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L. E. Lindberg · S. M. Willför · B. R. Holmbom

Antibacterial effects of knotwood extractives on paper mill bacteria

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Abstract Hydrophilic knotwood extracts from 18 wood species were assessed in disc diffusion and liquid culture tests for antibacterial effects against three species of paper mill bacteria. The *Pinus sylvestris*, *P. resinosa*, *P. contorta*, and *P. banksiana* extracts decreased or inhibited bacterial growth. The susceptibility order was *P. sylvestris* > *P. resinosa* > *P. contorta* > *P. banksiana*, correlating with the concentrations of pinosylvin and pinosylvin monomethyl ether in these wood species. Also, *Pseudotsuga menziesii* and *Thuja occidentalis* extracts had a small inhibitory effect. The Gram-positive *Bacillus coagulans* was more susceptible to the extracts than the Gram-negative *Burkholderia multivorans* and *Alcaligenes xylosoxydans*. The main components in the *Pinus* knotwood extracts were pinosylvin monomethyl ether and pinosylvin, suggesting these to be the active components. Therefore, pure pinosylvin, pinosylvin monomethyl ether, and dihydro-pinosylvin monomethyl ether were also tested. All compounds showed antibacterial effects. However, higher concentrations were needed for these pure compounds than for the knotwood extracts. Pinosylvin had stronger antibacterial effects than pinosylvin monomethyl ether. This work shows that knotwood extracts, especially from *Pinus* species, have a potential for use as natural biocides in papermaking.

Keywords *Alcaligenes xylosoxydans* · *Bacillus coagulans* · *Burkholderia multivorans* · Hydrophilic extractives · Knotwood · Natural biocides · Pinosylvin · Pinosylvin monomethyl ether · Stilbenes

L. E. Lindberg (✉) · S. M. Willför · B. R. Holmbom
c/o Laboratory of Wood and Paper Chemistry,
Åbo Akademi Process Chemistry Centre,
Porthansgatan 3, 20500 Turku/Åbo, Finland
E-mail: Lenita.Lindberg@abo.fi
Tel.: +358-2-2154887
Fax: +358-2-2154868

Introduction

In papermaking, bacteria cause runnability problems and have a negative effect on paper quality. Bacteria may cause, e.g., paper web breakage, biofouling, deterioration of raw materials, bad odors, spots and holes in the product, and biocorrosion. This forces mills to use biocides. The increase in paper mill water system closure and, at the same time, greater environmental concerns, which call for restrictions on biocide usage, have led to a search for natural biocides as alternatives to the commonly used synthetic ones.

A wood knot is the part of a branch that is encased in the tree stem. Especially softwood knots, but also knots in some hardwoods, contain exceptionally large amounts of phenolic substances, such as lignans, flavonoids, and stilbenes [1–4]. Knotwood has a negative impact on both mechanical and chemical pulping [5–7]. However, knotwood is potentially valuable as a source of natural biocides due to the extremely high content of bioactive phenolic substances. The knots can be separated from the over-sized chip fraction in a pulp mill [8], in order to increase pulp quality or to utilize the bioactive substances found in the knots, e.g., as active substances in technical antioxidants, biological antioxidants in foodstuffs, or as natural biocides.

In response to microbial attack, plants synthesize low-molecular-mass antibacterial compounds, phytoalexins, as a defense mechanism. There are numerous studies on the bioactivity of natural phenolic compounds. For example, the antimicrobial properties of phenolic compounds against multi-drug-resistant human pathogens [9–13], oral pathogens [14], and intestinal bacteria [15] have been studied. Also, the fungicidal activities of phenolic compounds [16] and the natural decay resistance of wood [17] have been extensively studied. Lately, the potential to use natural bioactive phenolic compounds for cancer chemoprevention, in food preservation, as functional food, in pharmaceutical

applications, as antioxidants, or as antibacterial agents has been investigated [18–23].

Flavonoids are a class of phytochemicals with potential antibacterial activity. Malterud et al. [24] tested the antibacterial effects of *Salix caprea* stemwood extracts and found that naringenin, a flavanone, showed the strongest inhibiting effect. Basile et al. [25] studied the antibacterial effects of *Castanea sativa* leaf extracts and found that the active fraction contained rutin, hesperidin, quercetin, apigenin, morin, naringin, galangin, and kaempferol. Quercetin and rutin were the most active compounds. From plant materials, kaempferol, quercetin, myricetin, and morin [26], as well as flavone, quercetin, and naringenin [12] have shown antibacterial effects.

Nitta et al. [10] screened for antibacterial compounds against pathogenic bacteria from tropical and subtropical plants and found that all active compounds were stilbene derivatives. Natural stilbenes isolated from *Shorea hemsleyana* and *Cyphostemma bainessi* exhibited strong inhibiting activity against methicillin-resistant *Staphylococcus aureus*, comparable with that of the antibacterial drug vancomycin. *Pinus* species have been shown to be more resistant to biodegradation than other wood species. Harju et al. [17] studied chemical factors affecting the decay resistance of *P. sylvestris* heartwood. The concentration of total phenolics was higher in the decay-resistant heartwood than in the decay-susceptible heartwood. The amount of acetone-soluble extractives and pinosylvin and its derivatives was higher in the resistant trees than in the susceptible ones. Stilbenes are known to be toxic to bacteria [27, 28]. Pinosylvins have been shown to exhibit antifungal activities [29, 30]. In a study by Celimene et al. [31] on the effects of stilbenes on wood decay fungi in vitro, the relative activities of three pinosylvin compounds were: pinosylvin > pinosylvin monomethyl ether > pinosylvin dimethyl ether.

