Difficult Substances as Challenge for the Algal Growth Inhibition Test According to OECD Test Guideline 201

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Background

Photosynthetically active organisms such as green algae, blue green algae and Diatoms are not only part of the risk assessment (RA) for plant protection products, but also standard test organisms for the RA for pharmaceuticals and chemicals (REACH). Especially chemicals show a broad variety of characteristics from well water soluble, stable and non-toxic to hardly water soluble, unstable, volatile and toxic for water organisms. The group of chemicals with one or more of the latter characteristics is a challenge for the toxicity test with aquatic organisms. The OECD guidance document on aquatic toxicity testing of difficult substances and mixtures (23) provides some hints how the “standard testing” with such difficult test items should be conducted, but due to the countless combinations of characteristics of these difficult substances, some innovation is required to find the best test design for the individual chemicals. Herewith presented is an example for the toxicity testing of a difficult test item starting with the investigation of the characteristics of the test item in the respective test water, followed by the development of a specific test design to determine the toxicity, the testing itself and finally the choice of the most suitable evaluation method within the various possibilities of calculation and interpretation of the results. To make it even more complicated, there are different ways to interpret the analytical data and the most appropriate one based on the prior conducted research and evaluation is chosen to provide the required endpoints. The different possibilities are also introduced and discussed.

Material & Methods

The setup follows the methodology descripted in the OECD 201 (Test species: *P. subcapitata;* Medium: AAP (+Hepes, +$NaHCO_3$); 3 replicates/concentration + 6 replicates/control; Start: 5’000 cells/mL; Static design; Duration 72h; Test flasks shaked in a temperature (23°C) controlled incubator; Illumination by LEDs (continuously and uniformly); 5-6 concentrations; Cell density determined by cell counter (Casy TT), algal biomass by fluorescence measurement (SpectraMax).

Results

The test substance is a volatile single compound with a water solubility > 100 mg/L. Pretests showed a decrease of the test substance over the test period, but it was unclear whether the reduction was caused by hydrolysis, photolysis or adsorption to test organisms (recovery too low during pretests). Volatility as reason for losses could be excluded as a closed system was used for the pretests. For the main test we followed the setup shown in **Table 2**.

Treatment	Measurement	0 h	24h	48h	72h
Control	BM	x	x	x	x
	ANA (%)	<LOQ	<LOQ	(-)	< LOQ
C1	BM	x	x	x	x
	ANA (%)	109	65	(-)	<LOQ
C2	BM	x	x	x	x
	ANA (%)	103	79	(-)	<LOQ
C3	BM	x	x	x	x
	ANA (%)	91	92	(-)	<LOQ
C4	BM	x	x	x	x
	ANA (%)	87	85	(-)	<LOQ
C4 w/o algae, w/o light	BM	(-)	(-)	(-)	(-)
	ANA (%)	87	(-)	(-)	21
C4 w/o algae	BM	(-)	(-)	(-)	(-)
	ANA (%)	87	(-)	(-)	22
C4 w/o sampl. 24/48h	BM	(-)	(-)	(-)	(-)
	ANA (%)	87	(-)	(-)	<LOQ
C5	BM	x	x	x	x
	ANA	92	100	(-)	8
C6	BM	x	x	x	x
	ANA	98	87	(-)	62

Table 2: Design of the study with all measuring points to determine the biomass (BM, x) and concentrations of the test substance (ANA). The measured concentrations are given in % of nominal. (-) = no measurement.

Challenge	Method
Stability in Water and under Light	Stirring experiments with/without light
Possible Adsorption on Glass Vessels and Test Organisms	Stirring experiments/ pretest/additional test vessels during main test
Toxicity	Range Finding Test
Solubility in Test Water	Stirring experiments (with or without filtration, slow stirring or intense depending on test substance characteristics
Single Substance or Multi Component	Single substance with impurities: standard procedure Multi component and not water soluble at 100 mg/L: Water Accommodated Fraction (WAF)
Freezing Stability	Pretests to check whether the analytical samples can be stored until analysis
Volatility	Henry’s law constant and pretests with open/closed system

Table 1: General procedure: the listed characteristics of a test substance influence the test design of an (algae) study, but have to be considered for the design of an aquatic Ecotox study in general.

