# Sequenced Anaerobic-Aerobic Treatment of Hemp Pulping Wastewaters

Sjon Kortekaas

**Proefschrift**

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
Dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 4 november 1998
des namiddags te vier uur in de Aula

7. Ook in gemeenschappen van apen zijn sex en politiek nauw met elkaar verweven.

*Frans de Waal (1989). Chimpanzee politics: power and sex among Apes. John Hopkins University Press, USA.*

8. Het maatschappelijk risico van het halfslachtige Nederlandse beleid ten opzichte van softdrugs is niet gelegen in het gevaar voor de volksgezondheid, maar in het tolereren van een crimineel zwart geld circuit.

9. Duurzame landbouw vraagt een doordachte aanwending van mineralen, wat niet noodzakelijkerwijs inhoudt dat elke vracht mest gewogen, bemonsterd en geanalyseerd zou behoeven te worden.

*Gewijzigde meststoffenwet, 2 mei 1997.*

10. De overdracht van genetisch materiaal vanuit transgene landbouwgewassen naar een wilde populatie door geslachtelijke vererving, geeft aan dat genetische manipulatie van landbouwgewassen een groot ecologisch risico inhoudt.

*Bergelson et al. (1998). Promiscuity in transgenic plants. Nature, vol. 395, pp. 25.*

11. Na perfectionering van de pinpasjescultuur zullen mensen zich identificeren met één enkele identiteitsdrager onlosmakelijk verbonden met de huid, in functie en gebruik vergelijkbaar met het oormerk van koeien.

12. De individualisering van de maatschappij biedt de mensen slechts schijnvrijheid.

13. Als het regent in mei is april voorbij.

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# STELLINGEN

1. Het lagere kleurniveau van papierindustrieafvalwater na aerobe zuivering in vergelijking tot anaerobe-aerobe zuivering, wordt niet veroorzaakt door een hoger lignineverwijderingsrendement, maar door een geringere toename in de kleurintensiteit van het lignine bij 'aerobe' omzettingen.

   *Dit proefschrift.*

2. De aanmaak van retene (1-methyl-7-methylethyl-phenanthrene) uit dehydroabietic acid in riviersedimenten duidt op een biologische route voor de vorming van polycyclische aromatische koolwaterstoffen (PAK's).

   *Tavendale et al. (1997). The fate of resin acids-2. The fate of resin acids and resin acid derived neutral compounds in anaerobic sediments. Chemosphere, 35, pp. 2153-2166.*

3. Dilution is the solution for pollution.

   *Dit proefschrift.*

4. Size exclusion chromatography technieken welke gebruik maken van niet-waterige loopvloeistoffen, zijn slechts zeer beperkt toepasbaar voor de evaluatie van de molecuulgewichtsverdeling van lignine bij biologische zuivering, omdat de oplosbaarheid van lignine in de loopvloeistof beïnvloed wordt door biotransformatie.

   *Jokela et al. (1993). Effect of biological treatment on halogenated organics in bleached kraft pulp mill effluents studied by molecular weight distribution analysis. Environ. Sci. Technol. 27(3), pp. 547-557.*

5. Het ei was er eerder dan de kip.

   *Gibbons, A. (1998). Dinosaur fossils, in fine feather, show link to birds. Science, vol. 280, pp. 2051.*

6. De fysiologie van de Parwa (*Avicennia germinans*) is in hoge mate gevoelig voor regen, hetgeen blijkt uit de sterke en langdurige afname van de nectarproduktie van deze mangroveboom na een enkele regenbui.

   *Kortekaas, S. (1985). Parwa drachtonderzoek te Coronie. Intern rapport voor het Ministerie van Landbouw, Veeteelt en Visserij, Paramaribo, Suriname.*

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# Sequenced Anaerobic-Aerobic Treatment of Hemp Pulping Wastewaters

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Bijzonder hoogleraar 'anaërobe zuiveringstechnologie en hergebruik'

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*Vermaak de wereld, begin bij jezelf.*
Simon Vinkenoog

## Dankwoord

Een mensenleven biedt een onbeperkt aantal keuzemomenten. Kruispunten in de tijd, waar je het leven kunt inkleuren naar jouw kleur. Voorliggend boekje markeert zo'n kruispunt in de tijd en biedt perspectief op nieuwe keuzes. Leven en werken doe je echter niet alleen en daarom wil ik de mensen bedanken die aan de voltooiing van dit proefschrift hebben bijgedragen.

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# 1 General Introduction.

## Framework of this thesis

In the 1980's, increasing problems with soil-borne pathogens due to intensive cultivation of a limited number of crops in short rotations forced Dutch arable farming to look for new profitable crops intended for large-scale non-food markets. Hemp was considered, due to the outstanding quality of hemp bark fibres for papermaking. The potential of fibre hemp as a raw material for the pulp and paper industry was subject of a comprehensive 4 year study, the Hemp Research Programme, which took place from 1990 to 1994. The aim of the programme was the development of economically feasible and environmentally safe pulping processes for hemp fibres grown in The Netherlands. Environmental demands were addressed by avoiding sulphur-based cooking chemicals and chlorine-based bleaching processes as well as research on wastewater treatment to minimize the environmental impact of discharged pulping effluents. The task of the Department of Environmental Technology was the characterization of hemp pulping wastewaters, evaluation of biological methods for the treatment of these wastewaters and development of adequate technology to overcome constraints for treatment.

In this chapter, the importance of hemp in the history of papermaking will be traced. Subsequently, the chemical composition of hemp feedstocks in relation to wastewater characteristics will be discussed. Thereafter, the perspectives of anaerobic and aerobic biological treatment methods will be evaluated, with a focus on the toxicity and biodegradability of pulp and paper industry wastewater constituents.

## History and future of hemp as a fibre crop

Hemp (*Cannabis sativa* L.) is an ancient crop, which has been cultivated for more than 6000 years. Originating from Central Asia, hemp has been grown for multiple purposes from the tropics to the polar circle. Bark fibre has been utilized to manufacture rope, fabric and paper. Preparations constituting of hemp resin and buds, commonly known as hashish and marihuana, were used for medical, spiritual and recreational purposes and hemp seed provided oil for food, lubricant, paint and varnish. Among the various usages, it's use as a fibre feedstock was the prime function of hemp throughout history. When paper was invented in the year 105 A.D. in China by Ts'ai Lun, marquis at the court of emperor Ho Ti, hemp

was one of the main ingredients, among mulberry bark, rags and fishing nets. Ts'ai Lun probably perfected a technique that had been in use for some time, since recently an even older piece of paper was discovered in a tomb in Shensi province. Also this sample of paper, which dated before 104-87 B.C., was shown to be made of hemp (Li, 1974). From the invention of paper, for over 1700 years, paper was entirely made from a variety of non-wood fibres. In Europe, paper mills using worn-out ropes and tissues made from hemp and flax, were established from the twelfth century onwards (Atchinson and McGovern, 1987). The first wood-based papers were only made after 1840, when shortages of rags were the incentive to develop the stone groundwood process for converting wood into pulp (Rydholm, 1965). From that time, the importance of hemp as a source of fibres for pulp and paper production gradually declined and wood has become the predominant raw material for paper. Nowadays hemp only plays a minor role as a feedstock for speciality papers, such as cigarette papers, bibles and banknotes.

Worldwide, about 5-11% of the fibrous raw material (FRM) used to make paper, originates from non-woody plants (PPI 1995, FAO 1995). In Europe and North America however, essentially all pulp is wood-based and the share of non-wood fibres is restricted to 1-2% (FAO, 1995). The estimates of the annual non-wood pulp production vary considerably and hide very significant regional differences. Globally, the most common non-wood fibre used in papermaking is wheat straw, accounting for 47% of total non-wood paper production in 1993. Estimates of the global production of fibre hemp range from 100,000 to 200,000 tonnes per annum, which accounts for less than 1% of the world non-wood pulp production (FAO, 1995; Bolton, 1994).

In many countries, cultivation of hemp is restricted by law. Being a source of the soft drugs marihuana and hashish, hemp is often regarded as an illicit plant. Therefore, breeding of fibre hemp is aimed at negligible psycho-activity. Delta-9-tetrahydrocannabinol (THC) is generally accepted as the major psycho-active compound in hemp. Discrimination between 'fibre phenotype' cultivars and 'drug phenotype' cultivars is based on THC content. European Union legislation restricts the THC content of fibre hemp to a maximum of 0.3%, as measured in the flowering parts.

**Table 1.** Chemical composition and characteristics of some wood and non-wood fibrous raw materials.

| | | Non-wood | | | Softwood | | Hardwood |
|---|---|---|---|---|---|---|---|
| | | Hemp | | Wheat straw | Pine | Spruce | Birch |
| | | *Cannabis sativa*[1] | | *Triticum sativum*[2] | *Pinus silvestris*[3] | *Picea abies*[3] | *Betula verrucosa*[3] |
| | | Bark | Stem wood | | | | |
| Cellulose | (%) | 69.7 | 37.0 | 40 | 41 | 41 | 38 |
| Hemicelluloses | (%) | 7.8 | 17.8 | 28 | 24 | 26 | 37 |
| Lignin | (%) | 3.6 | 21.7 | 17 | 28 | 29 | 20 |
| Extractives EtOH-Benzene | (%) | 3.7 | 4.6 | 3.7 | 5.3 | 1.8 | 3.1 |
| 1% NaOH solubility | (%) | 31.4 | 32.3 | NA | 10-15 | 12-13 | 14-20 |
| Fibre length[4] | (mm) | 8-22 | 0.6 | 1.0-1.4 | 3.0 | 3.5 | 1.1 |
| Fibre width[4] | ($\mu$m) | 22 | 25 | 10-20 | 38 | 36 | 20 |

[1] van der Werf *et al.*, 1994; Nieschlag *et al.*, 1960;

[2] Misra, 1987; Bolton, 1995.

[3] Rydholm, 1965; Biermann, 1993.

[4] McDougall *et al.*, 1993, Catling & Grayson, 1982.

## Characteristics of hemp in relation to wastewater characteristics

Compared to wood, non-wood fibres are more much heterogeneous in their chemistry and fibre characteristics. The hemp stem provides two distinct fibre fractions. Outside the vascular cambium one finds the bark tissues, which make up 30 to 35% of the stem dry weight. The remaining dry weight is comprised of the stem wood tissues located in the core within the vascular cambium (van der Werf *et al.*, 1994). The most valuable fibre fraction for papermaking is the hemp bark fraction, which consists of very long fibres and is low in lignin content (4%) (see Table 1). Hemp stem wood on the other hand, consists of rather short fibres and a high lignin content (21%) similar to hardwood.

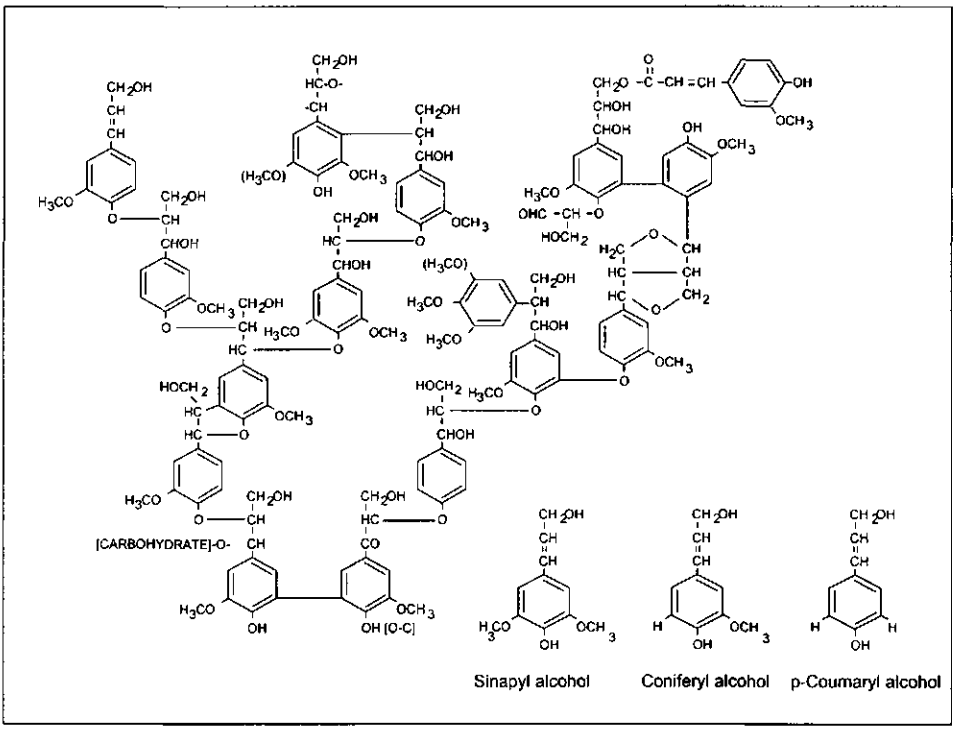


**Figure 1.** Schematic structural formula for lignin, adapted from Adler (1977). The three precursor alcohols are shown at the lower corner.

Plant fibres essentially consists of cell walls, which are composed of cellulose, hemicellulose, lignin and to a lesser extent extractives. Lignin is a highly branched, undefined aromatic polymer composed of phenylpropane subunits, which are randomly linked by a

variety carbon-carbon and ether bonds (Fengel and Wegener, 1984; Colberg, 1988). There are three basic monomers that are found in lignins, i.e. p-coumaryl alcohol, coniferyl alcohol (guaiacyl) and sinapyl alcohol (syringyl) (Figure 1). The relative proportion of the monomers depends on plant family. Hemp lignin resembles hardwood lignin and belongs to the class of guaiacyl-syringyl lignins, which are polymerizates of predominantly coniferyl alcohol and sinapyl alcohol. Characterization of hemp by nitrobenzene oxidation showed a ratio guaiacyl:syringyl of 1:0.9 for hemp bark and 1:1.4 for hemp stem wood lignin (Anonymous, 1992).

Extractives are compounds of diverse nature, which by definition are soluble in organic solvents or water. In this thesis however, the term extractives will be used in the narrow sense only, i.e. for those compounds soluble in organic solvents. According to this definition, extractives essentially constitute the compounds found in wood resin, including fatty and resin acids, and their esters, as well as apolar phenolic compounds and neutral components such as fatty alcohols, terpenes and sterols (Fengel and Wegener, 1984). Reports on the overall composition of hemp extractives are very seldom. Although the extractive fraction of hemp has been extensively studied, most research had been performed with the flowering parts of the drug-type cultivars and was focused on the isolation and identification of psycho-active compounds, in particular cannabinoids. Research by Brenneisen and ElSohly (1988), who studied the overall composition of the methanol-chloroform extractable fraction of various drug-type hemp samples, showed that terpenes and cannabinoids are the major constituents of the hemp extractive fraction. Terpene and cannabinoid contents were similar and together they constitute more than 90% of the extracted material, balanced with non-cannabinoid phenols and alkanes as minor fractions. Structures of representative hemp extractives are shown in Figure 2.

Terpenes are hydrocarbon compounds consisting of multiple isoprene units, $(C_5H_8)_n$. A total of 58 monoterpenes and 38 sesquiterpenes was reported to have been identified from different cannabis preparations (Turner *et al.*, 1980). Major terpene components present in the essential oil of hemp are monoterpenes like ß-myrcene, limonene and to a lesser degree $\alpha$-pinene and ß-pinene, and sesquiterpenes like ß-caryophyllene, ß-humulene and ß-farnesene (Nigam *et al.*, 1965; Hood *et al.*, 1973; Hendriks *et al.*, 1975; Ross and ElSohly, 1996). The data presented in Table 2, shows that the hemp terpene composition as reported in literature, varies considerably. Drying and storage of hemp is known to cause a relative increase of the fraction sesquiterpenes in the overall composition of the essential oil (Ross and ElSohly, 1996). It might be therefore, that in most studies the fraction of sesquiterpenes appears to overshadow the fraction monoterpenes, due to a significantly greater loss of the volatile

**Table 2.** Mono and sesquiterpene hydrocarbons in the essential oil of *Cannabis sativa* L[a].

| Component | Nigam *et al.*, 1965 | Hood *et al.*, 1973 | Hendriks *et al.*, 1975 | Ross & ElSohly, 1996 |
|---|---|---|---|---|
| | %[b] | % | % | % |
| Camphene | 0.1 | 0.7 | 0.1 | 0.4 |
| ß-Fenchol | - | - | - | 1.0 |
| Limonene | 2.8 | 1.0[b] | 0.5 | 16.3 |
| Linalool | - | - | - | 5.1 |
| *Myrcene* | 1.3 | 1.0 | 1.0 | 32.9 |
| ß-Phellandrene | 2.7 | 1.0[c] | 0.1 | - |
| α-Pinene | 1.3 | 3.9 | 1.5 | 1.6 |
| ß-Pinene | 0.8 | 2.2 | 1.5 | 2.5 |
| trans-α-Bergamotene | 5.0 | 8.0 | 36.9[d] | 0.5 |
| ß-Bisabolene | - | 3.2 | - | 0.2 |
| trans-γ-Bisabolene | - | - | - | 3.9 |
| epi-α-Bisabolol | - | - | - | 1.2 |
| ß-Caryophyllene | 45.7 | 37.5 | 36.9[d] | 5.5 |
| iso-Caryophyllene | - | - | 1.3 | - |
| Curcumene | 1.4 | 1.4 | - | 0.2 |
| ß-Eudesmol | - | - | - | 1.1 |
| α-Guaiene | - | - | - | 1.2 |
| Guaiol | - | - | - | 1.8 |
| trans-α-Farnesene | - | - | - | 2.7 |
| ß-Farnesene | 5.1 | 9.8 | 2.5 | 0.2 |
| α-Humulene | - | - | - | 2.1 |
| ß-Humulene | 16.0 | 13.9 | 9.4 | - |
| Selina-3,7(11)diene | - | - | 5.2 | - |
| Selina-4(14),7(11)diene | - | - | 3.2 | - |
| α-Selinene | 8.6 | 2.2 | 1.2 | 0.6 |
| ß-Selinene | - | - | 3.3 | 0.6 |
| Unindentified | - | - | 14.0 | 10.6 |
| Total mono and sesquiterpene hydrocarbons | 92.6 | 86.5 | 83.5 | 97.7 |

\- Not reported.
[a] Volatile oil had been prepared from dry hemp material. No information is available on age and storage conditions of the hemp samples, except for Ross & ElSohly, where hemp buds first have been dried at room temperature and then stored in the dark for three months.
[b] Percentage of the essential oil.
[c] Combined content Limonene + ß-Phellandrene.
[d] Combined content trans-α-Bergamotene + ß-Caryophyllene.

monoterpenes compared to the sesquiterpenes. Ross and ElSohly (1996) however, show that the essential oil obtained from fresh hemp, is composed mainly of monoterpenes (92%). After drying and storage, monoterpenes account for 62% of the hemp essential oil with 36% sesquiterpenes.

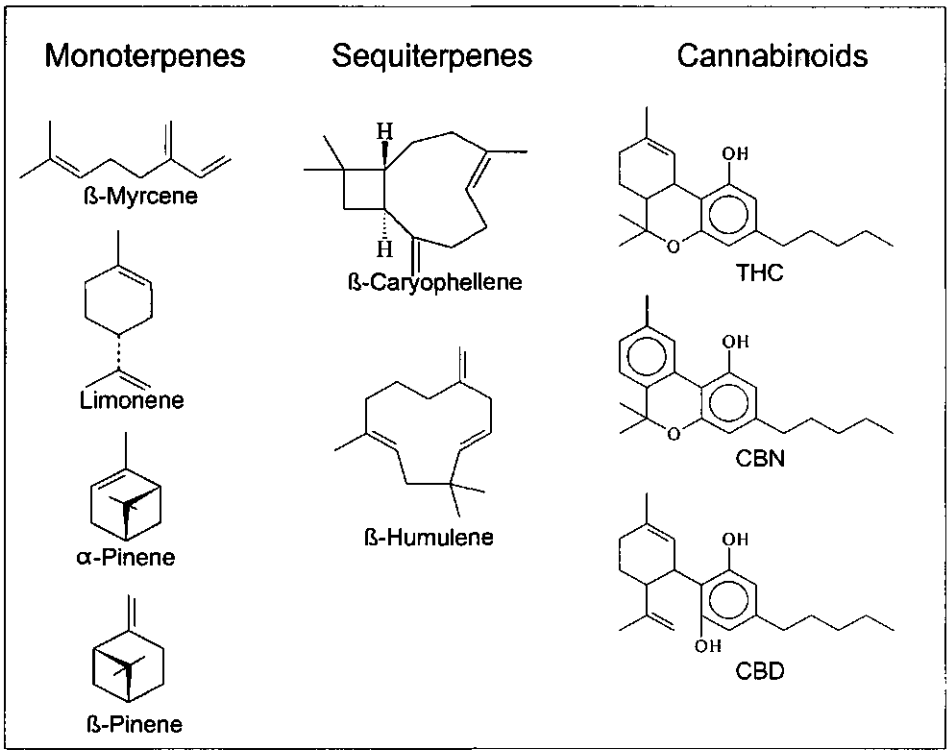


**Figure 2.** Chemical structure of representative hemp extractive components. THC: delta-9-tetrahydrocannabinol, CBN: cannabinol, CBD: cannabidiol.

Cannabinoids represent a distinctive class of compounds, found only in *Cannabis sativa* L. (Mechoulam and Gaoni, 1967). Cannabinoids are $C_{21}$-compounds belonging to the chemical class of the terpenophenolics and are responsible for the hallucinating effect of *Cannabis* preparations. More than 60 cannabinoid compounds have been isolated (Turner *et al.*, 1980), although the majority of the isolated compounds are only present at trace concentrations. Major cannabinoids in fibre hemp are cannabidiol (CBD), delta-9-tetrahydrocannabinol (THC) and cannabinol (CBN). The THC content of various fibre hemp cultivars was studied by de Meijer *et al.*, (1992) and ranged globally from 0.10-0.35% in the flowering parts. While the CBD content, in fibre hemp typically higher than the THC content, ranged from 0.70-1.70%. Reported cannabinoid contents overestimate the THC

content in the whole plant, since cannabinoids generally are accumulated in the flowering parts. Aside from terpenes and cannabinoids, numerous minor constituents of the hemp extractive fraction have been identified, among others terpenols, sterols, fatty acids, alkanes, apolar phenols and dihydrostilbenes (Turner *et al.*, 1980; ElSohly *et al.*, 1984).

## Environmental impact of pulp and paper industry effluents

### Wastewater composition

Pulp and paper mill effluents are highly heterogeneous. Their composition may vary dramatically from mill to mill depending on the feedstocks and processes utilized. The production of paper from wood or other lignocellulosic material, like straw or hemp, involves various operations including raw material preparation, mechanical or chemical pulping, pulp bleaching and papermaking. During conversion of the lignocellulosic feedstock, lignin, hemicelluloses and extractives are selectively removed to provide a cellulose enriched fibrous mass, known as pulp. Effluent from pulp and paper mills includes dissolved material from the pulping feedstock, residual process chemicals and compounds produced by reactions between process chemicals and pulping feedstock constituents. Pulping and bleaching processes play a central role in the pollution load and wastewater composition of a pulp and paper mill. In contrast, only minor loads are generated in the paper machines. A global overview of the pollution load of various pulping processes is given in Figure 3.

During pulp processing, more or less discrete fibres are liberated from the lignocellulosic feedstock by means of physical and/or chemical action. There are four categories of pulping processes, listed in order of decreasing reliance on physical action and increasing chemical action: mechanical, chemi-mechanical, semi-chemical and chemical pulping. Mechanical and thermomechanical pulps with high pulp yields produce low pollution loads and the wastewaters is usually composed mostly of biodegradable matter such as carbohydrates and organic acids. Thermomechanical pulping (TMP) wastewater differs from mechanical pulping (MP) wastewater in that the heating of the wood chips, prior to mechanical pulping, solubilizes lignin. Chemo-thermo-mechanical pulping (CTMP) and semichemical (eg. neutral sulphite semi-chemical, NSSC) wastewaters are of intermediate strength and contain more

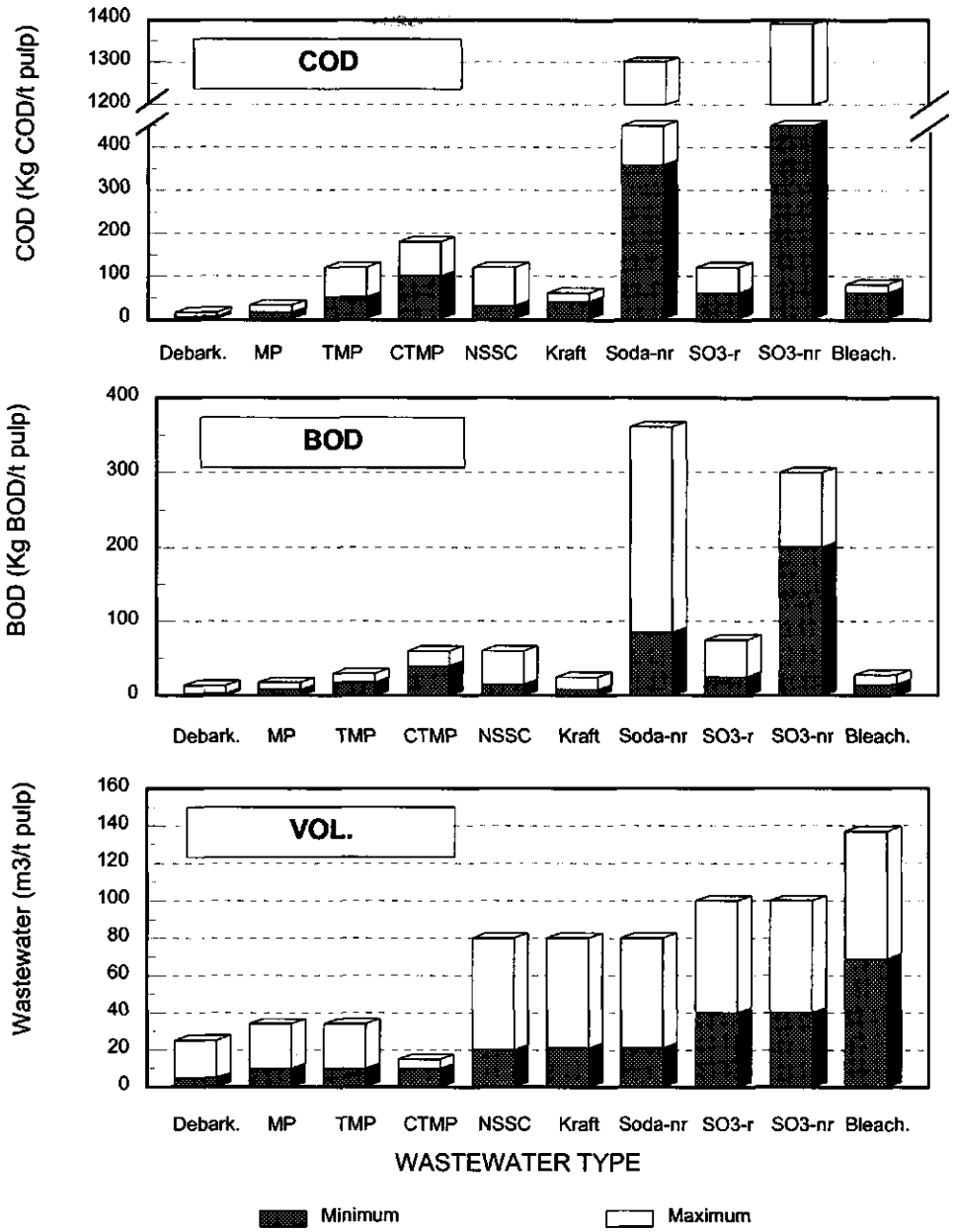


**Figure 3.** The specific pollution load of various wood-based pulping processes. Debark.= debarking; MP= mechanical pulping; TMP= thermomechanical pulping; CTMP= chemothermomechanical pulping; NSSC= neutral sulfite semi-chemical pulping; SO3= sulfite pulping; nr = no recovery; r = recovery (Calber, 1993; Chakrabarti and Kumar, 1986; Folke et al., 1993; Gullichsen, 1991; Rintala and Puhakka, 1994; Sierra-Alvarez, 1991; Walden et al., 1986).

lignin. The more that chemicals are involved, the lower the pulp yield and the lignin content of the pulp, therefore the higher the pollution load. Full chemical pulping (CP) promotes extensive decomposition of hemicellulose to soluble carbohydrates and organic acids as well as causes a severe dissolution of lignin. Furthermore, inorganic additives, such as alkali and sulphur containing cooking chemicals may be present at high concentrations in chemical pulping liquors. Therefore, at many large-scale CP mills (kraft and sulfite) pulping liquors are evaporated and burned in order to recover pulping chemicals and energy, which accounts for a considerable reduction of the pollution loads.

Extractives are lipophilic wood constituents, generally characterized by a poor water solubility at neutral to acidic pH. At more elevated pH however, the solubility of various resinous compounds, such as resin acids, increases, due to dissociation of carboxylic groups. Therefore, during alkaline pulping, aside from lignin a considerable portion of wood resinous compounds is extracted into the process water.

In general, non-wood fibres contain lower amounts of lignin and higher amounts of hot water and alkali soluble compounds. Due to the low lignin content of non-wood fibres, less energy and chemicals are required for pulping and bleaching, which makes them more environmentally friendly papermaking materials than wood. On the other hand, the pulp yield of non-wood fibres is generally lower compared to wood, due to the high content hot water and alkaline soluble material in non-woody fibres. For example, 1% NaOH solubility in hemp fibres is 31-33% (van de Werf *et al.*, 1994), whereas for soft woods values of 10-15% are reported (Rydholm, 1965; Biermann, 1993). The relatively high amounts of hot water and alkali soluble material in non-wood fibres can be regarded as disadvantageous, signifying elevated losses of raw material by extraction into the process waters, reduced pulp yields and increased organic loads to be removed by effluent treatment. Moreover, the absence of a dependable and economic chemical recovery system for small-scale non-wood pulp mills complicates effective recovery of pulping chemicals, which leads to relatively high pollution loads, in comparison to wood-based pulp mills with recovery (Figure 3). Pulping processes being investigated within the Hemp Research Programme, included thermomechanical pulping (TMP), alkaline peroxide mechanical pulping (APMP) (van Roekel *et al.*, 1995), and alkaline pulping (de Groot *et al.*, 1994, 1995, 1997). APMP applying a co-rotating twin-screw extruder, was evaluated as a sulphite-free alternative for conventional chemi-thermomechanical pulping (CTMP). NaOH-based soda pulping was considered as a chemical pulping method for hemp stem wood fibres. Application of sulphur-based pulping additives and chlorine bleaching processes was considered obsolete, due to environmental demands.

### Environmental Effects

The environmental impact of forest industry effluents can be attributed to three wastewater characteristics, i.e. 1) biological oxygen demand (BOD); 2) color; and 3) toxicity. Traditionally, BOD has been used as a parameter for regulating pollution at pulp and paper mills. BOD primarily results from the easily biodegradable components in the wastewater such as carbohydrates, organic acids and alcohols. Essentially all wastewaters of the forest industry contain to at least a certain extent BOD. Removal of BOD is required to prevent oxygen depletion and starvation in the aquatic ecosystems of receiving waters. Color in pulp and paper mill effluents is related to the presence of lignin compounds in the wastewater. Color levels are particularly high in chemical pulping and bleaching effluents, where lignin is effectively extracted into the wastewater. The color bearing compounds are for the most part high MW polymers, which are highly recalcitrant during biological treatment. Therefore, COD norms for discharge of pulping effluents essentially refer to the same compounds as the color norms.

Untreated pulp and paper mill effluents display acute lethal and chronic inhibitory effects to fish and aquatic organisms in the ecosystems of freshwater recipients. Natural wood constituents, like resin acids and tannins, have been shown to be major factors in effluent toxicity. Chlorophenols, formed by the interaction of lignin with bleaching chemicals, represent a third group of wastewater constituents, notorious for their ecotoxicological impact.

## Biological wastewater treatment

Biological wastewater treatment methods are highly effective for the removal of BOD containing dissolved organic compounds. Moreover, significant detoxification of toxic compounds can be achieved by biological means. The technologies which are presently available for biological treatment are firstly aimed at BOD removal and based on the activity of natural mixed cultures of bacteria present in either aerobic or anaerobic wastewater treatment plants. During biological treatment, organic pollutants are removed by metabolic conversion, occurring inside cells which release mineralization products. Additionally, also inorganic wastewater constituents, like nutrients as nitrate and phosphate, can be removed biologically, either by metabolic conversion or enhanced microbial uptake.

## Aerobic treatment

In the 1970's, enforcement of environmental regulations on BOD discharge in the USA, Canada and Scandinavia led to the implementation of aerobic biological treatment methods for the treatment of pulp and paper industry wastewaters (Virkola and Honkanen, 1985). Natural waters or ponds were provided with aerators, constructing aerated lagoons and aerated stabilization basins. The BOD reductions achieved in aerated lagoons range from 40-70% (Luonsi *et al.*, 1988; Ozturk *et al.*, 1990; Palabiyikoglu and Krogerus, 1989; Stuthridge *et al.*, 1991). Up till then, settling basins and mechanical clarifiers, build to recover fibrous material and suspended solids, were the principal measures for external treatment of pulp and paper industry wastewaters (Luonsi *et al.*, 1988; Biermann, 1993).

Tightening of environmental regulations in the early 1980's and space limitations on site, promoted the introduction of the activated sludge process, which provided better performance by the recirculation of biomass. At present, there is an important number of activated sludge plants in operation, treating effluents from mechanical and chemical paper mills. The loading rates achieved in activated sludge plants range from 0.2 to 4.9 g BOD/*l*·d (Palabiyikoglu and Krogerus, 1989; Saunamäki, 1989, 1997). However, the tendency is to operate activated sludge plants at low loadings, as is indicated by an average sludge loading of 0.15 g BOD/g MLSS·d for Finnish pulp mills (Saunimäki, 1997). The BOD removal efficiencies are high, in most cases between 65 and 99% (Easty *et al.*, 1978; Junna and Rintala, 1990; Palabiyikoglu and Krogerus, 1989; Viitasaari and Vuoriranta, 1991).

The COD reductions, however, vary between 40-92% at paper board mills and range between 25-65% at chemical pulp mills (Junna and Rintala, 1990; Saunamäki, 1989, 1997; Viitasaari and Vuoriranta, 1991). Generally, little or no color removal is obtained by activated sludge treatment (Walden, 1980; Yin *et al.*, 1989). During aerobic post-treatment of anaerobic treated effluents, color is actually increased (Rintala and Lepistö, 1992; Schnell *et al.*, 1990). The poor COD and color reduction observed during aerobic treatment of chemical pulping wastewaters is due to the high content poorly biodegradable lignin in these effluents. Aerobic treatment has been shown to be effective in reducing the aquatic toxicity from various types of forest industry wastewaters (Galvao *et al.*, 1987; Junna *et al.*, 1982; Mueller *et al.*, 1976; Wilson *et al.*, 1987).

An important drawback of aerobic wastewater treatment is, however, that they are expensive to operate. Considerable energy must be used to aerate the system (0.5 kWh $kg^{-1}$ $COD_{removed}$) (Rintala, 1992). Additionally, the high sludge yields, up to 0.5 kg sludge solids

$kg^{-1}$ $COD_{removed}$ (Eckenfelder *et al.*, 1988, Ramanathan and Gaudy, 1971; Saunamäki, 1989 & 1997) causes excessive costs for sludge processing, like disposal or incineration, and makes sludge management a major source of the cost for aerobic treatment systems (Kyllönen *et al.*, 1988).

**Anaerobic treatment**

Anaerobic degradation of organic material depends on the combined activity of various bacterial trophic groups, coexisting within a microbial consortium. First steps in the anaerobic decomposition of complex organic matter comprises of hydrolysis and fermentation reactions, mainly accomplished by fermentative bacteria (Gujer and Zehnder, 1983). The products of these degradation reactions are either acetate and hydrogen or intermediate compounds such as volatile fatty acids (propionate and butyrate), which may be further degraded to acetate and hydrogen by acetogenic bacteria. In the degradation chain, methanogenic bacteria are the last trophic group, comprised of acetotrophic (acetoclastic) methanogens, which produce methane by means of acetate decarboxylation, and hydrogenotrophic methanogens, involved in the formation of methane through the reduction of carbon dioxide by hydrogen. For the performance of anaerobic wastewater treatment systems, the activity of the methanogenic population is essential, since actual COD removal is achieved by the formation of methane, which is released from the wastewater.

Anaerobic wastewater treatment was introduced in the pulp and paper industry in 1983 (Lettinga *et al.*, 1991) with reasonable success, as is illustrated by the increase of anaerobic reactor volume in last decade (Figure 4). To date, the functioning reactor volume in the pulp and paper industry is estimated at 115,000 $m^3$. From the various anaerobic reactor concepts available, the upflow anaerobic sludge blanket (UASB) reactor is clearly the most successful with an estimated market share of 75%, followed by the contact reactor with 14% market share (Figure 5).

The basic idea behind the UASB concept is that anaerobic bacterial consortia are able to immobilize themselves by the formation of bacterial aggregates, given that good settling conditions are provided. Self immobilization of anaerobic biomass in aggregates or granules, provokes high sludge retention inside UASB reactors, facilitating high organic loading rates (Lettinga, 1995). The contact process on the other hand, is essentially an anaerobic design of the activated sludge process with corresponding flocculant sludge characteristics. A great

majority of the existing anaerobic full-scale plants are treating non-inhibitory forest industry wastewaters rich in readily biodegradable organic matter such as paper recycling wastewaters, evaporator condensates, mechanical pulping and thermomechanical pulping effluents (Frostell *et al.*, 1985; Habets and Knelissen, 1985; Maat and Habets, 1987; Pearson, 1989; Rosen and Gunnarsson, 1986). The organic loading rates achieved for these types of forest industry effluents in full scale UASB plants range from 5 to 27 g $COD \cdot l^{-1} \cdot d^{-1}$. The BOD efficiencies are high, in most cases between 75 and 99%, indicating that anaerobic treatment is particulary useful for the elimination of readily biodegradable organic matter. In contrast to the readily biodegradable effluents, the number of anaerobic full scale plants applications to chemical, semichemical, and chemo-thermomechanical pulping liquors, as well as bleaching and debarking effluents, is limited (Pearson, 1989). In any case, some full scale plants are in successful operation with these kinds of wastewater (Habets and de Vegt, 1991; Prong *et al.*, 1987; Velasco *et al.*, 1985). The average COD efficiencies obtained with CTMP, semichemical, black liquors and bleaching effluents range from 29-56%. The low COD removal efficiency reflects the fact that lignin in these wastewaters is not significantly degraded by anaerobic bacteria (Benner and Hodson, 1985; Hackett *et al.*, 1977; Odier and Monties, 1983; Zeikus *et al.*, 1982).

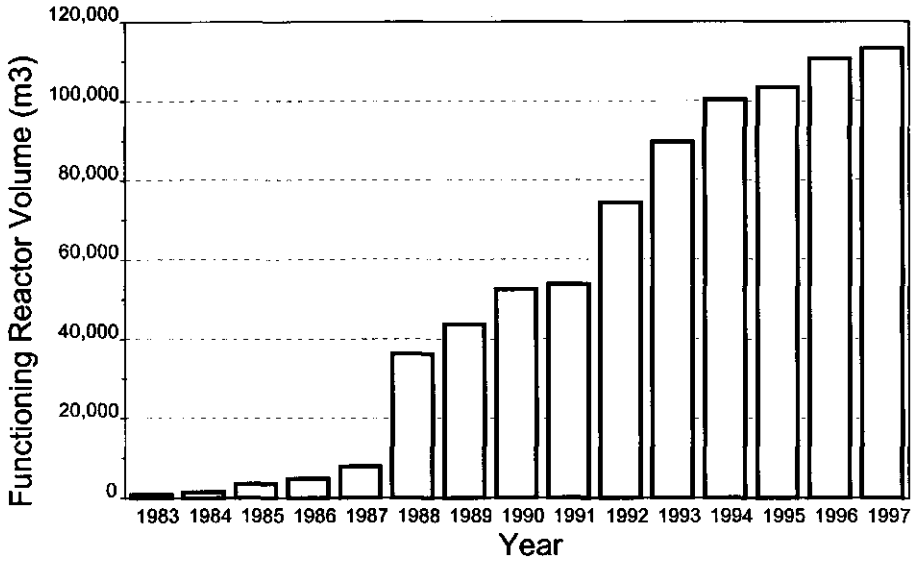


**Figure 4.** Functioning volume of anaerobic reactors in pulp and paper industry.

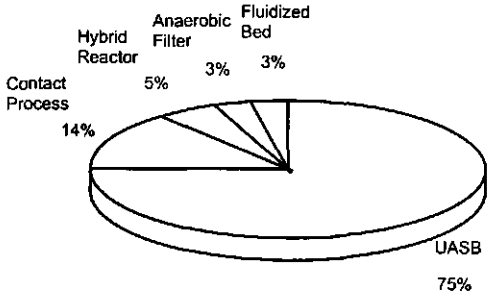


**Figure 5.** Market share of various types of anaerobic reactors in pulp and paper industry.

The limitations of anaerobic treatment are strongly related to the low growth rate and yields of anaerobic bacteria. Therefore, the recovery of the methanogenic population after a toxic shock or the acclimatization to specific persistent compounds may be quite time consuming. Likewise, long periods are required for 'first' start-up of anaerobic reactors, seeded with a low quality sludge. To date however, large quantities of highly active anaerobic sludge from existing full-scale installations are available, which can shorten start-up periods considerably.

Compared to conventional aerobic methods, the implementation of an anaerobic wastewater treatment step offers some important benefits, which are reflected in the treatment costs. Including an aerobic polishing step to remove residual VFA and compounds recalcitrant to anaerobic mineralization, the investment cost of a sequential anaerobic-aerobic treatment plant are generally in the same range as an activated sludge plant alone. The advantage of an anaerobic-aerobic treatment system however is due to their lower operational cost compared to aerobic treatment systems. Lower operational costs can be attributed to reduced energy requirements for aeration, reduced excess sludge production and potential fuel savings from the recovery of methane.

# Toxicity and biodegradability of pulping wastewater constituents

## Introduction

The presumed susceptibility of anaerobic bacteria, particulary methanogens, for toxic substances has been a key factor for pulp and paper mills to opt for aerobic effluent treatment systems in spite of their higher operating costs. Toxicity studies which compare the tolerance of anaerobes with aerobes, however, reveal that methanogens and aerobic heterotrophs, in general, show similar sensitivities to toxicants (Blum and Speece, 1991). Moreover, the identification of toxic compounds in pulp and paper industry wastewaters and the present state of knowledge on biodegradative potential and toxicity tolerance of anaerobic microorganisms enables us to select the wastewaters suitable for anaerobic treatment and to define criteria for effective detoxification.

Research on the toxicity of pulp and paper industry wastewaters has been based predominantly on wood-derived pulping effluents, whereas this thesis is the first report which deals with the toxicity of hemp pulping wastewaters. Resinous compounds and lignin are the major constituents of hemp with potential impacts on the toxicity and biodegradability of pulping wastewaters (Table 3).

## Resinous compounds

The apolar extractives of wood are known as resin. Coniferous wood resin has been demonstrated to be highly inhibitory to methanogenic bacteria (Field *et al.*, 1988; Sierra-Alvarez and Lettinga, 1990). Alkaline extracted crude pine and spruce resin caused 50% inhibition of methanogenic activity around 50 mg/*l*. The composition of wood extractives strongly depends on plant family. Terpenic compounds are important constituents of softwood as well as hemp extractives. Softwood extractives contain volatile monoterpenes, sesquiterpenes and resin acids, while hemp extractives, apart from volatile monoterpenes and sesquiterpenes also contain cannabinoids (Sierra-Alvarez *et al.*, 1994; Brenneisen and ElSohly, 1988).

**Table 3.** The methanogenic toxicity of wood constituents relevant for hemp pulping wastewaters.

| Compound | 50%IC (mg/*l*) | references |
|---|---|---|
| Wood Extractives | | |
| LCFA | 250 - 1235 | [1-4] |
| Resin acids | 21 - 400 | [5-8] |
| Volatile terpenes | 42 - 500 | [8-12] |
| Triterpenes | > 1000 | [8] |
| Hydroxystilbene | 25 | [8] |
| Monomeric lignin derivatives | | |
| Phenolic acids | 1170 - >10500 | [7,11] |
| Phenolic aldehydes | 1800 - 4400 | [11] |
| Methoxyphenols | 498 - 2200 | [9,12] |
| Alkylguaiacols | 140 - 250 | [9,11] |
| Lignin | 3320 - >15000 | [11,13] |

1-Chou *et al.*, 1978; 2-Hanaki *et al.*, 1981; 3-Koster & Kramer, 1987; 4-Prins *et al.*, 1972; 5-Field *et al.*, 1988; 6-McCarthy *et al.*, 1991, 7-Patel *et al.*, 1991; 8-Sierra-Alvarez & Lettinga, 1990; 9-Benjamin *et al.*, 1984; 10-Crane *et al.*, 1957; 11-Sierra-Alvarez & Lettinga, 1991b; 12-Sierra-Alvarez & Lettinga, 1991a; 13-Puhakka *et al.*, 1985.

