LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by roman numerals in the text.


# List of Contents

1. Introduction .................................................................................................................. 5  
   1.1 Background ............................................................................................................... 5  
   1.2 Research aim .......................................................................................................... 6  
2. Dyes - different classes and field of application ....................................................... 7  
   2.2 Dye classification according to chemical structure .............................................. 8  
   2.3 Dye classification according to method of application ........................................ 10  
3. Textile fabric and dyeing processes ........................................................................... 14  
4. Water and environmental impact ............................................................................... 20  
5. Different wastewater treatment methods ................................................................. 23  
   5.1 Physical ................................................................................................................ 23  
      5.1.1 Flocculation .................................................................................................... 23  
      5.1.2 Adsorption ..................................................................................................... 23  
      5.1.3 Filtration ....................................................................................................... 24  
   5.2 Chemical ............................................................................................................. 24  
5.3 Biological processes ............................................................................................... 25  
   5.3.1 Bioremediation of aromatic hydrocarbons ...................................................... 28  
   5.3.2 Aerobic conditions .......................................................................................... 29  
   5.3.3 Anaerobic or anoxic conditions ...................................................................... 30  
   5.3.4 Anaerobic and aerobic treatment combined ................................................... 31  
   5.3.5 Fungi .............................................................................................................. 32  
   5.3.6 Nutrient and carbon sources .......................................................................... 33  
   5.3.7 Reactor design ................................................................................................. 34  
   5.3.8 Toxicity ........................................................................................................... 36  
6. Chemical analysis ....................................................................................................... 39  
7. Microbial analysis ....................................................................................................... 42  
   7.1 Fungi ................................................................................................................... 42  
   7.2 Microscopy and fluorescent in situ hybridization ............................................... 43  
   7.3 Amplification of microbial DNA ......................................................................... 43  
   7.4 DGGE ................................................................................................................ 44  
   7.5 DNA sequencing .................................................................................................. 44  
8. Presentation of included papers ................................................................................ 46  
   Paper I: Decolourization of reactive azo dyes with microorganisms growing on soft wood chips ............................................................................. 46  
   Paper II: Biodegradation of Azo and Anthraquinone dyes in batch and continuous systems ....................................................................................... 46  
   Paper III: Biodegradation of the Azo dyes Reactive Red 2 and Reactive Black 5 in a continuous system based on rice husks and its microbial diversity ................................................................. 47
Paper IV: The treatment of azo dyes found in textile industry wastewater by anaerobic biological method and chemical oxidation..........................48
Manuscript V: Biotreatment of actual textile wastewater in a continuous biofilter and the associated bacterial and fungal microflora.................49
9. Discussion ..................................................................................................50
10. Conclusions ..............................................................................................55
11. Future research .........................................................................................57
12. Acknowledgement ....................................................................................60
Author’s contribution to presented papers: ...............................................61
References .......................................................................................................63
1. INTRODUCTION

1.1 Background

Even though we can travel to the Moon, send robots to Mars, make super computers and clone organisms we still have difficulties to clean the water we use. In many parts of the world the availability of water is a crucial issue, and even more so, clean water.

Textile production was one of the first areas where industrial processes developed in the early-nineteenth century. Textiles and clothing are still necessary and have developed as a means of fashion and a way to display one’s identity in the twenty-first century. There is also a branch of high technological textiles with advanced polymers and coatings of fibres (which is not discussed in this thesis).

Most textiles are coloured in some way. With a few exceptions, extracting intense colour from natural sources is quite rare. The intense red colour Carmine, extracted from Cochineal lice (Dactylopius coccus), was imported from Mexico, but remained secret for a long time. This colour contributed towards and was used to display the wealth of the Spanish empire. It was not until the Mexican war of independence (1810-1821) that this knowledge spread and production started in other countries. Indigo, another natural dye with great historical importance, was originally extracted from plants of the genus Indigofera; native to the tropics, the species Indigofera suffruticosa and Indigofera arrecta being the most important. Indigo has been used to dye textile and clothing since the Mesopotamia culture and was considered valuable as merchandise, with both the Greeks and Romans considering it a luxury product. Both of these dyes are today approved as food dyes; Carmine is labelled E120 and Indigo as E132. Furthermore, some dyes can even be used as drugs, for instance the azo substance Balsalazide is used as a drug in the treatment of ulcerative colitis (Tursi, 2009).

Of course, chemists have tried to develop synthetic methods to produce dyes for commercial use. The first synthetic colour of significance was Aniline
Purple (Mauveine), discovered in 1856 by William Henry Perkin, which led to the start of the organic chemical industry. Today, chemists have developed dyes that are durable against washing, sunlight, enzymes and ageing. The process of dyeing is usually performed in water, with part of the dye inevitably ending up in the water and thus fouling it. Today authorities within the EU demand that water used by the textile industry be cleaned. Water and chemicals used in the process of manufacturing textile fabrics is an increasing environmental issue of concern. In 2000, the EU production of fabrics represented 3.4% of the manufacturing industry’s turnover and 6.9% of industrial employment (Commission, 2003).

Increased environmental requirements together with a high degree of associated manual work have resulted in the textile industries locating their production to developing countries. However, with a rapidly growing population, developing countries are struggling to reach higher standards and therefore face urbanization and an increased industrial sector. This process places great pressure on the limited available resources in these countries, causing major environmental concerns. For example, in the Gujarat region of India, the water in the major river “Tapti” has a Water Quality Index so poor that it is unsuitable for neither drinking nor irrigation (Sousa et al., 2008). In Tirapur “the textile region” of India, the entire textile sector with over 700 textile processing units was closed down in February 2011 by the Madras High Court. In 2012, some 100 units have reopened, now able to meet the zero liquid discharge.

1.2 Research aim

The overall aim of this research is to develop a method to degrade dyes and chemicals released from the textile process, in a cost efficient and environmentally safe process. To achieve this, microorganisms were employed and the treated water was monitored through analytical methods. A further goal was to monitor the decolourization and the degradation of metabolites. Molecular fingerprinting techniques were employed to gain insights into the microbial species that constitutes the mixed communities engaged in the degradation of the different molecules in textile wastewater. The research started with controlled environments and dye solutions in batch and developed into continuous systems with anaerobic and aerobic filters treating the actual textile wastewater.
2. DYES - DIFFERENT CLASSES AND FIELD OF APPLICATION

Textile fibres are all constructs of polymeric organic fibres, and vary in physical and chemical compositions. The fiber material to be coloured can differ in origin; whether protein fibers from wool or silk or cellulose fibers from cotton, all are natural fibers. There are also semi-synthetic fibres such as viscose rayon made from cellulose in wood and cellulose acetate made from modified cotton. Because of these different fibre types, various dyes and processes have evolved to colour the material (Christie, 2007). This work concentrates on addressing issues related to the treatment of natural fibres. Colour is when electrons in molecules become excited to a higher energy level and return to their ground state, light of a certain wavelength is transmitted and colour is perceived. Molecules with these properties are often found at specific metal ions or in conjugated organic systems where electrons are more movable.

Colorants are divided into dyes that are soluble and pigments that are insoluble in its vehicle, i.e. dyes become a solution and pigments become a suspension. A substance can be either a dye or pigment depending on the solvent used. The limit between these concepts is a little fluid, since occasionally soluble dyes become insoluble in the dyeing process. The most important reference work on dyes and pigments is the Colour Index (C.I.), a publication produced by the Society of Dyers and Colourists, Bradford England. The Colour Index lists all commercial dyes and pigments and gives them a C.I. Generic name. The Colour Index also lists information about chemical constitution, range of fastness properties, methods of application, uses, and trade names and manufacturers.

There are two ways to classify dyes, by chemical structure or upon their dyeing process. The classification of dyes according to the method of application is of greater interest for the textile dyer. However, with this method of classification azo dyes can, for example, belong to several different classes. (Christie, 2007)
2.2 Dye classification according to chemical structure

The classifications used follow those outlined in Industrial dyes by Hunger (2003).

Azo Chromophore
Azo compounds are distinguished by the double azo bound between two nitrogen atoms (Fig. 1). Each nitrogen atom is bound to another group, usually an aromatic group. There are dyes with one, two, three and even four azo groups in the molecule. The aromatic rings usually have chloride, hydroxyl, sulphate or nitro groups attached to increase solubility in water and enhance interactions with the substrate. Azo dyes are today widely used because of their good performance and cost effectiveness. (Hunger, 2003)

Anthraquinone Chromophore
Anthraquinone compounds (Fig. 2) are today considered as the second most important textile dye after azo dyes. The oldest known dye is over 4,000 years old, found in the wrappings of mummies. Anthraquinones do not have the broad variety of colours that azo dyes can perform, though they are known for their good fastness and light fastness. The drawback is that Anthraquinone dyes are expensive (Christie, 2007; Hunger, 2003).

Indigoid Chromophore
Indigo (Fig. 3), a natural dye originally extracted from the plant *Indigofera tinctoria*, has been traditionally used in dyeing for 5,000 years. In 1878, Adolf von Bayer managed to synthesize indigo from phenylacetic acid. Indigo is still widely used to colour denim, mostly due to its gradual characteristic fading blue shades. This class is essentially the same as “vat dyes” discussed under method class. (Christie, 2007; Hunger, 2003)
Cathionic Dyes and Chromophores
See “basic dyes” under method class

Polymethine and related Chromophores
This group include dyes of aliphatic molecules with conjugated systems, the most well-known representative β-Carotene having a straight conjugated system. However, there are also dyes with aromatic structures in this class (Christie, 2007).

Di- and Triarylcarnenium and related Chromophores.
The first dye ever synthesised, Mauveine, belongs to this class. Their significance today has decreased since the early-20th century. However, some dyes still have their uses, especially for colouring acrylic fibres and paper. Several arylcarbenium dyes (Fig. 4) are used in the basic process (cationic), and there are also neutral and anionic dyes.

Phthalocyanine Chromophore
This group of dyes is constituted by a ring structure of aromatic or benzopyrrole (indole) rings (Fig. 5), usually with a metal ion in the centre, that are slightly reminiscent of chlorophyll and haem structures. Phthalocyanines can give colour from reddish blue to yellowish green through complexes with metal, metalloids or even non-metals such as Phosphorous. The most important in this class is Copperphthalocyanine, which is widely used in plastics, paints and inks, as well as the textile industry for dyeing and printing. The colours are extremely stable and strong, making them cost effective (Hunger, 2003).
Sulphur compounds and Chromophore
If aromatic compounds are heated together with sulphur, where sulphur dyes are formed out of polymers of heterocyclic rings and thiophenolic sulphur (Fig. 6). To apply this dye to cellulosic fibres, the polymer is dissolved by heating it in a sodium sulphide solution. The uncoloured solution is applied to the fibre and colour is induced through oxidation, which makes the polymer reform. Sulphur black is today the textile dye with highest tonnage (Hunger, 2003).

