Study Title

ACTIVATED SLUDGE RESPIRATION INHIBITION TEST OF ZONYL® [redacted] (OECD 209)

Test Guideline(s)

OECD (1984) Guideline for Testing of Chemicals, Section 2, No. 209: "Activated Sludge, Respiration Inhibition Test", adopted April 4, 1984

Author(s)

William R. Berti, Ph.D.

Study Completion Date

30-August-2004

Test Facility

E. I. du Pont de Nemours and Company
Environmental and Microbiological Sciences & Engineering
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Central Research & Development
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Wilmington, Delaware 19898
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EMSER Study / Project Number

97-03/ 4842

Report Number

EMSER 97-03

PAGE RESERVED FOR SPECIFIC COUNTRY REQUIREMENTS

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The study described in this report, with the exception of the items listed below was conducted in compliance with the following GLP Standards:

- United States Environmental Protection Agency, (FIFRA), Title 40 Code of Federal Regulations Part 160 (effective October 16, 1989)

which are consistent with:

- The OECD Principles of Good Laboratory Practice (as revised 1997), ENV/MC/CHEM/98(17), OECD, Paris, 1998
- MAFF Japan Good Laboratory Practice Standards (11 NohSan Number 6283).

The following items are exceptions to the GLP Standards. These items do not impact the validity of the study.

The test chemical was not characterized under GLP. It is a commercially available material that was provided by the submitter. The certificate of analysis (COA) was provided by the manufacturer and the accuracy of the data is considered sufficient for the purposes of this study.

The reference chemical was not characterized under GLP. It is a commercially available material. The COA was provided by the supplier and the accuracy of the data is considered sufficient for the purposes of this study.

The Study Protocol was not signed by the study director prior to the start of the study. The study director gave approval to start the study by an electronic message.

Analysis to confirm concentration and uniformity of the test solutions was not performed. This is believed not to affect the validity of the study because the test chemical was accurately measured and added to the test vessels.

Study Director

William R. Berti, Ph.D.
Senior Research Biologist
DuPont Environmental and Microbiological Sciences & Engineering

30-Aug-2004
Date

Submitter

QUALITY ASSURANCE STATEMENT

Study Number
97-03/4842

Study Title
Activated Sludge Respiration Inhibition Test of Zonyl® [redacted] (OECD 209)

The conduct of this study was subjected to periodic Quality Assurance inspections. The dates of inspection are indicated below:

Study Phase Inspected	Inspection/Audit Dates	Dates Findings Reported to Study Director	Dates Findings Reported to Management
Study Conduct	14 Nov 2003	19 Nov 2003	19 Nov 2003
Report	07-08 Jul 2004	12 Jul 2004	12 Jul 2004

Maryanne B. Oster
Staff Quality Assurance Auditor
DuPont Haskell Laboratory for Health & Environmental Services

27 August 2004
Date

CERTIFICATION OF AUTHENTICITY

ACTIVATED SLUDGE RESPIRATION INHIBITION TEST OF ZONYL® [redacted] (OECD 209)

We, the undersigned, declare that the work described in this report was performed under our supervision, and that this report provides an accurate record of the procedures and results.

Report by:

William R. Berti, Ph.D.
Senior Research Biologist

30-Aug-2004
Date

Approved by:

John T. Gannon, Ph.D.
Research Manager

08-30-04
Date

Study Initiation Date:
17-Nov-2003

Date Study Completed:
30-August-2004

Submitter:
E. I. du Pont de Nemours and Company
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U.S.A.

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ACTIVATED SLUDGE RESPIRATION INHIBITION TEST OF ZONYL®FCC (OECD 209)

1.0 SUMMARY

Test System:

Zonyl®FCC was tested for toxicity towards activated sludge according to OECD Guideline 209 in the version dated 4-April-1984. For the determination of the toxic behavior of the test substance, activated sludge from the aeration tank of a municipal sewage treatment plant was exposed to the test chemical over a range of 10 to 1000 mg L^{-1}. After 30 minutes of incubation, the respiration rate of the activated sludge was determined and compared to the respiration rate of the controls.

Findings:

Under the conditions of the test, the highest test concentration of the test substance, 1000 mg L^{-1}, exhibited <20% inhibition of the respiration rate (the detection limit of the system).