## Terpenes

Terpenes are among the most potent inhibitors of methanogenic bacteria present in forest industry wastewaters (Table 3). Terpenes are commonly found in pulp and paper industry wastewaters, such as black liquors and foul condensates (Hrutfiord *et al.*, 1975; Wilson and Hrutfiord, 1975; Keith, 1976; Landry *et al.*, 1985 ). 50% inhibitory concentrations range for volatile terpenes from 42-110 mg/*l* (Sierra-Alvarez and Lettinga, 1990). Terpenic hydrocarbons are not degraded in anaerobic environments (Sierra-Alvarez *et al.*, 1990; Liver and Hall, 1996). However, various studies have shown degradation of monoterpenes under

aerobic conditions (Wilson and Hrutfiord, 1975; Trudgill, 1990, 1994; Misra *et al.*, 1996; Misra and Pavlostathis, 1997; van der Werf *et al.*, 1997). Recently, also degradation of monoterpenes under denitrifying conditions in absence of molecular oxygen was demonstrated (Harder and Probain, 1995). Presence of molecular oxygen or 'oxygen carriers' as nitrate, are considered to be essential for microbial degradation of terpenes lacking ogygenated functional groups.

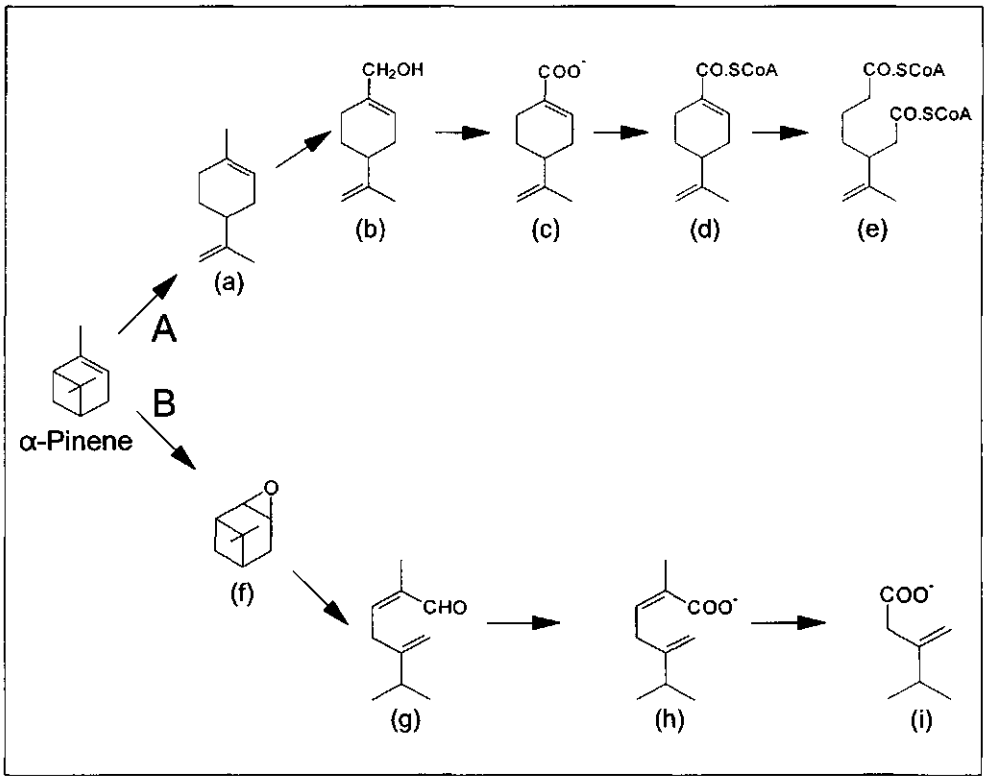


**Figure 6.** Two proposed metabolic pathways for the aerobic degradation of a-pinene.
*Pathway A:* Proposed pathway for cleavage of α-pinene by *Pseudomonas* PL. A prototrophic rearrangement of α-pinene to form limonene (a), followed by methyl group oxidation to perillic acid (c) and ring cleavage mediated by a ß-oxidation cycle to 3-isopropenylpimelyl-CoA (e). Based on the results of Shukla and Bhattacharyya (1968) and Shukla et al. (1968).
*Pathway B:* Partial catabolism of α-pinene by *Nocardia* sp. strain P18.3, *P. fluorescens* NCIMB 11671 and *P. putida* NCIMB 10684. α-pinene biotransformation involving the formation of α-pinene epoxide (f), ring cleavage and formation of *cis*-2-methyl-5-isopropylhexa-2,5-dienal (g), formation of *cis*-2-methyl-5-isopropylhexa-2,5-dienoic acid (h) and ß-oxidation to 3-isopropylbut-3-enoic acid (i) (Trudgill, 1994).

Acyclic monoterpene hydrocarbons (open chain) are typically attacked by oxygenation of a terminal methyl group. Further oxydation of this yields a terminal carboxyl group, amendable for ß-oxidation. Ring cleavage presents an additional problem to overcome for decomposition of the numerous cyclic monoterpenes and sesquiterpenes (Trudgill, 1986). Investigations on the biodegradation of cyclic monoterpenes have shown various aerobic transformations, not necessarily leading to converging metabolic pathways (Dhavalikar and Bhattacharyya, 1966; Trudgill, 1990, 1994). Examples of two distinct pathways for aerobic degradation of $\alpha$-pinene are illustrated in Figure 6.

**Resin acids**

Resin acids are monocarboxylic tricyclic diterpenes, typical constituents for the extractive fraction of softwoods. In pulp and paper industry wastewaters, resin acids are found in concentrations globally ranging from 2-50 $mg \cdot l^{-1}$ (Kennedy *et al.*, 1992, Kovacs and Voss, 1992; Liu *et al.*, 1993b; Lo *et al.*, 1994a). Concentrations however, can reach up to several hundreds of mg per litre in effluents from alkaline pulping processes, such as CTMP (McFarlane and O'Kelly, 1988; Habets and de Vegt, 1991).

Resin acids cause methanogenic inhibition at low concentrations. The reported 50% inhibitory concentrations for acetoclastic methanogenesis range from 21-235 mg/*l* (Field *et al.*, 1988; McCarthy *et al.*, 1991; Patel *et al.*, 1991; Sierra-Alvarez and Lettinga, 1990). Within the anaerobic microbial consortium, acetoclastic methanogens, which account for approximately 70% of the methane produced during anaerobic fermentation (Gujer and Zehnder, 1983), have been shown to be the trophic group most sensitive for resin acid inhibition, while acetogens and hydrogenotrophic methanogens seem to be less inhibited or even unaffected (Patel *et al.*, 1991; Patoine *et al.*, 1997).

Resin acids are lipophilic compounds, poorly soluble in water. Concentrations of resin acids encountered in pulp and paper industry wastewaters exceed by far the aqueous solubilities of the individual resin acids (Nyren and Back, 1958). The major quantity of resin acids in pulp and paper industry wastewaters therefore, is not solubilized, but present as a separate phase, accumulating in micelles (Drobosyuk *et al.*, 1982) or adsorbed on fines (McCarthy *et al.*, 1990) and colloidal particles (Hoel and Aarsand, 1995).

Aerobic treatment of pulp and paper industry wastewaters has proven to be successful in the extensive elimination of resin acids (Lo *et al.*, 1994a, 1994b; Liu *et al.*, 1993b; Yuan

*et al.*, 1993; Zender *et al.*, 1994). Typically, more than 90% of the resin acids are removed using activated sludge systems (Liu *et al.*, 1996) or aerated stabilization basins (Stuthridge *et al.*, 1991). For anaerobic treatment however, incomplete removal of resin acids is reported (Bissaillon et al, 1991; Andersson *et al.*, 1987; McFarlane and O'Kelly, 1988; Schnell *et al.*, 1990), ranging from 44-76% (Stuthridge *et al.*, 1991; Patoine *et al.*, 1997).

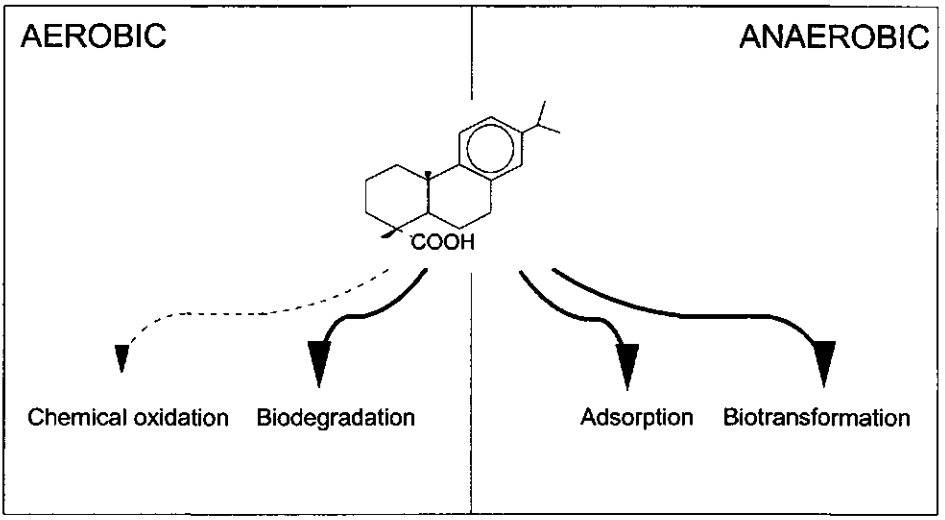


**Figure 7.** Fate of resin acids in aerobic and anaerobic treatment plants.

Due to the limited solubilities and their hydrophobic nature, resin acids are easily retained on suspended solids. Sorption onto biomass therefore, is the first step in the removal of resin acids in biological treatment systems (Hall and Liver, 1996). During aerobic treatment, subsequently, biodegradation is the main removal mechanism, while adsorption of resin acids onto the biomass is only important, when short sludge retention times are attained (Liu *et al.*, 1993a, 1996). Chemical oxidation of resin acids during aeration is confirmed, but has been shown to be of minor importance (Figure 7).

Non-acclimated activated sludge inocula and mixed consortia of river water microorganisms have been shown to be capable for aerobic biodegradation of resin acids (Hemmingway and Greaves, 1973; Liver and Hall, 1996). Additionally, various resin acid degrading bacterial strains have been isolated (Bicho *et al.*, 1995; Mohn, 1995; Wilson *et al.*, 1996; Morgan and Wyndham, 1996; Zhang *et al.*, 1997), as well as some fungi capable for resin acid degradation (Kutney *et al.*, 1981a, 1985, 1988; Blanchette *et al.*, 1992; Wang *et al.*,

1995). Some metabolic routes proposed for the aerobic bacterial degradation of resin acids are shown in Figure 8. Aerobic degradation, globally, concerns the activation of the aromatic rings by oxidation and hydroxylation reactions at C-3, C-7, C-11 and C-12 positions, followed by aromatic ring cleavage, yielding intermediates which are amendable for further degradation.

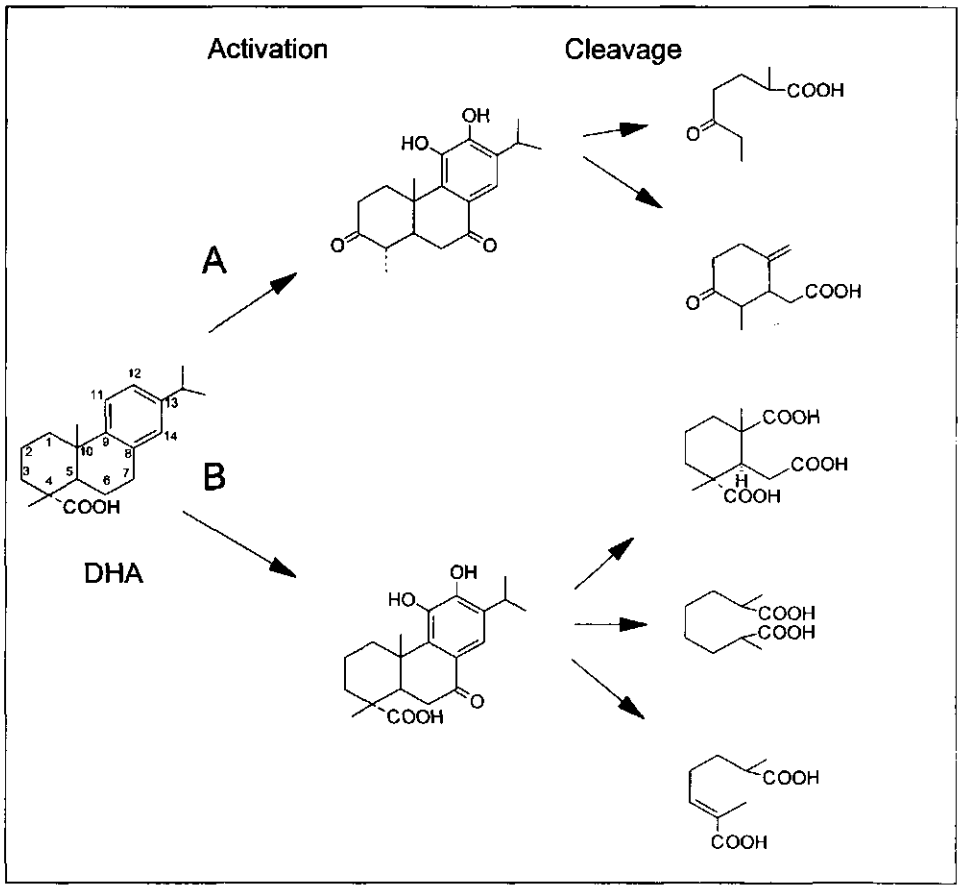


**Figure 8.** Proposed pathways for aerobic bacterial degradation of resin acids.
*Pathway A:* Degradation of dehydroabietic acid by *Flavobacterium resinovorum* (adapted from Biellmann et al., 1973a).
*Pathway B:* Degradation of dehydroabietic acid by *Pseudomonas* sp. and *Alcaligenes eutropus* (adapted from Biellmann et al., 1973b).

The literature does not provide a clear consensus on the fate of resin acids during anaerobic treatment (Figure 7). Adsorption onto the biomass has shown to be a major removal mechanism (Hall and Liver, 1996; McFarlane and Clark, 1988; Patoine *et al.*,

1997). Applying non-acclimated sludge inocula, no significant mineralization of resin acids was observed (Liver and Hall, 1996; Sierra-Alvarez *et al.*, 1990). Biodegradation of resin acids in batch assays as well as continuous anaerobic treatment has been claimed by others, but the claim was not supported by conclusive data on mineralization products (Tavendale *et al.*, 1997a; Tavendale *et al.*, 1997b; Patoine *et al.*, 1997). Several biotransformation products were observed during anaerobic incubation of deuterium labelled DHA with lake sediment collected downstream from a bleach kraft mill (Tavendale *et al.*, 1997a; Tavendale *et al.*, 1997b). Some of these biotransformation products were observed also during continuous anaerobic treatment of a resin acid containing wastewaters (McFarlane and Clark, 1988). The biotransformation products detected, indicate decarboxylation and hydrogenation of double bonds as well as aromatization reactions (see Figure 9). Additionally, a pathway for the anaerobic microbial formation of retene from DHA has been proposed (Figure 10).

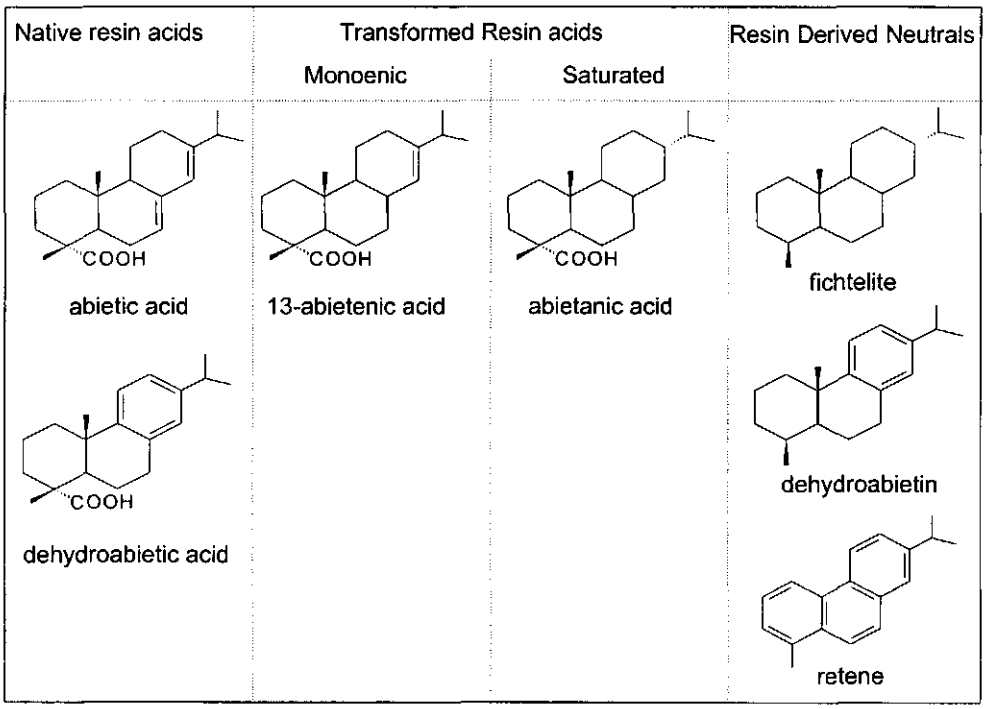


**Figure 9.** Structures of anaerobic biotransformation products of resin acids (McFarlane and Clark, 1988; Tavendale et al., 1997a).

## Cannabinoids

Cannabinoids are terpenophenolic compounds typical for the hemp extractive fraction, with a demonstrated antibiotic activity towards gram positive bacteria (Krejči, 1958; Kabelik *et al.*, 1960; van Klingeren and Ten Ham, 1976; ElSohly *et al.*, 1982). The minimum inhibiting concentrations of delta-9-tetrahydrocannabinol and cannabidiol for staphylococci and streptococci in broth were shown to be in the range of 1-5 mg/*l*. In the same range, both compounds are also bactericidal for *Staphylococcus aureus* (van Klingeren and ten Ham, 1976). The lipophilicity of cannabinoids is illustrated by the log octanol/water partition coefficient of delta-9-tetrahydrocannabinol, which is reported to be 3.20 (Anonymous, 1979). Being highly lipophilic and practically insoluble in water (Merck & Co, 1989; PSGB, 1978), cannabinoids will tend to partition onto organic material and bacterial membranes.

Biotransformation of cannabinoids, aimed at evaluating their pharmacological properties, was studied in mammals and microorganisms (Archer et al, 1979; Rosazza and Smith, 1979). Mammalian metabolic conversion of THC leads to a number of mono- and dihydroxylated derivatives. Microbial transformation studies have shown a large number of microorganisms capable for aerobic transformation of THC, whereas the yields of cannabinoid conversion products indicate that at least some organisms are capable for considerable conversion of THC (up to 75%) to noncannabinoid degradation products (Binder and Meisenberg, 1978).

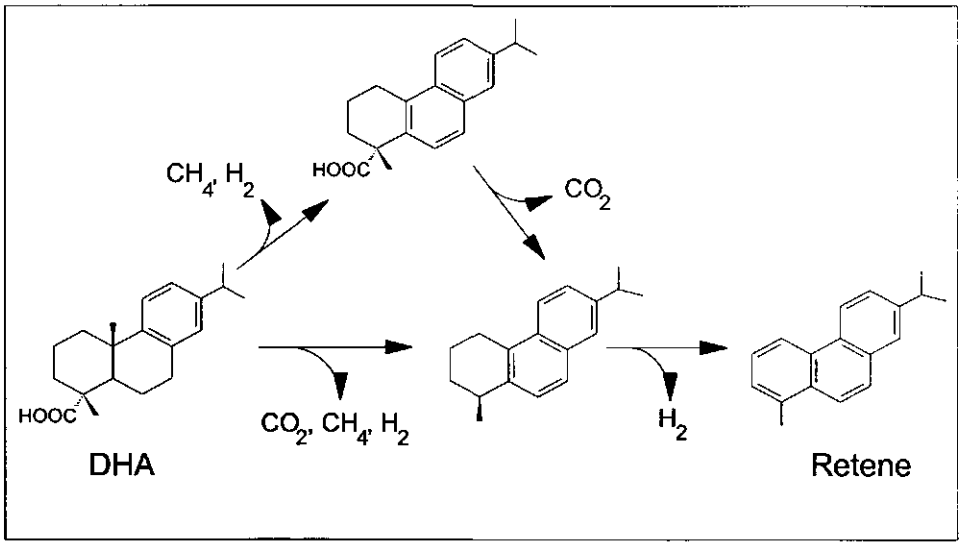


**Figure 10.** Anaerobic microbial transformation of DHA to retene (Tavendale et al., 1997a).

## Lignin

Lignin content is the main factor determining the biodegradability of pulping wastewaters. Native lignin is highly resistant to bacterial degradation under anaerobic as well as aerobic conditions. Molecular weight is an important factor determining the recalcitrance of lignin towards biological degradation. In nature, ligninolytic fungi are almost exclusively involved in the initial attack of high MW lignin, utilizing extracellular oxidative enzymes and active oxygen species for cleavage of intermonomeric bonds, yielding depolymerized products of lower molecular weight, accessible for further degradation (Kirk and Farrell, 1987; Young and Frazer, 1987; Schick-Zapanta and Tien, 1997).

The distribution of dissolved lignin fragments in pulp and paper mill wastewaters covers a broad molecular weight range from 100 up to sometimes 100,000 daltons (D). In anaerobic habitats, monomeric (Healy and Young, 1979; Kaiser and Hanselmann, 1982; Grbic-galic, 1983; Sierra-Alvarez *et al.*, 1990) and dimeric (Zeikus *et al.*, 1982; Chen *et al.*, 1985a, 1985b, 1987) lignin derivatives are readily degraded by methanogenic consortia. Various studies with lignin dimers show that all intermonomeric bonds, present in lignin, can be cleaved anaerobically (Zeikus *et al.*, 1982; Grbic-Galic, 1983; Chen *et al.*, 1985a, 1985b, 1987). Recently, also the degradation of lignin monomers under nitrate and sulphate reducing conditions was demonstrated (Phelps and Young, 1997). Anaerobic degradation of oligomeric lignin model compounds on the other hand, has been demonstrated, but was restricted to an upper limit of 600-1000 D, corresponding to 3-7 monomeric units (Zeikus *et al.*, 1982; Colberg and Young, 1982; Colberg and Young, 1985). The results suggest that as long as there is cell uptake these compounds can be metabolized.

Aerobic bacterial degradation of lignin, like anaerobic biodegradation, is also restricted by the size of the lignin oligomers. Complete degradation of lignin tetramers (MW 650 D) was reported during aerobic incubation with a mixed bacterial enrichment culture (Jokela *et al.*, 1985), while a maximum molecular weight of 600 to 1000 D was reported by Kern and Kirk (1984) for partial mineralization of radiolabelled lignin oligomers by a *Xanthomonas* strain. An overview of various aerobic bacterial strains capable of degradation of lignin derivatives, has been presented by Zimmermann (1990).

Only low molecular weight lignin derivatives are expected to exert noteworthy inhibition of methanogenic activity. Separation of wastewater lignins by ultrafiltration, showed that all methanogenic toxicity was present in the low molecular weight fraction. The lignin fraction over 10,000 D was completely free of methanogenic toxicity (Sierra-Alvarez and Lettinga,

1991b). Analogous with the effect of MW on tannin toxicity, 1000 to 3000 $g \cdot mol^{-1}$ is considered the upper lignin MW limit for inhibition of the methanogenic activity by lignin derivatives (Field *et al.*, 1990). High molecular weight compounds are considered to be too large for penetration of bacterial cell wall and adjacent slime layers and therefore relatively non-toxic for methanogens. Low molecular weight lignin compounds differ considerably in their inhibitory potential, as is indicated by the wide range of 50% IC data for monomeric lignin derivatives listed in Table 3, ranging from 140 to more than 10,000 mg/*l*. Lignin derivatives with aldehyde groups or apolar substituents are the most toxic to methanogens, those with carboxylic acid groups on the other hand are only mildly toxic.

Depending upon the structural properties of lignin derivatives, two mechanisms of bacterial inhibition can be observed. Polyphenolic lignin derivatives, readily available in the bacterial micro-environment due to their relatively high aqueous solubility, are considered to constrain metabolic processes by the formation of H-bridges with enzyme proteins, in a similar fashion as tannins. More lipophilic phenolic compounds on the other hand, may partition into the cell membranes, where they can cause damage to the membrane structure. In support of the latter mechanism, are the strong correlations between the hydrophobicity of different phenolic compounds and their toxicity towards methanogenic bacteria and bacteria present in activated sludge systems (Sierra-Alvarez and Lettinga, 1991a; Beltrame *et al.*, 1988).

## Scope and structure of the thesis

This thesis describes research on the sequenced anaerobic-aerobic treatment of hemp pulping wastewaters. Discharge of non-wood pulp mill effluents comprise a considerable load with organic material for receiving surface waters and aquifers. The absence of a dependable and cost-effective chemical recovery system for the treatment of non-wood pulping liquors emphasises the importance of biological treatment methods. The main objective of the study reported in this thesis was to investigate the suitability of biological methods for the treatment of hemp pulping wastewaters.

Hemp pulping wastewaters contain various toxic and recalcitrant compounds. Regarding the treatment of pulping wastewaters, the biodegradative capacity of anaerobic and aerobic microorganisms are different. Whereas terpenes are readily degraded aerobically, anaerobic microorganisms lack the potential for biodegradation of these inhibitory compounds.

Sequential anaerobic and aerobic treatment of hemp pulping liquors was studied for the removal of BOD, COD and toxicity. Research was focused on the fate of natural hemp constituents during biological treatment steps, with emphasis on the methanogenic toxicity of hemp extractives and the modification of lignin.

The papers of this thesis demonstrate the suitability of sequenced anaerobic-aerobic treatment. The combined biodegradative capacities of anaerobic and aerobic bacterial communities for removal of persistent organic compounds and toxicity are utilized, while maintaining the benefits of anaerobic fermentation for the bulk of the BOD removal.

Chapter 2 examines the contribution of extractives to the toxicity of hemp black liquor. Chapter 3 and 4 evaluate sequenced anaerobic-aerobic treatment for the removal of organic pollutants and detoxification of hemp black liquors. Upfront dilution of the inhibitory influent with effluent of the aerobic post-treatment, was applied as a detoxification measure. Additionally, Chapter 4 examines modification of lignin during anaerobic-aerobic treatment. In chapter 5, the continuous anaerobic treatment of non-inhibitory hemp TMP wastewaters is described, including the modification of lignin molecular weight during anaerobic and aerobic post-treatment. Finally, in chapter 6, the implications from this research are discussed with regard to detoxification and modification of lignin during biological treatment of hemp pulping wastewaters.

## Acknowledgements

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## References

**Adler, E.** (1977). Lignin chemistry. Past, present and future. Wood Sci. Technol., 11, 169-218.

**Andersson, P-E., Gunnarsson, L., Olsson, G., Welander, T., and Wikström, Å.** (1987). Anaerobic treatment of CTMP effluent. Pulp Paper Can., 88(7), T233-T236.

**Anonymous.** (1979). Marihuana biological effects. In: Nahas G.G. and Paton W.D.M. (eds). Proceedings of the satellite symposium of the 7th International congress of Pharmacology, Paris, 22-23 July 1978, Pergamon Press Oxford.

**Anonymous.** (1992). Halfjaarlijks verslag hennep onderzoek, No 5 [Biannual research report hemp research programme, No 5]. DLO Research Institute, Wageningen, The Netherlands, 64-69.

**Archer, R.A., Fukuda, D.S., Kossoy, A.D., and Abbott, B.J.** (1979). Microbiological transformations of Nabilone, a synthetic cannabinoid. Appl. Environ. Microbiol., 37, 965-971.

**Atchison, J.E., and McGovern, J.N.** (1987). History of paper and the importance of non-wood plant fibers. In: Hamilton, F. and Leopold, B. (Eds). Pulp and Paper Manufacture, Vol. 3 - Secondary fibers and non-wood pulping. Joint Textbook Committee of the Paper Industry of the United States and Canada. TAPPI-Atlanta, CPPA-Montreal.

**Beltrame, P., Beltrame, P.L., Carniti, P., Guardione, D., and Lanzetta, C.** (1988). Inhibiting action of chlorophenols on biodegradation of phenol and its correlation with structural properties of inhibitors. Biotechnol. Bioeng., 31, 821-828.

**Benjamin, M.M., Woods, S.L., and Ferguson, J.F.** (1984). Anaerobic toxicity and biodegradability of pulp mill waste constituents. Wat. Res., 18, 601-607.

**Benner, R., and Hodson, R.E. (1985).** Thermophilic anaerobic biodegradation of [14C]lignin, [14C]cellulose and [14C]lignocellulose preparations. Appl. Environ. Microbiol., 50, 971-976.

**Bicho, P.A., Martin, V., and Saddler, J.N.** (1995). Growth, Induction, and substrate specificy of dehydroabietic acid-degrading bacteria isolated from kraft mill effluent enrichment. Appl. Environ. Microbiol., 61, 3245-3250.

**Biellmann, J.F., Branlant, G., Gero-Robert, M., and Poiret, M.** (1973a). Dégradation bactérienne de l'acide déhydroabiétique par *Flavobacterium resinovorum*. Tetrahedron, 29, 1227-1236.

**Biellmann, J.F., Branlant, G., Gero-Robert, M., and Poiret, M.** (1973b). Dégradation bactérienne de l'acide déhydroabiétique par un *Pseudomonas* et une *Alcaligenes*. Tetrahedron, 29, 1237-1241.

**Biermann, C.J.** (1993). Essentials of pulping and papermaking. Academic Press Inc., London., 42-50.

**Binder, M., and Meisenberg, G.** (1978). Microbial transformation of cannabinoids. European J. Appl. Microbiol. Biotechnol., 5, 37-50.

**Bissaillon, J-G., Perron, J., Paquette, G., Lépine, F., and Beaudet, R.** (1991). Effet d'un traitement anaérobie sur la charge organique et la toxicité d'un effluent thermoméchanique, Sci. Tech. Eau, 24, 137-142.

**Blanchette, R.A., Farrell, R.L., Burnesw, T.A., Wendler, P.A., Zimmermann, W., Brush, T.S., and Snyder, R.A.** (1992). Biological control of pitch in pulp and paper production by *Ophiostoma piliferum*. TAPPI J., 75(12), 102-106.

**Blum, D.J.W., and Speece, R.E.** (1991). A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. Res. JWPCF 63, 198-207.

**Bolton, A.J.** (1994). Natural fibres for plastic reinforcement. Materials Technology, 9, 12-20.

**Bolton, A.J.** (1995). The potential of plant fibres as crops for industrial use. Outlook on Agriculture, 24(2), 85-89.

**Brenneisen, R., and ElSohly, M.A.** (1988). Chromatographic and spectroscopic profiles of *Cannabis* of different origins; part I. Journal of Forensic Sciences, JFSCA, 33(6), 1385-1404.

**Calber, G.E.** (1993). Characterization of effluents from mechanical pulp production. Proceedings of the EUCEPA International Environmental Symposium, Paris, April 27-29th, 1993, Vol. 1, 45-52.

**Catling, D., and Grayson, J.** (1982). Identification of vegetable fibres. Chapman & Hall, London, UK., 71-79.

**Chakrabarti, S.P., and Kumar, A.** (1986). Comprehensive Industry Document Small Scale Pulp and Paper Industry. Series COINDS 22. Centralo Board for the Prevention and Control of Water Pollution. New Delhi, India.

**Chen, W., Ohmiya, K., Shimizu, S., and Kawakami, H.** (1985a). Degradation of dehydrovanillin by anaerobic bacteria from cow rumen fluid. Appl. Environm. Microbiol., 50, 211-216.

**Chen, W., Supanwong, K., Ohmiya, K., Shimizu, S., and Kawakami, H.** (1985b). Anaerobic degradation of veratrylglycerol-beta-guaiacyl ether and guaiacoxyacetic acid by mixed rumen bacteria. Appl. Environm. Microbiol., 50, 1451-1456.

**Chen, W., Ohmiya, K., Shimizu, S., and Kawakami, H.** (1987). Anaerobic degradation of dehydrodiisoeugenol by rumen bacteria. J. Ferment. Technol., 65(2), 221-224.

**Chou, W.L., Speece, R.E., Siddiqi, R.H., and McKeon, K.** (1978). The effect of petrochemical structure on methane fermentation toxicity. Prog. Wat. Tech. 10, 545-558.

**Colberg, P.J., and Young, L.Y.** (1982), Biodegradation of lignin-derived molecules under anaerobic conditions. Can. J. Microbiol., 28, 886-889.

**Colberg, P.J., and Young, L.Y.** (1985). Anaerobic degradation of soluble fractions of [14C-lignin]lignocellulose. Appl. Environ. Microbiol. 49(2), 345-349.

**Colberg, P.J.** (1988). Anaerobic microbial degradation of cellulose, lignin, oligolignols and monoaromatic lignin derivatives. In: Zehnder, A.J.B. (ed). Biology of anaerobic microorganisms. John Wiley & Sons, New York, 333-372.

**Cocaign, M., Wilberg, E., and Lindley, N.** (1991). Sequential demethoxylation reactions during methylotrophic growth of methoxylated aromatic substrates with *Eubacterium limosum*. Arch. Microbiol., 155, 496-499.

**Crane, A., Nelson, W.O., and Brown, R.E.** (1957). Effects of d-limonene and α-d-pinene on in vitro carbohydrate dissimilation and methane formation by rumen bacteria. J. Dairy Sci., 40, 1317-1323.

**Dhavalikar, R.S., and Bhattacharyya, P.K.** (1966). Micobial transformations of terpenes: Part VII - fermentation of limonene by a soil pseudomonad. Indian J. Biochem., 3, 144-157.

**Drobosyuk, V.M., Borodulina, M.R., Krylatov, Y.A., and Talmud S.L.** (1982). The dispersive power of resin acids and fatty acids. J. Appl. Chem. (russian), 55, 1935.

**Easty D.B., Borchardt, G., and Wabers, A.** (1978). Wood-derived toxic compounds. TAPPI J., 61(10), 57-60.

**Eckenfelder, W.W., Patoczka, J.B., and Pulliam, G.W.** (1988). Anaerobic versus aerobic treatment in the U.S.A. In: Hall, E. R. and Hobson, P. N. (eds)., Anaerobic Digestion 1988., Pergamon Press, Oxford, GB., 105-114.

**ElSohly, H.N., Turner, C.E., Clark A.M., and ElSohly, M.A.** (1982). Synthesis and antimicrobial activity of certain cannabichromene and cannabigerol related compounds. Journal of Pharmaceutical Sciences, 71, 1319-1323.

**ElSohly, H.N., Ma, G.E., Turner, C.E., and ElSohly, M.A.** (1984). Constituents of *Cannabis sativa*, XXV. Isolation of two new dihydrostilbenes from a Panamanian variant. Journal of Natural Products, 47(3), 445-452.

**FAO** (1995). FAOSTAT statistical database on forestry, Food and Agricultural Organization of the United Nations, Geneva.

**Fengel, D., and Wegener, G.** (1984). Wood: Chemistry, Ultrastructure, Reactions, Walter de Gruyter, Berlin.

**Field, J.A., Leyendeckers, M.J.H., Sierra-Alvarez, R., Lettinga, G., and Habets, L.H.A.** (1988). The methanogenic toxicity of bark tannins and the anaerobic biodegradability of water soluble bark matter. Wat. Sci. Tech., 20(1), 219-240.

**Field, J.A., Lettinga, G., and Habets, L.H.A.** (1990). Measurement of low molecular weight tannins: Indicators of methanogenic toxic tannins. J. Fermentation Bioengineer., 69(3), 148-153.

**Folke, J., Mannisto, E., Lethinen K.J., and McCubbin, N.** (1993). Chlorine dioxide in pulp bleaching: Technical aspects and environmental effects. European Environmental Research Group, Ltd, Allerod, Denmark.

**Frostell, B., Bonkoski, W., and Sointio, J.E.** (1985). Full scale anaerobic treatment of a pulp and paper industry wastewater. In: Proc. 39th Industrial Waste Conf., Butterworth Publishers, Boston, 687-696.

**Galvao, J.B., Grieco, V.M., Araújo, R.P.A., Ortelano, M.R., Bertoletti, E., and Ramos, M.L.L.C.** (1987). Treatability studies and toxicity reduction in pulp and paper mill effluents. In: Proc. Second IAWPRC Symposium on Forest Industry Wastewaters, Tampere Univ. of Technology, Tampere, Finland.

**Grbic-Galic, D.** (1983). Anaerobic degradation of coniferyl alcohol by methanogenic consortia. Appl. Environ. Microbiol., 46, 1442-1446.

**de Groot, B, van Dam, J.E.G., van der Zwan, R.P., and van 't Riet, K.** (1994). Simplified kinetic modelling of alkaline delignification of hemp woody core. Holzforschung, 48, 207-214.

**de Groot, B, van Dam, J.E.G., and van 't Riet, K.** (1995). Alkaline pulping of hemp wood core: kinetic modelling of lignin and cellulose extraction and degradation. Holzforschung, 49, 332-342.

**de Groot, B., van der Kolk, J.C., van der Meer, P., van Dam, J.E.G. and van 't Riet, K.** (1997). Alkaline swelling of hemp woody core chips. Journal of Wood Chemistry and Technology, 17(1-2), 187-208.

**Gujer, W., and Zehnder, A.J.B.** (1983). Conversion processes in anaerobic digestion. Wat. Sci. Tech., 15(8/9), 127-167.

**Gullichsen, J.** (1991). Process internal measures to reduce pulp mill pollution load. Wat. Sci. Tech., 24(3/4), 45-53.

**Habets, L.H.A., and Knelissen, J.H.** (1985). Application of the UASB-reactor for anaerobic treatment of

paper and board mill effluent. Wat. Sci. Tech., 17(1), 61-76.

**Habets, L.H.A., and de Vegt, A.L.** (1991). Anaerobic treatment of bleached TMP and CTMP effluent in the BIOPAQ UASB system, Wat. Sci. Tech., 24(3/4), 331-345.

**Hackett, W.F., Connors, W.J., Kirk, T.K., and Zeikus, J.G.** (1977). Microbial decomposition of synthetic 14C-labeled lignins in nature: Lignin biodegradation in a variety of natural materials. Appl. Environ. Microbiol., 39, 43-51.

**Hall, E.R., and Liver, S.F.** (1996). Interactions of resin acids with aerobic and anaerobic biomass-II. Partitioning on biosolids. Wat. Res., 30(3), 672-678.

**Hanaki, K., Matsuo, T., and Nagase, M.** (1981). Mechanism of inhibition caused by long-chain fatty acids in anaerobic process. Biotechnol. Bioeng. 23, 1591-1610.

**Harder, J., and Probain, C.** (1995). Microbial degradation of monoterpenes in the absence of molecular oxygen. Appl. Environ. Microbiol., 61, 3804-3808.

**Healy, J.B., and Young, L.Y.** (1979). Anaerobic degradation of eleven aromatic compounds to methane. Appl. Environ. Microbiol., 38, 84-89.

**Hemmingway, R.W., and Greaves, H.** (1973). Biodegradation of resin acid sodium salts. TAPPI J., 56(12), 189-192.

**Hendriks, H., Malingré, T.M., Batterman, S., and Bos, R.** (1975). Mono- and sesqui-terpene hydrocarbons of the essential oil of *Cannabis sativa*. Phytochemistry, 14, 814-815.

**Hoel, H., and Aarsand, R.** (1995). Acute toxicity of colloidal and dissolved material in TMP effluents. Nord. Pulp Pap. J., 2, 98-103.

**Hood, L.V.S., Dames, M.E., and Barry, G.T.** (1973). Headspace volatiles of Marijuana. Nature, 242, 402-403.

**Hrutfiord, B.F., Friberg, T.S., Wilson, D.F., and Wilson, J.R.** (1975). Organic compounds in aerated stabilization basin discharge. TAPPI J., 58(10), 98-100.

**Jokela, J., Pellinen, J., Salkinoja-Salonen, M., and Brunow, G.** (1985). Biodegradation of two tetrameric lignin compounds by a mixed bacterial culture. Appl. Microbiol. Biotechnol., 23, 38-46.

**Junna, J., Lammi, R., and Miettinen, V.** (1982). Removal of organic and toxic substances from debarking and kraft pulp bleaching effluents by activated sludge treatment. In: Publ. of the Water Research Institute, National Board of Waters, Finland. No. 49, 17-28.

**Junna, J., and Rintala, J.** (1990). Evaluation of purification efficiency of activated sludge treatment plants for pulp and paper industry wastewaters in Finland. Wat. Sci. Tech., 22(9), 199-206.

**Kaiser, J., and Hanselmann, K.W.** (1982). Fermentative metabolism of substituted monoaromatic compounds by bacterial community from anaerobic sediments. Arch. Microbiol., 133, 185-194.

**Kennedy, K.J., McCarthy, P.J., and Droste, R.L.** (1992). Batch and continuous anaerobic toxicity of resin acids from chemothermo-mechanical pulp wastewater. J. Ferm. Bioeng., 73, 206-212.

**Kern, H.W., and Kirk, T.K.** (1987). Influence of molecular size and ligninase pretreatment on the degradation of lignins by *Xanthomonas* sp. strain 99. Appl. Environ. Microbiol., 53, 2242-2246.

**Kirk, T.K., and Farrell, R.L.** (1987). Enzymatic "combustion": The microbial degradation of lignin. Ann.

Rev. Microbiol., 41, 465-505.

**van Klingeren, B., and ten Ham, M.** (1976). Antibacterial activity of delta-9-tetracannabinol and cannabidiol. Antonie van Leeuwenhoek 42, 9-12.

**Koster, I.W., and Kramer, A.** (1987). Inhibition of methanogenesis from acetate in granular sludge by long-chain fatty acids. Appl. Environ. Microbiol., 53, 403-409.

**Kovacs, T.G., and Voss, R.H.** (1992). Biological and chemical characterization of newsprint/specialty mill effluents. Wat. Res., 26(6), 771-780.

**Krejči, Z.** (1958). Hanf (*Cannabis sativa*) - Antibiotisches heilmittel. 2. Mitteilung: Methodik und ergebnisse der bakteriologischen untersuchungen und vorläufige klinische erfahrungen. Pharmazie, 13, 155-166.

**Kabelik, J., Krejči, Z., and Santavy, F.** (1960). Cannabis as a medicament. Bulletin on Narcotics, July-September 1960, 5-23.

**Keith, L.H.** (1976). Identification of organic compounds in unbleached treated kraft paper mill wastewaters. Environ. Sci. Technol., 10, 555-564.

**Kyllönen, H.L., Lappi, M.K., Khun, R.T., and Mustranta, A.H.** (1988). Treatment and characterization of biological sludges from the pulp and paper industry. Wat. Sci. Tech., 20(1), 183-192.

**Kutney, J.P., Singh, M., Hewitt, G., Salisbury, P.J., Worth, B.R., Servizi, J.A., Martens, D.W. and Gordon, R.W.** (1981). Studies related to biological detoxification of kraft pulp mill effluent I. The biodegradation of dehydroabietic acid with *Mortierella isabellina*. Can. J. Chem., 59, 2334-2341.

**Kutney, J.P., Lewis, S.L., Hewitt, G., Salisbury, P.J., and Singh, M.** (1985). Biotransformation of dehydroabietic acid with resting cell suspensions and calcium algimate-immobilized cells of *Mortierella isabellina*. Appl. Environ. Microbiol., 49, 96-100.

**Kutney, J.P., Berset, J-D., Hewitt, G., Salisbury, P.J., and Singh, M.** (1988). Biotransformation of dehydroabietic, abietic and isopimaric acids by *Mortierella isabellina* immobilized in polyurethane foam. Appl. Environ. Microbiol., 54, 1015-1022.

**Landry, G.C., Rockwell, J.N., and Steltenkamp, M.S.** (1985). Analysis of mill-recovered and laboratory-recovered turpentine. TAPPI J., 68(4), 98-102.

**Lettinga, G., Field, J.A., Sierra-Alvarez, R., van Lier, J.B., and Rintala, J.** (1991). Future perspectives for the anaerobic treatment of forest industry wastewaters. Wat. Sci. Tech., 24, 91-102.

