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ENVIRONMENTAL STUDY REPORT

The Bioconcentration and Elimination
of ^{14}C -Bis(2-ethylhexyl) Terephthalate by
Eastern Oysters (Crassostrea virginica)

Final Report For:

Di(2-ethylhexyl) Terephthalate (DOTP)

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The work described in this report was done under contract for
Eastman Kodak Company, Rochester, New York 14650

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HEALTH AND ENVIRONMENT LABORATORIES
EASTMAN KODAK COMPANY, Rochester, N.Y. 14650

FINAL REPORT

**THE BIOCONCENTRATION AND ELIMINATION
OF ^{14}C -BIS(2-ETHYLHEXYL) TEREPHTHALATE BY
EASTERN OYSTERS (Crassostrea virginica)**

Submitted to

**Health and Environment Laboratories
Building 320, Kodak Park
Eastman Kodak Company
Rochester, New York 14650**

September 1986

by

**BATTELLE
New England Marine Research Laboratory
397 Washington Street
Duxbury, Massachusetts 02332**

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QUALITY ASSURANCE STATEMENT

FOR

**N-0950-2203, The Bioconcentration and
Elimination of ^{14}C -Bis (2-ethylhexyl)
Terephthalate by Eastern Oysters (Crassostrea virginica)**

In accordance with Good Laboratory Practice standards (EPA and TSCA, 40 CFR Part 792), this study has been monitored by Battelle's Quality Assurance Office. Monitoring included surveillance, audits, report preparation, and conference with the Study Director and Laboratory management.

Study audits which resulted in reports to management are listed in the attached table.

Christine Werme
10 September 1986

Christine E. Werme, Ph.D.
Manager,
Quality Assurance Office
Battelle New England
Marine Research Laboratory

QUALITY ASSURANCE AUDITS FOR N-0950-2203, The BIOCONCENTRATION AND ELIMINATION of ^{14}C -Bis (2-ethylhexyl) Terephthalate by Eastern Oysters (Crassostrea virginica)

Subject Audited	Date of Audit	Date of Report to Management
Initiation	3 January 1986	8 January 1986
Test conduct	21 January 1986	29 January 1986
Raw vs. reported data	22 August 1986 5,7 September 1986	25 August 1986 8 September 1986
Data file	25 August 1986 3 September 1986 8 September 1986	25 August 1986 4 September 1986 8 September 1986

The work conducted throughout the Eastman Kodak Study, "The Bioconcentration and Elimination of ^{14}C -Bis (2-ethylhexyl) Terephthalate by Eastern Oysters (Crassostrea virginica)," was performed in compliance with good laboratory practice (GLP). (Federal Register. Part III. Environmental Protection Agency: Toxic Substances Control; Good Laboratory Practice Standards; Final Rule. 40 CFR Part 792, 1983).



Sam R. Petrocelli, Study Director

9 September 1986
Date

Signature Page

September 1986

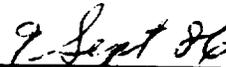
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Eastman Kodak Company
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Title of Study: The Bioconcentration and Elimination of ^{14}C -Bis(2-ethylhexyl) Terephthalate by Eastern Oysters (Crassostrea virginica).

Study Number: N-0950-2203



Michael E. Barrows, Author



Date



Sam R. Petrocelli, Study Director



Date

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FINAL REPORT

on

**THE BIOCONCENTRATION AND ELIMINATION
OF ^{14}C -BIS(2-ETHYLHEXYL) TEREPHTHALATE BY
EASTERN OYSTERS (Crassostrea virginica)**

to

**Health and Environment Laboratories
Building 320, Kodak Park
Eastman Kodak Company
Rochester, New York 14650**

September 1986

by

Michael E. Barrows

**For the Period
December 17, 1985 to January 24, 1986**

**BATTELLE
New England Marine Research Laboratory
397 Washington Street
Duxbury, Massachusetts 02332**

INTRODUCTION

The bioconcentration test described in this report was conducted at Battelle New England Marine Research Laboratory (BNEMRL), Duxbury, Massachusetts. The general objective of this investigation was to define the kinetics of the uptake (bioconcentration) and elimination (depuration) of ^{14}C -residues in the soft tissues of eastern oysters (Crassostrea virginica) continuously exposed to ^{14}C -bis(2-ethylhexyl) terephthalate at a nominal concentration of 50 micrograms per liter ($\mu\text{g/L}$). Specifically, three major areas were investigated:

The rate and extent of accumulation of ^{14}C -residues (i.e., parent compound, degradation products and metabolites calculated as bis(2-ethylhexyl) terephthalate) in the soft tissue portions of eastern oysters during 24 days of continuous aqueous exposure to ^{14}C -bis(2-ethylhexyl) terephthalate.

- B. The rate and extent of elimination of ^{14}C -residues from the soft tissue portion of oysters after transfer to flowing, uncontaminated salt water for a 14-day depuration period.
- C. The characterization of metabolites of ^{14}C -bis(2-ethylhexyl) terephthalate in oysters.

MATERIALS AND METHODS

The overall study organization, technical development, and project staff coordination were the responsibility of the Study Director and Technical Advisor, Dr. Sam R. Petrocelli. Project audits were conducted by Debra McGrath, project Quality Assurance Monitor, under the direction of Dr. Christine E. Werme, Director of Battelle New England's Quality Assurance Unit.

The Biology Task Leader was Michael Barrows, with the technical assistance of Rosanna Buhl. Dr. Robert Hillman conducted the histopathological examination and species verification of the oysters used in the test, with the technical assistance of Joanne Lahey. The analytical chemistry Task Leader was William Steinhauer, with the technical assistance of Elisabeth Smolski and John Bassett.

Test Material

The test material, bis(2-ethylhexyl) terephthalate, also known as dioctyl terephthalate, with a product name "Kodaflex DOTP," will be referred to as BEHTP throughout this report. The test material was supplied to BNEMRL by Eastman Kodak Company in two forms: bis(2-ethylhexyl) terephthalate (100%), SRID No. 84-6-20, HAEL No. 840090, a clear, viscous liquid; and bis(2-ethylhexyl) terephthalate (hexyl-2- ^{14}C), Lot No. 1249-297, >98.5% pure, a clear liquid dissolved in methanol. Both materials were used to prepare the primary stock and dosing material for the test. Verification of the identity and stability of each test material was the responsibility of Eastman Kodak. The test material was not characterized by Battelle. The bis(2-ethylhexyl) terephthalate was

stored at room temperature in a locked cabinet in the Biochemistry Laboratory, accessible only by the Project Task Leader, Analytical Chemistry Task Leader, or the Technical Supervisor. The ^{14}C -BEHTP was stored in the Radioactive Materials section of the Biochemistry Laboratory refrigerator, accessible solely by the authorized personnel identified above. Test concentrations are reported as micrograms (μg) of ^{14}C -BEHTP per liter (L) of diluent or parts per billion.

Test Animals

Test animals were received from Aquaculture Research Corporation, East Dennis, Mass., a commercial mariculture organization, and were acclimated for 62-89 days prior to testing. Oysters were cultured singles, between 3.5 and 5.6 centimeters (cm) from umbo to distal valve edge. Histological examination verified that the oysters were free of disease and that the gonads were in prespawning condition.

The ambient water characteristics of Duxbury Bay were similar to those of the culturing source, thus there was no need to adjust the oysters to either a new salinity or temperature during the acclimation period. Oysters were held in unfiltered Duxbury Bay salt water until 12 days before the test start. At that time they were moved to a tray receiving test dilution water, which was coarsely ($30\ \mu\text{m}$) filtered. During the acclimation period, oysters were observed to be actively filtering and free from epifaunal organisms.

Test Conditions

The test methods for the 38-day bioconcentration test were based on Battelle's SOP No. 5-083-01, "Performance of Bioconcentration Test with Oysters (Crassostrea virginica)," an adaptation of EPA protocol No. EG-6, Oyster Bioconcentration Test, August, 1982, from the Office of Pesticides and Toxic Substances, EPA, Washington, D.C. (EPA 560/6-82-002), with amendments previously approved by Kodak.

