

International Molybdenum Association

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FYI-0497-1265

2nd April 1997

FYI Co-ordinator, FYI Submissions
Office of Toxic Substances (TS-778)
U S. Environmental Protection Agency
401 M Street, SW
Washington DC 20460, USA

97 APR 15 PM 3:22



FYI-96-001265

Dear Sir

Testing for Skin Sensitisation of Molybdenum Compounds

In 1991 and 1994, this Association published reports on the acute toxicity and ecotoxicity of certain molybdenum compounds. In 1996, reports on tests for skin sensitisation of the same molybdenum compounds were sent to you. As a final part of that programme, a further test was conducted on sodium molybdate in relation to algal growth inhibition and a copy of the laboratory's report is enclosed.

I would be grateful if you would ensure that the test results are fed into existing databases and given as wide a distribution as possible.

Yours faithfully

pp Michael Maby
SECRETARY-GENERAL

Contains No CBI



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Huntingdon Life Sciences

SODIUM MOLYBDATE 241/32

ALGAL GROWTH INHIBITION

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Report

CONFIDENTIAL

IMA 20/960915

**SODIUM MOLYBDATE 241/32
ALGAL GROWTH INHIBITION**

Sponsor

International Molybdenum Association,
Unit 7,
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Testing facility

Huntingdon Life Sciences Ltd.,
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Report issued: 31 July 1996

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health & Social Security 1986 and subsequent revision, Department of Health 1989.

EC Council Directive, 87/18 EEC of 18 December 1986, (No. L 15/29).

Good Laboratory Practice in the testing of Chemicals OECD, ISBN 92-64-12367-9, Paris 1982, subsequently republished OECD Environment Monograph No. 45, 1992.

United States Environmental Protection Agency, (FIFRA), Title 40 Code of Federal Regulations Part 160, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Japan Ministry of Agriculture, Forestry and Fisheries, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984.

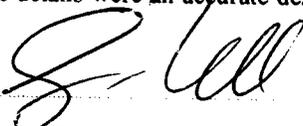
United States Environmental Protection Agency, (TSCA), Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Federal Register, 22 December 1978, and subsequent amendments.

Japan Ministry of Health and Welfare, Notification No. Yakuhatu 313 Pharmaceutical Affairs Bureau, 31 March 1982 and subsequent amendment Notification No. Yakuhatu 870, Pharmaceutical Affairs Bureau, 5 October 1988.

The test substance was assumed to be stable for the duration of the study, since the expiry date of the test substance was not supplied. It remains the responsibility of the Sponsor to ensure the test substance details were an accurate description of the test substance test.


Graeme Bell, M.Sc.,
Study Director,
Huntingdon Life Sciences Ltd.

31 July 1996
Date

QUALITY ASSURANCE STATEMENT

This report has been audited by Huntingdon Life Sciences Quality Assurance Department (Huntingdon). The methods, practices and procedures reported herein are an accurate description of those employed at Huntingdon during the course of the study. Observations and results presented in this final report form a true and accurate representation of the raw data generated during the conduct of the study at Huntingdon.

Certain studies such as that described in this report, are conducted at Huntingdon in a setting which involves frequent repetition of similar or identical procedures. At or about the time the study described in this report was in progress, 'process-based' inspections were made by the Quality Assurance Department of critical procedures relevant to this study type. The findings of these inspections were reported promptly to the Study Director and to Management, Huntingdon Life Sciences.

Dates of inspection 4 December 1995 - 12 January 1996

Dates of reporting inspection findings to the Study Director and Management 24 January 1996

Date of reporting audit findings to the Study Director and Management 15 July 1996

C. Sheets

Caroline Sheets
 Audit Team Supervisor,
 Department of Quality Assurance,
 Huntingdon Life Sciences Ltd

29.7.96

Date

RESPONSIBLE PERSONNEL

STUDY MANAGEMENT

Graeme Bell, M.Sc.,
Senior Study Director,
Department of Ecotoxicology.



Simon N. Groom, B.Sc. (Hons.),
Scientific Officer,
Department of Ecotoxicology.



STATISTICAL ANALYSIS

Graham F. Healey, B.Sc., M.Sc., A.R.C.S.,
Head, Department of Statistics.