**Lettinga, G.** (1995). Anaerobic digestion and wastewater treatment systems. Antonie van Leeuwenhoek, 67, 3-28.

**Li, H-L.** (1974). An archaeological and historical account of *Cannabis* in China. Economic Botany, 28, 437-448.

**Liu, H.W., Lavaleé, H.C., and Lo, S.N.** (1993a). Mechanisms and kinetics of removal of resin acids, toxic compounds, in CTMP effluents during aerobic biological treatment. Proceedings of the 7th Int. Symposium on wood and pulping chemistry. May 25-28, 1993, Beijing, P.R. China.

**Liu, H.W., Lo, S.N., and Lavalleé, H.C.** (1993b). Study of the performance and kinetics of aerobic biological treatment of a CTMP effluent. Pulp Paper Can., 94, T502-T507.

**Liu, H.W., Lo, S.N., and Lavaleé, H.C.** (1996). Mechanisms of removing of resin and fatty acids in CTMP effluent during aerobic biological treatment. TAPPI. J., 79, 145-154.

**Liver, S.F., and Hall, E.R.** (1996). Interactions of resin acids with aerobic and anaerobic biomass-I. Degradation by non-acclimated inocula. Wat. Res., 30(3), 663-671.

**Lo, S.N., Lavaleé, H.C., Rowbottom, R.S., Meunier, M.A., and Zaloum, R.** (1994a). Activated sludge treatment of TMP mill effluents. Part 1: Effluent characterization and treatability study. TAPPI J., 77(11), 167-178.

**Lo, S.N., Lavaleé, H.C., Rowbottom, R.S., Meunier, M.A., and Zaloum, R.** (1994b). Activated sludge treatment of TMP mill effluents. Part 2: Biokinetic parameters and effluent treatment strategies. TAPPI J., 77(11), 169-184.

**Luonsi, A., Junna, J., and Nevlainen, I.** (1988). The development of waste water treatment in the finnish pulp and paper industry. Wat. Sci. Tech., 20(1), 25-36.

**Maat, D.Z., and Habets, L.H.A.** (1987). The upflow anaerobic sludge blanket wastewater treatment system: A technological review. Pulp Paper Can. 88(11): T410-414.

**McCarthy, P.J., Kennedy, K.J., Droste, R.L.** (1990). The role of resin acids in the anaerobic toxicity of chemithermomechanical pulp wastewater. Wat. Res., 24, 1401-1405.

**McCarthy, P.J., Landine, R.C., Kennedy, K.J., and Droste, R.L.** (1991). Anaerobic toxicity of resin acids in chemithermomechanical pulp wastewater. In: J.M. Bell (ed.). Proceedings of the 45th Industrial Waste Conference, May 8-10, 1990, Lewis Publishers, Inc. Chelsea, MI, 435-440.

**McDougall, G.J., Morrison, I.M., Stewart, D., Weyers, J.D.B., and Hillman, J.R.** (1993). Plant fibers: chemistry and processing for industrial use. J. Sci. Food Agric., 62, 1-20.

**McFarlane, P., and O'Kelly, N.** (1988). Treatment of CTMP effluents in New Zealand. Proc. Technology transfer workshop on CTMP effluents. Report WTC-Bio-07-1988, Wastewater Technology Centre, Burlington, Ontario, Canada.

**Mechoulam, R., and Gaoni, Y.** (1967). Recent advances in the chemistry of hashish. Fortsch. Chem. Org. Naturst., 25, 175.

**de Meijer, E.P.M., van der Kamp, H.J., and van Eeuwijk, F.A.** (1992). Characterisation of *Cannabis* accessions with regard to cannabinoid content in relation to other plant characters. Euphytica, 62, 187-200.

**Merck & Co. Inc.** (1989). Cannabidiol, p. 264; Tetrahydrocannabinol, p. 1451. In: Budavari, S. *et al.* (eds.), The Merck Index, 11th edition. Rahway, N.J., U.S.A.

**Misra, D.K.** (1987). Cereal straw. In: Hamilton, F. and Leopold, B. (eds.). Pulp and Paper manufacture, Vol. 3 -Secondary fibers and non-wood pulping. pp 82-93. Joint Textbook Committee of the paper industry of the United States and Canada. TAPPI-Atlanta, CPPA-Montreal.

**Misra, G., Pavlostathis, S.G., Perdue, E M., and Araujo, R.** (1996). Aerobic degradation of selected monoterpenes. Appl. Microbiol. Biotechnol. 45, 831-838.

**Misra, G., and Pavlostathis, S.G.** (1997). Biodegradation kinetics of monoterpenes in liquid and soil-slurry systems. Appl. Microbiol. Biotechnol., 47, 572-577.

**Mohn, W.W.** (1995). Bacteria obtained from a sequencing batch reactor that are capable of growth on dehydroabietic acid. Appl. Environ. Microbiol., 61, 2145-2150.

**Morgan, C.A., and Wyndham, R.C.** (1996). Isolation and characterization of resin acid degrading bacteria found in effluent from a bleached kraft pulp mill. Can. J. Microbiol., 42(5), 423-430.

**Mueller, J.C., Leach, J.M., and Walden, C.C.** (1976). Biological detoxification of pulp and paper mill effluents. In: Proc. 5th International Fermentation Symposium, Berlin.

**Nieschlag, H.J., Nelson, G.H., Wolff, I.A., and Perdue, R.E.** (1960). A search for new fibre crops. TAPPI J., 43, 193-201.

**Nigam, M.C., Handa, K.L., Nigam, I.C., and Levi, L.** (1965). Essential oils and their constituents. XXIX, The essential oil of marihuana: composition of genuine indian cannabis sativa L., Canadian Journal of Chemistry, 43, 3372-3376.

**Nyren, V., and Back, E.** (1958). The ionization constant, solubility product and solubility of abietic and dehydroabietic acid. Acta Chem. Scand., 12(7), 1516-1520.

**Odier, E., and Monties, B.** (1983). Absence of microbial mineralization of lignin in anaerobic enrichment cultures. Appl. Environ. Microbiol., 46, 661-665.

**Ozturk, I., Eroglu, V., and Toroz, I.** (1990). Pilot and full scale treatment experiences on paper mill effluents. Wat. Sci. Tech., 22(9), 255-263.

**Palabiyikoglu, N., and Krogerus, M.** (1989). Wastewater tretment of pulp and paper mill effluents. In: Proc. Int. Symp. on Waste Management Problems in Agro-Industry, September 1989, Istanbul, Turkey., 245-252.

**Patel, G.B., Agnew, B.J., and Dicaire, C.J.** (1991). Inhibition of pure cultures of methanogens by benzene ring compounds. Appl. Environ. Microbiol., 57, 2969-2974.

**Patoine, A., Manuel, M.F., Hawari, J.A., and Guiot, S.R.** (1997). Toxicity reduction and removal of dehydrobietic and abietic acids in a continuous anaerobic reactor. Wat. Res., 31, 825-831.

**Pearson, J.** (1989). Major anaerobic plants start up. Pulp Pap. Int. , March 1989., 57-60.

**Phelps, C.D., and Young, L.Y.** (1997). Microbial metabolism of the plant phenolic compounds ferulic and syringic acids under three anaerobic conditions. Microb. Ecol., 33, 206-215.

**PPI** (1995). International fact and price book 1995, Pulp and Paper International, miller Freeman, Brussels, Belgium.

**Prins, R.A., van Nevel, C.J., and Demeyer, D.I.** (1972). Pure culture studies of inhibitors for methanogenic bacteria. Antonie van Leeuwenhoek, 38, 281-287.

**Prong, C.F., Ferguson, A.M.D., Chmelauskas, A.J., and Tielbaard, M.H.** (1987). High rate UASB anaerobic demonstration plant confirms the feasability of full-scale treatment of NSSC. TAPPI Proceedings Environmental Conference. TAPPI, Atlanta, GA, USA, 219- 226.

**PSGB** (1978). Cannabidiol and Tetrahydrocannabinol, p. 1014. In: Clarke, E.G.C. *et al.* (eds.), Isolation and identification of drugs, vol. 2. The Pharmaceutical Society of Great Britain. The Pharmaceutical Press, London.

**Puhakka, J.A., Rintala, J.A., and Vuoriranta, P.** (1985). Influence of sulfur compounds on biogas production from forest industry wastewater. Wat. Sci. Tech., 17, 281-288.

**Ramanathan, M., and Gaudy, A.F.** (1971). Studies on sludge yield in aerobic systems: In: Proceedings of the 26th Industrial Waste Conference, May 4-6th, 1971, Purdue Univ., Lafayette, Indiana, USA, 665-675.

**Rintala, J.** (1992). Thermophilic and mesophilic anaerobic treatment of pulp and paper industry wastewaters. Ph.D. Thesis. Tampere University of Technology, Tampere, Finland.

**Rintala, J., and Lepistö, S.** (1992). Anaerobic treatment of thermomechanical pulping whitewater at 35-70°C.

Wat. Res., 26, 1297-1305.

**Rintala, J.A., and Puhakka, J.A.** (1994). Anaerobic treatment in pulp- and paper-mill waste management - a review. Bioresource Technol., 47, 1-18.

**van Roekel, G.J., Lips, S.J.J., op den Kamp, R.G.M., and Baron, G.** (1995). Extrusion pulping of true hemp bast fibre (*Cannabis sativa* L.). Proceedings of the 1995 TAPPI pulping conference, October 2-6, Chicago, USA, 477-485.

**Rosazza, J.P., and Smith, R.V.** (1979). Microbial models for drug metabolism. Adv. Appl. Microbiol., 25, 169-208.

**Rosen, R., and Gunnarsson, L.** (1986). Treatment of mill wastewaters with anaerobic fixed bed reactors. In: Proc. PIRA Paper and Board Division Key-Event, Cost Effective Treatment of Papermill Effluents Using Anaerobic Technologies, Leatherhead, England.

**Ross, S.A., and ElSohly, M.A.** (1996). The volatile oil composition of fresh and air-dried buds of *Cannabis sativa*. J. Nat. Prod., 59, 49-51.

**Rydholm, S.A.** (1965). Pulping processes. Interscience publishers, John Wiley & Sons Inc., New York., 50, 96.

**Saunamäki, R.** (1989). Biological waste water treatment in the Finnish pulp and paper industry. Paperi ja Puu - Paper and Timber 1989/2, 158-164.

**Saunamäki, R.** (1997). Activated sludge plants in Finland. Wat. Sci. Tech., 35(2\3), 235-243.

**Schick-Zapanta, L. and Tien, M.** (1997). The roles of veratryl alcohol and oxalate in fungal lignin degradation. J. Biotechnol., 53, 93-102.

**Schnell, A., Dorica, J., Ho, C., Ashikawa, M., Munnoch, G. and Hall, E.R.** (1990). Anaerobic and aerobic pilot-scale effluent detoxification studies at an integrated newsprint mill. Pulp Pap. Can., 91, T347-T352.

**Shukla, O.P., and Bhattacharyya, P.K.** (1968). Microbial transformations of terpenes: Part IX - pathways of degradation of α- and ß-pinenes in a soil pseudomonad (PL-strain). Indian J. Biochem., 5, 92-101.

**Shukla, O.P., Moholay, M.N., and Bhattacharyya, P.K.** (1968). Microbial transformations of terpenes: Part X --fermentation of α- and ß-pinenes in a soil pseudomonad (PL-strain). Indian J. Biochem., 5, 79-91.

**Sierra-Alvarez, R.** (1991). The role of natural wood constituents on the anaerobic treatability of forest industry wastewaters. Ph.D. Thesis. Agricultural University, Wageningen, The Netherlands.

**Sierra-Alvarez, R., and Lettinga, G.** (1990). The methanogenic toxicity of wood resin compounds. Biol. Wastes, 33, 211-226.

**Sierra-Alvarez, R., Kato, M., and Lettinga, G.** (1990). The anaerobic biodegradability of paper mill wastewater constituents. Environ. Technol., 11, 891-898.

**Sierra-Alvarez, R., and Lettinga, G.** (1991a). The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. Appl. Microbiol. Biotechnol. 34, 544-550.

**Sierra-Alvarez, R., and Lettinga, G.** (1991b). The methanogenic toxicity of wastewater lignins and lignin related compounds. J. Chem. Techn. Biotechn., 50, 443-455.

**Sierra-Alvarez, R., Field, J.A., Kortekaas, S., and Lettinga, G.** (1994). Overview of the anaerobic toxicity caused by organic forest industry wastewater pollutants. Wat. Sci. Tech., 29 (5/6), 353-363.

**Stuthridge, T.R., Campin, D.N., Langdon, A.G., Mackie, K.L., McFarlane, P.N., and Wilkins, A.L.** (1991). Treatability of bleached kraft pulp and paper mill wastewaters in a New Zealand aerated lagoon treatment system, Wat. Sci. Tech., 24(3/4), 309-317.

**Tavendale, M.H., McFarlane, P.N., Mackie, K.L., Wilkins, A.L., and Langdon, A.G.** (1997a). The fate of resin acids-1. The biotransformation and degradation of deuterium labelled dehydroabietic acid in anaerobic sediments. Chemosphere, 35, 2137-2151.

**Tavendale, M.H., McFarlane, P.N., Mackie, K.L., Wilkins, A.L., and Langdon, A.G.** (1997b). The fate of resin acids-2. The fate of resin acids and resin acid derived neutral compounds in anaerobic sediments. Chemosphere, 35, 2153-2166.

**Trudgill, P.W.** (1986). Terpenoid metabolism of *Pseudomonas*, p. 483-525. *In* Sokatch J. R. (ed.), The bacteria, a treatise on structure and function, Vol X. Academic Press, New York.

**Trudgill, P.W.** (1990). Microbial metabolism of monoterpenes - recent developments. Biodegradation, 1, 93-105.

**Trudgill, P.W.** (1994). Microbial metabolism and transformation of selected monoterpenes, p. 33-61. *In* Ratledge C. (ed.), Biochemistry of microbial degradation. Kluwer Academic Publishers.

**Turner, C.E., ElSohly, M.A., and Boeren, E.G.** (1980). Constituents of *Cannabis sativa* L. XVII. A review of the natural constituents. J. Nat. Prod., 43, 169-234.

**Velasco, A.A., Frostell, B., and Greene, M.** (1985). Full scale anaerobic-aerobic biological treatment of a semichemical pulping wastewater. Proc. 40th Industrial Waste Conf., Purdue University, 297-304.

**Viitasaari, M., and Vuoriranta, P.** (1991). Biological waste water treatment in the finnish pulp and paper industry. In: Mikkanen, P. and S. Poyry (eds.), ENTREE: A Development Conference in Environmental Training in Engineering Education, October 21-25, 1991, Laxenburg, Austria, Conference Proceedings.

**Virkola, N.E., and Honkanen, K.** (1985). Wastewater characteristics. Wat. Sci. Tech., 17(1), 1-28.

**Walden, C.C.** (1980). Biological effects of pulp and papaer mill effluents. In: Moo-Young, M. and Campbell, W.R. (eds)., Proc. of the Sixth International Fermentation Symposium. v.2. Fuels, chemicals, foods and waste treatment. July 20- 25, London, Canada., 669-676.

**Walden, C.C., McLeay, D.J., and McKague, A.B.** (1986). Cellulose production processes. In: Hutzinger, O. (ed.). The Handbook of Environmental Chemistry, Vol. 3, Part D, Anthropogenic Compounds. Springer-Verlag, Berlin, 1-34.

**Wang, Z., Chen, T., Gao, Y., Breuil, C., and Hiratsuka, Y.** (1995). Biological degradation of resin acids in wood chips by wood-inhabiting fungi. Appl. Environ. Microbiol., 61(1), 222-225.

**van der Werf, H.M.G., Harsveld van der Veen, J.E., Bouma, A.T.M. and ten Cate, M.** (1994). Quality of hemp (*Cannabis sativa* L.) stems as a raw material for paper. Industrial Crops and Products, 2, 219-227.

**van der Werf, M.J., de Bont, J.A.M., and Leak, D.J.** (1997). Opportunities in microbial biotransformation of monoterpenes. Adv. Biochem. Engineer./Biotechnol., 55, 148-177.

**Wilson, D., and Hrutfiord, B.F.** (1975). The fate of turpentine in aerated lagoons, Paper Mag. Can., 76(6), T195-T197.

**Wilson, R.W., Murphy, K.L., and Frenette, E.G.** (1987). Aerobic and anaerobic treatment of NSSC and CTMP effluent. Pulp Pap. Can., 88, T4-T8.

**Wilson, A.E.J., Moore, E.R.B., and Mohn, W.W.** (1996). Isolation and characterization of isopimaric acid-degrading bacteria from a sequencing batch reactor. Appl. Environ. Microbiol., 62, 3146-3151.

**Yin, C-F., Joyce, T.W., and Chang, H-M.** (1989). Dechlorination of conventional softwood bleaching effluent by sequential biological treatment. In: T.K. Kirk and H-M. Chang (eds.), Biotechnology in Pulp and Paper Manufacture: Proceedings of the Fourth International Conference on Biotechnology in the Pulp and Paper Industry, Butterworth-Heinemann, Boston, 231-24.

**Yuan, L.Q., Lo, S.N., and Lavalleé, H.C.** (1993). Study of activated sludge for the treatment of TMP effluent. Proceedings of the 7th Int. Symposium on wood and pulping chemistry. May 25-28, 1993, Beijing, P.R. China.

**Young, L.Y., and Frazer, A.C.** (1987). The fate of lignin and lignin-derived compounds in anaerobic environments. Geomicrobiology J., 5(3/4), 261-293.

**Zeikus, J.G., Wellstein, A.L., and Kirk, T.K.** (1982). Molecular basis for the biodegradative recalcitrance of lignin in anaerobic environments. FEMS Microbiology Letters. 15, 193-197.

**Zender, J.A., Stuthridge, T.R., Langdon, A.G., Wilkins, A.L., Mackie, K.L., and McFarlane, P.N.** (1994). Removal of resin acids during secondary treatment at a New Zealand bleached kraft pulp and paper mill. Wat. Sci. Tech., 29(5/6), 105-121.

**Zhang, Y., Bicho, P.A., Breuil, C., Saddler, J.N., and Liss, S.N.** (1997). Resin acid degradation by bacterial strains grown on CTMP effluent. Wat. Sci. Tech., 35(2-3), 33-39.

**Zimmermann, W.** (1990). Degradation of lignin by bacteria. J. Biotechnol., 13, 119-130.

# The Contribution of Extractives to the Methanogenic Toxicity of Hemp Black Liquor.

# 2

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## Abstract

The objective of this research was to identify the major sources of methanogenic toxicity in hemp black liquors. As many toxic compounds in forest industry effluents are derived from the pulping feedstock, specific compounds were removed from the hemp fibres prior to pulping and in additional experiments also from the black liquor. The effect of the treatments on the methanogenic toxicity of the black liquors was evaluated. Hemp extractives were shown to be highly toxic. The stem wood extractives, which had a 50% IC corresponding to 0.25 g COD/*l*, were somewhat more toxic than the bark extractives with a 50% IC corresponding to 0.65 g COD/*l*. The COD content of the extractive fractions averaged 2.80 g COD/g extractive dry weight. The methanogenic toxicity of black liquors from hemp were comparable with the toxicity found with black liquors from woody feedstocks and ranged between 2.8 and 4.8 g COD/*l*. Removal of extractives from the hemp fibre prior to pulping only partly reduced methanogenic toxicity although the extractive removal efficiencies were relatively high. The residual extractives remaining in the feedstocks after fibre extraction (10 to 30% of the soxhlet determined extractives) were apparently released into the black liquor during pulping. To enable improved removal of the extractive fraction, hemp black liquor was subjected to several detoxification treatments. Ethyl ether extraction and Amberlite XAD-2 treatment achieved complete wastewater detoxification. The importance of the extractive fraction on the overall toxicity of hemp black liquor was further confirmed by the high toxicity of the recovered extractive fraction. These results indicate that extractives are the principal toxic substances in hemp black liquors, like in wood derived soda pulping wastewaters.

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## Introduction

During the last decade, anaerobic treatment technology has been successfully applied to various types of pulp and paper industry effluents. Full-scale plants are in operation for the treatment of paper recycling wastewaters, evaporator condensates and mechanical pulping effluents (Sierra-Alvarez *et al.*, 1994; Lettinga *et al.*, 1991). Despite its increasing popularity, the application of anaerobic treatment has been restricted mainly to non-inhibitory and highly biodegradable pulp and paper industry effluents. The extension of anaerobic treatment technology to the treatment of chemical pulping effluents is hampered by the methanogenic toxicity of these wastewaters. Literature reports on wood-derived wastewaters show that effluents from chemical pulping processes are highly toxic wastewaters. The concentration causing 50% inhibition of methanogenic activity ranges between 2 and 8 g COD/*l* for soda black liquors and chemithermomechanical pulping (CTMP) effluents (Sierra-Alvarez *et al.*, 1994), and is usually much lower than the actual concentration of these wastewaters. Wood extractives, which is the apolar solvent extractable material in wood, are suggested to be one of the major sources of methanogenic toxicity in black liquors (Sierra-Alvarez *et al.*, 1991) and CTMP effluents (Welander and Andersson, 1985; Richardson *et al.*, 1991; McCarthy *et al.*, 1990). Aside from the wood extractives, various low-molecular-weight fragments of lignin may also be implicated to a certain extent in the inhibitory characteristics of these wastewaters (Sierra-Alvarez and Lettinga, 1991).

Many toxic compounds in forest industry effluents are derived from the fibre source utilized for pulping. Hemp is a non-woody fibre. Approximately 94% of the virgin fibres used in the world in 1990 for the manufacture of paper came from wood, and the remaining 6% was from annual crops (FAO, 1991). Although non-woody fibres are a minor feedstock for paper production, they are used for the production of specialty paper internationally and are inevitable fibre sources for many developing countries. Pulp mills using non-woody fibres are usually not large enough to justify the capital expenditures for chemical recovery. Chemical recovery units enable utilization of chemicals and energy from the combustion of spent liquors. As a consequence, wastewater treatment is required to diminish the environmental impact of discharge of these wastewaters.

In previous research (Kortekaas *et al.*, 1994) we have demonstrated that black liquors from hemp are as toxic as those from woody feedstocks. The objective of this research was to localize the major sources of methanogenic toxicity in hemp black liquors. To quantify the relative toxicity of hemp fibre components, specific wastewater compounds were selectively

removed. Resinous compounds were removed by dichloromethane extraction of hemp fibres, after which the raw material was subjected to soda pulping. Additionally, resinous compounds were removed from industrial black liquor by ethyl ether liquid-liquid extraction and adsorption onto XAD-2. Subsequently, the methanogenic toxicity of the black liquors was determined in batch assays. The adsorbent XAD-2 previously has been used to isolate wood resin compounds from pulp and paper effluents (Rogers, 1973; Leach and Thakore, 1976), as well as to remove methanogenic inhibitory substances from pine- and spruce-wood-derived black liquors (Sierra-Alvarez *et al.*, 1990b; Sierra-Alvarez *et al.*, 1991). In methanogenic toxicity assays, the XAD-2 adsorbent was able to detoxify pine black liquors completely. The extraction of wood fibres or wood-derived wastewaters with organic solvents leads to the removal of hydrophobic resin components, such as fatty acids, resin acids, esters waxes and sterols (Bjorklund-Jansson, 1980; Voss and Rapsomatiotis, 1985). Sierra-Alvarez *et al.* (1991) demonstrated a significant decrease of methanogenic toxicity when the pine fibres were subjected to extraction with diethyl ether prior to pulping.

## Materials and Methods

### Detoxification treatments

In order to evaluate the relative toxicity of hemp fibre components, specific compounds were removed either from the pulp feedstock or from the black liquor. The detoxification treatments are outlined as follows.

*Fibre extraction method I.* A 1 *l* serum flask was filled with 60 g hemp fibres and 1.2 g $Na_2SO_4$. Two consecutive extractions with dichloromethane (800 m*l* each) were applied. The extract was dried in the rotary evaporator under vacuum at 40 °C and the residue obtained was dried at 105 °C for two hours. The extractives recovered were redissolved in a 1 g/*l* NaOH solution. Fibres were dried at 40 °C and consequently used for soda pulping.

*Fibre extraction method II.* A 1 *l* serum flask was filled with 60 g hemp fibres, 800 g demineralized water and 120 g dichloromethane. The serum bottle was sealed with a Teflon-lined septum and placed on a shake table for one hour (300 strokes $min^{-1}$; stroke = 2 cm). After settling, the organic fraction was collected and dichloromethane replenished. The

extraction procedure was repeated ten times. The organic fraction was dried in the rotary evaporator under vacuum at 40 °C. The residue was dried with $N_2$ in a bath at 90 °C. Fibres were dried at 40 °C and used for soda pulping.

*Ethyl ether liquid-liquid extraction.* To 800 m*l* of black liquor adjusted to a concentration of 10 g COD/*l* and pH 8.5, two consecutive liquid-liquid extractions of ethyl ether were applied. The residual ethyl ether in the decanted black liquor was cleaned in a rotary evaporator under vacuum at 40 °C, and rediluted with distilled water to 800 m*l*. The extractive fraction recovered from the ethyl ether was dried in the rotary evaporator under vacuum at 40 °C, and then redissolved in 800 m*l* of 1 g/*l* NaOH.

*Amberlite XAD-2 treatment.* To 1 *l* of black liquor adjusted to pH 8, 70 g/*l* XAD-2 was added. This adsorbent was agitated with the black liquor with a magnetic stirring device for 5 hours. Afterwards, the treated black liquor was passed through a paper filter.

*HCl precipitation.* To 1 *l* of black liquor, 7 m*l* of 35% HCl was added to reduce the pH to 1.8. This reaction mixture was allowed to stand overnight and the following day it was passed through a paper filter.

*Calcium precipitation.* To 1 *l* of black liquor (no pH adjustment, pH = 11.7), 1 g/*l* of $Ca^{2+}$ (as $CaCl_2$) was added. This reaction mixture was allowed to stand overnight and the following day it was passed through a paper filter.

**Preparation of the Black Liquors**

The hemp black liquors used in this study originated from two sources. Aside from black liquors derived from Celesa pulp mill, Tortosa, Spain, black liquors were prepared from fibre hemp (*Cannabis sativa L.*, cultivar Kompolti Hybrid TC), grown in the Netherlands. The hemp stem was mechanically divided into a hemp bark and a hemp stem wood fraction, both fractions were processed separately. Per litre of tap water 20 g NaOH and 100 g of dry hemp fibre was added. The mixture was cooked at 165 °C for a period of 30 minutes.

**Biomass**

The anaerobic granular sludge used in these experiments was obtained from a full-scale anaerobic reactor treating wheat starch wastewater (Latenstein, Nijmegen, the Netherlands). The sludge was elutriated to remove fines and stored at 4 °C under nitrogen gas.

**Anaerobic Toxicity Assay**

All assays contained essential inorganic macro and micro nutrients as outlined previously (Kortekaas *et al.*, 1998) and were placed in a temperature controlled room at 30 ± 2°C. In this study, two types of toxicity experiments were conducted as outlined as follows:

*Type 1.* This method was applied to assay the methanogenic toxicity of hemp black liquor, ethyl ether extracted hemp black liquor and the recovered extractive fraction from hemp black liquor. The black liquor was derived from Celesa pulp mill, Tortosa, Spain. Toxicity assay type 1 was conducted in duplicate. The assays were carried out in two consecutive feedings, each lasting 10 to 14 days. The first feeding is the exposure feeding; distilled water, granular sludge (1.5 g VSS/*l*), neutralized acetic acid (4 g COD/*l*) and a known amount of wastewater COD were transferred to the serum flasks containing nutrient solution. No wastewater was added to the substrate controls. To each bioassay 1 g $NaHCO_3$ was added per gram of biodegradable COD present in the treatment with the highest COD concentration. Subsequently, distilled water was added to complete a medium volume of 0.25 *l*, and a concentration equal to 8.17 g COD/*l* black liquor. Methane production was monitored periodically during the assays with modified mariotte flasks, filled with a 3% NaOH solution. At the end of the first feeding, all serum flasks were provided with a second feeding in order to evaluate the residual activity after exposure to the wastewater. The supernatants were carefully decanted to avoid losses of methanogenic sludge and replaced with a medium containing 4 g neutralized acetic acid per litre. No wastewater was included in the replacement medium.

*Type 2.* This method was used to determine the methanogenic toxicity of the extractive fraction recovered from hemp fibres, the black liquor prepared from untreated and extracted fibres and the hemp extractive compounds cannabidiol, caryophellene and humulene. Extractives were solubilized in 1% NaOH solution, which was neutralized after addition. Cannabidiol was solubilized in methanol and added by micropipet. The methanol added to the medium with the transfer of cannabidiol was 100 $\mu l/l$. The methanogenic toxicity assay was conducted in triplicate in 0.6 *l* glass serum flasks. The anaerobic sludge (1.5 g VSS/*l*) was transferred to a flask containing 0.05 *l* medium, which consisted of nutrients, $NaHCO_3$ (8 g/*l*), neutralized acetate (3 g COD/*l*) and wastewater. The wastewater concentrations supplied were chosen to provide an inhibition of methanogenic activity ranging from 0% to 100%. Substrate controls were based on assays were no wastewater was added. The flasks were sealed with a rubber septum and a screw cap. Before incubation the headspace of the

flasks was flushed with a gas mixture containing 70% $N_2$ and 30% $CO_2$ and $Na_2S$ (39 mg/*l*) was added to remove oxygen. The bottles were placed in a reciprocating shaker. After 6 days incubation, the assays were provided with a second feeding lacking the wastewater; this was done in order to determine the residual activity of the sludge after exposure to the wastewater. The supernatant was carefully decanted and replaced with a medium containing nutrients, $NaHCO_3$ (4 g/*l*) and neutralized acetate (3 g COD/*l*). After 24 h incubation, the headspace was flushed with the gas mixture and the accumulation of methane in the headspace was determined during a period of 3-6 h. The inhibited activity was expressed as a percentage of the control activity and is abbreviated to "%ACT". The percentage inhibition, "%I", was defined as: %I = 100 - %ACT. The wastewater concentration that caused 50% inhibition of the acetoclastic activity is referred to as "50% IC".

**Analyses**

The methane concentration in the headspace of the serum flasks was determined by gaschromatography using a Packard-Becker 438/S. The 2 m * 2 mm steel column was packed with Poropak Q (80-100 mesh). The temperatures of the column, injection port, and flame ionization detector were 60, 200 and 220 °C, respectively. Nitrogen was the carrier gas at a flow rate of 20 ml/min. Volatile fatty acids (VFA) were analyzed by gas chromatography using a Hewlett Packard 5890 equipped with 2 m x 2 mm glass column packed with Supelcoport (100-120 mesh) coated with 10% Fluorad FC 431. The temperatures of the column, injection port and flame ionization detector were 130, 200, 280 °C, respectively. The carrier gas was nitrogen saturated with formic acid (40 ml/min). Wastewater samples were analyzed for COD (the micro method with dichromate), volatile suspended solids (VSS), total suspended solids (TSS) according to APHA standard methods (APHA, 1989). Samples for the determination of COD, $UV_{280}$ and $VIS_{440}$ were membrane filtered (0.45 $\mu$m). The ultraviolet absorbance at 280 nm ($UV_{280}$) and color at 440 nm ($VIS_{440}$) were determined in a 1 cm quartz cuvette by diluting the samples to less than 0.8 absorbance units in a 0.02 M borate buffer providing a pH 9.1. Extractive content in the hemp fibres was determined by extraction of a known amount of milled fibre with dichloromethane in a Soxhlet apparatus for 8 hours and subsequent gravimetric determination of the amount of extractives after drying the residue at 105 °C. Cannabinoids were determined by gas chromatography (de Meijer *et al.*, 1992).

### Chemicals

Cannabidiol was purchased from HortaPharm B.V. (Amsterdam, The Netherlands). ß-Caryophellene and α-humulene were obtained from Sigma Chemical Co., St. Louis, USA.

## Results

### Methanogenic toxicity of hemp black liquors

Hemp black liquors exert a high toxicity on methanogenic bacteria. Figure 1 displays the methanogenic activity during the first and second feeding of anaerobic sludge exposed to hemp black liquor obtained from a full scale pulp mill. The COD concentrations resulting in 50% inhibition of methanogenic activity was 4.5 g COD/*l* during the first feeding and 4.8 g COD/*l* during the second feeding.

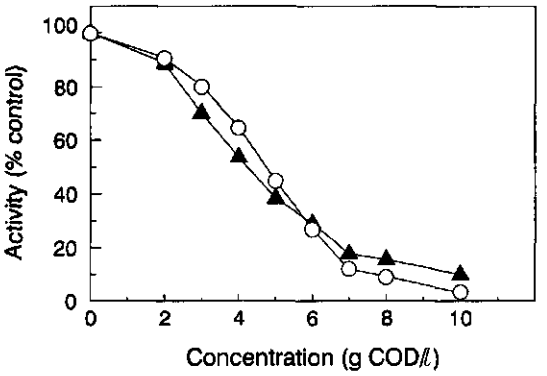


**Figure 1.** The methanogenic activity of granular sludge exposed to hemp black liquor during the first (▲) and second (○) feeding of an anaerobic toxicity assay type 1. The hemp black liquor, obtained from a full scale pulp mill, had the following characteristics: $COD_{tot}$ = 150.6 g COD/*l*, $COD_{inf}$ = 143.1 g COD/*l*, $UV_{280}$ = 84 AU280(1x,1cm), $VIS_{440}$ = 60.5 AU440(1x,1cm), pH = 13.3.

**Dichloromethane extraction of hemp fibres**

To evaluate the contribution of extractives to the total toxicity of hemp black liquors the extractive fraction was removed from the fibre material by extraction with dichloromethane. Subsequently, the treated and untreated fibres were subjected to soda pulping and the toxicity of the black liquor was evaluated in a anaerobic toxicity assay type 2. Two extraction methods were used to remove the extractives from the raw material, which, as a consequence, had different extractive yields. The removal efficiencies of both extraction methods are described in Table 1. As the chemical composition of the hemp bark and hemp stem wood fibres is rather different, both raw material fractions were tested, separately. Hemp stem wood contained more extractives than hemp bark. The COD content of the recovered extractive fractions averaged 2.80 g COD/g extractive dry weight. Two compounds typical for the hemp extractive fraction are delta-9-tetrahydrocannabinol and cannabidiol. The hemp extractive fraction recovered in this research, contained only minor concentrations cannabinoids, since a fibre hemp cultivar was utilized. The sum of delta-9-tetrahydrocannabinol and cannabidiol fraction in hemp stem wood and bark extractives were 2% and 1%, respectively.

**Table 1.** Extractive removal during dichloromethane extraction of hemp fibre material.

| Material | Extraction[a] | Extractives removed | |
|---|---|---|---|
| | | g/kg fibre | expressed as % soxhlet-extractives |
| Hemp stem wood | Method I | 1.40 | 73 |
| | Method II | 1.68 | 88 |
| Hemp bark | Method I | 0.51 | 68 |
| | Method II | 0.69 | 92 |

[a] Experimental conditions for method I and method II fibre extractions are described in Materials and Methods.

## The methanogenic toxicity of hemp extractives

The methanogenic toxicity of the extractive fraction recovered from hemp bark and hemp stem wood is displayed in Figure 2. Hemp stem wood extractives displayed the highest toxicity with 50% inhibition of methanogenic activity at a concentration of 0.25 g COD/*l*, where as the 50% IC value of hemp bark extractives was 0.65 g COD/*l*. To elucidate the contribution of cannabinoids to the methanogenic toxicity of hemp extractives, cannabidiol was tested. Cannabidiol showed to be non-toxic to concentrations up till 200 mg/*l* (results not shown). Two hemp sesquiterpenes were tested and proved to be only mildly toxic. The methanogenic toxicity of ß-caryophellene and α-humulene at a concentrations up to 800 mg COD/*l* was less than 30% (results not shown).

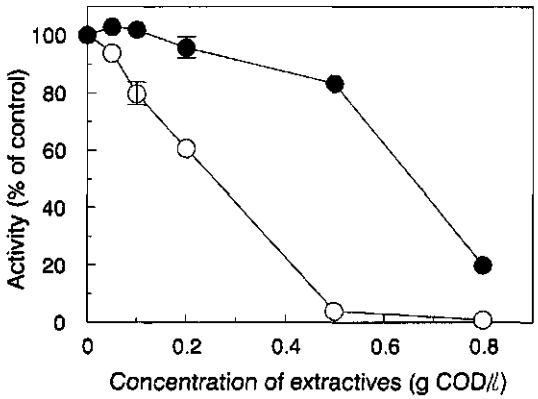


**Figure 2.** The methanogenic toxicity of hemp bark (•) and hemp stem wood (○) extractives. The extractives were recovered after method I extraction of hemp fibre material; bars represent standard deviations.

## Methanogenic toxicity of hemp black liquors prepared from treated and untreated fibres

In addition to the assessment of the methanogenic toxicity of hemp extractives, the methanogenic toxicity of hemp stem wood and bark black liquor was determined. Hemp stem wood black liquor, which had a 50% IC corresponding to 2.6 g COD/*l*, was slightly more toxic than hemp bark black liquor with a 50% IC corresponding to 4.5 g COD/*l*. Figure 3 and 4 demonstrate that a decrease of the extractive content in the raw material utilized for pulping strongly influences the methanogenic toxicity of black liquors. However, removal

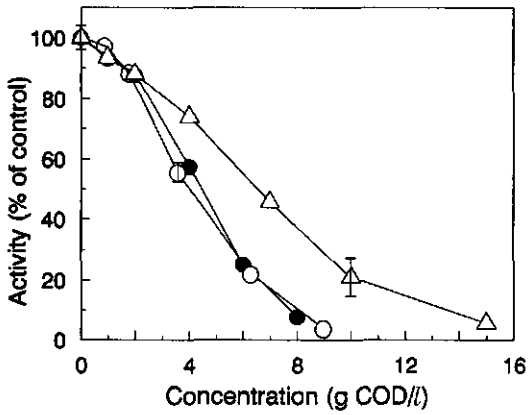


**Figure 3.** The methanogenic toxicity of black liquors prepared from extracted and untreated hemp bark fibres. Symbols: •, untreated; ○, method I extraction, 70% extractive removal; △, method II extraction, 90% extractive removal (removal efficiency relative to Soxhlet extraction); bars represent standard deviations.

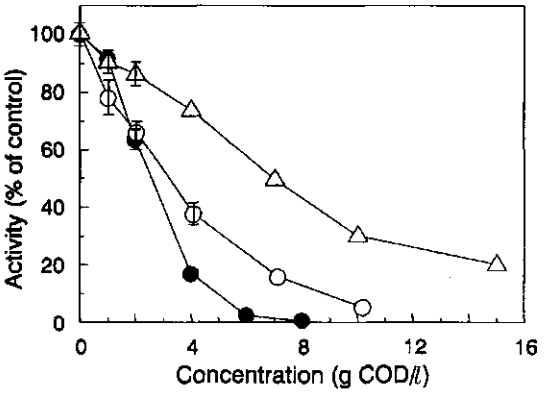


**Figure 4.** The methanogenic toxicity of black liquors prepared from extracted and untreated hemp stem wood fibres. Symbols: •, untreated; ○, method I extraction, 70% extractive removal; △, method II extraction, 90% extractive removal (removal efficiency relative to Soxhlet extraction); bars represent standard deviations.

of 70% of the extractive fraction from the pulp feedstock hardly influenced the methanogenic toxicity of the black liquor, 90% removal of the extractive fraction clearly led to detoxification of the wastewaters. A survey of the 50% IC values is listed in Table 2.

**Table 2.** The 50% IC of hemp extractives and black liquors.

| | 50% IC (g COD/*l*) | | |
|---|---|---|---|
| | Laboratory prepared wastewaters | | Pulp mill wastewater |
| | Bark | Stem wood | Whole stem |
| Black liquor | 4.5 | 2.6 | 4.8 |
| Black liquor, fibre extraction method I[a] | 4.0 | 3.2 | ND[c] |
| Black liquor, fibre extraction method II[a] | 6.5 | 6.9 | ND |
| Recovered extractives[b] | 0.65 | 0.25 | ND |

[a] Experimental conditions for method I and method II fibre extractions are described in Materials and Methods.

[b] Extractives recovered from dichloromethane after fibre extraction method I.

[c] ND, not determined.

## Effect of detoxification treatments on the methanogenic toxicity of hemp black liquor

Several detoxification methods were utilized to remove the methanogenic toxicity from the pulp mill hemp black liquor. The treatments applied, included XAD-2 adsorption and ethyl ether liquid-liquid extraction. The XAD-2 treatment removed about 25% of the COD and 33% of the $UV_{280}$ and visible absorbance. XAD-2 therefore was able to adsorb a significant fraction of the organic material in the black liquors, which aside from the extractive fraction probably included some lignic matter. Ethyl ether liquid-liquid extraction, which was applied to more specifically remove fatty and resinous compounds, caused approximately 10% decrease in the COD and 15% of the $UV_{280}$ absorbance from hemp black liquor. The recovered extractive fraction in turn contained 13% of the COD.

The effect of the XAD-2 treatment, the ethyl ether liquid-liquid extraction and the recovered extractive fraction on the methanogenic toxicity of hemp black liquor is shown in Figure 5. The XAD-2 treatment and the ethyl ether extraction of black liquor resulted in the complete removal of toxicity. Other detoxification treatments which were able to promote complete detoxification of hemp black liquor, were calcium and acid precipitation (results not shown). Both treatments remove extractives as well as lignin, which was indicated by high removal of COD, $UV_{280}$ and $VIS_{440}$, which was approx. 35%, 65% and 80%, respectively.

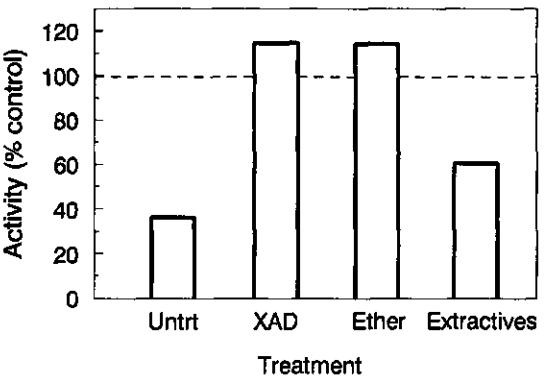


**Figure 5.** The effect of several detoxification treatments on the first feeding methanogenic activity of anaerobic sludge exposed to hemp black liquor in an anaerobic toxicity assay type 1. Untreated black liquor (Untrt); black liquor treated with Amberlite XAD-2 (XAD); black liquor subjected to ethyl ether liquid-liquid extraction (Ether); extractives recovered after ethyl ether liquid-liquid extraction of black liquor (Extractives). Prior to the detoxification treatments, the black liquor, obtained from a full scale pulp mill, was diluted to a concentration of 10 g COD/*l*.

## Discussion

Black liquors derived from wood are highly inhibitory to methanogenic organisms. Previous research shows that the concentrations causing 50% inhibition of methanogenic bacteria ranges from 2 to 6 g COD/*l* (Sierra-Alvarez *et al.*, 1991). Although hemp is a non-woody feedstock, the methanogenic toxicity of the hemp black liquors was comparable with the toxicity found with black liquors of woody feedstocks.

In this research, the high methanogenic toxicity of hemp extractives was demonstrated. The 50% IC value for hemp stem wood extractives and hemp bark extractives was 250 mg COD/*l* and 650 mg COD/*l*, respectively. The hemp extractives were found to be only a little less toxic than softwood resin. Alkaline extracted spruce and pine resin caused 50% inhibition of methanogenic activity at a concentration of 130 and 160 mg COD/*l*, respectively (Sierra-Alvarez and Lettinga, 1990; Field *et al.*, 1988). The COD content of the material in the recovered extractive fraction was 2.80 g COD/g extractive dry weight on average, which is similar to the theoretical value for the fatty and terpenic compounds expected in the extractive fraction.

Moreover it is known that hemp contains many classes of resinous compounds, among which are several types of terpenes, terpenols, fatty acids, cannabinoids and dihydrostilbenes (Turner *et al.*, 1980). Cannabinoids are compounds typical for the genus *Cannabis* with reported antibiotic activity (Krejči, 1970). These compounds however, are expected to be of minor importance for the methanogenic toxicity of hemp extractives, due to their poor solubility (Merck & Co, 1989; PSGB, 1978). Cannabidiol did not cause methanogenic inhibition at concentrations up to 200 mg/*l*. This concentration is much higher than the theoretical maximum, assuming 100% extraction. The most important terpenes in the essential oil of hemp, α-pinene, ß-pinene and limonene, α-selinen, ß-caryophyllene and ß-humulene (Turner *et al.*, 1980; Hendriks *et al.*, 1975), are also commonly found in softwood resin (Fengel and Wegener, 1984). The sesquiterpenes caryophyllene and humulene were found to be only mildly inhibitory to methanogenic bacteria. Apolar phenols, monoterpenes and terpenols however, exert a high methanogenic toxicity with 50% IC values which range from 20-330 mg/*l* (Sierra-Alvarez and Lettinga, 1990) and are expected to be the main toxic compounds in hemp extractives.

