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CHD-FA:

ASSESSMENT OF READY BIODEGRADABILITY; CO_2 EVOLUTION TEST

PROJECT NUMBER: 41004595

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QUALITY ASSURANCE REPORT

This study type is classed as short-term. The general study plan for this study type was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress. In addition, inspection of general facilities not specifically related to this study are done monthly or annually in accordance with QA Standard Procedure.

This report has been audited by the Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

	26 October 2010	General Study Plan Compliance Audit
	09 November 2010	Test Item Preparation
	08, 09 November 2010	Test System Preparation
	09 November 2010	Exposure
	10 December 2010	Assessment of Response
§	12 January 2011	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: 08 FEB 2011

For the Quality Assurance Unit*

***Authorised QA Signatures:**
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GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

This report fully and accurately reflects the procedures used and data generated.

..... Date: 2 FEB 2011

N Clarke BSc
Study Director

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ASSESSMENT OF READY BIODEGRADABILITY; CO_2 EVOLUTION TEST

SUMMARY

Introduction. A study was performed to assess the ready biodegradability of the test item in an aerobic aqueous medium. The method followed was designed to be compatible with the OECD Guidelines for Testing of Chemicals (1992) No 301B, "Ready Biodegradability; CO_2 Evolution Test" referenced as Method C.4-C of Commission Regulation (EC) No. 440/2008 and US EPA Fate, Transport, and Transformation Test Guidelines OPPTS 835.3110 (Paragraph (M)).

Methods. The test item, at a concentration of 10 mg Carbon/l, was exposed to activated sewage sludge micro-organisms with culture medium in sealed culture vessels in the dark at temperatures of between 19 to 21°C for 28 days.

The degradation of the test item was assessed by the determination of carbon dioxide produced. Control solutions with inoculum and the reference item, sodium benzoate, together with a toxicity control were used for validation purposes.

Results. The test item attained 97% degradation after 28 days and satisfied the 10-Day window validation criterion, whereby 60% degradation must be attained within 10 days of the degradation exceeding 10%. The test item can therefore be considered to be readily biodegradable under the strict terms and conditions of OECD Guideline No 301B.

CHD-FA:

ASSESSMENT OF READY BIODEGRADABILITY; CO_2 EVOLUTION TEST

1. INTRODUCTION

This report contains a description of the methods used and results obtained during a study to investigate the ready biodegradability of the test item when exposed to sewage sludge micro-organisms under aerobic conditions. The method followed was designed to be compatible with the OECD Guidelines for Testing of Chemicals (1992) No 301B "Ready Biodegradability; CO_2 Evolution Test" referenced as Method C.4-C of Commission Regulation (EC) No. 440/2008 and US EPA Fate, Transport, and Transformation Test Guidelines OPPTS 835.3110 (Paragraph (M)).

The results of the study are of value in the assessment of the persistence of the test item in the aquatic environment.

The study was conducted between 24 November 2010 and 29 December 2010.

2. TEST ITEM

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	CHD-FA
Description	:	pale yellow liquid
Purity	:	4.39%
Batch number	:	API 14.1
Date received	:	15 November 2010
Expiry date	:	09 April 2012
Storage conditions	:	approximately 4°C in the dark until 16 November, thereafter, room temperature in the dark

The integrity of supplied data relating to the identity, purity and stability of the test item is the responsibility of the Sponsor.

3. METHODS

3.1 Experimental Preparation

For the purpose of the test, the test item was dissolved directly in culture medium.

An amount of test item (6000 mg) was dissolved in culture medium and the volume adjusted to 1 litre to give a 6000 mg/l stock solution. An aliquot (303.1 ml) of this stock solution was dispersed in inoculated culture medium and the volume adjusted to 3 litres to give a final concentration of 606.1 mg/l, equivalent to 10 mg carbon/l. The volumetric flask containing the test item was inverted several times to ensure homogeneity of the solution.

A test concentration of 10 mg carbon/l was employed in the test following the recommendations of the Test Guideline.

Data from the control vessels was shared with similar concurrent studies.

