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Acute and Chronic Toxicity of Alcohol Ethoxylates to the Green Alga, *Desmodesmus (=Scenedesmus) subspicatus,* and the Subsequent Development of Structure Activity Relationships

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Alcohol Ethoxylates (AE) are nonionic surfactants produced by the reaction of fatty alcohol and ethylene oxide and are common components of detergent formulations with approximately 1.1 million metric tons produced worldwide in 2004 (Hauthal 2004). AEs used in consumer product and industrial applications are actually complex mixtures of various compositions. AE molecules (also called ethoxymers) have the general formula $CH_3(CH_2)_n(OCH_2\ CH_2)_yOH$, where n is generally 11-15 and 17and y is 0-18. Physico-chemical behavior is somewhat predictable as a function of the hydrophobic (alkyl) and hydrophilic (ethoxylate) moieties. Once AEs are discharged to the environment, these same properties affect sorption (Van Compernolle et al. 2005), biodegradation (Wind et al. 2005; Federle and Itrich 2005), and ecotoxicity (Boeije et al. 2005; Belanger et al. 2005).

Aquatic toxicity of AE mixtures has recently been explored by Boeije et al. (2005) who developed chronic mixture toxicity QSARs (quantitative structure activity relationship) for invertebrates (*Daphnia magna*) and fish (*Pimephales promelas*). The fundamental property linking AE homologue structure to toxicity is hydrophobicity (Roberts 1991). Because direct measurement of surfactant hydrophobicity by octanol/water partitioning is difficult due to their surface-active nature, log K_{ow} can be well estimated mathematically by knowing molecular fragments and branching positions (Leo and Hansch 1979; Roberts 1991; Roberts and Marshall 1995). Thus, mathematically determined log K_{ow} can be used as a hydrophobicity parameter when developing ecotoxicity QSARs for AE homologues (Boeije et al. 2005).

Almost all studies on AE toxicity have been performed on commercial mixtures. The presented research had the goal of determining the toxicity of pure AE homologues to algae that span a wide range of hydrophobicity in order to derive a QSAR for the log K_{ow}-toxicity relationship. A standardized and consistent testing approach was taken to develop structure-activity-relationships for several common endpoints. These analyses would then allow ecotoxicity of AEs to algae to be properly incorporated in full-scale environmental risk assessments of complex AE mixtures.

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MATERIALS AND METHODS

Axenic cultures of the green alga *Desmodesmus* (= *Scenedesmus*) *subspicatus* were used throughout all tests. The source of the initial culture was from the Department of Plant Physiology (University of Goettingen / Germany).

Desmodesmus was cultured in mineral salt media according to Directive 92/69/EWG, Annex C3 corresponding to OECD 201 (OECD 1984). The culture medium was also used in performing toxicity tests.

Pure grade homogeneous AEs were purchased from Nikko Chemicals LtD. (Japan). Some physico-chemical characteristics are given in Table 1. Purity was confirmed by GC analysis (www.Nikkol.co.jp). Log K_{ow} for the test materials ranged from 3.45-7.29 based on the calculation methods of Leo and Hansch (1979) and Roberts (1991).

Table 1. Alcohol ethoxylates used in algal toxicity tests.

AE	Trade name (Lot#)	Chemical name	Purity (%)	Log K_{ow}	Solubility (mM)
$C_{10}EO_8$	BD-8SY (Lot 6003)	octaethylenglycol – decylether	100	3.45	4.59×10^{-1}
$C_{12}EO_2$	BL-2SY (Lot 1005)	diethylenglycol – dodecylether	100	5.13	4.59×10^{-3}
$C_{12}EO_4$	BL-4SY (Lot 6003)	tetraethylenglycol-dodecylether	100	4.93	7.33×10^{-3}
$C_{12}EO_8$	BL-8SY (Lot 6015)	octaethylenglycol-dodecylether	100	4.53	2.24×10^{-2}
$C_{16}EO_2$	BC-2SY (Lot 6001)	diethylenglycol – hexadecylether	100	7.29	1.00×10^{-5}
$C_{16}EO_8$	BC-8SY (Lot 6001)	octaethylenglycol-hexadecylether	100	6.69	5.35×10^{-5}

Standardized ecotoxicity test conditions followed those described in OECD Method 201 (OECD 1984) or EC Directive 92/69/EWG and are summarized in Table 2. Tests were begun when cells in culture were in exponential growth phase with initial cell densities of 1×10^4 cells/mL. Five to seven test concentrations were employed in each test with controls, all in triplicate. Test flasks (500mL) were held on a Clim-o-Shake® (horizontal shaker) at 100 rpm under constant illumination at approximately 2000 Lux. Cell density was confirmed at every time point (24, 48 and 72 h) by Coulter Counter, which was calibrated by manual cell counting in a Thoma-chamber before each counting routine.