Metal Complexes and Chromophores
Inducing colour with metal complexes dates back to the Middle Ages, when a method of mordant dyeing was developed. Metal complexes are often formed with azo dyes. The most used metals in metal complex dyes are chromium and copper, iron, cobalt and nickel. The most important groups involved in complex forming around the metal ion are hydroxyl (-OH), carboxy (-CO2) and amino groups (-NH-). (See also mordant dyes under method classifications) (Christie, 2007; Hunger, 2003).

Fluorescent dyes and other chromophores are not widely used, e.g. quinophthalone dyes, nitro- & nitroso dyes, stilbene dyes, formazan dyes and triphenodioxazine dyes. These are not discussed further in this thesis.

2.3 Dye classification according to method of application
The dye classes presented here are those considered most interesting for dyeing different textile fibres. The classification is basically the same as in the EU report for work package 1 - “Industrial needs analysis and target(s) selection” in the project SOPHIED, where the textile industry in Europe is described (Vanhulle, 2004).

Acid dye (Anionic Azo Dyes)*
Acid dyes are low molecular dyes usually with a monoazo, diazo or anthraquinone structure and are known for their capacity to perform bright shades. Their small sizes allow them to diffuse rapidly into complex materials
such as leather. They are sometimes referred to as *penetrating dyes*. The dyeing process involves an acidic step, hence the name of this class. Acid azo dyes are used for dyeing and printing mainly wool and polyamide, along with materials such as silk, leather, paper and food (Hunger, 2003).

*Basic dye (cationic Azo dyes and cationic Methine dyes)*
Various types of dye molecules can have cationic functionality, though it is most common in Azo dyes and methine dyes. Today, they are mainly used for dyeing polyacrylonitrile (acrylic) fibres and bleached sulphite cellulose. They are even used to a lesser degree for dyeing leather, paper, plastics and waxes, and as constituents of graphic arts colours (Hunger, 2003).

*Direct dye*
Direct dyes are used to dye natural or regenerated cellulose; mordants are not used in the process. Their quality in wetfastness is not the best, but their reasonable price and ease of application make them one of the largest azo dye groups used today. Cotton is dyed in neutral or alkaline process, and paper in an often-weak acidic process. Fibres are dyed at hot temperatures, allowing the dye to penetrate and form a dye aggregate within the fibre and then resist being washed out (Hunger, 2003).

*Mordant dye*
Mordant dyes are a group of dyes that need mordants for their application on fibres. Mordants are used to enhance the colour, considered important for most natural dyes. The material is usually pre-treated with metal salts that are used as mordants, and a metal compound is then formed on the fibre during the process. The metal compound can form insoluble coloured complexes with certain dyes (often azo or anthraquinone) (Hunger, 2003).

*Vat dye***
Vat dyes are essentially insoluble in water. The dye is treated by alkaline liquor that reduces it to a leuco form, which has a good affinity for cotton and other cellulose fibres. When the dye oxidises, colour is developed. This group of dyes is dominated by anthraquinone and indigoid dyes (Christie, 2007; Vanhulle, 2004).

*Reactive dye*
The development of reactive dyes had a major impact on the dyeing industry, with its colour-fastness and outstanding wash-fastness properties. Reactive dyes react chemically to the fibre and form a covalent bond between the dye molecule and the fibre. This covalent bond is formed between a carbon atom in the dye molecule and an oxygen, nitrogen or sulphur atom of a hydroxyl, amino or thiol group on the polymer (Christie, 2007). There are reactive dyes for cellulosic, protein and polyamide fibres. To initiate the reaction between
fibre and dye an alkali environment is used. This transforms, for example, the hydroxy groups (–OH) on the cellulose fibre into the more reactive nucleophilic anions (–O\(^-\)). The reaction with the dye forms a covalent bond: Cell-O-Dye.

Unfortunately, the dye molecules can also react with the alkaline ions in the solution and form a hydroxy group. The formed hydrolysed dye is no longer capable of reacting with the fibre and must be washed out of the fibre before dyeing is complete (Christie, 2007). The drawback with reactive dyes is that 10-40% of the dyes never react with the fibre, requiring a wash-off step in the process. One step to improve the dyes further is by developing dyes with two reactive groups: bifunctional reactive dyes, which improve the fixation. Efforts are also being made on designing dyes with a lower sulphur content (Commission, 2003).

**Disperse dye**

Disperse dyes with their low water solubility are well suited for colouring hydrophobic fibres such as polyester. Most of these dyes come from the azo class, but there are anthraquinons and some minor representatives from nitro, methine, naphthamide and quinophthalone dyes (Christie, 2007; Hunger, 2003).

**Metal-Complex dyes**

Metal-complex dyes, often a metal complex with azo dyes, show good affinity to most fibre materials. Commercially, the most interesting colours are obtained through complex dyes with chromium, cobalt, copper, iron and nickel. Metal-complex dyes are often applied to protein fibres, where they provide dyeing with excellent fastness properties (Christie, 2007; Hunger, 2003).

* Water soluble
** Soluble in water at high temperature
*** Insoluble in water, converted chemically to soluble form-return to insoluble at fibre.

In the case of vat, sulphur and azoic dyes, an insoluble pigment is formed within the fibre and retained with mechanical forces (entrainment). In most other cases, intermolecular forces such as dipolar, van der Waal’s forces, ionic and hydrogen bonding retain the dye molecules. Reactive dyes form a covalent bond to the fibre.
3. TEXTILE FABRIC AND DYEING PROCESSES

To make dyed textiles, a chain of processes, which are basically the same for the different fibres, is required. Natural fibres are prepared; synthetic fibres are manufactured. Natural fibres contain more impurities that need to be treated. During these processes a substantial amount of water and a palette of different chemicals are used, which end up in the wastewater leaving the facility. In Fig. 7 below a process flow over a typical textile mill is presented (Alex, 2009; Balachandran & Rudramoorthy, 2008; Bisschops & Spanjers, 2003; ReiFe & Freeman, 1996; Savin & Butnaru, 2008).

Each process has its special function and leads to a wastewater stream with a specific composition. Usually, these streams are mixed together. However, in the pursuit to re-use water and decrease wastewater, separated flows have their advantages. A lot of water is also used outside the process, merely to wash and clean buckets, baths and pipes.

The processes can be either batch or continuous, and many different chemicals are used to obtain the desired results. Dyes are not the biggest contributor to the Chemical Oxygen Demand (COD) values in wastewater streams, but are regarded as one of the trickier ones to deal with. Certain chemicals such as insecticides and pesticides are not used in the fabric processes, however, attached on the fibre material and end up in the wastewater from textile mills.
Fig. 7 Process flow chart over textile manufacturing.
Fibre preparation
In this step natural fibres are separated from plant residues and formed into yarn.

Sizing
The sizing step is about preparing fibres for mechanical warping and weaving. The most common sizing agents used are, synthetic: poly vinyl alcohols (PVA), polyacrylates, polyesters, and native polysaccharides: CMC and starch. The type of sizing agents used is connected to fibre types and processes. As well, chemicals such as peroxosulphates (viscosity regulators), polyglycol ethers (antistatic agents), fatty alcohols (wetting agents), paraffin or silicon oils (de-foaming agents), formaldehyde, phenol derivates and fungi/bactericides (preservatives) are used. The fibre is oiled or waxed, usually with sulphated fats or fatty acid esters. Of the chemicals mentioned above mineral oil stands out as not biodegradable. In Europe technologies have been somewhat developed to recycle some of these chemicals (Bisschops & Spanjers, 2003; Commission, 2003).

Weaving
The yarn is weaved into textiles without any additives or water.

Knitting
If the yarn is knitted instead of woven, paraffin oil is often added to allow for a higher knitting speed. Mineral oils are widely used to lubricate the knitting machinery, which may add 4-8% of the fabric weight.

Desizing
Combined with the desizing step the fabric is often treated with a flame to remove fibre ends sticking up from the surface, called singeing. To prepare the textiles for dyeing, all additives are removed, done by enzymatic or oxidative (Hydrogen Peroxide) desizing and washing (95°C) (Commission, 2003). In this process, all chemicals added in the preceding steps and detergents are released into the water, resulting in an extensive COD load (Bisschops & Spanjers, 2003; Commission, 2003).

Scouring
Scouring can also be a part of the desizing or bleaching process and is applied to remove impurities in the fibres, such as pectins, fats and waxes, proteins, inorganic substances and sizing degradation products. Scouring is performed in an alkali environment, together with reducing agents (sulphite & hydrosulphite), dispersing agents (polyacrylates & phosphonates), complex binders (EDTA, NTA, DTPA) and some non-ionic (alkyl phenol & ethoxylates) and anionic (sulphonates, carboxylates & phosphates) additives (Bisschops & Spanjers, 2003; Commission, 2003).
Bleaching
Textiles dyed in dark colours can be dyed directly, but for textiles intended for bright and pastel colours, bleaching is obligatory. Today, chemicals like hydrogen peroxide (H₂O₂), sodium hypochlorite (NaClO), sodium chlorite (NaClO₂) are most widely used. In some cases additional optical brightening agents are also used. Applying hydrogen peroxide is preferred during alkaline conditions, with additional complex former agents (EDTA), to bind metal ions that can catalyse H₂O₂. Sodium hypochlorite is applied during pH 9-11 and sodium chlorite is applied during acidic conditions (pH 3.5-4) (Bisschops & Spanjers, 2003; Commission, 2003).

Mercerising
The mercerising step (the stretching of fabric during alkaline conditions) is applied on cotton. Generally, this is performed with a sodium hydroxide solution (170-350g NaOH/kg fabric) for 40-50 seconds. The reaction is exothermic and needs cooling. Mercerising improves dye uptake and reduces the amount of dye needed by 30-50%. Ammonia is an alternative chemical that can be used in this process (Commission, 2003).

Dyeing
Various types of dyes are described in previous sections. Christie (2007) stated that azo dyes represented 60-70% of the commercial dye market, and Vandevivere et al. (1998) estimated that anthraquinone dyes accounted for 20-30% of the market. Besides dyes, dispersing agents are also used, particularly in vat, disperse and sulphur dyes processes. Traditionally, naphthalene sulphonic acids with formaldehyde have been used for this purpose, though these can be exchanged for aromatic sulphonic acids or sometimes more environmentally friendlier products based on fatty acids and esters (EU-commission, 2003). The widely-used reactive dyes have problems with poor dye fixation, but the new design of bifunctional reactive dyes can attain >95% fixation on the fibre. Poor fixation of mono reactive dyes is often compensated with high concentrations of salts, compared to the low salt concentrations used with bifunctional dyes. The process of applying High-affinity dyes requires 10-50 g salt l⁻¹; low-affinity dyes demand 60-100g salt l⁻¹, the higher value is for deep shades. In Industrial Dyes under “Reactive dyes on Cellulose” (Hunger, 2003), an example from Exhaustion Dyeing is described: “The optimum dyeing conditions depend on the reactivity of the dyes. Cold dyers are dyed at 30-50 °C, pH 10-11; hot dyers at 70-90 °C and pH 11-12. For pH control normally mixtures of sodium carbonate and sodium hydroxide are used. To enhance substantivity of the dye sodium sulphate or sodium chloride is added. For less than 0.5 % dye based on textile weight, salt concentrations of 10-30 g l⁻¹ are
recommended. For deep shades (more than 4%), ca 50 g l\(^{-1}\) is used; with vinylsulphone dyes having low substantivity, up to 80 g l\(^{-1}\).”