The methanogenic toxicity of the resinous fraction is attributed to the lipophilic character of the extractives. Lipophilic compounds tend to accumulate in the cytoplasmic membranes of the bacterial cell. The resulting changes in the membrane structure affect the functioning of membrane embedded proteins, as well as the barrier function of the membrane (Sikkema *et al.*, 1994), which results in deterioration of activity.

Removal of the extractives from the fibres prior to pulping indicated that the extractive fraction plays an important role in the methanogenic toxicity of hemp black liquor. A decreasing extractive content in the pulp feedstock is paralleled by a decreasing toxicity of the resulting wastewater. The residual toxicity in the wastewater at relatively high extractive removal efficiencies in the feedstock indicates that the extractive fraction which survived the extraction is suitably released during subsequent pulping to exert high methanogenic toxicity.

In order to display the impact of the extractive fraction on the methanogenic toxicity of hemp black liquors, complete elimination of the extractive fraction from the pulp feedstock is required. As complete elimination of the extractives from the fibre material proved to be rather difficult; therefore, detoxification treatments were used to remove specific compounds from the black liquor itself. The XAD-2 treatment and the ethyl ether extraction of black liquor resulted in the complete removal of toxicity. This indicates that the extractive fraction which is responsible for about one tenth of the black liquor COD is the main source of inhibitory substances in hemp black liquor. The importance of the extractive fraction on the

overall toxicity of this black liquor is further confirmed by the high toxicity of the recovered extractive fraction. While it is true that this fraction was not as toxic as the untreated wastewater itself, the difference might possibly be attributable to modifications of the compounds (e.g. oxidation or volatilization) during their residence in the ethyl ether and subsequent residence in the rotary evaporator used to vaporize it. In an additional experiment, volatile compounds were not found to be responsible for the toxicity in the black liquors (results not shown). The figures reveal that the sludge exposed to some of the detoxified black liquors had slightly higher activities than the control sludge, fed only acetate substrate. A mild stimulatory effect of detoxified black liquor on the sludge activity is therefore indicated.

The identification of the major sources of methanogenic toxicity in black liquors will enable further research on the development of detoxification treatments. One option for the detoxification of black liquors is based on biodegradation of the extractive fraction. Wood extractives are not suitably degraded anaerobically (Sierra-Alvarez *et al.*, 1990a), but they are eliminated during aerobic treatment (Mueller *et al.*, 1976; Easty *et al.*, 1978; Servizi and Gordon, 1986; Habets and de Vegt, 1991). This observation enables detoxification during an aerobic post-treatment step. Subsequently, the detoxified effluent of the aerobic treatment can be utilized for "upfront dilution" of the influent of an anaerobic treatment system.

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## References

**APHA** (1989). Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association Inc., Washington D.C., USA.

**Bjorklund-Jansson, M.** (1980). Chemistry of fiber building board mill effluent before and after biological treatment. TAPPI, 63 (7), 78-80.

**Easty, D.B., Borchardt, G. and Wabers, A.** (1978). Wood-derived toxic compounds. TAPPI J., 61 (10), 57-60.

**FAO** (1991). The outlook for pulp and paper to 1995. Paper products: an industrial update. Food and Agricultural Organisation of the United Nations, Rome.

**Fengel, D. and Wegener, G.** (1984). Extractives, p. 182-221. Wood: Chemistry, Ultrastructure, Reactions, Walter de Gruyter, Berlin.

**Field, J.A., Leyendeckers, M.J.H., Sierra-Alvarez, R., Lettinga, G. and Habets, L.H.A.** (1988). The methanogenic toxicity of bark tannins and the anaerobic biodegradability of water soluble bark matter. Wat. Sci. Tech., 20(1), 219-240.

**Habets, L.H.A. and de Vegt, A.L.** (1991). Anaerobic treatment of bleached TMP and CTMP effluent in the BIOPAQ UASB system, Wat. Sci. Tech., 24(3/4), 331-345.

**Hendriks, H., Malingré, T.M., Batterman, S. and Bos, R.** (1975). Mono- and sesqui-terpene hydrocarbons of the essential oil of *Cannabis sativa*. Phytochemistry, 14, 814-815.

**Kortekaas, S., Doma, H.S., Potapenko, S.A., Field, J.A. and Lettinga, G.** (1994). Sequenced anaerobic-aerobic treatment of hemp black liquors. Wat. Sci. Tech., 29(5/6), 409-419.

**Kortekaas, S., Vidal, G., Yan-Ling, H., Lettinga, G., and Field, J.A.** (1998). Anaerobic-aerobic treatment of toxic pulping black liquor with upfront effluent recirculation. J. Ferment. Bioeng., 86(1), 97-110.

**Krejči, Z.** (1970). Changes with maturation in the amounts of biologically interesting substances of *Cannabis*, p. 49-55. *In*: C.R.B. Joyce and Curry S.H. (eds), The Botany and Chemistry of *Cannabis*, J.& A. Churchill-London.

**Leach, J.M. and Thakore, A.N.** (1976). Toxic constituents of mechanical pulping effluents. TAPPI, 59, 129-132.

**Lettinga, G., Field, J.A., Sierra-Alvarez, R., van Lier, J.B., and Rintala, J.** (1991). Future perspectives for the treatment of forest industry wastewaters. Wat. Sci. Tech., 24 (3/4), 91-102.

**McCarthy, P.J., Kennedy, K.J. and Droste, R.L.** (1990). The role of resin acids in the anaerobic toxicity of chemithermomechanical pulp wastewater. Wat. Res., 24, 1401-1405.

**de Meijer, E.P.M., van der Kamp, H.J. and van Eeuwijk, F.A.** (1992). Characterisation of *Cannabis* accessions with regard to cannabinoid content in relation to other plant characters. Euphytica, 62, 187-200.

**Merck & Co., Inc.** (1989). Cannabidiol p. 264, Tetrahydrocannabinol p. 1451. *In* Budavari, S. *et al.* (ed.), The Merck Index, 11th edition. Rahway, N.J., U.S.A..

**Mueller, J.C., Leach, J.M. and Walden, C.C.** (1976). Biological detoxification of pulp and paper mill effluents. *In*: Proc. 5th International Fermentation Symposium, Berlin.

**PSGB** (1978). Cannabidiol and Tetrahydrocannabinol, p. 1014. *In* Clarke, E.G.C. *et al.* (ed.), Isolation and Identification of Drugs, Volume 2. The Pharmaceutical Society of Great Britain. The Pharmaceutical Press, London, Great Britain.

**Richardson, D.A., Andras, E. and Kennedy, K.J.** (1991). Anaerobic toxicity of fines in chemi-thermomechanical pulp wastewaters: A batch assay-reactor study comparison. Wat. Sci. Tech., 24, 103-112.

**Rogers, I.H.** (1973). Isolation and Chemical identification of toxic components of kraft mill wastes. Pulp and Paper Mag. Can., 74, 111-116.

**Servizi, J.A. and Gordon, R.W.** (1986). Detoxification of TMP and CTMP effluents alternating in a pilot scale aerated lagoon. Pulp and Paper Canada, 87, T405-T409.

**Sierra-Alvarez, R. and Lettinga, G.** (1990). The methanogenic toxicity of wood resin compounds. Biol. Wastes, 33, 211-226.

**Sierra-Alvarez, R. and Lettinga, G.** (1991). The methanogenic toxicity of wastewater lignins and lignin related compounds. J. Chem. Technol. Biotechnol., 50, 443-455.

**Sierra-Alvarez, R., Kato, M. and Lettinga, G.** (1990a). The anaerobic biodegradability of paper mill wastewater constituents. Environ. Technol., 11, 891-898.

**Sierra-Alvarez, R., Kortekaas, S., Van Eekert, M., Harbrecht, J. and Lettinga, G.** (1990b). The continuous anaerobic treatment of pulping wastewaters. J. Ferment. Bioengineer., 70 (2), 119-127.

**Sierra-Alvarez, R., Kortekaas, S., Van Eekert, M. and Lettinga, G.** (1991). The anaerobic biodegradability and methanogenic toxicity of pulping wastewaters. Wat. Sci. Tech., 24(3/4), 113-125.

**Sierra-Alvarez, R., Field, J.A., Kortekaas, S. and Lettinga, G.** (1994). Overview of the anaerobic toxicity caused by organic forest industry wastewater pollutants. Wat. Sci. Tech., 29(5/6), 353-363.

**Sikkema, J., de Bont, J.A.M. and Poolman, B.** (1994). Interactions of cyclic hydrocarbons with biological membranes. J. Biol. Chem., 269, 8022-8028.

**Turner, C.E., ElSohly, M.A. and Boeren, E.G.** (1980). Constituents of *Cannabis sativa* L. XVII. A review of the natural constituents. J. Nat. Prod., 43, 169-234.

**Voss, R.H. and Rapsomatiotis, A.** (1985). An improved solvent-extraction based procedure for the gas-chromatographic analysis of resin and fatty acids in pulp mill effluents. J. Chromatogr, 346, 205-214.

**Welander, T. and Andersson, P.E.** (1985). Anaerobic treatment of wastewater from the production of chemi-thermomechanical pulp. Wat. Sci. Tech., 17, 103-112.

# Sequenced Anaerobic-Aerobic Treatment of Hemp Black Liquors.

# 3

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## Abstract

This study evaluates the sequenced anaerobic-aerobic treatment of hemp bark and hemp stem wood black liquors with respect to COD removal efficiency and detoxification. Anaerobic toxicity assays revealed that soda pulping liquors derived from hemp are just as toxic as those derived from wood. Hemp bark and stem wood black liquors caused 50% inhibition at concentrations of 5.9 and 4.5 g COD/*l*, respectively. Long term experiments were conducted in lab-scale upflow anaerobic sludge blanket (UASB) reactors at 30 °C. Black liquor was fed at sub-toxic concentrations (< 4 g COD/*l*). In the bark column loadings were applied up to 17.5 g COD/*l*·d with COD and $BOD_5$ efficiencies of 56.0 and 87.9%, respectively. In the stem wood column loadings up to 18.4 g COD/*l*·d were reached with COD and $BOD_5$ efficiencies of 42.3 and 81.0%, respectively. Aerobic post-treatment displayed only minor extra COD removal and a strong increase of color levels. After sequenced anaerobic-aerobic treatment, the COD removal was 70.9 and 58.4% for bark and stem wood liquor, respectively, whereas the $BOD_5$ removal exceeded 98%. Strong detoxification was accomplished after anaerobic-aerobic treatment, as was demonstrated by anaerobic toxicity assays. After anaerobic-aerobic treatment hemp bark and stem wood black liquors caused 50% inhibition at concentrations of 13.5 and 21.5 g COD/*l*, respectively.

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## Introduction

The treatment of wastewater derived from the pulping of hemp (*Cannabis sativa* L.) is part of a national research programme, which studies the technical and economical feasibility of producing paper from hemp fibres. The Dutch Department of Agriculture initiated the research to improve pest prevention in arable farmland by enhancing crop rotation. Hemp is an annual crop with a long history in paper production (Bosia, 1975; Gilabert-Peréz, 1983; Sandermann, 1988). The hemp stem consists of a bark and a stem wood fraction, which will be processed separately, because of their different fibre qualities. The research encompasses chemical pulping methods to produce high quality paper. Soda pulping was chosen, as environmental requirements demanded a sulphur-free pulping method.

Soda pulping processes generate highly concentrated liquors. Usually these wastewaters are burnt to recover chemicals and energy. Pulp mills using non-woody feedstocks are often too small to apply chemical recovery. Biological treatment of black liquors derived from wood, shows that these wastewaters are poorly biodegradable, highly toxic and contain high color levels. Hemp is a non-woody lignocellulosic feedstock. One may question if black liquors from non-woody feedstocks are equally problematic as those from woody feedstocks. The composition of hemp and two commonly used woody feedstocks is listed in Table 1.

**Table 1.** Composition of hemp bark, hemp stem wood, birch and pine (Rydholm, 1965; Fengel and Wegener, 1984; van der Werf, 1994).

| | Composition (% total dry weight) | | | |
|---|---|---|---|---|
| | Hemp | | Birch | Pine |
| | Bark | Stem Wood | *Betula verrucosa* | *Pinus sylvestris* |
| Lignin | 4 | 21 | 20 | 28 |
| Hemicellulose | 8 | 17 | 36 | 25 |
| Cellulose | 68 | 35 | 37 | 42 |
| Extractives | 3.7# | 4.6# | 3.1# | 5.3# |
| | 0.8* | 1.9* | 2.0** | 2.3** |

# Ethanol-benzene extraction.

* Dichloromethane extraction.

** Petroleum ether extraction.

The objective of the present research was to evaluate the efficiency of sequential anaerobic-aerobic treatment of hemp bark and stem wood black liquors. An additional objective was to determine the detoxification achieved by anaerobic and combined anaerobic-aerobic treatment.

## Materials and Methods

### Analyses

Wastewater samples were analyzed for COD (the micro method with dichromate), $BOD_5$, VSS, TSS according to APHA standard methods (APHA, 1989). Samples for the determination of COD, $VIS_{440}$ and $UV_{280}$ were membrane filtered (0.45 $\mu$m). $VIS_{440}$ and $UV_{280}$ were determined in a 1 cm quartz cuvette by diluting the samples to less than 0.8 absorbance units in a 0.02 M. borate buffer providing a pH 9.1. The lignin content of a wastewater was estimated from the ultraviolet absorbance of the sample at 280 nm ($UV_{280}$) using an absorbance coefficient of 20.6 *l*/g·cm (present research). VFA were analyzed by gas chromatography using a Hewlett Packard 5890 equipped with 2 m x 4 mm glass column packed with Supelcoport (100-120 mesh) coated with 10% Fluorad FC 431. The temperatures of the column, the injection port and the flame ionization detector were 130, 200, 280 °C, respectively. The carrier gas was nitrogen saturated with formic acid (30 m*l*/min).

### Preparation of the black liquors

Black liquors were prepared from fibre hemp (*Cannabis sativa* L.), cultivar Kompolti Hybrid TC), grown in the Netherlands. The hemp stem was mechanically divided into a hemp bark and a hemp stem wood fraction, both fractions were processed separately. Per litre of tap water 20 g NaOH and 100 g of dry hemp fibre was added. The mixture was cooked at 165 °C for a period of 30 minutes. Typically, the bark black liquor showed a concentration of 39 g COD/*l*; $BOD_5$/COD = 41%; pH = 12.5; $UV_{280}$ = 171.0 absorbtion units 1x,1cm and $VIS_{440}$ = 14.2 absorbtion units 1x,1cm. Typically, the stem wood black

liquor showed a concentration of 55 g COD/*l*; $BOD_5$/COD = 30%; pH = 12.5; $UV_{280}$ = 321.3 absorbtion units 1x,1cm and $VIS_{440}$ = 20.6 absorbtion units 1x,1cm.

**Biomass**

The anaerobic granular sludge used in these experiments was obtained from a full-scale anaerobic reactor treating wheat starch wastewater (Latenstein, Nijmegen, the Netherlands). The sludge was elutriated to remove fines and stored at 4 °C under nitrogen gas.

**Batch assays**

All assays contained essential inorganic macro and micro nutrients as outlined previously (Kortekaas *et al.*, 1998) and were placed in a temperature controlled room at 30 ± 2°C. The methanogenic activity and toxicity assays were conducted in 0.6 *l* glass serum flasks, sealed with a rubber septum and a screw cap. Before incubation the headspace of the flasks was flushed with a gas mixture containing 70% $N_2$ and 30% $CO_2$ and $Na_2S$ (39 mg/*l*) was added to remove oxygen. The bottles were placed in a reciprocating shaker.

*Methanogenic activity assay.* The specific acetoclastic activity was determined utilizing 0.6 *l* glass serum flasks. Anaerobic sludge (1.5 g VSS/*l*) was transferred from the column to the serum flask containing 0.05 *l* medium, which consisted of nutrients, $NaHCO_3$ (4 g/*l*) and neutralized acetate (3 g COD/*l*). After 24 h incubation the headspace was flushed with the gas mixture and the accumulation of methane in the headspace was determined during a period of 3-6 h. The specific acetoclastic activity was calculated from the slope of the methane production versus time curve and the quantity of VSS.

*Anaerobic toxicity assay.* The methanogenic toxicity assay was conducted in a similar fashion as the methanogenic activity assay. The anaerobic sludge (1.5 g VSS/*l*) was transferred to a serum flask containing 0.05 *l* medium, which consisted of nutrients, $NaHCO_3$ (8 g/*l*), neutralized acetate (2 g COD/*l*) and wastewater. The wastewater concentrations supplied were chosen to provide an inhibition of methanogenic activity ranging from 0% to 100%. Substrate controls were based on assays were no wastewater was added. After 7 days incubation, the assays were provided with a second feeding lacking the wastewater; this was done in order to determine the residual activity of the sludge after exposure to the

wastewater. The supernatant was carefully decanted and replaced with a medium containing nutrients, $NaHCO_3$ (4 g/*l*) and neutralized acetate (3 g COD/*l*). After 24 h incubation, the headspace was flushed with the gas mixture and the accumulation of methane in the headspace was determined during a period of 3-6 h. The inhibited activity was expressed as a percentage of the control activity and is abbreviated to "%ACT". The percentage inhibition, "%I", was defined as: %I = 100 - %ACT. The wastewater concentration that caused 50% inhibition of the acetoclastic activity is referred to as "50% IC". To perform anaerobic toxicity assays with influent and effluent of the different treatment steps, concentration of the wastewater was required. The wastewater was concentrated by evaporating the wastewater under a constant flow of $N_2$.

*Anaerobic biodegradability assay.* The anaerobic biodegradability assay was conducted in triplicate in 1 *l* serum flasks containing granular sludge (5 g VSS/*l*) and nutrients. The treatments consisted of 4 g COD/*l* black liquor. Sludge blanks, to correct for gas production from the sludge, were based on assays were no wastewater was provided. Both the sludge blanks and the treatments received 2 g $NaHCO_3$/*l* as buffer. Methane production was monitored periodically during the assay with modified Mariotte flasks. These flasks were filled with a 3% NaOH solution, which served to remove the carbon dioxide from the biogas. Daily, the serum flasks were manually shaken. The biodegradability assay was carried out for a period of 14 days. Parameters to describe the biodegradability of the wastewater are defined below in the section " Continuous anaerobic treatment".

**Continuous anaerobic treatment**

The continuous experiments were performed in UASB reactors with a liquid volume of 2 l, placed in a temperature controlled room at 30 ± 2°C. Due to sludge floatation problems, effluent recirculation was applied to improve degasification of the sludge. The superficial velocity owing to recirculation was 0.94 m/h. Baker's Antifoam B silicone emulsion (0.1 g/*l*) was used to diminish foam problems. The reactors were inoculated with 20 g VSS/*l* granular sludge. Diluted hemp black liquor influents were neutralized with HCl to approximately pH 8.2. Nutrients were added as outlined previously for the bioassays. No buffer was added due to the alkaline nature of the wastewater. The influent was fed directly to the reactor from a refrigerator (5°C). Prior to measurement in a wet gas meter, the biogas evolved passed through a 10% NaOH solution and a column filled with soda lime pellets.

The following parameters were used to indicate the performance of the UASB reactors. $COD_{in}$ = Influent COD (g COD/*l*); M = % conversion $COD_{in}$ to methane; VFA = VFA remaining in the effluent as % of $COD_{in}$; A = % conversion $COD_{in}$ to VFA (A = M + VFA); E = % $COD_{in}$ removed based on the membrane filtered effluent COD concentration; Cells = % conversion of $COD_{in}$ to cells (estimation of Cells = E - M); BD = % biodegradable $COD_{in}$ (BD = A + Cells); Y = specific cell yield in COD per unit COD biodegraded (Cells/BD); $E_{bd}$ = the elimination of the biodegradable COD = [1 - (VFA/BD)] · 100; OLR = Organic Loading Rate (g COD/*l*·d); $OLR_{bd}$ = BD/100 · OLR (g $COD_{bd}/l$·d); $E_{bod}$ = % influent $BOD_5$ removed based on the membrane filtered effluent $BOD_5$ concentration; $E_{uv}$ = % $UV_{280}$ elimination based on the membrane filtered effluent $UV_{280}$; $E_{vis}$ = % $VIS_{440}$ elimination based on the membrane filtered effluent $VIS_{440}$.

**Aerobic treatment**

During period II (day 221-316) the anaerobic treatment was extended with a aerobic post-treatment. The aerobic post-treatment, which was inoculated with activated sludge from a pilot scale activated sludge plant treating domestic sewage, took place in a 3 *l* aeration tanks at a hydraulic retention time (HRT) of 1.3 days. Daily the aeration was stopped to let the sludge settle, and 2 *l* of the content was removed and replaced by effluent of the anaerobic reactor.

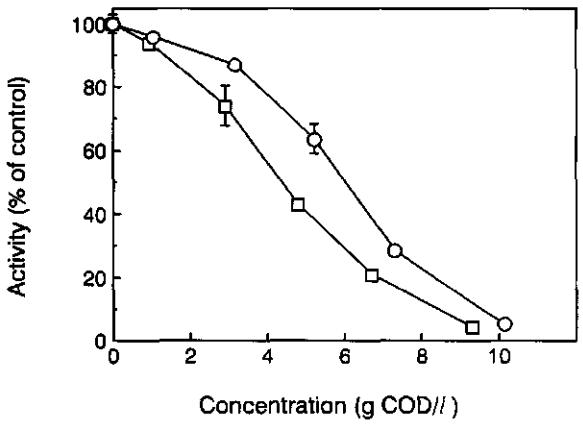


**Figure 1.** The methanogenic activity (as percentage of the control activity) of granular sludge exposed to hemp bark (○) and hemp stem wood (□) black liquors. Bars represent standard deviation.

## Results

The anaerobic biodegradability was studied in a 14 day batch assay. Table 2 shows that hemp black liquors are only partially biodegradable, which points to the presence of recalcitrant organic matter. Bark black liquor was the more biodegradable wastewater with a biodegradability of 68% compared to 59% for stem wood black liquor. Apart from the low biodegradability, Figure 1 shows that these wastewaters are also highly toxic. Hemp stem wood black liquor, which had a 50% IC corresponding to 4.5 g COD/*l*, was slightly more toxic than hemp bark black liquor with a 50% IC corresponding to 5.9 g COD/*l* (Table 6).

**Table 2.** The average methanogenesis, acidification, biodegradability, and cell yield of hemp bark and stem wood black liquor determined in anaerobic biodegradability assay on day 14.

| | | Bark Black Liquor | Stem Wood Black Liquor |
|---|---|---|---|
| | | Average ± S.D. (%) | Average ± S.D. (%) |
| M | (% $COD_{in}$) | 53.6 ± 0.5 | 40.3 ± 0.6 |
| A | (% $COD_{in}$) | 54.0 ± 0.5 | 40.6 ± 0.6 |
| BD | (% $COD_{in}$) | 67.9 ± 0.7 | 59.2 ± 0.8 |
| Y | (% $COD_{bd}$) | 20.7 ± 1.4 | 31.6 ± 2.0 |

Abbreviations are defined in Materials and Methods.

Two laboratory columns were used to study the continuous anaerobic treatment of hemp bark and stem wood black liquor in a long term experiment. A diluted influent was fed to prevent methanogenic inhibition, since our results demonstrated a high toxicity of hemp black liquors. During period I (day 0-220) the average influent concentration was 1.9 g COD/*l* and during period II (day 221-316) it was 3.9 g COD/*l*. The operation and performance of the reactors during the course of the experiment are illustrated in Figure 2 and 3 and summarized in Table 3. The average OLR during period II was about 17.5 g COD/*l*·d (9.9 g $COD_{bd}$/*l*·d) and 18.4 g COD/*l*·d (8.5 g $COD_{bd}$/*l*·d) for the bark and stem wood column; with an average COD elimination of 56.0% and 42.3% respectively,. The removal of biodegradable COD was for the bark and stem wood column 95.2% and 91.1%, respectively. The corresponding $BOD_5$ removal efficiencies were 87.9% and 81.0 %, respectively (Table 4). The elimination of $UV_{280}$ was for both columns about 10% (Table 4), which indicates only a minor removal of some lignin components. The VFA concentrations in the effluent were always low, except

for the period between days 283-316, when loadings of 13.3 g $COD_{bd}/l \cdot d$ were applied. For the bark column, these higher VFA concentrations were only temporary. For the stem wood column, the increasing VFA level indicated that the maximum methanogenic capacity of the reactor had been reached. This is confirmed by the sludge VSS and activity data, which are given in Table 5.

**Table 3.** Average influent concentration, OLR, hydraulic retention time, %E, %M, $\%E_{bd}$ during continuous operation of UASB reactors fed with bark and stem wood black liquors.

| | | Bark Column | | Stem Wood Column | |
|---|---|---|---|---|---|
| | | Period I# | Period II* | Period I# | Period II* |
| Operational parameters: | | | | | |
| $COD_{in}$ | (g COD/*l*) | 1.89 | 3.88 | 1.93 | 3.86 |
| OLR | (g $COD/l \cdot d$) | 9.8 | 17.5 | 9.2 | 18.4 |
| $OLR_{bd}$ | (g $COD_{bd}/l \cdot d$) | 5.9 | 9.9 | 4.2 | 8.5 |
| HRT | (h) | 5.4 | 6.0 | 5.9 | 6.0 |
| Efficiency: | | | | | |
| E | (%) | 57.7 | 56.0 | 42.8 | 42.3 |
| M | (%) | 46.9 | 48.9 | 33.4 | 38.5 |
| $E_{bd}$ | (%) | 97.3 | 95.2 | 96.0 | 91.1 |

Abbreviations are defined in Materials and Methods.

# Experimental period I: day 0 - 220.

* Experimental period II: day 221 - 316.

The VSS concentration and the specific methanogenic activity of the sludge were monitored regularly during the experiment. Table 5 shows that the activity of the bark column sludge remained high during the course of the experiment. However in contrast, the activity of the stem wood column sludge shows a drastic 60% drop after start up and stabilizes at this level. The VSS content of the bark column fluctuated heavily during the course of the experiment, owing to temporarily sludge wash-out, which was accompanied by foam formation in the reactor and floating pistons of sludge.

**Table 4.** Average treatment efficiency during sequenced anaerobic-aerobic treatment of hemp bark and stem wood black liquors (Period II; day 221 -316).

| | E (%) | $E_{bod}$ (%) | $E_{uv}$ (%) | $E_{vis}$ (%) |
|---|---|---|---|---|
| Bark: | | | | |
| Anaerobic | 56.0 | 87.9 | 11.8 | 9.0 |
| Anaerobic-aerobic | 70.9 | 98.6 | 22.3 | -32.2# |
| Stem wood: | | | | |
| Anaerobic | 42.3 | 81 | 9.5 | 5.0 |
| Anaerobic-aerobic | 58.4 | 99.2 | 18.6 | -82.2# |

Abbreviations are defined in Materials and Methods.

# Negative values indicate increase of color.

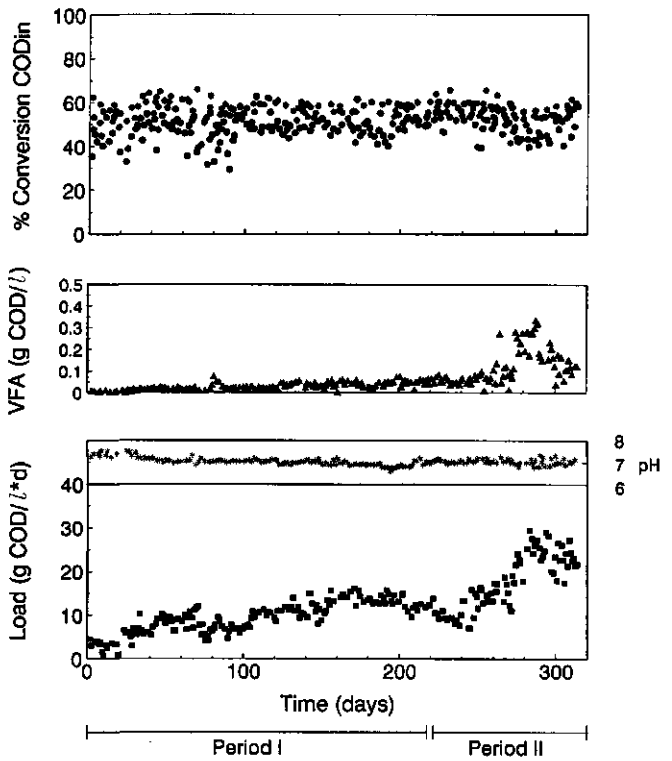


**Figure 2.** Operation and efficiency during anaerobic treatment of hemp bark black liquor. •) COD removal, % $COD_{in}$; ○) methanogenesis, % $COD_{in}$; ▲) VFA in effluent, % $COD_{in}$; *) pH in UASB reactor; ▪) OLR, g $COD \cdot l^{-1}d^{-1}$. Period I: day 0-220, $COD_{in} \approx$ 1.9 g COD/*l*; Period II: day 221-316, $COD_{in} \approx$ 3.9 g COD/*l*.

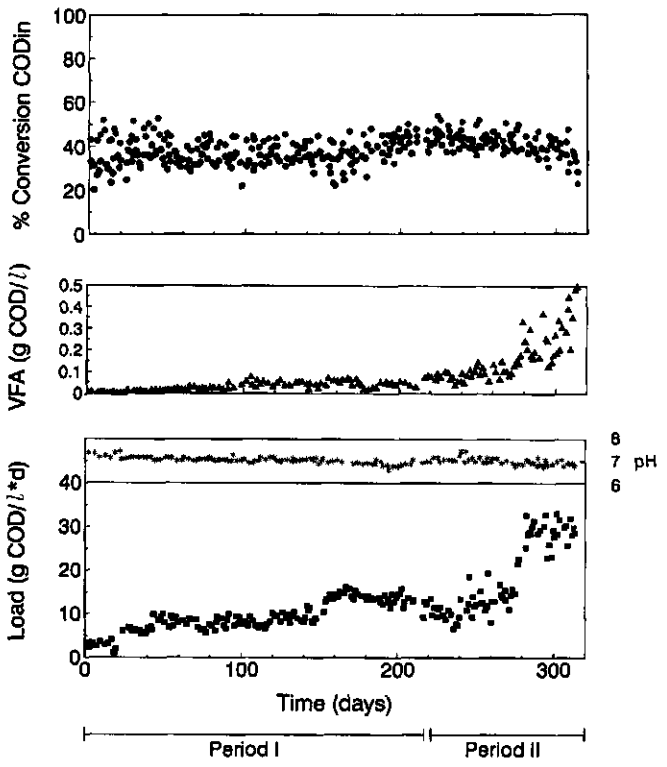


**Figure 3.** Operation and efficiency during anaerobic treatment of hemp stem wood black liquor. •) COD removal, % $COD_{in}$; ○) methanogenesis, % $COD_{in}$; ▲) VFA in effluent, % $COD_{in}$; *) pH in UASB reactor; ■) OLR, g $COD \cdot l^{-1}d^{-1}$. Period I: day 0-220, $COD_{in} \approx 1.9$ g COD/*l*; Period II: day 221-316, $COD_{in} \approx 3.9$ g COD/*l*.

To study the potential of aerobic treatment to enhance COD removal, $BOD_5$ removal and detoxification, the anaerobic treatment was extended with a batch-wise operated aerobic treatment (HRT 1.3 d) in period II. The elimination of COD, $BOD_5$, $UV_{280}$ and $VIS_{440}$ during the sequential anaerobic-aerobic treatment is listed in Table 4. The extra COD removal achieved by aerobic post-treatment was little. A higher COD removal was demonstrated for the bark black liquor compared to the stem wood black liquor. Despite the limited COD removal during anaerobic-aerobic treatment, the $BOD_5$ removal exceeded 98%. Color removal did not occur; on the contrary, the aerobic post-treatment demonstrated a strong increase in color levels. This phenomenon was previously observed by Rintala and Vuoriranta (1988), Rintala and Lepistő (1992) and Sierra-Alvarez *et al.* (1990b).

**Table 5.** The sludge concentration and specific methanogenic activity during continuous operation of UASB reactors fed with hemp bark and stem wood black liquor.

| Day | Bark Column | | Stem Wood Column | |
|---|---|---|---|---|
| | VSS (g/*l*) | Methanogenic Activity (g COD/g VSS·d) | VSS (g/*l*) | Methanogenic Activity (g COD/g VSS·d) |
| 0 | 20.0 | 1.22 | 20.0 | 1.22 |
| 98 | 9.4 | 0.79 | 22.7 | 0.33 |
| 149 | 17.2 | 0.98 | 28.7 | 0.26 |
| 211 | 21.5 | 0.98 | 31.0[#] | 0.38 |
| 261 | 15.4 | 0.73 | 21.0 | 0.34 |
| 316 | 29.9 | 0.96 | 20.8 | 0.32 |

[#] On day 211, the sludge concentration was adjusted to 20 g VSS/*l*.

The detoxification during anaerobic-aerobic treatment was studied with methanogenic bacteria as test organisms. Figure 4 shows the acetoclastic methanogenic inhibition of bark and stem wood black liquor during anaerobic-aerobic treatment. The 50% inhibition concentration estimated in these experiments is summarized in Table 6.

**Table 6.** The 50% IC of hemp bark and stem wood black liquor during sequenced anaerobic-aerobic treatment.

| Wastewater | 50% IC (g COD/*l*) | |
|---|---|---|
| | Bark Black Liquor | Stem wood Black Liquor |
| Untreated | 5.9 | 4.5 |
| Untreated[*] | 6.3 | 6.4 |
| After anaerobic treatment[*] | 11.5 | 9.9 |
| After anaerobic-aerobic treatment[*] | 13.5 | 21.5 |

[*] Wastewater concentrated by evaporation.

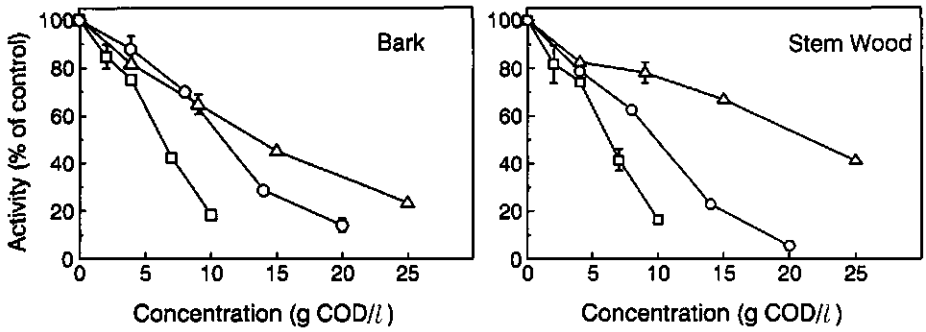


**Figure 4.** The effect of anaerobic and anaerobic-aerobic treatment on the methanogenic toxicity of hemp black liquors. □) untreated; ○) after anaerobic treatment; △) after anaerobic-aerobic treatment. COD refers to influent concentrations.

It is obvious that an increased detoxification takes place in the successive treatment steps, and that the detoxification in the stem wood column is bigger than in the bark column. The major part of this detoxification is accomplished during aerobic treatment of the stem wood black liquor. Concentration of the wastewater by evaporation was required in order to perform the anaerobic toxicity assay. Besides the concentrated samples, a non-concentrated control sample was tested to estimate the effect of concentration on the toxicity of the wastewaters. In the case of influent samples, the non-concentrated samples were slightly more toxic than the concentrated samples, which indicated that during concentration of the influent some unstable or volatile toxic compounds were removed. After anaerobic and aerobic treatment, the differences between concentrated and non-concentrated samples were negligible.

## Discussion

Little is known about the treatability of hemp soda pulping wastewaters. Research on the treatment of wood derived black liquors shows that these wastewaters are troublesome for anaerobic treatment, owing to their low biodegradability and high toxicity (Sierra-Alvarez *et al.*, 1990b, 1991). The low biodegradability of black liquors is due to the high lignin fraction. Lignin is a very recalcitrant material. Hardly any lignin degradation is observed during anaerobic or aerobic treatment (Colberg and Young, 1985; Sierra-Alvarez *et al.*,

1990a). Apart from the low biodegradability, lignin causes the high color levels of these wastewaters. The major toxic substances in wood derived black liquors were identified as resin compounds. These compounds are responsible for inhibition of methanogenic bacteria (Benjamin *et al.*, 1984; Field *et al.*, 1988; Sierra-Alvarez and Lettinga, 1990; McCarthy *et al.* 1990) and have toxic effects on fish and aquatic organisms in the recipient (Leach and Thakore, 1976; Walden *et al.*, 1986). Anaerobically, resin compounds are not suitably degraded (Sierra-Alvarez *et al.*, 1990a), but they are eliminated in aerated active sludge plants (Mueller *et al.*, 1976; Easty *et al.*, 1978).

The results of anaerobic batch toxicity assays indicate that soda pulping liquors derived from hemp are just as toxic as those derived from wood. The methanogenic 50% IC value of hemp black liquors is comparable with that found by others with wood derived black liquors (Sierra-Alvarez *et al.*, 1991). This is not surprising as hemp resin contains, like wood resin, volatile terpenes (Hood *et al.*, 1973; Hendriks *et al.*, 1975; Turner *et al.*, 1980), furthermore the concentration of resin in hemp is comparable with that of wood (Table 1). The hemp black liquors were poorly biodegradable in anaerobic batch assays as well as in continuous anaerobic-aerobic treatment. These results are in accordance to the biodegradability data of the continuous experiment and the data found by others with wood derived black liquors (Sierra-Alvarez *et al.*, 1991). Therefore, hemp black liquors are not necessarily an easier wastewater for biological treatment.

Due to the high toxicity, it is necessary to dilute the black liquor to subtoxic concentrations for continuous anaerobic treatment. Previously Field *et al.* (1991) found that the maximal tolerable concentration of hemp black liquor during continuous treatment was between 4-6 g COD/*l*. Total failure of the system occurred, when higher influent concentrations were fed. Therefore, we evaluated the treatability at concentrations diluted down to 2 to 4 g COD/*l*. In this concentration range, a good removal of the readily biodegradable components of the wastewater was found. Although the removal efficiencies were good, the specific methanogenic activity of the sludge from the stem wood column had dropped to about 40% of the activity of the bark column. We asked ourselves if this difference in activity was due to toxicity of the stem wood liquor or to dilution of the active biomass by lignin precipitates. The lignin content of the sludge was in both columns approximately 24% of the VSS. So the difference in sludge activity reflects the fact that stem wood black liquor is more toxic. Despite the toxicity of the stem wood black liquor, the sludge content of the stem wood column increased steadily (Table 5), which indicated that bacterial growth in the granules was occurring. The average specific cell yield of the stem

wood column was 0.10 g COD/g $COD_{bd}$. The sludge concentration of the bark column varied quite strongly, owing to temporarily sludge wash-out. Despite the fluctuations in sludge concentration, the stable treatment efficiencies of the bark column indicate that the organic loading never exceeded the methanogenic capacity. In this study we have shown, that even for these toxic wastewaters it is still feasible to obtain high loadings combined with very high $BOD_5$ removal rates, provided that the dilution requirements are fulfilled.

However, in practice dilution may be difficult to realize. Black liquors are highly concentrated wastewaters and dilution rates of 10-100 may be necessary. Other waste streams of the paper mill may contribute to the dilution water, but may be insufficient. Cold dilution water should be heated up to mesophillic temperatures and this will entail considerable costs. Dilution water may simply not be available. And lastly, it may be a politically sensitive matter to use clean water to dilute wastewater.

In case that sufficient dilution cannot be achieved, detoxification measures should be considered. Several detoxification methods have been recommended in different studies on forest industry wastewaters. Welander (1988) successfully used aluminum, calcium and iron salts to precipitate long chain fatty acids and resin acids to remove the high toxicity of CTMP effluents. Acid precipitation was tested by Field *et al.* (1991) and Sierra-Alvarez *et al.* (1991) and proved to be effective in the detoxification of black liquors. A disadvantage of precipitation methods is that the precipitated sludges have to be separated from the wastewater and disposed of. Another detoxification method was based on the aerobic biodegradation of resin compounds. This observation has led to the development of a detoxification system for CTMP wastewater in which the effluent of the aerobic post-treatment is recirculated to dilute the influent of the anaerobic reactor (Habets and De Vegt, 1991). The detoxifying potential of the anaerobic-aerobic treatment was studied by performing anaerobic toxicity assays with the untreated, anaerobically treated and anaerobic-aerobically treated hemp black liquors. The results display a strong detoxification during the successive treatment steps. The anaerobic-aerobic detoxification makes it hypothetically possible to treat stem wood black liquor as high as 18 g COD/*l*, provided that the aerobic effluent is recirculated and an inhibition of 40% of the sludge activity is accepted.

In this study, the aerobic post-treatment provided little extra COD removal, owing to removal of most of the biodegradable matter during anaerobic treatment and the highly recalcitrant lignin fraction, which also persists aerobic post-treatment. However, the small additional COD removal significantly enhanced the elimination of $BOD_5$. As a result, almost complete $BOD_5$ removal ($> 98\%$) was obtained after sequenced anaerobic-aerobic treatment of hemp black liquors.

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## References

**APHA** (1989). Standard methods for the examination of water and wastewater. 17th edn, American Public Health Assoc., Washington D.C., USA.

**Benjamin, M.M., Woods, S.L., and Ferguson, J.F.** (1984). Anaerobic toxicity and biodegradability of pulp mill waste constituents. Wat. Res., 18, 601-607.

**Bosia, A.** (1975). Hemp for refiner mechanical pulp. Paper, World Research and Development number, 37-41.

**Colberg, P.J., and Young, L.Y.** (1985). Anaerobic degradation of soluble fractions of [14 C-lignin]lignocellulose. Appl. Environ. Microbiol., 49(2), 345-349.

**Easty, D.B., Borchardt, G., and Wabers, A.** (1978). Wood-derived toxic compounds. TAPPI J., 61(10), 57-60.

**Fengel, D., and Wegener, G.** (1984). Extractives. In: Wood: Chemistry, Ultrastructure, Reactions, Chp. 7, Walter de Gruyter, Berlin.

**Field, J.A., Leyendeckers, M.J.H., Sierra-Alvarez, R., Lettinga, G., and Habets, L.H.A.** (1988). The methanogenic toxicity of bark tannins and the anaerobic biodegradability of water soluble bark matter. Wat. Sci. Tech., 20(1), 219-240.

**Field, J.A., Vicent, T., Sierra-Alvarez, R., and Paris, J.** (1991). The anaerobic treatment and detoxification of black liquors derived from non-woody feedstocks. Internal report: Department of Chemical Engineering, Autonomous University of Barcelona, Bellaterra, Spain.

**Gilabert-Peréz, E.J.** (1983). Las fibras no madreras y el cañamo en la industria papelera. Invest. Téc. Papel., 20(76), 465-474.

**Habets, L.H.A., and De Vegt, A.L.** (1991), Anaerobic treatment of bleached TMP and CTMP effluent in the BIOPAQ UASB system, Wat. Sci. Tech., 24(3/4), 331-345.

**Hendriks, H., Malingré, T.M., Batterman, S., and Bos, R.** (1975). Mono- and sesqui-terpene Hydrocarbons of the essential oil of *Cannabis sativa*. Phytochemistry, 14, 814-815.

**Hood, L.V.S., Dames, M.E., and Barry, G.T.** (1973), Headspace volatiles of marijuana. Nature, 242, 402-403.

**Leach, J.M., and Thakore, A.N.** (1976). Toxic constituents of mechanical pulping effluents. TAPPI J., 59, 129-132.

**Kortekaas, S., Vidal, G., Yan-Ling, H., Lettinga, G., and Field, J.A.** (1998). Anaerobic-aerobic treatment of toxic pulping black liquor with upfront effluent recirculation. J. Ferment. Bioeng., 86(1), 97-110.

**McCarthy, P.J., Kennedy, K.J., and Droste, R.L.** (1990). The role of resin acids in the anaerobic toxicity of chemithermomechanical pulp wastewater. Wat. Res., 24, 1401-1405.

**Mueller, J.C., Leach, J.M., and Walden, C.C.** (1976). Biological detoxification of pulp and paper mill effluents. In: Proc. 5th International Fermentation Symposium, Berlin.

**Rintala, J.A., and Vuoriranta, P.** (1988). Anaerobic-aerobic treatment of thermomechanical pulping effluents. TAPPI J., 71, 201-207.

**Rintala, J.A., and Lepistő, S.L.** (1992). Anaerobic treatment of thermomechanical pulping whitewater at 35-70°C. Wat. Res., 26(10), 1297-1305.

