The Effect of Tannic Compounds on Anaerobic Wastewater Treatment

Promotor: dr. ir. G. Lettinga,
bijzonder hoogleraar in de anaerobe waterzuivering

Jim A. Field

The Effect of Tannic Compounds on Anaerobic Wastewater Treatment

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op vrijdag 6 oktober 1989
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

ISN 27/3014

NN08201, 1299

STELLINGEN

1. De konklusies van het onderzoek van Stuckey and McCarty (1984) betreffende de methanogene toxiciteit van aangebrande eiwitten en suikers zijn zeer aanvechtbaar.

   Stuckey and McCarty. 1984. Wat. Res. 18: 1343-1353.

2. Aan de octrooiaanvraag (European Patent Application # 85850078.8, 1985) betreffende de vermindering van de methanogene toxiciteit van vetzuren door middel van de toevoeging van calcium, ijzer of aluminium, worden zeer ten onrechte rechten ontleend aangezien de methode reeds in 1981 uitgebreid is gepubliceerd.

   Hanaki et al. 1981. Biotechnol. Bioengineer. 23: 1591-1610.

3. Naast lignine moeten vooral flavonoiden uit naalden, bladeren en de bast van bomen worden beschouwd als belangrijke precursors van humus.

   Hurst and Burges. 1967. Soil Biochemistry Marcel Dekker Inc., N.Y. pp. 260-286.

4. Noch de Nederlandse noch de Amerikaanse wetgeving betreffende de lozing van afvalwater beschermt het leefmilieu in voldoende mate tegen het vrijkomen van toxische verbindingen.

5. Het voorstel van de kunstmestfabrikant Kemira, om d.m.v. toevoeging van salpeterzuur aan mest de luchtverontreiniging met ammoniak te verminderen, kan worden aangemerkt als een typisch voorbeeld van een werkwijze, waarbij de oplossing van één milieuprobleem een ander veroorzaakt.

   Volkskrant 3 Juni, 1989 b.z. 19

6. Gezien de unieke rol die schimmels in de natuur spelen bij de afbraak van complexe polyfenolen moet meer aandacht worden besteed aan de toepassingsmogelijkheden van deze microorganismen in de milieubiotechnologie.

7. Tanninen zijn slechts effectieve beschermers van kernhout tegen schimmelaantasting, voor zolang ze niet zijn geautoöxideerd.

   Lyr. 1962. Nature 195: 289-290.

8. Overplukken wordt vaak genoemd als de oorzaak voor het verdwijnen van paddestoelen in Nederland. Ontbossing en verontreiniging spelen waarschijnlijk een grotere rol.

9. Het publiekelijke openbaar maken van een z.g. wetenschappelijke of technische doorbraak, zonder dat daarbij een controleerbaar basis wordt verstrekt in de vorm van toegankelijke wetenschappelijke publikaties of octrooiën, is een weinig elegante handelswijze van de zijde van een wetenschappelijke instelling.

   LUW en Ecotechniek, B.V. NOS Journal, 20 Juni, 1989; en de brochure "De Voordelen van het Mestoverschot"

10. De vaste fraktie van anaeroob uitgegiste mest mag niet worden aangemerkt als "single cell protein".

    LUW en Ecotechniek, de brochure "De Voordelen van het Mestoverschot"

11. Het selektieve krimp en groei beleid van Nederlandse universiteiten, dat onder andere is bedoeld om een meer markt gerichte universiteit te krijgen, brengt het grote gevaar met zich mee, dat de universiteit steeds meer als een goedkoop adviesbureau gaat functioneren voor het bedrijfsleven.

12. De risicos van atoomenergie worden ook in een z.g. vrije markteconomie - uiteindelijk grotendeels afgewenteld op de totale gemeenschap, aangezien geen verzekeringsmaatschappij de gevolgen van een nucleair ongeluk durft te dekken.

13. Het was in Spanje gedurende de middeleeuwen al mogelijk een telegram te versturen.

Stellingen behorende bij het proefschrift "The Effect of Tannic Compounds on Anaerobic Wastewater Treatment" van J. A. Field.

Wageningen, 6 oktober 1989

Acknowledgements

The research presented in this dissertation was supported in part by PAQUES, BV., Balk, The Netherlands. I would like to express my gratitude to Joost Paques for funding the research, and to his employee, Leo Habets, and former employee, Anton van der Vlugt, who cooperated in the planning of the research.

Part of the research in this dissertation was the result of the efforts of several students and colleagues of the department of water pollution control. I am grateful to Marc Leyendeckers for his nine months of hard experimental work and to Sjon Kortekaas for helping me conduct some of the key experiments. I thank Reyes Sierra, for conducting some of the experimental work and Math Geurts, for helping identify intermediates of phenolic degradation. I am grateful to Hans Brons, for getting me started in the topic of humus.

I give my special thanks to the analyst, Paul de Jong, who worked together with me in conducting numerous experiments that are described throughout this dissertation, without his help this dissertation would never have been completed. I also am grateful to other members of the analytical staff. Firstly, to Johannes van der Laan and Arjen van de Peppel whose efforts made the chromatographic determinations possible. Secondly, to Jo Jacobs, Sjoerd Hobma and Theo Ijwema who were always willing to help when asked.

It was a great pleasure to work together with Hans Temmink of the department of toxicology and his students, Jos van Haastrecht and Rob Merckelbach, on experiments concerning fish toxicity.

Acknowledgement should be made to the staff of the technical services at the Biotechnion, stockroom, drawing room, photographic services, maintenance and the shop.

I am grateful to PARENCO in Renkum for allowing me to take bark samples from the trees delivered at their factory and their continued willingness to help other colleagues of the department in the collection of wastewater and wood samples.

I thank René Wijffels and Gert van de Bergh for helping with the Dutch translation of the "stellingen" and summary and conclusions.

I express my gratitude to Gatze Lettinga for serving as my "promotor" and for his efforts in coordinating the research project that led to this dissertation. I also thank Dora Lettinga whose hospitality has made my stay in the Netherlands enjoyable.

I give my thanks to my former professor in the United States, Dr. W. Kroontje, who sent me to Holland in the first place. Additionally, I express my appreciation to all the members of the staff at the department of water pollution control for having me at the department.

Last but not least, I thank my parents, Milton and Isabel Field, for their support throughout my university education.

Abstract

Field, J. A. (1989) The Effect of Tannic Compounds on Anaerobic Wastewater Treatment. Doctoral Thesis. Wageningen Agricultural University. Wageningen, The Netherlands.

Anaerobic wastewater treatment is an alternative to the conventional aerobic treatment processes for the removal of easily biodegradable organic matter in medium to high strength industrial wastestreams. Anaerobic treatment has several advantages, however one important disadvantage is the high sensitivity of the anaerobic bacteria (ie. methanogenic bacteria) to toxic compounds. The anaerobic technologies were initially developed for the treatment of non-toxic organic wastewaters. As the technology matured, the limits of its application to toxic wastewaters were studied. Past research has been mostly directed towards the toxic effects of compounds introduced by man into the industrial process rather than natural constituents present in agricultural wastewaters.

This dissertation investigates the role of natural polar phenolics (ie. tannins and related compounds) on anaerobic digestion. Tannins are important constituents of certain types of agro-industrial wastewaters such as vegetable tannery effluent; olive oil mill effluent; wine vinasse; coffee pulp water; debarking wastewater; and masonite (fiber board) wastewater. A distinct feature of highly hydroxylated phenolics is that they are readily oxidized to darkly colored humic compounds. Such transformations can generate products which differ in toxicity and biodegradability compared to the original tannic compounds. Industrial process waters are often exposed to conditions which promote phenol oxidation, therefore the role of humus forming processes was a major consideration included in this study.

The toxicity of tannin compounds to anaerobic bacteria was determined. The concentration of tannins found to cause 50% inhibition to methanogenic bacteria was 350 and 700 mg L^{-1} of condensed and hydrolyzable tannins, respectively. The condensed tannins were the major inhibitors present in wastewater derived from the debarking of wood at pulping factories.

The effects of oxidation treatments on the methanogenic toxicity of phenolic compounds was evaluated. The initial polymerization of monomers led to a higher toxicity due to an increase in tannic qualities. The oligomers formed have stronger hydrogen bonds with proteins than the monomers. They are thus more likely to react with the functional proteins of bacteria. If the polymerization was continued, a decrease in toxicity occurred due to a lower effectiveness of high MW compounds to penetrate bacteria.

These results indicated that toxic oligomeric tannins can be detoxified by oxidative polymerization. The application of autoxidation (aeration at a high pH) as a pretreatment prior to anaerobic digestion of tannin containing wastewater was tested. Debarking wastewaters of coniferous trees were successfully detoxified by autoxidation pretreatments. The tannins were converted to poorly degradable humic compounds that were non-toxic. During anaerobic treatment, no inhibition occurred and the fermentable fraction of the wastewater was converted to methane. The high MW humic products were non-toxic for aquatic organisms and thus could be discharged to the surface waters with considerably less environmental impact as compared to the unoxidized tannins.

Up to date, methods of combatting toxic organic pollutants have been largely based on microbial degradation or physical-chemical removal. A viable alternative approach to these methods that potentially is applicable for certain aromatic compounds, could be polymerizing these inhibitory compounds to non-toxic humus. The humus forming process is a natural mechanism in the forest environment that detoxifies tannic compounds before such compounds are released into the surface waters. The humus forming reactions were imitated in this study and were an effective method for eliminating the environmental impact of tannins in wastewater. Research should be continued to determine the extent to which humus forming processes can be applied for the treatment of other toxic organic contaminants.

Contents

CHAPTER 1

The Effect of Tannic Compounds on Anaerobic Wastewater Treatment: General Introduction.

THE EFFECT OF TANNIC COMPOUNDS ON ANAEROBIC WASTEWATER TREATMENT: GENERAL INTRODUCTION

J. A. Field

Dept. of Water Pollution Control
Wageningen Agricultural University
Bomenweg 2, 6703 HD, Wageningen
The Netherlands

ABSTRACT - Little is known about the effect of tannic compounds on anaerobic wastewater treatment. The tannins, in any case are important constituents of certain types of agro-industrial wastewaters. Since the toxicity of tannins to enzymes and aerobic microorganisms has been reported, their inhibitory effects on the bacteria involved in the anaerobic treatment process should be considered. A distinct characteristic of tannic and related monomeric phenolic compounds is their high susceptibility to become polymerized to darkly colored humic compounds upon exposure to air during the handling of process waters and wastewaters. Reactions which cause the oxidative polymerization potentially can effect the biodegradability and toxicity of the phenolic fraction. The literature reveals that the anaerobic biodegradability of phenolic compounds decreases with the increasing molecular weights. Therefore the humus forming reactions are expected to decrease the biodegradability of the phenolic fraction during subsequent anaerobic wastewater treatment. The oxidative polymerization of monomeric phenols to tannic substances has been reported to increase the toxicity of the phenols towards enzymes and microorganisms. Whereas, in contrast, the polymerization of tannins to humic-like compounds has been shown to decrease the inhibitory characteristics. The possibility that the humus forming reactions can be imitated in order to detoxify tannins in wastewater is discussed.

1. BACKGROUND

The anaerobic technologies of wastewater treatment are an alternative to aerobic biological treatment processes for the removal of easily biodegradable organic matter in medium to high strength industrial waste streams. The advantages of the anaerobic approach can be outlined as follows (McCarty, 1964a; Lettinga et al., 1980; Speece, 1983): (1) anaerobic bacteria have a lower cell yield than aerobic bacteria, thus anaerobic technologies are responsible for less excess sludge production; (2) aerobic bacteria must be supplied with air, thus operating costs associated with aeration are eliminated; (3) anaerobic bacteria can be immobilized in dense biofilms, enabling the application of more compact wastewater treatment facilities; (4) the production of a methane gas, a fuel which can be utilized by industry to lower energy costs. The anaerobic treatment alternative, however has several important disadvantageous. Namely, the higher sensitivity of the most important bacteria involved, methanogenic bacteria, to toxic compounds. Additionally, the slow growth rate of methane bacteria, which implies long recovery times are required if the treatment process is severely upset.

The anaerobic method was initially developed for the treatment of non-toxic organic wastewaters of the food industry. As the technology matured, the limits of its application to toxic wastewaters have been studied. The effects of ammonia, sulfur compounds, salt, heavy metals, volatile fatty acids, long chain fatty acids, surfactants, antibiotics, cyanide, halogenated hydrocarbons, formaldehyde and furfurals have received considerable research

attention (see Table 1). From these studies, it appears that the presence of such toxic compounds does not necessarily rule out the possibility for anaerobic treatment (McCarty, 1964b; Yang et al., 1979; Speece, 1983): in many cases, subtoxic concentrations of such compounds can be tolerated; anaerobic bacteria can adapt to toxic concentrations; the toxic compounds may undergo transformations by anaerobic bacteria that lead to compounds of decreased toxicity; and for certain toxins, adjusting the environmental conditions (for example pH) can minimize the inhibiting effect.

Past research has been mostly directed towards the toxic effects of compounds introduced by man into the industrial process rather than the natural constituents of the agricultural feedstocks which ultimately are extracted into the wastewater. Perhaps the natural phenolic constituents of plants (lignin, tannins) deserve more attention since these are abundant in agricultural wastewaters, especially from the forest industries. Since the purpose of tannins in plants is to inhibit pathogenic microorganisms, the possibility that they could disturb the efficiency of anaerobic bacteria cannot be overlooked (White, 1957). Therefore tannic compounds should be the first of the natural phenolic constituents to be studied further.

A distinct feature of tannic compounds is that they are readily oxidized to darkly colored humic compounds. Such transformations can produce products which differ in toxicity and biodegradability compared to the original tannic compounds present in the industrial feedstocks. Industrial process waters are often exposed to conditions which promote phenol oxidation, therefore the role of the humus forming processes is an essential consideration that needs to be included in the study of tannins. In some cases, the humus forming reactions are an unavoidable consequence inherent to a specific industrial process; however in other cases, the humus forming reactions can be applied as part of the overall wastewater treatment scheme.

Table 1. Studies on the Methanogenic Toxicity of Ammonia, Sulfur Compounds, Salt, Heavy Metals, Volatile Fatty Acids, Long Chain Fatty Acids, Surfactants, Antibiotics, Cyanide, Halogenated Hydrocarbons, Formaldehyde and Furfurals.

Compounds	References
ammonia	3, 5, 174, 176, 178, 216, 239, 303
sulfur compounds	70, 137, 154, 175, 181, 224, 239, 248, 250
salt	184, 185, 212, 251
heavy metals	99, 138, 149, 191, 221, 222, 223, 226, 239, 242, 273
volatile fatty acids	10, 54, 138, 179, 180, 211
long chain fatty acids	54, 116, 177, 252
surfactants	238, 283, 310
antibiotics	29, 42, 322
cyanide	79, 239, 269, 323
halogenated hydrocarbons	22, 54, 134, 182, 239, 256, 277, 288, 301, 320, 323, 323
formaldehyde	54, 134, 239, 241, 322
furfurals	23, 325

2. SOURCES AND CHEMISTRY OF TANNINS

Tannins are polar phenolic compounds in plant extracts that are recognized based on their reactivity with proteins or related polyamide polymers. Tannins that are used by the tanning industry normally are oligomeric, ranging in MW's from 500 to 3000 g $mole^{-1}$ (White, 1957). However, tannins up to MW of 5000 to 28,000 g $mole^{-1}$ have been described (Jones et al., 1976). Since tannins are polar compounds, they are easily extracted into the process water (ie. eventually the wastewater) by contact of the feedstocks with water.

Hydrolyzable tannins, which occur only in the wood or leaves of very specific species of woody plants, are polyesters of trihydroxybenzoic acid (gallic acid) and/or a dimer, ellagic acid (Figure 1). They are readily hydrolyzed by acid or enzymes (Haslam, 1966). Since hydrolyzable tannins are not common to a wide variety of agricultural products, their presence in wastewater would be limited to that of vegetable tanneries which use extracts of rather uncommon plants that contain hydrolyzable tannins. However, it should be stated that some tanneries may use condensed tannins instead.

Condensed tannins are polymers of flavonoid type monomers that are linked together by covalent bonds between the C4 and C8 (Figure 1), that are not readily hydrolyzed (Gupta and Haslam, 1980; Haslam, 1966; Porter, 1974; Karchesy and Hemmingway, 1980; Hemingway et al., 1982). The procyanidins, polymers of catechins, are perhaps the most classic example of condensed tannins. The condensed tannins are more widespread than hydrolyzable tannins, as they are common to fruits (grapes and apples), tree bark, olives, beans, coffee and sorghum grains. The concentration of tannins in various feedstocks are indicated in Table 2. The presence of condensed tannins would be expected in the wastes or wastewaters generated from the feedstocks listed in Table 2, namely apple pulp, cannery wastewaters (apples and beans), sorghum and wine vinasses, coffee pulping wastewaters, edible oil industry wastewater (olives) and debarking wastewaters (paper industry). The tannin concentrations listed are quite high, often the tannins account for 1 to 10% of the feedstock dry weight or one to several grams per liter in liquid samples. Therefore, the wastewaters originating from tannin containing feedstocks could potentially contain significantly high concentrations of tannins. In certain cases, like debarking wastewater, the tannin fraction is estimated to be responsible for 50% or more of the COD, based on the available literature data concerning the composition of bark extracts (Hegert et al, 1965; Karchesy and Hemmingway, 1980; Markham and Porter, 1973; Updegraff and Grant, 1975).

Since lignin is also a natural polymeric constituent of plants, consideration should be given to its chemistry for several reasons. Firstly, the structural features of lignin should be highlighted that are distinct from tannins. Secondly, lignin also plays an important role in the humus forming processes. Lignins are complex polymers found in wood and grasses. The lignin content of wood generally ranges from 17 to 30% (Higuchi, 1980; Fengel and Wegner, 1984). The content in various grasses (straw) ranges from 8 to 21% (Misra, 1980; Hartley, 1983; Lindberg et al., 1984). The monomeric units of lignin are primarily coniferyl, sinapyl and p-coumaryl alcohols (Higuchi, 1980), which are illustrated in Figure 2. These monomers are connected by a variety of intermonomeric bonds. The most predominant intermonomeric linkages are the ether bonds (ß-O-4, see Figure 2) which connect the side chain with the ring (Glasser and Kelley, 1987). Various direct carbon-to-carbon bonds between two side chains, between two rings or between the side chain and the ring, are responsible for most of the other intermonomeric connections. Lignins are heterogeneous mixtures of high MW polymers ranging from 1,000 to greater than 100,000 g $mole^{-1}$. The averaged MW values reported for isolated lignin extracts range from 7,000 to 85,000 depending on the isolation method and source (Fengel and Wegner, 1984).

Due to its high content of methoxy groups, lignin is an apolar polymer. Therefore simple contact between lignocellulosic feedstocks and water is not sufficient to extract lignin in the process water. However during chemical pulping and bleaching of lignocellulose, alkali conditions are employed to partially depolymerize and modify the native lignin in order to solubilize it; thereby extracting a considerable amount of lignin in forest industry process waters (Forss, 1982; NEERI, 1986; Sagfors and Stark, 1988; Lindstorm and Osterberg, 1984; Osterberg and Lindstrom, 1985; Priha, 1985).

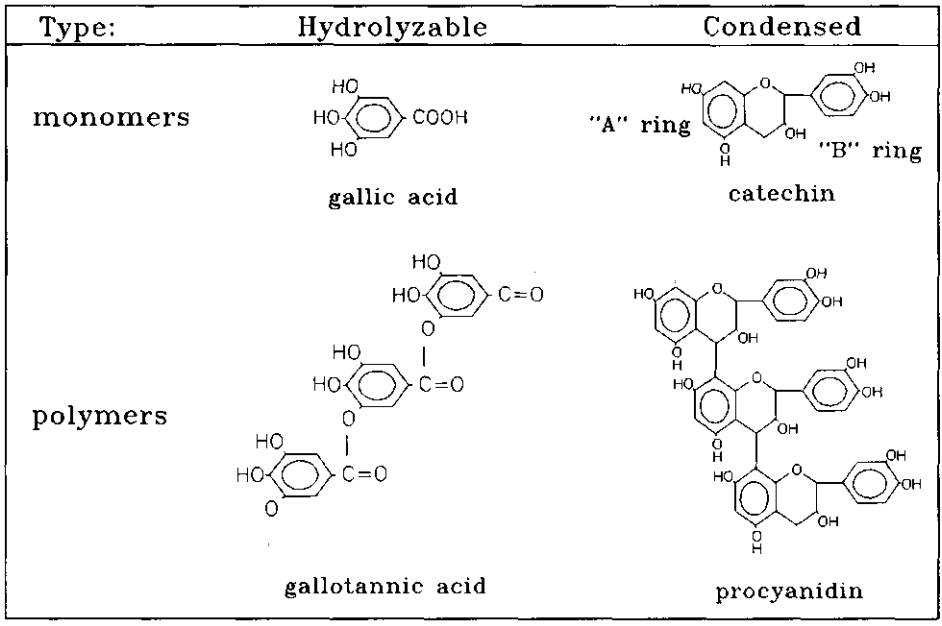


Figure 1. Tannic monomers and intermonomeric bonds of tannins.

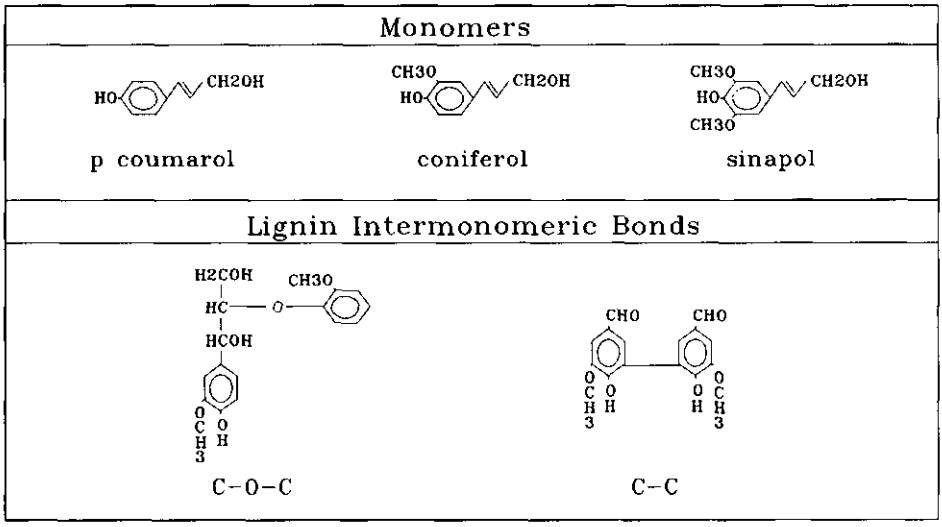


Figure 2. Monomers and intermonomeric bonds of lignin.

Under certain conditions, the modifications of lignin can include the demethylization of methoxy groups or cleavage of ether bonds resulting in lignins enriched in phenolic hydroxyl groups. Such lignins have tannic properties since they are capable of precipitating proteins (Augustin and Puls, 1982; Bryce, 1980) and they are susceptible to the polymerization reactions of the humus forming process (Martin and Haider, 1980; Salkinoja-Salonen, 1980; Huttermann et al., 1980).

3. HUMUS FORMING PROCESSES

Humic compounds can be regarded as poorly biodegradable darkly colored high MW compounds composed of enzymatically or autoxidatively polymerized phenolic units (Stevenson, 1982; Martin et al., 1972; Hurst and Burges, 1967; Haider et al., 1975; Sjoblad and Bollag, 1981). Historically, several theories on the genesis of humus have been developed. The original concept of humus, "lignin theory," postulates that humus is a modified lignin polymer. Modifications of the lignin structure include the demethylization of methoxy groups (producing hydroxyl groups) and the oxidation of terminal aliphatic side chains (producing carboxylic acid groups). This form of humus genesis is probably important in water logged soils and aquatic environments (Stevenson, 1982). The modern day concept of humus, "polyphenol theory," postulates that plant polymeric phenols such as lignin and condensed tannins are oxidatively attacked by microorganisms, producing low molecular weight phenols, phenolic acids and flavonoids that undergo enzymatic or autoxidative conversion to quinones. The quinones polymerize to form humic compounds (Stevenson, 1982; Haider et al., 1975; Martin et al., 1982; Hurst and Burges; 1967; Sjoblad and Bollag, 1981). During soil humus genesis, copolymerization of the quinones with amino compounds can

Table 2. Tannin Concentration in Various Agricultural Feedstocks.

Feedstock	Concentration	units	References
GRAPES			
red wines	1 to 5	g L^{-1}	266
white wines	0.2 to 2	g L^{-1}	266
APPLES			
apple cider	3.5	g L^{-1}	192
BEANS			
red beans	0.3 to 1.3	% dry wt.	35 36
black beans	0.6 to 1.2	% dry wt.	35
white beans	0.3 to 0.4	% dry wt.	35
SORGHUM			
sorghum grain	0.2 to 3.0	% dry wt.	62 106
COFFEE			
coffee pulp	1.8 to 8.6	% dry wt.	71 220 225
OLIVES			
olive oil wastewater	2 to 6	g L^{-1}	17
BARK			
pine bark	5 to 20	% dry wt.	126 153 206 298 319
spruce bark	6 to 37	% dry wt.	126 186
larch bark	6 to 20	% dry wt.	126 186 319
douglas fir bark	5 to 25	% dry wt.	126 319
hemlock bark	9 to 18	% dry wt.	126 130
birch bark	10 to 15	% dry wt.	126
oak bark	8 to 16	% dry wt.	126 259
chestnut bark	8 to 14	% dry wt.	126

account for the low to moderate concentrations of organic nitrogen in humus, ranging from 0.4 to 6% of the dry weight (Stevenson, 1982; Martin et al., 1972; Hurst and Burges; 1967). Originally, lignin was considered the most important parent material to humus. However, the analysis of humus structures reveals a high content of phloroglucinol units, which can only be derived from flavonoid compounds (Hurst and Burges, 1967). Therefore, not only lignin, but also the condensed tannins are important sources of phenols in naturally occurring humus. Finally, it has been shown that microorganisms themselves can synthesize low MW phenolic compounds from simple carbohydrate substrates. These too can eventually be incorporated in the humus polymer (Haider et al., 1972). The various pathways of humus genesis are outlined in Figure 3.

Humus genesis is produced by a complex set of reactions, that potentially involve both depolymerization and polymerization of plant polyphenols. The humus forming reactions are not limited to soil environments, analogous reactions can take place in process waters and wastewaters if the phenols present undergo oxidative reactions. The oxidative reactions may occur by: simple autoxidation; the addition of oxidants (H_2O_2, O_3); extracellular phenol oxidizing enzymes of plants and fungi.

3.1. Autoxidation

Autoxidation of phenolic compounds can occur if three conditions are met: (1) the phenol contains at least two neighboring hydroxyl groups on the ring(s); (2) neutral to alkaline pH; (3) exposure to air or oxygen. Ortho-dihydroxy phenols are converted to quinones which condense with one another, eventually becoming extensively polymerized to darkly colored humus-like high MW compounds, as illustrated in Figure 4 (Singleton, 1972; Hathway and Seakins, 1955 and 1957). The autoxidative polymerization of catechin and the related condensed tannins is perhaps the most important example of autoxidation which can take place in wastewaters. The condensed tannins are important constituents of many types of wastewaters and their autoxidation readily occurs with neutral to alkaline pH conditions.

Autoxidation of trihydroxy phenols leads to the formation of benzotropolones which are not extensively polymerized (Singelton, 1972; Mathew and Parpia, 1971). As illustrated by the formation of purpurogallin from pyrogallol (Figure 5), the destruction of some of the aromatic rings is already evident at an early stage of the autoxidation. With gallocatechins of green tea (trihydroxy B rings), autoxidation may produce both high MW pigments (like phlobatannins in Figure 4) and benzotropolones (like purpurogallin in Figure 5), the latter are eventually destructively oxidized to aliphatic acids and CO_2 (Singleton, 1972).

With high alkali supply, phenols will largely be destructively oxidized. In order to gain more insight in the types of reactions that can occur, the destructive oxidation of lignin during oxygen bleaching should be considered. This process which involves aerating lignocellulosic materials in hot alkaline solutions can hydrolyze ether intermonomeric bonds of lignin and cleave aromatic rings to aliphatic carboxylic acids (Glasser, 1980; Glasser and Kelley, 1987).

3.2. Oxidants

Certain oxidants like H_2O_2 and O_3 might be added to wastewaters to transform phenolic compounds as part of a treatment process. Generally, they are applied at high rates that cause destructive oxidations with the formation of phenolic acids, phenols with increased hydroxylation, and aliphatic carboxylic acids as well as CO_2 in extreme cases (Glasser, 1980; Glasser and Keller, 1987; Mallevialle, 1975; Keating et al., 1978; Gilbert, 1983; Dore et al, 1980; Duguet et al., 1987). The general reaction leading to the aromatic ring cleavage are shown in Figure 6. Extensive oxidations with H_2O_2 and O_3 have been used to decolorize lignin and humic compounds in wastewaters and drinking water supplies (Paice and Jurasek, 1984; Flogstad and Odegard, 1985; Mallevialle, 1975; Gilbert, 1988). Additionally, these oxidants have been used to destructively oxidize monomeric xenobiotic aromatic compounds (Keating et al., 1978; Gilbert 1983, and 1987).

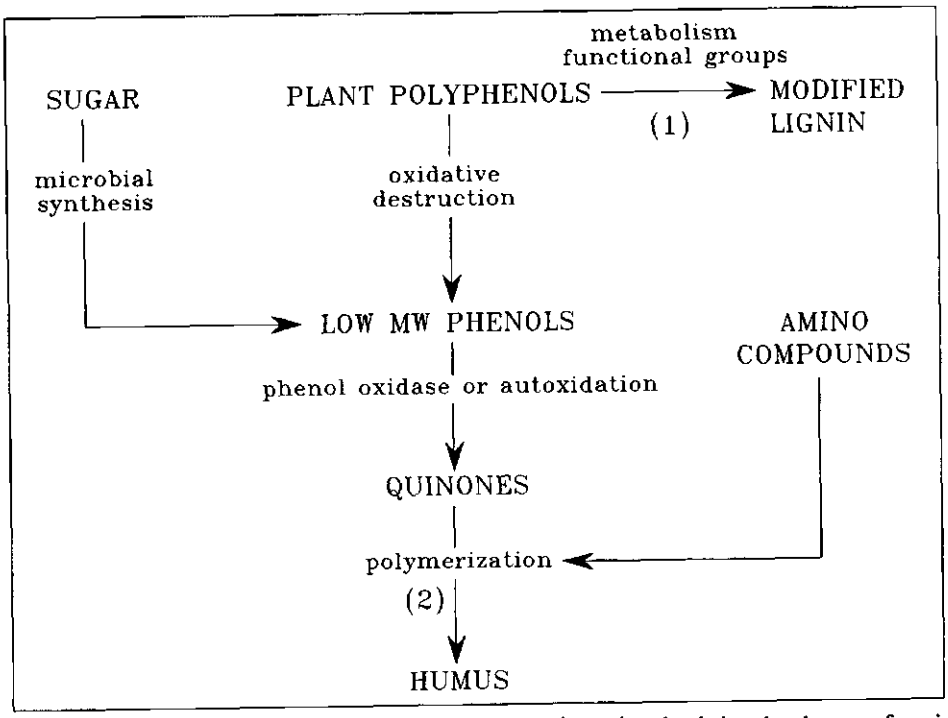


Figure 3. Schematic representation of the mechanisms involved in the humus forming process. (1) = Lignin Theory; (2) = Polyphenol Theory. Adapted from Stevenson (1982).

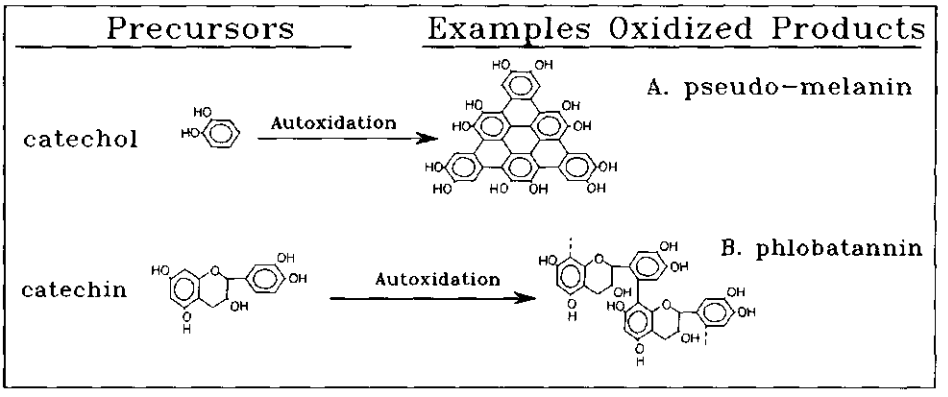


Figure 4. Autoxidative polymerization of ortho-dihydroxy phenols (Singleton, 1972; Hathway and Seakins, 1955 and 1957).

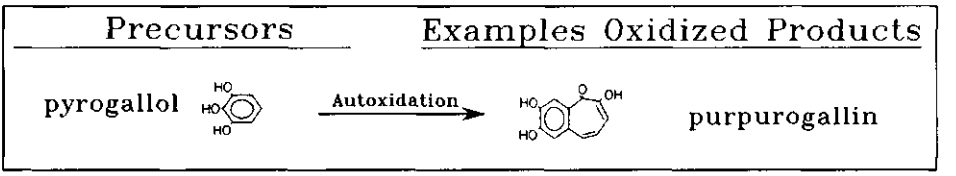


Figure 5. Autoxidation of trihydroxy phenols (Singelton, 1972; Mathew and Parpia, 1971).

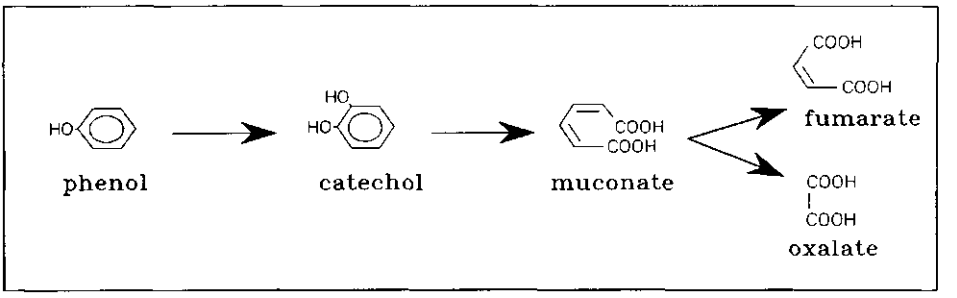


Figure 6. Aromatic cleavage by ozonation. Adapted from Mallvialle (1975).

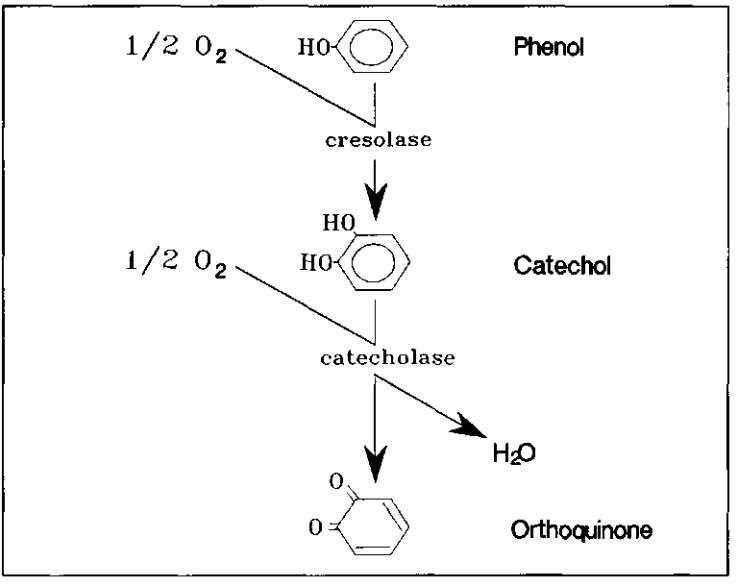


Figure 7. Stoichiometry of tyrosinase (Mathew and Parpia, 1971; Singelton, 1972; Walker, 1975).

The oxidation reactions can also polymerize the phenolic compounds if low rates of oxidants are supplied (Duguet et al. 1987; Chrostowski et al., 1983).

3.3. Phenol Oxidizing Enzymes

Phenol oxidizing enzymes are present in plants. Perhaps the most important phenol oxidizing enzyme of plants is tyrosinase. During agricultural processing, the physical destruction of plant cells allows the phenolic compounds, tyrosinase and air to come into contact with each other, whereby the resulting oxidation process leads to coloration. The phenomenon, commonly observed in food is called "food browning" and may be responsible for the coloration of certain wastewaters and wastes where tyrosinase and phenols are present (like potato starch wastewater and apple pulp).

Tyrosinase has two activities: (1) the catecholase activity is only reactive with simple phenols, phenolic acids and flavonoids with neighboring hydroxyl groups (ortho-dihydroxy rings), that are oxidized to quinones; (2) the creosolase activity hydroxylates monohydroxy phenolic compounds to their dihydroxy counterparts, which are in turn suitable substrates for the catecholase activity (Baruah and Swain, 1959; Kendal, 1949). The stoichiometry of the enzyme's activities is given in Figure 7. The enzymatic step is necessary to oxidize phenols to their respective ortho-quinones, thereafter the reaction is a non-enzymatic condensation of quinones with amino compounds and other quinones or phenols (Hess, 1958; Jackson and Kendal, 1949; Loomis and Battaile, 1966; Mason and Peterson, 1965; Mathew and Parpia, 1971; Sarkar and Burns, 1984; Singleton, 1972), eventually forming darkly colored high MW melanin polymers. Based on their genesis, N content, high MW and dark color, these polymers are almost completely analogous to the concept of humus according to the polyphenol theory (Haider et al., 1972; Stevenson, 1982). Melanin is a complex three dimensional polymer, composed of indole-type units. A proposed conceptual structure is given in Figure 8.

Fungi are the most important group of microorganisms involved in the genesis of humus in soils (Haider et al., 1972 and 1975; Martin et al., 1972; Sjoblad and Bollag, 1981) as well as in the biodegradation of lignin (Kirk et al., 1977; Kirk and Fenn, 1982; Kirk and Farrell, 1987; Haars et al., 1987; Haider and Trojanowski, 1980; Iwahara, 1980). They are involved in the depolymerization and modification of lignin and tannins, producing intermediates that are susceptible to polymerization to humic substances. The polymerization of the low MW phenols may occur as a result of extracellular fungal phenol oxidizing enzymes or as a the result of autoxidation (Haider et al., 1972; Martin and Haider, 1980). The latter mechanism of polymerization is quite important since the low MW phenols resulting from the degradation of lignin are highly hydroxylated.

Wood degrading fungi can be classified into two groups depending on the type of decay: white-rot, in which both lignin and polysaccharides are metabolized; and brown-rot in which only polysaccharides are metabolized and the lignin is modified (Kirk and Fenn, 1982; Kirk, 1971; Kirk and Shimada, 1985). The primary modification of lignin by brown-rot fungi is the demethylization of aromatic methoxy groups (Kirk and Shimada, 1985; Kirk 1971; Haider and Trojanowski, 1980). As a result there is an increase in phenolic hydroxyl groups. Although white-rot fungi can ultimately degrade lignin completely, they too can initially modify lignin by demethylating the methoxy groups (Chen and Chang, 1985; Kirk et al., 1977; Haider and Trojanowski, 1980).

Numerous enzymes of fungi are involved in the degradation and modification of lignin. The lignin degrading activity of white-rot fungi has been attributed to an H_2O_2 dependent enzyme, ligninase (Tien and Kirk, 1983 and 1984). White-rot fungi contain enzymes that produce H_2O_2 by the oxidation of easy substrates like glucose (Highley, 1987; Eriksson et al., 1986; Kelley et al., 1986; Kelley and Reddy, 1986). The major activity of ligninase is the cleavage between side chain carbons that results in lignin depolymerization (Figure 9). Other reactions associated with the degradation of lignin, such as aromatic ring cleavage (see Figure 10), have been reported (Umezawa and Higuchi, 1987 and 1986; Umezawa et al., 1986; Miki et al., 1987; Kirk and Fenn, 1982; Chen and Chang, 1985). Ligninase can also oxidize phenols to quinones, suggesting that oxidative coupling and thus polymerization can occur (Tien and Kirk, 1984) as shown in Figure 9. Research with isolated ligninase, has produced

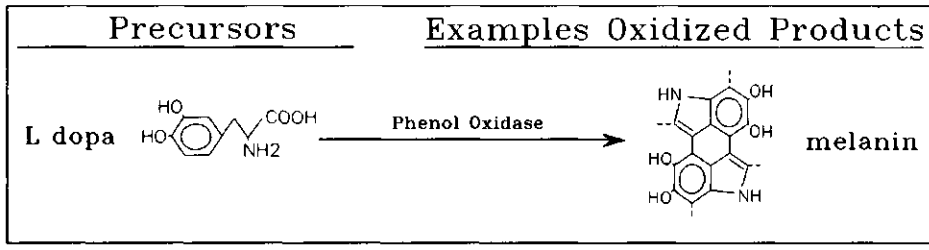


Figure 8. Conceptual structure of melanin (Singelton, 1972; Mathew and Parpia, 1971).

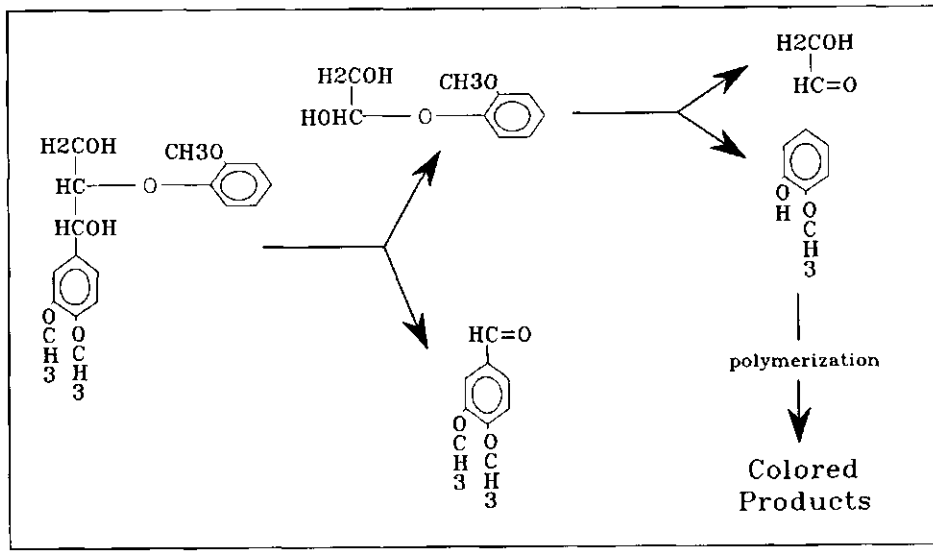


Figure 9. Intermonomeric cleavage of a lignin model dimeric compound by white-rot fungi and some of the depolymerized products (Tien and Kirk, 1984).

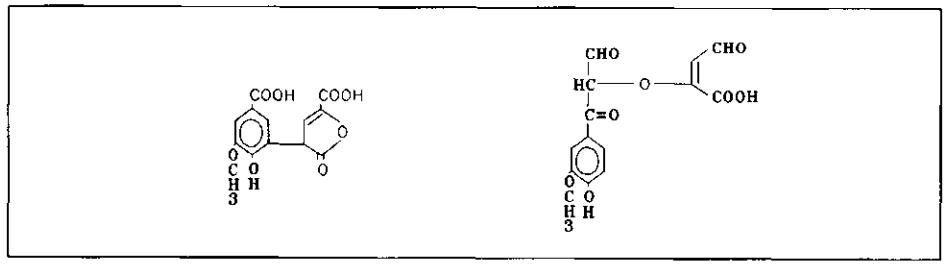


Figure 10. Some aromatic ring cleavage products identified in lignin degrading white-rot culture media (Kirk and Fenn, 1982).

predominantly depolymerization reactions of lignin if the lignin was previously methylated (Tien and Kirk, 1983), however, natural lignins (with phenol groups) are actually polymerized by isolated ligninase (Haemmerli et al., 1986a). Cultures of white-rot fungi depolymerize natural lignins if easily biodegradable carbohydrate substrates are available (Fukuzumi et al., 1977, Eaton et al., 1980; Milstein et al., 1983; Livernoche et al., 1983; Kirk 1980; Leatham, 1986). This is most likely attributable to the presence of enzymes that reduce the quinones at the expense of carbohydrate oxidation, thereby preventing the polymerization (Kirk et al., 1977; Ander et al., 1980; Kirk and Shimada, 1985; Haemmerli et al., 1986a).

The lignolytic activities of fungi are associated with enzymes that primarily have a polymerizing effect on phenols. These are referred to as phenol oxidases and include tyrosinase, peroxidase and laccase (Kirk and Shimada, 1985; Ishihara, 1980; Sjoblad and Bollag, 1981; Martin and Haider, 1980). The major difference between phenol oxidases and ligninase is that phenol oxidases are only effective on phenolic moieties, whereas ligninase oxidizes both phenolic and non-phenolic sections of the lignin polymer (Harvey et al., 1985). The distinction between tyrosinase and laccase is that the latter can oxidize phenols with methoxy groups, peroxidase is also effective with methoxy substituted phenols but requires H_2O_2 as an oxidant (Sjoblad and Bollag, 1981).

Considering that fungi are the best suited organisms for degrading or modifying recalcitrant and toxic phenolic compounds, their direct use or the use phenol oxidizing enzymes (from fungi or plants) in wastewater treatment should not be overlooked. In this regard, already several research groups have examined using white-rot fungi; (1) for the decolorization of forest industry bleaching wastewaters by degradation of the lignin fraction (Fukuzumi, 1980; Paice and Jurasek, 1984; Livernoche et al., 1983; Royer et al., 1983; Eriksson and Kolar, 1985; Eaton et al., 1980 and 1982; Sundman et al., 1981); (2) for the decolorization of industrial polymeric dyes (Glenn and Gold, 1983); and (3) for the degradation of environmental chemicals like chlorinated phenols and polycyclic aromatics (Eaton, 1985; Bumpus et al., 1985; Arjmand and Sandermann, 1985; Kohler et al., 1988; Bumpus and Aus, 1987; Huynh et al., 1985; Haemmerli et al., 1986b; Hammel et al., 1986). Additionally, soil fungi are applicable for the degradation of the condensed tannins (Chandra et al., 1973; Grant, 1976). While the former applications are based on the destructive action of white-rotters and soil fungi, other applications take advantage of phenol oxidase enzymes for polymerization of low MW phenols. Laccase isolated from fungi (Hakulinen, 1987; Campbell and Joyce., 1983) or peroxidase isolated from horseradish roots (Schmidt and Joyce, 1980) can be utilized to improve the lime precipitability of lignin derived phenols in forest industry wastewaters. Likewise, laccase of fungi have been applied to the polymerization of wastewater lignosulfonates to very high MW compounds that were suitable for use as commercial binding materials (Haars et al., 1987). Atlow et al. (1984) demonstrated the utilization of mushroom tyrosinase for the treatment of phenol containing coking wastewaters. The phenols were oxidatively polymerized to high MW insoluble pseudo-melanin which were removed from the wastewater by settling or filtration. The same research group has produced similar polymerizations of coking wastewater phenols and aromatics with horseradish peroxidase (Alberti and Klibanov, 1981; Klibanov et al., 1980). Even earlier research, has demonstrated the use of fungal phenol oxidizing enzymes for the transformation of toxic monomeric chlorinated phenols to colored nontoxic polymers (Lyr, 1963). Finally, tyrosinase, peroxidase and laccase are also capable of polymerizing condensed tannins (Hathway and Seakins, 1957; Updegraff and Grant, 1975; Weinges et al., 1969). This type of polymerization can potentially be applied to debarking wastewaters in order to eliminate the tannin fraction by their conversion to humic compounds.

4. ANAEROBIC BIODEGRADATION OF PHENOLIC COMPOUNDS

The degradation of aromatic compounds in anaerobic environments is different from the degradation in aerobic environments for a number of reasons (Schink, 1988). Firstly, aerobic microorganisms utilize O_2 as a terminal acceptor of electrons, while anaerobic microorganisms can only utilize NO_3, SO_4 and CO_2 for this purpose. Secondly, aerobic

organisms have enzyme systems (oxygenases) that can incorporate oxygen atoms (from O_2) into aromatic substrates; thereby, producing less inert aliphatic acids. Furthermore, aerobic organisms utilize the high reactivity of superoxides, peroxides and hydroxyl radicals generated from O_2 as a method of primary attack to destroy polymeric phenolic structures in the extracellular environment.

Essentially all natural phenolic compounds are ultimately degradable in aerobic environments. This would even include the most stable forms of humus, although these may have half-lives in aerobic soils ranging from 300 to 3000 years (Hurst and Burges, 1967). The geological accumulation of the so called "fossil fuels" provides evidence that certain phenolic compounds are in fact not biodegradable in anaerobic environments. The fossil fuels are derived from lignin and humus that survived degradation in ancient anaerobic environments. Therefore, it is important to ascertain the limits of phenolic biodegradability in anaerobic environments. From a technological point of view, it is important to determine which structural features of phenolic compounds are related to recalcitrance in anaerobic microbial communities.

4.1. Monomeric Phenols

The recalcitrant nature of phenolic compounds in the anaerobic environment is not limited by the metabolism of the aromatic ring. Already in the 1930's, evidence that simple phenolic monomeric compounds are mineralized to CH_4 and CO_2 by anaerobic microorganisms was reported (Tarvin and Buswell, 1934). Since that time, numerous experiments have demonstrated that the monomeric units of lignin, hydrolyzable tannins and condensed tannins; as well as other simple phenolic compounds are biodegradable in anaerobic environments. These experiments are summarized in Table 3. The table also illustrates that even aromatic amino acids and humic-like monomers (indole) are biodegradable. Furthermore, even benzene rings that lack polar substitutions, like benzene itself and toluene, can be hydroxylated by anaerobic microorganisms (Vogel and Grbic-Galic, 1986) to phenol and p cresol; respectively, which are compounds that are otherwise known to be mineralized anaerobically.

The important events in the anaerobic degradation of monomeric phenols are illustrated in Figure 11. Initially, the lignin and tannin monomers are simplified by several catabolic steps. During the simplification process, numerous types of fermentative bacteria are involved. The activities of these bacteria cause: (1) cleavage of flavonoids in A and B rings; (2) saturation of double bonds on the side chains; (3) demethylization of methoxy groups; (4) dehydroxylation of phenolic groups; (5) removal of aliphatic side chains; (6) decarboxylation; and (7) ß oxidation or oxidative decarboxylation of aliphatic side chains. Finally, several simple aromatic compounds with one or two functional groups can be recognized just prior to point that the aromatic rings are broken open. Two possibilities exists for the decomposition of the ring, these shall be denominated as the "phenol" and "trihydroxy" pathways.

During the decomposition of mono- and dihydroxy (or methoxy) flavonoid B rings and lignin monomers, phenol and benzoic acid are the most common simplified aromatic degradation intermediates found. Their aromatic rings are saturated to form cyclohexane type compounds which are cleaved to higher fatty acids of 5 to 7 carbons, acetic acid and H_2. The fatty acids in turn are degraded by acetogenic organisms to acetic acid and H_2. This sequence of reactions is thermodynamically unfavorable (ie.$\Delta G°$ benzoic acid = +70 kJ mol^{-1}). The "phenol" pathway is a syntrophic pathway in which the acidification of the aromatic compounds and fatty acid intermediates to acetic acids and H_2 is made thermodynamically favorable by the methanogenic removal of acetic acid and H_2 (Ferry and Wolfe, 1976; McInerney et al., 1981). This is in correspondence with the observation that adding methanogenic inhibitors to cultures actively degrading mono- or dihydroxy (or methoxy) phenolic compounds causes an accumulation of simplified aromatic intermediates, cyclohexane compounds and fatty acids (Grbic-Galic, 1986; Lane, 1980; Colberg and Young 1985b; Healy et al., 1980; Fedorak et al., 1986). Furthermore, addition of acetic acids to the cultures can drastically decrease the anaerobic degradation rate of simplified phenolic compounds, like phenol and p cresol (Fedorak et al., 1986). Therefore, in the absence of

Table 3. The Anaerobic Biodegradability of Monomeric Phenolic Compounds Reported in the Literature.

Compound	Inoculum	Evidence Degradation*	Reference
LIGNIN MONOMERS			
p coumaric acid	anaerobic filter	I-VFA, Disapp, CH4	286
p coumaric acid	isolated bacteria	I-arom	49
p coumaric acid	isolated bacteria	I-arom	145
p coumaric acid	isolated bacteria	I-arom	83
p coumaric acid	isolated bacteria	Disapp	305
p coumaric acid	pig manure	I-arom	271
ferulic acid	enrich. culture	I-arom, I-aliph, I-VFA	103
ferulic acid	sewage sludge	Disapp, CH4	128
ferulic acid	enrich. culture	I-arom, Disapp, CH4	101
ferulic acid	isolated bacteria	I-arom, Disapp	232
ferulic acid	isolated bacteria	I-arom	49
ferulic acid	isolated bacteria	I-arom, Disapp, Reduc-NO3	287
ferulic acid	isolated bacteria	I-arom	12
ferulic acid	isolated bacteria	I-arom	145
ferulic acid	isolated bacteria	I-arom	83
ferulic acid	isolated bacteria	Disapp	305
ferulic acid	enrich. culture	I-arom, I-aliph, I-VFA, Disapp, CH4	129
ferulic acid	isolated bacteria	I-arom, Disapp	102
ferulic acid	CSTR	Disapp, CH4	98
coniferyl alcohol	enrich. culture	I-arom, Disapp, CH4	101
caffeic acid	isolated bacteria	I-arom	145
caffeic acid	rat faeces	I-arom	31
caffeic acid	isolated bacteria	I-arom	243
caffeic acid	isolated bacteria	I-arom	83
caffeic acid	isolated bacteria	I-arom, Disapp	102
caffeic acid	CSTR	Disapp, CH4	98
sinapic acid	isolated bacteria	I-arom	12
guaiacol	isolated bacteria	I-arom, Disapp, Reduc NO3	287
guaiacol	anaerobic sludge	Disapp, CH4	23
vanillic acid	sewage sludge	Disapp, CH4	128
vanillic acid	isolated bacteria	Disapp, Reduc-NO3	287
vanillic acid	isolated bacteria	I-arom	12
vanillic acid	isolated bacteria	I-arom, I-VFA, Disapp	88
vanillic acid	anoxic lake sediments	CH4	328
vanillic acid	sewage sludge	CH4	140
vanillin	sewage sludge	CH4	128
vanillin	isolated bacteria	I-arom	12
vanillin	isolated bacteria	Disapp	305
syringic acid	sewage sludge	CH4	128
syringic acid	isolated bacteria	I-arom	12
syringic acid	enrich. culture	I-arom, I-VFA, Disapp, CH4	151
syringaldehyde	sewage sludge	CH4	128
syringaldehyde	isolated bacteria	I-arom	12
HYDROLYZABLE TANNIN MONOMERS			
gallic acid	isolated bacteria	I-VFA, Disapp	183
gallic acid	enrich. culture	I-VFA, Disapp, CH4	151
gallic acid	isolated bacteria	I-VFA, Disapp	261

* Abbreviations: **I-arom** = aromatic degradation intermediates; **I-aliph** = aliphatic degradation intermediates; **I-VFA** = volatile fatty acid degradation intermediates; **Disapp** = disappearance of phenolic compound; **CH4** = formation methane from phenolic compound; **Reduc-NO3** = anaerobic respiration nitrate.

Table 3. The Anaerobic Biodegradability of Monomeric Phenolic Compounds (Continued).

Compound	Inoculum	Evidence Degradation	Reference
gallic acid	CSTR	Disapp, CH4	98
pyrogallol	isolated bacteria	I-VFA, Disapp	183
pyrogallol	sewage sludge	CH4	50
pyrogallol	enrich. culture	I-VFA, Disapp, CH4	151
pyrogallol	isolated bacteria	I-VFA, Disapp	261
pyrogallol	sewage sludge	CH4	140
pyrogallol	CSTR	Disapp, CH4	98
pyrogallol	sewage sludge	CH4	264
CONDENSED TANNIN MONOMERS (FLAVONOIDS)			
catechin	rat intestinal contents	I-arom, I-aliph, Disapp	63
catechin	rat faeces	I-arom	31
catechin	enrich. culture	I-arom, I-aliph, I-VFA, CH4	15
quercetin	isolated bacteria	I-arom, I-VFA, Disapp	183
quercetin	rumen fluid	I-arom, Disapp	265
naringin	rumen fluid	I-arom, Disapp	265
apigenin	rat intestinal contents	I-arom, I-aliph, Disapp	105
myricetin	rat intestinal contents	I-arom, I-aliph, Disapp	104
rutin	isolated bacteria	Disapp	48
rutin	rumen fluid	I-arom, Disapp	265
rutin	rat faeces	I-arom	31
hesperetin	rat intestinal contents	I-arom, Disapp	139
mixed flavonoids	UASB, contact process	Disapp	17
SIMPLE PHENOLS			
phenol	sewage sludge	CH4	50
phenol	sewage sludge	Disapp, CH4	52
phenol	sewage sludge	CH4	128
phenol	sewage sludge	Disapp, CH4	127
phenol	digested pig manure	I-VFA, Disapp, CH4	304
phenol	anaerobic sludge	Disapp, CH4	55
phenol	anaerobic filter	Disapp	55
phenol	anoxic lake sediments	CH4	328
phenol	sewage sludge	Disapp, CH4	327
phenol	sewage sludge	Disapp, CH4	34
phenol	sewage sludge	CH4	140
phenol	immob. enrich. culture	Disapp, CH4	66
phenol	enrich. culture	I-VFA, Disapp, CH4	79
phenol	enrich. culture	CH4	78
phenol	CSTR	I-VFA, Disapp	228
phenol	CSTR	Disapp, CH4	98
phenol	sewage sludge	CH4	30
phenol	anaerobic filter	Disapp, CH4	30
phenol	sewage sludge	CH4	77
phenol	enrich. culture	Disapp, CH4	314
phenol	anaerobic AC filter	I-VFA, Disapp, CH4	313
phenol	anaerobic AC filter	Disapp, CH4	161
phenol	anaerobic fixed film	I-VFA, Disapp, CH4	308
phenol	anaerobic AC filter	Disapp, CH4	163
phenol	sewage sludge	I-arom, I-VFA, Disapp, CH4	171
phenol	sewage sludge	CH4	264
phenol	enrich. culture	Disapp, I-VFA, CH4	284
p cresol	sewage sludge	CH4	50

Table 3. The Anaerobic Biodegradability of Monomeric Phenolic Compounds (Continued).

Compound	Inoculum	Evidence Degradation	Reference
p cresol	sewage sludge	Disapp, CH4	52
p cresol	digested pig manure	I-VFA, Disapp, CH4	304
p cresol	sewage sludge	I-arom, Disapp, CH4	327
p cresol	sewage sludge	Disapp, CH4	34
p cresol	sewage sludge	CH4	140
p cresol	enrich. culture	I-VFA, Disapp, CH4	79
p cresol	enrich. culture	CH4	78
p cresol	sewage sludge	CH4	30
p cresol	anaerobic filter	Disapp, CH4	30
p cresol	sewage sludge	CH4	77
p cresol	enrich. culture	Disapp, CH4	314
p cresol	anaerobic fixed film	I-VFA, Disapp, CH4	308
p cresol	sewage sludge	CH4	264
m cresol	sewage sludge	CH4	140
m cresol	enrich. culture	I-VFA, Disapp, CH4	79
m cresol	enrich. culture	Disapp, CH4	314
m cresol	sewage sludge	CH4	264
ethylphenol	anaerobic filter	Disapp, CH4	30
catechol	enrich. culture	I-arom, I-aliph, CH4	15
catechol	sewage sludge	CH4	128
catechol	sewage sludge	Disapp, CH4	127
catechol	anaerobic filter	Disapp	55
catechol	sewage sludge	CH4	140
catechol	enrich. culture	I-VFA, Disapp, CH4	79
catechol	sewage sludge	CH4	30
catechol	anaerobic filter	Disapp, CH4	30
catechol	sewage sludge	CH4	264
catechol	anaerobic AC filter	Disapp, CH4	278
catechol	enrich. culture	Disapp, I-VFA, CH4	284
hydroquinone	anaerobic filter	Disapp	55
hydroquinone	sewage sludge	I-arom, Disapp, CH4	327
hydroquinone	enrich. culture	I-VFA, Disapp, CH4	79
hydroquinone	enrich. culture	Disapp, I-arom, I-VFA, CH4	284
"A" RING OF FLAVONOIDS			
resorcinol	sewage sludge	CH4	50
resorcinol	sewage sludge	Disapp, CH4	52
resorcinol	anaerobic filter	Disapp	55
resorcinol	sewage sludge	CH4	140
resorcinol	sewage sludge	CH4	30
resorcinol	anaerobic filter	Disapp, CH4	30
resorcinol	isolated bacteria	Disapp, I-VFA	293
phloroglucinol	isolated bacteria	I-VFA, Disapp	183
phloroglucinol	sewage sludge	CH4	50
phloroglucinol	rumen fluid	I-aliph, Disapp, CH4	13
phloroglucinol	isolated bacteria	I-VFA, Disapp	261
phloroglucinol	rumen fluid	Disapp	265
phloroglucinol	isolated bacteria	Disapp	292
phloroglucinol	isolated bacteria	Disapp	240
phloroglucinol	sewage sludge	I-arom, I-VFA, Disapp, CH4	327
phloroglucinol	sewage sludge	CH4	140
phloroglucinol	CSTR	Disapp, CH4	98
phloroglucinol	sewage sludge	CH4	264

Table 3. The Anaerobic Biodegradability of Monomeric Phenolic Compounds (Continued).

Compound	Inoculum	Evidence Degradation	Reference
SIMPLE BENZOIC ACIDS			
benzoic acid	sewage sludge	CH4	128
benzoic acid	rumen fluid	I-aliph, Disapp, CH4	13
benzoic acid	anaerobic filter	Disapp, CH4	286
benzoic acid	enrich. culture	I-aliph, I-VFA, Disapp, CH4	81
benzoic acid	enrich. culture	Disapp, CH4	82
benzoic acid	isolated bacteria	Reduc-NO3	287
benzoic acid	anaerobic sludge	Disapp, CH4	55
benzoic acid	anoxic lake sediments	CH4	328
benzoic acid	anoxic lake sediments	CH4	140
benzoic acid	sewage sludge	CH4	140
benzoic acid	CSTR	Disapp, CH4	98
benzoic acid	sewage sludge	CH4	30
benzoic acid	enrich. culture	I-aliph, I-VFA, CH4	156
benzoic acid	sewage sludge	CH4	264
benzoic acid	enrich. culture	I-VFA, Disapp, CH4	294
p hydroxybenzoic acid	sewage sludge	CH4	50
p hydroxybenzoic acid	sewage sludge	Disapp, CH4	128
p hydroxybenzoic acid	enrich. culture	I-arom, I-aliph, Disapp, CH4	14
p hydroxybenzoic acid	isolated bacteria	Reduc-NO3	287
p hydroxybenzoic acid	anoxic lake sediments	CH4	140
p hydroxybenzoic acid	sewage sludge	CH4	140
p hydroxybenzoic acid	pig manure	I-arom	271
p hydroxybenzoic acid	sewage sludge	CH4	264
p hydroxybenzoic acid	enrich. culture	I-VFA, Disapp, CH4	294
protocatechuic acid	sewage sludge	CH4	128
protocatechuic acid	enrich. culture	I-arom, I-aliph, Disapp, CH4	14
protocatechuic acid	isolated bacteria	Reduc-NO3	287
protocatechuic acid	anoxic lake sediments	CH4	140
protocatechuic acid	sewage sludge	CH4	140
protocatechuic acid	sewage sludge	CH4	264
AROMATIC ACIDS			
phenylacetic acid	anaerobic filter	Disapp, CH4	286
phenylpropionic acid	isolated bacteria	I-arom, Disapp	102
cinnamic acid	sewage sludge	CH4	128
cinnamic acid	rumen fluid	I-aliph, Disapp, CH4	13
cinnamic acid	anaerobic filter	Disapp, CH4	286
cinnamic acid	isolated bacteria	Disapp	305
cinnamic acid	isolated bacteria	I-arom, Disapp	102
AROMATIC AMINO ACIDS			
phenylalanine	rumen fluid	I-aliph, Disapp, CH4	13
phenylalanine	enrich. culture	I-arom, I-aliph, Disapp, CH4	16
phenylalanine	isolated bacteria	I-arom	72
phenylalanine	rumen	I-arom, I-VFA	263
tyrosine	anaerobic filter	I-arom, Disapp, CH4	286
tyrosine	enrich. culture	I-arom, I-aliph, I-VFA, Disapp, CH4	16
tyrosine	isolated bacteria	I-arom	72
tyrosine	pig manure	I-arom	271
tyrosine	rumen	I-arom, I-VFA	263
L dopa	rat faeces	I-arom	31

Table 3. The Anaerobic Biodegradability of Monomeric Phenolic Compounds (Continued).

Compound	Inoculum	Evidence Degradation	Reference
HUMIC-LIKE MONOMERIC UNITS			
tryptophan	enrich. culture	I-arom, Disapp, CH4	16
tryptophan	isolated bacteria	I-arom	72
tryptophan	pig manure	I-arom	271
tryptophan	rumen	I-arom, I-VFA	263
indole	sewage sludge	I-arom, Disapp, CH4	27
indole	sewage sludge	I-arom, Disapp, Reduc-NO3	203
indole	enrich. culture	Disapp, CH4	312
APOLAR BENZENES			
toluene	enrich. culture	I-arom, Disapp	309
benzene	enrich. culture	I-arom, Disapp	309

methanogenesis (or anaerobic respiration), mono and dihydroxy phenolic monomers may behave as recalcitrant compounds.

During the decomposition of hydrolyzable tannin monomers, flavonoid A rings, trihydroxy flavonoid B rings and trihydroxy (or methoxy) lignin monomers, the most likely end products of the aromatic simplification process are pyrogallol and phloroglucinol. These simple trihydroxy compounds are cleaved into acetic acid and the reaction is thermodynamically favorable (ie. $\Delta G°$ pyrogallol = -158 kJ mol^{-1}). Addition of methanogenic inhibitors to methanogenic consortia degrading trihydroxy (or methoxy) phenolics has no effect on the rate of acidification to acetic acid (Kaiser and Hanselmann, 1982), and even pure cultures of isolated strictly anaerobic fermentative bacteria can completely acidify the trihydroxy phenols in the absence of methane bacteria (Schink and Pfennig, 1982). Therefore, the "trihydroxy" pathway is not a syntrophic pathway. The acidification of trihydroxy phenolics is not inhibited by the accumulation of methanogenic substrates. The trihydroxy phenolics compounds should be regarded as easy substrates.

In anaerobic microbial environments with active methanogenesis, most monomeric phenolic compounds can be degraded rapidly after adequate acclimation. The fact that phenolic monomeric substrates can be degraded at moderate to high loading rates in reactors with immobilized sludge has been demonstrated on numerous occasions (Chmielowski and Kusznik, 1966; Suidan et al., 1983; Khan et al., 1981; Khan et al., 1982; Chou et al., 1979; Wang et al., 1986; Dwyer et al., 1986; Suidan et al., 1980b; Suidan et al., 1988; Fox et al., 1988; Kim et al., 1986).

4.2. Polymeric Lignin and Tannins

Natural lignins, which are high molecular weight (MW) polymers, in contrast to monomers are not biodegradable in anaerobic environments (Figure 12). The fact that the lignin polymer is not an available anaerobic substrate is not due to the cleavage of lignin intermonomeric bonds. Each type of intermonomeric bond present in lignin can be cleaved by anaerobic microorganisms fed dimeric model lignins (Chen et al., 1985a; Chen et al., 1985b; Chen et al., 1987; Zeikus et al., 1982). Even lignin oligomers of 3 to 7 monomeric units are partly biodegradable in anaerobic environments (Figure 12). It is also evident that such oligomeric lignins are somewhat depolymerized during anaerobic digestion (Colberg and Young, 1982) indicating that intermonomeric cleavage of such compounds can also occur. There appears to be an inverse correlation between lignin polymer size and its anaerobic biodegradability. Therefore it is the high MW quality of natural lignins that is responsible for its recalcitrance in anaerobic environments. Large polymers cannot be taken-up by microorganisms to be attacked intracellularly, and the only extracellular enzymes in nature

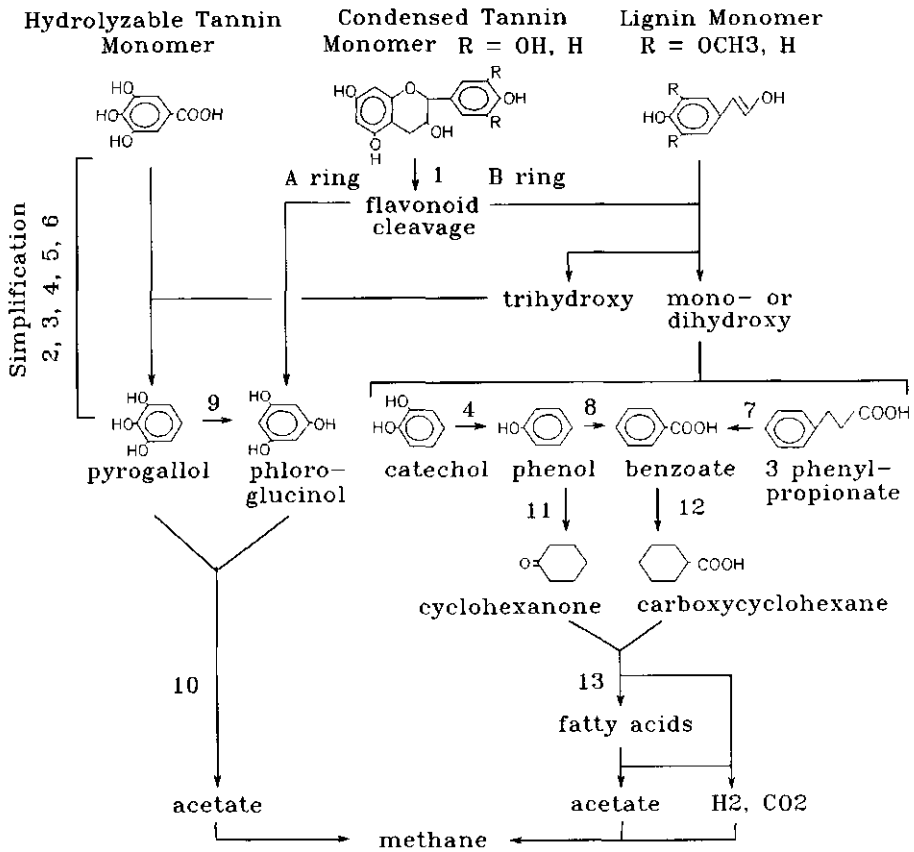


Figure 11. Anaerobic degradation pathways of tannin and lignin monomers.

References: (1) cleavage of flavanoids in A and B rings Griffiths and Smith, 1972a and b; Honohan et al., 1976; Brown, 1977; Simpson et al, 1969; Balba and Evans, 1980a; Lane, 1980; Krumholz and Bryant, 1986; Das, 1969; (2) Hydrogenation of double bonds on the side chain Grbic-Galic, 1985; Chesson, 1982; Peppercorn and Goldman, 1971; Healy et al., 1980; Ohmiya et al., 1986; Griffiths and Smith, 1972b; (3) demethylization of methoxy groups Frazer and Young, 1986; Schink and Pfennig, 1982; Bach and Pfennig, 1981; Kaiser and Hanselmann, 1982a and b; Boyd et al., 1983; Taylor, 1983; Balba and Evans, 1980a; Grbic-Galic, 1985; Balba et al, 1979; (4) Dehydroxylation Peppercorn and Goldman, 1971; Booth and Williams, 1963; Scott et al., 1963; Healy et al., 1980; Grbic-Galic, 1983; Grbic-Galic, 1985; Grbic-Galic, 1986; Griffiths and Smith, 1972a; Balba et al., 1979; Szewzyk et al., 1985; (5) removal aliphatic side chains Kaiser and Hanselmann, 1982b; Balba and Evans, 1980a; Tarvin and Buswell, 1934; Young and Rivera, 1985; (6) decarboxylation Finkle et al., 1962; Balba and Evans, 1980a; Balba et al., 1979; Indhal and Scheline, 1968; Steinke and Paulson, 1964; Grbic-Galic, 1986; (7) ß oxidation or oxidative decarboxylation aliphatic side chains Berry et al., 1987b; Young, 1984; Healy et al., 1980; Grbic-Galic, 1983; Grbic-Galic, 1985; Grbic-Galic, 1986; Colberg, 1988; (8) phenol to benzoate Neufeld et al., 1980; Knoll and Winter, 1987; (9) pyrogallol to phloroglucinol Haddock and Ferry, personal communication; (10) trihydroxy benzenes to acetate Kaiser and Hanselmann, 1982a; Schink and Pfennig, 1982; Schink, 1988; Patel et al., 1981; (11) phenol to cyclohexanone Balba et al., 1979; Balba and Evans 1980a; Evans, 1977; Schink, 1988; Colberg, 1988; (12) benzoate to carboxy cyclohexane Healy et al., 1980; Balba and Evans, 1977; Colberg, 1988; Keith et al., 1978; Szewzyk et al., 1985; (13) cyclohexanes to fatty acids Healy et al., 1980; Balba and Evans, 1977; Balba et al., 1979; Evans, 1977; Colberg, 1988; Keith et al., 1978.

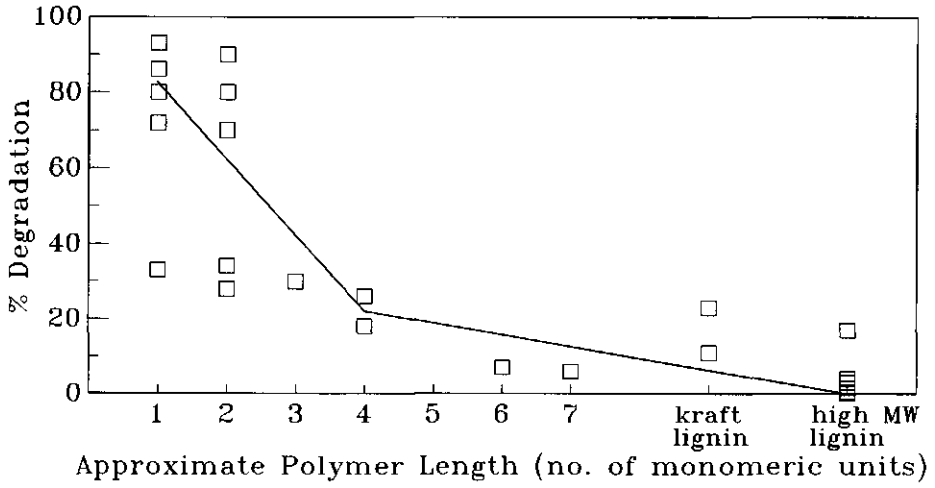


Figure 12. The role of polymer size on the biodegradability of lignin.

Data: type lignin, (number of monomeric units or approximation), days of digestion, inoculum, measurement of degradation, percent degraded, reference; 1. syringic acid, (1), 17 days, sewage sludge, gas production, 80%, Healy and Young, 1979; 2. vanillin, (1), 28 days, sewage sludge, gas production, 72%, Healy and Young, 1979; 3. vanillic acid, (1), 28 days, sewage sludge, gas production, 86%, Healy and Young, 1979; 4. vanillic, (1), 16 days, anoxic lake sediment, 14C in gas, 33%, Zeikus et al., 1982; 5. ferulic acid, (1), 34 days, sewage sludge, gas production, 86%, Healy and Young, 1979; 6. coniferyl alcohol, (1), 8 days, enrich. culture, gas production, 93%, Grbic-Galic, 1983; 7. ferulic acid, (1), 8 days, enrich. culture, gas poroduction, 93%, Grbic-Galic, 1983; 8. dehydrodivanillin, (2), 8 days, rumen fluid, disapp, I-VFA, 80%, Chen et al., 1985a; dehydrodiisoeugenol, (2), 8 days, rumen bacteria, disappearance, 70%, Chen et al., 1987; 9. veratrylglycol-beta-guaiacyl ether, (2), 4 days, rumen bacteria, disappearance, 90%, Chen et al., 1985b; 10. guaiacylglycol-beta-guaiacyl ether, (2), 16 days, anoxic lake sediment, 14C in gas, 28%, Zeikus et al., 1982; 11. kraft lignin, (mixture oligomeric and high MW lignin), 30 days, anoxic lake sediment, 14C in gas, 11%, Zeikus et al., 1982; 12. high MW fraction heat alkali extracted lignin, (high MW lignin), 30 days, anoxic lake sediment, 14C in gas, 0%, Zeikus et al., 1982; 13. lignin poplar wood, (high MW lignin), 180 days, soil, 14C in gas, 0%, Odier and Monties, 1983; 14. natural lignin unspecified source, (high MW lignin), 41 days, anoxic sediments, rumen contents, 14C in gas, 0%, Hackett et al., 1977; 15. lignin pine wood, (high MW lignin), 60 days, thermophilic enrich. culture, 14C in gas, 3%, Benner and Hodson, 1985; 16. synthetic lignin, (high MW lignin), 60 days, thermophilic enrich. culture, 14C in gas, 4%, Benner and Hodson, 1985; 17. kraft lignin, (mixture oligomeric and high MW lignin), 60 days, thermophilic enrich. culture, 14C in gas, 23%, Benner and Hodson, 1985; 18. heat alkali extracted lignin douglas fir wood, (fraction average = 7), 43 days, enrich. culture, 14C in gas, 6%, Colberg and Young, 1985a; 19. heat alkali extracted lignin douglas fir wood, (fraction average = 4), 43 days, enrich. culture, 14C in gas, 26%, Colberg and Young, 1985a; 19. heat alkali extracted lignin douglas fir wood, (fraction average = 2), 43 days, enrich. culture, 14C in gas, 34%, Colberg and Young, 1985a; 20. heat alkali extracted lignin douglas fir wood, (average = 4), 30 days, sewage sludge, 14C in gas, 18%, Colberg and Young, 1982; 21. lignin grass, (high MW lignin), 294 days, marsh anoxic sediments, 14C in gas, 17%, Benner et al., 1984; 22. lignin red mangrove wood, (high MW lignin), 246 days, marsh anoxic sediments, 14C in gas, 1.5%, Benner et al., 1984; 23. synthetic lignin, (high MW lignin), 276 days, marsh anoxic sediments, 14C in gas, 4%, Benner et al., 1984; 24. lignin grass, (high MW lignin), 7 days, rumen fluid, 14C in gas, 0.3%, Akin and Benner, 1988; 25. heat alkali extracted lignin peat, (fraction average = 3), ND days, sewage sludge, gas production, 30%, Young and McCarty, 1981; 26. heat alkali extracted lignin peat, (fraction average = 6), ND days, sewage sludge, gas production, 7%, Young and McCarty, 1981.

capable of depolymerizing lignin promote a nonspecific oxidative erosion, whereby oxygen derived radicals play a key role (Kirk et al.,, 1987).

The anaerobic biodegradability of tannins has not been as thoroughly studied. In many respects, condensed tannins are similar to lignins since their intermonomeric bonds involve C to C linkages between branch and ring carbons. Unaltered condensed tannins of plants, however have a distinctly lower MW than lignin. The tannins are most commonly present as oligomeric constituents. In one previous study, the disappearance (82%) of oligomeric condensed tannins in olive oil mill wastewater during anaerobic treatment by a contact reactor has been reported (Balice, et al., 1988), however poor removal efficiencies (5%) were observed in a UASB reactor. The difference was attributed to the longer hydraulic retention times (9 days versus 1) in the contact reactor. In any case, the monomeric condensed tannins were removed with a higher efficiency in the UASB reactor, indicating that they are more readily biodegradable than the oligomers.