One hundred twenty (120) individual oysters were cleaned of all attached organisms and measured from umbo to distal valve edge prior to test initiation. Sixty oysters were randomly placed in each container. Two all-glass 20-gallon aquaria were used in the bioconcentration test. Each aquarium was fitted with a stand-pipe drainage system to maintain a water depth of 15 cm and a test solution volume of approximately 40 liters. During the exposure period, one aquarium received $50\ \mu\text{g}$ of ^{14}C -BEHTP per liter

of dilution salt water and the other aquarium (solvent control) received a combination of solvent and salt water, but no BEHTP. Both aquaria received dilution salt water at a continuous flow rate of 60 liters/hour.

The nominal concentration of 50 µg/L ¹⁴C-BEHTP was selected based on the results of the oyster shell deposition test and represented a concentration equivalent to one-tenth of the reported solubility of BEHTP in salt water. The ¹⁴C-BEHTP was dissolved in distilled-in-glass acetone and metered to the test aquarium at a rate of 0.057 milliliters (ml) per liter of salt water using a peristaltic pump. Similarly, 838 ml of acetone was combined with 162 ml of deionized water and metered to the solvent control at a rate of 0.068 ml per liter of salt water. A chemical characterization of Duxbury Bay salt water is presented in Table 1.

Since phthalate esters readily adsorb to solid surfaces from aqueous solution, the ¹⁴C-BEHTP was allowed to equilibrate with the physical system for a three-day period prior to test initiation. At the end of this time, duplicate 1-ml water samples were taken to verify that the actual exposure concentration was within 30 percent of the nominal concentration. The exposure of eastern oysters to ¹⁴C-BEHTP at a nominal concentration of 50 µg/L was continuous for 24 days. After 24 days of exposure, all remaining oysters from both test aquaria were transferred to clean aquaria in which toxicant-free salt water was introduced at a rate equal to the flow rate during exposure. The depuration period continued for 14 days to estimate half-life (i.e., the period of time required for the elimination of 50% of the accumulated ¹⁴C-residues present in the soft tissue of oysters at termination of exposure).

During the test, temperature, salinity, dissolved oxygen, and pH were measured in both test aquaria at a minimum of once a week. In addition, temperature in the solvent control aquarium was recorded hourly throughout the test with the use of an Omega Data Logger. A photoperiod of 14 hours light : 10 hours dark with a 30-minute phase-in period was maintained throughout the test. A saltwater alga, Isochrysis galbana, was cultured and used as a food supplement during the test. Fecal material was siphoned from each test aquarium when necessary throughout the testing period.

Analytical Methods

Duplicate 1-ml water samples were taken from the middle of the exposure and solvent control aquaria on Days 1, 3, 6, 10, 14, 17, 21, and 24 of the bioconcentration

phase, and on Days 1, 3, 7, 11, and 14 of the depuration phase. Each water sample was immediately placed in a glass vial containing 15-ml of Aquasol-2, a liquid scintillation counting medium, and analyzed for ^{14}C -activity with a Beckman LS-7500 Liquid Scintillation Counter. Samples (5 μl , 100 μl) of the ^{14}C -BEHTP stock solution were collected during the bioconcentration phase at the same time as collection of water samples and analyzed for ^{14}C -activity to verify stock solution stability.

Concentrations of ^{14}C -BEHTP in test water were measured according to procedures described in Battelle New England SOP Nos. 5-094-02 (Extraction and Analysis of Phthalate Esters in Water) and 5-025-01 (Gas Chromatography Protocols). In brief, duplicate 20-ml water samples were collected from the exposure aquarium on Days 0, 6 and 24 and a single 20-ml sample from the solvent control aquarium on Days 0 and 24 of the bioconcentration phase. After collection, each water sample was transferred to the chemistry laboratory and extracted with hexane in a separatory apparatus. All extractions were completed within 24 hours of sample receipt. The extracts were analyzed on a Shimadzu Gas Chromatograph (Model GC-9A) with a wide-base fused silica capillary column. The internal-standard method was used for calibration (SOP No. 5-025-01). No attempt was made to verify the stability of the acetone carrier in the test system because it is commonly recognized as the solvent of choice for aquatic toxicology tests.

Four replicate oysters were sampled at random from the exposure and solvent control aquaria on Days 1, 3, 6, 10, 14, 17, 21, and 24 of the bioconcentration phase and on Days 1, 3, 7, 11, and 14 of the depuration phase. The shell height of each oyster was measured to the nearest 1.0 mm and recorded. At each sampling interval, oysters exposed to ^{14}C -BEHTP were shucked and the soft tissues blotted with a Kimwipe^R to remove excess moisture. Oysters were weighed to the nearest 0.01 mg on a Mettler balance. The soft tissue portion of each oyster was then placed in an all-glass tissue grinder and homogenized at 3500 rpm. A 100-150 mg aliquot of each homogenate was then placed in a glass scintillation vial and 1.0 ml of Protosol, a tissue solubilizer, was added. Each vial was then sealed tightly with a polyethylene-lined cap and heated overnight at 55°C. The following day, a 0.1-ml volume of 30% hydrogen peroxide was added to each sample and reheated to 55°C for a 30-minute period to remove any color. After each sample was allowed to cool for 15 minutes, 12 ml of Econofluor, a liquid scintillation medium for organic samples, was added to each vial. The vials were then sealed tightly, shaken, and allowed to equilibrate in the dark for at least 60 minutes prior to analyses for ^{14}C -activity. Oysters from the solvent control aquarium were processed similarly, but only on

Days 1 and 24 of the bioconcentration phase and Day 14 of the depuration phase. The remaining control oysters were removed at each sampling interval to maintain equality with respect to the loading rate of biomass per unit volume of oysters exposed to ^{14}C -BEHTP.

To determine if the oysters had metabolized the ^{14}C -BEHTP, four oysters were removed from the exposure aquarium at the end of both the bioconcentration and depuration phases. Tissue from these oysters was pooled and analyzed for the presence of phthalate metabolites according to the procedures in draft SOP No. 5-110-01, a modification to the method of Wofford et al. (1981). Metabolites were separated by thin layer chromatography on 0.25-mm silica gel plates (silica gel 60 F-254) using a solvent system of methylene chloride:methanol (2:1, v/v). The developed plates were divided into three fractions: F1—from the origin to just below the BEHTP zone, corresponding to material more polar than BEHTP; F2—the zone containing only BEHTP; and F3—from above the BEHTP zone to the solvent front, corresponding to material less polar than BEHTP. Each fraction was scraped off the TLC plate and placed into liquid scintillation vials containing 15 ml of an Aquasol-2/Milli-Q water mixture (77%: 23%, v:v). All samples were analyzed within 24 hours after preparation and the ^{14}C -activity was measured by liquid scintillation counting techniques on the Beckman LS-7500 Liquid Scintillation Counter (SOP No. 5-092-01).

RESULTS

^{14}C -Residues in Water

The concentrations of ^{14}C -residues, calculated as BEHTP, measured in the water of the exposure aquarium during the 24-day exposure and the 14-day depuration period are presented in Table 2. These data indicate that the mean (\pm standard deviation) concentrations of ^{14}C -residues in aqueous solution prior to the introduction of oysters into the system (i.e., equilibration period) was $47.6 \pm 3.39 \mu\text{g/L}$, 95% of the nominal concentration of $50 \mu\text{g/L}$. Based on this observation, the oyster bioconcentration test was initiated. Radiometric analyses indicate that the replicate values measured at each sample interval were relatively consistent throughout the exposure period. Despite a few minor fluctuations, the ^{14}C -residue concentration in the water of the treated system remained relatively constant throughout the 24-day exposure period and was calculated to be $48.4 \pm 7.56 \mu\text{g/L}$, 97% of nominal (Table 3).

Radiometric analyses performed on samples of the ^{14}C -BEHTP stock solution collected periodically during the exposure period indicate that the mean measured concentration was $858 \pm 80.9 \mu\text{g/L}$ throughout the exposure period. This value represents 98% of the nominal stock concentration of $877 \mu\text{g/L}$, calculated prior to test initiation and verifies the stability of the stock solution in the acetone carrier. These data are presented in Appendix 4 of the data package.

Radiometric analyses performed on water samples collected during the depuration phase in which previously exposed oysters were held in ^{14}C -BEHTP free-flowing water are presented in Table 2. These data indicate measurable amounts of ^{14}C -BEHTP in some of the water samples collected during the initial 11 days of depuration. By Day 14, the ^{14}C -residues in the water had decreased to below minimum detectable limits ($0.68 \mu\text{g/L}$). These very low concentrations of aqueous ^{14}C -residues were presumed to be a result of the elimination of the ^{14}C -residues previously accumulated in the oyster tissues.

