The willow tree acute toxicity test

Laboratory manual to the RECETO PhD course
Uptake and Effects of Xenobiotics in Plants, 14-18 of June, 2004, Copenhagen, KVL


**Principle: Healthy trees transpire more!**

The willow tox tests determines the acute toxicity of a substrate (water of any kind, toxic solutions, or solids like soil, waste, sludge). The toxicity is measured by the inhibition of the transpiration. The effect usually occurs latest after a few days, followed by other effects, such as necrosis and growth reduction, general decay and (at lethal conditions) by the death of the plant. The test is fast (1-2 weeks), cheap and easy. The replicability is high, if performed in our laboratory.

The test was designed for phytoremediation and ecotoxicity and was used on many different compounds, polluted soils, wastewater, rainwater and other substrate.

**MATERIALS AND METHODS**

Needed are 17 trees (for 3 doses: 5 controls, 4 replicates per dose), 17 Erlenmeyer flasks (500 mL), 17 cork stoppers, aluminum foliage, nutrient solution, artificial light (eventually daylight) and a balance to measure the weight with an accuracy of 0.1 gram.

**Willows.** Basket willow trees *Salix viminalis* or hybrids of this are used. Cuttings of 40 cm length and approx. 1 cm diameter are provided from nature, plantations, phytoremediation sites or ordered from Aage Bach, "Ny Vraa", Gl. Vråvej 31, 9382 Tylstrup, Denmark, Tlf: +45 98 26 17 00, Fax: +45 98 26 11 18, Mobil: 40 18 99 00, [http://home2.inet.tele.dk/abach/](http://home2.inet.tele.dk/abach/) email bach@ny-vraa.dk

Best quality have cuttings cutted after the winter. Plants are stored in the dark at 4 degree Celsius in plastic bags (add some water to keep them fresh), not longer than 1 year. Poplar cuttings can also be used.

**Nutrient solution.** We use a modified ISO Standard 8692 solution (recepy see below).

**Light and growth chamber.** We use artificial light, a rack of ten fluorescent tubes 36 W/33 with 20 cm distance between each at a height of 65 cm with 24 hours light per day. Other well-suited light sources are energy saving lamps, such as Osram Dulux, which have a very good light spectrum. However, homogenous light distribution is more difficult to achieve. We have a room with constant temperature (about 26 degrees) and humidity (50% to 65% relative humidity). The toxic effect is lower at lower temperatures.
RUN THE TEST

1) Approx. 3 weeks before the test shall start, willow cuttings are placed in a bucket with tap water and placed at a sunny window. The temperature should be between 10 and 20 degrees Celsius. The number of cuttings per buckets should not be more than 35. Take care, there is an upside and a downside (look at the buds). Remove trees that seem to be sick. In summer, check for insects.

2) Once the trees have roots and leaves (about 10 leaves), select 17 well-developed healthy trees of similar size. Give each tree a number. **Do not forget to weigh each cutting.**

3) Take the 17 cork stoppers, drill holes of a diameter similar to the willow stem diameter, and make a half cut with a knife, so that the cork rings can be fitted around the trees.

4) Prepare the nutrient solution as described below, and fill in 400 mL in 17 Erlenmeyer flasks. Fit the cork rings around the trees, in a height that the roots dip into the nutrient solution. Put the trees into the nutrient solution. Wrap aluminum foliage around the flasks (to prevent algae growth).

If you work with volatile compounds, use additionally teflon tape, silicone or else.

5) Put the trees for 1 day or longer under the light in the growth chamber. The trees need some time to adapt to the new conditions.

6) After 1-3 days, **measure the weight** of the whole apparatus again. Make marks, where the trees are placed, and take care to put them **exactly back into this place** (same direction / same distance to the light! – all the times). Best make a mark on the tree and on the table.

