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Acute Toxicity of the Dissociating Veterinary Antibiotics Trimethoprim to Willow Trees at Varying pH

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Abstract The willow tree acute toxicity test was applied to determine the acute toxicity of the neutral and the ionic form of the antibiotic trimethoprim (TMP) to trees. The pKa of the weak base TMP is at 7.2, and the toxicity testing was done at low (pH 4.3), medium (pH 6.4) and high pH (pH 8.15). The test gave toxic effects, i.e. reduced transpiration at an external concentration of 100 mg/L TMP at medium and high pH, where the neutral form of TMP dominates. At an external concentration of 1,000 mg/L, also at low pH an effect occurred, but it was less pronounced than at medium pH.

Keywords Base · Ion · Pharmaceuticals · Plants

Most pharmaceuticals (77%) are ionisable compounds, the majority weak bases (Manallack 2007). Trimethoprim (TMP) is also a weak base with pKa at 7.2 (ACD 2008). The question arises how dissociation influences the toxicity. Most standard toxicity tests require a pH in the neutral range (near 7) and do not make an attempt to discriminate between the toxicity of the various species at a wider pH range. The willow tree test was used earlier to measure the toxicity of a weak base (tributyltin) at low and high pH (Trapp et al. 2004). Therefore, we decided to apply the willow tree toxicity test (Trapp et al. 2000) to TMP. Due to the ionizing nature of TMP, we performed the test at different pH levels, so that TMP was present either as ionic or as neutral molecule.

TMP is one of the most widely applied veterinary antibiotics in Denmark and other parts of the world (EMEA 2007; Emborg and Hammerum 2007. Trimethoprim is mainly applied in the treatment of livestock, such as pigs, cattle, poultry and aquaculture, but also for human use (Emborg and Hammerum 2007). A relevant fraction of the chemical is excreted unchanged [18% (Nielsen 1975) to 22% (EMEA 2007) by pigs, 80% by humans (EMEA 2007)], and may thus reach farmland and arable land either by direct excretion via urine and faeces, or by application of manure, sludge and slurry from husbandry as fertilizer to fields, but also via irrigation with river water and wastewater. TMP was also found relatively persistent in the activated sludge reactor, with half-lifes between 22 and 41 days (Halling-Sørensen et al. 2000). Measured concentrations of TMP in soils were between 0.072 and 8.07 mg/kg; in groundwater, concentrations between 4×10^{-5} and 0.044 mg/L were found (Boxall et al. 2005). Reported environmental concentrations are 0.5 μg/kg for soil and 0.02 μg/L for water (Boxall et al. 2005).

TMP, as antibiotic compound, is targeting bacteria, but it reaches also non-target organisms, such as crops. In humans and animals, TMP can cause effects like changes in the bone marrow and significant effects on some organ weights (EMEA 2007). TMP has been prioritised among other veterinary medicines for detailed risk assessment (Capleton et al. 2006; Holmes et al. 2007). Some toxicity data for the compound are available. An $EC_{50} > 100$ mg/L was found for the aquatic animal species *Hydra attenuate*. A microbial ADI (acceptable daily intake) of 4.2 μg/kg bw

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per day was found (EMEA 2007). The European Union has arbitrarily proposed that concentrations of pharmaceutical compounds shall not exceed 10 µg/kg in the soil before further evaluation of a specific compound is considered. This proposed limit was set based on known concentrations in the environment and thus lacks the consideration of the potential impact on crops or other organisms (Spaepen et al. 1997; Kühne et al. 2000; Jjemba 2002).

The purpose of this study was to discriminate between the toxicity of the various species of TMP at a wider pH range.

Materials and Methods

Crystalline Trimethoprim (5-(3,4,5-trimethoxybenzyl) pyrimidine-2,4-diamine) was obtained from Sigma–Aldrich in ≥98% purity. Trimethoprim (TMP) is a polar, non-volatile weak base with pK_a at 7.2 (Table 1; Fig. 1). This means TMP is mostly neutral under basic conditions, but ionized (protonised) at acidic pH. The neutral and the ionic form of the compound differ in polarity (i.e. log K_{OW}) and vapor pressure (which is essentially zero for the ionic form) (ACD, 2008). Thus, it is likely that differences in uptake and therefore also toxicity exist between neutral and ionic compound (Trapp et al. 2000; Trapp et al. 2004). The apparent log K_{OW}, also known as log D, is 0.4 at neutral pH (pH 7), but ranges from −1.7 at low pH (pH ≤ 3, cation) to 0.79 at high pH (pH ≥ 10, neutral molecule). The water solubility of TMP also varies strongly with pH.