Analyses of solvent control water samples indicated that no measurable ^{14}C -residues were present above background radiation throughout the study period. The background radiation associated with water from the solvent control ranged from 19 to 28 disintegrations per minute (dpm) throughout the study period. These data are presented in Appendix 4 of the data package.

Gas Chromatographic Analysis of BEHTP in Water

The concentrations of BEHTP measured by gas chromatography (GC) in water samples collected from the test system on Days 0, 6 and 24 of the exposure period are presented in Table 4. These data indicate that the measured concentrations of BEHTP varied by a factor of 2x to 5x within sample replicates on Days 0 and 6 of the exposure period which was not corroborated by the ^{14}C -measurements. The mean measured concentration of $18 \pm 2.1 \mu\text{g/L}$, for water sampled on Day 24, represented 32% of the mean ^{14}C -residue concentration measured by LSC techniques on Day 24 ($56.5 \mu\text{g/L}$). The variability in the GC analysis results maybe an artifact of the extraction and analysis procedure.

GC analysis of the water from the solvent control aquarium indicated a measurable concentration of BEHTP present on Day 24 of the exposure period. No measurable amounts of ^{14}C -residues were observed in the solvent control water on Day 24 of exposure. These data are documented in Appendix 4 of the data package.

¹⁴C-Residues in Oyster Tissue

The mean ¹⁴C-residue concentrations measured in homogenized soft tissues of eastern oysters sampled during 24 days of continuous aqueous exposure to ¹⁴C-BEHTP and during the 14-day depuration period are presented in Tables 2 and 3, and Figure 1. These data indicate that the concentration of ¹⁴C-residues measured in the homogenized oyster tissues increased substantially during the first three days of exposure. This was the period of maximum accumulation of ¹⁴C-residues, calculated as ¹⁴C-BEHTP. The mean concentration of 36.2 ± 16.5 $\mu\text{g/g}$ measured in the oyster tissues sampled on Day 3 of exposure represented a maximum bioconcentration factor of 790X. Between Days 3 and 10 of exposure, the ¹⁴C-residue concentration in oyster tissues decreased substantially (50%). At this point (Day 10) and throughout the remaining 14 days of exposure, an apparent equilibrium between the rates of accumulation and elimination of ¹⁴C-residues in oyster tissues existed. Based on a mean measured concentration of 19.0 ± 2.65 $\mu\text{g/g}$ in the oyster tissue sampled on Days 10 to 24 of exposure and a mean aqueous concentration of 48.4 ± 7.56 $\mu\text{g/L}$ ¹⁴C-BEHTP measured in the water during the 24-day exposure period, the mean equilibrium bioconcentration factor for ¹⁴C-BEHTP in oyster tissues was 393X.

On January 8, 1986 (Day 22), a one-way analysis of variance was performed on the results of analyses of the ¹⁴C-residue content in oyster tissues on Days 14, 17 and 21 of exposure as stipulated in the test protocol. The result of the ANOVA test indicated that there was no statistical difference in the ¹⁴C-residue concentrations in oyster tissues over the three sampling periods. Based on these results, the exposure phase of the bioconcentration test was terminated at Day 24.

Depuration of ¹⁴C-Residues from Oyster Tissue

Radiometric analyses of the homogenized tissues of oysters transferred to flowing, uncontaminated water after 24 days of continuous aqueous exposure to ¹⁴C-BEHTP indicate that there was significant elimination of ¹⁴C-residues from oyster tissues during the initial 7 days of the depuration phase. Half-life of the ¹⁴C-residue content present in oyster tissues at the end of the exposure period occurred between Days 3 and 7 of the depuration period. Between Days 7 and 14 of depuration, no further elimination of ¹⁴C-residues from the oyster tissues was observed. The mean concentration of 4.70 ± 0.25 $\mu\text{g/g}$, measured in the oyster tissues during Days 7 to 14 of depuration, represented

an elimination of approximately 70% of the ^{14}C -residue content present in the oyster tissues at the termination of the exposure period.

Some measurable ^{14}C -residue concentrations were detected in the homogenized tissue of two of the four oysters from the solvent control aquarium on Day 24 of exposure. These levels, 0.11 and 0.18 $\mu\text{g/g}$, were attributed to contamination of the samples during preparation for analysis rather than to any ^{14}C -residues actually being present in the animals prior to sampling. No measurable ^{14}C -residues were present in the two remaining oysters from that sample set or at any other sampling of solvent control oysters. The background radiation associated with oyster tissues from the solvent control ranged from 40 to 49 dpm during the exposure period and 25 to 26 dpm on Day 14 of the depuration period.

Metabolism of ^{14}C -BEHTP by Oysters

Thin layer chromatographic analyses (TLC) combined with liquid scintillation counting techniques (LSC) were used to determine the concentration of ^{14}C -BEHTP and its metabolites in the homogenized soft tissue of oysters after 24 days of exposure to a mean aqueous concentration of $48.4 \pm 7.56 \mu\text{g/L}$ ^{14}C -BEHTP and after an additional 14 days of depuration in flowing, uncontaminated water. Each sample was extracted and developed on three separate TLC plates that were subsequently analyzed by separate LSC determinations. One plate was used for methods development and the remaining two plates were used to generate the results presented in Table 5. The mean background radiation of the blank samples for each sample run was calculated and the results were used as the sample correction value. These data are documented in Appendix 4 of the data package.

The results of the TLC analyses indicate that there were visible phthalates in the F1 and F2 fractions, corresponding to the locator standards dibutyl phthalate ester and BEHTP, for the Day 24 exposure and Day 14 depuration samples. A measureable amount of activity was indicated in the F3 fraction, corresponding to material less polar than BEHTP. Of the total ^{14}C -activity measured from the two TLC plates, 16.1 to 17.3% was associated with the F1 fraction, 79.4 to 80.7% was associated with the F2 fraction (BEHTP), and 3.2 to 3.4% was associated with the F3 fraction for Day 24 of exposure. Based on these results, the adjustment factor for the concentration of ^{14}C -residues (as BEHTP) measured in oyster tissues on Day 24 of exposure is 80.0%. The corresponding

results for Day 14 of depuration are 22 to 24.9% (F1 fraction), 72.9 to 75.5% (F2 BEHTP) and 2.1 to 2.5% (F3 fraction). Based on these results, the adjustment factor for the concentration of ^{14}C -residues (as BEHTP) measured in oyster tissues on Day 14 of depuration is 74.2%.

Therefore, the mean measured concentration of $15.6 \pm 8.11 \mu\text{g/g}$ observed in the soft tissues of oysters after 24 days of exposure contained 20.0% as metabolites of BEHTP and a mean concentration of $12.5 \pm 6.51 \mu\text{g/g}$ as BEHTP (Table 6). Corresponding results for Day 14 of depuration indicate that the mean concentration of $4.92 \pm 5.43 \mu\text{g/g}$ contained 25.8% as metabolites of BEHTP and a mean concentration of $3.65 \pm 4.03 \mu\text{g/g}$ as BEHTP.

Water Quality

Means, standard deviations, and ranges were calculated for the following parameters measured during the test: temperature, salinity, pH, and dissolved oxygen. These data are summarized in Table 7 and indicate that for the test water of the solvent control system, the salinity ranged from 32.5 to 34.0 parts per thousand (‰), the temperature from 19.5 to 23.4°C, the pH from 7.79 to 8.07 units, and the dissolved oxygen from 6.8 to 8.8 mg/L. For the exposed aquarium, the test water measured throughout the testing period indicated that the salinity ranged from 32.5 to 34.0 ‰, the temperature from 19.4 to 22.7°C, the pH from 7.86 to 8.07 units, and the dissolved oxygen from 6.8 to 8.9 mg/L.

The shell growth of oysters from both test populations was monitored throughout the 38-day testing period and is presented in Section F of Appendix 3. These data indicate that no difference was observed in the mean shell growth of oysters from the two test populations, although some variability in shell growth was observed between individual oysters in some sample sets. At the end of the 38-day testing period, the maximum mean shell growth of oysters from the ^{14}C -BEHTP-exposed population was 7 ± 4 mm with a range of 0 to 12 mm. The corresponding data for oysters from the solvent control system were 7 ± 3 mm with a range of 0 to 11 mm.

