

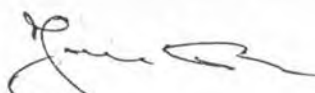
Test report
Stormøllen A/S
Algal growth inhibition test

February 2004

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Sample material

On a request by the client the laboratory bought 25 kg of "Stalosan F" at a local pet shop on 21 May 2003. Stalosan F is used as a stable bedding material.

The sample was stored dark at room temperature until test.

The test was carried out during the period 13 - 16 January 2004.

Purpose

The purpose of the test is to detect a possible effect of Stalosan F on the growth rate of the freshwater alga *Chlorella vulgaris*.

Test methods

Stalosan F: Physical/chemical properties

Description:	Powder, light brown
pH:	3.6 (according to MSDS)
Batch:	Not given
Expiry date:	Not given
Storage:	In original container in dimmed light at room temperature
Solubility:	Slightly soluble in water at 25°C
Stability of test concentrations:	The stability was not verified by chemical analysis

Test performance

Source and Culturing of *Chlorella vulgaris*

For the test was used *Chlorella vulgaris* from NIVA, Norway, (clone NIVA CHL 19). The basis culture was kept at the temperature $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and in a light/dark cycle of 16/8 hours and an intensity of light between $60\text{-}120 \mu\text{mol m}^{-2} \text{s}^{-1}$. The algae were kept in 250 ml Erlenmeyer flasks covered with rubber stoppers, and they were continuously aerated with humidified atmospheric air passing through a $0.45 \mu\text{m}$ membrane filter. The algae were grown in a fresh water medium based on a modification of the OECD Guideline.

Three days before the start of the test, the algae were transferred to a 250 ml Erlenmeyer flask with fresh medium giving a cell concentration of 10^4 cells/ml. Cells were counted in a haemocytometer. The preculture was incubated in a temperature controlled room on a orbital shaker with a continuous light intensity of $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ and a temperature of $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$, where it was shaken at 50-70 rpm.

Preparation of the test material

All test concentrations are prepared directly. The test products are added to the medium, stirred for 18 hours and pH adjusted to 7.9 ± 0.1 . After the adjustment the test solutions were filtrated through ignited GF/A filters. Using this preparation of the test product, all the test solutions were clear.

As dilution water was used an alga medium identical to the type used to maintain the culture of *Chlorella vulgaris*.

Test procedure

The study was carried out under static conditions with 6 controls and 5 concentrations of Stalosan F in triplicates. A blind concentration (without the algae) was prepared in duplicate for each concentration. Test conditions similar to those used for the preculture of *Chlorella vulgaris* were used. The temperature was monitored by a thermologger (Delphi). The test concentrations used were:

0 mg/l (control); 40 mg/l; 100 mg/l; 250 mg/l; 600 mg/l and 1,600 mg/l.

The initial cell concentration of *Chlorella vulgaris* was 10^3 cells/ml. The pH was measured at the start of the test and after 72 hours.

At the start of the test and after 24, 48 and 72 hours of exposure, the cell mass was determined by fluorescence analysis.

Calculations

The growth rate was calculated as follows:

$$\text{Growthrate} = \frac{\ln(\text{fluorescence}_{72h} - \text{fluorescence}_{\text{blind},72h}) - \ln(\text{fluorescence}_{0h} - \text{fluorescence}_{\text{blind},0h})}{72h}$$

The inhibition in percent of the control was calculated after 72 hours of exposure:

$$\% \text{ Inhibition} = \left(\frac{\text{growth rate}_{\text{control}} - \text{growth rate}_{\text{sample}}}{\text{growth rate}_{\text{control}}} \right) \times 100\%$$

Results

The fluorescence signals for the control group from which has been subtracted the blind values as a reflection of the exposure time are visualized in Figure 1. The test system should give the control group optimal conditions. Therefore, the control group must have exponential growth as visualized in Figure 1. The exponential growth was controlled statistically.