For the toxicity test, basket willow *Salix viminalis* was used. Willow cuttings of 40 cm length and approximately 1 cm diameter were placed in a basket with tap water and exposed to natural sunlight until about ten leaves and roots were sprouted. Well-developed healthy trees of similar size were selected for the testing under artificial conditions. Trees were placed into a growth chamber with constant temperature (about 26°C) and humidity (50% to 65% relative humidity). Fluorescent tubes of 36 W with 20 cm distance between each at a height of 65 cm with 24 h light per day were used as constant source of artificial light. The willow tree acute toxicity test was applied to determine the acute toxicity of TMP to trees (Trapp et al. 2000). The basic principle of the test is that healthy trees transpire more water. The toxic end-point is the loss of water from the nutrient solution. This loss is determined by measuring the weight of the glass bottle with the nutrient solution and the tree in it. The weight loss is compared to that of controls. The result is expressed as "Normalized Relative Transpiration" NRT, defined as:

$$NRT(C,t) = 100x\frac{\frac{1}{n}\sum_{i-1}^{n} T_i(C,t)/T_i(C,0)}{\frac{1}{m}\sum_{j-1}^{m} Tj(0,t)/T_j(0,0)} \quad [\%] \qquad \text{(Eq.1)}$$

Table 1 Physico-chemical properties of trimethoprim

Species	z	pK_a	log K_{OW} (L/L)	S (mol/m^3)	S (g/L)	p_S (Pa)	K_{AW} (L/L)
Neutral	0	7.2	0.79	1.31	0.38 (pH 9)	2.35×10^{-10}	7.34×10^{-14}
Cation	+1	7.2	−1.7	410	100 (pH 4)	0	0

z valency; *log* K_{OW} partition coefficient octanol to water; S solubility in water; p_S saturation vapour pressure at 12°C; K_{AW} partition coefficient between air and water, calculated from S and p_S. All properties were estimated using ACD (2008)

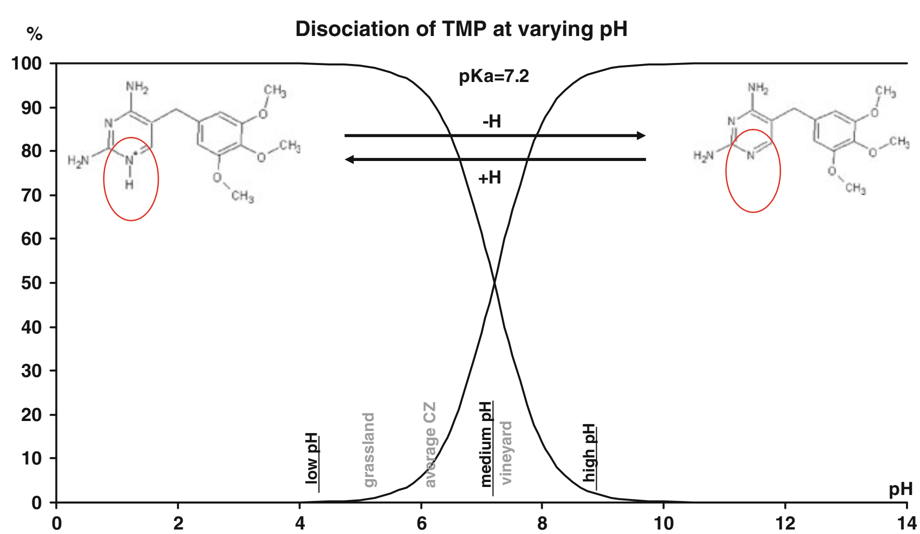

Fig. 1 Dissociation of trimethoprim at varying pH (pKa ACD, 2008). Median pH of different treatments are highlighted by underlining. Grey text is visualization of the typical pH of various Czech soils (CISTA 2009)

where C is concentration (mg/L), t is time period (h), T is absolute transpiration (g/h), i is replicate 1, 2, …, n and j is control 1, 2, …, m.