Conclusions:

The EC_{50} of the test chemical could not be quantified because the highest nominal test concentration of 1000 mg/L resulted in less than 20% inhibition after 30 m of contact time. The EC_{50} of the test chemical is estimated to be greater than 1000 mg L^{-1}.

2.0 GENERAL STUDY INFORMATION

Study Objectives

The aim of this study was the determination of the acute toxic behaviour of Zonyl®[redacted] towards the microorganisms of activated sludge according to OECD guideline 209 in the version of April 4, 1984. The objectives of this study were to:

- Determine the effect of the test substance, Zonyl®[redacted] on microorganisms from municipal sewage sludge and
- Assess the potential of the test chemical to adversely affect aerobic municipal sewage treatment plants

The testing protocol used a microbial inoculum derived from a municipal wastewater treatment plant and an artificial sewage feed. The respiration rate of the test system was determined after a 30 m time period under controlled laboratory conditions.

Test System Justification

The test system is outlined by the OECD guideline 209 and was requested by the submitter.

Study Personnel

E. I. du Pont de Nemours and Company
Environmental and Microbiological Sciences & Engineering

Management:	John T. Gannon, Ph.D.
Study Director:	William R. Berti, Ph.D. E. I. du Pont de Nemours and Company Environmental and Microbiological Sciences & Engineering Central Research & Development, Glasgow Building 300 P.O. Box 6101 Newark, DE 19714-6101 USA
Principle Investigator:	William R. Berti, Ph.D. Study Phase(s): All Same as above
Technical Personnel:	Lynn Ann Dekleva Patrick W. Folsom Mark Starr Lisa M. Sulecki

Study Execution Dates

Study Initiation Date:	17-Nov-2003
Experimental Start Date:	13-Nov-2003
Experimental Termination Date:	14-Nov-2003
Study Completion Date:	30-Aug-2004

3.0 MATERIALS AND METHODS

3.1 Test Guidelines

The purpose of the procedure is to provide a rapid screening method whereby substances that may adversely affect aerobic microorganisms from municipal sewage treatment plants can be identified and to indicate the suitable non-inhibitory concentrations of test substances to be used in biodegradability tests. The test uses a microbial inoculum and a synthetic sewage feed. The respiration rate of the test system is measured after an exposure period of 30 minutes under controlled laboratory conditions. The inhibitory effect of the test chemical at the particular concentrations is expressed as percentage of the mean respiration rate of the controls.

3.2 *Test System*

3.2.1 *Chemical System*

3.2.1.1 *Test Item*

Name:	Zonyl®[redacted] ✓
Synonyms:	NA
Active substance:	Zonyl®[redacted] ✓
CAS Name:	NA
CAS Number:	NA
EMSE Sample Number:	CRD12005
Supplier	Scott Krenzel DuPont Haskell Laboratory Newark, DE
Submitter Sample:	H-24616
Lot Number:	436

3.2.1.2 *Reference Item*

Toxic Reference

Name:	3,5-dichlorophenol
Active substance(s):	3,5-dichlorophenol
CAS Number(s):	591-35-5
Product Number:	Aldrich D70600
Lot Number:	15809KI
EMSE Sample No.	M57657
Concentration of a.s., nominal:	97%
Concentration of a.s., analyzed:	99.7%
Certificate of Analysis Date:	August 2000
Expiry Date:	August 2005
Date Received:	January 2002
Appearance/Color:	White Powder
Storage Conditions:	Store in a cool dry place; keep tightly closed
Safety Precautions:	Irritant; wear suitable gloves and eye/face protection.
Manufacturer:	Aldrich Chemical Co., Inc. 1001 West St. Paul Milwaukee, WI 53233 USA

3.2.1.3 Test Vehicle

No test vehicle was used in this study for the test substance. The test chemical was mixed with synthetic sewage feed, water, and inoculum before addition to the test vessel.

3.2.1.4 Application Information

The test chemical was added to the test vessels from a stock solution at the following concentrations: 1000, 320, 100, 32 and 10 mg L^{-1}.

3.2.2 Biological System

Secondary activated sludge from Cecil County Fletchwood Facility, Maryland (USA) Publically Owned Treatment Works (POTW) was used as the microbial inoculum. The activated sludge was aerated and used within 24 h of collection. The solids were separated by coarse filtration, centrifuged at 10,000 m s^{-2} for approximately 10 minutes, washed twice, and resuspended in tap water. The solids concentration was determined by dry weight and adjusted to yield an inoculum concentration equivalent to about 4 g per liter (on dry weight basis).

