

**Section A7.4.1.3 Growth inhibition test on algae****Annex Point IIA7.3 *Selenastrum capricornutum***Official  
use only

		<b>1 REFERENCE</b>
<b>1.1 Reference</b>		Hanstveit, A.O., Oldersma, H., 1992 Effect of L(+) lactic acid on the growth of the alga <i>Selenastrum capricornutum</i> (OECD 201). TNO, report nr. IMW-91-0076-05. GLP, Unpublished
<b>1.2 Data protection</b>		Yes
1.2.1 Data owner		Purac Biochem
1.2.2 Companies with letter of access		No
1.2.3 Criteria for data protection		Data submitted to the MS after 13 May 2000 on existing [a.s. / b.p.] for the purpose of its [entry into Annex I/IA / authorisation]
		<b>2 GUIDELINES AND QUALITY ASSURANCE</b>
<b>2.1 Guideline study</b>		Yes, OECD 201
<b>2.2 GLP</b>		Yes
<b>2.3 Deviations</b>		The test was ended after 70,5 h instead of 72 h. Furthermore, no EC50 based on biomass was calculated, which was not common at the time the study was performed.
		<b>3 MATERIALS AND METHODS</b>
<b>3.1 Test material</b>		As given in section 2
3.1.1 Lot/Batch number		Batch no.: ZO 3456
3.1.2 Specification		As given in section 2
3.1.3 Purity		About 80% L(+) lactic acid
3.1.4 Composition of Product		Not applicable
3.1.5 Further relevant properties		Not applicable
3.1.6 Method of analysis		Enzymic analysis with a Boehringer Mannheim test kit (cat. no. 1 112 821).
<b>3.2 Preparation of TS solution for poorly soluble or volatile test substances</b>		Not applicable
<b>3.3 Reference substance</b>		No
3.3.1 Method of analysis for reference substance		Not applicable
<b>3.4 Testing procedure</b>		

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3.4.1	Culture medium	Medium was prepared from concentrated stock solutions in Milli-Q filtered water and sterilized by micropore filtration. NaHCO <sub>3</sub> content is 150 mg/L, CaCl <sub>2</sub> ·2H <sub>2</sub> O content 18 mg/L, MgCl <sub>2</sub> ·6H <sub>2</sub> O content 12 mg/L and MgSO <sub>4</sub> ·7H <sub>2</sub> O content 15 mg/L.
3.4.2	Test organisms	For details see table A7_4_1_3-2.
3.4.3	Test system	For details see table A7_4_1_3-3
3.4.4	Test conditions	For details see table A7_4_1_3-4
3.4.5	Duration of the test	70.5 h
3.4.6	Test parameter	Growth inhibition
3.4.7	Sampling	One sample taken from each flask after 0, 23.5, 48 and 70.5 h.
3.4.8	Monitoring of TS concentration	Yes, at start and end of the test.
3.4.9	Statistics	EC values with respect to the area under the growth curve were calculated according to the method given in OECD 201. EC values with respect to the inoculum viability followed by exponential growth were calculated according to a parametric model developed by Kooijman et al., assuming an error proportional to the number of cells.

**4 RESULTS**

<b>4.1</b>	<b>Limit Test</b>	Not performed		
4.1.1	Concentration	Not applicable		
4.1.2	Number/ percentage of animals showing adverse effects	Not applicable		
<b>4.2</b>	<b>Results test substance</b>			
4.2.1	Initial concentrations of test substance	Nominal test substance	Nominal lactic acid	Measured lactic acid
		0	0	<5 mg/L
		100	80	65 mg/L
		1000	800	620 mg/L
		2800	2240	1595 mg/L
4.2.2	Actual concentrations of test substance	Nominal test substance	Nominal lactic acid	Measured lactic acid
		0	0	<5 mg/L
		100	80	12 mg/L
		1000	800	455 mg/L
		2800	2240	1645 mg/L

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4.2.3 Growth curves

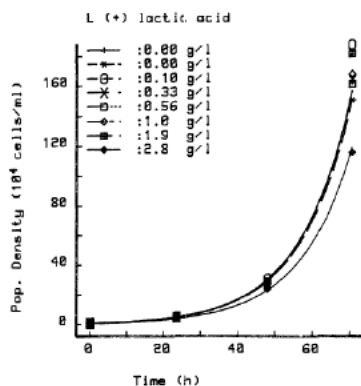


Figure 1 Growth curves for *Selenastrum capricornutum* exposed to a range of concentrations of an 80% aqueous solution of L(+)-lactic acid.

