Research Articles

Phytotoxicity of Polycyclic Aromatic Hydrocarbons to Willow Trees

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Abstract. The toxicity of PAH to willow trees (*Salix alba, S. viminalis, S. viminalis x schwerinii*) was investigated. Willow cuttings were grown in PAH-saturated hydroponic solution (naphthalene NAP, phenanthrene PHEN and benzo(a)pyrene BaP). Toxicity was related to aqueous solubility and was highest for NAP. PHEN did not show significant effects, except in one case. Exposure of trees to BaP showed no effect in two cases, but increased transpiration and growth in two others. High dosages of NAP were fatal for the trees, the lowest dosage significantly stimulated growth.

Soil samples were taken from several PAH contaminated sites, among them gas works sites and a former sludge basin. The PAH contents ranged from 1.76 mg/kg to 1451 mg/kg. None of the soils was lethally toxic to the trees, and difference between growth in control soils and growth in PAH contaminated soils was not apparent. Growth and water use efficiency were positively, but not significantly correlated to the PAH content of the soils. Outdoor growth of willows and poplars on the former sludge basin in Valby was monitored, with willows growing faster than poplars (*Populus trichocarpa*). Phytotoxic effects could be observed at some willows at the Valby sludge basin, but it is not sure whether these effects can be contributed to PAH.

Keywords: Benzo(a)pyrene; naphthalene; PAH; phenanthrene; phytoremediation; plants; toxicity; willow, *Salix*

Introduction

Polycyclic aromatic hydrocarbons (PAH) are compounds with two or more (up to seven) condensed aromatic and other cyclic rings. PAH are a by-product of incomplete combustion, e.g., from traffic, coal fires, heating, and are frequent environmental pollutants. Some members of this chemical class, e.g., benzo(a)pyrene, are prominent and strong carcinogens. Therefore, PAH are considered a serious soil pollution problem. Recently, phytoremediation has been shown to be an effective way of reducing concentration levels in soil.

Pradhan et al. (1998) tested six different plant species for a clean-up of a former gas works site contaminated with PAH (in total concentrations of 200 mg/kg to 1000 mg/kg). Alfalfa (*Medicago sativa*) and switchgrass (*Panicum virgatum*) removed 57% of total PAH in six months, compared to 26% in the controls. Reilley et al. (1996) compared the changes of PAH concentrations in soil in a simulated landfarming with and without vegetation in a greenhouse experiment. Elimination of anthracene (3-ring-PAH) from soil was rapid, best stimulated by alfalfa (*Medicago sativa*), compared to fescue *Festuca arundinacea*, sudangrass *Sorghum vulgare*, switchgrass *Panicum virgatum* and no vegetation. The elimination was presumed to be mainly by rhizodegradation. In a study by Qui et al. (1997) with clay soil at a former olefins production area, Verde kleingrass *Panicum coloratum* was found to be superior compared to all other plant species and non-vegetated soil. The measured concentrations of PAH for the plots of Verde kleingrass was either non-detectable or one or two orders of magnitude lower than in non-vegetated soil and other grass species. However, the initial concentration of PAH was 0.1 to 5 mg/kg, which is low for a contaminated site.

A highly PAH contaminated former sludge basin in the US had undergone fast and substantial ecological recovery, but the structure of the biodiverse plant community (51 species and 22 families) was different from that at a recovering non-polluted disturbed site (Olson and Fletcher 2000). In a follow-up study, a significant decrease of PAH concentrations in the root zone was found, from an average of 16854 mg/kg PAH (sum of 16 EPA PAH) in the parent sludge to 1121 mg/kg in the upper root zone (Olson et al. 2001).

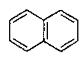
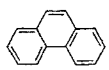
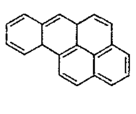
The Valby sludge basin, Denmark, Copenhagen, was planted with willow and poplar trees in May 1999 and April 2000. The sludge originated from a wastewater treatment plant and is contaminated with several pollutants, among them PAH, phthalates and heavy metals (L. Andersen, DHI Hørsholm, pers. comm.). This study investigates whether the content of PAH in the sludge has an influence on the growth of the trees. A second site in Denmark, where poplar trees had been planted in PAH-containing soil is the former gas works at Holte, Søllerød. Additional soils originate from a former asphalt works site. The question was whether the toxicity of soil pollutants, among them PAH, would allow phytoremediation.