3.2 Reference Item

For the purposes of the test, a reference item, sodium benzoate (C_6H_5COONa) (Sigma Aldrich Lot No 096K1231), was used. An initial stock solution of 1000 mg/l was prepared by dissolving the reference item directly in culture medium with the aid of ultrasonication for approximately 10 minutes. An aliquot (51.4 ml) of this stock solution was added to the test vessel containing inoculated culture medium and the volume adjusted to 3 litres to give a final test concentration of 17.1 mg/l, equivalent to 10 mg carbon/l. The volumetric flask containing the reference item was inverted several times to ensure homogeneity of the solution.

Data from the reference item vessels was shared with similar concurrent studies.

3.3 Toxicity Control

For the purposes of the test, a toxicity control, containing the test item and sodium benzoate, was prepared in order to assess any toxic effect of the test item on the sewage sludge micro-organisms used in the test.

An aliquot (303.1 ml) of the test item stock solution was dispersed in inoculated culture medium along with an aliquot (51.4 ml) of the sodium benzoate stock solution. The volume was adjusted to 3 litres to give a final concentration of 606.1 mg test item/l plus 17.1 mg sodium benzoate/l, equivalent to a total of 20 mg carbon/l.

3.4 Preliminary Investigational Work

During the study, samples are taken for Dissolved Organic Carbon (DOC) analysis and as part of the sample preparation the samples are either filtered or centrifuged to remove the sewage sludge solids. Due to a shortage of 0.45 µm filters, no samples were fitered for analysis. Thus the following work was conducted and samples analysed for Dissolved Organic Carbon (DOC) using either a Shimadzu TOC-V_{CSH} TOC analyser or a Shimadzu TOC-V_{CPH} TOC analyser (see Appendix 1).

An amount of test item (1000 mg) was dissolved in culture medium (500 ml) to give a 2000 mg/l stock solution. One untreated sample was taken for DOC analysis. A further amount of test item (1000 mg) was dissolved in culture medium and inoculated at a concentration of 30 mg suspended solids (ss)/l prior to adjusting to a final volume of 500 ml. One sample was taken for DOC analysis after centrifugation at 3500 rpm for 15 minutes. A control sample was prepared by inoculating culture medium (500 ml) at a suspended solids level of 30 mg ss/l and then centrifuging as per the test item samples.

The results of this preliminary investigational work showed that, based on the representative chemical structure provided by the Sponsor, measured concentrations of below 80% of nominal were obtained. As such, in order to determine the exact carbon content of the test item sample received, three 2000 mg/l stock solutions were prepared by dissolving 1000 mg of test item in a final volume of 500 ml of culture medium. A sample was taken from each of the replicate flasks for DOC analysis without any pre-treatment. The initial preliminary investigational work was also repeated in a similar manner as previously conducted in order to check the results obtained.

A mixed population of sewage treatment micro-organisms was obtained on 23 and 24 November 2010 from the aeration stage of the Severn Trent Water Plc sewage treatment plant at Loughborough, Leicestershire, UK, which treats predominantly domestic sewage.

3.5 Test Species

A mixed population of activated sewage sludge micro-organisms was obtained on 29 November 2010 from the aeration stage of the Severn Trent Water Plc sewage treatment plant at Loughborough, Leicestershire, UK, which treats predominantly domestic sewage.

3.6 Procedure

3.6.1 Preparation of inoculum

The activated sewage sludge sample was washed three times by settlement and resuspension in culture medium to remove any excessive amounts of dissolved organic carbon (DOC) that may have been present. The washed sample was then maintained on continuous aeration in the laboratory at a temperature of approximately 21°C and used on the day of collection. Determination of the suspended solids level of the activated sewage sludge was carried out by filtering a sample (100 ml) of the washed activated sewage sludge by suction through pre-weighed GF/A filter paper* using a Buchner funnel. Filtration was then continued for a further 3 minutes after rinsing the filter three successive times with 10 ml of deionised reverse osmosis water. The filter paper was then dried in an oven at approximately 105°C for at least 1 hour and allowed to cool before weighing. This process was repeated until a constant weight was attained. The suspended solids concentration was equal to 3.4 g/l prior to use.