Extracts from several plants, but only from some wood species, have been studied for antibacterial effects against mainly clinically important microorganisms. However, the antibacterial effects of wood extracts at large have not been studied, especially not against industrially important microorganisms, such as paper mill bacteria, although wood extracts could be produced in large amounts. The activity of natural biocides against sessile organisms has been studied by Gomes de Saravia and Gaylarde [32]. Biofilm formers, such as some of the bacteria present in paper mills, are particularly important since the sessile bacteria present in surface-associated aggregates are much more problematic, i.e., are more resistant to antibacterial agents than freely swimming planktonic bacteria [33].

In this work, hydrophilic extracts from the knot heartwood of 18 wood species (16 softwoods, two hardwoods) were tested in disc diffusion and liquid culture tests for antibacterial effects against three species of paper mill bacteria. Two of the bacterial species tested, *Burkholderia multivorans* and *Bacillus coagulans*, are dominant biofilm-formers isolated from paper mill surfaces. *Burkholderia* is both clinically and industrially important. The third bacterium studied was *Alcaligenes xylosoxydans*.

Materials and methods

Knotwood extracts

The wood species, 16 coniferous and two deciduous, and their sampling sites are presented in Table 1. Wood discs containing knots were sawn from freshly felled trees and delivered in a frozen state. Knot heartwood, here called knotwood, was separated from ordinary stemwood. The knotwood was splintered manually, freeze-dried, and ground in a Wiley mill, producing particles passing a 10-mesh screen. A second freeze-drying step after the milling ensured almost complete removal of volatile compounds. The wood meals were stored at −18°C until extraction. Crude extracts from knots were obtained using an accelerated solvent extractor (ASE 200; Dionex

Table 1 Wood species and their sampling sites

	Wood species	Common name	Sampling site
1	*Abies balsamea*	Balsam fir	Itasca County, Blandin Land, Minn., USA
2	*A. lasiocarpa*	Alpine fir	Solböle, Bromarf, Finland
3	*A. pectinata*	Silver fir	Saint-Die, Vosges, France
4	*A. sibirica*	Siberian fir	St. Petersburg region, Russia
5	*Picea glauca*	White spruce	Itasca County, Blandin Land, Minn., USA
6	*P. mariana*	Black spruce	Solböle, Bromarf, Finland
7	*P. sitchensis*	Sitka spruce	Llandeglam, north Wales, UK
8	*P. abies*	Norway spruce	Southwest Finland
9	*Pseudotsuga menziesii*	Douglas fir	Solböle, Bromarf, Finland
10	*Pinus contorta*	Lodgepole pine	Sävar, Sweden
11	*P. banksiana*	Jack pine	Itasca County, Blandin Land, Minn., USA
12	*P. resinosa*	Norway pine	Itasca County, Blandin Land, Minn., USA
13	*P. sylvestris*	Scots pine	Southwest Finland
14	*Larix decidua*	European larch	Solböle, Bromarf, Finland
15	*L. lariciana*	Tamarack	Itasca County, Blandin Land, Minn., USA
16	*Thuja occidentalis*	Northern white-cedar	Itasca County, Blandin Land, Minn., USA
17	*Fagus sylvatica*	Beech	Slovenia
18	*Betula pendula*	Silver birch	Ekenäs, Finland

Corp., Sunnyvale, Calif.). The sample cell was filled with 7–16 g of knotwood meal which was submitted to sequential extraction, first with hexane to remove lipophilic extractives and then with acetone:water (95:5, v/v) to collect hydrophilic extractives [3]. All extracts were evaporated to dryness, vacuum oven-dried, diluted with acetone:water (9:1, v/v) to a gravimetric concentration of 64 g l^{-1}, and stored at −18°C until tested.

Pure stilbenes were prepared from pine wood extracts by chromatographic separation and crystallization. The gas chromatographic (GC) purities were: pinosylvin (PS) 95%, pinosylvin monomethyl ether (PSM) 95%, and dihydro-pinosylvin monomethyl ether (DPSM) 98%.

Bacterial strains and media

The bacterial strains were isolated from a paper mill in Finland producing mineral-coated magazine paper from mechanical and chemical pulp; and they were identified as described by Väisänen et al. [34]. The strains and their sampling sites are shown in Table 2. The strains were stored at −70°C in Nutrient broth (Difco, Becton, Dickinson Co., Sparks, Md.) containing 15:85 (v/v) glycerol. For inoculation of the growth media, the strains were grown on Plate count agar (Difco, Becton, Dickinson Co.) at 40°C for 1–2 days. One loopful of biomass was suspended per milliliter of 0.1% sterile peptone water [consisting of 1 g of proteose peptone number 3 (Bacto, Becton, Dickinson Co.) in 1 l of distilled water]. A volume of 70–100 µl of the suspension was added to agar or liquid growth medium.

Disc diffusion tests

Discs (10 mm diam.) were punched from filter paper (white ribbon, 110 mm diam.; Schleicher & Schuell, Dassel, Germany) and dipped into a hydrophilic knotwood extract, giving a concentration of about 1 mg of extract per disc. The discs were then vacuum oven-dried and placed on the surface of agar plates (Plate count agar) seeded with the test bacteria. The agar plates were incubated at 40°C overnight. Antimicrobial activity was assessed as the diameter of the inhibition zones around the paper discs containing the extract.