This section describes the different parameters involved in the process of applying different dyes very well. The dyes have various numbers and abilities of reactive groups, requiring various degrees of enhancement in the chemical process. The material and the dyes in the process will affect the level of chemicals and pH in the wastewater stream. Wastewater loads of 5,000 mg COD/l are common in processes with mono reactive dyes. The salts involved are often sodium sulphite, sodium hydrosulphite and sodium chloride. In pad-bath dyeing, some dyes used contain additional urea, sodium silicate and salt. New dyes without these additives are available, but require a high capital investment in technical equipment.

Another problem is that a part of the dyeing industry is still traditionally conservative and prefers chromium (mordant) dyes rather than new more environmentally friendlier dyes (Bisschops & Spanjers, 2003; Christie, 2007; Commission, 2003; Hunger, 2003).

**Printing**

In printing, the dyes are applied to the fabric in the form of pastes, though dyes and auxiliaries are basically the same as in dyeing processes above. The pastes consist of dye, water, thickeners, urea, defoamers (e.g. silicon compounds, esters), surfactants and solvents (e.g. glycol, glycerine, and ethylene). Cleaning the printing equipment with large amounts of leftover printing pastes leads to a wastewater fraction (Bisschops & Spanjers, 2003; EU-commission, 2003).

**Finishing**

In the finishing process, certain textiles go through an “easy-care treatment”, where compounds such as dihydroxietylene urea are applied and formaldehyde is often used as a solvent. There can also be treatments such as water-repellency, softening, flame retardancy, antistatic, or bactericidal and fungicidal. Several of the treatments are applied using organic solvent and dried, which produce small volumes of wastewater when the equipment is washed. Still, biocides are chemicals that lead to environmental concern due to their toxicity to aquatic life. However, there are very few reports on data concerning wastewater from this step (Bisschops & Spanjers, 2003; Commission, 2003).

Technology development in Europe in these areas is heading towards a decreased consumption of dyes and chemicals, and the reuse of water and processes to waste less dyes and chemicals. For example, shorter pipes for dye solutions in printing processes leads to less water needed for cleaning (Commission, 2003).
4. WATER AND ENVIRONMENTAL IMPACT

The water used in textile mill processes ends up in the wastewater streams and different approaches exist to deal with this contamination. If the wastewater flows are mixed together, the resulting water will contain oils, fats, dyes, different salts and other chemicals as described in the previous section. If the process involves wool, the load of fats will be even heavier. The pH of the water depends on the mixing of flows, but will usually be between 7-10 in combined water (Bisschops & Spanjers, 2003).

The dye contents in waters treated in previous studies differ quite notably; from 20 mg l^{-1} to 2.5 g l^{-1} in artificial waters compared to 10-50mg l^{-1} are usually found in dye house effluents (O'Neill et al., 1999). O’Neill also states that a typical dye effluent from a textile mill contains several dyes; the best simulation was attained by mixing yellow, red and blue dyes. Literature on dyes (Hunger, 2003) also states that colour is produced by mixing different dyes in the dye bath.

The treatment of water effluents from dyeing industries and textile mills has been an area of extensive research during the last two decades and hundreds of articles have been produced. The ultimate goal must be to circulate all water in the process and become independent of external water. However, this is technically very complicated and in some parts expensive. One method is to divide different flows in the process to clean and reuse as much chemicals and water as possible. Still, this approach is maybe more for industry in the developed world. In developing countries the goal is to have the best possible wastewater treatment at the lowest conceivable cost. The quality of water or degree of pollution in wastewaters is often measured in Biological Oxygen Demand for 5 or 7 days (BOD5/BOD7), and Chemical Oxygen Demand (COD) to measure the amount of oxygen needed for degradation or oxidation of the molecules (mostly organic) in the water. BOD and COD are good parameters, but they do not indicate anything about the composition of the
molecules in the water. To find out more about the biological and chemical properties of the molecules in the water or monitor a degradation process, more advanced analytical equipment is needed. Suitable methods to investigate and detect molecules in water solutions are High-Performance Liquid Chromatograph (HPLC), coupled with mass detection (LC/MS or LC/MS/MS). This is further discussed in the analytical section.
5. DIFFERENT WASTEWATER TREATMENT METHODS

5.1 Physical

5.1.1 Flocculation
A common first step in wastewater treatment is flocculation, where particles in the water coagulate to floccs, usually enhanced with additional Iron(III) chloride (FeCl₃) or Aluminium chloride (AlCl₃). Studies of flocculation have shown that the number of sulphonic and amino groups of the dye molecule correlate to the ability to flocculate (Szygula, a et al., 2008).

5.1.2 Adsorption
Adsorption is a way to trap chemical molecules onto the surfaces of solid material and thus extract them from the water phase. One of the most well-known adsorbents is activated carbon with its good adsorption values, though it is also expensive. Natural materials have also a proven capability to adsorb different dyes. The most cost efficient method is to use waste fractions such as bark, sawdust, rice husks or other type of husks. The different chemical parameters of different dyes affect the adsorption efficiency of the materials. Mondal (2008) summarises the adsorption capability for some cellulosic material for a handful of different dyes; about 200-500 mg of dye/g of material is outlined from the data.
Ash is another waste fraction that can adsorb textile dyes. Note that the adsorption capacity of fly ash is affected by pH and the presence of inorganic salts. Other adsorbents such as silica, Corynebacterium glutamicum, starch, zeolite (natural or modified), polyether foam and different fungi have also proven effective in adsorbing dyes (Chiavola, 2009). Several other materials have also been tested in adsorption studies, such as cells, where both bacteria and fungi can adsorb dyes to the surface. All of these materials can adsorb dyes to various degrees. The drawback is that the problem transfers to another
fraction, which needs disposal. To completely oxidise organic molecules with aromatic structure combustion over 800°C is necessary (Lewtas, 2007).

5.1.3 Filtration
Treatment using different filters is very attractive, as this opens up the possibility to reuse water. How the filters perform depends on parameters such as pH and concentrations. Filtration is divided into three categories: Ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). The membranes are constructed from several different polymer materials (e.g. polyamides, polyester, polycarbonates, fluor-carbon based polymers, polysulphones, polyacrylonitriles). Membrane information was collected from DOW and Koch industries (Dow, 2012; Koch, 2012).

All filter types work with pressure as the driving force and their performance depends on temperature, concentrations and type of molecule. Other parameters, such as available surface for separation and fouling of pores, also concern the separation.

Ultrafiltration
Ultrafilters have pore sizes in the range of 0.01 - 0.3 µm and can separate molecules down to molecular weights of approximately 10,000 g mol⁻¹ (Dow, 2012; Koch, 2012).

Nanofiltration
Nanofilters have pore sizes in the range 0.001 - 0.01 µm and can separate molecules down to molecular weights of approximately 100 g mol⁻¹. Nanofilter can separate some salts (ions), but not all (Dow, 2012; Koch, 2012).

Reverse osmosis
Reverse osmosis is based on a semi-permeable membrane that allows water molecules to pass, but not ions. Osmosis is a natural force where water wants to neutralise the differences in ion concentrations and thus water diffuses over membranes. Reverse osmosis is when pressure is applied on one side of the membrane (this process can be driven against the diffusion coefficient) and pure water obtained and the ions are retained. The membrane is sensitive to chlorine (Dow, 2012; Koch, 2012).

5.2 Chemical
Oxidation with ultra violet light (UV), electrochemical, hydrogen peroxide (H₂O₂) and ozone (O₃)
Oxidation processes that use UV are often called Photodegradation processes. These are often combined with a catalyst and hydrogen peroxide (H₂O₂) or
sometimes with ozone ($O_3$). Different catalysts are used, such as Co-ZSM-5 zeolites, $Fe_2O_3$, CdS, ZnO, and $WO_3$, but the most preferred is $TiO_2$. (Chiavola, 2009)

Another chemical degradation method is the Fenton process, which uses iron ions, such as a catalyst with hydrogen peroxide, to create hydroxyl radical that is known to degrade organic compounds (Matilainen & Sillanpää, 2010). A similar mechanism is also performed by electrochemical degradation method. The most well-established is the Electro-Fenton process, which can use Pt or Boron-doped diamond anode and a stainless steel or carbon-felt cathode (Martínez-Huitle & Brillas, 2009; Umar et al., 2010). Certain advantages are obtained by combing these methods, e.g. Wu and Ng (2008) received the most energy efficient degradation by combining $Fe^{3+}$/H$_2$O$_2$ with O$_3$ or UV/O$_3$ (pH4 or 7). Their system with UV/O$_3$/H$_2$O$_2$/Fe$^{3+}$ used only 15% of the energy needed in the UV/O$_3$ system.

5.3 Biological processes

Biological processes can be performed through different living organisms. In sewage treatment bacteria (Hesham et al., 2011) microscopic animal and fungi play important roles. Previous experiments showed that most dyes pass relatively unaffected through traditional active sludge treatment. Scientists from the Environmental Protection Agency’s (EPA’s) Water Engineering Research Laboratory in Cincinnati, Ohio studied the fate of water-soluble acid and direct dyes in the activated sludge process. Screened raw wastewater was used from a local sewage treatment plant, while influent to three pilot-scale activated sludge biological treatment systems operated in parallel. The wastewater influent to the treatment system was spiked with various commercial dyes in concentrations of either 1 or 5 mg l$^{-1}$. Dye analyses were conducted on the wastewater effluent and activated sludge. Data obtained from this study demonstrated that 11 of 18 dyes passed through an activated sludge process in pilot-scale untreated, 4 adsorbed onto the activated sludge and 3 underwent biodegradation (Shaul et al., 1991).

Different dye classes show different grades of adsorption, which seems to depend on the dyes’ solubility. For example, acid dyes with more sulphonic groups (higher solubility) tend to adsorb to a lesser degree (Shaul et al., 1991). The amount of dye adsorbed was found to be linear to the number of cells in the activated sludge (Ledakowicz et al., 2001; ReiFe & Freeman, 1996).

There are two major groups of enzymes used by bacteria in the degradation of azo dyes: reductive or oxidative enzymes (Saratale et al., 2011). Example of the oxidative enzyme activities reported were lignin and manganese peroxidase, laccase and tyrosinase (Saratale et al., 2011). Very few studies
have reported activity of oxidative enzymes in bacteria, though *Streptomyces* (dos Santos, 2007) and *Sphingomonas* produce an unspecific extracellular enzyme peroxidase (Stolz, 2001). The lignolytic enzyme system is further discussed in the white-rot fungi section.

