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November 12, 1997

OPPT Document Control Officer – TSCA 8(e)
U.S. Environmental Protection Agency
401 M. Street, SW
Washington, DC 20460

Via Certified Mail
Return Receipt Requested

**Subject: Substantial Risk Notification under TSCA § 8(e) for
Irgasan® DP300R (Generic Name: triclosan; CAS No. 3380-34-5)**

Dear Sir or Madam:

In accordance with the reporting requirements of TSCA § 8(e) and FIFRA § 6(a)(2), the Consumer Care Division of Ciba Specialty Chemicals Corporation ("Ciba") is hereby providing to the U.S. Environmental Protection Agency notification of scientific results indicating previously unobserved environmental effects of triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol).

Triclosan is a FIFRA registered antimicrobial substance (EPA Registration No. 70404-2). However, since triclosan is also listed under the TSCA Inventory, and may therefore also be subject to the requirements of TSCA § 8(e), we are submitting this information to EPA within the 15-day TSCA § 8(e) notification period and have requested that OPP forward a copy of our 6(a)(2) transmittal to OPPT.

A copy of this transmittal and associated reports is enclosed for your convenience. If you should have any questions regarding this submission, please contact me at (910) 801-2493.

Sincerely,



Contains No C.

Carl D. D'Ruiz, MPH
Director, Regulatory Affairs & Product Stewardship



8EHQ-97-14064

Attachments

CC: J. Plautz
K. Hostetler
A. Wiedow

4090 Premier Drive
P.O. Box 2444
High Point, NC 27261-2444



8898000033

Tel. 910 801 2000

November 12, 1997

Document Processing Desk - 6(a)(2)
Office of Pesticide Programs
U.S. Environmental Protection Agency
Room 266A
Crystal Mall #2
1921 Jefferson Davis Highway
Arlington, VA 22202

Ciba

Via Next Day Courier

9710117 P1 8-35

Subject: FIFRA Section 6(a)(2) Adverse Environmental Effects Report for Irgasan® DP300R (Generic Name: triclosan; EPA Registration No. 70404-2)

Dear Sir or Madam:

In accordance with the reporting requirements of FIFRA Section 6(a)(2), the Consumer Care Division of Ciba Specialty Chemicals Corporation ("Ciba") is hereby providing to the U.S. Environmental Protection Agency notification of scientific results indicating previously unobserved environmental effects of triclosan. Ciba's Consumer Care Division sells this antimicrobial active ingredient under the trade name Irgasan DP300R (CAS No. 3380-34-5, chemical name: 5-chloro-2-(2,4-dichlorophenoxy)phenol).

Results from a study of the effects of triclosan on growth and reproduction in five species of aquatic plants indicated growth inhibition at exposure concentrations lower than those tested previously. In the present study (Carolina Ecotox, Inc., Study No. 21-02-1; 13 October 1997), four species of microalgae (*Anabaena flos-aquae*, *Navicula pelliculosa*, *Selenastrum capricornutum*, *Skeletonema costatum*) and one vascular plant (*Lemna gibba*) were exposed to triclosan nominal concentrations of 0, 0.5, 2.5, 12.5, or 62.5 ug/L.

For *Anabaena flos-aquae* and *Navicula pelliculosa* the growth inhibition EC_{50} was 0.996 ug/L (95% confidence interval: 0.72 - 1.3 ug/L). The other species showed much higher growth inhibition effective concentrations (4.4 up to 66 ug/L). A copy of this study is included with this submission.

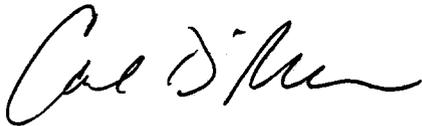
Under the recently revised requirements for FIFRA 6(a)(2) reporting of environmental effects, we are herewith also reporting our preliminary data for the detection of triclosan in the final effluent of two municipal wastewater treatment plants. Analytical results from this study revealed triclosan concentrations of 0.2 and 0.9 ug/L. We have not yet received the final reports for these non-routine monitoring studies.

Irgasan® DP300R – FIFRA 6(a)(2)
Page 2 of 2

Since triclosan is also listed under the TSCA Chemical Inventory, and may therefore also be subject to the requirements of TSCA § 8(e), we are submitting this information to EPA within the 15-day TSCA § 8(e) notification period and request that OPP forward a copy of this transmittal to OPPT.

A copy of the above-mentioned reports is enclosed as required. If you should have any questions about this submission, please contact me at (910) 801-2493.

Sincerely,



Carl D. D'Ruiz, MPH
Director, Regulatory Affairs & Product Stewardship

Attachments

Cc:

E. Finkelman
K. Hostetler
A. Wiedow
J. Plautz



US Monitoring Columbus, Ohio Results Report

Authors: Dr. Bert Schatowitz, Dr. Armin Hauk, Martin Jacob
Date: 04 August 1997
Test No.: 242.96.255

1. Samples

Sampling location: Columbus, Ohio, sewage treatment plant.
Sampling Date: 10/8/96.

1. Raw influent (2 bottles), 24 h composite
2. Primary effluent (2 bottles), 24 h composite
3. Final effluent (2 bottles), 24 h composite
4. Primary sludge (2 bottles), grab sample
5. Activated sludge (2 bottles), grab sample

Temperature of all samples on receipt: 6°C. pH of water samples: 2-3 for raw influent, 2 for primary and final effluent. 3 mL of conc. phosphoric acid were added to raw influent samples and 1 mL to primary and final effluent samples.

On-site spiking of the samples according to the attached spiking protocol "Addition of the ¹³C-Triclosan Spike to Monitoring Samples". Concentration of the spike solution: 2.99 mg ¹³C-TCS/50 mL ethanol.

Dry weight of sludges determined after freeze-drying: Primary sludge: 5.2% (bottle 1) and 5.4% (bottle 2); activated sludge: 1.4% (bottle 1) and 0.9% (bottle 2).

2. Results

Raw influent:

| Sample | µg/L | | | | |
|------------|------|----------|----------|----------|------|
| | TCS | TetraII | TetraIII | Penta | O-Me |
| Bottle 1 | 4.31 | (0.010)* | (0.020) | (0.016) | nq** |
| Bottle 1 | 4.27 | (0.010) | (0.015) | (0.012) | nq |
| Bottle 2 | 7.69 | (0.010) | (0.020) | (0.016) | nq |
| Bottle 1+2 | 5.39 | (0.020) | (0.025) | (0.025) | nq |
| Bottle 1+2 | 5.93 | (0.015) | (0.025) | (0.020) | nq |
| Mean | 5.87 | (0.0125) | (0.021) | (0.0175) | nq |

* values in parenthesis: below the validated limit of quantitation of 0.05 µg/L, but the higher chlorinated closans were detectable at the given approx. concentrations.

** nq: not quantifiable. TCS-O-methyl ether was qualitatively detectable in all samples, but the method was not validated for the determination of the methyl ether. By using the standard addition method for quantitation of TCS and the other closans also TCS-O-Me was added as a standard and recoveries were acceptable (around 50%). Calculations of the concentration of the O-methyl ether resulted in approx. 0.02-0.05 µg/L.

Primary effluent:

| <i>Sample</i> | <i>µg/L</i> | | | | |
|---------------|-------------|----------------|--------------------|--------------------|-------------|
| | <i>TCS</i> | <i>TetraI</i> | <i>TetraIII</i> | <i>Penta</i> | <i>O-Me</i> |
| Bottle 1 | 1.91 | <LOD* | (0.010)** | (0.020) | nq*** |
| Bottle 2 | 2.05 | <LOD | <LOD | <LOD | nq |
| Mean | 1.98 | <LOD | (<0.010) | (<0.020) | nq |

* <LOD: below limit of detection of 0.01 µg/L.

** values in parenthesis: below the validated limit of quantitation of 0.05 µg/L, but the higher chlorinated closans were detectable at the given approx. concentrations.

*** nq: not quantifiable. TCS-O-methyl ether was qualitatively detectable in all samples, but the method was not validated for the determination of the methyl ether. By using the standard addition method for quantitation of TCS and the other closans also TCS-O-Me was added as a standard and recoveries were acceptable (around 50%). Calculations of the concentration of the O-methyl ether resulted in approx. 0.02-0.05 µg/L.

Final effluent:

| <i>Sample</i> | <i>µg/L</i> | | | | |
|---------------|--------------|----------------|-----------------|----------------|-------------|
| | <i>TCS</i> | <i>TetraI</i> | <i>TetraIII</i> | <i>Penta</i> | <i>O-Me</i> |
| Bottle 1 | 0.285 | <LOD* | <LOD | <LOD | nq*** |
| Bottle 2 | 0.125 | <LOD | <LOD | <LOD | nq |
| Bottle 1+2 | 0.119 | (0.020)** | (0.025) | (0.025) | nq |
| Bottle 1+2 | 0.151 | <LOD | <LOD | <LOD | nq |
| Mean | 0.182 | <LOD | <LOD | <LOD | nq |

**Primary sludge:**

| <i>Sample</i> | $\mu\text{g/g}$ dry weight | | | | |
|---------------|----------------------------|----------------|-----------------|--------------|-------------|
| | <i>TCS</i> | <i>TetraII</i> | <i>TetraIII</i> | <i>Penta</i> | <i>O-Me</i> |
| Bottle 1 (IS) | 4.71 | 0.12 | 0.16 | 0.38 | 0.15 |
| Bottle 2 (IS) | 4.99 | 0.07 | 0.08 | 0.09 | 0.14 |
| Bottle 1 (SA) | 5.03 | 0.07 | 0.07 | 0.09 | 0.16 |
| Bottle 2 (SA) | 5.28 | 0.07 | 0.07 | 0.08 | 0.16 |
| Mean | 5.00 | 0.08 | 0.10 | 0.16 | 0.15 |

Activated sludge:

| | $\mu\text{g/g}$ dry weight | | | | |
|---------------|----------------------------|----------------|-----------------|---------------|-------------|
| | <i>TCS</i> | <i>TetraII</i> | <i>TetraIII</i> | <i>Penta</i> | <i>O-Me</i> |
| Bottle 1 (IS) | 0.49 | (0.03)* | (0.01) | (0.03) | 0.13 |
| Bottle 2 (IS) | 0.51 | (0.03) | (0.01) | (0.04) | 0.21 |
| Bottle 1 (SA) | 0.55 | (0.03) | (0.01) | (0.02) | 0.20 |
| Bottle 2 (SA) | 0.57 | (0.03) | (0.01) | (0.04) | 0.23 |
| Mean | 0.53 | (0.03) | (0.01) | (0.03) | 0.19 |

* values in parenthesis: below the validated limit of quantitation of 0.04-0.07 $\mu\text{g/g}$, but the higher chlorinated closans were detectable at the given approx. concentrations.

IS: Quantitation by internal standard method using the on-site spiked ^{13}C -TCS.

SA: Quantitation by standard addition method using additional ^{13}C -TCS spiked in the lab before sample preparation.

Recoveries**Raw influent:**

| <i>Sample</i> | %Recovery | | | | |
|---------------|---------------------------------------|--------------------------|------------------------------|-------------------------------|----------------------------|
| | <i>¹³C-TCS on-site</i> | <i>TCS lab spike</i> | <i>TetraII lab spike</i> | <i>TetraIII lab spike</i> | <i>Penta lab spike</i> |
| Bottle 1 | 144 | 91 | 77 | 82 | 65 |
| Bottle 1 | 181 | 108 | 85 | 93 | 72 |
| Bottle 2 | 183 | 72 | 92 | 93 | 86 |
| Bottle 1+2 | 174 | 94 | 103 | 101 | 86 |
| Bottle 1+2 | 171 | 87 | 91 | 88 | 69 |

Primary effluent:

| <i>Sample</i> | %Recovery | | | | |
|---------------|---------------------------------------|--------------------------|------------------------------|-------------------------------|----------------------------|
| | <i>¹³C-TCS on-site</i> | <i>TCS lab spike</i> | <i>TetraII lab spike</i> | <i>TetraIII lab spike</i> | <i>Penta lab spike</i> |
| Bottle 1 | 85 | 79 | 53 | 61 | 52 |
| Bottle 2 | 81 | 72 | 50 | 62 | 58 |

Final effluent:

| <i>Sample</i> | %Recovery | | | | |
|---------------|---------------------------------------|--------------------------|------------------------------|-------------------------------|----------------------------|
| | <i>¹³C-TCS on-site</i> | <i>TCS lab spike</i> | <i>TetraII lab spike</i> | <i>TetraIII lab spike</i> | <i>Penta lab spike</i> |
| Bottle 1 | 96 | 69 | 54 | 58 | 32 |
| Bottle 2 | 89 | 66 | 47 | 55 | 30 |
| Bottle 1+2 | 105 | 83 | 69 | 66 | 28 |
| Bottle 1+2 | 112 | 91 | 95 | 102 | 51 |

Sludges:

| <i>Sample</i> | % Recovery | |
|----------------------------|---|---|
| | ¹³ C-TCS <i>on-site spike</i> | ¹³ C-TCS <i>lab spike</i> |
| Primary sludge, bottle 1 | ≈10 | 116 |
| Primary sludge, bottle 2 | 120 | 102 |
| Activated sludge, bottle 1 | 91 | 110 |
| Activated sludge, bottle 2 | 118 | 117 |

3. Remarks

Quantitations of data of the water samples were done by the standard addition method using the corresponding reference compounds. Data of the sludge samples were quantified by the internal standard method using the on-site spiked ¹³C-TCS as internal standard as well as by the standard addition method using additional ¹³C-TCS spiked before sample preparation.