3.6.2 Culture medium

The culture medium used in this study (see Appendix 2) was that recommended in the OECD Guidelines.

3.6.3 Preparation of test system

The following test preparations were prepared and inoculated in 5 litre glass culture vessels each containing 3 litres of solution:

a) A control, in duplicate, consisting of inoculated culture medium.

* Rinsed three times with 20 ml deionised reverse osmosis water prior to drying in an oven

b) The reference item (sodium benzoate), in duplicate, in inoculated culture medium to give a final concentration of 10 mg carbon/l.

c) The test item, in duplicate, in inoculated culture medium to give a final concentration of 10 mg carbon/l.

d) The test item plus the reference item in inoculated culture medium to give a final concentration of 20 mg carbon/l to act as a toxicity control (one vessel only).

Each test vessel was inoculated with the prepared inoculum at a final concentration of 30 mg suspended solids (ss)/l. The test was carried out in a temperature controlled room at temperatures of between 19 to 21°C, in darkness. This was a deviation to the study plan which states that the temperature should be 22 ± 2°C. This slight deviation on Days 19, 25 and 26 of the study was considered to have had no adverse effect given that all validation criterion were satisfied.

Approximately 24 hours prior to addition of the test and reference items the vessels were filled with 2400 ml of culture medium and 26.5 ml of inoculum and aerated overnight. On Day 0 the test and reference items were added and the volume in all the vessels adjusted to 3 litres by the addition of culture medium.

The culture vessels were sealed and CO_2-free air bubbled through the solution at a rate of approximately 40 ml/minute and stirred continuously by magnetic stirrer.

The CO_2-free air was produced by passing compressed air through a glass column containing self-indicating soda lime (Carbosorb®) granules.

The CO_2 produced by degradation was collected in two 500 ml Dreschel bottles containing 350 ml of 0.05 M NaOH. The CO_2 absorbing solutions were prepared using purified de-gassed water.

3.6.4 Sampling and analysis

3.6.4.1 CO_2 analysis

Samples (2 ml) were taken from the control, reference and test item first CO_2 absorber vessels on Days 0, 2, 6, 8, 10, 14, 21, 28 and 29 and from the toxicity control first CO_2

absorber vessel on Days 0, 2, 6, 8, 10 and 14. The second absorber vessel was sampled on Days 0 and 29 for the control, reference and test item and on Day 0 for the toxicity control.

The samples taken on Days 0, 2, 6, 8, 10, 14, 21, 28 and 29 were analysed for CO_2 immediately.

On Day 28, 1 ml of concentrated hydrochloric acid was added to each vessel to drive off any inorganic carbonates formed. The vessels were resealed, aerated overnight and the final samples taken from both absorber vessels on Day 29.

The samples were analysed for CO_2 using a Tekmar-Dohrmann Apollo 9000 TOC analyser and a Shimadzu TOC-V_{CSH} TOC analyser. Samples (300 or 50 µl) were injected into the IC (Inorganic Carbon) channel of the TOC analyser. Inorganic carbon analysis occurs by means of the conversion of an aqueous sample to CO_2 by orthophosphoric acid using zero grade air as the carrier gas. Calibration was by reference solutions of sodium carbonate (Na_2CO_3). Each analysis was carried out in triplicate.

3.6.4.2 Dissolved organic carbon (DOC) analysis

Samples (30 ml) were removed from all culture vessels on Day 0 and from the control, reference and test item culture vessels on Day 28 and centrifuged (3500 rpm, 15 minutes) prior to DOC analysis.

The samples were analysed for DOC using a Shimadzu TOC-V_{CPH} TOC Analyser. Samples (50 µl) were injected into the Total Carbon (TC) and Inorganic Carbon (IC) channels of the TOC analyser. Total carbon analysis is carried out at 680°C using a platinum based catalyst and zero grade air as the carrier gas. Inorganic carbon analysis involves conversion by orthophosphoric acid at ambient temperature. Calibration was performed using reference solutions of potassium hydrogen phthalate ($C_8H_5KO_4$) and sodium carbonate (Na_2CO_3) in deionised water. Each analysis was carried out in triplicate.