3.2.3 Physical System

3.2.3.1 Test Units

The test vessels used in the incubation period were glass flasks of approximately 1-liter. For the measurement of the respiration rate, samples were transferred to 300-mL Biological Oxygen Demand (BOD) glass bottles.

3.2.3.2 Test Conditions

Test solutions were aerated for a 30-minute contact period at room temperature.

3.3 Test Conduct

All test vessels were 1-liter glass flasks and contained a final volume of 500 mL. The test solution contained 16 mL of synthetic sewage feed, 200 mL microbial inoculum (containing approximately 4 g dry weight/L), the test chemical at the appropriate test solution concentration, and tap water to a final test volume of 500 mL. The test was initiated with the first positive control by combining the sewage feed, tap water, and inoculum in a 500 mL graduated cylinder and transferring it to the appropriately labeled test vessel. Aeration at 0.5 to 1.0 liter per minute initiated the test. The test vessel was continuously stirred using a stir bar and stir plate. The procedure was repeated at approximately 15-minute intervals with the reference chemical (at three concentrations) and then the test chemical to give a series of test vessels containing different concentrations of the test substance. A negative (abiotic) control at the highest test concentration was initiated by combining sewage feed, tap water, and test substance stock solution at the highest concentration in a 500 mL graduated cylinder. The solution was transferred to the test vessel to begin aeration. The final flask was a second positive control, prepared exactly as the first. The test was conducted so that the reference and test substances were bracketed by the two controls:

1. first positive control
2. reference chemical at 3 concentrations
3. test chemical at 5 concentrations

4. negative (abiotic) control at highest concentration of test substance
5. second positive control

3.4 *Parameters Observed*

3.4.1 *Analysis of Test Systems*

The respiration rate of a well-mixed sample of each treatment was determined after exactly 30 minutes of incubation time with aeration. The oxygen concentration was measured with an oxygen electrode and recorded over a period of up to about 10 minutes. During measurement, the samples were continuously stirred with a stirrer built into the oxygen electrode and measurements were recorded using an attached printer. The rate of oxygen consumption (in mg O_2 L^{-1} h^{-1}) was determined from the most linear part of the respiration curve.

The temperature was measured in the positive control flasks at the start and the end of the incubation period.

3.5 *Result Analysis*

The respiration rate was calculated from the recorder output as mg O_2 L^{-1} h^{-1} over a period of approximately 10 minutes or less. The portion of the respiration curve over which the respiration rate was determined should be linear.

The inhibitory effects were determined by comparing the rates at each concentration of either test or reference chemical to the rates in the controls. The results are expressed as percentage of the mean value of the respiration rates of the two controls according to:

$$\left[1 - \frac{2R_S}{R_{c1} + R_{c2}}\right] * 100 = \text{Percent inhibition} \qquad (1)$$

where

Rs = oxygen consumption rate at tested concentration of test substance

R_{c1} = oxygen consumption rate of positive control 1

R_{c2} = oxygen consumption rate of positive control 2

The percent inhibition was calculated at each test concentration as above.

A linear regression analysis was performed using the Regression Analysis Tool in Microsoft® Excel 2000. Percent inhibition was used as the input y-values. Log10 of the test or reference chemical concentration was used as the input x-values. From this analysis, an equation relating chemical concentration to percent inhibition was determined at the 95% confidence level (p = 0.05)

$$\text{Percent Inhibition} = A * [\log10\ (EC)] + B \qquad (2)$$

where:

A = X Variable 1, determined by the regression analysis

EC = Effective Concentration of the Test or Reference Chemical, in mg/L

B = Intercept, determined by the regression analysis

To calculate an EC_{50}, equation (2) was solved for $[\log_{10}(EC)]$. Percent Inhibition was then set equal to 50.

$$\text{Log}_{10}(EC_{50}) = (\text{Percent Inhibition} - B)/ A \qquad (3)$$

For A = 50.933 and B = -5.404

$$\text{Log}_{10}(EC_{50}) = (50 - (-5.404))/ 50.933)$$

$$= 1.088$$

$$EC_{50} = 10^{1.088} = 12 \text{ mg/L}$$

Similarly, an EC20 and EC80 can be calculated by using a Percent Inhibition equal to 20 or 80, respectively, in equation (3). For example:

$$\text{Log}_{10}(EC_{20}) = (20 - (-5.404))/ 50.933)$$

$$= 0.4988$$

$$EC_{20} = 10^{0.4988} = 3 \text{ mg/L}$$

The percent inhibition also was plotted against concentration as a log-normal (or log-probability) graph, from which an EC_{50} value can be derived graphically.