Exposure concentrations were generally chosen based on experience and range finding tests. Dilution series were constructed using factors between 2 to 3 depending on the substance and test.

AE exposure concentrations were confirmed using the BiAS method (German Industry Norm 1980) in 2-3 treatments of each test. This method is specific for the major non-ionic surfactants of the EO type (>4EO) by analyzing bismuth after complexing the surfactant with barium and bismuth (BiAS=Bismuth Active Substance). Non-ionic surfactants <4EO are not quantitatively analyzed and therefore need to be adjusted by internal standards (eg. Nonylphenol EO_{10}). The analytical limit of detection is around 5-10µg/L. Because analytical recovery of $C_{12}EO_4$ dropped below 80% during the test, the effects in this test were based on the arithmetic mean of the nominal concentrations (e.g. 65.5%). Due to solubility limits the $C_{16}EO_2$ AE was sonicated (ultrasound) in the test medium at nominal concentrations of 0.1-10 mg/L in order to enhance bioavailability. No additional carrier solvent was used. No analytical confirmation was possible for this test compound and effects were calculated on nominal concentrations.

Table 2. Algal ecotoxicity test conditions.

Parameter	**Value**
Duration	72 h
Light	2000 Lux, full spectrum
Initial inoculum	1 x 10^4 cells/mL
Counting method	Calibrated Coulter counter
Temperature	23 +/- 2° C
Shaker rotation	100 rpm
Test medium	Mineral Salt (EU 1992)
Frequency of counts	Every 24 h

Several endpoints were evaluated based on cell count data and included population growth rate (0 to 72 h) and yield (area under the growth curve, 0 to 72 h) as described in standard protocols (OECD 1984; EU 1992).

Endpoints were statistically evaluated by exposure-response modeling (point estimate by regression) and threshold estimation (analysis of variance) techniques. Exposure-response modeling employed the non-linear, iterative regression technique of Bruce and Versteeg (1992). This technique has the advantage of reliably estimating EC_x values where x denotes a specified effect level between 1 and 99 while including the common non-monotonic trends observed at low exposure concentrations in algal tests (such as the hormetic response). Both 72-h EC_{50} and EC_{20} levels were determined to estimate acute and chronic responses, respectively. Thresholds for the no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) were concluded based on one-way analysis of variance (ANOVA) followed by an *a posteriori* Dunnet's multiple range test. In all cases significance was inferred at $\alpha = 0.05$. All statistical analyses were conducted in SAS (1999).

RESULTS AND DISCUSSION

The six pure AE homologues varied in toxicity by approximately two orders of magnitude across the log K_{ow} range of 3.45-7.29 (Figure 1, Tables 3 and 4). $C_{10}EO_8$ was the least toxic and $C_{16}EO_8$ was the most toxic. In general, AE toxicity increases with increasing alkyl chain length and decreases with increasing ethoxylate chain length (Wong et al. 1997; Boeije et al. 2005). This is directly related to the influence of these factors on the hydrophobicity of the molecule.

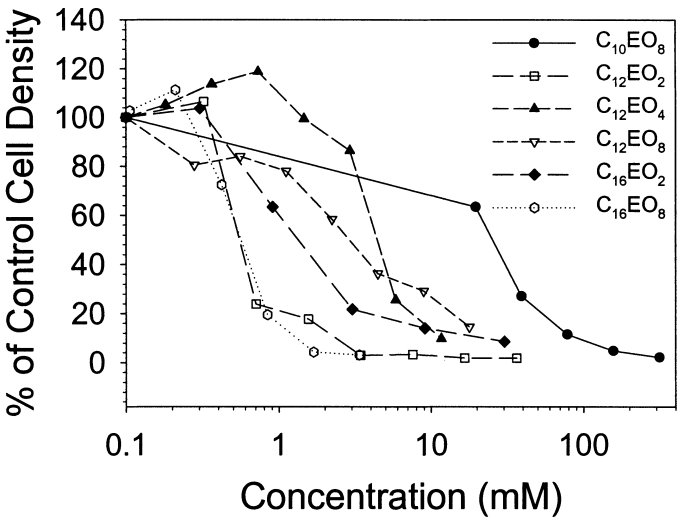

Figure 1. Response curves for all tests

Table 3. Acute 72-h EC_{50} (in mM) estimations for various pure AE homologues.