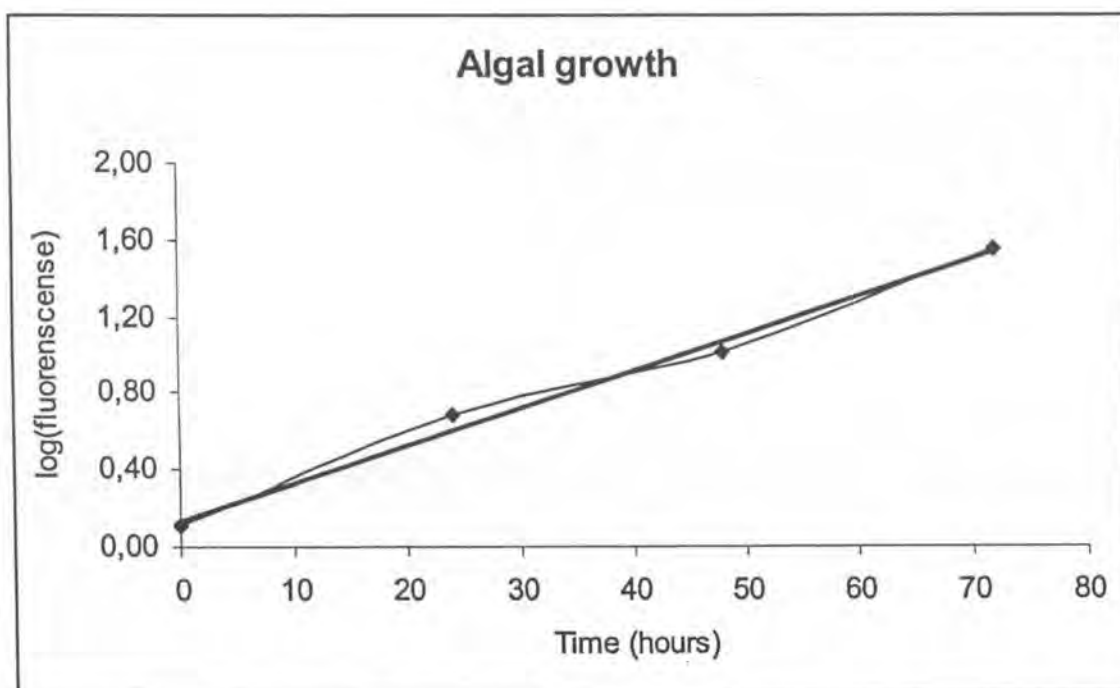


Figure 1. The growth of *Chlorella vulgaris* in the control group after 1, 2 and 3 days. The values are averages of the replicates and have been subtracted the blind values. The growth are shown as the logarithmic to the fluorescence.

Table 1 lists the pH value, the calculated growth rate and the inhibition after 72 hours of exposure for all the test concentrations. The raw data are given in the appendix.

Table 1. The pH in the test containers after 96 hours of exposure, growth rate and inhibition.

Test concentration (mg/l)	pH*	Growth rate (hours ⁻¹)	Inhibition (%)
0	8.3 (8.2-8.4)	0.04 – 0.05	
40	8.4	0.03	25 – 38
100	8.5 (8.4-8.5)	0.03 – 0.04	12 – 24
250	8.4	0.03 – 0.04	23 – 40
600	8.4 (8.3–8.5)	0.04 – 0.05	-7 – 14
1,600	8.4	0.04	13 – 20

*: the values given in brackets indicate the range of the pH values for the three determinations.

The inhibition varies a lot within the triple determination of each test concentration, and the inhibition does not increase as the test concentration increases. Compared to the lack of changes in the pH values and in the growth rates, the data indicate that the inhibition is not significant.

Since the inhibitions do not increase as the test concentrations increase there is no possibility of calculating the effect concentrations using statistical methods. The effect concentration, EC20, cannot be assessed from these test data. However, the effect concentration, EC50, can be assessed to be:

EC50-72h > 1,600 mg/l

Conclusion

The alga growth test was performed with a range of test concentrations of Stalosan F. The inhibition values did not indicate a toxicity relationship between the growth rate of *Chlorella vulgaris* and Stalosan F.

The effect concentration, EC20, could not be assessed on the test data given, but EC50 was assessed to the following:

EC50-72h > 1,600 mg/l

Appendix – raw data

Væksthæmningstest
Indtastningsbillede version: 2.1

 Reg. nr: Projektnr.: Forsøgsplannr.:

 Enhed: Dato Start: Dato slut:

 Init. tekniker: Init forsøgst.: Startbiomasse:

 Dato tekniker: Dato forsøgst.:

 Coulter Counter Flourometer Mikroskopi
Resultater blindprøver

T = 0		T = 1		T = 2		T = 3	
Koncentration	Respons	Koncentration	Respons	Koncentration	Respons	Koncentration	Respons
0	3,1715	0	2,0505	0	2,527	0	2,0895
0,04	2,499	0,04	2,4375	0,04	3,0575	0,04	2,622
0,1	2,5715	0,1	2,4135	0,1	2,6215	0,1	2,609
0,25	2,779	0,25	2,675	0,25	3,0445	0,25	2,6595
0,6	3,1015	0,6	2,5025	0,6	2,5145	0,6	2,553
1,6	2,7805	1,6	2,627	1,6	2,5315	1,6	2,9385
		0		0		0	
		0		0		0	

Resultater testopløsninger

Konc:	0	Replik:	6	Konc:	0,04	Replik:	3
0	24	48	72	0	24	48	72
4,179	6,625	18,93	46,5	4,812	6,906	9,78	24,4
5,215	5,897	13,9	38,05	5,167	8,069	16,01	34,72
4,117	9,288	14,07	41,81	5,246	6,751	10,28	24,3
4,358	6,187	9,071	28,45				
4,641	5,997	8,587	23,21				
4,191	6,994	11,54	46,99				
Konc:	0,1	Replik:	3	Konc:	0,25	Replik:	3
0	24	48	72	0	24	48	72
5,443	8,865	18,27	55,55	6,149	8,056	21,22	45,73
5,374	8,424	15,38	38,93	6,268	7,846	17,8	28,57
5,055	6,088	9,752	33,27	6,119	7,711	13,84	35,13
Konc:	0,6	Replik:	3	Konc:	1,6	Replik:	3
0	24	48	72	0	24	48	72
4,961	8,054	23,35	68,02	4,923	7,701	14,09	41,29
5,565	7,108	18,36	45,68	5,373	7,426	17,74	47,42
4,944	7,101	13,74	48,68	5,906	5,63	16,17	47,78
Konc:	0	Replik:		Konc:	0	Replik:	
0	24	48	72	0	24	48	72

The test results relate only to the items tested.

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Resultater testopløsninger - korrigeret for blind respons

Konc: 0				Konc: 0,04			
0	24	48	72	0	24	48	72
1,0075	4,5745	16,403	44,4105	2,313	4,4685	6,7225	21,778
2,0435	3,8465	11,373	35,9605	2,668	5,6315	12,9525	32,098
0,9455	7,2375	11,543	39,7205	2,747	4,3135	7,2225	21,678
1,1865	4,1365	6,544	26,3605				
1,4695	3,9465	6,06	21,1205	2,576	4,8045	8,96583333	25,18466667
1,0195	4,9435	9,013	44,9005				

Konc: 0,1				Konc: 0,25			
0	24	48	72	0	24	48	72
2,8715	6,4515	15,6485	52,941	3,37	5,381	18,1755	43,0705
2,8025	6,0105	12,7585	36,321	3,489	5,171	14,7555	25,9105
2,4835	3,6745	7,1305	30,661	3,34	5,036	10,7955	32,4705
2,71916667	5,3788333	11,8458333	39,974333	3,39966667	5,196	14,5755	33,81716667

Konc: 0,6				Konc: 1,6			
0	24	48	72	0	24	48	72
1,8595	5,5515	20,8355	65,467	2,1425	5,074	11,5585	38,3515
2,4635	4,6055	15,8455	43,127	2,5925	4,799	15,2085	44,4815
1,8425	4,5985	11,2255	46,127	3,1255	3,003	13,6385	44,8415
2,05516667	4,9185	15,9688333	51,573667	2,62016667	4,292	13,4685	42,55816667

Konc: 0				Konc: 0			
0	24	48	72	0	24	48	72
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Beregning af hæmning

Replikatno.	μ (kontrol)	μ (K1)	μ (K2)	μ (K3)	μ (K4)	μ (K5)	μ (K6)	μ (K7)
1	0,05	0,04	0,03	0,04	0,04	0,05	0,04	#NUM!
2	0,04	0,04	0,03	0,04	0,03	0,04	0,04	#NUM!
3	0,05	0,05	0,03	0,03	0,03	0,04	0,04	#NUM!
	gennemsnit	0,05						
			$l(\mu_1)$	$l(\mu_2)$	$l(\mu_3)$	$l(\mu_4)$	$l(\mu_5)$	$l(\mu_6)$
1			32,48	12,25	23,28	-7,23	13,14	#NUM!
2			25,10	22,86	39,63	13,81	14,42	#NUM!
3			37,80	24,33	31,52	3,04	19,80	#NUM!
			Gennemsnit:	31,80	19,81	31,48	3,21	15,79
								#NUM!