4. Abbreviations

| | |
|----------|--|
| O-Me | triclosan-O-methyl ether (2,4,4'-trichloro-2'-methoxydiphenyl ether) |
| Penta | pentaclosan (2,3',4,4',5'-pentachloro-2'-hydroxydiphenyl ether) |
| TCS | triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) |
| TetraII | tetraclosan (2,3',4,4'-tetrachloro-2'-hydroxydiphenyl ether) |
| TetraIII | tetraclosan (2,4,4',5'-tetrachloro-2'-hydroxydiphenyl ether) |

Attachment Remelts Report Columbus

Addition of the 13C-Triclosan Spike to Monitoring Samples

| Sample Type | Amount of Sample collected at STP | Volume of Sample Aliquot * | Volume of 13C Spike added |
|--------------------|-----------------------------------|----------------------------|---------------------------|
| Raw Influent @ | approx. 2700 mL. | 2-500mL aliquots | 100 uL / aliquot |
| Primary Effluent @ | approx. 2700 mL | 2-500mL aliquots | 20 uL / aliquot + |
| Final Effluent @ | approx. 2700 mL | 2-500mL aliquots | 5 uL / aliquot + |
| Primary Sludge | approx. 1000 mL | 2-500 mL aliquots | 2 mL / aliquot |
| Activated Sludge | approx. 1000 mL | 2-500 mL aliquots | 200 uL / aliquot |

* All sample aliquots were measured with a 500 mL graduated cylinder and poured into a 1 liter jar that had been previously rinsed with ethanol.

@ Aliquots of raw, prim. eff., and final eff. were taken directly from the large volume of sample after it had been vigorously mixed.

+ Spiking solution was added using a transfer pipett with a plastic tip. All other spiking solution additions were made per Ciba's request.



US Monitoring Glendale, Ohio Results Report

Authors: Dr. Bert Schatowitz, Dr. Armin Hauk, Martin Jacob
 Date: 06 August 1997
 Test No.: 97.056

1. Samples

Sampling location: Glendale, Ohio, sewage treatment plant (trickling filter).

Sampling Date: April 11, 1997.

1. Influent (2 bottles)
2. Primary effluent (2 bottles)
3. Secondary effluent (2 bottles)
4. Primary sludge (2 bottles)
5. Digester sludge (2 bottles)

Temperature of all samples on receipt: 7°C, pH of water samples: 1.5.

On-site spiking of the samples according to the attached spiking protocol "Addition of the ¹³C-Triclosan Spike to Glendale Monitoring Samples". Concentration of the spike solution: 2.99 mg ¹³C-TCS/50 mL ethanol (same spike solution as used for the Columbus plant).

Dry weight of sludges determined after freeze-drying: Primary sludge bottle 1 and 2: each 3.2%; digester sludge bottle 1 and 2: each 3.8%.

2. Results

Influent:

| Sample | µg/L | | | | |
|------------|------|---------|----------|-----------|-------|
| | TCS | TetraII | TetraIII | Penta | O-Me |
| Bottle 1+2 | 2.13 | <LOD* | <LOD* | (0.013)** | nq*** |
| Bottle 1+2 | 2.13 | <LOD* | <LOD* | (0.006) | nq |
| Mean | 2.13 | <LOD* | <LOD* | (0.010) | nq |

* <LOD: below limit of detection of 0.01 µg/L.

** values in parenthesis: below the validated limit of quantitation of 0.05 µg/L, but the higher chlorinated closans were detectable at the given approx. concentrations.

*** nq: not quantifiable. TCS-O-methyl ether was qualitatively detectable in all samples, but the method was not validated for the determination of the methyl ether. By using the standard addition method for quantitation of TCS and the other closans also TCS-O-Me was added as a standard and recoveries were acceptable (50-70%). Calculations of the concentration of the O-methyl ether resulted in approx. 0.002-0.05 µg/L.

Primary effluent:

| <i>Sample</i> | µg/L | | | | |
|---------------|-------------|----------------|-----------------|----------------|----------------|
| | <i>TCS</i> | <i>TetraII</i> | <i>TetraIII</i> | <i>Penta</i> | <i>O-Me</i> |
| Bottle 1 + 2 | 1.99 | <LOD* | <LOD* | <LOD* | <LOD* |
| Bottle 1 + 2 | 1.97 | <LOD | <LOD | <LOD | <LOD |
| Mean | 1.98 | <LOD | <LOD | <LOD | <LOD |

* <LOD: below limit of detection of 0.01 µg/L.

Secondary effluent:

| <i>Sample</i> | µg/L | | | | |
|---------------|-------------|----------------|-----------------|----------------|----------------|
| | <i>TCS</i> | <i>TetraII</i> | <i>TetraIII</i> | <i>Penta</i> | <i>O-Me</i> |
| Bottle 1+2 | 0.88 | <LOD* | <LOD* | <LOD* | <LOD* |
| Bottle 1+2 | 0.90 | <LOD | <LOD | <LOD | <LOD |
| Mean | 0.89 | <LOD | <LOD | <LOD | <LOD |

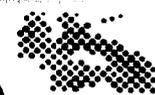
* <LOD: below limit of detection of 0.01 µg/L.

Primary sludge:

| <i>Sample</i> | µg/g dry weight | | | | |
|---------------|-----------------|----------------|-----------------|----------------|-------------|
| | <i>TCS</i> | <i>TetraII</i> | <i>TetraIII</i> | <i>Penta</i> | <i>O-Me</i> |
| Bottle 1 | 4.1 | <LOD* | <LOD* | <LOD* | nq** |
| Bottle 2 | 4.2 | <LOD | <LOD | <LOD | nq |
| Mean | 4.2 | <LOD | <LOD | <LOD | nq |

* <LOD: below limit of detection of 0.04 µg/g (Tetra) and 0.35 µg/g (Penta).

** not quantified, identification criteria (chlorine isotope ratio) not fulfilled.

**Digester sludge:**

| | $\mu\text{g/g}$ dry weight | | | | |
|-------------|----------------------------|----------------|-----------------|---------------|-------------|
| | <i>TCS</i> | <i>TetraII</i> | <i>TetraIII</i> | <i>Penta</i> | <i>O-Me</i> |
| Bottle 1 | 9.5 | 0.06 | 0.06 | (0.05) | 0.08 |
| Bottle 2 | 7.8 | 0.06 | 0.05 | (0.05) | 0.06 |
| Mean | 8.7 | 0.06 | 0.06 | (0.05) | 0.07 |

* values in parenthesis: below the validated limit of quantitation of $0.07 \mu\text{g/g}$, but the pentachloran was detectable at the given approx. concentrations.

Recoveries**Influent:**

| <i>Sample</i> | ¹³ C-TCS <i>on-site</i> | %Recovery | | | |
|---------------|---------------------------------------|--------------------------------|------------------------------------|-------------------------------------|----------------------------------|
| | | <i>TCS</i> <i>lab spike</i> | <i>TetraII</i> <i>lab spike</i> | <i>TetraIII</i> <i>lab spike</i> | <i>Penta</i> <i>lab spike</i> |
| Bottle 1+2 | 121 | 82 | 79 | 69 | 107 |
| Bottle 1+2 | 129 | 85 | 79 | 71 | 99 |

Primary effluent:

| <i>Sample</i> | ¹³ C-TCS <i>on-site</i> | %Recovery | | | |
|---------------|---------------------------------------|--------------------------------|------------------------------------|-------------------------------------|----------------------------------|
| | | <i>TCS</i> <i>lab spike</i> | <i>TetraII</i> <i>lab spike</i> | <i>TetraIII</i> <i>lab spike</i> | <i>Penta</i> <i>lab spike</i> |
| Bottle 1 + 2 | 119 | 84 | 87 | 85 | 148 |
| Bottle 1 + 2 | 121 | 7 | 73 | 74 | 91 |

Secondary effluent:

| <i>Sample</i> | ¹³ C-TCS <i>on-site</i> | %Recovery | | | |
|---------------|---------------------------------------|--------------------------------|------------------------------------|-------------------------------------|----------------------------------|
| | | <i>TCS</i> <i>lab spike</i> | <i>TetraII</i> <i>lab spike</i> | <i>TetraIII</i> <i>lab spike</i> | <i>Penta</i> <i>lab spike</i> |
| Bottle 1+2 | 137 | 27 | 104 | 100 | 78 |
| Bottle 1+2 | 104 | 44 | 106 | 103 | 83 |

Sludges:

| <i>Sample</i> | %Recovery | | | | | |
|---------------|---------------------------------------|--------------------------|------------------------------|-------------------------------|----------------------------|---------------------------|
| | <i>¹³C-TCS on-site</i> | <i>TCS lab spike</i> | <i>TetraII lab spike</i> | <i>TetraIII lab spike</i> | <i>Penta lab spike</i> | <i>O-Me lab spike</i> |
| Primary | 50 | 70 | 30 | 30 | 13 | 90 |
| Digester | 100 | 70 | 80 | 80 | 75 | 100 |

3. Remarks

All quantitations were done by the internal standard method using the on-site added ¹³C-TCS as internal standard (concentrations of the ¹³C-TCS were based on the informations provided by the sampling responsables), except data of the higher chlorinated closans in the water samples, which were quantified by the standard addition method using the corresponding reference compounds.

The recoveries of TCS of the water samples of this treatment plant were inconsistent. Furthermore, recoveries of the ¹³C-TCS spiked on-site and the TCS spiked in the lab before sample preparation were different. Both effects - related to the characteristics of the samples - resulted in inconsistent results when using the quantitation via standard addition method.

Therefore it is recommended to continue on-site spiking with ¹³C-TCS for all further plants to enable quantitation of target compound via the internal standard method. This method gives correct results based on the more realistic on-site added TCS and avoids errors due to different extraction behaviours of on-site and lab spiked TCS.

4. Abbreviations

| | |
|----------|--|
| O-Me | triclosan-O-methyl ether (2,4,4'-trichloro-2'-methoxydiphenyl ether) |
| Penta | pentaclosan (2,3',4,4',5'-pentachloro-2'-hydroxydiphenyl ether) |
| TCS | triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) |
| TetraII | tetraclosan (2,3',4,4'-tetrachloro-2'-hydroxydiphenyl ether) |
| TetraIII | tetraclosan (2,4,4',5'-tetrachloro-2'-hydroxydiphenyl ether) |