Liquid culture tests

The extracts were pipetted into 8 ml of Plate count broth, resulting in a final extract concentration of 100 mg l^{-1}. The mixtures were inoculated with the test bacteria and incubated on a rotary shaker (126 rpm, 40°C) for 24 h. Bacterial growth was measured as turbidity at 620 nm (Lambda 40 UV-VIS spectrophotometer; Perkin Elmer Instruments, Shelton, Conn.). Antimicrobial activity was expressed as a decreased turbidity in a culture containing extract, as compared with one without extract. After the liquid culture test, the viability of the cells was tested by cultivation on Plate count agar.

Analysis of extracts

The extracts were analyzed by GC after evaporation and silylation. Heneicosanoic acid, betulinol, cholesteryl heptadecanoate, and 1,3-dipalmitoyl-2-oleoyl glycerol were used as internal standards. The individual phenolic components were analyzed on a column (25 m × 0.20 mm i.d.) coated with crosslinked methyl polysiloxane (HP-1, 0.11 µm film thickness). The method used was according to Ekman and Holmbom [36] and Willför et al. [3]. Oligomeric aromatic compounds were analyzed on a column (6 m × 0.53 mm i.d.) coated with crosslinked dimethyl polysiloxane (HP-1, 0.15 µm film thickness) according to Örså and Holmbom [37]. Identification of individual components was performed by gas chromatography-mass spectrometry (GC-MS) analysis of the silylated components with a HP 6890-5973 GC-MSD instrument, using a GC column (25 m) similar to the above.

Results and discussion

Disc diffusion tests

Hydrophilic knotwood extracts from 18 wood species were screened for antibacterial effects against three species of paper mill bacteria. Pure cultures of bacteria were cultivated overnight on agar plates with paper discs containing extract. The antibacterial activity was assessed as an inhibition zone around the paper discs containing extract (Table 3).

All *Pinus* extracts were active against the tested strains. This was probably due to the presence of stilbenes in these extracts. The *P. sylvestris*, *P. resinosa*, and *P. contorta* extracts had a stronger effect than the *P. banksiana* extract. Also, the *Pseudotsuga menziesii* and *Thuja occidentalis* extracts had a small inhibitory effect. For the *Bac. coagulans* samples, two inhibition zones were present, a clear zone totally devoid of growth

Table 2 Bacterial strains and their sampling sites in the paper mill

Bacterial species	Strain(s)	Sampling site
Bur. multivorans[a]	F45L5, F453DL1	Biofilm on steel surface in paper machine wire water
Bac. coagulans	E50L1	Biofilm on steel surface in paper machine process water
Alc. xylosoxydans	A50182	Headbox

[a]Formerly classified as *Bur. cepacia* [35]

Table 3 Susceptibility of *Bur. multivorans*, *Bac. coagulans*, and *Alc. xylosoxydans* to hydrophilic knotwood extracts. The repeatability of the tests was within ±20%. – No visible effect, + small effect [inhibition zone (i.z.) diam. 11–15 mm], + + moderate effect (i.z. diam. 16–19 mm), + + + strong effect (i.z. diam. 20 mm or more)

	Knotwood extract	*Bur. multivorans*		*Bac. coagulans* E50L1	*Alc. xylosoxydans* A50182
		F45L5	F453DL1		
1	*Abies balsamea*	–	–	–	–
2	*A. lasiocarpa*	–	–	–	–
3	*A. pectinata*	–	–	–	–
4	*A. sibirica*	–	–	–	–
5	*Picea glauca*	–	–	–	–
6	*P. mariana*	–	–	–	–
7	*P. sitchensis*	–	–	–	–
8	*P. abies*	–	–	+	–
9	*Pseudotsuga menziesii*	+	+	+	–
10	*Pinus contorta*	+ + /+ + +[a]	+ + +	+ + /+ + +[a]	+ +
11	*P. banksiana*	+	+ +	+ +	+
12	*P. resinosa*	+ + +	+ + +	+ + +[a]	+
13	*P. sylvestris*	+ + +	+ + +	+ + +[a]	+ +
14	*Larix decidua*	–	+	+	+
15	*L. lariciana*	–	–	+	+
16	*Thuja occidentalis*	+	+	+/+ +[b]	+
17	*Fagus sylvatica*	–	–	+	+
18	*Betula pendula*	–	–	+	–

[a]Two inhibition zones: one absolute inhibition zone totally devoid of growth and one relative inhibition zone with distinctly inhibited growth

[b]Only decreased growth, no zone totally devoid of growth

and another with only decreased growth. Additionally, the border of growth inhibition was diffuse. Beyond the clear zone, growth increased gradually with increasing distance from the paper disc. All the other bacterial species showed a sharp border between the clear inhibition zone and normal growth elsewhere on the agar plate. Color changes occurred on the agar plates around the paper discs containing extracts of *P. menziesii*, *L. decidua*, *L. lariciana*, *F. sylvatica*, *T. occidentalis*, and *Betula pendula*. For the *Pseudotsuga* and *Larix* extracts, the color may be due to the presence of taxifolin [38].

Lipophilic knotwood extracts were also tested for antibacterial effects (data not shown). In this case, the *Pinus* extracts had a small effect, probably due to the presence of small amounts of stilbenes in the lipophilic extracts. This supports the hypothesis that stilbenes are the compounds causing the antibacterial effects. Additionally, a small inhibitory effect against *Bac. coagulans* was seen for the *P. menziesii*, *T. occidentalis*, and *Abies* extracts.