3.6 *Validity Criteria of the Study*

The test results are valid if:

- The two positive control respiration rates (PCRR) rates are within 15 percent of each other.
- The EC_{50} of reference chemical 3,5-dichlorophenol is in the accepted range of 5 to 30 mg L^{-1}.

4.0 RESULTS AND DISCUSSION

- The test chemical showed no acute toxic effect towards the microbial inoculum (activated sludge) at the highest concentration of 1000 mg L^{-1} that was tested.
- EC_{50} of the reference chemical was 12 mg 3,5-dichlorophenol per liter, which was within the acceptable range of from 5 to 30 mg L^{-1}.
- The respiration rates of the two positive controls were within 4%.

5.0 CONCLUSIONS

- The EC_{50} of the test chemical Zonyl®([redacted]) is estimated to be greater than 1000 mg L^{-1}.
- The EC_{50} of the reference chemical 3,5-dichlorophenol is 12 mg L^{-1}.
- The respiration rates of the two positive controls were within 15% of each other.
- The test is valid.

6.0 RETENTION OF RECORDS

For the periods demanded by GLP guidelines and specific country requirements, study documents and materials will be stored in the archives of the DuPont CR&D Environmental & Microbiological Sciences and Engineering (EMSE) archives and/or Iron Mountain Records Management, Wilmington, Delaware 19802 USA, including but not limited to:

- study protocol;
- any protocol and/or report amendments or addenda or SOP deviations;
- all raw data;
- one original signed copy of the final report;
- all documentation generated by the Quality Assurance Unit (to be archived by the Quality Assurance Unit, separate from study records);
- laboratory-specific or site-specific raw data such as personnel files, instrument, equipment, refrigerator, and/or freezer raw data.

Documents and materials are archived according to the principles of Good Laboratory Practice in the organization of the testing facility.

7.0 DISPOSAL OF TEST ITEM

After issuance of the final report, the remaining test chemical will be stored at the DuPont EMSE laboratory until its expiration date and then destroyed by burning, unless other arrangements are made between the submitter and the Test Facility.

8.0 REFERENCES

1. OECD (1984) Guideline for Testing of Chemicals, Section 2, No. 209: "Activated Sludge, Respiration Inhibition Test", adopted April 04, 1984
2. International Standard ISO 8192: "Water quality – Test for inhibition of oxygen consumption by activated sludge". First edition 1986-07-15. Ref. No. ISO 8192-1986 (E).

TABLE 1:
INFLUENCE OF TEST CHEMICAL ZONYL® [redacted] AND REFERENCE CHEMICAL 3, 5-DICHLOROPHENOL ON THE O_2 CONSUMPTION OF ACTIVATED SLUDGE

No.	Treatment	Concentration	O_2 Concentration†		O_2 Consumption	Inhibition‡
			Start	End		
		mg/L	*mg O_2/L*		*mg O_2/L/h*	*%*
1	Positive Control 1	0	6.32	2.72	44.0	NA¶
11	Positive Control 2	0	4.37	2.61	42.6	NA
	Mean				43.3	
	Standard deviation				1.02	
2	3,5-dicholorophenol	3.2	6.26	3.19	33.4	22.9
3	3,5-dicholorophenol	10	6.50	3.54	23.5	45.7
4	3,5-dicholorophenol	32	8.00	6.89	12.2	71.9
5	Zonyl® [redacted]	10	4.98	2.53	41.8	3.58
6	Zonyl® [redacted]	32	5.18	2.74	41.9	3.31
7	Zonyl® [redacted]	100	6.52	2.64	42.1	2.73
8	Zonyl® [redacted]	320	6.38	2.52	41.9	3.27
9	Zonyl® [redacted]	1000	6.35	2.38	39.7	8.25
10	Zonyl(R) [redacted] (negative control)	1000	8.69	8.71	-0.11	100

†The readings of the Dissolved O_2 meter at start and end of linear portion during the period over which respiration rate was calculated between approximately 6.5 and 2.5 mg O_2/L or over a 10 min period when the repiration rate was low.