Attachment Recs Report Glendale

Addition of the 13C-Triclosan Spike to Glendale Monitoring Samples

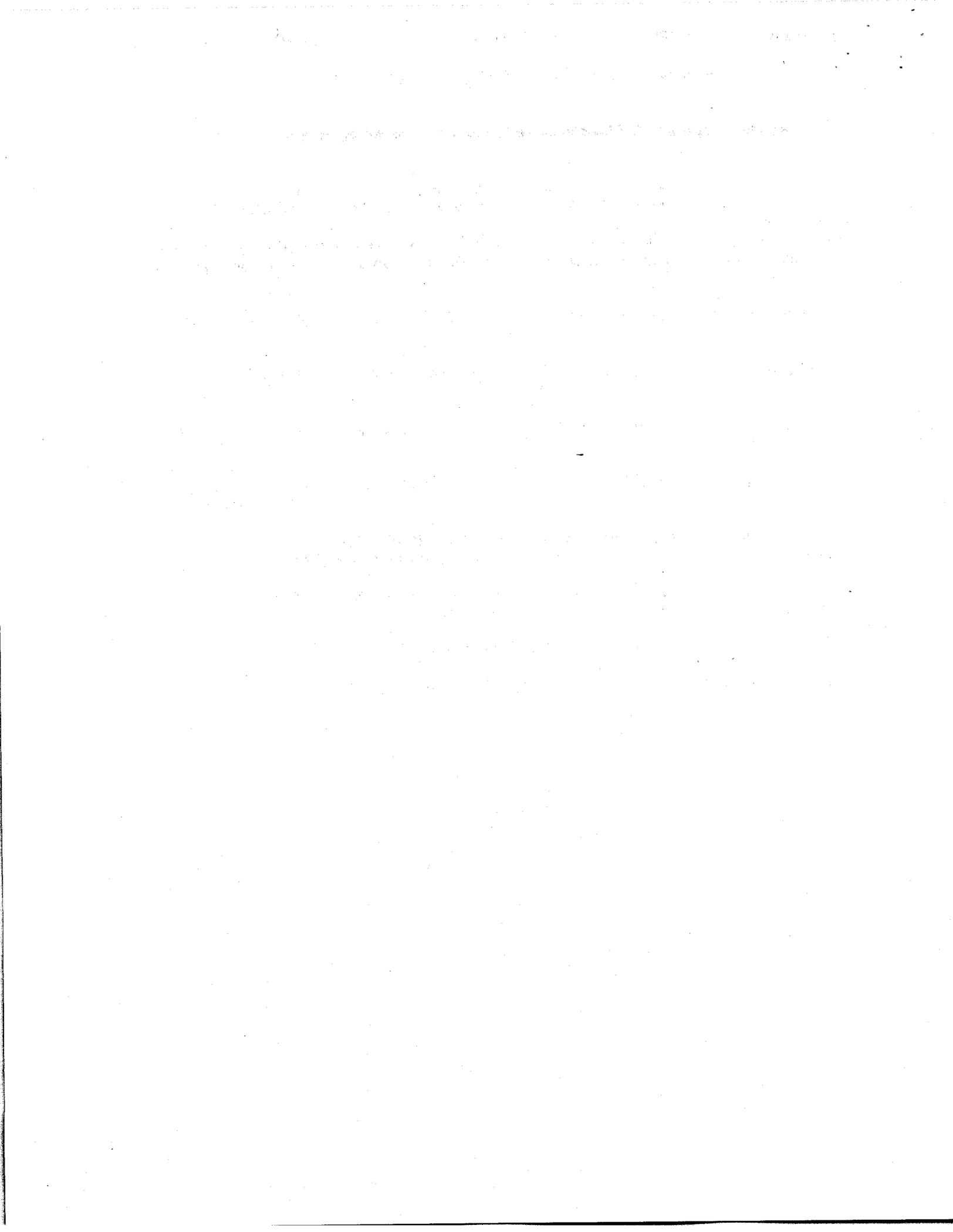
| Sample Type | Amount of Sample collected at STP | Volume of Sample Aliquot * | Volume of 13C Spike added |
|--------------------|-----------------------------------|----------------------------|--|
| Raw Influent @ | approx. 2700 mL. | 2-500mL aliquots | 100 uL / aliquot + |
| Primary Effluent @ | approx. 2700 mL | 2-500mL aliquots | 20 uL / aliquot + |
| Final Effluent @ | approx. 2700 mL | 2-500mL aliquots | 5 uL / aliquot + |
| Primary Sludge | approx. 1000 mL | 2-500 mL aliquots | 1.0 mL / aliquot # |
| Digester Sludge | approx. 1000 mL | 2-500 mL aliquots | 1.0 mL / aliquot - 1 # 0.3 mL / aliquot - 2 # |

* All sample aliquots were measured with a 500 mL graduated cylinder and poured into a 1 liter jar that had been previously rinsed with ethanol.

@ Aliquots of raw, prim. eff., and final eff. were taken directly from the large volume of sample after it had been vigorously mixed.

+ Spiking solution was added using a glass HPLC syringe.

Spiking solution additions were made using a 1.0 mL glass pipett.



Study Title

Effect of Triclosan on the Growth and
Reproduction of Aquatic Plants

Authors

Jane P. Staveley, Carolina Ecotox, Inc.
Toni L. Williams, Carolina Ecotox, Inc.

Study Completion Date

October 13, 1997

Sponsor

Ciba-Geigy Corporation
Greensboro, NC 27419

Testing Facility

Carolina Ecotox, Inc.
710 West Main Street
Durham, NC 27701

Carolina Ecotox, Inc. Study No.

21-02-1

Total Pages

57

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Test Substance: Triclosan

Title of Report: Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

The study described in this report was conducted in accordance with the following Good Laboratory Practice Standards:

United States Environmental Protection Agency,
Title 40 Code of Federal Regulations Part 160
Final Rule

United States Environmental Protection Agency,
Title 40 Code of Federal Regulations Part 792
Final Rule

Organization for Economic Co-operation and Development
"The OECD Principles of Good Laboratory Practice"

Toni L. Williams

Toni L. Williams
Study Director

10/13/97
(Date)

Sponsored by:

Per Hans Stensby

Per Stensby
Ciba-Geigy Corporation

10/29/97
(Date)

Submitted by:

Carl D'Pau

11/4/97
(Date)

SIGNATURE PAGE

Carolina Ecotox, Inc.
710 West Main Street
Durham, NC 27701
(919) 956-9036

Report Title: Effect of Triclosan on the Growth and Reproduction
of Aquatic Plants

Test Substance: Triclosan

Test Species: *Selenastrum capricornutum*, *Anabaena flos-aquae*,
Navicula pelliculosa, *Skeletonema costatum*,
and *Lemna gibba*

Sponsor: Ciba-Geigy Corporation
Greensboro, NC 27419

Test Dates: Test Initiation: 4 February 1997
Experimental Start Date (begin first definitive test):
10 February 1997
Experimental Termination Date (last date of data
collection): 26 February 1997

Study No.: 21-02-1

Report prepared by:

Toni L. Williams
Toni L. Williams
Carolina Ecotox, Inc.

Date: 10/13/97

Jane P. Staveley
Jane P. Staveley
Carolina Ecotox, Inc.

Date: 10/13/97

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QUALITY ASSURANCE INSPECTION STATEMENT

Title: Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Sponsor: Ciba-Geigy Corporation

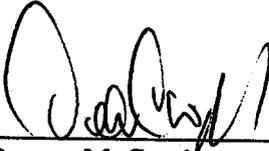
Inspections were conducted during the course of this study for GLP compliance verification by the Quality Assurance Unit.

| Date of Inspection | Study Aspect Inspected | Inspector | Date Findings Reported to Study Director | Date Findings Reported to Testing Facility Management |
|--------------------|---|-------------|--|---|
| 2/26/97 | Froned counts on day 7 of duckweed test | S. Sherrill | 2/26/97 | 2/26/97 |
| 4/23/97 | Raw data and draft final report | S. Sherrill | 4/23/97 | 4/23/97 |

Comments: None

The final report was reviewed and accurately reflects the methods, standard operating procedures and raw data.

Quality Assurance: _____


Deanna M. Croghan

Date: 10-13-97

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I. SUMMARY

Aquatic plant toxicity tests were conducted for Ciba-Geigy Corporation by Carolina Ecotox, Inc., Durham, NC. The objective of the study was to assess the effects of Triclosan upon the growth and reproduction of four microalgal species and an aquatic vascular plant. Testing was performed with *Selenastrum capricornutum* (recently renamed *Raphidocelis subcapitata*), *Anabaena flos-aquae*, *Navicula pelliculosa*, *Skeletonema costatum* and *Lemna gibba*, using preselected test concentrations. The targeted nominal concentrations were 0.5, 2.5, 12.5 and 62.5 µg/L. The test substance was prepared using acetone as a solvent. Population growth was measured by cell counts or frond counts at the end of the exposure period (96 hours for the algal species and 7 days for *Lemna gibba*).

The population growth data were used to determine the EC25 and EC50 values for each species, which are reported based upon the nominal concentrations. For each species, percent inhibition of growth for each test concentration was calculated relative to the solvent control (if there were statistically significant differences between the no-treatment control and the solvent control) or relative to the combined control (in the absence of such statistically significant differences). The EC25 and EC50 values, with 95% confidence intervals, were calculated by weighted least squares non-linear regression of the log of the nominal test concentrations against the replicate values for cell counts (or frond counts). The results are tabulated below.

| Species | EC25 (95% confidence interval) | EC50 (95% confidence interval) |
|-------------------------|------------------------------------|-----------------------------------|
| <i>S. capricornutum</i> | 2.44 µg/L (0.817 - 7.29 µg/L) | 4.46 µg/L (2.06 - 9.66 µg/L) |
| <i>A. flos-aquae</i> | 0.666 µg/L (0.450 - 0.985 µg/L) | 0.966 µg/L (0.720 - 1.30 µg/L) |
| <i>N. pelliculosa</i> | 10.7 µg/L (7.93 - 14.5 µg/L) | 0.966 µg/L (0.720 - 1.30 µg/L) |
| <i>S. costatum</i> | > 66.0 µg/L | > 66.0 µg/L |
| <i>L. gibba</i> | > 62.5 µg/L | > 62.5 µg/L |

II. INTRODUCTION

Aquatic plants are ubiquitous in aquatic ecosystems, where they incorporate solar energy into biomass, produce oxygen, function in nutrient cycling, and serve as food for animals. Because of their ecological importance, sensitivity to many test materials, ready availability, ease of culture, and fast growth rates, aquatic plants (microalgae and macrophytes) are often used in toxicity testing.

Aquatic plant growth is expressed in terms of mean cell counts or mean frond counts after a specified period of exposure to the test substance. Percent inhibition or stimulation, relative to the solvent control (or combined controls), for each test concentration is determined based upon mean cell counts or mean frond counts, as appropriate. The concentrations causing 25 and 50 percent inhibition of growth for each species are designated the EC25 and EC50, respectively.

A study consisting of a series of aquatic plant toxicity tests was conducted by Carolina Ecotox, Inc. for Ciba-Geigy Corporation under study number 21-02-1. Toxicity tests were conducted with Triclosan on test species selected to be representative of various taxonomic groups of freshwater and marine aquatic plants. The methods utilized in the study were specified in the protocol for Study No. 21-02-1 and are based on methods originally developed by the EPA (EPA, 1971; EPA, 1974; Miller et al., 1978; EPA, 1978; Holst and Ellwanger, 1982; EPA, 1985; EPA, 1987) and the American Society for Testing and Materials (ASTM, 1990; ASTM, 1991a). The protocol was also supplemented by Carolina Ecotox, Inc.'s current Standard Operating Procedures. The plants were directly exposed to the test substance, consequently maximizing the phytotoxic potential of the test substance.

Purpose of Study

The objective of this study was to assess the effects of Triclosan upon a variety of aquatic microalgae and an aquatic vascular plant. Toxicity tests were performed with *Selenastrum capricornutum* (recently renamed *Raphidocelis subcapitata*), *Anabaena flos-aquae*, *Navicula pelliculosa*, *Skeletonema costatum*, and *Lemna gibba*. The objective of these tests was to determine the EC50 and EC25 values of the test substance for each of the selected test species.

III. METHODS AND MATERIALS

Test Organisms

Three species of freshwater algae, one species of marine algae and one aquatic vascular plant were used: *Selenastrum capricornutum* (recently renamed *Raphidocelis subcapitata*), a unicellular, non-motile green alga; *Anabaena flos-aquae*, a nitrogen-fixing, filamentous blue-green alga; *Navicula pelliculosa*, a freshwater diatom; *Skeletonema costatum*, a marine diatom; and the duckweed, *Lemna gibba*. Stock cultures were maintained according to facility SOPs. Transfers were made regularly into fresh medium to provide cultures in the logarithmic phase of growth for toxicity test inoculations. All organisms used for a particular test were from the same source and same stock culture, and were not used in a previous test. Refer to Table 1 for specific culturing requirements, i.e., lighting, medium, temperature, and shaking, for each species.

Preparation of Glassware

All glassware used in testing was prepared according to facility SOP. The glassware was thoroughly scrubbed with non-phosphate detergent and rinsed with tap water. This was followed by a rinse with acetone, further rinses with deionized water, a rinse in 10% reagent grade hydrochloric acid, and thorough rinsing in deionized water. A final rinse was performed with ASTM Type I (ASTM, 1991b) reagent grade water (Type I water). Glassware was dried, in an oven at 50 - 70°C or air-dried, and autoclaved for 20 minutes at 1.1 kg/cm² and 121°C. Test vessels were 125-mL or 500-mL Erlenmeyer flasks fitted with foam stoppers to permit gas exchange and prepared as described above. Each test vessel was uniquely identified as to test concentration, replicate and study number.

Preparation of Medium

Several types of media were used: AAP, AAP/Si, MAA, and 20X-AAP. Refer to Table 1 for media requirements for each species. All medium was prepared according to facility SOPs. Medium for freshwater species was prepared by adding, for each liter, aliquots of the appropriate stock solutions to Type I water. The pH was adjusted to 7.5 ± 0.1 with 0.1 N or 1.0 N sodium hydroxide or hydrochloric acid as necessary and the volume brought to one liter. Medium was then filtered through a 0.22 μ filter into a sterile container.

To prepare medium for the marine species, a commercial salt mix was added to Type I water to reach a salinity of approximately 30‰ and then filtered as described above. Nutrients were added to the sterile synthetic salt water. All medium was prepared in advance of test initiation and stored under refrigeration prior to use. The composition of the various media types are given in Appendices A through D.