Archive Location

All raw data collected during the test period and the final report will be submitted to Eastman Kodak Company. A copy of the raw data will be stored in Battelle New England's limited-access archives, maintained by Debra McGrath. Original instrument calibration and organism culture records are stored in the Battelle Toxicology Laboratory files.

CONCLUSIONS

The following conclusions are based on the analyses performed throughout the 38-day oyster bioconcentration test with ^{14}C -BEHTP:

1. The aqueous ^{14}C -residue concentrations, calculated as BEHTP, remained relatively constant throughout the exposure period. The mean concentration of $48.4 \pm 7.56 \mu\text{g/L}$ represented 97% of the nominal concentration of $50 \mu\text{g/L}$.
2. Analyses of the ^{14}C -BEHTP stock solution indicate that the mean measured concentration was $858 \pm 80.9 \mu\text{g/L}$ throughout the exposure period; this verified the stability of the stock solution in the acetone carrier.
3. Measurable amounts of ^{14}C -residues were observed in the depuration water of the oysters previously exposed to ^{14}C -BEHTP; this verified the elimination of ^{14}C -residues that had been accumulated by the oysters during the exposure period.
4. No measurable ^{14}C -activity was observed in the water of the solvent control system throughout the study period.
5. GC analyses of the water from the exposure aquarium indicate variability between sample replicates on Days 0 and 6 of the exposure period which was not corroborated by the ^{14}C -measurements. The mean concentration of $18 \pm 2.1 \mu\text{g/L}$ measured by GC analyses on Day 24 represented 32% of the ^{14}C -residue concentration measured by LSC techniques on Day 24 of exposure. The variability in the GC analysis results maybe an artifact of the extraction and analysis procedure.
6. The maximum bioconcentration factor of ^{14}C -BEHTP in oyster tissue was 790x. The maximum ^{14}C -BEHTP concentration in oyster tissue was observed on Day 3 of the exposure period.

7. The mean equilibrium bioconcentration factor of ^{14}C -BEHTP in oyster tissue was 393x. The apparent equilibrium of ^{14}C -BEHTP in oyster tissues was observed between Days 10 and 24 of the exposure period.
8. A one-way analysis of variance indicated that there was no statistical difference between the mean ^{14}C -residue content measured in oysters on Days 14, 17, and 21 of the exposure period.
9. Half-life of the accumulated ^{14}C -residue content present in oyster tissues at the end of the exposure period occurred between Days 3 and 7 of the depuration period.
10. TLC analyses indicate that 79.4 to 80.7% of the accumulated ^{14}C -residue content present in oyster tissues at the end of the exposure period was the parent compound BEHTP and the remaining 19.3 to 20.6% were metabolites and/or degradation products.
11. TLC analyses indicate that 72.9 to 75.5% of the ^{14}C -residues remaining in the tissue of oysters after 14 days of depuration was the parent compound BEHTP and the remaining 24.5 to 27.1% were metabolites and/or degradation products.
12. Water quality analyses performed during testing indicate that all parameters measured were within the ranges specified in the test protocol.
13. No difference was observed in the mean shell growth of oysters from the two test populations at the end of the testing period.

Table 1. Chemical Characterization of Duxbury Bay Seawater.

Parameter Description	Measured Concentration ^a
Total Copper	0.053 mg/L
Ammonia	0.10 mg/L
Residual Chlorine	<0.1 mg/L
Pesticides	ND ^b
PCBs	ND ^b
Total Organic Carbon	0.77 mg/L
Suspended Solids	19 mg/L

^a Water samples for ammonia, pesticides, and PCB analyses were collected in June 1985. Water samples for the other analyses were collected in November 1985.

^b ND = not detected (detection limits were pesticides = 0.1 µg/L; PCBs = 0.1 µg/L)

Table 2. Measured ^{14}C -residue concentrations, calculated as BEHTP, in the soft tissue portion of eastern oysters (*Crassostrea virginica*) during 24 days of continuous aqueous exposure to ^{14}C -BEHTP at a mean measured concentration of $48.4 \pm 7.56 \mu\text{g/L}$ and during an additional 14 days of depuration in flowing, toxicant-free salt water.

Period	Day	^{14}C -Residue Concentration	
		Salt Water ($\mu\text{g/L}$) ^a	Oyster Tissue ($\mu\text{g/g}$) ^b
Equilibration	-1	47.6 \pm 3.39	--
Exposure	0	45.9 \pm 8.77	--
	1	46.6 \pm 0.99	5.72 \pm 3.36
	3	44.8 \pm 0.49	36.2 \pm 16.5
	6	44.8 \pm 5.30	27.2 \pm 5.63
	10	52.4 \pm 2.40	18.4 \pm 3.57
	14	34.6 \pm 0.49	22.2 \pm 4.44
	17	52.0 \pm 1.91	21.0 \pm 9.85
	21	57.5 \pm 6.79	17.6 \pm 6.89
	24	56.5 \pm 1.41	15.6 \pm 8.11
Depuration	1	<1.02 \pm 0.49	14.6 \pm 5.44
	3	2.06 \pm 0.97	8.23 \pm 3.38
	7	<1.36 \pm 0.97	4.43 \pm 3.49
	11	<1.36 \pm 0.97	4.76 \pm 3.37
	14	<0.68 \pm 0.00	4.92 \pm 5.43

^a Mean and standard deviation based on the radiometric analyses of duplicate 1-ml samples.

^b Mean and standard deviation based on the radiometric analyses of four 100-150 mg aliquots of homogenized oyster tissue.

Table 3. Bioconcentration of ^{14}C -residues, calculated as BEHTP, in the soft tissue portion of eastern oysters (*Crassostrea virginica*) during 24 days of continuous aqueous exposure to ^{14}C -BEHTP.

Exposure Period Day	^{14}C -Residue Concentration			Bioconcentration Factor ^d (x)
	Salt Water ^a ($\mu\text{g/L}$)	Cumulative Mean ^b Salt Water ($\mu\text{g/L}$)	Oyster Tissue ^c ($\mu\text{g/kg}$)	
0	45.9 \pm 8.77	—	—	—
1	46.6 \pm 0.99	46.2 \pm 5.11	5,720	124x
3	44.8 \pm 0.49	45.8 \pm 4.03	36,200	790x
6	44.8 \pm 5.30	45.6 \pm 3.98	27,200	596x
10	52.4 \pm 2.40	46.9 \pm 4.61	18,400	392x
14	34.6 \pm 0.49	44.9 \pm 6.37	22,200	494x
17	52.0 \pm 1.91	45.9 \pm 6.44	21,000	458x
21	57.5 \pm 6.79	47.3 \pm 7.40	17,600	372x
24	56.5 \pm 1.41	48.4 \pm 7.56	15,600	322x

^a Mean and standard deviation based on the radiometric analyses of duplicate water samples (N = 2).

^b Cumulative mean concentration in salt water based on progressive data (Days 0-24); N = 2 + 2...N = 18.

^c Mean based on the radiometric analyses of four homogenized tissue samples (See Table 2).

^d Bioconcentration factor is the mean measured ^{14}C -residues of BEHTP in oyster tissues divided by the cumulative mean measured concentration of ^{14}C -BEHTP in exposure water (\bar{x} $\mu\text{g/Kg}$ \div \bar{x} $\mu\text{g/L}$).