7) Next day, measure the weight again. Determine the weight loss. The weight loss should be around 20 g/day. Rank the trees for their weight loss from 1 to 17. Highest transpiration: control. Second highest transpiration: highest dose. 3rd highest transpiration: second highest dose. And so on.

8) Prepare your toxic solution. Is nothing is known about the tree toxicity of that compound, use a logarithmic scale – e.g., 0, 0.1, 1 and 10 mg/L dosis.

9) Fill in toxic solutions. **Use 5 (or 6) trees for controls and 4 (or 5) trees for each dose.** The tree with the highest transpiration (No 1) is a control. No 2 gets the highest, dose, etc. The last tree (with lowest transpiration) is again a control. By this method, you guarantee an equal distribution of stronger and weaker trees. Measure the weight again and place the trees **exactly** back in place!
10) Two days later, measure the weight (you may also wish to measure the first day after dosage – gives a better graph).

11) Three days later, measure the weight again. If there is a clear toxic effect, the test can be finished. Otherwise, place the trees exactly back in place!

12) Measure the weight again after 3 days and after 4 days (second week). It usually makes no sense to continue the test for longer, because the standard deviation of the result increases, and the risk of diseases increases. If the test has to be prolonged, take care to refill the proper solution.

13) Measure the weight of the whole apparatus again before you stop. Then take the trees out of the flasks (take care! you used toxic solution!), remove the cork, use a tissue paper to dry the roots and the lower stem, and measure the weight of each tree (without flask).

14) The test is finished. Trees and remaining solution might be analyzed for chemicals to see loss and uptake.

15) How to calculate the result

It is recommended to use the “Normalized Relative Transpiration NRT”. To consider the fact that healthy trees grow quickly and thereby increase transpiration, the normalized relative transpiration (NRT) is calculated. The NRT is the change of transpiration of exposed trees divided by the change of control trees:

\[
NRT(C,t) \, (\%) = \frac{1}{n} \cdot \frac{\sum_{i=1}^{n} T_i(C,t)/T_i(C,0)}{\sum_{j=1}^{m} T_j(0,t)/T_j(0,0)} \times 100
\]

, where \( C \) is the concentration (mg/L), \( t \) is the time period (h, from 0 to 24 h, from 24 h to 48 h, from 48 h to 72 h and so on), \( T \) is the absolute transpiration (g/h), \( i \) is replicate 1, 2,…, \( n \) and \( j \) is control 1, 2,…, \( m \) (Trapp et al. 2000).

The NRT of the controls is always 100%. Values of the treated trees below 100% are caused by an inhibition effect of the treatment. As second toxicity parameter, the growth of the trees is determined from the weight difference of the tree before and after the test.
Example calculation for the NRT

Table: Measured absolute transpiration (g/h), mean.

<table>
<thead>
<tr>
<th>Dose</th>
<th>t = 0 h</th>
<th>t = 24 h</th>
<th>t = 48 h</th>
<th>t = 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 0</td>
<td>0.92</td>
<td>0.99</td>
<td>1.07</td>
<td>1.08</td>
</tr>
<tr>
<td>C = 1.0</td>
<td>1.07</td>
<td>1.08</td>
<td>1.19</td>
<td>1.28</td>
</tr>
<tr>
<td>C = 10</td>
<td>1.12</td>
<td>0.74</td>
<td>0.38</td>
<td>0.33</td>
</tr>
<tr>
<td>C = 100</td>
<td>1.00</td>
<td>0.44</td>
<td>0.25</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table: Normalized relative transpiration NRT (%).