SUMMARY

A study was performed to assess the inhibitory effect of sodium molybdate 241/32 on the growth of the unicellular green alga *Selenastrum capricornutum*, Strain No. CCAP 278/4.

The study was conducted in accordance with EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J. No. L383A, 29.12.92) Part C, Method 3 "Algal Inhibition Test" and the OECD Guideline for Testing of Chemicals No. 201 "Alga, Growth Inhibition Test".

Algal cultures exposed to five test concentrations of sodium molybdate 241/32 plus one untreated control were incubated on an orbital shaker under continuous illumination at $23 \pm 1^\circ\text{C}$ for 72 hours. Growth was monitored daily by determining the cell density of each culture by direct counts.

The following values were derived from the data:

E_bC_{50} (72 h): > 100 mg/l.

E_rC_{50} (0-72 h): > 100 mg/l.

"No-observed effect level": 4.6 mg/l.

All results are based on nominal concentrations.

E_bC_{50} ("x" h): The median effective concentration for inhibition of growth based on a comparison of areas under the growth curves after "x" hours

E_rC_{50} ("x" - "y" h): The median effective concentration for inhibition of growth based on a comparison of growth rates (from "x" to "y" hours)

INTRODUCTION

This study was designed to assess the inhibitory effect of sodium molybdate 241/32 on the growth of the unicellular green alga *Selenastrum capricornutum*, Strain No. CCAP 278/4.

The study was conducted in accordance with EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J. No. L383A, 29.12.92) Part C, Method 3 "Algal Inhibition Test" and the OECD Guideline for Testing of Chemicals No. 201 "Alga, Growth Inhibition Test".

The protocol was approved by Huntingdon Life Sciences Management on 5 February 1996, by the Sponsor on 4 March 1996 and by the Study Director on 5 March 1996.

The experimental phase of the study was conducted between 5 and 8 March 1996.

TEST SUBSTANCE

Identity: Sodium molybdate 241/32

Batch number: 241/32

Expiry: Assumed to be stable for duration of study

Composition: Molybdenum (40.52%)
Sodium (19.29%)

Appearance: White powder

Storage conditions: In darkness at room temperature

Date received: 24 March 1993

EXPERIMENTAL PROCEDURE

TEST SPECIES

Name

Selenastrum capricornutum. Strain No. CCAP 278/4.

Source

Culture Centre of Algae & Protozoa c/o Freshwater Biological Association, Cumbria, UK.

Pre-culture

Sterile nutrient medium (Appendix 1) was inoculated from a master culture and incubated under continuous illumination (≈ 7000 lux) and stirring (orbital shaker) at $24 \pm 1^\circ\text{C}$ to give an algal suspension in log phase growth characterised by a cell density of 4.5×10^6 cells per ml.

The suspension was diluted using sterile nutrient medium to a cell density of 3.3×10^6 cells per ml prior to use.

TEST WATER

Sterile nutrient medium as recommended in Official Journal No. L383A Part C.3 (see Appendix 1). The EDTA solution was omitted from this medium due to the presence of the heavy metal, molybdenum.

TEST SUBSTANCE PREPARATION

Method of preparation

The test substance was dispersed in sterile nutrient medium to give an initial stock solution of 200 mg/l. This stock solution was further diluted with sterile nutrient medium to produce a series of solutions exactly twice the concentration of the intended exposure levels. 200 ml of algal pre-culture was mixed with 200 ml of each of these solutions to give the final test series.

Stability of test concentrations

Test concentrations were not verified by chemical analysis, at the request of the Sponsor.

EXPOSURE CONDITIONS

Experimental design

Five test concentrations plus one untreated control each in triplicate

Test concentrations

4.6, 10, 22, 46 and 100 mg/l.

Culture conditions

Conical flasks (250 ml) each containing 100 ml of test or control culture were loosely stoppered and placed at random in a Gallenkamp Illuminated Orbital Incubator. The cultures were incubated, without media renewal, for 72 hours under continuous illumination of approximately 7000 lux provided by 7 x 30 W "universal white" 1 metre fluorescent tubes. The temperature was maintained at 23 ± 1 °C and gaseous exchange and suspension of the algal cells was ensured by the action of the orbital shaker oscillating at 120 cycles per minute.