**Rydholm, S.A.** (1965), Wood chemistry, In: Pulping processes, Chp. 4. Intersci. Publ., New York, London, Sydney, 96-97.

**Sandermann, W.** (1988). Die kulturgeschichte des papiers. Springer Verlag, Berlin, New York.

**Sierra-Alvarez, R., and Lettinga, G.** (1990). The methanogenic toxicity of wood resin compounds, Biol. Wastes, 33, 211-226.

**Sierra-Alvarez, R., Kato, M., and Lettinga, G.** (1990a). The anaerobic biodegradability of paper mill wastewater constituents. Environ. Technol., 11, 891-898.

**Sierra-Alvarez, R., Kortekaas, S., Van Eekert, M., Harbrecht, J., and Lettinga, G.** (1990b). The continuous anaerobic treatment of pulping wastewaters. J. Ferment. Bioengineer., 70 (2), 119-127.

**Sierra-Alvarez, R., Kortekaas, S., Van Eekert, M., and Lettinga, G.** (1991). The anaerobic biodegradability and methanogenic toxicity of pulping wastewaters. Wat. Sci. Tech., 24(3/4), 113-125.

**Turner, C.E., ElSohly, M.A., and Boeren, E.G.** (1980). Constituents of *Cannabis sativa* L. XVII. A review of the natural constituents. J. Nat. Prod., 43, 169-234.

**Van der Werf, H.M.G., Harsveld van der Veen, J.E., Bouma, A.T.M., and Ten Cate, M.** (1994). Quality of hemp (*Cannabis sativa* L.) stems as raw material for paper. Industrial Crops and Products, 2, 219-227.

**Walden, C.C., McLeay, D.J., and McKague, A.B.** (1986), Cellulose production processes. In: The Handbook of Environmental Chemistry, O. Hutzinger (Ed)., Vol. 3, Part D, Anthropogenic Compounds, Springer-Verlag, Berlin, 1-34.

**Welander, T.** (1988), An anaerobic process for treatment of CTMP effluent. Wat. Sci. Tech., 20(1), 143-147.

# Anaerobic-Aerobic Treatment of Toxic Pulping Black Liquor with Upfront Effluent Recirculation.

# 4

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*He Yan-Ling*
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*Jim Field*

## Abstract

Alkaline pulping liquors are problematic for anaerobic treatment due to their toxicity to methanogens and their relatively large fraction of inert lignin. In previous research, toxicity due to wood extractives was shown to be highly eliminated during aerobic wastewater treatment, but not during anaerobic treatment. These observations have led to the proposal of a detoxification strategy denominated upfront dilution, based on the sequenced anaerobic-aerobic treatment of the pulping liquor, recirculating the aerobic effluent to dilute the incoming influent to sub-toxic concentrations.

In this study, the treatment of highly toxic hemp stem wood black liquor (HSWBL) in lab-scale UASB reactors with upfront dilution was compared with direct anaerobic treatment and with direct aerobic treatment. Direct anaerobic treatment of 12 g COD/*l* HSWBL led to almost complete inhibition of the methanogenic activity within 14 days. However, recirculation of 75% of the aerobic post-treatment effluent for upfront dilution of the toxic HSWBL, enabled anaerobic treatment at loading rates up to 21.5 g COD/$l_{UASB}$·d without significant inhibition of the methanogenic activity. Extensive detoxification was confirmed during anaerobic-aerobic treatment of 20 g COD/*l* HSWBL recirculating 86% of the aerobic effluent. COD and BOD removal was 72% and 97%, respectively, after anaerobic-aerobic treatment at an overall loading rate of 3.6 g COD/*l*·d, while 30-35% of the incoming COD was recovered as methane. Batch-assays demonstrated significant detoxification after anaerobic-aerobic treatment of HSWBL. Treatment efficiencies and detoxification during anaerobic-aerobic and aerobic treatment were similar. However, the anaerobic-aerobic treatment system provided 50% lower surplus sludge production, production of 0.16 $m^3$ methane/kg $COD_{removed}$ as an energy source, less nutrient dosage and substantial reductions in aeration costs.

During anaerobic-aerobic treatment as well as aerobic treatment significant lignin removal was obtained, ranging from 28-58%. Lignin removal could be attributed to biodegradation of low molecular weight lignin (MW<2.2 kD). The lignin which survived biological treatment was extensively polymerized to MW of >34 kD.

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## Introduction

Anaerobic wastewater treatment technology has been successfully applied for the treatment of non-toxic and highly biodegradable pulp and paper industry effluents, like mechanical pulping effluents, paper recycling wastewaters and evaporator condensates. The development of upflow anaerobic sludge blanket (UASB) reactors (Lettinga *et al.*, 1980), which are characterized by high sludge retention, due to the formation of highly settleable sludge aggregates, enables anaerobic treatment of concentrated effluents at high volumetric loading rates. Major benefits of anaerobic treatment over aerobic treatment are the significantly lower energy consumption, since no energy is required for aeration, production of methane as a useful byproduct and the low surplus sludge production. The slow growth rate of methanogens however, can also be regarded as a drawback, since it makes anaerobic treatment susceptible for toxic shocks, due to the long recovery periods required. A broader application of anaerobic technology for the treatment of more complex forest industry wastewaters, like chemothermomechanical pulping (CTMP), semichemical pulping effluents and black liquors, is restricted due to toxicity problems and the poor biodegradability of those effluents (Lettinga *et al.*, 1991).

In this study, we describe the anaerobic-aerobic treatment of black liquor derived from the chemical pulping of hemp (*Cannabis sativa* L.) stem wood. Hemp is a non-woody fibre, which recently has been reintroduced in Dutch arable land farming as a fibre crop for paper production. Hemp black liquors (HSWBL's) are highly toxic wastewaters, which are just as toxic as those derived from woody feedstocks (Kortekaas *et al.*, 1994). The concentration resulting in a 50% inhibition of methanogenic activity ranges for hemp and wood derived black liquors from 2 to 7 g COD/*l* (Sierra-Alvarez *et al.*, 1991; Kortekaas *et al.*, 1995). The apolar extractive fraction in hemp black liquors as well as wood pulps was identified as the major source of methanogenic toxicity (Sierra-Alvarez *et al.*, 1991; Kortekaas *et al.*, 1995). Resinous compounds, which are released from the fibre feedstock under alkaline pulping conditions, are important constituents of the apolar extractive fraction in black liquors. The resin fraction from wood as well as non-woody fibres, has been demonstrated to be highly inhibitory to methanogenic bacteria (Kortekaas *et al.*, 1995; Sierra-Alvarez and Lettinga, 1990). Hemp resin contains many classes of compounds (Turner *et al.*, 1980), among which apolar phenols, terpenes and terpenols are expected to be the main toxic compounds, causing 50% inhibition of methanogenic bacteria at concentrations ranging from 20-330 mg/*l* (Sierra-Alvarez and Lettinga, 1990). In addition to the inhibition of methanogenic bacteria, resinous

compounds display severe toxicity to fish and other aquatic organisms (Leach and Thakore, 1976).

Various detoxification methods have been developed to decrease the toxicity of the extractive fraction towards methanogenic bacteria during the anaerobic treatment of forest industry wastewaters. Several precipitation methods, which aim to remove the extractive fraction by addition of divalent metal salts or acids, proved to be effective in the detoxification of CTMP effluents and black liquors (Sierra-Alvarez *et al.*, 1991; Welander, 1988; Stephenson and Duff, 1996). However, a disadvantage of the precipitation methods is that the precipitates form a light poorly settleable sludge which has to be separated from the wastewater and disposed of. The observation that resin acids were predominantly adsorbed to fine suspended matter in the wastewater, has led to the development of a detoxification method where the fines are removed prior to anaerobic treatment by sedimentation or dissolved air flotation (Kennedy *et al.*, 1992; Richardson *et al.*, 1991). An additional effect of the fines removal was the improvement of the methanogenic sludge settleability, due to the decreased supply of suspended solids.

In previous works, we have observed a strong elimination of methanogenic toxicity of hemp black liquor after sequenced anaerobic-aerobic treatment (Kortekaas *et al.*, 1994), in which the major detoxification was accomplished during aerobic post-treatment. Several studies show effective elimination of aquatic toxicity during aerobic treatment of different forest industry wastewaters (Servizi and Gordon, 1986; Wilson *et al.*, 1987). The detoxification during aerobic treatment, can be attributed to the removal of resinous compounds during aerobic treatment (Easty *et al.*, 1978; Leach *et al.*, 1978); whereas, the removal of resinous compounds during anaerobic treatment was rather poor (Sierra-Alvarez *et al.*, 1990a; Liver and Hall, 1996).

Generally, anaerobic treatment is followed by an aerobic post-treatment polishing step (Zitomer and Speece, 1993). The strong reduction of methanogenic toxicity obtained during aerobic treatment of pulping effluents enables the utilization of aerobically detoxified effluent as a means of diluting an inhibitory influent to sub-toxic concentrations. In previous research, we demonstrated that dilution is a suitable tool to combat methanogenic toxicity in black liquors (Kortekaas *et al.*, 1994). However in practice, dilution is difficult to realize, since black liquors are concentrated wastewaters and other waste streams of the paper mill will be insufficient to obtain proper detoxification. Utilization of ground or surface water requires heating up to mesophyllic temperatures and it is also politically sensitive to use communal water resources to dilute wastewater. The recirculation of effluent from the aerobic post-

treatment for upfront dilution of toxic pulping liquors prior to anaerobic treatment, enables detoxification without the above mentioned drawbacks. This detoxification method, firstly described by Habets & de Vegt (1991) for the treatment of CTMP effluent, is denominated as the upfront dilution method.

Aside from the toxicity problems, the biological treatment of black liquors is hampered by the poor biodegradability of these wastewaters. The biggest organic fraction in black liquors is lignin, which may account up to 40-60% of the wastewater COD (Sierra-Alvarez *et al.*, 1991; Anonymous, 1986). Lignin, a heterogenous three-dimensional biopolymer composed of phenylpropane units (Fengel and Wegener, 1984), is poorly biodegradable and only partly removed during anaerobic as well as aerobic wastewater treatment (Sierra-Alvarez *et al.*, 1990a; Walden, 1980; Colberg and Young, 1985). The lignin fraction which survives biological treatment gives rise to recalcitrant COD and high color levels in the final discharged effluent.

In the present study, we describe the continuous anaerobic-aerobic treatment of toxic hemp stem wood black liquor (HSWBL) with recirculation of aerobic effluent for upfront dilution. The performance during anaerobic-aerobic treatment of HSWBL with upfront dilution was compared to anaerobic treatment only and aerobic treatment only. The objective of this research was to demonstrate the feasibility of the upfront dilution method for detoxifying HSWBL, enabling anaerobic treatment. Additionally, the removal and modifications of lignin and during anaerobic and aerobic treatment steps were evaluated.

## Materials and Methods

### Experimental Set-Up

Research on the anaerobic-aerobic treatment of hemp stem wood black liquor with upfront dilution experiment II & III) was compared with two control experiments, considering anaerobic treatment without upfront dilution (experiment I) and aerobic treatment only (experiment IV).

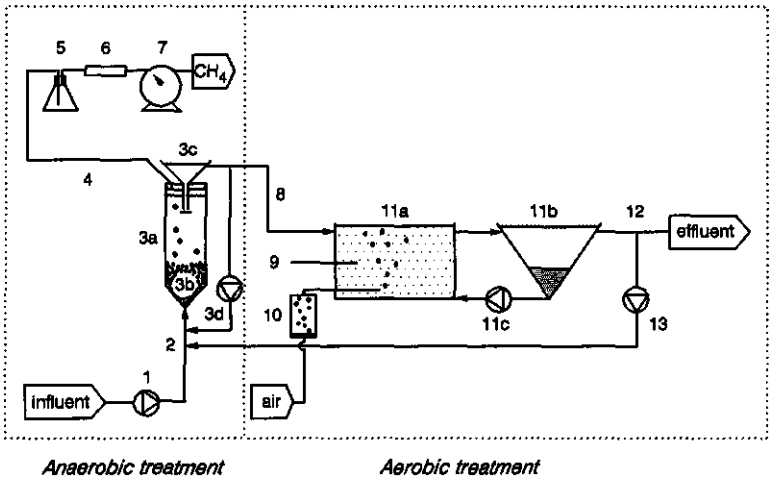


**Figure 1.** Flow sheet of the experimental set-up for anaerobic-aerobic treatment with recirculation of aerobic effluent for upfront dilution (experiment II and III). 1) incoming influent; 2) dilution of incoming influent with recirculated effluent from aerobic post-treatment; 3a) UASB reactor; 3b) anaerobic sludge bed; 3c) gas-solids-separator; 3d) internal recirculation; 4) biogas; 5) NaOH-solution; 6) column with soda-lime pellets; 7) wet-test gas meter; 8) effluent from anaerobic treatment; 9) pH-controller; 10) temperature controlled bubble column to moisturize air supplied to aeration tank (was not installed during experiment II); 11a) aeration tank; 11b) settler; 11c) sludge recycle; 12) effluent from aerobic post-treatment; 13) recirculation of effluent from aerobic post-treatment.

The reactor set-up for anaerobic-aerobic treatment with upfront dilution is shown in Figure 1. The set-up consists of a UASB reactor followed by an aerobic activated sludge reactor. The liquid volume of the UASB was 2 *l*. The liquid volume of the aerobic reactor (AR) in experiment II was 10 *l* and in experiment III was 3.3 *l*. The UASB reactor had an internal recirculation loop to increase the superficial velocity in the UASB to 0.94 m/h. Biogas was led through a 10% NaOH solution and a column filled with soda-lime pellets, after which methane was measured in a wet-test gas meter. In experiment III, air was saturated with $H_2O$ in a temperature controlled bubble column prior to supplementation to the aeration tank. The oxygen concentration in the aeration tank ranged between 2-4 mg/*l*. The pH in the aeration tank was regulated by addition of HCl by a pH controller with a set-point of 8.4. Once a week, the surplus sludge was removed from the aeration tank. Effluent from the aerobic post-treatment was partly recirculated for upfront dilution of the influent fed to the UASB reactor. The recirculation rate was defined as the fraction of aerobic effluent, which was recirculated to dilute the incoming black liquor; recirculation rate =

$100 \cdot Vol_{rae}/(Vol_{rae}+Vol_{in})$, where as $Vol_{rae}$ = volume of recirculated aerobic effluent and $Vol_{in}$ = volume influent. The following parameters were used to indicate the performance of the UASB reactors. $COD_{in}$ = influent COD (untreated) fed to the treatment system; M = methanogenesis, % conversion $COD_{in}$ to methane; VFA = volatile fatty acids remaining in the effluent of the UASB reactor as a percentage of $COD_{in}$; A = acidification, % conversion $COD_{in}$ to volatile fatty acids and methane (A = M + VFA); OLR = organic loading rate (g COD/*l*·d). Treatment efficiencies attributed to anaerobic step were calculated as follows: $100 \cdot (Vol_{in} \cdot Conc_{in} + Vol_{rae} \cdot Conc_{rae} - Vol_{an} \cdot Conc_{an})/(Vol_{in} \cdot Conc_{in})$, in which: $Conc_{in}$ = concentration influent; $Conc_{an}$ = concentration anaerobic effluent; $Conc_{rae}$ = concentration recirculated aerobic effluent.

In experiment I, continuous anaerobic treatment of HSWBL without upfront dilution was performed in a glass upflow anaerobic sludge blanket reactor (UASB) with a liquid volume of 0.11 *l*. The methane production was measured with 10 *l* Mariotte flask filled with 3% (w/v) NaOH solution to remove carbon dioxide from the biogas.

The operation and design of the aerobic activated sludge reactor applied in experiment IV was the same as that used for the aerobic post-treatment applied in experiment III.

**Biomass**

Methanogenic granular sludge was utilized to seed the UASB reactors and to perform anaerobic toxicity assays. Two types of methanogenic sludge were used:

- Methanogenic sludge adapted to HSWBL, cultivated in UASB reactor fed with 2 g COD/*l* HSWBL for over 10 months (Kortekaas *et al.*, 1994).
- Methanogenic sludge unadapted to HSWBL, obtained from full-scale anaerobic reactor treating wheat starch wastewater (Latenstein, Nijmegen, The Netherlands).

Activated sludge inoculum was obtained from a full-scale activated sludge plant treating thermomechanical pulping (TMP) effluent (Parenco Newsprint, Renkum, The Netherlands).

**Start-up**

The UASB reactors in experiment I and II were seeded with methanogenic sludge (approximately 20 g VSS/*l*) adapted to HSWBL. The aerobic reactor in experiment II was seeded with activated sludge (4 g VSS/*l*). During a one week start-up period, the reactors

were fed HSWBL at sub-toxic concentrations (2 g COD/*l*). Subsequently, the feeding of 12 g COD/*l* HSWBL was started. The UASB reactor and the aerobic reactor in experiment III were seeded with respectively, methanogenic sludge and activated sludge, obtained from experiment II. In experiment IV, the aerobic reactor, seeded with activated sludge (4 g VSS/*l*), was fed during a one week start-up period 2 g COD/*l* HSWBL, after which the influent concentration was increased to 20 g COD/*l*.

**Preparation of Influent**

Black liquors were prepared from hemp fibre (*Cannabis sativa* L., cultivar Kompolti Hybrid TC), grown in The Netherlands by DLO Research Institute for Agrobiology and Soil Fertility, Wageningen. The hemp stem was mechanically divided into a hemp bark and a hemp stem wood fraction from which the stem wood fraction was utilized for chemical pulping. Per litre of tap water 20 g NaOH and 100 g of dry hemp stem wood fibre was added. The mixture was cooked at 165 °C for a period of 30 minutes. Typically, the stem wood black liquor showed a concentration of 55 g COD/*l*; $BOD_5$/COD = 30%; pH = 12.5; $UV_{280}$ = 321 absorbtion units 1x,1cm and $VIS_{440}$ = 20.6 absorbtion units 1x,1cm. The lignin content of untreated HSWBL accounted for 50% of the wastewater COD and was estimated from the ultraviolet absorbence at 280 nm, using an absorbance coefficient of 20.6 *l*/g·cm (Kortekaas *et al.*, 1994).
Influent was prepared by diluting black liquors with demineralized water and neutralization with HCl to approximately 7.5. The supplementation of macro- and micro-nutrients was related to the influent concentration. For each 5 g COD/*l* HSWBL one dosage of nutrients (described under batch assays) was supplied. No buffer was added due to the alkaline nature of the wastewater. 0.1 g/*l* Baker's Antifoam B silicone emulsion (Boom B.V., Meppel, The Netherlands) was used to prevent foam problems.

**Batch Assays**

All assays contained essential inorganic macro- and micro-nutrients (mg/*l*): $NH_4Cl$ (280), $CaCl_2 \cdot 2H_2O$ (10), $K_2HPO_4$ (250), $MgSO_4 \cdot 7H_2O$ (100), yeast extract (100), $H_3BO_4$ (0.05), $FeCl_2 \cdot 4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2 \cdot 4H_2O$ (0.05), $(NH_4)6Mo_7O_{24} \cdot 4H_2O$ (0.05),

$AlCl_3 \cdot 6H_2O$ (0.09), $CoCl_2 \cdot 6H_2O$ (2), $NiCl_2 \cdot 6H_2O$ (0.05), $CuCl_2 \cdot 2H_2O$ (0.03), $Na_2SeO_3 \cdot 5H_2O$ (0.1), EDTA (1), resazurin (0.2) and 36% HCl (0.001 ml/*l*). The methanogenic activity and toxicity assays were conducted in 0.315 *l* glass serum flasks, sealed with a rubber septum and a screw cap. Before incubation the headspace of the flasks was flushed with a gas mixture containing 70% $N_2$ and 30% $CO_2$, and $Na_2S$ (39 mg/*l*) was added to remove oxygen. The bottles were placed on a reciprocating shaker in a temperature controlled room at 30 ± 2°C.

*Methanogenic activity assay.* Anaerobic sludge (1.5 g VSS/*l*) was transferred from the UASB to the serum flask containing 0.05 *l* medium, which consisted of nutrients, $NaHCO_3$ (4 g/*l*) and neutralized acetate (2.5 g COD/*l*). After 24 h incubation the headspace was flushed with the gas mixture and the accumulation of methane in the headspace was determined during a period of 3-6 h. The specific acetoclastic activity was calculated from the slope of the methane production versus time curve and the quantity of VSS.

*Methanogenic toxicity assay.* The methanogenic toxicity assay was conducted in a similar fashion as the methanogenic activity assay. The anaerobic sludge (1.5 g VSS/*l*) was transferred to a serum flask containing 0.05 *l* medium, which consisted of nutrients, $NaHCO_3$ (8 g/*l*), neutralized acetate (2 g COD/*l*) and wastewater. The wastewater concentrations supplied were chosen to provide an inhibition of methanogenic activity ranging from 0% to 100%. Substrate controls were based on assays were no wastewater was added. After 7 days of incubation, the assays were provided with a second feeding lacking the wastewater; this was done in order to determine the residual activity of the sludge after exposure to the wastewater. The supernatant was carefully decanted while purging with $N_2$ gas, and replaced with a medium containing nutrients, $NaHCO_3$ (4 g/*l*) and neutralized acetate (2 g COD/*l*). After 24 h incubation, the headspace was flushed with the gas mixture and the accumulation of methane in the headspace was determined during a period of 3-6 h. The inhibited activity was expressed as a percentage of the control activity. The wastewater concentration that caused 50% inhibition of the acetoclastic activity is referred to as "50% IC". To perform anaerobic toxicity assays with influent and effluent of anaerobic-aerobic treatment with upfront dilution, concentration of the wastewater was required. The wastewater was concentrated by evaporating the wastewater under a constant flow of $N_2$. Control samples, which had not been concentrated, showed that concentration of the wastewater samples, had only minor effect on the toxicity observed.

## Analytical Methods

Wastewater samples were analyzed for COD (the micro method with dichromate), $BOD_5$, MLVSS, VSS, TSS according to American Public Health Association standard methods (APHA, 1989). Samples for the determination of COD, $VIS_{440}$ and $UV_{280}$ were membrane filtered (0.45 $\mu$m). $VIS_{440}$ and $UV_{280}$ were determined in a 1 cm quartz cuvette by diluting the samples to less than 0.8 absorbance units in a 0.02 M borate buffer providing a pH 9.1. VFA were analyzed by gas chromatography using a Hewlett Packard 5890 equipped with 2 m x 4 mm glass column packed with Supelcoport (100-120 mesh) coated with 10% Fluorad FC 431. The temperatures of the column, the injection port and the flame ionization detector were 130, 200, 280 °C, respectively. The carrier gas was nitrogen saturated with formic acid (30 m*l*/min).

Methane was determined by gas chromatography with a Hewlett Packard 589/S gas chromatograph (Delft, The Netherlands). The 2 m * 2 mm steel column was packed with Poropak Q (80-100 mesh). The temperatures of the column, injection port, and flame ionization detector were 60, 200 and 220 °C, respectively. Nitrogen was the carrier gas at a flow rate of 20 m*l*/min. Injection volume was 100 $\mu l$.

Ultrafiltration experiments were conducted at room temperature using a 50 m*l* stirred cell (Amicon model 8050) with an exposed membrane surface area of 12.6 $cm^2$. The regenerated cellulose membranes were YM100 and YM10 (Amicon, Capelle aan de Ijssel, The Netherlands), which nominal molecular weight cut-off (MWCO) values for ultrafiltration of kraft lignin were 34 kD and 2.2 kD, respectively (Li *et al.*, 1996). Nitrogen was applied over the liquid in the stirred cell. Operating pressures were 70 and 360 kPa for YM100 and YM10, respectively. Prior to ultrafiltration, the pH of the wastewater was adjusted to 10.5 and subsequently the sample was filtered through a 0.2 $\mu$m membrane filter. To fractionate the lignin, 40 m*l* of wastewater sample was filtered through a YM100 membrane to give approximately 37 m*l* of permeate and 3 m*l* of retentate. This filtration procedure was repeated twice after addition of 50 m*l* of $NaHCO_3$ buffer solution (0.025 M, pH 10.5) to the retentate. Subsequently, the permeates were pooled and filtered through a YM10 membrane to give a retentate of 3 m*l*. This filtration procedure was repeated twice after addition of 50 m*l* of $NaHCO_3$ buffer solution to the retentate. After ultrafiltration the following three UF samples were obtained: a YM100 retentate sample, MW > 34 kD, denominated as > YM100; a sample which passed by the YM100 membrane, but was rejected by the YM10 membrane, 2.2 kD < MW < 34 kD, denominated as YM100-YM10; and a YM10 permeate sample,

MW < 2.2 kD, denominated as < YM10.

The molecular weight distribution of black liquor lignins was determined by gel filtration chromatography on a standard chromatography Hiload™ system (Pharmacia LKB, Roosendaal, The Netherlands) equipped with a XK16 column (1.6 x 90 cm) packed with Sephacryl S-100 (Pharmacia Biotech). A 500 $\mu l$ membrane filtered (0.45 $\mu$m) wastewater sample was applied to the column and eluted with a $NaHCO_3$ (0.025 M)-NaOH buffer solution (pH 10.5) containing 0.5 g/*l* of polyethylene glycol (MW 6000) (Pellinen and Salkinoja-Salonen, 1985). Void and retention volume of the gel filtration column, determined with Blue dextran (Pharmacia Biotech) and guaiacol, was 74 m*l* and 190 m*l*, respectively.

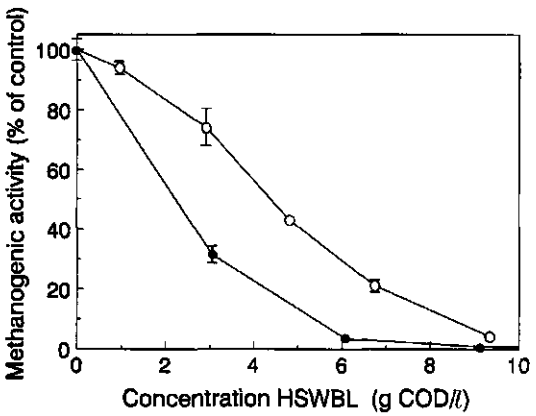


**Figure 2.** Inhibition of acetoclastic activity in unadapted sludge and adapted granular sludge exposed to increasing concentrations of hemp stem wood black liquor. ○) activity of unadapted methanogenic sludge, cultivated in a full-scale UASB treating starch manufacturing wastewater. The control activity was 0.88 g COD/g VSS·d. •) activity of adapted methanogenic sludge, cultivated in a laboratory scale UASB fed with HSWBL at sub-toxic concentrations. The control methanogenic activity of adapted sludge was 0.61 g COD/g VSS·d. Bars represent standard deviation.

## Results

### Methanogenic toxicity of hemp stem wood black liquor

Hemp stem wood black liquor (HSWBL) was assayed for it's toxicity to acetoclastic methanogenic bacteria in unadapted sludge obtained from a full-scale UASB reactor treating starch manufacturing wastewater. Figure 2 indicates that the methanogenic toxicity of HSWBL was high. The HSWBL concentration causing 50% inhibition of acetoclastic activity (50%IC) was 4.5 g COD/*l*. In order to evaluate the adaptation potential of methanogenic bacteria to the toxicity exerted by HSWBL, sludge obtained from a UASB reactor treating 2 g COD/*l* HSWBL for over 10 months, was also tested. As can be seen in Figure 2, adapted sludge was inhibited at even lower HSWBL concentrations (*50%IC* = 2.2 g COD/*l*) than the unadapted sludge. The results indicate that long term anaerobic treatment of HSWBL at sub-toxic concentrations, did not increase it's tolerance to the inhibitory compounds in HSWBL. On the contrary, the susceptibility of methanogenic bacteria to the toxicity of HSWBL increased to a small extent.

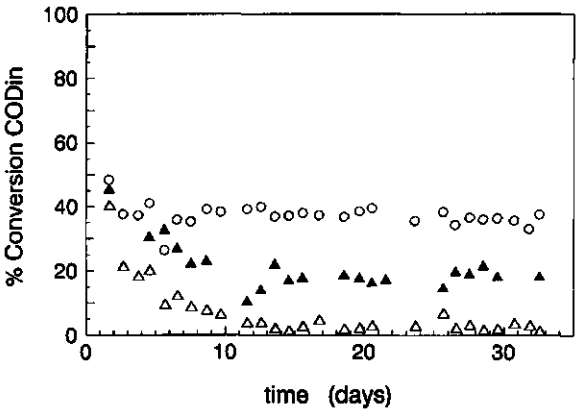


**Figure 3.** Performance of UASB reactor during anaerobic treatment of 12 g COD/*l* hemp stem wood black liquor without upfront dilution (experiment I). △) Methanogenesis, % $COD_{in}$; ○) Acidification, % $COD_{in}$; ▲) COD removal, % $COD_{in}$; average OLR: 13.4 g COD/*l*·d.

**Table 1.** Operational parameters and average treatment efficiency during anaerobic treatment of HSWBL with and without recirculation of aerobic effluent for upfront dilution.

| | | attributed to anaerobic treatment[a] | | |
|---|---|---|---|---|
| | | Experiment I without upfront dilution[b] | Experiment II with upfront dilution | |
| | | | Period I[c] | Period II[d] |
| Operational parameters: | | | | |
| $COD_{in}$ | (g COD/*l*) | 11.9 | 11.7 | 11.7 |
| OLR | (g COD/*l*·d) | 13.0 | 13.0 | 21.5 |
| Recirculation rate[e] | (%) | 0 | 75 | 75 |
| Removal efficiency: | | | | |
| COD | (%) | 18.2 | 40.9 | 42.6 |
| Methanogenesis | (%) | 2.5 | 35.3 | 31.6 |
| Acidification | (%) | 36.8 | 36.8 | 35.2 |
| $BOD_5$ | (%) | ND[f] | 83.2 | 81.1 |

[a] share attributed to anaerobic treatment: $100 \cdot (Vol_{in} \cdot Conc_{in} + Vol_{rae} \cdot Conc_{ae} - Vol_{an} \cdot Conc_{an}) / (Vol_{in} \cdot Conc_{in})$.

[b] anaerobic treatment without upfront dilution: day 13-34.

[c] anaerobic treatment with upfront dilution, period I: day 28-53.

[d] anaerobic treatment with upfront dilution, period II: day 53-74.

[e] recirculation rate: $100 \cdot Vol_{rae} / (Vol_{rae} + Vol_{in})$.

[f] ND, not determined.

**Feasibility of upfront dilution for detoxifying hemp stem wood black liquor**

The feasibility of detoxifying HSWBL by an aerobic post-treatment step was studied by comparing the performance of UASB reactors fed 12 g COD/*l* HSWBL with and without upfront dilution. One UASB reactor was fed 12 g COD/*l* HSWBL directly (experiment I) and another UASB reactor was fed the same influent, but diluted upfront with effluent from the aerobic post-treatment step (experiment II). Figure 3 shows the performance of the UASB reactor fed undiluted influent. The high methanogenic toxicity of 12 g COD/*l* HSWBL is evident from the strong decrease in the conversion of influent COD to methane, which was observed immediately after introducing the undiluted influent (day 0). Within 14 days, the methane production became negligible, indicating an almost complete inhibition of

methanogenic bacterial activity. In contrast, the acidification was not influenced and was stable at approximately 37% of the $COD_{in}$ (Table 1). As a consequence, VFA accumulated in the effluent of the UASB and the COD removal was rather poor (approximately 18%). The COD removed is probably attributable to the cell yield of fermentative bacteria. The severe methanogenic toxicity of 12 g COD/*l* HSWBL is also evident from the extremely low specific acetoclastic activity (< 0.01 g COD/g VSS·d) of the sludge sampled from the UASB reactor on day 34 (Table 2).

**Table 2.** The sludge concentration and specific methanogenic activity during continuous operation of UASB-reactors fed with hemp stem wood black liquor (12 g COD/*l*) with and without recirculation of aerobic effluent for upfront dilution.

| Day | experiment I without upfront dilution | | experiment II with upfront dilution[a] | |
|---|---|---|---|---|
| | VSS (g/*l*) | Methanogenic activity (g COD/g VSS·d) | VSS (g/*l*) | Methanogenic Activity (g COD/g VSS·d) |
| start-up | 20.0 | 0.40 | 18.6 | 0.42 |
| 34 | 24.5 | < 0.01 | ND[b] | ND |
| 74 | ND | ND | 29.0 | 0.57 |

[a] Recirculation rate: $100 \cdot Vol_{rac}/(Vol_{rac} + Vol_{in}) = 75\%$.

[b] ND, not determined.

In experiment II, 75% of the aerobic effluent was recirculated for upfront dilution of the incoming 12 g COD/*l* HSWBL influent prior to feeding to the UASB reactor. A 4 week transition period was needed to reach steady treatment conditions, in order to allow the dilution water of the reactor system on day 0 to become replaced by treated wastewater which originated from the feeding of 12 g COD/*l* HSWBL. Results of the UASB reactor performance are shown in Figure 4, starting on day 28. Methane production was stable for the entire study period of 74 days. Table 1 shows that the fraction fermentable substrates in HSWBL (approximately 36% of $COD_{in}$) was converted for the major part to methane (32-35% of $COD_{in}$), indicating effective reduction of methanogenic toxicity by the upfront dilution method. The absence of methanogenic inhibition is also evident by comparing the

specific methanogenic activity of the anaerobic sludge from the reactors in experiment I and II. The sludge sampled from the UASB of experiment II, which had been exposed to HSWBL upfront diluted with aerobic effluent, had a high methanogenic activity (0.57 g COD/g VSS·d), which was even better than the activity at reactor start-up. On the other hand, the sludge sampled from the UASB of experiment I, which had been exposed directly to HSWBL, had essentially no methanogenic activity.

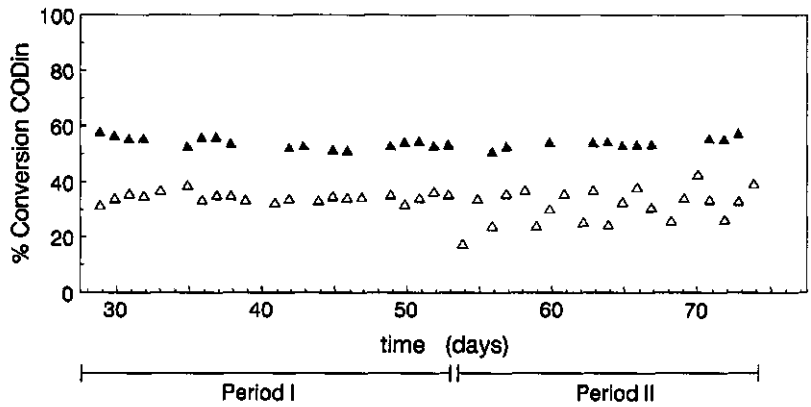


**Figure 4.** Performance during anaerobic-aerobic treatment of 12 g COD/*l* hemp stem wood black liquor with upfront dilution (experiment II). 75% of the aerobic effluent was recirculated for dilution of the 12 g COD/*l* influent prior to feeding to the UASB. ▲) COD removal during anaerobic-aerobic treatment uncorrected for evaporation losses, % $COD_{in}$; △) Methanogenesis, % $COD_{in}$; Period I: day 28 - 53, average OLR = 13.0 g COD/*l*·d. Period II: day 53 - 74, average OLR = 21.5 g COD/*l*·d.

The share of anaerobic treatment in the overall treatment efficiency was calculated from the mass flow which entered the UASB reactor (influent and recirculated aerobic effluent) and the mass flow which left the UASB reactor (anaerobic effluent). Treatment efficiencies attributed to the anaerobic step are expressed as a fraction of the undiluted influent. In experiment II, two periods can be distinguished with average OLR's in the anaerobic step of 13 and 21.5 g COD/$l_{UASB}$·d. Treatment efficiencies are summarized in Table 1. The COD removal attributed to anaerobic treatment was in both periods approximately 42% and the BOD removal attributed to anaerobic treatment was 81-83%. The recirculation of aerobic effluent for upfront dilution of the toxic HSWBL, significantly increased the COD removal efficiency attributed to anaerobic treatment from 18% in experiment I to 43% in experiment II. During experiment II, it was observed that evaporation losses during aerobic post-

treatment caused increased aerobic effluent concentrations and an underestimation of the overall treatment efficiency. The evaporation losses however, did not affect the removal efficiency attributed to the anaerobic step. The overall COD removal in experiment II, uncorrected for evaporation losses, accomplished by anaerobic-aerobic treatment, was approximately 54% and was accompanied by an almost complete BOD removal (> 99%).

**Anaerobic-aerobic treatment of highly concentrated hemp stem wood black liquor with upfront dilution**

The upfront dilution method was evaluated for the treatment of even more toxic black liquors in experiment III, utilizing an influent concentration of 20 g COD/*l* HSWBL. The experimental set-up was slightly modified compared to experiment II. A humidifier was installed in the air supply of the aerobic post-treatment to minimize evaporation losses, and the volume of the aerobic reactor was decreased to 3.3 *l*. Additionally, the recirculation of the aerobic effluent for upfront dilution was increased to 86%.

Due to the increased influent concentration, a transition period of 50 days was required before steady state treatment conditions were achieved. The treatment efficiencies achieved during anaerobic-aerobic treatment of 20 g COD/*l* HSWBL with upfront dilution are shown in Figure 5. Up to day 85, stable treatment efficiencies were obtained. During this initial period (period I), the average COD removal efficiency after anaerobic-aerobic treatment was 73.8% (Table 3). The anaerobic reactor was responsible for 46.6% removal of COD, and 34.0% of the incoming COD was recovered as methane. Indicating that the methanogenesis proceeded relatively well, despite the increased influent concentration. The specific acetoclastic activity of the sludge in the UASB reactor however, showed some decrease to 0.39 g COD/g VSS·d on day 71, which was reflected in a small increase of the volatile fatty acids concentration in the effluent of the UASB to 230 mg COD/*l*. The BOD removal attributed to anaerobic treatment was 67%.

On day 86, the methane production of the UASB reactor suddenly decreased dramatically. The origin of this failure was due to high sludge concentrations in the aerobic post-treatment reactor, which caused washout of aerobic sludge. It was observed that the flocculent aerobic sludge entered into the UASB reactor, resulting in a gradual replacement of the methanogenic biomass. The washout of methanogenic sludge is evident from the very low acetoclastic activity of 0.12 g COD/g VSS·d and the low sludge concentration in the UASB reactor

which accounted for only 10.5 g VSS/*l* on day 89. The washout of methanogenic sludge was confirmed by the unusually high methanogenic activity of the aerobic sludge (0.06 g COD/g VSS·d).

On day 89, the settler was modified in order to improve the removal of surplus sludge from the aerobic treatment, preventing transport of aerobic sludge to the UASB reactor. The organic loading rate of the UASB reactor however, was unchanged. The first two weeks after the accidental washout of methanogenic sludge, the performance of the UASB was rather poor. The average methanogenesis accounted for only 12% of the incoming COD. Volatile fatty acids accumulated in the effluent of the UASB reactor up to 800 mg COD/*l*, while the COD removal attributed to anaerobic treatment dropped to below 30%. The overall COD removal in this period, however, was 68%, indicating almost complete compensation of the decrease in anaerobic removal efficiency by the aerobic post-treatment.

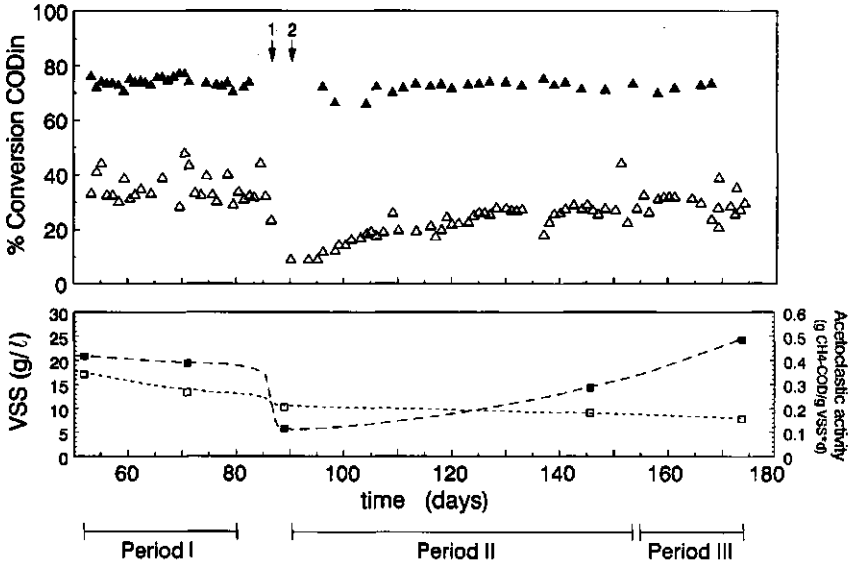


**Figure 5.** Treatment efficiencies during anaerobic-aerobic treatment of 20 g COD/*l* hemp stem wood black liquor with upfront dilution (experiment III). The recirculation rate was 86%. ▲) COD removal after anaerobic-aerobic treatment, % $COD_{in}$; △) Methanogenesis, % $COD_{in}$; -□-) sludge concentration in UASB, g VSS/*l*; —■—) specific acetoclastic activity, g $CH_4$-COD/g VSS·d. Period I: day 52-80, average OLR: 9.5 g $COD/l_{UASB}$·d; Period II: day 89-154, average OLR: 9.3 g $COD/l_{UASB}$·d; Period III: day 155-174, average OLR: 9.5 g $COD/l_{UASB}$·d. Arrow 1 indicates accidental washout of activated sludge from the aerobic post-treatment, which was recirculated to the UASB. Arrow 2 indicates modification of the settler of the aerobic post-treatment to prevent washout of aerobic sludge.

**Table 3.** Operational parameters and average treatment efficiency during anaerobic-aerobic treatment of hemp stem wood black liquor with recirculation of aerobic effluent for upfront dilution (experiment III).

| | | Period I[a] | | Period III[b] | |
|---|---|---|---|---|---|
| | | attributed to anaerobic treatment[c] | complete treatment (anaerobic + aerobic) | attributed to anaerobic treatment | complete treatment (anaerobic + aerobic) |
| Operational parameters: | | | | | |
| $COD_{in}$ | (g COD/*l*) | 19.3 | | 19.7 | |
| OLR | (g COD/*l*·d) | 9.5 | 3.6 | 9.5 | 3.6 |
| Recirculation rate[d] | (%) | 86 | | 86 | |
| Removal efficiency: | | | | | |
| COD | (%) | 46.6 | 73.8 | 49.3 | 71.9 |
| Methanogenesis | (%) | 34.0 | | 29.3 | |
| Acidification | (%) | 42.6 | | 35.6 | |
| $BOD_5$ | (%) | 67.2 | 96.7 | ND[e] | ND |
| $UV_{280}$ | (%) | 9.0 | 43.4 | 21.0 | 39.5 |
| $VIS_{440}$ | (%) | NA[f] | -11.8[g] | NA | -15.7 |

[a] Period I: day 52-80.
[b] Period III: day 155-174.
[c] Share attributed to anaerobic treatment: $100 \cdot (Vol_{in} \cdot Conc_{in} + Vol_{rae} \cdot Conc_{rae} - Vol_{an} \cdot Conc_{an})/(Vol_{in} \cdot Conc_{in})$.
[d] Recirculation rate: $100 \cdot Vol_{rae}/(Vol_{rae} + Vol_{in})$.
[e] ND, not determined.
[f] NA, not applicable.
[g] Negative values indicate increase of color.

As can be seen in Figure 5, during period II a steady recovery of the methanogenic activity of the UASB reactor was obtained. Methanogenesis gradually improved and an increase of the specific acetoclastic activity of the sludge in the UASB was also observed. The concentration volatile fatty acids in the effluent of the UASB decreased to below 250 mg COD/*l* after day 140, which resulted in 54% BOD removal after anaerobic treatment and 99% after anaerobic-aerobic treatment. The recovery of the UASB reactor is evident from the high specific acetoclastic activity of the sludge (0.49 g COD/g VSS·d) determined on day 174. Despite the fact that the sludge concentration in the UASB at the end of period III was still relatively low (8.1 g VSS/*l*), excellent anaerobic removal efficiencies were obtained. The concentration volatile fatty acids in the effluent of the UASB reactor decreased further to 161 mg COD/*l*, while the COD removal attributed to the anaerobic treatment step increased to 49% and the fraction of incoming COD converted to methane increased to 29%.

In Table 3 the treatment efficiencies of the combined anaerobic-aerobic treatment during experiment III are shown. The overall COD removal efficiency after anaerobic-aerobic treatment averaged 72%, with 48% COD removal being attributable to the anaerobic treatment step. Methane production accounted for 30-35% of the incoming COD, which is equal to 59-72% of the COD removed during anaerobic treatment. Surplus sludge production during the anaerobic and aerobic treatment step accounted for 0.04 and 0.18 g VSS/g $COD_{removed}$, respectively, with an average of 0.09 g VSS/g $COD_{removed}$ over the complete treatment system.