<table>
<thead>
<tr>
<th>Dose</th>
<th>t = 0 h</th>
<th>t = 24 h</th>
<th>t = 48 h</th>
<th>t = 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>C = 1.0</td>
<td>100.0</td>
<td>93.8</td>
<td>96.4</td>
<td>102.1</td>
</tr>
<tr>
<td>C = 10</td>
<td>100.0</td>
<td>61.3</td>
<td>29.2</td>
<td>24.9</td>
</tr>
<tr>
<td>C = 100</td>
<td>100.0</td>
<td>41.3</td>
<td>21.8</td>
<td>13.8</td>
</tr>
</tbody>
</table>

The effect is better seen and quantified by the NRT.
The time scale again

Spring: Order trees and store them cold, humid and dark.

minus 3 weeks: Wake the trees up. Place the cuttings into a bucket with water, give them light.

minus 2 to 4 days: Adapt the trees in the nutrient chamber (friday is a good day)

minus 1 day: Measure initial transpiration in nutrient solution (monday)

Start (day 0): Fill in toxic solutions (tuesday)

Day 2: Measure the weights. Place the trees exactly back (thursday)

Day 3: Measure the weight again (friday morning)

It is recommended to put the trees back in place and let them there over weekend. Eventually (if the trees are big and transpire much) refill solution before you leave into weekend (friday lunch time).

Optional to continue the next week (12)

End day (day 3 or later) (13): Measure the weight of the whole flasks again, then of the tree alone (monday or later)

Total time scale: 1 – 2 weeks; Day 0 is a full working day. The other days, 1 hour is sufficient. The last day is a ½ working day.

Modification when working with soil or sand

Hydroponic solution is somewhat artificial for trees, and some effects (e.g., root damage) may be more severe in soil or sand. Roots do not develop “root hairs” in hydroponic solution, but in sand and soil. Therefore, the trees need shelter against drying out when transferred into solid (put a transparent plastic bag over).

The sand or soil should be transferred to the Erlenmeyer flasks at the same time with the cutting. At this moment, weigh the sand contained in the flask in order to determine the weight without nutrient solution. An optimum volume (about 150 mL) of nutrient solution is added to the medium and then, the Erlenmeyer flask is sealed with a cork stopper. In addition, place a transparent plastic bag over each willow cuttings for the first three days in order to decrease transpiration and let the roots develop root hairs as well as to avoid the drying of leaves. After 3 days, do not forget to remove the plastic bag and continue as above.

For cuttings in the sand, the test should be finished when less than 30 to 50 mL of water is remaining in the flask (you find this out by weighing the loss).
TROUBLESHOOTING

The cuttings make no leaves and roots
Several reasons: the cuttings were to old; get new ones. Or the cuttings were cut in autumn (before winter). It is difficult to convince the cuttings that the winter is over when they think it was not yet there (do trees have an inner clock?). Solutions: wait longer; eventually store cuttings from autumn cold; try to use rooting hormones; best: cuttings in spring.

I forgot to measure a day // I could only come in the afternoon // it is weekend
Don’t panic. That’s why we use continuous light and calculate the result as transpiration per hour. It is almost irrelevant then whether you measure after 22 or 26 hours, or a day later. Also, the end does not need to be after 72 hours (although then you have a longer weekend).

Controls decay, while dosed samples grow fine
This is a phenomena we observed repeatedly. Probably, the toxic chemical is more toxic to pests than to trees.

All trees decay
Maybe sick, or something else. Stop the test.

Brown color appears on stems and leaves. Transpiration goes down.
This might be a fungal attack, e.g., the Virosa disease. More frequent with poplars than with willows. Fungicides usually don’t help. Remove infected trees quickly (in particular when pre-growing trees together in a bucket). If too many trees are infected, carefully clean your equipment and restart.

Insects appear
Aphids, the white fly, caterpillar larvae – the list of animals that wish to eat your trees is long, and they find their way. Sucking insects can be removed successfully with a soap solution. Rinse the trees with fresh water, they survive. We also use anti-insect strips, eventually insecticides.

The water is out. All trees dried out.
This is the end of your trees and your test. Remember: trees need light and water. If they have no light, they do not need much water. If you are away for a longer period (holidays or so) it is better to close the light, or bring the trees into shadow. Best is of course, if someone regularly refills water.