In this study, the Gram-positive bacterium *Bac. coagulans* was more susceptible to the tested extracts than the Gram-negative *Bur. multivorans*, which is in accordance with earlier studies [11, 39]. Also, some slight differences in susceptibility between the two strains of *Bur. multivorans* were seen. Strain F45L5 was less susceptible to extracts than strain F453DL1. *Alc. xylosoxydans* was the least susceptible bacterial species.

When the antimicrobial effects of the extracts are expressed as inhibition zones, a wider zone is assumed to indicate higher susceptibility. However, when directly comparing the effectiveness of two different components, it should be remembered that the width of the inhibition zone is also a function of the initial concentration of the component, its solubility, and its diffusion rate through agar.

Liquid culture tests

Since toxicity depends both on the compound being tested and on the conditions under which the test is performed [40], conclusions should rely on more than one type of bioassay. Therefore, the antibacterial effects of the extracts were studied also in liquid cultures. In a preliminary test, bacteria were cultivated for 24 h in Plate count broth containing 1, 5, 30, 60, and 100 mg l^{-1} of *P. resinosa* extract. The bacterial growth, measured as turbidity, is shown in Fig. 1.

None of the tested concentrations inhibited bacterial growth totally, even at the highest level. However, 100 mg l^{-1} was sufficient to indicate an antibacterial effect and was therefore used in further tests. Bacteria were cultivated for 24 h in Plate count broth containing knotwood extracts from 18 wood species at a concentration of 100 mg l^{-1}. The antimicrobial activity, expressed as a decreased turbidity in a culture containing extract, as compared with that of one without extract, is presented in Fig. 2.

For *Bur. multivorans* strain F45L5 and *Alc. xylosoxydans*, the bioactive compounds only decreased growth, whereas for *Bur. multivorans* strain F453DL1 and *Bac. coagulans*, growth was totally inhibited. The growth of *Bac. coagulans* was inhibited by extracts from *P. sylvestris*, *P. contorta*, *P. resinosa*, and *P. banksiana* (some turbidity). The growth of *Bur. multivorans* strain

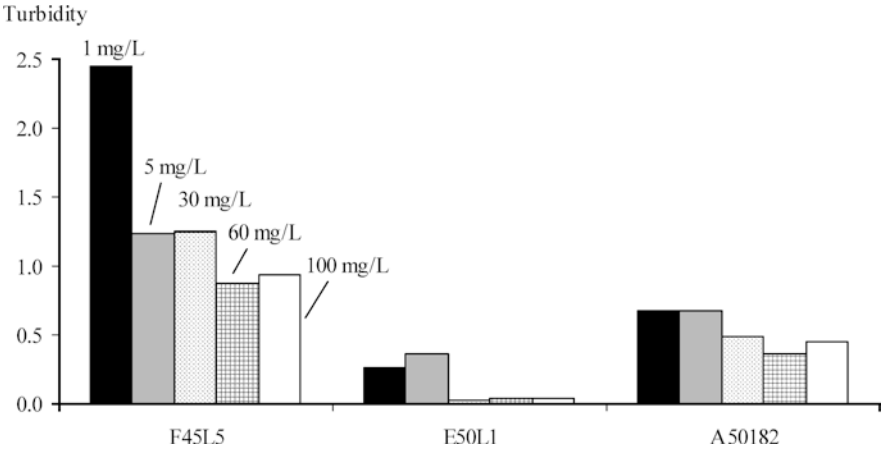

Fig. 1 Growth of the *Bur. multivorans* strain F45L5, *Bac. coagulans* strain E50L1, and *Alc. xylosoxydans* strain A50182 in Plate count broth containing hydrophilic *Pinus resinosa* knotwood extract at concentrations of 1, 5, 30, 60, and 100 mg l^{-1}

F453DL1 was inhibited by the *P. contorta*, *P. resinosa*, and *P. sylvestris* extracts. The growth of *Bur. multivorans* strain F45L5 and *Alc. xylosoxydans* was lower in the presence of the *P. contorta*, *P. resinosa*, and *P. sylvestris* extracts, as compared with a culture without extract.

Also in the liquid culture test, *Bac. coagulans* was more susceptible to the extracts than *Bur. multivorans* and *Alc. xylosoxydans*; and *Bur. multivorans* strain F453DL1 was more susceptible than strain F45L5. None of the extracts tested inhibited the growth of *Alc. xylosoxydans* totally.

Biofilm was formed in all *Bur. multivorans* strain F45L5 samples. Bacteria attached to the walls of the test tubes at the air/liquid interface. Also, *Bur. multivorans* strain F453DL1 formed a biofilm. *Bac. coagulans* did not form a biofilm, even though it had been isolated from biofilms in the paper mill. This is in accordance with Kolari et al. [41], who noticed that *Bacillus* does not form biofilm in monoculture, even though it forms biofilms in paper mills.

After the liquid culture test, the bacteria were cultivated on agar plates in order to determine whether the extract killed the bacteria or only inhibited their growth. If inhibited liquid cultures grew when cultivated on agar, the extract only inhibited growth, or for spore-forming species, it perhaps killed the bacteria but not the spores. All samples grew, even *Bur. multivorans* and the spore-forming *Bac. coagulans* strain E50L1 which had been cultivated in the presence of *P. sylvestris*, *P. contorta*, and *P. resinosa* extracts, although their growth was totally inhibited by extracts in liquid culture.