MEASUREMENT OF GROWTH

Samples were taken at 0, 24, 48 and 72 hours and the cell density determined by direct counting with the aid of a Coulter® Multisizer II particle counter.

EVALUATION OF DATA

The area under each growth curve (cell density v time) is taken to be an index of growth and is calculated using the equation:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times [t_2 - t_1] + \dots + \frac{N_{n-1} + N_n - 2N_0}{2} \times [t_n - t_{n-1}]$$

- where
- A = area
 - N₀ = cell density at t₀
 - N₁ = cell density at t₁
 - N_n = cell density at t_n
 - t₁ = time of first measurement (hours from start)
 - t_n = time of n_{th} measurement (hours from start)
 - n = number of measurements taken after the beginning of the test

Percentage inhibition of growth at each test concentration (I_A) is calculated by comparing the area under the test curve (A_t) with that under the control curve (A_c) using the equation:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

I_A values are plotted against test concentration, a line fitted by logistic regression (see STATISTICAL ANALYSIS) and the $E_b C_{50}$ estimated by interpolation of the fitted curve. The $E_b C_{50}$ ("x" h) is the median effective concentration for inhibition of growth based on a comparison of areas under the growth curves after "x" hours.

The average specific growth rate (μ) for each exponentially growing culture is also calculated from the appropriate section of the growth curve by the equation:

$$\mu = \frac{\ln N_n - \ln N_0}{t_n - t_0}$$

where t_0 is the time at the beginning of the test.

Percentage reductions in growth rate and the $E_r C_{50}$ value are calculated as for the "area under the curve" data. The $E_r C_{50}$ ("x"- "y" h) is the median effective concentration for inhibition of growth based on a comparison of growth rates from "x" to "y" hours.

The "no-observed effect level" (NOEL) is obtained using Williams' test to compare the percentage inhibition in each treated group with that for the control cultures (Williams' D.A., 1971/72, biometrics 27; 107 - 117 and 28; 519 - 531).

ARCHIVES

All specimens, raw data and study related documents generated during the course of the study at Huntingdon Life Sciences, together with a copy of the final report will be lodged in the Huntingdon Life Sciences archive.

Such specimens and records will be retained for a minimum period of five years from the date of issue of the final report. At the end of the five year retention period the client will be contacted and advice sought on the future requirements. Under no circumstances will any item be discarded without the client's knowledge.

RESULTS

Individual and mean cell densities for each exposure level are given in Table 1 and the mean values are presented graphically in Figure 1. All results are expressed in terms of nominal concentration.

The calculated "area under the curve" and "specific growth rate" values are given in Table 2 and are expressed in terms of percentage inhibition by comparing each value with that of the control curve.

Throughout the study, the cell density of replicate 1 at the 22 mg/l exposure level cultures was continually lower than the cell densities in replicates 2 and 3. This replicate is therefore considered anomalous and has not been included in any subsequent calculations.

The estimated E_bC_{50} and E_rC_{50} values, together with the calculated NOEL are given below:

E_bC_{50} (72 h):	> 100 mg/l
E_rC_{50} (0-72 h):	> 100 mg/l
"No-observed effect level":	4.6 mg/l.
Mean cell density of control @ 0 h:	1.6×10^4 cells/ml.
Mean cell density of control @72 h:	9.0×10^5 cells/ml.

OBSERVATIONS

All test and control cultures were inspected microscopically at 72 hours. There were no abnormalities detected in any cultures although the cells in all the test levels were paler than the control cultures.

No cultures showed any signs of contamination by foreign algal cells or protozoa.

CONCLUSION

Sodium molybdate 241/32 is inhibitory to the growth of *Selenastrum capricornutum*, Strain No. CCAP 278/4 at concentrations in excess of 4.6 mg/l. The E_bC_{50} (72 h) is > 100 mg/l and the E_rC_{50} (0-72 h) is > 100 mg/l.