The reductive enzymes azoreductases are classified as flavin dependent (FMN) or flavin independent, and NADH or NADPH or both can be used as a cofactor (Saratale et al., 2011). Azoreductase activity was reported from both intracellular and extracellular degradation, even though either sulfonatedazo dyes or flavin dependent reductases should be able to pass the plasma membrane (Erkurt, 2010). Presently, exact mechanisms remain unclear. Several bacteria have a proven degradation ability and capability to produce azoreductase. Species known to utilize insoluble materials use what is sometimes called extracellular respiration to donate electrons to the outside of the cell. Electron shuttles (redox mediators) can help transfer electrons to the substrate (electron acceptor) and enhance reactions by increasing the activity sphere of the microbe. The substrate or acceptor can be oxygen, nitrate, sulphate, metal oxides, halogenated organics or azo dyes. The electrons can be either donated directly by the shuttle, or transferred by a secondary mediator (Watanabe et al., 2009). Quinone, indophenol, humic acids or flavin groups in the environment often function as secondary electron shuttles (Chen, 2006; Watanabe et al., 2009). Anaerobic bacterial processes were proven to degrade azo dyes. This is probably due to the electrochemical unstable azo bond (-N=N-) that can act as an electron receiver. However, oxygen is a better electron acceptor and the potential of the azo bonds to receive electrons is reduced in the presence of oxygen; hence, the azo bond is better degraded in anaerobic environment (Table 1) (Van Der Zee et al., 2003; Watanabe et al., 2009). Dubin and Wright (1975) stated that azo dyes usually have $E_0$ values between -0.430 and -0.180 V. NAD(P)H have $E_0$ -0.320, which limits the reducing system below this value. Redox mediators, i.e. molecules that can transfer electrons, play an important role if they have $E_0$ between the $E_0$ region for NAD(P)H and azo dyes.
Table 1: RedOx potentials

<table>
<thead>
<tr>
<th>Redox potential</th>
<th>( E_0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{O}_2/\text{H}_2\text{O} )</td>
<td>0.8'</td>
</tr>
<tr>
<td>( \text{NO}^-/\text{N}_2 )</td>
<td>0.74'</td>
</tr>
<tr>
<td>( \text{NO}^-/\text{NO}_2^- )</td>
<td>0.42'</td>
</tr>
<tr>
<td>( \text{Fe}^{3+}/\text{Fe}^{2+} ) (pH7)</td>
<td>0.2'</td>
</tr>
<tr>
<td>( \text{SO}_3^{2-}/\text{H}_2\text{S} )</td>
<td>-0.17'</td>
</tr>
<tr>
<td>( \text{FAD}^+/\text{FADH}^- )</td>
<td>-0.22'</td>
</tr>
<tr>
<td>( \text{NAD}^+/\text{NADH}^- )</td>
<td>-0.32'</td>
</tr>
<tr>
<td>Azo dyes</td>
<td>-0.18'-0.43'</td>
</tr>
<tr>
<td>RM</td>
<td>-0.5'</td>
</tr>
</tbody>
</table>

(Madigan et al., 2009)
(Dubin & Wright, 1975)
1Redox Mediators: several different quiniones, flavins etc. (dos Santos, 2007; Van der Zee & Cervantes, 2009)

Chang et al. (2001) found that azoreductase performance was affected by pH, with 2.5 times better dye reduction at pH 7-9 than below pH 7. Morrison et al. (2012) investigated this in *Clostridium perifringens* and determined the optimal pH for the azoreductase enzyme activity to pH 9. These findings corresponded well to the best decolorization found between pH 7-9.5 (Saratale et al., 2011). Furthermore, the azo bond reduction rate rose with an increased temperature, a maximum rate around 40°C, 3-5 times faster than at 20°C (Angelova et al., 2008).

Certain classes of aerobic azoreductase are considered homologous: flavin free azoreductases with cofactor NADPH, NADH or both; flavin dependent azoreductases with cofactor NADPH, NADH or both (Chen, 2006; Stolz, 2001). Chen (2006) listed eleven different azoreductases, several from aerobic or facultative anaerobic bacteria such as *Pigmentiphaga kullae, Xenophilus azovorans, bacillus* sp. (Table 2). Investigations on intestinal bacteria, such as *Clostridium, Enterococcus* and *Bifidobacterium*, have a proven capability to split the azo dye direct blue 15 during anaerobic conditions. Also, species such as *Staphylococci aureus, Staphylococci epidermis, Micrococcus luteus* living in environments like the surface of human skin showed the ability to decolourize the azo dyes (Stingley et al., 2010). The azoreductases from these bacteria seem to be more specific about the dyes’ molecular structure than the electron shuttles in anaerobic bacteria.

However, many investigations have indicated uncertainties of how aerobic the environment was in the experiments performed. In several cases cells were...
grown with mixed media in batch experiments without shaking, probably resulting in an oxygen free environment after a short time (Stolz, 2001). The last year’s rapid development in genome sequencing has provided powerful tools to identify different genes in bacteria, e.g. over 100 bacteria were identified to carry genes for one or several azoreductases (UniProt Consortium, 2002–2012).

5.3.1. Bioremediation of aromatic hydrocarbons

From extensive research about pesticides and poly aromatic hydrocarbons (PAH), information concerning microbial degradation of aromatic molecules can be extracted. Chemicals based on an aromatic ring structure, either accidently or intentionally spread in nature, have become an environmental problem due to their recalcitrant structure (Gevao et al., 2000; Seo et al., 2009).

Benzene was found to initially degrade when oxidized to catechol (Fig. 8), before the ring structure is fractured through several enzymatic steps into either succinic acid and acetyl-CoA or pyruvic acid and acetaldehyde (Cerniglia, 1984; Juhasz & Naidu, 2000). Bacteria use similar degradation pathways to degrade and utilize chemicals such as nitroaromatic compounds, nitrobenzene, nitrobenzoates, nitrophenols, nitrotoluenes (Ju & Parales, 2010). In more complex ring structures, bacteria degraded the complex ring structure first and then the same pathway as for benzene (Haritash & Kaushik, 2009). Juhasz and Naidu (2000) listed about 20 bacteria able to oxidize naphthalene, which is one of the core structures in the molecule for both reactive red 2 and reactive black 5. Interestingly, Juhasz and Naidu (2000) includes *Acinetobacter* and *Pseudomonas*, close relatives to the bacteria identified in papers III and V. Furthermore, if we include investigations from the biodegradation of Aceanaphthene and Anthracene, *Sphingomonas* had a proven degradation capability. This supported the results in papers III and V, where the dye is degraded without any accumulation of aromatic metabolites. It seems that bacteria capable of PAH degradation are often present in sediments. Bacteria degrade PAH faster with two and tree ring structures than four and five and degradation is more effectively performed in water environments than in soil (Haritash & Kaushik, 2009).

However, aromatics can have different structures (the cleavage of the azo bond in the dye molecules often results in amino benzenesulfonates and naphthalenesulfonates) that are known to be quite persistent to degradation (Lourenço et al., 2003).

Anaerobic and aerobic bacteria seem to share similarities in their degradation strategy of aromatic hydrocarbons (e.g. phenols, toluene, benzoates, anilines,
creosols, benzene, xylenes, naphthalene, PAH, Nitroaromatic and chlorinated compounds); the diverse structure is degraded into a few central structures. Thereafter, the ring structure is activated and cleaved resulting in short organic acids (Haritash & Kaushik, 2009).

A Verhagen et al. (2011b) study on the biodegradation of pesticides supports that large molecules and the produced metabolites can be completely mineralized by a biological consortium. In fact, several times, the microbial consortium has shown to degrade the aromatic amines produced by azo cleavage (Saratale et al., 2011) to a much larger extent than single species.

5.3.2. Aerobic conditions

There are bacterial strains known to decolourize azo dyes under aerobic conditions (Pandey et al., 2007). Pourbabae et al (2006) showed good degradation of some disperse dyes and actual effluent from a textile mill by Bacillus sp. Resmi and Senan (2003) used an aerobic reactor with a consortium of bacteria immobilised on laterite pebbles to successfully degrade dye in a continuous system.

A possible criticism from these studies is that cells can adsorb dye on their surface and no real degradation takes place. This is difficult to rule out with short batch experiments. However, in the case of Resmi’s reactor, which ran continuously for 14 days with residence time between 0.78 and 6.23 h and dye concentrations up to 100 mg l⁻¹, degradation was probably performed. The Laccase enzyme could also be identified in the effluent of their reactor; still, the pebbles may have provided support for the biofilm with anaerobic environment in some parts.

Several species have been reported to actively degrade azo dyes in aerobic environments (Table 2). Some species have several azoreductases, e.g. Pseudomonas aeruginosa have three azoreductases: paAzoR1, paAzoR2, and paAzoR3, known to degrade various azo dyes (Ryan et al., 2010).
Table 2: Example of bacteria with azoreductases insensitive to oxygen, compiled by Chengalroyen and Dabbs (2012)

<table>
<thead>
<tr>
<th>Family/species</th>
<th>Enzyme class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus sp.</td>
<td>NADH reductase</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>FMN dependent NAD(P)H reductase</td>
</tr>
<tr>
<td>Gracilibacter</td>
<td>NADH reductase</td>
</tr>
<tr>
<td>Klebsella sp.</td>
<td>FMN dependent NADH reductase</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>NADH reductase</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>FMN dependent NADPH reductase</td>
</tr>
<tr>
<td>Xenophilus azovorans KF46F</td>
<td>Flavin free reductase</td>
</tr>
</tbody>
</table>

1 some species display oxygen sensitive azoreductases.