Controls always have an NRT of 100%. Inhibition of transpiration leads to NRT < 100%. Dead trees have NRT values at about 10%. This acute tree toxicity test has been used in several studies before (Trapp et al. 2000, 2004; Larsen et al. 2005; Seeger et al. 2009). The first set of experiment (dose 1–100 mg/L) was done with four replicates and five controls for each concentration and pH. The experiments at the highest dose (1 g/L TMP) were done with three replicates and four control trees per test at low and medium pH. The experiment with 1 g/L dose could not be preformed at the highest pH, because the solubility of TMP at this pH is below that concentration (Table 1).

The toxicity testing was at low, medium and high pH levels, to determine the toxicity for both neutral and cationic TMP three modified ISO 8692 nutrient solutions (ISO 1997) (Table 2). To obtain a low pH, NH_4Cl was used instead of $NaNO_3$. When plants take up the charged NH_4^+, they have to excrete a H_3O^+-ion, in order to keep electroneutrality, hereby lowering the pH of the solution. To obtain the high pH, 0.01 M KOH (approx. 290 ml) was added to the solution. The resulting pH levels (see Fig. 2) were about 4.30 (low pH), 6.40 (medium pH) and 8.15 (high pH).

Table 2 Composition of the modified ISO 8692 nutrient solutions

Chemical	Final concentration (mg/L)
$NaNO_3$	240[a,c]
NH_4Cl	53[b]
KOH	Variable[c]
$MgCl_2.6H_2O$	12
$CaCl_2.2H_2O$	18
$MgSO_4.7H_2O$	15
KH_2PO_4	32
	(μg/L)
$FeCl_3.6H_2O$	100
$Na_2EDTA.2H_2O$	200
H_3BO_3	185
$MnCl_2.4H_2O$	415
$ZnCl_2$	3
$CoCl_2.6H_2O$	1.5
$CuCl_2.2H_2O$	0.01
$Na_2MoO_4.2H_2O$	7

[a] Medium pH

[b] Low pH

[c] High pH

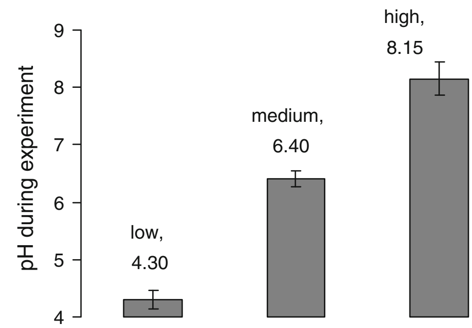

Fig. 2 pH measured in the nutrient solution (median and 99% CI) during the experiment. Each median is based on 50 (*low* and *medium pH*) or 100 measurements (*high pH*)

The results were statistically tested with the Dean-Dixon-outlier test. The two-sided Student *t* test was done to compare different sets of samples (i.e. pH, concentration, control). The significance level was chosen to 5%.

Results

The normalized transpiration of willows exposed to 1 mg/L, 10 mg/L and 100 mg/L TMP at low, medium and high pH over a period of 281 h is shown in Fig. 3. Detailed results with standard deviations for those treatment are shown in Table 3. There is no statistical difference between controls and mean transpiration of exposed trees at any pH level, neither at 1 mg/L nor at 10 mg/L exposure ($p < 0.01$). At 100 mg/L TMP exposure and low pH, the effect on trees is not statistically different to controls. At high pH, the toxic effect on trees is significant ($p < 0.05$), and trees at medium pH showed even higher statistical difference ($p < 0.01$).

Figure 4 shows the normalized transpiration of willows exposed to 1,000 mg/L TMP. The mean NRT at low pH is 42% at the end of the test, while at medium pH, the mean NRT is only 18%, and trees had decayed in a visible manner. This confirms the results obtained at lower concentration, namely that TMP at medium pH is more toxic than at low pH.