Hydrolyzable tannins are quite distinct from lignin because they are composed of easily hydrolyzable ester bonds. These bonds are cleaved by a hydrolytic extracellular enzymes, called tannase, of aerobic microorganisms (Yamada et al., 1968, Aoki et al., 1976). The hydrolytic activity of such enzymes presumably does not require oxygen. Experiments with gallotannic acid as a sole carbon source during anaerobic digestion have demonstrated a high conversion (68% of the COD) to methane (Gollakota and Sarada, 1988). The anaerobic bacteria are therefore capable of hydrolyzing the ester intermonomeric bonds of this hydrolyzable tannin (of nine monomeric units). Likewise, high removal efficiencies of hydrolyzable tannins in olive oil mill effluents (67 to 73%) have been observed during anaerobic treatment in both a UASB and a contact reactor (Balice et al., 1988).

4.3. Humus Polymers

Humus is similar to lignin due to its high MW. However, humic polymers are richer in direct C to C bonds between aromatic rings, and to some extent certain humic compounds, melanin, have amino compounds incorporated in the polymer structure. They are highly condensed polymers which possibly contributes to their recalcitrance.

Humic substances are clearly less biodegradable than various types of high MW lignins in aerobic environments. Lignins incubated in aerobic soils are mineralized for 10 to 20% after 60 to 175 day incubations; whereas in parallel experiments, peat and melanin were only mineralized for about 5% and phenolase polymers, autoxidation polymers or natural humus for only 1% or less (Martin and Haider, 1980; Martin et al., 1972). Even white rot fungi, which were capable of decolorizing lignin polymers, were not able to decolorize humic polymers prepared from the oxidation of ortho-dihydroxy phenols (Fukuzumi, 1980).

Very few experiments have been conducted to study the anaerobic biodegradability of humic substances. The biodegradability of peat was investigated using anaerobic sludge adapted to lignocellulosic substrate for 1.5 years. Absolutely no conversion of the peat COD was observed in 31 days (Owens et al., 1979), the sludge was in any case able to partially decompose depolymerized peat samples. Likewise it has been observed that the autoxidation products of pyrogallol were not degraded by anoxic muds capable of degrading unoxidized pyrogallol under anaerobic conditions (Schink and Pfennig, 1982).

Therefore, humus forming processes, like autoxidation and phenol oxidase oxidations, which can occur rather rapidly in the handling of process waters and wastewaters, have a significant implication on the biodegradability of phenolic fraction for both aerobic as well as anaerobic treatment systems. These polymerization reactions can transform biologically degradable substrates to colorful recalcitrant organic substances. In this regard, anaerobic treatment may be advantageous to aerobic treatment with respect to the COD efficiency for wastewaters that contain highly hydroxylated phenolic substances prone to autoxidative or phenol oxidase polymerization. The condensed tannins are a good example of such compounds. During their fungal decomposition in aerated cultures, a large fraction of the tannins are polymerized to nonbiodegradable humic substances (Updegraff and Grant, 1975; Grant, 1976; Chandra et al., 1973). Significant degradation was only obtained if the polymerization reactions were carefully prevented by not agitating the culture medium or lowering the pH to 3. With bacterial cultures, Das (1969) could only obtain degradation of

condensed tannin monomers under anaerobic conditions, because the humic substances produced by aeration were not degraded by aerobic bacteria.

5. TOXICITY OF PHENOLIC COMPOUNDS

The biodegradability of phenolic compounds is important for understanding the ultimate efficiency of phenolic compound removal during biological waste treatment. Considering that the phenolic compounds are often present in the wastewater with other substrates, than it is also important to evaluate the inhibitory effects of the phenolic compounds on the anaerobic metabolism of easily biodegradable organic matter. Generally, the rate limiting stage of the biodegradation is the methanogenesis of acidified substrates. Therefore, the inhibiting effects of phenolic compounds on the activity and growth of methanogenic bacteria deserves close attention. Additionally, the inhibiting effect on extracellular hydrolytic enzymes will also be of importance in cases where the polymeric substrates are major constituents of the wastewater. If inhibition occurs, the phenolic compounds than play a role in reducing the rate at which wastewater can be treated. The possibility that phenolic compounds are exposed to humus forming processes should also be considered, since the chemical transformation of phenols during such processes may have a distinct impact on their inhibitory characteristics.

5.1. The Methanogenic Inhibition of Monomeric Phenols

In the past, considerable research has been directed towards the inhibiting effect of numerous compounds on the activity of methanogenic bacteria. Among the compounds, several monomeric phenols and related aromatics have been investigated. The inhibiting concentrations found are summarized in Table 4. The inhibitory nature of monomeric phenols is highly related to the apolarity of the compounds. Phenolic acids and the polar phenols with several hydroxyl group substitutions are the least toxic; whereas increasing toxicity is evident with increasing alkyl substitutions and with decreasing number of hydroxyl groups. The apolarity perhaps enables the compounds to become partially solubilized in the membranes of bacteria, which would damage the membrane's function. The degree of inhibition observed for any given phenolic compound varies with different assay substrates. Lower inhibition is observed with acetic acid that only involves acetoclastic methanogenesis. The highest inhibitions are evident where propionic acid or phenol are utilized as assay substrates. In addition to acetoclastic methanogens, these are metabolized by acetogenic bacteria and autotrophic methanogens. Therefore, the latter bacteria are more sensitive to the toxicity of phenolic compounds.

5.2. The Toxicity of Tannic Compounds

The toxicity of tannic compounds to methane bacteria has not been studied. However, numerous indications of tannin inhibition are evident from the literature. The high methanogenic toxicity of debarking wastewater, which is similar to a crude tannin extract, is reported from two previous studies (Latola, 1985; Rekunen, 1986). Similarly, a water extraction of bark, which removes tannins, greatly increased the convertibility of bark to methane by anaerobic digestion (Kuwahara et al., 1984). Inhibition during the anaerobic treatment of vegetable tannery wastewater applied at high concentrations was attributed to the tannins present, 320 mg L^{-1} (Arora et al., 1975). The fact that tannins are toxic to methane bacteria could be expected, considering the inhibitory characteristics tannins have towards a wide variety of enzymes (Table 5) and microorganisms (Table 6). Tannins are also inhibitory to viruses (Thung and van der Want, 1951; van Schreven, 1941) and plants (Percuoco et al., 1973). Furthermore, with regard to aquatic toxicity of tannins, studies cited by Mahadevan and Muthukumar (1980) report that tannins are lethal to fresh water fish at

Table 4. The Methanogenic Toxicity of Monomeric Phenolic Compounds Reported in the Literature.

Compounds[+]	Concentration	Substrate[*]	Inhibition	Reference
	mg L^{-1}		%	
Phenolic acids				
p coumaric acid	3300[**]	cellulose	50	234
ferulic acid	4850[**]	cellulose	0	234
vanillic acid	5040	cellulose	0	234
benzoic acid	4880	C_2	35	54
Polar simple phenols				
hydroquinone	4400	C_2	35	54
resorcinol	3190	C_2	50	54
catechol	2640	C_2	50	54
catechol	3000	C_2	50	30
catechol	1500	C_3	50	30
Simple phenols				
guaiacol	2200[**]	C_2	50	23
phenol	2444	C_2	50	54
phenol	2250	C_2	50	30
phenol	1800[**]	C_2,C_3	50	77
phenol	875	C_3	50	30
phenol	900	phenol	50	66
phenol	700[**]	sewage sludge	50	241
Apolar simple phenols				
p cresol	1000	C_2	50	30
p cresol	800[**]	C_2,C_3	50	77
p cresol	500	C_3	50	30
p cresol	500	phenol	50	314
napthol	650	C_2	50	30
napthol	300	C_3	50	30
eugenol	250[**]	C_2	50	23
p ethylphenol	250	phenol	50	314
Apolar simple aromatics				
p cymene	500[**]	C_2	50	23
ethylbenzene	340	C_2	50	54

[+] from top to bottom the compounds are approximately placed in order of increasing apolarity.

[*] substrates: C2 = acetate; C3 = propionate

[**] indicates 50% inhibitory concentration was estimated from data.

Table 5. Tannin Inihibition of Enzymes Reported in the Literature.

Tannin Type (or source)	Concentration $g\ L^{-1}$	Enzyme	Inhibition %	Reference
commercial tannin	1.7	pectinase	84	202
commercial tannin	0.004	peroxidase	70	202
wattle tannin	10.0	pectinase	70	26
wattle tannin	10.0	cellulase	80	26
wattle tannin	0.17	urease	50	26
grape leaf tannin	0.16	pectinase	98	246
commercial tannin	0.17	xylanase	50	200
commercial tannin	0.51	pectinase	50	200
commercial tannin	0.003	peroxidase	50	200
commercial tannin	0.034	laccase	50	200
chinese gallotannin	1.8	pectinase	85	31
turkish gallotannin	1.8	pectinase	75	31
pentogalloyl glucose	1.8	pectinase	96	31
theaflavin	7.6	pectinase	72	31
thearubigen	7.0	pectinase	73	31
tannic acid	1.7	succinoxidase	97	141
tannic acid	0.85	malate decarboxylase	90	141
tannic acid	0.4	alcohol dehydrogenase	29	97
wattle tannin	0.4	alcohol dehydrogenase	76	97
tannic acid	1.0	lactate dehydrogenase	100	97
wattle tannin	1.0	lactate dehydrogenase	100	97
tannic acid	1.0	peroxidase	41	97
wattle tannin	1.0	peroxidase	50	97
tannic acid	1.0	catalase	42	97
wattle tannin	2.0	catalase	80	97
tannic acid	2.0	β-glucosidase	48	97
wattle tannin	2.0	β-glucosidase	47	97
sorghum tannin	0.14	α-amylase	68	62
carob pod tannin	0.267	trypsin	55	285
m-digallic acid	0.267	trypsin	30	285
carob pod tannin	0.016	α-amylase	80	285
m-digallic acid	0.016	α-amylase	70	285
carob pod tannin	0.107	lipase	60	285
m-digallic acid	0.107	lipase	90	285
tannic acid	0.12	malate dehydrogenase	100	84
tannic acid	0.12	isocitrate dehydrogenase	100	84
tanic acid	0.12	G-6-P dehydrogenase	100	84
acid condensed epicatechin	0.087	malate dehydrogenase	98	33
acid condensed epicatechin	0.087	G-6-P dehydrogenase	96	33
sorghum tannin	0.030	α-amylase (pancrease)	95	276
sorghum tannin	0.006	α-amylase (bacteria)	56	276
sorghum tannin	0.050	α-amylase (barley)	65	276
sorghum tannin	0.033	pectinase-methyl esterase	50	276
sorghum tannin	0.050	pectinase-lyase	100	276
sorghum tannin	0.011	ribulosediphosphatase	97	276

Table 6. The Tannin Inhibition of Microorganisms Reported in the Literature.

Type Tannin	Concentration	Microorganism	Observation	Inhibition	Ref.
(or source)	g L^{-1}			%	
		FUNGI			
white oak heartwood	1.0	Polyporus versicolor	growth	5	119
white oak heartwood	1.0	Poria monticola	growth	92	119
elligotannins	1.0	Poria monticola	growth	28	119
chinese chestnut bark	12.0	Endothia parasitica	growth	100	229
american chestnut bark	12.0	Endothia parasitica	growth	20	229
japanese chestnut bark	12.0	Endothia parasitica	growth	20	229
chestnut wood	0.31	Fusarium solani	germination	100	195
gallotannin	0.31	Fusarium solani	germination	100	195
wattle tannin	0.31	Fusarium solani	germination	100	195
chestnut wood	0.31	Verticillium albo-atrum	germination	100	195
gallotannin	0.31	Verticillium albo-atrum	germination	84	195
wattle tannin	0.31	Verticillium albo-atrum	germination	100	195
chestnut wood	1.0	Fusarium solani	germination	100	195
gallotannin	1.0	Fusarium solani	germination	100	195
wattle tannin	1.0	Fusarium solani	germination	0	195
chestnut wood	1.0	Verticillium albo-atrum	germination	100	195
gallotannin	1.0	Verticillium albo-atrum	germination	100	195
wattle tannin	1.0	Verticillium albo-atrum	germination	50	195
oak acorn	2.2	Calvatia gigantea	growth	50	157
commercial tannin	7.7	Aspergillus fumigatus	growth	99	61
commercial tannin	7.7	Aspergillus terreus	growth	66	61
commercial tannin	7.7	Fusarium oxysporum	growth	100	61
commercial tannin	7.7	Absidia glauca	growth	100	61
commercial tannin	7.7	Oospora sulphurea	growth	100	61
commercial tannin	7.7	Penicillium granulatus	growth	0	61
commercial tannin	7.7	Penicillium implicatum	growth	0	61
commercial tannin	7.7	Penicillium nigricans	growth	0	61
commercial tannin	7.7	Penicillium lilacinum	growth	100	61
chestnut wood	4.6	Saccharomyces cerevisiae	respiration	20	20
wattle tannin	4.6	Saccharomyces cerevisiae	respiration	10	20
		BACTERIA			
oak wood	5.0	Nitrifying bacteria	nitrification	50	19
wattle tannin	5.0	Nitrifying bacteria	nitrification	48	19
chestnut wood	4.6	Azotobacter vinelandii	respiration	51	20
wattle tannin	4.6	Azotobacter vinelandii	respiration	84	20
chestnut wood	4.6	Azotobacter chroococcum	respiration	99	20
wattle tannin	4.6	Azotobacter chroococcum	respiration	99	20
chestnut wood	4.6	Rhizobium meliloti	respiration	0	20
wattle tannin	4.6	Rhizobium meliloti	respiration	5	20
chestnut wood	4.6	Pseudomonas flourescens	respiration	0	20
wattle tannin	4.6	Pseudomonas flourescens	respiration	0	20
chestnut wood	4.6	Escherichia coli	respiration	39	20
wattle tannin	4.6	Escherichia coli	respiration	23	20

Table 6. The Tannin Inhibition of Microorganisms Reported In the Literature (Continued).

Type Tannin (or source)	Concentration $g\ L^{-1}$	Microorganism	Observation	Inhibition %	Ref.
tannic acid	0.012	Cellvibrio fulvus	growth	100	132
carob pod	0.015	Cellvibrio fulvus	growth	100	132
tannic acid	0.045	Sporocytophaga myxococcoides	growth	100	132
tannic acid	0.012	Cellvibrio fulvus	growth	100	132
carob pod	0.015	Cellvibrio fulvus	growth	100	132
tannic acid	0.045	Sporocytophaga myxococcoides	growth	100	132
carob pod	0.075	Sporocytophaga myxococcoides	growth	100	132
tannic acid	0.010	Clostridium cellulosolvens	growth	100	132
carob pod	0.060	Clostridium cellulosolvens	growth	100	132
tannic acid	0.030	Bacillus subtilis	growth	100	132
carob pod	0.075	Bacillus subtilis	growth	100	132
tannic acid	0.250	Streptococcus cremoris	growth	100	132
carob pod	0.600	Streptococcus cremoris	growth	100	132
tannic acid	0.100	Escherichia coli	respiration	15	132
carob pod	0.100	Escherichia coli	respiration	4	132
tannic acid	0.100	Cellvibrio fulvus	respiration	29	132
carob pod	0.100	Cellvibrio fulvus	respiration	26	132
tannic acid	10.0	Desulphovibrio desulphuricans	growth	100	32
myrobolam tannin	20.0	Desulphovibrio desulphuricans	growth	100	32
quebracho tannin	20.0	Desulphovibrio desulphuricans	growth	100	32
chestnut wood	20.0	Desulphovibrio desulphuricans	growth	100	32
mimosa tannin	5.0	Desulphovibrio desulphuricans	growth	100	32

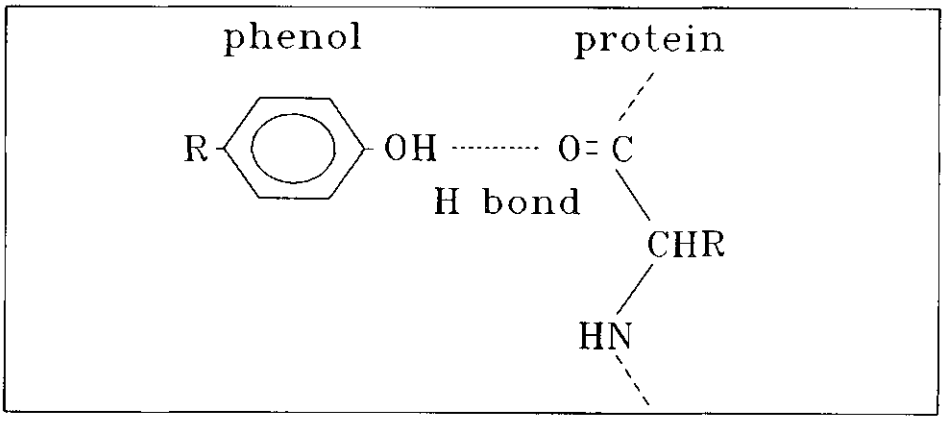


Figure 13. The hydrogen bond between phenol and proteins.

Table 7. Effect of Humus Forming Reactions on the Toxicity of Polar Phenolic Monomers and Tannins.

Compound	Conc.	Polymerization	Microorganism	Observation	Inhibition %		Ref.
	g L^{-1}	method	or Enzyme		before oxid.	after oxid.	
PART A. INCREASE IN INHIBITION BY POLYMERIZING MONOMERIC PHENOLS							
catechol	2.200	phenol oxidase	pectinase	activity	64	97	202
catechol	0.110	phenol oxidase	pectinase	activity	0	28	202
o cresol	0.025	phenol oxidase	pectinase	activity	70	95	202
protocatechuate	0.154	phenol oxidase	pectinase	activity	0	36	202
protocatechuate	0.154	phenol oxidase	cellulase	activity	0	33	202
catechol	1.1	phenol oxidase	pectinase	activity	18	63	31
catechin	2.9	phenol oxidase	pectinase	activity	19	80	31
epicatechin	2.9	phenol oxidase	pectinase	activity	0	93	31
epigallocatechin	3.1	phenol oxidase	pectinase	activity	0	63	31
EPG[a]	4.6	phenol oxidase	pectinase	activity	35	74	31
lueco-anthocyanidin	2.9	phenol oxidase	pectinase	activity	11	95	31
catechol	0.110	phenol oxidase	polygalacturonase	activity	0	68	272
catechol	0.110	phenol oxidase	PM[b] esterase	activity	0	75	272
catechol	NS[+]	phenol oxidase	Fusarium oxysporum	growth	low	high	272
catechol	NS	phenol oxidase	Fusarium oxysporum	germination	low	high	272
epicatechin	0.087	condensation[++]	malate dehydrogenase	activity	0	98	33
epicatechin	0.087	condensation[++]	G-6-P dehydrogenase	activity	0	96	33
catechol	0.330	phenol oxidase	C. miyabeanus[c]	growth	0	100	233
catechol	0.330	phenol oxidase	C. miyabeanus	germination	94	100	233
chlorogenate	0.944	phenol oxidase	CM virus[d]	infectivity	13	97	118
chlorogenate	0.944	phenol oxidase	TN virus[e]	infectivity	13	97	118
catechin	1.429	autoxidized	bacteria (rat gut)	growth	low	high	63
PART B. DECREASE IN INHIBITION BY POLYMERIZING TANNINS (LOW MW POLYMERIC PHENOLS)							
commercial tannin	1.7	autoxidation	pectinase	activity	84	0	202
commercial tannin	0.004	autoxidation	peroxidase	activity	70	0	202
oxid. catechol*	2.200	phenol oxidase	pectinase	activity	97	65	202
oxid. catechol*	0.110	phenol oxidase	pectinase	activity	28	0	202
oxid. o cresol*	0.025	phenol oxidase	pectinase	activity	95	70	202
oxid. protocatechuate*	0.154	phenol oxidase	pectinase	activity	36	0	202
oxid. protocatechuate*	0.154	phenol oxidase	cellulase	activity	33	0	202
apple tannins	NS	phenol oxidase	pectinase	activity	45	0	306
tallowwood tannin	NS	phenoloxidase	Coriolus versicolor	activity	95	19	254
canaigre tannin	1.0	autoxidation	Fusarium solani	growth	100	0	195
chestnut wood tannin	1.0	autoxidation	Fusarium solani	growth	100	0	195
canaigre tannin	1.0	autoxidation	V. albo-atrum[f]	growth	100	0	195
chestnut wood tannin	1.0	autoxidation	V. albo-atrum	growth	100	0	195
wattle tannin	1.0	autoxidation	V. albo-atrum	growth	50	0	195

[a] EPG = epigallocatechin-gallate, [b] PM = pectin-methyl, [c] C. = Cochliobolus, [d] CM = Cucumber Mosaic, [e] TN = Tobacco Necrosis, [f] V. = Verticillium

* partially polymerized monomeric phenols (20 to 60 minutes of oxidation) were polymerized further up to 1020 minutes.

[+] NS = not specified, [++] acid catalyzed condensation

concentrations ranging from 6.5 to 320 mg L^{-1}. This corresponds to the very high fish toxicity generally observed for debarking wastewater (Virkola and Honkanen, 1985). Concentrations as low as 25 to 100 mg COD L^{-1} were responsible for 50% fish (rainbow trout) kills.

Although the apolarity of phenolic compounds is a characteristic correlated to increasing methanogenic toxicity, tannins appear to be highly toxic regardless of the fact that they are highly polar compounds. The ambiguity perhaps is attributable to a unique mechanism of inhibition. Tannin compounds are noted for their sorptive reactions with proteins, called tanning (White, 1957). The hydrogen (H) bonding reactions with proteins (Figure 13) that are necessary for tanning are postulated to cause toxicity to bacteria because such interactions interfere with the functioning of enzymes (Goldstein and Swain, 1965; Gupta and Haslam, 1980; Haslam, 1974; White, 1957; Ladd and Butler, 1975; Loomis and Battaile, 1966; Strumeyer and Malin, 1969; Daiber, 1975; Tamir and Alumot, 1969; Hart and Hillis, 1972). The polar monomeric phenols have limited H bonding capacity, while the oligomeric tannins have a far superior H bonding capacity because of their ability to form multiple H bonds, called,"cross-linking" (Bate-Smith, 1973; Haslam, 1974; Loomis and Battaile, 1966; Endres and Hormann, 1963; Williams, 1963). Likewise we can expect that the toxicity of the oligomeric tannins is then far superior to their monomeric counterparts, as has been shown to be the case with metabolic enzymes (Boser, 1961; Firenzuoli et al., 1969; Hulmes and Jones, 1963), extracellular enzymes (Haslam, 1974; Lyr, 1965; Lyr, 1961; Williams, 1963) and eubacteria (Henis et al., 1964). The minimum MW for effective tannins is approximately 500 g $mole^{-1}$, which are equivalent to dimers (White, 1957; Williams, 1963). Therefore the low methanogenic toxicity of polar monomeric phenolic compounds can be explained by the fact that these are poor tanning substances.

5.3. Effect of Humus Forming Reactions on the Toxicity of Polar Phenolic Compounds

During the humus forming process, low MW phenolic substances are subjected to oxidative polymerization reactions. The reactivity of monomeric phenolics with proteins has been shown to increase as they are polymerized to oligomeric tannins (Chollot et al. 1961). This corresponds to the increase in the toxicity of monomeric phenols to enzymes, microorganisms and viruses (Table 7A), as they are oxidized to low MW polymers. Thus during the early stages of humus development, the nontannic polar phenolic monomers of low toxicity can be potentially converted to tannic substances of higher toxicity.

The tannins which are already quite toxic on the other hand become less toxic as they are oxidatively polymerized to high MW polymers of humic qualities (Table 7B). Thus there appears to be a maximum effective tannin size. For the effective tanning of hides, a maximum MW of 3,000 g $mole^{-1}$ has been found (White, 1957). The limitation is due to the poor penetration of high MW tannins into the interfiber spacings of the hide protein fibers. Likewise, we can expect that a similar maximum size must not be exceeded for penetration to bacterial enzymes or other functional proteins. The penetration barrier may consist of the outer membranes of gram negative bacteria (Nikaido and Vaara, 1985), cell envelopes of archaeobacteria (Konig, 1988), extracellular polysaccharides (Whitfield, 1988) forming slime layers around bacteria or glycocalyx (a polysaccharide) that surrounds the cell membranes of higher organisms. Considering reconstituted outer membranes of gram negative bacteria as a model, exclusion limits to passive diffusion have been reported to range between 600 and 3000 g $mole^{-1}$ (Vachon et al., 1985; Hancock and Nikaido, 1978; Vachon et al., 1988). Thus oligomeric tannins would be the most effective tannins for inhibiting the growth and metabolism of bacteria.

In any case, tannins are reactive with soluble proteins up to a MW of approximately 20,000 g $mole^{-1}$, which is equivalent to polymers of about 70 monomeric units (Jones et al. 1976). This would indicate the existence of high MW tannins that can potentially react with freely soluble extracellular enzymes, although they may not be inhibitory to microorganisms. The final products of phenol oxidation are high MW humic compounds that are even non-toxic towards extracellular enzymes (Table 7B). In this case, a loss in tanning capacity is perhaps due to an excessive size, in which the majority of the phenolic groups are buried too deep in the molecule or are too stabilized for effective H bonding interactions.

Additionally, it has been suggested that during autoxidation some ring opening may occur, producing polymers with increased carboxylic acid groups and thus contributing to a decrease in the tannic properties (Lewis and Papavizas, 1967).

The final end products of phenol oxidation are thus nontoxic humic compounds. The non-toxicity of at least two types of humic compounds towards methanogenic bacteria has been reported, these include: peat up to 16 g L^{-1} (Owens et al., 1979); and an extract of melanin (Lane, 1983).

The non-toxicity of humic compounds reveals a natural mechanism in the environment to detoxify tannic compounds of plants between the time that tannins are released to the forest floor and the time they enter ground and surface waters. This mechanism is the natural humus forming processes which occur in the forest soil. Potentially the humus forming reactions can be imitated in order to detoxify toxic concentrations of tannins in wastewater by their polymerization. In fact, this principle has already been applied to detoxify tannin inhibitors of pectinase during apple pulp fermentation by a simple oxidation pre-treatment (Verspuy and Pilnik, 1970).

6. SCOPE OF THIS DISSERTATION

The objectives of this dissertation are to define the anaerobic biodegradability and methanogenic toxicity of tannic compounds and to determine the extent to which these are altered by the humus forming process.

Chapters 2 - 4 investigate the biodegradability and toxicity of tannic substances and related compounds during anaerobic conditions. Chapters 5 - 7 describe the effects of oxidative reactions on the biodegradability and toxicity of defined tannic and monomeric phenolic compounds. Chapters 8 - 10 explore the possibility to utilize the humus forming reactions to detoxify the methanogenic inhibition caused by tannins in wastewater. Chapter 11 compares the anaerobic treatment performance of unoxidized and oxidatively detoxified tannin containing wastewater, fed continuously to laboratory scale reactors.

7. LITERATURE CITED

1. Akin, D. E. and R. Benner. 1988. Degradation of polysaccharides and lignin by ruminal bacteria and fungi. Appl. Environ. Microbiol. 54: 1117-1125.
2. Alberti, B. N. and A. M. Klibanov. 1981. Enzymatic removal of dissolved aromatics from industrial aqueous effluents. Biotechnol. Bioengineer. Symposium No. 11. John Wiley and Sons, Inc., N.Y. pp. 373-379.
3. Albertson, O. E. 1961. Ammonia nitrogen and the anaerobic environment. JWPCF 33(9): 978-995.
4. Ander, P., A. Hatakka and K-. E. Eriksson. 1980. Degradation of lignin and lignin related substances by Sporotrichum pulverulentum (Phanerochaete chrysosporium). in T. K. Kirk, T. Higuchi and H. M. Chang (eds.) Lignin Biodegradation: Microbiology, Chemistry and Potential Applications , Vol. 2. CRC Press, Boca Raton, Florida, USA. pp. 1-15.
5. Anderson, G. K., T. Donnelly and K. J. McKeown. 1982. Identification and control of inhibition in the anaerobic treatment of industrial wastewaters. Process Biochemistry July/August 1982 pp. 28-32, 41.
6. Aoki, K., R. Shinke and H. Nishira. 1976. Purification and some properties of yeast tannase. Agr. Biol. Chem. 40(1): 79-85.
7. Arjmand, M. and H. Sandermann, Jr. 1985. Mineralization of chloroaniline/lignin conjugates and of free chloroanilines by the white rot fungus Phanerochaete chrysosporium. J. Agric. Food Chem. 33: 1055-1060.
8. Arora, H. C., S. N. Chattopadhya and T. Routh. 1975. Treatment of vegetable tanning effluent by anaerobic contact filter process. Wat. Pollut. Control (UK), 74: 584-596.
9. Atlow, S. C., L. C. Bonadonna-Aparo and A. M. Klibanov. 1984. Dephenolization of industrial wastewaters catalyzed by polyphenol oxidase. Biotechnol. Bioeng. 26: 599-603.
10. Attal, A., F. Ehlinger, J. M. Audic and G. M. Faup. 1988. PH inhibition mechanisms of acetogenic, acetoclastic and hydrogenophilic populations. in E. R. Hall and P. N. Hobson (eds.) Anaerobic Digestion 1988, pp. 71-77. Pergamon Press, Oxford.
11. Augustin, H. and J. Puls. 1982. Perspectives on the production of chemicals from wood. Chemical Processing of Wood: Supplement 13 to Vol. 34. of Timber Bulletin for Europe, United Nations Office, Geneva. pp. 8-31.

12. Bache, R. and N. Pfennig. 1981. Selective isolation of Acetobacterium woodii on methoxylated aromatic acids and determination of growth yields. Arch. Microbiol. 130: 255-261.
13. Balba, M. T. and W. C. Evans. 1977. The methanogenic fermentation of aromatic substrates. Biochemical Soc. Trans. 5: 302-304.
14. Balba, M. T., N. A. Clarke and W. C. Evans. 1979. The methanogenic fermentation of plant phenolics. Biochemical Soc. Trans. 7: 1115-1116.
15. Balba, M. T. and W. C. Evans. 1980a. The methanogenic biodegradation of catechol by a microbial consortium: Evidence for the production of phenol through cis-benzenediol. Biochemical Soc. Trans. 8: 452-453.
16. Balba, M. T. and W. C. Evans. 1980b. Methanogenic fermentation of the naturally occuring aromatic amino acids by a microbial consortium. Biochemical Soc. Trans. 8: 625-627.
17. Balice, V., C. Carrieri, O. Cera and R. Rindone. 1988. The fate of tannin-like compounds from olive mill effluents in biological treatments. in E. R. Hall and P. N. Hobson (eds.) Anaerobic Digestion 1988. Pergamon Press, Oxford. pp. 275-279.
18. Baruah, P. and T. Swain. 1959. The action of potato phenolase on flavanoid compounds. J. Sci. Food Agric. 10: 125-129.
19. Basaraba, J. 1964. Influence of vegetable tannins on nitrification in soil. Plant and Soil, 21(1): 8-16.
20. Basaraba, J. 1966. Effect of vegetable tannins on glucose oxidation by various microorganisms. Can. J. Microbiol. 12: 787-794.
21. Bate-Smith, E. C. 1973. Haemanalysis of tannins: The concept of relative astringency. Phytochemistry, 12: 907-912.
22. Bauchop, T. 1967. Inhibition rumen methanogenesis by methane analogues. J. Bacteriology 94(1): 171-175.
23. Benjamin, M. M., S. L. Woods and J. F. Ferguson. 1984. Anaerobic toxicity and biodegradability of pulp mill waste constituents. Water Res. 18: 601-607.
24. Benner, R., A. E. Maccubbin and R. E. Hodson. 1984. Anaerobic biodegradation of the lignin and polysaccharide components of lignocellulose and synthetic lignin by sediment microflora. Appl. Environ. Microbiol. 47: 998-1004.
25. Benner, R. and R. E. Hodson. 1985. Thermophilic anaerobic biodegradation of (^{14}C)lignin, (^{14}C)cellulose and (^{14}C)lignocellulose preparations. Appl. Environ. Microbiol. 50:971-976.
26. Benoit, R. E. and R. L. Starkey. 1968. Enzyme inactivation as a factor in the inhibition of decomposition of organic matter by tannins. Soil Sci. 105: 203-208.
27. Berry, D. F., E. L. Madsen and J. M. Bollag. 1987a. Conversion of indole to oxindole under methanogenic conditions. Appl. Environ. Microbiol. 53: 180-182.
28. Berry, D. F., A. J. Francis and J. M. Bollag. 1987b. Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. Microbiol. Rev. 51(1): 43-59.
29. Blotevogel, K. H. and S. Jannsen. 1988. The influence of common feed antibiotics on methane fermentation. in A. Tilche and A. Rozzi (eds.) Anaerobic Digestion 1988 Poster-Papers, pp. 829-832. Monduzzi Editore, Bologna, Italy.
30. Blum, J. W., R. Hergenroeder, G. F. Parkin and R. E. Speece. 1986. Anaerobic treatment of coal conversion wastewater constituents: biodegradability and toxicity. JWPCF, 58(2): 122-131.
31. Booth, A. N. and R. T. Williams. 1963. Dehydroxylation of catechol acids by intestinal contents. Biochem. J. 88: 66p-67p.
32. Booth, G. H. 1960. A study of the effect of tannins on the growth of sulphate-reducing bacteria. J. Appl. Bact. 23(1): 125-129.
33. Boser, H. 1961. Modellversuche zur beeinflussing des zellstoffweschsel durch pflanzeninhaltstoffe, insbesondere flavonoide. Planta Med. 9: 456-465.
34. Boyd, S. A., D. R. Shelton, D. Berry and J. M. Tiedje. 1983. Anaerobic biodegradation of phenolic compounds in digested sludge. Appl. Environ. Microbiol. 46: 50-54.
35. Bressani, R. and L. G. Elias. 1980. The nutritional role of polyphenols in beans. in J.H. Hulse (ed.), Polyphenols in Cereals and Legumes. Int. Develop. Research Centre, Ottawa, Canada. pp. 61-68.
36. Bressani, R., L. G. Elias, A. Wolzak, A. E. Hagerman and L. G. Butler. 1983. Tannin in common beans: Methods of analysis and effects on proteins quality. J Food Sci. 48: 1000-1001, 1003.
37. Brown, J. P. 1977. Role of gut bacterial flora in nutrition and health: A review of recent advances in bacteriological techniques, metabolism and factors effecting flora composition. Critical Reviews in Food Science and Nutrition 8: 229-336.
38. Bryce, J. G. R. 1980. Alkaline pulping. in J. P. Casey (ed.) Pulp and Paper Chemistry and Chemical Technology Vol. 1., John Wiley and Sons N.Y. pp. 377-492.

39. Bumpus, J. A., M. Tien, D. Wright and S. D. Aust. 1985. Oxidation of persistent environmental pollutants by a white rot fungus. Science, 228: 1434-1436.

40. Bumpus, J. A. and S. D. Aus. 1987. Biodegradation of environmental pollutants by the white rot fungus Phanerochaete chrysosporium: Involvement of the lignin degrading system. BioEssays, 6(4): 166-170.

41. Campbell, J. C., and T. W. Joyce. 1983. Investigations of the potential of an enzymatic treatment of pulp and paper mill effluent to facilitate decolorization by lime precipitation. Proc. 38th Industrial Waste Conf., Purdue University Butterworth, Boston. pp. 67-73.

42. Camprubi, M., J. M. Paris and C. Casas. 1988. Effects of antimicrobial agents and feed additives on the performance of piggery waste anaerobic treatment. in E. R. Hall and P. N. Hobson (eds.) Anaerobic Digestion 1988. Pergamon Press, Oxford. pp. 239-248.

43. Chandra, T., V. Krishnamurty, W. Madhavakrishna and Y. Nayudamma. 1973. Astringency in fruits - 5: Microbial degradation of wood apple (Feronia elephantum) tannin. Leather Sci. 20: 269-273.

44. Chen, C-. L. and H-. M. Chang. 1985. Chemistry of lignin biodegradation. in T. Higuchi (ed.) Biosynthesis and Biodegradation of Wood Components, Academic Press, Inc., N.Y. pp. 535-556.

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

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

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

48. Cheng, K-. J., G. A. Jones, F. J. Simpson and M. P. Bryant. 1969. Isolation and identification of rumen bacteria capable of anaerobic rutin degradation. Can. J. Microbiol. 15: 1365-1371.

49. Chesson, A., C. S. Stewart and R. J. Wallace. 1982. Influence of plant phenolic acids on growth and cellulytic activity of rumen bacteria. Appl. and Environ. Microbiol. 44: 597-603.

50. Chmielowski, J., A. Grossman and S. Labuzek. 1965. Biochemical degradation of some phenols during the methane fermentation. Zeszyty Naukowe Politechniki Slaskiej: Inzynieria Sanitarna. 8: 97-122.

51. Chmielowski, J. A. and W. Kusznik. 1966. Preliminary trials on the methane fermentation of some phenolic wastewaters. Zeszyty Naukowe Politechniki Slaskiej: Inzynieria Sanitarna. 9: 123-144.

52. Chmielowski, J. A. and W. Wasilewski. 1966. An investigation of the dynamics of anaerobic decomposition of some phenols in the methane fermentation. Zeszyty Naukowe Politechniki Slaskiej: Inzynieria Sanitarna. 9: 95-122.

53. Chollot, B., L. Chapon and E. Urion. 1961. Polyphenols and proteins as precursors of peroxidase haze formation. Proceedings of the European Brewery Convention, Vienna, 1961 Elsevier. pp. 334-350.

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

55. Chou, W. L., R. E. Speece and R. H. Siddiqi. 1979. Acclimation and degradation of petrochemical wastewater components by methane fermentation. Biotechnology and Bioengineering Symp. No. 8. John Wiley and Sons, Inc., N.Y. pp. 391-414.

56. Chrostowski, P. C., A. M. Dietrich and I. H. Suffet. 1983. Ozone and oxygen induced oxidative coupling of aqueous phenolics. Wat. Res. 17: 1627-1633.

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

58. Colberg, P. J. and L. Y. Young. 1985a. Anaerobic degradation of soluble fractions of (^{14}-lignin)lignocellulose. Appl. Environ. Microbiol. 49: 345-349.

59. Colberg, P. J. and L. Y. Young. 1985b. Aromatic and volatile acid intermediates observed during anaerobic metabolism of lignin-derived. Appl. Environ. Microbiol. 49: 350-358.

60. Colberg, P. J. 1988. Anaerobic microbial degradation of cellulose, lignin, oligolignols, and monoaromatic lignin derivatives. in A. J. B. Zehnder (ed.) Biology of Anaerobic Microorganisms, John Wiley and Sons, Inc., N.Y. pp. 333-372.

61. Cowley, G. T. and W. F. Whittingham. 1961. The effect of tannin on the growth of selected soil microfungi in culture. Mycologia, 53: 539-542.

62. Daiber, K. H. 1975. Enzyme inhibition by polyphenols of sorghum grain and malt. J. Sci. Food Agric. 26: 1399-1411.

63. Das, N. P. 1969. Studies on flavanoid metabolism. Degradation of (+)-catechin by rat intestinal contents. Biochim. Biophys. Acta, 177: 668-670.

64. Dore, M., B. Langlais and B. Legube. 1980. Mechanism of the reaction of ozone with soluble aromatic pollutants. Ozone: Science and Engineering, 2: 39-54.

65. Duguet, J. P., B. Dussert, J. Mallavialle and F. Fiessinger. 1986. Polymerization effects of ozone: Applications to the removal of phenolic compounds from industrial wastewaters. Wat. Sci. Tech. 19: 919-930.

66. Dwyer, D. F., M. L. Krumme, S. A. Boyd and J. M. Tiedje. 1986. Kinetics of phenol biodegradation by an immobilized methanogenic consortium. Appl. Environ. Microbiol. 52: 345-351.

67. Eaton, D. C., H-. M. Chang, T. W. Joyce, T. W. Jeffries and T. K. Kirk. 1982. Method obtains fungal reduction of the color of extraction-stage kraft bleach effluents. Tappi, 65(6): 89-92.

68. Eaton, D. C. 1985. Mineralization of polychlorinated biphenyls by Phanerochaete chrysosporium: a lignolytic fungus. Enzyme Microb. Technol. 7(5): 194-196.

69. Eaton, D., H-. M. Chang and T. K. Kirk. 1980. Fungal decolorization of kraft bleach plant effluents. Tappi, 63(10): 103-106.

70. Eis, J., J. F. Ferguson and M. M. Benjamin. 1983. The fate and effect of bisulfate in anaerobic treatment. JWPCF 55(11): 1355-1365.

71. Elias, L. G. 1979. Chemical composition of coffee-berry by-products. in J. E. Braham and R. Bressani (eds.), Coffee Pulp: Composition, Technology and Utilization, Int. Develop. Research Centre, Ottawa, Canada. pp. 11-16.

72. Elsden, S. R., M. G. Hilton and J. W. Waller. 1976. The end products of the metabolism of aromatic amino acids by Clostridia. Arch. Microbiol. 107: 283-288.

73. Endres, H. and H. Hörmann. 1963. Praparative und analytische Trennung organischer Verbindungen durch Chromatographie an Polyamid. Angew. Chem. 75(6): 288-294.

74. Eriksson, K-. E. and M-. C. Kolar. 1985. Microbial degradation of chlorolignins. Environ. Sci. Technol. 19: 1086-1089.

75. Eriksson, K-. E., B. Pettersson, J. Volc and V. Musilek. 1986. Formation and partial characterization of glucose-2-oxidase, a H_2O_2 producing enzyme in Phanerochaete chrysosporium. Appl. Microbiol. Biotechnol. 23: 257-262.

76. Evans, W. C. 1977. Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Nature 270(3): 17-22.

77. Fedorak, P. M. and S. E. Hrudey. 1984. The effects of some alkyl phenolics on batch anaerobic methanogenesis. Wat. Res. 18: 361-367.

78. Fedorak, P. M. and S. E. Hrudey. 1986. Nutrient requirements for the methanogenic degradation of phenol and p-cresol in anaerobic draw and feed cultures. Wat. Res. 20: 929-933.

79. Fedorak, P. M.,D. J. Roberts and S. E. Hrudey. 1986. The effects of cyanide on the methanogenic degradation of phenolic compounds. Wat. Res. 20: 1315-1320.

80. Fengel, D. and G. Wegner. 1984. Lignin. Chp. 2 in Wood: Chemistry, Ultrastructure, Reactions, Walter de Gruyter, Berlin.

81. Ferry, J. G. and R. S. Wolfe. 1976. Anaerobic degradation of benzoate to methane by a microbiol consortium. Arch. Microbiol. 107: 33-40.

82. Fina, L. R. and A. M. Fiskin. 1960. The anaerobic decomposition of benzoic acid during methane fermentation: 2. Fate of carbons one and seven. Arch. Biochem. Biophys. 91: 163-165.

83. Finkle, B. J., J. C. Lewis, J. W. Corse and R. E. Lundin. 1962. Enzyme reactions with phenolic compounds: Formation of hydroxystyrenes through the decarboxylation of 4-hydroxycinnamic acids by Aerobacter. J. Biol. Chem. 9: 2926-2931.

84. Firenzuoli, A. M., P. Vanni and E. Mastronuzzi. 1969. The effect of some aromatic compounds on pure enzymes and their subsequent reactivation by PVP and Tween 80. Phytochemistry, 8: 61-64.

85. Flogstad, H. and H. Odegaard. 1987. Treatment of humic waters by ozone. Ozone: Science and Engineering, 7: 121-136.

86. Forss, K. 1982. Chemical processing of wood in Finland. Chemical Processing of Wood: Supplement 13 to Vol. 34. of Timber Bulletin for Europe, United Nations Office, Geneva. pp. 1-7.

87. Fox, P., M. T. Suidan and J. T. Pfeffer. 1988. Anaerobic treatment of a biologically inhibitory wastewater. JWPCF, 60(1): 86-92.

88. Frazer, A. C. and L. Y. Young. 1986. Anaerobic C_1 metabolisim of the O-methyl-^{14}C-labeled substituent of vanillate. Appl. and Environ. Microbiol. 51: 84-87.

89. Fukuzumi, T. 1980. Microbial decolorization and defoaming of pulping liquors. in T. K. Kirk, T. Higuchi and H. M. Chang (eds.) Lignin Biodegradation: Microbiology, Chemistry and Potential Applications , Vol. 2. CRC Press, Boca Raton, Florida, USA. pp. 161-177.

90. Fukuzumi, T., A. Nishida, K. Aoshima and K. Minami. 1977. Decolourization of kraft waste liquor with white rot fungi: 1. screening of the fungi and culturing condition for decolourization of kraft waste liquor. Mokuzai Gakkaishi, 23(6): 290-298.

91. Gilbert, E. 1983. Investigations on the changes of biological degradability of single substances induced by ozonation. Ozone: Science and Engineering, 5: 137-149.
92. Gilbert, E. 1987. Biodegradability of ozonation products as a function of COD and DOC elimination by example of substituted aromatic substances. Wat. Res. 21: 1273-1278.
93. Gilbert, E. 1988. Biodegradability of ozonation products as a function of COD and DOC elimination by the example of humic acids. Wat. Res. 22: 123-126.
94. Glasser, W. G. 1980. Lignin. in Casey, J. P. (ed.) Pulp and Paper Chemistry and Chemical Technology. John Wiley and Sons, NY. pp. 39-113.
95. Glasser, W. G. and S. S. Kelley. 1987 Lignin. Encyclopedia of Polymer Science and Engineering Vol 8. John Wiley and Sons, Inc. N.Y. pp. 795-852.
96. Glenn, J. K. and M. H. Gold. 1983. Decolorization of several polymeric dyes by the lignin-degrading basidiomycete Phanerochaete chrysosporium. Appl. Environ. Microbiol. 45: 1741-1747.
97. Goldstein, J. L. and T. Swain. 1965. The inhibition of enzymes by tannins. Phytochemistry, 4: 185-192.
98. Gollakota, K. G. and R. Sarada. 1988. Anaerobic degradation of tea wastes and some aromatic compounds by a methanogenic consortium. in E. R. Hall and P. N. Hobson (eds.) Anaerobic Digestion 1988, Pergamon Press, Oxford, England. pp. 281-285.
99. Gould, M. S. and E. J. Genetelli. 1978. Heavy metal complexation behavior in anaerobically digested sludges. Wat. Res. 12: 505-512.
100. Grant, W. D. 1976. Microbial degradation of condensed tannins. Science, 17: 1137-1138.
101. Grbic-galic, D. 1983. Anaerobic degradation of coniferyl alcohol by methanogenic consortia. Appl. Environ. Microbiol. 46: 1442-1446.
102. Grbic-galic, D. 1985. Fermentative and oxidative transformation of ferulate by a facultatively anaerobic bacterium isolated from sewage sludge. Appl. and Environ. Microbiol. 50: 1052-1057.
103. Grbic-Galic, D. 1986. Anaerobic production and transformation of aromatic hydrocarbons and substituted phenols by ferulic acid-degrading BESA-inhibited methanogenic consortia. FEMS Microbiol. Ecology, 38: 161-169.
104. Griffiths, L. A. and G. E. Smith. 1972a. Metabolism of myricetin and related compounds in the rat: metabolite formation in vivo and by the intestinal microflora in vitro. Biochem. J. 130: 141-151.
105. Griffiths, L. A. and G. E. Smith. 1972b. Metabolism of apigenin and related compounds in the rat: metabolite formation in vivo and by the intestinal microflora in vitro. Biochem J. 128: 901-911.
106. Gupta, R. K. and E. Haslam. 1980. Vegetable tannins structure and biosynthesis. in J.H. Hulse (ed.), Polyphenols in Cereals and Legumes. Int. Develop. Research Centre, Ottawa, Canada. pp. 15-24.
107. Haars, A., J. Trojanowski and A. Huttermann. 1987. Lignin bioconversion and its technical applications. in D. L. Wise (ed.) Bioenvironmental Systems Vol. 1., CRC Press, Inc., Boca Raton, Florida, USA. pp. 89-129.
108. Hackett, W. F., W. J. Connors, T. K. Kirk and J. G. Zeikus. 1977. Microbial decomposition of synthetic ^{14}C-labeled lignins in nature: Lignin biodegradation in a variety of natural materials. Appl. Environ. Microbiol. 39: 43-51.
109. Haemmerli, S. D., M. S. A. Leisola and A. Fiechter. 1986a. Polymerisation of lignins by ligninases from Phanerochaete chrysosporium. FEMS Microbiol. Letters, 35: 33-36.
110. Haemmerli, S. D., M. S. A. Leisola, D. Sanglard and A. Fiechter. 1986b. Oxidation of benzo(a)pyrene by extracellular ligninase of Phanerochaete chrysosporium. J. Biol. Chem. 261: 6900-6903.
111. Haider, K. and J. Trojanowski. 1980. A comparison of the degradation of 14C-labelled DHP and corn stalk lignins by micro- and macrofungi and bacteria. in T. K. Kirk, T. Higuchi and H. M. Chang (eds.) Lignin Biodegradation: Microbiology, Chemistry and Potential Applications , Vol. 1. CRC Press, Boca Raton, Florida, USA. pp. 111-134.
112. Haider, K., J. P. Martin, Z. Filip and E. Fustec-Mathon. 1972. Contribution of soil microbes to the formation of humic compounds. Humic Substances: Their Structure and Function in the Biosphere. Proceedings of an International Meeting Held at Nieuwersluis, The Netherlands May 29-31, 1972. PUDOC - Centre for Agricultural Publishing and Documentation (1975), Wageningen, The Netherlands. pp. 71-85.
113. Haider, K., J. P. Martin and Z. Filip. 1975. Humus biochemistry. in E. A. Paul and A. D. McLaren (eds.) Soil Biochemistry Vol. 4. Marcel Dekker, Inc., N.Y. pp. 196-244.
114. Hakulinen, R. 1987. The use of enzymes in the wastewater treatment of pulp and paper industry - a new possibility. Paper presented at The Second IAWPRC Symposium on Forest Industry Wastewaters. Tampere Univ. of Technology, Tampere, Finland.
115. Hammel, K. E., B. Kalyanaraman and T. K. Kirk. 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo(p)-dioxins by Phanerochaete chrysosporium ligninase. J. Biol. Chem. 261: 16948-16952.
116. Hanaki, K., T. Matsuo and N. Nagase. 1981. Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. Biotechnol. Bioengineer. 23: 1591-1610.

117. Hancock, R. E. and H. Nikaido. 1978. Outer membranes of gram-negative bacteria: 19. Isolation from Pseudomonas aeruginosa PAO1 and use in reconstitution and definition of permeability barrier. J. Bacteriol. 136: 381-390.

118. Harrison, B. D. and W. S. Pierpoint. 1963. The relation of polyphenoloxidase in leaf extracts to the instability of cucumber mosaic and other plant viruses. J. Gen. Microbiol. 32: 417-427.

119. Hart, J. H. and W. E. Hillis. 1972. Inhibition of wood-rotting fungi by ellagitannins in the heartwood of Quercus alba. Phytopathology, 62: 620-626.

120. Hartley, R. D. 1983. Degradation of cell walls of forages by sequential treatment with sodium hydroxide and a commercial cellulase preparation. J. Sci. Food Agric. 34: 29-36.

121. Harvey, P. J., H. E. Schoemaker and J. M. Palmer. 1985. Enzymatic degradation of lignin and its potential to supply chemicals. Ann. Proc. Phytochem. Soc. Eur. 26: 249-266.

122. Haslam, E. 1966. The Scope of Vegetable Tannins. Academic Press, N.Y.

123. Haslam, E. 1974. Polyphenol-protein interactions. Biochem J. 139: 285-288.

124. Hathway, D. E. and J. W. T. Seakins. 1955. Autoxidation of catechin. Nature, 176: 218.

125. Hathway, D. E. and J. W. T. Seakins. 1957. Enzymatic oxidation of catechin to a polymer structurally related to some phlobatannins. Biochem. J., 67: 239.

126. Hathway, D. E. 1962. The condensed tannins. in W. E. Hillis (ed.) Wood Extractives and their Significance to the Pulp and Paper Industry, Academic Press, N. Y. pp. 191-228.

127. Healy, J. B. Jr. and L. Y. Young. 1978. Catechol and phenol degradation by a methanogenic population of bacteria. Appl. Environ. Microbiol. 35: 216-218.

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

129. Healy, J. B. Jr., L. Y. Young and M. Reinhard. 1980. Methanogenic decomposition of ferulic acid, a model lignin derivative. Appl. Environ. Microbiol. 39: 436-444.

130. Hegert, H. L., L. E. van Blaricom, J. C. Steinberg and K. R. Gray. 1965. Isolation and properties of dispersants from western hemlock bark. Forest Prod. J. 15: 485-491.

131. Hemingway, R. W., L. Y. Foo and L. J. Porter. 1982. Linkage isomerism in trimeric and polymeric 2,3-cis-procyanidins. J. Chem. Soc., Perkin Trans. 1, 1209-1216.

132. Henis, Y., H. Tagari and R. Volcani. 1964. Effect of water extracts of carob pods, tannic acid, and their derivatives on the morphology and growth of microorganisms. Appl. Microbiol. 12(3): 204-209.

133. Hess, E. H. 1958. The polyphenolase of tobacco and its participation in amino acid metabolism. 1. Manometric studies. Arch. Biochem. Biophys. 74: 198-208.

134. Hickey, R. F., J. Vanderwielen and M. Switzenbaum. 1987. The effects of organic toxicants on methane production and hydrogen gas levels during the anaerobic digestion of waste activated sludge. Wat. Res. 21: 1417-1427.

135. Highley, T. L. 1987. Effect of carbohydrate and nitrogen on hydrogen peroxide formation by wood decay decay fungi in solid medium. FEMS Microbiol. Letters, 48: 373-377.

136. Higuchi, T. 1980. Lignin structure and morphological distribution in plant cell walls. in T. K. Kirk, T. Higuchi and H. M. Chang (eds.) Lignin Biodegradation: Microbiology, Chemistry and Potential Applications , Vol. 1. CRC Press, Boca Raton, Florida, USA. pp. 2.

137. Hilton, B. L. and J. A. Oleszkiewiez. 1987. Toxicity of sulfides to the anaerobic treatment process. Proceedings of the 42nd Industrial Waste Conference, Purdue Univ., West Lafayette, Indiana, USA, pp. 739-747.

138. Hobson, P. N. and B. G. Shaw. 1976. Inhibition of methane production by Methanobacterium formicicum. Water Res. 10:849-852.

139. Honohan, T., R. L. Hale, J. P. Brown and R. E. Wingard Jr. 1976. Synthesis and metabolic fate of hespertin-3-^{14}C. J. Agric. Food Chem. 24: 906-911.

140. Horowitz, A., D. R. Shelton, C. P. Cornell and J. M. Tiedje. 1981. Anaerobic degradation of aromatic compounds in sediments and digested sludge. Developments Industrial Microbiol. 23: 435-444.

141. Hulmes, A. C. and J. D. Jones. 1963. Tannin inhibition of plant mitochandria. in J. B. Pridham Enzyme Chemistry of Phenolic Compounds, Pergamon Press, N.Y. pp. 97-120.

142. Hurst, H. M. and N. A. Burges. 1967. Lignin and humic acids. in A. D. McLaren and G. H. Peterson (eds.) Soil Biochemistry Marcel Dekker, Inc. N.Y. pp. 260-286.

143. Huttermann, A., C. Herche and A. Haars. 1980. Polymerization of water-insoluble lignins by Fomes annosus. Holzforschung 34(2): 64-66.

144. Huynh, V-. B., H-. M. Chang, T. W. Joyce and T. K. Kirk. 1985. Dechlorination of chloro-organics by a white-rot fungus. Tappi, 68(7): 98-102.

145. Indahl, S. R. and R. R. Schleline. 1968. Decarboxylation of 4-hydroxy cinnamic acids by Bacillus strains isolated from rat intestine. Appl. Microbiol. 15: 667.

146. Ishihara, T. 1980. The role of laccase in lignin biodegradation. in T. K. Kirk, T. Higuchi and H. M. Chang (eds.) Lignin Biodegradation: Microbiology, Chemistry and Potential Applications , Vol. 2. CRC Press, Boca Raton, Florida, USA. pp. 18-31.

147. Iwahara, S. 1980. Microbial degradation of DHP. in T. K. Kirk, T. Higuchi and H. M. Chang (eds.) Lignin Biodegradation: Microbiology, Chemistry and Potential Applications , Vol. 1. CRC Press, Boca Raton, Florida, USA. pp. 151-171.

148. Jackson, H. and L. P. Kendal. 1949. The oxidation of catechol and homocatechol by tyrosinase in the presence of amino acids. Biochem. J. 44: 477-487.

149. Jarrel, K. F. and M. Saulnier. 1987. Inhibition of methanogenesis in pure cultures by ammonia, fatty acids and heavy metals, and protection against heavy metal toxicity by sewage sludge. Can. J. Microbiol. 33: 551-554.

150. Jones, W. T., R. B. Broadhurst and J. W. Lyttleton. 1976. The condensed tannins of pasture legume species. Phytochemistry 15:1407-1409.

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

152. Kaiser, J. P. and K. W. Hanselmann. 1982b. Aromatic chemicals through anaerobic microbial conversion of lignin monomers. Experientia. 38: 167-176.

153. Karchesy, J. J. and R. W. Hemingway. 1980. Loblolly pine bark polyflavonoids. J. Agric. Food Chem. 28: 222-228.

154. Karhadkar, P. P., J. M. Audic, G. M. Faup and P. Khanna. 1987. Sulfide and sulfate inhibition of methanogenesis. Water Res. 21(9): 1061-1066.

155. Keating, J. E., R. A. Brown and E. S. Greenberg. 1979. Phenolic problems solved with hydrogen peroxide oxidation. Proceedings of the 33rd Industrial Waste Conference, May 9, 10 and 11, 1978, Purdue Univ. Lafayette, Indiana, Ann Arbor Science Publ. Inc., Ann Arbor, USA. pp. 464-470.

156. Keith, C. L., R. L. Bridges, L. R. Fina, K. L. Iverson and J. A. Cloran. 1978. The anaerobic decomposition of benzoic acid during methane fermentation. Arch. Microbiol. 118: 173-176.

157. Kekos, D. and B. J. Macris. 1987. Effect of tannins on growth and amylase production by Calvatia gigantea. Enzyme Microb. Technol. 9(2): 94-96.

158. Kelley, R. L., K. Ramasamy and A. Reddy. 1986. Characterization of glucose oxidase-negative mutants of a lignin degrading basidomycete Phanerochaete chrysosporium. Arch. Microbiol. 144: 254-257.

159. Kelley, R. L. and A. Reddy. 1986. Identification of glucose oxidase activity as the primary source of hydrogen peroxide production in lignolytic cultures of Phanerochaete chrysosporium. Arch. Microbiol. 144: 248-253.

160. Kendal, L. P. 1949. The action of tyrosinase on monophenols. Biochem. J. 44: 442-454.

161. Khan, K. A., M. T. Suidan, W. H. Cross. 1981. Anaerobic activated carbon filter for the treatment of phenol-bearing wastewater. JWPCF, 53(10): 1519-1532.

162. Khan, K. A., M. T. Suidan and W. H. Cross. 1982. Role of surface active media in anaerobic filters. J. Environ. Engineer. Division: Proceedings Am. Soc. Civil Engineers, 108(EE2): 269-285.

163. Kim, B. R., E. S. K. Chian, W. H. Cross and S-. S. Cheng. 1986. Adsorption, desorption, and bioregeneration in an anaerobic, granular activated carbon reactor for the removal of phenol. JWPCF, 58(1): 35-40.

164. Kirk, T. K. 1971. Effects of microorganisms on lignin. Ann. Rev. Phytopathol. 9: 185-210.

165. Kirk, T. K., H. H. Yang and P. Keyser. 1977. The chemistry and physiology of the fungal degradation of lignin. Developments in Industrial Microbiology 19: 51-61.

166. Kirk, T. K. 1980. Physiology of lignin metabolism by white-rot fungi. in T. K. Kirk, T. Higuchi and H. M. Chang (eds.) Lignin Biodegradation: Microbiology, Chemistry and Potential Applications , Vol. 2. CRC Press, Boca Raton, Florida, USA. pp. 51-63.

167. Kirk, T. K. and P. Fenn. 1982. Formation and action of the lignolytic system in basidiomycetes. Frankland, Hedger and Swift (eds.) Decomposer Basidiomycetes, British Mycological Society Symposium 4., Cambridge Univ. Press, Cambridge, England. pp. 67-90.

168. Kirk, T. K. and M. Shimada. 1985. Lignin biodegradation: The microorganisms involved and the physiology and biochemistry of degradation by white-rot fungi. in T. Higuchi (ed.) Biosynthesis and Biodegradation of Wood Components, Academic Press, Inc., N.Y. pp. 579-605.

169. Kirk, T. K. and R. L. Farrell. 1987. Enzymatic "combustion": The microbial degradation of lignin. Ann. Rev. Microbiol., 41: 465-505.

170. Klibanov, A. M., B. N. Alberti, E. D. Morris and L. M. Felshin. 1980. Enzymatic removal of toxic phenols and anilines from waste waters. J. Appl. Biochem. 2: 414-421.
171. Knoll, G. and J. Winter. 1987. Anaerobic degradation of phenol in sewage sludge: Benzoate formation from phenol and CO_2 in the presence of hydrogen. Appl. Microbiol. Biotechnol. 25: 384-391.
172. Kohler, A., A. Jager, H. Willershausen and H. Graf. 1988. Extracellular ligninase of Phanerochaete chrysosporium burdsall has no role in the degradation of DDT. Appl. Microbiol. Biotechnol. 29: 618-620.
173. Konig, H. 1988. Archaeobacterial cell envelopes. Can. J. Microbiol. 34: 395-406.
174. Koster, I. W. and G. Lettinga. 1983. Ammonium toxicity in anaerobic digestion. Proceedings of the Anaerobic Wastewater Treatment Symposium, Noordwijkerhout. pp. 553. TNO Corporate Communication Dept., the Hague, The Netherlands.
175. Koster, I. W., A. Rinzema, A. L. De Vegt and G. Lettinga. 1986. Sulfide inhibition of the methanogenic activity of granular sludge at various pH-levels. Water Res. 20: 1561-1567.
176. Koster, I. W. 1986. Characteristics of the pH influenced adaption of methanogenic sludge to ammonium toxicity. J. Chem. Tech. Biotechnol. 36: 445-455.
177. Koster, I. W. and A. Cramer. 1987. Inhibition of methanogenesis from acetate in granular sludge by long-chain fatty acids. Appl. Environ. Microbiol. 53: 403-409.
178. Koster, I. W. and E. Koomen. 1988. Ammonia inhibition of the maximum growth rate of hydrogenotrophic methanogens at various pH levels and temperatures. Appl. Microbiol. Biotechnol. 28: 500-505.
179. Kroeker, E. J., D. D. Schulte, A. B. Sparling and H. M. Lapp. 1979. Anaerobic treatment process stability. JWPCF 51(4): 718-727.
180. Kroiss, H. 1985. Toxicity problems during the anaerobic treatment of wastewater. Hanover Industrieabwasser Tagung: Anaerobe Reinigung Industrieller Abwässer. pp. 12.1 - 12.19.
181. Kroiss, H. and F. Plahl-Wabnegg. 1983. Sulfide toxicity with anaerobic waste water treatment. Proceedings of the Anaerobic Wastewater Treatment Symposium, Noordwijkerhout. pp. 72-85. TNO Corporate Communication Dept., the Hague, The Netherlands.
182. Kruize, R. R., V. W. J. van den Berg and H. Haartjes. 1983. Vergiftiging van de slibgisting op rwzi Amsterdam-noord. H2O 16(5).
183. Krumholz, L. R. and M. P. Bryant. 1986. Eubacterium oxidoreducens sp. nov. requiring H_2 or formate to degrade gallate, pyrogallol, phloroglucinol and quercetin. Arch. Microbiol 144: 8-14.
184. Kugelman, I. J. and P. L. McCarty. 1965. Cation toxicity and stimulation in anaerobic waste treatment. JWPCF 37(1): 97-116.
185. Kugelman, I. J. and K. K. Chin. 1971. Toxicity, synergism, and antagonism in anaerobic waste treatment processes. Adv. Chem. Ser. 105: 55-90.
186. Kuwahara, M., T. Kagimura and K. Takagi. 1984. Anaerobic fermentation of bark. 1. Effect of treatment of bark with white-rot fungi and chemicals on the production of methane. Mokuzai Gakkaishi 30: 769-776.
187. Ladd, J. N. and J. H. A. Butler. 1975. Humus-enzyme systems and synthetic organic polymer-enzyme analogs. in E. A. Paul and A. D. McLaren (eds.) Soil Biochemistry, Marcel Dekker, Inc., NY. pp. 143-193.
188. Lane, A. G. 1980. Production of aromatic acids during anaerobic digestion of citrus peel. J. Chem. Tech. Biotechnol. 30: 345-350.
189. Lane, A. G. 1983. Anaerobic digestion of spent coffee grounds. Biomass 3: 247-268.
190. Latola, P. K. 1985. Treatment of different wastewaters from pulp and paper industry in methane reactors. Wat. Sci. Tech. 17: 223-230.
191. Lawerence, A. W. and P. L. McCarty. 1965. The role of sulfide in preventing heavy metal toxicity in anaerobic treatment. JWPCF 37(3): 392-406.
192. Lea, A. G. H. and G. M. Arnold. 1978. The phenolics of ciders: Bitterness and astringency. J. Sci. Food Agric. 29: 478-483.
193. Leatham, G. F. 1986. The lignolytic activities of Lentinus edodes and Phanerochaete chrysosporium. Appl. Microbiol. Biotechnol. 24: 51-58.
194. Lettinga, G., A. F. M. van Velsen, S. W. Hobma, De W. Zeeuw and A. Klapwijk. 1980. Use of the Upflow-Sludge-Blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol. Bioengineer. 22(4): 699-734.
195. Lewis, J. A. and G. C. Papavizas. 1967. Effects of tannins on spore germination and growth of Fusarium solani f. phaseoli and Verticillium albo-atrum. Can. J. Microbiol. 13: 1655-1661.
196. Lindberg, J., I. E. Tenrud and O. Theander. 1984. Degradation rate and chemical composition of different types of alkali-treated straws during rumen digestion. J. Sci. Food Agric. 35: 500-506
197. Lindstorm, K. and F. Osterberg. 1984. Characterization of high molecular mass chlorinated matter in spent bleach liquors (SBL). Part 1. Alkaline (SBL). Holzforschung 38(4): 201-212.

198. Livernoche, D., L. Jurasek, M. Desrochers, J Dorica and I. A. Veliky. 1983. Removal of color from kraft mill wastewaters with cultures of white-rot fungi and with immobilized mycelium of Coriolus versicolor. Biotechnol. Bioengineer. 25: 2055-2065.

199. Loomis, W. D. and J. Battaile. 1966. Plant phenoliccompounds and the isolation of plant enzymes. Phytochemistry, 5: 423-438.

200. Lyr, H. 1961. Hemmungsanalytische untersuchungen an einigen ektoenzymen holzzerstorender pilze. Enzymologia, 23: 231-248.

201. Lyr, H. 1963. Enzymatische detoxifikation chlorierter phenole. Phytopath. Z. 47: 73-83.

202. Lyr, H. 1965. On the toxicity of oxidized polyphenols. Phytopath. Z. 52: 229-240.

203. Madsen, E. L. and J. M. Bollag. 1989. Pathway of indole metabolism by a denitrifying microbial community. Arch. Microbiol. 151: 71-76.

204. Mahadevan, A. and G. Muthukumar. 1980. Aquatic microbiology with reference to tannin degradation. Hydrobiologia, 72: 73-79.

205. Mallevialle, J. 1975. Action de l'ozone dans la degradation des composes phenoliques simples et polymerrises: Application aux matieres humiques contenues dans les eaux. T.S.M. L'Eau, 70(3): 107-113.

206. Markham, K. R. and L. J. Porter. 1973. Extractives of Pinus radiata bark. 1. Phenolic components. New Zealand J. Sci. 16: 751-761.

207. Martin, J. P., K. Haider and Bondeietti. 1972. Properties of model humic acids synthesized by phenoloxidase and autoxidation of phenols and other compounds formed by soil fungi. Humic Substances: Their Structure and Function in the Biosphere. Proceedings of an International Meeting Held at Nieuwersluis, The Netherlands May 29-31, 1972. PUDOC - Centre for Agricultural Publishing and Documentation (1975), Wageningen, The Netherlands. pp. 171-186.

208. Martin, J. P. and K. Haider. 1980. Microbial degradation and stabilization of 14C-labelled lignins, phenols, and phenolic polymers in relation to soil humus formation. in T. K. Kirk, T. Higuchi and H. M. Chang (eds.) Lignin Biodegradation: Microbiology, Chemistry and Potential Applications , Vol. 1. CRC Press, Boca Raton, Florida, USA. pp. 77-100.

209. Mason, H. S. and E. W. Peterson. 1965. Melanoproteins: 1. Reactions between enzyme generated quinones and amino acids. Biochim. Biophys. Acta, 111: 134-136.

210. Mathew, A. G. and H. A. B. Parpia. 1971. Food browning as a polyphenol reaction. C. O. Chichester, E. M. Mrak and G. F. Stewart Adv. Food Research. Vol 19. Acadenmic Press, N.Y. pp. 75 - 145.

211. McCarty, P. L. and R. E. McKinney. 1961a. Volatile-acid toxicity in anaerobic digestion. JWPCF 33: 223.

212. McCarty, P. L. and R. E. McKinney. 1961b. Salt toxicity in anaerobic digestion. JWPCF 33(4): 399-415.

213. McCarty, P. L. 1964a. Anaerobic wastetreatment fundamentals. Part 1: Chemistry and microbiology. Public Works September 1964 pp. 107-112.

214. McCarty, P. L. 1964b. Anaerobic waste treatment fundamentals. Part 3: Toxic materials and their control. Public Works November 1964 pp. 91-94.

215. McInerney, M. J., M. P. Bryant, R. B. Hespell and J. W. Costerton. 1981. Syntrophomonas wolfei gen. nov. sp. nov., an anaerobic syntrophic, fatty acid-oxidising bacterium. Appl. Environ. Microbiol. 41: 1029-1039.

216. Melbinger, N. R. and J. Donnellon. 1971. Toxic effects of ammonia nitrogen in high-rate digestion. JWPCF 43(8): 1658-1670.

217. Miki, K., V. Renganathan, M. B. Mayfield and M. H. Gold. 1987. Aromatic ring cleavage of a ß-biphenyl ether dimer catalyzed by lignin peroxidase of Phanerochaete chrysosporium. FEBS Letters, 210(2): 199-203.

218. Milstein, O. A., Y. Vered, A. Sharma, J. Gressel and H. M. Flowers. 1983. Fungal biodegradation and biotransformation of soluble lignocarbohydrate complexes from straw. Appl. Env. Microb. 46(2):55-61.

219. Misra, D. K. 1980. Pulping and Bleaching of nonwood fibers. in J. P. Casey (ed.) Pulp and Paper Chemistry and Chemical Technology Vol. 1., John Wiley and Sons N.Y. pp. 504-568.

220. Molina, M. R., G. de la Fuente, M. A. Batten and R. Bressani. 1974. Decaffeination: A process to detoxify coffee pulp. J. Agric. Food Chem. 22: 1055-1059.

221. Mosey, F. E., J. D. Swanwick and D. A. Hughes. 1971a. Factors affecting the availability of heavy metales to inhibit anaerobic digestion. Wat. Pollut. Control 70:668-680.

222. Mosey, F. E. 1971b. The toxicity of cadmium to anaerobic digestion: Its modification by inorganic anions. Wat. Pollut. Control. 70:584-598.

223. Mosey, F. E. and D. A. Hughes. 1975. The toxicity of heavy metal ions to anaerobic digestion. J. Institute Wat. Pollut. Control No. 1. 3-24.

224. Mulder, A. 1984. The effects of high sulphate concentrations on the methane fermentation of wastewater. in Houwink, E. H., Van der Meer, R. R., (eds.) Innovations in Biotechnology, Elsevier Science Publ. B.V., Amsterdam, The Netherlands, pp. 133-143.

225. Murillo, B., M. T. Cabezas, R. Jarquin and R. Bressani. 1977, Effect of bisulfite addition on the chemical composition and cellular content fractions of dehydrated coffee pulp. J. Agric. Food Chem. 25: 1090-1092.

226. Nasr, F. A. and H. I. Abdel Shafy. 1988. The toxicity of heavy metal ions on anaerobic digestion. in A. Tilche and A. Rozzi (eds.) Anaerobic Digestion 1988 Poster-Papers, pp. 133-138. Monduzzi Editore, Bologna, Italy.

227. National Environmental Engineering Research Institute. 1986. Comprehensive Industry Document: Small Pulp and Paper Industry. Series: COINDS-22-1986. Central Board for the Prevention and Control of Water Pollution, New Delhi.

228. Neufeld, R. D., J. D. Mack and J. P. Strakey. 1980. Anaerobic phenol biokinetics. JWPCF, 52(9): 2367-2377.

229. Nienstaedt, H. 1953. Tannin as a factor in the resistance of chestnut, Castanea spp., to the chestnut blight fungus, Endothia parasitica. Phytopathology, 43: 32-38.

230. Nikaido, H. and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49(1): 1-32.

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

232. Ohmiya, K., M. Takeuchi, W. Chen, S. Shimizu and H. Kawakami. 1986. Anaerobic reduction of ferulic acid to dihydroferulic acid by Wolinella succinogenes from cow rumen. Appl. Microbiol. Biotechnol. 23: 274-279.

233. Oku, H. 1960. Biochemical studies on Cochliobolus miyabeanus: 4. Fungicidal action of polyphenols and the role of polyphenoloxidase of the fungus. Phytopath. Z. 38: 343-354.

234. Op den Camp, H. J. M., F. J. M. Verhagen, A. K. Kivaisi, F. E. de Windt, H. J. Lubberding, H. Gijzen and G. D. Vogel. 1988. Effects of lignin on the anaerobic degradation of (ligno) cellulosic wastes by rumen microorganisms. Appl. Microbiol. Biotechnol. 29: 408-412.

235. Osterberg, F. and K. Lindstorm. 1985. Characterization of high molecular mass chlorinated matter in spent bleach liquors (SBL). Part 2. Acidic SBL. Holzforschung 39(3): 149-158.

236. Owen, W. F., D. C. Stuckey, J. B. Healy, Jr., L. Y. Young and P. L. McCarty. 1979. Bioassays for monitoring biochemical methane potential and anaerobic toxicity. Wat. Res. 13: 485-492.

237. Paice, M. G. and L. Jurasek. 1984. Peroxidase-catalysed color removal from bleach plant effluent. Biotechnol. Bioengineer. 26: 477-480.

238. Parkin, G. F., W. M. Kocher and S. W. Miller. 1981. Microbial methane fermentation kinetics for toxicant exposure. Final Report Prepared for the Air Force Office of Scientific Research, Research Contract F 49620-79-C-0190.

239. Parkin, G. F. and R. E. Speece. 1983. Attached versus suspended growth anaerobic reactors: response to toxic substances. Wat. Sci. Tech. 15: 261-289.

240. Patel, T. R., K. G. Jure and G. A. Jones. 1981. Catabolism of phloroglucinol by the rumen anaerobe Coprococcus. Appl. Environ. Microbiol. 42: 1010-1017.

241. Pearson, F., C. Shium-Chung and M. Gautier. 1980. Toxic inhibition of anaerobic biodegradation. JWPCF 52: 473-478.

242. Peiffer, S. and K. Pecher. 1988. Monitoring anaerobic digestion processes of domestic waste and their relation to the dynamic behaviour of environmental pollutants. in A. Tilche and A. Rozzi (eds.) Anaerobic Digestion 1988 Poster-Papers, pp. 851-856. Monduzzi Editore, Bologna, Italy.

243. Peppercorn, M. A. and P. Goldman. 1971. Caffeic acid metabolisim by bacteria of the human gastro-intestinal tract. J. Bacteriol. 108: 996-1000.

244. Percuoco, G., S. Coppola, A. Zonia and G. Picci. 1973. Azione della microflora del suolo sulla fitotossicita di alcuni composti tannici. Agri. Ital. 73: 298-309.

245. Porter, L. J. 1974. Extractives of Pinus radiata bark. 2. Procyanidin constituents. New Zealand J. Sci. 17: 213-218.

246. Porter, W. L. and J. H. Schwartz. 1962. Isolation and description of the pectinase-inhibiting tannins of grape leaves. J. Food Sci. 27: 416-418.

247. Priha, M. 1985. Toxicity and mutagenicity of pulp and paper mill effluents. in Proceedings EUCEPA Symposium, Helsinki. pp. 40-49.

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

249. Rekunen, S. 1986. Taman process and its applicability to wastewaters from papermaking. Proceedings: PIRA Paper and Board Division Key-Event, Cost Effective Treatment of Papermill Effluents Using Anaerobic Technologies, Leatherhead, England.

250. Rinzema, A. and G. Lettinga. 1988. The effect of sulphide on the anaerobic degradation of propionate. Environ. Technol. Lett. 9: 83-88.

251. Rinzema, A., J. van Lier and G. Lettinga. 1988a. Sodium inhibition of acetoclastic methanogens in granular sludge from a UASB reactor. Enzyme Microb. Technol. 10: 24-32.

252. Rinzema, A., M. Boone, J. Keurentjes, K. van Knippenberg and G. Lettinga. 1988b. Bactericidal effect of long chain fatty acids in anaerobic digestion. Chapter 2 in Anaerobic Treatment of Wastewater with High Concentrations of Lipids or Sulfates, Ph.D. Dissertation, Dept. Water Pollution Control, Agricultural Univ., Wageningen, The Netherlands, pp. 31-48.

253. Royer, G., D. Livernoche, M. Desrochers, L. Jurasek, D. Rouleau and R. C. Mayer. 1983. Decolorization of kraft mill effluent: Kinetics of a continuous process using immobilized Coriolus versicolor. Biotechnol. Letters, 5(5): 321-326.

254. Rudman, P. 1962. The causes of natural durability in timber: 8. The causes of decay resistance in tallowwood (Eucalyptus Microcrys F. Meull.), white mahogany (Eucalyptus triantha Link.) and mountain ash (Eucalyptus regnans F. Muell.). Holsforschung, 16(2): 56-61.

255. Sagfors, P. -E. and B. Starck. 1988. High molar mass lignin in bleached kraft pulp mill effluents. Wat. Sci. Tech.^R 20: 49-58.

256. Salenieks, S. E. and J. G. Henry. 1986. Response of anaerobic filters to toxic organics. Water Poll. Res. J. Canada 21: 547-559.

257. Salkinoja-Salonen, M. and V. Sundman. 1980. Regulation and genetics of the biodegradation of lignin derivatives in pulp mill effluents. in T. K. Kirk, T. Higuchi and H. M. Chang (eds.) Lignin Biodegradation: Microbiology, Chemistry and Potential Applications , Vol. 2. CRC Press, Boca Raton, Florida, USA. pp. 179-198.

258. Sarkar, J. M. and R. G. Burns. 1984. Characterization of cellulase in soils and sediments and the evaluation of synthetic humic-cellulase complexes. in G. L. Ferrero, M. P. Ferranti and H. Naveau (eds.) Anaerobic Digestion and Carbohydrate Hydrolysis of Waste, Elsevier Applied Science Publishers, London. pp.169-178.

259. Scalbert, A., B. Monties and J-., M. Favre. 1988. Polyphenols ofQuercus robur: Adult tree and in vitro grown calli and shoots. Phytochemistry 27: 3483-3488.

260. Schink, B. 1988. Principles and limits of anaerobic degradation: Environmental and technological aspects. in A. J. B. Zehnder (ed.) Biology of Anaerobic Microorganisms, John Wiley and Sons, Inc., N.Y. pp. 771-846.

261. Schink, B. and Pfennig, 1982. Fermentation of trihydroxybenzenes by Pelobacter acidgallici gen. nov. sp. nov., a new strictly anaerobic, non-sporeforming bacterium. Arch. Microbiol. 133: 195-201.

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

263. Scott, T. W., P. F. V. Ward and R. M. C. Dawson. 1963. Formation of aromatic acids from amino acids by rumen micro-organisms. Biochem. J. 87: 3p-4p.

264. Shelton, D. R. and J. M. Tiedje. 1984. General method for determining anaerobic biodegradation potential. Appl. Environ. Microbiol. 47: 850-857.

265. Simpson, F. J., G. A. Jones and E. A. Wolin. 1969. Anaerobic degradation of some bioflavonoids by microflora of the rumen. Can. J. Microbiol. 15: 972-974.

266. Singleton, V. L., P. Esau. 1969. Phenolic substances in grapes and wine, and their significance. in C. D. Chichester (ed.), Advances in Food Research, Supplement 1. Academic Press, NY. pp. 1-281.