1 Methods

1.1 Chemicals

With increasing number of rings, PAH become less water soluble and more lipophilic. For this study, naphthalene (two rings) phenanthrene (3 rings) and benzo(a)pyrene (5 rings)

Table 1: Properties of the investigated PAH (Rippen 2001)

Structure	Name	Abbr.	Molar Mass g/mol	log K_{ow}	P (Pa)	S (mg/L)
	Naphthalene	NAP	128.18	3.36	11.3	32
	Phenanthrene	PHEN	178.24	4.45	0.022	1.6
	Benzo(a)pyrene	BaP	254.33	6.13	7×10^{-7}	4.5×10^{-3}

P: saturation vapor pressure; S: water solubility; K_{OW}: partition coefficient between n-octanol and water

were selected as model compounds (**Table 1**). Phenanthrene and naphthalene were provided from MERCK-Schuchardt, Hohenbrunn, Germany, and benzo(a)pyrene from Sigma-Aldrich Chemie, Germany.

1.2 Spiked hydroponic solutions

The problem was to get a constant concentration of PAH in the hydroponic solutions, despite the fact that chemicals sorb to the glass wall and the roots, and are taken up into plants, translocated and metabolized. Therefore, PAH were added in amounts above water solubility. Phenanthrene and benzo(a)-pyrene were dissolved in acetone and then mixed into the nutrient solution. Nominal concentrations were 325 mg/L naphthalene (>10 times water solubility), 5 mg/L phenanthrene (>3 times water solubility) and 0.55 mg/L benzo(a)pyrene (122 times water solubility). The solutions were equilibrated using a magnetic stirrer and stored at 5 degrees.

1.3 Soils

Several PAH contaminated soils were used for the toxicity determination. The soils were taken at the Valby sludge basin (Copenhagen-Valby, DK); the Holte gas works site (commune Søllerød, DK); and the former asphalt works at Ringe, DK. The samples were taken with a grabber from the top soil or from less than 40 cm depth, and stored at 4°C in plastic bags or closed plastic tubes until the start of the experiments. Control soils were taken from the DTU campus in Lyngby, DK. The control soils had been previously analyzed by GC/FID for hydrocarbons, but no detectable amounts (<5 mg/kg) had been found. The Holte control soil was taken from the gas works site, but from an area free of PAH contamination.

1.4 Chemical analysis

Naphthalene and phenanthrene were extracted from hydroponic solution using pentane, which contained bromonaphthalene as internal standard and analyzed by GC/FID. The analysis of benzo(a)pyrene in hydroponic solution with this standard method failed, due to the very high boiling point. Soil samples were analyzed for PAH according to the method described by the Danish EPA (Miljøstyrelsen 2000). Ten g samples were given into a 250 mL flask; 0.1 mL internal standard (236 mg d_{10}-pyrene/L and 115 mg d_{10}-phenanthrene/L) were added, followed after 10 min by 40 mL demineralized water. The pH was adjusted to 10–12 with 10 M NaOH. Then, 40 mL pentane-diethyl ether mix (1:1) were added and the sample was sonicated for 10 min and left standing overnight. Hereafter, the pentane-diethyl ether phase was taken and evaporated to about 150 µg. The sample was analyzed using a GC-MS HP 5973 Varian 8200CX autosampler and a 25 m aluminia column with 0.22 mm diameter and 5% phenyl polysiloxan carboran layer and 0.1 µm film thickness. Helium was used as carrier gas with 1.0 mL/min. The samples were splitless injected. The temperature was 2 min at 50°C, increasing with 40°C/min to 140°C and then with 10°C/min to 310°C, remaining there for 12 min. Single ion monitoring was used for identification of compounds, with primary and secondary ions as described in Miljøstyrelsen (2000). The result was calculated with the software HP ChemStation. The Valby samples were analyzed with the same method at the DHI in Hørsholm.

1.5 Plants and toxicity testing

Willows and willow hybrids were used for the tests. *Salix alba* (Silver willow, wild species) was collected on the bank of a lake in Hørsholm, Denmark, early March 2001. Basket willows (*Salix viminalis* var. Maria) and willow hybrids (*Salix viminalis x schwerinii* var. Aage) were provided from Aage Bach, Tylstrup, Denmark. Branches of the trees were cut into 40 cm pieces, with a diameter of 1–2 cm, and pre-rooted for several weeks indoors in buckets under natural light. When roots and leaves were fully developed, plants were transferred into Erlenmeyer flasks filled with 400 mL ISO 8692 standard nutrient solution and put under artificial light in a climate chamber. After two days, the nutrient solution was exchanged against PAH solution or soils, except for controls. The test is described in detail in Trapp et al. (2000).