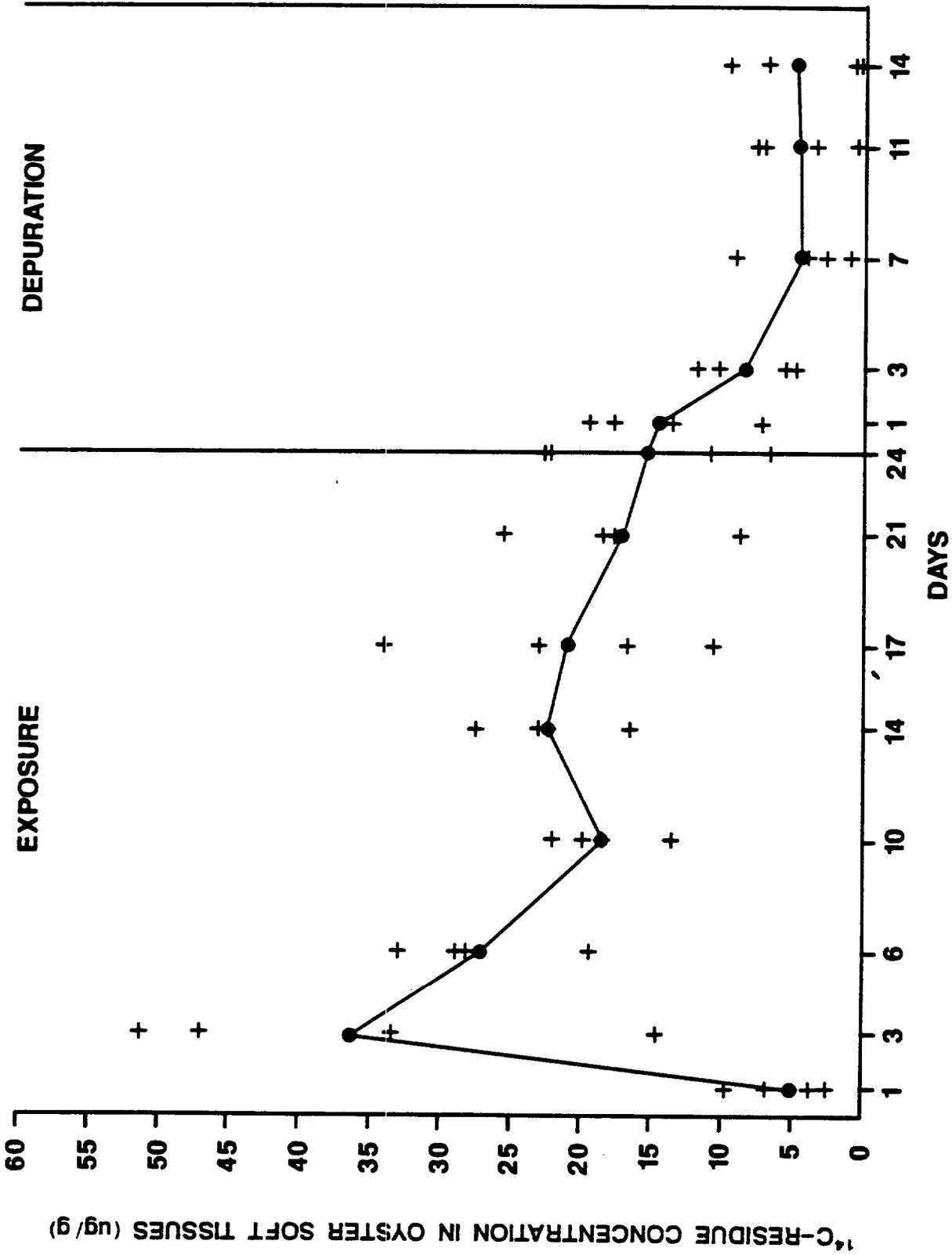


Figure 1. Mean measured ¹⁴C-residue concentrations, calculated as BEHTP, in the soft tissues of eastern oysters (*Crassostrea virginica*) during 24 days continuous exposure to ¹⁴C-BEHTP at a mean measured concentration of 48.4 µg/L and during an additional 14 days depuration in flowing, toxicant-free seawater.

Table 4. Concentrations of BEHTP measured by gas chromatography in the water during 24 days of continuous aqueous exposure of eastern oysters (*Crassostrea virginica*) to ^{14}C -BEHTP at a mean measured concentration of 48.4 ± 7.56 $\mu\text{g/L}$.

Nominal Concentration ($\mu\text{g/L}$)		Measured Concentration ($\mu\text{g/L}$) ^a		
		Day 0	Day 6	Day 24
Solvent Control		ND ^b	NA ^c	1.6
50.0	Rep A	20	4.2	16
	Rep B	46	20	19
$\bar{x} \pm \text{S.D.}$		33 ± 18	12 ± 11	18 ± 2.1

^a Analyses performed on a Shimadzu Gas Chromatograph (Model GC-9A) with a wide-base fused silica capillary column.

^b Not detected.

^c Not analyzed.

Table 5. Results of thin layer chromatographic analyses performed on the homogenized soft tissues of eastern oysters (*Crassostrea virginica*) exposed for 24 days to a mean aqueous concentration of $48.4 \pm 7.56 \mu\text{g/L}$ ^{14}C -BEHTP and after an additional 14 days in flowing, uncontaminated water.

	Net Disintegrations Per Minute ^a			% Metabolite ^b
	F1 Fraction Metabolite	F2 Fraction BEHTP	F3 Fraction Metabolite	
Exposure Period				
Day 24				
Plate A	38.6	193.2	7.6	19.3
Plate C	254.9	1169.7	49.4	<u>20.6</u>
				\bar{x} 20.0 \pm 0.92
Depuration Period				
Day 14				
Plate A	27.4	94.1	3.1	24.5
Plate C	87.2	255.0	7.4	<u>27.1</u>
				\bar{x} 25.8 \pm 1.84

^a Net disintegrations per minute (dpm) determined by subtracting the mean background dpm value for each LSC run from the sample dpm value.

^b Percentage metabolite = $\frac{F1+F3}{F1+F2+F3}$

Table 6. Adjusted concentrations of ^{14}C -BEHTP, as parent compound, in the homogenized soft tissues of eastern oysters (*Crassostrea virginica*).

	LSC ^a Concentration ($\mu\text{g/g}$)	Correction ^b Factor (%)	Adjusted Concentration as BEHTP ($\mu\text{g/g}$)
<u>Exposure Period</u>			
Day 24			
Oyster Sample			
KOBT No. 115	22.7	80.0	18.2
KOBT No. 116	10.8	80.0	8.64
KOBT No. 117	6.63	80.0	5.30
KOBT No. 118	22.2	80.0	17.8
	\bar{x} 15.6 \pm 8.11		\bar{x} 12.5 \pm 6.51
<u>Depuration Period</u>			
Day 14			
Oyster Sample			
KOBT No. 169	11.7	74.2	8.68
KOBT No. 170	0.426	74.2	0.316
KOBT No. 171	0.649	74.2	0.482
KOBT No. 172	6.92	74.2	5.13
	\bar{x} 4.92 \pm 5.43		\bar{x} 3.65 \pm 4.03

^a Concentration measured by liquid scintillation counting (LSC) techniques.

^b Percentage parent compound derived by thin layer chromatographic analyses (See Table 5).

Table 7. Water quality of test treatments during a 38-day bioconcentration test of ^{14}C -BEHTP to eastern oysters (*Crassostrea virginica*) in a flow-through system.

Concentration		Water Quality Parameter ^a			
Test Concentration	Test Date	Temperature (°C)	Salinity (‰)	pH	Dissolved Oxygen (ppm)
Solvent	12/17/85	21.1	32.5	7.99	7.6
Control	12/26/85	19.5	33.0	8.07	7.5
	12/31/85	21.3	33.0	8.06	7.3
	1/7/86	21.2	33.0	7.85	6.8
	1/10/86	21.7	33.0	7.86	7.1
	1/10/86	21.1	33.0	7.91	8.8
	1/17/86	23.4	34.0	7.79	7.6
	1/24/86	20.7	33.0	8.05	7.8
	\bar{x}		21.2 ± 1.1	33.0 ± 0.4	7.95 ± 0.11
Range		19.5 - 23.4	32.5 - 34.0	7.85 - 8.07	6.8 - 8.8
^{14}C -BEHTP 50 µg/L	12/17/85	21.1	32.5	8.00	7.5
	12/26/85	19.4	33.0	8.06	7.4
	12/31/85	21.2	33.0	8.04	7.2
	1/7/86	21.2	33.0	7.86	6.8
	1/10/86	21.7	33.0	7.88	7.1
	1/10/86	21.2	33.0	7.92	8.9
	1/17/86	22.7	34.0	8.00	7.1
	1/24/86	20.5	33.0	8.07	7.6
\bar{x}		21.1 ± 0.9	33.0 ± 0.4	7.98 ± 0.08	7.4 ± 0.6
Range		19.4 - 22.7	32.5 - 34.0	7.86 - 8.07	6.8 - 8.9

^a Water quality parameters measured periodically throughout the testing period.