267. Singleton, V. L. 1972. Common plant phenols other than anthocyanins contribution to coloration and discoloration. in C. O Chichester (ed.) Advances in Food Research Supplement 3. The Chemistry of Plant Pigments, Academic Press, N.Y. pp. 143 - 191.

268. Sjoblad, R. D. and J. M. Bollag. 1981. Oxidative coupling of aromatic compounds by enzymes from soil microorganisms. in E. A. Paul and J. N. Ladd (eds.) Soil Biochemistry Vol. 5 Marcel Dekker, Inc., New York. pp. 113-152.

269. Smith, M. R., J. L. Lequerica and M. R. Hart. 1985. Inhibition of methanogenesis and carbon metabolism in Methanosarcina sp. by Cyanide. J. Bacteriol. 162: 67-71.

270. Speece, R. E. 1983. Anaerobic biotechnology for industrial wastewater treatment. Environ. Sci. Technol. 17(9): 416A-427A.

271. Spoelstra, S. F. 1978. Microbial aspects of the formation of malodorous compounds in anaerobically stored piggery wastes. Dissertation Dept. Microbiology, Agricultural University , Wageningen, The Netherlands.

272. Stahmann, M. A. 1965. The biochemistry of proteins of the host and parasite in some plant diseases. Tagungs Berichte: Deutsche Demokratische Republik Deutsche Akademie Der Landwirtschaftswissenschaften Zu Berlin, 74: 9-40.

273. Steiner, A. and R. Muller. 1988. Influence of nickel and copper on anaerobic sludge digestion. in A. Tilche and A. Rozzi (eds.) Anaerobic Digestion 1988 Poster-Papers, pp. 141-144. Monduzzi Editore, Bologna, Italy.

274. Steinke, R. D. and M. C. Paulson. 1964. The production of steam-volatile during cooking and alcoholic fermentation of grain. Agric. Food Chem. 12: 381-387.

275. Stevenson, F. J. 1982. Biochemistry of the formation of humic substances. Chp 8 in Humus Chemistry: Genesis, Composition, Reactions John Wiley and Sons, N.Y. pp. 195-220.

276. Strumeyer, D. H. and M. J. Malin. 1969. Identification of the amylase inhibitor from seeds of Leoti sorghum. Biochim. Biophys. Acta. 184: 643-645

277. Stuckey, D. C., W. F. Owen, P. L. McCarty and G. F. Parkin. 1980. Anaerobic toxicity evaluation by batch and semi-continuous assays. JWPCF 52(4): 720-729.

278. Suidan, M. T., W. H. Cross and M. Fong. 1980a. Continuous bioregeneration of granular activated carbon during the anaerobic degradation of catechol. Prog. Wat. Tech. 12: 203-214.

279. Suidan, M. T., G. L. Siekerka, S. Kao and J. T. Pfeffer. 1980b. Anaerobic filters for the treatment of coal gasification wastewater. Biotechnol. Bioengineer. 25: 1581-1596.

280. Suidan, M. T., G. L. Siekerka, S-. W. Kao and J. T. Pfeffer. 1983. Anaerobic filters for the treatment of coal gasification wastewater. Biotechnol. Bioengineer. 25: 1581-1596.

281. Suidan, M. T., J. T. Pfeffer and G. F. Nakhla. 1988. Anaerobic expanded-bed GAC reactor for the treatment of biologically inhibiting wastes generated during coal and petroleum distillation. in E. R. Hall and P. N. Hobson (eds.) Anaerobic Digestion 1988, Pergamon Press, N.Y. pp. 249-257.

282. Sundman, G., T. K. Kirk and H-. M. Chang. 1981. Fungal decolorization of kraft bleach plant effluents: Fate of the chromophoric material. Tappi, 64(9): 145-149.

283. Swanwick, J. D. and D. G. Shurben. 1969. Effective chemical treatment for inhibition of anaerobic sewage sludge digestion due to anionic detergents. J. Institute Wat. Pollut. Control No. 2 pp. 3-15.

284. Szewzyk, U., R. Szewzyk and B. Schink. 1985. Methanogenic degradation of hydroquinone and catechol via reductive dehydroxylation to phenol. FEMS Microbiol. Ecology, 31: 79-87.

285. Tamir, M. and E. Alumot. 1969. Inhibition of digestive enzymes by condensed tannins from green and ripe tannins. J. Sci. Food Agric. 20: 199-202.

286. Tarvin, D. and A. M. Buswell. 1934. The methane fermentation of organic acids and carbohydrates. J. Amer. Chem. Soc. 56: 1751-1755.

287. Taylor, B. F. 1983. Aerobic and anaerobic catabolism of vanillic acid and some other methoxy-aromatic compounds by Pseudomonas sp. strain PN-1. Appl. Environ. Microbiol. 46: 1286-1292.

288. Thiel, P. G. 1969. The effect of methane analogues on methanogenesis in anaerobic digestion. Water Res. 3: 215-223.

289. Thung, T. H. and J. P. H. van der Want. 1951. Viren en looistoffen. Tijdschrift Over Plantenziekten, 57(4): 173-174.

290. Tien, M., and T. K. Kirk. 1983. Lignin-degrading enzyme from the hymenomycete Phanerochaete chrysosporium burds.. Science, 221: 661-663.

291. Tien, M., and T. K. Kirk. 1984. Lignin-degrading enzymes from Phanerochaete chrysosporium: Purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. Proc. Natl. Acad. Sci. USA, 81: 2280-2284.

292. Tsai, C. and G. A. Jones. 1975. Isolation and identification of rumen bacteria capable of anaerobic phloroglucinol degradation. Can. J. Microbiol. 21: 794-801.

293. Tschech, A. and B. Schink. 1985. Fermentative degradation of resorcinol and resorcylic acids. Arch. Microbiol. 143: 52-59.

294. Tschech, A. and B. Schink. 1986. Fermentative degradation of monohydroxybenzoates by defined syntrophic cocultures. Arch. Microbiol. 145: 396-402.

295. Umezawa, T. and T. Higuchi. 1986. Aromatic ring cleavage of ß-O-4 lignin model dimers without prior demeth(ox)ylation by lignin peroxidase. FEBS Letters, 205(2): 293-298.

296. Umezawa, T. and T. Higuchi. 1987. Mechanisms of aromatic ring cleavage of ß-O-4 lignin substructure models by lignin peroxidase. FEBS Letters, 218(2): 255-260.

297. Umezawa, T., M. Shimada, T. Higuchi and K. Kusai. 1986. Aromatic ring cleavage of ß-O-4 lignin substructure model dimers by lignin peroxidase of Phanerochaete chrysosporium. FEBS Letters, 205(2): 287-292.

298. Updegraff, D. M. and W. D. Grant. 1975. Microbial utilization of Pinus radiata bark. Appl. Microbiol. 30: 722-723.

299. Vachon, V., D. J. Darwin and J. W. Coulton. 1985. Transmembrane permeability channels across the outer membrane of Haemophilus influenzae type b. J. Bacteriol. 162: 918-924.

300. Vachon, V., D. N. Kristjanson and J. W. Coulton. 1988. Outer membrane porin protein of Haemophilus influenzae type b: Pore size and subunit structure. Can. J. Microbiol. 34: 134-140.

301. Van Nevel, C. J., H. K. Henderickx, D. I. Demeyer and J. Martin. 1969. Effect of chloral hydrate on methane and propionic acid in the rumen. Appl. Microbiol. 17: 695-700.

302. Van Schreven, D. A. 1941. Control of tobacco mosaic by means of extracts of tanning substances. Natuurwetenschappelijk Tijdschrift voor Ned. Indie, 101(4): 113-114.

303. Van Velsen, A. F. M. 1979. Adaption of methanogenic sludge to high ammonia-nitrogen concentrations. Water Res. 13: 995-999.

304. Van Velsen, A. F. M. 1981. Preliminary research into the anaerobic decomposition of phenol and p-cresol. appendix in Anaerobic Digestion of Piggery Wastes, Dissertation, Dept. Water Pollution Control, Agricultural Univ., Wageningen, The Netherlands. pp. 81-89.

305. Varel, V. H. and H-. J. G. Jung. 1986. Influence of forage phenolics on ruminal fibrolytic bacteria and in vitro fiber degradation. Appl. Environ. Microbiol. 52: 275-280.

306. Verspuy, A. and W. Pilnik. 1970. Recovery of apple juice by pulp fermentation II: Shortening the fermentation time. Flussiges Obst. 37: 518-519.

307. Virkola, N. E. and Honkanen, K. 1985. Wastewater characteristics. Water Sci. Technol. 17: 1-28.

308. Vogel, P. and J. Winter. 1988. Anaerobic degradation of phenol and cresol in petrochemical wastewater. in A. Tilche and A. Rozzi (eds.) Anaerobic Digestion 1988 Poster Papers, Monduzzi Editore, Bologna, Italy. pp. 829-832.

309. Vogel, T. M. and D. Grbici-galic. 1986. Incorperation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Appl. Environ. Microbiol. 52: 200-202.

310. Wagner, S. and B. Schink. 1987. Anaerobic degradation of nonionic and anionic surfactants in enrichment cultures and fixed-bed reactors. Water Res. 21: 615-622.

311. Walker, J. L. R. 1975. Enzymatic browning in foods: A review. Enzyme Technol. Dig. 4: 89-100.

312. Wang, Y. T., M. T. Suidan and J. T. Pfeffer. 1984. Anaerobic biodegradation of indole to methane. Appl. Environ. Microbiol. 48: 1058-1060.

313. Wang, Y-. T., M. T. Suidan and B. E. Rittman. 1986. Anaerobic treatment of phenol by an expanded-bed reactor. JWPCF, 58(3): 227-233.

314. Wang, Y-. T., M. T. Suidan, J. T. Pfeffer and I. Najm. 1988. Effects of some alkyl phenols on methanogenic degradation of phenol. Appl. Environ. Microbiol. 54: 1277-1279.

315. Weinges, K., W. Ebert, D. Huthwelker, H. Mattauch and J. Perner. 1969. Oxydative kupplung von phenolen, 2. Konstitution und bildungsmechanismus des dehydro-dicatechins A. Liebigs Ann. Chem. 726: 114-124.

316. White, T. 1957. Tannins - their occurence and significance. J. Sci. Food Agric. 8: 377-384.

317. Whitfield, C. 1988. Bacterial extracellular polysaccharides. Can. J. Microbiol. 34: 415-420.

318. Williams, A. H. 1963. Enzyme inhibition by phenolic compounds. in J. B. Pridham Enzyme Chemistry of Phenolic Compounds, Pergamon Press, N.Y. pp. 87-95.

319. Wise, L. E. 1946. Wood Chemistry. Reinhold Publ. Corp., N.Y.

320. Witkowski, J. P. and J. S. Jeris. 1983. Anaerobic toxicity testing of several soft drink additives. in Bell, J. M. (ed.) Proc. 38th Annual Purdue Ind. Waste Conf. pp. 839-845. Butterworth, Boston.

321. Yamada, H., O. Adachi, M. Watanabe and N. Sato. 1968. Studies on fungal tannase: Part 1. Formation, purification and catalytic properties of tannase of Aspergillus flavus. Agr. Biol. Chem. 32(9): 1070-1078.

322. Yang, C. H. J., G. F. Parkin and R. E. Speece. 1979. Recovery of anaerobic digestion after exposure to toxicants. Final Report prepared for the US Dept. of Energy, Contract no. EC-77-S-02-4391.

323. Yang, J. and R. E. Speece. 1986. The effects of chloroform toxicity on methane fermentation. Water Res. 10: 1273-1279.

324. Yang, J., R. E. Speece, G. F. Parkin, J. Gossett and W. Kocher. 1980. The response of methane fermentation to cyanide and chloroform. Prog. Wat. Tech. 12: 977-989.

325. Young, L. Y. and P. L. McCarty. 1981. Heat treatment of organic materials for increasing anaerobic biodegradability. in L. W. Wise (ed.) Fuel Gas Production from Biomass: Volume 2. CRC Press, Boca Raton, Florida, USA. pp. 134-176.

326. Young, L. Y. 1984. Anaerobic degradation of aromatic compounds. in D. F. Gibson (ed.) Microbial Degradation of Organic Compounds. Marcel Dekker, Inc., NY. pp. 487-523.

327. Young, L. Y. and M. D. Rivera. 1985. Methanogenic degradation of four phenolic compounds. Water Res. 19: 1325-1332.

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

CHAPTER 2

The Methanogenic Toxicity and Anaerobic Degradability of Potato Starch Wastewater Phenolic Amino Acids.

Published in: Biological Wastes. (1987) 21: 37-54.

ERRATUM:
the units of methanogenic activity were printed as "mg COD liter^{-1} day^{-1}" they should be "mg COD g^{-1} VSS day^{-1}"

Biological Wastes **21** (1987) 37–54

The Methanogenic Toxicity and Anaerobic Degradability of Potato Starch Wastewater Phenolic Amino Acids

J. A. Field, G. Lettinga & M. Geurts

Department of Water Pollution Control, Agricultural University, De Dreijen 12, 6703 BC, Wageningen, The Netherlands

(Received 5 June, 1986; accepted 25 October, 1986)

ABSTRACT

Potatoes, which are important agricultural feedstocks for the starch industry, contain tyrosine and phenol oxidase. Since L-*dopa can be formed from tyrosine by phenol oxidase, both tyrosine and* L-*dopa are presumably present in potato starch wastewaters. The purpose of this study was to evaluate the methanogenic toxicity and anaerobic degradability of these two phenolic amino acids. Tyrosine was found to be negligibly toxic to methane bacteria, while* L-*dopa caused from 40% to 50% inhibitions of the methanogenic activity at a concentration of 327 mg liter*$^{-1}$. *The toxicity of* L-*dopa occurred only if anaerobic sludge was exposed to* L-*dopa in the presence of Volatile Fatty Acids (VFA). The* L-*dopa toxicity could be minimized by maintaining low VFA concentrations in the media and by adapting the sludge to VFA prior to* L-*dopa exposure.*

Both tyrosine and L-*dopa were anaerobically degraded to* CH_4; *however, only tyrosine was degradable after prolonged operation of continuously VFA-fed, granular sludge, packed columns. Phenol and* p-*cresol were identified as phenolic intermediates of anaerobic tyrosine degradation. Both* p-*cresol and* m-*cresol were identified as phenolic intermediates of anaerobic* L-*dopa degradation.*

INTRODUCTION

Potatoes are an important agricultural feedstock of the starch industry. They contain both tyrosine and the phenol oxidase known as tyrosinase (Mathew & Parpia, 1971; Baruah & Swain, 1959; Mapson *et al.*, 1963). This

Biological Wastes 0269-7483/87/$03·50 © Elsevier Applied Science Publishers Ltd, England, 1987. Printed in Great Britain

suggests the presence of both tyrosine and oxidation products of tyrosine in the wastewater generated from the processing of potatoes for starch. Tyrosinase plays an important role in the browning of potatoes which occurs when the tissue is damaged. Figure 1 illustrates the oxidation of tyrosine to the brown polymeric products, referred to as melanine, by the enzymatic action of tyrosinase (Nelson & Dawson, 1944; Mason, 1955; Rolland & Lissitzky, 1962; Mason & Peterson, 1965; Mathew & Parpia, 1971; Singleton, 1972). Tyrosinase has two enzyme activities. The first activity, cresolase, hydroxylates monohydroxy phenolic compounds to *o*-dihydroxy phenolic compounds. With tyrosine as substrate, the cresolase activity results in the formation of L-dopa. The second activity, catecholase, oxidizes the *o*-dihydroxy phenolic compounds to their respective quinones. The oxidation of L-dopa by the catecholase activity produces dopaquinone. In

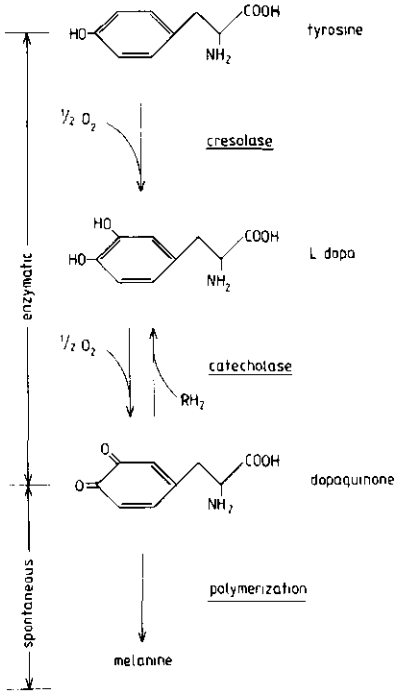


Fig. 1. The cresolase and catecholase activities of tyrosinase (a phenol oxidase) and subsequent polymerization of dopaquinone to form brown melanine compounds.

the presence of air dopaquinones polymerize spontaneously to form melanine.

The cresolase activity is not reversible. The catecholase activity, on the other hand, is reversible (Fig. 1) in the presence of reducing agents (Nelson & Dawson, 1944; Mathew & Parpia, 1971; Walker, 1975; Muneta, 1981). The reducing agents reduce the quinones to their *o*-dihydroxy phenolic precursors. Therefore, the aeration of tyrosine in the presence of both tyrosinase and reducing agents should lead to the accumulation of L-dopa. This has been confirmed by several researchers using ascorbate or dihydroxyfumarate as the reducing agent (Kendal, 1949; Rolland and Lissitzky, 1962; Muneta, 1981). Sulfur dioxide is most commonly used in the potato starch industry to minimize coloration of the process water. While sulfur dioxide partially functions to prevent browning by inactivation of tyrosinase (Diemar *et al.*, 1960), it also functions like ascorbate by reducing quinones back to *o*-dihydroxy phenolic compounds (Mathew & Parpia, 1971). The aeration of potato process water during the manufacture of starch may lead to the accumulation of L-dopa from tyrosine, since reducing agents ensure that dopaquinone is reduced to L-dopa.

Tyrosine is present in potatoes in significant concentrations. The literature average tyrosine concentration of potatoes is 5750 mg kg^{-1} dry tuber solids (Labib, 1962; Mapson *et al.*, 1963; FAO, 1970; Kaldy & Markakis, 1972; Davies & Laird, 1976; Livingstone *et al.*, 1980; Samotus *et al.*, 1982). Taking into account the removal of starch during processing and that two-thirds of the potato wastewater amino acids are present in the free form (Heisler *et al.*, 1972), we have calculated that an average of 15 mg free tyrosine would be expected per gram of potato-starch wastewater COD. The percentage conversion of tyrosine to L-dopa will largely depend on the amount of cresolase activity present in the potato process water and the reducing agents available to prevent further oxidation of L-dopa to melanine pigments.

The methanogenic toxicity of several phenolic compounds has been established (Chou *et al.*, 1978; Pearson *et al.*, 1980; Fedorak & Hrudey, 1984; Benjamin *et al.*, 1984). However, the toxicity of tyrosine and L-dopa have not been studied. The purpose of this study was to evaluate the methanogenic toxicity and anaerobic degradability of tyrosine and L-dopa since these compounds may be present in toxic concentrations in potato starch wastewaters.

METHODS

Media contained the following nutrients (per liter): H_3BO_3, 0·05 mg; $FeCl_2 . 4H_2O$, 2 mg; $ZnCl_2$, 0·05 mg; $MnCl_2 . 4H_2O$, 0·5 mg; $CuCl_2 . 2H_2O$,

0·03 mg; $(NH_4)_6$ $Mo_7O_{24}.4H_2O$, 0·05 mg; $AlCl_3.6H_2O$, 0·09 mg; $CoCl_2.6H_2O$, 2 mg; $NiCl_2.6H_2O$, 0·05 mg; Na_2Se $O_3.5H_2O$, 0·1 mg; EDTA, 1 mg; resazurine, 0·2 mg; 36% HCl, 0·001 ml; NH_4Cl, 0·28 g; K_2HPO_4, 0·25 g; $MgSO_4.7H_2O$, 0·1 g; $NaHCO_3$, 0·4 g; yeast extract, 0·1 g and $CaCl_2.2H_2O$, 0·01 g. Batch-fed experiments were conducted in 0·25- or 0·5-liter serum flasks or 2·5 liter pots. Cultures were either unshaken, shaken (serum flasks placed in a reciprocal shaker; 1 min, shaking, 4 min, rest cycles), or stirred (2·5-liter pots with an agitator that rotated in the sludge bed at about 0·5 cycles s^{-1}; (1 min, stirring, 4 min, rest cycles)). Granular sludge (1·5 years in 4°C storage) used in the experiments was originally obtained from an UASB reactor treating potato-derived wastewater. Sludge concentrations used were between 1·0 and 1·5 g organic solids (OS) $liter^{-1}$. Exact OS concentrations are reported in Figure and Table captions. The Volatile Fatty Acid (VFA) substrates used throughout the experiments were obtained from a stock solution containing 100:100:100 g acetate:propionate:butyrate per kilogram of pH 6·8 neutralized (with NaOH) solution. The chemical oxygen demand ratio of the VFA stock was 24·3:34·4:41·3% of the total COD for C_2, C_3 and C_4, respectively. Exact concentrations of VFA used during the experiments are listed with the Tables and Figures. The temperature of all experiments was 30°C. Continuously fed experiments were conducted in 0·2-liter columns packed with 3·4 g OS granular sludge, which was previously adapted for 5 weeks on a continuous feed of 5 g COD per liter VFA. The columns contained a sieve (1 mm openings) between the top of the sludge bed and effluent discharge to prevent granular sludge wash-out. The columns were operated for 3 months with hydraulic retention times (HRT) ranging from 11 to 2·4 h and VFA influent concentrations ranging from 4·7 to 24·7 g COD per liter. The VFA loadings and operating modes are indicated in the Figure with the column results. The feed of all columns contained the nutrient supplementation used in the batch assays. One column was a VFA-fed control, while 300 mg per liter tyrosine and 327 mg per liter L-dopa were present in the feeds of the second and third columns.

The phenolic amino acids were tyrosine (MW = 181 and COD = 1·724 g O_2 per gram of tyrosine) and L-dopa (MW = 197 and COD = 1·503 g O_2 per gram of L-dopa) obtained from Janssen Chimica (Beerse, Belgium). Both amino acids are illustrated in Fig. 1. In all cases (except where otherwise indicated), the nutrient supplement, sludge and VFA were brought together, adjusted to pH 7·4 with NaOH and predigested overnight prior to addition of the phenolic amino acids. This addition was conducted under N_2 flushing conditions.

Methane production was monitored with modified mariotte flasks containing 3% NaOH to remove CO_2 from the gas. Volatile Fatty Acids

(VFA) were analyzed on a Packard 417 gas chromatograph equipped with a 2 m × 2 mm ID column packed with 10% Fluorad FC 431 on supelcoat (100–120 mesh). The carrier gas (N_2) was saturated with formic acid (flow, 35 ml min^{-1}). The oven temperature was set at 130°C. The FID detector signal was processed with a SP41 Spectra Physics integrator. The VFA standards (C_2–C_5) were obtained from Merck. Several aromatic compounds could be detected by this chromatography procedure without derivatization. Standards tested included phenol, *p*-cresol, *o*-cresol, *m*-cresol, benzoic acid, *p*-ethylphenol, *m*-ethylphenol, phenylacetic acid and 3-phenylpropionic acid. The *p*-cresol and *m*-cresol could not be separated into distinct peaks when injected together. The two components, however, gave unique peaks when injected alone. The pH was monitored with a Knick 511 meter and Schot Gerate N61 double electrode. The phenolic amino acids have UV light absorbing properties. The elimination of UV absorbence from the media is indicative of the phenolic's disappearance from the media. UV absorption was measured with a Perkin-Elmer 550 A spectrophotometer and a Hellma 100–QS 1 cm quartz cuvette. Absorption is reported as the absorption of the media containing phenolic amino acids less the absorption of the control media (which contained no UV absorbing compounds).

Methanogenic activities are reported for batch assays as a percentage ratio of the treatment CH_4 production rate and control CH_4 production rate at the time period of maximal control activity. Inhibition is defined as: 100 – activity. The methanogenic inhibition caused by the phenolic amino acids in the continuously VFA fed experiments was calculated as follows:

$$(1 - (E \text{ paa}/E \text{ control})) \times 100;$$

where E paa = COD removal efficiency of phenolic amino acid treated column which received the same VFA loading as the control; E control = COD removal efficiency of control column.

RESULTS

Toxicity of tyrosine and L-dopa

Figure 2A indicates the cumulative methane production from four consecutive VFA feedings of granular sludge, with 300 mg $liter^{-1}$ tyrosine or 327 mg $liter^{-1}$ L-dopa (concentration of equivalent molarity) and without any phenolic amino acids present in the media. Tyrosine was not significantly toxic to methanogenesis. Only a 4% inhibitition of the CH_4 production rate was evident in the first VFA feeding, which became 2% or less in the consecutive feedings. While L-dopa appeared non-toxic during the

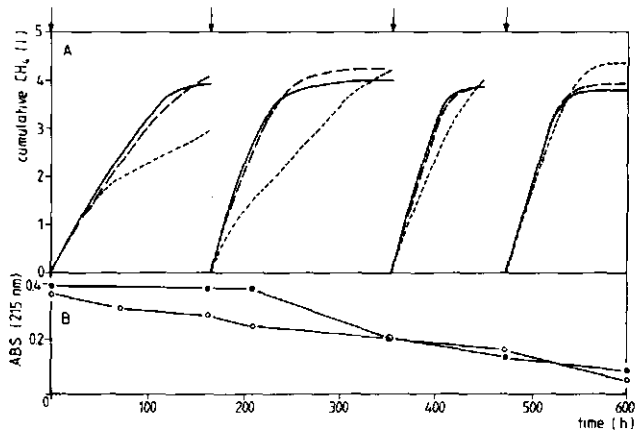


Fig. 2A. The cumulative CH_4 production from stirred VFA batch-fed cultures (2·5 liters) with 1·36 g OS per liter granular sludge and 4·17 g COD per liter VFA (C_2:C_3:C_4) added on days 0, 7, 15 and 20 as indicated by the arrows. The media included either 300 mg liter^{-1} tyrosine (– –) or 327 mg liter^{-1} L-dopa (-----). The VFA-fed control is indicated by the unbroken line (——). New VFA solution for each additional feeding was added to existing media. Tyrosine and L-dopa were added only once at time 0. **Fig. 2B.** The UV absorbance of 30× diluted media at 215 nm with tyrosine (○) or L-dopa (●).

first 50 h of assay, a 49% inhibition of the CH_4 production was suddenly evident in the remainder of the first feeding: this increased to a 53% inhibition in the second feeding. The L-dopa toxicity decreased in the third and fourth VFA feedings, when 17% and 6% inhibition of the CH_4 production rates were observed.

Figure 3A-D illustrates the decreases of methanogenic activity resulting from increasing concentrations of L-dopa in four consecutive VFA feedings. The toxicity of L-dopa reached its maximum in the second feeding. A large loss (43%) of methanogenic activity was evident on increasing the L-dopa concentration from 0 to 400 mg liter^{-1}. Increasing the L-dopa concentration beyond 400 mg liter^{-1} only increased the inhibition to 52% (Fig. 3B). The L-dopa toxicity decreased in the third and fourth feedings if the L-dopa concentration was 1000 mg liter^{-1} or less. No, or little, decrease in L-dopa toxicity was observed in the third and fourth feedings for treatments which received 1400 mg liter^{-1} or more L-dopa.

The effect of shaking during the assay period on the L-dopa toxicity is shown in Fig. 4A-B for the first and second VFA feedings. The toxicity of 200 to 400 mg per liter of L-dopa could be decreased by not shaking. The

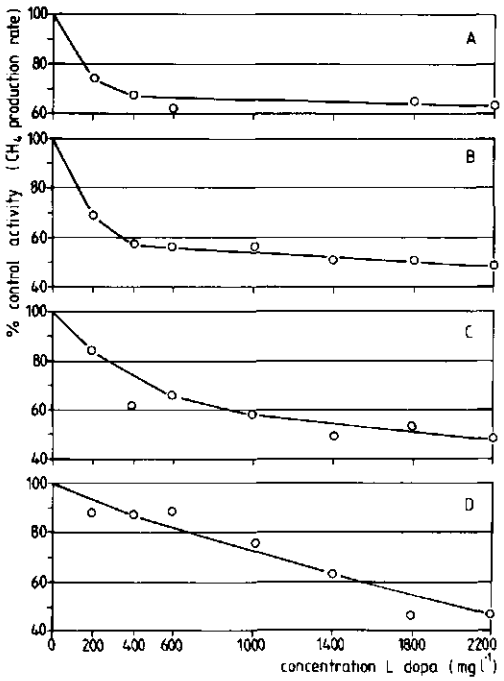


Fig. 3. The activity of CH_4 production in shaken VFA batch-fed cultures (0·25 liter) with various concentrations of L-dopa added only at the beginning of the experiment. The sludge concentration was 1·46 g OS liter^{-1} and the VFA were added at 4·17 g COD liter^{-1} to the existing supernatant on days 0, 8, 14 and 41 for activity assays shown in Figs 3A, B, C and D, respectively. The control activities were 399, 833, 847 and 839 mg COD liter^{-1} day^{-1} in parts A, B, C and D.

shaking most likely brought the sludge into better contact with both VFA and L-dopa.

The effect of VFA concentration during a 3-day exposure of sludge with 327 mg per liter of L-dopa on the activity of the sludge following the shaken exposure periods is shown in Fig. 5. L-dopa was not toxic if no VFA were present during the exposure period. So just the better contact of L-dopa with the sludge was not the reason for increased toxicity on shaking. Increasing the VFA concentration to 2 g COD litre^{-1}, during the L-dopa exposure period, increased the L-dopa toxicity to 39% inhibition. The highest concentration of VFA, 4 g COD liter^{-1}, increased the L-dopa toxicity to

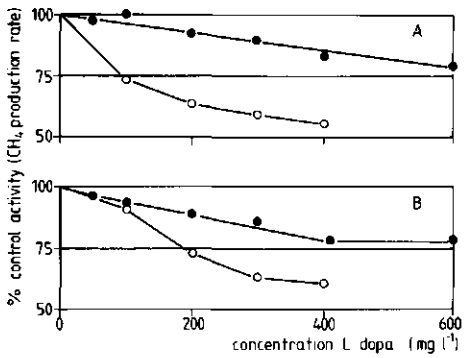


Fig. 4. The activity of CH_4 production in shaken (○) and unshaken (●) VFA batch-fed cultures (0·25 liter) with various concentrations of L-dopa added only at the beginning of the experiment. The sludge concentration was 1·27 g OS per liter and the VFA were added at 4·17 g COD per liter to existing supernatant on days 0 and 9 for the activity assays indicated in Figs 3A and B, respectively. The control activities of the shaken cultures were 437 and 885 mg COD $liter^{-1}$ day^{-1} and the control activities of the unshaken cultures were 490 and 915 mg COD $liter^{-1}$ day^{-1} in parts A and B, respectively.

47%. The better contact of the sludge with the VFA must have caused the increased toxicity on shaking. The presence of VFA in the media appears to have a synergistic toxicity with L-dopa. Adapting the sludge on 4 g COD per liter VFA for 5 days prior to L-dopa exposure reduced the synergistic effect of the VFA on the L-dopa toxicity. The maximum level of VFA, 4 g COD $liter^{-1}$, used during the 3-day L-dopa exposure period caused 33% inhibition with VFA-adapted sludge (Fig. 5). The toxicity was not related to the VFA alone, since the controls showed increasing activity with increasing VFA concentrations during the exposure period.

The toxic effect of L-dopa with VFA present was damaging to the methane bacteria. This was evident since exposing the sludge to L-dopa and VFA for 3 days caused the sludge to lose its activity when assayed on replaced media which contained no L-dopa (Fig. 5). The damage caused by 1000 mg $liter^{-1}$, or less, L-dopa was not permanent since the lost activity was partially recovered after 20 to 41 days (Figs 2 and 3D).

The performance of VFA-fed columns with 327 mg $liter^{-1}$ L-dopa present in the feed is compared with 300 mg $liter^{-1}$ tyrosine and a VFA control in Fig. 6. The columns in this experiment were packed with sludge which was previously adapted to a continuous feed of 5 g COD per liter VFA. The column receiving L-dopa had lower levels of COD removal efficiencies than the control column, indicating that the methanogenic activity was inhibited by the L-dopa. The maximum L-dopa inhibition of 14% (occurring between

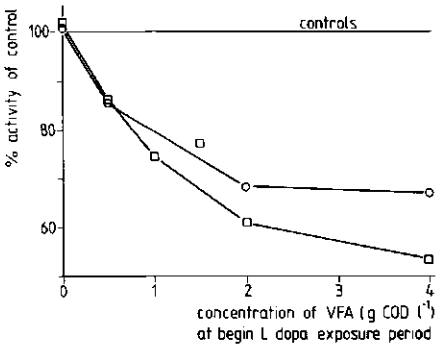


Fig. 5. The CH_4 production in shaken VFA batch-fed cultures (0·25 liter) as affected by exposure of the sludge (1·36 g OS per liter) to 327 mg liter^{-1} L-dopa at various concentrations of VFA during a 3-day pre-incubation period. The activities are reported for the 3-day assay period, which followed, on replaced medium with 4·17 g COD per liter VFA and without L-dopa. Activities are reported as a percentage of the respective control activities which were exposed to the same concentrations of VFA during pre-incubation as the L-dopa treatments. Before pre-incubation, the sludge was either pre-adapted to 4 g COD per liter VFA for 5 days (○) or not pre-adapted to VFA (□). The sludge which was not adapted to VFA before pre-incubation had control activities of 335, 491, 607, 686, 683 and 750 mg COD liter^{-1} day^{-1} when 0, 0·5, 1·0, 1·5, 2·0 and 4·0 g COD per liter, respectively, of VFA were supplied to the media in the pre-incubation period. The sludge which was pre-adapted to VFA before the pre-incubation period had control activities of 833, 856, 960 and 859 mg COD liter^{-1} day^{-1} when 0, 0·5, 2 and 4 g COD liter^{-1}, respectively, of VFA were supplied to the media in the pre-incubation period.

days 3 to 8; 71–189 h) was much lower than the L-dopa toxicity in batch-fed assays with lower concentrations of unadapted (to VFA) sludge (Fig. 2A). After 20 days (490 h), the inhibition decreased to 3·0%. After 68 days (1636 h), the inhibition increased to 12% when the columns were overloaded with VFA for 3 days. Sludge recovered from the L-dopa column after 74 (1776 h) days was exposed to 327 mg liter^{-1} L-dopa and 6 g COD per liter VFA for 5 days with shaking. Following the exposure, 1·5 g OS per liter of this sludge had 7% less activity than the same sludge only exposed to 6 g COD per liter of VFA.

The column receiving tyrosine showed only negligible inhibitions in the first week. After 3 weeks (500 h), the COD removal efficiency of the tyrosine-treated column was slightly greater than the control.

Anaerobic degradation of tyrosine and L-dopa

During the VFA-fed assays, certain modifications of both tyrosine and L-dopa were evident from the disappearance of UV absorption in the media

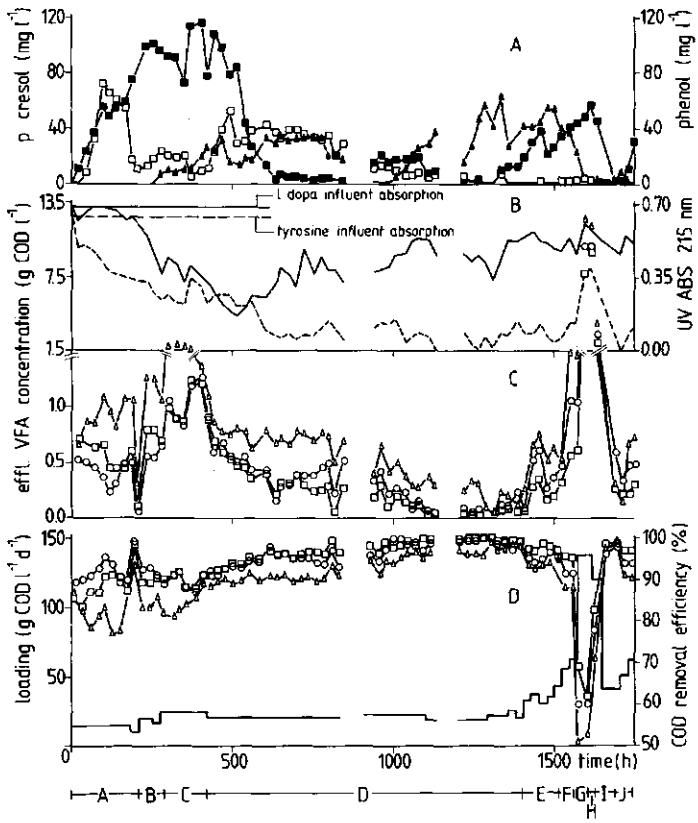


Fig. 6. Results from continuously VFA fed columns. A. The phenol (□) and *p*-cresol (■) concentration observed in the effluent of the column fed VFA and 300 mg per liter tyrosine. The *p*-cresol (▲) concentration observed in the effluent of the column fed VFA and 327 mg per liter L-dopa. B. The UV absorbance of the effluents (diluted 16×) from the VFA-fed columns receiving tyrosine (-----) and L-dopa (——). C. The VFA concentrations in the effluents of the VFA-fed columns receiving tyrosine (□), L-dopa (△) and no phenolic amino acids (○). D. The COD removal efficiency of the VFA-fed columns receiving tyrosine (□), L-dopa (△) and no phenolic amino acids (○). The VFA space loading of the columns ( ). The loading was approximately the same for all of the columns. At time 0, the columns (each 0·2 liter) were filled with 3·4 g OS granular sludge which was previously adapted to a continuous feed of 5 g COD per liter of VFA for 5 weeks. At the end of the experiment the VFA-fed control column contained 9·28 g OS. The operation mode of the columns in a given time period is summarized by the letter codes at the bottom of the Figure: (A) HRT 6·1 to 10·7 h, influent COD 4·7 to 4·9 g liter^{-1}; (B) HRT 7·4 to 10·0 h, influent COD 7·4 g liter^{-1}; (C) HRT 7·7 to 10·9 h, influent COD 9·9 g liter^{-1}; (D) HRT 6·8 to 10·0 h, influent COD 7·4 g liter^{-1}; (E) HRT 5·3 to 7·8 h, influent COD 9·9 g liter^{-1}; (F) HRT 4·4 to 5·4 h, influent COD 12·3 g liter^{-1}; (G) VFA overload period HRT 4·1 to 4·7 h, influent COD 24·7 g liter^{-1}; (H) VFA overload period HRT 2·4 to 2·7 h, influent COD 12·3 g liter^{-1}; (I) HRT 8·2 to 14·0 h, influent COD 14·8 g liter^{-1}; (J) HRT 2·7 to 3·8 h, influent COD 7·4 g liter^{-1}.

(Figs 2B and 7, Table 2). The appearance of phenolic intermediates (Tables 1 and 2 and Fig. 6A) indicates that at least some biological transformations of tyrosine and L-dopa were occurring during digestion. Phenol and *p*-cresol identified in the tyrosine treatments accounted for 55% of the initial tyrosine COD (Table 1). The *p*-cresol identified in the L-dopa treatments accounted for 33% to 37% of the initial L-dopa COD (Tables 1 and 2). In the effluent of the continuously fed columns the maximum concentrations of phenol and *p*-cresol from the tyrosine treatments occurred between 4 and 21 days (96 and 504 h) of operation and were approximately equivalent to 60% of the initial tyrosine COD. The maximum concentrations of *p*-cresol from the L-dopa treatment occurred between 55 and 66 days (1320 and 1584 h) of operation and were equivalent to approximately 33% of the initial L-dopa COD. After 30 days of the L-dopa column operation, *m*-cresol was present in the effluent in a proportion ranging from 25% to 50% of the total *m*-cresol plus *p*-cresol concentration. The combined concentrations of the two cresols are reported as *p*-cresol in Fig. 6A (*m*-cresol and *p*-cresol peaks could not be distinguished by the integrator).

TABLE 1

The Concentrations of Phenol and *p*-Cresol in Tyrosine and L-Dopa Supplied Media at Several Selected Sampling Times Following Initiation of VFA-Fed Batch Digestions. Details of This Experiment are Described in the Legend to Fig. 2.

Time of digestion (h)	*Treatment*		
	Control	*300 mg liter*$^{-1}$ *tyrosine*	*327 mg liter*$^{-1}$ L-*dopa*
A. Concentration of phenol in media (mg liter^{-1})			
72	0	0	0
165	0	37·8	0
211	0	37·1	0
354	0	42·7	0
475	0	25·5	0
593	0	0	0
B. Concentration of *p*-cresol in media (mg liter^{-1})			
72	0	27·5	0
165	0	65·4	0
211	0	77·4	0
354	0	64·2	33·4
475	0	78·4	58·0
593	0	78·6	67·2
C. Phenol + *p*-cresol COD expressed as a percentage of initial amino acid COD			
211	—	54·8	—
593	—	—	34·5

TABLE 2

The UV Absorbance and *p*-Cresol Concentration in VFA-Fed, Shaken, Batch Cultures at Selected Sampling Times with Various L-Dopa Concentrations. Details of the Experiment are Reported in the Legend to Fig. 4.

Time of digestion (h)	*Initial L-dopa concentration (mg liter^{-1})*				
	0	*100*	*200*	*300*	*400*
A. UV absorption (205 nm) in 31× diluted media[a]					
24	—	0·303	0·603	0·860	1·090
216	—	0·038	0·096	0·388	0·627
384	—	0·034	0·088	0·119	0·165
B. Concentration of *p*-cresol in media (mg liter^{-1})					
24	0	0	0	0	0
216	0	11·7	43·0	26·4	45·7
384	0	10·4	44·6	58·7	84·4
C. *p*-Cresol COD expressed as a percentage of the initial L-dopa COD					
384	—	17·4	37·4	32·8	35·4

[a] Absorbance data reported as treatment less control (no L-dopa).

TABLE 3

The Cumulative CH_4 Production and UV Absorption After a 51-Day Digestion of 327 mg liter^{-1} L-Dopa As Sole Substrate with 1·3 g OS Granular Sludge per Liter. The Experiment was Conducted in 0·5-Liter Serum Flasks Filled to 0·25-Liter Culture Volume. No Shaking was Employed during the Assay Period.

	L-Dopa (327 mg liter^{-1})		*Controls (no L-dopa)*	
	1	*2*[a]	*1*	*2*
Cumulative CH_4[b] ml per liter of culture (30°C at atmospheric pressure)	205	209	88	102
UV absorbance[c] at beginning of assay, 205 nm, dilution = 31×	1·031	1·062	—	—
UV absorbance after 51 days' digestion, 205 nm, dilution = 31×	0·370	0·461	—	—

[a] 1 and 2 are replicates.

[b] If all of the L-dopa were converted to CH_4, then the treatments would have 191 ml more CH_4 than the controls.

[c] The absorbance data reported as treatment less control.

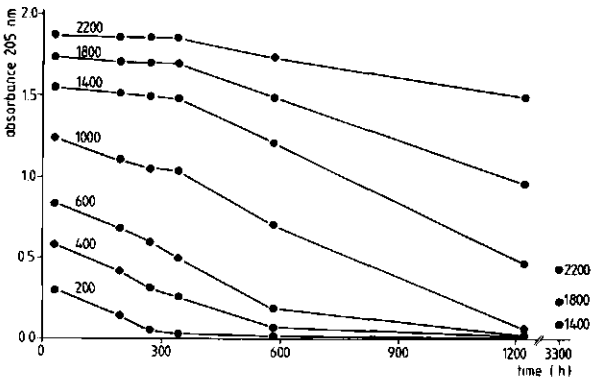


Fig. 7. The disappearance of UV absorbance from the media (diluted 67 ×) with various concentrations (mg liter^{-1}) of L-dopa supplied at the beginning of a VFA-fed batch experiment. Details of the experiment are described in the legend to Fig. 3.

The anaerobic degradation of L-dopa to CH_4 was confirmed by providing L-dopa as the sole substrate for granular sludge. Granular sludge (1·3 g OS sludge per liter) was allowed to digest with only 327 mg per liter L-dopa as substrate. After a 51-day digestion period the L-dopa treatments produced about twice as much CH_4 as the control sludge (Table 3). The extra production was approximately equal to 60% of the maximum CH_4 production expected, based on the theoretical COD content of the L-dopa. A lower than 100% recovery of the COD was expected since some UV absorbance remained in the treatments (Table 3).

The disappearance of UV absorption in VFA batch-fed cultures supplied only once with L-dopa was usually significant. In many cases (L-dopa < 1000 mg liter^{-1}) greater than 90% disappearance was evident in the treatments (Fig. 7). The continuously fed columns, which received a constant supply of 327 mg per liter of L-dopa were, however, not able to reliably remove UV absorbance (Fig. 6B). While UV absorption elimination increased to 75% in the first 21 days (500 h) of operation, the elimination decreased to 33% ± 9% after about 56 days (1350 h) of operation (Table 4) and remained low for the rest of the experiment (Fig. 6B). Low conversion of the L-dopa to CH_4 + VFA COD was also observed (Table 4). The tyrosine-fed column had increasing levels of UV absorption elimination with time (Fig. 6B). After about 56 days (1350 h) 90% ± 7% of the UV absorbance was eliminated and the extra COD of the CH_4 and the effluent VFA was equivalent to 73% of the tyrosine COD (Table 4). This is strong evidence that tyrosine was degraded to CH_4 during digestion in the continuous

TABLE 4

The Recovery of Tyrosine and L-Dopa COD as CH_4 + VFA COD and the Disappearance of UV Absorption (215 nm, Media Diluted 16×) from the 52nd (1244 h) to 60th Day (1452 h) of Continuously VFA-Fed Column Operation with 300 or 327 mg liter^{-1} Tyrosine or L-Dopa Present in the Influents. Hydraulic Retention Times were Between 7·5 and 10 h. Other Details of the Experiment are Reported in the Legend to Fig. 6.

	300 mg liter^{-1} tyrosine	*327 mg liter^{-1} L-dopa*
COD recovery %[a]	73·4 ± 16·7[b]	23·0 ± 15·9
Disappearance UV ABS%	90·0 ± 6·8	33·0 ± 9·2

[a] $[(CH_4 + VFA)COD_{treatment} - (CH_4 + VFA)COD_{control}] \times 100/COD_{paa}$, where

$(CH_4 + VFA)COD_{treatment}$ = the quantity of COD leaving the phenolic amino acid-treated, VFA-fed column as CH_4 in the gas or VFA (C_2 to C_5) in the effluent for a given time interval

$(CH_4 + VFA)COD_{control}$ = the quantity of COD leaving control VFA-fed column as CH_4 in the gas or VFA (C_2 to C_5) in the effluent for a given time interval

COD_{paa} = the quantity of COD entering the phenolic amino acid-treated, VFA-fed column as phenolic amino acid COD

[b] ± one standard deviation.

column, since almost no VFA nor phenol or *p*-cresol intermediates were apparent in the effluent (during the period evaluated in Table 4).

The elimination of UV absorbance in the tyrosine column was greatly disturbed when the column was severely overloaded with VFA, allowing high concentrations of VFA in the effluent (Fig. 6B). The *p*-cresol intermediate of anaerobic tyrosine degradation also reappeared in the

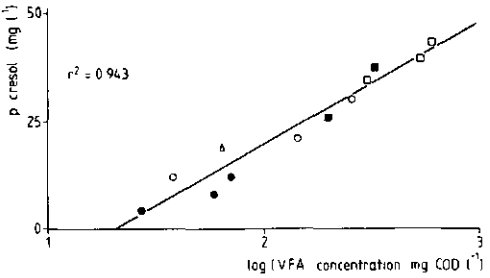


Fig. 8. The relationship between effluent *p*-cresol and VFA concentrations of the VFA-fed column with 300 mg liter^{-1} tyrosine in the feed. Data are reported from the time period representing the 52nd (1244 h) to 70th (1689 h) day of column operation. The sludge was adapted to anaerobic tyrosine degradation by the 52nd day. The HRT associated with each reported value of effluent *p*-cresol are coded to demonstrate that HRT was not the only factor determining the *p*-cresol concentration: (□) = 4·9 to 5·4 h HRT; (■) = 6·4 to 6·5 h HRT; (○) = 7·3 to 7·4 h HRT; (●) = 8·2 to 8·8 h HRT; (△) = 9·1 h HRT.

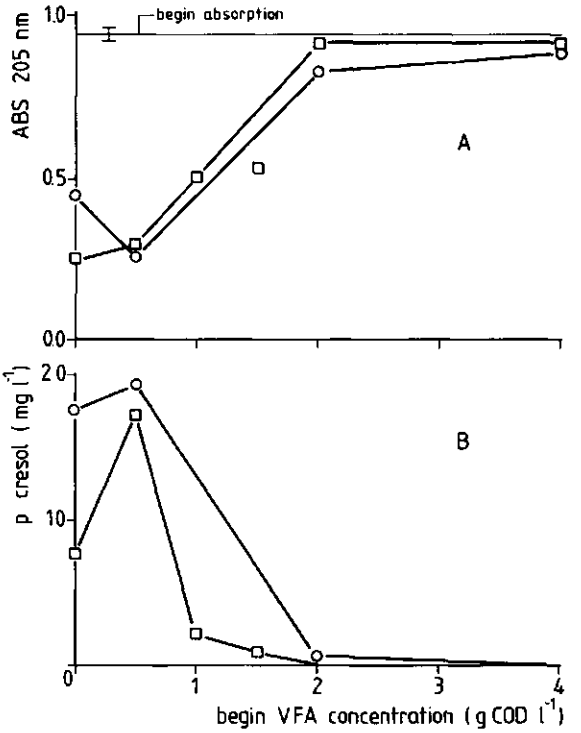


Fig. 9A. The UV absorption of L-dopa (327 mg liter^{-1}) supplied media (diluted 31×) following 3 days of batch digestion with various concentrations of VFA present. B. The concentration of *p*-cresol present in the L-dopa supplied media after 3 days of digestion. Data in both Figs 9A and B are reported for sludge which was previously adapted on 4 g COD per liter VFA for 5 days (○) or sludge which was not previously adapted (□). Details of this experiment are described in the legend to Fig. 5.

effluent. This suggested a relationship between the anaerobic degradation of *p*-cresol and the effluent VFA concentration. The level of formed *p*-cresol left undegraded could be linearly correlated with the log of the VFA concentration in the effluent (Fig. 8). The high concentrations of VFA may lower the free energy available for *p*-cresol degradation, since VFA are important intermediates of this degradation (Young & Rivera, 1985). The logarithmic relationship supports this theory. Shortening the hydraulic retention time (HRT) to increase the VFA loading could also contribute to increases in the effluent *p*-cresol concentration by decreasing the contact time with the bacteria. The data, presented in Fig. 8, are coded for HRT to

demonstrate that, at any given HRT, a linear relationship between *p*-cresol and log VFA concentrations was still evident, confirming that the presence of VFA in the media is unfavourable for the anaerobic *p*-cresol degradation.

The formation of the *p*-cresol intermediate of anaerobic L-dopa degradation was drastically reduced during the VFA overload period (Fig. 6A). The high concentrations of VFA in this case prevented the formation of *p*-cresol from L-dopa. Likewise, low L-dopa UV absorption elimination during the VFA overload period was evident (Fig. 6B). The unfavourable effect of high VFA concentration on the formation of *p*-cresol from L-dopa was also observed in batch-fed experiments (Fig. 9).

DISCUSSION

Tyrosine was found not to be toxic to methanogenic bacteria and easily degraded to CH_4 after 8 weeks operation of a granular-sludge column. Therefore, its presence in potato starch wastewater should not significantly affect anaerobic waste treatment. However, prior conversion of tyrosine to L-dopa by potato tyrosinase could affect the anaerobic waste-treatment, since L-dopa was found to be toxic to methanogenic bacteria and less degradable than tyrosine.

The toxicity of L-dopa was significantly greater in cultures which were shaken with VFA present. The shaking provided good contact between the granular sludge and the VFA in the media. The fact that L-dopa was not toxic if no VFA were present indicated that the toxicity of L-dopa is synergistic with VFA. The synergistic influence of VFA on the L-dopa toxicity could be reduced by adapting the sludge to VFA before allowing the sludge to come into contact with the L-dopa. Therefore, maintaing low VFA concentrations in the media and adapting the sludge to VFA are two measures which can be utilized to minimize the toxicity of L-dopa, if it is present in the wastewater.

The anaerobic degradation of tyrosine to CH_4 was observed for the first time many years ago (Tarvin & Buswell, 1934). The observation that phenol and *p*-cresol are intermediates of anaerobic tyrosine degradation has already been reported (Tarvin & Buswell, 1934; Elsden *et al.*, 1976; Brown, 1977; Spoelstra, 1978; Balba & Evans, 1980). The results of this study are in agreement with these previous observations, which establish the anaerobic degradability of the most commonly occurring phenolic amino acid, tyrosine. The operation of a continuous VFA-fed column with tyrosine present in concentrations expected in potato starch wastewater indicates that a large percentage of the tyrosine can be completely degraded after the sludge has had 2 months' experience with the tyrosine.

The anaerobic degradation of L-dopa, another phenolic amino acid, to CH_4, is established in this study. One intermediate, *p*-cresol, was observed during the batch degradation of L-dopa (*m*-cresol was also observed in continuous cultures). However, the continuous operation of a VFA-fed column with L-dopa present indicated that only a small percentage of the L-dopa COD is degraded to VFA and CH_4 after a prolonged experience of the sludge with the L-dopa. Therefore, the removal of phenolic amino acid COD in potato wastewater will be greatly affected if the tyrosine is converted to L-dopa by potato tyrosinase before the wastewater enters anaerobic waste-treatment.

REFERENCES

Balba, M. T. & Evans, W. (1980). Methanogenic fermentation of the naturally occurring aromatic amino acids by a microbial consortum. *Biochem. Soc. Trans.*, **8**, 625–7.

Baruah, P. & Swain, T. (1959). The action of potato phenolase on flavanoid compounds. *J. Sci. Food Agric.*, **10**, 125–9.

Benjamin, M. M., Woods, S. L. & Ferguson, J. F. (1984). Anaerobic toxicity and biodegradability of pulp mill waste constituents. *Water Res.* **18**, 601–7.

Brown, J. P. (1977). Role of gut bacterial flora in nutrition and health: A review of recent advances in bacteriological techniques, metabolism, and factors affecting flora composition. *Critical Reviews in Food Science and Nutrition.*, **8**, 229–336.

Chou, L. W., Speece, R. E., Siddigi, R. H. & McKeon, K. (1978). The effect of petrochemical structure on methane fermentation toxicity. *Prog. Wat. Tech.*, **10**, 545–58.

Davies, A. M. C. & Laird, W. M. (1976). Changes in some nitrogenous constituents of potato tubers during aerobic autolysis. *J. Sci. Fd Agric.* **27**, 377–82.

Diemar, W., Koch, J. & Hess, D. (1960). Einfluss der schwefligen Säure und L-Ascorbinsäure bei der weinbereitung. *Z. Lebensm. Untersuch U. Forsch.* **113**, 381–7.

Elsden, S. R., Hilton, M. G. & Waller, J. M. (1976). The end products of the metabolism of aromatic amino acids by clostridia. *Arch. Microbiol.*, **107**, 283–8.

FAO (1970). Amino acid content of foods and biological data on proteins. Food Policy and Food Science Service. Nutrition Division. FAO. Rome, Italy.

Fedorak, P. M. & Hrudey, S. E. (1984). The effects of phenol and some alkyl phenolics on batch anaerobic methanogenisis. *Water Res.*, **18**, 361–7.

Heisler, E. G., Siciliano, J. & Krulick, S. (1972). Potato starch factory waste effluents. II. Development of a process for recovery of amino acids, protein and potassium. *J. Sci. Fd. Agric.*, **23**, 745–62.

Kaldy, M. S. & Markakis, P. (1972). Amino acid composition of selected potato varieties. *J. of Fd Sci.*, **37**, 375–7.

Kendal, L. P. (1949). The action of tyrosinase on monophenols. *Biochem. J.*, **44**, 442–54.

Labib, A. I. (1962). *Potato proteins: Their properties and nutritional value.* Doctoral dissertation, Agricultural University Wageningen, The Netherlands.

Livingstone, R. M., Baird, B. A., Atkinson, T. & Crofts, R. M. J. (1980). The effect of either raw or boiled liquid extract from potato (*Solanum tuberosum*) on the digestibility of a diet based on barley in pigs. *J. Sci. Fd Agric.*, **31**, 695–700.

Mapson, L. W., Swain, T. & Tomlin, A. W. (1963). Influence of variety, cultural conditions and temperature of storage on enzymic browning of potato tubers. *J. Sci. Fd Agric.*, **14**, 673–84.

Mason, H. S. (1955). Comparative biochemistry of the phenolase complex. *Advances in Enzymology*, **16**, 105–84.

Mason, H. S. & Peterson, E. W. (1965). Melanaproteins: I. Reactions between enzyme-generated quinones and amino acids. *Biochem. Biophys. Acta*, **III**, 134–46.

Mathew, A. G. & Parpia, H. A. B. (1971). Food browning as a polyphenol reaction. *Adv. Fd Res.*, **19**, 75–145.

Muneta, P. (1981). Comparison of inhibitors of tyrosine oxidation in the enzymatic blackening of potatoes. *American Potato J.*, **58**, 85–92.

Nelson, J. M. & Dawson, C. R. (1944). Tyrosinase. *Advances in Enzymology*, **4**, 99–152.

Pearson, F., Shium-Chung, C. & Gavtier, M. (1980). Toxic inhibition of anaerobic biodegradation. *J. WPCF*, **52**, 473–82.

Rolland, M. & Lissitzky, S. (1962). Oxydation de la tyrosine et de peptides ou protéines la contenant, par la polyphénoloxydase de champignon: I. Oxydation de la tyrosine et de peptides la contenant, en présence d'acide ascorbique. *Biochim. Biophys. Acta.*, **56**, 83–94.

Samotus, B., Leja, M., Scigalski, A., Dulinski, J. & Siwanowicz, R. (1982). Determination of tyrosine by a modified Millon's reaction and its application to potato tuber extracts. *J. Sci. Fd Agric.* **33**, 617–22.

Singleton, V. L. (1972). Common plant phenols other than anthocyanins contributions to coloration and discoloration. *Advances in Food Research Supplement*, **3**, 143–91.

Spoelstra, S. F. (1978). *Microbial aspects of the formation of malodorous compounds in anaerobically stored piggery wastes.* Dissertion, Dept. Microbiology, Agricultural University. Wageningen, The Netherlands.

Tarvin, D. & Buswell, A. M. (1934). The methane fermentation of organic acids and carbohydrates. *J. Amer. Chem. Soc.*, **56**, 1751–5.

Walker, J. L. R. (1975). Enzymatic browning in foods: A review. *Enzyme Technology Digest*, **4**, 89–100.

Young, L. Y. & Rivera, M. D. (1985). Methanogenic degradation of four phenolic compounds. *Water Res.*, **19**, 1325–32.

CHAPTER 3

The Methanogenic Toxicity and Anaerobic Degradability of a Hydrolyzable Tannin.

Published in: Wat. Res. (1987) 21: 367-374.

Wat. Res. Vol. 21, No. 3, pp. 367–374, 1987
Printed in Great Britain.

0043-1354/87 $3.00 + 0.00

THE METHANOGENIC TOXICITY AND ANAEROBIC DEGRADABILITY OF A HYDROLYZABLE TANNIN

J. A. FIELD and G. LETTINGA
Department of Water Pollution Control, Agricultural University, De Driejen 12, Wageningen, The Netherlands

(*Received June 1986*)

Abstract—Gallotannic acid was found to be highly toxic to methanogenic activity. Concentrations, representing 50% inhibition, approximated 700 mg l^{-1}. The toxicity was persistent despite the rapid degradation of gallotannic acid to volatile fatty acids and methane. A 72.5% loss of sludge activity was associated with a 1 day exposure of methanogenic granular sludge to 1000 mg l^{-1} gallotannic acid. The toxicity of gallotannic acid was persistent over 2 month assay periods. The monomeric derivatives of gallotannic acid, gallic acid and pyrogallol were much less toxic. The 50% inhibition concentration of the monomers approximated 3000 mg l^{-1} and their toxicities were not persistent. No activity losses were evident after sludge was exposed to 3000 mg l^{-1} gallic acid for 19 days.

The lower toxicities of the monomers compared to the gallotannic acid polymer suggests that the mechanism of toxicity was "tanning", since data in the literature indicate that tannin polymers are more effectively adsorbed and precipitated with proteins compared to their monomeric counterparts. Functional proteins (enzymes) located at accessible sites in or on the methane bacteria are most likely disturbed by the tanning action.

Key words—methanogenic toxicity, anaerobic phenolic degradation, tannic acid, gallic acid, pyrogallol

INTRODUCTION

Tannins are by definition phenolic compounds which are highly reactive with proteins (Gupta and Haslam, 1980; Haslam, 1966) and are polymers ranging in mol. wt from 500 to 3000 g mol^{-1} (White, 1957). They may be entering anaerobic digestion processes where wastewaters utilized are derived from sources highly concentrated with tannins. Several examples of such sources include apples (Lea, 1978), sorghum (Gupta and Haslam, 1980), grapes (Singleton and Esau, 1969; Van Buren, 1970), banana (Goldstein and Swain, 1963), coffee (Elias, 1979), cacao (Goldstein and Swain, 1963), beans (Bressani and Elias, 1980), and bark (Niiranen in Virkola and Honkanen, 1985; Karchesy and Hemingway, 1980; Herrick, 1980; Haslam, 1966; Wise, 1946). While the toxicity of several simple phenols on methanogenesis has previously been investigated (Chou *et al.*, 1978; Fedorak and Hrudey, 1984; Pearson *et al.*, 1980; Benjamin *et al.*, 1984), there is little information available regarding the influence of tannins. The toxicity of tannins on several enzymes, however, has been established (Loomis and Battaile, 1966; Daiber, 1975; Tamir and Alumot, 1969; Gupta and Haslam, 1980).

The purpose of this study was to evaluate the effect of a tannin on the methane production from granular anaerobic sludge. Common occurring tannins, known as condensed tannins, are polymers of flavanols (Gupta and Haslam, 1980). Figure 1 illustrates the structure of procyanidins which are common condensed tannins. Condensed tannins are not readily available in pure form. Therefore, gallotannic acid, a hydrolyzable tannin, was chosen as a tannin for study, since it is a commercially available reference compound. Hydrolyzable tannins are polyesters of gallic acid (Haslam, 1966). Figure 1 illustrates the structure of gallotannins which are common hydrolyzable tannins. Gallic acid and pyrogallol (Fig. 1) were also studied, since they are monomeric derivatives of gallotannic acid.

MATERIALS AND METHODS

Media contained the following nutrients (per liter): H_3BO_3, 0.05 mg; $FeCl_2 \cdot 4H_2O$, 2 mg; $ZnCl_2$, 0.05 mg; $MnCl_2 \cdot 4H_2O$, 0.05 mg; $CuCl_2 \cdot 2H_2O$, 0.03 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.05 mg; $AlCl_3 \cdot 6H_2O$, 0.09 mg; $CoCl_2 \cdot 6H_2O$, 2 mg; $NiCl_2 \cdot 6H_2O$, 0.05 mg; $Na_2SeO_3 \cdot 5H_2O$, 0.1 mg; EDTA, 1 mg; resazurine, 0.2 mg; 36% HCl, 0.001 ml; NH_4Cl, 0.28 g; K_2HPO_4, 0.25 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $NaHCO_3$, 0.4 g; yeast extract, 0.1 g and $CaCl_2 \cdot 2H_2O$, 0.01 g. Batch fed experiments were conducted in 0.25 or 0.5 l. serum flasks. Two shaking regimes were utilized: unshaken cultures and shaken cultures, which were reciprocally shaken with 1 min shaking per 4 min rest cycle. Granulated sludge (1.5 years in 4°C storage) used in the experiments, was originally obtained from an UASB reactor treating potato derived wastewater. Sludge concentrations utilized ranged from 1.0 to 1.5 g organic solids (OS) l^{-1}. Exact OS concentrations are reported in figure and table captions. The volatile fatty acid (VFA) substrates utilized throughout most of the experiments were obtained from a stock solution containing 100:100:100 g acetate:propionate:butyrate per kg of pH 6.8 neutralized (with NaOH) solution. The chemical oxygen demand ratio of the VFA stock was 24.3:34.4:41.3% of the total COD for C_2,

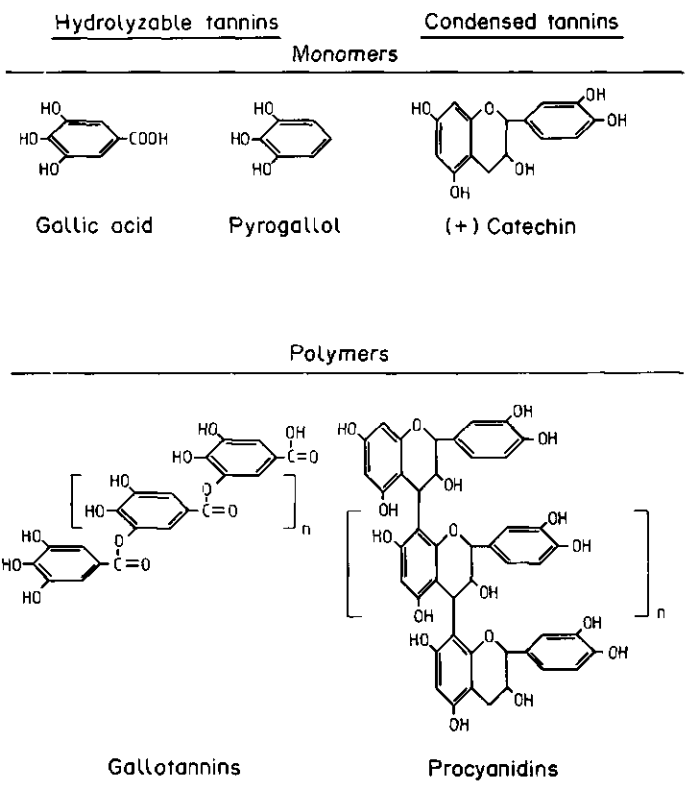


Fig. 1. Tannins and their monomeric derivatives.

C_3 and C_4, respectively. Exact concentrations of VFA used during the experiments are listed in tables and figures. The temperature used in all assays was 30°C.

The phenolic compounds utilized were obtained from Janssen Chimica, Boom BV and British Drug House for gallic acid (3,4,5-trihydroxybenzoic acid), pyrogallol (1,2,3-trihydroxybenzene) and gallotannic acid (a polyester of 9 gallic acid units and 1 sugar moiety), respectively. Gallotannic acid (MW = 1701) has the following formula: $C_{76}H_{52}O_{46}$.

Methane production was monitored with modified Mariotte flasks containing 3% NaOH (which served to remove CO_2 from the gas). Volatile fatty acids (VFA) were analyzed with a Packard 417 gas chromatograph equipped with a 2 m × 2 mm i.d. column packed with 10% Flourad FC 431 on supelcoat (100–200 mesh). The carrier gas (N_2) was saturated with formic acid (flow 35 ml min^{-1}). The oven temperature was set at 130°C. The FID detector signal was processed with a SP41 Spectra Physics integrator. The VFA standards (all isomers of C_2 through C_5). Several aromatic compounds could be detected by this chromatography procedure without derivatization. Standards tested included phenol, *p*-cresol, *o*-cresol, *m*-cresol, benzoic acid, *p*-ethylphenol, *m*-ethylphenol, phenylacetic acid, and 3-phenylpropionic acid. None of these aromatic compounds, however, were identified in cultures which degraded pyrogallol, gallic acid and gallotannic acid. The pH was monitored with a Knick 511 meters and a Schot Gerate N61 double electrode. Phenolic compounds have u.v. absorbing properties. The elimination of u.v. absorbance from the media is indicative of the phenolic compound's disappearance. Ultraviolet absorption was measured with a Perkin–Elmer 550 A spectrophotometer and a Hellma 100-QS 1 cm quartz cuvette. Absorption is reported as the absorption of the media containing phenolic compounds less the absorption of the control media (which contained no u.v. absorbing compounds).

Methanogenic activities are reported for batch assays as a percentage ratio of treatment CH_4 production rate and control CH_4 production rate. The percentage inhibition is defined as: 100 – activity; where activity is expressed as a percent of the control.

The recovery of phenolic COD as VFA and CH_4 COD (indicative of the percent conversion of phenolics to fermentation end products) was calculated by summing up the COD present as produced CH_4 and as VFA in the media for both the treatment and the VFA fed controls and taking the difference of these two sums and dividing by COD added with the phenolic compound treatment.

RESULTS

Anaerobic degradation of gallotannic acid

The disappearance of u.v. absorption during VFA fed serum flask batch digestion with gallotannic acid present in the media is illustrated in Fig. 2. At almost

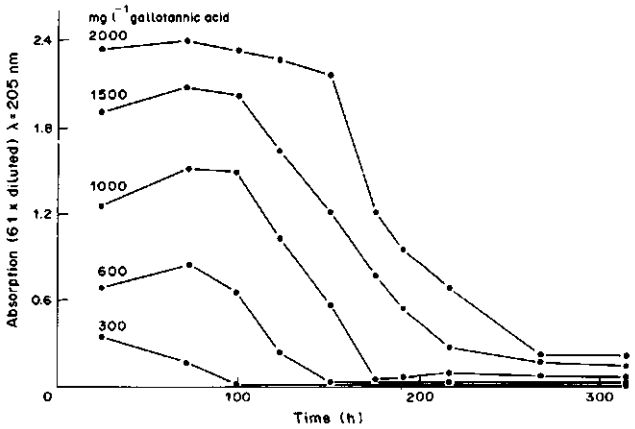


Fig. 2. The disappearance of u.v. absorption in 0.5 l. serum flask digestions of gallotannic acid and 4.17 g COD l^{-1} VFA with 1.11 g OS l^{-1} granulated sludge (unshaken).

all of the initial gallotannic acid concentrations rapid disappearance of u.v. absorption commenced after 100 h. Most of the u.v. loss is related to biological anaerobic degradation of gallotannic acid since COD balancing results (Fig. 3) indicate that large percentages of the gallotannic acid COD were recovered as CH_4 and VFA. Where high concentrations of gallotannic acid were utilized, the inhibition of methanogenesis was extreme (Fig. 4). In one case, the methanogenic activity was so low that increases of the acetate concentration in the media could be observed as a result of gallotannic acid degradation (Table 1). Acetate is therefore an important intermediate of anaerobic gallotannic acid degradation to CH_4 and CO_2.

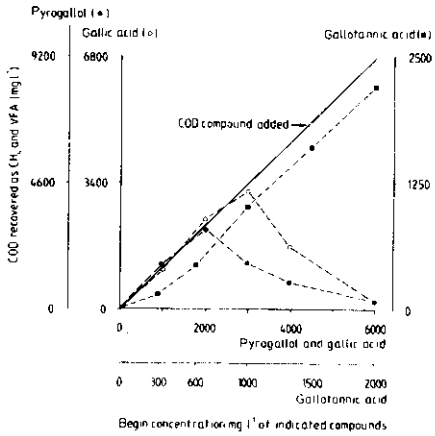


Fig. 3. The recovery of gallotannic acid and hydrolyzable tannin monomer COD in VFA + CH_4 fractions following 19 days of digestion (details of experiment reported in Fig. 2 caption).

Anaerobic degradation of hydrolyzable tannin monomers

Losses (or partial losses) of u.v. absorption were observed during VFA fed serum flask batch digestions with gallic acid or pyrogallol added at concentrations up to 4 g l^{-1}. This corresponded to the recovery (or partial recovery) of the COD added with these compounds in CH_4 and VFA (Fig. 3). Where initial concentrations were $\leqslant 2$ g l^{-1} pyrogallol or 3 g l^{-1} gallic acids, >90% phenolic COD conversions to CH_4 and VFA and >90% u.v. absorption losses were observed. With 4 g l^{-1} gallic acid, the methanogenesis was extremely inhibited (Fig. 4) and it was possible to observe an increase of the acetate concentration in the media (Table 2) resulting from gallic acid degradation. This indicates that acetic acid is an important intermediate of anaerobic gallic acid degradation.

Low concentrations of gallic acid amended VFA fed batch cultures, were not inhibitory but were stimulatory to the CH_4 production (Fig. 4). The stimulatory effect is related to the fermentation of the phenolic compound. The VFA added to the medium by degrading a non-toxic phenolic compound would not be expected to increase the methane production rate based on Monod kinetics. This is because the concentrations of VFA in the VFA fed media were in large excess of K_s values reported for Methanotrix. The added VFA contributes to a stimulatory effect, however, due to several other reasons. Firstly, diffusional limitations in unshaken or intermittently shaken cultures would be compensated by increasing the VFA concentration of the bulk solution. Secondly, the sludge has a higher activity on acetate than propionate and butyrate (the latter two make up 76% of the VFA stock solution COD) and the VFA added to the culture media by degrading trihydroxy pheno-

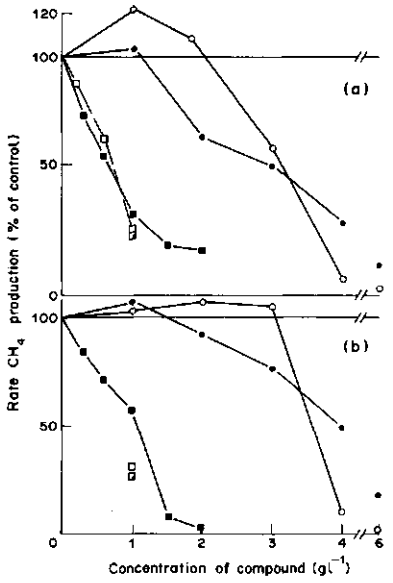


Fig. 4. (a) The *in situ* activity of sludge expressed as a percentage of the control activity during the first VFA (4.17 g COD l^{-1}) feeding for various concentrations of gallic acid (○), pyrogallol (●) and gallotannic acid (■) in still standing (unshaken) 0.5 l. batch serum flask digestions with 1.11 g OS l^{-1} granulated sludge; gallotannic acid (□) with 1.05 OS l^{-1} granulated sludge in unshaken experiments; and gallotannic acid (◪) with 1.05 g OS l^{-1} granulated sludge in mechanically shaken (reciprocal shaking for 1 min every 5 min) experiments. The absolute activities of the controls were: 497.3, 471.9, 477.5, 522.0 and 504.3 mg COD g^{-1} OS added d^{-1}, for ○, ●, ■, □ and ◪, respectively. (b) The sludge activity following 19 days (○, ●, ■), 25 days (◪) or 54 days (□) of digestion. Supernatants were decanted under N_2 flushing and replaced with nutrient supplemented medium (pH 7.4) containing 4.17 g COD l^{-1} VFA, inhibitors were not included in replacement medium. The absolute activities of the controls were 961.1, 918.5, 868.9, 971.0 and 1052 mg COD g^{-1} OS added d^{-1} for ○, ●, ■, □ and ◪, respectively.

lics was observed to be acetate. The moment that the acetic acid from the phenolic compound fermentation was released into the bulk media of VFA fed cultures there was a simultaneous stimulation of the methane production beyond the rate of the VFA fed control. Therefore, the time when the methane production rate of the phenolic treated VFA cultures increased beyond the control is indicative of the time needed for the cultures to adapt to the phenolic substrate. This adaption time could be referred to as the lag period for phenolic compound degradation. The short lag periods (several days) required for anaerobic decomposition of pyrogallol and gallic acid are reported in Table 3 for initial concentrations of these compounds which were not toxic to methanogenesis.

Toxicity of gallotannic acid

Gallotannic was observed to inhibit the activity of methanogenic bacteria (Fig. 4). The concentration of gallotannic acid corresponding to 50% inhibition approximated 700 mg l^{-1} in various experiments. The toxicity of gallotannic acid was persistent. The increases of the activity which were observed after approx. 800 h (1 month) of digestion were proportional to the increases in the control activity (Fig. 5). There was little recovery of the relative activity (percentage of the control activity). Even when supernatant was removed and replaced with new medium (lacking gallotannic acid), only partial recovery (in experiments without shaking) or no recovery (in experiments with shaking) of residual sludge activity were observed in assays conducted after 19, 25 or 54 days of digestion (Fig. 4). Relative activity of recovered sludge was actually lost at high concentrations of gallotannic acid (1.5–2.0 g l^{-1}) compared to the *in situ* activity of the first VFA feeding (Fig. 4). Cultures subjected to these high concentrations were also associated with pH drops in the media (Table 4).

The observation that activity was not recovered after gallotannic acid was degraded, indicates that the initial exposure of the sludge to gallotannic acid prior to degradation was sufficient to impart a damaging effect. Table 5 confirms this hypothesis, since 1 day exposure of the sludge to gallotannic acid resulted in large losses of activity. The toxicity of gallotannic acid was greatest for the metabolism of butyrate compared to acetate and propionate (Table 5).

Toxicity of hydrolyzable tannin monomers

The two monomeric derivatives of gallotannic acid (gallic acid and pyrogallol) were found to be much less toxic than gallotannic acid (Fig. 4). The residual sludge activity, following 19 days exposure to either 2 g l^{-1} of gallic acid or pyrogallol with VFA substrate was similar to the control residual sludge activity. The control sludge was exposed for 19 days to only the VFA substrate. In contrast to the monomeric derivatives, the residual sludge activity remaining after 19 days exposure to 2 g l^{-1} gallotannic acid with VFA substrate was only 3% of the control. The methanogenic toxicities observed at 4 g l^{-1} gallic acid and pyrogallol (Fig. 4) were not completely due to the compounds themselves. The high concentration of gallic acid caused a severe drop in medium pH (Table 4), which undoubtedly contributed to lower methanogenic acid. The pyrogallol stock solution contained low concentrations of oxidized impurities. These impurities are known to cause more toxicity than pyrogallol itself (unpublished data).

DISCUSSION

Degradation of trihydroxy phenolic compounds and hydrolyzable tannin

The anaerobic degradation of gallotannic acid occurred rapidly and was associated with a rather

Table 1. Changes in VFA and CH_4 COD at selected time intervals of methanogenesis inhibited anaerobic digestion with 4.17 g COD l^{-1} VFA and 2 g l^{-1} gallotannic acid (2.48 g COD l^{-1}) in a 0.5 l. (contents) serum containing 1.11 g OS l^{-1} granulated sludge. The VFA substrate was added to the culture first to allow the medium to become anaerobic by a 16 h predigestion. Following the predigestion, the gallotannic acid was added to initiate the assay. The COD of the VFA at the beginning of the assay is equal to the VFA COD added before predigestion minus the COD of the CH_4 evolved during predigestion

		COD (mg l^{-1})				
		VFA				
Event	Time (h)	C_2	C_3	C_4	C_{total}*	CH_4 evolved
Addition of VFA to the culture						
Predigestion (overnight)						
Begin predigestion	0	1014	1434	1722	4172	0
End predigestion	16	ND	ND	ND	3822	350
Addition of gallotannic acid to the culture						
Assay						
Begin assay	0	ND	ND	ND	3822†	0‡
First sampling	217	1596	1288	1676	4656	582
Second sampling	459	1942	1228	1602	4868	932

*C_{total} = C_2 through C_5.
†Same as the end of predigestion.
‡CH_4 COD data of the assay is reported as CH_4 accumulated from the beginning of the assay (and does not include CH_4 produced during predigestion).
ND = no data.

Table 2. Changes in VFA and CH_4 COD at selected time intervals of methanogenesis inhibited anaerobic digestion with 4.17 g COD l^{-1} VFA and 4 g l^{-1} gallic acid (4.52 g COD l^{-1}) in 0.5 l. (contents) serum flasks containing 1.11 g OS l^{-1} granulated sludge. The VFA substrate was added to the culture first to allow the medium to become anaerobic by a 16 h predigestion. Following the predigestion, the gallic acid was added to initiate the assay. The COD of the VFA at the beginning of the assay is equal to the VFA COD added before predigestion minus the COD of the CH_4 evolved during predigestion

		COD (mg l^{-1})				
		VFA				
Event	Time (h)	C_2	C_3	C_4	C_{total}*	CH_4 evolved
Addition of VFA to the culture						
Predigestion (overnight)						
Begin predigestion	0	1014	1434	1722	4172	0
End predigestion	16	ND	ND	ND	3776	396
Addition of gallic acid to the culture						
Assay						
Addition gallic acid	0	ND	ND	ND	3776†	0‡
End digestion	456	2224	1332	1576	5216	412

*C_{total} = C_2 through C_5.
†Same as the end of predigestion.
‡CH_4 COD data of the assay is reported as CH_4 accumulated from the beginning of the assay (and does not include CH_4 produced during predigestion).
ND = no data.

short (4 days) lag phase with untried granular methanogenic sludge. The ester intermonomeric bond of hydrolyzable tannins is therefore, readily degradable under anaerobic conditions. However, no conclusions can be made from this study regarding the degradability of condensed tannins, since these are polymers of flavanoid compounds connected by ring C–branch C intermonomeric bonds (Gupta and Haslam, 1980; Haslam, 1966). There is very little information available in the literature concerning the anaerobic degradability of condensed tannins except to note that comparable intermonomeric linkages of dehydrovanillin (Chen *et al.*, 1985) and Douglas fir lignin (Colberg and Young, 1985b) are anaerobically degradable with adapted incolum. Additionally, condensed tannin monomers (flavanoids) are eliminated by rumen fluid (Simpson *et al.*, 1969) or are converted in anaerobic environments by digester sludge (Balba and Evans, 1980) and digestive tract flora (Brown, 1977) to aromatic intermediates, which are otherwise known to be degradable to CH_4 in methanogenic sludges.