Discussion

The phytotoxicity of antibiotics to vegetation was reviewed by Jjemba (2002). The toxic concentrations of various classes of therapeutic compounds ranged from 10 μg/L for oxytetracycline to bean plants, *Phaseolus vulgaris* L., to 300 mg/L for other compounds and plants. The phytotoxicity of trimethoprim was measured in the seed germination test for sweet oat, rice and cucumber, and in the elongation test for rice and cucumber (Liu et al. 2009). The EC_{50} of

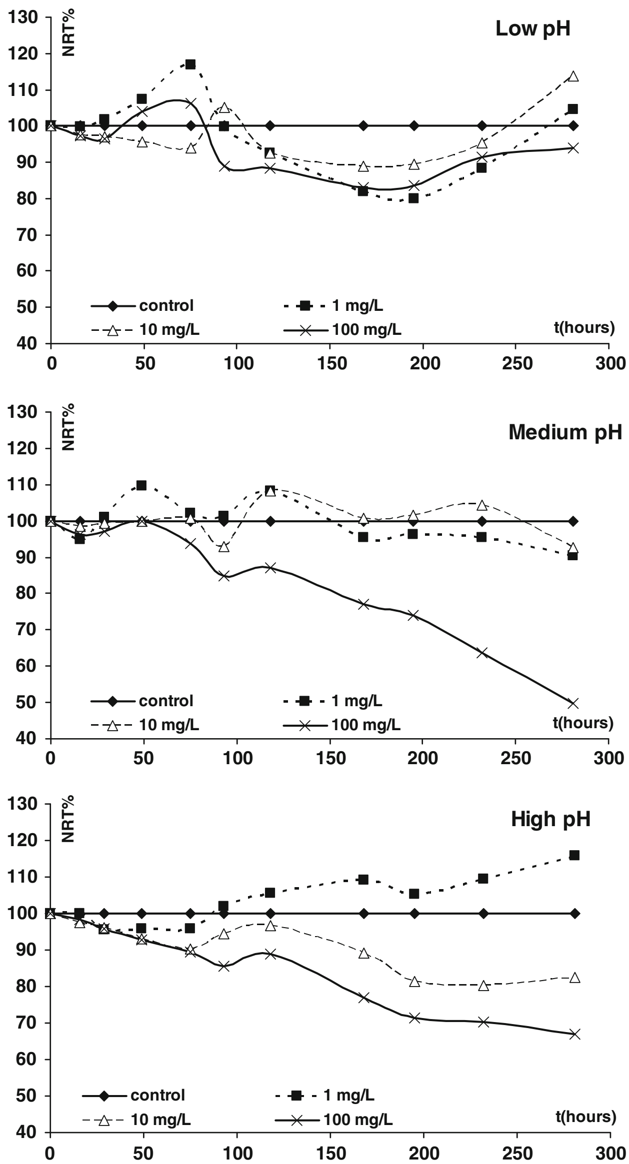

Fig. 3 Normalized relative transpiration (NRT) versus time (hours) of willow trees exposed at *low pH* to 1,10 and 100 mg/L TMP, *medium pH* and *high pH*

TMP in the seed germination test was 86 mg/L, 118 mg/L and >300 mg/L. In the (shoot and root) elongation test, the EC_{50} of TMP was above 300 mg/L for both rice and cucumber. The pH of the tests was not given, but was probably near neutral. These results are similar to those obtained in our study (toxic effect between 100 mg/L and 1,000 mg/L).

Compared to other compounds (Larsen et al. 2005; Ucisik and Trapp 2006, 2008, 2007) the toxicity of TMP to willow trees ranges in the lower range.

Measured concentrations of TMP in soils were between 0.072 and 8.07 mg/kg; in groundwater, concentrations between 4×10^{-5} and 0.044 mg/L were found (Boxall et al. 2005). Reported environmental concentrations are 0.5 µg/kg for soil and 0.02 µg/L for water (Boxall et al. 2005). Acute toxicity of this antibiotic for higher plants seems therefore very unlikely with the given environmental concentrations. Boxall et al. (2005) proposed not to treat TMP as a high priority compound in the future. However, in the study by Holmes et al. (2007) TMP was prioritised as a potentially highly recalcitrant antibiotic. Average concentrations found in the environment may be much smaller than those immediately after the application of contaminated manure or water, because dissipation half-lives of TMP range between 2 and 5 days (Liu et al. 2009) and up to 110 days (Boxall et al. 2005) for soils.