FIGURE 1

Mean cell densities v time

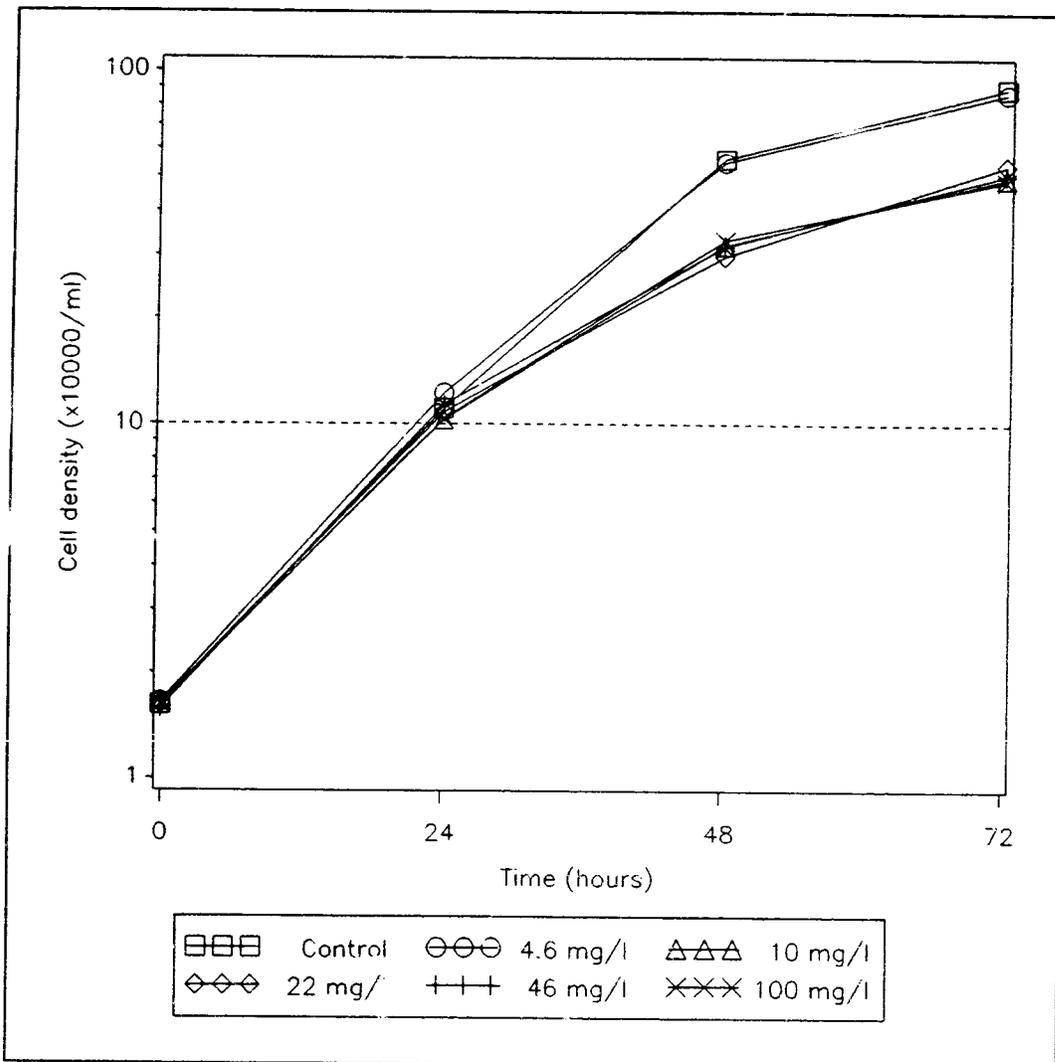


TABLE 1
Cell densities

Nominal concentration mg/l	pH	Cell densities (cells/ml)				pH	
		0 h	24 h	48 h	72 h		
Control	R ₁	7.6	1.6x10 ⁴	1.0x10 ⁵	5.6x10 ⁵	9.5x10 ⁵	7.6
	R ₂	7.6	1.6x10 ⁴	1.3x10 ⁵	5.5x10 ⁵	9.4x10 ⁵	7.7
	R ₃	7.6	1.6x10 ⁴	1.0x10 ⁵	5.6x10 ⁵	8.0x10 ⁵	7.8
	\bar{x}	7.6	1.6x10 ⁴	1.1x10 ⁵	5.6x10 ⁵	9.0x10 ⁵	7.7
4.6	R ₁	7.7	1.7x10 ⁴	1.2x10 ⁵	5.4x10 ⁵	9.0x10 ⁵	7.7
	R ₂	7.6	1.6x10 ⁴	1.2x10 ⁵	5.8x10 ⁵	9.1x10 ⁵	7.7
	R ₃	7.7	1.7x10 ⁴	1.2x10 ⁵	5.2x10 ⁵	7.8x10 ⁵	7.7
	\bar{x}	7.7	1.7x10 ⁴	1.2x10 ⁵	5.5x10 ⁵	8.7x10 ⁵	7.7
10	R ₁	7.6	1.6x10 ⁴	1.1x10 ⁵	3.4x10 ⁵	4.7x10 ⁵	7.7
	R ₂	7.6	1.6x10 ⁴	1.1x10 ⁵	3.2x10 ⁵	5.4x10 ⁵	7.7
	R ₃	7.6	1.7x10 ⁴	9.6x10 ⁴	3.0x10 ⁵	4.8x10 ⁵	7.7
	\bar{x}	7.6	1.6x10 ⁴	1.0x10 ⁵	3.2x10 ⁵	5.0x10 ⁵	7.7
22	†R ₁	7.6	1.7x10 ⁴	9.5x10 ⁴	2.2x10 ⁵	3.0x10 ⁵	7.7
	R ₂	7.6	1.6x10 ⁴	1.2x10 ⁵	3.3x10 ⁵	5.4x10 ⁵	7.7
	R ₃	7.6	1.6x10 ⁴	1.1x10 ⁵	3.4x10 ⁵	5.4x10 ⁵	7.7
	\bar{x}	7.6	1.6x10 ⁴	1.2x10 ⁵	3.4x10 ⁵	5.4x10 ⁵	7.7
46	R ₁	7.6	1.6x10 ⁴	1.1x10 ⁵	3.2x10 ⁵	5.1x10 ⁵	7.7
	R ₂	7.7	1.6x10 ⁴	1.1x10 ⁵	3.1x10 ⁵	5.1x10 ⁵	7.7
	R ₃	7.6	1.6x10 ⁴	1.2x10 ⁵	3.3x10 ⁵	5.0x10 ⁵	7.7
	\bar{x}	7.6	1.6x10 ⁴	1.1x10 ⁵	3.2x10 ⁵	5.1x10 ⁵	7.7
100	R ₁	7.7	1.6x10 ⁴	1.0x10 ⁵	3.2x10 ⁵	5.0x10 ⁵	7.9