5.3.3. Anaerobic or anoxic conditions

Today, the biodegradation of textile dyes and especially azo dyes focuses on anaerobic treatment (Dafale et al., 2010; Mondal, 2008; Saratale et al., 2011). In this field, extensive research has been mostly performed on artificial waters with one or more dyes mixed in distilled water. Rafii (1990) reported the presence of azoreductases in the anaerobic bacteria *Clostridium* and *Eubacterium*. Further investigations showed that most azoreductases are known to be sensitive to oxygen, and Rafii et Cerniglia (1995) also showed that azoreductase were capable of degrading nitro aromatic compounds. Many researchers have conducted interesting experiments with anaerobic degradation. Many of the investigations focused on one or several species isolated from an environment considered to be interesting, e.g. textile effluent or contaminated soil. Their degradation performances were tested under different conditions (Kalyani et al., 2008; Khalid et al., 2008). These studies were usually performed during sterile laboratory conditions on artificial waters. Different strains of *Pseudomonas* and *Bacillus* were found to degrade several dyes (Dafale et al., 2010). In many anaerobic studies decolourization were performed successfully; examples of bacteria species capable of degrading different azo dyes are compiled in Table 3. However, colour reduction is not enough and metabolites and chemicals can still linger in the water. The dyes are often cleaved into aromatic amines, which absorb light in other wave lengths than most dyes (Pinheiro et al., 2004). The presence of sulphate in dye molecules and in the water will affect the degradation process. The presence of sulphate reducing bacteria can contribute to release sulphide, which can reduce some of the dyes chemically (Mondal, 2008).
### Table 3: Example of anaerobic bacteria capable of degrading azo dyes

<table>
<thead>
<tr>
<th>Family/species</th>
<th>Enzyme class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridium</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Sphingomonas Xenophaga</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Eubacterium</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Klebsiella sp.</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Shewanella decolotionis</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Citrobacter sp.</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>FMN-dependent NADPH alt. NADH-azoreductase^4</td>
</tr>
<tr>
<td>Paenibacillus</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Proteus</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Kerstersia</td>
<td>-</td>
</tr>
<tr>
<td>Alcaligenes faecalis</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Comamonas acidovorans</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Halomonas</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Rhizobium</td>
<td>Oxidative and reductive</td>
</tr>
<tr>
<td>Aeromonas hydrophila</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
<tr>
<td>Desulfovibrio sp.</td>
<td>FMN-dependent NADH-azoreductase^4</td>
</tr>
</tbody>
</table>

^1 Compiled by Cervantes and Dos Santos (2011)
^2 Compiled by Khalid et al. (2010).
^3 Compiled by Saratale et al. (2011)

#### 5.3.4. Anaerobic and aerobic treatment combined

Colour fades when the azo dyes are split, though the metabolites, usually aromatic amines, are often even more toxic than the dye they originate from (Stolz, 2001). To decolourize, water is just one part of the purification process and the next step is to mineralise the aromatic amines formed. Aromatic amines are smaller colourless molecules that can enter the bacterial cell to a great extent to be mineralised through oxidative pathways (Ju & Parales, 2010). Several researchers have seen a major decrease in toxicity from the effluent when the anaerobic treatment was followed by an aerobic step (Libra et al., 2004; O’Neill et al., 2000). Van der Zee and Villaverde (2005) concluded in their review article that most aromatic amines were mineralised.
in the aerobic step. However, they also noticed that some amines appeared to be recalcitrant to degradation.

One of the most promising approaches to the biodegradation of textile dyes is therefore to use a combined anaerobic and aerobic system. The anaerobic step splits the azo bond and forms aromatic amines in the process, which can be mineralised in the following aerobic step. Similar environments can also be created with carriers in the same system, creating possibilities for development of biofilms. Kudlich et al. (1997) immobilised *Sphingomonas* sp. in alginate beads in an aerobic reactor. However, the beads maintained an anoxic environment in their centre, which allowed for a reductive cleavage, and the bacteria on the aerobic surface of the beads mineralised the metabolites.

The author developed systems with anaerobic reactors followed by one aerobic reactor (Papers II and V). In paper II, the reactors had wood shavings as carriers and were fed with artificial wastewater containing 200 mg l\(^{-1}\) Reactive Black 5 and 200 mg l\(^{-1}\) Reactive Red 2. The system decolourized over 90% of the colour in the anaerobic reactors. The metabolites identified after the anaerobic treatment could not be retrieved in the water following the aerobic reactor. The biofilter system was further developed articles III, IV and V, with rice husks as carrier in the biobeds and more complex wastewaters containing dye and other chemicals such as cotton fats, salts and detergents. Furthermore, our biofilter in manuscript V performed robust degradation (over 90% degradation at 20 degrees) of an actual textile wastewater containing dye and additional chemicals, without any nutrient amendment or added redox mediators. In view of this data and compared to values reviewed by (dos Santos, 2007) of mostly lower concentrations, we consider our developed system to perform at the high end of efficiencies.

5.3.5. Fungi

Fungi are an evolutionary old organism, and can occur in various forms and in nearly all habitats. Fungi grow better in low pH and can endure lower humidity and higher osmolality than most bacteria (Tortora, 2011). The kingdom of fungi is divided in five phyla: Ascomycota, Basidiomycota, Zygomycota, Chytridiomycota, and Glomeromycota (Madigan et al., 2009). In water environments, fungi are known to infect fish, but we seem to lack more detailed knowledge about fungi in water environments. For example, in 2008 only Sweden had any specifications for fungi in drinking water (Hageskal et al., 2009). Hageskal et al (2009) also stated that species like *Trichoderma viride*, *Aspergillus fumigatus*, *Mucor*, *Absidia*, and *Candida* are known to inhabit tap water. Fungi are not known to be invasive through water to healthy people, but might be involved in skin irritations and allergenic responses (Hageskal et al., 2009).
In environmental water treatment, several fungi with interesting qualifications occur in wastewater treatment, and most fungi studied are white-rot fungi with lignolytic enzymes. Different strains of white-rot fungi have been tested in the field of biological degradation due to their ability to release enzymes extracellular. Fungus has developed this ability in nature to degrade large complex molecules extracellular, e.g. lignin or cellulose. This ability can be used to degrade large organic molecules that have difficulty in passing the cell membrane. There are several enzymes of interest; cellulases are more specific than the enzymes developed to degrade lignin. The lignin degrading enzymes, e.g. Laccase, manganese peroxidise and lignin peroxidase, are unspecific but potent decomposers of organic molecules when combined with hydrogen peroxide (Chen, 2006; Cullen & Kersten, 1992; Srinivasan & Viraraghavan, 2010; Zhao, 2004). Fungi degrading lignin are named white-rot fungi. The white-rot fungi enzyme production is limited by nutrients (nitrogen or carbon) (Ge et al., 2004). The lignolytic enzymes perform their best degradations at low pH and several fungi have their optimum growth at pH 4 (Kaushik & Malik, 2009).

Fungi can function as dye degrading organisms, and species such as *Pleurotus Ostreatu*, *Tramete Versicolor*, *Candida Tropicalis*, *Phanerochaete chrysosporium* have been used successfully in experiments in controlled environments. The author performed experiments with the white-rot fungi *Bjerkandera sp.* in a contactor that treated artificial textile wastewater successfully in sterile conditions. However, the degradation did not turn out well in unsterile environment since the fungi were sensitive to contamination and got knocked out. (Forss unpublished results; Libra et al., 2003).

5.3.6. Nutrient and carbon sources

To enhance the degradation an electron donor can be added, e.g. glucose, hydrolysed starch, yeast extract, acetate. This is needed for both anaerobic and aerobic degradation. Alex (2009) worked with a microbial consortium that was isolated from effluent water from a Urafiki Textile factory and investigated the effect of different carbon sources, e.g. glucose, starch, fructose, lactose, galactose, maltose, sucrose, on degrading Rhemazol Brilliant Blue (RBB) during shaking. Glucose, galactose and maltose enhanced the decolourization equally and further investigations determined the concentration 0.3% (glucose) to be most effective. Alex also investigated the effect of organic (peptone, tryptone, yeast-, beef-, malt extract) and inorganic (ammonium sulphate, ammonium chloride, ammonium nitrate and sodium nitrate) nitrogen sources. The best enhancement in decolourisation was obtained by 0.2% yeast extract (70% decolourization) for the organic and 0.2% sodium nitrate (45% degradation) for the inorganic nitrogen. The addition of yeast extract obtained better degradation than inorganic nitrogen, and others have reported similar results (Baêta et al., 2012; Moosvi et al.,
This may be due to the yeast containing many other minerals and carbon sources in addition to nitrogen. Of note, the nitrogen per cent in yeast extract is only about 11% of dry weight (BD, 2010). Other carbon and nitrogen sources are also available. Fats and waxes and impurities from the fibers, together with chemicals such as detergents, dyes and additives from the process, also end up in the effluent. Proteins and amino acids can contribute with nitrogen, and oil, fats, proteins and detergents can provide carbon sources. Carbon can also be attained from lignocellulosic material of the carrier constituting the biobeds and aromatic amines become available if the dye molecules are degraded. However, enhancing the degradation capacity is important, to keep operational costs as low as possible with the prospect of the technology being used in developing countries.

5.3.7. Reactor design

Bioreactor design is a key aspect in treating textile wastewater, especially if operated in an actual situation. The wastewater streams at textile mills are irregular, where a stream from a bleaching step reaches the treating facility one moment and a stream from the dyeing step the next. This means that the treating facility usually get periods of high flows with occasional extreme values in pH, temperature, chemical and dye concentrations. Wastewater treatment with active sludge systems based on suspended or flocculent biomass can risk being washed out (Sipma et al., 2010). One attempt to neutralize the extremes is to have an equalization tank or pond before the treatment process.

Systems with immobilised cells or biofilm formations have better resistance to wash outs and fluctuations (Olivieri et al., 2010). For example, immobilised cells of *Rhodococcus erythropolis* have shown a shorter lag phase and a rate 8.5 times higher when degrading phenol, compared to suspended solution of the same strain (Prieto et al., 2002). Amorim (2005) reported that immobilized cells handled organic shock loads and toxic pollutants better. Furthermore, immobilized cells were also better in recovering their performance after extreme events (Amorim et al., 2005). Cells in immobilised systems were found to remain more active during starvation periods, and proved to have a shorter lag phase when the organic source was reintroduced (Jorge & Livingston, 2000; Nicolella et al., 2005). Additionally, cell systems with high continuity are vital for slow growing organisms, which include anaerobes. Moreover, Verhagen et al. (2011a) found that biofilm formation results in different bacteria species and a lower accumulation of metabolites when degrading the pesticide Chloropropham.
Cells can be immobilized by polymers or form biofilms on suitable materials, such as glass beads, plastic, perlite mineral, bamboo and wood. Many types of plastic carriers are available commercially (Lenntech, 2012; Veolia, 2012). Bacteria living on surfaces can build biofilm structures up to 100 nm thick (Madigan et al., 2009). There are different reactor designs to immobilise bacteria or fungi cells (Fig. 9). However, when applying segregated biophases, the mass transport phenomena become relevant to reactor performance. Compounds diffuse from the liquid bulk towards the biophase and continue to intra biofilm/particle diffusion. Chen et al. (2003) investigated the optimal bead size for cells (*Aeromonas hydrophila*, *Comamonas testosteroni*, and

---

Fig. 9: a) Bioreactor with moving bed, b) bioreactor with immobilized bed, c) contactor with rotating wood discs.

---

Cells can be immobilized by polymers or form biofilms on suitable materials, such as glass beads, plastic, perlite mineral, bamboo and wood. Many types of plastic carriers are available commercially (Lenntech, 2012; Veolia, 2012). Bacteria living on surfaces can build biofilm structures up to 100 nm thick (Madigan et al., 2009). There are different reactor designs to immobilise bacteria or fungi cells (Fig. 9). However, when applying segregated biophases, the mass transport phenomena become relevant to reactor performance. Compounds diffuse from the liquid bulk towards the biophase and continue to intra biofilm/particle diffusion. Chen et al. (2003) investigated the optimal bead size for cells (*Aeromonas hydrophila*, *Comamonas testosteroni*, and
Acinetobacter baumannii) immobilized in Polyvinyl Alcohol (PVA) beads and reported a model to calculate kinetics and dye diffusion combined. Furthermore, from their results they recommended PVA gel beads with a diameter of 2.7–3.7 mm for best efficiency.