Five test series (1a, b, c, d, e) were carried out to determine the toxicity of PAH to willow trees in hydroponic solution. In Test 1 a–d, over-saturated solutions were used. In the first two test series, the toxicity of a mix of all three PAH was also investigated. In Test 1 e, nominal concentrations below water saturation were used. For the soil toxicity tests, *Salix viminalis x schwerinii* were used, three trees for each soil. The tests with samples from the Valby sludge basin were repeated. Parameters to describe toxicity were transpiration, growth and water use efficiency of the production (gram of growth per liter of water transpired). Trees were pre-selected for similar transpiration in each group. To eliminate the influence of size, growth was given in % per test period, and transpiration was normalized with respect to the initial transpiration of the individual tree.

2 Results and Discussion

2.1 Tests in hydroponic solution

Naphthalene and phenanthrene concentrations in the solutions at the beginning of the experiments are given in **Table 2**. Although theoretically, the samples 1 a–d should have the same dissolved concentration in water (the water solubility), there were differences between the tests, and most measured concentrations were lower than the water solubility given in the literature (Rippen 2001). This was although crystalline, non-dissolved NAP could be observed at the bottom of the samples 1 a–d. In the test series 1 e, nominal concentrations of NAP were water solubility (NAP 32) or half of water solubility (NAP 16). Both NAP concentrations were lower than expected, and were lower than in test 1a–d. Test PHEN 1 and PHEN 0.5 should have aqueous concentrations of 1 and 0.5 mg/L, but measured concentrations were above, and in the range of the measured concentrations in the test 1a–d.

Growth and transpiration of PAH exposed trees compared to controls. Due to limited resources, the number of trees per test was small, between three and five. This, together with the occurring biological variation of the trees, made the statistical interpretation of the data difficult. A one-tailed

Table 2: Conditions of the PAH exposure tests and measured initial PAH concentrations in solution (n=3)

Test	Variant	Species	Duration (h)	Concentration mean (mg/L)	Concentration s (mg/L)
1 a	Control	*Salix alba*	302	na	na
1 a	NAP	*S. alba*	302	8.9	2.2
1 a	PHEN	*S. alba*	302	0.49	0.04
1 a	BaP	*S. alba*	302	na	na
1 a	Mix	*S. alba*	302	NAP 11.25 PHEN 0.49 BaP na	NAP 7.84 PHEN 0.20 BaP na
1 b	Controls	*Salix viminalis*	527	na	na
1 b	NAP	*S. viminalis*	527	23.2	2.7
1 b	PHEN	*S. viminalis*	527	0.63	0.15
1 b	BaP	*S. viminalis*	527	na	na
1 b	Mix	*S. viminalis*	527	NAP 19.44 PHEN 0.51 BaP na	NAP 3.10 PHEN 0.09 BaP na
1 c	Controls	*S. viminalis*	502.5	na	na
1 c	NAP	*S. viminalis*	502.5	9.9	3.30
1 c	PHEN	*S. viminalis*	502.5	1.88	1.17
1 c	BaP	*S. viminalis*	502.5	na	na
1 d	Controls	*S. viminalis x schwerinii*	385	na	na
1 d	NAP	*S. viminalis x schwerinii*	385	7.3	0.66
1 d	PHEN	*S. viminalis x schwerinii*	385	0.44	0.10
1 d	BaP	*S. viminalis x schwerinii*	385	na	na
1 e	Controls	*S. viminalis x schwerinii*	145.5	na	na
1 e	NAP 32	*S. viminalis x schwerinii*	145.5	3.67	3.56
1 e	NAP 16	*S. viminalis x schwerinii*	145.5	0.61	0.10
1 e	PHEN 1	*S. viminalis x schwerinii*	145.5	1.09	0.63
1 e	PHEN 0.5	*S. viminalis x schwerinii*	145.5	0.78	0.56

s: standard deviation; na: not analyzed

t-test with $\alpha = 5\%$ was used to decide, whether PAH exposed trees showed significant differences to the controls. Trees exposed to various levels of phenanthrene showed in none but one case (smaller transpiration 1c) a significant difference to controls. The only statistically significant effects of benzo-(a)pyrene were an increase in growth (1 a) and transpiration (1 c). Naphthalene showed a dose-dependent response: During test 1 a–d, with the high dose, in all except one case, growth and transpiration of trees were significantly smaller in the tests 1a–d. At the lower doses (1 e), transpiration and growth were increased, and for the lowest dose (NAP 16), the increase in growth was significant (Table 3 and 4).