3.6.4.3 pH measurements

The pH of the test preparations was determined on Day 28, prior to acidification with hydrochloric acid, using a WTW pH/Oxi 340I pH and dissolved oxygen meter.

3.7 Evaluation of Data

3.7.1 Determination of carbon content

The structural formula for the test item supplied by the Sponsor was for a representative structure. Therefore in order to determine the carbon content of the test item, DOC analysis was performed on three replicate 2000 mg/l stock solutions.

From DOC analysis conducted on three separate 2000 mg/l stock solutions (see Appendix 1), the percentage carbon content of the test item was determined to be 1.65%.

The theoretical amount of carbon present in the reference item, sodium benzoate (C_6H_5COONa) was calculated as follows:

$$\frac{\text{No of C atoms x mol wt of C}}{\text{mol wt of sodium benzoate}} \text{ x } 100\%$$

$$\frac{7 \text{ x } 12.011}{144.11} \text{ x } 100 = 58.34\%$$

Thus for a 10 mg C/l test concentration (a total of 51.4 mg of sodium benzoate in 3 litres) the total organic carbon present for sodium benzoate was 30 mg C.

3.7.2 Percentage degradation

The percentage degradation or percentage of Theoretical Amount of Carbon Dioxide ($ThCO_2$) produced is calculated by substituting the inorganic carbon values, given in Table 1, in the following equation:

The values of Replicates R_1 and R_2 are meaned for the control, test and reference items before substitution in the equation.

$$\%\ ThCO_2 (= \%\ \text{degradation}) = \frac{\text{mg IC in test flask} - \text{mg IC in control}}{\text{mg TOC as test chemical}} \times 100\%$$

The percentage degradation from the results of the DOC analysis, see Table 4, is calculated from the equation below. Replicate values are corrected for the mean control value prior to calculation of percentage degradation.

$$\text{Percentage degradation} = \left[1 - \frac{\text{mg DOC in test flask on Day 28}}{\text{mg DOC in test flask on Day 0}}\right] \times 100\%$$

The total CO_2 evolution in the control vessels at the end of the test is calculated from the equation below. The inorganic carbon values for Replicates R_1 and R_2 on Day 28 are meaned before substitution into the equation.

$$\text{Total } CO_2 \text{ evolution} = \text{mg IC in control} \times \frac{100}{\%C \text{ of } CO_2} \times \frac{1}{\text{test volume}}$$

$$= \text{mg IC in control} \times \frac{100}{27.29} \times \frac{1}{3}$$

3.7.3 Validation criteria

The results of the degradation test are considered valid if in the same test the reference item yields ≥ 60% degradation by Day 14.

The test item may be considered to be readily biodegradable if ≥ 60% degradation is attained within 28 days. This level of degradation must be reached within 10 days of biodegradation exceeding 10%.

The toxicity control (test item and sodium benzoate) should attain ≥ 25% degradation by Day 14 for the test item to be considered as non-inhibitory.

The test is considered valid if the difference of the extremes of replicate values of production of CO_2 at the end of the test is less than 20%.

The total CO_2 evolution in the control vessels at the end of the test should not normally exceed 40 mg/l medium.

The IC content of the test item suspension in the mineral medium at the beginning of the test should be < 5% of the TC.

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Harlan Laboratories Ltd, Shardlow, UK archives for five years, after which instructions will be sought as to further retention or disposal.

5. RESULTS

5.1 Preliminary Investigational Work

The results obtained from the samples taken for DOC analysis from the preliminary investigational work indicated that the test item did not adsorb to activated sewage sludge (see Appendix 1). Therefore, for the purpose of the study, the samples taken for DOC analysis were centrifuged to remove the suspended solids present without the loss of any test item.

5.2 Definitive Test

Inorganic carbon values for the test item, reference item, toxicity control and control vessels at each analysis occasion are given in Table 1. Percentage biodegradation values of the test and reference items and the toxicity control are given in Table 2 and the biodegradation curves are presented in Figure 1. Total and Inorganic Carbon values in the culture vessels on Day 0 are given in Table 3, and the results of the Dissolved Organic Carbon analyses performed on Days 0 and 28 are given in Table 4. The pH values of the test preparations on Day 28 are given in Table 5. Observations made on the contents of the test vessels are given in Table 6.