Table 3. Lag period for pyrogallol and gallic acid anaerobic degradation during VFA (4.17 g COD l^{-1}) digestion (1.11 g OS l^{-1}) based on gas yield data from methanogenesis uninhibited cultures

Compound	Concentration (mg l^{-1})	Observations	Lag period (days)*
Gallic acid	1000	2	2
	2000	1	5
Pyrogallol	1000	2	4–5

*Based on time when methane production rate of VFA fed treatment increased beyond the rate observed for the VFA fed control.

The gallic acid monomer of hydrolyzable tannins and pyrogallol (the decarboxylated form of the monomer) were also readily degraded by methanogenic granular sludge. The rapid anaerobic fermentation of gallic acid, pyrogallol and other methoxy substituent variations of the galloyl group

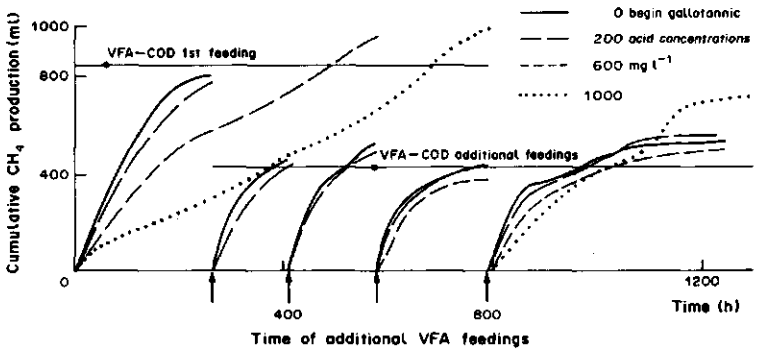


Fig. 5. Cumulative methane production from gallotannic acid and VFA (4.17 g COD l^{-1}) fed batch serum flask (0.5 l.) digestions with 1.05 g OS l^{-1} granular sludge. Horizontal lines indicate the COD equivalent (in gas production) of added VFA substrates. VFA (2.09 g COD l^{-1}) of additional feedings (without gallotannic acid) was added directly to existing supernatant under N_2 flushing. The shaking regime was performed twice daily by hand for 2 min.

Table 4. Original and final pH following 19 days of anaerobic digestion (1.11 g OS l^{-1} granular sludge) with VFA (4.17 g COD l^{-1}) substrate and added phenolic compounds

Original concentration (mg l^{-1})	Pyrogallol		Gallic acid		Gallotannic acid	
	Before*	After	Before*	After	Before†	After
0	7.40	7.43	7.40	7.40	7.57	7.45
300	—	—	—	—	7.40	7.35
600	—	—	—	—	7.43	7.20
1000	7.50	7.25	6.35	7.18	7.40	7.10
1500	—	—	—	—	7.30	6.55
2000	7.43	6.80	5.50	7.05	7.38	5.72
3000	7.39	7.05	5.18	7.05	—	—
4000	7.32	7.45	4.95	5.25	—	—
6000	7.22	7.45	4.61	5.00	—	—

*The begin pH was not adjusted in the culture media. The original pH was also not measured *in situ* but in solutions made to the same composition as in the experiments.
†Begin pH adjusted with NaOH under N_2 flushing and then measured *in situ*.

(1,2,3-trihydroxybenzene) has previously been described by Schink and Pfennig (1982) and Kaiser and Hanselmann (1982a,b). These studies have indicated as our study that anaerobic fermentation of galloyl derivatives is non-obligatory syntrophic with methanogenesis. In this study, gallic acid and gallotannic acid were readily degraded to acetate at gallic acid and gallotannic acid concentrations which were toxic to methanogenesis. Kaiser and Hanselmann (1982a) have observed that adding specific methanogenesis inhibitors to syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid) degrading cultures resulted in no delay in the syringic acid fermentation; however, acetate accumulated instead of being converted to CH_4.

Table 5. Inhibition caused by 24 h exposure (reciprocal shaking 1 min every 5 min) of 1.11 g OS l^{-1} granulated sludge to 1 g l^{-1} gallotannic acid. Following exposure gallotannic acid was decanted, sludge was rinsed with H_2O and sludge activity determined on replaced media (not containing gallotannic acid) with begin VFA concentration of 4 g COD l^{-1} (pH 7.4). The COD based average inhibition (for comparison with others reported in this article with C_2:C_3:C_4 substrate) was 72.5%

VFA	Absolute activity of control (mg COD g^{-1} OS d^{-1})	Inhibition by gallotannic acid (%)
C_2	1053	69.0
C_3	503	62.4
C_4	446	82.6

The mechanism(s) involved in anaerobic galloyl derivative degradation appear to differ largely from those involved in the decomposition of other simple phenolic and aromatic compounds. The addition of specific methanogenic inhibitors to cultures degrading other kinds of natural phenolic compounds results in the accumulation of *o*-dihydroxy (or methoxy) compounds or aromatic compounds lacking hydroxyl groups (Healy *et al.*, 1980; Colberg and Young, 1980b). Additionally adaption of sludge to methanogenesis has been reported to be beneficial for phenol and *p*-cresol (Van Velsen, 1981) as well as benzoate (Ferry and Wolfe, 1976; Balba and Evans, 1977; Clark and Fina, 1952) fermentation. While it is not necessarily true that the fermentation of non-galloyl type phenolics is obligatory syntrophic with methanogenesis (Young, 1984), disturbing methanogenesis, nonetheless, disturbs the fermentation of non-galloyl type phenolics but does not disturb the fermentation of galloyl type phenolics. Cultures enriched on galloyl (or methoxy substituents) type phenolics do not cross acclimate with benzoic acid

and other non-galloyl type phenolics, but they do cross acclimate with other galloyl types (Kaiser and Hanselmann, 1982; Schink and Pfennig, 1982). This suggests that the population of organisms responsible for the anaerobic degradation of trihydroxybenzenes are different from the degraders of other simple phenols.

Additional evidence for separate organisms involved in the anaerobic degradation of 1,2,3-trihydroxy vs *o*-dihydroxy and monohydroxy type phenolics is evident from the short lag periods required by our granular sludge to initiate the degradation of pyrogallol, gallic acid and gallotannic acid (2–5 days), while the same sludge needed 31 days to initiate the degradation of phenol and more than 56 days to initiate the degradation of catechol (1,2-dihydroxybenzene) under the same experimental conditions (unpublished data). Similar results have also been reported in the literature. Horowitz *et al.* (1981) observed that digester sludge needed 1 week to degrade 50 mg Cl^{-1} pyrogallol while 2 and 3 weeks were required for the same amount of phenol and catechol, respectively. Healy and Young (1979) observed that 300 mg l^{-1} syringic acid (a methoxylated galloyl type compound) degradation was initiated after 2 day lag periods by digester sludge, while from 8 to 21 day lag periods were required for 300 mg l^{-1} of 9 other non-galloyl type aromatic compounds tested.

Tannin toxicity

Gallotannic acid, while rapidly biodegraded, is still highly toxic to methanogenic activity. The toxicity in severe cases results in the loss of activity which is slowly or completely not recovered over long assay periods (2 months). A plausible mechanism for the toxicity may involve the "tanning" of proteins (such as enzymes) located at accessible sites in the methane bacteria. The observation that a hydrolyzable tannin polymer was significantly more toxic than its monomeric derivatives corresponds closely to observations in the literature that tannin polymers (and dimers) are more effectively adsorbed or precipitated with proteins than their lower molecular weight counterparts (Haslam, 1974; McGuiness *et al.*, 1975; Bate-Smith, 1973).

This study indicates that tannins are potent inhibitors of methanogenesis. Therefore, their presence in wastewater should be considered when evaluating the feasibility of anaerobic waste treatment processes.

REFERENCES

Balba M. T. and Evans W. C. (1977) The metanogenic fermentation of aromatic substrates. *Trans. Biochem. Soc.* **5**, 302–304.

Balba M. T. and Evans W. C. (1980) The methanogenic biodegradation of catechol by a microbial consortium: evidence for the production of phenol through cis-benzenediol. *Trans. Biochem. Soc.* **8**, 452–453.

Bate-Smith E. C. (1973) Haemanalysis of tannins: the concept of relative astringency. *Phytochemistry* **12**, 907–912.

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.

Bressani R. and Elias L. G. (1980) The nutritional role of polyphenols in beans. In *Polyphenols in Cereals and Legumes* (Edited by Hulse J. H.), pp. 61–68. International Development Research Centre, Ottawa, Canada.

Brown J. P. (1977) Role of gut bacterial flora in nutrition and health: a review of recent advances in bacteriological techniques, metabolism, and factors affecting flora composition. *Crit. Revs Fd Sci. Nutrition* **8**, 229–336.

Chen W., Ohmiya K., Shimizu S. and Kawakami H. (1985) Degradation of dehydrodivanillin by anaerobic bacteria from cow rumin fluid. *Appl. envir. Microbiol.* **49**, 211–216.

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

Clark F. M. and Fina L. R. (1952) The anaerobic decomposition of benzoic acid during methane fermentation. *Arch. Biochem.* **36**, 26–32.

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

Colberg P. J. and Young L. Y. (1985b) Aromatic and volatile acid intermediates observed during anaerobic metabolism of lignin-derived oligomers. *Appl. envir. Microbiol.* **49**, 350–358.

Daiber K. H. (1975) Enzyme inhibition by polyphenols of sorghum grain and malt. *J. Sci. Fd Agric.* **26**, 1399–1411.

Elias L. G. (1979) Chemical composition of coffee-berry by-products. In *Coffee Pulp: Composition, Technology and Utilization* (Edited by Braham J. E. and Bressani R.), pp. 11–16. International Development Research Centre, Ottawa, Canada.

Fedorak P. M. and Hrudey S. E. (1984) The effects of phenol and some alkyl phenolics on batch anaerobic methanogenesis. *Wat. Res.* **18**, 361–367.

Ferry J. G. and Wolfe R. S. (1976) Anaerobic degradation of benzoate to methane by a microbial consortium. *Arch. Microbiol.* **107**, 33–40.

Goldstein J. L. and Swain T. (1963) Changes in tannins in ripening fruits. *Phytochemistry* **2**, 371–383.

Gupta R. K. and Haslam E. (1980) Vegetable tannins—structure and bionsynthesis. In *Polyphenols in Cereals and Legumes* (Edited by Hulse J. H.), pp. 15–24. International Development Research Centre, Ottowa, Canada.

Haslam E. (1966) *Chemistry of Vegetable Tannins*. Academic Press, New York.

Haslam E. (1974) Polyphenol protein interactions. *Biochem. J.* **139**, 285–288.

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

Healy J. B., Young L. Y. and Reinhard M. (1980) Methanogenic decomposition of ferulic acid, a model lignin derivative. *Appl. envir. Microbiol.* **39**, 436–444.

Herrick F. W. (1980) Chemistry and utilization of western hemlock bark extractives. *J. agric. Fd Chem.* **28**, 228–237.

Horowitz A., Shelton D. R., Cornell C. P. and Tiedje J. M. (1981) Anaerobic degradation of aromatic compounds in sediments and digested sludge. *Devs ind. Microbiol.* **23**, 435–444.

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

Kaiser J. P. and Hanselmann K. W. (1982b) Aromatic chemicals through anaerobic microbial conversion of lignin monomers. *Experientia* **38**, 167–176.

Karchesy J. J. and Hemingway R. W. (1980) Loblolly pine bark polyflavanoids. *J. agric. Fd Chem.* **26**, 222–228.

Lea A. G. (1978) The phenolics of ciders: oligomeric and polymeric procyanidins. *J. Sci. Fd Agric.* **29**, 471–477.

Loomis W. D. and Battaile J. (1966) Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* **5**, 423–438.

McGuinness J. D., Eastmond R., Laws D. R. J. and Gardner R. J. (1975) The use of ^{14}C-labelled polyphenols to study haze formation in beer. *J. Inst. Brew.* **81**, 287–292.

Pearson F., Shium-Chung C. and Gavtier M. (1980) Toxic inhibition of anaerobic biodegradation. *J. Wat. Pollut. Control Fed.* **52**, 473–482.

Schink B. and Pfennig N. (1982) Fermentation of trihydroxybenzenes by *Pelobacter acidgallici* gen. nov. sp. nov., a new strictly anaerobic, non-sporeforming bacterium. *Arch. Microbiol.* **133**, 195–201.

Simpson F. J., Jones G. A. and Wolin E. A. (1969) Anaerobic degradation of some biflavonoids by microflora of the rumen. *Can. J. Microbiol.* **15**, 972–974.

Singleton V. L. and Esau P. (1969) Phenolic substances in grapes and wine, and their significance. *Advances in Food Research, Supplement 1.* (Edited by Chichester C. D. *et al.*), pp. 1–281. Academic Press, New York.

Tamir M. and Alumot E. (1969) Inhibition of digestive enzymes by condensed tannins from green and ripe carobs. *J. Sci. Fd Agric.* **20**, 199–202.

Van Buren J. (1970) Fruit phenolics. *The Biochemistry of Fruits and Their Products* (Edited by Hulme A. C.), pp. 269–304. Academic Press, New York.

Van Velsen A. F. M. (1981) Anaerobic digestion of piggery waste. Ph.D. dissertation. Department of Water Pollution Control, Agricultural University, Wageningen, The Netherlands.

Virkola N. E. and Honkannen K. (1985) Waste water characteristics. *Wat. Sci. Technol.* **17**, 1–28.

White T. (1957) Tannins—their occurrence and significance. *J. Sci. Fd Agric.* **8**, 377–384.

Wise L. E. (1946) *Wood Chemistry*. Reinhold, New York.

Young L. Y. (1984) Anaerobic degradation of aromatic compounds. In *Microbial Degradation of Organic Compounds* (Edited by Gibson D. T.), pp. 487–523. Marcel Dekker, Inc., New York.

CHAPTER 4

The Methanogenic Toxicity of Bark Tannins and the Anaerobic Biodegradability of Water Soluble Bark Matter.

Published in: Wat. Sci. Tech. (1988) 20(1): 219 -240.
An updated edition is printed here.

THE METHANOGENIC TOXICITY OF BARK TANNINS AND THE ANAEROBIC BIODEGRADABILITY OF WATER SOLUBLE BARK MATTER

J. A. Field[1], M. J. H. Leyendeckers[1], R. Sierra-Alvarez[1], G. Lettinga[1] and L. H. A. Habets[2]

[1]Dept. of Water Pollution Control
Wageningen Agricultural University
Bomenweg 2, 6703 HD, Wageningen
The Netherlands

[2]Paques B.V.
P.O. Box 52, 8560 AB Balk
The Netherlands

ABSTRACT - The principal methanogenic toxins of bark soluble matter were identified as the tannins. The tannins, which were measured with a selective tannin adsorbent, called polyvinylpyrrolidone, accounted for about half of the aqueous extractable COD of tree bark. The 50% inhibitory concentration of bark tannins averaged approximately 600 mg COD L^{-1} (350 mg tannin solids L^{-1}). The toxicity caused by the resin fraction of bark was demonstrated not to be very important to the methanogenic toxicity of aqueous extracts. While tree resin compounds were found to be very toxic to methanogenic bacteria, the solubility of the resin fraction was very poor due to the low natural pH during the aqueous extraction. Additionally, those compounds aqueous extracted from tree resin at the natural pH were not as toxic as all the tree resin compounds solubilized by alkali and supplied at similar concentrations. Anaerobic biodegradability results indicated that 30 to 50% of the bark water soluble COD can be acidified to methanogenic substrates during short term digestion. Pine and birch bark water soluble COD acidified up to 70% after long term batch digestion of 7 weeks. The high level of bark aqueous extract UV absorbance elimination by anaerobic digestion and the appearance of phenolic degradation intermediates indicated that at least some of the bark phenolic compounds were degraded.

KEYWORDS - Tannins, procyanidins, flavonoid compounds, phenolic compounds, polyvinylpyrrolidone, anaerobic digestion, methanogenesis, bark wastewater, methanogenic toxicity, resin, resin acid.

1. INTRODUCTION

1.1. Source and Composition of Bark Wastewater

Bark wastewater is principally derived from the contact of water with mechanically damaged bark. The major sources of such wastewater in the forest industry are associated with wet and half-wet debarking and bark pressing operations as outlined in Figure 1. Debarking removes from 96.0 to 99.7% of the bark from the wood (Hatton, 1987), therefore small amounts (from 0.3 to 4%) of the bark may enter the pulping process. Bark soluble matter can account for important contributions to pulping wastewater if bark is pulped together with the wood (masonite, fiber board, card board etc.). Generally, 3 to 9 m^3 of water are used to debark 1 metric ton of harvested tree (dry weight basis) with wet debarking processes. This corresponds to approximately 20 m^3 to 60 m^3 of water brought

into contact with each metric ton of bark (dry weight). If bark presses are utilized, an additional 0.5 to 0.8 m^3 of bark juice can be squeezed out of each metric ton of bark (dry weight). Some reported values of debarking and bark pressing effluent COD are cited in Table 1.

The compounds released from bark into wastewater are the water soluble compounds. The truly soluble compounds included simple carbohydrates (sugar), polar phenolic monomers and polymeric tannins. Apolar resin compounds are only slightly extractable from bark with water (at the natural pH of the extracts 3.5 to 5.5). Based on the available literature data, the predicted average composition of bark water soluble COD is outlined in Figure 2 (Hegert, 1960; Hathway, 1962; Wise, 1946; Kuwahara et al., 1984; Karchesy and Hemingway, 1980; Hegert et al., 1965; Pearl and Buchanan, 1976; Herrick, 1980; Fengel and Wegener, 1984; Markham and Porter, 1973; Updegraff and Grant, 1975; Junna, 1983).

Tannins are clearly the most dominant constituents of bark water soluble COD. This is not surprising due to the high tannin contents of whole bark (soluble + insoluble matter) previously reported in the literature, Table 2. Tannins are phenolic compounds that display sorptive reactions with proteins (Haslam, 1966; Loomis and Battaile, 1966; White, 1957) through hydrogen bonding. Two general classes of tannins are recognized, the hydrolyzable tannins and condensed tannins, Figure 3. The hydrolyzable tannins are not very common in bark. They are polymers of gallic acid linked together by easily hydrolyzed ester bonds. They are also easily hydrolyzed and degraded during anaerobic digestion (Field and Lettinga, 1987). The condensed tannins are very common in bark. They are polymers of flavonoid compounds linked together by difficult to hydrolyze C-C bonds. The predominant types of condensed tannins isolated from bark are the procyanidins (Karchesy and Hemingway, 1980; Hemingway et al., 1982) which are polymers of catechin and its isomer, epicatechin. Other flavonoids, including leucocyanidins, dihydroquercetin, gallocatechin and epigallocatechin, have been identified in bark extracts (Hegert, 1960) and are undoubtedly involved in the polymeric condensed tannins of bark. Piceatannol (tetrahydroxy stilbene) is reported to be an important monomeric unit of bark tannins isolated from spruce (Hegert, 1960; Cunningham et al., 1963) and white pine (Hegert, 1960). Effective tannins, with regard to the tanning of hides, are low molecular weight polymers (oligomers) ranging from 500 to 3000 g $mole^{-1}$ in size (White, 1957). The majority of bark tannins correspond to this size class (Karchesy and Hemingway, 1980; Hemingway et al., 1981, Hegert et al., 1965). To a lesser extent monomeric tannins as well as high molecular weight tannins (phlobaphenes) are present in the total bark tannin fraction. Not all of the water soluble monomeric phenols present in bark extracts are tannic. Some of the monomers have structures characteristic of lignin, for example guaiacol, vanillin and ferulic acid (Hegert, 1960).

Resin is a mixture of diverse compounds that together make up the oily (fatty) constituents of wood and bark. An appropriate definition for resin is the apolar solvent extractable fraction of wood or bark. Resin is poorly soluble by aqueous extraction; however under alkaline conditions, resin can be dissolved in water. The major constituents of coniferous wood resin are resin acids, long chain fatty acids (LCFA), fat (LCFA esters of glycerol), apolar phenols, lignans and volatile terpenes (Rudloff and Sato, 1963; Sato and Rudloff, 1964; Fengel and Wegener, 1984). Typical resin compounds are illustrated in Figure 4. The resin acids account for about 25 to 40% of coniferous wood resin but are distinctly less important in bark resin (Fengel and Wegener, 1984). Birch wood resin contains no resin acids and is dominated by neutral fats and triterpenol compounds. Betulinol derivatives are typical examples of birch triterpenols (Fengel and Wegener, 1984; Ekman and Sjoholm, 1983).

1.2. Toxicity of Bark Soluble Matter

Bark soluble matter is highly toxic. One review article (Virkola and Honkanen, 1985) cited finish literature which indicated that the fish LC50 96h toxicity concentration of wet debarking effluent corresponds to an effluent dilution of 50 to 100 times. Resin acids have been identified as important fish toxins in forest industry wastewater (Leach and Thakore, 1976; Rogers, 1973). The resin acid concentration reported for a finish wet debarking effluent (Junna et al., 1983) was much lower than the reported resin acid LC50 96h

Table 1. Some COD Concentrations Previously Reported from Bark Wastewater.

Wastewater	COD mg L^{-1}		Reference[&]	Wastewater	COD mg L^{-1}		Reference
Wet Debarking	4111	Cr[+]	W84	Half-wet Debarking	2000	Cr	W84
	1333	Cr	W84		889	Mn	W84
	1034	Mn	W84		500	Mn	V+H84
	940	Mn	V+H84	Bark Press	20,000 to 60,000	Cr	ACpc

[+] Cr = COD determined with the chromate method; Mn = COD determined with the permanganate method
[&] W84 = Wirkkala, 1984; V+H84 = Virkola and Honkanen, 1984; ACpc = Ahlstrom Corp., Finland

Table 2. Literature Reported Extractable Tannin Yields of Bark

Species Bark	Tannin Conc. % bark dry wt.	Reference[&]	Species Bark	Tannin Conc. % bark dry wt.	Reference[&]
Pine (Pinus)			**Douglas Fir (Pseudotsuga)**		
P. sylvestris	16	H62	P. menziesii	5 to 25	H62
P. taeda	10	K+H80	P. menziesii	6 to 9	W46
P. ponderosa	5 to 11	H62	**Hemlock (Tsuga)**		
P. ponderosa	6 to 9	W46	T. canadensis	10 to 11	H62
P. radiata	17 to 18	H62	T. heterophylla	15 to 16	H62
P. radiata	17 to 21	M+P73	T. heterophylla	9 to 18	H65
P. radiata	16	U+G75	**Birch (Betula)**		
Spruce (Picea)			B. alba	10 to 15	H62
P. jezoensis	6	K84	**Oak (Quercus)**		
P. abies	5 to 18	H62	Q. robur	12 to 16	H62
P. stichensis	11 to 37	H62	Q. robur	8	S88
Larch (Larix)			**Chestnut (Castanea)**		
L. gmelinii	12	K84	C. sativa	8 to 14	H62
L. decidua	5 to 20	H62			
L. occidentalis	6 to 9	W46			

[&] H62 = Hathway, 1962; K+H80 = Karchesy and Hemmingway, 1980; W46 = Wise, 1946; M+P73 = Markham and Porter, 1973; U+G75 = Updegraff and Grant, 1975; K84 = Kuwahara et al., 1984; H65 = Hegert et al., 1965; S88 = Scalbert et al., 1988

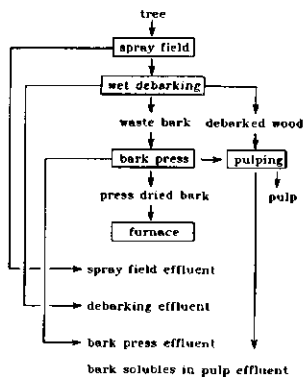


Figure 1. Possible sources of bark soluble matter in forest industry wastewater.

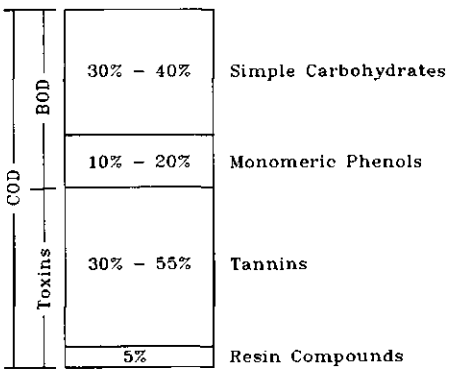


Figure 2. Model composition of bark water soluble COD based on the available literature data cited in the text.

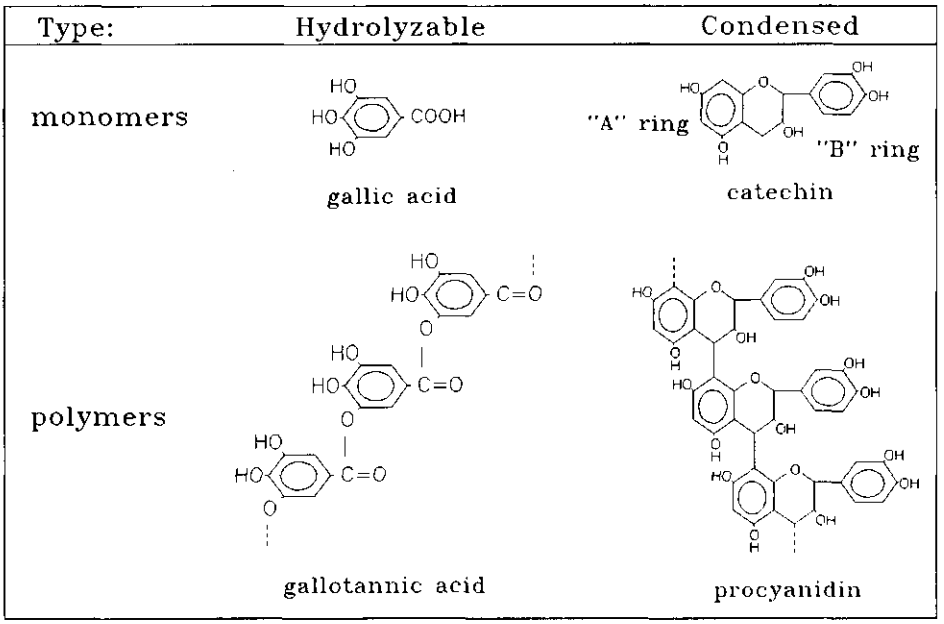


Figure 3. The structure of tannins.

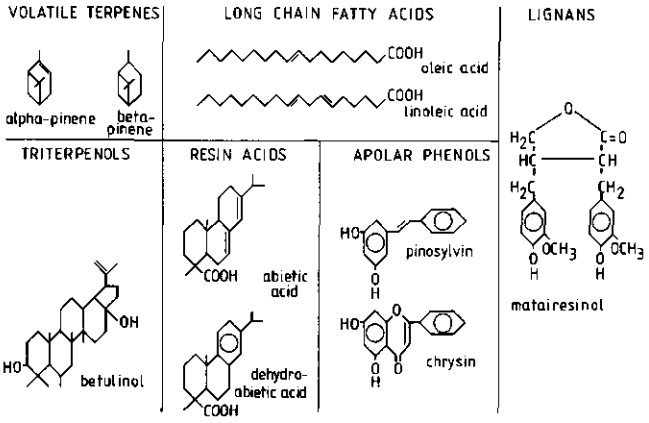


Figure 4. The structure of selected tree resin compounds.

Table 3. The Inhibition of Methanogenic Activity by Defined Compounds which are Related to Extractable Bark Compounds.

Compound	Concentration mg L^{-1}	Substrate[+]	Inhibition[*] %	Reference
Part A. Compounds related to resin and lignin				
LCFA				
lauric acid	525	C_2	50	Chou et al., 1978
capric acid	1027	C_2	50	Koster and Cramer, 1987
lauric acid	869	C_2	50	Koster and Cramer, 1987
myristic acid	1104	C_2	50	Koster and Cramer, 1987
oleic acid	1235	C_2	50	Koster and Cramer, 1987
mixture LCFA	250**	C_4	50[R]	Hanaki et al., 1981
mixture LCFA	250**	H_2	50	Hanaki et al., 1981
Resin Acids				
abietic:oleic acids[1]	1178	C_2	99[NR]	Andersson and Welander, 1985
Volatile Terpenes				
p cymene	500**	C_2	50[R]	Benjamin et al., 1984
limonene	250**	C_2	50[R]	Benjamin et al., 1984
Lignin Related Monomers				
guaiacol	2200**	C_2	50[R]	Benjamin et al., 1984
eugenol	250**	C_2	50[R]	Benjamin et al., 1984
Miscellaneous				
ethylbenzene	340	C_2	50	Chou et al., 1978
p cresol	800**	C_2, C_3	50	Fedorak and Hrudey, 1984
phenol	1500**	C_2, C_3, C_4	50	Field and Lettinga, 1989
phenol	1800**	C_2, C_3	50	Fedorak and Hrudey, 1984
phenol	2444	C_2	50	Chou et al., 1978
Part B. Compounds related to monomeric and polymeric tannins				
Polar Monomeric Phenols				
catechol	1755**	C_2, C_3, C_4	50[R]	Field and Lettinga, 1989
catechol	2640	C_2	50	Chou et al., 1978
pyrogallol	3000	C_2, C_3, C_4	50[R]	Field and Lettinga, 1987
gallic acid	3200	C_2, C_3, C_4	50[R]	Field and Lettinga, 1987
resorcinol	3190	C_2	50	Chou et al., 1978
L dopa	1000	C_2, C_3, C_4	50[R]	Field et al., 1987
Hydrolyzable Tannins				
gallotannic acid	700	C_2, C_3, C_4	50	Field and Lettinga, 1987

* percent inhibition compared to substrate control

** approx. 50% inhibition conc. estimated from available reported data

+ the assay substrate: C_2 = acetate; C_3 = propionate; C_4 = butyrate; H_2 = hydrogen

R recovery of at least some of the inhibited activity before completion of a long term assay

NR no recovery of the inhibited activity during long term assays

1 the abietic:oleic acid mixture was 52:48% on a dry weight basis

concentration if this bark wastewater were diluted to the wastewater LC50 96h concentration (50 to 100 times). This indicates that resin acids are not likely the principal fish toxins of debarking effluents. Clearly, another group of toxins are important in bark.

Bark soluble matter is also highly toxic to methane bacteria. Severe inhibitions of methane bacteria by bark soluble matter during anaerobic digestion are evident in several studies (Latola, 1985; Rekunen, 1986; Wirkkala, 1984; Kuwahara et al. 1984). In these studies, removal of bark soluble matter from the whole wastewater (or waste) influent was associated with large improvements of methanogenic activity in separate start up attempts with new sludge. While resin compounds are highly toxic to methane bacteria, Table 3A, their concentration in bark wastewater is expected to be lower than the 50% toxicity values reported. Resin compounds are also not unique to bark, therefore other compounds unique to the bark are expected to account for improved methanogenic anaerobic digestion of total forest industry wastewater when the bark substream is excluded.

The compounds which are unique to bark are tannins. They are the suspect toxicants of bark soluble matter for two reasons. Firstly, they are highly concentrated in bark. Secondly, they are known to be toxic to enzymes and microorganisms (Gupta and Haslam, 1980; Haslam, 1974; White, 1957; Ladd and Butler, 1975; Loomis and Battaile, 1966; Singleton and Esau, 1969; Strumeyer and Malin, 1969; Daiber, 1975; Tamir and Alumot, 1969; Verspuy and Pilinik, 1970). Methanogenic toxicity from tannins in tannery wastewater is also evident from one previous study (Arora et al., 1975). We have confirmed the methanogenic toxicity of a hydrolyzable tannin, gallotannic acid (Field and Lettinga, 1987). This oligomeric tannin was distinctly more toxic than polar phenolic monomers including tannin monomeric derivatives, Table 3B.

The purpose of this study was to determine the fermentable COD substrate of bark soluble matter and determine if the bark tannins are responsible for the previously indicated methanogenic toxicity.

2. MATERIALS AND METHODS

2.1. Biological Assays

All assays contained essential inorganic macro and micro nutrients as outlined previously (Field et al., 1987). Batch fed assays were conducted in 0.5 or 1.0 L serum flasks. The assay temperature was 30±2°C. For experiments described in this study, the serum flasks were not shaken during the assay period.

Anaerobic bark extract acidification experiments were conducted with approximately 5 g VSS L^{-1} granular sludge which was originally cultivated in a UASB for recycle paper wastewater (Roermond). Acidification assays were supplied with approximately 2 g COD L^{-1} bark extract and 2 g L^{-1} $NaHCO_3$ to buffer eventual VFA accumulations. The percent acidification of the COD was calculated by the summation of cumulative CH_4 COD and media VFA COD for a given assay period. The acidification results are corrected for the acidification of sludge controls. In certain examples, the acidification during the toxicity assays is reported; in which case, the results are corrected for VFA substrate fed sludge controls. Additional feedings during acidification assays are initiated by removing the anaerobically digested medium from the sludge and replacing it with a freshly prepared bark extract containing medium.

Anaerobic bark extract methanogenic toxicity assays were supplied with approximately 4 g COD L^{-1} neutralized with NaOH 100:100:100 g kg^{-1} acetate (C_2), propionate (C_3),and butyrate (C_4) VFA stock solution. This stock solution COD is 24.3:34.4:41.3% C_2, C_3 and C_4 COD; respectively. The VFA stock solution served as the substrate for the toxicity assays which were also fed variable concentrations of the bark extract. All treatments and substrate controls were supplied with 2 g L^{-1} $NaHCO_3$. From 1.0 to 1.5 g VSS L^{-1} granular sludge was used, that was either obtained from a UASB treating potato derived wastewater (Aviko) or vinasse (Nedalco).

Following the first feeding, medium was removed and the second feeding is initiated with new medium containing only VFA substrate. This is done in order to evaluate the residual activity of the sludge following a defined exposure (approximately 2 weeks) to the bark extract. The first feeding activity is less reliable than the second feeding activity because the bark extract is not only a toxin but also a substrate and because the toxins do not express their full toxicity prior to the time period used to calculate the methanogenic activity. The activity was evaluated in the time period when 60 to 80% of the supplied VFA substrate was most rapidly used by the VFA fed control. The activity of the control is expressed as the amount of CH_4, as COD, produced by 1 g VSS sludge per day (mg COD g^{-1} VSS d^{-1}). The activity of the bark extract treatments (extract and VFA) are reported as a percent of the control (VFA) methanogenic activity.

2.2. Extract Preparation

Warm water extracts of bark were prepared from 18 g of air dried (70°C for 2 days) cross milled bark per L of 60°C tap water containing 250 mg L^{-1} ascorbic acid. The bark and water were placed in a N_2 filled gas tight container and shaken at 30°C for 3 hours, paper filtered and stored under N_2 at 4°C. Extracts were not stored longer than 4 days.

Bark was collected from freshly cut trees at a local forest or from delivered logs at Parenco paper factory (Renkum, the Netherlands). The common name, latin name (and average air dried moisture content) of species used in this study were as follows: scot's pine, Pinus sylvestris (2.63%); norway spruce, Picea abies (10.30%); fir, Abies alba (2.60%); douglas fir, Pseudotsuga menziessi (2.58%); european white birch, Betula verrucosa (5.82%); larch, Larix decidua (2.86%). and european beech, Fagus sylvatica (2.45%). The whole bark was always sampled at the part of the trees that ranged from 10 to 20 cm in diameter. The air dried bark was stored from 0.5 to 6.0 months prior to use for preparing extracts.

2.3. Methods

UV absorbance was measured in a 1 cm quartz cuvette by diluting extracts (to < 0.8 absorbance units) in pH 6, 0.2 M KH_2PO_4 buffer as described earlier (Field et al., 1987). The UV absorbance was measured at 215 nm (UV_{215}) and is reported as 1x absorbance units of the sample. The UV_{215} is indicative of the phenol compound concentration of bark extracts because their aromatic structure absorbs UV light.

COD (the micro method with dichromate) and VSS were determined according to standard methods (American Public Health Assoc., New York).

VFA and the same gas chromatographic procedure for the determination of anaerobic intermediates of phenolic compound degradation (carboxycyclohexane, p cresol, phenol) is previously described (Field et al., 1987).

The reversed phase HPLC chromatography method for bark tannins was adapted from previously reported methods designed for condensed tannins in apple juice and wine (Lea, 1982; Lea, 1980; Wilson, 1981). The column used in this study was a 200 mm x 3 mm ID C_{18} Chromosphere. A gradient was used with 4% (v/v) acetate in water (A) or in methanol (B). The A:B ratio was 98:2 at 0 minutes, 75:25 at 23 minutes and 2:98 at 33 minutes to 45 minutes. The sample size was 0.02 mL. The total solvent flow was 0.6 mL $minute^{-1}$. UV absorbance was detected at 280 nm.

The development of the polyvinylpyrrolidone (PVP) method for tannin determination of bark extracts and wastewater is described in the results. The standard method developed is applicable to extracts and wastewater that contain from 2 to 7 g COD L^{-1} bark soluble matter. The standard tannin determination, called the PVP method, was with 7 ml extract added to 0.1 g cross-linked PVP (Janssen Chimica, Beers, Belgium), capped in a N_2 flushed test tube, shaken intensely for 1 hour in a 30°C bath. After shaking, samples were either glass fiber filtered (extracts) or membrane filtered (wastewater). Both COD and UV_{215} were measured from the filtered PVP untreated and treated samples. The tannin COD and tannin UV_{215} were both determined by difference of PVP untreated and treated samples corrected for COD and UV_{215} of a PVP blank. Ideally, the quantity of tannin COD should be 5 to 20%

of the quantity of PVP. If samples were too concentrated than they were diluted or if samples were too dilute than less PVP was used in order to arrange this tannin COD to PVP percentage.

The development of a standard active carbon (AC) test, for determining bark extract AC adsorbable matter, is described in the results. The standard method developed called, the exhaustive AC treatment, was 7 ml extract added to 0.2 g granular AC (Darco, 20-40 mesh, Aldrich Chemie, West Germany) capped in a N_2 flushed test tube and shaken intensely for 2 hours. Another method called, the half exhaustive AC treatment, was the same except the shaking was mild (the AC granules were not suspended by the shaking). The calculation of AC adsorbable matter was the same as described for PVP.

3. RESULTS AND DISCUSSION

3.1. Polyvinylpyrrolidone Determination of Tannins

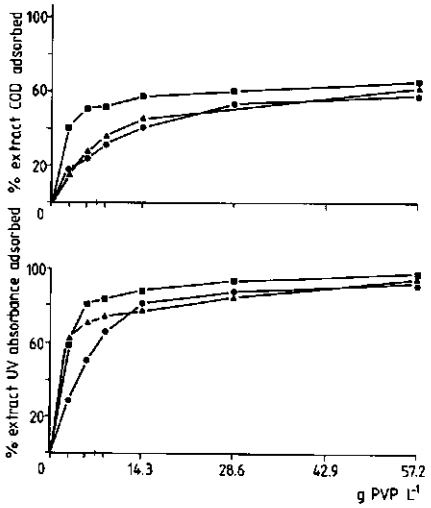
One approach for determining tannins is to measure those compounds that have hydrogen bonding affinities with proteins and polyamides (Gupta and Haslam, 1980; Bate-Smith, 1973; Hathway, 1962; Wise, 1946). In many earlier studies the removal of enzyme inhibitors from plant extracts has been achieved by adding protein or polyamide, which serves as an indicator for tannin toxicity (Loomis and Battaile, 1966; Ladd and Butler, 1975; Verspuy and Pilnik, 1970; Strumeyer and Malin, 1969; Tamir and Alumot, 1969). We have chosen for the use of an insoluble cross-linked PVP as a specific adsorbent of tannins in bark extracts. The tannin adsorbing properties of PVP are considered better than most other polyamides (Loomis and Battaile, 1966). In order to develop the guidelines for the tannin determination, we have examined the adsorption of bark extract COD and UV_{215} by PVP under variable conditions; such as PVP concentration (Figure 5) and contact time (Figure 6). The figures illustrate that the ultimate COD and UV_{215} adsorption from pine, spruce and birch bark extracts by PVP ranged from 35 to 65% (COD) and 82 to 97% (UV). This indicates that tannins account for about half of all the bark extract compounds and a large majority of the UV light absorbing aromatic compounds.

The PVP determination developed from these results utilizes the contact time and PVP concentration which correspond to approximately 90% of the ultimate adsorption. This is one hour contact of the extract with 14.3 g PVP L^{-1}, as described in the methods. The pH has no effect on the tannin determination as long as the pH is between 3 and 8 (Table 4). The COD and UV_{215} of a pure tannic monomer (catechin) solution (Figure 6), and pure tannin extracts (Table 5) were almost completely adsorbed by PVP during the tannin determination. Indicating that tannins are effectively detected by PVP.

The PVP tannin determination was applied to the measurement of tannins of numerous bark extracts, reported in Tables 4 and 5. Repeated measurements of similarly prepared extracts indicate that the results are very reproducible, including the relationship between measured tannin UV and tannin COD. The percentage of bark extract COD and UV_{215} that was detected as tannins as well as the tannin UV:COD ratio varied between species.

Tannins were also determined from several real wastewaters which contain bark soluble matter (Table 6). The proportion of membrane filtered COD and UV_{215} of the wastewater, which was tannin, was lower than those of laboratory made aqueous bark extracts. However, the tannin fraction was still quite large ranging from 13 to 30% of the soluble COD. Considered as a fraction of the soluble wastewater aromatic compounds (UV data), the tannins account for nearly half to more than half of these compounds in both bark and bark + wood derived wastewaters.

A small percentage of the bark soluble matter is expected to be composed of resin compounds (Figure 2). The toxicity of some resin compounds to methane bacteria has been reported (Table 3), which led us to evaluate the PVP adsorption of resin compounds, in order to confirm that only the toxicity caused by tannins is removed by PVP adsorption treatments. The percentage COD and UV_{215} adsorption of an abietic acid standard solution and a crude pine resin extract by PVP was very marginal (Table 7), indicating that resin



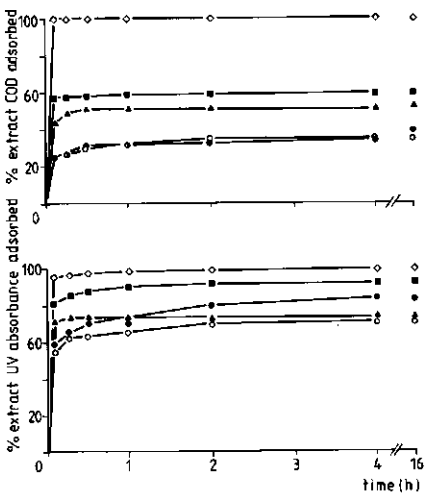


Figure 5 (left). Bark extract COD and UV (215nm) adsorption after 1 hour contact time with variable PVP concentrations. Pine bark extract (lot 1 bark) was diluted 1.6x (●). Spruce bark extract (lot 2 bark) was diluted 1.6x (■). Birch bark extract was not diluted (▲). The mg COD L^{-1} (and UV 215nm, 1cm,1x) of the extracts, after dilution, was 2893 (86.4), 3129 (89.0), and 2657 (69.4); respectively, for pine, spruce and birch.

Figure 6 (right). Bark extract COD and UV (215nm) adsorption by 14.3 g L^{-1} PVP with variable contact times. Pine bark extract (lot 2 bark) was either diluted 1.6x (●) or was undiluted (○). Spruce bark extract (lot 2 bark) was diluted 1.6x (■). Birch bark extract was not diluted (▲). The adsorption of the bark extracts is compared with the adsorption of a condensed tannin monomer, catechin (◇). The mg COD L^{-1} (and UV 215nm, 1cm,1x) of the extracts, after dilution, was 2928 (85.4), 4751 (146.5), 3115 (100.3), 3198 (64.1), and 1276 (70.8); respectively, for pine (1.6x), pine (1x), spruce, birch and the catechin solution.

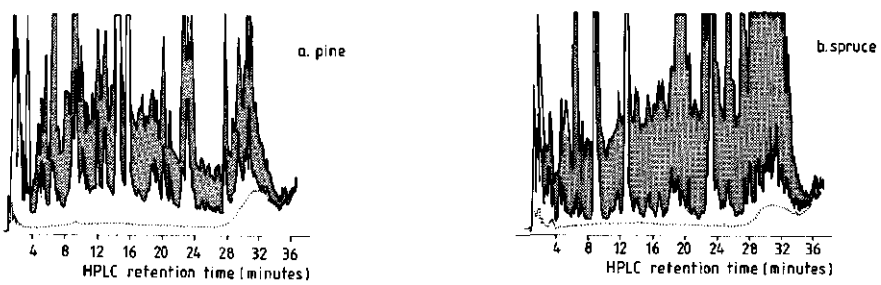


Figure 7. The reversed phase HPLC chromatograms of pine (a.) and spruce (b.) bark extracts. The top solid line is the untreated extract. The bottom solid line is the PVP treated extract. The shaded area between these lines is the tannin peak area. The dotted line is the HPLC solvent background.

Table 4. The Percentage Bark Extract COD and UV (215nm) Adsorbed by the PVP Determination at pH Values Ranging from 3 to 8.

pH*	COD		UV		UV:COD
	total mg L^{-1}	tannin %	total 1cm,1x	tannin %	tannin 1cm,1x mg^{-1}
Pine (lot 1)[1]					
3	3066	31.5	95.5	73.1	0.0721
4	3066	30.9	93.3	74.1	0.0729
5	3050	28.5	94.3	72.1	0.0782
6	3066	29.9	92.5	72.2	0.0728
7	3050	28.5	91.1	71.7	0.0752
8[D]	2756	33.2	67.9	76.3	0.0566
average	3060	29.9	93.3	72.6	0.0742
Spruce (lot 2)[1]					
3	3097	58.3	114.2	91.5	0.0579
4	3113	56.0	103.1	88.9	0.0526
5	3229	59.3	102.9	89.3	0.0465
6	3066	55.8	99.7	89.9	0.0524
7	3082	57.0	102.5	86.5	0.0504
8	3019	55.8	97.1	88.6	0.0510
average	3118	57.0	103.1	89.1	0.0518
Birch[2]					
3	3978	46.3	86.0	77.6	0.0362
4	3726	42.6	83.0	76.5	0.0400
5	3821	44.1	85.0	78.7	0.0397
6	3805	43.8	88.8	77.1	0.0411
7	3758	43.1	84.2	76.4	0.0397
8	3774	43.5	85.8	76.6	0.0400
average	3810	43.9	85.4	77.2	0.0395

* pH after adjustment with HCl or NaOH extract diluted 1.6x in 0.2 M KH_2PO_4

[1] undiluted bark extract prepared with 18 g air dried bark L^{-1}

[2] undiluted bark extract prepared with 36 g air dried bark L^{-1}

[D] pine bark pH 8 data discarded from the calculation of the average

compounds are not adsorbed by PVP. The UV:COD ratio of the unadsorbed resin compounds is distinctly lower than those reported for tannins.

The HPLC chromatograms obtained from PVP untreated and treated bark extracts, presented in Figure 7 indicate that bark tannins are a complex mixture of numerous tannic compounds. The tannins with retention times between 8 and 28 minutes correspond to monomeric (300 g $mole^{-1}$) and oligomeric (500-3000 g $mole^{-1}$) procyanidins (condensed tannins) previously identified with a similar HPLC method in apple juices (Lea, 1982; Lea, 1980; Wilson, 1981). The tannins of greater than 28 minutes retention time correspond to high molecular weight tannins. The majority of the bark tannins are oligomers. A comparison of the chromatograms from the two species analyzed, pine and spruce, indicates that the different species do not have the same tannin composition.

3.2. Active Carbon Adsorbable Matter of Bark Extracts

The adsorption of bark extract COD and UV_{215} by granular AC is presented in Table 8 for variable conditions. The ultimate elimination of COD and UV_{215} with AC was 96% (COD)

Table 5. The Total COD and UV (215nm) of Glass Fiber Filtered Aqueous Extracts of Bark or Purified Hydrolyzable Tannins.

Extract[1]	n*	COD		UV		UV:COD	Tannin Yield+
		total[SD] mg L^{-1}	tannin[SD] %	total[SD] 1cm,1x	tannin[SD] %	tannin[SD] 1cm,1x mg^{-1}	percent of bark dry wt.
BARK							
spruce (lot 1)	4	5793 (228)	51.1 (3.2)	156 (3)	88.4 (0.9)	0.0466 (0.0016)	10.8
spruce (lot 2)	3	5013 (91)	57.9 (0.7)	156 (11)	89.0 (0.9)	0.0474 (0.0030)	10.7
pine (lot 1)	3	4166 (408)	42.0 (2.3)	128 (9)	73.7 (8.7)	0.0538 (0.0054)	5.9
pine (lot 2)	3	4772 (126)	31.3 (0.2)	140 (5)	66.6 (3.1)	0.0625 (0.0033)	5.7
birch	7	3519 (450)	48.0 (4.0)	89 (10)	81.3 (2.4)	0.0434 (0.0039)	5.9
larch	1	7193	49.3	309	77.9	0.0685	11.8
douglas fir	1	7226	52.4	279	90.9	0.0671	12.6
fir	1	7611	58.5	316	88.6	0.0630	14.9
beech	1	3324	29.9	77	36.6	0.0283	3.3
bark average:			46.7 (10.5)		77.0 (17.2)	0.0534 (0.0132)	9.1
HYDROLYZABLE TANNIN**							
aleppo	1	3597	92.8	263	94.4	0.0792	
tarra	1	3735	83.9	242	97.0	0.0821	

1 *all bark extracts were prepared with 18 g air dried bark* L^{-1}

* n = number of samples analyzed

** the hydrolyzable tannin extracts were obtained in dry powder form from OmniChem (Wetteren, Belgium), aleppo tannins were isolated from the galls of Quercus infectoria
tarra tannins were isolated from the pods of Caesalpinia spinosa

\+ the tannin yield from the bark (dry weight tannins extracted/dry weight bark) was estimated assuming the COD:dry weight ratio of bark tannins is equal to that of the condensed tannin monomer, catechin (1.7).

SD the values reported in the parenthesis are the standard deviation units.

and 100% (UV) of the extract values with an excess of AC. This indicates high removal of all bark compounds and complete removal of the complex UV light absorbing organic matter (including tannins). The AC should be regarded as a "universal" adsorbent. Based on these results the standard treatment as described in the methods was chosen based on the conditions that correspond to 90% of the ultimate adsorption. The AC adsorption of bark soluble matter was not affected by pH between 3 and 8. It was influenced by the shaking intensity employed. Only about a third of the extract matter was adsorbed with mild shaking that only swirled the extract above a stationary bed of AC granules instead of throwing the AC granules into suspension as was the case with intense shaking. The resin extracts, which were not adsorbed by PVP were adsorbed by AC as will be discussed later.

3.3. The Anaerobic Biodegradability of Bark Extracts

The anaerobic biodegradability of bark extracts is reported in Table 9. The conversion of bark extract COD to VFA and CH_4 (called acidification) reached very high percentages (approximately 70%) after long term (7 to 8 weeks) batch anaerobic digestions of pine and

Table 6. The Unfiltered and Membrane Filtered Total and Tannin COD and UV (215nm) of Clarified Wastewaters Containing Bark Soluble Matter.

Wastewater*	site**	COD			UV			UV:COD
		UFtotal[1]	MFtotal[2]	tannin[2]	UFtotal[1]	MFtotal[2]	tannin[2]	tannin
		----- mg L^{-1} -----		%	----- mg L^{-1} -----		%	1cm,1x mg^{-1}
Debarking Wastewaters								
PW	1	1464	1047	29.8	ND+	16	55.6	0.029
BHW-R	2	9763	5437	13.0	114	66	54.0	0.050
BPr	2	25314	17847	29.3	379	225	70.8	0.031
PPr	1	9662	5865	20.4	113	61	42.4	0.022
P/SPr	3	24900	20700	19.8	ND	168	65.5	0.027
Masonite Wastewaters								
SMPlp	4	15784	13084	5.9	ND	106	40.8	0.056
SMPr	4	19427	19427	11.0	ND	176	46.4	0.038

* wastewaters: PW = pine wet debarking; BHW = birch half wet debarking with partial wastewater recirculation; BPr = birch bark pressing effluent; PPr = pine bark pressing effluent; P/SPr = 70% pine and 30% spruce bark pressing; SMPlp = spruce wood and bark pulping; SMPr spruce wood and bark pressing

** one of four forest industries visited in central and northern europe

1 UF = unfiltered; 2 MF = membrane filtered (tannin COD and UV expressed as a ratio of the MFtotal)

\+ ND = no data

Table 7. The Poor Adsorption of Resin Compound COD and UV (215nm) by PVP

Resin Solution	pH*	COD		UV		UV:COD	
		MFtotal[1] mg L^{-1}	adsorbed %	MFtotal[1] mg L^{-1}	adsorbed %	unadsorbed	adsorbed
						----- 1cm,1x mg^{-1} ----	
crude fresh pine resin	5.5	604	0.0	2.83	13.1	0.0041	NA+
abietic acid	5.5	642	0.0	2.43	0.7	0.0038	NA+
crude fresh pine resin	8.0	1333	5.3	10.65	19.1	0.0068	0.0287
abietic acid	8.0	1498	1.4	11.00	13.3	0.0065	0.0695

* solutions were prepared from 2 g L^{-1} indicated resin compound at pH 11 (with NaOH), the pH was lowered (with HCl) to the indicated pH value

1 solutions were membrane filtered

\+ NA = not applicable because no COD was adsorbed

Table 8. The Adsorption of Filtered Bark Extract COD and UV (215nm) by Granular Active Carbon (AC) under Variable Conditions.

Sample	Adsorption Conditions		Pine*		Spruce*	
	constant	variable	COD	UV	COD	UV
			-- % adsorbed --		-- % adsorbed --	
A.	28.6 g AC L^{-1}	Time (h)				
	pH ≈ 5	0.1	50.9	58.9	36.6	36.5
	intense shaking	0.3	66.9	77.9	47.2	46.0
		1.0	84.2	96.6	77.1	83.6
		2.0	91.7	100.0	86.0	95.7
		4.0	91.7	100.0	90.4	99.5
		16.0	90.1	97.5	94.2	99.1
A.	2.0 h	AC (g L^{-1})				
	ph ≈ 5	4.3	32.7	47.4	23.5	27.4
	intense shaking	8.6	51.9	53.6	46.2	57.9
		14.3	69.6	88.8	62.7	68.1
		28.6	91.7	100.0	86.0	94.6
		42.9	93.4	100.0	94.7	99.5
		57.1	95.1	100.0	95.8	100.0
B.	28.6 g AC L^{-1}	pH				
	2.0 h	3	85.7	99.0	84.5	90.0
	intense shaking	4	88.2	100.0	84.2	92.2
		5	88.5	100.0	85.1	94.7
		6	87.5	96.9	83.8	92.4
		7	87.5	100.0	82.0	90.8
		8	84.6	98.6	79.1	86.6
C.	28.6 g AC L^{-1}	Shaking**				
	2.0 h	mild	36.3	41.1	32.2	28.4
	pH ≈ 5	intense	89.6	100.0	87.5	93.5

* The 1.6x diluted extracts used had the following COD as mg L^{-1} (and UV 215nm as 1cm,1x): 2569 (64.5), 2351 (59.5), and 2531 (56.3) with pine; and 4006 (111.4), 3928 (111.6), and 4182 (107.5) with spruce bark from samples A, B, and C; respectively.

** intense shaking = exhaustive AC treatment; mild shaking = half exhaustive AC treatment

birch bark extracts. The long term acidification of these extract was sustained during repeated feedings. The acidification of spruce bark extract was distinctly lower (44%). These results indicate that large fractions of the bark extracts are potentially convertible to CH_4 in anaerobic environments. The UV light absorbing matter of the extracts was also eliminated by long term anaerobic digestions. The ultimate percentage eliminated reached 91, 62 and 74% of pine, spruce and birch bark extract UV_{215}; respectively. These results indicate that many bark phenolic compounds, responsible for the UV light absorbance, were biologically modified by anaerobic digestion.

The COD which could be expected to be acidified in anaerobic wastewater treatment reactors, generally corresponds to the short term (1 week) acidification. The short term acidification of bark extracts ranged from 40 to 50% of the total COD in first, second and third feedings. The short term acidifiable COD accounted for about 70% of the long term acidifiable COD. The short term acidification of bark extract COD was less when VFA stock solutions were fed to the medium together with the extracts (Table 10). The presence of the added VFA prevented the acidification of about 13% (spruce) to 27% (pine) of the COD which was acidified in 1 to 2 weeks when no VFA was added. The fact, that VFA is known to hinder the degradation of phenolic compounds (Field et al., 1987), serves as an indication

Table 9. The acidification (A), Methanogenesis (M), Elimination (ECOD) of Bark Extract COD and Elimination of Bark Extract UV, 215 nm, (EUV) by Anaerobic Digestion. Assays Were Conducted with 5 g VSS L^{-1} Granular Sludge.

Days[+]	Pine				Spruce				Birch			
	M[*]	A[*]	EUV[*]	ECOD[*]	M	A	EUV	ECOD	M	A	EUV	ECOD
	%											
First Feeding												
4	17.2	32.7	54.3		16.5	34.0	34.0		15.4	39.1	45.0	
8	30.4	40.7	66.3		18.1	49.1	49.1		25.5	47.9	55.3	
12	49.0	51.2	74.4		21.5	54.7	54.7		34.4	56.0	56.8	
16	56.8	59.4	79.4		23.6	58.4	58.4		39.4	56.0	56.2	
24	63.6	64.3	83.2		ND	ND	ND		ND	ND	ND	
32	65.3	65.3	86.0		34.6	61.0	61.0		51.6	65.2	65.1	
48	69.3	69.3	91.1	87.1[a]	37.2	61.8	61.8	44.2	ND	ND	ND	
57	ND[+]	ND	ND		ND	ND	ND		65.3	77.6	73.5	58.3
Second Feeding												
1	8.6	20.0	18.3				8.1		4.6	16.9	15.1	
3	25.6	50.9	52.2				28.7		9.4	30.5	33.5	
4	29.7	55.5	57.9				32.0		12.2	41.8	40.1	
8	36.4	60.8	70.0				42.6		19.9	56.1	56.7	
12	55.8	74.2	74.0				45.5		17.2	56.5	62.5	
32	ND	ND	87.9	84.6[a]			ND		ND	ND	ND	
41	ND	ND	ND				ND		21.1	71.6	71.6	21.6
Third Feeding												
1	5.3	18.0	21.1						0.0	6.4	8.2	
3	10.8	36.5	49.4						1.6	16.6	18.4	
4	11.4	38.9	53.8						3.2	25.3	26.8	
9	16.8	46.7	63.0						6.9	43.8	43.6	
12	21.8	54.2	68.2						ND	ND	ND	
24	52.1	56.3	79.5						6.9	52.2	62.2	

* M = % conversion extract COD to CH_4; A = % conversion extract COD to VFA+CH_4; EUV = % elimination of extract UV; ECOD = % elimination of extract COD where final COD was measured from the unfiltered settled supernatant of the medium. The initial extract COD as mg L^{-1} (and UV, 215 nm, as 1cm,1x) in the assays was 1885 (58.4), 1992 (69.1), and 2238 (69.9) for pine; 2037 (54.1), and 1914 (52.4) for spruce; and 1804 (46.1), 1675 (47.4), and 1882 (51.6) for birch at the start of the first, second and third feedings; respectively. The values reported are triplicate averages.

[+] days of anaerobic digestion

[a] no residual VFA in assay medium when COD of supernatant was measured.

that some phenolic compounds are included in the acidifiable substrate. On many occasions small amounts (< 1% extract COD) of p cresol, phenol and carboxycyclohexane were identified in the assay media of bark extract digestions. These are reported to be anaerobic intermediates of plant phenolic compounds (Balba and Evans, 1980; Young, 1984; Field et al., 1987). Their maximum concentration after 2 weeks of digestion was higher (1 to 4% extract COD) when VFA was added to the assay together with bark extracts.

The quantity of extract COD acidified was always a little less if the extract was previously treated with PVP to remove the tannins (Table 10). This indicates that at least some of the short term acidifiable COD originates from the tannins, which are possibly

simple monomeric phenolic compounds and sugar glycosides of the tannin fraction. The average bark extract COD is approximately half tannins and half non-tannins (Table 5). The majority, about 80%, of the short term acidifiable substrate originates from the non-tannin half and the remaining (20%) short term substrate originates from the tannin half.

3.4. The Methanogenic Toxicity of Bark Extracts

The effect of bark extract soluble matter on the methanogenic activity of granular methanogenic sludge is shown in Figure 8. The bark extracts were very toxic, the soluble bark COD concentrations corresponding to 50% inhibition of methanogenic activity ranged from 880 to 1929 mg COD L^{-1} and were less than the soluble COD concentrations generally observed in most bark wastewater. Therefore, the high methanogenic toxicity of bark wastewaters is indicated.

3.5. The Methanogenic Toxicity of Bark Tannins

In order to evaluate the role of tannins in the observed toxicity, the effect of both untreated and PVP treated extracts on the methanogenic activity was tested as shown in Figure 9, 10, 11 and Tables 11 and 13. A complete (spruce, douglas fir) or almost complete (pine, larch) removal of the methanogenic toxicity was observed by specifically removing the tannins from the extract with PVP. The toxicity of coniferous bark water soluble matter is primarily caused by tannins. The coniferous bark non-tannin fraction was non-toxic to mildly toxic. In these cases, the resin compounds are either not responsible or are only responsible for mild toxicity, since PVP which removes most of the toxicity does not remove resin compounds (Table 7). The hard wood extracts ranged from the least toxic bark (beech) to the most toxic bark (birch). The PVP treatment of highly toxic birch bark, indicated that the tannins were only responsible for about half of the methanogenic toxicity. Based on these results the 50% (and 80%) inhibitory concentration of pine, spruce and birch bark

Table 10. The Percentage Acidification of Extract COD during Short Term Anaerobic Digestion with (+) or without (-) Supplementation of 4.0 g COD L^{-1} as VFA.

Extract			Acidification**		
Type	VFA added	Assay concentration* mg COD L^{-1}	Days+	Untreated	PVP Treated
				----- % extract COD -----	
Pine	(-)	5000	7	45.2	36.4
Pine	(+)	5430	15	32.8	28.9
Pine	(+)	1885	14	33.2	ND+
Spruce	(-)	1437	14	42.0	29.1
Spruce	(+)	2298	15	36.4	30.1
Larch	(+)	3597	13	35.1	26.6
Douglas Fir	(+)	3613	13	46.4	38.1
Beech	(+)	2327	13	54.3	45.8

* The assays reported in this Table were with 1.4 g VSS L^{-1} granular sludge and 2.0 to 2.5 g $NaHCO_3$ L^{-1} as buffer. The COD values reported are the untreated bark extract COD concentrations in the assay medium.

** Acidification is the conversion of the COD to VFA+CH_4, expressed as percentage of the untreated bark extract COD. The COD removed from the bark extract by PVP treatment is reported in Table 5.

\+ ND = no data

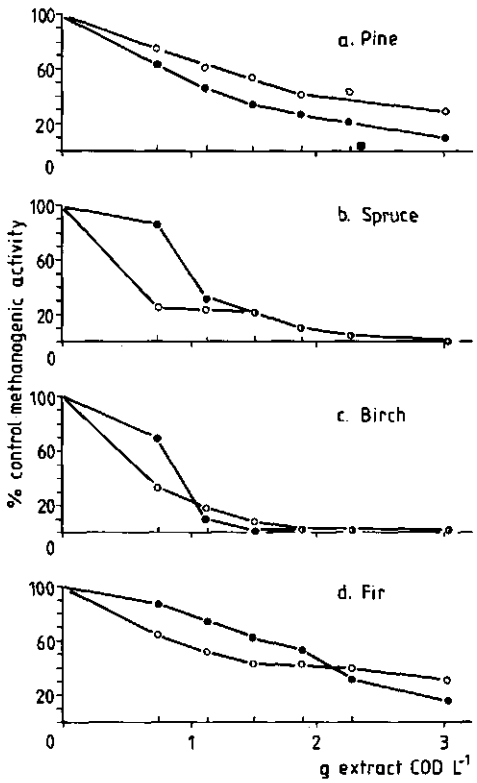


Figure 8. The first VFA feeding activity (○) and the residual sludge activity (●) of the second VFA feeding after 14 days exposure to various concentrations of bark extract COD. During the first feeding, 2 g $NaHCO_3$ L^{-1} was added as buffer (pH 7.2 to 7.8). The toxicity assays utilized 1.4 g VSS L^{-1} granular sludge and 4.1 g COD L^{-1} as VFA substrate per feeding. The tannin COD was 44.8, 55.4 52.1 and 58.5% of the total extract COD; the tannin UV (215nm) was 75.6, 89.9, 83.2 and 88.6% of the total extract UV; and the UV:COD ratio of the tannins was 0.0534, 0.0467, 0.0412 and 0.0630 1cm,1x mg^{-1} for pine, spruce, birch and fir bark extracts; respectively. The control methanogenic activities during the first (and second) VFA feedings were 266 (689), 350 (555), 368 (553), and 383 (797) mg COD g^{-1} VSS d^{-1} for the experiments with pine, spruce, birch, and fir bark extracts; respectively. In one duplicated toxicity assay with pine bark extract, the residual sludge activity of the second VFA feeding (■) is shown following a first feeding when the assay pH dropped to 6.7 (because the buffer was added after 4 days of digestion). The 50% (80%) methanogenic inhibition concentrations of bark extract COD were 1040 (2350), 1000 (1530), 880 (1080), and 1929 (2807) mg L^{-1}. for pine, spruce, birch, and fir; respectively, based on the second feeding activity results.

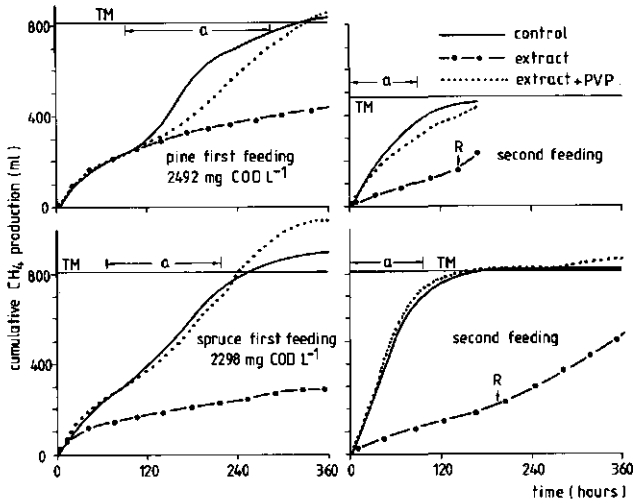


Figure 9. The cumulative CH_4 production of VFA fed assays, containing pine and spruce bark extracts, treated with PVP or untreated. Details of this experiment are described in Table 11. a = time period used to determine activity; TM = theoretical maximum methane production from VFA feed COD; R = recovery, distinct increase of activity during the residual sludge activity assay (second feeding).

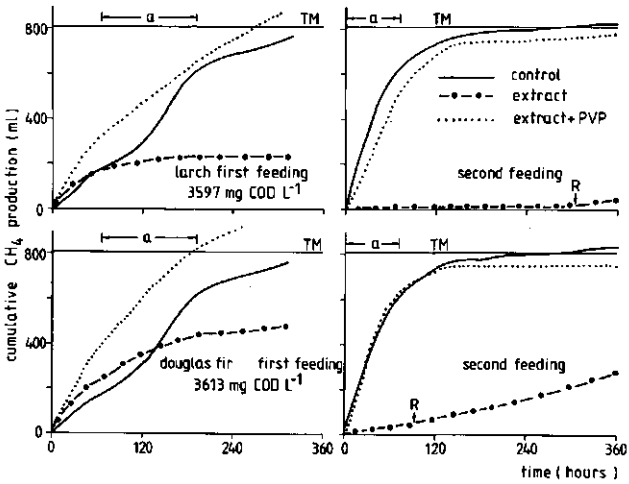


Figure 10. The cumulative CH_4 production of VFA fed assays, containing larch and douglas fir bark extracts, treated with PVP or untreated. Details of this experiment are described in Table 11. The figure legend can be found in the caption of Figure 9.

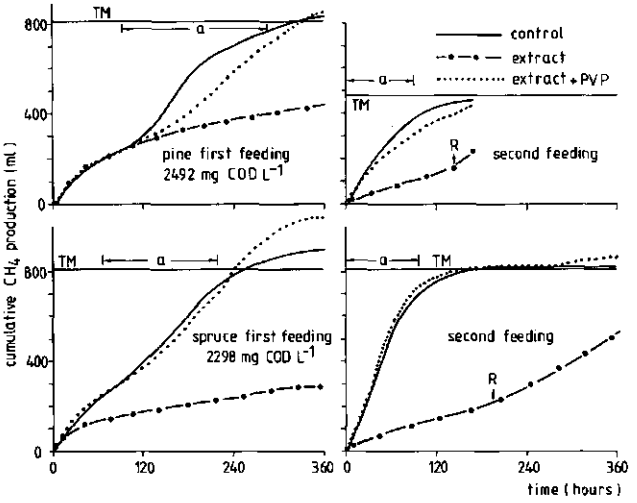


Figure 11. The cumulative CH_4 production of VFA fed assays, containing birch bark extract, treated with PVP or untreated. Details of this experiment are described in Table 11. The figure legend can be found in the caption of Figure 9.

Table 11. Methanogenic Activity of Granular Sludge with Bark Extracts and Tannin Free Bark Extracts (PVP Treated) Present in the First and Second VFA Feeding Media.

Assay*	Bark Extract		Activity with Extract		Activity with PVP Treated Extract	
	Type	Assay conc.** mg L^{-1}	First Feed	Second Feed	First Feed	Second Feed
			% control activity			
A	pine (lot 2)	2942	26.6	28.5	68.4	82.2
B	spruce (lot 1)	2298	17.6	14.8	90.1	102.7
C	larch	3597	13.4	1.9	70.1	83.1
C	douglas fir	3613	42.0	5.4	92.4	102.0
D	birch	1968	4.3	0.9	16.9	30.7
E	birch	1396	4.5	1.2	38.5	51.1
F	birch	1225	5.6	0.7	47.2	53.7
C	beech	2327	84.5	93.1	97.6	99.1

* Assays were conducted with 1.4 g VSS L^{-1} granular sludge fed VFA at 4.1 g COD L^{-1} per feeding (with the exception of assay A, where the second feeding VFA concentration was 2.5 g COD L^{-1}), the first feeding media contained 2.0 to 2.5 g L^{-1} $NaHCO_3$ as buffer, the second feeding was conducted after 13 to 15 days of the first feeding to determine the residual sludge activity after replacing the supernatant with a VFA containing medium. The first (and second) feeding control activities were 301 (330), 294 (665), 333 (801), 267 (504), 195 (562), and 285 (719) mg COD g^{-1} VSS d^{-1}; respectively, for assay A, B, C, D, E, and F.

** The COD concentrations of the untreated bark extracts in the assay medium are reported. The COD removed from the bark extract by PVP treatment is reported in Table 5.

tannins was estimated to be about 410 (920), 510 (780), and 845 (1040) mg COD L^{-1}; respectively (1 mg condensed tannin = 1.7 mg COD). The condensed tannins of bark are about twice as toxic as a standard hydrolyzable tannin studied previously (Field and Lettinga, 1987).

3.6. The Methanogenic Toxicity of Coniferous Resin

Figure 12 illustrates that filtered extracts of alkaline extracted resin compounds are highly toxic to methane bacteria. The 50% inhibitory concentrations were 160 and 320 mg COD L^{-1} for crude pine resin and abietic acid (1 mg resin = 2.8 mg COD). However, when 30 g COD L^{-1} of crude pine resin was extracted with water, only 2% of the COD was solubilized in 2 days because the natural pH was low (6.3). The highest concentration (520 mg COD L^{-1}) of the low pH extracted resin tested for toxicity only caused about a 15% inhibition. The resin material extracted at a low pH (similar to bark extracts) have distinctly lower toxicity than alkaline extracted resin. The toxicity of low pH extracted resin compounds in bark extracts can be expected to be low even though conifer bark may contain high levels of resin (about 3 to 5% of the bark dry weight, Fengel and Wegener, 1984). We did not study hardwood resin. The methanogenic toxicity of low pH aqueous extracts of birch resin should be evaluated since they are unique in composition and because non-tannin compounds were recognized as important toxins in birch bark extracts.

Table 12. The Granular Active Carbon (AC) Adsorption and Calcium (Ca) Precipitation of Resin COD and UV (215nm), and the Influence of these Treatments on the Methanogenic Activity of Sludge Exposed to the Resin.

Resin Extract*	pH	COD+		UV+		Activity**			
		total	adsorbed++	total	adsorbed++	Untreated Resin		Treated Resin	
						First[a]	Second[b]	First	Second
		mg L^{-1}	%	1cm,1x	%	% control activity			
A. AC Treatments of Resin Extracts									
fresh pine resin[F]	7.9	910	75.6	6.1	78.0	8.5	1.4	91.1	83.7
abietic acid[F]	11.3	1002	94.7	8.6	94.1	6.4	5.2	74.7	87.8
B. Ca Treatments of Resin Extracts									
abietic acid[F]	11.6	1128	63.1	8.8	63.1	3.0	7.9	40.0	48.7
abietic acid[UF]								3.2	3.9

* The AC treatment was the half exhaustive method described previously. The Ca treatment was with 250 mg L^{-1} Ca^{2+}. The fresh pine resin extract was prepared from 2 g L^{-1} at pH 10.0. The abietic acid extract was prepared from 0.36 g L^{-1} at pH 11.5. The extracts were diluted 1.25x into pH 7.8 media containing 1 g $NaHCO_3$ L^{-1} (note the reported concentrations are given for the undiluted extract).

** Assays were conducted with 1.4 g VSS L^{-1} granular sludge fed VFA at 4.1 g COD L^{-1} per feeding. The second feeding was conducted after 13 to 15 days of the first feeding to determine the residual sludge activity after replacing the supernatant with a VFA containing medium. The control methanogenic activities during the first (and second) feedings were 373 (931), 441 (1117), and 429 (833) mg COD g^{-1} VSS d^{-1}, for pine resin AC, abietic acid AC and abietic acid Ca experiments; respectively.

\+ The COD and UV of the extracts were measured from membrane filtered samples

++ Adsorbed by active carbon or precipitated by calcium

a First = first feeding of activity assay; b Second = second feeding of activity assay

F Extracts were centrifuged and filtered before diluting them into the assay medium

UF Extracts were not centrifuged nor filtered before diluting them into assay, ie. the assay contained the calcium abietic acid floccules

The half exhaustive treatment of AC could remove high levels of COD and UV_{215} from alkaline resin extracts, Table 12. The AC treated resin extracts gave mild toxicity while the untreated resin extracts were highly toxic. Precipitations with 250 mg L^{-1} Ca^{2+} partially removed abietic acid toxicity (Table 12) but only if the Ca^{2+}-abietic acid floccules were filtered out of the solution.

3.7. Toxicity of AC versus PVP Adsorbable Bark Matter

Extracts of pine and spruce bark were treated with PVP or with the half exhaustive treatments of AC. The AC treatment was arranged so that it adsorbed the same amount of COD as the PVP (Table 13). However, the AC had only a small effect on removing the observed methanogenic toxicity because it removed only some and not all of the tannins. The compounds determined by PVP are therefore specific for the toxic factors (tannins) in these coniferous barks.

4. CONCLUSIONS

The average bark extract is composed approximately half out of tannins and half out of non-tannins. The non-tannin compounds are responsible for the majority of the readily available anaerobic digestion substrate. The tannin compounds are responsible for the

Table 13. The Adsorption of Bark Extract COD and UV (215nm and 280nm) by PVP and Granular AC, and the Influence of these Treatments on the Methanogenic Activity of Sludge Exposed to the Bark Extracts.

Treatment*	COD		UV 215nm		UV 280nm		Activity**	
	total mg L^{-1}	adsorbed %	total 1cm,1x	adsorbed %	total HPLC+ signalx10^6	adsorbed %	First[a] % control	Second[b] activity
A. Pine (lot 2) Bark Extract								
untreated	5663		166.8		4.70		33.8	2.5
PVP		30.7		67.3		58.1	94.3	83.3
AC		35.5		45.7		48.9	62.4	26.1
PVP + AC		66.9		72.8		97.9	102.7	92.7
B. Spruce (lot 1) Bark Extract								
untreated	5760		170.4		9.19		8.9	0.9
PVP		52.0		88.0		83.6	116.3	115.4
AC		60.1		68.0		68.6	27.9	2.7
AC + PVP		85.4		97.4		95.3	ND+	ND+

* PVP = extract treated with PVP in accordance with the tannin determination; AC = extract treated with granular AC in accordance with the half exhaustive method; PVP+AC = extract first treated with PVP followed by AC; AC+PVP = extract first treated with AC followed by PVP

** Assays were conducted with 1.4 g VSS L^{-1} granular sludge fed VFA at 4.1 g COD L^{-1} per feeding, the extracts were diluted 1.25x into the first feeding media which contained 2.5 g L^{-1} $NaHCO_3$ as buffer. The second feeding was conducted after 13 to 15 days of the first feeding to determine the residual sludge activity after replacing the supernatant with a VFA containing medium. The control methanogenic activities during the first (and second) feedings were 367 (961), and 337 (722) mg COD g^{-1} VSS d^{-1}, for pine (A.) and spruce (B.) experiments; respectively.

\+ the integrator units of the HPLC chromatograms reported in Figure 7 based on the 280 nm signal.

++ ND = no data; [a] First = first feeding of activity assay; [b] Second = second feeding of activity assay

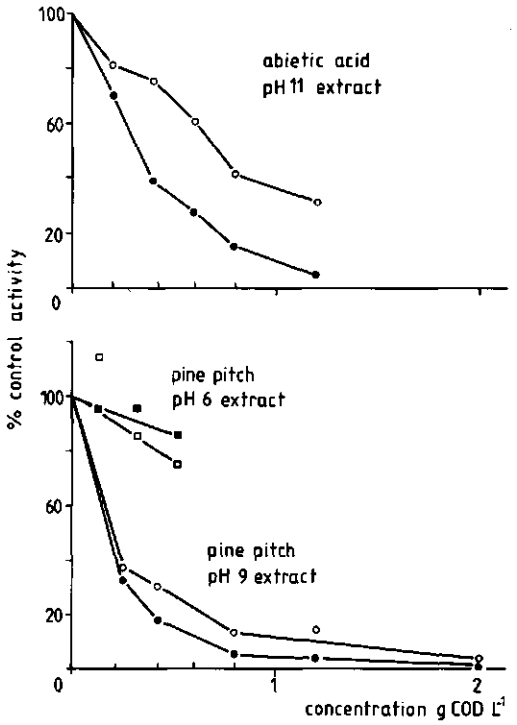


Figure 12A. The methanogenic activity of the first (○) and second (●) VFA (4.1 g COD L^{-1}) feedings of 1.4 g VSS L^{-1} granular sludge with variable concentrations of an alkaline abietic acid extract (PH 11 during extraction) present in the first feeding media for 14 days. The control methanogenic activities were 275 and 577 mg COD g^{-1} VSS d^{-1} during the first and second feedings, respectively. The abietic acid extract was adjusted to pH 8, paper filtered and diluted into pH 7.8 assay media.