**Aerobic treatment of hemp stem wood black liquor**

As a reference to the anaerobic-aerobic treatment of HSWBL with upfront dilution, the direct aerobic treatment of 20 g COD/*l* HSWBL in a laboratory scale activated sludge reactor was also studied (experiment IV) with a loading rate of approximately 3.7 g COD/*l*·d, which is comparable to the overall loading of the anaerobic-aerobic treatment in experiment III. Treatment efficiencies during aerobic treatment of HSWBL apparently were related to the concentration of activated sludge in the aerobic reactor. Table 4 shows an average COD removal of 78% obtained in period II, when the activated sludge concentration averaged 9.4 g MLVSS/*l*, while only approximately 62% COD removal was obtained in period I, when the sludge concentration averaged 6.2 g MLVSS/*l*. The COD and BOD removal efficiencies during aerobic treatment of HSWBL were comparable with the treatment efficiencies achieved during the anaerobic-aerobic treatment of HSWBL in experiment III, when an

average activated sludge concentration of approximately 8.0 g MLVSS was present in the aerobic post-treatment reactor. The surplus sludge production during aerobic treatment, 0.18 g VSS/g $COD_{removed}$, was twice as high as that produced during anaerobic-aerobic treatment.

**Table 4.** Operational parameters and average treatment efficiency during aerobic treatment of hemp stem wood black liquor (experiment IV).

| | | Period I[a] | Period II[b] |
|---|---|---|---|
| Operational parameters: | | | |
| $COD_{in}$ | (g COD/*l*) | 19.9 | 20.1 |
| OLR | (g COD/*l*·d) | 3.8 | 3.7 |
| sludge concentration | (g MLVSS/*l*) | 6.2 | 9.4 |
| Removal efficiency: | | | |
| COD | (%) | 61.7 | 77.6 |
| $BOD_5$ | (%) | ND[c] | 97.6 |
| $UV_{280}$ | (%) | 27.5 | 57.7 |
| $VIS_{440}$ | (%) | -5.8[d] | 38.8 |

[a] Period I: day 63-88.
[b] Period II: day 100-142.
[c] ND, not determined.
[d] Negative values indicate increase of color.

## Detoxification during anaerobic-aerobic and aerobic treatment of hemp stem wood black liquor

In order to illustrate the detoxification achieved during biological treatment, the methanogenic toxicity of untreated, anaerobic-aerobically treated (experiment III) and aerobically treated (experiment IV) HSWBL was assayed. Figure 6 clearly displays the decreased toxicity of HSWBL after anaerobic-aerobic and aerobic treatment. While untreated HSWBL exerted 50% inhibition at a concentration of 7.6 g COD/*l*, effluents from anaerobic-aerobic and aerobic treatment were only responsible for 30% inhibition of methanogenic activity at concentrations which correspond to a 23 g COD/*l* influent. The methanogenic inhibition of the untreated HSWBL in this experiment is somewhat less than that observed

in the experiment exhibited in Figure 2. The reason for this is attributed to a partial decrease in HSWBL toxicity, observed after prolonged storage times, due to autoxidation (Field *et al.*, unpublished data).

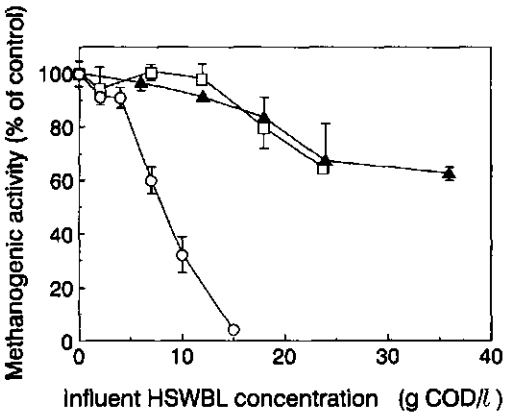


**Figure 6.** The acetoclastic methanogenic activity of Latenstein granular sludge exposed to untreated (○), anaerobic-aerobically treated (□) and aerobically treated (▲) hemp stem wood black liquor. The concentration of the effluent samples refers to the corresponding influent COD concentration. Effluents of anaerobic-aerobic treatment and aerobic treatment were concentrated by evaporation. Control assays with non concentrated samples (results not shown) demonstrated that concentrated samples were slightly less toxic than the original samples, but that the differences were negligible. Bars represent standard deviation.

## Lignin removal during anaerobic-aerobic and aerobic treatment of hemp stem wood black liquor

Lignin removal during biological treatment of black liquors is generally regarded to be low. In this research however, remarkably high lignin removal rates were obtained. Lignin removal after anaerobic-aerobic treatment, measured as $UV_{280}$ reduction, was approximately 40% (Table 3), of which up to half was obtained anaerobically. In contrast to the removal of $UV_{280}$, color levels increased after anaerobic-aerobic treatment. After aerobic treatment of HSWBL, average $UV_{280}$ removal efficiencies ranged between 28% and 51% (Table 4), better results were obtained when the activated sludge concentration was higher.

The effect of biological treatment on the molecular weight distribution of wastewater lignin was studied by a combination of ultrafiltration (UF) and gel permeation chromatography

(GPC) techniques. Untreated, anaerobic-aerobically treated and aerobically treated HSWBL's were fractionated by ultrafiltration. The characteristics of the UF samples are listed in Table 5 and the GPC profiles of the UF samples, indicating the molecular weight distribution of wastewater lignins, are shown in Figure 7 and Figure 8.

**Table 5.** Characteristics of by ultrafiltration fractionated samples of untreated, anaerobically-aerobically treated and aerobically treated hemp stem wood black liquor. COD, $UV_{280}$ and $VIS_{440}$ of the different UF samples is expressed as a percentage of the concentration in the untreated HSWBL.

| | | sample < YM10 | sample YM100-YM10 | sample > YM100 | total |
|---|---|---|---|---|---|
| untreated HSWBL[a,b] | | | | | |
| % $COD_{in}$ | (-) | 77 | 11 | 12 | 100 |
| % $UV_{280,in}$ | (-) | 63 | 17 | 20 | 100 |
| % $VIS_{440,in}$ | (-) | 53 | 23 | 24 | 100 |
| UV/COD | (-) | 4.28 | 7.84 | 8.81 | 6.29 |
| VIS/COD | (-) | 0.17 | 0.50 | 0.50 | 0.39 |
| VIS/UV | (-) | 0.040 | 0.064 | 0.057 | 0.062 |
| anaerobic-aerobically treated HSWBL | | | | | |
| % $COD_{in}$ | (-) | 10 | 4 | 10 | 25 |
| % $UV_{280,in}$ | (-) | 14 | 11 | 23 | 48 |
| % $VIS_{440,in}$ | (-) | 29 | 26 | 51 | 106 |
| UV/COD | (-) | 7.97 | 13.57 | 12.39 | 11.90 |
| VIS/COD | (-) | 0.98 | 2.05 | 1.70 | 1.64 |
| VIS/UV | (-) | 0.123 | 0.151 | 0.137 | 0.137 |
| aerobically treated HSWBL | | | | | |
| % $COD_{in}$ | (-) | 8 | 4 | 6 | 18 |
| % $UV_{280,in}$ | (-) | 11 | 7 | 11 | 29 |
| % $VIS_{440,in}$ | (-) | 15 | 13 | 19 | 47 |
| UV/COD | (-) | 7.26 | 10.80 | 10.97 | 10.34 |
| VIS/COD | (-) | 0.66 | 1.21 | 1.10 | 1.04 |
| VIS/UV | (-) | 0.089 | 0.112 | 0.100 | 0.101 |

[a] Untreated HSWBL: $COD_{in}$ = 20.4 g COD/*l*; $UV_{280,in}$ = 128.3 AU280(1x,1cm); $VIS_{440,in}$ = 7.9 AU440(1x,1cm).

[b] >YM100 and YM100-YM10 samples fractionated by ultrafiltration of untreated HSWBL, were not completely free of LMW material.

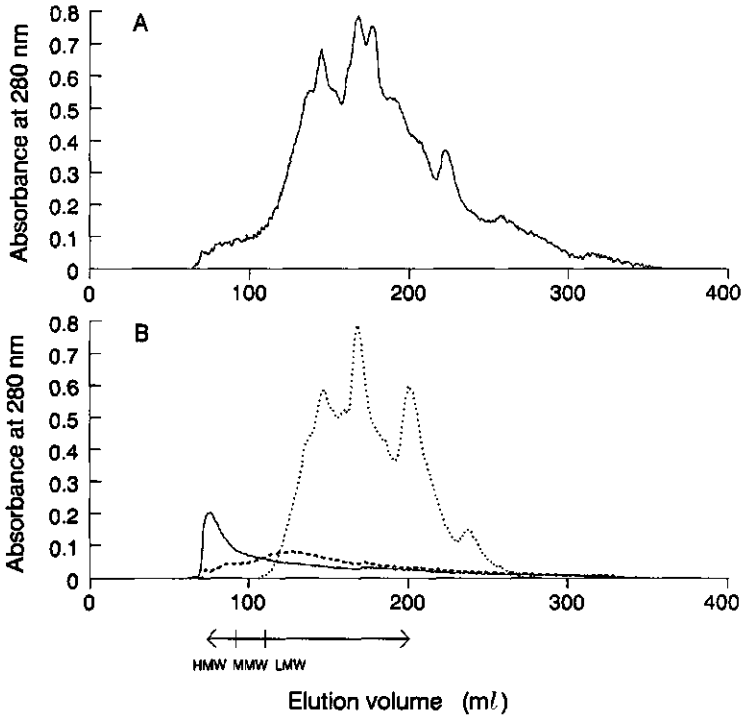


**Figure 7.** Molecular weight distribution of lignin (measured as $UV_{280}$) in untreated HSWBL. Panel A: unfractionated HSWBL. Panel B, HSWBL fractionated by ultrafiltration: —) sample > YM100 (MW > 34 kD); ---) sample YM100-YM10 (2.2 kD < MW < 34 kD); ···) sample < YM10 (MW < 2.2 kD). HMW lignin < 92 m*l*; MMW lignin 92-110 m*l* and LMW lignin > 110 m*l*.

It can be seen from Figure 7B, that the sample < YM10 undoubtedly is the biggest $UV_{280}$-absorbing fraction in untreated HSWBL, which proves that untreated HSWBL lignin predominantly consists of compounds with a MW smaller than 2.2 kD. The exclusion peak at the left side of the sample > YM100 most likely reveals an artefact due to autoxidation, since this peak is not present in the unfractionated sample. The tailing in the profile of the samples > YM100 and YM100-YM10 is attributed to contamination of these higher molecular weight UF samples with low molecular weight material. This contamination is due to the relatively low concentration of high MW lignin in the untreated HSWBL, which hinders a good separation from the low MW material. The presence of low MW material in the samples > YM100 and YM100-YM10, does not permit the use of HSWBL UF samples for the assignment of high molecular weight (HMW; MW > 34 kD), medium molecular weight (MMW; 2.2 kD < MW < 34 kD) and low molecular weight (LMW; MW < 2.2 kD) lignin fractions. On the other hand, high molecular weight, medium molecular weight and

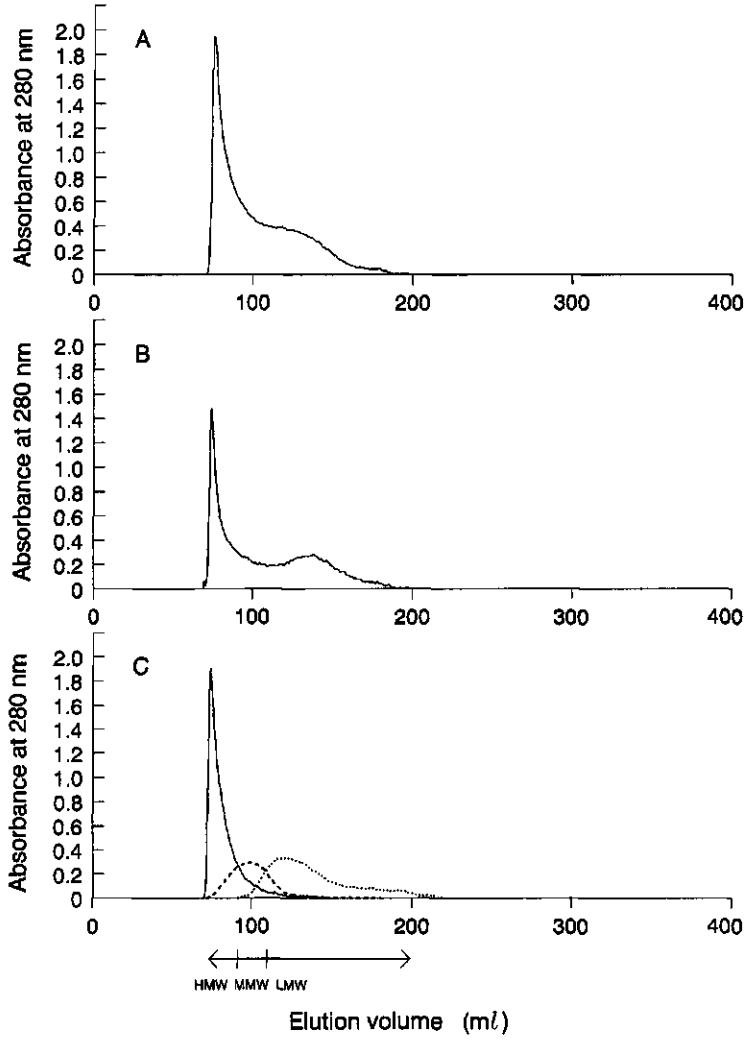


**Figure 8.** Molecular weight distribution of lignin (measured as $UV_{280}$) in anaerobic-aerobically treated and aerobically treated HSWBL. Panel A: anaerobic-aerobically treated HSWBL. Panel B: aerobically treated HSWBL. Panel C: anaerobic-aerobically treated HSWBL fractionated by ultrafiltration; —) sample > YM100 (MW > 34 kD); ---) sample YM100-YM10 (2.2 kD < MW < 34 kD); ···) sample < YM10 (MW < 2.2 kD). HMW lignin < 92 m*l*; MMW lignin 92-110 m*l* and LMW lignin > 110 m*l*.

low molecular weight lignins in biologically treated effluents were adequately separated by the ultrafiltration technique, as can be seen in Figure 8C. No unproportional overlap of the profiles of UF samples was observed, and the summation of the three UF samples corresponded well with the profile of the unfractionated sample. Profiles of the UF samples from the anaerobic-aerobically treated HSWBL were used to assign the three molecular

weight lignin fractions in the GPC profile (HMW fraction < 92 m*l*, MMW fraction 92-110 m*l*, LMW fraction > 110 m*l*). Based on these assignments, the size of the HMW, MMW and LMW lignin fractions were quantified according their $UV_{280}$ absorbance in the GPC profile. The results show that almost all of the lignin (95%) in untreated HSWBL belonged to the LMW fraction, while HMW and MMW lignin accounted each for approximately 2.5%.

The MW distribution of lignin was observed to undergo dramatic changes during biological treatment. These changes are due in part to the removal of lignin by biodegradation or adsorption onto the biomass, resulting in a decrease of LMW lignin. Additionally, polymerization of lignin occurs by a combination of microbially or chemically catalyzed transformations, resulting in an increase of the HMW lignin fractions. Figure 8 shows the MW distribution of the lignin in unfractionated effluents of HSWBL after anaerobic-aerobic treatment and aerobic treatment and the profiles of the three UF samples in anaerobic-aerobically treated HSWBL. GPC profiles after biological treatment of HSWBL indicate a strong decrease of LMW lignin compared to the GPC profile of the untreated HSWBL and polymerization as indicated by the formation of the exclusion peak at the left side of the chromatogram. During anaerobic-aerobic treatment, LMW lignin was removed by 83%. On the other hand polymerization is evident from the 3.8 and 9.2-fold increase of MMW and HMW lignin, respectively. The share of HMW, MMW and LMW lignin observed in the anaerobic-aerobic treated HSWBL was 46%, 20% and 34%, respectively, which is more or less the same as the $UV_{280}$ attributed to the UF samples >YM100, YM100-YM10 and <YM10 in Table 5. Aerobic treatment provided 88% removal of LMW lignin, while MMW and HMW lignin increased 1.9 and 5.1-fold, respectively, indicating that during aerobic treatment polymerization also occurred. The share of the HMW, MMW and LMW lignin after aerobic treatment of HSWBL was 43%, 16% and 41%, respectively, which was also comparable to the $UV_{280}$ attributed to the UF samples >YM100, YM100-YM10 and <YM10 (Table 5).

Comparison of the average spectrophotometric data listed in Table 5, reveals modification of lignin. Lignin which is subjected to anaerobic-aerobic treatment, developed more color than when it was subjected to aerobic treatment. Color bearing properties of lignin changed from 0.062 $VIS_{440}(1x,1cm)/UV_{280}(1x,1cm)$ for the untreated HSWBL to 0.101 and 0.137 $VIS_{440}(1x,1cm)/UV_{280}(1x,1cm)$ for the aerobically treated and anaerobic-aerobically treated HSWBL, respectively. Lignin in YM100-retentate and YM100-YM10 samples contained somewhat more color than the lignin in the YM10-permeate sample. However, differences within the three UF samples of one type of effluent were generally smaller than the

differences between the three types of effluents, indicating that the modification of the color bearing properties of lignin within one treatment, anaerobic-aerobic or aerobic, was similar in all molecular weight fractions.

## Discussion

### Detoxification

Anaerobic bioreactors are an attractive alternative for biological treatment of pulping effluents, offering several advantages compared to aerobic treatment systems (Lettinga *et al.*, 1991; Rintala and Puhakka, 1994). The most important benefits are reduced energy consumption, lowered surplus sludge production and compact reactor designs. Furthermore the utilization of the methane gas, the main byproduct of the process, can displace fuel costs and $CO_2$ emission at the mill.

Nonetheless, the high toxicity of effluents from chemical, semichemical and chemothermomechanical pulping has hampered a broad application of anaerobic technologies for the treatment of pulp and paper industry wastewaters. Anaerobic treatment systems rely on slow growing microorganisms exemplified by methanogens, the key trophic group in the microbial community, having doubling times of 0.5 to 9 days (Henze and Harremoes, 1983; Vogels *et al.*, 1988; Pavlostathis and Giraldo-Gomez, 1991). Through the immobilization of the sludge in biofilms or aggregates, dense populations of anaerobic microorganisms can be maintained inside the reactors, permitting the application of high organic loading rates. Inhibitory wastewater components that lower the growth rate of methanogens to less than their decay rate, ultimately will lead to deterioration of the active biomass in the immobilized sludge. Moreover, toxic incidents that kill off the active biomass are particulary problematic for anaerobic treatment systems since long periods of time (several months) are required for regrowth of the methanogens.

Effluents from chemical pulping processes, like black liquors, cause severe methanogenic toxicity at concentrations which are much lower than the actual wastewater concentration. In this study, we determined a 50%IC for HSWBL of 4.5 COD/*l* to methanogenic bacteria, which is within the range reported for wood derived black liquors (Sierra-Alvarez *et al.*,

1991). From these observations, black liquor typically occurring at concentrations of 50-170 g COD/*l* (Anonymous, 1986; Marton, 1971) would not be feasible to treat in anaerobic treatment systems, unless they are detoxified or diluted. The first step in developing a detoxification strategy is to identify the compounds responsible for toxicity. Terpenoid compounds, referred to as the resin fraction of the wastewater, have been shown to be the major source of methanogenic toxicity in hemp black liquors (Kortekaas *et al.*, 1995), wood derived black liquors (Sierra-Alvarez *et al.*, 1991) and CTMP effluents (Richardson *et al.*, 1991; McCarthy *et al.*, 1990). During anaerobic treatment, no significant degradation of resinous compounds is observed (Sierra-Alvarez *et al.*, 1990a; Liver and Hall, 1996). This recalcitrance of resinous compounds to biodegradation under anaerobic conditions, explains why no tolerance to the toxic compounds in HSWBL was developed in the adapted sludge, which had been cultivated in a UASB reactor fed with HSWBL at sub-lethal concentrations.

On the other hand, aerobic microbial degradation of resinous compounds has been demonstrated for terpenes and resin acids (Liver *et al.*, 1996; Bicho *et al.*, 1995; Mohn, 1995; Wilson *et al.*, 1996; Misra *et al.*, 1996). Molecular oxygen has been considered to be essential for microbial utilization of terpenes lacking oxygenated functional groups. Pathways of biological degradation of terpenes and alicyclic hydrocarbons under aerobic conditions have been reviewed by Trudgill (Trudgill, 1986, 1994).

Biodegradation of resinous compounds under aerobic conditions, probably accounts for the substantial detoxification of pulp and paper industry wastewaters during aerobic treatment (Servizi and Gordon, 1986; Easty *et al.*, 1978; Mueller *et al.*, 1976; Liu *et al.*, 1993; Zender *et al.*, 1994). Anaerobic reactors are typically placed in sequence with aerobic post-treatment reactors to polish the final effluent (Zitomer and Speece, 1993). Consequently, aerobically detoxified effluents can be used for upfront dilution of the incoming pulping wastewater to enable anaerobic treatment at sub-toxic concentrations. Based on this concept, a detoxification method was developed for the treatment of CTMP effluents, in which effluent of the aerobic post-treatment is recirculated for dilution of the influent of the anaerobic reactor (Habets and de Vegt, 1991).

In this study, we demonstrated the principle of the upfront dilution method for the treatment of black liquors. Direct feeding of 12 g COD/*l* HSWBL to adapted sludge in an UASB reactor killed off almost all methanogenic activity within 14 days, confirming the high methanogenic toxicity of HSWBL. While recirculation of 75% of the aerobic effluent, enabled anaerobic treatment of 12 g COD/*l* HSWBL at high loadings without noticeable inhibition of the methanogenesis. The specific methanogenic activity of the sludge even

increased somewhat after prolonged exposure to the upfront diluted wastewater. The potential of the upfront dilution method for detoxification of toxic black liquors during anaerobic-aerobic treatment was confirmed further in an experiment feeding 20 g COD/*l* HSWBL at a loading rate of 3.6 g COD/*l*·d and recirculating 86% of the aerobic effluent. The anaerobic treatment functioned well despite the high influent concentration. The major part of fermentable substrate in HSWBL was converted to methane, while the specific methanogenic activity stabilized at an activity of 0.49 g COD/g VSS·d, which is more or less equal to the methanogenic activity found after anaerobic treatment of 1.9 g COD/*l* HSWBL diluted with tap water (Kortekaas *et al.*, 1994) and just slightly lower than the 0.53 g COD/g VSS·d reported by Habets & de Vegt (1991) for the activity of the methanogenic sludge during anaerobic-aerobic treatment of 4-7 g COD/*l* CTMP wastewater with upfront dilution. The low toxicity of anaerobic-aerobic treated HSWBL was confirmed in batch methanogenic toxicity assays. In previous research it was demonstrated that detoxification for the most part occurred during the aerobic post-treatment step (Kortekaas *et al.*, 1994). Detoxification achieved during anaerobic-aerobic treatment of HSWBL was equal to that achieved during aerobic treatment. These effluents only caused minor inhibition of the methanogenic activity (30%) at the highest concentration tested, corresponding to influent concentrations of 23 g COD/*l*.

The recirculation of aerobic effluents to dilute anaerobic influents would introduce some dissolved oxygen into the anaerobic reactor and may be a reason for some concern for oxygen toxicity to methanogens. However, previous research has demonstrated the unusually high tolerance of granular sludge to oxygen exposure (Shen and Guiot, 1996; Kato *et al.*, 1993a). Kato *et al.* (1993b) have demonstrated that facultative bacteria in the methanogenic sludge sufficiently remove oxygen, preventing direct contact of methanogens inside the biofilm with oxygen.

**Reactor performance**

The treatment efficiency and loading rate achieved in this study during anaerobic treatment of 12 to 20 g COD/*l* HSWBL with upfront dilution were comparable to those found for wood derived black liquors diluted to sub-toxic concentrations of 2.0-3.9 g COD/*l* (Sierra-Alvarez *et al.*, 1990b). BOD removal attributed to the anaerobic step accounted for 67-83%, while COD removal attributed to the anaerobic step was 41-49%. These COD removal rates are

typical for this type of wastewater, which contains a large fraction poorly biodegradable lignin. Sierra-Alvarez *et al.* (1990b) reported 46-47% COD removal during anaerobic treatment of diluted pine and spruce black liquors. Aerobic post-treatment improved BOD removal to 97% and COD removal to 72%. Therefore, the anaerobic reactor contributed approximately two-thirds of the overall COD removal obtained during anaerobic-aerobic treatment of HSWBL; while, approximately half of the COD removed was recovered as methane.

Direct aerobic treatment of HSWBL was studied for comparison with anaerobic-aerobic treatment. Treatment efficiencies during aerobic treatment of HSWBL were comparable with those obtained during anaerobic-aerobic treatment. COD removal during aerobic treatment of 20 g COD/*l* HSWBL ranged from 62-78%, while the BOD removal was maximally 97-98%.

The overall sludge production during anaerobic-aerobic treatment of HSWBL was 0.09 g VSS/g $COD_{removed}$, which is within the range of 0.07-0.12 kg VSS/g $COD_{removed}$, reported by Huss *et al.* (1986) based on pilot-scale experiments with various types of pulp and paper industry effluents. During aerobic treatment of HSWBL, significantly more surplus sludge was produced, accounting for 0.18 g VSS/g $COD_{removed}$. This value is equal to the sludge production reported by Liu *et al.* (1993) and within the range of 0.2-0.6 g TSS/g $BOD_7$ reported by Saunamäki (1988) for low loaded activated sludge treatment plants in the Finish pulp and paper industry.

The upfront dilution system for anaerobic-aerobic treatment of pulping liquors, was found to be sensitive for washout of aerobic sludge, which upon entering the anaerobic reactor with recirculated effluent caused serious deterioration of the methanogenic activity in the sludge of the anaerobic reactor. Incidental washout of voluminous aerobic sludge in experiment III replenished the methanogenic sludge by aerobic sludge, resulting in a sharp decrease of the methanogenic capacity in the anaerobic reactor. Therefore, special attention should be paid to the design and operation of the aerobic post-treatment to prevent sludge bulking. The overall treatment efficiency during anaerobic-aerobic treatment, however, was hardly influenced, since the aerobic post-treatment was able to compensate for the decreased performance of the anaerobic reactor. Once aerobic sludge washout was prevented by modifying the settler, the anaerobic reactor was able to recover within a period of 12 weeks.

The results of this study demonstrate that the recirculation of effluent from the aerobic post-treatment for upfront dilution of toxic black liquors is a suitable method to reduce the inhibition of methanogenic activity during anaerobic treatment of black liquors. Treatment efficiencies during anaerobic-aerobic and aerobic treatment were similar. However, the

anaerobic-aerobic treatment system provided 50% lower surplus sludge production, the production of 0.16 $m^3$ methane/kg $COD_{removed, UASB+AR}$ as an energy source, less nutrient dosage and substantial reductions in aeration costs, since at least two-thirds of the BOD was removed anaerobically.

**Lignin removal**

Lignin is the main organic constituent in black liquor. In HSWBL, lignin is responsible for approximately 50% of the COD (Kortekaas *et al.*, 1994). During aerobic or anaerobic wastewater treatment lignin is poorly removed, accounting for the high residual COD and color in biologically treated wood pulping effluents (Walden, 1980; Sierra-Alvarez *et al.*, 1990b).

In this study, HSWBL was extensively removed and modified by biological treatment. The lignin removal efficiency based on $UV_{280}$ absorbance measurements reached values up to 43 and 58% during anaerobic-aerobic treatment and during direct aerobic treatment, respectively. Furthermore, the COD eliminations ranging from 72 to 78%, exceeded the COD of the non-lignin fraction, confirming that at least part of the lignin COD was removed. Anaerobic treatment accounted for up to half of the lignin removal achieved during anaerobic-aerobic treatment, which is in similar to previous findings (Kortekaas *et al.*, 1994). The elimination of lignin can be attributed to either biological degradation or physical removal mechanisms such as precipitation or adsorption. However, the sludge yield of 0.09 g VSS/g $COD_{removed}$ during anaerobic-aerobic treatment and 0.18 g VSS/g $COD_{removed}$ during direct aerobic treatment, was too low to fully account for all the lignin removed. If all of the lignin were removed by physical mechanisms, a sludge yield of 0.49 and 0.69 g COD/g $COD_{removed}$ would be expected, respectively assuming a cell yield of 20 and 50% for anaerobic and aerobic metabolism of the biodegradable fraction in black liquor, respectively, and a sludge yield of 100% for the removed lignin fraction. Consequently, we conclude that a significant fraction of the lignin removed was biodegraded.

A major factor determining the rate of bacterial lignin degradation is the molecular weight. While the biodegradation of lignin by bacteria is known to be very limited (Benner *et al.*, 1984; Crawford *et al.*, 1977), both aerobic and anaerobic bacteria can cause extensive degradation of lignin derivatives with molecular weights of less than 1000 D (Colberg and Young, 1985; Jokela *et al.*, 1985). GPC and ultrafiltration experiments demonstrate that

lignin in HSWBL consists almost completely of low molecular weight lignin with a molecular weight smaller than 2.2 kD.

Anaerobic degradation studies using radiolabelled lignin, show lignin degradation up to molecular weights of 1400 D and the extent of mineralization was inversely correlated to the lignin molecular weight (Colberg and Young, 1985). Lignin monomers are readily anaerobically mineralized (Healy and Young, 1979; Zeikus *et al.*, 1982; Grbic-Galic, 1983) and many studies with lignin dimers show that all intermonomeric bonds present in lignin can be cleaved anaerobically (Zeikus *et al.*, 1982; Chen *et al.*, 1985a; Chen *et al.*, 1985b; Chen *et al.*, 1987). Lignin oligomers consisting of 3-7 monomeric units on the other hand, are only partly biodegradable (Colberg and Young, 1985; Zeikus *et al.*, 1982; Colberg and Young, 1982).

Aerobic bacterial biodegradation of lignin, like anaerobic biodegradation, is also restricted by the size of the lignin oligomers. Complete degradation of lignin tetramers (MW 650 D) was reported during aerobic incubation with a mixed bacterial enrichment culture (Jokela *et al.*, 1985), while a maximum molecular weight of 600 to 1000 D was reported by Kern & Kirk (1987) for partial mineralization of radiolabelled lignin oligomers by a *Xanthomonas* strain.

GPC and ultrafiltration experiments clearly demonstrate removal of the lower molecular weight lignin during anaerobic and aerobic treatment steps. Partial lignin removal was restricted to the lower molecular weight fraction, indicating its biodegradation as was previously observed after anaerobic treatment of straw and pine black liquors (Sierra-Alvarez *et al.*, 1990b).

**Lignin polymerization**

Apart from the partial biodegradation of low molecular weight lignin, extensive polymerization was observed during both anaerobic-aerobic and direct aerobic treatment. Lignin of less than 2.2 kD was polymerized to lignin of more than 34 kD. Associated with the polymerization were increases in the lignin color. Similar increases in color have been reported previously during sequenced anaerobic-aerobic treatment of pulping wastewaters (Kortekaas *et al.*, 1994; Rintala and Vuoriranta, 1988; Rintala and Lepistö, 1992) or upon exposure of anaerobic pulping effluent to air (Sierra-Alvarez *et al.*, 1990b).

The increase in lignin molecular weight and color can be explained by a combination of

biologically mediated modifications followed by autoxidative polymerization. It is known that acetogenic bacteria, such as *Clostridium Thermoaceticum, Acetobacterium woodii* and *Eubacterium limosum*, present in anaerobic treatment systems are capable to demethoxylating lignin model compounds, leaving the hyroxylated aromatic structure intact (Daniel *et al.*, 1988; Kaiser and Hanselmann, 1982; Bache and Pfenning, 1981; Cocaign *et al.*, 1991). The increase of ortho-dihydroxy groups formed after demethoxylation makes the lignin susceptible for autoxidation. Providing the presence of air and neutral to alkaline conditions, lignin fragments with neighbouring hydroxy groups are abiotically converted to quinones, which condense with one other, forming darkly colored high molecular weight lignin compounds (Field *et al.*, 1989; Field *et al.*, 1991).

The polymerization of lignin may offer some benefits for subsequent physical-chemical lignin removal. Precipitation of lignin with lime or sodium sulphate, is known to be more effective for high molecular weight lignin, which provoked the utilization of enzymatic pretreatments involving peroxidases and laccases, to enhance lignin precipitation by polymerization (Forss *et al.*, 1989; Schmidt and Joyce, 1980). The observed increase of lignin molecular weight after biological treatment may also be beneficial for ultrafiltration during tertiary treatment, such as improved efficiency compared to untreated effluents and decrease of membrane fouling caused by intermediate size lignin and biodegradable constituents.

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## References

**Anonymous** (1986) Comprehensive industry document for small pulp and paper industry. *In* Chakrabarti, S.P. and Kumar, A. (eds.), Comprehensive industry document series: COINDS/22/1986, Central Board for Prevention and Control of Water Pollution, New Delhi, India.

**APHA** (1989). Standard methods for examination of water and wastewater, 17th edn., American Public Health Association, Washington D.C., USA.

**Bache, R., and Pfenning, N.** (1981). Selective isolation of *Acetobacterium woodii* on methoxylated aromatic acids and determination of growth yields. Arch. Microbiol., 130, 255-261.

**Benner, R., Maccubbin, A.E., and Hodson, R.E.** (1984). Anaerobic biodegradation of the lignin and polysacharide components of lignocellulose and synthetic lignin by sediment microflora. Appl. Environ. Microbiol., 47, 998-1004.

**Bicho, P.A., Martin, V., and Saddler, J.N.** (1995). Growth, Induction, and substrate specificy of dehydroabietic acid-degrading bacteria isolated from kraft mill effluent enrichment. Appl. Environ. Microbiol., 61, 3245-3250.

**Chen, W., Ohmiya, K., Shimizu, S., and Kawakami, H.** (1985a). Degradation of dehydrovanillin by anaerobic bacteria from cow rumen fluid. Appl. Environm. Microbiol., 50: 211-216.

**Chen, W., Supanwong, K., Ohmiya, K., Shimizu, S., and Kawakami, H.** (1985b). Anaerobic degradation of veratrylglycerol-beta-guaiacyl ether and guaiacoxyacetic acid by mixed rumen bacteria. Appl. Environm. Microbiol., 50, 1451-1456.

**Chen, W., Ohmiya, K., Shimizu, S., and Kawakami, H.** (1987). Anaerobic degradation of dehydrodiisoeugenol by rumen bacteria. J. Ferment. Technol., 65(2), 221-224.

**Cocaign, M., Wilberg, E., and Lindley, N.** 1991). Sequential demethoxylation reactions during methylotrophic growth of methoxylated aromatic substrates with *Eubacterium limosum*. Arch. Microbiol., 155, 496-499.

**Colberg, P.J., and Young, L.Y.** (1982). Biodegradation of lignin-derived molecules under anaerobic conditions. Can. J. Microbiol., 28, 886-889.

**Colberg, P.J., and Young, L.Y.** (1985). Anaerobic degradation of soluble fractions of [14 C lignin] lignocellulose. Appl. Environ. Microbiol., 49(2), 345-349.

**Crawford, D.L. Floyd, S., Pometto III, A.L., Crawford, R.L.** (1977). Degradation of kraft lignins by the microflora of soil and water. Can. J. Microbiol., 23, 434-440.

**Daniel, S.L., Wu, Z., and Drake, H.L.** (1988). Growth of thermophilic acetogenic bacteria on methoxylated aromatic acids. FEMS Microbiol. Lett., 52, 25-28.

**Easty, D.B., Borchardt, G., and Wabers, A.** (1978). Wood-derived toxic compounds. TAPPI J., 61(10), 57-60.

**Fengel, D., and Wegener, G.** (1984). Wood: Chemistry, Ultrastructure, Reactions. Walter de Gruyter, Berlin.

**Field, J.A., Kortekaas, S., and Lettinga, G.** (1989). The tannin theory of methanogenic toxicity. Biological Wastes, 29, 241-262.

**Field, J.A., Leyendeckers, M.J.H., Sierra-Alvarez, R., Lettinga, G., and Habets, L.H.A.** (1991).

Continuous anaerobic treatment of autoxidized bark extracts in laboratory-scale columns. Biotechnol. Bioeng., 37, 247-255.

**Forss, K., Jokinen, K., Savolainen, M., and Williamson, H.** (1989). Utilization of enzymes for effluent treatment in the pulp and paper industry. Paperi ja Puu, 10, 1108-1112.

**Grbic-Galic, D.** (1983). Anaerobic degradation of coniferyl alcohol by methanogenic consortia. Appl. Environ. Microbiol., 46, 1442-1446.

**Habets, L.H.A., and de Vegt, A.L.** (1991). Anaerobic treatment of bleached TMP and CTMP effluent in the BIOPAQ UASB system, Wat. Sci. Tech., 24(3/4), 331-345.

**Healy, J.B. Jr., and Young, L.Y.** (1979). Anaerobic biodegradation of eleven aromatic compounds to methane. Appl. Environ. Microbiol., 38, 84-89.

**Henze, M., and Harremoes, P.** (1983). Anaerobic treatment of wastewater in fixed film reactors -a literature review. Wat. Sci. Techn., 15(8/9), 1-100.

**Huss, L., Sievert, P., and Särner, E.** (1986). Anamet full-scale anaerobic treatment experiences at three pulp and paper mills. *In* PIRA Paper and board division seminar cost effective treatment of papermill effluents using anaerobic technologies, 14-15 Jan. 1986, Leatherhead, UK.

**Jokela, J., Pellinen, J., Salkinoja-Salonen, M., and Brunow, G.** (1985). Biodegradation of two tetrameric lignin compounds by a mixed bacterial culture. Appl. Microbiol. Biotechnol. 23, 38-46.

**Kaiser, J., and Hanselmann, K.W.** (1982). Fermentative metabolism of substituted monoaromatic compounds by bacterial community from anaerobic sediments. Arch. Microbiol., 133, 185-194.

**Kato, M.T., Field, J.A., and Lettinga, G.** (1993a). High tolerance of methanogens in granular sludge to oxygen. Biotechnol. Bioeng., 42, 1360-1366.

**Kato, M.T., Field, J.A., and Lettinga, G.** (1993b). Methanogenesis in granular sludge exposed to oxygen. FEMS Microbiology Letters, 114, 317-324.

**Kennedy, K.J., McCarthy, P.J., and Droste, R.L.** (1992). Batch and continuous anaerobic toxicity of resin acids from chemithermomechanical pulp wastewater. J. Ferment. Bioeng., 73, 206-212.

**Kern, H.W., and Kirk, T.K.** (1987). Influence of molecular size and ligninase pretreatment on the degradation of lignins by *Xanthomonas* sp. strain 99. Appl. Environm. Microbiol., 53, 2242-2246.

**Kortekaas, S., Doma, H.S., Potapenko, S.A., Field, J.A., and Lettinga, G.** (1994). Sequenced anaerobic-aerobic treatment of hemp black liquors. Wat. Sci. Tech., 29, 409-419.

**Kortekaas, S., Soto, M., Vicent, T., Field, J.A., and Lettinga, G.** (1995). Contribution of extractives to methanogenic toxicity of hemp black liquor. J. Ferment. Bioeng., 80, 383-388.

**Leach, J.M., and Thakore, A.N.** (1976). Toxic constituents of mechanical pulping effluents. TAPPI J., 59, 129-132.

**Leach, J.M., Mueller, J.C., and Walden, C.C.** (1978). Biological detoxification of pulp mill effluents. Process Biochemistry. January 1978, 18-21.

**Lettinga, G., van Velzen, A.F.M., Hobma, S.W., de Zeeuw, W., and Klapwijk, A.** (1980). Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially anaerobic treatment. Biotechnol. Bioeng., 22, 699-734.

**Lettinga, G., Field, J.A., Sierra-Alvarez, R., van Lier, J.B., and Rintala, J.** (1991). Future perspectives for the treatment of forest industry wastewaters. Wat. Sci. Tech., 24, 91-102.

**Li, J., O'Hagan, T., and MacLeod, J.M.** (1996). Using ultrafiltration to analyze the molecular mass distribution of kraft lignin at pH 13. Can. J. Chem. Engineer., 74(1), 110-117.

**Liu, H.W., Lavallée, H.C., and Lo, S.N.** (1993). Mechanisms and kinetics of removal of resin and fatty acids, toxic compounds, in CTMP effluents during aerobic biological treatment, p. 911-919. *In* Proceedings of the seventh international symposium on wood and pulping chemistry. China technical association of paper industry, Beijing.

**Liver, S.F., and Hall, E.R.** (1996). Interactions of resin acids with aerobic and anaerobic biomass-I. Degradation by non-acclimated inocula. Wat. Res., 30(3), 663-671.

**Marton, J.** (1971). Reactions in alkaline pulping, p. 639-694. *In* Sarkanen K. V. and Ludwig C. H. (eds.), Lignins, occurence, formation, structure and reactions. Wiley-interscience, New York.

**McCarthy, P.J., Kennedy, K.J., and Droste, R.L.** (1990). The role of resin acids in the anaerobic toxicity of chemithermomechanical pulp wastewater. Wat. Res., 24, 1401-1405.

**Misra, G., Pavlostathis, S.G., Perdue, E.M., and Araujo, R.** (1996). Aerobic degradation of selected monoterpenes. Appl. Microbiol. Biotechnol., 45, 831-838.

**Mohn, W.W.** (1995). Bacteria obtained from a sequencing batch reactor that are capable of growth on dehydroabietic acid. Appl. Environ. Microbiol., 61, 2145-2150.

**Mueller, J.C., Leach, J.M., and Walden, C.C.** (1976). Biological detoxification of pulp and paper mill effluents. *In* Proc. 5th international fermentation symposium, Berlin.

**Pavlostathis, S.G., and Giraldo-Gomez, E.** (1991). Kinethics of anaerobic treatment: A critical review. Crit. Rev. in Environ. Control, 21(5,6), 411-490.

**Pellinen, J., and Salkinoja-Salonen, M.S.** (1985). Aqueous size exclusion chromatography of industrial lignins. J. Chromatogr., 322, 129-138.

**Richardson, D.A., Andras, E., and Kennedy, K.J.** (1991). Anaerobic toxicity of fines in chemi-thermomechanical pulp wastewaters, a batch assay - reactor study comparison. Wat. Sci. tech., 24, 103-112.

**Rintala, J.A., and Lepistö, S.** (1992). Anaerobic treatment of thermomechanical pulping whitewater at 35-70°C. Wat. Res., 26, 1297-1305.

**Rintala, J.A., and Puhakka, J.A.** (1994). Anaerobic treatment in pulp and paper-mill waste management: a review. Biores. Technol., 47, 1-18.

**Rintala, J.A., and Vuoriranta, P.** (1988). Anaerobic-aerobic treatment of thermomechanical pulping effluents. TAPPI J., 71(9), 201-207.

**Saunamäki, R.** (1988). Sludge handling and disposal at Finish activated sludge plants. Wat. Sci. Tech., 20, 171-182.

**Schmidt, R.L., and Joyce, T.W.** (1980). An enzymatic pretreatment to enhance the lime precipitability of pulp mill effluents. TAPPI J., 63(12), 63-67.

**Servizi, J.A., and Gordon, R.W.** (1986). Detoxification of TMP and CTMP effluents alternating in a pilot scale aerated lagoon. Pulp Paper Can., 87, T405-T409.

**Shen, C.F., and Guiot, S.R.** (1996). Long-term impact of dissolved O2 on the activity of anaerobic granules. Biotechnol. Bioeng., 49, 611-620.

**Sierra-Alvarez, R., and Lettinga, G.** (1990). The methanogenic toxicity of wood resin compounds. Biol. Wastes, 33, 211-226.

**Sierra-Alvarez, R., Kato, M., and Lettinga, G.** (1990a). The anaerobic biodegradability of paper mill wastewater constituents. Environ. Technol., 11, 891-898.

**Sierra-Alvarez, R., Kortekaas, S., van Eekert, M., Harbrecht, J., and Lettinga, G.** (1990b). The continuous anaerobic treatment of pulping wastewaters. J. Ferment. Bioengineer., 70(2), 119-127.

**Sierra-Alvarez, R., Kortekaas, S., van Eekert, M., and Lettinga, G.** (1991). The anaerobic biodegradability and methanogenic toxicity of pulping wastewaters. Wat. Sci. Tech., 24(3/4), 113-125.

**Stephenson, R.J., and Duff, S.J.B.** (1996). Coagulation and precipitation of a mechanical pulping effluent-II. Toxicity removal and metal salt recovery. Wat. Res., 30(4), 793-798.

**Trudgill, P.W.** (1986). Terpenoid metabolism of *Pseudomonas*, p. 483-525. *In* Sokatch J. R. (ed.), The bacteria, a treatise on structure and function, Vol X. Academic Press, New York.