Textile wastewater can be either treated in batch or in continuous applications. Several designs of bioreactors are available for different applications: Rotating drum, Up flow anaerobic sludge bed (UASB), fixed bed reactor, sequencing batch reactor (SBR), continuous stirred tank reactor (CSTR), plug flow reactor (PFR), up flow packed bed reactor, fluidized bed reactor (Olivieri et al., 2010). The SBR is one of the most frequently used for textile wastewater treatment (Lu & Liu, 2010). Treatment is performed in one reactor that is filled, the water is treated first anaerobically and then aerobically. After the active treatment, there is a calm phase where particles and floccs can settle, followed by the phases draw and idle, and then the cycle is restarted. Another reactor type is the UASB, where the wastewater is fed to an anaerobic zone at the bottom and gas and treated water are collected at the top. In some cases, an aerobic zone is implemented at the top of the reactor (Olivieri et al., 2010). Treatment can also be performed with a packed bed reactor or fluidized bed reactors usually with anaerobic environments (Sipma et al., 2010). The rotating drum and rotating contactor use the same principle with part of the system in water and part in the air, presumably performing aerobic degradation. However, further investigations of biofilm performance by Zhang et al. (1995) showed that the biofilms in these applications displayed anaerobic environments where the azo bonds were split.

A rotating contactor with wood discs (fig. 9) partially in water was used by the author in application with white-rot fungus to treat dye solutions (Forss, 2006 unpublished results). The immobilised bed system is suitable for anaerobic biofilters, e.g. to treat textile dye in continuous systems. The author has developed an anaerobic biofilter with packed beds of wood shavings (Paper II) and rice husks (papers III, IV and V), followed by an aerobic treatment (paper II and V).

5.3.8. Toxicity

Analyzing molecules in wastewater is a complex undertaking, and testing for toxicity is often used instead. Toxicity test are developed from different water living species such as Vibrio fisheri, Rhaphidocelis, Ceriodaphnia, Nitocra or Zebra fish (Rizzo, 2011). Toxicity is measured as LC/EC 50 values for mg/L of substance or % of wastewater (Öman et al., 2000). Different organisms are suitable for different applications, e.g. Ceriodaphnia is suitable for fresh water and Nitocra for brackish or salt water. The procedures are described in standards. In this work, the SS028106 for Nitocra was suitable due to the textile wastewater having a salinity of 4‰ (article V).

Although toxicity test are good, it is well known that high levels of ions such as ammonium/ammonia are difficult on the test organisms and are known to
disturb the tests (Swedish Environmental Protection Agency, 2008). Therefore, the tests are not always applicable on landfilling leach water, etc. The textile effluent water used in article V had a total nitrogen concentration of 41 mg l\(^{-1}\). Experiments on frogs have shown that tadpoles are sensitive to nitrogen, with 96 hr-LC50 values of around 20 mg l\(^{-1}\) of nitrogen for several species (Rouse et al., 1999). However, *Nitocra Spinipes* is considered a suitable organism to characterize landfill leachates (Assarsson, 2003). A preliminary test with *Nitocra spinipes* was used to evaluate the treated water in manuscript V, though the results were not clear enough to be included in the article. The 48h test showed that *Nitocra* was sensitive to the treated water, though when diluted to 50%, most of the crustaceans survived.
6. CHEMICAL ANALYSIS

The analytical field contains diverse methods, with many highly advanced and demanding special skills. Several of these methods, e.g. 3D gas chromatography, could probably expand and deepen the research on the enzymatic steps of degradation. However, due to the high level of knowledge needed and expense for advanced analytical instruments, these methods are not yet employed. The methods used in today’s articles are spectrophotometer, Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis. The latter three techniques can be coupled with Mass Detection (MS) in one or two steps (GC/MS, GC/MS/MS, HPLC/MS, HPLC/MS/MS, electrophoresis/MS). Spectrophotometric analyses are widely used to observe changes in colour at specific wave lengths, thus providing information about the degree of decolourization performed by the biological system. Yet, this gives little or no information about the metabolites formed from degradation, which in some cases are even more toxic than the dye. If the samples are scanned over a broader range, some information about the metabolites can be observed, e.g. aromatic amines absorbs light between 200 to 350 nm (Pinheiro et al., 2004). Spectrophotometric analysis provides information on how something absorbs at a certain wave length. To determine how many different molecules there are behind the absorption, a separation in GC or HPLC is needed. HPLC is preferred because the dye and their metabolites are solved in water. HPLC can be used to identify substances if the standards are known. However, in most cases concerning biological degradation the molecular structure of the metabolites is unknown and there are no standards. To gain knowledge about the molecules in the water LC/MS or LC/MS/MS is preferred. GC/MS can also be applied, but requires an extraction step that is more difficult and whose results are more uncertain. This is unfortunate, as LC/MS have no connected molecular libraries as is usually the case with GC/MS (Bianchi et al., 2005; Pielesz et al., 2002; Pinheiro et al., 2004; Plum & Rehorek, 2005; Sanz Alaejos et al., 2008; Weschenfelder et al., 2007).
The author used the HPLC analysis in paper I and LC/MS in paper II (Fig. 10) to monitor the degradation process. Of note, the metabolites could become hydrophobic and absorb onto the packing material in the biofilters or the container. To rule out this possibility, the filling material was tested over a long series for several experiments. The long operating periods should have saturated the filling material and these metabolites should have leaked to be eventually observed in the outlet. The containers for the biofilter have also been used over long series of experiments (paper II).

Fig. 10: LC/MS scan with mass spectra for the peaks at 31.69 and 37.74 with the suggested molecular structure (paper II).
7. MICROBIAL ANALYSIS

7.1 Fungi

In the environment, microorganisms work together in a microcosm of interactions, and different organisms such as archea, protista, fungi and bacteria closely interact with each other and plants in symbiosis, commensalisms or competition. Biomolecular techniques have been widely used and successfully on different bacteria in diverse environments. However, it seems that fungi and archea in environmental samples have not been thoroughly investigated.

Fig. 11: Epifluorescence microscopy, of cells from the biofilter used in paper III, stained with SYBR Gold.
7.2 Microscopy and fluorescent in situ hybridization

Using a microscope to visualize cells in samples taken from the solution in biofilters is good, but difficulties arise with biofilms and fungus. Cells can be stained with probes to be visualized, their morphology can be observed and their number counted (Fig. 11, paper III and V). Cells can also be visualized in more detail with different probes specific to species and structures. Staining cells with fluorescent DNA probes, e. g. Fluorescent in situ hybridization (FISH), is an excellent way to attain information about the species in a microbial population. The probes used can be directed at different targets and information can be extracted from different levels in taxonomy and cell functions (Sanz & Köchling, 2007). FISH can complement the information from sequencing by displaying the species’ locations and provide information about the interaction in biological systems.

7.3 Amplification of microbial DNA

Samples from biobeds can be grown and isolated on agar plates to receive information at the species level, though this is time consuming work and several species are difficult to cultivate (Dubey et al., 2006). A better way is to use PCR amplification and Denaturing Gradient Gel Electrophoresis (DGGE) analysis (Talbot et al., 2008). The genes of microorganisms can be amplified through PCR with primers usually targeted on bacteria 16S rDNA (Sanz & Köchling, 2007).

The bacteria genes for the ribosomal unit is evolutionary conserved; therefore, the 16S region is well suited to describe the phylogenetic relationship between different species. To separate and analyze the organisms, primers are targeted towards variable DNA regions close to the preserved ones, receiving a molecular fingerprint (Gilbride et al., 2006). However, fungi’s corresponding 18S rRNA genes do not display the same variation, limiting identification to the genus or family level. To receive greater taxonomic resolution the internal transcribed spacer (ITS) region, with a greater sequence variation, is often targeted (Anderson & Cairney, 2004). Much knowledge about fungi is found in mycorrhiza research, however, mycorrhiza samples are differ in the parts of roots to consider in the extraction. In water environments, a handful of articles describing molecular applications investigate the presence or development of fungi. Fungi are analyzed through amplification of the 18S or the ITS region, since fungi are eukaryote. Several suitable primers are used for amplification, targeted towards the ITS region (Martin & Rygiewicz, 2005). Nikolcheva et al. (2005), Das et al. (2007; 2012) and Buesing et al. (2009) investigated fungi in environmental samples from different water habitats, and Pereira et al. (2010) attempted to identify the presence of fungi in drinking water. Nikolcheva and Bärlocher’s (2004) investigation cross referenced several other types of organisms to check their expression from the ITS primers used.
Based on these experiences, the primers ITS 3 with a GC-clamp and ITS 4 were selected for the amplification of fungi in article V.

7.4 DGGE

DGGE separates the various DNA in the sample and displays a pattern of specific bands from the microbes. The patterns similarities can be compared with computer analysis and the development between samples can be overviewed. The similarities and differences are often presented in a dendrogram. The bands in the DGGE can be cut out and the DNA be sequenced. These molecular techniques were used to retrieve information in paper III and follow the development of bacterial composition in the anaerobic biofilters treating Reactive Black 5 and Reactive Red 2. The composition of the microbial community was further investigated in manuscript V, where both bacteria and fungi from a biofilter treating actual textile wastewater were analysed.

7.5 DNA sequencing

Amplified DNA can be sequenced through molecular techniques and is usually performed by specialized companies. The base pairs of the extracted DNA are determined and the amplified DNA is compared using BLAST (Basic Local Alignment Search Tool) algorithm and comparing to databases e.g. U.S. National Center for Biotechnology Information (NCBI) where suggestions of probable species are interpreted. Sequencing information is usually presented with the genetic similarity to both the closest relative and closest cultured relative in the GenBank. From the closest cultured relative in the GenBank valuable information is often retrieved from databanks such as the NBCI. NBCI’s information provides insight into the microorganisms’ capabilities and preferred environments. Substantial information about bacteria is available, because the methods were developed earlier and more uniform in this field. The fungal biomolecular taxonomic development was first based on 18S rRNA, as for certain phyla, though researchers now prefer investigations targeted towards the ITS-region for its better resolution (Anderson & Cairney, 2004). Therefore, the information retrieved from databases to construct the collective knowledge about microorganisms, their habitat and capabilities is much more detailed for bacteria than for fungi.
8. PRESENTATION OF INCLUDED PAPERS

Paper I: Decolourization of reactive azo dyes with microorganisms growing on soft wood chips

The decolourization of 200 mg l\(^{-1}\) Reactive Black 5 and 200 mg l\(^{-1}\) Reactive Red 2 dye mixed together was studied in batch experiments using microorganisms growing on wood chips from forest residues combined with or without added white-rot fungus, *Bjerkandera sp.* BOL 13. The study was performed as a first stage in the development of a relatively simple treatment process for textile wastewater, designed to work in developing countries. Wood chips of forest residues are populated by a mixture of fungi and bacteria, which is an advantage when complex molecules need to be degraded. The wood chips might also provide the microorganisms with a source of carbon, possibly making the addition of, e.g. glucose unnecessary. The results showed that *Bjerkandera sp.* had trouble in competing in a non-sterile environment. The microorganisms growing on the wood chips of forest residues performed better. They decolourized the mixture of the two dyes; adding extra nutrients approximately doubled the decolourization rate. The time needed for decolourization in batch experiment was approximately 18 days when nutrients were added. Lignocellulosic material is complex and substances dissolved in the water, disturbing the analysis. Microorganisms were therefore transferred to ordinary soft wood chips from forest residue wood chips. Decolourization was measured with a spectrophotometer and monitored with HPLC.