Table 3: Growth of trees exposed to PAH and statistically significant differences compared to controls

Test	Test ID	# of Trees	Mean growth (g)	Growth %	s %	Difference
1 a	Controls	5	0.136	0.28	0.12	
1 a	NAP	4	–3.5	–10.31	7.69	smaller
1 a	PHEN	4	0.72	2.33	4.71	none
1 a	BaP	4	0.40	0.99	0.71	larger
1 a	Mix	4	–6.72	–18.44	4.07	smaller
1 b	Controls	5	2.05	5.14	3.03	
1 b	NAP	4	–3.19	–8.08	5.89	smaller
1 b	PHEN	4	1.50	3.37	4.80	none
1 b	BaP	4	1.50	3.56	2.15	none
1 b	Mix	4	–7.01	–14.8	3.78	smaller
1 c	Controls	4	4.04	7.14	5.11	
1 c	NAP	4	1.59	1.90	5.30	none
1 c	PHEN	4	2.19	4.24	2.12	none
1 c	BaP	4	3.39	6.48	3.19	none
1 d	Controls	4	2.19	4.61	3.56	
1 d	NAP	4	–5.32	–9.09	3.83	smaller
1 d	PHEN	4	2.58	4.77	1.95	none
1 d	BaP	4	3.42	5.93	1.67	none
1 e	Controls	4	0.93	1.30	0.61	
1 e	NAP 32	4	1.51	1.93	1.32	none
1 e	NAP 16	3	1.86	2.35	0.54	larger
1 e	PHEN 1	4	1.12	1.50	0.37	none
1 e	PHEN 0.5	3	0.32	0.47	0.50	none

Table 4: Relative transpiration of trees (after one week) exposed to PAH and statistically significant differences compared to controls

Test	Test ID	Transpiration %	s %	Difference
1 a	Controls	100	21.4	
1 a	NAP	51.6	17.8	smaller
1 a	PHEN	81.5	27.4	none
1 a	BaP	95.9	16.3	none
1 a	Mix	14.4	34.0	smaller
1 b	Controls	100	20.3	
1 b	NAP	58.4	12.7	smaller
1 b	PHEN	109.6	14.9	none
1 b	BaP	117.7	25.0	none
1 b	Mix	25.1	4.7	smaller
1 c	Controls	100	7.8	
1 c	NAP	66.8	17.7	smaller
1 c	PHEN	80.2	12.0	Smaller
1 c	BAP	119.9	14.3	Larger
1 d	Controls	100	4.6	
1 d	Nap	70.1	19.4	Smaller
1 d	PHEN	104.5	6.7	None
1 d	BAP	102.4	8.4	None
1 e	Controls	100	33.1	
1 e	NAP 32	106.1	14.1	None
1 e	NAP 16	116.0	36.0	None
1 e	PHEN 1	121.4	24.3	None
1 e	PHEN 0.5	122.6	10.5	None

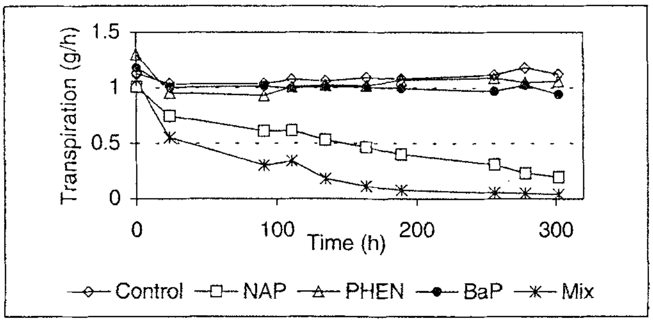


Fig. 1: Transpiration of silver willow trees (*Salix alba*) in hydroponic solution spiked with PAH; test 1a

Fig. 1 shows the normalized transpiration of silver willows in test 1 a over time. The transpiration of controls and of the PHEN and BaP variants remained almost constant, whereas the transpiration of NAP exposed trees decreased, and faster when mixed with BaP and PHEN.