	R ₂	7.6	1.6x10 ⁴	1.0x10 ⁵	3.5x10 ⁵	4.9x10 ⁵	7.9
	R ₃	7.6	1.6x10 ⁴	1.0x10 ⁵	3.2x10 ⁵	4.8x10 ⁵	8
	\bar{x}	7.6	1.6x10 ⁴	1.0x10 ⁵	3.3x10 ⁵	4.9x10 ⁵	7.9
Temperature °C	-	24	22	24	24	-	-

† Replicate considered anomalous and not used in calculation of mean

R₁, R₂, R₃ Replicates 1, 2 and 3
Particle counter: Coulter® Multisizer II
pH meter: Sentron 1001

TABLE 2

Inhibition of growth

Nominal concentration mg/l	Area under curve @ 72 h	% Inhibition*	Growth rate (0-72 h)	% Inhibition*
Control	2588	-	0.05570	-
4.6	2549	2	0.05496	1
10	1514	41	0.04753	15
22	1639	37	0.04868	13
46	1552	40	0.04818	13
100	1532	41	0.04734	15

* Percentage inhibition values calculated using non-rounded data

APPENDIX 1

Nutrient medium

Four stock solutions are prepared according to the following table, using reverse osmosis purified water. Stock solutions are sterilised by autoclaving (solutions 1-3) or by membrane filtration (solution 4) before being stored at +4°C in the dark.

Aliquots of stock solutions 1-3 are further diluted with reverse osmosis purified water and autoclaved again to produce the working strength nutrient medium. Prior to use, an aliquot of stock solution 4 is added aseptically to the medium via a membrane filter. The pH of the medium after equilibration with air is approximately 8.