EASTMAN KODAK COMPANY

Oyster Bioconcentration Test

N-0950-2203

APPENDIX I

Test Protocol and Amendment

Subject: Oyster Shell Deposition and Bioconcentration Test with BEHTP (bis-(2 ethylhexyl) terephthalate) Amendments to Revised Protocol Dated 19 September 1985

Page 2: Cognizant technical managers are:
Battelle: change Jerry Neff to Sam R. Petrocelli

Page 2: Study Director: change Dr. Jerry M. Neff to Dr. Sam R. Petrocelli

Page 3: First line should read as follows:

The proposed duration of this investigation is three months, from October 28, 1985 to January 31, 1986. The proposed schedule is as follows:

Week of:

October 28 Acquisition/acclimation of oysters. Analytical chemistry protocol verification. Equilibration of diluter system with test compound

November 4 Oyster Shell deposition bioassay initiated

November 25 Calibration/verification of dosing system

December 2 Oyster bioconcentration test, exposure phase

December 30 Oyster bioconcentration test, depuration phase

January 13 Data analysis and synthesis

January 27 Submission of draft final report

Page 5: First paragraph; line 7; should have read: we will acclimate oysters obtained from Dennis for a minimum of four days in the Battelle laboratory.....

Page 6: First paragraph, line 3; should have read: The stock solution will be delivered by a metering pump through Teflon tubing from a 4 liter amber stock bottle to the mixing chamber of the diluter system.

Page 6: First paragraph, line 8; should have read: Four liters of the 1000 ppm stock solution will be sufficient to provide test compound to the diluter system for the four days of equilibration and the four days of testing. Note: Delete last sentence - stock solution was not renewed daily.

Page 6: Second paragraph, line 5; should have read: The nominal exposure concentrations will be: 31.2, 62.5, 125, 250 and 500 µg BEHTP/liter seawater.

Page 6: Third paragraph, line 3; change from protocol: On day 4 of equilibration, duplicate 20 ml water samples were collected from the high, middle and low test concentrations. Single 20 ml water samples were collected from the control and solvent controls. Each sample was a composite of 10 mls from the A and B replicates. On day 8 of equilibration, triplicate 20 ml water samples were collected from the high test concentration and single 20 ml water samples were collected from the control, low and middle test concentrations. Each sample was a composite of 10 mls from the A and B replicates.

Note: Due to a miscalculation in the preparation of the initial stock solution, the integrity of the aforementioned samples were deemed inadequate as 0-hour samples for the initiation of the oyster shell deposition test. Following the preparation of a new stock solution and prior to the introduction of oysters into the test system, duplicate 20 ml water samples were collected from each replicate of the high, middle and low test concentrations to confirm that the desired test concentrations were established. A single 20 ml water sample was collected from each replicate of the solvent control.

Page 7: First Paragraph, line 2; should have read: Triplicate 20 ml samples will be taken from each replicate of the low, middle and high test concentrations at test termination (96-hours) of the shell deposition test. One 20 ml water sample will be taken from each replicate of the solvent control.

Note: Based on the changes made in the sampling schedule, a total of 43 water samples were collected for analysis during the equilibration and definitive shell deposition test.

Page 8: First paragraph, line 3; Delete: a seawater control

Page 8: Third Paragraph: Change: Assuming an exposure concentration of 50 µg/liter, the stock solution will be prepared at a concentration appropriate to the toxicant delivery method. This method will provide a 50 µg BEHTP/liter exposure solution containing \leq 0.1 ml/l acetone carrier.

Page 9: First paragraph, line 1; amend: The solvent control tank will receive seawater containing the maximum concentration of acetone used in the exposure tank. Line 2: delete.

Page 9: Second paragraph, line 1; amend: Seawater containing the test material in solution will be delivered to the exposure chamber at a rate of 60 liters per hour.

- Page 9: Third paragraph, line 1; amend: Sixty small oysters in the size range of 40-60 mm..... in each the exposure and solvent control chambers. Exposure chambers will be all glass 30-gallon aquaria fitted with a stand-pipe drainage system to maintain water depth at 16 cm.
- Page 9: Fifth paragraph, line 1; amend: Duplicate 1 to 2-ml water samples.....on days 0, 3, 7, 10.....
- Page 10: First paragraph, line 1; amend: In addition, duplicate 20 ml water samples.....
- Page 10: Second paragraph, line 2; delete: two and amend to read: exposure chamber and the solvent control chamber on days 1, 3, 7, 10.....
- Page 10: Section 4.4. First paragraph, line 1; amend to read: Duplicate 2-ml water samples
- Page 10: Section 4.4. Second paragraph, line 1: amend to read: In addition, duplicate 20-ml water samples will be collected from the exposure tank on day 0 and 28 of the bioconcentration phase.....
- Page 11: Section 4.5. First paragraph, line 2; change: 1.0 mm to read 0.1 mm.
- Page 12: Section 5.1. First paragraph; change: SOP No. 5-094-02.....In brief, a 20 ml water sample will be transferred to the laboratory and extracted with hexane in a separatory apparatus.
- Page 12: Section 5.1. First paragraph, line 6; change: The hexane extract is dried and concentrated to an appropriate volume.
- Page 13: Amend: SOP No. 5-094-01 to 5-094-02.
- Page 14: Section 5.2. First paragraph, line 3; amend to read: through sample preparation and analysis, and up to archival of sample extracts pending review of analytical data.
- Page 15: Section 5.2. Third paragraph, line 5; amend to read: Upon completion of sample analyses, custody of sample data will be relinquished to the Toxicology Study Director for archival. Sample extracts will be discarded in an approved manner.

Approval

Study Director:

S R Procell

Date:

28 Aug. 1986

STUDY PROTOCOL APPROVALS

OYSTER, Crassostrea virginica, SHELL DEPOSITION AND
BIOCONCENTRATION TEST WITH BIS (2-ETHYLHEXYL) TEREPHTHALATE

Eastman Kodak Company

Program Monitor: Rodney J. Boatman
Title: Chemist
Date: Oct. 7, 1985

Battelle New England Marine Research Laboratory

Study Director: Jerry M. Hoff
Title: Research Leader and Assistant Director for Research
Date: Sept. 17, 1985

Director, Marine Toxicology Section: S R Petrucci
Title: Director of Marine Toxicology
Date: 19 September 1985

Director, Analytical Chemistry Section: F. DeLuca
Title: Director of Marine Chem
Date: 19 Sept 1985

Director, Quality Assurance: Christine Warner
Title: Director, Quality Assurance
Date: 18 September 1985

Director, Battelle
New England Marine Research Laboratory: Paul G. Gifford
Title: Director
Date: 19 Sept 1985

STUDY PROTOCOL

OYSTER, Crassostrea virginica, SHELL DEPOSITION AND BIOCONCENTRATION TESTS WITH BIS (2-ETHYLHEXYL) TEREPHTHALATE

1.0 Statement of Work

Two marine toxicity/biological effects tests will be performed with the American oyster Crassostrea virginica and the client's organic industrial chemical, bis (2-ethylhexyl) terephthalate. The oyster shell deposition test will be performed to provide a short-term assessment of the hazard of the test chemical to a commercially important marine animal and as a range-finder to establish the appropriate exposure concentration for the oyster bioconcentration test. The oyster bioconcentration test will be performed to provide an assessment of the potential for and magnitude of bioconcentration and subsequent depuration of the test chemical during and following exposure to the test chemical in solution in the ambient seawater medium.

The test chemical, bis (2-ethylhexyl) terephthalate (BEHTP), is an isomer of the widely-used plasticizer di (2-ethylhexyl) phthalate (DEHP). In BEHTP, the carboxylic acid esters are in the para position of the benzene nucleus; in DEHP, the carboxylic acid esters are in the ortho position. There is a fairly extensive published literature on the toxicity and biological effects of DEHP (reviewed by Thomas et al., 1978), including data on bioconcentration from seawater by marine mussels, Mytilus edulis (Brown and Thompson, 1982). However, there is little information about the marine toxicology of BEHTP.

BEHTP will be supplied by Eastman Kodak Corporation in an unlabelled and ^{14}C -labelled form. Phthalate esters are hydrophobic nonpolar liquid organic compounds which have low aqueous solubilities and tend to absorb strongly on solid surfaces.