‡Inhibition of the reference and test substances was calculated using the average value of the positive control solutions. A negative value indicates that respiration was enhanced, not inhibited.

¶ NA = Not Applicable.

FIGURE 1:
INFLUENCE OF THE REFERENCE CHEMICAL 3,5-DICHLOROPHENOL ON THE RESPIRATION RATE OF AEROBIC WASTEWATER MICROORGANISMS AFTER 30 MINUTES OF EXPOSURE

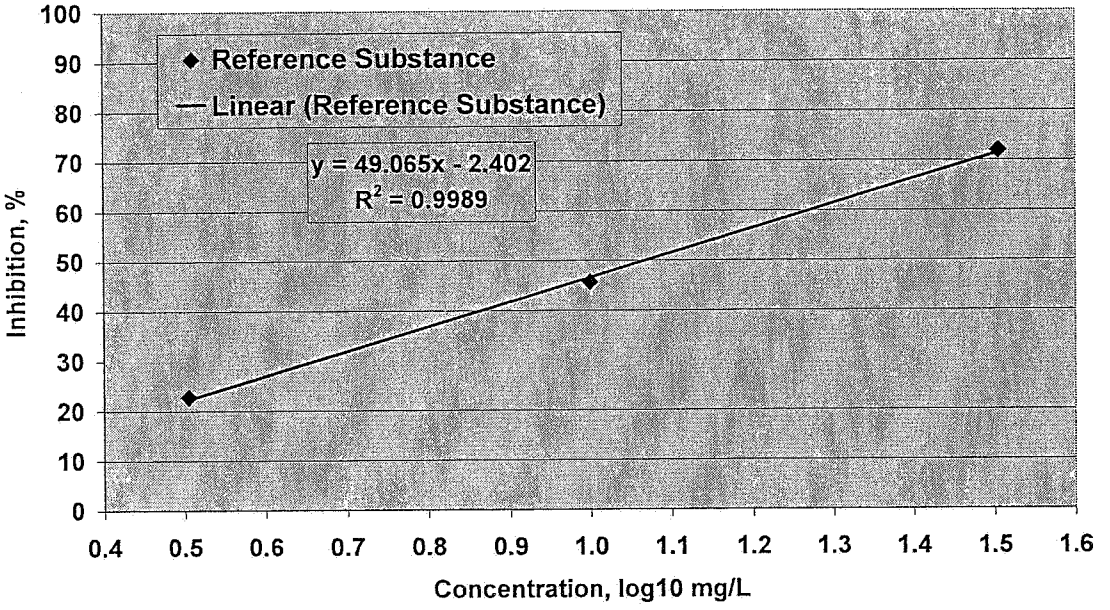
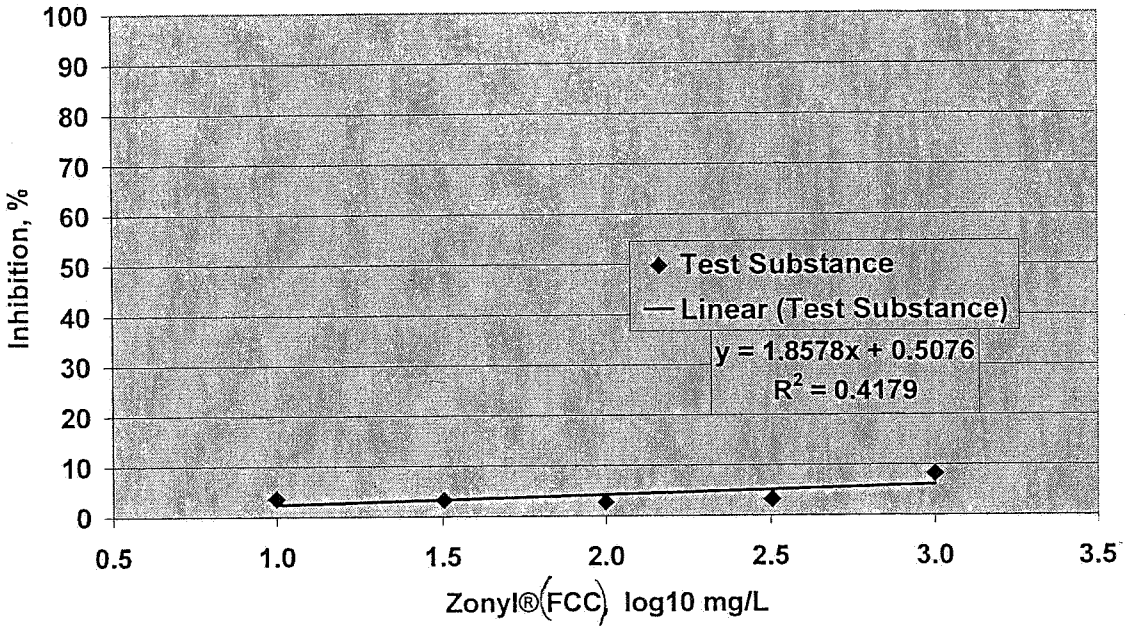