Other trouble?
Contact to the author via stt@er.dtu.dk
Example applications of the tree toxicity test


Modified ISO 8692 nutrient solution

<table>
<thead>
<tr>
<th>Modified</th>
<th>ISO 8692</th>
<th>Final conc.</th>
<th>Mol.</th>
<th>g/l</th>
<th>µMol/L</th>
<th>ml/l</th>
<th>Stock conc (Mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAM A</td>
<td>NaNO3</td>
<td>240,00</td>
<td>84,99</td>
<td>2823,71</td>
<td>48,00</td>
<td>5 ml in 1 L</td>
<td>0,56</td>
</tr>
<tr>
<td>A</td>
<td>MgCl2.6H2O</td>
<td>12,00</td>
<td>203,30</td>
<td>59,03</td>
<td>12,00</td>
<td>1 ml in 1 L</td>
<td>0,06</td>
</tr>
<tr>
<td>B</td>
<td>CaCl2.2H2O</td>
<td>18,00</td>
<td>147,02</td>
<td>122,44</td>
<td>18,00</td>
<td>1 ml in 1 L</td>
<td>0,12</td>
</tr>
<tr>
<td>C</td>
<td>MgSO4.7H2O</td>
<td>15,00</td>
<td>246,47</td>
<td>60,86</td>
<td>15,00</td>
<td>1 ml in 1 L</td>
<td>0,06</td>
</tr>
<tr>
<td>D</td>
<td>KH2PO4</td>
<td>32,00</td>
<td>136,09</td>
<td>235,15</td>
<td>6,40</td>
<td>5 ml in 1 L</td>
<td>0,05</td>
</tr>
<tr>
<td>E</td>
<td>FeCl3.6H2O</td>
<td>100,00</td>
<td>270,30</td>
<td>369,96</td>
<td>50,00</td>
<td></td>
<td>0,18</td>
</tr>
<tr>
<td>mod. F</td>
<td>Na2EDTA.2H2O</td>
<td>200,00</td>
<td>372,24</td>
<td>537,29</td>
<td>100,00</td>
<td>2 ml in 1 L</td>
<td>0,27</td>
</tr>
<tr>
<td>mod. G</td>
<td>H3BO3</td>
<td>185,00</td>
<td>61,83</td>
<td>2991,98</td>
<td>185,00</td>
<td></td>
<td>2,99</td>
</tr>
<tr>
<td></td>
<td>MnCl2.4H2O</td>
<td>415,00</td>
<td>197,90</td>
<td>2096,97</td>
<td>415,00</td>
<td></td>
<td>2,10</td>
</tr>
<tr>
<td>G</td>
<td>ZnCl2</td>
<td>3,00</td>
<td>136,29</td>
<td>22,01</td>
<td>3,00</td>
<td></td>
<td>0,02</td>
</tr>
<tr>
<td></td>
<td>CoCl2.6H2O</td>
<td>1,50</td>
<td>237,93</td>
<td>6,30</td>
<td>1,50</td>
<td>1 ml in 1 L</td>
<td>0,01</td>
</tr>
<tr>
<td></td>
<td>CuCl2.2H2O</td>
<td>0,01</td>
<td>170,48</td>
<td>0,06</td>
<td>0,01</td>
<td></td>
<td>0,00</td>
</tr>
<tr>
<td></td>
<td>Na2MoO4.2H2O</td>
<td>7,00</td>
<td>241,95</td>
<td>28,93</td>
<td>7,00</td>
<td></td>
<td>0,03</td>
</tr>
<tr>
<td>H</td>
<td>NaHCO3</td>
<td>150,00</td>
<td>84,01</td>
<td>1,79</td>
<td>15,00</td>
<td>5 ml in 1 L</td>
<td>0,18</td>
</tr>
</tbody>
</table>

A, B, C, D and G are added to medium before sterilisation. E, F and H are added after sterilisation. H is sterilised in a closed bottle to prevent escape of CO2.