2.2 Tests with PAH-contaminated soils

Table 5 gives the test conditions and the PAH concentrations in soil (sum of 16 EPA PAH). The PAH concentrations ranged from 1.76 to 1451 mg/(kg dw). Oil content (alkane fraction C_{10}–C_{28}) of the soil samples from the Valby sludge basin was analyzed by GC/FID, but no oil was found (<5 mg/kg).

Growth of willow hybrids in PAH contaminated soil. The willows grew very well in the PAH polluted soil samples, independent of the PAH level. Significant differences between PAH contaminated soils and controls were not observed, with one exception: willows were growing much better in the PAH soil from Holte than in the control soil from that site. Neither absolute and relative growth, nor transpiration and water use efficiency were significantly correlated to the PAH concentration in soil (5% level), although all correlation was positive. Even in the sample UKA 1 with a PAH content of about 1.5 g/kg, the willows were growing, not only surviving. Figs. 2 and 3 show the log PAH concentration versus the growth rate and the water use efficiency. Note that the trendlines are not significant.

Comparison to outdoor growth. The Valby sludge basin is a test area for phytoremediation and has been planted with

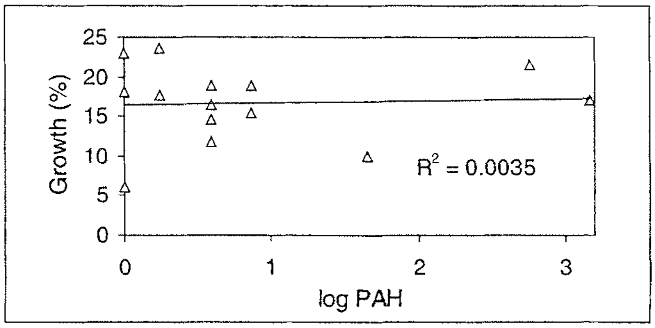


Fig. 2: Plot of log PAH concentration [log mg PAH/(kg dw)] versus growth (%) of willow hybrids growing in PAH contaminated soils

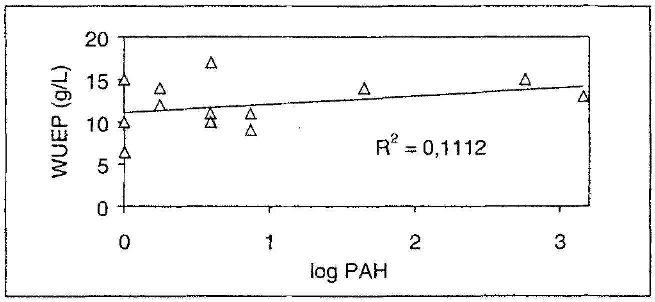


Fig. 3: Plot of log PAH concentration [log mg PAH/(kg dw)] versus water use efficiency of production WUEP (g/L) of willow hybrids growing in PAH contaminated soils

basket willow hybrids (*S. viminalis x schwerinii*) and poplar trees (*Populus trichocarpa*) in 1999 and replanted in April 2000, because many trees died, due to massive weed growth. In June 2001, the area planted with poplars was covered to 94.9% by thistles (*Carduus crispus*), and the poplars were no longer visible (1.5% coverage). Despite that, the poplars did not show signs of sickness, such as yellow or hanging leaves. Willows grew better (9.8% coverage in June 2001), with only 41.2% *Carduus crispus*, beneath some other weed (*Stellaria media* 18.8%, *Urtica dioica* 7.1%, *Tripleurospermum inodorum* 6.8%, *Lamium purpureum* 4.4%, and others). Willows showed signs of sickness at spot K 7, the soil with highest measured PAH concentration. When we reanalyze the laboratory data by plotting only the Valby samples,

Table 5: Conditions for growth of willow hybrids in soil samples with varying PAH content