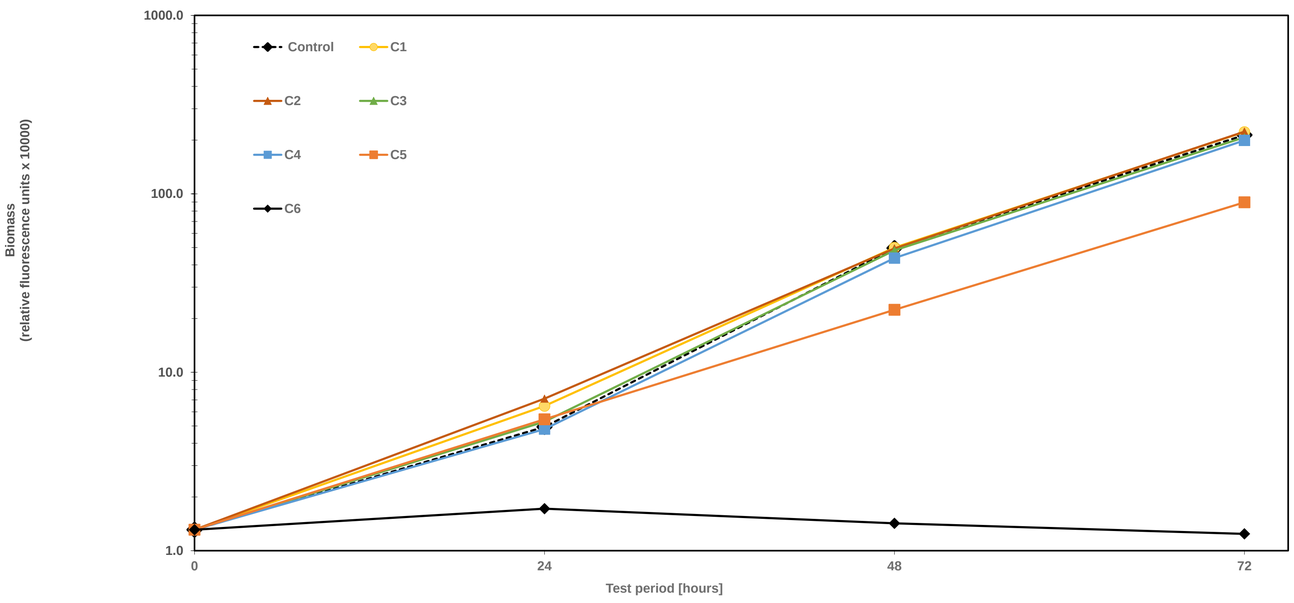

Figure 1: Concentration-response relationship over the test period of 72h. Clear effect of the two highest concentrations, minor effect of the third concentration (C4, blue) and no effect of the three lower concentrations.

Treatment	TWM (211)	TWM (23)	GM
C1	55%	49%	49%
C2	50%	37%	27%
C3	46%	26%	14%
C4	39%	17%	7%
C5	56%	42%	27%
C6	80%	79%	78%

Table 3: Comparison of three possible calculation models to calculate the average concentration: Time Weighted Mean (TWM) 211 is based on the proposal mentioned in the OECD 211 (Daphnia reproduction), TWM 23 on the OECD no. 23 (Difficult Substances) and Geometric Mean is the simple calculation of the geometric mean based on the measured concentration at the beginning and at the end of the exposure period (square root of conc1 * conc2).

EC_{50} (mg/L)	TWM (211)	TWM (23)	GM
Growth Rate	28 (25-33)	22 (19-27)	18 (17-19)
Yield	15 (14-16)	10 (9.2-11)	6.3 (5.8-6.9)

Table 4: Comparison of the EC_{50} values based on the calculated means of TWM 211,TWM 23 and GM.

Summary & Discussion

- The chosen test design resulted in valid data.
- The liquid and volatile substance could be dissolved in the test water within a few minutes (80-120% of nominal).
- The closed system guaranteed a minimum loss of test substance during the testing period.
- A clear concentration - response relationship could be shown (Figure 1).
- The decrease of the substance was not related to light => substance is not highly photosensitive (**Table 2**, C4 w/o algae/light). In addition, the closing and opening of the test vessels had no critical influence on the decrease (**Table 2**, C4 w/o sampling after 24 and 48h).
- The presence of algae influences the decrease of the substance. Either the substance adsorbs to the algae or the substance is taken up and maybe metabolized by the algae.
- There is a distinct difference in terms of the resulting endpoints depending on the number of samples analyzed (sampling interval) and the method of calculation **(Table 3, 4).** Especially for substances with detectable concentrations after 24h, but values <LOQ after 72h, the endpoints can differ significantly.

The presented working procedures demonstrate that every test item - independent from its characteristics - can be tested according to established OECD Test Guidelines, but in some cases extensive biological and chemical background and innovation is required to find the best test design.

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