Chemical composition of extracts

In order to find out the possibly active compounds, the chemical composition of the extracts was determined by GC after silylation. The main components are listed in Table 4, and the structure for some of them is shown in Fig. 3. The chemical composition of the extracts will be reported in detail elsewhere (Willför et al., personal communication).

The main components in the hydrophilic *P. contorta* and *P. banksiana* knotwood extract solutions were the stilbenes PS and PSM, the flavanones pinocembrin and pinobanksin, and the lignan nortrachelogenin. The *P. resinosa* extract contained mainly PS and PSM. The main component in the *Abies* and *Picea* extracts was secoisolariciresinol and hydroxymatairesinol, respectively. The main component in the *Pseudotsuga* extract was taxifolin, in the *Larix* extracts secoisolariciresinol and taxifolin, in the *Fagus* extract catechin and in the *Thuja* extract thujalignans. The hydrophilic extracts also contained small amounts of lipophilic extractives.

The main components in the hydrophilic *Pinus* extracts inhibiting bacterial growth were PS and PSM, suggesting that these components would be at least partially antibacterial substances. When comparing the toxicity of extracts from different *Pinus* species in this study, the *P. sylvestris* extract was the most toxic, followed by the *P. resinosa*, *P. contorta*, and *P. banksiana* extracts, which correlates with the concentrations of PS and PSM.

Tests with stilbenes

In order to support the hypothesis of pinosylvins being compounds responsible for the antibacterial effect, PS, PSM, and DPSM (Fig. 3) were studied at three concentration levels in both disc diffusion and liquid culture tests. For comparison purposes, the *P. sylvestris* extract was also tested again in the same experiment. The results are presented in Table 5.

All compounds inhibited bacterial growth, but none of them at the level of 0.2 g l^{-1} in the extract solutions (0.004 mg per paper disc). However, in the *P. sylvestris* knotwood extract, this concentration level of PS and PSM was enough to inhibit bacterial growth. PS was the most effective compound against all bacteria; and the susceptibility order was PS > PSM > DPSM at the middle concentration level and was PS > DPSM > PSM at the highest concentration level. The small differences in concentrations within the solutions might be the explanation for this change in susceptibility order.