Test Substance

The test substance was identified as Triclosan and is also known as Irgasan DP 300 or Irgacare MP, Batch/Lot No. EN:275927.26, CAS No. 3380-34-5. The test substance, a white powder, has a molecular weight of 289.55 and a reported water solubility of 0.01 g/L (10 mg/L) at 20°C. The test substance was stored at room temperature. The expiration date of the test substance was September 1999. All calculations were corrected for the reported purity of the test substance, 99.5% active ingredient (a.i.) (Reference: Quality Control Report, Ciba-Geigy Ltd., Basle, dated September 18, 1992). Test substance remaining after the study was returned to the Sponsor.

Preparation of Test Solutions

Toxicity tests for each of the five test species were conducted during the period from February 10 through February 26, 1997. Test concentrations were pre-selected; therefore, no range-finding tests were performed. For each species, four test substance concentrations, a no-treatment control and a solvent control were tested. The targeted nominal concentrations were: 0.5, 2.5, 12.5, and 62.5 µg/L.

To begin each test, a fresh primary stock solution was prepared by weighing an appropriate amount of Triclosan and diluting with the solvent acetone. The primary stock solution was mixed by inversion or by sonication for five minutes, resulting in a clear liquid. Secondary stock solutions for each test were prepared, in acetone, by serial dilution at concentrations such that an equivalent amount of solvent was used in each test treatment. The nominal concentrations for the primary stock solutions and the date of preparation were as follows:

For *Selenastrum capricornutum* and *Anabaena flos-aquae*:

138 µg/mL, prepared on February 10, 1997

For *Navicula pelliculosa* and *Skeletonema costatum*:

132 µg/mL, prepared on February 21, 1997

For *Lemna gibba*:

1330 µg/mL, prepared February 19, 1997

For each toxicity test, the test treatments were prepared by quantitatively diluting aliquots of the appropriate stock solutions, with medium in volumetric flasks, to yield nominal test solution concentrations. Actual nominal concentrations for each definitive test were as follows:

For *Selenastrum capricornutum* and *Anabaena flos-aquae*:

0.496, 2.48, 12.4, and 69.0 µg/L

For *Navicula pelliculosa* and *Skeletonema costatum*:

0.500, 2.51, 12.6, and 66.0 µg/L

For *Lemna gibba*:

0.500, 2.50, 12.5, and 62.5 µg/L

The no-treatment controls contained medium only. The solvent controls contained an amount of solvent equivalent to the amount present in each test treatment, i.e., 0.5 mL/L. After thorough mixing of the test substance treatments, appropriate volumes of the no-treatment control, solvent control, and each test substance treatment were added to each of the replicate test vessels. For the algal toxicity tests, 25 mL of test solution was used in each 125-mL Erlenmeyer flask. Two hundred mL of test solution was used in each 500-mL Erlenmeyer flask for the duckweed test. For each toxicity test, there were three replicate flasks for each test substance treatment, no-treatment control, and solvent control. For each toxicity test, pH was measured at test initiation and test termination (Tables 2 - 6).

Inoculation

To start each algal toxicity test, an algal inoculum was taken from a logarithmically-growing stock culture (7 days old). For *Selenastrum capricornutum*, 3 mL of the stock culture was taken and diluted with approximately 18 mL AAP medium. Cell density in the diluted stock culture was determined with an electronic particle counter (Model ZM). The diluted stock culture contained 1,217,280 cells/mL. A 0.205-mL volume of the diluted stock culture was added aseptically, using an automatic pipette with a sterile tip, to 25 mL of solution in each of the replicate flasks, yielding nominal initial concentrations of approximately 10,000 cells/mL.

For *Anabaena flos-aquae*, 5 mL of the stock culture was taken and diluted with approximately 15 mL AAP medium. Cell density in the diluted stock culture was determined with an electronic particle counter (Model ZBI Coulter Counter). The diluted stock culture contained 1,072,000 cells/mL. A 0.233-mL volume of the diluted stock culture was added aseptically, using an automatic pipette with a sterile tip, to 25 mL of solution in each of the replicate flasks, yielding nominal initial concentrations of approximately 10,000 cells/mL.

Three mL of the *Navicula pelliculosa* stock culture was taken and diluted with approximately 11 mL AAP/Si medium. Cell density in the diluted stock culture was determined with an electronic particle counter (Model ZM Coulter Counter). The diluted stock culture contained 992,600 cells/mL. A 0.252-mL volume of the diluted stock culture was added aseptically, using an automatic pipette with a sterile tip, to 25 mL of solution in each of the replicate flasks, yielding nominal initial concentrations of approximately 10,000 cells/mL.

For *Skeletonema costatum*, cell density was determined in the undiluted stock culture with an electronic particle counter (Model ZM Coulter Counter). The stock culture contained 919,520 cells/mL. A 2.1-mL volume of the stock culture was added aseptically, using a sterile serological pipette (2 mL), to 25 mL of solution in each of the replicate flasks, yielding nominal initial concentrations of approximately 77,000 cells/mL.

The inoculum of *Lemna gibba* used to begin the test was taken from healthy 12-day old stock cultures. Three plants, consisting of four fronds each (for a total of 12 fronds) were added aseptically to each test vessel using a sterile inoculating hook. Plants were selected and assigned to randomly selected test vessels.

Incubation

Specific environmental conditions for each species are presented in Table 1. For the tests with *Selenastrum capricornutum* and *Navicula pelliculosa*, flasks were kept in a Psychrotherm Controlled Environment Incubator Shaker, Model G-27, designed to maintain a temperature of $24 \pm 2^\circ\text{C}$. Flasks were continuously shaken at 100 oscillations per minute. Continuous illumination of $4306 \pm 15\%$ lux was provided by overhead cool-white fluorescent lights.

For the test with *Anabaena flos-aquae*, flasks were also kept in a Psychrotherm Controlled Environment Incubator Shaker, Model G-27, as described above. However, flasks were manually shaken once each working day and continuous illumination was at $2153 \pm 15\%$ lux. Flasks for the definitive test with *Lemna gibba* were kept in a pH Environmental Incubator, Model CEC-32TC, designed to maintain a temperature of $25 \pm 2^\circ\text{C}$. Continuous illumination of $5000 \pm 15\%$ lux was provided by overhead warm-white fluorescent lights.

Flasks for the definitive test with *Skeletonema costatum* were kept in a Percival Incubator, Model I-60LLX, designed to maintain a temperature of $20 \pm 2^\circ\text{C}$. Illumination of $4306 \pm 15\%$ lux on a photoperiod of 14 hours light: 10 hours dark was provided by overhead cool white fluorescent lights. Flasks were manually shaken once each working day.

For all five tests, the instantaneous temperature was manually read in each incubator and recorded each day, while an automated system recorded temperature continuously. Flasks were randomly repositioned each working day to minimize potential bias in the incubators.

Observations: Algal Toxicity Tests

Cell counts were made using a Coulter Counter (Model ZBI with MHR Computer or Model ZM) at 96 hours after test initiation. The operating parameters for the Coulter Counters are presented in Appendices E and F. The Coulter Counter operates on the principle that cells are poor electrical conductors. The algal cells, suspended in an electrolyte, can be counted by passing through an aperture with a specific path of current flow. As cells pass through the aperture and displace a volume of electrolyte equal to their volume, the resistance changes, causing current and voltage changes which are translated into numbers of cells.

Samples of 0.1 to 2.0 mL were collected aseptically from the test flasks using an automatic pipette with a disposable tip. For counting, sample dilutions were made with the electrolyte Isoton II (Coulter Electronics, Inc.) as the diluent. Samples were not returned to the test flasks. Three counts per replicate were made. All counts were multiplied by the appropriate conversion factors (for sample dilution and volume counted) to yield cells/mL.

A. flos-aquae grows in filaments which, during the logarithmic phase of growth, may be hundreds of cells long. An effective method for reducing the length of the filaments without rupturing the cells is

sonication. At 96 hours, a 5.0-mL algal sample was collected aseptically from each test flask using an automatic pipette with a sterile tip. The samples were placed in individual disposable containers and sonicated in a water bath ultrasonic cleaning machine. Samples were sonicated for a sufficient duration (at least five minutes) to reduce the filaments to a length that was consistent between samples.

Observations: Duckweed Toxicity Test

Fronde counts for *Lemna gibba* were made using a lighted magnifying lens 7 days after test initiation. In order to eliminate subjective decisions on frond maturity, every frond visibly projecting beyond the edge of the parent frond was counted. Fronds which lost their pigmentation were noted but not included in the frond counts.

Data Analysis

Statistical analyses were performed using spreadsheets or SAS software (SAS Institute, Cary, NC). The level of significance was at $\alpha = 0.05$ for all analyses. A two-tailed t-test was used to determine any significant differences between the counts for the no-treatment control and solvent control for each species. If there were no statistically significant differences, the data from the two controls were pooled. Otherwise, all comparisons were made against the solvent control.

For the toxicity tests with *Selenastrum capricornutum*, *Navicula pelliculosa*, and *Skeletonema costatum*, the mean cell count values at 96 hours for each test concentration were expressed as a percentage relative to the mean in the control (solvent control or combined control). Percent inhibition for these toxicity tests, % I, was calculated according to Equation 1:

Equation 1

$$\%I = \frac{C - T}{C} \times 100\%$$

where: %I = percent inhibition (a negative value indicates stimulation)
C = mean count in the control
T = mean count in test substance treatment

Since *Anabaena flos-aquae* and *Lemna gibba* grow more slowly than the other test species, the mean cell or frond count values at test termination were expressed as a percentage relative to the mean in the solvent control or combined control less the original inoculum level. Percent inhibition for these two toxicity tests was calculated according to Equation 2:

Equation 2

$$\%I = \frac{(C - O) - (T - O)}{(C - O)} \times 100\%$$

where: %I = percent inhibition (a negative value indicates stimulation)

C = mean count in the control

O = original inoculum level

T = mean count in test substance treatment

The EC25 and EC50 values and associated 95% confidence limits were calculated using weighted least squares nonlinear regression of the log of test concentration against the 96 hour mean cell counts or the 7-day frond counts (Bruce and Versteeg, 1992). Due to the preselection of the test concentrations and the spacing interval between the exposure concentrations (factor of 5), some of the EC values could not be determined with great precision.

Data Reporting and Presentation

All final values in laboratory worksheets, spreadsheets, and in the report are expressed as per facility SOP. Standard rules of rounding are used. To accurately express a final value when using a calculator, it may be necessary to carry all starting and intermediate data to at least three places beyond the last significant digit, continuing through each step of the computations, before rounding for the final value.

Amendments and Deviations from Protocol

The study protocol, amendments, and deviations are included as Appendix G.

IV. RESULTS

Selenastrum capricornutum

A cool-white illumination range of 3790 - 4780 lux, as measured on February 10, 1997, was the actual illumination range used during the test. The pH of the test solutions at day 0 ranged from 7.32 to 7.53. At test termination, the pH values ranged from 7.34 to 8.26 (Table 2). As indicated by the temperature monitoring system, the temperature range for the test period was 23.20 - 23.83°C, with a mean temperature of 23.52°C.

Cell counts at test termination are given in Table 7. The solvent appeared to enhance growth relative to that in the no-treatment control. Since the cell count in the solvent control was statistically significantly different from that in the no-treatment control, effects of the test substance are calculated relative to the solvent control. Exposure of *S. capricornutum* to Triclosan resulted in inhibition of population growth ranging from 22.5% to 98.8%. The calculated EC25 was 2.44 µg/L (95% confidence interval 0.817 - 7.29 µg/L) and the calculated EC50 was 4.46 µg/L (95% confidence interval 2.06 - 9.66 µg/L).

Anabaena flos-aquae

A cool-white illumination range of 1960 - 2260 lux, as measured on February 10, 1997, was the actual illumination range used during the test. The pH of the test solutions at day 0 ranged from 7.32 to 7.53. At test termination, the pH values ranged from 7.26 to 7.40 (see Table 3). As indicated by the temperature monitoring system, the temperature range for the test period was 22.88 - 24.82°C, with a mean temperature of 23.48°C.

Cell counts at test termination are given in Table 8. The solvent appeared to have an adverse effect on growth relative to that in the no-treatment control. Since the cell counts in the solvent control were statistically significantly different from that in the no-treatment control, effects of the test substance are calculated relative to the solvent control. Exposure of *A. flos-aquae* to Triclosan resulted in inhibition of population growth ranging from 11.4% to 103%. The calculated EC25 was 0.666 µg/L (95% confidence interval 0.450 - 0.985 µg/L) and the calculated EC50 was 0.966 µg/L (95% confidence interval 0.720 - 1.30 µg/L).

Navicula pelliculosa

A cool-white illumination range of 3740 - 4640 lux, as measured on February 21, 1997, was the actual illumination range used during the test. The pH of the test solutions at day 0 ranged from 7.15 to 7.46. At test termination, the pH values ranged from 7.41 to 7.84 (see Table 4). As indicated by the temperature monitoring system, the temperature range for the test period was 22.62 - 24.87°C, with a mean temperature of 23.93°C.