Figure 12B. The methanogenic activity of the first (□) and second (■) VFA (4.1 g COD L^{-1}) feedings of 1.4 g VSS L^{-1} granular sludge with variable concentrations of fresh pine resin aqueous extract (pH 6.3 during extraction) present in the first feeding media for 13 days. The control methanogenic activities were 230 and 676 mg COD g^{-1} VSS d^{-1} during the first and second feedings, respectively. The extract was diluted into pH 7.8 assay media. The methanogenic activity of the first (○) and second (●) VFA feedings of a similar assay with variable concentrations of fresh pine resin alkaline extract (pH 9.2 during extraction) present in the first feeding media for 14 days. The control methanogenic activities were 276 and 719 mg COD g^{-1} VSS d^{-1} during the first and second feedings, respectively. The extract was adjusted to pH 8, paper filtered and diluted into pH 7.8 assay media.

majority of the methanogenic toxicity. These observations indicate that the tannins must be eliminated or detoxified if anaerobic treatment of bark wastewater is expected to function properly. In so far as the anaerobic digestion substrate and tannin methanogenic toxicity can serve to be indicative of BOD and aquatic toxicity, both bark BOD and tannin toxicity need to be eliminated to relieve the environmental impact of bark wastewater.

5. LITERATURE CITED

Andersson, P. and T. Welander. 1985. Wastewater treatment method. European Patent Application. Publ. No. EP O-159-972-A1. European Patent Office, Munich, Germany.

Arora, H. C., S. N. Chattopadhya and T. Routh. 1975. Treatment of vegetable tanning effluent by anaerobic contact filter process. Wat. Pollut. Control (UK), 74: 584-596.

Balba, M. T. and W. C. Evans. 1980. The methanogenic biodegradation of catechol by a microbial consortium: Evidence for the production of phenol through cis-benzenediol. Biochemical Soc. Trans. 8: 452-453.

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

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

Cunningham, J., E. Haslam and R. D. Haworth. 1963. The constitution of piceatannol. J. Chem. Soc. 2875.

Daiber, K. H. 1975. Enzyme inhibition by polyphenols of sorghum grain and malt. J. Sci. Food Agric. 26: 1399-1411.

Ekman, R. and R. Sjoholm. 1983. Betulinol-3-caffeate in outer bark of Betula verrucosa ehrh. Finn. Chem. Lett. 5/6: 134-136.

Fedorak, P. M. and S. E. Hrudey. 1984. The effects of some alkyl phenolics on batch anaerobic methanogenesis. Wat. Res. 18: 361-367.

Fengel, J. D. and G. Wegener. 1984. Wood. Walter De Gruyter, NY.

Field, J. A. and G. Lettinga. 1987. The methanogenic toxicity and anaerobic degradability of a hydrolyzable tannin. Wat. Res. 21: 367-374.

Field, J. A., G. Lettinga and M. Geurts. 1987. The methanogenic toxicity and anaerobic degradability of potato starch wastewater phenolic amino acids. Biological Wastes. 21: 37-54.

Field, J. A. and G. Lettinga. 1989. The effect of oxidative coloration on the methanogenic toxicity and anaerobic biodegradability of phenols. Biological Wastes in press.

Gupta, R. K. and E. Haslam. 1980. Vegetable tannins structure and biosynthesis. in J.H. Hulse (ed.), Polyphenols in Cereals and Legumes. Int. Develop. Research Centre, Ottawa, Canada. pp. 15-24.

Hanaki, K., T. Matsuo and N. Nagase. 1981. Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. Biotechnol. Bioengineer. 23: 1591-1610.

Haslam, E. 1966. The Scope of Vegetable Tannins. Academic Press, N.Y.

Haslam, E. 1974. Polyphenol-protein interactions. Biochem J. 139: 285-288.

Hathway, D. E. 1962. The condensed tannins. in W. E. Hillis (ed.) Wood Extractives and their Significance to the Pulp and Paper Industry, Academic Press, N. Y. pp. 191-228.

Hegert, H. L. 1960. Chemical composition of tannins and polyphenols in bark and wood. Forest Prod. J. 10: 610-617.

Hegert, H. L., L. E. van Blaricom, J. C. Steinberg and K. R. Gray. 1965. Isolation and properties of dispersants from western hemlock bark. Forest Prod. J. 15: 485-491.

Hemingway, R. W., L. Yeap-Foo and L. J. Porter. 1981. Polymeric proanthocyanidins: interflavonoid linkage isomerism in (epicatechin-4)-(epicatechin-4)-catechin procyanidins. J. Chem. Soc. Chem. Comm. 320.

Herrick, F. W. 1980. Chemistry and utilization of western hemlock bark extractives. J. Agric. Food Chem. 28: 228-237.

Junna, J. L., R. Lammi and V. Miettinen. 1983. Removal of organic and toxic substances from debarking and kraft pulp bleaching effluents by activated sludge treatment. Publications of the Water Research Institute, National Board Of Waters, Finland. 49: 17-29.

Karchesy, J. J. and R. W. Hemingway. 1980. Loblolly pine bark polyflavonoids. J. Agric. Food Chem. 28: 222-228.

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

Kuwahara, M., T. Kagimura and K. Takagi. 1984. Anaerobic fermentation of bark. 1. Effect of treatment of bark with white-rot fungi and chemicals on the production of methane. Mokusai Gakkaishi 30: 769-776.

Ladd, J. N. and J. H. A. Butler. 1975. Humus-enzyme systems and synthetic organic polymer-enzyme analogs. in E. A. Paul and A. D. McLaren (eds.) Soil Biochemistry, Marcel Dekker, Inc., NY. pp. 143-193.

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

Lea, G. H. A. 1980. Reversed-phase gradient high performance liquid chromatography of procyanidins and their oxidation products in ciders and wines, optimised by Snyder's procedures. J. Chromatogr. 194: 62-68.

Lea, G. H. A. 1982. Reversed-phase high performance liquid chromatography of procyanidins and other phenolics in fresh and oxidising apple juices using a pH shift technique. J. Chromatogr. 238: 253-257.

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

Loomis, W. D. and J. Battaile. 1966. Plant phenolic compounds and the isolation of plant enzymes. Phytochemistry, 5: 423-438.

Markham, K. R. and L. J. Porter. 1973. Extractives of Pinus radiata bark. 1. Phenolic components. New Zealand J. Sci. 16: 751-761.

Pearl, I. A. and M. A. Buchanan. 1976. Tappi J. 59: 136-139.

Rekunen, S. 1986. Taman process and its applicability to wastewaters from papermaking. Proceedings: PIRA Paper and Board Division Key-Event, Cost Effective Treatment of Papermill Effluents Using Anaerobic Technologies, Leatherhead, England.

Rogers, I. H. 1973. Isolation and chemicals identification of toxic components of kraft mill wastes. Pulp and Paper Mag. Can. 74: 111-116.

Rudloff, E. and A. Sato. 1963. The heartwood extractives of Pinus banksiana lamb. Canadian J. Chem. 41: 2165-2174.

Sato, A. and E. Rudloff. 1964. The heartwood extractives of Pinus resinosa ait. Canadian J. Chem. 42: 635-641.

Scalbert, A., B. Monties and J-., M. Favre. 1988. Polyphenols of Quercus robur: Adult tree and in vitro grown calli and shoots. Phytochemistry 27: 3483-3488.

Singleton, V. L., P. Esau. 1969. Phenolic substances in grapes and wine, and their significance. in C. D. Chichester (ed.), Advances in Food Research, Supplement 1. Academic Press, NY. pp. 1-281.

Strumeyer, D. H. and M. J. Malin. 1969. Identification of the amylase inhibitor from seeds of Leoti sorghum. Biochim. Biophys. Acta. 184: 643-645

Tamir, M. and E. Alumot. 1969. Inhibition of digestive enzymes by condensed tannins from green and ripe tannins. J. Sci. Food Agric. 20: 199-202.

Updegraff, D. M. and W. D. Grant. 1975. Microbial utilization of Pinus radiata bark. Appl. Microbiol. 30: 722-723.

Verspuy, A. and W. Pilnik. 1970. Recovery of apple juice by pulp fermentation II: Shortening the fermentation time. Flussiges Obst. 37: 518-519.

Virkola, N. E. and Honkanen, K. 1985. Wastewater characteristics. Water Sci. Technol. 17: 1-28.

White, T. 1957. Tannins - their occurrence and significance. J. Sci. Food Agric. 8: 377-384.

Wilson, E. L. 1981. High-pressure liquid chromatography of apple juice phenolic compounds. J. Sci. Food Agric. 32: 257-264.

Wirkkala, A. 1984. Debarking effluents from the pulp and paper mills and their anaerobic treatment. Mimeographs of the National Board of Waters, Finland. 259: 1-73. (in finnish).

Wise, L. E. 1946. Wood Chemistry. Reinhold Publ. Corp., N.Y.

Young, L. Y. 1984. Anaerobic degradation of aromatic compounds. in D. F. Gibson (ed.) Microbial Degradation of Organic Compounds. Marcel Dekker, Inc., NY. pp. 487-523.

CHAPTER 5

The Effect of Oxidative Coloration on the Methanogenic Toxicity and Anaerobic Biodegradability of Phenols.

Accepted for publication in: Biological Wastes.

The Effect of Oxidative Coloration on the Methanogenic Toxicity and Anaerobic Biodegradability of Phenols.

J. A. Field and G. Lettinga

Dept. of Water Pollution Control
Wageningen Agricultural University
Bomenweg 2, 6703 HD, Wageningen
The Netherlands

ABSTRACT - Phenolic compounds are often present in agricultural wastewater intended for anaerobic treatment. During industrial processing, storage or wastewater pretreatments, these compounds are susceptible to oxidative modifications leading to the formation of colored oxidized products. In this study, the role of the oxidative coloration on changing the methanogenic toxicity and anaerobic biodegradability of phenolic compounds was examined. Short exposures to air increased the methanogenic toxicity of monomeric phenols provided these compounds have neighboring hydroxyl groups, necessary for the coloration, and if their oxidized products lack free carboxylic acid groups. In contrast, a methanogenic toxic tannin, gallotannic acid (oligomeric), was detoxified by oxidative coloration. The extensive oxidation of a monomer, L_dopa, produced a dark colored precipitate which was nontoxic. These results indicated that the initial products of the oxidation of monomers are toxic whereas highly polymerized products are nontoxic. The colorless phenolic compounds tested in this study were either partially or fully degraded (with the exception of catechol). The trihydroxy phenols were the most readily biodegradable compounds. The oxidized solutions of trihydroxy phenols were less biodegradable in proportion to their color.

KEY WORDS - phenolic compounds, phenol, catechol, pyrogallol, phloroglucinol, gallic acid, gallotannic acid, L_dopa, caffeic acid, p_coumaric acid, methanogenic bacteria, methanogenic toxicity, autoxidation, tyrosinase, anaerobic biodegradability phenolic compounds, anaerobic digestion

1. INTRODUCTION

Phenolic compounds are important constituents of various agricultural products used as feedstocks in industry. They eventually can become incorporated in wastewater if the feedstocks are processed under wet conditions. The natural occurrence of phenolic compounds in wastewater may affect the efficiency of anaerobic waste treatment since phenols are toxic to methanogenic bacteria (Chou et al., 1978; Fedorak and Hrudey, 1984; Pearson et al., 1980; Benjamin et al., 1984; Blum et al., 1986). Phenols are susceptible to oxidative modifications that lead to the formation of colored compounds. The possibility that their toxicity and biodegradability are altered by short exposures to air, therefore cannot be ignored.

Figure 1, illustrates some examples of oxidation products derived from various phenolic monomers. Autoxidative alterations occur if the phenols have at least two neighboring hydroxyl (ortho) groups (Mathew and Parpia, 1971; Singleton, 1972). The ortho-dihydroxy phenols, which are the most common type in agricultural products, are generally polymerized in a fashion analogous to humus forming reactions. Under neutral to alkaline conditions, the exposure to air will cause the formation of unstable ortho-quinones. The subsequent condensation reactions polymerize the phenols (Hathway and Seakins, 1955; and 1957) to compounds with stable color. Ultimately, darkly colored humic-like compounds such as melanin are formed. The same type of oxidation can be catalyzed more rapidly and at lower pH values with phenol oxidases (for example tyrosinase). The enzymes have the capability of hydroxylating mono-hydroxy phenols to ortho-dihydroxy phenols which are then converted

to ortho-quinones by a second activity of the enzyme. Tyrosinase has been applied to polymerize simple monomeric phenols to insoluble humic compounds that can be separated from the wastewater (Atlow et al., 1984).

The trihydroxy phenols are even more reactive with oxygen by autoxidation as compared to the dihydroxy phenols, but their polymerization is limited and ultimately they are depolymerized by partial destruction of the aromatic structure. This type of oxidation can be characterized by the formation of purpurogallin (Figure 1), its structure illustrates the ambiguous reactions that lead to its formation: condensation of two pyrogallol rings; and the destruction of the aromaticity of one of the rings.

The impact of phenol coloration on the methanogenic toxicity and anaerobic biodegradability of the phenols was investigated in this study by aerating solutions of simple phenolic compounds under autoxidative conditions and in one case with a crude extract of tyrosinase.

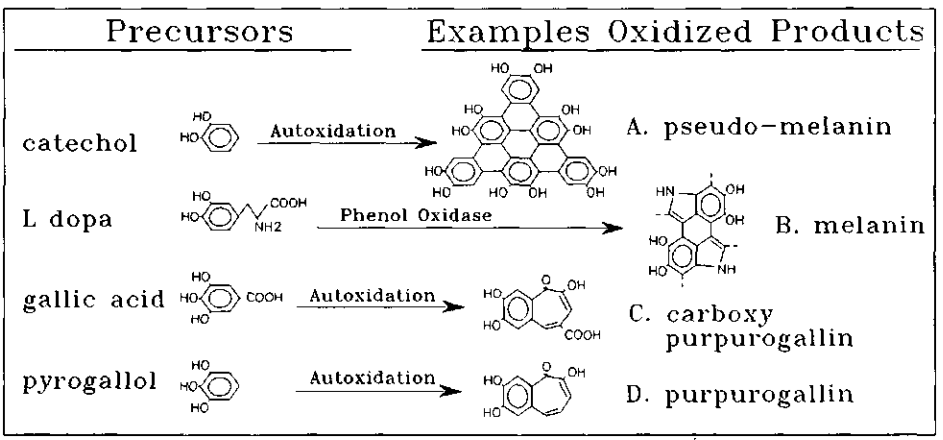


Figure 1. Oxidative modification of phenolic compounds. References: A. Singleton, 1972; B. Singleton, 1972; C. Sjoblad and Bollag, 1981; D. Mathew and Parpia, 1971.

2. MATERIALS AND METHODS

2.1. Analytical Methods

2.1.1. UV Absorbance and Color of the Extracts

The ultra violent (UV) and visible light absorbance (color) of the extracts was measured in a 1 cm quartz cuvette by diluting to less than 0.8 absorbance units in pH 6, 0.2 M KH_2PO_4 buffer as described earlier (Field et al., 1987; Field and Lettinga, 1987). The UV absorbance was usually measured at 215 nm. The UV light absorbance is indicative of the total phenolic concentration.

2.1.2. Color

The term color was quantified by the absorbance of visible light. Generally color was based on the absorbance at 440 or 340 nm at a dilution in 0.2M KH_2PO_4 pH 6 buffer to an absorbance less than 0.8 units. Most compounds used did not have any absorption at 340 nm and only the oxidized color products gave absorbance at 340 nm in close association with the development of visible absorbance at higher wavelengths.

Usually, color based on 340 nm absorbance was reported for the samples obtained after anaerobic digestion, in which case it is called, "anaerobic digestion stable" (ADS) color.

2.1.3. COD, VSS and VFA Determinations

The COD (the micro method with dichromate) and VSS were determined according to standard methods (American Public Health Assoc., New York). The method for determining the volatile fatty acids by gas chromatography were described previously (Field and Lettinga, 1987). The same gas chromatographic procedure was used to determine certain intermediates of phenolic degradation by extending the retention time to 2 hours. The compounds tested are listed in the Tables.

2.2. Oxidation Treatments

2.2.1. Autoxidation Phenolic Compounds

An initial set of experiments were conducted by preparing the anaerobic assay medium (nutrients and substrate), including the phenolic compound, and aerating the medium with pressurized air through a porous stone. Generally, the pH during autoxidation was 7.4 and the time of aeration ranged from 1 second to 15 minutes. The autoxidation was stopped by placing the sludge in the assay bottles and flushing the head space with N_2.

In other experiments, the phenolic compounds were in most cases autoxidized at higher pH values (7.4 to 12) and as a pure stock solution. The pH was adjusted to the initial value with NaOH and the autoxidation was stopped by readjusting the pH to 6.5 with HCl. The stock solution was then immediately diluted in the assay medium and the assay was started after adding the sludge and flushing the head space with N_2.

2.2.2 Oxidation L Dopa with Potato Tyrosinase

In two experiments L dopa (1.818 g L^{-1}) was oxidized at pH 6.5 with 0.1M KH_2PO_4 buffer extracts of potato (Doré) peels. The potato peels are known to contain tyrosinase (Mathew and Parpia, 1971). In one experiment designated, "mild oxidation," the L_dopa was treated with a tyrosinase at a concentration diluted 255x from the fresh potato peel while exposed to air (for 0 to 69 hours) in an open shake flask (contents 0.11 L) using 0.25 rotations per second. The colored products of this oxidation were completely soluble. In a second experiment designated, "intense oxidation," the L_dopa was treated with a tyrosinase concentration diluted 58x from the fresh potato peel, using 1 rotation per second and shaking (for 0 to 49 hours). The colored products of this oxidation were soluble but precipitated to a large extent in the first 24 hours of the anaerobic assay. An additional treatment was aerated with 2 rotations per second shaking for 49 h. The colored products were completely insoluble and this treatment was designated "pre-flocculated."

2.3. Bioassays

All assays contained essential inorganic macro and micro nutrients as outlined previously (Field and Lettinga, 1987). The batch fed assays were conducted in 0.5 L serum flasks. The assay temperature was 30 ± 2° C. The serum flasks were not shaken during the assay period.

The anaerobic toxicity assays of oxidized and unoxidized phenolic solutions were supplied at the start of each feeding substrate of approximately 4 g COD L^{-1} neutralized with NaOH VFA stock solution, 100:100:100 g kg^{-1} acetate (C_2), propionate (C_3) and butyrate (C_4). The stock solution COD ratio is 24:34:41 C_2:C_3:C_4. From 1 to 1.5 g VSS L^{-1} granular sludge was used in the assays, that was either obtained from a UASB treating potato derived wastewater ("AVIKO") or vinasse ("NEDALCO").

The assays were carried out in one, two or three consecutive feedings. The first feeding always supplied the VFA substrate to the treatments (assays with phenolic compounds) and the control. The first feeding generally lasted for one to three weeks. In this study, numerous types of experiments were conducted as outlined follows: Type 1 Only the first feeding in the presence of the phenolic solution; Type 2I First feeding with phenolic solution present, the additional feedings were initiated by adding VFA to the existing

medium; Type 2R first feeding with phenolic solution present, the second feeding was initiated after decanting the first feeding medium and replacing it with VFA medium not containing phenolic solutions (residual activity).

Methane production was monitored with a modified mariotte flask constructed from serum flasks. These flasks were filled with 3 to 5% NaOH solutions to dissolve all CO_2.

The activity of the controls is expressed as the amount of CH_4 in COD produced by one gram of VSS per day (mg COD g^{-1} VSS d^{-1}). The activity of the phenolic solution supplied assays is reported as the percentage of the control activity. The activities are based on the methane production rates during the activity period; where, the activity period is the time interval of maximum control activity. The activity period was usually a 4 to 5 day period within the first 6 days of the first feeding and the first 2 to 3 days of the consecutive feedings. Inhibition is equal to 100 minus the percent of control activity. The specific assay conditions and control methanogenic activities are reported in Table 1 for each of the experiments in this study.

The recovery of phenolic COD in the form of VFA and CH_4 was calculated by summing up the COD present as produced CH_4 and as VFA in the media. The VFA control sum was subtracted from the phenol treated assay sum. The difference was labelled "recovery COD" (RCOD).

2.4. Materials

The phenolic compounds were obtained from Janssen Chimica, Boom BV and British Drug House.

Table 1. The Assay Parameters of the Experiments Conducted in this Study.

Experiment			Assay+						
Number	Compound	Concentration* in Assay mg L^{-1}	Type**	Sludge VSS g L^{-1}	Duration Time++ First	Second	Activity Control First	Second	Third
					--- days ----		(mg COD g^{-1} VSS d^{-1})		
1.	catechol	351 to 1755	2I	1.46	10	21	389	762	693
	and phenol	300 to 1500							
2.	pyrogallol	1000	1	1.05			440		
3.	pyrogallol	1000	2R[b]	1.05	1		reported in Table		
4.	catechol	1000	2I	1.10	9	17	540	1050	962
5.	gallic acid	1000	1	1.05			440		
6.	gallic acid	750	1	1.82			314		
7.	gallotannic acid	1000	2R	1.05	25		453	971	
8.	phloroglucinol	500 to 1500	1	1.46			385		
9.	p coumaric acid	1000	2I	1.46	10		445	931	
	and caffeic acid	1000							
10.	L dopa	1000	2I	1.50	7		686	839	
11.	L dopa	800	2R	1.50	22		385	550	

* Concentration unoxidized phenolic compound (mg dry matter per liter) in assay media, the oxidized phenolic solutions were diluted in the same fashion as the unoxidized compound.

** Type of toxicity assay (see text methods).

\+ Unless otherwise stated, the VFA spikes used in the experiments supplied 4 g COD L^{-1} (C_2:C_3:C_4 = 24:34:41 % of the COD).

++ Duration time of the feedings are reported to indicate the exposure time prior to the feedings which followed.

b Assays of experiment 3 were supplied with 4 g COD L^{-1} of either acetate, propionate or butyrate.

3. RESULTS

3.1. Effect of Oxidation on the Methanogenic Toxicity of Phenolic Compounds

Table 2 illustrates the role of the hydroxylation pattern of phenols on their susceptibility to coloration by autoxidation. The absence of colored compounds illustrates how the simplest monohydroxy phenolic compound, phenol itself, did not react with the oxygen. In contrast, the simplest ortho dihydroxy phenol, catechol, did produce colored products upon autoxidation. The autoxidation, however, did not affect the UV absorbance, indicating the phenolic characteristic of the colored compounds.

The autoxidation applied was mild to imitate the conditions that might be expected if phenolic wastewaters were exposed to air. The exposure to air had no consequence to the methanogenic toxicity of phenol which was expected as this compound was not affected by the autoxidation. The coloration of the catechol solution had a significant effect on increasing its methanogenic toxicity (Figure 2). Based on the activity during the second VFA feeding, the 50% inhibiting concentration of catechol was decreased by 33%.

Autoxidation treatments were applied to a variety of phenolic compounds (Table 3), the results of these experiments are summarized in Figure 3. The phenols which lacked the ortho hydroxyl groups (p coumaric acid, phloroglucinol), like phenol, were not colorized or otherwise not colorized to a large extent by autoxidation. Likewise, their methanogenic toxicity was not affected. Although, caffeic acid has the ortho dihydroxy substitution, it did not form a colored solution by the autoxidation applied and therefore, its autoxidation also did not affect its methanogenic toxicity. Pyrogallol, with three neighboring hydroxyl groups, was dramatically colored by the short autoxidation treatment applied. Although, the unoxidized pyrogallol was completely nontoxic to methane bacteria, the pyrogallol solutions recovered after short aeration periods of one second to 15 minutes were highly toxic. This indicated the high toxicity of the first autoxidation product produced, purpurogallin (Figure 1). An additional experiment demonstrated that the autoxidized pyrogallol solution was able to damage the methanogenic activity of sludge even after only a one day contact with the

Table 2. The UV absorbance (at the Start of the Experiment) and the Anaerobic Digestion Stable Color (Day 9 of the Experiment) of Unoxidized and Autoxidized Phenol and Catechol

Compound	Concentration	ADS* Color		UV Absorbance	
		unox+	auto+	unox	auto
		340 nm		215 nm	
	mg L^{-1}	5x, 1cm		1x, 1cm	
Phenol	300	0.000	0.016	14.7	15.8
	600	0.000	0.022	28.2	31.2
	1000	0.000	0.040	50.2	51.6
	1500	0.000	ND**	77.3	ND
Catechol	351	0.000	0.256	17.3	19.3
	702	0.000	0.360	35.4	38.7
	1170	0.000	0.390	60.0	61.4
	1755	0.000	ND	87.2	ND

* ADS Color = anaerobic digestion stable color

** ND = no data

\+ unox = the unoxidized phenolic solution; auto = the autoxidized phenolic solution, the solution was aerated at a pH of 7.4 for 15 minutes and then allowed to stand for 5 days in an open flask exposed to the atmosphere

Table 3. Influence of Aerating Phenolic Solutions (pH = 7.4) on the Methanogenic Activity of Sludge.

Experiment			Type[&] Autoxidation	ADS Color[*]	Methanogenic Activity		
Number	Compound	Concentration[+] g L^{-1}		340nm 5x,1cm	First[**]	Second	Third
					----- percent control -----		
2.	pyrogallol	1.00	0	0.000	138.0		
			A (1 sec)	0.276	57.0		
			A (1 min)	0.486	41.0		
			A (15 min)	0.895	32.4		
4.	catechol	1.00	0	0.003	62.3	60.1	78.8
			A (1 sec)	0.000	61.0	64.7	80.2
			A (1 min)	0.026	60.8	65.9	77.9
			A (15 min)	0.159	47.9	47.8	56.9
5.	gallic acid	1.00	0	0.000	124.0		
			A (1 min)	0.070	118.0		
			A (15 min)	0.135	119.0		
7.	gallotannic acid	1.00	0	0.085	28.9	25.8	
			A (1 min)	0.245	39.2	46.6	
			A (15 min)	0.320	55.4	64.2	
8.	phloroglucinol	1.00	0	0.021	128.4		
			B	0.137	113.4		
	phloroglucinol	0.50	0	0.013	119.5		
			B	0.038	106.5		
9.	caffeic acid	1.00	0	0.000	82.5	88.6	
			C	0.038	83.8	88.2	
	p coumaric acid	1.00	0	0.000	74.0	82.5	
			C	0.004	92.0	87.1	
6.	gallic acid	0.75	0	0.015	141.2		
			D (1 min)	0.043	136.1		
			D (5 hour)	1.040	99.0		
			D (73 hour)	1.265	91.9		

[+] Concentration of the unoxidized and oxidized phenolic compound (dry matter basis) in the assay media.

[*] Anaerobic Digestion Stable Color of the media after 58, 1, 12, 25, 9, 18 and 9 days of digestion, respectively for experiments 2, 4, 5, 7, 8, 9 and 6.

[**] First, Second and Third VFA Feedings (spikes) as outlined in Table 1.

[&] Type Autoxidation: O = unoxidized, A = assay medium with phenolic compounds aerated at pH 7.4 for 1 second to 15 minutes (the 15 minute aerated control had on the average 98% of the unaerated control activity), B = phenolic stock solution aerated for 2 hours at pH 11.5, C = phenolic stock solution aerated at pH 8 for 15 minutes followed by aerating at pH 10 for 5 minutes, D = phenolic stock solution aerated for 1 minute to 73 hours at pH 12

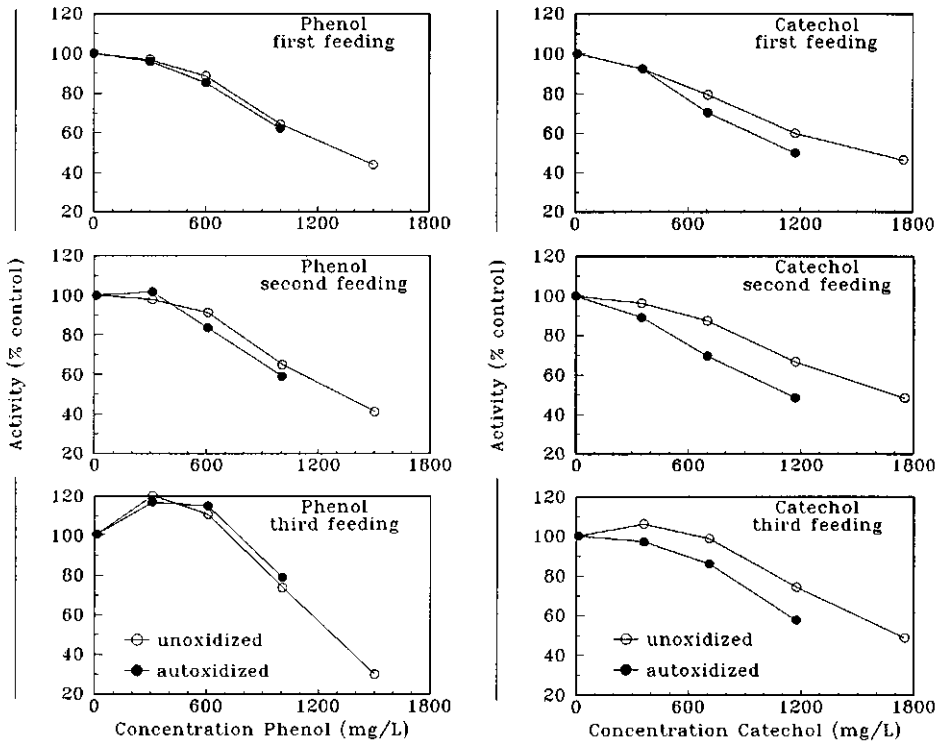


Figure 2. The effect of a mild autoxidation on the methanogenic activity of granular sludge exposed to phenol and catechol in three consecutive VFA feedings (Experiment 1). The autoxidation treatment and formation of colored products are described in Table 2.

sludge (Table 4), and that its toxicity was effective on both the metabolism of acetoclastic (acetate) as well as acetogenic substrates (propionate and butyrate). Gallic acid which has a very similar structure to pyrogallol except for its free carboxylic acid group, was not colored as extensively which might serve as an explanation for why it did not increase in methanogenic toxicity by short autoxidation treatments. When a severer autoxidation was applied, producing comparable levels of color, the autoxidized solutions were still nontoxic. Thus a large difference in methanogenic toxicity is indicated if the autoxidized products contain free carboxylic acids, like carboxy-purpurogallin (Figure 1), as compared to purpurogallin which lacks the free carboxylic acid group. Perhaps the fact that this group would be dissociated at the assay pH (7.4) plays a role in decreasing the toxicity.

In contrast to the monomers tested, the autoxidative coloration of gallotannic acid led to a decrease in its methanogenic toxicity (Table 3). It was not clear which mechanism led to the detoxification effect. The experiment was repeated but efforts to reproduce the brown colored solution with autoxidation failed, finally autoxidation was conducted at a higher pH (11) but in these cases only a green colored extract that produced a yellow precipitate when the pH was again adjusted to 7 was evident. The only difference, in the way in which the autoxidation was performed in the latter attempts, is that, in the original experiment, the gallotannic acid was autoxidized together with the nutrient and substrate solutions of the anaerobic assay. Since the nutrient solution used in this particular experiment was an old unsterile solution, it was speculated that the nutrient solution was contaminated with some sort of microorganism that influenced the oxidative alterations of the tannin.

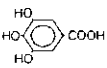
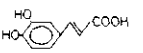
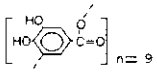
COMPOUND	STRUCTURE	COLORATION	EFFECT ON TOXICITY
phenol		no	no change
catechol		yes	increased
pyrogallol		yes	increased
phloroglucinol		yes (low)	no change
gallic acid		yes	no change
p coumaric acid		no	no change
caffeic acid		no	no change
gallotannic acid		yes	decreased
L dopa		A.* yes B. yes	increased decreased

Figure 3. Summary of the effect of oxidation treatments on the coloration and methanogenic toxicity of phenolic compounds examined in this study. *A and B refer to the "mild" and "intense" oxidations of L_dopa with tyrosinase (see Figure 4).

In addition to the autoxidative methods, the phenol oxidase (tyrosinase) catalyzed oxidation of phenols was studied using L dopa. This compound is suspected to be present in potato starch wastewaters as a product of the naturally present tyrosine (a phenolic amino acid) resulting from the monohydroxylase activity of the potato tyrosinase. While tyrosine was not toxic, L dopa was found to be a methanogenic inhibitor (Field et al., 1987), indicating the role of the monohydroxylase activity on changing the methanogenic toxicity of tyrosine. In this study the role of the dihydroxylase activity was considered by oxidizing solutions of L-dopa with tyrosinase. With mild oxidation conditions, the L-dopa was polymerized to soluble colored solutions with a low but significantly increased methanogenic toxicity (Figure 4a). However, when a more extensive oxidation was performed, the colored products precipitated in the anaerobic assay media and were less toxic than L dopa (Figure 4b). In one case, the data point labeled "pre-flocculated", the oxidation was so extensive that all the L dopa was converted to darkly colored compounds that were already insoluble before mixing them together with the anaerobic assay media. These were completely nontoxic. This indicated that the intermediate products of the melanin formation were the most effective inhibitors, whereas mature melanin was nontoxic.

3.2. Effect of the Oxidation on the Anaerobic Biodegradability of Phenolic Compounds

3.2.1. Biodegradability of Unoxidized Phenolic Solutions

Unoxidized catechol was not degraded anaerobically during the toxicity assays of this study as is evident from the absence of UV absorbance elimination after 50 days of anaerobic digestion (Figure 5), likewise not any recovery of the catechol in the form of

Table 4. Influence of a One Day Exposure of Sludge to 15 Minute Autoxidized 1 g L^{-1} Pyrogallol on the Methanogenic Activity Following the Exposure. The Autoxidized Pyrogallol Containing Medium was Decanted after One Day and the Sludge Was Supplied with Individual VFA Substrates (4 g COD L^{-1}) in a Medium Lacking Autoxidized Pyrogallol to Determine the Residual Activity.

VFA*	Absolute Activity of the control mg COD g^{-1} VSS d^{-1}	Exposed Sludge Activity percent control
C_2	1031	52.0
C_3	499	32.6
C_4	428	40.5

* experiment 3 substrate: C_2 = acetate, C_3 = propionate; C_4 = butyrate

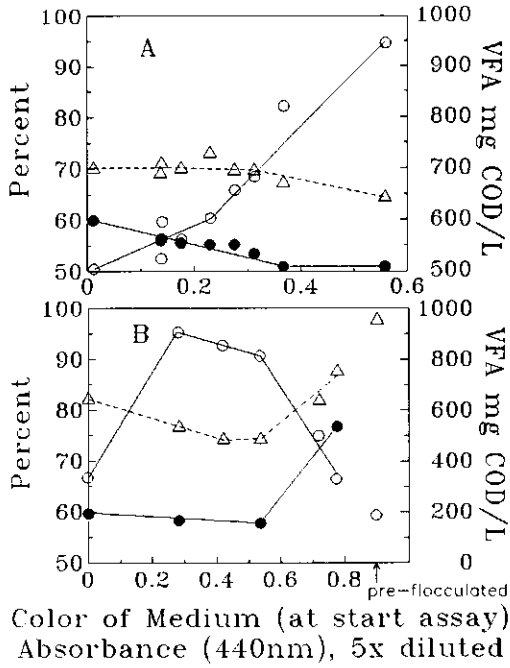


Figure 4. The methanogenic activity of sludge exposed to potato phenol oxidase (tyrosinase) oxidized L dopa. A. Mild oxidation and B. Intense oxidation. Methanogenic activity during the 1st (△) and 2nd (●) feeding and the VFA concentration of the media (○) at the end of the 1st feeding. Experimental conditions are described in Table 1 (experiments 10 and 11 for A. and B., respectively) and oxidation conditions are described in Materials and Methods.

VFA and CH_4 was observed. Although no degradation occurred in this study, the anaerobic biodegradability of catechol has been confirmed in several studies (Balba and Evans, 1980; Healy and Young, 1978 and 1979; Horowitz et al., 1981; Blum et al., 1986; Suidan et al., 1980). Of all the simple phenolic compounds, inoculum appears to need more time to acclimate to catechol (Healy and Young, 1978 and 1979) or in studies with short term assays, the acclimation does not occur (Simpson et al., 1969; Chmielowski et al., 1965; Kaiser and Hanselmann, 1982a and b; Clark and Fina, 1952). Additionally, in the other studies, the biodegradation of catechol was investigated without a substrate VFA spike. Since the biodegradation of some simple phenolic compounds are inhibited by high VFA concentrations (Fedorak et al., 1986), this would indicate that the conditions of a toxicity assay (ie. VFA added) are not the most favorable for catechol degradation.

The cinnamic acids studied, caffeic and p coumaric acids, were biologically transformed to intermediates of lower UV absorbance but no recovery of the phenolic COD in the form of VFA and CH_4 was observed after 18 days of digestion (Table 5).

Unoxidized phenol, although not readily degraded initially, was finally degraded by the sludge after a certain lag phase which was increasingly longer with the increasing phenol concentrations (Figure 5). The accumulation of two intermediates of its biodegradation, benzoic acid and carboxycyclohexane, were observed (Table 6), as has been observed previously (Neufeld et al., 1980; Knoll and Winter, 1987; Keith et al., 1978). With the highest concentration of phenol tested, 1500 mg L^{-1}, an almost complete conversion of the phenol to benzoic acid occurred, although no elimination of the UV absorbance was apparent.

In contrast to the mono- and dihydroxy phenols, the unoxidized trihydroxy phenols were degraded readily in short time periods of digestion as is evident from the rapid elimination of the UV absorbance and recovery of the phenolic COD in the form of VFA and CH_4 during the toxicity assays with pyrogallol and phloroglucinol (Figure 6 and Table 7, respectively). This indicates, that the mechanisms of anaerobic trihydroxy phenolic biodegradation are quite distinct from those of the mono- and dihydroxy phenolic biodegradation.

Table 5. The Elimination of UV Absorbance and the Appearance of Phenolic Intermediates during the Toxicity Assay with Hydroxy Cinnamic Acids (Experiment 9).

Cinnamic Acids Supplied			Intermediates*		RCOD**	EUV**
Type	Concentration mg L-1	mg COD L-1	Type	Concentration mg COD L-1	------- % -------	
p coumaric acid (4 hydroxy)	1000	1854	phenol	684	0.0	65.6
			4 ethylphenol	3		
			3 phenylpropionate	627		
caffeic acid (3,4 dihydroxy)	1000	1600	phenol	14	0.0	62.3
			3 ethylphenol	3		

* The intermediates of the hydroxy cinnamic acid degradation observed in the medium after 24 days of digestion during the toxicity assay. Only the following intermediates were investigated with the GC procedure utilized: phenol; p,m and o cresol; 4 and 3 ethylphenol; benzoate; carboxycyclohexane; phenylacetate; and 3 phenylpropionate. Potential intermediates such as catechol, hydroxy- 3 phenylpropionates and cinnamic acids were not determinable. The COD/DS ratio of the phenols mentioned in the Table: p coumaric acid = 1.854; caffeic acid = 1.600; phenol = 2.383; ethylphenol = 2.623; 3 phenylpropionate = 2.240.

** RCOD = the recovery of the phenolic COD as VFA and CH_4 after 18 days of digestion; EUV = the elimination of the UV absorbance after 24 days of digestion (day 0 UV215nm 1x, 1cm = 77.2 and 87.7, respectively for p coumaric and caffeic acid).

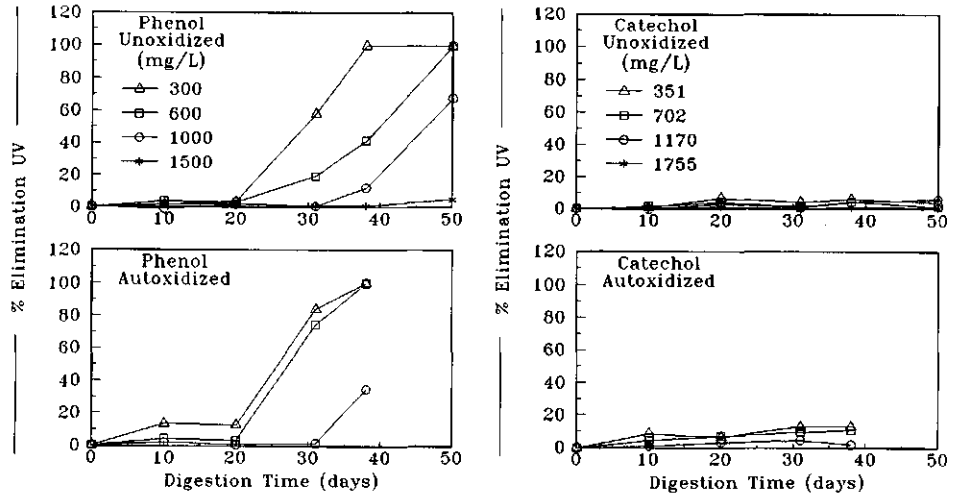


Figure 5. The elimination of UV absorbance from the media during the toxicity assay with unoxidized and autoxidized phenol and catechol (Experiment 1). The initial UV absorbance of the media are reported in Table 2.

Table 6. Concentration of Phenol, Anaerobic Degradation Intermediates of Phenol in the Media and the Recovery of Phenol COD as CH_4 and VFA at Selected Sampling Times of the Toxicity Assay (Experiment 1).

Day 0		Day 38					Day 50				
Phenol		Phenol*	Benzoate	CCH*	RCOD**	COD Balance&	Phenol	Benzoate	CCH*	RCOD**	COD Balance
mg L^{-1}	mg COD L^{-1}	mg COD L^{-1}				%	mg COD L^{-1}				%
0	0	0	0	0	0	NA	0	0	0	0	NA
300	715	0	0	0	662	92.6	0	0	0	792	110.8
600	1430	519	100	0	592	84.8	0	0	0	1374	96.1
1000	2383	1177	472	57	310	84.6	291	376	145	1692	105.1
1500	3575	2917	234	0	102	91.0	436	3350	0	102	108.8

* CCH = carboxycyclohexane, the phenolic intermediates investigated with the GC procedure are listed in the Footnote of Table 5

** RCOD = phenolic COD converted to VFA and CH_4; + COD/DS Ratios: phenol = 2.383; benzoate = 1.967; CCH = 2.250

& COD Balance = (phenol + intermediates + RCOD)/$phenol_{added}$

3.2.2. Biodegradability of Oxidized Phenolic Solutions

Since unoxidized catechol was not biodegradable in this experiment, the effect of the coloration on its biodegradability could not be determined. Although, unoxidized phenol and the cinnamic acids were biodegraded or at least transformed to intermediates during the anaerobic digestion, since the autoxidation of these compounds did not produce color, their autoxidation had no effect on their biodegradability.

The autoxidation of the trihydroxy phenolics decreased their conversion to VFA and CH_4 and decreased the elimination of UV absorbance (Figure 6 and Table 7). In all cases, the

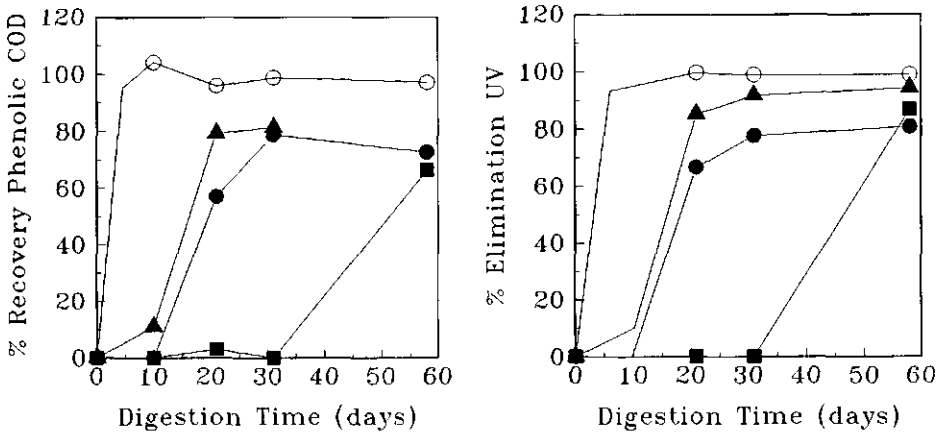


Figure 6. The recovery of phenolic COD as VFA and CH_4 and the elimination of UV absorbance from the media during the toxicity assay with unoxidized pyrogallol (○) and with pyrogallol autoxidized for 1 second (▲), 1 minute (■), and 15 minutes (●). Results were obtained from Experiment 2. The initial UV absorbance of the assay media at the start of the experiment was 60.8, 64.8, 68.8 and 68.9 1x, 1cm 215nm absorbance units, respectively for the pyrogallol which was unoxidized, autoxidized for 1 second, 1 minute and 15 minutes.

Table 7. The Anaerobic Biodegradability of Phloroglucinol and Autoxidized Phloroglucinol.

	Concentration Phloroglucinol					
Days of Digestion	0.5 (g L^{-1}) RCOD*	EUV**	1.0 (g L^{-1}) RCOD	EUV	1.5 (g L^{-1}) RCOD	EUV
	%					
unox+						
4	75.0	93.0	63.5	63.8	27.3	25.8
9	101.8	98.0	103.0	98.9	95.6	98.4
auto+						
4	78.4	71.3	62.9	59.1	ND++	ND
9	85.3	86.2	86.1	73.3	ND	ND

* Recovery of phenolic COD as VFA and CH_4

** Anaerobic elimination of UV_{215} absorbance (the 1x, 1cm absorbance at the start of the experiment was 30.5, 62.0 and 92.4, respectively for 0.5, 1.0 and 1.5 g L^{-1} phloroglucinol). The results were obtained from experiment 8.

\+ unox = the unoxidized phenolic solution, auto = the autoxidized phenolic solution (see Table 3 for description of the autoxidation).

++ ND = no data

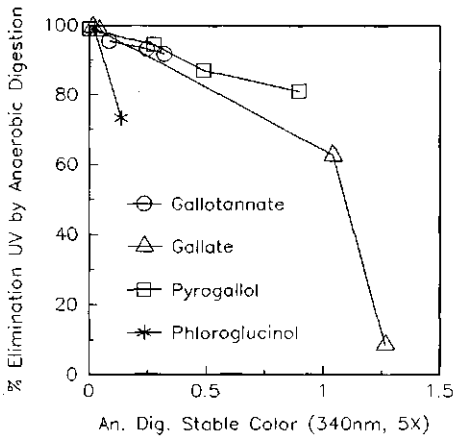


Figure 7. The elimination of UV by anaerobic digestion of autoxidized trihydroxy phenols as a function of the anaerobic digestion stable color. The duration of the digestion was for 58, 9, 25, and 9 days for pyrogallol (1 g L^{-1}, experiment 2), phloroglucinal (1 g L^{-1}, experiment 8), gallotannic acid (1 g L^{-1}, experiment 7), and gallic acid (0.75 g L^{-1}, experiment 6), respectively.

decrease in the ultimate biodegradability corresponded to the level of coloration (Figure 7), indicating that the colored oxidation compounds were not biodegradable. The biodegradation which did occur from the colored phenolic solutions, was most likely related to the fraction of the colorless precursors which escaped transformation during autoxidation as has been reported previously in one study with partially colored solutions of pyrogallol (Schink and Pfennig, 1982).

The colored products of gallic acid, gallotannic acid and phloroglucinol did not affect the rate at which the biodegradation of the colorless fraction occurred. However, the colored products of pyrogallol autoxidation were unique, since they inhibited the rate of biodegradation (Figure 6). Since the pyrogallol solution which was autoxidized for only one minute lowered this rate even more than the solution which was autoxidized for 15 minutes, the most likely explanation for the inhibition is that the first autoxidation product, purpurogallin, was the inhibitor of the anaerobic pyrogallol degradation.

4. DISCUSSION

Phenolic compounds are often present in agricultural wastewater intended for anaerobic treatment. These compounds are susceptible to oxidative coloration during industrial processing, storage or wastewater pretreatments which inevitably could contribute to changes in their methanogenic toxicity. In this study we have observed that an increase in the toxicity occurred under mild conditions of autoxidation if the phenolic compound tested has neighboring hydroxyl groups and lacks free carboxylic acid groups. A similar increase in toxicity also occurred when L_dopa was oxidized mildly with potato tyrosinase. Although, this appears to form an exception to the trend, since L_dopa contains a free carboxylic acid group, this group is actually cleaved during the initial stages of the oxidation (Singleton, 1972). Therefore the initial products of oxidative coloration are toxic to methane bacteria, provided that these products are devoid of free carboxylic acid groups.

The oxidative modifications of phenols did not always lead to an increased methanogenic toxicity. When a methanogenic toxic oligomer, gallotannic acid, was successfully oxidized to

soluble colored products, a distinct detoxification was observed. Detoxification of L_dopa occurred when an extensive oxidation with tyrosinase was applied, producing a darkly colored melanin precipitate. These latter experiments suggest that highly polymerized products of the oxidation are nontoxic.

The oxidative coloration also affected the biodegradability of the phenolics. Trihydroxy phenolic solutions which were readily biodegradable during the toxicity assays, became less biodegradable after autoxidation in proportion to the formation of colored compounds. In most cases, the autoxidized products did not affect the rate at which the biodegradable fraction was degraded. However, the initial autoxidation product of pyrogallol was inhibitory to the biodegradation of the pyrogallol.

The oxidative coloration of phenols therefore cannot be ignored. The results of this study demonstrate that short exposure of phenol bearing wastewaters to air prior to anaerobic treatment can have a drastic effect on both the methanogenic activity of the reactor sludge and the anaerobic biodegradability of the phenolic wastewater components.

5. LITERATURE CITED

Atlow, S. C., L. C. Bonadonna-Aparo and A. M. Klibanov. 1984. Dephenolization of industrial wastewaters catalyzed by polyphenol oxidase. Biotechnol. Bioeng. 26: 599-603.

Balba, M. T. and W. C. Evans. 1980. The methanogenic biodegradation of catechol by a microbial consortium: Evidence for the production of phenol through cis-benzenediol. Biochemical Soc. Trans. 8: 452-453.

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

Blum, J. W., R. Hergenroeder, G. F. Parkin and R. E. Speece. 1986. Anaerobic treatment of coal conversion wastewater constituents: biodegradability and toxicity. J. WPCF. 58(2): 122-131.

Chmielowski, J., A. Grossman and S. Labuzek. 1965. Biochemical degradation of some phenols during the methane fermentation. Zeszyty Naukowe Politechniki Slaskiej; Inzynieria Sanitarna. 8: 97-122. (in polish).

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

Clark, F. M. and L. R. Fina. 1952. The anaerobic decomposition of benzoic acid during methane fermentation. Archs. Biochem. Biophys. 36: 26-32.

Fedorak, P. M. and S. E. Hrudey. 1984a. The effects of some alkyl phenolics on batch anaerobic methanogenesis. Wat. Res. 18: 361-367.

Fedorak, P. M.,D. J. Roberts and S. E. Hrudey. 1986. The effects of cyanide on the methanogenic degradation of phenolic compounds. Wat. Res. 20: 1315-1320.

Field, J. A. and G. Lettinga. 1987. The methanogenic toxicity and anaerobic degradability of a hydrolyzable tannin. Wat. Res. 21: 367-374.

Field, J. A., G. Lettinga and M. Geurts. 1987a. The methanogenic toxicity and anaerobic degradability of potato starch wastewater phenolic amino acids. Biological Wastes. 21: 37-54.

Hathway, D. E. and J. W. T. Seakins. 1955. Autoxidation of catechin. Nature 176: 218.

Hathway, D. E. and J. W. T. Seakins. 1957. Enzymatic oxidation of catechin to a polymer structurally related to some phlobatannins. Biochem. J. 67: 239.

Healy, J. B. Jr. and L. Y. Young. 1978. Catechol and phenol degradation by a methanogenic population of bacteria. Appl. and Environ. Microbiol. 35: 216-218.

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

Horowitz, A., D. R. Shelton, C. P. Cornell and J. M. Tiedje. 1981. Anaerobic degradation of aromatic compounds in sediments and digested sludge. Developments Industrial Microbiol. 23: 435-444.

Kaiser, J. P. and K. W. Hanselmann. 1982b. Aromatic chemicals through anaerobic microbial conversion of lignin monomers. Experientia. 38: 167-176.

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

Keith, C. L., R. L. Bridges, L. R. Fina, K. L. Iverson and J. A. Cloran. 1978. The anaerobic decomposition of benzoic acid during methane fermentation: IV Dearomization of the ring and volatile fatty acids formed on ring rupture. Arch. Microbiol. 118: 173-176.

Knoll, G. and J. Winter. 1987. Anaerobic degradation of phenol in sewage sludge: Benzoate formation from phenol and CO_2 in the presence of hydrogen. Appl. Microbiol. Biotechnol. 25: 384-391.

Mathew, A. G. and H. A. B. Parpia. 1971. Food browning as a polyphenol reaction. Adv. Food Research 19: 75-145.

Neufeld, R. D., J. D. Mack and J. P. Strakey. 1980. Anaerobic phenol biokinetics. J. WPCF. 52: 2367-2377.

Pearson, F., C. Shium-Chung and M. Gavtier. 1980. Toxic inhibition of anaerobic biodegradation. J. WPCF 52: 473-478.

Schink, B. and N. Pfennig, 1982. Fermentation of trihydroxybenzenes by Pelobacter acidgallici gen. nov. sp. nov., a new strictly anaerobic, non-sporeforming bacterium. Arch. Microbiol. 133: 195-201.

Simpson, F. J., G. A. Jones and E. A. Wolin. 1969. Anaerobic degradation of some bioflavonoids by microflora of the rumen. Can. J. Microbiol. 15: 972-974.

Singleton, V. L. 1972. Common plant phenols other than anthocyanins contribution to coloration and discoloration. in: C. O. Chichester (ed.) Advances in Food Research Supplement 3 pp. 143-191.

Sjoblad, R. D. and J. M. Bollag. 1981. Oxidative coupling of aromatic compounds by enzymes from soil microorganisms. In: E. A. Paul and J. N. Ladd (eds.), Soil Biochemistry Vol. 5 pp. 113 - 152. Marcel Dekker, Inc., New York.

Suidan, M. T., W. H. Cross and M. Fong. 1980. Continuous bioregeneration of granular activated carbon during the anaerobic degradation of catechol. Prog. Wat. Tech. 12: 203-214.

CHAPTER 6

The Tannin Theory of Methanogenic Toxicity.

Accepted for publication in: Biological Wastes.

THE TANNIN THEORY OF METHANOGENIC TOXICITY

J. A. Field, S. Kortekaas and G. Lettinga

Dept. of Water Pollution Control
Wageningen Agricultural University
Bomenweg 2, 6703 HD Wageningen
The Netherlands

ABSTRACT - The tannin theory describes the effectiveness of tannic compounds as bacterial inhibitors based on their MW. The inhibition is postulated to result from the hydrogen bonding of the tannins with bacterial proteins (ie. enzymes). Oligomeric tannins are expected to be the most effective inhibitors. The oligomeric tannins form stronger hydrogen bonds with proteins as compared to their monomers. The high MW tannins are also reactive with proteins in the bulk solution; however, they are not able to penetrate to bacterial proteins. In this study, tannins were polymerized with autoxidation to colored compounds to investigate the role of tannin MW on methanogenic toxicity. The autoxidation of catechin, a tannin monomer, paralleled the expected toxicity as predicted by the tannin theory. The toxicity increased as it was polymerized to oligomeric tannins and decreased as the oligomers were converted to nontoxic high MW tannins. The autoxidation of green tea tannins, oligomeric tannins, did not lead to extensive polymerization reactions and no accumulation of high MW tannins were observed. Instead, the toxic green tea tannins were transformed to colored nontannic compounds of variable MW, which were nontoxic.

KEY WORDS - tannin, catechin, phenolic compounds, flavonoid compounds, anaerobic digestion, methanogenic bacteria, methanogenic toxicity, anaerobic biodegradability phenolic compounds, autoxidation, detoxification, HPLC tannins

1. INTRODUCTION

1.1. The Tannin Theory

The tannin theory model for methanogenic toxicity is based on the relationship found between tannin polymer size and quality as a tanning agent for the preparation of leather from hide. White (1957) recognizes tanning agents with maximal quality for the leather industry to be polar oligomeric phenols of 2 to 10 monomeric units in length (ie. MW = 500 to 3000). The hydrogen (H) bonding reactions with proteins (Figure 1) that are necessary for tanning are postulated to cause toxicity to bacteria because such interactions interfere with the functioning of enzymes (Gupta and Haslam, 1980; Haslam, 1974; White, 1957; Ladd and Butler, 1975; Loomis and Battaile, 1966; Strumeyer and Malin, 1969; Daiber, 1975; Tamir and Alumot, 1969). The polar monomeric phenols have limited H bonding capacity, while the

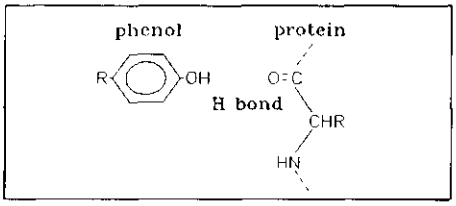
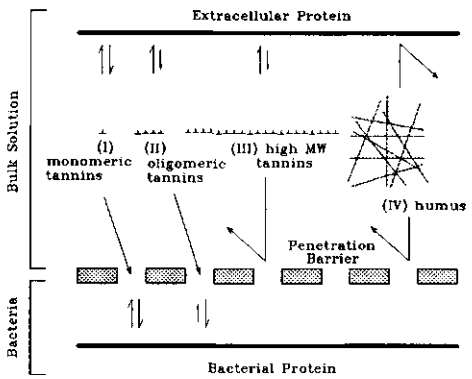


Figure 1. The hydrogen bond between phenol and proteins.

oligomeric tannins have a far superior H bonding capacity because of their ability to form multiple H bonds, called,"cross-linking" (Bate-Smith, 1973; Haslam, 1974; Loomis and Battaile, 1966). The reactivity of monomeric phenolics with proteins has been shown to increase as they are polymerized to oligomeric tannins (Chollot et al. 1961). Likewise we can expect that the toxicity of the oligomeric tannins is then far superior to their monomeric counterparts, as has been shown to be the case with metabolic enzymes (Boser, 1961; Firenzuoli et al., 1969), eubacteria (Singleton and Esau, 1969) and methanogenic bacteria (Field and Lettinga, 1987).

Tannins used for preparing leather must not exceed a maximum size (approx. 3000 g $mole^{-1}$) because the tannins must penetrate into the interfiber spacings of the hide proteins (White, 1957). Likewise, we can expect that a similar maximum size must not be exceeded for penetration to bacterial enzymes. Therefore we can recognize high MW tannins that are still good tannins in the sense that they react effectively with proteins available in the bulk solution but fail as tanning agents for preparing leather and fail as toxins to bacteria because in both cases they are too large for penetration. Polymers yet even larger than high MW tannins have no H bonding affinities towards proteins. Based on the protein precipitation data of Jones et al. (1976), the polymer size associated with a loss in reactivity towards soluble extracellular proteins is approximately 20,000 g $mole^{-1}$. In this case, perhaps there is no capacity for H bonding because the molecule has such an excessive size that the majority of the phenolic groups are buried too deep in the polymer to be effective. These very high molecular weight nontannic polymers can be called humus because they have many characteristics associated with humic compounds: darkly colored; phenolic; high MW; oxidative genesis; and poor biodegradability (White, 1957).

Evidence for the detoxification of tannins by their polymerization to high MW compounds is reported in the literature. The oxidative polymerization of apple tannins (procyanidins) with phenolase has been found to eliminate the inhibitory effect of the tannins on pectinase. The decrease in tannin toxicity was attributed to the high MW tannins formed (Verspuy and Pilnik, 1970). The antibiotic properties of wine are generally reported to be due to the wine tannins (anthocyanidins). Old wine aged for 82 years was less than one third as bactericidal as the younger wines, aged for only 9 to 10 years. The loss of antibiotic properties was suggested to be due to the slow polymerative oxidation of the tannins (ie. from oxygen



	(I)	(II)	(III)	(IV)
H Bond	weak	strong	strong	none
Penetration	yes	yes	none	none
Toxicity	low	high	none	none

Polymerization →
Humification →

Figure 2. Conceptual overview of the tannin theory.

leaking through the wood of the storage barrels), eventually polymerizing them to excessive sizes (Singleton and Esau, 1969). Generally, high MW phenolic compounds have not been observed to cause methanogenic toxicity. Humic acid isolated from peat was not found to cause toxicity to methane bacteria at 1 g L^{-1} (Brons et al., 1985) nor peat up to 16 g L^{-1} (Young and McCarty, 1981). Likewise, a highly oxidized soluble extract of melanin pigments was not toxic to methane bacteria (Lane, 1983).

A conceptual overview of the tannin theory is presented in Figure 2. The figure illustrates the theorized toxicity of tannins on either bacterial enzymes or free extracellular enzymes based on the expected H bonding strength as well as the penetration limitations. In this figure four categories of soluble polar phenolic compounds are recognized, the group (I), (II), (III) and (IV) compounds. These are; respectively, monomeric tannins, oligomeric tannins, high MW tannins and humus in increasing order of MW.

1.2. Objectives

Since the molecular weight plays such an important role in the behaviour of tannic compounds, the purpose of this study was to determine if coloration of tannic compounds by oxidative polymerization can lead to changes in the methanogenic toxicity that correlated to the basic trends recognized by the tannin theory. Catechin was chosen as a model compound since it is a typical monomeric unit of condensed tannins, procyanidins (flavonoids with dihydroxy rings), that are expected in certain forest industry wastewater that contain bark tannins (Field et al., 1988). Catechin also fulfills the structural requirements suggested to be necessary for the alteration of phenolic compound toxicity by autoxidation (Field and Lettinga, 1989). Green tea was chosen since its tannins have a similar flavonoid structure but are unique in that they have trihydroxy rings, which could potentially effect their behaviour during autoxidation. An additional objective was to determine if the coloration products of the autoxidations were in fact polymerized products.

1.3. Oxidative Polymerization of Tannic Compounds

The polymerization reactions of the tannins are illustrated in Figure 3 with classic examples of the products obtained by autoxidation or phenolase oxidation.

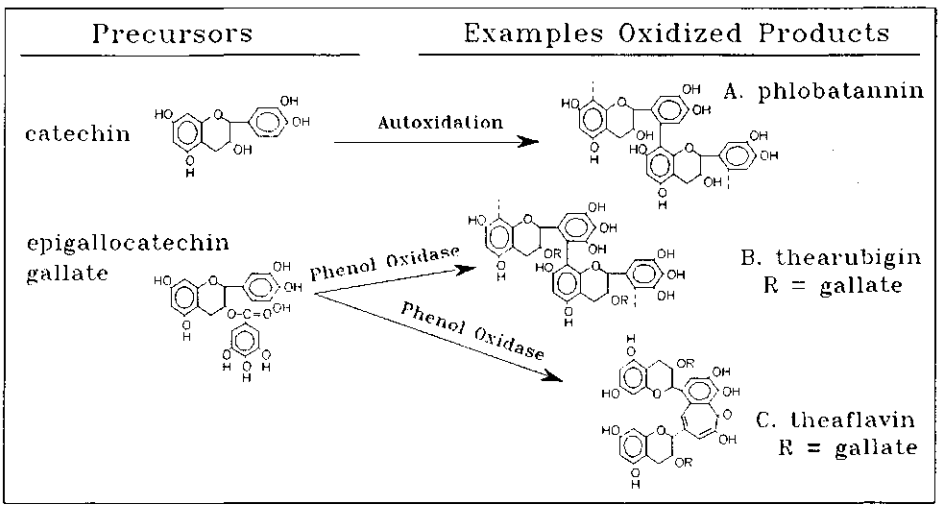


Figure 3. Oxidative polymerization of flavonoid tannin monomers. References: A. Hathway and Seakins, 1955 and 1957; B. Brown et al., 1969; C. Singleton, 1972.

2. MATERIALS AND METHODS

2.1. Analytical Methods

2.1.1. Standard Determinations

The UV absorbance, Color, COD, VSS and VFA determinations were described in the previous article (Field and Lettinga, 1989).

2.1.2. PVP Determination of Tannins

The determination of the total tannin concentration by adsorption on the insoluble polyamide, polyvinylpyrrolidone (PVP), was described previously (Field et al., 1988). In this study, the tannin concentration of the extracts were based on the COD and UV_{215} light absorbance units adsorbed on the PVP.

2.1.3. HPLC of Tannins and Oxidized Tannins

Part of the results presented in this article are based on the high performance liquid chromatography (HPLC) characterization of the tannic compounds. The technique utilized is a reverse phase system with a solvent gradient that runs from a highly aqueous solution to methanol. Several other research groups have utilized similar methods for the analysis of tannins (Wulf and Nagel, 1976; Rosten and Kissinger, 1982; Wilson, 1981; Lea, 1980; and 1982). Generally two factors play an important role in determining the retention times: (1) increasing MW can be associated with increasing retention time; (2) increasing apolarity can be associated with increasing retention time. Plant tannins in their unaltered form are mixtures of oligomers and each MW fraction may be composed of several compounds that have a mean retention time. This median value has been shown to increase with increasing MW of the oligomeric and highly polymeric condensed tannins isolated from apple juice and white wine (Wilson, 1981; Lea, 1980; and 1982). HPLC chromatograms of unoxidized and oxidized apple juice presented by Lea (1982) are shown in Figure 4. The unoxidized chromatogram illustrates the specific peaks of the colorless natural monomeric and oligomeric condensed tannins (procyanidins) which are the group (I) and (II) tannins of the tannin theory, respectively. After the oxidation these compounds disappeared to a large extent and there appeared a colored broad high MW peak. The broad peak indicates a heterogeneous mixture expected from a random polymerization process. The high MW peak eluted when the solvent gradient changed rapidly over to methanol. This peak is most likely associated with the group (III) and (IV) tannins of the tannin theory.

The hydrolyzable tannins do not have the same behaviour as the condensed tannins with the reversed phase HPLC method. Verzele and Delahaye (1983) observed that the oligomeric hydrolyzable tannins eluted when the solvent gradient changed mostly over to methanol, which coincided with the point where the high MW condensed tannins elute. Therefore, the aforementioned HPLC method for indicating the MW of tannins is limited to the condensed tannins.

The reversed phase high pressure liquid chromatographic method of characterizing condensed tannins was adapted from previously reported methods (Lea, 1982; and 1980; Wilson, 1981). The column used in this study was 200 mm x 3mm ID with C_{18} Chromsphere packing. A gradient was used with 4% acetate (v/v) in water (A) or in methanol (B). The A:B ratio was 98:2 at 0 minutes, 75:25 at 23 minutes and 2:98 at 33 through 45 minutes. The sample size was 0.02 mL. The total solvent flow was 0.6 mL per minute. The UV absorbance was detected at 280 nm. Under the conditions utilized, the elution of the broad high MW peak started at 28 minutes retention time. Recently we have introduced the concept of treating samples with polyvinylpyrrolidone to specifically remove the tannins and thereafter process a chromatogram of the non-tannin fraction (Field et al., 1988). This enables the possibility of distinguishing tannic and non-tannic peak areas. The total extract as well as the PVP treated extract are plotted together, the difference in peak area between the two represents the tannin peak area.

The four important categories of compounds that can be recognized in the HPLC chromatograms are the monomeric phenolic oxidation precursors (I) which are located by a standard, the oligomeric tannins (II) which are represented by newly formed tannic peak

area of less than 28 minutes, the high MW tannins (III) which are located in the tannic peak area of the high MW peak and high MW humus (IV) in the non-tannic peak area of the high MW peak.

The low MW tannin concentration (monomeric plus oligomeric) was estimated by taking the ratio of tannin peak area < 28 minutes : total tannin peak area from the HPLC chromatograms and multiplying it by the total PVP determined tannin concentration.

2.2. Autoxidation Phenolic Compounds

The phenolic compounds were autoxidized at high pH values (9 to 11.5) by aerating the phenolic stock solutions. The stock solutions were prepared fresh and with 250 mg L^{-1} ascorbic acid to prevent premature coloration. The pH was adjusted to the initial value with NaOH and the autoxidation was stopped by readjusting the pH to 6.5 with HCl. The stock solutions were than immediately diluted in the assay media and the assays were started after adding the sludge and flushing the head space with N_2.

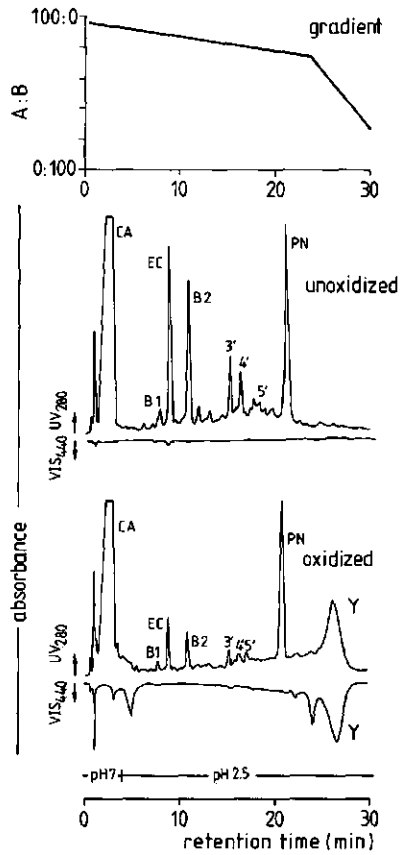


Figure 4. The HPLC of apple tannins in normal and oxidized apple juice as reported by Lea (1982). Procyanidin Tannins: (EC) epicatechin, a monomer; (B1, B2) procyanidin dimers; (3', 4', 5') oligomeric procyanidins; (Y) oxidatively polymerized procyanidins. Miscellaneous Monomeric Compounds: (CA) Chlorogenic acid; (PN) Phloridzin. (This Figure is a modified reprint with permission of the publisher, Elsevier Science Publishers B.V., Amsterdam).

2.3. Bioassays

The bioassay used in this study for determining the methanogenic toxicity are described in the previous study (Field and Lettinga, 1989). The specific assay conditions and control methanogenic activities are reported in Table 1 for each of the experiments in this study. On certain occasions, the method was modified, as described in the footnote of Table 1.

Table 1. The Assay Parameters of the Experiments Conducted in this Study.

Experiment			Assay+					
Number	Compound	Concentration* in Assay mg L^{-1}	Type**	Sludge VSS g L^{-1}	Buffer $NaHCO_3$ g L^{-1}	Duration++ days	Activity Control First (mg COD g^{-1}	Second VSS d^{-1})
1.	catechin	1535	2R[a]	1.38	1.0	21	318	868
2.	catechin	1433	P2R[a]	1.38	1.0	14	768	1002
3.	green tea	3370 (COD)	2R	1.38	3.0	8	357	831

* Concentration unoxidized phenolic compound (mg dry matter per liter unless COD is indicated) in assay media, the oxidized phenolic solutions were diluted in the same fashion as the unoxidized compound.

** Type of toxicity assay: Type 2R: First feeding with phenolic solution present. The second feeding was initiated after decanting the first feeding medium and replacing it with VFA medium not containing phenolic solutions (residual activity). Type P2R: The sludge was given a (1 week) prefeeding of VFA without phenolic solution. The prefeeding media was decanted and replaced with the phenolic solution containing first feeding media. The second feeding was the same as type 2R.

\+ The VFA spikes used in the experiments supplied 4 g COD L^{-1} (C_2:C_3:C_4 = 24:34:41 % of the COD).

++ Duration time of the first feeding, to indicate the exposure time prior to the second feeding which followed.

[a] The first feeding activity results of experiments 1 and 2 are reported for the initial 2 (to 3) days of the assay before unoxidized catechin degradation.

Table 2. Characteristics of Phenolic Stock Solutions Used in the Autoxidation Experiments.

Experiment		Phenolic Stock Solution					
		Untreated				Autoxidized	
		COD		UV			
Number	Compound	Total	Tannin*	Total	Tannin*	Max. Color** Total	Dilution Stock Solution in Assay Media
		mg COD L^{-1}		Abs 215nm, 1x		Abs 440nm, 11x	x
1.	catechin	3727	2539	197	181	1.569	1.43
2.	catechin	3481	3048	156	145	1.780	1.43
3.	green tea	4815	2338	254	196	0.646	1.43

* The PVP determinable tannins.

** The maximum color development by autoxidation, the color of the stock solutions before anaerobic treatment.

2.4. Materials

Catechin was obtained from Janssen Chimica. Chinese green tea extract was prepared by shaking 16 g dry tea per liter hot (60°) water (containing 250 mg L^{-1} ascorbic acid) for 3 hours. The characteristics of the stock phenolic solutions that were autoxidized and their dilution into the assay media are reported in Table 2.

3. RESULTS

3.1. Catechin Autoxidation

3.1.1. Changes in Catechin Characteristics

Catechin solutions were autoxidized for periods between 5 minutes and 22 hours during two separate experiments, labelled series 1 and 2. The catechin used in series 2 contained more impurities and was more susceptible to foaming compared to the catechin of series 1. During series 2, a lower intensity of aeration had to be applied. Consequently, the rate of coloration was slower than the rate obtained during series 1. The autoxidation increased both the anaerobic digestion stable color (340nm) of the media as well as the color (440nm) measured directly from the autoxidation solutions (Figure 5A). The color increased rapidly in the initial phase of autoxidation but the rate decreased as the autoxidation continued. While dramatic changes in the solution color were evident, very minor changes occurred with respect to the UV light absorbance and COD of the catechin solution (Figure 5A). This indicated that the colored products were not suspended solids but instead soluble products of the oxidation.

During the initial phases of the autoxidation no losses in the PVP determinable tannins were evident (Figure 5B), indicating that the initial color products formed were still reactive with PVP as catechin is. However as the autoxidation was continued to very high levels of color, a noticeable decrease in the PVP determinable tannins was evident (Figure 5B), which indicates the point when the polymerization had advanced so far that the compounds loose their tannic quality, called "detannification." The detannification begins when the first high MW tannins are polymerized to humus.

The PVP tannin determination can detect all compounds that have at least a minimal tanning capacity as represented by compounds with at least one cross-linking possibility via H bonds with the polyamide (Endres and Hörmann, 1963; Olsson and Samuelson, 1974). Figure 6 illustrates that single ring compounds like phloroglucinol with spread-out hydroxyl groups or compounds like catechin with multiple hydroxylated rings have the minimum tannin quality whereas other types of monomers do not. Unfortunately, the PVP method cannot detect the difference in tanning power that is expected between the monomeric tannin (ie. the catechin precursor) and the polymeric tannins formed by the autoxidation (oligomeric and high MW tannins). The difference in tanning power is indicated by the greater resistance of the polymeric tannins against desorption with polar organic solvents. Table 3 shows that a greater percentage of autoxidized tannins (ie. polymeric tannins) resist desorption from PVP when treated for one hour with methanol. The greater resistance against desorption of the polymeric tannins is expected due to their greater H bonding strength in comparison with a tannin monomer. The desorption of the tannins was not complete even in the case of catechin, therefore the difference in tanning strength could perhaps be better indicated with a stronger stripping solvent.

The fact that the autoxidation coloration truly caused polymerization reactions was confirmed by HPLC determinations of the autoxidized solutions recovered after different autoxidation periods (Figures 5B and 7). During the course of the autoxidation, the catechin peak decreased and was paralleled by the formation of oligomeric tannins that upon further autoxidation were polymerized to the high MW peak. The final stage of polymerization, when high MW tannins are converted to humus is indicated by the increase of material in the high MW peak which is not adsorbed onto PVP.

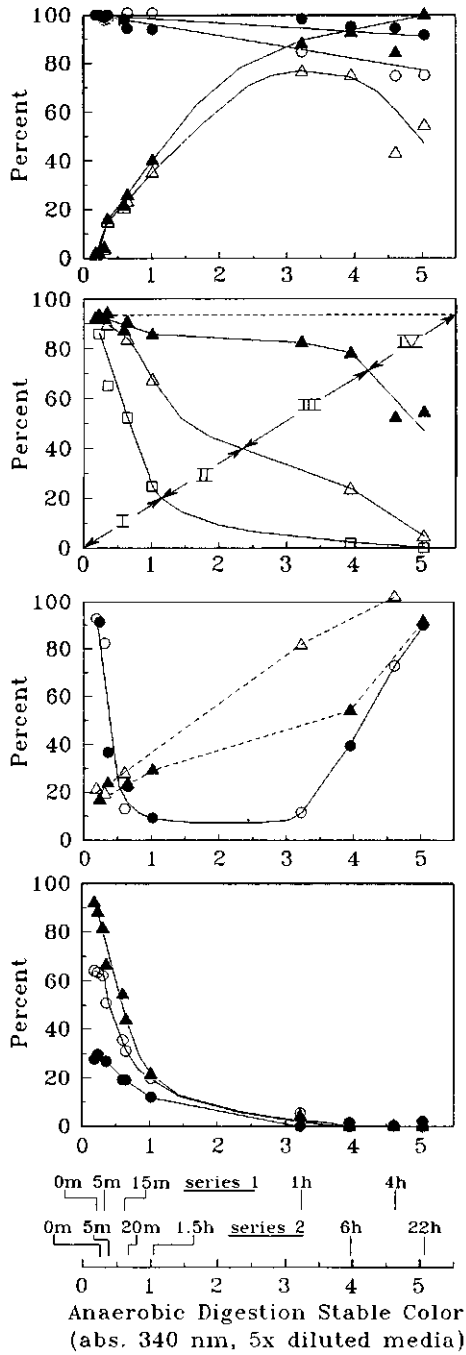


Figure 5. The autoxidation of catechin. Where: COLTf = maximum color developed by autoxidation, CODT0 and UVT0 = COD and UV of the unoxidized stock solution (Table 2), and UVTx = total UV of the sample. Assay conditions: experiments 1 and 2 in Table 1 for series 1 and 2, respectively.

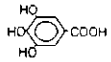
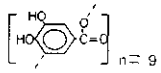
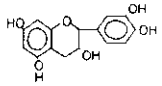
Compound	Structure	Adsorption by PVP (%)
phenol		22.6
catechol		29.6
pyrogallol		38.1
phloro-glucinol		82.5
gallic acid		15.2
gallotannic acid		96.2
caffeic acid		18.1
L dopa		18.3
catechin		98.5

Figure 6. The adsorption of selected phenolic compounds (1 g L^{-1}) on PVP (using the PVP determinable tannin method). The minimal tanning quality is indicated by a high level (> 80%) of adsorption on the PVP.

Table 3. The Desorption of Catechin and Catechin Autoxidation Tannin Products from PVP by Methanol.

Autoxidation Time m = minutes h = hours	Adsorbed Tannins Desorbed from PVP by Methanol* % all tannins adsorbed
0 m	27.9
5 m	25.0
15 m	18.8
1 h	11.4
4 h	10.4

* 0.1 g PVP with adsorbed tannins treated with 7ml methanol for 1 h. This method was not very exhaustive because methanol is a weak stripping solvent. The tannins were measured with UV light absorbance at 280 nm because methanol has absorbance at 215 nm.

The samples tested represented all stages of the autoxidation, these included points where the group (I), (II), or (III) tannins were dominant. Therefore, the catechin autoxidation series is a continuum of analogous tannic compounds that vary only in MW which gives an ideal bases for testing the validity of the tannin theory for methanogenic toxicity.

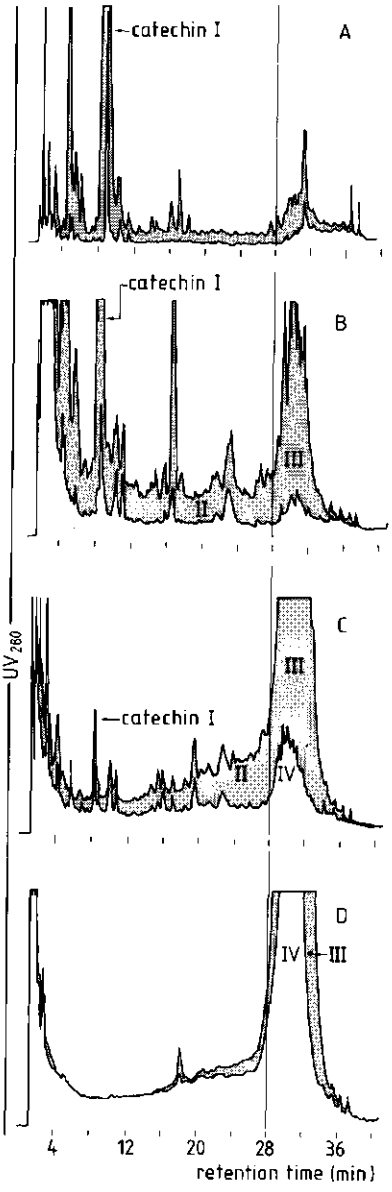


Figure 7. The HPLC of catechin after (A) 0, (B) 1.5, (C) 6 and (D) 22 hours of autoxidation (starting pH 11). The top chromatogram was the total UV of the sample. The bottom chromatogram was the UV remaining after removing the PVP determinable tannins. The shaded area between the two chromatograms represents the tannin peak area.