Characteristics of the test compound are as follows:

Compound: bis (2-ethylhexyl) terephthalate

Molecular Weight: 391

Solubility (seawater): 610 $\mu\text{g/l}$

Octanol/Water Partition Coefficient: seawater 1.8×10^5 , \log_{10} 5.26
freshwater 5.2×10^5 , \log_{10} 5.72

For radiolabelled compound:

Vial No. Information will be added as an amendment
Purity: Information will be added as an amendment
Amount: Information will be added as an amendment
Solvent: acetone
Specific activity: approx. 0.0222 mCi/mg
Concentration: Information will be added as an amendment

The study will be conducted for:

Health and Environment Laboratories
Building 320, Kodak Park
Eastman Kodak Company
Rochester, New York 14650

and will be performed by:

Battelle New England Marine Research Laboratory
397 Washington Street
Duxbury, Massachusetts 02332

Cognizant technical managers are:

Kodak: Rodney Boatman (716) 588-5343
Battelle: Jerry Neff (617) 934-5682

Key Battelle staff who will perform this investigation are:

Study Director: Dr. Jerry M. Neff

Toxicity Test Task Leader: Mr. Michael Barrows

Analytical Chemistry Task Leader and
Chemistry Laboratory Manager: Mr. William Steinhauer

Chemistry Laboratory Sample Custody: Mr. William Steinhauer
and Ms. Elisabeth Smolski

Quality Assurance Officer: Dr. Christine Werme

Laboratory Director: Dr. Anthony Graffeo

The proposed duration of this investigation is three months, from September 23, 1985 to December 20, 1985. The proposed schedule is as follows:

Week of:

- | | |
|--------------|--|
| September 23 | Aquisition/acclimation of oysters. Analytical chemistry protocol verification. Equilibration of dilutor system with test compound. |
| September 30 | Oyster shell deposition bioassay initiated. |
| October 7 | Calibration/verification of dosing system. |
| October 14 | Oyster bioconcentration test, bioconcentration phase. |
| November 11 | Oyster bioconcentration test, depuration phase. |
| November 25 | Data analysis and synthesis. |
| December 16 | Submission of draft final report. |

Battelle New England Standard Operating Procedures (SOPs) which will be used in this study are listed in Tables 1.1 and 5.1.

2.0 Dilution Water

Test water will be obtained from Duxbury Bay, an arm of Plymouth Bay which derives water from Cape Cod Bay, Massachusetts. Seawater used as dilution water will be pumped continuously from the bay and, if necessary, passed through a heat exchanger which will heat the water to approximately 20°C. Information regarding the laboratory's seawater system and heat exchanger can be found in Battelle New England SOPs Nos. 3-022-01 and 3-062-01, respectively.

The annual salinity range of Battelle's seawater is approximately 28-34 parts per thousand (ppt). The pH is approximately 7.9 ± 0.3 . Dilution water will meet specifications for contaminant levels as outlined in Battelle New England SOP No. 4-011-01, entitled "Quality of Natural Dilution Seawater Used in Bioassays and Toxicological Investigations."

TABLE I.1. STANDARD OPERATING PROCEDURES WHICH WILL BE FOLLOWED IN PERFORMANCE OF TESTS DESCRIBED IN THIS PROTOCOL OTHER THAN SUPPORT ANALYTICAL CHEMISTRY.

Battelle New England SOP Number	Title
3-006-01	Use of Mettler Balance Serial No. 224228
3-007-01	Use of Mettler Balance Serial No. 334722
3-009-01	Use and Maintenance of Deionized Water System in the Toxicology and the Biochemistry Laboratories
3-010-01	Use of Adjustable and Eppendorf Single Volume Pipets
3-011-01	Mettler and Ohaus Top-Loading Balances
3-013-02	YSI Model 51B Dissolved Oxygen Meter
3-016-01	Sandfilter Maintenance
3-021-01	Glassware and Equipment Cleaning Procedures - Toxicology and Wet Laboratories
3-022-01	Operation of Seawater Supply to Laboratory Buildings
3-024-01	Glassware Cleaning for Algal Cultures and Algal Bioassays
3-025-01	The Algal Culture Lab
3-045-01	OMEGA Model OM-205 Data Logger
3-046-01	Special Maintenance of the Toxicology Wet laboratory
3-047-02	Use of pH Meters with Ag/Ag Cl Electrode
3-048-02	Digital Thermometers
3-051-03	Use of the ATAGO and CHEMTRIX Sodium Chloride Refractometer
3-052-01	Aquatic Test Monitoring and Emergency Alarm System
3-062-01	The Seawater Heating System
3-065-01	All-Glass Continuous Flow Serial Diluters
4-006-01	Quality Assurance Audit of Final Reports of Studies Conducted Under "Good Laboratory Practices"
4-007-01	Quality Assurance Audits of Studies Conducted under "Good Laboratory Practices"
4-008-01	Conduct of Simultaneous Experiments in a Confined Work Area
4-010-01	Performance of Toxicological Tests in the Bioassay Room
4-011-01	Quality of Natural Dilution Seawater Used in Bioassays and Toxicological Investigations
4-013-01	Response to Water Level or Temperature Alarm in the Toxicology Laboratory
5-003-01	Water Quality Monitoring for Bioassays and Toxicological Investigations
5-008-01	Maintenance of Axenic Phytoplankton Cultures
5-027-01	Stock Solution Preparation
5-032-01	Oyster Shell Deposition Bioassay
5-041-01	Preparation of Artificial Seawater Medium for Algal Culture
5-083-01	Performance of Bioconcentration Test With Oysters (<i>Crassostrea virginica</i>)
5-092-01	Analysis of Radiolabelled Test Materials by Liquid Scintillation
6-004-01	Study Plans for Projects Conducted Under "Good Laboratory Practices"
6-005-01	Final Reports of Studies Conducted Under "Good Laboratory Practices"
6-009-01	Data Collection and Archival for Projects Conducted Under "Good Laboratory Practices"
6-014-01	Tracking of Test Material in the Toxicology Laboratory
7-006-01	Calculation of LC50 and EC50 values in Toxicological Studies
7-010-01	Random Assignment and Sampling in Toxicological Testing
7-011-01	Quality Control of Numerical Data Entries, Transcriptions, Calculations and Reductions in the Toxicology Group
8-001-01	Safety in the Laboratory
8-002-01	Radiation Safety at Battelle New England Marine Research Laboratory

2.1 Acquisition and Acclimation of Oysters

Juvenile oysters, Crassostrea virginica for use in the shell deposition test and bioconcentration test will be obtained from an oyster mariculture facility in Dennis, Massachusetts, a short distance from the Battelle Laboratory. Temperature and salinity of seawater at the Dennis facility are within 1°C and 1 ‰ of that of the flowing seawater in our laboratory, and holding conditions for oysters at both facilities are similar. Therefore, in accordance with ASTM minimum acclimation requirements (ASTM, E729-80), we will acclimate oysters obtained from Dennis for four days in the Battelle laboratory to dilution waters to be used in the tests.

3.0 Oyster Shell Deposition Test

A 96-hour oyster shell deposition test will be performed in a flowing seawater exposure system with five concentrations of BEHTP, a solvent control and a seawater control. Results of this bioassay (96-h EC₅₀, if obtained) will be used to establish the exposure concentration for the oyster bioconcentration test. Analytical confirmation of nominal concentrations of test material in exposure chambers will be performed by gas chromatography with electron capture detection (Battelle New England (SOP 5-094-01). The oyster shell deposition test will be performed as described in detail in the attached Battelle New England SOP 5-032-01, "Oyster Shell Deposition Bioassay". This SOP provides a detailed description of all aspects of the oyster shell deposition test, including: procurement and acclimation of test animals; grinding of shell edge of living test oysters; design of the dosing system, and test chambers; performance of the range-finder and definitive test; routine water quality monitoring; measurement of shell growth; data analysis and reporting; and quality control. Only those aspects of the test unique to this test protocol for BEHTP will be described here.

3.1 Test System

An all-glass, continuous flow serial diluter (Battelle New England SOP 3-065-01) will be used to deliver five toxicant concentrations, in a geometric series, a solvent control and a seawater control to the test.