Cell counts at test termination are given in Table 9. One replicate (C) at the highest test concentration was a statistical outlier according to Dixon's criteria (Dixon, 1953), and therefore, was not included in determination of the mean for that test concentration. There was no statistically significant difference between the cell counts in the no-treatment control and the solvent control, so the effects of the test substance are calculated relative to the combined control. Exposure of *N. pelliculosa* to Triclosan resulted in inhibition of population growth ranging from 10.8 to 93.2%. The calculated EC25 was 10.7 µg/L (95% confidence interval 7.93 - 14.5 µg/L) and the calculated EC50 was 19.1 µg/L (95% confidence interval 15.3 - 23.8 µg/L).

Skeletonema costatum

A cool-white illumination range of 3720 - 4720 lux, as measured on February 21, 1997, was the actual illumination range used during the test. The pH of the test solutions at day 0 ranged from 7.99 to 8.23. At test termination, the pH values ranged from 8.51 to 8.54 (see Table 5). As indicated by the temperature monitoring system, the temperature range for the test period was 20.90 - 22.61°C, with a mean temperature of 21.56°C.

Cell counts at test termination are given in Table 10. There was no statistically significant difference between the cell counts in the no-treatment control and the solvent control, so the effects of the test substance are calculated relative to the combined control. Exposure of *S. costatum* to Triclosan resulted in inhibition of population growth ranging from 2.71% to 13.2%. Since no test concentration resulted in inhibition greater than 25%, the EC25 and EC50 values could not be determined and are reported to be >66.0 µg/L.

Lemna gibba

A warm-white illumination range of 4540 - 5680 lux, as measured on February 19, 1997, was the actual illumination range used during the test. The pH of the test solutions at day 0 ranged from 7.71 to 7.76. At test termination, the pH values ranged from 7.58 to 8.92 (see Table 6). As indicated by the temperature monitoring system, the temperature range for the test period was 24.10 - 24.76 °C, with a mean temperature of 24.51 °C.

Fronnd counts at test termination are given in Table 11. There was no statistically significant difference between the frond counts in the no-treatment control and the solvent control, so the effects of the test substance are calculated relative to the combined control. Exposure of *L. gibba* to Triclosan resulted in effects ranging from 16.2% stimulation to 20.9% inhibition. Since no test concentration resulted in inhibition greater than 25%, the EC25 and EC50 values could not be determined and are reported to be >62.5 µg/L.

V. DISCUSSION

The aquatic plant species used in this study demonstrated varying sensitivities to Triclosan. The blue-green alga, *A. flos-aquae*, was the most sensitive species, with an EC50 of 0.966 µg/L. The next most sensitive species was the green alga, *S. capricornutum* (EC50 of 4.46 µg/L), followed by the freshwater diatom, *N. pelliculosa* (EC50 of 19.1 µg/L). Both the marine diatom, *S. costatum* and the duckweed, *L. gibba*, were relatively insensitive to Triclosan, with EC50 values in excess of the highest test concentration used (66.0 or 62.5 µg/L, respectively).

VI. STUDY INTEGRITY

There were no known circumstances that may have adversely affected the quality or integrity of the data.

VII. ARCHIVAL OF RAW DATA

All original raw data, protocol, amendments, and deviations, documentation and records related to the study, and the final report are stored in the Archives of Carolina Ecotox, Inc.

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X. TABLES

Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Table 1. Environmental conditions for each species.

| Species | Medium | Temp | Shaking | Illumination | Photoperiod |
|----------------------------------|---------|----------|--------------------|--------------------------------------|---------------------|
| <i>Selenastrum capricornutum</i> | AAP | 24 ± 2°C | 100 RPMs | 4306 ± 646 lux; cool white lights | continuous light |
| <i>Anabaena flos-aquae</i> | AAP | 24 ± 2°C | manually 1x/day | 2153 ± 323 lux; cool white lights | continuous light |
| <i>Navicula pelliculosa</i> | AAP/Si | 24 ± 2°C | 100 RPMs | 4306 ± 646 lux; cool white lights | continuous light |
| <i>Skeletonema costatum</i> | MAA | 20 ± 2°C | manually 1x/day | 4306 ± 646 lux; cool white lights | 14L:10D |
| <i>Lemna gibba</i> | 20X-AAP | 25 ± 2°C | not applicable | 5000 ± 750 lux; warm white lights | continuous light |

Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Table 2. pH values recorded during test: *Selenastrum capricornutum*.

| Nominal Concentration $\mu\text{g/L}$ | pH value | |
|--|----------|----------|
| | 0 Hours | 96 Hours |
| No-treatment Control | 7.53 | 8.26 |
| Solvent Control | 7.48 | 7.90 |
| 0.496 | 7.34 | 7.70 |
| 2.48 | 7.32 | 7.69 |
| 12.4 | 7.33 | 7.36 |
| 69.0 | 7.33 | 7.34 |

Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Table 3. pH values recorded during test: *Anabaena flos-aquae*.

| Nominal Concentration $\mu\text{g/L}$ | pH value | |
|--|----------|----------|
| | 0 Hours | 96 Hours |
| No-treatment Control | 7.53 | 7.34 |
| Solvent Control | 7.48 | 7.33 |
| 0.496 | 7.34 | 7.40 |
| 2.48 | 7.32 | 7.36 |
| 12.4 | 7.33 | 7.33 |
| 69.0 | 7.33 | 7.26 |

Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Table 4. pH values recorded during test: *Navicula pelliculosa*.

| Nominal Concentration $\mu\text{g/L}$ | pH value | |
|--|----------|----------|
| | 0 Hours | 96 Hours |
| No-treatment Control | 7.46 | 7.84 |
| Solvent Control | 7.27 | 7.68 |
| 0.500 | 7.22 | 7.68 |
| 2.51 | 7.17 | 7.72 |
| 12.6 | 7.15 | 7.67 |
| 66.0 | 7.15 | 7.41 |

Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Table 5. pH values recorded during test: *Skeletonema costatum*.

| Nominal Concentration µg/L | pH value | |
|-------------------------------|----------|----------|
| | 0 Hours | 96 Hours |
| No-treatment Control | 7.99 | 8.54 |
| Solvent Control | 8.20 | 8.52 |
| 0.500 | 8.21 | 8.52 |
| 2.51 | 8.22 | 8.54 |
| 12.6 | 8.23 | 8.53 |
| 66.0 | 8.23 | 8.51 |

Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Table 6. pH values recorded during test: *Lemna gibba*.

| Nominal Concentration $\mu\text{g/L}$ | pH value | |
|--|----------|-------|
| | Day 0 | Day 7 |
| No-treatment Control | 7.74 | 8.92 |
| Solvent Control | 7.76 | 8.27 |
| 0.500 | 7.71 | 8.46 |
| 2.50 | 7.72 | 8.66 |
| 12.5 | 7.71 | 8.61 |
| 62.5 | 7.72 | 7.58 |

Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Table 7. Cell counts at test termination: *Selenastrum capricornutum*.

| Nominal Concentration µg/L | | Counts at 96 hours cells/mL | % Inhibition ¹ relative to mean in solvent control |
|-------------------------------|------|--------------------------------|--|
| Solvent control | A | 3,291,000 | --- |
| | B | 3,566,300 | |
| | C | 3,077,200 | |
| | Mean | 3,311,500 | |
| | SD | 2.45E+05 | |
| | Var | 6.01E+10 | |
| No-treatment control | A | 2,063,400 | 45.0 |
| | B | 1,741,000 | |
| | C | 1,662,400 | |
| | Mean | 1,822,267 | |
| | SD | 2.12E+05 | |
| | Var | 4.52E+10 | |
| 0.496 | A | 2,455,800 | 27.8 |
| | B | 2,918,700 | |
| | C | 1,797,100 | |
| | Mean | 2,390,533 | |
| | SD | 5.64E+05 | |
| | Var | 3.18E+11 | |
| 2.48 | A | 3,729,600 | 22.5 |
| | B | 2,140,300 | |
| | C | 1,828,400 | |
| | Mean | 2,566,100 | |
| | SD | 1.02E+06 | |
| | Var | 1.04E+12 | |
| 12.4 | A | 118,030 | 93.4 |
| | B | 442,560 | |
| | C | 93,730 | |
| | Mean | 218,107 | |
| | SD | 1.95E+05 | |
| | Var | 3.79E+10 | |
| 69.0 | A | 42,690 | 98.8 |
| | B | 41,640 | |
| | C | 35,010 | |
| | Mean | 39,780 | |
| | SD | 4.16E+03 | |
| | Var | 1.73E+07 | |

¹ Calculated according to Equation 1 on page 15.

SD = standard deviation

Var = variance

Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Table 8. Cell counts at test termination: *Anabaena flos-aquae*.

| Nominal Concentration µg/L | | Counts at 96 hours cells/mL | % Inhibition ¹ relative to mean in solvent control |
|-------------------------------|------|--------------------------------|--|
| Solvent control | A | 100,000 | --- |
| | B | 100,000 | |
| | C | 103,000 | |
| | Mean | 101,000 | |
| | SD | 1.73E+03 | |
| | Var | 3.00E+06 | |
| No-treatment control | A | 134,000 | -32.2 |
| | B | 128,000 | |
| | C | 129,000 | |
| | Mean | 130,333 | |
| | SD | 3.21E+03 | |
| | Var | 1.03E+07 | |
| 0.496 | A | 105,000 | 11.4 |
| | B | 82,000 | |
| | C | 85,000 | |
| | Mean | 90,667 | |
| | SD | 1.25E+04 | |
| | Var | 1.56E+08 | |
| 2.48 | A | 13,000 | 95.6 |
| | B | 14,000 | |
| | C | 15,000 | |
| | Mean | 14,000 | |
| | SD | 1.00E+03 | |
| | Var | 1.00E+06 | |
| 12.4 | A | 7,000 | 103 |
| | B | 10,000 | |
| | C | 6,000 | |
| | Mean | 7,667 | |
| | SD | 2.08E+03 | |
| | Var | 4.33E+06 | |
| 69.0 | A | 11,000 | 102 |
| | B | 9,000 | |
| | C | 5,000 | |
| | Mean | 8,333 | |
| | SD | 3.06E+03 | |
| | Var | 9.33E+06 | |

¹ Calculated according to Equation 2 on page 16.

A negative percent inhibition indicates stimulation.

SD = standard deviation

Var = variance

Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Table 9. Cell counts at test termination: *Navicula pelliculosa*.

| Nominal Concentration µg/L | | Counts at 96 Hours cells/mL | % Inhibition ¹ relative to mean in combined control |
|-------------------------------|------|--------------------------------|---|
| Combined control | A | 1,281,200 | --- |
| | B | 1,184,100 | |
| | C | 1,255,300 | |
| | D | 1,329,100 | |
| | E | 1,469,600 | |
| | F | 1,190,300 | |
| | Mean | 1,282,117 | |
| | SD | 1.07E+05 | |
| 0.500 | A | 1,256,100 | 10.8 |
| | B | 1,059,200 | |
| | C | 1,116,200 | |
| | Mean | 1,143,833 | |
| | SD | 1.01E+05 | |
| | Var | 1.03E+10 | |
| 2.51 | A | 1,055,000 | 14.6 |
| | B | 1,234,300 | |
| | C | 997,200 | |
| | Mean | 1,095,500 | |
| | SD | 1.24E+05 | |
| | Var | 1.53E+10 | |
| 12.6 | A | 794,680 | 34.7 |
| | B | 936,160 | |
| | C | 779,800 | |
| | Mean | 836,880 | |
| | SD | 8.63E+04 | |
| | Var | 7.45E+09 | |
| 66.0 | A | 89,680 | 93.2 |
| | B | 83,740 | |
| | C | 387,820* | |
| | Mean | 86,710 | |
| | SD | 4.20E+03 | |
| | Var | 1.76E+07 | |

¹ Calculated according to Equation 1 on page 15.

For the combined control, replicates A, B, and C are the solvent control and replicates D, E, and F are the no-treatment control.