**Trudgill, P.W.** (1994). Microbial metabolism and transformation of selected monoterpenes, p. 33-61. *In* Ratledge C. (ed.), Biochemistry of microbial degradation. Kluwer Academic Publishers.

**Turner, C.E., ElSohly, M.A., and Boeren, E.G.** (1980). Constituents of *Cannabis sativa* L. XVII. A review of the natural constituents. J. Nat. Prod., 43, 169-234.

**Vogels, G.D., Keltjens, J.T., and van der Drift, C.** (1988). Biochemistry of methane production, p. 707-770. *In* A.J.B. Zehnder (ed.), Biology of Anaerobic Microorganisms, Wiley, New York.

**Walden, C.C.** (1980). Biological effects of pulp and paper mill effluents, p.669-676. *In* Moo-Young M. and Campbell W. R. (eds.), Proc. of the 6th International Fermentation Symposium. V.2. Fuels, chemicals, foods and waste treatment. July 20-25, London, Canada.

**Welander, T.** (1988). Anaerobic process for treatment of CTMP effluent. Wat. Sci. Tech., 20(1), 143-147.

**Wilson, R.W., Murphy, K.L., and Frenette, E.G.** (1987). Aerobic and anaerobic treatment of NSSC and CTMP effluent. Pulp Paper Can., 88(1), T4-8.

**Wilson, A.E.J., Moore, E.R.B., and Mohn, W.W.** (1996). Isolation and characterization of isopimaric acid-degrading bacteria from a sequencing batch reactor. Appl. Environ. Microbiol., 62, 3146-3151.

**Zeikus, J.G., Wellstein, A.L., and Kirk, T.K.** (1982). Molecular basis for the biodegradative recalcitrance of lignin in anaerobic environments. FEMS Microbiology Letters, 15, 193-197.

**Zender, J.A., Stuthridge, T.R., Langdon, A.G., Wilkins, A.L., Mackie, K.L., and McFarlane, P.N.** (1994). Removal and transformation of resin acids during secondary treatment at new zealand bleached kraft pulp and paper mill. Wat. Sci. Tech., 29, 105-121.

**Zitomer, D.H., and Speece, R.E.** (1993). Sequential environments for enhanced biotransformation of aqueous contaminants. Environ. Sci. Technol., 27(2), 227-244.

# Anaerobic Treatment of Hemp Thermomechanical Pulping Wastewater.

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## Abstract

Biological treatment is an indispensable instrument for water management of non-wood pulp mills, either as internal measure or end of pipe. In this paper, anaerobic treatment of wastewaters derived from the thermomechanical pulping (TMP) of hemp (*Cannabis sativa L.*) is described. Hemp stem wood and hemp bark TMP wastewaters were treated in laboratory scale upflow anaerobic sludge blanket (UASB) reactors. For both types of wastewater, maximal COD removal of 72% were obtained at loading rates of 13-16 g COD·$l^{-1}d^{-1}$, providing 59-63% recovery of the influent COD as methane. The reactors continued to provide excellent COD removal efficiencies of 63-66% up to a loading rate of 27 g COD·$l^{-1}d^{-1}$, being the highest loading rate tested. Batch toxicity assays revealed the absence of methanogenic inhibition by hemp TMP wastewaters, coinciding with the high acetoclastic activity of the reactor sludge of approximately 1 g COD·g $VSS^{-1}d^{-1}$. Due to the relatively low molecular weight of hemp TMP lignin, its removal (measured as $UV_{280}$) during anaerobic treatment was remarkably high and averaged 45 and 31% for the hemp stem wood and the hemp bark TMP UASB reactors, respectively. Gel permeation chromatography revealed that the lignin removed corresponded to the lowest molecular weight derivatives. Subsequent batch aerobic post-treatment led to a considerable increase of color levels and polymerization of the residual lignin to molecular weights in excess of 34 kD.

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## Introduction

Historically, paper was entirely made from a variety of non-wood fibres, yet today in Europe and North America the vast majority of paper is produced from wood. Worldwide however, significant regional differences occur, as can be seen from the 49% and 86% market share of non-wood pulping in India and China, respectively (FAO, 1995). One of the pulping fibres with a very long history in paper production is hemp (*Cannabis sativa L.*). Hemp is an annual fibre crop, belonging to the *Cannabaceae* family and has a relatively low lignin content as well as a different extractive composition compared to wood. The lignin content in hemp varies from 4% in the bark fraction to 21% in the stem wood fraction (van der Werf *et al.*, 1994). Since the fibre characteristics are rather different, separate processing of both fibre fractions is recommended.

Anaerobic treatment of hemp thermomechanical pulping (TMP) wastewater was studied as part of a research programme, initiated by the Dutch government to stimulate the cultivation of non-food crops in arable lands. During thermomechanical pulping, fibres are separated in a refiner by means of mechanical energy at temperatures above 100°C, providing high pulp yields and minor losses of soluble organic compounds to the process water. TMP has become one of the major processes for the manufacturing of ultra-high yield pulps, accounting for 20% of the world pulp production together with other mechanical pulps (FAO, 1995).

TMP mills are typically wood-based. Effluent characterization studies (Stenberg and Norberg, 1977; Jurgensen *et al.*, 1985) show that TMP effluents are medium strength wastewaters with concentrations ranging from 1000-6000 mg COD·$l^{-1}$. The major fraction of TMP wastewaters consists of easy biodegradable material, such as carbohydrates and organic acids, which account for 40-60% of the wastewater COD, while the lignin content is generally low, ranging from 15-30%. Aquatic toxicity displayed by TMP wastewaters is attributed mainly to resin acids (Leach and Thakore, 1976; Kovacs and Voss, 1992). Resin acids are a major constituent of wood resin, generally found in TMP wastewaters at concentrations about 10-50 mg·$l^{-1}$ (Kovacs and Voss, 1992; Lo *et al.*, 1994); however, concentrations up to 550 mg·$l^{-1}$ have been reported for combined wastewaters of TMP and chemothermomechanical pulping (CTMP) (Habets and de Vegt, 1991).

Like in wood-derived wastewaters, methanogenic toxicity in hemp pulping wastewaters is attributed to resinous compounds originating from the pulp feedstock (Sierra-Alvarez and Lettinga, 1990; Kortekaas *et al.*, 1995). Hemp soda pulping wastewaters were found to be

as toxic as wood-derived soda pulping wastewaters (Kortekaas *et al.*, 1994 & 1995). However, since relatively little resinous compounds are extracted into the wastewater during mechanical pulping, TMP wastewaters are expected to be non-toxic in anaerobic treatment systems, irrespective of the fibre feedstock, hemp or wood.

Economic and environmental pressures have led to a marked reduction in water use in the pulping industry. Mechanical pulping wastewaters however, are too diluted to facilitate cost-effective concentration and burning in recovery boilers, while alternative techniques such as membrane filtration are highly costly (Gerbasi *et al.*, 1993). Therefore, biological treatment of TMP wastewaters is regarded to be the only viable option to meet effluent discharge regulations and should also be considered as a part of internal treatment systems for closing the water circuits.

TMP wastewaters are known to be highly biodegradable during anaerobic digestion and non-toxic to methanogenic bacteria, which makes them highly suitable for anaerobic wastewater treatment (Sierra-Alvarez *et al.*, 1990b). After the development of high rate anaerobic treatment systems, such as the upflow anaerobic sludge blanket (UASB) reactor, in the early 1980's, an increasing application of anaerobic technologies for the treatment of forest industry wastewaters has been observed (Lettinga *et al.*, 1991). Benefits of anaerobic treatment over aerobic treatment are the high biomass retention, which enables compact reactor designs and high loading rates, low energy consumption, generation of energy rich biogas, minimal nutrient requirements and low surplus sludge production.

Since TMP mills are typically wood-based, little is known about the toxicity and biodegradability of non-wood TMP wastewaters. In this research, the continuous anaerobic treatment of hemp TMP wastewaters in laboratory scale UASB reactors was studied. The objective of this research was to demonstrate the feasibility of anaerobic treatment of hemp TMP wastewaters. Additionally, the modification of lignin during aerobic post-treatment was evaluated.

## Materials and methods

### Anaerobic reactor studies

Continuous anaerobic treatment of hemp TMP wastewaters was studied in two laboratory scale UASB reactors. UASB reactors, made of glass with a liquid volume of 0.15 *l* (previously described by Sierra-Alvarez *et al.*, 1990b), were placed in a temperature controlled room at 30 ± 2°C. Both reactors were inoculated with anaerobic granular sludge, 20 g volatile suspended solids (VSS) per liter reactor volume. During the first 7 days, the reactors were fed with a neutralized volatile fatty acid (VFA) solution ($C_2$:$C_3$:$C_4$ = 50:25:25 on COD basis) at a concentration of 2 g COD·$l^{-1}$. Subsequently, the reactor experiment was started by replacing the VFA solution with the TMP wastewater. Methane production was measured with 10 *l* Mariotte flasks filled with 3% (w/v) NaOH solution to scrub out the carbon dioxide from the biogas. The following parameters were used to indicate the performance of the UASB reactors. $COD_{in}$ = influent COD (g·$l^{-1}$); E = % $COD_{in}$ removed based on the membrane filtered effluent COD concentration; M = methanogenesis, % conversion $COD_{in}$ to methane; VFA = VFA remaining in the effluent as % of $COD_{in}$; A = acidification, % conversion $COD_{in}$ to VFA (A = M + VFA); Cells = % conversion of $COD_{in}$ to cells (estimation of Cells = E - M); BD = % anaerobically biodegradable $COD_{in}$ (BD = A + Cells); Y = specific cell yield in COD per unit COD biodegraded (Cells/BD); $E_{bd}$ = the elimination of the anaerobically biodegradable COD = [1 - (VFA/BD)]·100; OLR = Organic Loading Rate (g COD·$l^{-1}$$d^{-1}$); $E_{uv}$ = % elimination of the ultraviolet absorbance at 280 nm, based on the membrane filtered effluent $UV_{280}$; $E_{vis}$ = % elimination of the visible light absorbance at 440 nm, based on the membrane filtered effluent $VIS_{440}$.

### Bio-assays

The toxicity assays and acetoclastic methanogenic activity assays were conducted in triplicate as outlined previously ("anaerobic toxicity assay *Type 2*", Kortekaas *et al.*, 1995) in 120 m*l* glass serum vials containing 25 m*l* medium. All assays were buffered with $CO_2$/$NaHCO_3$ (Donlon *et al.*, 1995) and contained acetate as a substrate and essential macro

and micro nutrients as described previously (Kortekaas *et al.*, 1998). In order to broaden the concentration range being assessed, hemp TMP effluents were concentrated by evaporation under a constant flow of $N_2$. Control samples, which had not been concentrated, showed that concentration procedure had only a minor effect on the toxicity observed.

**Batch aeration experiments**

Modification of lignin during aerobic post-treatment of anaerobically treated TMP wastewater was studied in batch aeration experiments. Activated sludge, sampled from an aerobic reactor fed with hemp stem wood TMP wastewater, was centrifugated to remove bulk water and transferred to a serum flask. Activated sludge (final concentration 4 g $VSS \cdot l^{-1}$) was resuspended in 50 m*l* of anaerobically treated hemp stem wood TMP wastewater and subjected to aeration by mechanical stirring. Within a 10 day experimental period, at regular time intervals samples were taken to examine pH, $UV_{280}$, $VIS_{440}$ and the lignin molecular weight distribution. Corrections for evaporation were made by addition of distilled water prior to sampling based on weight losses. Additionally, a control experiment was performed in which anaerobically treated hemp stem wood TMP effluent was incubated with activated sludge and subjected to mechanical stirring under an atmosphere of $N_2$.

**Source of inoculum**

Anaerobic granular sludge was obtained from a full-scale anaerobic reactor treating wheat starch wastewater (Latenstein, Nijmegen, The Netherlands). The sludge utilized to seed the UASB reactors and to perform anaerobic toxicity assays, was elutriated to remove fines and stored at 4 °C under $N_2$ gas.

Activated sludge was cultivated in an sequencing batch reactor fed with hemp stem wood TMP wastewater at a loading rate of 0.25 g $BOD_5 \cdot g\ VSS^{-1} \cdot d^{-1}$ for a period of 4 months.

**Preparation of influent**

TMP wastewaters were prepared from hemp (*Cannabis sativa L.*, cultivar Kompolti Hybrid TC), grown in the Netherlands by DLO Research Institute for Agrobiology and Soil Fertility, Wageningen. The hemp stems were mechanically divided into a hemp bark and a hemp stem wood fraction, both fractions were processed separately. Pulping conditions applied were representative for TMP. A slurry containing 100 g of dry hemp fibre was cooked at 120 °C for a period of 2 h. The wastewater was separated from the remaining pulp by centrifugation and subsequent filtration. VSS content in TMP wastewaters was negligible. Average characteristics of hemp stem wood TMP wastewater were as follows: $COD_{in}$ = 7.70 $g \cdot l^{-1}$; VFA = 1.00 g $COD \cdot l^{-1}$; $UV_{280}$ = 39.6 absorbance units 1x,1cm; $VIS_{440}$ = 24.0 absorbance units 1x,1cm; pH = 6.1. Average characteristics of hemp bark TMP wastewater were as follows: $COD_{in}$ = 8.7 $g \cdot l^{-1}$; VFA = 1.04 g $COD \cdot l^{-1}$; $UV_{280}$ = 25.2 absorbance units 1x,1cm; $VIS_{440}$ = 24.0 absorbance units 1x,1cm; pH = 6.2.

Influent solutions were prepared by addition of all essential macro and micro nutrients as described previously (Kortekaas *et al.*, 1998) and dilution with tap water to adjust the influent concentration to 3-4 g $COD \cdot l^{-1}$. 1 g of $NaHCO_3$ was added per gram of biodegradable wastewater COD to buffer eventual accumulation of VFA. The influent solutions were stored under $N_2$ gas in a refrigerator (5°C).

**Analytical methods**

Wastewater samples were analyzed for COD (the micro method with dichromate), $BOD_5$, VSS, TSS according to American Public Health Association standard methods (APHA, 1992). The pH was determined immediately after sampling with a Knick 511 pH-meter (Berlin, Germany) and a Scot Gerade N61 double electrode (Hofheim, Germany). Sludge volume index (SVI) was determined according a modified APHA (1992) procedure after 10 minutes of settling. Samples for the determination of COD, $VIS_{440}$ and $UV_{280}$ were membrane filtered (pore size = 0.45 $\mu$m). $VIS_{440}$ and $UV_{280}$ were determined in a 1 cm quartz cuvette by diluting the samples to less than 0.8 absorbance units in a 0.02 M borate buffer providing a pH 9.1. The lignin content of the TMP wastewaters was estimated from $UV_{280}$ using an absorbance coefficient of 20.6 $l \cdot g^{-1} cm^{-1}$ (Kortekaas *et al.*, 1994). VFA and methane were analyzed by gas chromatography as described previously (Kortekaas *et al.*, 1994).

The molecular weight (MW) distribution of TMP wastewater lignins was determined by gel permeation chromatography (GPC) on a standard chromatography Hiload™ system (Pharmacia LKB, Roosendaal, The Netherlands) equipped with a XK16 column (1.6 x 90 cm) packed with Sephacryl S-100 (Pharmacia Biotech). A 500 $\mu l$ membrane filtered (0.45 $\mu$m) wastewater sample was applied to the column and eluted with a $NaHCO_3$ (0.025 M)-NaOH buffer solution (pH 10.5) containing 0.5 $g \cdot l^{-1}$ of polyethylene glycol (MW 6000) (Pellinen and Salkinoja-Salonen, 1985a). Void and retention volume of the gel filtration column, determined with Blue dextran [$> 10^6$ daltons (D)] (Pharmacia Biotech) and guaiacol, was 74 m$l$ and 190 m$l$, respectively. GPC profiles were calibrated by elution of three lignin molecular weight samples obtained by ultrafiltration of biologically treated soda pulping black liquor. Elution volumes attributed to different molecular weight fractions are listed in Table 1.

**Table 1.** Calibration of gel permeation chromatography with three molecular weight fractions of lignin obtained by ultrafiltration of biologically treated soda pulping black liquor[a].

| | Fractions | | |
|---|---|---|---|
| | High molecular weight (HMW) | Medium molecular weight (MMW) | Low molecular weight (LMW) |
| Elution volume (m$l$) | < 92 | 92 - 110 | > 110 |
| Molecular weight | HMW > 34 kD | 2.2 kD < MMW < 34 kD | LMW < 2.2 kD |

[a] Li *et al.*, 1996; Kortekaas *et al.*, 1998

## Results

Anaerobic treatment of hemp stem wood and hemp bark TMP wastewaters was studied separately. Prior to the continuous experiments, the methanogenic toxicity of the separate TMP wastewaters was evaluated in batch anaerobic toxicity assays. No significant inhibition of the acetoclastic methanogenic activity was observed at the highest concentration of each hemp TMP wastewater tested (8 g COD·$l^{-1}$). The hemp stem wood and hemp bark TMP wastewaters were therefore not toxic towards methanogens in granular sludge (Figure 1).

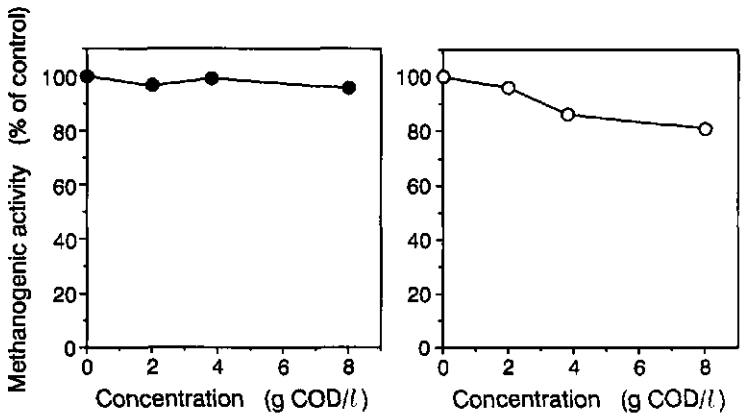


**Figure 1.** The acetoclastic methanogenic activity of granular sludge exposed to hemp stem wood (•) and hemp bark (○) TMP wastewaters.

The feasibility of anaerobic treatment of hemp stem wood and hemp bark TMP wastewaters was studied in two laboratory scale UASB reactors. The reactors were seeded with unadapted granular sludge and were fed with TMP wastewater at a concentration of 3-4 g COD·$l^{-1}$, a concentration which is comparable to wastewaters generated at full-scale TMP mills. In order to give the seed sludge opportunity to accommodate to the substrate, data collecting was preceded by a 35 day start-up period, applying an OLR of 7-8 g COD·$l^{-1}d^{-1}$.

Daily treatment efficiencies and operational parameters during anaerobic treatment of hemp stem wood and hemp bark TMP wastewaters are shown in Figure 2 and Figure 3. Treatment efficiencies obtained from day 35-52 (Period I) at an OLR of 13-16 g COD·$l^{-1}d^{-1}$, showed that the anaerobic biodegradability of hemp stem wood and hemp bark TMP wastewaters was similar (Table 2). The acidifiable fraction (conversion to VFA and $CH_4$) in both types of wastewater accounted for approx. 65% of the COD on the average, while the conversion of incoming COD to methane was 59-63% and the COD removal efficiency was 71-73%.

**Table 2.** Average operational parameters and treatment efficiencies during anaerobic treatment of hemp TMP wastewaters.

| | | Hemp stem wood | | | Hemp bark | | |
|---|---|---|---|---|---|---|---|
| | | Period I[a] | Period II | Period III[b] | Period I | Period II | Period III[b] |
| Operational parameters: | | | | | | | |
| $COD_{in}$ | g $COD \cdot l^{-1}$ | 3.4 | 4.3 | 4.1 | 3.5 | 4.1 | 3.7 |
| OLR | g $COD \cdot l^{-1} d^{-1}$ | 12.6 | 26.6 | 24.7 | 16.3 | 25.8 | 21.2 |
| HRT | h | 7.1 | 3.6 | 4.1 | 4.6 | 3.6 | 4.4 |
| Efficiency: | | | | | | | |
| COD removal | % $COD_{in}$ | 73 | 69 | 66 | 71 | 69 | 63 |
| Methanogenesis | % $COD_{in}$ | 63 | 57 | 53 | 59 | 57 | 52 |
| Acidification | % $COD_{in}$ | 64 | 62 | 62 | 65 | 68 | 64 |
| VFA in effluent | % $COD_{in}$ | 2 | 5 | 9 | 3 | 10 | 13 |
| $UV_{280}$ removal | % | 28 | 45 | 45 | 21 | 31 | 31 |
| $VIS_{440}$ removal | % | 5 | 35 | 32 | 5 | 33 | 31 |

[a] Period I: day 35-52.
Period II: day 52-67.
Period III: day 67-80.

[b] At day 67, excess sludge had been removed.

During the course of the experiment the sludge loading was increased stepwise. Increasing of the OLR to approximately 26 g $COD \cdot l^{-1}d^{-1}$ in Period II caused an increase in the VFA levels measured in the anaerobic effluent to 5-10% of the influent COD, which was reflected by a slight drop in the average COD removal efficiency to 69% (Table 2). Further increases in the sludge loading occurred in Period III after removal on day 67 of 51 and 25% excess sludge from the UASB reactors, fed with stem wood and bark TMP wastewaters, respectively. Compared to Period II, a small decrease in methanogenesis and COD removal was observed during Period III, while VFA levels in the anaerobic effluent rose to 9-13% of the influent COD, indicating a slight overloading of the UASB reactors. The average COD removal efficiency in this period was 63-66% with 52 to 53% of the incoming COD being converted to methane.

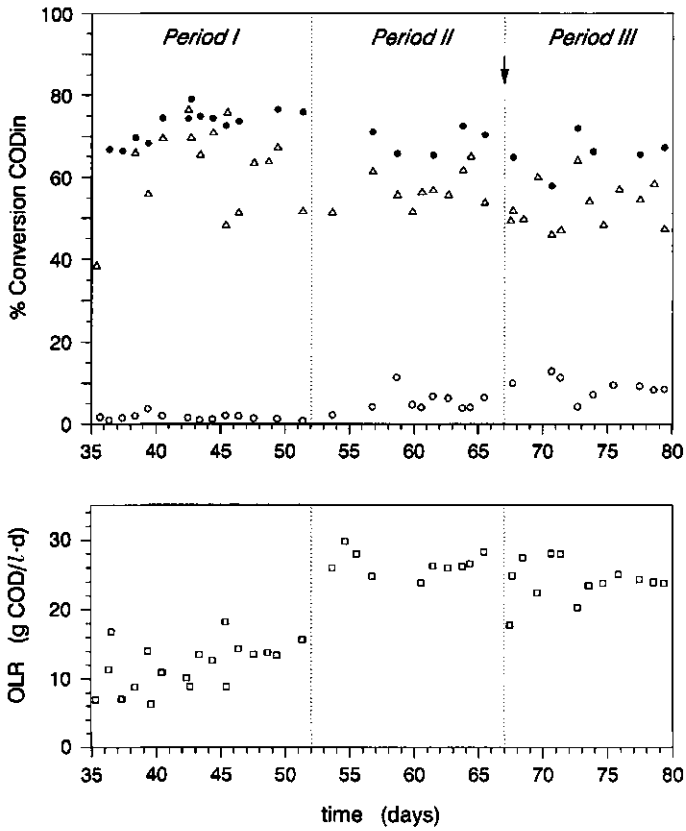


**Figure 2.** Performance of UASB reactor fed with hemp stem wood TMP wastewater. •) COD removal, % $COD_{in}$; ▵) methanogenesis, % $COD_{in}$; ○) VFA in effluent, % $COD_{in}$; □) OLR, g $COD \cdot l^{-1}d^{-1}$. Arrow indicates partial removal of sludge from the UASB reactor (see Table 3).

The specific acetoclastic activity of the sludge in both UASB reactors, sampled on days 67 and 80, was high, averaging approximately 1 g COD·g $VSS^{-1}d^{-1}$ during anaerobic treatment of both hemp stem wood and hemp bark TMP effluent (Table 3). Sludge yield during anaerobic treatment of hemp stem wood TMP wastewater based on the net increase in VSS of the retained sludge, corresponded to approximately 0.15 g VSS·g $COD_{removed}^{-1}$, while on basis of a COD balance a 0.18 g $COD_{cells}$·g $COD_{bd}^{-1}$ cell yield was calculated.

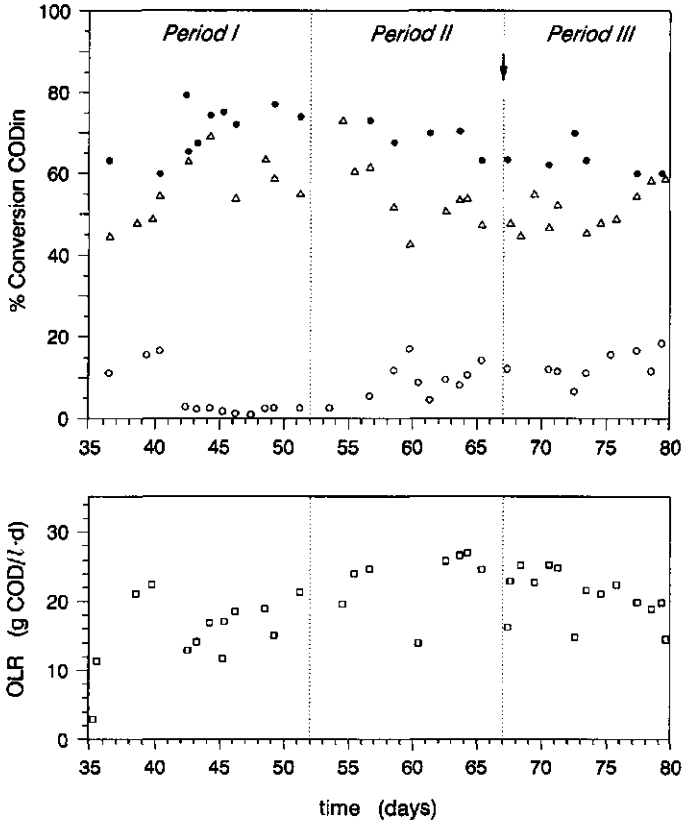


**Figure 3.** Performance of UASB reactor fed with hemp bark TMP wastewater. •) COD removal, % $COD_{in}$; ▵) methanogenesis, % $COD_{in}$; ○) VFA in effluent, % $COD_{in}$; □) OLR, g $COD \cdot l^{-1}d^{-1}$. Arrow indicates partial removal of sludge from the UASB reactor (see Table 3).

During the course of the experiment with hemp bark TMP wastewater, the granules were gradually converted to a more flocculent sludge. This effect was quantified by measuring the sludge volume index (SVI). The original seed sludge had an SVI of 24 $ml \cdot g\ VSS^{-1}$, while at day 80 an SVI of 31 and 45 $ml \cdot g\ VSS^{-1}$ was measured for the sludge cultivated in the

hemp stem wood and hemp bark TMP wastewater-fed UASB reactors, respectively. The higher SVI value for the sludge cultivated on hemp bark TMP corresponded to poor retention of newly grown biomass in the UASB reactor. The calculated cell yield during anaerobic treatment of hemp bark TMP wastewater accounted for 0.16 g $COD_{cells}$·g $COD_{bd}^{-1}$. Nonetheless, no increase in the retained sludge VSS was observed during the first 67 days, while a net loss of biomass was observed over Period III (Table 3). Wash-out of small sludge particles from the UASB reactor was observed occasionally after day 63. Increasing VFA levels in the anaerobic effluent of the hemp bark TMP wastewater-fed UASB reactor, illustrate the decreasing methanogenic capacity, resulting from the gradual loss in biomass (Figure 3).

**Table 3.** Sludge concentration and specific acetoclastic methanogenic activity during continuous anaerobic treatment of hemp TMP wastewaters.

| | Stem wood TMP wastewater | | Bark TMP wastewater | |
|---|---|---|---|---|
| day | VSS | Acetoclastic Activity | VSS | Acetoclastic Activity |
| | ($g \cdot l^{-1}$) | (g COD·g $VSS^{-1}d^{-1}$) | ($g \cdot l^{-1}$) | (g COD·g $VSS^{-1}d^{-1}$) |
| 0 | 20.0 | 0.96 | 20.0 | 0.96 |
| 67 | 28.1[a] | 0.88 | 19.9[b] | 1.12 |
| 80 | 17.4 | 0.95 | 12.1 | 1.05 |

[a] Sludge concentration adjusted to 13.8 g $VSS \cdot l^{-1}$.
[b] Sludge concentration adjusted to 14.9 g $VSS \cdot l^{-1}$.

Lignin concentrations in hemp TMP wastewaters were substantial, accounting for approximately 43% and 24% of the influent COD in hemp stem wood and hemp bark TMP wastewater, respectively. Lignin removal, monitored by the loss of $UV_{280}$ during passage of the anaerobic reactor, increased gradually during start-up of the UASB reactors (Table 2). Maximal $UV_{280}$ removal was obtained after 7-8 weeks and averaged 45 and 31% for the hemp stem wood and hemp bark TMP wastewater-fed UASB reactors, respectively. Color removal, measured as $VIS_{440}$, was for both columns minimal at start-up and after prolonged operation reached a plateau ranging between 31-35% (Table 2).

The molecular weight distribution of the lignin fraction removed during anaerobic treatment was studied by GPC. Elution profiles of untreated and anaerobically treated hemp stem wood TMP wastewater are displayed in Figure 4. From this figure it can be seen that the lignin present in untreated hemp stem wood TMP wastewater consists almost exclusively of low molecular weight material (<2.2 kD). During anaerobic treatment, major removal of lignin is obtained in the lower LMW fraction, which corresponds to the removal of monomeric lignin derivatives, having a similar elution pattern from the GPC as guaiacol.

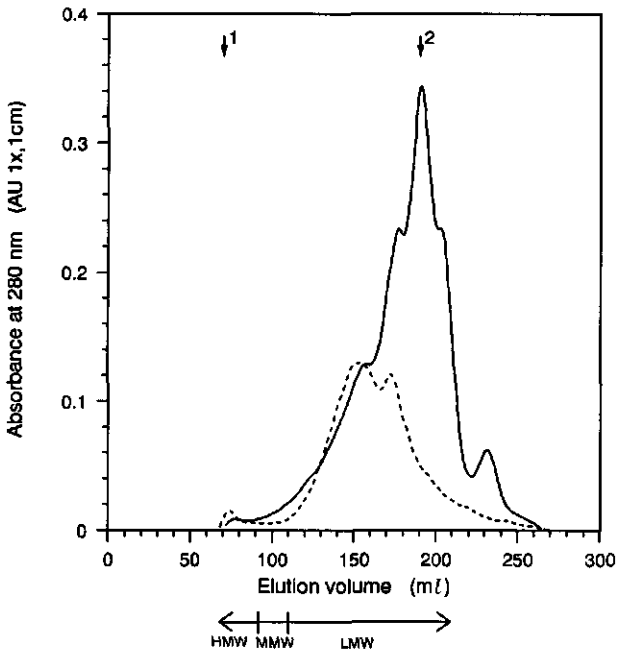


**Figure 4.** The molecular weight distribution of lignin (measured as $UV_{280}$) in untreated (——) and anaerobically treated (----) hemp stem wood TMP wastewater. Calibration of GPC profiles was obtained by elution of three lignin molecular weight fractions previously separated by ultrafiltration of biologically treated soda pulping black liquor. LMW (<2.2 kD); MMW (2.2 to 34 kD); HMW(>34 kD). Arrows indicate the elution volumes of high and low molecular weight reference compounds: arrow 1, blue dextran (>$10^3$ kD); arrow 2, guaiacol (a monomeric lignin model compound).

Subsequent modification of lignin during aerobic post-treatment was studied in batch aeration assays. Figure 5 illustrates the gradual increase in molecular weight of the lignin during aerobic post-treatment. Prior to exposure upon air, the lignin in the effluent of the anaerobic reactor consisted almost completely of LMW material. During aeration, low

molecular weight lignin is gradually transformed to medium molecular weight lignin, as can be seen from the slow drift of the major peak to the left, while at the same time an exclusion peak is formed at the outer most left side of the elution profile, indicating extensive polymerization to high molecular weight (HMW) lignin in excess of 34 kD. During this test a small increase in $UV_{280}$ was observed (9%), while the color of wastewater increased significantly by 211%.

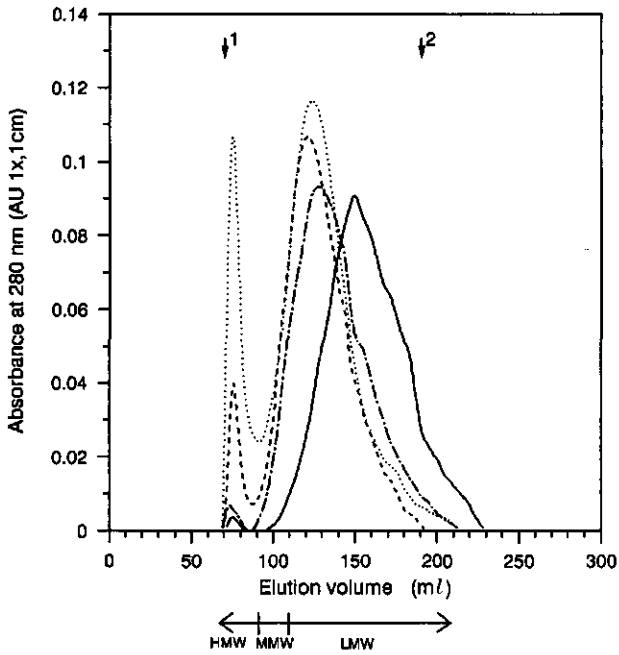


**Figure 5.** The molecular weight distribution of lignin (measured as $UV_{280}$) during aerobic activated sludge treatment of anaerobically treated hemp stem wood TMP wastewater. (——) day 0; (— · — ·) day 1; (— — —) day 4; (····) day 10. Calibration of GPC profiles was obtained by elution of three lignin molecular weight fractions previously separated by ultrafiltration of biologically treated soda pulping black liquor. LMW (< 2.2 kD); MMW (2.2 to 34 kD); HMW (> 34 kD). Arrows indicate the elution volumes of high and low molecular weight reference compounds: arrow 1, blue dextran (> $10^3$ kD); arrow 2, guaiacol (a monomeric lignin model compound).

Incubation of anaerobically treated hemp stem wood TMP wastewater with activated sludge in absence of oxygen in a control experiment flushed with $N_2$ gas, showed no change in the molecular weight distribution, indicating that oxygen is a prerequisite for the observed polymerization (results not shown).

## Discussion

Discharge of untreated TMP wastewaters leads to oxygen deficiencies and toxicity problems in the recipient water bodies (Leach and Thakore, 1976; Walden 1980). Biological wastewater treatment therefore, should aim at *i*) the reduction of the organic load, which upon decomposition will result in oxygen consumption from the aquatic environment and *ii*) the removal of wood extractives, which account for the major source of aquatic toxicity.

TMP wastewaters are generally highly biodegradable and not inhibitory to methanogens (Jurgensen *et al.*, 1985; Sierra-Alvarez *et al.*, 1990b) and therefore are highly suitable for anaerobic treatment. During anaerobic treatment of TMP wastewaters, BOD is removed to a high extent (Habets and de Vegt, 1991; Latola 1985), while no energy is required, as would be necessary for aerobic treatment using aeration. Wood extractives however, are not suitably degraded during anaerobic treatment (Liver and Hall, 1996; Sierra-Alvarez *et al.*, 1990a), albeit that some biotransformation reactions (hydrogenation of double bonds) have been noted (McFarlane and Clark, 1988). On the other hand, wood extractives are removed during aerobic post-treatment (Easty *et al.*, 1978; Leach *et al.*, 1978) and by aerobic bacterial cultures (Hemingway and Greaves, 1973; Bicho *et al.*, 1995; Mohn, 1995; Wilson *et al.*, 1996). Sequenced anaerobic-aerobic treatment of pulping liquors therefore, enables substantial reduction of BOD as well as aquatic toxicity at relatively low cost (Kortekaas *et al.*, 1994).

As hemp is a non-woody fibre, one may question if the anaerobic treatability of hemp TMP wastewaters is comparable to the treatment efficiencies previously obtained with wood-derived TMP wastewaters. Laboratory-scale continuous experiments have shown that hemp TMP wastewaters were somewhat more biodegradable than wood-derived TMP wastewater. The acidifiable fractions of hemp stem wood, hemp bark, and spruce TMP wastewater were found to be approximately 62-64%, 64-68% and 56% (Sierra-Alvarez *et al.*, 1990b), respectively. The COD removal efficiencies during anaerobic treatment of hemp TMP wastewaters and wood-derived TMP wastewaters were similar. Maximal COD removal during anaerobic treatment of hemp stem wood and hemp bark TMP wastewater was on average 71-73%, which is only slightly higher than the efficiency of 68% reported for anaerobic treatment of spruce TMP wastewater (Sierra-Alvarez *et al.*, 1990b).

Excellent COD removal efficiencies, obtained at high loading rates, demonstrate the suitability of anaerobic systems for the treatment of hemp TMP pulping wastewaters. The average removal efficiency of the anaerobically biodegradable COD ($E_{bd}$) was 97 and 93%

during anaerobic treatment of hemp stem wood TMP wastewater at loading rates up to 13 and 27 g $COD \cdot l^{-1}d^{-1}$, respectively. The average removal efficiency of the anaerobically biodegradable COD was 96 and 87% during anaerobic treatment of hemp bark TMP wastewater at loading rates up to 16 and 26 g $COD \cdot l^{-1}d^{-1}$, respectively.

Increases of the sludge loading by removal of biomass from the reactors on day 67, caused a slight overloading of the methanogenic capacity of the reactors, as is indicated by the increase of VFA levels in the anaerobic effluent. During Period III (day 67-80), the conversion to methane approximates to the maximal acetoclastic capacity (specific activity x VSS content) of the reactors (Table 3).

The observed acetoclastic activity of the sludges from the hemp stem wood TMP and hemp bark TMP-fed reactors, approximately 1 g $COD \cdot g\ VSS^{-1}d^{-1}$, compares well to the methanogenic activity of 1.4 g $COD \cdot g\ VSS^{-1}d^{-1}$ determined on a mixture of acetate, propionate and butyrate for anaerobic sludge sampled from a spruce TMP wastewater-fed UASB reactor (Sierra-Alvarez *et al.*, 1990b). The high acetoclastic activity of the anaerobic sludge demonstrates the absence of methanogenic inhibition exerted by hemp TMP wastewaters and confirms the results obtained in batch methanogenic toxicity assays and previous reports on wood-derived TMP wastewaters (Sierra-Alvarez *et al.*, 1991; Sierra-Alvarez *et al.*, 1994).

Evaluation of the COD balance over the UASB reactors demonstrated a steady sludge growth during anaerobic treatment of hemp TMP wastewaters. The calculated cell yields accounted 0.18 g $COD_{cells} \cdot g\ COD_{bd}^{-1}$ and 0.16 g $COD_{cells} \cdot g\ COD_{bd}^{-1}$ for the hemp stem wood and the hemp bark reactor, respectively. Calculated cell yields correspond well to literature data for non-acidified wastewaters (Sierra-Alvarez *et al.*, 1988; Hulshoff Pol, 1989) and are supported by the determination of 0.15 g $VSS \cdot g\ COD_{removed}^{-1}$ of retained sludge during anaerobic treatment of hemp stem wood TMP wastewater.

During the course of the experiment in the hemp bark TMP wastewater-fed UASB reactor, gradually a more flocculent sludge had developed. Decrease of the settling characteristics, as indicated by an increase of SVI, led to some wash-out of biomass from the hemp bark TMP wastewater-fed UASB reactor, which did not allow for any retention of newly grown cells and caused a small net decrease of biomass when high loading rates were applied. Better settling characteristics of hemp stem wood TMP-fed sludge indicates, that the development of a more flocculent sludge in the hemp bark TMP-fed reactor was related to wastewater composition. Sludge wash-out from one-step UASB reactors treating non-acidified carbohydrate wastewaters was observed previously (Sierra-Alvarez *et al.*, 1988; Hulshoff Pol, 1989) and was attributed to the formation of loosely attached acidogenic bacteria

(Alphenaar, 1994). Pre-acidification of carbohydrate-rich wastewaters and increase of upflow velocity, conditions which are typically fulfilled in full-scale UASB systems, contribute to preservation of granular sludge settling characteristics.

A major factor governing the COD efficiency during treatment of pulping wastewaters, is the share of lignin which survives biological treatment. Nonetheless, so far little attention has been paid to the fate of lignin during biological treatment of pulping wastewaters. Degradation studies show that bacterial degradation of lignin under anaerobic as well as aerobic conditions, is limited and highly governed by molecular weight. Bacterial mineralization is observed only for low molecular weight lignin (MW < 1000 D) (Zeikus *et al.*, 1982; Colberg and Young, 1985; Kern and Kirk, 1987). In correspondance, removal of low molecular weight lignin during anaerobic and aerobic treatment of pulping wastewaters has been demonstrated in several studies (Sierra-Alvarez *et al.*, 1990b; Kortekaas *et al.*, 1998; Pellinen and Salkinoja-Salonen, 1985b; Jørgensen *et al.*, 1995). The lignin removal during biological treatment of wood pulping wastewaters is generally low, due to the relatively high share of high MW lignin. Reported lignin removal efficiencies, measured as $UV_{280}$, range from 4-26% during thermophilic anaerobic treatment of wood-derived TMP wastewaters (Rintala and Lepistö, 1992), 15-20% during anaerobic treatment of wood-derived soda pulping liquors (Sierra-Alvarez *et al.*, 1990b) and 10-20% during activated sludge treatment of kraft and sulfite pulping effluents (Pandila, 1973; Ganczarczyk and Obiaga, 1974). Wastewater lignin is a polydispers material, which varies considerably in molecular size. In chemical pulping wastewaters, the molecular weight distribution of lignin generally ranges from $10^2$ to $10^5$ D (Fengel and Wegener, 1984; Forss *et al.*, 1989b). Lignin encountered in mechanical pulping wastewaters, however, has considerably lower molecular weight (Forss *et al.*, 1989a). In this study, we demonstrated that hemp stem wood TMP lignin consisted almost completely of lignin derivatives smaller than approximately $2 \cdot 10^3$ D, indicating a relatively large fraction of low molecular weight lignin, amendable for biodegradation.

Lignin removal during anaerobic treatment of hemp TMP pulping wastewaters was remarkably high, averaging 45 and 31% for the hemp stem wood and hemp bark TMP wastewater-fed reactor, respectively. The lignin fraction removed during anaerobic treatment of hemp stem wood TMP wastewater, corresponded to lowest molecular weight derivatives, having an elution pattern corresponding to the monomeric lignin model compound, guaiacol. Further evidence for lignin degradation is the observation that the COD removal (73%) exceeded the COD of the non-lignin fraction (57%) in the UASB treating hemp wood TMP

wastewater.

During aerobic post-treatment of anaerobically treated hemp stem wood TMP wastewater, an increase of color levels was observed. Strong coloration during aerobic post-treatment of anaerobically treated pulping wastewaters was reported previously (Rintala and Vuoriranta, 1988; Rintala and Lepistö, 1992) and was attributed to increase of phenolic groups by demethylation of lignin during anaerobic treatment and subsequent autoxidation of lignin-derived phenolic compounds during exposure upon air (Field *et al.*, 1989; Sierra-Alvarez *et al.*, 1990b). In this study, it was demonstrated that color formation during aerobic post-treatment of anaerobically treated pulping wastewater is accompanied by a dramatic increase of lignin molecular weight from < 2.2 kD to greater than 34 kD, which supports the hypothesis of oxidative polymerization of lignin derived phenolic compounds.

The observed increase of lignin MW after anaerobic-aerobic treatment shall provide improved performance of ultrafiltration membranes during optional tertiary treatment. Also the elimination of BOD prior to filtration shall reduce membrane fouling problems.

## References

**Alphenaar, P.A.** (1994). Anaerobic granular sludge: Characterization and factors affecting its performance. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.

**APHA** (1992). Standard methods for examination of water and wastewater, 18th edn., American Public Health Association, Washington D.C., USA.

**Bicho, P.A., Martin, V., and Saddler, J.N.** (1995). Growth, Induction, and substrate specificy of dehydroabietic acid-degrading bacteria isolated from kraft mill effluent enrichment. Appl. Environ. Microbiol., 61, 3245-3250.

**Colberg, P.J., and Young, L.Y.** (1985). Anaerobic degradation of soluble fractions of [14 C lignin] lignocellulose. Appl. Environ. Microbiol., 49, 345-349.

**Donlon, B., Razo-Flores, E., Lettinga, G., and Field, J.A.** (1995). Toxicity of N-substituted aromatics to acetoclastic methanogenic activity in granular sludge. Appl. Environ. Microbiol., 61, 3889-3893.