Soil origin	Variant	Period (h)	Sum of PAH [mg/(kg dw)]
Holte	Control soil	336	na
Holte	PAH soil	336	45
Valby	Controls	381.5	na
Valby	K 1	381.5	3.95
Valby	K 3	381.5	1.76
Valby	K7	381.5	7.42
Valby	D1	381.5	3.91
Asphalt works	UKA 2	381.5	582
Valby	Controls	361.5	na
Valby	K 1	361.5	3.95
Valby	K 3	361.5	1.76
Valby	K7	361.5	7.42
Valby	D1	361.5	3.91
Asphalt works	UKA 1	361.5	1451

na: not analyzed

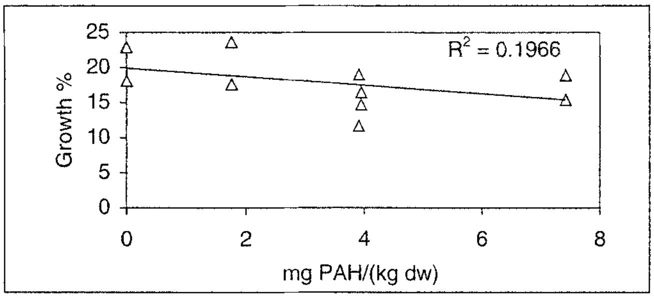


Fig. 4: Plot of PAH concentration [mg PAH/(kg dw)] versus the growth of willow hybrids (%) growing in the Valby soil samples

there is a negative, but non-significant trend to reduced growth at higher PAH concentrations (Fig. 4).

According to our tests in hydroponic solution, this decrease in growth is probably not due to the toxicity of PAH. Several other organic and inorganic compounds have been found at elevated levels at the sludge basin. Analyses are only available for the part of the area, which is planted with poplars, including sample D1 (Lehmann, DHI, personal communication). Heavy metals were found in quite high concentrations in sample D1. Lead was 410 to 500 mg/(kg dw), cadmium was 8.8 to 11 mg/(kg dw), chromium was 880 to 970 mg/(kg dw), copper was 580 to 730 mg/(kg dw), mercury was 1.4 to 2.6 mg/(kg dw), and zinc was 2700 to 4000 mg/(kg dw). Sample K7 was not analyzed for heavy metals.

2.3 Comparison to other findings

Gräf and Novak (1966) investigated the effect of growth stimulation of small amounts of BaP and other PAH on plants. Growth promotion was found for algae in aquatic solutions, but also for tobacco and other plants growing in soil. The growth promotion of PAH was positively correlated to their cancerogenic activity, with BaP having the highest effect. Sims and Overcash (1983) concluded from these and other experiments that PAH have hormonal activity to plants. Walton and Hoylman (1992) reviewed uptake, translocation, and accumulation of polycyclic aromatic hydrocarbons in vegetation. Concerning phytotoxicity, the authors came to the conclusion that "The lack of evidence for toxicity of PAH to vegetation and the anecdotal nature of the existing reports of phytotoxicity to whole plants are perhaps the strongest evidence that PAH do not produce profound toxic effects in vegetation."

3 Conclusion

The toxicity tests in hydroponic solution showed that the short term acute toxicity of PAH to all trees investigated in this study was strongly related to the dosage that could be taken up from water. This dosage was limited by the water solubility of the PAH. The toxicity of over-saturated hydroponic solution was highest for naphthalene, followed by phenanthrene, which showed significant signs of toxicity only in one case. Benzo(a)pyrene over-saturated solutions did not seem to be toxic to the trees, but had a stimulating effect on transpiration and growth in some cases. We observed growth stimulation for lower concentrations of naphthalene, too, but our measurements did not give the clear growth-promoting effect reported earlier (Gräf and Nowak 1966). The mixture of the three PAH was more toxic any of the PAH alone, indicating that phenanthrene and/or benzo(a)pyrene contributed to the toxicity.

Growth of willows in soils was not correlated to the content of PAH, if a broad range of samples was used. However, in samples from one particular site, a trend to reduced growth with higher PAH content was observed. At the moment, it cannot be answered whether this is due to PAH toxicity, or due to other compounds.

Several willow species (wild *S. alba*, *S. viminalis* and hybrid *S. viminalis x schwerinii*) were tested in this study, with the goal to identify the best species for phytoremediation purposes. Concerning the toxicity, the reaction of all species was similar, with the wild *S. alba* being slightly more sensitive. No choice can be made for the other two species. But the willow hybrid *S. viminalis x schwerinii* is growing faster than the other two species, which is an advantage both for phytoremediation and for the later use as renewable resource for energy production.

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