SD = standard deviation

Var = variance

* = statistical outlier ($\alpha = 0.05$) according to Dixon's criteria; not included in mean

Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Table 10. Cell counts at test termination: *Skeletonema costatum*.

| Nominal Concentration μg/L | | Counts at 96 hours cells/mL | % Inhibition ¹ relative to mean in combined control |
|-------------------------------|------|--------------------------------|---|
| Combined control | A | 547,400 | --- |
| | B | 588,760 | |
| | C | 565,960 | |
| | D | 584,680 | |
| | E | 610,000 | |
| | F | 593,720 | |
| | Mean | 581,753 | |
| | SD | 2.20E+04 | |
| 0.500 | A | 566,080 | 2.71 |
| | B | 557,160 | |
| | C | 574,760 | |
| | Mean | 566,000 | |
| | SD | 8.80E+03 | |
| | Var | 7.74E+07 | |
| 2.51 | A | 551,200 | 4.92 |
| | B | 559,280 | |
| | C | 548,920 | |
| | Mean | 553,133 | |
| | SD | 5.44E+03 | |
| | Var | 2.96E+07 | |
| 12.6 | A | 570,280 | 4.47 |
| | B | 541,960 | |
| | C | 555,040 | |
| | Mean | 555,760 | |
| | SD | 1.42E+04 | |
| | Var | 2.01E+08 | |
| 66.0 | A | 530,960 | 13.2 |
| | B | 486,960 | |
| | C | 496,880 | |
| | Mean | 504,933 | |
| | SD | 2.31E+04 | |
| | Var | 5.33E+08 | |

¹ Calculated according to Equation 1 on page 15.
 For the combined control, replicates A, B, and C are the solvent control and replicates D, E, and F are the no-treatment control.
 SD = standard deviation
 Var = variance

Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Table 11. Frond counts at test termination: *Lemna gibba*.

| Nominal Concentration $\mu\text{g/L}$ | | Frond counts on Day 7 | % Inhibition ¹ relative to mean in combined controls |
|--|------|--------------------------|--|
| Combined control | A | 147 | --- |
| | B | 151 | |
| | C | 185 | |
| | D | 178 | |
| | E | 159 | |
| | F | 157 | |
| | Mean | 163 | |
| | SD | 12 | |
| 0.500 | A | 190 | -10.3 |
| | B | 178 | |
| | C | 167 | |
| | Mean | 178 | |
| | SD | 12 | |
| | Var | 132 | |
| 2.50 | A | 168 | -6.08 |
| | B | 184 | |
| | C | 164 | |
| | Mean | 172 | |
| | SD | 11 | |
| | Var | 112 | |
| 12.5 | A | 182 | -16.2 |
| | B | 192 | |
| | C | 188 | |
| | Mean | 187 | |
| | SD | 5 | |
| | Var | 25 | |
| 62.5 | A | 136 | 20.9 |
| | B | 145 | |
| | C | 113 | |
| | Mean | 131 | |
| | SD | 17 | |
| | Var | 272 | |

¹ Calculated according to Equation 2 on page 16.

For the combined control, replicates A, B, and C are the solvent control and replicates D, E, and F are the no-treatment control.

A negative percent inhibition indicates stimulation.

SD = standard deviation

Var = variance

XI. APPENDICES

| APPENDIX A. Composition of synthetic algal assay procedure nutrient medium (AAP) (after Miller et al., 1978) | | | |
|--|----------------------|---|------------------------------|
| a. Macronutrient Stock Solutions | | | |
| Compound | Concentration (g/L) | Nutrient composition of prepared medium | |
| | | Element | Nominal concentration (mg/L) |
| NaHCO ₃ | 15.000 | Na | 11.001 |
| | | C | 2.143 |
| K ₂ HPO ₄ | 1.044 | K | 0.469 |
| | | P | 0.186 |
| MgSO ₄ ·7H ₂ O | 14.700 | S | 1.911 |
| *NaNO ₃ | 25.500 | N | 4.200 |
| *MgCl ₂ ·6H ₂ O | 12.164 | Mg | 2.904 |
| *CaCl ₂ ·2H ₂ O | 4.410 | Ca | 1.202 |
| b. Micronutrient Stock Solution | | | |
| Compound | Concentration (g/L) | Nutrient composition of prepared medium | |
| | | Element | Nominal concentration (µg/L) |
| H ₃ BO ₃ | 0.1855 | B | 32.460 |
| MnCl ₂ ·4H ₂ O | 0.4154 | Mn | 115.374 |
| ZnCl ₂ | 0.0033 | Zn | 1.570 |
| CoCl ₂ ·6H ₂ O | 0.0014 | Co | 0.354 |
| CuCl ₂ ·2H ₂ O | 1.2x10 ⁻⁵ | Cu | 0.004 |
| Na ₂ MoO ₄ ·2H ₂ O | 0.0073 | Mo | 2.878 |
| FeCl ₃ ·6H ₂ O | 0.1600 | Fe | 33.051 |
| Na ₂ EDTA·2H ₂ O | 0.3000 | --- | --- |

* Components of these stocks may be combined with the micronutrient stock solution to form a single micro/macronutrient stock solution

| APPENDIX B. Composition of synthetic algal assay procedure nutrient medium with silicon (AAP/Si) (after Payne and Hall, 1979) | | | |
|---|----------------------|---|------------------------------|
| a. Macronutrient Stock Solution | | | |
| Compound | Concentration (g/L) | Nutrient composition of prepared medium | |
| | | Element | Nominal concentration (mg/L) |
| NaHCO ₃ | 15.000 | Na | 11.001 |
| K ₂ HPO ₄ | 1.044 | C | 2.143 |
| | | K | 0.469 |
| | | P | 0.186 |
| MgSO ₄ ·7H ₂ O | 14.700 | S | 1.911 |
| ⊕NaNO ₃ | 25.500 | N | 4.200 |
| ⊕MgCl ₂ ·6H ₂ O | 12.164 | Mg | 2.904 |
| ⊕CaCl ₂ ·2H ₂ O | 4.410 | Ca | 1.202 |
| *Na ₂ SiO ₃ ·9H ₂ O | | Si | 20.0 |
| **Na ₂ SeO ₄ | 0.0018 | Se | 0.000752 |
| b. Micronutrient Stock Solution | | | |
| Compound | Concentration (g/L) | Nutrient composition of prepared medium | |
| | | Element | Nominal concentration (μg/L) |
| H ₃ BO ₃ | 0.1855 | B | 32.460 |
| MnCl ₂ ·4H ₂ O | 0.4154 | Mn | 115.374 |
| ZnCl ₂ | 0.0033 | Zn | 1.570 |
| CoCl ₂ ·6H ₂ O | 0.0014 | Co | 0.354 |
| CuCl ₂ ·2H ₂ O | 1.2×10 ⁻⁵ | Cu | 0.004 |
| Na ₂ MoO ₄ ·2H ₂ O | 0.0073 | Mo | 2.878 |
| FeCl ₃ ·6H ₂ O | 0.1600 | Fe | 33.051 |
| Na ₂ EDTA·2H ₂ O | 0.3000 | — | — |

* 202.4 mg may be added directly, or 10 mL of 20,240 mg/L stock

** added to medium for stock cultures only

⊕ Components of these stocks may be combined with the micronutrient stock solution to form a single micro/macronutrient stock solution.

| APPENDIX C. Composition of synthetic marine algal assay nutrient medium (MAA)(Walsh, et al., 1980) | | |
|--|----------------------|--|
| A. Metal Mix Stock Solution | | |
| Compound | Concentration (g/L) | Concentration in Prepared Medium** (mg/L) |
| FeCl ₃ .6H ₂ O | 0.048 | 0.720 |
| MnCl ₂ .4H ₂ O | 0.144 | 2.16 |
| ZnSO ₄ .7H ₂ O | 0.045 | 0.675 |
| CuSO ₄ .5H ₂ O | 0.000157 | 0.00236 |
| CoCl ₂ .6H ₂ O | 0.000404 | 0.00606 |
| H ₃ BO ₃ | 1.140 | 17.10 |
| Na ₂ EDTA.2H ₂ O * | 1.000 | --- |
| B. Minor Salt Mix Stock Solution | | |
| Compound | Concentration (g/L) | Concentration in Prepared Medium (mg/L) ** |
| K ₃ PO ₄ | 0.3 | 3 |
| NaNO ₃ | 5.0 | 50 |
| Na ₂ SiO ₃ .9H ₂ O | 2.0 | 20 |
| C. Vitamin Mix Stock Solution | | |
| Compound | Concentration (mg/L) | Concentration in Prepared Medium (µg/L) ** |
| Thiamine hydrochloride | 500 | 250 |
| Biotin | 0.1 | 0.05 |
| B ₁₂ (Cyanocobalamin) | 1.0 | 0.5 |

* EDTA added only to metal mix used for stock culture medium

** Medium for stock cultures contains twice these amounts

| APPENDIX D. Composition of twenty-strength synthetic algal assay procedure nutrient medium (20X-AAP) (after ASTM, 1991a) | | | |
|--|----------------------|---|------------------------------|
| a. Macronutrient Stock Solutions | | | |
| Compound | Concentration (g/L) | Nutrient composition of prepared medium | |
| | | Element | Nominal concentration (mg/L) |
| NaHCO ₃ | 15.000 | Na | 220.02 |
| K ₂ HPO ₄ | 1.044 | C | 42.86 |
| | | K | 9.38 |
| | | P | 3.72 |
| MgSO ₄ ·7H ₂ O | 14.700 | S | 38.22 |
| *NaNO ₃ | 25.500 | N | 84.00 |
| *MgCl ₂ ·6H ₂ O | 12.164 | Mg | 58.08 |
| *CaCl ₂ ·2H ₂ O | 4.410 | Ca | 24.04 |
| b. Micronutrient Stock Solution | | | |
| Compound | Concentration (g/L) | Nutrient composition of prepared medium | |
| | | Element | Nominal concentration (µg/L) |
| H ₃ BO ₃ | 0.1855 | B | 649.20 |
| MnCl ₂ ·4H ₂ O | 0.4154 | Mn | 2307.48 |
| ZnCl ₂ | 0.0033 | Zn | 31.40 |
| CoCl ₂ ·6H ₂ O | 0.0014 | Co | 7.08 |
| CuCl ₂ ·2H ₂ O | 1.2x10 ⁻⁵ | Cu | 0.08 |
| Na ₂ MoO ₄ ·2H ₂ O | 0.0073 | Mo | 57.56 |
| FeCl ₃ ·6H ₂ O | 0.1600 | Fe | 661.02 |
| Na ₂ EDTA·2H ₂ O | 0.3000 | --- | --- |

* Components of these stocks may be combined with the micronutrient stock solution to form a single micro/macronutrient stock solution

APPENDIX E. Operating parameters for the Coulter Counter, Model ZBI

For counting *Anabaena flos-aquae* using the Model ZBI Coulter Counter with MHR Computer, the following parameters were used:

| | |
|------------------------|---------------|
| Aperture tube | 100 μ m |
| Volume | 500 μ L |
| Separate/Locked Switch | Separate |
| 1/aperture | $\frac{1}{2}$ |
| 1/amplification | $\frac{1}{2}$ |
| Matching Switch | 20K |
| Gain Trim | 3 |
| Lower Threshold | 8 |
| Upper Threshold | 100 |
| MHR Threshold | 8 |
| Threshold Factor | 2.62 |

APPENDIX F. Operating parameters for the Coulter Counter, Model ZM

For counting *Selenastrum capricornutum*, *Navicula pelliculosa*, and *Skeletonema costatum* using the Coulter Counter Model ZM, the following parameters were used:

| | |
|--------------------------|---|
| Aperture tube | 100 μ m |
| Volume | 500 μ L |
| I | 1 |
| A (Attenuation) | 1 (<i>S. capricornutum</i> and <i>N. pelliculosa</i>) 16 (<i>S. costatum</i>) |
| mA | 10 |
| Preset gain | 1 |
| Lower threshold | 6.5 (<i>S. capricornutum</i> and <i>S. costatum</i>) 7 (<i>N. pelliculosa</i>) |
| Upper threshold | 99.8 (<i>S. capricornutum</i> and <i>N. pelliculosa</i>) 99.9 (<i>S. costatum</i>) |
| Polarity | Auto |
| Alarm threshold | Off |
| Calibration threshold | 21.3 |
| Calibration constant, Kd | 14.69 |

APPENDIX G. Protocol, Amendments, and Deviations

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Study No. 21-02-1

Protocol for:

**Effect of Triclosan on the Growth
and Reproduction of Aquatic Plants**

Sponsor:

Ciba-Geigy Corporation
Greensboro, NC 27419

January 1997

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Protocol for Study No. 21-02-1
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Study: Carolina Ecotox No. 21-02-1

Protocol for: Effect of Triclosan on the Growth and Reproduction of Aquatic Plants

Sponsor: Ciba-Geigy Corporation

Greensboro, NC 27419

Testing Facility: Carolina Ecotox, Inc.

710 West Main Street, Durham, NC 27701

Sponsor's

Representative:

Per Stensby

Ciba-Geigy Corporation

1/31/97
Date

Technical

Representative:

Dr. Donald J. Versteeg

The Procter and Gamble Company

1/29/97
Date

Study Director:

Toni L. Williams

Carolina Ecotox, Inc.

2/4/97
Date

Proposed experimental start date: January 30, 1997

Proposed experimental termination date: March 18, 1997

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INTRODUCTION

Aquatic plants are ubiquitous in aquatic ecosystems, where they incorporate solar energy into biomass, produce oxygen, function in nutrient cycling and serve as food for animals. Because of their ecological importance, sensitivity to many toxicants, ready availability, ease of culture, and fast growth rates, aquatic plants (microalgae and macrophytes) are important organisms to use in ecotoxicity testing.