Paper II: Biodegradation of Azo and Anthraquinone dyes in batch and continuous systems

The purpose was to develop a complete microbiological model system for the treatment of wastewater from textile mills in developing countries. Artificial
wastewater was treated by microorganisms growing on wood shavings from Norway spruce in non-sterile conditions. The microorganisms were inoculated from forest residues. Mixtures of the azo dyes Reactive Black 5 and Reactive Red 2 were degraded in batch and continuous experiments. Reactive Red 2 mixed with the anthraquinone dye Reactive Blue 4 was also treated in the continuous system. The system consisted of three reactors – the first two with an anaerobic environment and the third with an aerobic. The dye concentrations were 200 mg l⁻¹ of each dye in the continuous system and the retention time was approximately 4 days and 20 h per reservoir. Samples from the process were analyzed with the spectrophotometer and LC/MS to monitor the degradation process. Between 86-90% of the colour was removed after a treatment of 4 days and 23 h in the continuous process. Two metabolites were found in the outlets of reactors one and two; a molecular structure is suggested for one of the metabolites. The metabolites were degraded to below the detection limit in the aerobic reactor.

Paper III: Biodegradation of the Azo dyes Reactive Red 2 and Reactive Black 5 in a continuous system based on rice husks and its microbial diversity

In the present study, the degradation of two common azo dyes used in dye houses today, Reactive Black 5 and Reactive Red 2, was evaluated in biofilters. In two experiments, bioreactors performed over 80% decolorization at a hydraulic retention time of only 28.4 h. The biofilter had reactors with biobeds of rice husks that contributed with microorganisms, in one of the experiment additional microorganisms were inoculated from forest residues. Treated water was analyzed by Liquid Chromatography coupled with mass spectroscopy (LC/MS), without any metabolites detected in the water leaving the system. Epifluorescence microscopy showed a bacterial abundance in the reactors of around 4 x 10⁸, and the diverse cell morphologies suggested a complex community in the reactors. The composition and development of the microflora in the biofilters was determined by polymerase chain reaction (PCR) amplification and denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA gene. The molecular analyses showed a diverse and dynamic bacterial community composition in the bioreactors, including members of the Bacteroidetes, Acinetobacter (Gammaproteobacteria) and Clostridium (Firmicutes), which possess the capacity to reduce azo dyes. Collectively, the results indicate that the development of mixed bacterial communities from natural biomaterials contributes to an efficient and robust degradation performance in bioreactors, even at a high dye concentration.
Paper IV: The treatment of azo dyes found in textile industry wastewater by anaerobic biological method and chemical oxidation

Synthetic wastewater containing azo dyes found in textile industry wastewater was treated through an anaerobic biological method and chemical oxidation. The main aim of this study was to compare different treatment methods and to evaluate the effect of different parameters on treatment effectiveness. Two different synthetic wastewaters were treated in continuous systems with biobeds of rice husks, which also contributed with microorganisms. The biofilter successfully treated a dye solution with two azo dyes, 79% decolorization with a residence time of 28.5h. However, when a more complex dye solution containing salts, cotton fats and detergents decolorization dropped to 29%. When the residence time was doubled with two additional reactors, the system attained 78% decolorization.

Collectively, in the microbial process, the results showed that an increase in the residence time and the amount of yeast extract and the addition of microorganisms originally growing on forest residues all had positive effects on the dye removal. The system obtained the best decolorization of 89% with 2 g/L of yeast extract (0.351 mL/min), though when the flow rate was doubled (0.803 mL/min), only a slight drop in performance to 82.7% decolorization was attained.

In the catalytic wet peroxide oxidation process, CWPO, the reaction conditions were optimized at 0.5 g/L activated carbon loading with 2mL H$_2$O$_2$/300mL (6.67ml/L) solution (35 wt%), at 80°C, in 2 h with pH 3. At the optimum conditions, approximately 93% of the dye was removed. At these optimized conditions, the CWPO process was tested with actual textile industry wastewater. The percentage of dye removal with this wastewater was 50%. The adsorption effect of the activated carbon was also investigated. At pH 7, the removal by adsorption was around 15%. But in acidic conditions (pH 3) and at higher temperatures the adsorption effect of activated carbon increased. Adsorption and oxidation performances were compatible at 80°C, whereas at lower temperatures the adsorption effect was greater than the oxidation. In conclusion, decolorization was generally accomplished by 60% adsorption and by 40% oxidation.
Textile processes use many different chemicals, most of which ends up in wastewater. Coloring of clothes is a particularly troublesome process since both azo and anthraquinone dyes are recalcitrant to degradation, causing environmental concerns. Hence, there is a great need to investigate and develop safe and applicable systems to the water demanding industry, such as textile mills in developing countries. In the present study biodegradation of actual textile wastewater (containing azo and anthraquinone dyes) was evaluated in biofilters. Indigenous decolourants from rice husks were used in bioreactors and the degradation was analyzed with spectrophotometer and liquid chromatography coupled with mass spectrometry (LC/MS/MS) to monitor metabolites, especially in the form of aromatic amines. Chemical characteristics of the water e.g. COD, volatile fatty acids, pH and oxygen were monitored during treatment.

The indigenous microflora from rice husks consistently performed over 90% decolorization at a hydraulic retention time of 67 h. Analysis by denaturing gradient gel electrophoresis (DGGE) analysis and subsequent sequencing of the 16S rRNA and ITS gene fragments revealed the presence of bacteria such as Clostridium, Pseudomonadales, Xenophilus, Paenibacillus, Acinetobacter and Sphingomonas, which are known to carry genes for azoreductases. Combined with previous results (article III) our results suggest that several bacterial species are preferentially selected in the biotreatments. Furthermore, results showed that fungi were present in the biofilter, and were predominant in the aerobic reactors. Collectively, these results indicate that the developed biofilter with rice husks support a mixed microbial community of both bacteria and fungi, with key features contributing to an efficient and reliable degradation performance of actual textile wastewater.
9. DISCUSSION

Solving substances in water is often an easy task, but removing recalcitrant organic molecules solved in water is often very complicated. This is troublesome because organic molecules can often be absorbed and accumulated in biological systems. Water in developing countries is a resource exposed to great demand from people, farming and industry. Clean water is a resource that is even more scarce, necessary and desired. A developing nation moving towards industrialism possesses a high risk to use and foul substantial water resources. Hence, there is a great need to research and develop methods and systems that are safe and applicable to the water demanding industry in developing countries. Extensive research in the area of water treatment has been done; where this thesis contributes is to the knowledge of textile wastewater treatment. The textile industry is slowly migrating to countries where cheap labour and resources are available. Textile processes use many different chemicals, most of which ending up in the wastewater.

Much research has been performed in a variety of different fields and presented in the literature. Because of the breadth of research, even review articles have trouble comparing the results from different methods due to the different values with parameters, units and incomplete data. Many researchers are engaged in finding a solution to the textile industry’s environmental influence, e.g. several chemical treatment methods such as precipitation or chemical degradation. Many cells utilize hydrogen peroxide (H₂O₂) for biological degradation, which therefore can be regarded as an environmental friendly chemical. Ozone is an even stronger oxidising agent, today widely used for environmental friendly bleaching in, for example, the pulp industry. Both chemicals can be used together with catalytic materials such as TiO₂, UV or both, or electrochemical input. In some cases, the methods are combined. However, some studies have used a great amount of H₂O₂ to degrade dye mixtures (Chiavola, 2009). Moreover, it can be difficult to implement these methods in an actual textile mill with complex wastewater and it may lead to high operational costs. Still, UV/electrochemical
Degradation is known to be efficient in colour removal at a low cost, but may leave high concentrations of chlorine in the water (Mondal, 2008). Overall, chemical methods should be considered as an important complement to biological treatment, especially if recalcitrant compounds are used in the process. However, to minimise the use of chemicals and operational costs, biological treatment is recommended as the main treatment.

Many factors affect the degradation of dyes, e.g. different dye classes and the dyes’ molecular structures influence water solubility, adsorption properties and cell transmembrane diffusion. The molecular structure also influences the stability, reactivity with electrons and the adherence to degrading enzymes (Pasti-Grigsby et al., 1992). This knowledge can also be used to design dye processes with dyes that microorganisms degrade easier (Chengalroyen & Dabbs, 2012). From the literature, biodegradation rates of azo dyes are known to increase with temperatures up to 40°C, 3-5 times faster than at 20°C (Angelova et al., 2008). The research from the papers in this thesis carry out degradation at 20°C, though heating of wastewater is not economical feasible. Moreover, isolated azo reductases display an optimum pH at 7-9.5 (Saratale et al., 2011). This was confirmed in manuscript V, where the first reactor had a pH of 6.6 and the degradation rate was low, while the second reactor pH increased to 7.9 and the degradation rate improved. Furthermore, to sustain activity most anaerobic bacteria utilise electrons from carbon sources other than the dye and its metabolites (Alex, 2009; Moosvi et al., 2007). In article IV, where a complex dye solution was degraded, the experiments showed that an increased nutrient amendment with yeast extract increased degradation rates. However, actual wastewater contains compounds from support chemicals, other processes, and the fibre material, which can constitute carbon and nutrient sources. A COD of 3,000 mg l⁻¹ characterised the actual wastewater in manuscript V, though all may not be biologically available. We hypothesise that the compounds in the wastewater and the rice husks in the filter will manage to sufficiently support the microorganisms with a carbon source and nutrients.

The presence of redox mediators such as flavins and quinones are known to improve decolorization (Saratale et al., 2011). This issue has not been addressed in the presented articles. Therefore, this work aspires towards cost efficiency and a minimum of additives. Although, if anthraquinone dyes are present they can probably provide some enhancement, since they are quinone derivates.

Aerobic biological treatment of wastewater is widely used in sewage treatment all over the world, where active sludge removes BOD, nitrogen and phosphorous from the water. It is cost-efficient and environmental friendly, qualities that are most desired in developing countries. However, most dyes pass this treatment unaffected, and the biological treatment of dyes needs
appropriate design to consider the molecules in the wastewater. Anaerobic treatment often degrades azo dyes to aromatic amines, posing an increased toxicity. Most aromatic amines degrade well in a following aerobic treatment. Furthermore, the use of HPLC and LC/MS analysis can give the researcher insights into degradation performance, metabolites and their further degradation.