4.2.4 Concentration / response curve

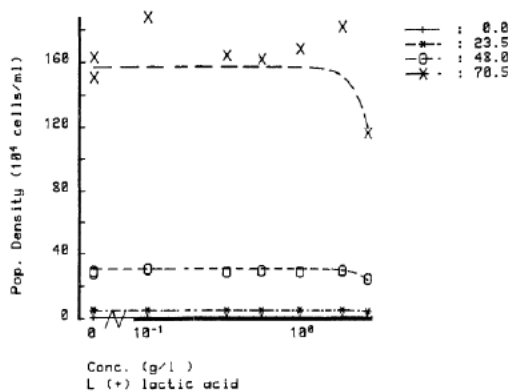


Figure 2 Concentration-effect curves for *Selenastrum capricornutum* exposed to a range of concentrations of an 80% aqueous solution of L(+)-lactic acid.

4.2.5 Cell concentration data See table A7\_4\_1\_3-5

4.2.6 Effect data (cell multiplication inhibition)

Effects on inoculum viability:

$E_cC_{50}$  >2.8 g/L (extrapolated  $E_cC_{50}$  3.5 g/L)

$E_cC_{10}$  2.3 g/L

$E_cC_{90}$  >2.8 g/L (extrapolated  $E_cC_{50}$  5.4 g/L)

Effects on area under the growth curve:

$E_bC_{50}$  >2.8 g/L

$E_bC_{10}$  2.4 g/L

$E_bC_{90}$  >2.8 g/L

Estimated NOEC: 1.9 g/L (by visual comparison of the measured and calculated growth curves of exposed and control algal suspensions)

4.2.7 Other observed effects Microscopic examinations of the cells revealed the presence of many bacteria in the cultures exposed to 1.0, 1.9, and 2.8 g/L

4.3 Results of controls See table A7\_4\_1\_3-5

4.4 Test with reference substance Not performed

4.4.1 Concentrations Not applicable

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4.4.2	Results	Not applicable	
<b>5 APPLICANT'S SUMMARY AND CONCLUSION</b>			
5.1	<b>Materials and methods</b>	Test performed according to OECD 201. A range-finding study was performed to determine the dose range in the study.	
5.2	<b>Results and discussion</b>	The range-finding test revealed that inhibiting effects could be expected at concentrations higher than 100 mg/L. Effects could be caused by the low pH level (pH 3.2 at 1008 mg/L). In the growth inhibition test, an effect on inoculum viability was observed. This effect may be expected when a test substance loses its toxicity during the test. The observed bacterial growth and the chemical analysis indicate degradation of the test substance during the test.	
5.2.1	NOE <sub>r</sub> C	1.9 g/L (estimated by visual comparison of the measured and calculated growth curves of exposed and control algal suspensions)	x
5.2.2	E <sub>e</sub> C <sub>50</sub>	>2.8 g/L (extrapolated E <sub>e</sub> C <sub>50</sub> 3.5 g/L)	x
5.2.3	E <sub>b</sub> C <sub>50</sub>	>2.8 g/L	
5.3	<b>Conclusion</b>	Validity criteria not fulfilled, due to biodegradation of the test substance, the concentration was <80% during the test.	x
5.3.1	Reliability	1	
5.3.2	Deficiencies	The test was ended after 70,5 h instead of 72 h. Furthermore, no EC <sub>50</sub> based on biomass was calculated, which was not common at the time the study was performed. Additionally, the concentrations tested were too low to derive a proper EC <sub>50</sub> , however, from the range-finding test it was concluded that the observed toxicity of higher doses is probably caused by the low pH level.	x

**Evaluation by Competent Authorities**

Use separate "evaluation boxes" to provide transparency as to the comments and views submitted

**EVALUATION BY RAPPORTEUR MEMBER STATE**

<b>Date</b>	2009/04/07
<b>Materials and Methods</b>	Applicant's version is acceptable