In an aquatic plant toxicity test, the growth of a selected species is monitored during a period of exposure to the test substance. Some measure of growth (such as population density) is used to calculate the percent inhibition or stimulation, relative to the control (or solvent control), for each test concentration. The concentrations causing 25 and 50 percent inhibition of growth are designated the EC25 and EC50, respectively. The highest concentration tested in which growth is not statistically significantly different from that in the control (or solvent control) is designated the no observed effect concentration (NOEC).

Purpose of Study

The objective of this study is to assess the effects of Triclosan upon a variety of aquatic microalgae and an aquatic vascular plant. Toxicity tests will be performed with *Selenastrum capricornutum* (recently renamed *Raphidocelis subcapitata*), *Anabaena flos-aquae*, *Navicula pelliculosa*, *Skeletonema costatum*, and *Lemna gibba*. The objective of these tests is to determine the EC50 and EC25 values of the test substance for each of the selected test species. Due to the preselection of the test concentrations and a wide spacing interval between exposure concentrations (factor of 5), precise estimation of the EC50 and EC25 values may not be possible. This would occur due to lack of toxicity at the highest concentration or a sudden increase in toxicity from approximately no inhibition to 100% inhibition between any two treatments.

Justification for Selection of Test System and Route of Administration

The method outlined below is based on methods originally developed by the U.S. Environmental Protection Agency (EPA, 1971; EPA, 1974; Miller et al., 1978; EPA, 1985; EPA, 1987) and the American Society for Testing and Materials (ASTM, 1990; ASTM, 1991a). This protocol is also

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supplemented by Carolina Ecotox, Inc.'s current Standard Operating Procedures. The selected test species are representative of various taxonomic groups of freshwater and marine aquatic plants. The test organisms will be directly exposed to the test substance in an aqueous matrix, consequently maximizing the phytotoxic potential of the test substance.

METHODS

Facilities

Toxicity testing will be conducted at the laboratory of Carolina Ecotox, Inc. in Durham, North Carolina, which is equipped and staffed to conduct aquatic plant toxicity tests.

Test Organisms

Three species of freshwater algae, one species of marine algae and one aquatic vascular plant will be used. Of the freshwater species, *Selenastrum capricornutum* (recently renamed *Raphidocelis subcapitata*) is a unicellular, non-motile green alga and *Anabaena flos-aquae* is a nitrogen-fixing, filamentous blue-green alga. The representative freshwater diatom that will be used is *Navicula pelliculosa*, and the representative marine species is also a diatom, *Skeletonema costatum*. The duckweed, *Lemna gibba*, is representative of the smallest and simplest aquatic vascular plants.

Stock Cultures

Carolina Ecotox's laboratory cultures of aquatic plants were originally obtained from a variety of commercial sources. Cultures are maintained in synthetic growth medium under appropriate conditions of lighting, temperature and shaking, as listed in Table 1. Stock cultures are routinely transferred into fresh medium to provide a healthy inoculum for toxicity test initiation. Cultures used to prepare inocula for toxicity tests will have been actively growing at the test conditions for at least two transfers prior to the start of the definitive test. All organisms used for a particular test will be from the same source and same stock culture, and will not have been used in a previous test. The source of each species will be documented in the raw data.

| Species | Medium | Temp | Shaking | Illumination | Photoperiod |
|----------------------------------|---------|----------|--------------------|--------------------------------------|------------------|
| <i>Selenastrum capricornutum</i> | AAP | 24 ± 2°C | 100 RPMs | 4306 ± 646 lux; cool white lights | continuous light |
| <i>Anabaena flos-aquae</i> | AAP | 24 ± 2°C | manually 1x/day | 2153 ± 323 lux; cool white lights | continuous light |
| <i>Navicula pelliculosa</i> | AAP/Si | 24 ± 2°C | 100 RPMs | 4306 ± 646 lux; cool white lights | continuous light |
| <i>Skeletonema costatum</i> | MAA | 20 ± 2°C | manually 1x/day | 4306 ± 646 lux; cool white lights | 14L: 10D |
| <i>Lemna gibba</i> | 20X-AAP | 25 ± 2°C | not applicable | 5000 ± 750 lux; warm white lights | continuous light |

Preparation of Glassware:

All glassware used in testing will be thoroughly scrubbed with non-phosphate detergent and rinsed with tap water. This will be followed by a rinse with acetone, further rinses with deionized water, a rinse in a 10 percent reagent grade hydrochloric acid, and thorough rinsing in deionized water. A final rinse will be performed with ASTM Type I reagent grade water (Type I water) (ASTM, 1991b). Glassware will be dried, at room temperature or in an oven at 50 - 70°C, and autoclaved for 20 minutes at 1.1 Kg/cm² and 121°C.

Preparation of Media

Several types of media will be used: AAP, AAP/Si, MAA, and 20X-AAP (see Table 1). Media will be prepared according to facility SOPs. Freshwater media will be prepared by adding specified amounts of stock solutions to Type I water, adjusting the pH, and filtering the medium through a 0.22 µ membrane filter into a sterile container. To prepare saltwater media, a commercial salt mix is added to Type I water to reach the desired salinity, and the salt water is then filtered as described above. Nutrients are then added to the sterile synthetic salt water. All media

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will be prepared in advance of test initiation and stored under refrigeration prior to use. The composition of the various types of media are given in Appendices A through D.

Test Vessels

Test vessels will be Erlenmeyer flasks fitted with foam stoppers which permit gas exchange. Flasks and stoppers will be autoclaved, as described above, prior to use. Flask sizes from 125 mL to 500 mL may be used, as long as the proper liquid-to-volume ratio is used. For algal toxicity tests, a liquid to flask size ratio of 1 to 5 is maintained, while for duckweed tests, the ratio is 2 to 5. Each test vessel will be uniquely identified as to test concentration, replicate and study number.

Test Substance

The test substance is identified as Triclosan, also known as Irgasan DP 300 or Irgacare MP, Batch/Lot No. EN:275927.26, CAS No. 3380-34-5. The test substance, a white powder, has a molecular weight of 289.55 and is reported to have a water solubility of 0.01 g/L (10 mg/L) at 20°C. The test substance will be stored at room temperature. The test substance sample has an expiration date of September, 1999. All calculations will be corrected for the reported purity of the test substance (99.5%).

A stock solution of the test substance will be prepared in the solvent acetone and aliquots added to test medium to obtain each test substance concentration. A solvent control will also be tested. The solvent control will contain an amount of solvent equivalent to the amount present in each test treatment, which must be less than or equal to 0.5 mL/L. If it is not possible to prepare a homogeneous solution of the test substance, it may be added directly into each replicate flask.

Preparation of Test Solutions

A toxicity test will be conducted for each species, employing four test concentrations, a control, and a solvent control, with three replicates of each. The targeted nominal concentrations will be as follows: 0.5, 2.5, 12.5, and 62.5 µg/L. The control treatment will consist of nutrient medium with no additions. A solvent control will also be included. The pH of the test concentrations and controls will be measured at the beginning and end of the test. Initial pH will be measured on the

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solutions used to begin the test, while final pH will be measured on an aliquot from the pooled replicate flasks. The test flasks will be inoculated and incubated as described below.

Inoculation

To start each algal toxicity test, an algal inoculum will be taken from a logarithmically-growing stock culture. A volume of inoculum calculated to yield the specified initial concentration, as listed below, will be aseptically added to each flask.

| <u>Species</u> | <u>Inoculum concentration</u> |
|-------------------------|-------------------------------|
| <i>S. capricornutum</i> | 10,000 cells/mL |
| <i>A. flos-aquae</i> | 10,000 cells/mL |
| <i>N. pelliculosa</i> | 10,000 cells/mL |
| <i>S. costatum</i> | 77,000 cells/mL |

To start the duckweed toxicity test, three to five plants, consisting of three or four fronds each (for a total of 12- 16 fronds) will be aseptically removed from seven- to twelve-day old stock cultures and added to each test vessel using a sterile inoculating loop. The same number of plants and fronds will be placed in each test vessel, and care will be taken to ensure that the plants are of a similar size.

Incubation

Test flasks will be kept in incubators designed to maintain constant temperature, under specified illumination conditions (described previously in Table 1). The instantaneous temperature will be manually read and recorded each working day, while an automated system will monitor temperature continuously. Flasks will be continuously shaken at approximately 100 oscillations per minute, shaken by hand once each working day, or not shaken, as listed in Table 1. Light intensity and oscillation rate will be measured and recorded prior to the beginning of each test. Each working day (excluding weekends) the flasks will be randomly repositioned to minimize spatial differences in the incubator, and any observations on apparent growth (from visual inspection) will be noted.

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Biomass Monitoring: Algal Toxicity Tests

The duration of the algal toxicity tests will be 96 hours, with biomass monitoring performed by cell counts at 96 hours in each replicate flask. Cell counts will be performed using an electronic particle counter (Model ZBI Coulter Counter with C-1000 Channelyzer and MHR Computer or a Model ZM Coulter Counter). The Coulter Counter operates on the principle that cells are poor electrical conductors. The algal cells, suspended in an electrolyte, can be counted by passing through an aperture with a specific path of current flow. As algal cells pass through the aperture and displace a volume of electrolyte equal to their volume, the resistance changes, causing current and voltage changes which are translated into number of cells.

Samples will be collected aseptically using an automatic pipetter with sterile tips, and sample dilutions will be made with the electrolyte Isoton II (Coulter Electronics, Inc.) as the diluent. Samples will not be returned to the test flasks. Three counts per replicate will be made. All counts will be multiplied by the appropriate conversion factors (for sample dilution and volume counted) to yield cells/mL. The Coulter Counter will be calibrated as per Standard Operating Procedures.

A. flos-aquae grows in filaments which, during the logarithmic phase of growth, may be hundreds of cells long. An effective method for reducing the length of the filaments without rupturing the cells is sonication. For counting, a 5.0-mL algal sample will be collected aseptically from each test flask using an automatic pipetter with a sterile tip. The samples will be placed in individual disposable containers and sonicated in an ultrasonic cleaning machine. Samples will be sonicated for a sufficient duration (generally five minutes) to reduce the filaments to a length that is consistent between samples.

If the test solutions contain particulates that interfere with the Coulter Counter, then a blank or blanks (test substance solution not inoculated with algae) will be prepared to assess potential interference with the Coulter Counter. If interference with electronic counts is observed (based on visual observations and/or counts in the blank(s) in apparent excess of obvious algal growth), then counts from the blank(s) may be subtracted from the counts of the treatment replicates. Alternatively, cell counts will be made using a microscope and an Improved Neubauer

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hemacytometer, 0.1 mm deep. Two samples will be taken from each flask and two counts will be made of each sample. Whenever feasible, at least 400 cells per flask will be counted in order to obtain $\pm 10\%$ accuracy at the 95% confidence level.

Biomass Monitoring: Duckweed Toxicity Test

The duration of the duckweed test will be 7 days, with frond counts performed at test termination. Frond production and appearance will be recorded. In order to eliminate subjective decisions on frond maturity, every frond visibly projecting beyond the edge of the parent frond will be counted. The numbers of chlorotic fronds, any flowering, and any gross abnormalities will also be recorded.

Data Analysis

A two-tailed t-test will be used to determine any significant differences between the control and solvent control. If there are no statistically significant differences, data from the two controls may be pooled. Otherwise, all comparisons will be made against the solvent control. Statistical analyses will be conducted manually, with the aid of spreadsheets, or using SAS software (SAS Institute, Cary, NC).

For each algal toxicity test, the mean cell count values at 96 hours for each test concentration will be expressed as percentage relative to that in the control. Percent inhibition will be calculated according to the following formula:

$$\%I = \frac{(C - T)}{C} \times 100\%$$

where: %I = percent inhibition (a negative value indicates stimulation)
 C = mean growth in the control
 T = mean growth in treated culture

For the duckweed toxicity test, the mean frond count values at test termination will be expressed as a percentage relative to that in the control, according to the following formula:

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$$\%I = \frac{(C-O) - (T-O)}{(C-O)} \times 100$$

where: %I = percent inhibition (a negative value indicates stimulation)
C = mean growth in the control
O = initial number of fronds in the control
T = mean growth in treated culture

The cell count or frond count data at test termination will be used to calculate the EC25 and EC50 values. These "effective concentrations" are defined as the concentrations producing an inhibition of growth, relative to the control, of 25 or 50 percent, respectively. The EC values and associated 95% confidence limits will be determined by weighted least squares non-linear regression (Bruce and Versteeg, 1992) of the log of test concentration against either the 96-hour cell counts or the 7-day frond counts.

QUALITY ASSURANCE

All test practices, procedures, records and reports will be examined by Quality Assurance personnel to ensure compliance with EPA Good Laboratory Practice Standards under the Federal Insecticide, Fungicide and Rodenticide Act (40 CFR Part 160).

REPORTING

A report will be issued describing the conduct and results of the study according to the Pesticide Assessment Guidelines (Holst and Ellwanger, 1982) and EPA's Pesticide Regulation Notice 86-5. This includes, but is not limited to, the following:

1. Identification of test substance as provided by the sponsor, including lot number and purity, and physical description including physical state and color.
2. Description of test organisms including species identification, source, culture practices, and holding conditions.
3. Dates of testing and name and address of testing facility, including the names of key personnel involved in the study.

