Huntingdon Life Sciences

[redacted]

ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

Company Sanitized. Does not contain TSCA CBI

Report

[redacted]

ACTIVATED SLUDGE - RESPIRATION INHIBITION TEST

Sponsor

Dupont Speciality Chemicals,
Jackson Laboratory,
Chambers Works,
Deepwater,
NJ 08023,
USA.

Research Laboratory

Huntingdon Life Sciences Limited,
Eye,
Suffolk ,
IP23 7PX,
ENGLAND.

Final report issued: 5 November 1998

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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.

United Kingdom Good Laboratory Practice Regulations (1997), Statutory Instrument No. 654.

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

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

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Christine E. Burwood, B.Sc. (Hons.),
Study Director,
Huntingdon Life Sciences Ltd.

5th November 1998

Date

QUALITY ASSURANCE STATEMENT

The following have been inspected or audited in relation to this study.

Study Phases Inspected	Date of Inspection	Date of Reporting
Protocol	11.08.98	11.08.98
Process Based Inspections		
Formulation	16.09.98	16.09.98
Measurement of dissolved oxygen	18.08.98	18.08.98
Collection of activated sludge	24.06.98	24.06.98
Audit	12.05.98	12.05.98
Report	14.10.98	15.10.98

Protocol: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Process based inspections: At or about the time this study was in progress inspections and audits of routine and repetitive procedures employed on this type of study were carried out. These were conducted and reported to appropriate Company Management as indicated above.

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results to reflect the raw data.

G. Goddard

Gregg I. Goddard,
Senior Auditor,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

30th OCTOBER 1998
Date

RESPONSIBLE PERSONNEL

Christine E. Burwood, B.Sc. (Hons.)
(Study Director)

Faye O. Shanahan
(Laboratory Technician)

SUMMARY

The effect of [redacted] on the respiration rate of activated sludge was assessed by the methods detailed in EC Directive 88/302, 'Biodegradation - Activated Sludge Respiration Inhibition test' and OECD Test Guideline 209, 'Activated Sludge, Respiration Inhibition test'.

Samples of activated sludge (suspended solids 1.6 g/l), fed with synthetic sewage, were exposed to the test substance at nominal concentrations of 1, 10 and 100 mg/l for three hours. Single mixtures were prepared at 1 and 10 mg/l and the highest level was prepared in triplicate. Their rates of oxygen consumption were determined using an oxygen electrode and compared with those of controls, containing activated sludge and synthetic sewage alone, which were established at the beginning and end of the culture series.

The reference inhibitor 3,5-dichlorophenol (3,5-DCP) was employed at 3.0, 10.0 and 32.0 mg/l, as a positive control.

The specific respiration rate of the control culture established at the end of the test series (32.6 $mgO_2/g/h$) was 96% of the rate of that established at the start (34.1 $mgO_2/g/h$). The three-hour 50% effect concentration (EC_{50}) for 3,5-DCP was calculated to be 15.1 mg/l (95% confidence limits 12.2 - 19.5 mg/l). These results show that the test was valid and that the sample of activated sludge employed was sensitive to inhibition.

[redacted] had no significant inhibitory effect on the respiration rate of activated sludge at any of the concentrations employed in the test. At nominal concentrations of 1 and 10 mg/l, respiration rates were reduced by 4% when compared to the mean control value; no effect on respiration was observed at 100 mg/l. The EC_{20}, EC_{50} and EC_{80} of the test substance could not, therefore, be calculated but these must be greater than 100 mg/l, the highest level tested.

INTRODUCTION

The objective of this study was to assess the effects of [redacted] on sewage micro-organisms by measuring the rate of oxygen uptake of activated sludge at 20 ± 2°C in its presence at a range of concentrations.

The methods employed were designed to meet the requirements of EC Directive 88/302, 'Biodegradation - Activated Sludge Respiration Inhibition test' and OECD test guideline 209, 'Activated Sludge, Respiration Inhibition test' adopted 4 April 1984.

The dechlorinated tap water used to prepare test mixtures had a measured hardness of 184 mg/l as $CaCO_3$. This deviated from the hardness specified in the protocol (200 - 250 mg/l as $CaCO_3$) but this is not considered to be significant, nor to have affected the integrity of the test.

The protocol was approved by Huntingdon Life Sciences Management on 7 July 1998, by the Sponsor on 17 July 1998, and by the Study Director on 10 August 1998.

The experimental phase of the study was conducted between 12 and 14 August 1998.

TEST SUBSTANCE

Identity:	[redacted]
Chemical name:	[redacted]
Lot number / Batch number:	[redacted]
Expiry date:	2 years from date of receipt
Purity / Composition:	25%
Appearance:	Pale Yellow Slurry
Storage conditions:	Room temperature
Date received:	23 June 1998

EXPERIMENTAL PROCEDURE

REFERENCE INHIBITOR

3,5-DCP, >97%, product number D7,060-0, was obtained from the Aldrich Chemical Company Ltd.