**Easty, D.B., Borchardt, G., and Wabers, A.** (1978). Wood-derived toxic compounds. TAPPI J., 61(10), 57-60.

**FAO** (1995). Forest products annual yearbook 1993, Food and Agricultural Organisation, Rome, Italy.

**Fengel, D., and Wegener, G.** (1984). Wood: Chemistry, Ultrastructure, Reactions. Walter de Gruyter, Berlin, 151-157.

**Field, J.A., Kortekaas, S., and Lettinga, G.** (1989). The tannin theory of methanogenic toxicity. Biol. Wastes, 29, 241-262.

**Forss, K., Jokinen, K., Savolainen, M., and Williamson, H.** (1989a). Utilization of enzymes for effluent treatment in pulp and paper industry. Paperi ja Puu, 71, 1108-1112.

**Forss, K., Kokkonen, R., and Sågfors, P-E.** (1989b). Determination of the molar mass distribution of lignins by gel permeation chromatography. In: Glasser W.G. and Sarkanen S. (eds). Lignin, properties and materials. American Chemical Society, Washington, 124-133.

**Ganczarczyk, J., and Obiaga, T.** (1974). Mechanism of lignin removal in activated sludge treatment of pulp mill effluents. Wat. Res., 8, 857-862.

**Gerbasi, B., Stuart, P.R., Arsenault, F., and Zaloum, R.** (1993). Techno-economic assessment of several closed cycle technology alternatives for an existing TMP-newsprint mill. Pulp Paper Canada, 94(12), 123-128.

**Habets, L.H.A., and de Vegt, A.L.** (1991). Anaerobic treatment of bleached TMP and CTMP effluent in the BIOPAQ UASB system, Wat. Sci. Tech., 24(3/4), 331-345.

**Hemingway, R.W., and Greaves, H.** (1973). Biodegradation of resin acid sodium salts. TAPPI J., 56(12), 189-192.

**Hulshoff Pol, L.W.** (1989). The phenomenon of granulation of anaerobic sludge. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.

**Jørgensen, G., Carlberg, G.E., Hoel, H., and Lystad, E.** (1995). Lignans in TMP effluents: fate and effects. TAPPI J., 78(8), 171-176.

**Jurgensen, S.J., Benjamin, M.M., and Ferguson, J.F.** (1985). Treatability of thermomechanical pulping process effluents with anaerobic biological reactor. In: Proceedings TAPPI 1985 Environmental Conference, TAPPI Press, Atlanta GA, 83-92.

**Kern, H.W., and Kirk, T.K.** (1987) Influence of molecular size and ligninase pretreatment on the degradation of lignins by *Xanthomonas* sp. strain 99. Appl. Environ. Microbiol., 53, 2242-2246.

**Kortekaas, S., Doma, H.S., Potapenko, S.A., Field, J.A., and Lettinga, G.** (1994). Sequenced anaerobic-aerobic treatment of hemp black liquors. Wat. Sci. Tech., 29(5-6), 409-419.

**Kortekaas, S., Soto, M., Vicent, T., Field, J.A., and Lettinga, G.** (1995). Contribution of extractives to methanogenic toxicity of hemp black liquor. J. Ferment. Bioeng., 80, 383-388.

**Kortekaas, S., Vidal, G., Yan-Ling, H., Lettinga, G., and Field, J.A.** (1998). Anaerobic-aerobic treatment of toxic pulping black liquor with upfront effluent recirculation. J. Ferment. Bioeng., 86(1), 97-110.

**Kovacs, T.G., and Voss, R.H.** (1992). Biological and chemical characterization of newsprint/specialty mill effluents. Wat. Res., 26, 771-780.

**Latola, P.K.** (1985). Treatment of different wastewaters from pulp and paper industry in methane reactors. Wat. Sci. Tech., 17(1), 223-230.

**Leach, J.M., and Thakore, A.N.** (1976). Toxic constituents of mechanical pulping effluents. TAPPI J., 59, 129-132.

**Leach, J.M., Mueller, J.C. and Walden, C.C.** (1978). Biological detoxification of pulp mill effluents. Process Biochemistry, 13(1), 18-21.

**Lettinga, G., Field, J.A., Sierra-Alvarez, R., van Lier, J.B., and Rintala, J.** (1991). Future perspectives for the treatment of forest industry wastewaters. Wat. Sci. Tech., 24(3/4), 91-102.

**Li, J., O'Hagan, T., and MacLeod, J.M.** (1996). Using ultrafiltration to analyze the molecular mass distribution of kraft lignin at pH 13. Can. J. Chem. Engineer., 74(1), 110-117.

**Liver, S.F., and Hall, E.R.** (1996). Interactions of resin acids with aerobic and anaerobic biomass-I. Degradation by non-acclimated inocula. Wat. Res., 30, 663-671.

**Lo, S.N., Lavallee, H.C., Rowbottom, R.S., Meunier, M.A., and Zaloum, R.** (1994). Activated sludge treatment of TMP mill effluents. Part 1: effluent characterization and treatability study. TAPPI J., 77(11), 167-178.

**McFarlane, P., and Clark, T.** (1988). Metabolism of resin acids in anaerobic systems. Wat. Sci. Tech., 20, 273-276.

**Mohn, W.W.** (1995). Bacteria obtained from a sequencing batch reactor that are capable of growth on dehydroabietic acid. Appl. Environ. Microbiol., 61, 2145-2150.

**Pandila, M.M.** (1973). Micro-organisms associated with microbiological degradation of lignosulphonates: a review of literature. Pulp Paper Mag. Can., 74, 80-84.

**Pellinen, J., and Salkinoja-Salonen, M.S.** (1985a). Aqueous size exclusion chromatography of industrial lignins. J. Chromatogr., 322, 129-138.

**Pellinen, J., and Salkinoja-Salonen, M.S.** (1985b). Characterization and biodegradability of lignin containing wastewater from forest industry. In: Bjørseth A. and Angeletti G. (eds). Organic micropollutants in the aquatic environment. Reidel Publishing Company, Dordrecht, 354-362.

**Rintala, J., and Vuoriranta, P.** (1988), Anaerobic-aerobic treatment of thermomechanical pulping effluents. TAPPI J., 71(9), 201-207.

**Rintala, J., and Lepistö, S.** (1992). Anaerobic treatment of thermomechanical pulping whitewater at 35-70°C. Wat. Res., 26, 1297-1305.

**Sierra-Alvarez, R., Hulshoff Pol, L.W., and Lettinga, G.** (1988). Start-up of a UASB reactor on carbohydrate substrate. In: Lettinga G., Zehnder A.J.B., Grotenhuis J.T.C., Hulshoff Pol L.W. (eds). Granular anaerobic sludge; microbiology and technology, Pudoc, Wageningen, The Netherlands, 223-229.

**Sierra-Alvarez, R., and Lettinga, G.** (1990). The methanogenic toxicity of wood resin compounds. Biol. Wastes, 33, 211-226.

**Sierra-Alvarez, R., Kato, M., and Lettinga, G.** (1990a). The anaerobic biodegradability of paper mill wastewater constituents. Environ. Technol., 11, 891-898.

**Sierra-Alvarez, R., Kortekaas, S., van Eekert, M., Harbrecht, J., and Lettinga, G.** (1990b). The continuous anaerobic treatment of pulping wastewaters. J. Ferment. Bioengineer., 70, 119-127.

**Sierra-Alvarez, R., Kortekaas, S., van Eekert, M., and Lettinga, G.** (1991). The anaerobic biodegradability and methanogenic toxicity of pulping wastewaters. Wat. Sci. Tech., 24(3/4), 113-125.

**Sierra-Alvarez, R., Field, J.A., Kortekaas, S., and Lettinga, G.** (1994). Overview of the anaerobic toxicity caused by organic forest industry wastewater pollutants. Wat. Sci. Tech., 29(5-6), 353-363.

**Stenberg, E., and Norberg, G.** (1977). Effluent from manufacturing of thermomechanical pulps and their treatment, In: Proc. Int. Mechanical Pulping Conference 1977, Vol 2, Canadian Pulp and Paper Association, Montreal, Quebec, Canada, 14:1-14:23.

**van der Werf, H.M.G., van der Veen, J.E.H., Bouma, A.T.M, and ten Cate, M.** (1994). Quality of hemp (*Cannabis sativa* L.) stems as raw material for paper. Ind. Crop. Prod., 2(3), 219-227.

**Walden, C.C.** (1980). Biological effects of pulp and paper mill effluents, In: Moo-Young M. and Campbell W. R. (eds.), Proceedings of the 6th International Fermentation Symposium. V.2. Fuels, chemicals, foods and waste treatment. July 20-25, London, Canada p.669-676.

**Wilson, A.E.J., Moore, E.R.B., and Mohn, W.W.** (1996). Isolation and characterization of isopimaric acid-degrading bacteria from a sequencing batch reactor. Appl. Environ. Microbiol., 62, 3146-3151.

**Zeikus, J.G., Wellstein, A.L., and Kirk, T.K.** (1982). Molecular basis for the biodegradative recalcitrance of lignin in anaerobic environments. FEMS Microbiol. Lett., 15, 193-197.

General Discussion.

# 6

## Introduction

The pulp and paper industry is a major consumer of natural resources, notably plant fibres, energy and water. In 1996, the production of pulp fibres worldwide was estimated at 413 x $10^{12}$ tons of wood and 25 x $10^{12}$ tons of nonwood fibres (FAO, 1998). Corresponding pulp production figures, however, are notably lower, as is illustrated by the estimated volume of 157 x $10^{12}$ tons of wood pulp produced in 1996, indicating that a considerable share of the raw material input of pulp manufacture is not incorporated in the final product. Major loss of organic material occurs in the pulping process; whereas, depending upon the type of process, from 5 up to 60% of the organic material present in the fibre feedstock is extracted into the process waters. The set-up of a sustainable Dutch agro-based pulp industry converting hemp should aim at negligible impact on the environment. Discharge of process waters from the pulp and paper manufacture is a major focus of concern. Well established standards for discharge of pulp and paper industry wastewaters concern chemical and biological oxygen demand (COD and BOD), adsorbable organic halogens (AOX) and aquatic toxicity. Since pulp liquor incineration is considered to be too expensive in relation to the size of the relatively small non-wood pulp mills, biological wastewater treatment methods are the first measures to meet environmental legislation standards.

Natural wood components are known to play a major role in the treatability of wood pulping wastewaters. Various classes of natural wood components, present in pulp and paper industry wastewaters, have been shown to be toxic and poorly biodegradable in biological treatment systems. Resinous compounds and low molecular weight apolar lignin derivatives are considered to be major contributors to the inhibition of the methanogenic population in anaerobic treatment systems. Additionally, lignin is known to be highly recalcitrant to bacterial degradation, under anaerobic as well as aerobic conditions.

In this dissertation, the results of studies are presented regarding the extent and source of toxicity in hemp pulping wastewaters. Subsequently, the suitability of anaerobic and aerobic treatment systems for removal of dissolved organic material and toxicity are discussed. Sequenced anaerobic-aerobic treatment of hemp pulping wastewaters was considered favourable, combining relatively low requirement of aeration energy, recovery of methane and low excess sludge production during anaerobic treatment on the one hand with aerobic detoxification and polishing on the other hand.

**Toxicity of hemp pulping liquors**

During the anaerobic degradation of complex organic material, 70% of the conversion of COD into methane occurs via acetoclastic methanogens. Therefore, the inhibition of acetoclastic methanogens was studied as a model for methanogenic toxicity exerted by hemp black liquors. Wood-derived black liquors are known to be highly toxic to methanogenic bacteria at relatively low concentrations. As hemp is a non-woody feedstock, one may question if black liquors from hemp are equally toxic as those from wood.

Hemp black liquors showed 50% inhibition towards acetoclastic methanogenic activity at concentrations ranging from 2 - 6 g COD/*l*. This concentration range is 10 to 100 times lower than the black liquor concentrations in industry and comparable to the methanogenic inhibition of wood pulping wastewaters. Black liquors originating from the pulping of hemp stem wood, were somewhat more toxic than black liquors originating from hemp bark fibres.

The results of chapter 2 demonstrate that extractives (resinous compounds) are the major source of methanogenic toxicity in hemp pulping liquors. After removal of the extractive fraction from hemp black liquor by ethyl ether extraction, complete wastewater detoxification was achieved, indicating that the extractive fraction, which is responsible for about one tenth of the COD, is the main source of inhibitory substances in hemp black liquors. Since ethyl ether extraction did not significantly remove lignin, it can be concluded that the major share of the lignin in hemp black liquor is non-toxic. The high toxicity of the hemp extractive fraction was confirmed in batch assays, which demonstrated that these compounds were mostly responsible for the toxicity removed during ethyl ether extraction and only slightly less toxic than softwood resin.

Hemp extractives are predominantly composed of terpenic compounds. Research on drug type hemp cultivars shows two major fractions, ie. cannabinoids and terpenes, with minor concentrations of non-cannabinoid phenols and alkanes; whereas, among the numerous compounds detected in hemp at trace concentrations also extremely toxic compounds such as dihydrostilbenes, are reported. Terpenes in hemp are mainly monoterpenes and sesquiterpenes, which are apolar hydrocarbon compounds with a demonstrated methanogenic inhibition. Monoterpenes are highly inhibitory towards methanogenic bacteria at concentrations of 100 mg/*l*. Our results indicate that sesquiterpenes present in hemp, are only mildly toxic (30% inhibition of acetoclastic methanogenic activity at 800 mg/*l*). Cannabinoids are reported to have a bactericidal effect against gram positive bacteria, however, no methanogenic inhibition was demonstrated in batch assays up to 200 mg/*l*, which might be

due to the poor solubility of these compounds.

Terpenic compounds in plants have an antimicrobial function. The methanogenic toxicity of terpenic compounds is attributed to the lipophilic character of these compounds, which causes them to partition into the bacterial membranes, affecting the membrane functions. With respect to the inhibition of methanogenic bacteria and structural features of apolar compounds like terpenes and chlorinated phenolics, some general principles can be recognized (Sierra-Alvarez et al.,1994). Structural features which enhance compound apolarity such as alkyl side chains, unsubstituted benzene rings or chloro groups contribute to increasing methanogenic toxicity (Sierra-Alvarez and Lettinga, 1991). In contrast, compounds rich in polar groups are not very inhibitory. Very apolar compounds however, such as triterpenes, tend to be less toxic, since with excessive apolarity, the dissolution of compounds becomes almost negligible, which makes these compounds virtually non-bioavailable and therefore non-toxic.

Regarding the treatment of pulp and paper industry wastewaters, the poor biodegradative capacity of anaerobic microorganisms for toxic terpenoid hydrocarbons reveals a basic difference between anaerobic and aerobic treatment systems. Whereas terpenes are readily aerobically degraded, anaerobic microorganisms lack the potential for biodegradation of terpenes. Adaptation of anaerobic microorganisms to the presence of toxic terpenes, therefore, is strongly hindered by the recalcitrance of toxic terpenoid hydrocarbons in anaerobic environments, obstructing adequate detoxification in the bacterial micro-environment.

New developments in industry indicate progressive closure of water-cycles and incorporation of biological treatment systems within the water-cycle of the pulp and paper mill, leading to increasing wastewater concentrations. Detoxification measures, therefore, will remain a vital topic for future research.

**Sequenced anaerobic-aerobic treatment**

The high methanogenic inhibition exerted by hemp bark and stem wood black liquor does not necessarily preclude anaerobic treatment. Identification of extractives as the major source of methanogenic toxicity in hemp black liquors enables determination of dilution requirements and development of detoxification methods. Long term continuous experiments in UASB reactors have shown, that anaerobic treatment of these toxic wastewaters is still feasible, provided that adequate dilution is applied. Chapter 3 demonstrates that during sequenced anaerobic-aerobic treatment of diluted hemp bark and stem wood black liquors, high organic loadings could be achieved. Very high BOD removal rates could be attributed to the

anaerobic step, while a substantial share of the BOD removed was recovered as methane. Despite the toxicity of hemp black liquors, growth and methanogenic activity of the biomass in the anaerobic reactors was stable throughout the course of the experiment. Aerobic post-treatment provided almost complete BOD elimination and substantial detoxification.

In case that sufficient dilution water cannot be obtained, detoxification measures should be considered. There are a number of effective detoxification methods based on the removal of the wood extractive fraction by precipitation with divalent salts (Welander, 1988) or lowering the pH (Field et al., 1991; Sierra-Alvarez et al., 1991). Additionally, it has been observed, that with CTMP wastewaters considerable detoxification of pulping effluents can be achieved by the removal of colloidal material on which extractives were adsorbed (McCarthy et al., 1990; Richardson et al., 1991). A disadvantage of these detoxification methods is that the precipitated sludges have to be separated from the wastewater and disposed of.

A highly elegant method for detoxification is the utilization of effluent from the aerobic post-treatment for dilution. The toxic extractive fraction in hemp black liquors is considered aerobically biodegradable and therefore suitably removed during aerobic biological treatment. Removal of the toxic extractive fraction during aerobic post-treatment provides substantial detoxification, which indeed has been observed during anaerobic-aerobic treatment of diluted hemp black liquors.

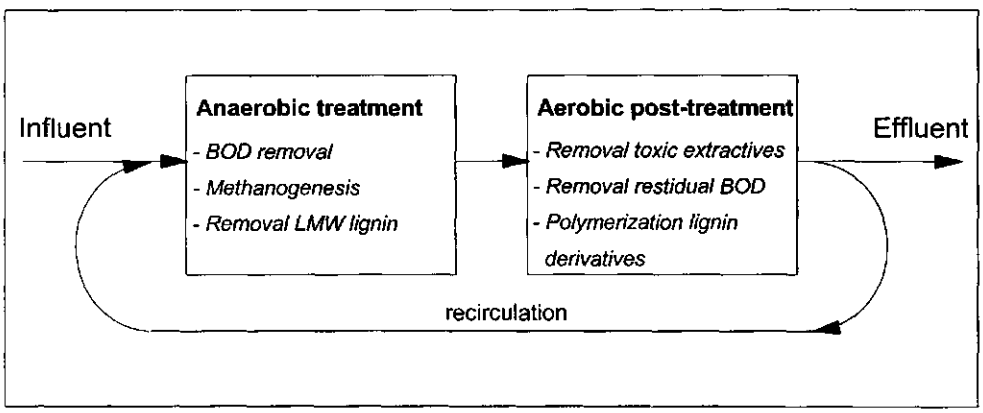


**Figure 1.** Flow sheet for anaerobic-aerobic treatment of toxic pulping liquors with recirculation of aerobic effluent for upfront dilution.

## Upfront dilution

The observation that methanogenic toxicity in pulping effluents was highly eliminated during aerobic wastewater treatment, but not during anaerobic treatment, has led to the proposal of a detoxification strategy denominated upfront dilution (Habets and de Vegt, 1991). This detoxification method is based on the sequenced anaerobic-aerobic treatment of the pulping wastewaters, recirculating the aerobic effluent for upfront dilution of the incoming influent to sub-toxic concentrations (Figure 1).

In chapter 4, the treatment of highly toxic hemp stem wood black liquor (HSWBL) in lab-scale UASB reactors with upfront dilution was compared with direct anaerobic treatment and with direct aerobic treatment. Direct anaerobic treatment of 12 g COD/*l* HSWBL led within 14 days to almost complete inhibition of the methanogenic biomass. However, recirculation of 75% of the aerobic post-treatment effluent for upfront dilution of the toxic HSWBL, enabled anaerobic treatment at high loading rates without significant inhibition of the methanogenic activity.

Extensive detoxification was confirmed during anaerobic-aerobic treatment of 20 g COD/*l* HSWBL recirculating 86% of the aerobic effluent. COD and BOD removal after anaerobic-aerobic treatment was 72% and 97%, respectively, at an overall loading rate of 3.6 g COD/*l*·d, while 30-35% of the incoming COD was recovered as methane. Batch methanogenic assays demonstrated significant detoxification after anaerobic-aerobic treatment of HSWBL.

Compared to conventional aerobic treatment in an activated sludge process, treatment efficiencies and detoxification during sequenced anaerobic-aerobic treatment were similar. The anaerobic-aerobic treatment system however, provided substantial lower surplus sludge production, recovery of methane as an energy source, less nutrient dosage and substantial reductions in aeration costs.

## Fate of lignin

Extent and rate of bacterial lignin degradation is restricted by molecular weight. Both anaerobic and aerobic bacteria can cause extensive degradation of lignin derivatives with molecular weights of less than 600 to 1000 D, which refers to 3 to 7 lignin monomer units. Higher molecular weight lignin appears to be relatively inert to bacterial degradation, suggesting that the responsible enzyme system is intracellular.

Lignin removal during biological treatment of pulping wastewaters is generally low. In this research however, remarkably high lignin removal rates were obtained. Lignin removal during anaerobic-aerobic treatment of hemp black liquors, was generally more than 20% and accounted up to 44%, of which globally about half was obtained anaerobically. During aerobic activated sludge treatment of hemp black liquors, lignin removal ranged between 28-58%, apparently influenced by fluctuations in biomass concentrations over the experimental period.

Molecular weight distribution profiles, obtained by gel permeation chromatography, demonstrated that the average molecular weight of lignin in hemp TMP and soda pulping black liquors was relatively small. Wastewater lignin in hemp mechanical and soda pulping liquors almost exclusively consisted of molecules smaller than 2.2 kD, indicating the presence of a considerable fraction amendable for bacterial degradation.

Results of our studies, presented in chapter 5, demonstrate that the removal of lignin during anaerobic biological treatment is related to the lowest molecular weight fraction, indicating that biodegradation is the major removal mechanism as opposed to physical removal mechanisms, like precipitation and adsorption processes. Subsequent aerobic post-treatment caused polymerization of lignin to molecular weights in excess of 34 kD, which was associated with strong increases in wastewater color.

Increase of color levels due to the formation of brown-colored substances is a well known phenomenon, occurring during humification processes in soil. According to current concepts on humification (Stevenson, 1994), polyphenols are the main building blocks from which humic substances are formed. The first step in the humification process is considered to be the breakdown of plant biopolymers, causing production of low molecular weight phenols and phenolic acids, that are susceptible for enzymatic or autoxidative polymerization. Lignin derived phenolic aldehydes and acids are enzymatically converted to quinones, which condense with one another, forming darkly colored high molecular weight humic compounds. Lignin fragments with neighbouring hydroxy groups on the other hand, may abiotically be converted to quinones, providing the presence of air and neutral to alkaline conditions (Field et al., 1989; Field et al., 1991), leading to polymerization.

The fate of hemp wastewater lignin during anaerobic and aerobic treatment steps is illustrated in Figure 2. High molecular weight lignin is highly recalcitrant towards bacterial degradation under aerobic as well as anaerobic conditions. Monomeric and oligomeric lignin however, is readily metabolized, eventually leading to complete mineralization. Lignin fragments are subject to depolymerization and autoxidative condensation reactions, simultaneously.

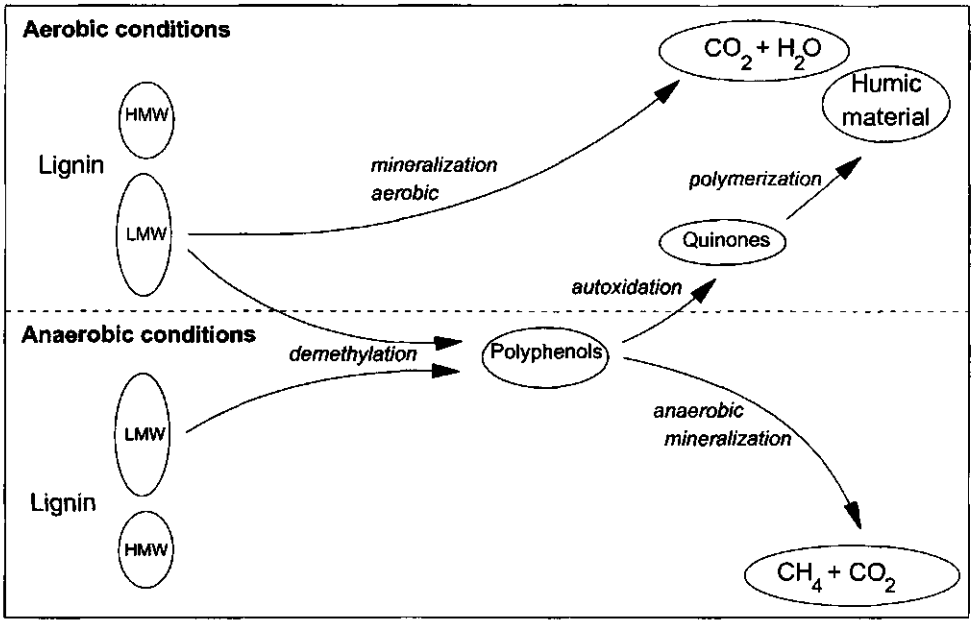


**Figure 2.** Fate of wastewater lignin during biological treatment.
HMW: high molecular weight lignin, LMW: low molecular weight lignin.

Following the example from nature, in Figure 3 a mechanism is proposed for polymerization of lignin during anaerobic-aerobic treatment of pulping wastewaters. Modification of wastewater lignin during anaerobic treatment includes loss of methoxy ($-OCH_3$) groups, generating polyphenolic compounds like o-hydroxyphenols. Acetogenic bacteria, such as *Clostridium Thermoaceticum*, *Acetobacterium woodii* and *Eubacterium limosum*, present in anaerobic treatment systems, are capable to demethoxylating lignin model compounds, leaving the hydroxylated aromatic structure intact (Daniel et al., 1988; Kaiser and Hanselmann, 1982; Bache and Pfenning, 1981; Cocaign et al., 1991). Moreover, demethoxylating activity of methanogenic biomass obtained from UASB reactors utilized in this study, has been demonstrated (Verhagen et al., 1998). Aerobic post-treatment thereupon, causes increase of pH due to stripping of carbon dioxide, and oxygenation of the water, thus fulfilling the prerequisites for autoxidative polymerization (Field et al., 1989; Field et al., 1991). In analogy to humification processes, wastewater lignin fragments will polymerize, forming inert high molecular weight polymers, which do not contain BOD nor display toxicity.

Subsequent discharge of pulping wastewaters leads to inclusion of lignin from anthropogenic source in the humic aquatic organic matter fraction of receiving water bodies (Santos and Duarte, 1998). The humic fraction of aquatic organic matter acts as sink for organic carbon, which by being of a high molecular weight and highly refractory is characterized by mean residence times ranging from several hundred to several thousand years (Stevenson, 1994).

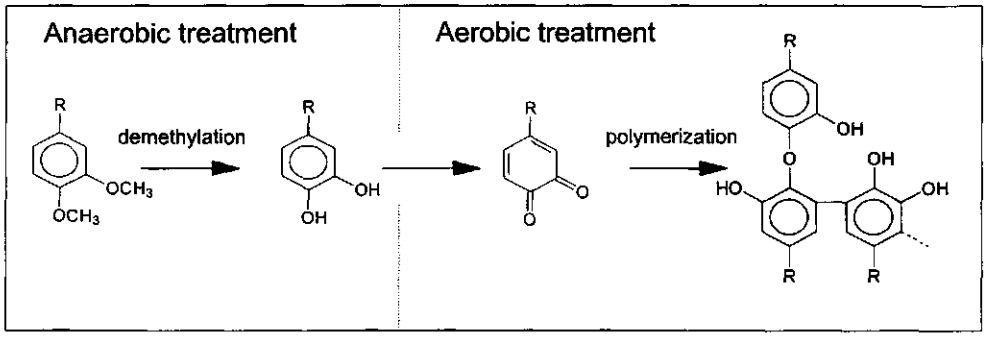


**Figure 3.** Concept for polymerization of lignin during anaerobic-aerobic treatment of hemp pulping liquors.

Also during direct aerobic treatment of hemp pulping wastewaters, polymerization of wastewater lignin accompanied by increase of color levels, was observed. The increase of color levels during aerobic treatment however, was less compared to anaerobic-aerobic treatment. Polymerization of lignin in aerobic treatment plants may proceed in analogy to the concept presented in Figure 3, since aerobic treatment systems contain numerous anaerobic microniches in biolayers and bacterial flocs, due to limitation of oxygen transport.

The polymerization of lignin during biological treatment offers benefits for subsequent physical-chemical lignin removal. Biological treatment leads to pulping wastewaters which are relatively clean, apart from the highly stable lignin-derived humic COD. The observed increase of lignin molecular weight during biological treatment will provide improved performance of ultrafiltration membranes due to decrease of membrane fouling, caused by intermediate size lignin and biodegradable constituents. Moreover, precipitation of lignin with lime or sodium sulphate is known to be more effective for high molecular weight lignin (Schmidt and Joyce, 1980).

## References

**Bache, R., and Pfenning, N.** (1981). Selective isolation of *Acetobacterium woodii* on methoxylated aromatic acids and determination of growth yields. Arch. Microbiol., 130: 255-261.

**Cocaign, M., Wilberg, E., and Lindley, N.** (1991). Sequential demethoxylation reactions during methylotrophic growth of methoxylated aromatic substrates with *Eubacterium limosum*. Arch. Microbiol. 155: 496-499.

**Daniel, S.L., Wu, Z., and Drake, H.L.** (1988). Growth of thermophilic acetogenic bacteria on methoxylated aromatic acids. FEMS Microbiol. Lett., 52: 25-28.

**FAO** (1998). FAOSTAT statistical database on forestry, Food and Agricultural Organization of the United Nations, Geneva.

**Field, J.A., Kortekaas, S., and Lettinga, G.** (1989). The tannin theory of methanogenic toxicity. Biological Wastes, 29: 241-262.

**Field, J.A., Leyendeckers, M.J.H., Sierra-Alvarez, R., Lettinga, G., and Habets, L.H.A.** (1991). Continuous anaerobic treatment of autoxidized bark extracts in laboratory-scale columns. Biotechnol. Bioeng., 37: 247-255.

**Habets, L.H.A., and de Vegt, A.L.** (1991). Anaerobic treatment of bleached TMP and CTMP effluent in the BIOPAQ UASB system, Wat. Sci. Tech., 24(3/4): 331-345.

**Kaiser, J., and Hanselmann, K.W.** (1982). Fermentative metabolism of substituted monoaromatic compounds by bacterial community from anaerobic sediments. Arch. Microbiol., 133: 185-194.

**McCarthy, P.J., Kennedy, K.J., and Droste, R.L.** (1990). The role of resin acids in the anaerobic toxicity of chemithermomechanical pulp wastewater. Wat. Res., 24, 1401-1405.

**Richardson, D.A., Andras, E., and Kennedy, K.J.** (1991). Anaerobic toxicity of fines in chemi-thermomechanical pulp wastewaters, a batch assay - reactor study comparison. Wat. Sci. tech., 24: 103-112.

**Santos, E.B.H., and Duarte, A.C.** (1998). The influence of pulp and paper mill effluents on the composition of the humic fraction of aquatic organic matter. Wat. Res., 32, 597-608.

**Schmidt, R.L., and Joyce, T.W.** (1980). An enzymatic pretreatment to enhance the lime precipitability of pulp mill effluents. TAPPI J., 63(12), 63-67.

**Sierra-Alvarez, R., and Lettinga, G.** (1991). The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. Appl. Microbiol. Biotechnol., 34, 544-550.

**Sierra-Alvarez, R., Kortekaas, S., van Eekert, M., and Lettinga, G.** (1991). The anaerobic biodegradability and methanogenic toxicity of pulping wastewaters. Wat. Sci. Technol. 24:113-125.

**Sierra-Alvarez, R., Field, J.A., Kortekaas, S., and Lettinga, G.** (1994). Overview of the anaerobic toxicity caused by organic forest industry wastewater pollutants. Wat. Sci. Tech., 29 (5/6), 353-363.

**Stevenson, F.J.** (1994). Humic chemistry: Genesis, Composition, Reactions, John Wiley & sons, New York, USA.

**Verhagen, F.J.M., Swarts, H.J., Wijnberg, J.B.P.A., and Field, J.A.** (1998). Biotransformation of the major fungal metabolite 3,5-dichloro-p-anisyl alcohol under anaerobic conditions and its role in the formation of bis(3,5-dichloro-4-hydroxyphenyl)methane. Appl. Environ. Microbiol., 64, 3225-3231.

**Welander, T.** (1988). An anaerobic process for treatment of CTMP effluent. Wat. Sci. Tech., 20 (1), 143-147.

Summary (English and Dutch).

# 7

## Summary

Biological treatment is an indispensable instrument for water management of non-wood pulp mills, either as internal measure to enable progressive closure of water cycles, or as end of pipe treatment. In this thesis, the sequenced anaerobic-aerobic treatment of hemp (*Cannabis sativa L.*) pulping wastewaters is described, with a focus on the treatability of thermomechanical pulping (TMP) effluents and soda pulping black liquors. The research was performed within the framework of the Dutch Hemp Programme, which aimed to develop environmentally safe and economically feasible pulping processes as a measure to explore non-food markets for arable farming.

Pulp and paper industry wastewaters are highly heterogeneous, depending on the feedstock and the pulping processes utilized. Hemp TMP wastewaters were found to be non-toxic to methanogens and highly suitable for sequenced anaerobic-aerobic treatment, which enabled 63-66% COD removal at loading rates up to 27 g COD/$l_{UASB}$·d. Hemp black liquors on the other hand, were somewhat more problematic, due to the high methanogenic toxicity, causing 50% inhibition of acetoclastic methanogenic activity (50% IC) at concentrations ranging from 2-6 g COD/*l*, which is 10 to 100 times lower than the black liquor concentrations in industry and comparable to the methanogenic inhibition of wood pulping wastewaters.

Apolar hemp extractives (resinous compounds) were observed to be the main source of inhibitory substances in hemp black liquors. Lignin derivatives on the other hand, were less important for methanogenic inhibition, since the main share of lignin in hemp black liquors was non-toxic. Despite the high methanogenic toxicity of hemp black liquors, anaerobic treatment was feasible, provided that adequate dilution was applied. Anaerobic treatment of diluted hemp stem wood black liquor (HSWBL) facilitated recovery of methane and high treatment efficiencies at high organic loading rates. Subsequent aerobic post-treatment provided almost complete removal of BOD, however COD-removal was limited due to the presence of recalcitrant lignin. Additionally, substantial detoxification was obtained after anaerobic-aerobic treatment. The major removal of inhibitory compounds was accomplished in the aerobic step.

The effective reduction of methanogenic toxicity during sequenced anaerobic-aerobic treatment of hemp black liquors was used as a detoxification strategy denominated upfront dilution, recirculating the aerobic effluent to dilute the incoming influent to sub-toxic concentrations. The feasibility of the upfront dilution method was demonstrated in an experiment, in which direct anaerobic treatment and sequenced anaerobic-aerobic treatment with upfront dilution were compared. Direct anaerobic treatment of 12 g COD/*l* HSWBL led to almost complete inhibition of the methanogenic activity within 14 days. While recirculation

of 75% of the aerobic post-treatment effluent for upfront dilution of the toxic HSWBL, enabled anaerobic treatment at loading rates up to 21.5 g COD/$l_{UASB}$·d without noticeable inhibition of methanogenesis. Extensive detoxification was confirmed during anaerobic-aerobic treatment of 20 g COD/*l* HSWBL recirculating 86% of the aerobic effluent. COD and BOD removal was 47% and 68%, respectively, after anaerobic treatment; and 74% and 97%, respectively, after anaerobic-aerobic treatment at an overall loading rate of 3.6 g COD/*l*·d, while 30-35% of the incoming COD was recovered as methane.

Lignin removal during anaerobic-aerobic treatment of hemp pulping wastewaters was remarkably high and ranged up to 44%, of which globally half was obtained anaerobically. Studies on hemp TMP wastewater revealed that lignin removal during anaerobic treatment corresponded to the lowest molecular weight derivatives, indicating that biodegradation was the major removal mechanism. Subsequently, aerobic post-treatment of hemp pulping wastewaters caused extensive polymerization of lignin to molecular weights in excess of 34 kD, which was associated with strong increases in wastewater color. Autoxidative polymerization of polyphenols formed out of lignin by anaerobic bioconversion is proposed as a mechanism for the observed increase in lignin molecular weight and color. Apart from the highly stable high molecular weight lignin-derived humic COD, biologically treated pulping effluents are relatively clean, which offers benefits for lignin removal during tertiary treatment, such as reduced fouling of ultrafiltration membranes and improved precipitation with divalent salts.

Comparison of anaerobic-aerobic treatment and aerobic treatment shows that treatment efficiencies and detoxification were similar. The anaerobic-aerobic treatment system however, provided 50% lower surplus sludge production, production of methane as an energy source (0.16 $m^3$/kg $COD_{removed}$), less nutrient dosage and substantial reductions in aeration costs.

The results of this research demonstrate that sequenced anaerobic-aerobic treatment is a suitable technology for the treatment of hemp pulping wastewaters. Upfront dilution effectively reduced inhibition of methanogenesis by extractive compounds during anaerobic treatment, whereas the observed increase in lignin molecular weight after biological treatment offers benefits for lignin removal during optional tertiary treatment.

## Samenvatting

Biologische zuiveringsmethoden vormen een essentieel instrument bij de behandeling van papierindustrieafvalwater, zowel voor interne behandeling bij het sluiten van waterkringlopen als voor 'end of pipe' behandeling. In dit proefschrift wordt de anaërobe-aërobe behandeling beschreven van afvalwater afkomstig van de pulping van hennep (*Cannabis sativa L.*), waarbij met name aandacht is besteed aan thermomechanical pulping (TMP) afvalwater en soda pulping black liquors (BL's). Het onderzoek is verricht in het kader van het hennepproject, dat tot doel had milieuvriendelijke en economisch renderende pulpprocessen te ontwikkelen op basis van hennep om afzet van landbouwgewassen buiten de voedselsector te stimuleren.

De samenstelling van afvalwaterstromen binnen de papierindustrie kan zeer sterk variëren, afhankelijk van de gebruikte vezelgrondstoffen en pulpprocessen. Hennep TMP-afvalwater was niet toxisch voor methanogene bacteriën en zeer geschikt voor anaërobe zuivering, zoals blijkt uit zuiveringsrendementen van 63 - 66% verwijdering van het chemisch zuurstof verbruik (CZV) bij organische belastingen tot 27 g CZV/$l_{UASB}$·d. Zuivering van afvalwater van soda pulping daarentegen gaf meer problemen, als gevolg van de hoge toxiciteit van dit type afvalwater voor methanogene bacteriën. Blootstelling van anaëroob korrelslib aan hennep soda pulping black liquor (BL) leidde tot 50% remming van de acetoclastische activiteit bij concentraties van 2-6 g CZV/*l*, wat circa 10 tot 100 maal lager is dan BL-concentraties gemeten in de industrie en vergelijkbaar met de methanogene toxiciteit van soda pulping afvalwater op basis van hout.

De harsfractie bleek de voornaamste bron van toxische verbindingen in hennep BL's. Lignine brokstukken daarentegen waren van minder belang voor de methanogene toxiciteit, omdat het overgrote deel van het lignine in hennep BL niet giftig was. Ondanks de hoge methanogene toxiciteit was anaërobe zuivering van hennep BL's mogelijk, mits voldoende verdunning werd toegepast. Anaërobe behandeling van hennep houtpijp BL verdund met kraanwater, verliep met hoge zuiveringsrendementen zonder remming van de methanogenese bij hoge belastingen. Aërobe nazuivering leidde vervolgens tot vrijwel volledige afbraak van het biologisch zuurstofverbruik (BZV), hoewel de verwijdering van CZV beperkt was door de hoge concentraties recalcitrant lignine. Bovendien werd gedurende anaërobe-aërobe zuivering een verregaande detoxificatie bereikt, welke voornamelijk toegeschreven kon worden aan de aërobe nazuivering.

De effectieve ontgifting van het afvalwater die wordt bereikt na anaërobe-aërobe zuivering, is de basis voor een detoxificatiestrategie, genaamd 'upfront dilution'. Hierbij is effluent van de aërobe nazuivering gerecirculeerd om hennep BL te verdunnen tot sub-toxische concentraties. Het principe van upfront dilution is gedemonstreerd in een experiment waar

directe voeding van hennep BL aan een anaërobe reactor vergeleken is met anaërobe-aërobe zuivering met upfront dilution. Directe voeding van 12 g CZV/*l* hennep houtpijp BL leidde binnen 14 dagen tot volledige remming van de methanogene activiteit. Terwijl recirculatie van 75% van het effluent van de aërobe nazuivering, anaërobe zuivering mogelijk maakte bij organische belastingen van 21.5 g CZV/*l* zonder merkbare remming van de methanogenese. Verregaande detoxificatie is bevestigd tijdens anaërobe-aërobe zuivering van 20 g CZV/*l* hennep houtpijp BL met 86% recirculatie van het effluent van de aërobe nazuivering voor upfront dilution. CZV-verwijdering na anaërobe en anaërobe-aërobe zuivering was respectievelijk 47 en 74% en BZV-verwijdering respectievelijk 68 en 97% bij een overall belasting van 3.6 g CZV/*l*·d, terwijl 30 - 35% van de CZV gevoed aan de zuiveringsinstallatie, teruggewonnen werd als methaan.

Lignineverwijdering tijdens anaërobe-aërobe behandeling van hennep pulping afvalwater was opmerkelijk hoog en bedroeg tot 44%, waarvan globaal de helft werd bereikt in de anaërobe zuiveringsstap. Experimenten met hennep TMP-afvalwater toonden aan dat de lignineverwijdering in de anaërobe zuiveringsstap, overeenkwam met de verwijdering van lignine brokstukken van de kleinste moleculairgewichtsklasse, hetgeen duidt op biologische afbraak als verwijderingsmechanisme. Vervolgens trad bij de aërobe nazuivering van hennep pulping afvalwater verregaande polymerisatie van lignine op tot molecuulgewichten boven de 34 kD, welke gepaard ging met een sterke kleuring van het afvalwater. Autoxidative polymerisatie van polyfenolen, gevormd door biotransformatie van lignine tijdens passage van de anaërobe zuiveringsstap, wordt aangegeven als mechanisme voor de sterke toename van het molecuulgewicht en de kleur van lignine. Na biologische zuivering resteert het CZV van de relatief inerte, hoog moleculaire humusachtige ligninefractie. Biologische zuivering vergemakkelijkt lignineverwijdering in een eventuele derde zuiveringsstap door vermindering van 'membrane fouling' bij ultrafiltratie en meer efficiënte precipitatie met tweewaardige metaalzouten.

Vergelijking van anaërobe-aërobe zuivering met aërobe behandeling van hennep BL toont aan dat beide processen globaal eenzelfde rendement hebben, aangaande zuivering en detoxificatie. Anaërobe-aërobe zuivering biedt echter als voordeel een 50% lagere slibproductie, productie van methaan als energiebron (0.16 $m^3$/kg $CZV_{verwijderd}$), lagere nutriëntenbehoefte en belangrijke besparingen aan beluchtingskosten.

De resultaten van dit onderzoek tonen aan dat anaërobe-aërobe zuivering een geschikte technologie is voor de behandeling van hennep pulping afvalwater. Upfront dilution bleek hierbij een nuttig instrument om remming van de methanogene activiteit door harsverbindingen tegen te gaan, terwijl de sterke toename in molecuulgewicht van lignine voordelen biedt voor lignineverwijdering in een eventuele derde zuiveringsstap.

## Curriculum vitae

Sjon Kortekaas is geboren te Castricum 20 mei 1959. In 1977 behaalde hij het Atheneum B diploma aan het Bonhoeffer College te Castricum en in datzelfde jaar werd begonnen aan de studie Milieuhygiëne aan de Landbouw Hogeschool te Wageningen. Deze studie werd in 1980 voor een periode van 7 jaar onderbroken, gedurende welke Sjon zich o.a. inzet voor het behoud van 'Huis ter Aa' en kookt in het vegetarisch specialiteitenrestaurant 'Nola Rae' te Arnhem, totdat hij, werkend op een bijenteeltproject in de Surinaamse mangrovebossen, het nut van zijn wageningse studie onderkent. Terug in Nederland bindt hij zich aan vrouw en kinderen en wordt de studie Milieuhygiëne hervat, welke in 1990 wordt voltooid. Vanaf 1991 is hij voor een periode van tweeënhalf jaar als tijdelijk wetenschapppelijk medewerker verbonden aan de vakgroep Milieuhygiëne, waar hij binnen het hennep-project onderzoek doet naar de zuivering van afvalwater en waarvan de resultaten de basis vormen voor onderhavig proefschrift. Als na wat korte projecten en een uitstapje naar de vakgroep Bosbouw in 1995 de financiering rondkomt voor een tweede onderzoeksproject naar de zuivering van papierindustrie afvalwater, ligt een promotieplaats binnen bereik. Echter door 'allocatie' van onderzoeksgelden valt de financiering weg, waarna het promotieonderzoek in 'eigen tijd' wordt afrond.