Nutrient	Concentration in stock solution	Volume of stock solution per litre of final medium	Final concentration in test solution
Stock solution 1: macro-nutrients			
NH ₄ Cl	1.5 g/l	10 ml	15 mg/l
MgCl ₂ .6H ₂ O	1.2 g/l		12 mg/l
CaCl ₂ .2H ₂ O	1.8 g/l		18 mg/l
MgSO ₄ .7H ₂ O	1.5 g/l		15 mg/l
KH ₂ PO ₄	0.16 g/l		1.6 mg/l
Stock solution 2: Fe-EDTA			
FeCl ₃ .6H ₂ O	80 mg/l	1 ml	0.08 mg/l
Na ₂ EDTA.2H ₂ O*	100 mg/l		0.1 mg/l
Stock solution 3: trace elements			
H ₃ BO ₃	185 mg/l	1 ml	0.185 mg/l
MnCl ₂ .4H ₂ O	415 mg/l		0.415 mg/l
ZnCl ₂	3 mg/l		3 × 10 ⁻³ mg/l
CoCl ₂ .6H ₂ O	1.5 mg/l		1.5 × 10 ⁻³ mg/l
CuCl ₂ .2H ₂ O	0.01 mg/l		10 ⁻⁵ mg/l
Na ₂ MoO ₄ .2H ₂ O	7 mg/l		7 × 10 ⁻³ mg/l
Stock solution 4: NaHCO₃			
NaHCO ₃	50 g/l	1 ml	50 mg/l

* solution omitted due to the presence of the heavy metal molybdenum.

IMA 20/960915

APPENDIX 2

Certificate of analysis

IMA 20/960915

STATISTICAL ANALYSIS OF GROWTH INHIBITION DATA

Author

M.H. Coates

Statistics Department

INTRODUCTION

Algal cell densities were available at 0, 24, 48 and 72 hours for three replicates in a control group and at each of five test concentrations. These were 4.6, 10, 22, 46 and 100 mg/l. For each replicate, the 'area under the curve' (AUC) and the growth rate were calculated, both between 0 and 72 hours. These were converted to percentage inhibition values by reference to the relevant control mean values.

METHODS

Williams' test (Williams, 1971, 1972) was used to compare the percentage inhibition in each treated group with the baseline (control) values. Bartlett's test for homogeneity of variance (Bartlett, 1937) was also applied. The first replicate in the 22 mg/l group was omitted from all calculations.

It was not possible to derive 50% growth inhibition points for this study since this level of inhibition was not attained for either AUC or growth rate over 0-72 hours.

DATA HANDLING

The data were entered by hand and analysed using Genstat 5 release 1.3 (Payne, *et al*, 1987).

RESULTS

The results of the Williams' tests are shown in Table 1. The highest concentration tested which resulted in no statistically significant inhibition of growth in comparison with the control group was 4.6 mg/l for both sets of data.

REFERENCES

- BARTLETT, M.S. (1937) Properties of sufficiency and statistical tests. *Proceedings of the Royal Society. Series A*, **160**, 268 - 282.
- PAYNE, R.W. *et al* (1987) *Genstat 5.1.3 Reference Manual*. Clarendon Press, Oxford.
- WILLIAMS, D.A. (1971) A test for differences between treatment means when several dose levels are compared with a zero dose control. *Biometrics*, **27**, 103 - 117.
- WILLIAMS, D.A. (1972) The comparison of several dose levels with a zero dose control. *Biometrics*, **28**, 519 - 531.

TABLE 1

Results of Williams' test for 'no observed effect level'

Data set	Nominal Concentration (mg/l)	Number of replicates	Mean % inhibition	MLE*	T-bar	Significance level
0-72 hour AUC	0.0	3	0.0			
	4.6	3	1.5	1.5	0.54	-
	10.0	3	41.5	39.6	14.11	<1%
	22.0	2	36.7	39.6	12.62	<1%
	46.0	3	40.0	40.0	14.28	<1%
	100.0	3	40.8	40.8	14.55	<1%
0-72 hour growth rate	0.0	3	0.0			
	4.6	3	1.3	1.3	0.88	-
	10.0	3	14.7	13.7	9.05	<1%
	22.0	2	12.6	13.7	8.10	<1%
	46.0	3	13.5	13.7	9.05	<1%
	100.0	3	15.0	15.0	9.91	<1%

* Maximum Likelihood Estimate of group mean under monotonicity constraint (used for Williams' test)

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