature of the system makes it difficult to ascertain the exact reactions involved in the decolourisation of NOM, however the structural similarity of lignin and NOM suggests that similar mechanisms might be involved in its depolymerisation by the three ligninolytic enzymes.

The decolourisation of Beaconsfield NOM and NOM concentrate is a complex process with significant interactions taking place between NOM, fungi, enzymes, pH, temperature and nutrients. The heterogeneous nature of the reaction system consequently makes it difficult to elucidate the mechanisms of NOM degradation, which for some of these WRF may involve different degrees of initial adsorption to the fungal mycelium followed by enzymatic action. Further studies are needed to fully clarify the physiological, physical and biochemical processes governing the biodegradation of NOM. A more comprehensive understanding of the degradation process would potentially lead to better process design. Despite the complexity of the system, this research was the first to illustrate the isolation and characterisation of a range of NOM-degrading fungi from several sources, and their potential to decolourise and depolymerise aquatic NOM concentrate and NOM from drinking water as a sole source of nutrients. Although several WRF were effective in decolourising NOM, especially *B. adusta*, they were limited in their decolourisation ability due to pellet disintegration caused by the low nutrient status of the water. An alternative treatment based on the use of a recycled flow-through system with immobilised WRF could prove to be effective in minimising or even eliminating the phenomenon of ‘negative decolourisation’ and thus maintaining a constant state of colour removal.

Chapter 10 Recommendations

Further work is suggested in the following areas:

As the biodegradation of NOM concentrate was conducted in shake flask cultures, the application of a bioreactor for the removal of NOM would be highly recommended. Operational conditions such as pH, dissolved oxygen level and agitation could be monitored and controlled for optimal NOM removal. Kirk *et al*. (1978) suggested that the lignin-degrading system of the WRF required high O_2 demand, and so an adequate supply of oxygen could facilitate in the treatment of NOM. Appropriate agitation would assist in maintaining a uniform environment by improving the mass transfer of oxygen and substrates. Controlling the culture pH by the incorporation of buffers or by batch feeding may enhance the removal process by maintaining maximum colour removal (which occurs at low pH) and by reducing negative decolourisation. By maintaining a low pH, the possibility of repolymerisation (due to high pH) may be avoided. Various types of bioreactor systems have been designed, including the use of *T. versicolor* cultures to form static biofilms on rotating discs for decolourisation reactors (Kapdan and Kargi, 2002) and a packed-bed bioreactor based on immobilised cells of *P. cinnabarinus* to treat the effluent from a pigment plant (Schliephake *et al*., 1993).

Immobilising the WRF onto a suitable support may be an option in minimizing the breakdown of fungi and the subsequent release of fungal products into the culture medium. Shim and Kawamoto (2002) observed that immobilization of mycelium on a bio-carrier was more effective in promoting cell growth and LiP production compared with stationary liquid culture. Immobilisation of the WRF on a steady-state support would allow the fungi to penetrate deeply into the intercellular and intracellular spaces of the support, thus reflecting the natural living conditions of ligninolytic fungi (Maceiras *et al*., 2001).

The decolourisation of NOM in the presence of various combinations of enzymes could help determine if the combined effect of the enzymes is additive or synergistic. Although these enzymes have shown potential in the decolourisation of NOM, particularly Lac, they are expensive and thus it may be more practical to immobilise whole organisms in preference to their enzymes. In addition the *in vitro* stability of lignin degrading enzymes will be an important factor in determining both the economic and technical feasibility of application for future uses (Aitken and Irvine, 1989).

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Appendices

Appendix 1. Measurement of enzyme activity

The activity of enzymes (U mL^{-1}) was measured by monitoring the increase in absorbance for 10 minutes, as exemplified for Lac in Figure A-1.

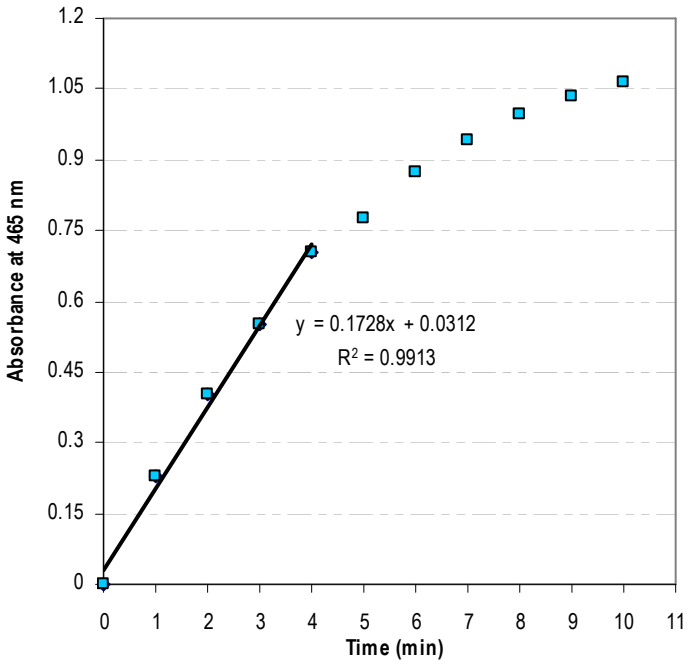

Figure A-1. Initial rate of guaiacol oxidation catalysed by Lac at pH 4.5, 50ºC.