The total CO_2 evolution in the control vessels on Day 28 was 28.33 mg/l and therefore satisfied the validation criterion given in the OECD Test Guidelines.

The IC content of the test item suspension in the mineral medium at the start of the test (see Table 3) was below 5% of the TC content and hence satisfied the validation criterion given in the OECD Test Guidelines.

The difference between the values for CO_2 production at the end of the test for the replicate vessels was <20% and hence satisfied the validation criterion given in the OECD Test Guidelines.

Acidification of the test vessels on Day 28 followed by the final analyses on Day 29 was conducted according to the methods specified in the Test Guidelines. This acidification effectively kills the micro-organisms present and drives off any dissolved CO_2 present in the test vessels. Therefore any additional CO_2 detected in the Day 29 samples originated from dissolved CO_2 that was present in the test vessels on Day 28 and hence

the biodegradation value calculated from the Day 29 analyses is taken as being the final biodegradation value for the test item.

The results of the inorganic carbon analysis of samples from the first absorber vessels on Day 29 showed an increase in all replicate vessels. Inorganic carbon analysis of the samples from the second absorber vessels on Day 29 confirmed that no significant carry-over of CO_2 into the second absorber vessels occurred.

The test item attained 97% degradation after 28 days and satisfied the 10-Day window validation criterion, whereby 60% degradation must be attained within 10 days of the degradation exceeding 10%. The test item can therefore be considered to be readily biodegradable under the strict terms and conditions of OECD Guideline No 301B.

The toxicity control attained 97% degradation after 14 days thereby confirming that the test item was not toxic to the sewage treatment micro-organisms used in the test.

Sodium benzoate attained 99% degradation after 14 days and 109% degradation after 28 days thereby confirming the suitability of the inoculum and test conditions. Degradation values in excess of 100% were considered to be due to sampling/analytical variation.

Analysis of the test media from the test item culture vessels on Days 0 and 28 for Dissolved Organic Carbon (DOC), see Table 4, gave percentage degradation values of 98% and 88% respectively for the test item Replicates R_1 and R_2. Sodium benzoate attained 99% degradation for Replicates R_1 and R_2 calculated from the results of the DOC analyses. The degradation rates calculated from the results of the DOC analyses were similar to those calculated from inorganic carbon analysis.

6. CONCLUSION

The test item attained 97% degradation after 28 days and satisfied the 10-Day window validation criterion, whereby 60% degradation must be attained within 10 days of the degradation exceeding 10%. The test item can therefore be considered to be readily biodegradable under the strict terms and conditions of OECD Guideline No 301B.

Table 1 Inorganic Carbon Values on Each Analysis Occasion

Day	Control (mg IC)				Sodium Benzoate (mg IC)				Test Item (mg IC)				Test Item plus Sodium Benzoate Toxicity Control (mg IC)	
	R_1		R_2		R_1		R_2		R_1		R_2		R_1	
	Abs 1	Abs 2	Abs 1	Abs 2	Abs 1	Abs 2	Abs 1	Abs 2	Abs 1	Abs 2	Abs 1	Abs 2	Abs 1	Abs 2
0	1.05	1.28	1.17	1.40	0.93	1.40	0.93	1.17	0.82	1.63	1.05	1.75	1.05	1.52
2	7.89	-	7.77	-	23.55	-	21.81	-	25.64	-	23.90	-	44.20	-
6	13.84	-	16.26	-	37.25	-	29.52	-	40.71	-	31.95	-	61.13	-
8	15.14	-	19.61	-	40.25	-	32.34	-	43.34	-	35.78	-	65.93	-
10	16.87	-	22.00	-	44.69	-	33.40	-	44.80	-	35.91	-	66.46	-
14	20.17	-	21.76*	-	52.02	-	49.07	-	51.11	-	48.17	-	79.45	-
21	20.62	-	20.73	-	52.28	-	52.95	-	52.50	-	48.45	-	-	-
28	23.63	-	22.74	-	53.09	-	55.78	-	49.73	-	52.64	-	-	-
29	23.71	1.85	23.05	1.63	55.55	1.74	56.89	1.63	51.21	1.63	53.66	1.63	-	-