The manipulation of pH by the change of the nitrogen source in the nutrient solution was successful. Figure 1 shows the measured pH during the experiment. The pH of the “low” pH test series dropped within a few hours to 4.3, with small variation. The pH in the “medium” pH experiments was at 6.40 and the pH of the “high” pH series at 8.15 in average during the experiments. 0.01 M KOH was used to achieve a high pH in the test. Continuous measurement of the pH showed that the pH constantly decreased in the “high” pH solution (from about nine after refilling KOH to values near 7.5 within a few hours). These solutions were titrated adding a few drops of KOH almost every day to rise the pH back to nine. The volume added by titration was considered in the calculations of transpiration. Potassium is a macro-nutrient. It did not seem to harm the trees, as the transpiration of the trees without added KOH (medium pH) was very similar to that of trees with KOH (high pH). Even though it was troublesome to refill so often KOH solution to the “high pH” test solutions, we preferred the physiologically achieved pH over buffer solutions. Buffers could be taken up by the trees (which transpire a large fraction of the nutrient solution during the experiment), and this could lead to unstable pH and, worse, toxic effects. Since trees consume more solution at lower dose, this could mask the toxicity of the compound.

The highest concentration used for the tests was 1 g/L. At pH 7, the solubility of TMP is only 0.98 g/L (ACD 2008). This was the reason why no toxicity test at high pH was done for 1 g/L. But also at medium pH, this concentration is very close to solubility. TMP might have precipitated or formed micro-crystals, thus reducing the actual concentration in the test solution.

The toxicity of TMP was clearly different at low and mean (or high) pH. Results of the “low” pH treatments at 1, 10 and 100 mg/L were not statistically different from controls, but results from medium and high pH treatments showed statistically significant toxic effects, compared to controls, already for 100 mg/L from the 3rd day ($p < 0.05$). The toxic effect was observed for low pH at the

Table 3 Normalized relative transpiration (NRT) and standard deviation (SD) at different pHs

1st experiment	Hours	16	29	49	75	93	118	168	195	232	281
Low pH (4.3)											
1 mg/L	NRT	99.9	101.7	107.4	116.7	99.8	92.5	81.8	79.9	88.3	104.5
	SD	4.68	8.60	8.93	10.42	14.65	21.41	20.60	9.77	2.33	11.04
10 mg/L	NRT	97.5	97.1	95.7	93.9	105.1	92.4	89.0	89.5	95.4	113.7
	SD	6.21	7.80	12.47	25.25	34.63	33.95	33.39	33.39	27.56	20.59
100 mg/L	NRT	97.3	96.4	104.0	106.4	89.0	88.4	83.1	83.5	91.5	94.0
	SD	9.52	8.32	11.18	16.87	38.54	40.01	34.46	30.04	10.34	6.20
Medium pH (6.4)											
1 mg/L	NRT	94.9	100.9	109.7	102.0	101.4	108.3	95.3	96.3	95.4	90.4
	SD	4.64	7.24	16.16	9.89	16.84	16.56	17.69	20.73	23.57	25.75
10 mg/L	NRT	98.4	99.5	100.0	100.7	92.9	108.2	100.7	101.7	104.5	92.6
	SD	15.39	7.56	11.24	10.61	3.84	6.76	7.54	12.34	3.40	8.83
100 mg/L	NRT	96.4	97.1	99.8	93.7	84.8	87.1	77.1	74.1	63.7	49.6
	SD	3.87	6.80	5.96	7.18	3.67	7.94	9.02	12.66	9.55	7.40
High pH (8.15)											
1 mg/L	NRT	99.9	95.4	95.8	95.7	102.1	105.6	109.1	105.3	109.3	115.9
	SD	6.15	5.20	7.64	8.26	8.91	10.96	6.43	8.22	11.39	12.10
10 mg/L	NRT	97.4	96.1	93.1	90.2	94.6	96.8	89.1	81.3	80.2	82.5
	SD	5.23	4.51	6.49	10.20	5.64	6.17	7.99	6.71	11.57	14.90
100 mg/L	NRT	98.4	95.8	92.7	89.4	85.4	88.8	77.1	71.3	70.2	66.8
	SD	10.95	9.47	11.35	10.06	9.63	7.01	11.18	17.15	16.98	12.27