Microbiological species, their development and community composition are known to be affected by changes in their environment (Madigan et al., 2009). There is a need for further research closer to the implementation phase, with microbes that are adapted to treat complicated wastewater and survive open competition. Both Dafale et al. (2010) and Saratale et al. (2011) emphasize that a microbial consortia achieve a higher degree of degradation and mineralization. There is presently no strain reported that can decolourize a broad range of dyes. Moreover, to treat the complexity of textile wastewaters, a biofilm community of microbes is better suited to handle fluctuations and, as reported, to degrade metabolites to a higher extent (Verhagen et al., 2011b). Our knowledge of these complex interactions between diverse species living in biofilms is scarce, and the usage of methods such as PCR, DGGE and sequencing increases our insights into their community. Furthermore, it is also probable that some microorganisms living in ponds outside the textile mills already possess the desired abilities and can be recruited to assist degradation.

In this thesis, a biological treatment from a controlled batch experiment was developed to degrade an actual textile wastewater in a continuous biofilter with anaerobic/aerobic biofilm treatment. The treatment was based on lignocellulosic materials such as rice husks to be cost-efficient enough for implementation in developing countries. The analytical analysis, though troublesome, showed in most of the performed experiments an absence of metabolites such as aromatic amines. The analyses were difficult, though in paper II the metabolites found were clearly metabolized in the aerobic step. The same methods were used in paper III and manuscript V, without any metabolites identified in the treated water. However, in paper V the COD analysis indicated organic or inorganic matter in the water that was recalcitrant to biological degradation. Whether or not the rice husks emit organic or siliconized compounds in the water can be further investigated. A deeper analysis of the samples in manuscript V, with a specialized acetylation of aromatic amines, revealed one compound that was perhaps an aromatic amine. The substance was increased during the three first reactors and then decreased by 60% in the last two reactors. When active coal was tested on the treated water the remaining indicated amine was absorbed and the COD was diminished to 173mg l\(^{-1}\). To conclude, the microbiology treatment decolorized the investigated dyes well, even though the COD indicated that some organic material was not degraded. Furthermore, the analytical results indicated that
the dyes in our experiment were degraded and most of the metabolites mineralized.

During this work, new insights were gained into the complexity of the microbial communities and the species supported by the biofilter. Several species with interesting characteristics were found in the biofilters. In article III bacteria like *Clostridium*, *Acinetobacter* and representatives from *Sphingobacteriales* were found to inhabit the filters. All of these species carry genes for azoreductases, a quality most desired to a bacterium in a biofilter degrading azo dyes. Furthermore, when actual textile wastewater was treated (manuscript V) the microbial consortium developed with the bacteria, such as *Clostridium*, *Pseudomonadales*, *Xenophilus*, *Acinetobacter* and *Sphingomonas*, also capable of produce azoreductases. The retrieval of such similar species confirms that these are competitive species and strengthens our belief that the developed biofilter can be used in actual applications. In the same study, several fungi were also found to inhabit the environment in the biofilter (manuscript V). The rice husks showed good qualities to recruit and support an actively degrading microbiological consortium. Collectively, this research showed potential that further scale-up studies could be applied at least as a vital part of a wastewater treatment in a textile industry.
10. CONCLUSIONS

From the papers in this thesis, several members of the indigenous flora on forest residues and rice husks possess the capacity to degrade azo dyes in a competitive environment. The continuous system with soft wood shavings inoculated with indigenous microorganisms from forest residues also displayed the ability to degrade anthraquinone dye.

Actively dye degrading microorganisms can be transferred and supported by forest residues, wood shavings and rice husks (papers I, II, III). However, the best degradation results were attained with microorganisms supported by rice husk (papers III and V). The recruited microorganisms were used to form a continuous system with anaerobic and aerobic reactors capable of reliably degrading dye solutions. Throughout the work in the following papers more complex dye solutions were investigated. Thus, in manuscript V indigenous microorganisms from rice husks treated actual textile wastewater successfully (at 20°C without any nutrient amendment), where 90% were decolourized in 67 h. Furthermore, the hydraulic retention time could be shortened, results indicate 80% decolorization within 27 h.

One of the most challenging tasks was to analyse the chemistry in the collected samples, from which bark included in the early experiments greatly disturbed analytical resolution. The HPLC analysis in paper I indicated that dye was degraded, metabolites formed and disappeared. In paper II, SP analysed the samples with LC/MS, resulting in one identified and one unknown metabolite after water passed the anaerobic reactors. Still, the metabolites were mineralised in the following aerobic reactor. The constituents in the actual textile wastewater samples (manuscript V) interfered with the LC/MS and complicated the analysis. An acetylation reaction was used to highlight the aromatic amines in the samples, and revealed that the first unknown peak in the diagrams was not caused by aromatic amines, strengthening earlier results. Furthermore, the analysis
showed that all but one of the metabolites was mineralized, with the remaining metabolite in the water decreased by 60% during treatment.

The microorganisms retrieved with PCR, DGGE and sequencing described in paper III confirm our interest in natural microorganisms from lignocellulosic materials. Several interesting species were identified, e.g. *Sphingobacterales*, *Clostridium*, and *Acinetobacter*, with known abilities to produce azoreductase. The mixed communities in the continuous biofilters performed a robust degradation successfully over several weeks. Furthermore, in the work that led to manuscript V our knowledge about the selected microbial community was greatly deepened by bacterial and fungal rDNA. The bacteria degrading the actual textile wastewater, such as *Clostridium*, *Pseudomonadales*, *Xenophilus*, *Acinetobacter* and *Sphingomonas*, carry genes for azoreductases. The composition of the bacteria consortium displayed several similarities to species retrieved earlier in article III. The results verified the potential and sturdiness of the species supported by the biofilter.

Overall, the results presented in this thesis indicate that a biofilter based on rice husks is a sustainable concept in treating textile wastewater. The studied biofilters showed great potential in selecting microorganisms that could possibly be used to treat textile wastewaters in a very cost-efficient way.
11. FUTURE RESEARCH

The next step would be to scale up the developed filter and test the technical design and function in a long-term study on site. To scale up a laboratory biofilter into a pilot plant or an actual treatment facility with complex wastewater containing different chemicals and fluctuating flows is a major challenge. Testing wood material from developing countries is interesting, wood chips are perhaps better suited in size to handle increased flows. The process of implementation should be divided into small steps. The studied biofilters have potential to treat textile wastewater successfully, much due to its cost-effective construction and therefore deserve to be developed further.

The analytical methods used need to be further developed concerning sampling and pre-treatment. The actual textile wastewater in manuscript V had a more complex background, influencing the analytical analysis more than previous dye solutions. Therefore, a further developed sampling procedure with Solid Phase Extraction (SPE) or similar method is needed.

In future studies, it could be sufficient to monitor the aromatic amines, which are volatile enough to extract with Solid Phase Micro Extraction fibre (SPME) and analyse on GC/MS. Here, an easier sampling and analysis with the possibility to compare mass spectrum with the library could be set up. There is potential to develop the analytical procedure further, which would help future studies on actual textile wastewaters.

There are new insights to be gained in the organization of the microbial community, e.g. the role of fungi needs further investigation. Furthermore, if Archea are present and if it contributes in dye degradation. Another area to examine further is collaboration between fungi, bacteria and archea.
Moreover, there are several new bio molecular tools, e.g. terminal restriction fragment length polymorphism (T-RFLP), amplified ribosomal intergenic spacer analysis (ARISA), and 454 pyrosequencing, that could be implemented to receive more detailed information.
Employing Fluorescence in situ hybridization (FISH) techniques to learn more about the construction of the microbial consortium and which species interact with one another would be interesting.

An increased knowledge of biological processes, complexity of wastewaters and the use of analytical methods would lead to new questions and further research. The horizon is always changing. We have taken small steps on the path to knowledge about the microbial world in wastewater treatment. Yet, we still have much to learn about the microorganisms’ interaction and influence in biofilm structures and biofilters.
12. ACKNOWLEDGEMENT

I would like to express my gratitude to everyone who has helped and contributed to my work with their knowledge, friendship and support:

My supervisor, Ulrika Welander, who has always found time to discuss scientific issues and read my texts.

My Co-supervisor, Magnus Christensson at Anox Kaldness, Veolia water, for valuable input and that you shared the technical and industrial aspects of wastewater treatment with me.

Jarone Pinhassi for being open minded and believing in our work and for all your time and patience in reviewing my texts to article III and manuscript V.

Markus Lindh for assisting me with the molecular analysis of microbes in the laboratory.

Lena Brive at SP for chemical analysis and her efforts in trying to teach me the “language of MS”.

Susanne Wikman for engaging in discussions on organic molecules and methods of their analysis.

My colleagues in Växjö and Kalmar for participating in discussions and all the knowledge they have shared.

My family for supporting me on my journey and filling my world with meaning.

The Swedish International Development Cooperation Agency (SIDA) is gratefully acknowledged for its financial support.
AUTHOR’S CONTRIBUTION TO PRESENTED PAPERS:

I. Decolourization of reactive azo dyes with microorganisms growing on soft wood chips
   Author contributed by invention of ideas of use of forest residues; designed experiment in discussion with supervisor, carried through experimental set up; developed HPLC analysis; and wrote the article.

II. Biodegradation of azo and anthraquinone dyes in batch and continuous systems
   Author designed, developed and ran the continuous system: experimental set up was partly run by diploma worker Sara Palacios from Spain. Author performed HPLC analysis and SP analysed samples on LC/MS. Author interpreted results and wrote the article.

III. Article III: Microbial diversity in a continuous system based on rice husks for biodegradation of the azo dyes Reactive Red 2 and Reactive Black 5.
    LC/MS analysis performed by SP, microbial analysis, PCR and DGGE performed in collaboration with Markus Lindh and Jarone Pinhassi, Linnaeus University. Author evaluated results and wrote major part of the article.

IV. The treatment of azo dyes found in textile industry wastewater by anaerobic biological method and chemical oxidation.
    Author developed experimental design and set up of microbiological treatment. Master student Orçun Türgay from Turkey ran major parts of the microbial experimental set up under the author’s supervision.

V. Manuscript: Biotreatment of actual textile wastewater in a continuous biofilter and the associated bacterial and fungal microflora.
Author designed and ran the experimental set up. SP Technical Research Institute of Sweden performed LC/MS analysis, all other chemical analysis were performed by the author. The author designed the molecular fungi analysis. Microbial analysis, PCR and DGGE were performed by author with support from Markus Lindh and Jarone Pinhassi, Linnaeus University. Markus Lindh has contributed with bioinformatic evaluation and support. Author evaluated results and wrote the manuscript.
REFERENCES


Swedish Environmental Protection Agency. 2008. Lakvatten från deponier (Swedish).


Tortora, F., Case. 2011. *An introduktion to mikrobiology*.