## Section A7.4.1.3

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<b>Results and discussion</b>	<p>Applicants version can be adopted with the following remarks:</p> <p>To 4.26, 5.2.1- 5.2.3:</p> <p>The estimated EC<sub>50</sub> value based on growth inhibition is higher than the highest test concentration. This value was obtained by extrapolation because no 50% inhibition was reached at the end of the test in the highest test concentrations. The re-calculation of EC<sub>50</sub> values and NOEC value by RMS results in effect values in the same order of magnitude:</p> <p><math>E_rC_{50} = 5.39 \text{ g a.s./L (nominal)}</math></p> <p><math>E_bC_{50} = 2.38 \text{ g a.s. /L (nominal)}</math></p> <p><math>NOE_rC = 1.52 \text{ g a.s. /L (nominal)}</math></p> <p>Effect values in the study are related to nominal concentrations although the measured values for three concentration levels show a decrease during the exposure period. Therefore a recalculation was conducted as follows: Because of the nominal <math>E_rC_{50}</math> (5.39 g a.s./L) is higher than the highest nominal test concentration (2.24 g a.s./L) for the calculation of the mean measured effect values the geometric mean from start to the end of the test from the highest test concentration of 2.24 g/L with 72.3 % will be used.</p> <p><math>E_rC_{50} (70,5 \text{ h}) = 3.90 \text{ g a.s./L}</math></p> <p><math>E_bC_{50} (70.5 \text{ h}) = 1.72 \text{ g a.s. /L NOE}_rC (70,5 \text{ h}) = 1.10 \text{ g a.s./L}</math></p> <p>5.3.: As possible biodegradation or transformation from Lactic acid to Lactate could cause the decrease in test substance concentration the effect values were estimated based on the actual measured concentration.</p> <p>5.3.2: In the test protocol it is stated that pH value was adjusted to pH 7.5-8 and that it remained constant during testing. In the presence of algae the pH value was found to increase just a little with algal cell density (pH 8.2-8.7).</p>
<b>Conclusion</b>	Applicant's version can be adopted
<b>Reliability</b>	2, see remarks
<b>Acceptability</b>	acceptable
<b>Remarks</b>	Deficiencies: Instead of 3 proposed replicates according to the current OECD guideline 201 only 2 replicates were tested.
<b>Date</b>	<b>COMMENTS FROM ...</b> <i>Give date of comments submitted</i>
<b>Materials and Methods</b>	<i>Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion. Discuss if deviating from view of rapporteur member state</i>
<b>Results and discussion</b>	<i>Discuss if deviating from view of rapporteur member state</i>
<b>Conclusion</b>	<i>Discuss if deviating from view of rapporteur member state</i>
<b>Reliability</b>	<i>Discuss if deviating from view of rapporteur member state</i>
<b>Acceptability</b>	<i>Discuss if deviating from view of rapporteur member state</i>
<b>Remarks</b>	

Table A7\_4\_1\_3-2: Test organisms

Criteria	Details
Species	<i>Selenastrum capricornutum</i>
Strain	ATCC 22662
Source	Culture was supplied by the "American Type Culture Collection", Rockville, Maryland, USA
Laboratory culture	Yes
Method of cultivation	Not reported
Pretreatment	According to OECD 201, a preculture of algae in the exponential growth phase was prepared in the medium used for the test.
Initial cell concentration	In the test flasks a mean inoculum cell density of $0.9 \times 10^4$ cells/mL was measured in the control cultures.

Table A7\_4\_1\_3-3: Test system

Criteria	Details
Volume of culture flasks	200 mL conical test flasks
Counting apparatus	Electronic particle counting with Coulter Counter model TAI
Light quality	Fluorescent lamps, light intensity within the standard range $120 \pm 20\% \mu\text{mol}\cdot\text{S}^{-1}\cdot\text{m}^{-2}$
Procedure for suspending algae	Shaking (100 rpm in a Gallenkamp orbital shaker)
Number of vessels/ concentration	Duplicate, four controls with algae only and a single background series containing test substance without algae
Test performed in closed vessels due to significant volatility of TS	No

Table A7\_4\_1\_3-4: Test conditions

Criteria	Details
Test temperature	$23 \pm 1^\circ\text{C}$
pH	Start of test: 7.5-8.0 End of test without algae: 8.0-8.1 End of test with algae: 8.2-8.7
Aeration of dilution water	No
Light intensity	$120 \pm 20\% \mu\text{mol}\cdot\text{S}^{-1}\cdot\text{m}^{-2}$
Photoperiod	Not reported

Table A7\_4\_1\_3-5: Cell concentration data

Test-Substance Concentration (nominal) [g/l]	Cell concentrations (mean values) [cells/ml]							
	measured				Percent of control			
	0 h	23.5 h	48 h	70.5 h	0 h	23.5 h	48 h	70.5 h
0	0.9	5.3	28.5	157.0	100	100	100	100
0.10	0.9	5.1	30.3	188.0	100	96	106	120
0.33	1.0	5.0	28.6	164.3	111	94	100	105
0.56	0.9	4.9	29.4	161.8	100	92	103	103
1.0	1.0	5.1	28.7	168.4	111	96	102	107
1.9	1.0	5.0	29.2	181.9	111	94	102	116
2.8	0.9	4.1	24.0	116.0	100	77	84	74
Temperature [°C]	23 ± 1°C							
pH	7.5-8.0	8.2-8.7						

### 3. Tables for Applicant's Summary and Conclusion

#### 3.1 Validity criteria for algal growth inhibition test according to OECD Guideline 201

	fulfilled	Not fulfilled
Cell concentration in control cultures increased at least by a factor of 16 within 3 days	x	
Concentration of test substance $\geq 80\%$ of initial concentration during test		x

Criteria for poorly soluble test substances	Not applicable	