3.1.2. Changes in Catechin Toxicity

The second feeding methanogenic sludge activity, that survived a two to three week exposure to the autoxidized catechin solutions, is shown in part C of Figure 5. The second feeding sludge activity followed the expected pattern according to the tannin theory. During the initial stages of autoxidation, when the monomeric tannin was polymerized to oligomeric tannins, an increased toxicity (decreased sludge activity) was observed, corresponding to the toxification phase of the tannin theory. When a toxic autoxidized sample was treated with PVP, a complete removal of the toxicity was evident (Figure 8) indicating that the tannins were responsible for the toxicity. At a later stage of autoxidation, a continuation of the autoxidation resulted in a decreased toxicity, corresponding to the detoxification phase of the tannin theory. At the end of the autoxidation, only about half the original tannin content was detannified and yet there was no residual toxicity remaining. This clearly demonstrates the existence of nontoxic high MW tannins, since these were the only tannins present in the most autoxidized solution. Therefore, the detoxication corresponded to the conversion of the oligomeric tannins to high MW tannins, as would be expected from a lowered penetration ability to the bacterial enzymes.

Certain changes in the catechin and autoxidized catechin toxicity were evident if the sludge activity during the initial three days of exposure (first feeding) is compared with the sludge activity in the second feeding, following two to three weeks of exposure (Figure 5C). During the initial period of exposure, catechin expressed an inhibitory effect on the sludge activity. After three days of anaerobic digestion the monomeric flavonoid, catechin, was biologically cleaved. Directly afterwards, complete reversal of the catechin toxicity was evident (Figure 8). The anaerobic degradation was certainly an important factor contributing to the reversal of the monomeric tannin toxicity. However, this behaviour is not the same as that of oligomeric tannins previously studied. Gallotannic acid, which like catechin is degraded anaerobically after several days of anaerobic digestion (Field and Lettinga, 1987), caused a residual inhibition to the sludge which was still evident several weeks after the biodegradation. Therefore catechin is not a strong inhibitor which can impart a residual damage to the sludge as the oligomeric tannins can. This may be due to the fact that monomeric tannins have a weaker H bonding with the proteins compared to the oligomeric tannins.

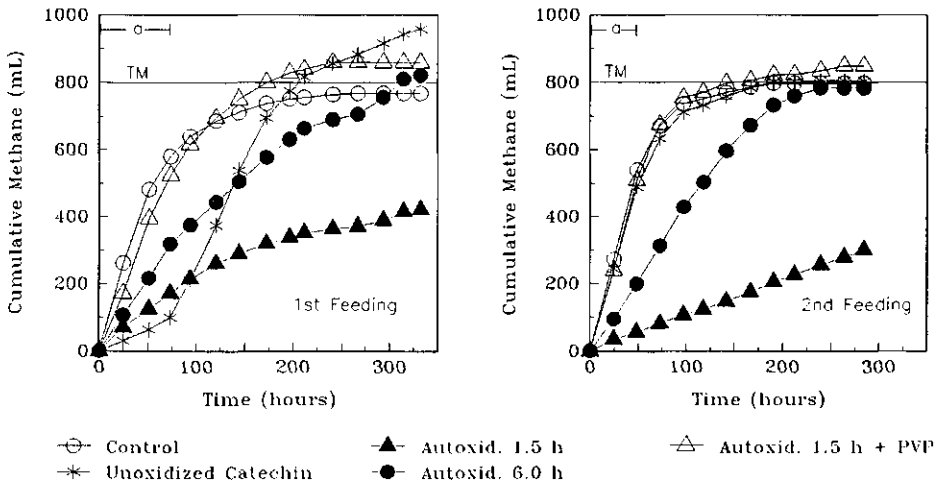


Figure 8. The cumulative methane production during the methanogenic toxicity assay of autoxidized catechin (experiment 2). Where: a = the activity period used for the reported activities in Figure 5; TM = the theoretical maximum CH_4 production based on the VFA supplied to the assay.

The oligomeric tannic products of the catechin autoxidation were effective methanogenic toxins that left a residual sludge toxicity following a two to three week exposure (Figure 5C). While the oligomeric products at various stages of the autoxidation caused similar levels of inhibition, a large difference in their initial toxicities was evident during the first three days of anaerobic digestion (Figure 5C) during the first feeding. During this initial exposure period, the early stage oligomers (small oligomers) expressed more toxicity than the late stage oligomers, ie. those which have a border-line size between oligomeric and high MW tannins. The delayed toxicity response of the anaerobic sludge to the late stage oligomers clearly demonstrates that penetration is an important factor. Those compounds that have a size just slightly less than the maximum effective size are exactly those compounds which are expected to take a long time to penetrate.

3.1.3. Changes in Catechin Biodegradability

The disappearance of PVP determinable tannins and media UV_{215} absorption as well as the recovery of phenolic COD as VFA and CH_4 during anaerobic digestion were found to decrease with the catechin concentration still remaining after autoxidation (Figure 5D). This indicated that the autoxidized products were not degraded and that only catechin was transformed anaerobically.

The complete loss of PVP determinable tannins by anaerobic digestion of unoxidized catechin, indicates that the catechin is cleaved, since the diphenolic structure is an essential attribute to the tannic quality of this monomer. The A ring intermediate was most likely phloroglucinol as observed by other researchers (Simpson et al., 1969; Brown, 1977). Phloroglucinol was most likely immediately acidified to VFA and CH_4 after cleavage as was the case in similar experiments supplied with phloroglucinol (Field and Lettinga, 1989). The residual UV_{215} absorption and incomplete acidification of the phenolic compounds indicate that the B ring remained in the media as an unidentified nontannic phenolic degradation intermediate. While it is known that catechol (benzene with the same substitution as the B ring) is not degraded under similar experimental conditions, the nontoxicity of the phenolic intermediates would indicate that catechol was not predominant. Catechol would have imparted a small level of toxicity, considering the results of previous experiments conducted under similar conditions (Field and Lettinga, 1989). In addition to catechol (Balba and Evans, 1980), 3 phenylpropionic acid and phenylacetic acid substituted intermediates are known to occur as the B ring intermediate during the anaerobic degradation of flavonoids (Brown, 1977; Lane, 1980; Krumholz and Bryant, 1986).

3.2. Green Tea Extract Autoxidation

Green tea was chosen, because it is known to contain high levels of polyphenolic compounds. Vuataz and Brandenberger (1961) found that 80% of green tea extractable matter is composed of polyphenols. Two tannic monomers, epigallocatechin gallic acid and epicatechin gallic acid were identified and accounted for about 25 and 5% of all the polyphenols, respectively. These monomeric compounds should be expected to behave as oligomeric tannins since they are composed of three hydroxylated rings.

3.2.1. Changes in Green Tea Characteristics

The results obtained from the autoxidation of green tea extracts are outlined in Figure 9. During the autoxidation, rapid coloration (based on 440nm) occurred, while little losses in COD were observed. The UV_{215} absorbance was decreased by nearly half and most of the PVP determinable tannins were eliminated. This indicated drastic modifications of the aromatic structures resulted from the autoxidation.

The HPLC chromatograms illustrate a few prominent tannin peaks with distinct retention times (Figure 10) in the unoxidized tea extract. At the early stages of the autoxidation, these peaks decrease, yielding a heterogeneous mixture of tannins that could be detected at almost all retention times. At the end of autoxidation, the tannin peak area was largely replaced by non-tannic peak area. There was some accumulation of compounds in the high MW humic peak area. These might correspond to thearubigins, which are high MW

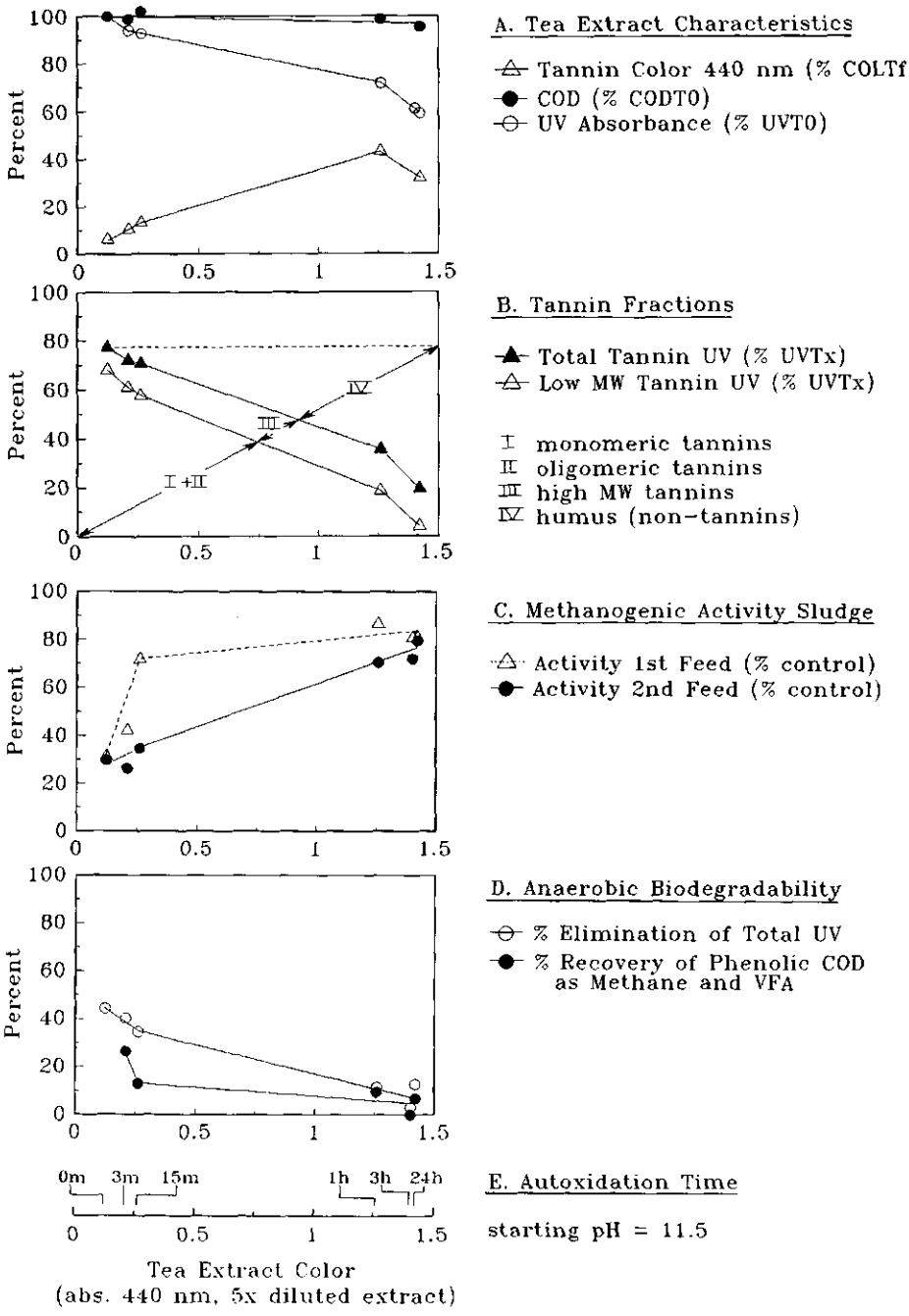


Figure 9. The autoxidation of green tea. Where: COLTf = maximum color developed by autoxidation, CODT0 and UVT0 = COD and UV of the unoxidized stock solution (Table 2), and UVTx = total UV of the sample. Assay conditions: experiment 3 in Table 1

polyphenols recovered from black tea (Brown et al., 1969; Crispin et al., 1968; Millin et al., 1969). However, for the most part, the autoxidation eliminated the tannins without drastically modifying the MW of the polyphenols. This may be due to the formation and subsequent destruction of theaflavins which is also reported to occur during the auto- and enzymatic oxidations of tea (Singleton, 1972; Mathew and Parpia, 1971).

3.2.2. Changes in Green Tea Toxicity

The unoxidized green tea caused methanogenic inhibition (Figure 9) that was still evident in the second feeding. Unlike the catechin monomer, which was only temporarily inhibitory, green tea tannins have an inhibition characteristic of oligomeric tannins. During the

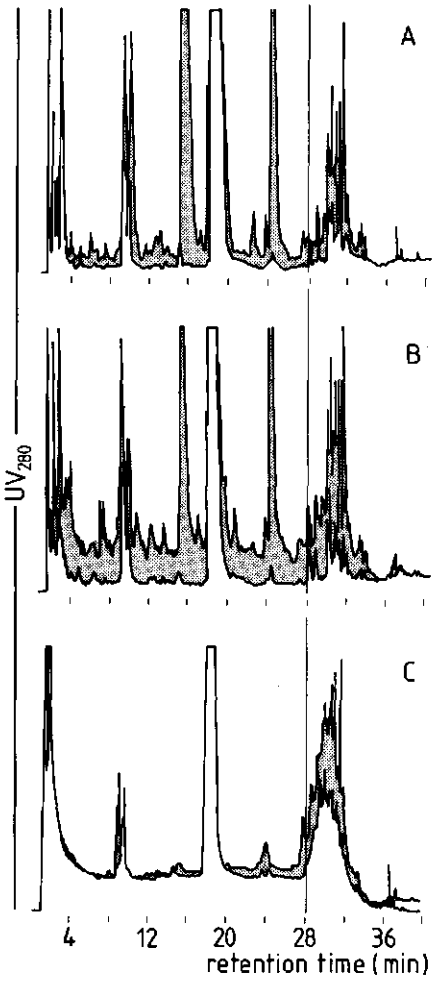


Figure 10. The HPLC of green tea extract after (A) 0, (B) 0.25 and (C) 24 hours of autoxidation (starting pH 11). The top chromatogram was the total UV of the sample. The bottom chromatogram was the UV remaining after removing the PVP determinable tannins. The shaded area between the two chromatograms represents the tannin peak area.

autoxidation, the detoxification of green tea paralleled the decrease in total tannin content. Since only part of the tannins were converted to high MW compounds, the detoxification of the green tea by autoxidation cannot be completely due to polymerization. The destruction of tannin structure seems to also have been involved.

3.5.3. Changes in Green Tea Biodegradability

During the 8 days of the first feeding, unoxidized green tea phenolics were biologically modified and partially converted to VFA and CH_4. This is illustrated in Figure 9D by the disappearance of UV_{215} absorbance and recovery of green tea COD as VFA and CH_4. The colored detoxified products of tea, in contrast were degraded to a much lesser extent.

4. DISCUSSION

4.1. Methanogenic Toxicity

The methanogenic toxicity of polar phenolic compounds depends on the tannic quality of these compounds and their capacity to penetrate. An important factor governing the tannin quality and penetration ability is the polymer size (ie. MW) of the tannic compounds. The relationship between tannin toxicity and MW has been outlined by the tannin theory. In this study, we have found that polymerizing phenolic compounds with oxidative reactions to colored compounds caused changes in methanogenic toxicity that paralleled the expected toxicity as predicted by the tannin theory. This was most clearly demonstrated by autoxidizing catechin, a tannin monomer, to tannins of increasing MW. Essentially the ideas expressed by White (1957) with regard to the tannin size ranges which are most effective for the preparation of leather from hide are applicable for understanding those tannin compounds which are the most effective inhibitors of methanogenesis. In both cases, the oligomeric tannins are the most effective. They constitute the best compromise between being large enough for effective tanning quality and being small enough for penetration.

Unlike catechin (dihydroxy rings), the autoxidation of green tea tannins (trihydroxy rings) did not cause extensive polymerization to high MW compounds. The autoxidation; however, did largely transform the tea tannins to colored oxidation products. These products did not have tannic qualities and they were nontoxic.

Phenolic compounds present in agricultural are susceptible to oxidative modifications during short exposures to air which will have an impact on their methanogenic toxicity. In cases where the predominant phenols are monomeric tannins, than the oxidative polymerization leads to oligomeric tannins, which would increase the wastewater toxicity. On the other hand, in cases where the predominant tannins are oligomers, than the initially toxic wastewater could potentially be detoxified by applying the concepts of the tannin theory. In this regard, oxidative polymerization can be applied to transform the toxic oligomers to nontoxic high MW tannins and humic compounds.

4.2. Anaerobic Biodegradability

The colorless phenolic compounds tested in this study were partially degraded. The flavonoid structure was cleaved and the A ring corresponding to phloroglucinol was converted to VFA and CH_4 while the B ring remained in the media and was not identified. The colored products of phenolic oxidation were not degraded anaerobically. These compounds are analogous to humic compounds, since they are the poorly degradable colored products of oxidatively modified phenolic compounds.

5. Literature Cited

Balba, T. M. and W. C. Evans. 1980. The methanogenic biodegradation of catechol by a microbial consortium: evidence for the production of phenol through cis-benzenediol. Biochem. Soc. Trans. 8: 452-453.

Bate-Smith, E. C. 1973. Haemanalysis of tannins: the concept of relative astringency. Phytochemistry 12: 907-912.

Boser, H. 1961. Modellversuche zur beeinflussing des zellstoffweschsel durch pflanzeninhaltsstoffe, insbesondere flavonoide. Planta Med. 9: 456-465.

Brons, J. B., J. A. Field, W. A. C. Lexmond and G. Lettinga. 1985. Influences of humic acids on the hydrolysis of potato protein during anaerobic digestion. Agric. Wastes 13: 105-114.

Brown, A. G., W. B. Eyton, A. Holmes and W. D. Ollis. 1969. The identification of the thearubigins as polymeric proanthocyanidins. Phytochemistry 8: 2333-2340.

Brown, J. P. 1977. *Role of gut bacterial flora in nutrition and health: A review of recent advances in* bacteriological techniques, metabolism and factors effecting flora composition. Critical Reviews in Food Science and Nutrition 8: 229-336.

Chollot, B., L. Chapon and E. Urion. 1961. Polyphenols and proteins as precursors of peroxidase haze formation. Proceedings of the European Brewery Convention, Vienna, 1961 pp. 334 - 350. Elsevier Publ. Co, Amsterdam.

Crispin, D. J., R. H. Payne and D. Swaine. 1968. Analysis of the pigments of black tea extracts by chromatography on acetylated Sephadex. J. Chromatog. 37: 118-119.

Daiber, K. H. 1975. Enzyme inhibition by polyphenols of sorghum grain and malt. J. Sci. Food Agric. 26: 1399-1411.

Endres, H. and H. Hörmann. 1963. Präparative und analytische Trennung organischer Verbindungen durch Chromatographie an Polyamid. Angew. Chem. 75(6): 288-294.

Field, J. A. and G. Lettinga. 1987. The methanogenic toxicity and anaerobic degradability of a hydrolyzable tannin. Wat. Res. 21: 367-374.

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

Field, J. A. and G. Lettinga. 1989. The effect of oxidative coloration on the methanogenic toxicity and anaerobic biodegradability of phenols. Biological Wastes In Press.

Firenzuoli, A. M., P. Vanni and E. Mastronuzzi. 1969. *The effect of some aromatic compounds on pure enzymes* and their subsequent reactivation by PVP and Tween 80. Phytochemistry 8: 61-64.

Gupta, R. K. and E. Haslam. 1980. Vegetable tannins structure and biosynthesis. in: J.H. Hulse (ed.), Polyphenols in Cereals and Legumes pp. 15-24. Int. Develop. Research Centre, Ottawa, Canada.

Haslam, E. 1974. Polyphenol-protein interactions. Biochem J. 139: 285-288.

Hathway, D. E. and J. W. T. Seakins. 1955. Autoxidation of catechin. Nature, 176: 218.

Hathway, D. E. and J. W. T. Seakins. 1957. Enzymatic oxidation of catechin to a polymer structurally related to some phlobatannins. Biochem. J., 67: 239.

Jones, W. T., R. B. Broadhurst and J. W. Lyttleton. 1976. The condensed tannins of pasture legume species. Phytochemistry 15: 1407-1409.

Krumholz, L. R. and M. P. Bryant. 1986. Eubacterium oxidoreducens sp. nov. requiring H_2 or formate to degrade gallate, pyrogallol, phloroglucinol and quercetin. Arch. Microbiol. 144: 8-14.

Ladd, J. N. and J. H. A. Butler. 1975. Humus-enzyme systems and synthetic organic polymer-enzyme analogs. in: Paul, E. A. and A. D. McLaren (eds.), Soil Biochemistry, Marcel Dekker, Inc., NY. pp. 143-193.

Lane, A. G. 1980. Production of aromatic acids during anaerobic digestion of citrus peel. J. Chem. Tech. Biotechnol. 30: 345-350.

Lane, A. G. 1983. Anaerobic digestion of spent coffee grounds. Biomass 3: 247-268.

Lea, G. H. A. 1980. Reversed-phase gradient high performance liquid chromatography of procyanidins and their oxidation products in ciders and wines, optimised by Snyder's procedures. J. Chromatogr. 194: 62-68.

Lea, G. H. A. 1982. Reversed-phase high performance liquid chromatography of procyanidins and other phenolics in fresh and oxidising apple juices using a pH shift technique. J. Chromatogr. 238: 253-257.

Loomis, W. D. and J. Battaile. 1966. Plant phenolic compounds and the isolation of plant enzymes. Phytochemistry, 5: 423-438.

Mathew, A. G. and H. A. B. Parpia. 1971. Food browning as a polyphenol reaction. in: C. O. Chichester, E. M. Mrak and G. F. Stewart (eds.) Adv. Food Research. Vol 19. Academmic Press, N.Y. pp. 75-145.

Millin, D. J., D. Swaine and P. L. Dix. 1969. Separation and classification of the brown pigments on aqueous infusions of black tea. J. Sci. Food Agric. 20: 296-302.

Olsson, L. and O. Samuelson. 1974. Chromatography of aromatic acids and aldehydes and phenols on crossed-linked polyvinylpyrrolidone. J. Chromatog. 93: 189-199.

Rosten, A. and T. Kissinger. 1982. Liquid chromatographic determination of phenolic acids of vegetable origin. J. Liquid Chromatog. 5(Suppl. 1): 75-103.

Simpson, F. J., G. A. Jones and E. A. Wolin. 1969. Anaerobic degradation of some bioflavonoids by microflora of the rumen. Can. J. Microbiol. 15: 972-974.

Singleton, V. L. and P. Esau. 1969. Phenolic substances in grapes and wine, and their significance. in: C. O. Chichester (ed.) Advances in Food Research, Supplement 1. Academic Press, NY. pp. 1-281.

Singleton, V. L. 1972. Common plant phenols other than anthocyanins contribution to coloration and discoloration. in: C. O. Chichester (ed.) Advances in Food Research Supplement 3 pp. 143-191.

Strumeyer, D. H. and M. J. Malin. 1969. Identification of the amylase inhibitor from seeds of Leoti sorghum. Biochim. Biophys. Acta. 184: 643-645.

Tamir, M. and E. Alumot. 1969. Inhibition of digestive enzymes by condensed tannins from green and ripe tannins. J. Sci. Food Agric. 20: 199-202.

Verspuy, A. and W. Pilnik. 1970. Recovery of apple juice by pulp fermentation II: shortening the fermentation time. Flussiges Obst. 37: 518-519.

Verzele, M. and P. Delahaye. 1983. Analysis of tannic acids by high-performance liquid chromatography. J. Chromatog. 268: 469-476.

Vuataz, L. and H. Brandenberger. 1961. Plant phenols III: separation of fermented and black tea polyphenols by cellulose column chromatography. J. Chromatog. 5: 17-31.

White, T. 1957. Tannins - their occurence and significance. J. Sci. Food Agric. 8: 377-384.

Wilson, E. L. 1981. High-pressure liquid chromatography of apple juice phenolic compounds. J. Sci. Food Agric. 32: 257-264.

Wulf, L. W. and C. W. Nagel. 1976. Analysis of phenolic acids and flavonoids by high-pressure liquid chromatography. J. Chromatog. 116: 271-279.

Young, L. Y. and P. L. McCarty. 1981. Heat treatment of organic materials for increasing anaerobic biodegradability. in: Wise, L. W. (ed.) Fuel Gas Production from Biomass: Volume 2. pp. 134-176. CRC Press, Boca Raton, Florida, USA.

CHAPTER 7

The Effect of Autoxidation on the Methanogenic Toxicity and Anaerobic Biodegradability of Pyrogallol.

Accepted for publication in: Biological Wastes.

The Effect of Autoxidation on the Methanogenic Toxicity and Anaerobic Biodegradability of Pyrogallol.

J. A. Field, S. Kortekaas and G. Lettinga

Dept. of Water Pollution Control
Wageningen Agricultural University
Bomenweg 2, 6703 HD, Wageningen
The Netherlands

ABSTRACT - In a previous study, catechin (a condensed tannin monomer) was polymerized by autoxidation treatments. The resulting oligomeric tannins were responsible for the methanogenic toxicity observed in the autoxidized catechin solutions (Field et al., 1989). In this study, the autoxidation of pyrogallol (a hydrolyzable tannin monomer) did not cause extensive polymerization. Initially some polymerization occurred producing toxic intermediates that were later destroyed by a destructive type of oxidation caused by prolonged autoxidation treatments. The first intermediate formed, purpurogallin (a dimer), caused a high level of toxicity to both the methanogenic activity and to the anaerobic degradation of pyrogallol. Since purpurogallin is a highly toxic autoxidation product that lacks tannic features, the changes in methanogenic toxicity induced by the autoxidation of pyrogallol cannot be estimated by changes in the oligomeric tannin concentration.

Regardless of the reactions that take place during the autoxidation of tannin monomeric derivatives, the initial reactions can potentially lead to colored products of increased methanogenic toxicity. These are later detoxified by prolonged autoxidation, either by polymerization to high MW compounds (with condensed tannin model compounds), or by destruction of the initially toxic intermediates to low MW compounds (with hydrolyzable tannin model compounds).

1. INTRODUCTION

Monomeric phenolic compounds are susceptible to oxidative alterations to colored products during short periods of exposure to air. These alterations can potentially affect the methanogenic toxicity of phenolic wastewaters intended for anaerobic treatment. The autoxidation of dihydroxy ring type phenols like catechin, a condensed tannin monomer, causes polymerization. Initially, methanogenic toxic oligomeric tannins are formed, but these are later polymerized further to nontoxic compounds of high MW. The tannin theory (Field et al., 1989) is applicable to describing the modifications in the methanogenic toxicity that result from the alterations in MW by polymerization. However with trihydroxy ring phenols, the oxidative reactions are not entirely polymerative, and the resulting products do not necessarily have tannic structures. Nonetheless, these modifications may be of great importance, since rather strong methanogenic toxins were produced by short autoxidation of the hydrolyzable tannin monomer model compound, pyrogallol (Field and Lettinga, 1989). The objective of this work was to study the methanogenic toxic products of pyrogallol autoxidation in more detail.

2. METHODS

The methods utilized for the measurement of COD, UV absorbance, color and tannins; the HPLC characterization of phenolic solutions; and the autoxidation method have been described in previous publications (Field and Lettinga, 1989; Field et al., 1989). The tannin

determination was based on the COD and UV of the phenolic stock solution which was adsorbed on an insoluble polyamide, polyvinylpyrrolidone (PVP) as described in detail in Field et al. (1988).

The general method utilized during the bioassays to determine the methanogenic toxicity of the phenolic solutions was described in the previous publications (Field and Lettinga, 1989; Field et al., 1989). In this study, granular sludge was first prefed for one week with 4 g COD L^{-1} of stock VFA solution to adapt the sludge to the VFA substrate. At the end of the prefeeding period, the supernatent was decanted, thereafter phenolic compounds were added together with 4 g COD L^{-1} VFA to initiate the first feeding of the toxicity assay. After two weeks of exposure to the phenolic medium, the supernatant was decanted and replaced with a 4 g COD L^{-1} VFA solution in order to determine the residual activity of the sludge during the second feeding of the toxicity assay in the absence of the phenolic solution. The specific conditions during the assays are reported in Table 1. Two experiments were conducted. In the first experiment the toxic effects of autoxidizing pyrogallol were examined. The characteristics of the pyrogallol stock solution are reported in Table 1. In the second experiment, the toxicity of purpurogallin was examined at various concentrations. The purpurogallin stock solutions were prepared in demineralized water, as they had lower solubility in tap water (presumably due to the interactions with Ca^{2+}).

The phenolic compounds, pyrogallol and purpurogallin were obtained from Janssen Chimica (Tilburg, The Netherlands).

Table 1. The Assay Parameters of the Experiments Conducted in this Study and the Pyrogallol Stock Solution Characteristics.

ASSAY CONDITIONS						PYROGALLOL STOCK SOLUTION					
Experiment[a]	Conc.[b] in assay	Sludge VSS	Buffer $NaHCO_3$	Activity Control		COD		UV 215nm		Max.[c] Color	Dilution factor
				First	Second	total	tannin	total	tannin		
										440nm	
#	mg L^{-1}	g L^{-1}	g L^{-1}	mg COD g^{-1}	VSS d^{-1}	mg COD L^{-1}		1cm,1x		1cm,11x	in assay
1.	1385	1.38	1.0	768	999	4223	1579	198	72	2.113	2.0 X
2.	25 to 200	1.50	1.0	771	1004						

[a] Experiment 1 = autoxidation series of pyrogallol; Experiment 2 = concentration series of unoxidized purpurogallin.

[b] For experiment 1 the concentration of unoxidized pyrogallol (mg dry matter per liter) in assay media is shown, the autoxidized pyrogallol solutions were diluted in the same fashion as the unoxidized pyrogallol solution. For experiment 2 the range of purpurogallin concentrations tested (mg dry matter per liter) are shown.

[c] the maximum color (based on absorbance at 440 nm) developed by autoxidation.

3. RESULTS

3.1. Changes in Pyrogallol Characteristics

The data collected at various stages of the pyrogallol autoxidation are summarized in Figure 1. During the initial stages of the autoxidation, the formation of color was associated with the conversion of pyrogallol to its first autoxidation intermediate, purpurogallin (Figure 1A, 2A and 2B). Associated with the formation of purpurogallin was a precipitate. It was first evident after 10 minutes of autoxidation when the pH of the solution was dropped to 7 by addition of HCl (ie. in order to render the solutions suitable

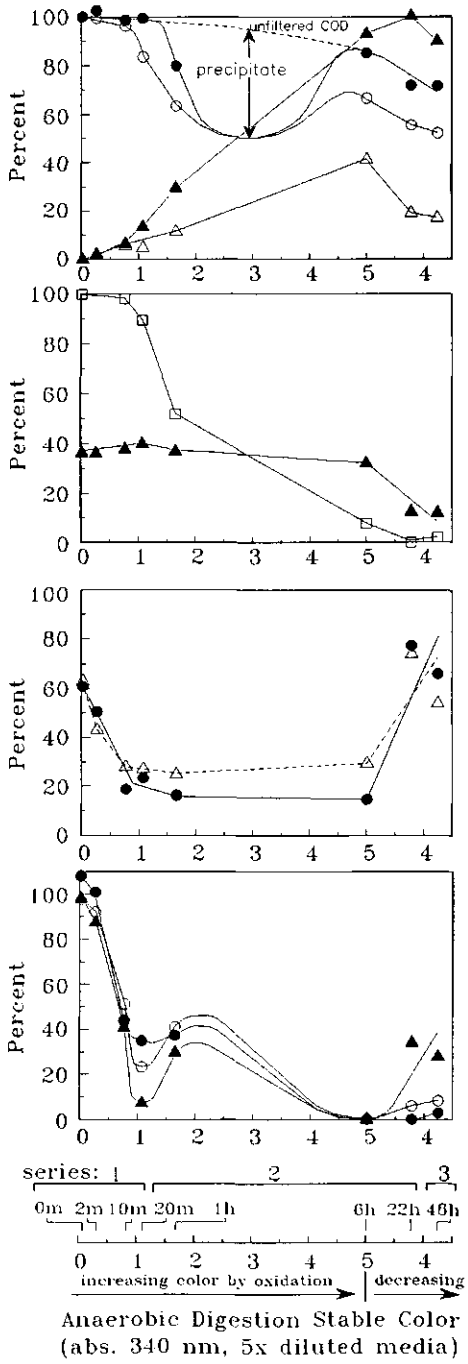


Figure 1. The autoxidation of pyrogallol. Where: COLTf = maximum color developed by autoxidation, CODT0, UVT0 and PYRTO = COD, UV and pyrogallol of the unoxidized stock solution (Table 1), and UVTx = total UV of the sample. Assay conditions: experiment 1 in Table 1. Note: the data are reported for the paper filtered solutions.

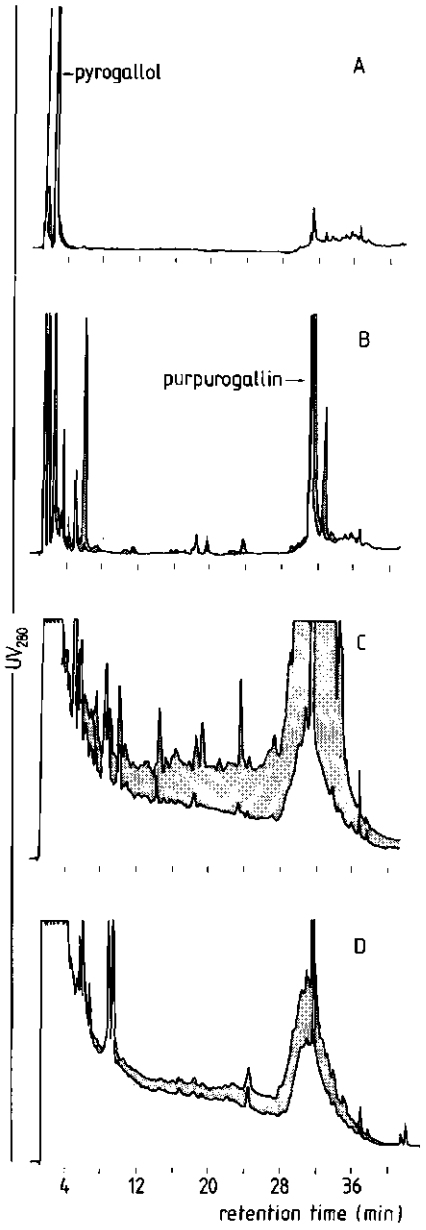


Figure 2. The HPLC of pyrogallol after (A) 0, (B) 0.17, (C) 6 and (D) 48 hours of autoxidation (starting pH 9 for B, 11 for C and 11.5 for D). The top chromatogram was the total UV of the sample. The bottom chromatogram was the UV remaining after removing the PVP determinable tannins. The shaded area between the two chromatograms represents the tannin peak area.

for the bioassays). A purpurogallin standard also had the same poor solubility in water at a neutral or acidic pH. The large decreases in UV and COD of the filtered solutions from 10 minutes to 1 hour of autoxidation are due to the formation of the purpurogallin precipitate rather than oxidative destruction of the phenolic compounds (Figure 1A). Eventually, the precipitate accumulated to up to approximately 50% of the original pyrogallol COD when the oxidation was stopped (pH lowered to 7) at time intervals between 1.5 and 3 hours (data not shown).

When the autoxidation was continued to 6 hours, the precipitate was no longer evident. Most of the purpurogallin was further oxidized to soluble products. These products were present in the HPLC chromatogram as nontannic peak area with a low retention time or as tannic peak area at high retention times (Figure 2C). The estimation of high MW tannins by assuming they occupy the peak area of more than 28 minute retention time, as was the case with catechin (Field et al., 1989), was not applicable for the autoxidation products of pyrogallol. Purpurogallin, a dimer, had a retention time of 31 minutes with the HPLC procedure. Therefore, it is conceivable that other products of the autoxidation with low MW also occupied the peak area at high retention times.

When the autoxidation was continued beyond 6 hours, a destructive effect on the intermediate autoxidation products was evident. A decrease in color, COD and UV_{215} was observed and most of the HPLC peak area was present at very low retention times (Figure 2D), which indicates polar low MW compounds.

Pyrogallol was not a good tannin since it was only partially (36%) adsorbed by PVP. Its first autoxidation product, purpurogallin was adsorbed by PVP to the same extent. In fact during the first 6 hours of the autoxidation, there was no significant change in the percentage of the phenolic compounds adsorbed by PVP (tannins) (Figure 1B). Since between 1 and 6 hours of autoxidation there was a considerable loss in pyrogallol and purpurogallin, the PVP determinable tannins remaining are attributable at least in part to some tannic compounds (with a high percentage adsorbance by PVP) as is evident in the HPLC plots (Figure 2C). During the destructive phase of the autoxidation (beyond 6 hours), the PVP determinable tannin concentration decreased. The tannic compounds formed were eventually destroyed (Figure 2D).

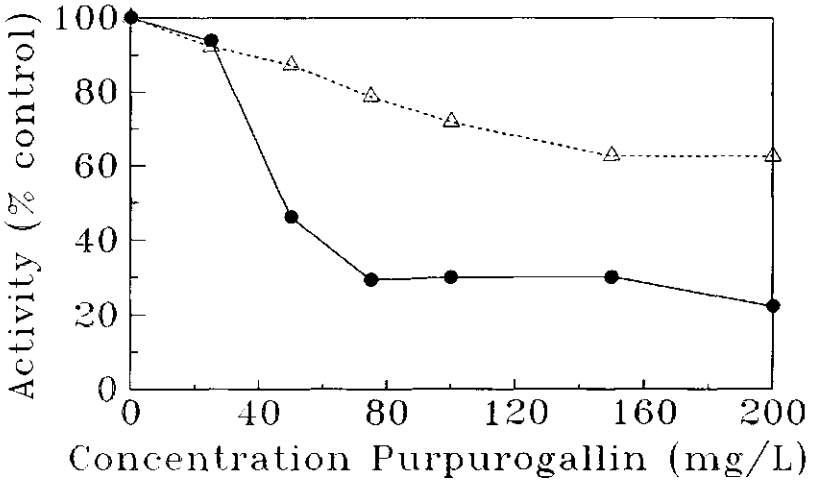


Figure 3. The methanogenic toxicity of purpurogallin in the first (△) and second (●) VFA fed assay. Assay conditions: experiment 2 in Table 1.

3.2. Changes in Pyrogallol Toxicity

The methanogenic toxicity of the pyrogallol solution increased rapidly during the first hour of autoxidation (Figure 1C). Since the initial phase of the autoxidation corresponded to the formation of purpurogallin, a high methanogenic toxicity of purpurogallin was suspected. This was confirmed by measuring the effect of variable concentrations of purpurogallin on the methanogenic activity of granular sludge in a separate experiment (Figure 3). In the second feeding, the concentration of purpurogallin that corresponded to 50% inhibition was 45 mg L^{-1}. The activity results reported in Figure 3 are based on the activity during the initial two days of the assay, when most of the VFA substrate of the controls are consumed. However, in this time period the purpurogallin treatments were only utilizing acetic acid. The toxicity of purpurogallin on the metabolism of propionic acid was more severe. Table 2 illustrates that very little propionic acid was consumed during the entire assay. Similar results were also observed with the pyrogallol treatments that were autoxidized from 10 minutes to 1 hour (data not shown). Although according to Figure 3, the purpurogallin toxicity in the first feeding appeared not to be strong, this was due to a three day delay in the full expression of its toxicity. The fact that the pyrogallol treatments autoxidized from 10 minutes to 1 hour did show strong toxicity in the first feeding (ie. without any delay) may be due to interactive effects between pyrogallol and purpurogallin.

The autoxidized solutions of pyrogallol were filtered before using them in the toxicity test reported in Figure 1C. Therefore, only the toxicity of the soluble purpurogallin was actually measured. The purpurogallin precipitate was also extremely toxic. The sludge exposed for two weeks to the unfiltered 1 hour oxidized solution (containing both insoluble and soluble fractions of purpurogallin) had only 4% of the control activity (data not shown). This was lower than the activity shown (16% of the control) for the sludge exposed to the filtered 1 hour autoxidized-pyrogallol-containing solution.

The high toxicity of purpurogallin exceeds the toxicity expected based on the measured tannin concentration. Therefore, the reason for its toxicity does not seem to be related to tannin qualities. This is further confirmed by its partial adsorption on PVP and low solubility in water, both characteristics are not associated with tannic compounds.

Between 1 and 6 hours of autoxidation, most of the purpurogallin was autoxidized further to soluble products which were also toxic (Figure 1C). At this stage, some of the tannic autoxidation products were possibly responsible for the toxicity.

Detoxification of the autoxidized solutions began at the point the autoxidation became a destructive oxidation (after 6 hours of autoxidation). The decrease in the inhibitory effect paralleled closely the decrease in PVP measured tannin concentration during this phase (Figure 1C).

Table 2. The VFA Concentration in the Medium and the Total Methane Production at the End of the First and Second VFA Feeding of the Purpurogallin Toxicity Assay.

PURPUROGALLIN	END FIRST FEEDING (11 DAYS)					END SECOND FEEDING (14 DAYS)				
Concentration	C_2	C_3	C_4	C_t	CH_4[a]	C_2	C_3	C_4	C_t	CH_4
mg L^{-1}	mg COD L^{-1}					mg COD L^{-1}				
0	13	18	0	31	3856	20	0	0	20	3649
100	13	950	89	1277	2461	9	1062	0	1082	2489
150	14	971	85	1292	2409	10	993	0	1180	2564
200	14	1000	80	1300	2428	15	1281	1071	3210	1153

[a] C_2 = acetate; C_3 = propionate; C_4 = butyrate; C_t = total volatile fatty acids (C_2 - C_5); CH_4 = metane produced per liter of assay medium. At the start of each VFA feeding 4000 mg COD L^{-1} were added as a VFA mixture (containing 972, 1376 and 1652 mg COD L^{-1} C_2, C_3 and C_4; respectively).

3.3. Changes in Pyrogallol Biodegradability

Pyrogallol is readily degraded in anaerobic environments (Field and Lettinga, 1987). After the unoxidized pyrogallol solution has been digested for 2 weeks a complete elimination of the UV_{215} absorption and PVP determined tannins, as well as a complete conversion of pyrogallol COD to VFA and CH_4, was observed (Figure 1D).

During the course of autoxidation, there was a decrease in the available level of pyrogallol (ie. pyrogallol surviving the oxidation treatment) (Figure 1B); however in the purpurogallin forming stage of the oxidation, the decrease in pyrogallol biodegradability was greater than the decrease in pyrogallol available (Figure 1D). This indicates that purpurogallin was toxic to the biodegradability of pyrogallol as was also indicated by similar observations in a previous study (Field and Lettinga, 1989). An improvement in the pyrogallol degradation did occur at a later point in the oxidation (at 1 hour) and may have resulted from a decrease in the soluble purpurogallin concentration. The purpurogallin peak area in the HPLC chromatograms at 1 hour autoxidation (data not shown) was in any case lower than that at 20 minutes autoxidation. At 6 hours of autoxidation, no available pyrogallol was present nor was there any degradation observed. Therefore, the autoxidized products were not biodegradable. Schink and Pfennig (1982) also observed that the colored compounds of autoxidized pyrogallol solutions were not degraded, while degradation of the colorless pyrogallol did occur.

During the destructive phase of the autoxidation, the colored solutions were still for the most part not degraded anaerobically. However, associated with the decrease in color was a very small increase in biodegradability (Figure 1D). The biodegradability was so low that it was impossible to determine if it was real or a result of experimental error. However, it is known that destructively oxidizing humus with ozone can increase its biodegradability (Gilbert, 1988), thus it is plausible that a continuation of the present destructive oxidation could have led to an increased biodegradability.

4. DISCUSSION

In a previous study, we have shown that changes in the methanogenic toxicity of catechin polymerized to colored compounds by autoxidation parallel the tannin quality of the compounds (Field et al., 1989). The tannin quality is related to the MW of the compounds. Since oligomers are the only effective tannins which can penetrate bacteria, they are the only significantly toxic tannins.

Unlike catechin, the autoxidation of pyrogallol did not cause extensive polymerization. Initially, some polymerization occurred producing toxic intermediates that were later destroyed by a destructive type of oxidation caused by prolonged autoxidation treatments. The first intermediate formed, purpurogallin (a dimer), caused a high level of methanogenic toxicity. As little as 45 mg L^{-1} of soluble purpurogallin, was shown to cause 50% inhibition of the methanogenic activity. The rapid increase of methanogenic toxicity during the initial period of pyrogallol autoxidation can therefore be explained by the formation of small amounts of purpurogallin. As the autoxidation proceeds, high levels of purpurogallin accumulate at concentrations that are no longer fully soluble in water. The unfiltered autoxidized solutions which contain these purpurogallin precipitates caused nearly complete inhibition of the methanogenic activity. Purpurogallin is more toxic to methane bacteria than the oligomeric tannins in autoxidized solutions of catechin (Field et al., 1989) and the tannins (primarily oligomeric) of aqueous tree bark extracts (Field et al., 1988). Purpurogallin also effectively inhibited the anaerobic degradation of pyrogallol. Since purpurogallin lacks tannin features and is highly toxic, the changes in the methanogenic toxicity induced by the autoxidation of pyrogallol cannot be estimated by the changes in the oligomeric tannin concentration. Clearly, other factors besides the tannin quality are involved in the methanogenic toxicity of autoxidized pyrogallol.

Regardless of the reactions that take place during the autoxidation of tannin monomeric derivatives, the initial reactions can potentially lead to colored products of increased

methanogenic toxicity. These are later detoxified by prolonged autoxidation, either by polymerization to high MW compounds (with condensed tannin model compounds), or by destruction of the initially toxic intermediates to low MW compounds (with hydrolyzable tannin model compounds).

5. LITERATURE CITED

Field, J. A. and Lettinga, G. (1987). The methanogenic toxicity and anaerobic degradability of a hydrolyzable tannin. Wat. Res. 21: 367-74.

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): 219-40.

Field, J. A. and Lettinga, G. (1989). The effect of oxidative coloration on the methanogenic toxicity and anaerobic biodegradability of phenols. Biological Wastes In Press.

Field, J. A., Kortekaas, S. and Lettinga, G. (1989). The tannin theory of methanogenic toxicity. Biological Wastes In Press.

Gilbert, E. (1988). Biodegradability of ozonation products as a function of COD and DOC elimination by the example of humic acids. Wat. Res. 22: 123-26.

Schink, B. and Pfennig, N. (1982). Fermentation of trihydroxybenzenes by Pelobacter acidgallici gen. nov. sp. nov., a new strictly anaerobic, non-sporeforming bacterium. Arch. Microbiol. 133: 195-201.

CHAPTER 8

Oxidative Detoxification of Aqueous Bark Extracts. Part I: Autoxidation.

(Submitted for publication)

OXIDATIVE DETOXIFICATION OF AQUEOUS BARK EXTRACTS. PART I: AUTOXIDATION

J. A. Field[1], G. Lettinga[1] and L. H. A. Habets[2]

[1]Dept. of Water Pollution Control
Wageningen Agricultural University
Bomenweg 2, 6703 HD Wageningen
The Netherlands

[2]Paques B.V.
P.O. Box 52, 8560 AB Balk
The Netherlands

ABSTRACT - The aqueous extracts of bark were studied as a model for wet debarking wastewater. These extracts are known to contain a high concentration of methanogenic toxic tannins. The objective of this study was to modify the native bark tannins (oligomers) with oxidative methods in order to decrease their methanogenic toxicity. The tannins were polymerized by autoxidation, forming colored high molecular weight tannins that were non-toxic to methanogenic bacteria. The autoxidation of pine bark extracts provided complete detoxification. In the case of spruce bark, which was responsible for extracts of higher toxicity, the detoxification was either partial or complete depending on the specific sample of spruce bark from which the extract was prepared. The autoxidation of birch bark did not result in significant detoxification. Although, the oligomeric tannins were effectively polymerized, birch bark extracts contain non-tannin toxins which were not affected by the autoxidation and evidence that highly toxic intermediates were formed during the high pH autoxidation of birch bark extracts is presented.

Autoxidative detoxification offers an interesting approach for pretreating coniferous bark wastewaters. The oxidation reactions of the pretreatment serve to detoxify the largely non-BOD tannin fraction. Thus the methanogenic bacteria are not inhibited during the anaerobic wastewater treatment for removing BOD.

KEY WORDS - tannin, procyanidin, phenolic compounds, anaerobic digestion, methanogenic bacteria, methanogenic toxicity, autoxidation, detoxification, bark, debarking wastewater

1. INTRODUCTION

1.1. Objectives

Evidence indicating the high methanogenic toxicity of aqueous bark extracts has previously been reported (Field et al., 1988). The majority of the methanogenic toxicity was attributable to the tannin fraction of the extract. The tannin fraction generally accounted for about half of the extract COD. Biodegradability studies indicated that about half of the COD was readily fermentable substrate. Most of this substrate was found to belong to the non-tannin fraction.

These extracts were studied because they were representative of wet debarking wastewater from the forest industry. The anaerobic treatability of debarking wastewater is expected to be limited by the severe methanogenic toxicity. Considering the importance of the tannin fraction to the methanogenic toxicity, improvement of debarking wastewater treatability might be expected if the toxicity of the tannins could in some way be eliminated from the wastewater prior to anaerobic treatment. In a previous study (Field et al., 1989a),

we have demonstrated the relationship between tannin MW and methanogenic toxicity by polymerizing the model bark tannin monomer, catechin. The oligomeric intermediates, which have an analogous structure to native bark tannins were found to have the strongest toxicity. The high MW end products of the autoxidation were not inhibitory. The objective of this study was to evaluate the role of modifying the native bark tannin polymer size with oxidative methods in order to decrease their toxicity.

1.2. Autoxidative Reactions of Tannins

The native tannins present in bark are predominantly oligomeric procyanidins (Porter, 1974; Karchesy and Hemingway, 1980). They are colorless polymers formed by biosynthetic pathways analogous to acid condensation reactions. The procyanidins are linked mainly by 4 to 8 intermonomeric bonds (Karchesy and Hemingway, 1980; Hemingway et al., 1982), see Figure 1.

Autoxidation of procyanidins can occur spontaneously under alkali conditions. The sequence of reactions is the formation of o-quinone on the B ring and subsequent condensation reactions (Hathway and Seakins, 1955 and 1957). The resulting polymers are pigments characterized by intermonomeric bonds formed between two aromatic rings (Figure 1), namely the 2', 5', or 6' position of the B ring is linked to the 6 or 8 position of the A ring (Hathway and Seakins, 1955 and 1957). The colorless native bark tannins can be polymerized oxidatively to produce high MW compounds with color. These high MW products of bark tannin oxidation are generally referred to as phlobatannins and phlobaphanes.

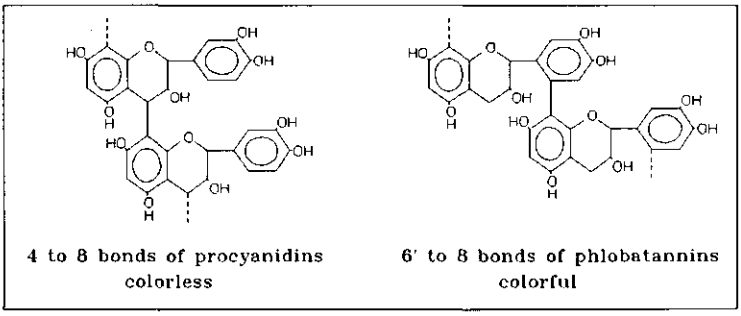


Figure 1. The intermonomeric bonds of native bark tannins (procyanidins) and oxidatively polymerized products of the procyanidins (phlobatannins).

2. MATERIALS AND METHODS

2.1. Bark Extract Preparation

Warm water extracts were prepared from 18 g of air dried milled bark per liter of 60° C tap water containing 250 mg L^{-1} ascorbic acid (to prevent premature oxidation). Bark was collected from freshly cut trees at a local forest or from logs delivered to Parenco paper factory (Renkum, the Netherlands). The following species were utilized in this study: scot's pine (Pinus sylvestris); norway spruce (Picea abies); and european white birch (Betula verrucosa). The extracts of spruce bark varied in toxicity and detoxification capacity depending on the lot of spruce bark collected. Three lots referred to as "lot 1", "lot 2" and "lot 3" were used in this study. The toxicity and detoxification capacity of pine and birch bark extracts were similar for the various lots of bark collected; therefore, no distinction between the various lots were made.

2.2. Methods

2.2.1. UV Absorbance and Color of the Extracts

The ultra violent (UV) and visible light absorbance (color) of the extracts was measured in a 1 cm quartz cuvette by diluting to less than 0.8 absorbance units in pH 6, 0.2 M KH_2PO_4 buffer. The UV absorbance was measured at 215 nm (UV_{215}). UV Absorbance is reported as 1x (ie. undiluted extract) absorbance units. The UV light absorbance is indicative of the total phenolic concentration. The color was indicated by measuring the visible light absorbance at 440 nm. The color is indicative of the oxidatively polymerized products.

2.2.2. COD and VSS Determinations

The COD (the micro method with dichromate) and VSS were determined according to standard methods (American Public Health Assoc., New York).

2.2.3. PVP Determination of Tannins

The determination of the total tannin concentration by adsorption on the insoluble polyamide, polyvinylpyrrolidone (PVP), was described previously (Field et al., 1988). The extracts were shaken with 14.3 g L^{-1} PVP for 1 h and filtered. In this study, the tannin concentration of the autoxidized extracts were based on the UV_{215} light absorbance units adsorbed on the PVP.

2.2.4 HPLC

The reversed phase high pressure liquid chromatographic method of characterizing bark extract tannins was adapted from previously reported methods designed for analyzing condensed tannins in apple juice and wine (Lea, 1982 and 1980; Wilson, 1981). The specific parameters and conditions of the HPLC procedure used in this study were described previously (Field et al., 1989a). The UV absorbance was detected at 280 nm. The total extract as well as the PVP treated extract (according to the tannin determination) are plotted together, the difference in peak area between the two represents the tannin peak area.

The three important categories of compounds that can be recognized in the HPLC chromatograms are the low MW tannins (oligomers), the high MW tannins and high MW humus (Field et al., 1989a). The low MW tannins are represented by the tannin peak area of a retention time less than 28 minutes. The high MW tannins are represented by the tannin peak area of a retention time greater than 28 minutes. The high MW humus is the non-tannin peak area of greater than 28 minutes.

The low MW tannin concentration was estimated by taking the ratio of tannin peak area < 28 minutes to total tannin peak area from the HPLC chromatograms and multiplying it by the total PVP determined tannin concentration.

2.3. Autoxidation Treatments of the Extracts

The bark extracts were aerated with pressurized air through a porous stone. The aeration rate applied during the autoxidation studies corresponded to approximately 30 vol. air per vol. extract per hour. The extracts were brought to a specific pH (between 10 and 12) with NaOH. For example, the NaOH consumed to raise 2 and 6 g COD L^{-1} spruce extracts to pH 10.2 was 0.13 and 0.44 g NaOH L^{-1}, respectively and to pH 11.7 was 0.44 and 1.0 g NaOH L^{-1}. Generally a drop in pH of 1 to 2 pH units was observed to occur during autoxidation. The autoxidation reactions were stopped at given time intervals by removing the aeration stone and lowering the pH to 6.5 with HCl.

2.4. Bioassays

All assays contained essential inorganic macro and micro nutrients as outlined previously (Field and Lettinga 1987). The batch fed assays were conducted in 0.5 L serum flasks. The assay temperature was 30 ± 2° C. The serum flasks were not shaken during the assay period.

The anaerobic toxicity assays of bark extract were supplied with approximately 4 g COD L^{-1} neutralized with NaOH VFA stock solution, 100:100:100 g kg^{-1} acetate (C_2), propionate (C_3) and butyrate (C_4). The stock solution COD ratio is 24:34:41 C_2:C_3:C_4. Usually, 1.4 g VSS L^{-1} granular sludge was used in the assays, that was either obtained from a UASB treating potato derived wastewater (Aviko) or vinasse (Nedalco). The sludge was previously rinsed with water in an upflow column to clean out the fine dispersed matter.

The assays were carried out in two consecutive feedings. The first feeding supplied the VFA substrate to the treatments and the control. The first feeding generally lasted for two weeks. Both treatments and controls received from 0.5 to 2.0 g $NaHCO_3$ L^{-1} as buffer to accommodate an eventual acidification of the substrate present in the bark extract of the treatments. Under the conditions of the assay, the activity of granular sludge is not largely affected by VFA starting concentrations between 4 to 10 g COD L^{-1}; therefore, the accumulation of additional VFA by the acidification of the extract treatments (generally less than 1 g COD L^{-1}) had little effect on the activity assayed. At the start of the assays, the pH ranged from 7.4 to 7.8. At the end of the experiments, the pH was 7.5 to 8.0 if VFA were consumed, and 6.9 to 7.1 if VFA were not significantly consumed (ie. in cases with highly inhibitory treatments).

The second feeding was designed to measure the residual activity of the sludge following the exposure of the extract to the sludge during the first feeding. To initiate the second feeding, the medium supernatant of the first feeding was decanted (through a tea sieve) while flushing the serum flask with N_2 gas, and the sludge from both the treatments and control was supplied with new media containing 4 g COD L^{-1} from the VFA stock solution. In the second feeding, the treatments were not supplied with bark extracts.

Methane production was monitored with serum flasks modified into mariotte flasks. These flasks were filled with 3 to 5% NaOH solutions to dissolve all CO_2.

The activity of the controls is expressed as the amount of CH_4 in COD produced by one gram of VSS per day (mg COD g^{-1} VSS d^{-1}). For a given sludge, the first feeding activities of the controls varied due to the variable periods of previous storage in refrigeration (4° C). After the sludge adapted to the VFA substrate in the first feeding, the control sludge activities from the second feedings were comparable for a given sludge in the various experiments. The activity of the extract supplied assays is reported as the percentage of the control activity. Inhibition is equal to 100 minus the percent of the control activity. Examples can be found in a previous publication (Field et al., 1988), which illustrate how the period for determining the activity is chosen during the first and second feedings.

2.5. Experiments

The specific extract parameters and assay conditions of most of the experiments conducted in this study are listed in Table 1. In an additional experiment, the effect of variable exposure times of sludge to spruce bark extract was investigated. During the exposure period the assays were supplied VFA substrate at 4 g COD L^{-1} and the unoxidized or autoxidized spruce bark (lot 2) extract at an assay concentration of 2.5 g COD L^{-1}. The control was only supplied with the VFA substrate. The granular methanogenic sludge concentration was 1.4 g VSS L^{-1} and 0.5 g $NaHCO_3$ L^{-1} was supplied during the exposure period as buffer. For exposure periods longer than 2 weeks, the exposure medium was exchanged with new medium as outlined above. At the end of the given exposure period, the exposure medium was exchanged for medium containing only 4 g COD L^{-1} VFA to assay the residual sludge activity.

Table 1. The Bark Extract Characteristics and Activity Assay Parameters of the Experiments.

Experiment	Extract					Assay[a]				
	Untreated				Autoxidized	Conditions			Control Activity	
	COD		UV		Max. Color	Buffer	Dilution	Exposure	First Feed	Second Feed
	Total	Tannin	Total	Tannin	Total	$NaHCO_3$	Extract[c]	Time[d]		
	(mg COD L^{-1})		(215nm,1x)		(440nm,11x)	(g L^{-1})	(X)	(days)	(mg COD g^{-1} VSS d^{-1})	
Figures										
2.pine	3546	1425	117.2	83.6	0.527	1.0	2.0	13	172	649[e]
2.spruce	5785	2694	154.8	137.9	0.677	2.0	2.1	13	411	739[e]
3.pine	4886	2097	126.0	103.7	0.417	2.0	1.67	14	263	534[f]
3.spruce	5997	3256	159.2	135.1	0.574	2.0	2.27	15	231	583[f]
4.birch	4069	1976	98.7	81.0	0.401	2.0	2.5	14	178	565[f]
9.pulp[b]	16039	895	112.4	46.7	0.144	10.0	1.67	15	350	560[f]

[a] In all assays the sludge concentration was 1.4 g VSS L^{-1} and 4 g COD L^{-1} as VFA was supplied.

[b] Pulp wastewater from a masonite factory (particle board). The filtered COD was 13661 mg L^{-1}. In the assay, approx. 75% of the wastewater COD acidified to methane and VFA. Since the wastewater alone supplied sufficient substrate, the standard toxicity was modified in the first feeding. No additional VFA was added to the assay treatments. The control received 4 g COD L^{-1} as VFA.

[c] The dilution factor of the extract in the assay media.

[d] Exposure time to the treatments during the first feeding

[e] "Aviko" granular sludge

[f] "Nedalco" granular sludge

3. RESULTS

3.1. Changes In Bark Extract Characteristics

Pine, spruce and birch bark extracts were autoxidized at pH 10.2 and 11.7 for various time periods. The development of color, change in the total UV light absorbance and tannin concentration during the course of pine, spruce and birch bark extract autoxidation are plotted in Figures 2, 3, and 4. The procyanidin tannins of the bark extract underwent extensive coloration, similar to the autoxidation of the procyanidin monomer, catechin (Field et al., 1989a). The visible absorbance of the different extracts tested increased 10 to 30 fold by autoxidation.

The autoxidation caused very minor losses (10 to 20%) of the total extract UV absorbance, which indicates the oxidation was not highly destructive to the aromatic rings. However a considerable decreases in the PVP measured tannins (detannification) indicates a change during autoxidation of at least some tannins to other aromatic structures that lack tannin quality. These colored non-tannic products of autoxidation will be referred to as humus. The detannification by autoxidation ranged from 30 to 50% in the various experiments. Therefore the autoxidation procedure could not serve to bring about a complete transformation of the tannins to humus.

3.2. Polymerization of the Oligomeric Bark Tannins

The bark extracts were analyzed with the HPLC to evaluate the MW classes of tannins that are originally present as well as those formed during the course of the oxidation. These

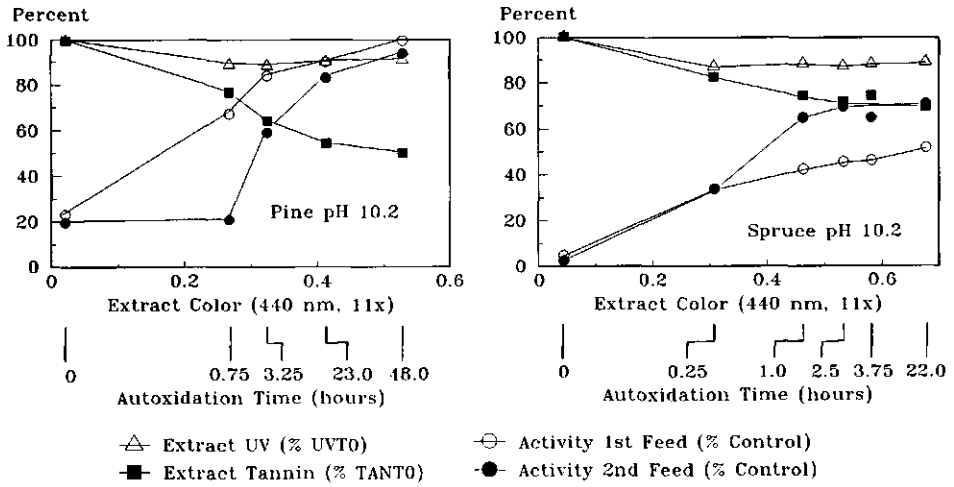


Figure 2. The methanogenic activity of sludge exposed to autoxidized (pH 10.2) extracts of pine and spruce (lot 1) bark as a function of the extract color. UVT0 = the total UV absorbance of the untreated extract (reported in Table 1); TANT0 = the PVP determined tannin concentration of the untreated extract (reported in Table 1).

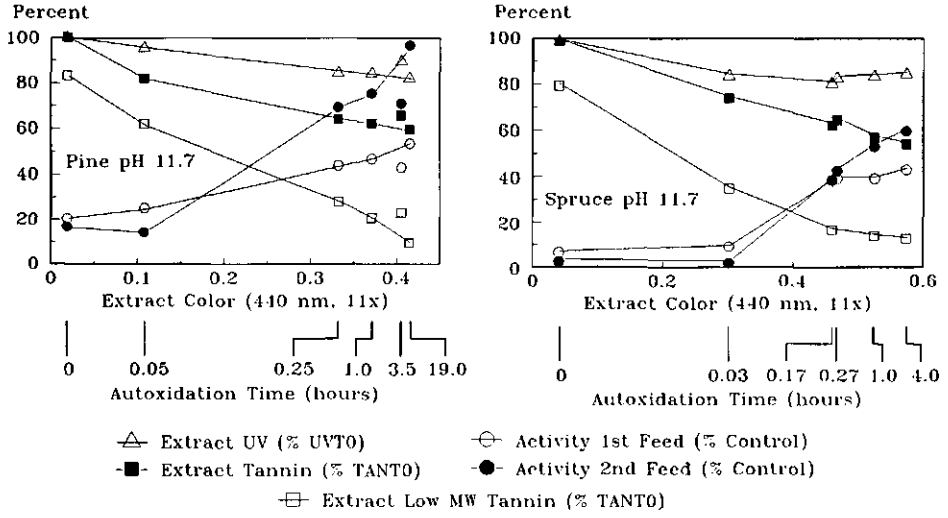


Figure 3. The methanogenic activity of sludge exposed to autoxidized (pH 11.7) extracts of pine and spruce (lot 1) bark as a function of the extract color. UVT0 and TANT0 are defined in the caption of Figure 2.

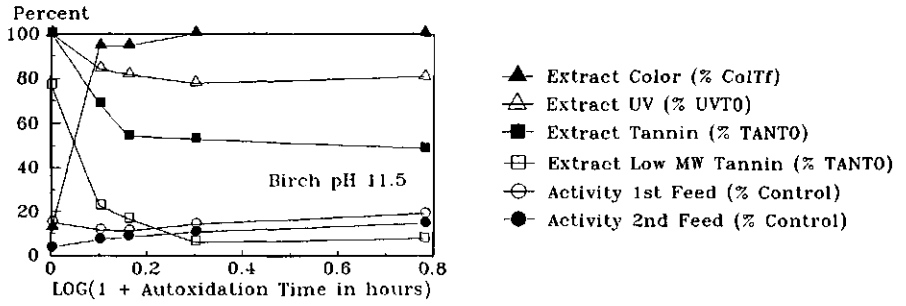


Figure 4. The methanogenic activity of sludge exposed to autoxidized (pH 11.5) extracts of birch. UVT0 and TANT0 are defined in the caption of Figure 2; COLTf = the maximum color (abs 440nm) obtained with the autoxidation (reported in Table 1).

chromatograms obtained from pine and spruce are shown in Figures 5, 6 respectively. The chromatograms reveal that the majority (~80%) of the original bark tannins belong to the low MW category. During the autoxidation, a rapid conversion of the oligomeric tannins to the high MW tannins occurred. By the end of the autoxidations some conversion of the high MW tannins to humus was evident from the increase in non-tannic material in the high MW peak.

The HPLC results enabled an estimation of the low MW tannins concentrations, which are plotted in Figures 3 and 4. The autoxidation decreased the low MW tannin concentration by 80 to 90%.

3.3. Autoxidative Detoxification of Bark Extracts

In Figures 2 and 3, the activity of methanogenic sludge after 2 weeks exposure to the coniferous bark extracts is illustrated. In the case of pine bark, the sludges exposed to the original extract were inhibited approximately 80% (ie. 20% of the control activity). Sludges exposed to highly autoxidized extracts only were inhibited by 5%. In the case of spruce bark (lot 1), the original extracts caused approximately 97% inhibition. The highly autoxidized extracts caused 30 to 40% inhibition. The coniferous bark extracts were therefore highly detoxified by the autoxidation method.

The low MW tannins concentrations during the autoxidation are plotted together with the methanogenic activity results in Figure 3. The observed decrease in the methanogenic toxicity by autoxidation closely corresponded to the decrease of the low MW tannin concentration. This indicated that the oligomeric tannins were responsible for the methanogenic toxicity as was observed in the autoxidation experiments with catechin (Field et al., 1989a).

As previously stated in the methods, the detoxification of spruce bark extracts varied depending on the specific lot of bark collected. The extracts prepared from lot 2 bark, were more toxic and more difficult to detoxify than those of lot 1. Figure 8 illustrates that the second feeding activity following a two week exposure to 2.5 g COD L^{-1} unoxidized spruce bark (lot 2) extract was inhibited by 100%. Autoxidation provided detoxification, but 87% inhibition was still evident after a similar exposure to 16 hour autoxidized (pH 11.5) spruce bark extract. The autoxidation reactions with extracts of lot 2 spruce bark were exhausted after one day, since in one experiment (results not shown), the extract autoxidized for one week at pH 11.5 was not less toxic nor contained less low MW tannins as compared to the extracts autoxidized for one day.

Finally, extracts prepared from another lot of spruce bark (lot 3) could be completely detoxified. The second feeding activity following a 14 day exposure to 2.6 g COD L^{-1}

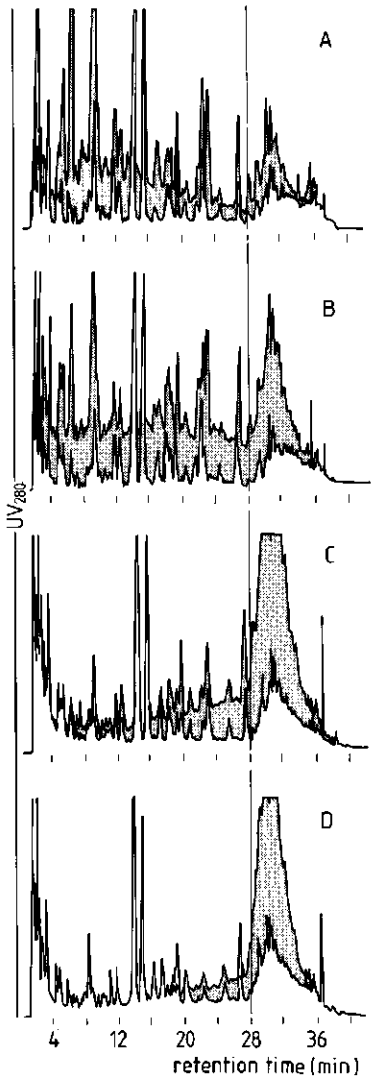


Figure 5. HPLC chromatograms during the course of pine bark extract autoxidation (for: A, 0.0; B, 0.05; C, 1.0; and D, 19.0 hours with a starting pH of 11.7). The boundary between low and high MW tannins is at 28 minutes retention time. The top chromatogram is the entire extract, the bottom chromatogram is the PVP treated extract. The tannin peak area is the shaded area between the two chromatograms.

spruce bark (lot 3) was inhibited by 98%, whereas no (ie. 0%) inhibition was evident from the extracts autoxidized for 16 hour at pH 11.5 and only 15% inhibition was observed from the extract autoxidized for 16 hour at pH 8 (results not shown).

No reason can be offered in order to explain the variable detoxification results obtained with different lots of spruce bark. Factors such as the different seasons when the bark was collected, variable periods of time between tree felling and collection of samples, and the variations in the spruce tree varieties from which different samples of bark were collected; undoubtedly, contributed to the variability.

On numerous occasions, a delayed response was observed in the full expression of the inhibition caused by partially detoxified coniferous bark extracts. For example in Figure 2, a large difference in activity was observed between the first and second feedings of the sludge exposed to the pine bark extract autoxidized for only 0.75 hours at pH 10.2. This delayed response was also observed with autoxidized catechin (Field et al., 1989a). The exposure time needed for spruce bark extract tannins to express their full toxicity was observed to increase with the degree of the autoxidation (Figure 7). In order to impose 50% of the eventual inhibition, 1, 2 and 6 days were required by unoxidized, 1 hour autoxidized and 16 hour autoxidized spruce bark (lot 2) extracts, respectively. For 80% of the eventual inhibition, 3, 6 and 12 days were required, respectively. The delay in the toxicity is perhaps due to the slow penetration of partially polymerized tannin oligomers to the bacteria.

In Figure 4, the activity of methanogenic sludge exposed to the birch bark extract for a two week period is illustrated. In this case, the original extract caused 97% inhibition. The highly autoxidized extract caused 85% inhibition. While some detoxification was evident, it was not as substantial as that observed with the coniferous barks. The autoxidation was in any case quite effective in decreasing the concentration of the original low MW procyanidin tannins; therefore, these low MW tannins were not responsible for the toxicity remaining after autoxidation. Birch bark extracts are unique from the coniferous bark extracts because the tannin fraction is only responsible for about half of the methanogenic toxicity (Field et al., 1988). Therefore, birch bark contains non-tannic toxins, which are not

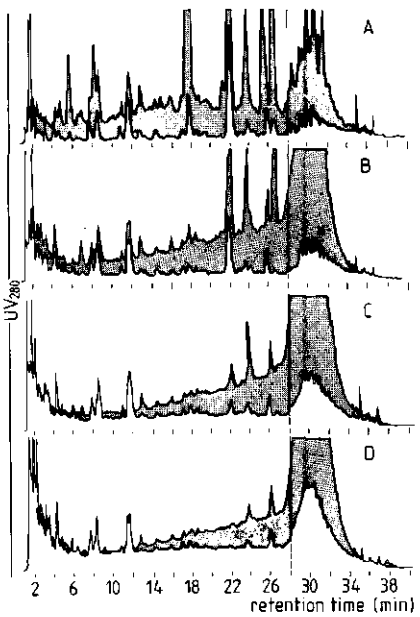


Figure 6. HPLC chromatograms during the course of spruce bark extract autoxidation (for: A, 0.0; B, 0.03; C, 0.17; and D, 4.0 hours with a starting pH of 11.7). See caption of Figure 5 for explanation of symbols.

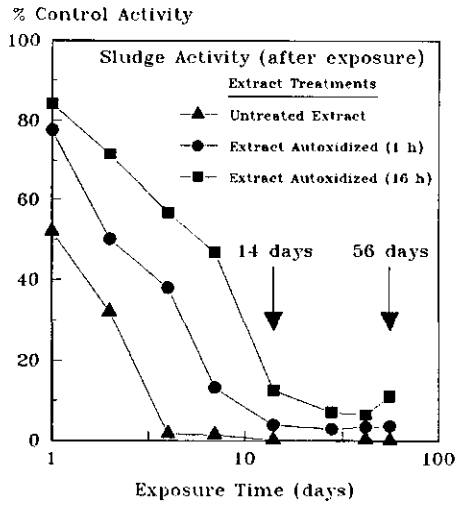


Figure 7. The methanogenic activity of sludge after various exposure periods to unoxidized and autoxidized (pH 11.5) spruce (lot 2) bark extracts, supplied at 2.5 g COD L^{-1} to the medium.

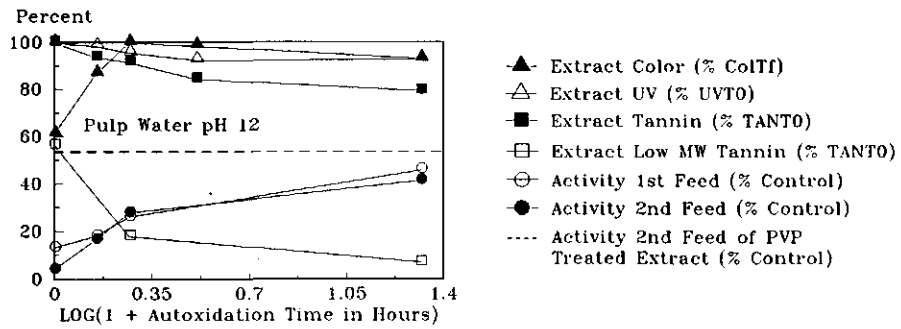


Figure 8. The methanogenic activity of sludge exposed to autoxidized (pH 12) masonite pulp wastewater as a function of the autoxidation time. The feedstock for the pulping was predominantly wood and bark of spruce. UVT0; TANT0 and COLTf are defined in the captions of Figure 2 and 4.

present in coniferous bark. However, the detoxification achieved with autoxidation was much lower than the detoxification achieved by treating the extract with PVP (according to the tannin determination) to remove the tannins. This indicates that after autoxidation additional toxins were present in the extract than just the original non-tannin toxins of the birch bark extract. The additional toxicity must have been produced during the autoxidation. The difficulty in detoxifying birch extracts may be due to a toxification process, that competes with the detoxification of the oligomeric tannins, either by raising the pH at the start of autoxidation or the autoxidation itself. In one experiment with lower concentrations of birch bark extract (results not shown), a distinct increase in the extract toxicity was observed during the initial stages of the autoxidation. Possibly, some trihydroxy residues present in birch bark extract produce highly toxic autoxidation intermediates as was previously observed with pyrogallol (1,2,3-trihydroxybenzene), which produced a highly methanogenic

toxic compound (purpurogallin) in the initial stages of the autoxidation (Field et al., 1989b). In any case, it is known that birch lignin contains syringyl residues (related to trihydroxy residues) and that these are generally absent in coniferous lignin.

3.4. Autoxidative Detoxification of Wastewater

One real wastewater was tested for autoxidative detoxification. It was the pulp water from a masonite (fiber board) factory which used predominantly spruce wood including bark. As shown in Figure 8, this wastewater caused 95% inhibition (only 5% of the control methanogenic activity). Removal of the tannins from the wastewater with PVP resulted in a detoxification of about half of the inhibition. The autoxidation of these tannins resulted in a similar level of detoxification and corresponded to the reduction in the low MW tannin concentration.

4. DISCUSSION

4.1. Autoxidative Detoxification

The autoxidation polymerized the tannic compounds in bark extracts leading to their detoxification as was anticipated from the tannin theory (Field et al., 1989a). The polymerization caused the formation of colored high molecular weight tannins that were too large to penetrate into (or on) cells as well as the formation of colored higher MW non-tannic humus compounds that were unreactive.

The autoxidation method is a simple technique because it only involves the addition of 0.2 to 1.0 g NaOH L^{-1} for debarking wastewater with oxidation times ranging from 1 to 20 hours depending on bark species and tannin concentration. The disadvantage of the autoxidation method is that it is only suitable for coniferous bark. Birch bark extracts are not highly detoxified by this method due to the presence of non-tannin toxins and the formation of toxic intermediates by high pH autoxidation. Therefore the applicability of autoxidation for bark wastewater detoxification should not be used where birch bark accounts for a large proportion of the bark extractives in the wastewater.

4.2. Ecological Significance

In this study, autoxidation was effective in eliminating the methanogenic toxicity of the tannins in aqueous extracts of coniferous bark. Recent studies have revealed that also the acute toxicity of spruce bark tannins to fish is drastically eliminated by the autoxidation treatment (Temmink et. al., 1989). These results establish that the high MW colored tannins and humic compounds formed during polymerative oxidation have considerably less aquatic toxicity compared to the original bark tannins. The dramatic change in the aquatic toxicity reveals a natural mechanism in the environment to detoxify tannic compounds between the time that tannins are released to the forest soil and the time they enter the ground and surface waters. This mechanism is perhaps the natural humus forming processes which occur in the forest soil.

The pollution problem of debarking wastewater tannins is due to the fact that the tannins, which serve to offer a pathological resistance in the bark, short-circuit the polymerization reactions of the humus forming processes in the soil. They are directly discharged in their toxic form into the surface waters. The role of oxidative detoxification should be viewed as a treatment method which imitates the natural detoxification mechanism of soil humus processes. In view of these results, special attention should be paid to the implication of color norms in debarking wastewater discharge control regulations. In

debarking wastewater, color is not necessarily a negative factor for the environment. The colored compounds are for the most part nontoxic and do not contain BOD.

The norms for debarking wastewater should be designed to eliminate those characteristics which can be damaging to aquatic life; namely, BOD and toxic compounds. Oxidative detoxification of bark wastewaters as a pretreatment to anaerobic treatment can offer an interesting approach to meeting both of these objectives. The oxidation reactions of the pretreatment serve to detoxify the largely non-BOD tannin fraction. Thus the methanogenic bacteria are not inhibited during the anaerobic wastewater treatment for removing BOD and the non-BOD fraction is distinctly less toxic for the aquatic organisms of the discharge environment.

5. LITERATURE

Field, J. A. and G. Lettinga. 1987. The methanogenic toxicity and anaerobic degradability of a hydrolyzable tannin. Wat. Res. 21: 367-374.

Field, J. A., M. J. H. Leyendeckers, R. Sierra-Alvarez, G. Lettinga and L. H. A. Habets. 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., S. Kortekaas and G. Lettinga. 1989a. The tannin theory of methanogenic toxicity. Biological Wastes (in press).

Field, J. A., S. Kortekaas and G. Lettinga. 1989b. The effect of autoxidation on the methanogenic toxicity and anaerobic biodegradability of pyrogallol. Biological Wastes (in press).

Hathway, D. E. and J. W. T. Seakins. 1955. Autoxidation of catechin. Nature, 176: 218.

Hathway, D. E. and J. W. T. Seakins. 1957. Enzymatic oxidation of catechin to a polymer structurally related to some phlobatannins. Biochem. J., 67: 239.