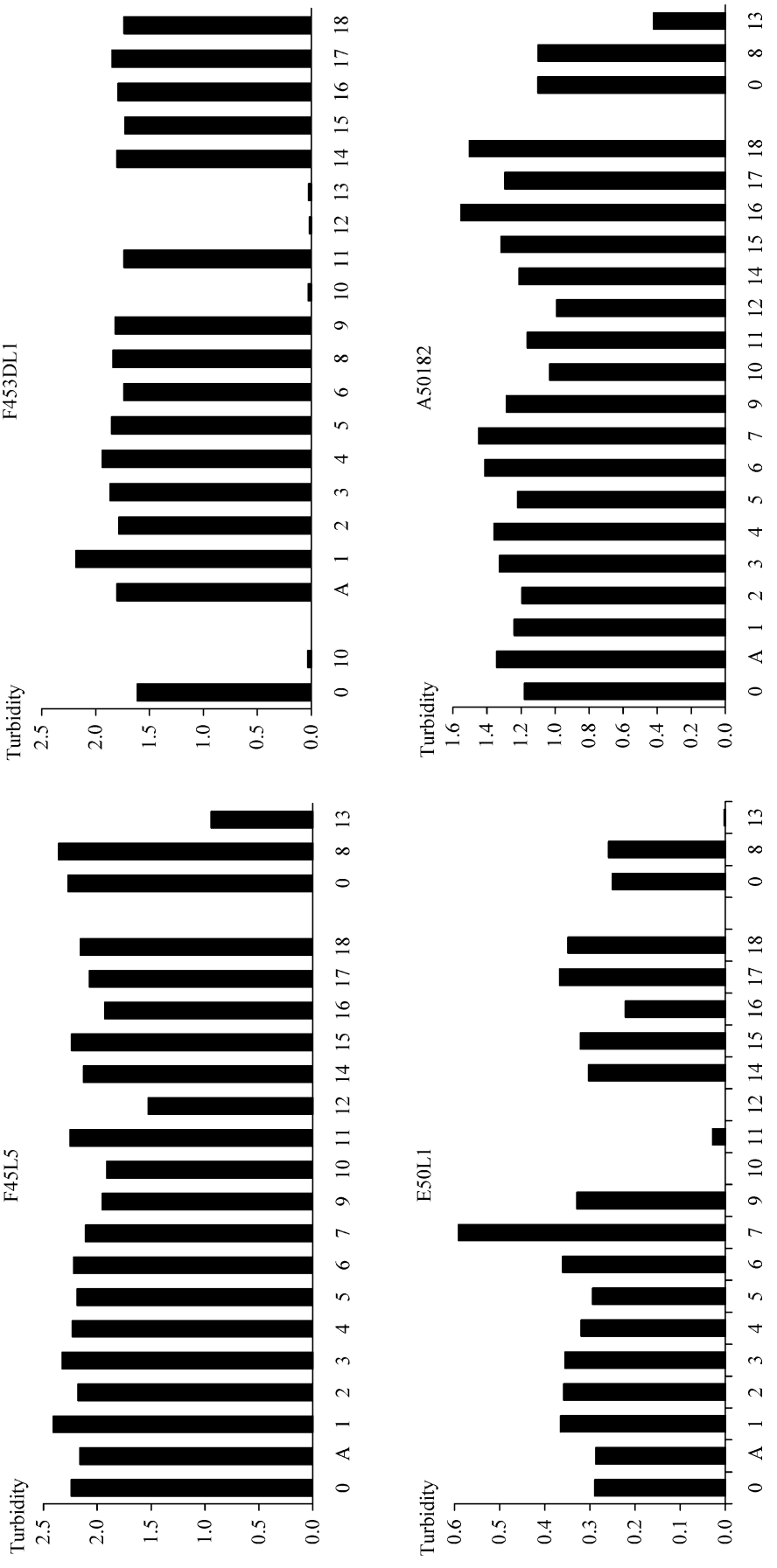

Fig. 2 Growth of *Bur. multivorans* strains F45L5 and F453DL1, *Bac. coagulans* strain E50L1, and *Alc. xylosoxydans* strain A50182 in Plate count broth containing hydrophilic knotwood extracts from 18 wood species at a concentration of 100 mg l^{-1}. The repeatability of the tests was within $\pm 20\%$. For the wood species numbers, see Table 3. *0* sample without extract, *A* acetone control

Table 4 Component groups and main individual components (mg l^{-1}) in the hydrophilic knotwood extract solutions. The concentration of the extract solution analyzed, was 64 g l^{-1}. The structure of some components is shown in Fig. 3

	Abies				*Picea*			*Pseudotsuga menziesii*	*Pinus*				*Larix*		*Thuja occidentalis*	*Fagus sylvatica*	*Betula pendula*
	balsamea 1	*lasiocarpa* 2	*pectinata* 3	*sibirica* 4	*glauca* 5	*mariana* 6	*sitchensis* 7	9	*contorta* 10	*banksiana* 11	*resinosa* 12	*sylvestris* 13	*decidua* 14	*lariciana* 15	16	17	18
Fatty acids	2.3	24	5.8	21	1.6	2.3	4.2	35	22	3.9	10	11	4.2	8.9	18	4.5	3.4
Resin acids	0.1	2.0	0.3	0.4	0.9	2.3	0.6	6.0	80	33	148	11	2.1	4.4	0.6	2.1	0.1
Abietic acid											94						
Dehydroabietic and levopimaric acid											21						
Sterols	2.4	3.5	2.0	4.5	10	14	8.6	2.3	0.4	0.9	1.0	0.5	4.2	5.4	77	7.3	77
Stilbenes	1.8	1.9	0.5	7.7	0.0	1.9	0.2	0.6	324	115	565	590	0.2	0.2	19	1.0	0.6
Pinosylvin									195	24	247	267					
Pinosylin monomethly ether									121	86	281	309					
Flavonoids	2.7	1.0	2.0	1.5	7.2	3.8	7.5	257	695	178	9.0	4.3	394	168	1.1	71	80
Pinocembrin									371	101							
Pinobanksin									208	66							
Dihydroka-empferol									64				171				
Pinobanksin 3-acetate									51								
Taxifolin								6.3					223	167			
Catechin																64	74
Juvabiones	5.3	64	63	51				63	9.1	20							
Lignans	674	324	652	524	1,049	392	384	397	188	274	491	2.7	381	329	14	8.4	8.2
Secoisolaric-iresinol	370	189	368	313		26		17					218	229			
Isolariciresinol and lariciresinol	200		140	114			45	45					118	75			
Hydroxyma-tairesinol					905	258	77										
α-conidendrin					50	56	125										
Pinoresinol					32												
Liovil							101										
Nortrachelogenin									131	254	469		32				
Oligomeric polyphenols[a]	378	288	372	532	183	232	346	198	132	84	63	56	157	212	104	162	368

[a]Mainly of lignan-type

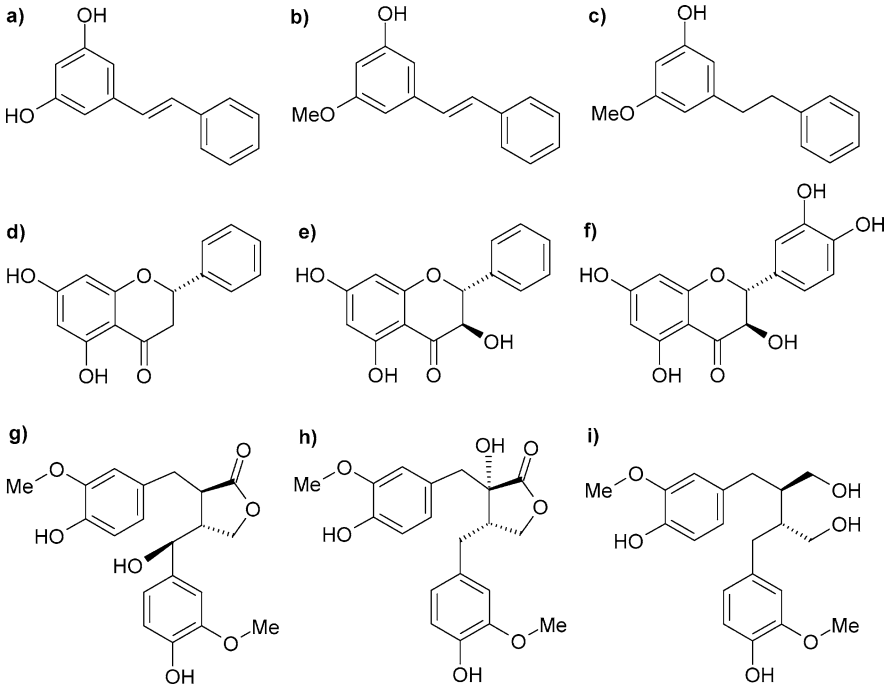

Fig. 3 a–I The structure of polyphenols found in wood. The structures shown are the stilbenes pinosylvin (**a**), pinosylvin monomethyl ether (**b**), and dihydro-pinosylvin monomethyl ether (**c**), the flavonoids pinocembrin (**d**), pinobanksin (**e**), and taxifolin (**f**), and the lignans hydroxymatairesinol (**g**), nortrachelogenin (**h**), and secoisolariciresinol (**I**)