R_1 – R_2 = Replicates 1 and 2
Abs = CO_2 absorber vessels
- = no value determined
* Duplicate sample analysed as the original result deemed anomalous

Table 2 Percentage Biodegradation Values

Day	% Degradation Sodium Benzoate	% Degradation Test Item	% Degradation Test Item plus Sodium Benzoate Toxicity Control
0	0	0	0
2	50	56	61
6	61	71	77
8	63	74	81
10	65	70	78
14	99	96	97
21	106	99	-
28	104	93	-
29*	109	97	-

- = No degradation result obtained due to toxicity control being terminated after 14 days.
* Day 29 values corrected to include any carry-over of CO_2 detected in Absorber 2

Table 3 Total and Inorganic Carbon Values in the Culture Vessels on Day 0

Test vessel	Total Carbon* (mg/l)	Inorganic Carbon* (mg/l)	IC Content (% of TC)
Sodium Benzoate 10 mg C/l R_1	9.45	0.00	0
Sodium Benzoate 10 mg C/l R_2	9.22	-0.03	0
Test Item 10 mg C/l R_1**	9.18	-0.05	0
Test Item 10 mg C/l R_2**	9.26	-0.08	0
Test Item plus Sodium Benzoate Toxicity Control 20 mg C/l**	18.37	-0.64	0

$R_1 – R_2$ = Replicates 1 and 2
* Corrected for control values. Negative values are due to measured concentrations being less than control values
** Duplicate sample analysed as the original result deemed anomalous

Table 4 Dissolved Organic Carbon (DOC) Values in the Culture Vessels on Days 0 and 28

Test Vessel	DOC* Concentration				
	Day 0		Day 28		
	mg C/l	% of Nominal Carbon Content	mg C/l	% of Initial Carbon Concentration	% Degradation
Sodium Benzoate 10 mg C/l R_1	9.45	95	0.05	1	99
Sodium Benzoate 10 mg C/l R_2	9.23	92	0.09	1	99
Test Item 10 mg C/l R_1	9.23	92	0.15	2	98
Test Item 10 mg C/l R_2	9.33	93	1.10	12	88

$R_1 - R_2$ = Replicates 1 and 2
* Corrected for control values.

Table 5 pH Values of the Test Preparations on Day 28

Test Vessel	pH
Control R_1	7.5
Control R_2	7.5
Sodium Benzoate 10 mg C/l R_1	7.5
Sodium Benzoate 10 mg C/l R_2	7.6
Test Item 10 mg C/l R_1	7.5
Test Item 10 mg C/l R_2	7.5