AE	72-h population growth rate		72-h yield or area under the curve	
	E_rC_{50}	95% Lower-Upper Confidence Limits	E_bC_{50}	95% Lower-Upper Confidence Limits
$C_{10}EO_8$	0.0859	0.0768-0.0958	0.0253	0.0186-0.0339
$C_{12}EO_2$	0.00103	0.00645-0.001653	0.000653	0.000369-0.001167
$C_{12}EO_4$	0.00704	0.00665-0.00751	0.00334	0.00296-0.00378
$C_{12}EO_8$	0.0287	0.0251-0.0331	0.00450	0.00368-0.00551
$C_{16}EO_2$	0.00900	0.00624-0.0130	0.00100	0.000545-0.00181
$C_{16}EO_8$	0.000976	0.00657-0.01447	0.000539	0.000387-0.000791

Table 4. Chronic 72-h EC_{20} endpoints (in mM) for various pure AE homologues.

AE	72-h population growth rate			72-h yield or area under the curve		
	E_rC_{20}	95% Conf Limits	NOEC	E_bC_{20}	95% Conf Limits	NOEC
$C_{10}EO_8$	0.0282	0.0233-0.0341	0.0196	0.00902	0.00569-0.01412	0.0196
$C_{12}EO_2$	0.00518	0.00255-0.01051	0.000321	0.000201	0.000088-0.000471	0.000279
$C_{12}EO_4$	0.00318	0.00282-0.00356	0.000718	0.00174	0.00141-0.00218	0.000718
$C_{12}EO_8$	0.00493	0.00405-0.00600	0.000279	0.00121	0.00084-0.00173	0.000321
$C_{16}EO_2$	0.00133	0.000667-0.00276	0.000303	0.000273	0.00091-0.00636	0.000421
$C_{16}EO_8$	0.000370	0.000185-0.000741	0.000421	0.000253	0.000135-0.000438	0.000303

From estimations based on Kow/toxicity correlations, $C_{16}EO_2$ should have been the most toxic AE. However, this AE is the most sorptive and least soluble homologue that was tested in this series. Analytical confirmation of exposure in the test also indicated $C_{16}EO_2$ was the most variable with declining test material concentrations as time progressed (Table 5). It is quite likely that the AE was sorbed to algal cells and this effect increased with time as population growth occurred. Still, the test was successful in that exposure-response was evident and both E_rC_x and E_bC_x endpoints were reliably quantified.

Table 5. Analytical confirmation of selected exposure concentrations in AE toxicity tests.

AE	Concentration (mM)	Percent of Nominal Mean ± SD (n = 4)
$C_{10}EO_8$	7.84×10^{-5}	99.4 ± 1.0
	3.14×10^{-5}	101.6 ± 1/6
$C_{12}EO_2$	7.55×10^{-7}	96.6 ± 39.4
	3.65×10^{-5}	105.0 ± 34.1
$C_{12}EO_4$	4.42×10^{-6}	65.5 ± 24.7
	8.84×10^{-6}	96.7 ± 14.1
$C_{12}EO_8$	8.92×10^{-6}	103.4 ± 2.6
	1.78×10^{-5}	102.4 ± 4.2
$C_{16}EO_2$	3.03×10^{-6}	65.0 ± 43.5
	9.09×10^{-6}	69.0 ± 44.9
$C_{16}EO_8$	3.37×10^{-6}	98.4 ± 4.6

Chronic toxicity was estimated as the EC_{20} and the mean ratio of the EC_{50}:EC_{20} was 3.7 and 2.8 for the E_rC_x and E_bC_x, respectively. This is in the range historically observed for acute EC_{50}/chronic NOEC values for algae (van de

Plassche et al. 1999; ECETOC 2003) and provides support for the EC_{20} as a reasonable replacement for the no-observed-effect-concentration.