2nd experiment		18	39	65	89	111	139	158	182	203
Low pH (4.3)										
1 g/L	NRT	88.8	78.0	68.2	58.8	53.2	48.9	44.9	43.3	41.8
	SD	16.62	13.54	12.70	11.22	10.72	7.64	4.16	4.31	6.89
Medium pH (6.4)										
1 g/L	NRT	113.1	99.3	71.8	53.0	39.6	31.8	24.9	21.1	18.5
	SD	18.96	28.57	34.02	27.60	20.01	16.34	14.70	13.63	13.07

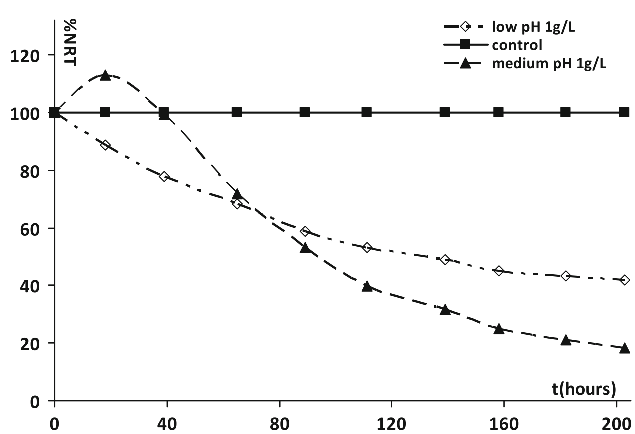

Fig. 4 Normalized relative transpiration (NRT) versus time (h) of willow trees exposed to 1,000 mg/L TMP at low pH and at medium pH

highest concentration treatment (1 g/L), but it was still much lower than for medium pH at 1 g/L ($p < 0.01$). Trimethoprim is a weak base, and very hydrophilic at low pH, where it is protonated and hence electrically charged (Table 1; Fig. 1). The toxicity was higher at high pH. This was to be expected, due to the so-called "ion trap effect" (De Duve et al. 1974; Trapp 2004; Trapp et al. 2008): if the outside pH is high, the compound enters the plant cells rapidly in neutral form. Inside, the pH is lower (pH 5.5 in vacuoles and pH 7 in cytosol), and the compound ionizes. Ions cross the lipophilic membrane barrier less rapidly (Trapp et al. 2008), and the ion gets "trapped". This process might well occur with the pK_a of TMP at 7.2.

The average pH of agricultural soil in CZ [provided by the Central Institute for Supervising and Testing in Agriculture in Czech Republic (CISTA 2009)] ranges from 5.6 (grasslands) to 7.3 (vineyards) with 6.1 as an overall average. The toxicity (and very likely also the uptake) of TMP to plants will be higher at the higher soil pH.

We speculate that this is a general trend, i.e. the toxicity (and the bioaccumulation) of most ionizable compounds is likely to be more pronounced when the compound is in neutral form. The common pH of surface waters is 6–9, but pH levels between 4 (acid lakes) and 10 (eutrophic lakes) may also occur. Ecotoxicity testing of ionizable compounds should be done at a pH where the compound can be expected to be neutral, as long as this pH is not outside the usual environmental pH.

The toxicity of the weak base trimethoprim to willow trees was tested with the willow tree transpiration test. The nutrient solutions were modified to achieve a low (pH 4), medium (pH 6–7) and a high pH (pH 8–9). The test gave toxic effects, i.e. reduced transpiration at an external concentration of 100 mg/L TMP at medium and high pH; at an external concentration of 1,000 mg/L, also at low pH an effect occurred, but it was less pronounced than at medium pH.

It can be concluded that

- the toxicity of the cationic species is lower than that of the neutral species, consequently
- the toxicity of the weak base TMP was higher at medium or high pH, which means that
- toxicity tests should be done at a pH where the organisms can survive and where the compound is as much neutral as possible.
- Concerning the test system,
- it is possible to achieve different pH levels in nutrient solutions for trees by modifying the nitrogen source
- a high pH can be established by titration of KOH into the nutrient solution, thus
- the willow tree toxicity test can be done in a range from pH 4 to 9, but with better replicability near neutral pH.

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