FIGURE 2:
INFLUENCE OF THE TEST CHEMICAL ZONYL® [redacted] ON THE RESPIRATION RATE OF AEROBIC WASTEWATER MICROORGANISMS AFTER 30 MINUTES OF EXPOSURE



APPENDIX A:
DATA TABLES AND CALCULATIONS FOR POSITIVE CONTROLS, REFERENCE AND TEST CHEMICALS

Positive Controls

Total Time	Positive Control 1	Positive Control 2
h	*Dissolved Oxygen, mg/L*	
0.00	8.96	8.94
0.01	6.69	4.48
0.02	6.32	4.37
0.03	5.97	4.04
0.03	5.63	3.64
0.04	5.31	3.29
0.05	4.68	2.95
0.06	4.34	2.61
0.07	4.02	2.24
0.08	3.70	1.91
0.08	3.35	1.50
0.09	3.06	1.20
0.10	2.72	0.87
0.11	2.40	
0.12	2.06	
0.13	1.77	

Reference Chemical: 3,5-dichlorophenol

Total Time	Reference 1: 3.2 mg/L	Reference 2: 10 mg/L	Reference 3: 32 mg/L
h	*Dissolved Oxygen, mg/L*		
0.00	9.07	9.36	8.09
0.01	7.89	6.50	8.00
0.02	7.65	6.28	7.91
0.03	7.36	6.07	7.78
0.03	7.16	5.88	7.68
0.04	6.83	5.68	7.59
0.05	6.55	5.48	7.50
0.06	6.26	5.27	7.39
0.07	5.96	5.08	7.30
0.08	5.68	4.87	7.19
0.08	5.38	4.71	7.07
0.09	5.10	4.50	6.98
0.10	4.83	4.32	6.89
0.11	4.56	4.12	
0.12	4.29	3.93	
0.13	4.01	3.73	
0.13	3.71	3.54	
0.14	3.46		
0.15	3.19		
0.16			

Test Chemical: Zonyl®[redacted]

Total Time	Test Chemical, 10 mg/L	Test Chemical, 32 mg/L	Test Chemical, 100 mg/L	Test Chemical, 320 mg/L	Test Chemical, 1000 mg/L	Abiotic Control, 1000 mg/L
h			*Dissolved Oxygen, mg/L*			
0.00	8.58	8.05	8.20	8.84	8.64	9.03
0.01	4.98	5.18	6.52	6.71	7.00	8.69
0.02	4.60	4.83	6.13	6.38	6.68	8.71
0.03	4.22	4.49	5.81	6.03	6.35	8.72
0.03	3.90	4.15	5.44	5.66	6.04	8.74
0.04	3.55	3.79	5.09	5.26	5.70	8.74
0.05	3.21	3.45	4.74	4.97	5.36	8.72
0.06	2.86	3.08	4.37	4.62	5.04	8.77
0.07	2.53	2.74	4.05	4.26	4.71	8.73
0.08	2.20	2.39	3.68	3.94	4.39	8.73
0.08	1.88	2.09	3.37	3.56	4.01	8.76
0.09	1.47	1.75	2.98	3.22	3.71	8.72
0.10	1.14	1.36	2.64	2.88	3.39	8.72
0.11	0.83	1.03	2.31	2.52	3.07	8.73
0.12			2.00	2.19	2.71	8.71
0.13			1.58	1.85	2.38	
0.13			1.25	1.48		
0.14				1.11		