Hemingway, R. W., L. Y. Foo and L. J. Porter. 1982. Linkage isomerism in trimeric and polymeric 2,3-cis-procyanidins. J. Chem. Soc., Perkin Trans. 1, 1209-1216.

Karchesy, J. J. and R. W. Hemingway. 1980. Loblolly pine bark polyflavonoids. J. Agric. Food Chem. 28: 222-228.

Lea, G. H. A. 1980. Reversed-phase gradient high performance liquid chromatography of procyanidins and their oxidation products in ciders and wines, optimised by Snyder's procedures. J. Chromatogr. 194: 62-68.

Lea, G. H. A. 1982. Reversed-phase high performance liquid chromatography of procyanidins and other phenolics in fresh and oxidising apple juices using a pH shift technique. J Chromatogr. 238: 253-257.

Porter, L. J. 1974. Extractives of Pinus radiata bark. 2. Procyanidin constituents. New Zealand J. Sci. 17: 213-218.

Temmink, J. H. M., J. A. Field, J. C. van Haastrecht and R. C. M. Merkelbach. 1989. Acute and sub-acute toxicity of bark tannins to carp (Cyprinus carpio L). Water Res. (in press).

Wilson, E. L. 1981. High-pressure liquid chromatography of apple juice phenolic compounds. J. Sci. Food Agric. 32: 257-264.

CHAPTER 9

Oxidative Detoxification of Aqueous Bark Extracts. Part II: Alternative Methods.

(Submitted for publication)

OXIDATIVE DETOXIFICATION OF AQUEOUS BARK EXTRACTS. PART II: ALTERNATIVE METHODS

J. A. Field[1], R. Sierra-Alvarez[1], G. Lettinga[1] and L. H. A. Habets[2]

[1]Dept. of Water Pollution Control
Wageningen Agricultural University
Bomenweg 2, 6703 HD Wageningen
The Netherlands

[2]Paques B.V.
P.O. Box 52, 8560 AB Balk
The Netherlands

ABSTRACT - The most important problem associated with the high pH autoxidative detoxification of methanogenic toxins in debarking wastewater was that its application was limited to the aqueous extractives of bark from specific species. This problem can potentially be resolved by applying alternative oxidation methods. In this study a high level of detoxification was obtained for all bark species tested by short term destructive oxidations with H_2O_2, or by long term aerobic biological treatments which caused high levels of polymerization. The applicability of high pH autoxidation was also made feasible for all species of bark by applying a granular active carbon treatment after the autoxidation. A second problem with the autoxidative detoxification method was the development of colored end products. Although these compounds are non-toxic and are non-biodegradable, their elimination from the wastewater would be necessary if discharge norms concerning color and non-biodegradable COD must be fulfilled. The destructive oxidation produced non-toxic low MW compounds which did not have much color. The autoxidation and long term aerobic biological treatments produced highly colored humic end products which could be eliminated by calcium precipitation.

KEY WORDS - tannin, procyanidin, phenolic compounds, anaerobic digestion, methanogenic bacteria, methanogenic toxicity, autoxidation, hydrogen peroxide, detoxification, bark, debarking wastewater, calcium precipitation, active carbon

1. INTRODUCTION

In the previous study (Field et al., 1990), high pH autoxidative methods were examined for the detoxification of debarking wastewaters, so that such wastewaters can be made suitable for anaerobic treatment. The method was based on modifying the toxic oligomeric procyanidins (tannins) of bark extractives, by their polymerization to nontoxic high MW compounds.

One disadvantage of the autoxidative detoxification was that its applicability was limited to the bark extractives of specific species. The autoxidation was effective for coniferous bark extracts, providing complete detoxification of pine bark extracts. However, the detoxification of spruce bark extracts was in some cases only partial and in other cases complete depending on the specific bark samples collected, from which the extracts were prepared. In contrast, the high pH autoxidation of birch bark extracts was not at all effective.

A second disadvantage of the autoxidative detoxification is that the polymerized end products are darkly colored non-biodegradable humic compounds. However, if no discharge requirements, regarding color and non biodegradable COD, have to be fulfilled, the humic

compounds can be discharged, having a much lower consequence for the environment as compared to the toxic tannic precursors. If these discharge requirements must be met, then it may be feasible to precipitate the humic compounds with calcium. Additionally, oxidative approaches, which cause destructive modifications of the phenols could be employed since these do not produce polymerized colored compounds. Destructive oxidation reactions generally lead to the fragmentation of the polyphenols to aliphatic carboxylic and phenolic acids (Glasser, 1980).

The objectives of this study were to determine if alternatives to high pH autoxidation could improve the reliability of the oxidative approach for the detoxification of bark extracts. Both destructive methods (H_2O_2 oxidation) and polymerative methods (long term aerobic biological treatment) were examined. The autoxidative method was also reevaluated in combination with active carbon treatments to determine if active carbon is capable of adsorbing the residual toxins present in autoxidized birch and spruce bark extracts. Finally, the effectiveness of calcium was studied, for precipitating the high MW colored products of the polymerative oxidations.

2. MATERIALS AND METHODS

2.1. Standard Methods, Analysis and Bioassays

The preparation of bark extracts, analysis of COD, VSS, UV absorbance, color and tannins as well as the HPLC procedure used to estimate the low and high MW tannin fractions were described in the previous publication (Field et al., 1990). The determination of granular active carbon (AC) adsorbable matter of bark extracts was previously described in detail in another publication (Field et al., 1988). The AC adsorbable matter was determined with 28.6 g L^{-1} of granular AC shaken with the extract for 2 hours, followed by filtering through a membrane filter.

The extracts prepared from spruce bark varied in toxicity and capacity to be detoxified by autoxidation, depending on the particular sample of bark collected. In this study, the most toxic and most difficult to detoxify sample of spruce bark was utilized, previously labelled "lot 2" (Field et al., 1990).

The bioassays used in this study to determine the methanogenic toxicity of the bark extracts are described in the previous publication (Field et al., 1990). The anaerobic granular sludge used in the assays was obtained from a UASB treating vinasse ("Nedalco"). The specific assay conditions of the experiments conducted in this study are listed in Table 1. All bioassay results reported in this study are the averaged data of duplicate run experiments.

2.2. Extract Treatments

2.2.1. Autoxidation

The high pH autoxidation treatment of the bark extracts was described in the previous study (Field et al., 1990). In this study, the starting pH of the autoxidation was always 11.5.

2.2.2. H_2O_2 Oxidation

Extracts were treated with 6 g H_2O_2 L^{-1} for spruce and 3 g H_2O_2 L^{-1} for birch bark extracts. The starting pH was adjusted to 11.5 with NaOH. Iron (10 mg Fe^{2+} L^{-1}) was added as oxidation catalyst. The contents were stirred with a magnetic stirrer. During the oxidation period, the pH dropped 4 to 5 units. If the iodometrically determined H_2O_2 was not completely eliminated after an overnight treatment, then the pH was again raised to 11 and the reaction was continued for a few more hours until the H_2O_2 concentration dropped below 30 mg L^{-1}. The total time period of the H_2O_2 oxidations were approximately 22 hours, after which time the pH was adjusted to 6.5 with HCl.

Table 1. The Bioassay Parameters of the Experiments Conducted in this Study.

Experiment[a]	Assay[b]				
	Conditions			Control Activity	
	Buffer	Dilution	Exposure	First Feed	Second Feed
	$NaHCO_3$	Extract[c]	Time[d]		
	(g L^{-1})	(X)	(days)	(mg COD g^{-1} VSS d^{-1})	
Tables					
2.spruce	2.0	2.5	14	285	719
2.birch	2.0	2.5	14	248	727
3.spruce	2.0	1.25	15	337	722
3.birch	1.0	2.22	10	380	920

[a] The extract COD and tannin concentrations are reported in the tables. [b] In all assays the sludge concentration was 1.4 g VSS L^{-1} and 4 g COD L^{-1} as VFA was supplied.
[c] The dilution factor of the extract in the assay media.
[d] Exposure time to the treatments during the first feeding.

2.2.3. Aerobic Biological Treatment

Extracts were aerated (30 v/v h^{-1} through a porous stone) after inoculating with 1 g L^{-1} (fresh weight) composted bark and supplying inorganic nutrients (250 mg L^{-1} NH_4^+N; and other nutrients supplied by adding a stock nutrient solution at one tenth the concentration prescribed for the anaerobic bioassays). The pH of the extracts at the start of aeration was 5.8, during the first week the pH dropped quickly to approximately 4. The period of aeration tested was 4 weeks for spruce and 3 weeks for birch. By the end of the experiments, the pH was approximately 6.5. No microbiological determinations were conducted. The purpose of applying the aerobic biological treatment was to produce highly polymerized samples of bark extract by imitating the conditions that the tannins might encounter in an aerobic forest soil.

2.2.4. PVP Treatment

Extracts were treated with an insoluble polyamide, polyvinylpyrrolidone (PVP), to specifically remove the tannin fraction. The treatment was in accordance to the tannin determination (14.3 g PVP L^{-1} for 1 hour shaking followed by filtering the extract).

2.2.5. AC Treatments

Extracts were treated with AC to remove the AC adsorbable matter. The treatment was in accordance with the exhaustive AC adsorbable matter determination (28.6 g AC L^{-1} for 2 hours intense shaking followed by centrifugation to remove AC). The half exhaustive granular active carbon treatment (1/2 AC) was conducted in the same fashion but only gentle shaking was employed in such a way that only about half of the COD and UV were adsorbed as was the case with the exhaustive method. Details of the 1/2 AC method are described in Field et al. (1988).

2.2.6. Ca^{2+} Precipitation

The precipitation of bark extract matter with calcium was conducted at various pH values (arranged with HCl and NaOH) with 50 to 1250 mg L^{-1} Ca^{2+} supplied as $CaCl_2$. The precipitation was allowed to occur for 1 hour after which the extracts were membrane filtered to separate the precipitate. The difference in the COD, UV absorbance and color before and after precipitation was calculated to be the Ca^{2+} precipitable matter.

3. RESULTS

3.1. Destructive Oxidative Detoxification with H_2O_2

Both the hydrogen peroxide oxidation of the untreated and autoxidized extracts of spruce and birch bark at a high pH were tested. The effect of these oxidations on the total, tannin and low MW tannin COD and UV as well as the color of the extract is shown in Table 2. The H_2O_2 treatments caused large compositional changes. The large loss of COD (14 to 22%), total UV_{215} (45 to 51%) and tannin (75 to 90%) from the original extract, as well as the loss of COD (7 to 14%), total UV_{215} (25 to 28%) and tannin (58 to 68%) from the autoxidized extracts, indicate the destructive nature of the H_2O_2 oxidation. These changes occurred without increasing the extract color to a large extent. In fact, the H_2O_2 treatment of the autoxidized extracts caused color to disappear (52 to 58%), which was quite distinct from the autoxidation (where color was formed). The HPLC chromatograms (Figure 1), reveal the absence of polymerization, since the characteristic high MW peak area (> 28 min.) was not formed. Instead a destructive oxidation occurred, which caused a decrease in MW. A large amount of the peak area was associated with very low retention times (< 4 min.), where almost no compounds were present in the original extract. The autoxidized extract was less effected by H_2O_2 in terms of COD and UV_{215} losses, indicating the greater resistance of the high MW autoxidation products to these destructive modifications. In any case, both the H_2O_2 treatments of the original and autoxidized extracts resulted in sufficient reductions of the low MW tannin concentration, that a complete removal of the methanogenic toxicity due to tannins was observed (Table 2 and Figure 2). The H_2O_2 treatments of the spruce bark extracts were able to reduce the low MW tannin concentration of the extracts better than by autoxidation alone. Consequently, H_2O_2 treated spruce bark extracts were less toxic than the autoxidized extract. The H_2O_2 treatment of the birch bark extracts decreased the methanogenic toxicity of the original extract to a level approximately equivalent to the detoxification achieved by selectively removing the tannins with PVP. This was a distinctly higher level of detoxification than that achieved with the autoxidation alone. The combined autoxidation and H_2O_2 treatment provided complete detoxification.

3.2. Aerobic Biological Treatment

After 3 to 4 weeks of aerobic biological treatment, the paper filtered COD, UV_{215} and tannin was decreased; respectively, by 29 to 33, 26 to 30 and 67 to 73% (Table 2) from the original spruce and birch bark extracts. While the COD elimination was slightly higher than observed by the H_2O_2 treatment, the loss of UV_{215} was distinctly lower. No oxidative changes (ie. coloration and UV losses) of the phenolic compounds occurred in the first week of the biological treatment, however this was the time period that most of the COD losses occurred. The oxidative coloration began after this first week. Therefore, the COD disappearance was probably BOD used to sustain primary growth of the inoculated organisms rather than destructive oxidative reactions of the phenols.

By the end of the biological treatment, highly polymerative oxidations of the spruce bark tannins occurred as can be witnessed from the HPLC chromatograms of Figure 1. Compared with the high pH autoxidized extract, the degree of polymerization was higher because the aerobic biological treatment produced more color and more high MW humus products (the PVP unreactive compounds of the high MW peak area). Likewise, a lower level of toxicity was evident as compared to the autoxidized extract (Table 2 and Figure 2).

The biological treatment of birch bark extract was unique because a large fraction of the oxidation products were colloidal. The colloidal matter of the biologically oxidized extract accounted for 58, 70 and 89% of the paper filtered COD, $UV2_{15}$ and color, respectively. The paper filtered biologically treated extract (including both the soluble and colloidal matter) was not toxic to the methane bacteria (Table 2 and Figure 2).

Table 2. The Influence of Various Treatments of Spruce and Birch Bark Extracts on the Methanogenic Activity of Granular Sludge Exposed to the Extracts.

Extract Treatment[a]	COD				UV_{215}			Color, Vis_{440}			Methanogenic Activity[f]	
	$Total_{mf}$	AC-ADS[b]	Tannin	LMW-Tannin[c]	$Total_{mf}$	AC-ADS	Tannin	$Total_{mf}$	AC-ADS	Tannin	First feed	Second feed
	mg COD L^{-1}				1x			1x			% Control Activity	
Spruce											extracts diluted 2.5x	
2. untrt	6601	5109	3544	2648	211.3	186.5	163.6	0.825	0.660	0.407	1.9	1.0 NR[g]
3. auto	6372	3326	2245	548	175.2	91.4	88.2	7.689	3.047	4.301	33.7	20.7 R
4. H_2O_2	5123	2484	359	120	102.7	61.0	15.6	1.232	0.660	0.253	80.0	103.4
5. auto+H_2O_2	5454	2739	720	107	126.3	67.8	27.7	3.047	1.452	0.924	76.2	104.2
6. biol	4148 (4712)[e]	1500	950	316	130.7 (146.8)[e]	35.3	32.5	9.768 (12.243)[e]	2.376	2.519	99.3	62.2
7. PVP[d]											90.1	102.7
Birch											extracts diluted 2.5x	
2. untrt	3062	2863	1620	1116	90.2	87.2	76.3	0.154	0.088	0.099	5.6	0.7 NR
3. auto	2808	2178	1048	118	68.9	46.8	36.3	4.180	2.310	2.387	17.1	4.0 R
4. H_2O_2	2629	1956	397	33	49.6	35.2	7.7	0.814	0.418	0.077	41.3	62.4
5. auto+H_2O_2	2604	1845	436	25	51.1	33.8	7.8	1.771	1.045	0.275	58.2	95.9
6. biol	878 (2066)[e]	819	539	4	20.3 (66.9)[e]	18.5	12.7	0.638 (5.830)[e]	0.550	0.484	52.5	88.4
7. PVP											47.2	53.7

[a] Treatments: 2. untrt = untreated bark extract; 3. auto = extract autoxidized for 22 hour with a starting pH of 11.5; 4. H202 = extract oxidized with H_2O_2; 5. auto+H202 = treatment 3. followed by treatment 4.; 6. biol = extract treated with the aerobic biological treatment; 7. PVP = extract PVP treated.

[b] AC-ADS: = the granular active carbon (AC) adsorbable matter utilizing the exhaustive method.

[c] LMW-Tannins: = the low molecular weight tannins.

[d] The activity results of PVP treated spruce extract were borrowed from a previously published experiment (Field et al.,1988).

[e] Only extracts of the biological aeration treatment (6.) contained significant amounts of colloidal suspended matter. In these cases, the paper filtered values are reported in the parenthesis. All other values reported in this table are based from membrane filtered (mf) samples.

[f] The second VFA feeding was started after removing the extract containing medium to determine the sludge activity remaining after the exposure to the extract (exposure period is reported in Table 1).

[g] NR = no recovery of indicated lost activity prior to the termination of the 2 week long 2nd feeding.
R = significant recovery of indicated lost activity prior to the termination of the 2 week long 2nd feeding.

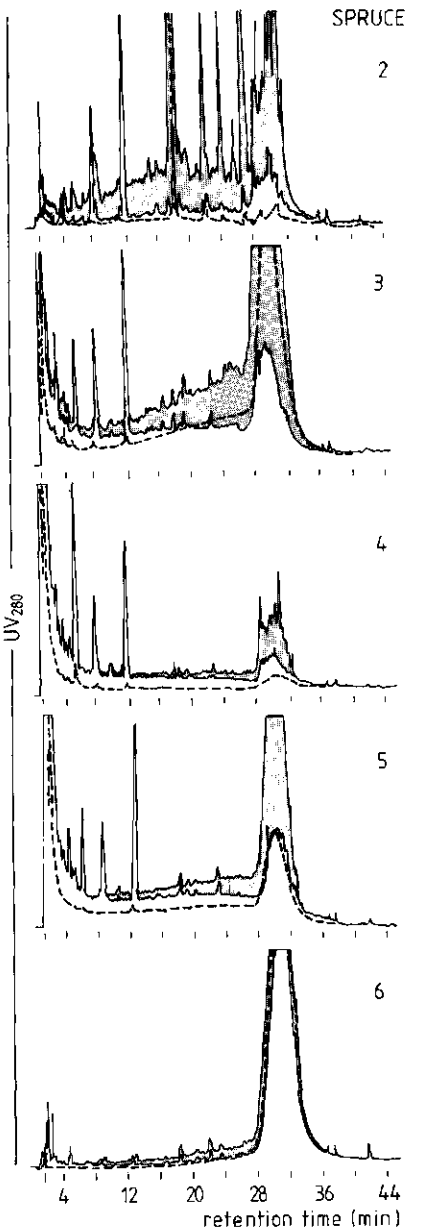


Figure 1. The HPLC chromatograms of untreated extract (2), autoxidized (3), H_2O_2 oxidized (4), autoxidized plus H_2O_2 oxidized (5), and aerobic biologically treated (6) extracts of spruce bark. The boundary between low and high MW tannins is at 28 minutes retention time. For each sample, the top solid line chromatogram is the entire extract, the bottom solid line chromatogram is the PVP treated extract. The tannin peak area is the shaded area between the two chromatograms. The dotted line chromatogram is the exhaustively AC treated extract.

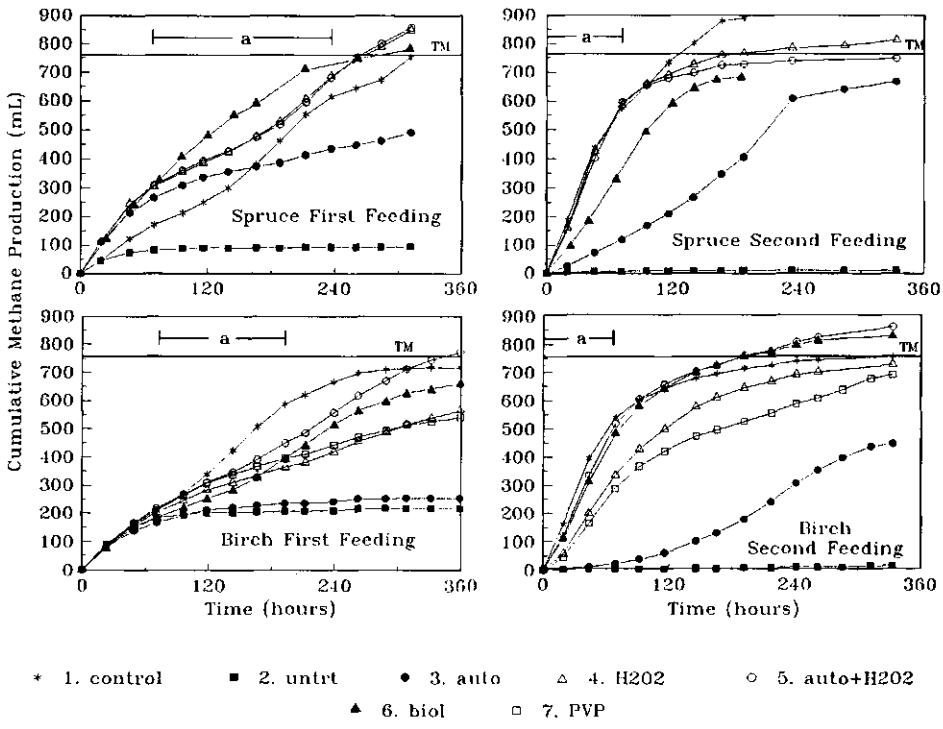


Figure 2. The cumulative CH_4 production during the digestion of VFA with 2.5x diluted spruce and birch bark extracts that were oxidized by various treatments. The second VFA feeding was started after removing the extract containing medium to determine the sludge activity remaining after the exposure to the extract. TM = the theoretical methane production if all the VFA supplied were converted to methane. "a" = the activity period chosen to determine the reported activities in Table 2. The abbreviations of the treatments are defined in the footnote of Table 2.

3.3. Granular Active Carbon Treatment of Bark Extracts

3.3.1 Half Exhaustive Treatments with Active Carbon

In a previous study, we have found that granular active carbon (AC) can adsorb considerable amounts of the bark extract COD (Field et al., 1988). The half exhaustive AC treatments removed significant amounts of COD (60%) and total UV_{215} (40 to 70%) from the extracts. The low MW tannins were insufficiently removed. Only 50% of the low MW tannins were eliminated from the unoxidized spruce bark extract. The low MW tannins that remained still had a sufficiently high enough concentration that the toxicity was still quite strong (Table 3).

Birch bark extracts were unique because they contained non-tannin toxins as is evident from the toxicity of the PVP treated extract (Table 3). The combined PVP and AC treatment of birch bark extract was non-toxic, indicating that AC could remove the non-tannin toxins.

Combinations of autoxidizing bark extracts followed by half exhaustive AC treatments provided complete detoxification that was higher than the sum of detoxification achieved from each individual treatment (Table 3). The PVP treated autoxidized spruce bark extract was completely non-toxic, indicating that the toxicity surviving autoxidation was entirely tannic. The toxic low MW tannins remaining after autoxidation were highly removed by the half exhaustive AC treatment. Likewise, the AC treated autoxidized extract was not very

Table 3. The Influence of PVP and Half Exhaustive AC Treatments of Spruce and Birch Bark Extracts and Autoxidized Extracts on the Methanogenic Activity of Granular Sludge Exposed to the Extracts.

Treatment[a]	COD			UV_{215}		Color, Vis_{440}		Methanogenic Activity[d]	
	$Total_{mf}$	Tannin	LMW-Tannin[b]	$Total_{mf}$	Tannin	$Total_{mf}$	Tannin	First Feeding	Second Feeding
	mg COD L^{-1}			1x		1x		% control activity	
Spruce Extract								extract diluted 1.25x	
2. untrt	5760	2998	2467	170.4	150.0	0.803	0.649	8.9	0.9 NR[e]
3. 1/2AC	2298	1458	1166	54.5	50.1	0.242	0.242	28.0	2.7 NR
4. auto	5760	2354	581	146.4	105.9	3.938	3.245	61.4	12.3 R
5. auto+1/2AC	2494	1295	199	68.2	53.3	2.013	1.232	97.6	75.5
6. PVP								116.3	115.4
7. auto+PVP								112.8	109.4
Birch Extract								extract diluted 2.22x	
2. untrt	3445	1637	1121	93.5	75.2	0.231	0.143	21.0	0.0 NR
3. AC1/2	1477	782	657	56.9	50.9	0.176	0.165	64.0	63.0
4. auto	3445	1291	125	71.6	35.2	4.455	2.332	32.6	2.1 R
5. auto+AC1/2	1760	894	0	49.6	32.8	4.323	ND[f]	114.2	105.8
6. PVP								79.3	66.8
8. PVP+AC1/2[c]								105.5	106.7

[a] Treatments: 2. untrt = untreated extract; 3. AC1/2 = extract treated with granular active carbon (AC) using the half exhaustive method; 4. auto = extract autoxidized for 1 hour (spruce) or 16 hours (birch) with a starting pH of 11.5; 5. auto+AC1/2 = treatment 4. followed by treatment 3.; 6. PVP = extract PVP treated; 7. auto+PVP = treatment 4. followed by treatment 6.; 8. PVP+AC1/2 = treatment 6. followed by treatment 3.

[b] The LMW-tannins = the low molecular weight tannins.

[c] The birch treatment 8. contained only 185 mg COD L^{-1} and 0.000 1x UV_{215}.

[d] The second VFA feeding was started after removing the extract containing medium to determine the sludge activity remaining after the exposure to the extract (exposure period is reported in Table 1).

[e] NR = no recovery of indicated lost activity prior to the termination of the 2 week long 2nd feeding.
R = significant recovery of indicated lost activity prior to the termination of the 2 week long 2nd feeding.

[f] ND = no data (not measured)

toxic. In the case of birch bark, the half exhaustive AC treatment was able to remove the toxic intermediates of autoxidation, because it relieved all of the toxicity from the autoxidized birch extracts (Table 3).

3.3.2. Exhaustive Treatments with Active Carbon

Experiments were continued with a more exhaustive AC treatment of the bark extracts. The AC was able to adsorb 77 to 94% of the COD and 88 to 97% of the total UV_{215} from the original spruce and birch bark extracts (Table 2). Since the exhaustive AC treatment removed essentially all the bark matter, one should assume that even the unoxidized extracts could potentially be detoxified with AC. However, the results with the half exhaustive AC treatments indicate that if the extract is first autoxidized, the detoxification can potentially be achieved with a less extensive AC treatment.

The amount of AC adsorbable matter decreased as a result of the oxidative polymerization (ie. autoxidation and aerobic biological treatments). The decrease resulted from an increase in the concentration of matter not adsorbed by AC. In Figure 1, the HPLC chromatograms of autoxidized and aerobic biological treated spruce bark extracts illustrate that the matter not adsorbed by AC was in fact high MW tannins and humus. Other research groups working with humic acids have also found that granular active carbon is increasingly less effective in adsorbing compounds with increasing MW (El-Rehaili and Weber, 1987).

3.4. Calcium Precipitation of Bark Extracts

Experiments with bark extracts treated with calcium indicate that calcium was only capable of precipitating UV light absorbing matter at a high pH from the unoxidized extracts. At neutral to low pH's, little or no precipitation occurred. The autoxidation of these extracts greatly improved the capacity of calcium to precipitate a significant amount of the UV light absorbing matter at the neutral to low pH's (Figure 3; Table 4). The precipitation was also effective in removing color from autoxidized extracts (Figure 4). The precipitation was even better with the highly polymerized aerobic biologically treated extract (Table 4), since complete removal of color was observed.

Oxidative polymerization of lignin containing wastewater is known to improve the lignin precipitation (Schmidt and Joyce, 1980; Milstein et al., 1988). Research with humic acids also indicates an increasing effectiveness of precipitation with increasing MW (El-Rehaili and Weber, 1987).

Table 4. The Effect of Polymerization on the pH 8 Calcium Precipitation (500 mg L^{-1}) of 1.5x Diluted Spruce Bark Etracts.

	COD		UV_{215}		Color	
Treatments[a]	total	tannin	total	tannin	total	tannin
	percent precipitated[b] by calcium					
2. untrt	5.0	1.2	3.8	3.6	NA[c]	NA
3. auto	29.1	30.0	51.1	40.8	53.5	39.0
6. biol	68.0	42.2	86.8	79.5	95.0	87.8

[a] Treatments: definition of abbreviations are given in footnote of Table 2.

[b] Calcium precipitation: = extract treated at pH 8 with 500 mg L^{-1} Ca^{2+} (as $CaCl_2$) for 2 hours and decanted through a paper filter.

[c] NA = not applicable, because the unoxidized extracts do not have significant color.

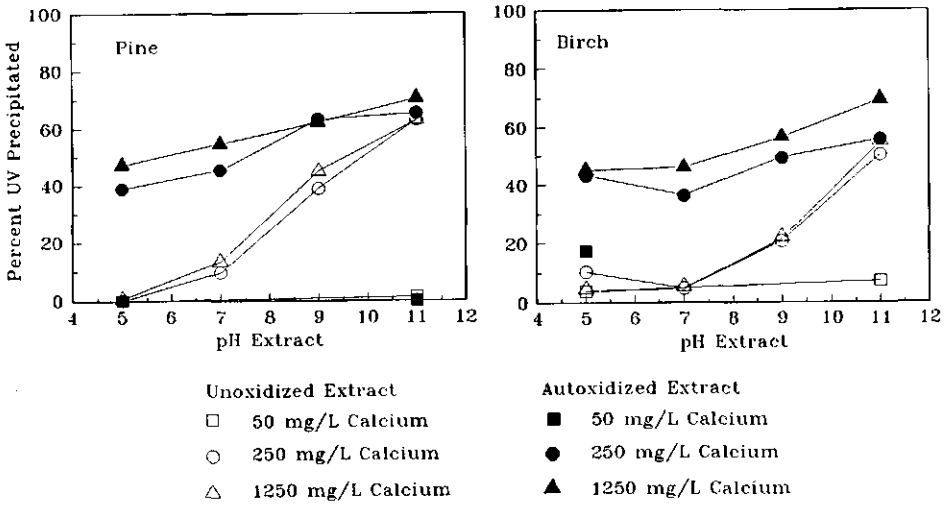


Figure 3. Calcium precipitation of UV_{215} absorbance from unoxidized pine and birch bark extract and autoxidized (16 hours, pH 11.5) extract. Extract Parameters: Pine: Total and tannin COD; 6914 and 1783 mg L^{-1}, respectively. Total and tannin UV; 189.7 and 141.6 (1x, absorbance units), respectively. The extract was diluted 3x for precipitation with calcium. Birch: Total and tannin COD; 4380 and 2112 mg L^{-1}, respectively. Total and tannin UV; 112.2 and 91.7 (1x, absorbance units), respectively. The extract was diluted 2x for precipitation with calcium.

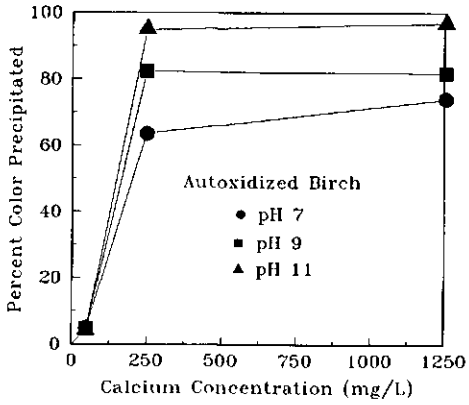


Figure 4. Calcium precipitation of autoxidized birch bark extract color with different pH regimes. Extract Parameters: The total and tannin color (440 nm); 6.09 and 3.88 (1x, absorbance units). The extract was diluted 2x for precipitation with calcium.

4. DISCUSSION

The problems associated with the autoxidative approach for detoxifying bark extracts, namely its application was limited for specific species of bark, were resolved by applying alternative oxidation methods. These were either based on the destruction of the toxic tannin structure with H_2O_2, or by the extensive polymerization of the tannins obtained with long term aerobic treatments. Additionally, reliable detoxification for all bark species tested was achieved with the autoxidation combined with a subsequent active carbon treatment.

The high level of detoxification achieved with the H_2O_2 oxidations indicates the non-toxicity of the end products obtained after destructive oxidation. The end products are not highly colored compounds, thus the destructive methods of oxidative detoxification are advantageous if color norms must be fulfilled. The application of H_2O_2 itself might not be economically feasible, considerable costs are expected to be involved in the H_2O_2 consumption. The H_2O_2 consumption needed for maximizing the detoxification was found to correspond to approximately 1g H_2O_2 g^{-1} COD. Future work in the area of destructive oxidations should examine less expensive oxidants which may give a similar result, like O_3. Additionally, white-rot fungi, deserve attention since their lignolytic activities (ligninase), which catalyze destructive reactions (Tien and Kirk, 1983 and 84), have already been applied to decolorize lignin containing wastewaters (Fukuzumi, 1980; Paice and Jurasek, 1984; Livernoche et al., 1983; Eaton et al., 1980 and 82; Sundman et al., 1982).

The long term aerobic treatments of bark extracts applied in this study polymerized the extracts beyond that which can be achieved by high pH autoxidation and were likewise less toxic. This indicates that the limitations of the autoxidation method can be resolved by developing methods which provide a more extensive polymerization. In this study, the aerobic treatments required 3 to 4 weeks to obtain the extensively polymerized extracts. Considering that the pH of the aerobically treated extracts had risen to 6.5, it is quite possible that the polymerization was the result of long term low pH autoxidation. Autoxidation of phenolic compounds is known to occur at pH values as low as 6 (Haider et al., 1975). In order to develop methods of rapid polymerization that are more extensive than high pH autoxidation, future work should investigate the possibilities of utilizing phenol oxidases of fungi and plants. However, one foreseeable problem is the high inhibition of such enzymes caused by tannins (Lyr, 1961; Goldstein and Swain, 1965). In any case, polymerization of tannins has been observed during the aeration of apple pulp at pH values lower than feasible for autoxidation, presumably the polymerization reactions were caused by phenol oxidases in the pulp (Lea, 1982; Verspuy and Pilnik, 1970). Polymerization of bark extract tannins by fungal cultures at low pH values has also been observed (Updegraff and Grant, 1975). Additionally, phenol oxidases have been used to polymerize monomeric model compounds of procyanidins (Hathway and Seakins, 1957).

The non-toxic soluble dark colored humic end products produced from the oxidative polymerization of tannins were precipitable with calcium. Therefore if color and non-biodegradable COD produced by polymerization methods, must be removed from the wastewater, calcium precipitation can be applied as part of the wastewater treatment system.

5. LITERATURE CITED

Eaton, D., H-. M. Chang and T. K. Kirk. 1980. Fungal decolorization of kraft bleach plant effluents. Tappi, 63(10): 103-106.

Eaton, D. C., H-. M. Chang, T. W. Joyce, T. W. Jeffries and T. K. Kirk. 1982. Method obtains fungal reduction of the color of extraction-stage kraft bleach effluents. Tappi, 65(6): 89-92.

El-Rehaili, A. M. and W. J. Weber, Jr. 1987. Correlation of humic substance trihalomethane formation potential and adsorption behavior to molecular weight distribution in raw and chemically treated waters. Wat. Res. 21: 573-582.

Field, J. A., M. J. H. Leyendeckers, R. Sierra-Alvarez, G. Lettinga and L. H. A. Habets. 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., G. Lettinga and L. H. A. Habets. 1990. Oxidative detoxification of aqueous bark extracts. Part I: Autoxidation. J. Chem. Tech. Biotechnol. (submitted; Chapter 8 dissertation).

Fukuzumi, T. 1980. Microbial decolorization and defoaming of pulping waste liquors. In: T. K. Kirk et al. (eds) Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications, Vol. 2, pp. 162-177, CRC Press, Boca Raton, Florida (USA).

Glasser, W. G. 1980. Lignin. In: Casey, J. P. (ed.) Pulp and Paper Chemistry and Chemical Technology. pp. 39-113. John Wiley and Sons, NY.

Goldstein, J. L. and T. Swain. 1965. The inhibition of enzymes by tannins. Phytochemistry, 4: 185-192.

Haider, K., J. P. Martin, Z. Filip and E. Fustec-Mathon. 1975. Contribution of soil microbes to the formation of humic compounds. In: D. Povoledo and H. L. Golterman (eds.) Humic Substances: Their Structure and Function in the Biosphere. Proceedings of an International Meeting Held at Nieuwersluis, The Netherlands May 29-31, 1972. PUDOC-Centre for Agricultural Publishing and Documentation (1975), Wageningen, The Netherlands. pp. 71-85.

Hathway, D. E. and J. W. T. Seakins. 1957. Enzymatic oxidation of catechin to a polymer structurally related to some phlobatannins. Biochem. J., 67: 239.

Lea, G. H. A. 1982. Reversed-phase high performance liquid chromatography of procyanidins and other phenolics in fresh and oxidising apple juices using a pH shift technique. J Chromatogr. 238: 253 - 257.

Livernoche, D., L. Jurasek, M. Desrochers, J Dorica and I. A. Veliky. 1983. Removal of color from kraft mill wastewaters with cultures of white-rot fungi and with immobilized mycelium of Coriolus versicolor. Biotechnol. Bioengineer. 25: 2055-2065.

Lyr, H. 1961. Hemmungsanalytische untersuchungen an einigen ektoenzymen holzzerstorender pilze. Enzymologia, 23: 231-248.

Milstein, O., A. Haars, A. Majcherczyk, J. Trojanowski, D. Tautz, H. Zanker and A. Huttermann. 1988. Removal of chlorophenols and chlorolignins from bleaching effluent by combined chemical and biological treatment. Wat. Sci. Tech. 20(1): 161-170.

Paice, M. G. and L. Jurasek. 1984. Peroxidase-catalyzed color removal from bleach plant effluent. Biotechnol. Bioengineer. 26:477-480.

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

Sundman, G., T. K. Kirk and H-. M. Chang. 1981. Fungal decolorization of kraft bleach plant effluents: Fate of the chromophoric material. Tappi, 64(9): 145-149.

Tien, M., and T. K. Kirk. 1983. Lignin-degrading enzyme from the hymenomycete Phanerochaete chrysosporium burds.. Science, 221: 661-663.

Tien, M., and T. K. Kirk. 1984. Lignin-degrading enzymes from Phanerochaete chrysosporium: Purification, characterization, and catalytic properties of a unique H_2O_2 requiring oxygenase. Proc. Natl. Acad. Sci. USA, 81: 2280-2284.

Updegraff, D. M. and W. D. Grant. 1975. Microbial utilization of Pinus radiata bark. Appl. Microbiol. 30: 722-723.

Verspuy, A. and W. Pilnik. 1970. Recovery of apple juice by pulp fermentation II: shortening the fermentation time. Flussiges Obst. 37:518 - 519.

CHAPTER 10

Measurement of Low Molecular Weight Tannins: Indicators of Methanogenic Toxic Tannins.

(Submitted for publication)

MEASUREMENT OF LOW MOLECULAR WEIGHT TANNINS: INDICATORS OF METHANOGENIC TOXIC TANNINS

J. Field[1], G. Lettinga[1] and L. H. A. Habets[2]

[1]Dept. of Water Pollution Control
Wageningen Agricultural University
Bomenweg 2, 6703 HD, Wageningen
The Netherlands

[2]Paques B.V.
P.O. Box 52, 8560 AB Balk
The Netherlands

ABSTRACT - The purpose of this study was to evaluate the effectiveness of several low MW tannin measurement methods for indicating the tannin toxicity. The methanogenic toxicity of the low and high MW tannins from autoxidized bark extracts was studied by selective removal of MW fractions from the extract with active carbon adsorption and calcium precipitation treatments. The toxicity of the low MW tannin fraction and the nontoxicity of the high MW tannin fraction were demonstrated. The low MW tannin concentration, determined by a HPLC method and a method based on the loss of tannins by treatment with granular active carbon (AC), had a very close relationship with the methanogenic toxicity; whereas, a poor relationship was found based on the total tannin concentration. The low MW tannins detected by the HPLC and AC methods had similar peak area positions in HPLC chromatograms as the tannins that were adsorbed by polyamide (trisacryl GF05) gel beads. These gel beads have an exclusion limit of 3000 g $mole^{-1}$, indicating that this is the approximate MW boundary between toxic and non-toxic tannins.

KEY WORDS - tannin, methanogenic toxicity, bark, HPLC, anaerobic digestion, granular active carbon, trisacryl GF05 gel, polyvinylpyrrolidone, molecular weight, autoxidation, calcium precipitation

1. INTRODUCTION

The coniferous bark extracts are useful for the study of tannin toxicity. The methanogenic toxicity of these extracts is almost entirely due to the tannin fraction (Field et al., 1988). The tannins of bark are predominantly oligomers (Porter, 1974; Karchesy and Hemingway, 1980; Hemingway et al., 1981 and 1982). According to the trends outlined by the tannin theory, only the oligomeric forms of tannins are effective inhibitors of methane bacteria (Field et al., 1989). The detoxification of coniferous bark extracts by their autoxidative polymerization was due to the conversion of the oligomeric tannins to high MW tannins (Field et al., 1990a). Therefore, the total tannin concentration cannot be used to estimate the residual toxicity of autoxidized extracts; since only, the oligomeric fraction is responsible for the toxicity.

The purpose of this study was to determine the applicability of measuring the low MW tannin fraction as an indicator of the methanogenic toxicity. Methods were investigated to distinguish low and high MW fractions of the tannins. The ability to distinguish between monomeric and oligomeric tannins of the low MW fraction is not considered necessary since the bark tannins contain relatively low contents of monomeric tannins. A method for determinig the low MW condensed tannins with a HPLC technique has previously been

described (Field et al., 1989). In this study, alternative methods were also investigated by use of adsorbants that were selective for the low MW compounds. The use of such methods could be advantageous by eliminating the need for a HPLC in order to determine the low MW tannins.

2. MATERIALS AND METHODS

2.1 Materials

Extracts were prepared from the bark of norway spruce (Picea abies), utilizing 18 g L^{-1} air dried milled bark in 60° C water and shaking for 3 hours. To avoid premature autoxidation of the extracts, 250 mg L^{-1} of ascorbic acid was added and the extraction was conducted with N_2 gas in the head space.

2.2. Analytical and Bioassays

The analytical methods regarding the measurement of COD, UV (215nm) and color (440nm) are described in previous articles (Field and Lettinga, 1987; Field et al., 1988; Field et al., 1990a). The total tannin determination was based on measuring the adsorption of soluble bark extract matter on an insoluble polyamide polymer called, "polyvinylpyrrolidone" (PVP). The PVP method of tannin determination is described in detail by Field et al. (1988). In this study, 14.3 g PVP L^{-1} bark extract was shaken for 1 hour at 30° C and filtered. The difference in COD, UV (215nm) and Color (440nm) before and after adsorption on PVP is taken as the total tannin concentration expressed either as a COD concentration or as absorbance units of UV or visible light.

The method used for determining methanogenic toxicity is described in previous articles (Field and Lettinga, 1987; Field et al., 1988; Field et al., 1990a). In this study, VFA substrate was supplied at 4.2 g COD L^{-1}. The granular methanogenic sludge concentration was 1.4 g VSS L^{-1} and 2 g L^{-1} $NaHCO_3$ was used as a buffer. A second VFA feeding was conducted after 14 days exposure to the extract containing media to determine the residual sludge activity in replaced media with extract not present. The control (fed VFA only) methanogenic activities were 764 and 952 mg COD g^{-1} VSS d^{-1} during the second VFA feeding of experiments labelled part "A." and "B.", respectively. All bioassay results reported in this study are the averaged data of duplicate run experiments.

2.3 Measuring the Low Molecular Weight Tannins

Three methods were utilized for quantifying the concentration of low MW tannins distinctly from the total tannin concentration: (1) HPLC method estimating the low MW tannin fraction from HPLC chromatograms; (2) AC method measuring the tannins that are adsorbed by granular active carbon; and (3) GG method measuring the tannins adsorbed by a polyamide gel bead adsorbant, trisacryl GF05. In all cases, the high MW tannins are calculated from the difference between total tannins (PVP determined) and the low MW tannins.

2.3.1 HPLC Method

The HPLC method has been previously described (Field et al., 1989). In this study, the peak area was detected at 280 nm. The low MW tannins are represented by the tannin peak area of less than 28 minutes retention time. The ratio of the low MW tannin peak area to total tannin peak area is multiplied by the total tannin concentration (PVP determined tannins) based on COD, UV 215nm or Color (440nm) to obtain the low MW tannin concentration estimate.

2.3.2. AC Method

The AC method was conducted by measuring the PVP determinable tannins in both a membrane filtered sample and a membrane filtered exhaustively AC treated (28 g L^{-1} granular AC for 2 hours) sample. Details of the exhaustive AC treatment were described previously (Field et al., 1988). The difference in the PVP determined tannin contents of the untreated and AC treated sample is equal to the low MW tannins. This method assumes that granular active carbon is only able to adsorb the low MW tannins. The ability of active carbon to adsorb the predominantly oligomeric bark tannins was established (Field et al., 1988). Additionally, a hydrolyzable tannin oligomer (gallotannic acid) and a condensed tannin monomer (catechin) were tested and adsorbed for 96.2 and 98.5%, respectively when treated with AC (as solutions of 1 g L^{-1}). The inability of active carbon to adsorb high MW tannins was indicated from the poor removal of high MW tannins by AC treatment of autoxidized spruce bark extracts (Field et al., 1990b).

2.3.3. GG Method

Trisacryl GF05 gel beads obtained from Société Chimique, Villeneuve-La-Garenne, France, were utilized as an adsorbant for low MW tannins. The gel beads are manufactured for gel permeation chromatography and have an exclusion limit of 3000 g $mole^{-1}$. Trisacryl GF05 is composed of an amide containing polymer that offers sites for hydrogen bonding with the low MW tannins that penetrate the beads.

The bark extract sample was shaken together with 35.8 g L^{-1} (dry weight) trisacryl GF05 beads for 1 hour, the membrane filtered filtrate was again treated with the same amount of trisacryl GF05 for another hour and then membrane filtered. A replicate of the sample was brought through the same procedure without any beads present. The difference in in COD concentration (or UV or color) between the untreated and trisacryl GF05 bead treated samples is equal to the low MW tannins COD (or UV or color). Distilled water was also treated with the gel beads to correct for COD and UV contributions originating from the gel beads. The method is abbreviated, "GG".

2.4. Treatment of Bark Extracts For Testing Toxicity

2.4.1. Autoxidation

The autoxidation was generally (unless otherwise stated) performed by aerating extracts at a high pH (11.5) for approximately one day as described in Field et al. (1990a).

2.4.2. PVP Treatment

Extracts were shaken with 14.3 g L^{-1} PVP for one hour and then paper filtered.

2.4.3. AC Treatment

Extracts were intensively shaken with 28.6 g L^{-1} granular AC for two hours then centrifuged followed by paper filtering.

2.4.4. Ca^{2+} Treatment

Extract treated at pH 8 with 500 mg L^{-1} of Ca^{2+} (as $CaCl_2$) for two hours, then decanted through a paper filter.

2.4.5. GG Treatment

Experiments were attempted by treating the bark extracts with trisacryl GF05 gel under the same conditions as the GG method for determining low MW tannins. However, the gel introduced toxicity in the liquid which came into contact with it, most likely related to the presence sodium azide in the gel (present in the supplied product). For this reason, the bioassay experiments were abandoned for investigating the removal of toxicity with trisacryl GF05 gel.

3. RESULTS

3.1. Effect of Treatments on COD, UV, Color and Total Tannins

The effect of various treatments on the basic parameters of the bark extracts are shown in Table 1. Autoxidation was responsible for drastically increasing the color of the extract, which resulted from the polymerization of the tannins. Only minor changes in the total extract COD and UV were observed. The autoxidation did cause a distinct decrease in the total tannin concentration (approximately 30% based on tannin COD and UV). The AC treatment removed 87 to 92% of the total extract COD and UV as well as remove 91% of the total tannin COD and UV. The AC treatment was thus capable of almost completely adsorbing the unoxidized bark matter. However, once the bark was autoxidized, the AC treatment was only able to adsorb 54 to 59% of the total COD and UV, and only 40% of the total tannin COD and UV. The lower adsorption of autoxidized bark matter on AC is perhaps due to a decreasing effectiveness of granular AC towards compounds of increasing MW (El-Rehaili and Weber, 1987). In contrast to AC, precipitation with calcium (Ca) was ineffective in eliminating total COD and UV and total tannins from the unoxidized extracts

Table 1. The COD, UV and Color of Untreated and Treated Spruce Bark Extracts and the Total Tannin Concentrations Based on COD, UV and Color Measurements.

Treatments		COD		UV (215nm)		Color (440nm)	
#	name[a]	total	tannin	total	tannin	total	tannin
		---- mg L^{-1} ----		---- 1x,1cm ----		---- 1x,1cm ----	
A. AC Exp.[b]							
2	untrt	4237	2397	109.5	88.4	0.51	0.24
3	untrt+Ac	543	205	8.4	7.6	0.10	ND[c]
4	auto	4127	1808	94.3	60.1	4.74	3.67
5	auto+AC	1679	1072	43.4	35.8	2.92	2.67
7	auto+PVP	2319	0	34.2	0.0	1.07	0.00
B. Ca Exp.[b]							
2	untrt	4533	2881	139.5	110.1	0.76	0.51
8	untrt+Ca	4120	2606	120.3	102.9	0.43	0.33
4	auto	4372	1877	124.6	75.7	6.20	4.03
9	auto+Ca	3099	1316	60.9	44.8	2.88	2.46
7	auto+PVP	2495	0	48.9	0.0	2.17	0.00

[a] Treatments: 2.= untreated spruce bark extract; 3.= extract treated with the exhaustive AC method; 4.= autoxidized extract (16 h, pH 11.5); 5.= autoxidized extract treated with the exhaustive AC treatment; 7.= autoxidized extract treated with PVP treatment; 8.= extract treated with Ca treatment; 9.= autoxidized extract treated with Ca treatment.

[b] The spruce bark extracts were diluted 1.6x (A) or 1.5x (B) to a stock extract for the analysis, the results given in this table are the concentrations measured in the diluted extract. The extracts used in the AC (A) and Ca (B) experiments were prepared separately

[c] ND = no data, not measured

(only 7 to 14% elimination). The Ca precipitation was effective with the autoxidized extract, in which case 29 and 51% of the total COD and UV, respectively were precipitated and 30 and 41% of the total tannin COD and UV were precipitated. The improvement is due to an increasing effectiveness of precipitation reactions with phenolic compounds of increasing MW (Schmidt and Joyce, 1980; Milstein et al., 1988; El-Rehaili and Weber, 1987).

3.2. Effect of Treatments on Low and High MW Tannins

Tables 2 and 3 present the low and high MW tannins measured by the three different methods in the untreated and treated bark extracts. The tables illustrate that the major effect of the autoxidation is to decrease the low MW tannin concentration. The average decrease was 70 and 74 % of the low MW tannin COD and UV. The decrease in low MW tannins coincided with an increase in the high MW tannin concentration. Therefore, the polymerization reactions of the autoxidation change the MW composition of the total tannins.

The residual low MW tannins in the autoxidized extract were highly adsorbed by the AC treatment. The average low MW tannin removal by AC was 79, 75 and 55% based on COD, UV and color, respectively. In contrast, the high MW tannins were not effectively adsorbed. The average high MW tannin removal was 14, 25 and 3% based on COD, UV and color, respectively. The Ca precipitation was not at all effective in precipitating the low MW tannins from the autoxidized extracts. However, the high MW tannins were eliminated on the average for 46, 50 and 50% based on COD, UV and color, respectively, by Ca precipitation. In conclusion, the results indicate that the AC treatment of autoxidized extracts selectively

Table 2. The Low Molecular Weight (MW) Tannins in Untreated and Treated Spruce Bark Extracts as Measured by Various Methods.

Extract	COD				UV (215 nm)				Color (440 nm)			
Treatments	HPLC[c]	GG[c]	AC[c]	AVG[d]	HPLC	GG	AC	AVG	HPLC	GG	AC	AVG
# name[a]	mg L^{-1}				1x,1cm				1x,1cm			
A. AC Exp.[b]												
2. untrt	1889	1665	2192	1915	69.7	63.7	80.8	71.4	0.19	0.29	ND[f]	0.24
4. auto	591	840	736	722	19.6	18.3	24.3	20.7	1.20	0.74	1.00	0.98
5. auto+AC	144	149	NA[e]	147	4.8	4.8	NA	4.8	0.36	0.44	NA	0.40
B. Ca Exp.[b]												
2. untrt	2161	1844	2751	2252	82.6	80.7	102.9	88.7	0.38	0.40	0.32	0.37
4. auto	460	346	638	481	18.6	18.0	28.2	21.6	0.99	0.65	1.30	0.98
9. auto+Ca	360	459	818	546	12.3	12.6	27.2	17.4	0.67	0.73	1.34	0.91

[a] Treatment name abbreviations are defined in the footnote of Table 1.

[b] See footnote "b" in Table 1.

[c] Low MW Tannins: HPLC = estimated from the HPLC data; GG = determined by adsorption on trisacryl GF05; AC = determined by PVP tannin measurements before and after AC treatment of extract.

[d] AVG = averaged low MW tannin concentration based on HPLC, GG, and AC methods.

[e] NA = not applicable

[f] ND = no data, not measured

removes the low MW tannins, whereas Ca precipitation selectively removes high MW tannins. These trends are seen in the HPLC chromatograms of Figures 1 and 2.

The three different methods of determining low MW tannins, basically predicted the same trends in the low MW tannin concentration with respect to the various treatments of the bark extract. The methods only differed to a small extent in the absolute concentration of low MW tannins measured. Generally, the AC method predicted the highest concentration, whereas the GG method predicted the lowest concentration. Figure 1 illustrates that the AC method exhaustively removed the low MW tannin peak area in the HPLC plots of the autoxidized extracts, but also illustrates that the AC method additionally removed a small part of the high MW tannin peak area. The GG method did not exhaustively remove the low MW tannin peak area of the HPLC plots, but it did not adsorb any high MW tannin peak area in the autoxidized extract.

3.3. Effect of Treatments on Methanogenic Toxicity

Figure 3, illustrates the effect of the various bark extract treatments on the methanogenic activity of sludge exposed to these extracts for 2 weeks. The activity results are plotted together with a COD balance of the bark extracts in order to illustrate which fraction of the COD is most likely responsible for the toxicity. The non-tannin fraction of the autoxidized extracts are not responsible for any toxicity. In a previous study, we have also shown that the non-tannin fraction of unoxidized spruce bark extracts are not responsible for any toxicity (Field et al., 1988). The autoxidation of the extracts dramatically reduces the toxicity, but partial inhibition is still evident. The partial inhibition

Table 3. The High Molecular Weight (MW) Tannins in Untreated and Treated Spruce Bark Extracts as Measured by Various Methods.

Extract	COD				UV (215 nm)				Color (440 nm)			
Treatments	HPLC[c]	GG[c]	AC[c]	AVG[d]	HPLC	GG	AC	AVG	HPLC	GG	AC	AVG
# name[a]	mg L^{-1}				1x,1cm				1x,1cm			
A. AC Exp.[b]												
2. untrt	508	732	205	482	18.7	24.7	7.6	17.0	0.05	-0.04	ND[f]	0.01
4. auto	1217	968	1072	1086	40.5	41.8	35.8	39.4	2.47	2.94	2.67	2.69
5. auto+AC	928	923	NA[e]	926	31.0	31.0	NA	31.0	2.31	2.23	ND	2.27
B. Ca Exp.[b]												
2. untrt	720	1037	130	629	27.5	29.4	7.2	21.4	0.13	0.11	0.19	0.14
4. auto	1417	1531	1239	1396	57.1	57.7	47.5	54.1	3.04	3.38	2.73	3.05
9. auto+Ca	956	857	498	770	32.5	32.2	17.6	27.4	1.78	1.73	1.12	1.54

[a] Treatment name abbreviations are defined in the footnote of Table 1.

[b] See footnote "b" in Table 1.

[c] High MW Tannins ((Total Tannin) - (Low MW Tannin)): definitions HPLC; GG and AC determined low MW tannin measurements are given in Table 2 footnote C.

[d] AVG = averaged high MW tannin concentration based on HPLC, GG, and AC methods.

[e] NA = not applicable

[f] ND = no data, not measured

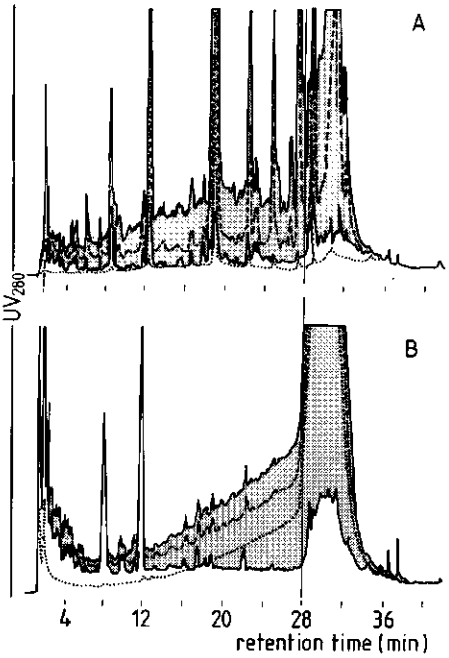


Figure 1. The HPLC chromatograms of untreated extract (A) and autoxidized (B) extracts of spruce bark. The boundary between low and high MW tannins is at 28 minutes retention time. For each sample, the top solid line is the entire extract, the bottom solid line is the PVP treated extract. The tannin peak area is the shaded area between the two chromatograms. The upper dotted line (long dashes) is the GG treated extract. The lower dotted line (short dashes) is the AC treated extract.

cannot be due to the high MW tannins as these increase in concentration as a result of the autoxidation. Therefore, the most likely fraction associated with residual toxicity in autoxidized spruce bark extracts is the low MW tannin fraction. The Ca precipitation, which has no effect on the low MW tannin fraction in autoxidized extract also had no effect in relieving the residual toxicity even though it was capable of removing about half of the high MW tannins. On the otherhand, the AC treatment of the autoxidized extract removed a majority of the low MW tannin fraction without altering the high MW tannin concentration and this corresponded to a distinct decrease in the methanogenic toxicity.

3.4. Tannins as an Indicator of Methanogenic Toxicity

3.4.1. Total (PVP) Tannin Data

The PVP determined tannin data from previous studies and this study are plotted in Figures 4(A) and 4(B) as a concentration in the toxicity assay media versus the activity of the exposed sludge. When the results were obtained from an experiment with a dilution series of unoxidized bark extract, a strong relationship between the sludge activity and PVP determined tannins was observed (Figure 4(A)). From this experiment, the 50% inhibiting concentration of spruce bark tannins was estimated to be 550 mg COD L^{-1}. In contrast, when the results obtained from experiments where the bark extracts were oxidatively treated or otherwise treated with AC or Ca^{2+}, than the relationship between the activity and PVP determined tannins was very poor (Figure 4(B)).

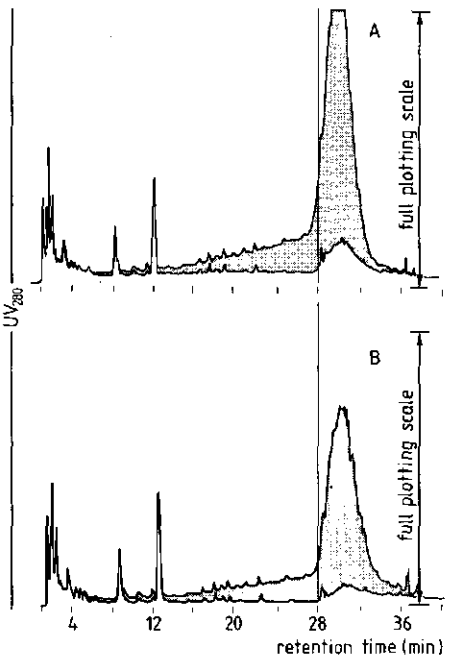


Figure 2. The HPLC chromatograms of autoxidized (A) and Ca treated autoxidized (B) extracts of spruce bark. The boundary between low and high MW tannins is at 28 minutes retention time. For each sample, the top solid line chromatogram is the entire extract, the bottom solid line chromatogram is the PVP treated extract. The tannin peak area is the shaded area between the two chromatograms.

The dilution experiments did not change the ratio of low MW to total tannins, while the oxidation experiments did. When the oxidation treatments alter the MW composition of the tannins, the total tannins are no longer good indicators of the methanogenic toxicity.

3.4.2. HPLC Low MW Tannin Data

The HPLC estimated low MW tannin data are plotted in Figure 5(A) as a concentration in the toxicity assay media versus the activity of the exposed sludge. The results obtained from the oxidation experiments showed a strong relationship between activity and low MW tannins. This can be attributed to the fact that only the low MW tannins are truely toxic and in this case the high MW tannins are discluded from the detection. Of the 24 experiments, 2 points (encircled in the graph) deviated to some extent from the average relationship found with the oxidized extracts. This was possibly due to the fact that these 2 points are from experiments that utilized extracts prepared from a different (older) sample of spruce bark.

The 50% inhibiting concentration of the HPLC determined low MW tannins from unoxidized extracts was 430 mg COD L^{-1} and from the oxidized extracts was 200 mg COD L^{-1}. The higher toxicity of low MW tannins from the oxidized extracts compared with those from the unoxidized extract indicates that the low MW tannins from these sources differ in toxicity. This behaviour is predicted by the tannin theory which postulates an increasing effectiveness of tannins as their size increases, at least as long as they are capable of penetrating bacteria (Field et al., 1989). Based on this postulate, we would expect the tannins with the largest size in the low MW tannin fraction to be the most toxic. The low MW tannins still present after polymerization treatments are more likely to have a greater size than the low MW tannins of the original unoxidized extract.

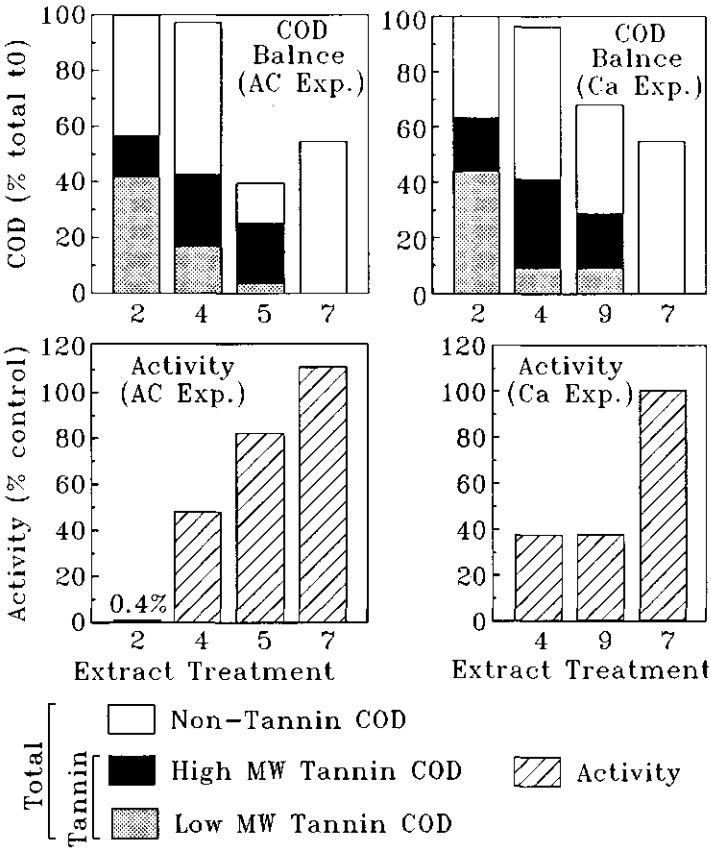


Figure 3. The methanogenic activity of sludge exposed for two weeks to unoxidized (2), autoxidized (4), autoxidized+AC (5), autoxidized+PVP (7), and autoxidized+Ca (9) treated bark extracts. The activity is expressed as a percentage of the activity obtained with a VFA fed control. The results are compared with the low and high MW tannin and non-tannin fractions expressed as a percent of the total COD in the unoxidized extract (% total t0). The stock extract concentrations reported in Tables 1, 2 and 3 were diluted 1.25 X in the assay media for Experiment A (AC) and diluted 1.16 X for Experiment B (Ca).

3.4.3. AC Low MW Tannin Data

The AC determined low MW tannin data are plotted in Figure 5(B) as a concentration in the toxicity assay media versus the activity of the exposed sludge. These results, obtained from the oxidation experiments, also showed a strong relationship between toxicity and low MW tannin concentration. The same two points which deviated in the figure with the HPLC data, also deviated to the same extent with the AC low MW tannin data which indicates that the anomalous behaviour is most likely due to the extract rather than the measurement methods.

The 50% inhibiting concentration of the AC determined low MW tannins from the oxidized extracts was 250 mg COD L^{-1}. This was slightly less toxic than the low MW tannins estimated from the HPLC method because the AC method also adsorbs to a small extent some high MW tannins. However the preferential adsorption for low MW tannins was high enough that this method is equally as useful for indicating the toxicity as the HPLC estimated low MW tannins.

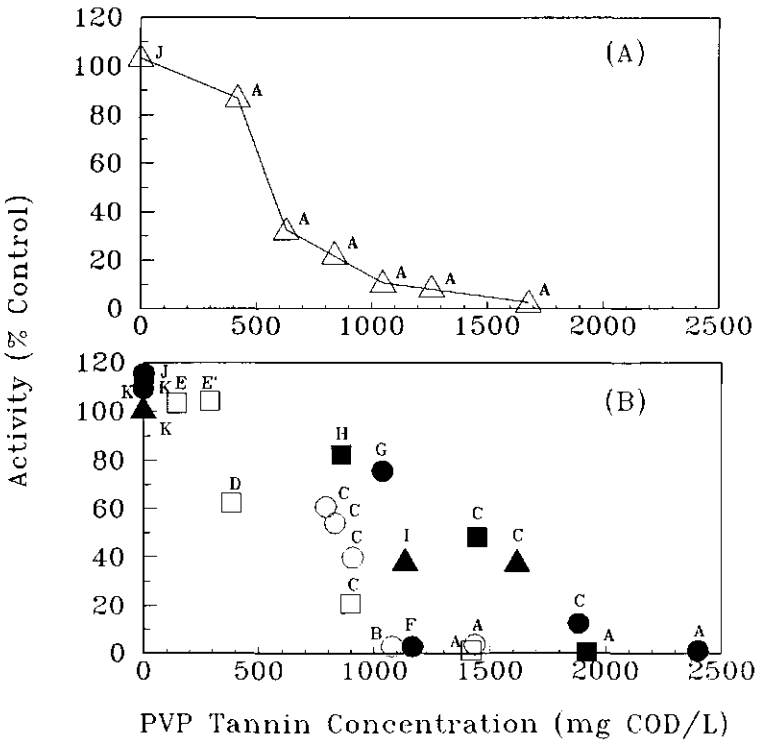


Figure 4. The methanogenic activity of sludge after two weeks of exposures to spruce bark extracts versus the assay concentration (during the exposure period) of the PVP determined tannins. Part A: Data from dilution of the unoxidized extracts. Part B: Data from oxidation treatments of undiluted extract. Experiments: 1 (△) = Field et al., 1988; 2 (○) = Field et al., 1990a; 3 (●) and 4 (□) = Field et al., 1990b; 5 (■) and 6 (▲) = this study (Figure 3); 7 (▬) and 8 (✱) = additional experiments of this study. Treatments: A, extract only; B, extract autoxidized (< 15 min.); C, extract autoxidized (> 15 min.); D, extract treated 4 weeks biologically (aerobic); E, extract oxidized with H_2O_2; E', autoxidized extract oxidized with H_2O_2; F, extract treated half exhaustively with AC; G, autoxidized extract treated half exhaustively with AC; H, autoxidized extract treated exhaustively with AC; I, autoxidized extract treated with Ca; J, extract treated with PVP; K, autoxidized extract treated with PVP.

4. DISCUSSION

The low MW fraction of tannins present in bark extracts was demonstrated to be the only tannin fraction responsible for toxicity to methanogenic bacteria. The high MW tannin fraction produced by the oxidative treatments of the extracts was likewise shown to not inhibit methane bacteria. Therefore an effective indication of tannin toxicity in bark extracts should limit itself to the detection of only the low MW tannin fraction as opposed to the total tannins. The low MW tannin concentration, determined by the HPLC and AC methods, had a very close relationship with the methanogenic toxicity; whereas, a poor relationship was found, based on the total (PVP) tannin concentration. The low MW tannins detected by the HPLC and AC methods had the same peak area positions (in the HPLC chromatograms) as those that were adsorbed by polyamide gel beads. The adsorption sites of

these beads are limited in availability to tannins with a MW less than 3000 g mole^{-1}. This MW constitutes the approximate upper limit of the various low MW tannins determinations proposed and likewise serves as an approximate boundry between non-toxic and toxic tannins. This MW has also been suggested to be the maximum effective tannin size for the tanning of hides (White, 1957).

The AC method of low MW tannin determination is recommended the most over the other methods. It would be the least expensive because chromatographic equipment would not be necessary. Additionally, the granular active carbon and insoluble polyvinylpyrrolidone adsorbants are widely available.

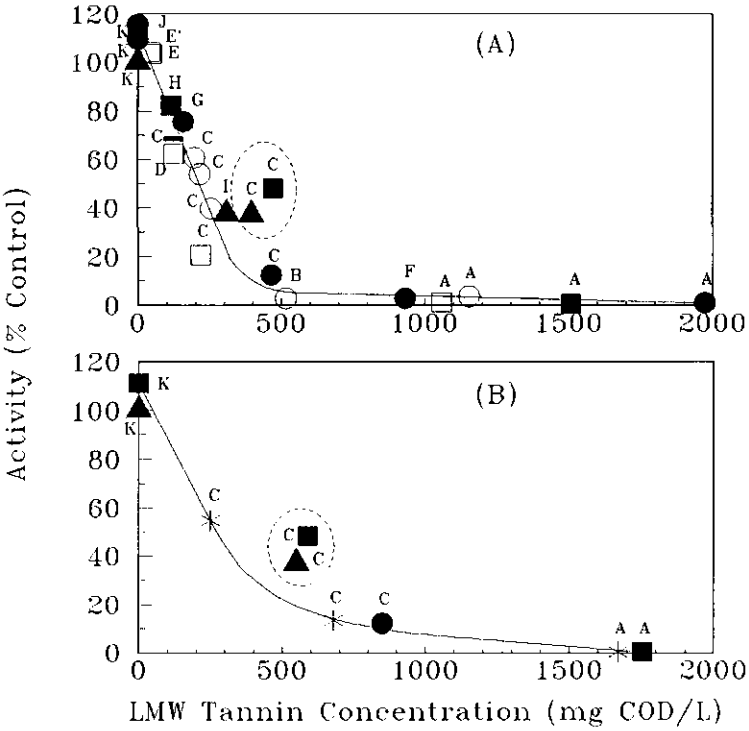


Figure 5. The methanogenic activity of sludge after two weeks of exposures to spruce bark extracts versus the assay concentration (during the exposure period) of the low MW tannins. Part A: Based on low MW tannin data of the HPLC method. Part B: Based on the low MW tannin data of the AC method. All the data in this figure was obtained from oxidation treatments of undiluted extract. Experiment and treatment legends are in caption of Figure 4.

5. LITERATURE CITED

Field, J. A. and G. Lettinga. 1987. The methanogenic toxicity and anaerobic degradability of a hydrolyzable tannin. Wat. Res. 21: 367-374.

Field, J. A., M. J. H. Leyendeckers, R. Sierra-Alvarez, G. Lettinga and L. H. A. Habets. 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., S. Kortekaas, and G. Lettinga. 1989. The tannin theory of methanogenic toxicity. submitted to Biological Wastes 29: in press.

Field, J. A., G. Lettinga and L. H. A. Habets. 1990a. Oxidative detoxification of aqueous bark extracts. Part I: Autoxidation. J. Chem. Tech. Biotechnol. (submitted; Chapter 8 dissertation).

Field, J. A., R. Sierra-Alvarez, G. Lettinga and L. H. A. Habets. 1990b. Oxidative detoxification of aqueous bark extracts. Part II: Alternative methods. J. Chem. Tech. Biotechnol. (submitted; Chapter 9 dissertation).

Hemingway, R. W., L. Yeap Foo and L. J. Porter. 1981. Polymeric proanthocyanidins: Interflavonoid linkage isomerism in (epicatechin-4) - (epicatechin-4) - catechin procyanidins. J. Chem. Soc. Chem. Commun., pp. 320-322.

Hemingway, R. W., L. Yeap Foo and L. J. Porter. 1982. Linkage isomerism in trimeric and polymeric 2,3 - cis-procyanidins. J. Chem. Soc. Perkin I., pp. 1209-1221.

Karchesy, J. J. and R. W. Hemingway. 1980. Loblolly pine bark polyflavonoids. J. Agric. Food Chem. 28: 222-228.

Porter, L. J. 1974. Extractives of Pinus radiata bark. 2. Procyanidin constituents. New Zealand J. Sci. 17: 213-218.

White, T. 1957. Tannins- their occurence and significance. J. Sci. Food Agric. 8: 377-384.

CHAPTER 11

Anaerobic Treatment of Autoxidized Bark Extracts in Continuous Laboratory-Scale Columns.

(Submitted for publication)

ANAEROBIC TREATMENT OF AUTOXIDIZED BARK EXTRACTS IN CONTINUOUS LABORATORY-SCALE COLUMNS

J. Field[1], M. J. H. Leyendeckers[1], R. Sierra-Alvarez[1], G. Lettinga[1] and L. H. A. Habets[2]

[1]Dept. of Water Pollution Control
Wageningen Agricultural University
Bomenweg 2, 6703 HD, Wageningen
The Netherlands

[2]Paques B.V.
P.O. Box 52, 8560 AB Balk
The Netherlands

ABSTRACT - Debarking wastewaters of the forest industry contain high concentrations of tannins that are inhibitory to methane bacteria. The tannins can be polymerized to non-toxic colored compounds by the application of an autoxidation pretreatment, enabling the anaerobic treatment of easily biodegradable components in the wastewater. The continuous anaerobic treatment of untreated and autoxidized pine bark extract was studied in laboratory scale granular sludge packed columns. The autoxidation doubled the conversion efficiency of bark extract COD to methane (from 19 to 40%). After five months of operation, anaerobic treatment of the autoxidized extracts was feasible at high influent concentrations (~14 g COD L^{-1}) and loading rates (40 g COD L^{-1} d^{-1}) with 98% elimination of the biodegradable fraction.

One possible consequence of autoxidizing the tannin fraction (37% of the extract COD) is a decrease in its biodegradability. This could potentially cause a decrease in the biodegradability of the entire extract. The autoxidation products of the tannins were not transformed during anaerobic treatment, whereas about half of the original tannins of the unoxidized extract were transformed to potentially biodegradable phenolic intermediates. However, the biodegradability of these intermediates was inhibited by a high reactor VFA concentration that resulted from the methanogenic inhibited sludge. Therefore, the average biodegradability (53%) of the entire extract COD during continuous anaerobic treatment was not decreased by the autoxidation pretreatment. The recalcitrant COD expected in the effluents of reactors treating autoxidized debarking wastewater can be effectively separated by calcium precipitation of the autoxidized products out of the influent prior to anaerobic treatment.

KEY WORDS - debarking wastewater, anaerobic treatment, tannin, detoxification, biodegradation

1. INTRODUCTION

Previous studies indicate that the debarking wastewaters of the forest industry are troublesome to treat by the anaerobic methods (Rekunen 1986; Latola 1985). The problems observed were due to their methanogenic toxicity. Aqueous extracts of bark were studied to determine the inhibiting compounds of debarking wastewaters. The tannins, which account for 30 to 60% of the COD and 70 to 90% of the UV absorbance, were shown to cause most of the methanogenic toxicity observed in coniferous bark extracts (Field et al., 1988). Autoxidative treatments can be applied to detoxify the tannins (Field et al., 1990a). The detoxification effect is due to the polymerization of the toxic low MW tannins to non-toxic high MW tannins and non-tannic compounds.

The previous work, however, is based on laboratory batch experiments conducted over relatively short time periods (~2 weeks). The purpose of this study was to evaluate the role of autoxidative detoxification methods in improving the long term anaerobic treatment of coniferous bark extract. Since the autoxidation pretreatment might change the biodegradability of the tannins and other phenols present in the debarking wastewater, an additional objective was to determine if the detoxification method lowers the available substrate for methane production.

2. MATERIALS AND METHODS

2.1. Bark Extracts

Throughout this study aqueous extracts of bark were prepared by adding 60° C tap water to ground air dried bark of scot's pine (Pinus sylvestris) and shaking for 3 hours with N_2 gas in the head space. The pine bark extracts were prepared to different concentrations by using different amounts of air dried bark which ranged from 18 to 144 g L^{-1}. The average characteristics of all the pine bark extracts used in this study are given in Table 1.

Table 1. The Average Tannin to Total COD and UV Ratios, the UV to COD Ratio of the Tannins, and the Yield of Tannins from the Bark Used in this Study for the Preparation of Aqueous Pine Bark Extracts .

PARAMETER	COD tan/tot %	UV215 tan/tot %	UV:COD tan 1x,1cm mg^{-1}	Yield[a] tan/bark %
AVERAGE	36.5	77.8	0.057	5.0
STD (±) n = 29	5.4	7.7	0.008	1.3

[a] yield of tannin dry weight (assuming 1.7 g COD g^{-1} tannin) per dry weight of bark

2.2. Analytical Methods

The UV absorbance of the extracts and samples were based on the absorbance at 215 nm in a 1cm quartz cuvette as described previously (Field et al, 1988).

The tannins were measured according to the polyvinylpyrrolidone (PVP) method, described in detail by Field et al. (1988). In summary, this method is based on the disappearance of COD and UV absorbance when the extract is shaken together with 14.3 g L^{-1} PVP for 1 hour. The HPLC chromatographic procedure and the low MW tannin determination based on the HPLC results were described in previous publications (Field et al., 1989 and 1990a).

The VFA analysis and the determination of phenol, p cresol and carboxycyclohexane were based on the gas chromatographic procedure described by Field et al. (1987). The analysis of trans cinnamic acid, 3 phenylpropionic acid and phenylacetic acid was based on a similar gas chromatographic procedure, using an oven temperature of 190° C instead of 130° C.

2.3. Anaerobic Assays

The toxicity and biodegradability assays with granular methanogenic used in this study were similar to those described previously (Field et al., 1988; Field et al., 1990a). The assays were conducted in 0.5 L serum flasks, which were incubated at 30° C. A new type of toxicity assay was also utilized in one experiment, designated "the repeated batch digestion". The activity was assayed using the substrate present in the bark extract (instead of a VFA spike). The alkalinity was provided by adding 1 g of $NaHCO_3$ per g biodegradable COD. The control assay was fed PVP treated extract. The PVP selectively removes the tannin fraction, which is responsible for the toxicity (Field et al., 1988) but does not contribute significantly to the readily biodegradable substrate. The feedings were repeated by decanting the old media (through a tea sieve), while flushing with N_2 gas, and replenishing the granular sludge with new extract containing media.

The acidification of the bark extract COD was calculated by the sum of COD converted to methane and VFA. This sum was corrected for the same sum obtained from a sludge control (no extract added).

The activity test was conducted for the granular sludge recovered from column experiments. The sludge (1.3 g VSS L^{-1}) was fed 4 g COD L^{-1} neutralized VFA substrate containing acetate; propionate and butyrate in a ratio of 1:1:1 dry wt. or 24:34:41 COD basis. The assay was conducted in two feedings. At the end of the first feeding, the old VFA media was replaced with new VFA media.

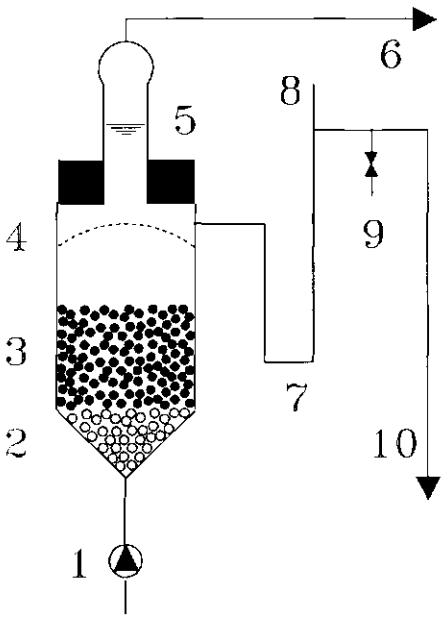


Figure 1. Design of the 0.15 L columns used in the continuous experiments of this study. **1.** influent; **2.** glass beads; **3.** granular sludge bed; **4.** screen; **5.** gas separator; **6.** biogas to mariotte flask; **7.** effluent water lock; **8.** syphon break; **9.** effluent sampling point; **10.** effluent discharge.