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4. Objectives and procedures as stated in the approved protocol.
5. Description of test system and operating parameters.
6. Description of the experimental design and procedures used during the conduct of the study, including administration to the test system, environmental parameter monitoring, and data collection.
7. For each test, cell counts or frond counts for each replicate at test termination, with means and standard deviations for each test concentration, control and solvent control.
9. Values for percent inhibition (or stimulation) at test termination. EC25 and EC50 values, with 95% confidence limits, as applicable.
10. Description of calculations performed on the data and statistical methods employed for analyzing the data; summary and conclusions drawn from the data analysis.
11. Certification documentation including laboratory and study identification; names and signatures of Study Director, Quality Assurance and Testing Facility Manager; protocol number and study number; data-archive reference; dates of testing and date of issue; GLP Compliance Statement and Quality Assurance Inspection Statement.
12. Deviations from protocol, if any.
13. A description of any factors that might affect the quality or integrity of the study.
14. Location of raw data and final report.

| APPENDIX A. COMPOSITION OF SYNTHETIC ALGAL ASSAY PROCEDURE NUTRIENT MEDIUM (AAP) (after Miller et al., 1978) | | | |
|--|----------------------|---|-----------------------------|
| a. Macronutrient Stock Solutions | | | |
| Compound | Concentration, g/L | Nutrient composition of prepared medium | |
| | | Element | Nominal concentration, mg/L |
| NaHCO ₃ | 15.000 | Na | 11.001 |
| | | C | 2.143 |
| K ₂ HPO ₄ | 1.044 | K | 0.469 |
| | | P | 0.186 |
| MgSO ₄ ·7H ₂ O | 14.700 | S | 1.911 |
| *NaNO ₃ | 25.500 | N | 4.200 |
| *MgCl ₂ ·6H ₂ O | 12.164 | Mg | 2.904 |
| *CaCl ₂ ·2H ₂ O | 4.410 | Ca | 1.202 |
| b. Micronutrient Stock Solution | | | |
| Compound | Concentration, g/L | Nutrient composition of prepared medium | |
| | | Element | Nominal concentration, µg/L |
| H ₃ BO ₃ | 0.1855 | B | 32.460 |
| MnCl ₂ ·4H ₂ O | 0.4154 | Mn | 115.374 |
| ZnCl ₂ | 0.0033 | Zn | 1.570 |
| CoCl ₂ ·6H ₂ O | 0.0014 | Co | 0.354 |
| CuCl ₂ ·2H ₂ O | 1.2×10 ⁻⁵ | Cu | 0.004 |
| Na ₂ MoO ₄ ·2H ₂ O | 0.0073 | Mo | 2.878 |
| FeCl ₃ ·6H ₂ O | 0.1600 | Fe | 33.051 |
| Na ₂ EDTA·2H ₂ O | 0.3000 | — | — |

* Components of these stocks may be combined with the micronutrient stock solution to form a single micro/macronutrient stock solution.

| APPENDIX C. COMPOSITION OF SYNTHETIC MARINE ALGAL ASSAY NUTRIENT MEDIUM (Walsh and Alexander, 1980) | | |
|---|---------------------|--|
| A. Metal Mix Stock Solution | | |
| Compound | Concentration, g/L | Concentration in Prepared Medium**, mg/L |
| FeCl ₃ .6H ₂ O | 0.048 | 0.720 |
| MnCl ₂ .4H ₂ O | 0.144 | 2.16 |
| ZnSO ₄ .7H ₂ O | 0.045 | 0.675 |
| CuSO ₄ .5H ₂ O | 0.157 | 2.36 |
| CoCl ₂ .6H ₂ O | 0.000404 | 0.00606 |
| H ₃ BO ₃ | 1.140 | 17.10 |
| Na ₂ EDTA.2H ₂ O * | 1.000 | --- |
| B. Minor Salt Mix Stock Solution | | |
| Compound | Concentration, g/L | Concentration in Prepared Medium**, mg/L |
| K ₃ PO ₄ | 0.3 | 3 |
| NaNO ₃ | 5.0 | 50 |
| Na ₂ SiO ₃ .9H ₂ O | 2.0 | 20 |
| C. Vitamin Mix Stock Solution | | |
| Compound | Concentration, mg/L | Concentration in Prepared Medium**, µg/L |
| Thiamine hydrochloride | 500 | 250 |
| Biotin | 0.1 | 0.05 |
| B ₁₂ (Cyanocobalamin) | 1.0 | 0.5 |

* EDTA added only to metal mix used for stock culture medium
 ** Medium for stock cultures contains twice these amounts

| APPENDIX B. COMPOSITION OF SYNTHETIC ALGAL ASSAY PROCEDURE NUTRIENT MEDIUM WITH SILICON (AAP/Si) (after Payne and Hall, 1979) | | | |
|---|----------------------|---|-----------------------------|
| a. Macronutrient Stock Solutions | | | |
| Compound | Concentration, g/L | Nutrient composition of prepared medium | |
| | | Element | Nominal concentration, mg/L |
| NaHCO ₃ | 15.000 | Na | 11.001 |
| K ₂ HPO ₄ | 1.044 | C | 2.143 |
| | | K | 0.469 |
| | | P | 0.186 |
| MgSO ₄ ·7H ₂ O | 14.700 | S | 1.911 |
| ○ NaNO ₃ | 25.500 | N | 4.200 |
| ○ MgCl ₂ ·6H ₂ O | 12.164 | Mg | 2.904 |
| ○ CaCl ₂ ·2H ₂ O | 4.410 | Ca | 1.202 |
| *Na ₂ SiO ₃ ·9H ₂ O | — | Si | 20.0 |
| **Na ₂ SeO ₄ | 0.0018 | Se | 0.000752 |
| b. Micronutrient Stock Solution | | | |
| Compound | Concentration, g/L | Nutrient composition of prepared medium | |
| | | Element | Nominal concentration, µg/L |
| H ₃ BO ₃ | 0.1855 | B | 32.460 |
| MnCl ₂ ·4H ₂ O | 0.4154 | Mn | 115.374 |
| ZnCl ₂ | 0.0033 | Zn | 1.570 |
| CoCl ₂ ·6H ₂ O | 0.0014 | Co | 0.354 |
| CuCl ₂ ·2H ₂ O | 1.2×10 ⁻⁵ | Cu | 0.004 |
| Na ₂ MoO ₄ ·2H ₂ O | 0.0073 | Mo | 2.878 |
| FeCl ₃ ·6H ₂ O | 0.1598 | Fe | 33.051 |
| Na ₂ EDTA·2H ₂ O | 0.3000 | — | — |

* 202.4 mg may be added directly, or 10 mL of 20,240 mg/L stock
 ** added to medium for stock cultures only
 ○ Components of these stocks may be combined with the micronutrient stock solution to form a single micro/macronutrient stock solution.

| APPENDIX D. COMPOSITION OF TWENTY-STRENGTH SYNTHETIC ALGAL ASSAY PROCEDURE NUTRIENT MEDIUM (20X-AAP) (after ASTM, 1991a) | | | |
|--|----------------------|---|-----------------------------|
| a. Macronutrient Stock Solutions | | | |
| Compound | Concentration, g/L | Nutrient composition of prepared medium | |
| | | Element | Nominal concentration, mg/L |
| NaHCO ₃ | 15.000 | Na | 220.02 |
| K ₂ HPO ₄ | 1.044 | C | 42.86 |
| | | K | 9.38 |
| MgSO ₄ ·7H ₂ O | 14.700 | P | 3.72 |
| | | S | 38.22 |
| *NaNO ₃ | 25.500 | N | 84.00 |
| *MgCl ₂ ·6H ₂ O | 12.164 | Mg | 58.08 |
| *CaCl ₂ ·2H ₂ O | 4.410 | Ca | 24.04 |
| b. Micronutrient Stock Solution | | | |
| Compound | Concentration, g/L | Nutrient composition of prepared medium | |
| | | Element | Nominal concentration, µg/L |
| H ₃ BO ₃ | 0.1855 | B | 649.20 |
| MnCl ₂ ·4H ₂ O | 0.4154 | Mn | 2307.48 |
| ZnCl ₂ | 0.0033 | Zn | 31.40 |
| CoCl ₂ ·6H ₂ O | 0.0014 | Co | 7.08 |
| CuCl ₂ ·2H ₂ O | 1.2×10 ⁻⁵ | Cu | 0.08 |
| Na ₂ MoO ₄ ·2H ₂ O | 0.0073 | Mo | 57.56 |
| FeCl ₃ ·6H ₂ O | 0.1600 | Fe | 661.02 |
| Na ₂ EDTA·2H ₂ O | 0.3000 | --- | --- |

* Components of these stocks may be combined with the micronutrient stock solution to form a single micro/macronutrient stock solution.

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ENTRY FORM

| CAPNUM | LTR | DATE | CBI | CASNO | CONCERN | AI | SOLUBILITY |
|--------|-----|------|-----|---------|---------|------|------------|
| 14064 | a | 1197 | | 3380345 | HIGH | 99.5 | 10mg/L |

CHEMNAME

5-chloro-2-(2,4-dichlorphenoxy)phenol, static

PHYSTATE

solid

| ORGANISM | DURATION | ENDPOINT | CODE | TOXVALUE | UNITS | MELTINGPT |
|------------------------------|----------|----------|------|----------|-------|-----------|
| Cyanobacteria, A. flos-aquae | 96h | EC50 | | 0.966 | ug/L | NS |

MELTINGPT

NS

COMMENTS

96hEC25=0.666ug/L (pop. growth)

nominal

acetone

ENTRY FORM

| CAPNUM | LTR | DATE | CBI | CASNO | CONCERN | AI | SOLUBILITY |
|--------|-----|------|-----|---------|---------|------|------------|
| 14064 | a | 1197 | | 3380345 | HIGH | 99.5 | 10mg/L |

CHEMNAME

5-chloro-2-(2,4-dichlorphenoxy)phenol, static

PHYSTATE

solid

| ORGANISM | DURATION | ENDPOINT | CODE | TOXVALUE | UNITS | MELTINGPT |
|-----------------------------|----------|----------|------|----------|-------|-----------|
| Algae, Navicula pelliculosa | 96h | EC50 | | 0.966 | ug/L | NS |

MELTINGPT

NS

COMMENTS

96hEC25=10.7ug/L (pop. growth)
nominal
acetone

ENTRY FORM

| CAPNUM | LTR | DATE | CBI | CASNO | CONCERN | AI | SOLUBILITY |
|--------|-----|------|-----|---------|---------|------|------------|
| 14064 | a | 1197 | | 3380345 | HIGH | 99.5 | 10mg/L |

CHEMNAME

5-chloro-2-(2,4-dichlorphenoxy)phenol, static

PHYSTATE

solid

| ORGANISM | DURATION | ENDPOINT | CODE | TOXVALUE | UNITS |
|----------------------------------|----------|----------|------|----------|-------|
| Algaefw, <i>S. capricornutum</i> | 96h | EC50 | | 4.46 | ug/L |

MELTINGPT

NS

COMMENTS

96hEC25=2.44ug/L (pop. growth)
nominal
acetone

ENTRY FORM

| CAPNUM | LTR | DATE | CBI | CASNO | CONCERN | AI | SOLUBILITY |
|--------|-----|------|-----|---------|---------|------|------------|
| 14064 | a | 1197 | | 3380345 | HIGH | 99.5 | 10mg/L |

CHEMNAME

5-chloro-2-(2,4-dichlorphenoxy)phenol, static

PHYSTATE

solid

| ORGANISM | DURATION | ENDPOINT | CODE | TOXVALUE | UNITS |
|----------|----------|----------|------|----------|-------|
|----------|----------|----------|------|----------|-------|

| | | | | | |
|-------------------------------|-----|------|---|------|------|
| Algaesw, Skeletonema costatum | 96h | EC50 | > | 66.0 | ug/L |
|-------------------------------|-----|------|---|------|------|

MELTINGPT

NS

COMMENTS

96hEC25>66.0ug/L (pop. growth)
nominal
acetone

ENTRY FORM

| CAPNUM | LTR | DATE | CBI | CASNO | CONCERN | AI | SOLUBILITY |
|--------|-----|------|-----|---------|---------|------|------------|
| 14064 | a | 1197 | | 3380345 | HIGH | 99.5 | 10mg/L |

CHEMNAME

5-chloro-2-(2,4-dichlorphenoxy)phenol, static

PHYSTATE

solid

| ORGANISM | DURATION | ENDPOINT | CODE | TOXVALUE | UNITS |
|-----------------------|----------|----------|------|----------|-------|
| Duckweed, Lemna gibba | 7d | EC50 | > | 62.5 | ug/L |

MELTINGPT

NS

COMMENTS

7dEC25>62.5ug/L (pop. growth)
nominal
acetone