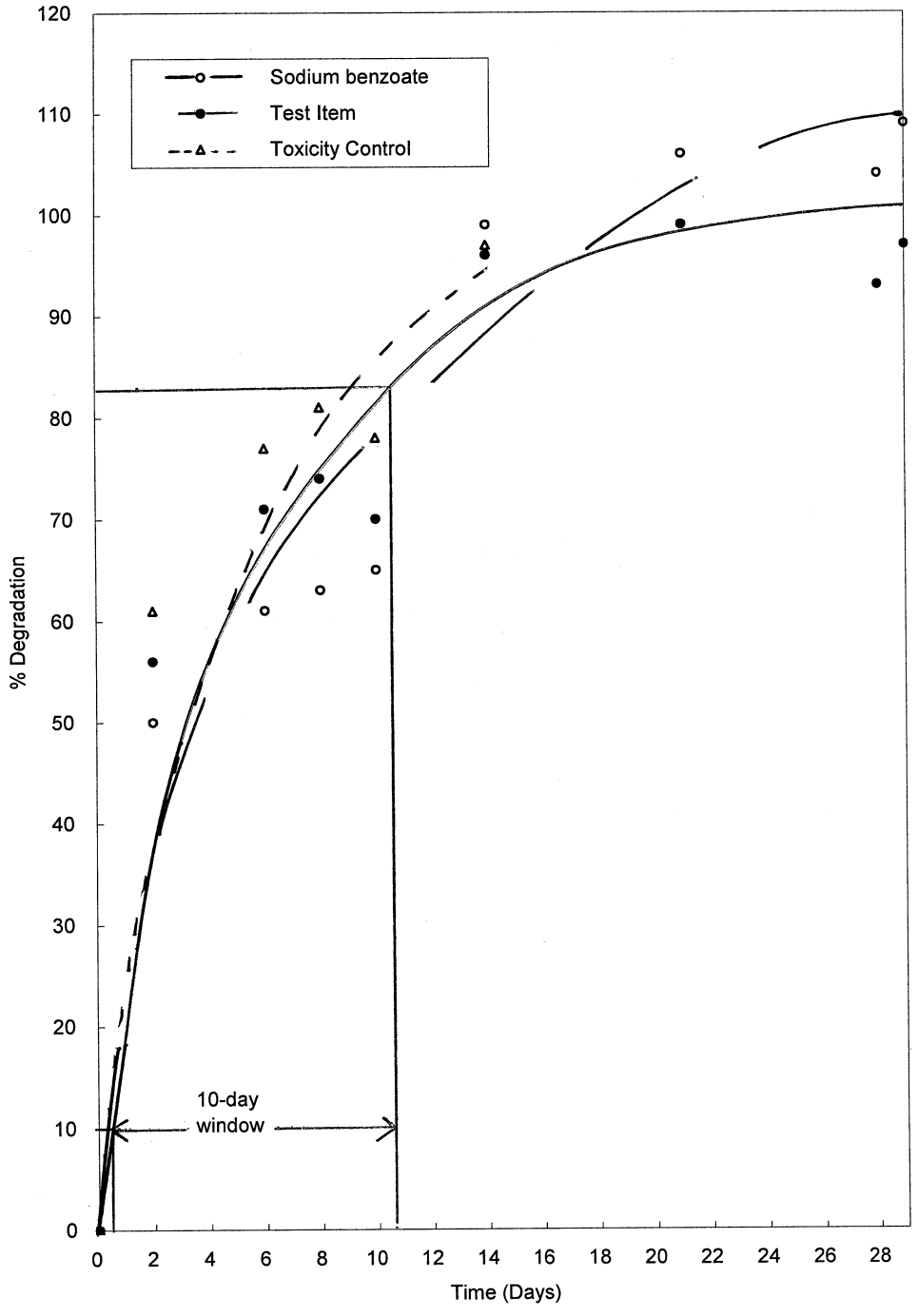
$R_1 – R_2$ = Replicates 1 and 2

Table 6 Observations on the Test Preparations Throughout the Test Period

Test Vessel		Observations on Test Preparations				
		Day 0	Day 6	Day 13	Day 20	Day 27
Control	R_1	Light brown dispersion	Light brown dispersion	Light brown dispersion	Light brown dispersion	Light brown dispersion
	R_2	Light brown dispersion	Light brown dispersion	Light brown dispersion	Light brown dispersion	Light brown dispersion
Reference Item	R_1	Light brown dispersion, no undissolved reference item visible	Light brown dispersion, no undissolved reference item visible	Light brown dispersion, no undissolved reference item visible	Light brown dispersion, no undissolved reference item visible	Light brown dispersion, no undissolved reference item visible
	R_2	Light brown dispersion, no undissolved reference item visible	Light brown dispersion, no undissolved reference item visible	Light brown dispersion, no undissolved reference item visible	Light brown dispersion, no undissolved reference item visible	Light brown dispersion, no undissolved reference item visible
Test Item	R_1	Light brown dispersion, no undissolved test item visible	Light brown dispersion, no undissolved test item visible	Light brown dispersion, no undissolved test item visible	Light brown dispersion, no undissolved test item visible	Light brown dispersion, no undissolved test item visible
	R_2	Light brown dispersion, no undissolved test item visible	Light brown dispersion, no undissolved test item visible	Light brown dispersion, no undissolved test item visible	Light brown dispersion, no undissolved test item visible	Light brown dispersion, no undissolved test item visible
Toxicity Control		Light brown dispersion, no undissolved test or reference item visible	Light brown dispersion, no undissolved test or reference item visible	Light brown dispersion, no undissolved test or reference item visible	-	-