2.4. Column Experiments

The continuous experiments were conducted in small glass columns which contained 0.15 L of liquid volume as illustrated in Figure 1. These were placed in a constant temperature room of 30 ±2° C. The influents of the columns were prepared from the bark extracts by adding required nutrients for bacterial growth as described in Field et al. (1987) and by adding alkalinity in the form of $NaHCO_3$ at approximately one gram per gram of biodegradable COD (~ 0.5 g g^{-1} extract COD). The influents which were prepared for 3 to 7 day intervals of operation were placed in a refrigerator. The head space of the influent containers were filled with N_2 gas, and as they were pumped empty, N_2 gas was provided from a gas bag to replenish the void volume.

The methane gas production was measured in mariotte flasks of 10 L volume filled with 5% NaOH to scrub out the CO_2 from the biogas.

In this study two separate experiments were conducted with the columns. The first experiment consisted of three columns treating: 1) untreated (unoxidized); 2) PVP treated; and 3) autoxidized pine bark extract. These columns were each seeded with 20 g VSS L^{-1} reactor with "AVIKO" sludge, a granular sludge originally cultivated on potato processing wastewater but was obtained after several months of storage in an anaerobic lagoon. After seeding, each of the columns were operated under identical conditions with a pH 7 neutralized VFA stock solution (C_2:C_3:C_4 = 24:34:41% COD) supplied at 4.2 g COD L^{-1} and an average loading of 12 g COD L^{-1} d^{-1} for a one month period, followed by an additional two weeks with 8.4 g COD L^{-1} and an average loading of 22 g COD L^{-1} at which time the COD elimination efficiency was 95% or greater. After this 6 week adaption period to the VFA substrate, the column experiment was started (day 0 of the experiment) by changing to the bark extract influents.

The second experiment consisted of one column treating calcium precipitated autoxidized pine bark extract. This column was seeded with 31 g VSS L^{-1} reactor with the sludge obtained from the column 3 (of the first experiment). The experiment was started (day 0) directly with the bark extract influent.

The biodegradability of the bark extracts during the column experiments was evaluated with the following parameters: M = % conversion influent COD to methane; VFA = VFA remaining in the effluent as % of the influent COD; ECOD = the % COD elimination based on the filtered effluent sample. Cells = % conversion of influent COD to cells (estimated from ECOD minus M); A = % conversion of influent COD to acidified products (the sum of M and VFA); BD = % biodegradability of the influent COD (sum of A and Cells); Y = specific cell yield in COD per unit of COD biodegraded; $ECOD_{BD}$ = the % elimination of the biodegradable COD = [1-(VFA/BD)]*100.

2.5. Bark Extracts Pretreatments

Details of the pretreatments applied to the bark extracts are described in the results. Autoxidation was conducted by raising the pH of the bark extract to pH 11.5 (requiring 0.7 to 1 g NaOH L^{-1}), and aerating with a porous aeration stone. The aeration rate applied was high (approximately 30 v/v per hour) to ensure that the supply of air was not limited. After autoxidation, the extract pH was readjusted to 6.5 with HCl.

3. RESULTS

3.1. Repeated Batch Digestion of Pine Bark Extract

3.1.1. Effect Autoxidation on Extract Characteristics

The changes in the pine bark extract characteristics as a function of the autoxidation pretreatment time are illustrated in Figure 2. The figure illustrates that the autoxidative polymerization caused coloration of the extract associated with a decrease in the tannin

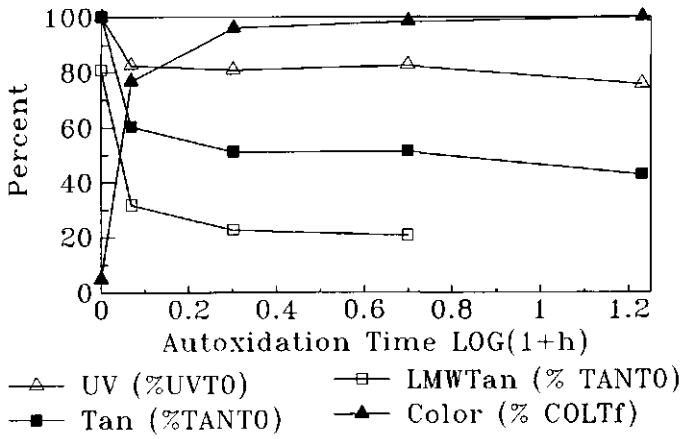


Figure 2. The changes in pine bark extract characteristics as a function of the autoxidation time (using a starting pH of 11.5). Where: UV = extract UV 215nm as a percent of the unoxidized extract UV 215nm (%UVT0), 128 1x,1cm; Tan = the tannin concentration expressed as a percent of the unoxidized extract tannin concentration (%TANT0), 2088 mg COD L^{-1} or 105 1x,1cm 215nm; LMWTan = the low MW tannin concentration expressed as a percent of the unoxidized tannin concentration; Color = extract VIS 440nm as a percent of the maximum VIS 440nm formation during autoxidation (%COLTf), 5.2 1x,1cm. The COD concentration of the extract was 6000 mg L^{-1}.

Table 2. Schedule of Feedings for the Repeated Batch Anaerobic Digestion of Pine Bark Extract and the Absolute Methanogenic Activities of the PVP Treated Extract (Substrate Control).

	FEEDING		BUFFER	COD		CONTROL ACTIVITY[a]
#	Started after	Length for	$NaHCO_3$	Extract[b]	PVP Extract[c]	PVP Extract[c]
	-- days --		g L^{-1}	----- g COD L^{-1} -----		mg COD g^{-1} VSS d^{-1}
1st	0	7	2.0	4.8	3.2	272
2nd	7	7	2.0	4.5	3.0	318
3rd	14	14	2.0	4.6	2.6	245
4th	28	14	4.0	9.4	6.3	409
5th	42	14	4.0	9.4	4.7	394

[a] The percent activity results in Figure 3 are expressed as percent of the activity of the sludge fed PVP treated extract.

[b] Assay concentration of the unoxidized and autoxidized pine bark extracts (no VFA substrate was added to these assays).

[c] Assay concentration of the PVP treated pine bark extracts present at the same dilution as the bark extracts (no VFA substrate was added to this assay). The PVP control was the tannin free extract which supplied 50 to 67% of the extract COD and approximately the same amount of biodegradable bark extract substrate.

content and an even larger decrease of the low MW tannin content. Most of the changes occurred after only one hour of autoxidation time, when the concentration of the toxic low MW tannins was reduced by 75%. The polymerization reactions cause a low decrease in the total UV (only 20%) and no decrease in the COD.

3.1.2. Effect Autoxidation on Extract Toxicity

The schedule of feedings utilized in the repeated batch anaerobic digestion of pine bark extract is listed in Table 2. The extracts provided the substrate for the toxicity test and the control activity was based on the sludge fed PVP treated extract. In a separate experiment with a 4 g COD L^{-1} VFA spike (data not shown), the non-toxicity of the PVP treated extract (unoxidized or autoxidized) was confirmed.

Figure 3 presents the methanogenic activity results of the sludge exposed to the unoxidized and autoxidized extracts during five consecutive feedings. In the first feeding, no toxicity was evident from any of the extracts. By the second feeding (after one week of exposure), the first signs of toxicity were evident from the unoxidized extract; which by the third feeding (after 2 weeks of exposure), caused about 85% inhibition. The autoxidized extracts, on the other hand, were not inhibitory. During the final two feedings when the concentration of the media was raised to 9.4 g COD L^{-1} the level of inhibition caused by the unoxidized extract increased to 90%. The 10 minute autoxidized extract started to cause inhibition which increased to 85% by the fifth feeding. The extracts autoxidized for 1 hour or more had low toxicities (< 25%) throughout all five feedings, indicating that their long term exposure to methanogenic sludge would not cause severe inhibition.

The concentration of individual VFA present in the media at the end of the feedings are reported in Table 3. These results show that the low methanogenic activity was due to toxicity, because the inhibited digestions left VFA unutilized. Particularly, propionate was poorly utilized.

3.1.3. Effect Autoxidation on Extract Acidification

The acidification of pine bark extract COD (to VFA and CH_4 COD) during the five consecutive feedings of the repeated batch digestion is reported in Figure 4. During the first three feedings, from 37 to 45% of the unoxidized and autoxidized extract COD was acidified. Initially, a higher acidification of the unoxidized extract was observed. However, in the second and third feedings, the percent acidification of the unoxidized bark extract decreased while the acidification of the PVP treated and autoxidized extracts was increasing.

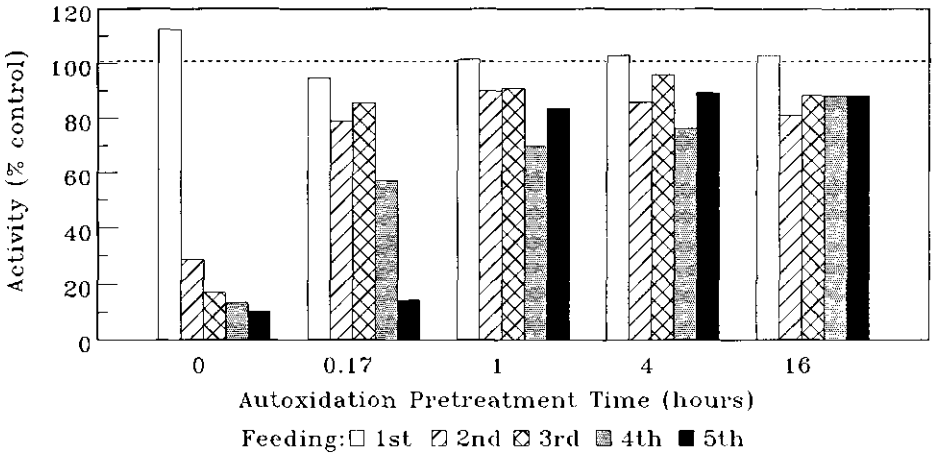


Figure 3. The methanogenic activity of granular sludge fed unoxidized and autoxidized pine bark extracts in 5 consecutive feedings during a repeated batch anaerobic digestion. The schedule of feedings and the extract concentrations are outlined in Table 2.

Table 3. The Acetate (C_2), Propionate (C_3) and Butyrate (C_4) Concentrations in the Media at the End of the Third, Fourth and Fifth Feedings of the Repeated Batch Anaerobic Digestion of Autoxidized Pine Bark Extract.

Extracts	VFA Concentration (mg COD L^{-1})								
	THIRD FEEDING			FOURTH FEEDING			FIFTH FEEDING		
Autoxidation Pretreatment	C_2	C_3	C_4	C_2	C_3	C_4	C_2	C_3	C_4
0 hours[a]	418	573	45	365	785	83	898	771	57
0.17 hours[a]	74	27	0	181	80	1	412	608	104
1 hours	61	20	0	144	48	0	102	75	3
4 hours	54	19	0	115	36	0	88	65	0
16 hours	77	23	0	94	34	0	72	50	0
PVP Control	80	34	0	91	31	0	159	46	0

[a] The unoxidized extract was inhibitory to the methanogenesis in the 3rd, 4th and 5th feedings. The Extract autoxidized for 0.17 h was inhibitory to the methanogenesis in the 5th feeding only.

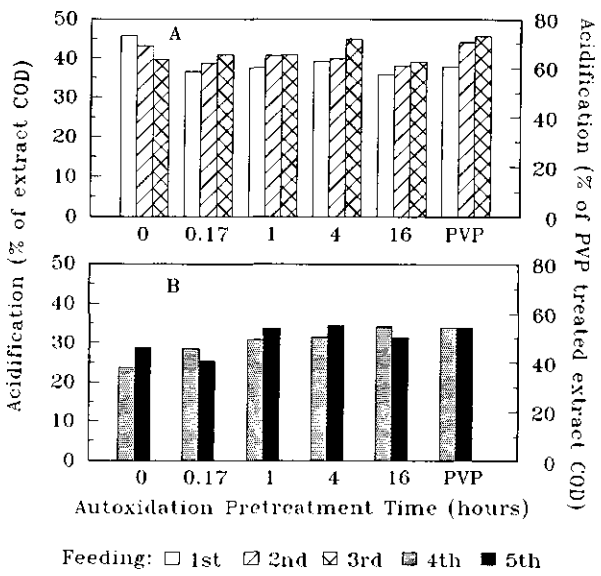


Figure 4. The acidification of unoxidized and autoxidized extracts of pine bark COD in 5 consecutive feedings during the repeated batch anaerobic digestion. Where: acidification = the % conversion of the COD supplied to VFA and CH_4. Part A. 1st to 3rd feeding with approximately 5 g COD L^{-1}. Part B. 4th to 5th feeding with approximately 10 g COD L^{-1}.

When the extract was supplied to the assays at higher concentrations in the fourth and fifth feedings, the autoxidized (≥ 1h) extracts acidified to a greater extent than the unoxidized extracts. At the end of the fourth and fifth feeding, the autoxidized extracts also contained lower concentrations of intermediates derived from the incomplete degradation of plant phenolics; such as phenol, cresol, and carboxycyclohexane (Figure 5).

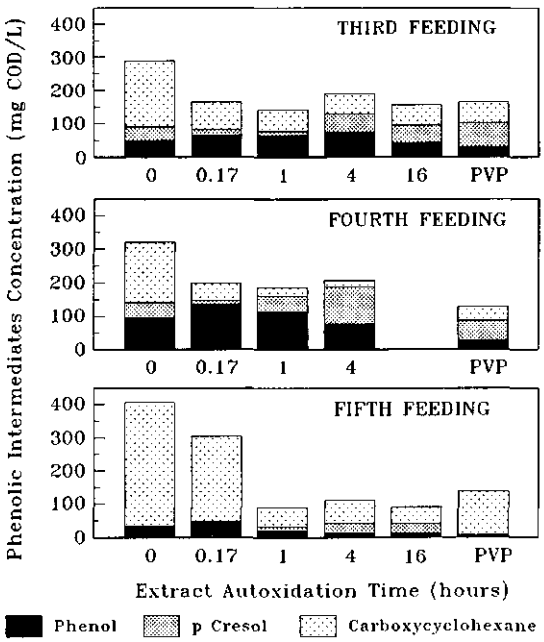


Figure 5. The concentration of phenol, p cresol and carboxycylohexane in the assay media at the end of the third, fourth and fifth feeding of the repeated batch digestion.

3.2. Continuous Treatment of Extract

3.2.1. Experimental Set-Up

Laboratory columns were utilized to investigate the continuous anaerobic treatment of pine bark extract, and pine bark extract autoxidized at a starting pH of 11.5 for 16 hours. In order to determine if the detoxification was complete the pine bark extract was treated with PVP (in accordance with the tannin determination) to prepare a third influent feed stock, which was free of tannins.

The autoxidation did not change the extract COD content and had only a small effect on the UV absorbance. The average decrease in the tannin content by the autoxidation performed on 19 extracts used to prepare influents in this study was 50.8% ±10.9%. The PVP treated extracts contained on the average 63.5% of the COD and only 22.2% of the UV absorbing material compared to the unoxidized extract.

The average load, HRT and influent concentration in various periods of the reactor operation are listed in Table 4.

Table 4. Period Averaged Operation Parameters of the Continuous Anaerobic Treatment of Pine Bark Extract.

Period	Day No. start/ end	Influent Conc. g COD L^{-1}			HRT hours			Load g COD L^{-1} d^{-1}		
		unox[a]	auto[a]	PVP[a]	unox	auto	PVP	unox	auto	PVP
A	0/16	6.5	7.1	4.3	10.6	10.3	10.3	14.8	16.7	10.0
B	17/21	10.2	9.9	7.8	6.4	5.9	5.7	41.4	44.2	34.7
C	22/56	3.9	3.8	2.8	6.7	4.8	5.6	16.4	20.1	13.1
D	57/75	4.8	4.7	3.4	5.7	5.5	7.1	20.1	20.4	12.5
E	76/93	5.9	5.7	4.2	5.1	4.8	5.2	32.1	30.8	22.7
F	94/119	10.1	9.5	6.6	6.2	6.8	7.8	42.1	43.3	23.5
G	120/133	8.2	14.0	4.0	7.0	14.8	7.2	34.6	26.9	19.8
H	134/155	12.9	13.9	9.8	7.0	8.4	7.3	46.8	41.3	33.3
EXP[b]	0/155	7.2	7.9	5.1	6.8	7.2	6.8	29.1	29.1	20.1

[a] unox = not pretreated; auto = pretreated by autoxidation for 16 hours at ph 11.5; and PVP = pretreated by removing the tannin fraction from the extract by adsorption on polyvinylpyrrolidone.

[b] EXP = experimental average

3.2.2. Treatment Performance

The autoxidation of the bark extracts increased the reactor performance. The column fed with the unoxidized extract provided lower levels of COD elimination and had higher effluent VFA concentrations compared to the column fed with autoxidized extract (Figures 6 and 7). This was the result of the methanogenic inhibition caused by the unoxidized extract tannins. The experiment averaged conversion of the COD to methane, was only 19% with the column fed unoxidized extract, whereas twice the yield of methane (40%) was obtained by autoxidizing the extract (Table 5). The low yield of methane from the unoxidized extracts was due to the incomplete utilization of VFA and likewise an incomplete elimination of biodegradable COD was observed. In contrast, the autoxidized extract fed column provided the maximum obtainable methane yield possible, since a complete elimination of the biodegradable substrate occurred.

The toxicity of the unoxidized pine bark extract was strong during the initial periods (A and B), when the methane production decreased to almost nothing. Afterwards (in period C), some adaption to the toxicity was evident. The COD loading was increased in the following periods (D to H), without a decrease in the COD conversion efficiency. Although adaption occurred, throughout the experiment high concentrations of VFA were present in the effluent. The effluent VFA was composed almost entirely of propionate (results not shown). Therefore, the unoxidized tannin toxicity was severe for the metabolism of propionate (as was also observed in the batch experiments). These results indicate that the improvement in methane production after period B was due to adaption of the acetoclastic methanogens.

The treatment of the unoxidized extract with PVP also increased the reactor performance to the same extent as the autoxidation treatment. Although the COD elimination and conversion of COD to methane was higher (Figure 8), this was only due to the fact that the PVP pretreatment removed the poorly biodegradable tannin COD fraction from the influent. The quantity of methane produced and COD eliminated was the same, and both columns provided the same percentage of biodegradable substrate elimination (Table 5).

Table 5. Average Treatment Efficiency and Biodegradation during the Continuous Column Experiments Fed Pine Bark Extract.

Parameter[a]	Units	Experimental Average		
		unox[b]	auto	PVP
ECOD	% infl. COD	37.4	51.8	74.3
M	"	18.8	39.5	56.5
VFA	"	15.8	1.4	2.4
Cells	"	18.4	12.3	16.4
A	"	34.7	40.7	59.0
BD	"	53.1	52.9	76.3
$ECOD_{BD}$	% infl. COD_{BD}	69.9	97.3	97.0
Y	g COD_{cells} g^{-1} COD_{BD}	0.33	0.22	0.21

[a] parameters are defined in Materials and Methods
[b] treatment definitions are defined in the footnote of Table 4

The pine bark tannins were highly detoxified by the autoxidation. The application of high organic loading rates of approximately 40 g COD L^{-1} d^{-1} (25 g biodegradable COD L^{-1} d^{-1}) and high influent concentrations 14 g COD L^{-1} was feasible by the end of the experiment with 98% removal of the biodegradable COD.

3.2.3. Sludge Activity

The sludge, obtained from each of the reactors at the end of the experiment, was assayed for specific methanogenic activity (Table 6). The specific methanogenic activity of the sludge from the column fed PVP treated extract was the highest. The sludge from the autoxidized and unoxidized extract fed columns had 34 and 80% less activity in the first VFA feeding of the assay, respectively. However, if the net methanogenic capacity of the columns are compared (activity multiplied by VSS content) than the autoxidized and unoxidized extracts fed columns had 25% and 77% less capacity, respectively, reflecting a low and high level of toxicity in these extracts. The low activity of the sludge from the column fed unoxidized extract was mostly due to a poor utilization of the propionate and butyrate (Table 7). The large increase in activity of this sludge in the second VFA feeding can be attributed to the adaption to the butyrate. No adaption to the metabolism of propionate was observed even after two weeks of assay in the absence of the toxins.

3.2.4. Biodegradability of Extracts during the Continuous Experiments

The experimental average acidification of the unoxidized pine bark extract was 35% (Table 5). This was lower than the average acidification of 41% from the autoxidized extract. This trend was already recognized in the repeated batch anaerobic digestions. The acidification of the PVP treated extract averaged 59% of the influent COD, but if expressed as a percentage of the whole extract COD (before PVP treatment) it was equal to 37.5%. The experiment averaged biodegradability of the extract COD was the same (53%) for the unoxidized and autoxidized extracts (Table 5). Although the average biodegradability of the PVP treated extract was higher, it was similar (49%) to the other extracts, if expressed as a percent of the original extract COD. Therefore, during continuous anaerobic treatment there was not any additional biodegradable substrate present in the tannin fraction.

The acidification and biodegradability of the extract by the uninhibited PVP treated and autoxidized extract fed columns increased during the course of the experiment (Figure 9). In the final periods of the experiment (periods F to H), the acidification was 39 to 45% greater and the biodegradability was 35 to 38% greater than at the beginning of the experiment

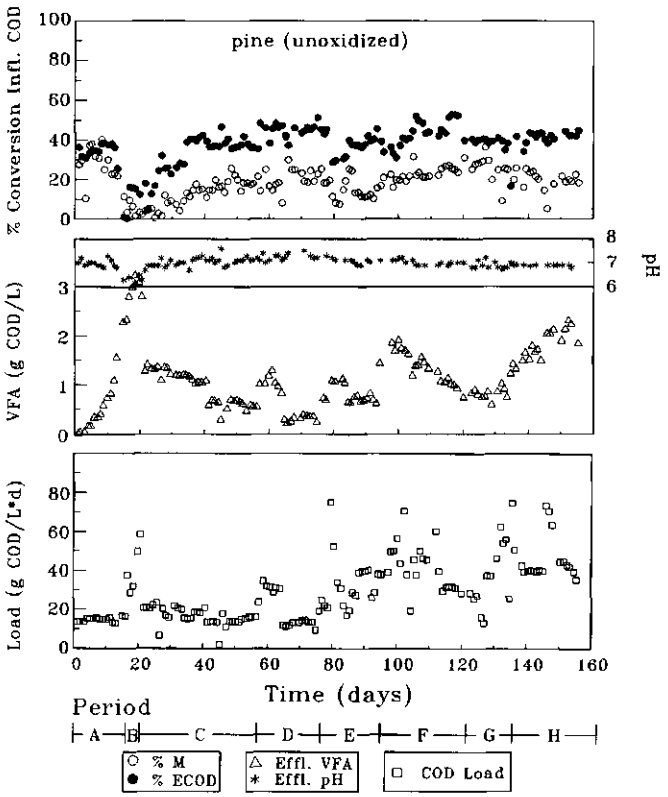


Figure 6. Operation and efficiency during the continuous anaerobic treatment of unoxidized pine bark extract. Where: %M = the percent conversion of the influent COD to CH_4; %ECOD = the elimination of COD based on the filtered effluent sample; Effl. VFA = the effluent VFA concentration in g COD L^{-1}; Effl. pH = the effluent pH; COD Load = the COD loading rate.

(periods A to B). In contrast, the acidification of the unoxidized extract remained largely unchanged and only a 27% increase in biodegradability was observed between the same periods. These results indicate that adaption to the acidification and biodegradability of certain components in the bark extracts occurred in the un-inhibited columns, while a similar level of adaption was not evident in the inhibited column.

The lower acidification of unoxidized compared to detoxified extracts might result from the incomplete biodegradation of phenolic intermediates by methanogenic inhibited digestions, as was observed in the batch digestion study. Their degradation is perhaps hindered by the high VFA concentrations that result from inhibited methanogenesis. Several studies (Lane, 1980; Fedorak et al., 1986; Field et al., 1987) have demonstrated that simple phenolic compounds are poorly degraded if high concentrations of VFA (and presumably H_2) are present in the media. The uninhibited methane bacteria maintain low VFA and H_2 concentrations in the media, which favours the degradation of phenols. This postulate is supported by the fact that these intermediates (phenol, p cresol and carboxycyclohexane) were linearly related to the logarithm of the effluent VFA concentration during the continuous column experiments (Figure 10). On one occasion (day 126) other intermediates of phenol degradation were investigated, these included trans cinnamate, phenylacetate and 3

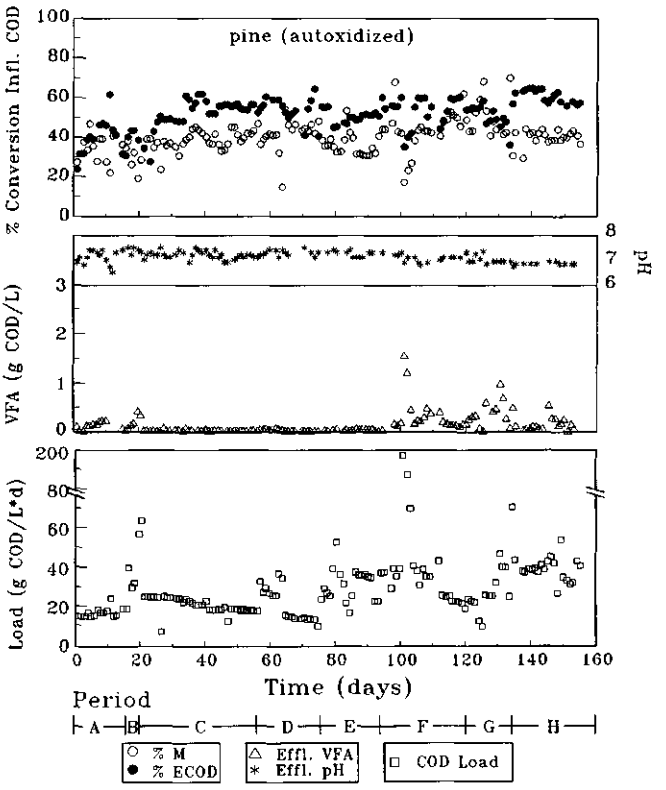


Figure 7. Operation and efficiency during the continuous anaerobic treatment of autoxidized bark extract. Definition of abbreviations are given in Figure 6 caption.

phenylpropionate, of the three only the latter was found (116 to 137 mg COD L^{-1}) in the columns which had significant levels of effluent VFA (600 to 800 mg COD L^{-1}) on the day of sampling.

During periods C to F of the continuous experiment, the elimination of UV absorbance and tannins by anaerobic treatment were measured (Table 8). About half of the UV absorbance and 60% of the tannins were eliminated from the unoxidized extract. Very low levels of UV absorbance were eliminated from the autoxidized extract which was expected assuming that the autoxidized tannin products would have increased recalcitrance (Table 8). The UV elimination that did occur corresponded to the quantity of UV absorbance units eliminated from the non-tannin fraction (PVP treated extract). No elimination of the autoxidized tannins was evident, and to a small extent their concentration increased (negative % elimination). The HPLC chromatograms of the influents and effluents, sampled on day 106, illustrate the partial elimination and biotransformations of the UV absorbing compounds in the unoxidized extracts by anaerobic treatment (Figure 11). In contrast, the high MW autoxidation products were not altered by anaerobic treatment. Although a few of the UV absorbing compounds were degraded in the autoxidized extract, these were the non-tannic phenolic compounds that were not altered by the autoxidation.

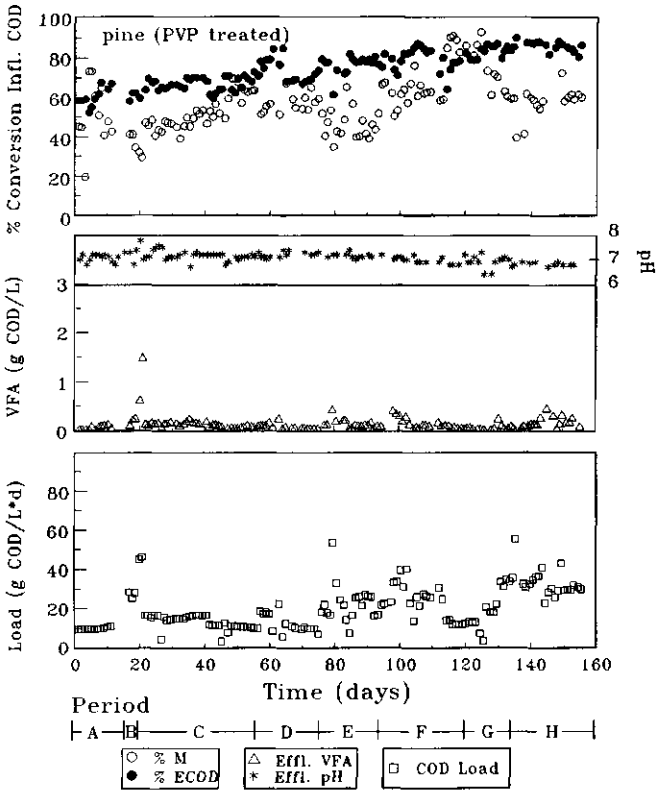


Figure 8. Operation and efficiency during the continuous anaerobic treatment of PVP treated pine bark extract. Definition of abbreviations are given in Figure 6 caption.

The elimination of tannins from the unoxidized extracts must have resulted from biological transformations to non-tannic intermediates of lower UV absorbance or to compounds not having UV absorbance. In any case, their elimination did not coincide to additional acidification nor to additional biodegradability beyond that observed in the autoxidized extract.

3.2.4. Biodegradability of Extracts by Sludge Recovered from the Continuous Experiments

At the end of the experiment, sludge from the column experiments was recovered to study the long term anaerobic biodegradability of bark extract. The sludge recovered from the PVP treated extract fed column was able to biodegrade (Figure 12) more bark extract COD as compared to the sludge obtained from the unoxidized extract fed column. The same trend between the sludges was also observed with respect to the UV elimination (Figure 13) and tannin elimination (Figure 14). Therefore, adapting the sludge to phenolic degradation in the absence of tannins was better for the degradation of the phenolic compounds in the whole bark extract (including tannins). This was probably due to the higher methanogenic activity of sludge cultivated on the non-tannin fraction, which served to maintain a lower VFA concentration in the media and thereby favour the degradation of phenolic compounds.

Table 6. The Sludge Concentration and the Sludge Activity at the End of the Continuous Column Experiments Fed Pine Bark Extract.

Sludge[a]	Sludge Concentration	Sludge Activity		Methanogenic Capacity
	in reactor g VSS L^{-1}	First[b] g COD g^{-1} VSS d^{-1}	Second[b]	of reactor[c] g COD L^{-1} d^{-1}
unox[d]	70	0.111	0.350	7.8
auto	68	0.370	0.561	25.2
PVP	60	0.559	0.797	33.5

[a] the experiment was started with 20 g VSS L^{-1} AVIKO granular sludge, having an activity of 0.62 g COD g^{-1} VSS d^{-1}.

[b] first and second VFA feedings

[c] the methanogenic capacity was calculated by multiplying the first feeding's activity by the VSS concentration in the reactor (the capacity refers to the acidifiable COD).

[d] unox = sludge from column fed unoxidized extract; auto = sludge from column fed autoxidized extract; PVP = sludge from column fed PVP treated extract.

Table 7. The Acetate (C_2), Propionate (C_3) and Butyrate (C_4) Concentrations in the Media at the End of the First and Second Feedings of the Sludge Activity Test Conducted for the Sludge Recovered at the End of the Continuous Pine Bark Extract Experiment.

Sludge	End Feedings[a]					
	FIRST			SECOND		
	C_2	C_3	C_4	C_2	C_3	C_4
	----- VFA concentration (mg COD L^{-1}) -----					
unox[b]	171	1389	1537	134	1436	99
auto	243	252	28	25	33	0
PVP	94	147	11	32	59	0

[a] each feeding lasted 7 days, the starting concentration of C_2, C_3 and C_4 was 979, 1387 and 1673 mg COD L^{-1}.

[b] abbreviations are defined in the footnote of Table 6.

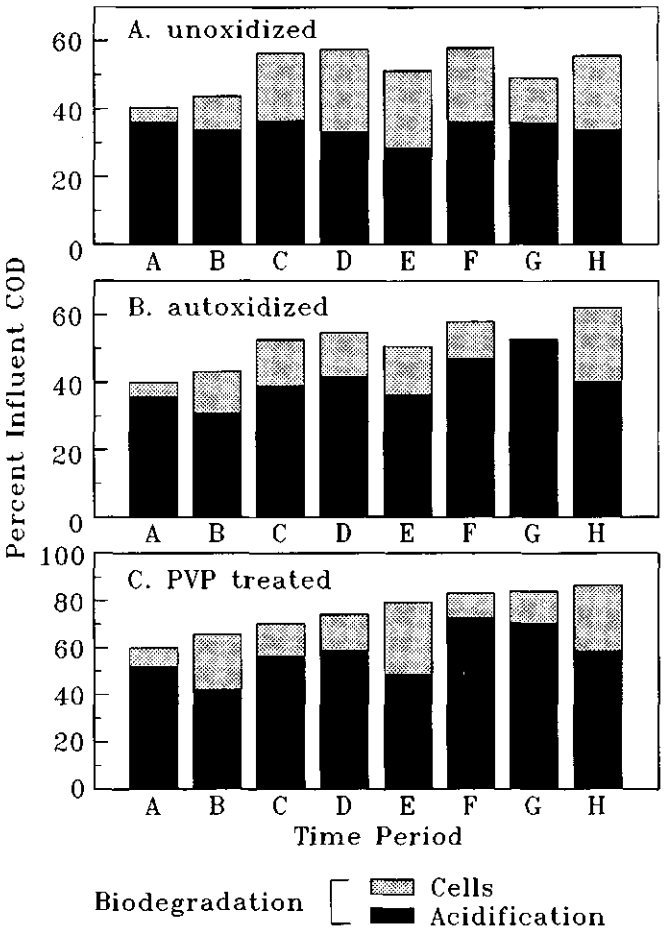


Figure 9. The period averaged acidification and biodegradation of the influent COD during the continuous anaerobic treatment pine bark extract. Where: acidification = the % conversion of the COD supplied to VFA and CH_4; biodegradation = acidification + cells. Part A. Unoxidized extract. Part B. Autoxidized extract. Part C. PVP treated extract.

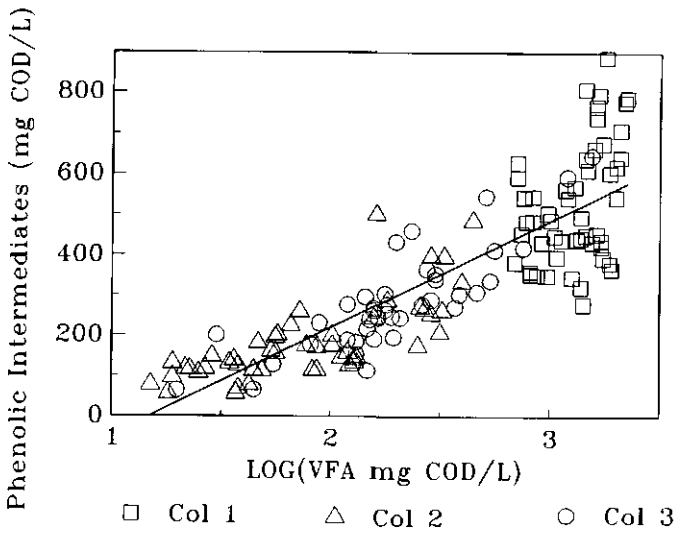


Figure 10. The sum of the effluent phenolic intermediate concentration (phenol, p cresol and carboxycyclohexane) as a function of the effluent VFA concentration during the continuous anaerobic treatment of unoxidized (Col 1), PVP treated (Col 2) and autoxidized (Col 3) extracts. The results were obtained between day 94 to day 155 of the experiment (R^2 = 0.72)

Table 8. Period Averaged Elimination of Total and Tannin UV (215 nm) Absorbance from Pine Bark Extracts by Continuous Anaerobic Treatment.

Per.	Day No.	Total UV						Tannin UV			
	start/ end	Infl. Absorbance 1cm,1x			Elimination % total UV			Infl. Absorbance 1cm,1x		Elimination % tannin UV	
		unox[a]	auto	PVP	unox	auto	PVP	unox	auto	unox	auto
C	22/56	87	69	21	48.5	9.9	34.5	70	27	65.0	-22.6
D	57/75	77	65	24	45.3	23.6	54.1	56	30	60.3	2.8
E	76/93	166	120	59	50.8	9.6	59.6	136	50	53.7	-11.2
F	94/119	257	212	55	53.3	15.9	65.5	198	120	62.0	-1.1
EXP	22/119	132	104	38	48.6	12.8	48.8	104	48	60.4	-12.2

[a] treatment definitions are defined in the footnote of Table 4

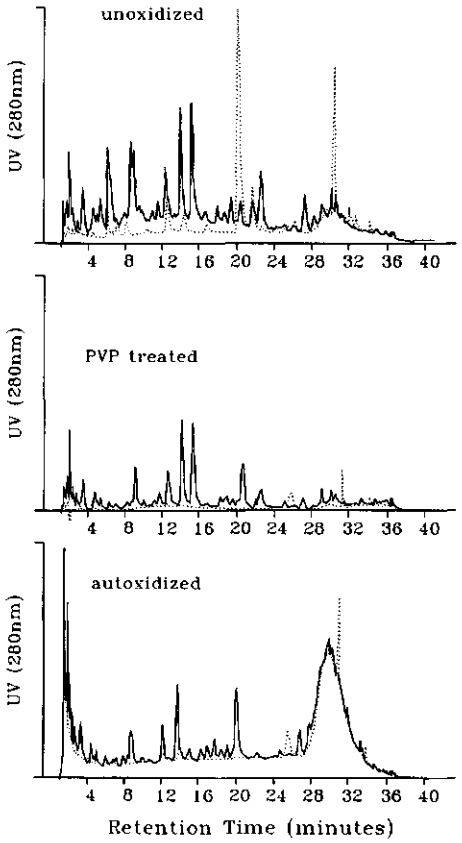


Figure 11. The HPLC chromatograms of the influents (———) and the effluents (·····), sampled on day 106, of the continuous anaerobic treatment of unoxidized, autoxidized and PVP treated pine bark extracts.

Based on the long term batch digestion experiments with the sludge cultivated on the non-tannin fraction of the wastewater, the ultimate COD biodegradability of the whole unoxidized bark extract was 85% after 30 days. The elimination of extract UV and tannins reached 80 and 75%, respectively. The whole pine bark extract contains more biodegradable substrate as compared to the PVP treated extract (Figure 9). This would indicate that at least some of the unoxidized tannins are potentially biodegradable to CH_4, VFA and cells if they have a sufficiently long residence time in anaerobic conditions.

3.3. Continuous Treatment of Precipitated Extracts

3.3.1. Set-Up

The sludge obtained at the end of the autoxidized extract fed column experiment was used to seed another column in order to investigate the anaerobic treatment of calcium precipitated autoxidized pine bark extract. In this experiment the pine bark extract was autoxidized for 1 h at a starting pH of 11.5, and then 500 mg L^{-1} of Ca^{2+} (in the form of

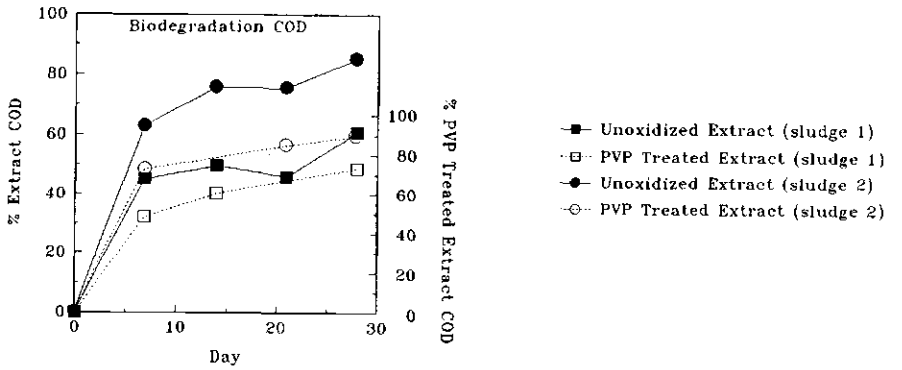


Figure 12. The biodegradability of unoxidized and PVP treated pine bark extract during batch anaerobic digestion by sludge recovered at the end of the continuous experiments fed unoxidized (sludge 1) and PVP treated pine bark (sludge 2) extracts. Sludge 1 and 2 concentrations were 1.25 and 1.09 g VSS L^{-1}, respectively. The unoxidized and PVP treated extract concentrations were 3.59 and 2.38 g COD L^{-1} at the start of the assay. Biodegradability = the % conversion of the COD supplied to CH_4, VFA and cells.

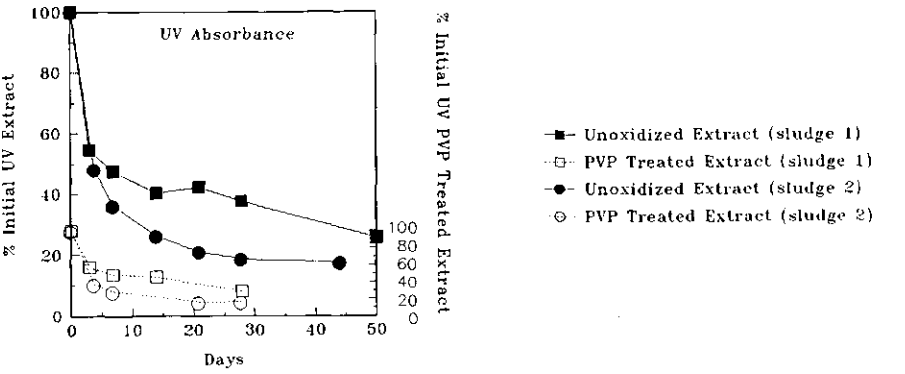


Figure 13. The UV absorbance (215nm) of the assay media during batch anaerobic digestion of unoxidized and PVP treated pine bark extracts by sludge recovered at the end of the continuous experiments fed unoxidized (sludge 1) and PVP treated (sludge 2) extracts. Details of this experiment are described in Figure 12 caption. The initial UV absorbance of the unoxidized and PVP treated extracts were 101 and 28 1x,1cm, respectively.

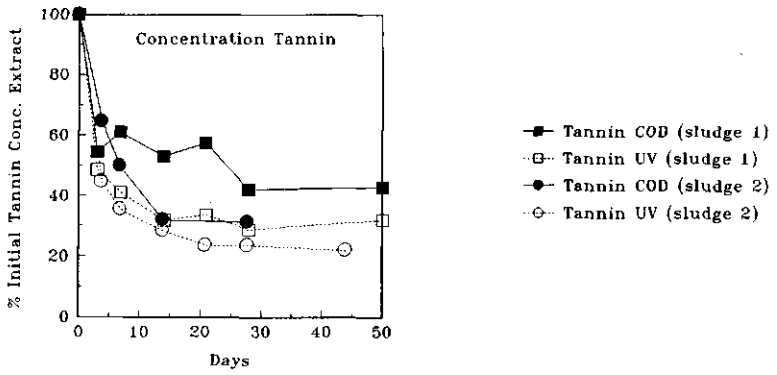


Figure 14. The tannin concentration based on COD and UV (215nm) measurements of the assay media during batch anaerobic digestion of unoxidized pine bark extracts by sludge recovered at the end of the continuous experiments fed unoxidized (sludge 1) and PVP treated (sludge 2) extracts. Details of this experiment are described in Figure 12 caption. The initial tannin concentration was 1.36 g COD L^{-1} or 75 1x,1cm 215nm absorbance units.

Table 9. The Average Removal of Extract COD, UV Absorbance and Tannins by 1 hour Autoxidation (with a Starting pH of 11.5) and by Autoxidation Followed by Precipitation with Calcium (500 mg L^{-1} for 2 hours, pH approx. 9).

Parameter	Autox. only		Autox. + Ca^{2+}	
	percent removed			
Total COD	4.0	(±3.8)[a]	29.6	(±2.1)
Total UV	16.3	(±3.2)	72.3	(±3.6)
Tannin COD	36.4	(±14.1)	71.6	(±6.2)
Tannin UV	43.5	(±11.3)	86.0	(±3.2)

[a] The values in parenthesis are the standard deviation units of the reported average. The values reported are the average of 5 extracts prepared for the influents of the calcium precipitated autoxidized bark extract fed columns.

Table 10. Period Averaged Operation Parameters of the Continuous Anaerobic Treatment of Pine Bark Extract Pretreated by Autoxidation and Calcium Precipitation.

Per.	Day No. start/ end	Infl Conc g COD L^{-1}	HRT hours	Load g COD L^{-1} d^{-1}
A	0/23	3.7	5.3	17.9
B	24/54	2.8	6.8	12.5
C	55/67	2.6	2.8	22.9
EXP[a]	0/67	3.1	5.6	16.2

[a] EXP = experimental average

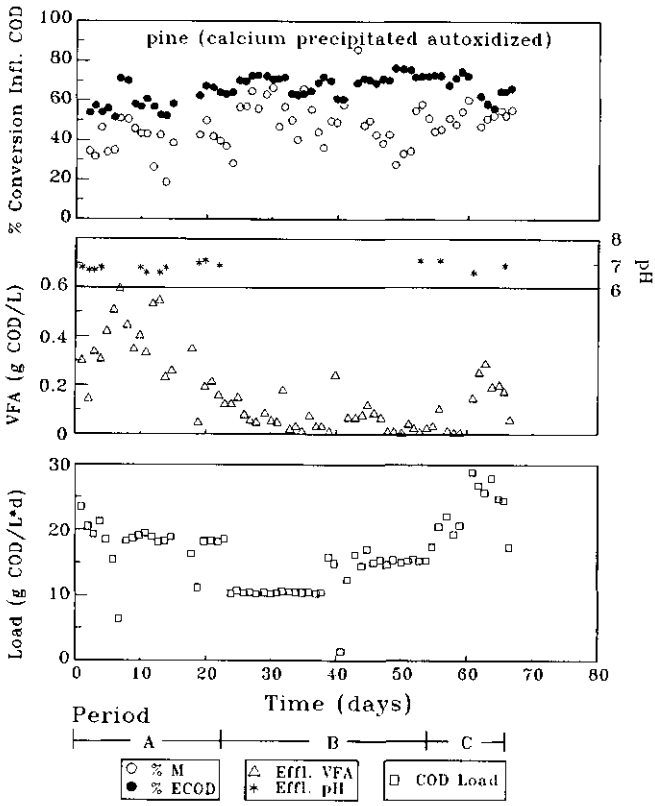


Figure 15. Operation and efficiency during the continuous anaerobic treatment of calcium (500 mg L^{-1}) precipitated autoxidized (starting pH of 11.5 for 1 hour) pine bark extract. Definition of abbreviations are given in Figure 6 caption.

Table 11. Average Treatment Efficiency and Biodegradation during the Continuous Column Experiments Fed Pine Bark Extract Pretreated by Autoxidation for 1 hour at pH 11.5 followed by Calcium Precipitation.

Parameter[a]	Units	Experimental Average
ECOD	% infl. COD	66.2
M	"	47.8
VFA	"	4.8
Cells	"	18.4
A	"	52.6
BD	"	71.1
$ECOD_{BD}$	% infl. COD_{BD}	93.1
Y	g COD_{cells} g^{-1} COD_{BD}	0.26

[a] parameters are defined in Materials and Methods

$CaCl_2$) was added. The precipitate was allowed to settle for 2 hours and the extract was then decanted over a paper filter. In a previous study, the ability of calcium to precipitate the high MW autoxidation products was observed (Field et al., 1990b). The pretreatment of the autoxidized extract by calcium precipitation prior to anaerobic treatment was applied to lower the concentration of recalcitrant COD in the anaerobic effluent. Based on the data presented in Table 9, the calcium precipitation of the autoxidized extracts removed 27% of the COD and 67% of the UV absorbance.

The average load, HRT and influent concentration in various periods of the reactor operation are listed in Table 10.

3.3.2. Treatment Performance and Biodegradability

The calcium precipitated autoxidized extract was treated with a higher COD elimination efficiency, acidification and was biodegraded to a greater extent (Figure 15 and Table 11) as compared to the treatment of the autoxidized extract. This was due to the precipitation of poorly degradable COD prior to the anaerobic treatment. However, the parameters were comparable to the those of the autoxidized extract fed column if they are expressed as a percent of the autoxidized extract COD before calcium precipitation. Therefore, calcium pretreatments of autoxidized extracts can be applied to physically remove some of the poorly biodegradable COD without decreasing the COD which is potentially convertible by biological treatment. The calcium treatment would also decrease the color of the autoxidized wastewater as was discussed previously (Field et al., 1990b).

Previously, an average of 53% biodegradation of the autoxidized extract was observed during anaerobic treatment, indicating that 47% of the extract COD would be present as recalcitrant COD in the effluent (Table 5). The combined precipitation and anaerobic treatment of this extract reduced the autoxidized COD by 79% on the average (27% and 52% of the original COD by Ca^{2+} and anaerobic treatment, respectively), leaving much less recalcitrant COD in the anaerobic effluent.

4. DISCUSSION

4.1. Effect Autoxidation on Treatment Efficiency

The debarking wastewaters of the forest industry are troublesome for anaerobic treatment due to their high content of methanogenic toxic tannins. An autoxidation pretreatment can be applied to polymerize the tannins to non-toxic colored compounds. In this study, the anaerobic treatment of aqueous pine bark extracts was improved by the autoxidation pretreatment. During anaerobic treatment of unoxidized extracts, incomplete conversion of the biodegradable substrates to methane was observed and high concentrations of VFA were present in the effluent. Whereas, anaerobic treatment of autoxidized extracts provided twice as much methane production. The absence of inhibitory factors in the autoxidized extracts was evident from the low effluent VFA concentrations during the continuous experiment and was also evident from long term batch toxicity tests. The autoxidation pretreatment permitted the anaerobic treatment of pine bark extracts at high influent concentrations (~14 g COD L^{-1}) and loading rates 40 g COD L^{-1} d^{-1} with 98% removal of the biodegradable COD.

4.2. Effect of Autoxidation on the Anaerobic Biodegradability

The unoxidized tannins, which are responsible for 37% of the extract COD, were eliminated 60% by continuous anaerobic treatment, whereas the autoxidized products of these tannins were not eliminated at all. The high MW products of autoxidation reactions increase the recalcitrance of the tannin fraction. Poor anaerobic biodegradability of high MW phenolic compounds has frequently been observed in studies with lignin and peat (Owen et al., 1979; Zeikus et al., 1982; Benner and Hodson, 1985; Colberg and Young, 1985). However,

the formation of high MW tannin and humic products by autoxidation did not correspond to a decrease in the whole extract biodegradability. The extract biodegradability during anaerobic treatment was 53% for both unoxidized and autoxidized influents. The elimination of tannins as well as other phenols from the unoxidized extracts was due to their partial degradation to non-tannic phenolic intermediates as opposed to their complete fermentation to VFA and CH_4. The methanogenic toxicity caused VFA from the acidification of the biodegradable fraction to accumulate. The high VFA concentration inhibited the biodegradation of the phenolic intermediates. Therefore, the application of the autoxidation pretreatment did not decrease the anaerobic biodegradability of the whole extract.

The recalcitrant COD expected in the effluents of anaerobic processes treating autoxidized bark wastewater can be effectively reduced by calcium precipitation prior to anaerobic treatment. The precipitation is also effective in reducing the color of the autoxidized extracts (Field et al., 1990b).

5. LITERATURE CITED

Benner, R. and R. E. Hodson. 1985. Thermophilic anaerobic biodegradation of (^{14}C)lignin, (^{14}C)cellulose and (^{14}C)lignocellulose preparations. Appl. Environ. Microbiol. 50:971-976.

Colberg, P. J. and L. Y. Young. 1985a. Anaerobic degradation of soluble fractions of (^{14}-lignin)lignocellulose. Appl. Environ. Microbiol. 49: 345-349.

Fedorak, P. M.,D. J. Roberts and S. E. Hrudey. 1986. The effects of cyanide on the methanogenic degradation of phenolic compounds. Wat. Res. 20: 1315-1320.

Field, J. A., G. Lettinga and M. Geurts. 1987. The methanogenic toxicity and anaerobic degradability of potato starch wastewater phenolic amino acids. Biological Wastes. 21: 37-54.

Field, J. A., M. J. H. Leyendeckers, R. Sierra-Alvarez, G. Lettinga and L. H. A. Habets. 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., S. Kortekaas and G. Lettinga. 1989. The tannin theory of methanogenic toxicity. Biological Wastes (in press).

Field, J. A., G. Lettinga and L. H. A. Habets. 1990a. Oxidative detoxification of aqueous bark extracts. Part I: Autoxidation. J. Chem. Tech. Biotechnol. (submitted; Chapter 8 in dissertation).

Field, J. A., R. Sierra-Alvarez, G. Lettinga and L. H. A. Habets. 1990b. Oxidative detoxification of aqueous bark extracts. Part II: alternative methods. J. Chem. Tech. Biotechnol. (submitted; Chapter 9 in dissertation).

Lane, A. G. 1980. Production of aromatic acids during anaerobic digestion of citrus peel. J. Chem. Tech. Biotechnol. 30: 345-350.

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

Owen, W. F., D. C. Stuckey, J. B. Healy, Jr., L. Y. Young and P. L. McCarty. 1979. Bioassays for monitoring biochemical methane potential and anaerobic toxicity. Wat. Res. 13: 485-492.

Rekunen, S. 1986. Taman process and its applicability to wastewaters from papermaking. Proceedings: PIRA Paper and Board Division Key-Event, Cost Effective Treatment of Papermill Effluents Using Anaerobic Technologies, Leatherhead, England.

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

CHAPTER 12

Summary and Conclusions

SUMMARY AND CONCLUSIONS

1. SUMMARY

Anaerobic wastewater treatment is an alternative to the aerobic treatment processes for the removal of easily biodegradable organic matter in medium to high strength industrial wastestreams. The application of anaerobic treatment has several advantages such as low excess sludge production, compact treatment facilities, low operation costs and a useful by-product (methane). However, one important disadvantage is the high sensitivity of the anaerobic bacteria (ie. methanogenic bacteria) to toxic compounds.

The anaerobic technologies were initially developed for the treatment of non-toxic organic wastewaters. As the technology matured, the limits of its application to toxic wastewaters were studied. Past research has been mostly directed towards the toxic effects of compounds introduced by man into the industrial process rather than natural constituents present in agricultural wastewaters.

This dissertation investigates the role of natural polar phenolics (ie. tannins and related compounds) on anaerobic digestion processes. A distinct feature of tannic compounds and other highly hydroxylated phenolics is that they are readily oxidized to darkly colored humic compounds. Such transformations can produce products which differ in toxicity and biodegradability compared to the original tannic compounds present in the industrial feedstocks. Industrial process waters are often exposed to conditions which promote phenol oxidation, therefore the role of humus forming processes was a major consideration included in this study.

Chapter 1

The available literature data regarding the toxicity and biodegradability of tannic compounds is reviewed in Chapter 1.

Chapter 2

Chapter 2 evaluated the effect of hydroxylation reactions on the methanogenic toxicity and anaerobic biodegradability of simple phenolic amino acids. The hydroxylation reactions are the first step in a series of reactions leading to darkly colored humic compounds known as melanin. Tyrosine (monohydroxy), present in potatoes can potentially be oxidized to L dopa (dihydroxy) during the processing of starch. The toxicity of tyrosine to methane bacteria was negligible, while L dopa caused from 40 to 50% inhibitions of the methanogenic activity at a concentration of 327 mg L^{-1}. The L dopa toxicity was synergistic with volatile fatty acids (VFA) and the inhibition thus could be minimized by maintaining low VFA concentrations in anaerobic reactors. In long term experiments with continuously operated anaerobic columns fed VFA substrates together with L dopa, the methanogenic bacteria adapted to the toxicity of L dopa. Both tyrosine and L dopa were anaerobically degradable in batch experiments. However, only tyrosine was degradable in continuously operated VFA-fed columns.

Chapter 3

In Chapter 3, the toxicity and biodegradability of a hydrolyzable tannin (gallotannic acid) and its monomeric counterparts (gallic acid and pyrogallol) were studied. Gallotannic acid was highly toxic to methane bacteria. Concentrations of 700 mg L^{-1} caused 50% inhibition. The toxicity was persistent over two month assay periods despite the rapid degradation of gallotannic acid. One day exposures of anaerobic sludge to gallotannic acid was sufficient to severely damage the sludge activity. In contrast, the monomeric derivatives were considerably less toxic. The 50% inhibiting concentrations of the monomers was 3000 mg L^{-1}, and their toxicity was not persistent. Gallotannic acid, gallic acid and pyrogallol were readily and completely biodegradable in batch anaerobic assays. Under conditions inhibitory to methane bacteria, these trihydroxy phenolic compounds were converted to acetic acid.

Chapter 4

The toxins of aqueous tree bark extracts were investigated in Chapter 4. The extracts were studied as a model for debarking wastewater of the forest industry. The polyflavonoids (condensed tannins), which were measured by their adsorption on an insoluble polyamide, polyvinylpyrrolidone (PVP), accounted for about half of the aqueous extractable COD of bark. The bark extracts caused severe inhibition to methane bacteria. The extracts that were treated with PVP to selectively remove tannins, were non-toxic to methane bacteria. This indicated that they were the principal toxins of bark soluble matter. The concentration of bark tannins causing 50% inhibition to methane bacteria averaged 600 mg COD L^{-1} (350 mg tannin solids L^{-1}).

Chapters 5 & 6

In Chapters 5 and 6, the effect of autoxidizing pure phenolic compounds to colored polymerized products was studied. Partial polymerization of relatively non-toxic polar monomeric phenolics caused the methanogenic toxicity to increase. In contrast, the polymerization of toxic tannic compounds led to detoxification. One compound, catechin (a condensed tannin monomer) first increased in toxicity and later decreased in toxicity as it was polymerized to darkly colored humic compounds. These results formed the basis for developing the tannin theory. The theory postulates that the initial polymerization of monomers leads to an increased toxicity due to an increase in tannic qualities. The oligomers formed are able to form stronger hydrogen bonds with proteins than the monomers. They are thus more likely to react with the functional proteins of bacteria. If the polymerization is continued, the theory postulates a decrease in toxicity due to a decreased effectiveness of high MW compounds to penetrate bacteria. The anaerobic biodegradability of the phenolic compounds was found to decreases with the degree in which colored products were formed by the oxidation. The end products of phenolic polymerization are high MW humic compounds that are non-toxic and non-biodegradable.

Chapter 7

In Chapter 7, we observed that pyrogallol (trihydroxy) was not highly polymerized by autoxidation. During the initial stages of pyrogallol autoxidation, a highly toxic compound was formed. This compound was identified as purpurogallin which is not a tannic compound. However it is more toxic than tannins. The concentration of purpurogallin causing 50% inhibition to methane bacteria was 45 mg L^{-1}. Purpurogallin was also toxic to the anaerobic biodegradation of pyrogallol. After long periods of autoxidation, purpurogallin is destructively oxidized and the methanogenic toxicity is eliminated.

Chapter 8

The detoxification of tannin containing wastewater was investigated in Chapter 8. Aqueous extracts of bark were treated by autoxidation (aeration at a high pH), in order to polymerize the toxic condensed tannins. The unoxidized and autoxidized extracts were tested for methanogenic toxicity in batch anaerobic assays fed VFA as substrate. The autoxidation of coniferous bark extracts caused high levels of detoxification. Complete detoxification of pine bark extracts was obtained. Extracts prepared from different samples of spruce bark were either partially or completely detoxified. The degree of detoxification corresponded to the decrease in the oligomeric tannin concentration as well as to the increase in color bearing polymerized products. Birch bark extracts could not be detoxified by autoxidation even though the tannins were successfully polymerized. The poor detoxification results were due to the presence of non-tannic toxins in the birch bark extracts.

Chapter 9

Alternative methods of oxidation were evaluated in Chapter 9. Both spruce and birch bark extracts were detoxified by destructive oxidations with H_2O_2. The H_2O_2 oxidation of bark extracts provides detoxification without producing the colored humic compounds. Extracts of both birch and spruce bark were also highly detoxified by long term aerobic biological treatments of 3 to 4 weeks, that were responsible for more extensive polymerization than could be achieved by high pH autoxidation. Neither H_2O_2 oxidation or long term aerobic

treatments are considered economically feasible. However, the results of this chapter indicate that reliable methods of detoxification for debarking wastewaters of all tree species can be achieved by the oxidative treatments that either extensively destroy or extensively polymerize the toxins.

Chapter 10

The toxicity of high and low MW tannin fractions in autoxidized spruce bark extracts was tested in Chapter 10. Calcium precipitation of the tannins only removed the high MW tannin fraction and this had no effect in relieving the methanogenic toxicity. In contrast, granular active carbon adsorption treatments were only effective in removing the low MW tannins and likewise the toxicity of the extract was removed. The oligomeric tannins, that remain after autoxidation treatments, are therefore responsible for the residual toxicity in partially detoxified extracts. The high MW tannins are non-toxic. In order to monitor the toxicity of autoxidized bark extracts, the oligomeric tannins instead of total tannins should be measured.

Chapter 11

The continuous laboratory-scale anaerobic treatment of untreated and autoxidized pine bark extracts was studied in Chapter 11, utilizing granular sludge packed columns. The autoxidation pretreatment doubled the conversion efficiency of bark extract COD to methane (from 19 to 40%). The improvement in the methane production was due to the better utilization of VFA formed from the easily fermentable substrate. After five months of operation, anaerobic treatment of the autoxidized extracts was feasible at high influent concentrations (~14 g COD L^{-1}) and loading rates (40 g COD L^{-1} d^{-1}) with 98% elimination of the biodegradable fraction. The autoxidation products of the tannins were not transformed during anaerobic treatment, whereas about half of the original tannins of the unoxidized extract were transformed to potentially biodegradable phenolic intermediates. However, the biodegradability of these intermediates was inhibited by a high reactor VFA concentration that resulted from the methanogenic inhibited sludge. Therefore, the average biodegradability (53%) of the entire extract COD during continuous anaerobic treatment was not decreased by the autoxidation pretreatment. The recalcitrant COD expected in the effluents of reactors treating autoxidized debarking wastewater can be effectively separated by calcium precipitation of the autoxidized products out of the influent prior to anaerobic treatment.

2. CONCLUSIONS

The methanogenic toxicity of polar phenolic compounds depends on the tannic quality of these compounds and their capacity to penetrate. An important factor governing the tannin quality and penetration ability is the polymer size (ie. MW) of the tannic compounds. The relationship between tannin toxicity and MW has been outlined by the tannin theory.

Phenolic compounds present in agricultural wastewaters are susceptible to oxidative modifications during short exposures to air which will have an impact on their methanogenic toxicity. In cases where the predominant phenols are monomeric tannins, than the oxidative polymerization leads to oligomeric tannins, which would increase the wastewater toxicity. On the other hand, in cases where the predominant tannins are oligomers, than the initially toxic wastewater could potentially be detoxified by oxidative polymerization.

Debarking wastewater of coniferous trees can successfully be detoxified by autoxidation pretreatments prior to anaerobic digestion. This pretreatment should be considered economically feasible since it only requires the addition of 0.2 to 1.0 g NaOH L^{-1} debarking wastewater with aeration periods ranging from 1 to 20 hours depending on bark species and tannin concentration. The tannins are converted to poorly degradable humic compounds that are non-toxic. During anaerobic treatment, no inhibition occurs and the fermentable fraction of the wastewater can be converted to methane. The high MW humic products are non-toxic

for aquatic organisms and thus can be discharged to the surface waters with considerably less environmental impact as compared to the unoxidized tannins. In a parallel study (Temmink, J. H. M., J. A. Field, J. C. van Haastrecht and R. C. M. Merckelbach. 1989. Acute and sub-acute toxicity of bark tannins to carp (Cyprinus carpio L). Wat. Res. 23(3): 341-344.), the high toxicity of unoxidized bark tannins to fish and the low toxicity of autoxidized tannins was clearly demonstrated.

Up to date, methods of combatting toxic organic pollutants have been largely based on microbial degradation or physical-chemical removal. A viable alternative approach to these methods that potentially is applicable for certain aromatic compounds, could be polymerizing the toxins to non-toxic humus. The humus forming process is a natural mechanism in the forest environment that detoxifies tannic compounds before such compounds are released into the surface waters. The humus forming reactions were imitated in this study and were an effective method for eliminating the environmental impact of tannins in wastewater. Research should be continued to determine the extent to which humus forming processes can be applied for the treatment of other toxic organic contaminants.

HOOFDSTUK 12

Samenvatting en Konklusies

SAMENVATTING EN KONKLUSIES

1. SAMENVATTING

Anaërobe behandeling van afvalwater is een alternatief voor aërobe afvalwaterzuivering om afbreekbare organische verontreiniging in industrieël afvalwater te verwijderen. Anaërobe zuivering heeft talrijke voordelen zoals bijvoorbeeld lage spuislib produktie, kompakte zuiveringsinstallaties, lage bedrijfkosten, en een bruikbaar bijprodukt (methaan). Echter, de hoge gevoeligheid van anaërobe bakteriën (met name methaanbakteriën) voor toxische stoffen vormt een belangrijk nadeel.

Aanvankelijk werd anaërobe zuivering op niet toxisch organisch afvalwater toegepast. Met het verdere ontwikkelen van de technologie, werden de grenzen van toepassing op toxisch afvalwater bestudeerd. Het voorgaande onderzoek was voornamelijke toegespitst op de toxische effekten van verbindingen toegevoegd door mensen in industriële processen en niet op toxische plantaardige verbindingen die aanwezig zijn in afvalwater vanuit de landbouwsektor.

Deze dissertatie evalueert de rol, die polaire plantaardige fenolen (looizuren, tanninen) op het anaërobe vergistingsproces hebben. Een opvallende eigenschap van tanninen is dat ze gemakkelijk tot donkere humus-achtige verbindingen worden geoxideerd. Dergelijke transformaties kunnen tot produkten leiden, die andere eigenschappen qua afbreekbaarheid en giftigheid hebben dan de oorspronkelijke fenolen in de industriële grondstoffen. Het proceswater van industrieën wordt vaak aan omstandigheden blootgesteld welke de oxidatie van fenolen bevorderen. Daarom is het humusvormende proces een hoofdzaak van deze studie geworden.

Hoofdstuk 1

De beschikbare gegevens uit de literatuur over de giftigheid en anaërobe afbreekbaarheid van tanninen worden samengevat in Hoofdstuk 1.

Hoofdstuk 2

Hoofdstuk 2 evalueert het effekt van hydroxylatiereakties op de toxische werking van fenolische aminozuren op methaanbakteriën en op de anaërobe afbreekbaarheid van deze verbindingen. De hydroxylatiereaktie is de eerste trap in een reeks die tot donkere gekleurde melanine leiden. Tyrosine (monohydroxy) is aanwezig in aardappelen. Het kan tot L dopa (dihydroxy) worden geoxideerd tijdens het verwerken van zetmeel. De toxiciteit van tyrosine op methaanbakteriën was gering, echter L dopa veroorzaakte 40 a 50% remming op de aktiviteit van methaanbakteriën bij 327 mg L^{-1}. De toxiciteit van L dopa was synergistisch met vluchtige vetzuren (VVZ) en de remming kan door het handhaven van lage VVZ koncentraties in anaërobe reaktoren worden verkleind. Tijdens langdurige kontinue vergisting in anaërobe kolommen gevoed met VVZ en L dopa, vond adaptatie aan L dopa plaats. Zowel tyrosine als L dopa werden tijdens batchproeven anaëroob afgebroken. Echter, alleen tyrosine was afbreekbaar in kontinu bedreven kolommen.

Hoofdstuk 3

In Hoofdstuk 3, worden de toxiciteit en afbreekbaarheid van hydroliseerbare looizuren (gallotanninezuur) en hun monomerische eenheden (galzuur en pyrogallol) beschreven. Gallotanninezuur was zeer toxisch voor methaanbakteriën. Een koncentratie van 700 mg L^{-1} veroorzaakte 50% verlaging van de methanogene aktiviteit. De toxische werking duurde twee maanden voort ondanks het feit dat al na enige dagen afbraak van gallotanninezuur werd waargenomen. Blootstelling met gallotanninezuur aan het slib gedurende 24 uur was al voldoende om de slibaktiviteit te verlagen. In tegenstelling tot dit, waren de monomeren minder toxisch. Koncentraties van 3000 mg L^{-1} veroorzaakte 50% remming en de slibaktiviteit werd snel hersteld na afbraak van de verbindingen. Gallotanninezuur, galzuur en pyrogallol werden gemakkelijk en volledig afgebroken in anaërobe batchproeven. Onder

omstandigheden, die toxisch op de methaanbakteriën waren, werden deze trihydroxy fenolen tot azijnzuur omgezet.

Hoofdstuk 4

De toxische stoffen in waterextrakten van boombast worden beschreven in Hoofdstuk 4. Deze extrakten dienden als een model voor ontbastingsafvalwater uit de papier- en pulpindustrie. De polyflavonoïden (gecondenseerde tanninen), die op basis van hun adsorptie op een niet oplosbaar polyamide (polyvinylpyrrolidone (PVP)) werden gemeten, waren verantwoordelijk voor ongeveer de helft van het extrakt chemisch zuurstof verbruik (CZV). De bastextrakten veroorzaakten sterke remmingen van de methaanbakteriën. Indien de extrakten werden voorbehandeld met PVP om de tanninen selektief te verwijderen, waren ze niet giftig. Dus waren de tanninen de primaire toxische stoffen die in de bastextrakten werden gevonden. De gemiddelde koncentratie aan basttanninen die 50% remming van de methaanbakteriën veroorzaakte was 600 mg CZV L^{-1} (ofwel 350 mg droog gewicht L^{-1}).

Hoofdstuken 5 & 6

In Hoofdstukken 5 en 6, wordt de invloed van het autöoxyderen van fenolen tot verkleurde en gepolymeriseerde verbindingen beschreven. Gedeeltelijke polymerisatie van relatief niet toxische polaire monomeren veroorzaakte een toename in hun toxische werking op methaanbakteriën. Polymerisatie van toxische tanninen leidde tot ontgiftiging. Polymerisatie van een verbinding, catechine, nam aanvankelijk in toxiciteit toe, maar als de polymerisatie werd voortgezet tot donkere humus-achtige verbindingen, werd een afname in toxiciteit waargenomen. Deze resultaten vormden de basis voor de looizuurtheorie. Volgens deze theorie, vindt een toename van de toxiciteit plaats tijdens de beginfase van de oxidatie door betere looiende eigenschappen van de fenolen. De oligomeren die geproduceerd worden, zijn in staat sterkere waterstofbruggen te vormen met eiwitten dan de monomeren. Daardoor reageren de oligomerische tanninen snel met functionele eiwitten van bakteriën. Indien de polymerisatie voortgezet wordt, is een afname in toxiciteit te verwachten wegens een verlaagd vermogen van de verbindingen met hoog molekuulgewicht (MW) de bakteriën binnen te dringen. De anaërobe afbreekbaarheid van de fenolen nam af in vergelijking met de produktie van kleurrijke geöxideerde verbindingen. De eindprodukten van fenoloxidatie zijn de hoog MW humus-achtige polymeren die niet giftig en niet biologisch afbreekbaar zijn.

Hoofdstuk 7

In Hoofdstuk 7, wordt beschreven dat pyrogallol (trihydroxy) slechts gedeeltelijk gepolymeriseerd wordt door autoöxidatie. Tijdens het aanvankelijke stadium van de oxidatie, ontstond een zeer giftige verbinding. Deze verbinding werd als purpurogalline geïdentificeerd. Purpurogalline heeft geen looiende eigenschappen. Echter, het was meer toxisch op methaanbakteriën dan tanninen. Een 50% reduktie van de methaanbakteriënaktiviteit werd bij 45 mg L^{-1} waargenomen. Purpurogalline remt ook de afbraak van pyrogallol. Na langdurige oxidatie van pyrogallol, werd purpurogalline destruktief geöxideerd en er werd geen toxisch effekt meer waargenomen.

Hoofdstuk 8

De ontgiftiging van looizuurhoudend afvalwater wordt in Hoofdstuk 8 beschreven. Waterextrakten van bast werden door autoöxidatie (beluchting bij een hoge pH) behandeld, om de toxische oligomerische tanninen te polymeriseren. De toxiciteit van niet geautoöxideerde en geautoöxideerde extrakten werden in anaërobe batchproeven gevoed met VVZ bepaald. De autoöxidatie van naaldboombastextrakten veroorzaakte een hoog niveau van ontgiftiging. Volledige ontgiftiging van de dennebastextrakten werd bereikt. Extrakten, die uit verschillende monsters fijnsparrebast voorbereid werden, konden gedeeltelijk tot volledig worden ontgiftigd. De ontgiftiging verliep in samenhang met afname in de koncentratie aan oligomerische tanninen en toename in de kleur van het extrakt. Het was niet mogelijk berkebastextrakten te ontgiftigen, ondanks dat de tanninen wel werden gepolymeriseerd. De slechte resultaten zijn te wijten aan de aanwezigheid van niet-looizuurachtige toxische verbindingen in de extrakten van berkebast.

Hoofdstuk 9

Alternatieve technieken van oxidatie werden in Hoofdstuk 9 geëvalueerd. Zowel fijnsparre- als berkebastextrakten konden door middel van destruktieve oxidaties met H_2O_2 worden ontgiftigd. Met de H_2O_2 oxidaties, vond de ontgiftiging plaats zonder verkleuring van de extrakten. Ook werden fijnsparre- en berkebastextrakten ontgiftigd door langdurige aërobe biologische behandeling van 3 à 4 weken. Er werden hogere niveaus van polymerisatie bereikt dan mogelijk was met autöxidaties bij hoge pH. Noch de H_2O_2 oxidatie noch de langdurige aërobe behandelingen kunnen worden gezien als ekonomisch haalbare technieken. Echter, de resultaten van dit hoofdstuk laten zien dat betrouwbare methoden van ontgiftigen voor alle soorten ontbastingsafvalwaters mogelijk zijn door middel van oxidatieve behandelingen, die intensieve destruktie of intensieve polymerisatie van de giftstoffen tot stand kunnen brengen.

Hoofdstuk 10

De toxiciteit van lage en hoge MW tannine frakties in geautoöxideerde fijnsparrebastextrakten wordt in Hoofdstuk 10 beschreven. Het neerslaan van de tanninen met kalk werkte uitsluitend op de hoge MW tannine fraktie. Dit had geen effekt op de toxiciteit. Korrelvormig aktief kool adsorbeerde alleen lage MW tannine wat wel tot vermindering van de toxiciteit leidde. De oligomerische tanninen, die na autoöxidatie nog overblijven, zijn verantwoordelijk voor de residuele toxiciteit in gedeeltelijk ontgiftigde geautoöxideerde extrakten. De hoge MW tanninen zijn niet toxisch. Men moet dus de lage MW tannine koncentratie controleren in plaats van de totale tannine koncentratie om de toxiciteit van het extrakt tijdens het verloop van de autoöxidatie in te kunnen schatten.

Hoofdstuk 11

Het kontinu anaëroob zuiveren van onbehandelde en geautoöxideerde dennebastextrakten werd onderzocht door middel van lab-schaal kolommen gevuld met korrelslib. De voorbehandeling van het extrakt door autoöxidatie verdubbelde de omzetting van het CZV naar methaan (van 19 tot 40%). De verbetering in de methaanproduktie was aan het verhoogde verbruik van VVZ (afkomstig van makkelijk te vergisten substraten) te wijten. Na vijf maanden bedrijf, was anaërobe zuivering van het geautoöxideerde extrakt mogelijk bij hoge influent koncentraties (~14 g COD L^{-1}) en hoge belastingen (40 g COD L^{-1} d^{-1}) met 98% verwijdering van het afbreekbare CZV. De humusachtige verbindingen gevormd uit de autoöxidatie van tanninen waren niet biologisch omzetbaar tijdens anaërobe vergisting. Ongeveer de helft van de niet geoxideerde tanninen werd wel tot potentiele afbreekbare fenolische intermediare verbindingen omgezet. De afbraak van deze intermediare verbindingen werd echter geremd door de hoge koncentratie aan VVZ die ophoopde als gevolg van de remming van methaanbakteriën. De autoöxidatieve voorbehandeling leidde dus niet tot een afname in de afbreekbaarheid van het totale extrakt CZV (53%). Het recalcitrante CZV, dat in geautoöxideerd ontbastingsafvalwater te verwachten is, kan makkelijk met kalk uit het influent worden geprecipiteerd voorafgaande aan anaërobe zuivering.

2. KONKLUSIES

De toxiciteit van polaire fenolen op methaanbakteriën is afhankelijk van het looizuur-gedrag van deze verbindingen en hun vermogen bakteriën binnen te dringen. De belangrijkste faktor, die een rol speelt voor zowel het looizuur-gedrag als het binnendringingsvermogen, is het molekuulgewicht van de tanninen. De verhouding tussen toxiciteit en MW wordt door de looizuurtheorie beschreven.

Fenolen in afvalwater zijn gevoelig voor oxidatieve omzettingen tijdens korte blootstellingen aan lucht. In het geval, de meeste fenolen in het afvalwater polaire monomeren zijn leidt de oxidatieve polymerisatie tot oligomerische tanninen, die verantwoordelijk zijn voor een toename in de toxiciteit. Echter, in gevallen waar de meeste fenolen oligomerische tanninen zijn, kan ontgiftiging plaatsvinden ten gevolge van oxidatieve polymerisatie.

Ontbastingsafvalwater afkomstig van naaldbomen kan met succes worden ontgiftigd door autoöxidatieve voorbehandelingen voorafgaande van de anaërobe zuivering. Deze soort voorbehandelingen moeten als ekonomisch haalbaar gezien worden, omdat slechts toevoeging van 0.2 à 1.0 g NaOH L^{-1} afvalwater en een aëratietijd van 1 à 20 uren nodig is. De tanninen worden tot slecht afbreekbare humus verbindingen omgezet die niet toxisch zijn. Tijdens anaërobe vergisting, vindt geen remming plaats en het makkelijk vergistbare substraat wordt volledig tot methaan afgebroken. Humus met hoog MW is ook niet toxisch voor aquatische organismen en kan dus op het oppervlaktewater geloosd worden. Het effekt op het milieu is dan veel kleiner in vergelijking met de niet geoxideerde tanninen. In een parallele studie (Temmink, J. H. M., J. A. Field, J. C. van Haastrecht and R. C. M. Merckelbach. 1989. Acute and sub-acute toxicity of bark tannins to carp (Cyprinus carpio L). Wat. Res. 23(3): 341-344.), werd de hoge toxiciteit van de niet geoxideerde tanninen op vissen en de lage toxiciteit van de geautoöxideerde tanninen duidelijk aangetoond.

Tot nu toe, worden technieken om de toxisch organische verontreiniging te bestrijden merendeels gebaseerd op microbiële afbraak of fysisch-chemische verwijderingen. Een waardevolle alternatieve techniek, die toepassing op aromatische verbindingen zou kunnen hebben, is de polymerisatie van dergelijke verbindingen naar het niet toxische humus. Het humusvormende proces is een natuurlijke mechanisme in het bosmilieu om tanninen te ontgiftigen voordat deze in het oppervlaktewater terecht komen. De humusvormende reakties werden in deze studie nagebootst en bleken zeer effektief de slechte uitwerking van tanninen in afvalwater op het aquatische milieu tegen te werken. Onderzoek moet worden voortgezet om vast te stellen in hoeverre het humusvormende proces toepasbaar is voor de behandeling van andere toxische organische verontreinigingen.

Curriculum vitae

The author of this dissertation received his high school diploma in 1977 from Groveton High School in Alexandria, Virginia (USA). In 1982 he obtained his bachelor of science degree with magna cum laude from the department of agronomy (soil science) at the Virginia Polytechnical Institute and State University in Blacksburg, Virginia (USA). From the same university, he was granted his master of science degree from the department agronomy (soil science) in 1983. The topic of his master thesis was the reutilization of anaerobically digested manure in agriculture. Between October, 1983 until October, 1985 and from May, 1987 until present the author has been a colleague of the department of water pollution control at the Agricultural University in Wageningen (The Netherlands). From October, 1985 until April, 1987, he was employed by the engineering firm, Paques B.V. in Balk (The Netherlands) to work at the department of water pollution control on a cooperative research project. In November, 1989, the author will go to the department of chemical engineering at the Autonomous University of Barcelona (Spain) to conduct post-graduate research on the topic of anaerobic wastewater treatment.