Table 5 The relationship between the concentration of PS, PSM, and DPSM and the inhibition zone diameter (mm), for *Bur. multivorans*, *Bac. coagulans*, and *Alc. xylosoxydans*. For the wood species, the PS/PSM concentration is given. The repeatability of the tests was within ±20%

Stilbene/wood extract	Concentration			*Bur. multivorans*		*Bac. coagulans* E50L1	*Alc. xylosoxydans* A50182
	[a] g l^{-1}	[b] mg	[c] mg l^{-1}	F45L5	F453DL1		
PS	0.24	0.004	0.37	0	0	0	0
PS	2.4	0.040	3.8	12.5	18	13/20[d]	19
PS	28.3	0.467	44.5	29	34	21/34[d]	22.5
PSM	0.21	0.004	0.33	0	0	0	0
PSM	1.8	0.029	2.8	11.5	13.5	13/15[d]	12
PSM	21.1	0.349	33.3	15	17	18/21[d]	13
DPSM	0.16	0.003	0.25	0	0	0	0
DPSM	1.8	0.002	2.9	12	12.5	12.5	0
DPSM	21.4	0.353	33.7	20	23	19/28[d]	13
13	0.27/0.31	0.003/0.002	0.42/0.49	24	30	21/28[d]	15.5
10	0.20/0.12	0.000/0.001	0.31/0.19	20	28	20	16
11	0.02/0.09	0.004/0.005	0.04/0.14	12	18	19	11
12	0.25/0.28	0.004/0.005	0.39/0.44	22	30	20/26[d]	14
13	0.27/0.31	0.003/0.002	0.42/0.49	24	28	20/28[d]	17

[a]In extract solutions
[b]In paper discs
[c]In liquid culture tests
[d]Totally devoid of growth/only decreased growth

Therefore, PS seems to be more toxic than PSM, which is in accordance with the report by Celimene et al. [31]. When comparing different bacteria, *Alc. xylosoxydans* was the least susceptible species to PS and PSM, but was equally or more susceptible to PS.

The results from the liquid culture tests are presented in Fig. 4. Antibacterial effects were obtained only at the highest concentration level. Contrary to the disc diffusion tests, PSM gave stronger effects than PS and DPSM was not antibacterial at all. For *Bur. multivorans* strain F45L5 and *Alc. xylosoxydans*, *P. sylvestris* was the most effective extract, followed by PSM and PS. For *Bac. coagulans*, PSM and *P. sylvestris* inhibited growth; and for *Bur. multivorans* strain F453DL1, PSM, PS, and *P. sylvestris* inhibited growth. *Bur. multivorans* strain F45L5 was the least susceptible to the stilbenes.

When comparing the *Pinus* species, it is notable that the susceptibility order correlated with the amount of PS and PSM present in these species. The susceptibility order was *P. sylvestris* > *P. resinosa* > *P. contorta* > *P. banksiana* and the amounts of PS/PSM were 0.42/0.49 > 0.39/0.44 > 0.31/0.19 > 0.04/0.14 (Table 5).