AE structure activity relationships were evaluated with log K_{ow} as the predictor variable (Table 6, Figure 2). The acute E_bC_{50} and chronic E_bC_{20} possessed the highest r^2 values (approximately 0.7, $p < 0.001$). The slope of the QSARs were close to -0.4 which is very similar to those previously determined for other AE QSARs with daphnids (-0.5) and fish (-0.3) (Boeije et al. 2005). The first acute algae toxicity QSAR for pure AE-homologues was reported by Willing (2000). While the author related toxicity (expressed as the E_rC_{50}) to the carbon chain-length and EO-moieties of AEs, the aquatic toxicity of AEs has been hypothesized to be driven by a non-polar narcotic mode of action and thus being related to log K_{ow} (Roberts 1991; Roberts and Marshall 1995). The r^2 of the K_{ow}-based ErC_{50} QSAR in Table 6 is not as high as that of Willing (2000, $r^2 = 0.994$).

Table 6. Regression coefficients for acute and chronic QSARs. All regressions were of the form: Log (Effect Endpoint in mM) = $\beta_0 + \beta_1$ * (log K_{ow}).

QSAR	Endpoint	Slope	Intercept	r^2	p-value
Acute	E_rC_{50}	-0.340	-3.315	0.586	0.003
	E_bC_{50}	-0.375	-3.646	0.699	< 0.001
Chronic	E_rC_{20}	-0.383	-3.610	0.609	< 0.001
	E_bC_{20}	-0.378	-4.072	0.720	< 0.001

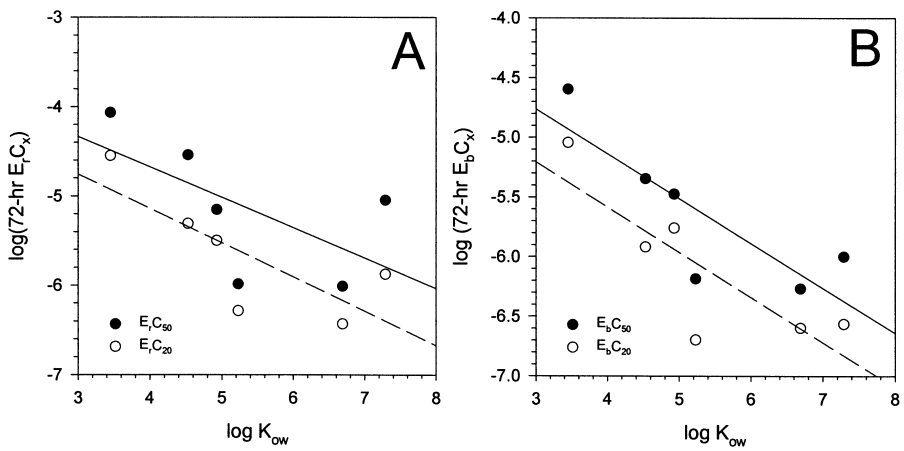

Figure 2. 72-h E_rC_x QSARs for growth rate (A) and E_bC_x for yield (area under the curve) (B).

However, the latter had the statistical advantage of a two-term model with only two remaining degrees of freedom which perhaps biased the r^2 value. Of the five studies, it is also relevant to note that the most hydrophobic AE that was sufficiently soluble to determine toxicity was $C_{16}EO_2$. The toxicity of this test

compound is much less than expected, perhaps indicating some solubility issues. If the data point is removed, the four QSARs above have r^2 values of 0.73-0.85. We have chosen not to cull values out of the data set as the tests were otherwise fairly well-behaved (such as good exposure-response profiles and low replicate variability). It is interesting to note that the slopes of all three taxonomic groups (fish, daphnia and algae) are similar and thus it can be hypothesized that the same mode of action applies. In addition, no one group is uniquely sensitive or insensitive as the intercepts also range between -0.3 to -0.4. Based on the data from this study, the E_bC_{20} QSAR is recommended for use in the evaluation of AE distributions in the aquatic environment and subsequent risk assessment (Belanger et al. 2005). This relationship is summarized as:

$\text{Log}(72\text{-h } E_bC_{20} \text{ in mM}) = -0.378 * \log K_{ow} - 4.072$

There is a lack of published data on toxicity of AEs to algae. Historically, the interpretation of toxicity of AEs to algae was hampered due to a lack of analytical confirmation of exposure (Yamane et al. 1984; Lewis and Hamm 1986). In fact, all published data on algal toxicity is based on nominal exposures of commercial mixtures. Previous historical summaries of algal sensitivity to AEs indicated that algae were perhaps an order of magnitude less sensitive than invertebrates and fish (Talmadge 1994; van de Plassche et al. 1999). Based on the new data this appears not to be the case.

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