The activity of the reconstituted enzymes was determined by using the Beer-Lambert law. Equation A1 was used to calculate the activity of enzymes in U mL^{-1}.

$$\frac{U}{mL} = \frac{(A_f - A_i)(V_t)(D_f)}{(V_s)(T_r)(\varepsilon)(L)} \qquad \text{Equation A1}$$

Where:

A_i = Absorbance at time 0, prior to the addition of enzyme

A_f = Absorbance at the end of the reaction

V_t = Total volume of assay mixture used (μL)

D_f = Dilution factor (for reconstituted enzyme)

V_s = Volume of enzyme solution (μL)

T_r = Reaction time (min)

ε = Molar extinction coefficient (mM^{-1} cm^{-1})

L = Path length (cm)

Appendix 2. Correlation between NOM concentration and A_{446}

The correlation between NOM concentration and A_{446} is represented in Figure A2 and by Equation A-2.

$$A_{446} = 0.0022 \times -0.0078 \qquad \text{Equation A2}$$

Where:

A_{446} = absorbance at 446 nm

x = initial NOM concentration (mg C L^{-1})

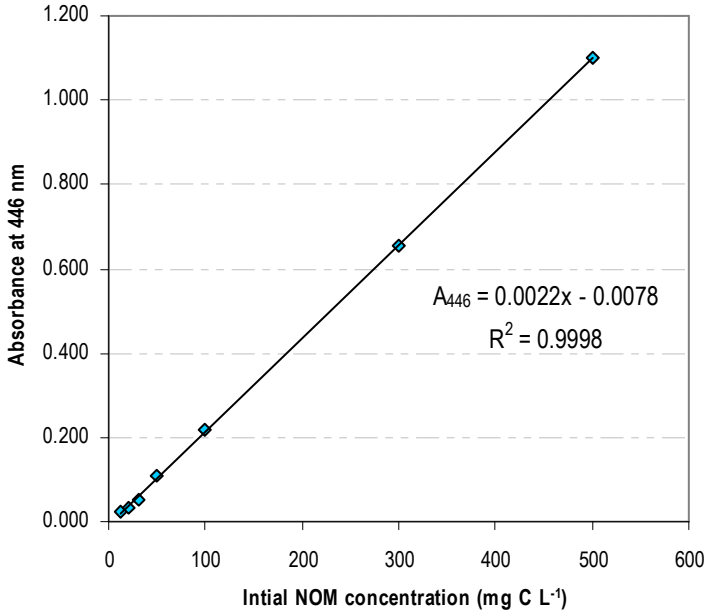

Figure A-2. Correlation between NOM concentration and A_{446}.

Appendix 3. Absorbance correction factor

The corrected absorbance relative to a particular change in pH can be calculated using the following formulas:

$$CF(\%) = \frac{(A_{ci} - A_i)}{A_i} \times 100 \qquad \text{Equation A3}$$

$$y = 5.2819x - 0.9718 \qquad \text{Equation A4}$$

$$pH = pH_0 - pH_i \qquad \text{Equation A5}$$

Where:

CF = correction factor (%)

A_{ci} = corrected absorbance on day i

A_i = measured absorbance on day i

y = correction factor

x = pH difference (pH_0-pH_i)

pH_0 = Initial pH

pH_i = pH on day i

Where MIEX NOM concentrate was used in the decolourisation studies, corrections were applied to all A_{446} and A_{254} measurements using Figure A3.

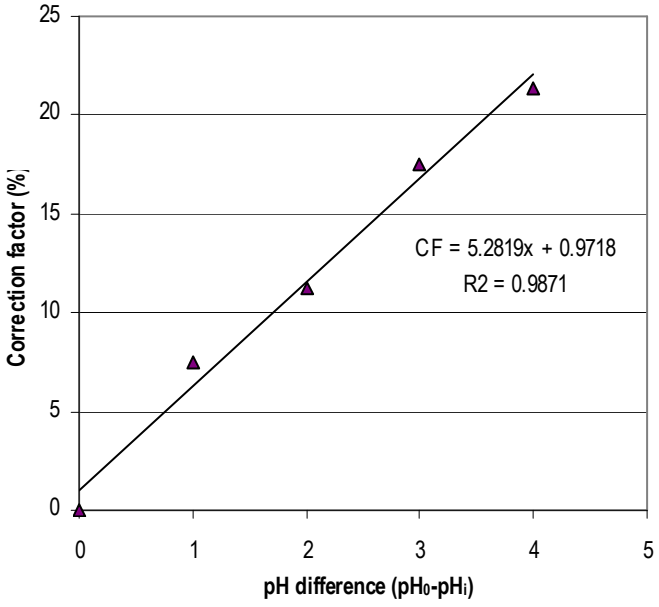

Figure A-3. Correction factor for A_{446} and A_{254} at different pH (Lee, 2005).