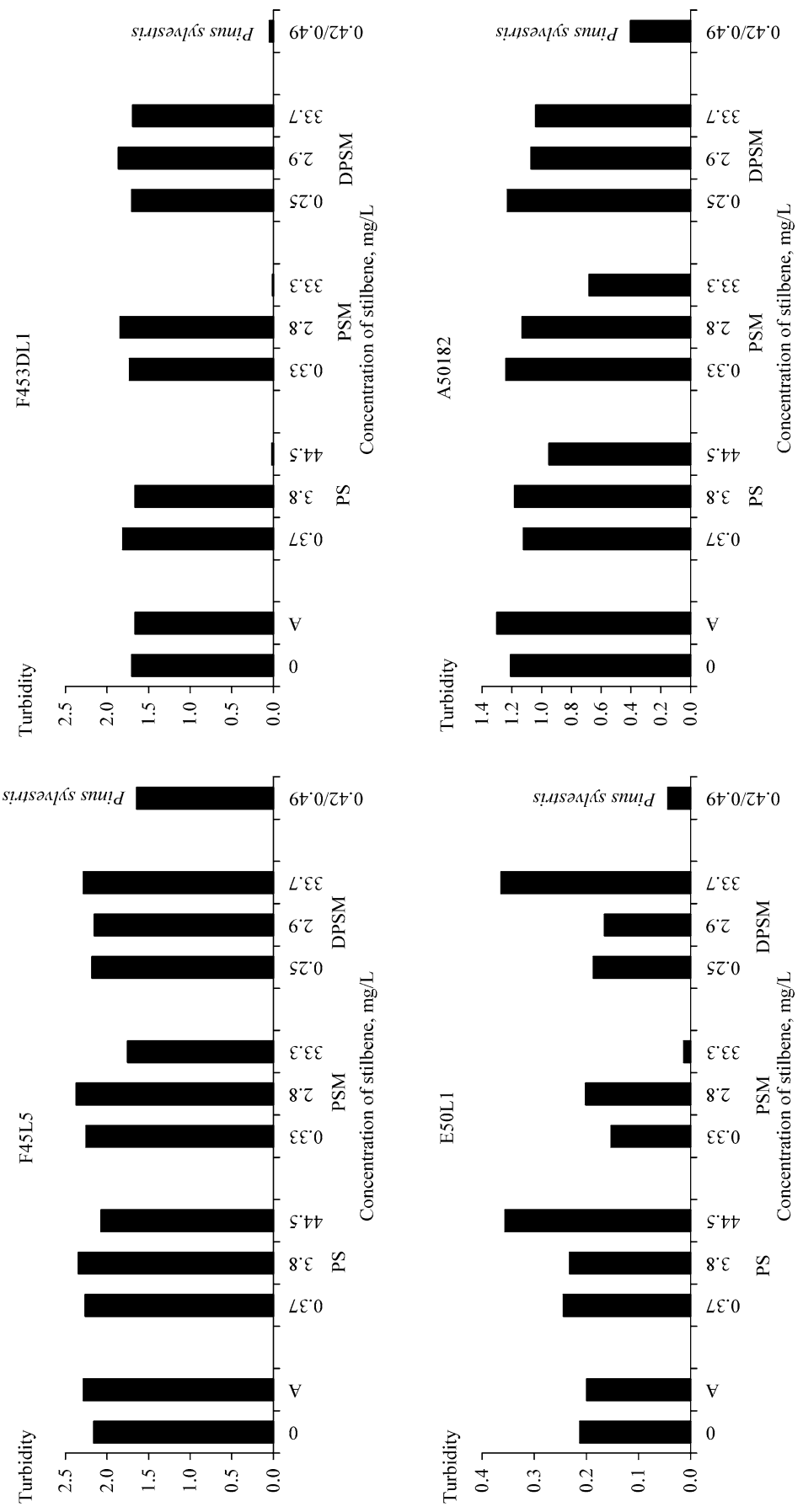

Fig. 4 Growth of *Bur. multivorans* strains F45L5 and F453DL1, *Bac. coagulans* strain E50L1, and *Alc. xylosoxydans* strain A50182 in Plate count broth containing PS, PSM, and DPSM at three different concentration levels (mg l^{-1}), and *P. sylvestris* crude extract at a concentration of 100 mg l^{-1} (0.4 mg PS l^{-1}, 0.5 mg PSM l^{-1}). The repeatability of the tests was within ±20%. *0* sample without extract, *A* acetone control

These results partly support the hypothesis of PS and PSM being the antibacterial compounds in *Pinus* species. But this is not totally true. The level of 0.4 mg PS l^{-1} and 0.5 mg PSM l^{-1} in *Pinus* species was growth-inhibiting, but when single compounds were used, this concentration level was not enough to inhibit bacterial growth. This might be due to the lack of synergism when using single compounds. Another explanation might be that minor extract components in *Pinus* species are also active at small concentrations.

The difference in results obtained with the disc diffusion and liquid culture tests might be explained by the difference in concentrations. In liquid culture tests, the concentrations were probably much lower than in the disc diffusion tests. Therefore, effects were seen only at the highest concentration level. Furthermore, with substances having only low water solubility, e.g., the stilbenes, nutrient agar is more useful than nutrient solution, but usually more extractive is required [42]. In a study by Rennerfelt [43], PS and PSM were approximately twice as toxic in a nutrient solution as in a nutrient agar. In addition, it should be kept in mind that, even though a compound is highly toxic when tested on agar, this does not necessarily mean that heartwood containing this compound is completely resistant to decay. Compounds can be far more toxic in an agar substrate than in a woody substrate [42], e.g., the fungitoxicity of stilbenes bioassayed in a woody substrate is reduced 90–99% [44].

PS and PSM are commonly assumed to be a factor influencing the durability of pine heartwood, implying that pine heartwood is durable, which it is not [42]. Wood extract is a complex mixture and the interaction of its components, both toxic and nontoxic, with each other needs consideration [44]. The decay resistance of pine heartwood is probably a multifunctional phenomenon of the lack of water up-take, the presence of resin acids, and the presence of pinosylvins; and it may be impossible to confer sole responsibility for resistance on one substance out of the entire heartwood. Very likely the compounds act synergistically. Testing compounds singly, even in wood, may yield very misleading data.

The *Pinus* extracts showed antibacterial effects against paper mill bacteria, but in general the other knotwood extracts did not inhibit bacterial growth at the concentration level tested. It is possible that other species would also have shown antibacterial effects if higher concentrations had been used. However, with higher extract concentrations, the solubility of the extracts in Plate count broth can become a limiting factor. Solubility could also be a limiting factor in the process water.

The antibacterial activity varied with the wood species, the stilbene, the concentration, the bacterial species, and the bioassay test used. These results for the inhibiting effects of knotwood extracts appear relevant since similar results were obtained both with disc diffusion and liquid culture tests. In the future, the most interesting extracts should be further fractionated, characterized, and tested in more detail, in order to determine the active compounds and their mechanisms of action. However, single compounds are probably not as effective inhibitors as a mixture of compounds. A mixture of compounds would probably be more efficient against bacteria than pure compounds. However, when using mixtures, it would be difficult to know which compounds are the active ones. This study has shown that hydrophilic knotwood extracts have a potential for use as natural biocides in paper mills. Especially, knotwood extracts from *Pinus* species are potential strong biocides.

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