$R_1 – R_2$ = Replicates 1 and 2
- = No observations made due to toxicity control being terminated after 14 days

Figure 1 Biodegradation Curves



Appendix 1 Dissolved Organic Carbon (DOC) Values from the Preliminary Investigational Work

In order to investigate whether the test item adsorbed to activated sewage sludge the following samples were analysed for Dissolved Organic Carbon (DOC) using a Shimadzu TOC-V_{CPH} TOC analyser.

Sample	DOC Concentration		% of Nominal Carbon Content compared to the representative structure
	mg C/l	mg C/l corrected for appropriate control	
Culture medium	<LOQ	-	-
Control, inoculated at 30 mg ss/l, Centrifuged	<LOQ	-	-
2000 mg/l Untreated	33.36	33.36	77
2000 mg/l, inoculated at 30 mg ss/l, Centrifuged	32.40	32.40	74

These results showed, based on the representative chemical structure supplied by the Sponsor, measured concentrations of below 80% of nominal were obtained. As such it was considered appropriate to repeat this work but to also determine the exact carbon content of the sample of test item as received.

The samples were analysed for Dissolved Organic Carbon (DOC) using a Shimadzu TOC-V_{CSH} TOC analyser.

LOQ = Limit of Quantitation (determined down to 1.0 mg C/l).

Appendix 1 (continued) Dissolved Organic Carbon (DOC) Values from Preliminary Investigational Work

Sample	DOC Concentration		% of Measured Carbon Content
	mg C/l	mg C/l corrected for appropriate control	
2000 mg/l Untreated A	32.73	32.73	-
2000 mg/l Untreated B	32.95	32.95	-
2000 mg/l Untreated C	33.07	33.07	-
Culture medium	<LOQ	-	-
Control, inoculated at 30 mg ss/l, Centrifuged	<LOQ	-	-
2000 mg/l Untreated	33.10	33.10	101
2000 mg/l, inoculated at 30 mg ss/l, Centrifuged	33.37	33.37	101

Analysis of the three replicate 2000 mg/l stock solutions showed a mean measured carbon concentration of 32.92 mg/l (1.65 %) was obtained. Comparison of the results obtained from the repeated preliminary investigational work against the actual measured carbon content of the test item showed near nominal concentrations were obtained indicating that the test item did not adsorb to activated sewage sludge. Therefore, for the purpose of the study, the test item concentration was calculated based on the carbon content obtained from DOC analysis and not the molecular formula of the representative structure. The samples taken for DOC analysis were centrifuged to remove the suspended solids present without causing a loss of any test item.

LOQ = Limit of Quantitation (determined down to 1.0 mg C/l).

Appendix 2 Culture Medium

Solution a	KH_2PO_4	8.50 g/l
	K_2HPO_4	21.75 g/l
	$Na_2HPO_4.2H_2O$	33.40 g/l
	NH_4Cl	0.50 g/l
	pH = 7.4	
Solution b	$CaCl_2$	27.50 g/l
Solution c	$MgSO_4.7H_2O$	22.50 g/l
Solution d	$FeCl_3.6H_2O$	0.25 g/l

To 1 litre (final volume) of purified water* was added the following volumes of solutions a – d.

10 ml of Solution a
1 ml of Solution b
1 ml of Solution c
1 ml of Solution d

* Reverse osmosis purified and deionised water (Elga Optima 15+ or Elga Purelab Option R-15 BP)

Appendix 3 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

DH Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

Harlan Laboratories Ltd.
Shardlow Business Park
London Road
Shardlow
Derby
DE72 2GD

TEST TYPE

Analytical Chemistry
Clinical Chemistry
Environmental Fate
Environmental Toxicity
Mutagenicity
Phys.Chem. Testing
Toxicology

DATE OF INSPECTION

20 July 2010

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

29/10/10

Dr. Andrew J. Gray
Head, UK GLP Monitoring Authority

MHRA