DILUTION WATER

The water used to prepare test mixtures was dechlorinated tap water (measured hardness, 184 mg/l as $CaCO_3$).

The dilution water used to prepare synthetic sewage was tap water that had been softened and treated by reverse osmosis (Elga Ltd, Prima 4 reverse osmosis unit) and then purified (Elga Ltd, UHP) to give a resistivity of 18 Megohm/cm. This water complies with the relevant standards (British Standard (BS) 3978 : 1987 (ISO 3696 : 1987)).

SYNTHETIC SEWAGE

Synthetic sewage feed for activated sludge was prepared by dissolving the following in one litre of ultrapure water (resistivity 18 Megohm/cm):

peptone	-	16.0 g
meat extract	-	11.0 g
urea	-	3.0 g
sodium chloride	-	0.7 g
calcium chloride dihydrate	-	0.4 g
magnesium sulphate heptahydrate	-	0.2 g
di-potassium hydrogen phosphate	-	2.8 g

PREPARATION OF THE MICROBIAL INOCULUM

A sample of activated sludge was obtained the day before the start of the test from Oakley Sewage Treatment Works, a sewage plant treating predominantly domestic waste. In the laboratory, the sample was maintained under aerobic conditions until required.

The concentration of suspended solids in a homogenised sample was determined on the day of collection and immediately before the start of the test.

On the day of collection, aliquots (25 ml) of the activated sludge were filtered through dried and preweighed Whatman's GFC filter papers which were then dried again at approximately 105°C for at least one hour, allowed to cool in a desiccator and reweighed. The mixed liquor suspended solids (MLSS) content of the activated sludge was then calculated. Synthetic sewage (50 ml/l) was added and the mixture aerated overnight. On the day of the test, the MLSS content of the sludge was determined and adjusted to 4 g/l by the addition of dechlorinated tap water. The pH of the sludge was also measured.

PREPARATION OF SOLUTIONS OF THE REFERENCE SUBSTANCE (3,5-DICHLOROPHENOL)

A concentrated solution of 3,5-DCP (500 mg/l) was prepared by dissolving 0.5 g in 10 ml of 1N sodium hydroxide and diluting to approximately 30 ml with ultrapure water. Sulphuric acid (1N) was added to the point of incipient precipitation and the solution made up to a final volume of one litre with ultrapure water. The pH of this solution was then measured.

Nominal concentrations of 3.0, 10.0 and 32.0 mg/l were prepared by dilution of this concentrated solution.

TEST METHODS

The results of a preliminary solubility trial showed that [redacted] was insufficiently soluble in water at room temperature to allow the preparation of a suitable stock solution, so appropriate weights contained in glass weighboats, were added directly to test beakers. The test material was provided as an aqueous slurry containing 25% active ingredient (A.I.) and 75% water. To promote dissolution, the slurry was gently heated in a water bath at 37.5°C until the solids had dissolved and a clear solution was obtained. An approximate amount of the warm test substance was transferred to a glass weighboat. The material was allowed to cool and the final weight recorded once a stable balance reading could be obtained. Dechlorinated tap water (284 ml) was added to each test beaker containing [redacted] and the mixtures were treated with ultrasound for 10 minutes. Additions of synthetic sewage and microbial inoculum were then made as detailed in the schedule below. The mixtures were prepared at 15 minute intervals.

Test mixture	Test substance* (mg) or reference (ml)	Synthetic sewage (ml)	Water (ml)	Microbial inoculum (ml)
Control (1)	0	16	284	200
Test substance (mg/l)				
1	2.0	16	284	200
10	20	16	284	200
100	200	16	284	200
100	200	16	284	200
100	200	16	284	200
3,5-DCP (mg/l)				
3.0	3.0	16	281	200
10.0	10	16	274	200
32.0	32	16	252	200
Control (2)	0	16	284	200

*The table quotes the intended addition of the test substance.

An allowance was made for the concentration of the active ingredient during the formulation of test mixtures. The actual weights of test substance added to each mixture were recorded and the achieved concentrations calculated.

Prepared mixtures were then aerated using a Pasteur pipette connected to a laboratory supply of oil-free compressed air for three hours.

Following the exposure period, a well-mixed sample of each mixture was transferred to a biochemical oxygen demand (BOD) bottle (capacity; 270 ml) and its rate of oxygen consumption over a period of approximately ten minutes was measured using a Yellow Springs Instruments (YSI) dissolved oxygen meter, with temperature probe and self-stirring bottle probe, connected to a chart recorder. The pH and temperature of the samples were measured at the start and end of the test.

CALCULATION OF RESULTS

The respiration rate of each test, reference and control mixture was calculated from oxygen levels in the following way:

$$\text{Respiration rate (r)} = \frac{DO_{(1)} - DO_{(2)}}{t} \text{ mg}O_2\text{/l/minute}$$

where:

$DO_{(1)}$ = initial oxygen level

$DO_{(2)}$ = final oxygen level

t = time over which measurements were made in minutes

The specific respiration rate of each mixture was calculated from respiration rate in the following way:

$$\text{SRR (mg}O_2\text{/g/h)} = \frac{r\ (\text{mg}O_2\text{/l/min}) \times 60}{\text{MLSS}}$$

where:

SRR = specific respiration rate

MLSS = concentration of mixed liquor suspended solids in the sample of activated sludge (g/l) in the test or control mixture.

The inhibitory effect of the test or reference substance at a particular concentration was calculated by expressing the specific respiration rate as a percentage of the mean of the respiration rates of the two controls in the following way:

$$\% \text{ inhibition} = 1 - \frac{(2Rs)}{Rc_1 + Rc_2} \times 100$$

where:

Rs = rate of oxygen consumption of test or reference substance

Rc_1 = rate of oxygen consumption of control 1

Rc_2 = rate of oxygen consumption of control 2

The EC_{50} and 95% confidence limits of the reference substance were calculated using the computer program of Stephan *et al* (1982).

MAINTENANCE OF RECORDS

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.

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

RESULTS

Temperature, pH and measurements of respiration rate in the test are given in Table 1.

Measurements of the pH of the aqueous stock solution of the reference substance and of the activated sludge before the start of the test, are given below.

Preparation	pH measurement
3,5-DCP stock solution	8.0
activated sludge	7.4

The weights of the test substance added to test beakers and the actual concentrations achieved are given below:

Beaker No.	Nominal Concentration (mg/l)	Weight Added (mg)	Actual Concentration (mg/l)	Actual as a Percentage of Nominal (%)
1	1	2.19	1.095	109.5
2	10	19.63	9.815	98.2
3	100	205.24	102.63	102.6
4	100	209.08	104.54	104.5
5	100	215.12	107.56	107.6

Sludge respiration rates were progressively reduced in the presence of increasing concentrations of 3,5-DCP. The three-hour 50% effect concentrations (EC_{50}) for 3,5-DCP was calculated, by the Moving Average method, to be 15.1 mg/l (95% confidence limits 12.2 - 19.5 mg/l).

The specific respiration rate of the control culture established at the end of the test (32.6 $mgO_2/g/h$) was 96% of the rate of that established at the start (34.1 $mgO_2/g/h$).

These results show that the test was valid and that the sample of activated sludge employed was sensitive to inhibition.

[redacted] had no significant inhibitory effect on the respiration rate of activated sludge at any of the concentrations employed in the test. In mixtures containing [redacted] at nominal concentrations of 1 and 10 mg/l, respiration rates were reduced by 4% when compared to the mean control value; no effect on respiration rate was observed at 100 mg/l. The EC_{20}, EC_{50} and EC_{80} of the test substance could not, therefore, be calculated but these must be greater than 100 mg/l, the highest level tested.

CONCLUSIONS

[redacted] had no significant inhibitory effect on the respiration rate of activated sludge at any of the concentrations employed in the test. The EC_{20}, EC_{50} and EC_{80} of the test substance could not, therefore, be calculated but these must be greater than 100 mg/l, the highest level tested.

The three-hour EC_{50} for 3,5-DCP (15.1 mg/l) fulfilled the validity criterion relating to sensitivity to inhibition (acceptable EC_{50} range; 5 to 30 mg/l), and that relating to the respiration rates in the control (variation not greater than 15%) was also satisfied.

REFERENCE

STEPHAN *et al* (1982). A computer program for calculating an LC_{50}. US Environmental Protection Agency.

TABLE 1

Temperature, pH and measurements of respiration rate

Test mixture	Temperature (°C)		pH		Respiration rate $mgO_2/g/h$	% inhibition
	Initial	Final	Initial	Final		
Control (1)	22.0	19.8	7.5	8.1	34.1	-
[redacted]						
1	21.2	19.6	7.5	8.1	31.9	4
10	21.0	19.8	7.5	8.0	32.0	4
100	22.0	20.8	7.2	7.8	33.8	0
100	21.6	19.6	7.1	7.9	34.1	0
100	21.2	20.0	7.1	7.9	35.6	0
3,5-DCP (mg/l)						
3.0	20.8	20.5	7.5	8.1	30.0	10
10.0	20.9	20.0	7.5	8.2	20.6	38
32.0	20.9	19.8	7.5	8.2	9.7	71
Control (2)	20.9	20.0	7.5	8.1	32.6	-