A stock solution of BEHTP will be prepared in nano-grade acetone at a concentration of 1000 mg/l, which is 2000-fold higher than the highest exposure concentration. The stock solution will be delivered by gravity-feed through Teflon tubing from a Mariotte bottle wrapped in aluminum foil to eliminate light to the mixing chamber of the diluter system. The minimum dilution (highest exposure concentration) will be 2000-fold, giving an exposure concentration of 500 µg BEHTP/liter and containing 0.5 ml/liter acetone carrier. This concentration is 82 percent of the solubility of BEHTP in seawater. One liter of the 1000 ppm stock solution will be sufficient to provide test compound to the diluter system for 27 hours. Therefore, the stock solution will be renewed daily during the four-day test.

3.2 Test Procedures

Because of the apparent low toxicity of BEHTP in relation to its solubility in seawater (H. Lockhart, Eastman Kodak, personal communication), a range-finder bioassay is not deemed necessary. Instead, the definitive test will be performed using a geometric series of exposure concentrations, the highest of which is 82 percent of the solubility of BEHTP in seawater. The nominal exposure concentrations will be: 25, 50, 100, 250 and 500 µg BEHTP/liter seawater.

Phthalate esters readily adsorb to solid surfaces from aqueous solution. Therefore, the complete diluter system including all test concentrations of BEHTP will be run for four days before oysters are added to the exposure tanks. Single one-liter water samples will be taken immediately after system start up when all tanks are full and again at 48 hours from both replicate low, median and high concentration exposure tanks. On day four, three replicate one-liter water samples will be taken from each replicate of the low, median and high concentration exposure tanks. These samples will be analyzed immediately (Section 3.3) to verify that the system has equilibrated and actual exposure concentrations are within 30 percent of nominal concentrations. If actual exposure concentrations differ from nominal concentrations by more than 30 percent, the reason for the deviation will be determined and corrected before the bioassay is initiated.

3.3 Analysis of Exposure Concentrations

Assuming that the diluter system does equilibrate and nominal and actual exposure concentrations match within $\pm 30\%$, the day four samples alluded to above

become the day 0 samples for the shell deposition test. Oysters are added to the test system after these samples are taken. An additional three replicate one-liter samples are taken from each replicate of the low, median and high concentration exposure tanks on day four (96 hours) of the shell deposition bioassay. Thus, a total of 48 water samples will be collected for analysis during the equilibration and definitive shell deposition test (equilibration: one sample X 2 replicate tanks X 3 concentrations X 2 times = 12) + (test: 3 replicate samples X 2 replicate tanks X 3 concentrations X 2 times = 36) = 48.

All one-liter water samples will be collected in precleaned amber glass bottles by staff of the chemistry laboratory under the direction of the chemistry task leader. The samples will be processed and analyzed by gas chromatography with electron capture detector according to Battelle New England SOP No. 5-094-01, "Extraction and Analysis of Phthalate Esters in Water".

3.4 Data Reduction

Since both a seawater (passive) and solvent (active) control are used in this test, the mean responses in the two controls are compared by a t-test for the shell growth rate data and Fisher's Exact Test for categorical data (percent oysters showing no shell growth). If the two control treatments produce significantly different responses, the solvent control treatment is used as control for comparison with other treatment groups. If the two controls are not statistically significantly different, they are combined and treated as a single test control. Similarly the response of the two replicate exposure chambers at each concentration are compared by a t-test or Fisher's Exact Test to determine if they are true replicates. If they are the same the mean values are used for the statistical analyses described in SOP No. 5-032-01. If they are not statistically similar, they are treated as separate treatments.

3.5 Documentation

All records of test results related to biological monitoring, analytical measurements, statistical computations, and any additional miscellaneous information will be kept on appropriate forms following the guidelines of Battelle New England SOP No. 6-009-01, "Data Collection and Archival for Projects Conducted Under Good Laboratory Practices." Examples of the data and information forms which will be used in this test have been included with this protocol as part of the appropriate SOPs.

4.0 Oyster Bioconcentration Test

A 42-day bioconcentration/depuration test with oysters Crassostrea virginica will be performed in a flowing seawater exposure system with a single concentration of BEHTP, a seawater control and a solvent control. Concentrations of test material in exposure and depuration water and in oyster tissues will be verified by liquid scintillation counting of the ^{14}C -labelled test compound (Battelle New England SOP 5-092-01). The oyster bioconcentration test will be performed as described in detail in the attached Battelle New England SOP 5-083-01, "Performance of Bioconcentration Test with Oysters (Crassostrea virginica)". This SOP provides a detailed description of all aspects of the oyster bioconcentration test, including: procurement and acclimation of test animals; design of the toxicant dosing system and test chambers; methods for selecting test chemical exposure concentration and duration of bioconcentration and depuration phases of test; performance of the test; routine water quality monitoring; schedule for sampling of oysters and test water; data analysis and reporting; and quality control. Only those aspects of the test unique to this test protocol for BEHTP will be described here.

4.1 Test System

A stock solution of BEHTP will be prepared in nano-grade acetone at a concentration at least 2000-fold higher than the exposure concentration. The exposure concentration will be chosen based on the results of the oyster shell deposition test. If an EC_{50} is determined, the exposure concentration for the bioconcentration test will be set at one-tenth the EC_{50} concentration. If the oyster shell deposition test does not yield a definitive EC_{50} because the toxic concentration was higher than the solubility of the test material in seawater, then a concentration of about one-tenth the solubility of the test compound in seawater will be used. In the case of BEHTP, this concentration would be set at 50 $\mu\text{g/liter}$, 8.2 percent of aqueous solubility if an EC_{50} can not be calculated. This is an environmentally realistic concentration.

Assuming an exposure concentration of 50 $\mu\text{g/liter}$, the stock solution will be prepared at 1000 ppm. The stock solution will be delivered via a Harvard syringe pump to the top of a one-meter vigreux column where it will be mixed in a constant ratio of one part stock solution to 20,000 parts seawater (0.05 ml stock/liter seawater). This preparation will provide a 50 μg BEHTP/liter exposure solution containing 0.05 ml/liter

acetone carrier. The solvent control tank will receive seawater containing 0.05 ml/liter acetone. The seawater control tank will receive seawater alone.

The BEHTP stock solution will be prepared by dissolving 50 mg of ^{14}C -BEHTP and 950 mg cold BEHTP in one liter nano-grade acetone. The stock solution will have a specific activity of 1.11 mCi/liter and a nominal counting rate of 2.46×10^6 DPM/ml. The exposure water containing 50 μg /liter BEHTP will have a counting rate of 123 DPM/ml. At this specific activity, a concentration of BEHTP in oyster tissue of 0.5 ppm should be detected easily in a tissue sample size of 100 mg wet weight.

Seawater containing the test material in solution will be delivered via Teflon tubing from the vigreux column to the exposure chamber at a rate of 60 liters per hour. At this rate of delivery, a one-liter stock solution will last for slightly more than 13 days. At this time it will be replaced with fresh solution of the same concentration.

4.2 Test Procedures

Sixty small oysters in the size range of 20-40 mm valve height (standard deviation of mean height less than 20%) will be placed in each the exposure, seawater control and solvent control chambers. Exposure chambers will be all glass 30-gallon aquaria fitted with a stand-pipe drainage system to maintain water depth at about 15 cm.

Preliminary calculations of equilibrium bioconcentration factor and time to steady state (Battelle New England SOP No. 5-083-01), based on the solubility and octanol/water partition coefficient of BEHTP, indicate that an equilibrium BCF of approximately 5,000 will be reached in 14-20 days. Therefore, the bioconcentration phase of the test will be scheduled to run for the full 28 days. However, oyster samples will be taken and analyzed immediately on days 14, 18, and 21 of the test. If body burdens of BEHTP are statistically indistinguishable over the three sampling periods, the bioconcentration phase will be terminated and the depuration phase initiated.

4.3 Sampling Strategy

Replicate 1 to 2-ml water samples will be taken from the middle of the exposure chamber and control chambers on days 1, 3, 7, 10, 14, 18, 21, 24 and 28 of the bioconcentration phase, and on days 1, 3, 7, 10 and 14 of the depuration phase of the test. These samples will be analyzed for ^{14}C activity by liquid scintillation (Battelle New

England SOP No. 5-092-01). In addition, replicate one-liter water samples will be collected from the center of the exposure chamber at the time of initiation of the test (day 0) and on day 28 or at the end of the bioconcentration phase for analysis by gas chromatography. This will be done to verify the values obtained by liquid scintillation and to determine if metabolism and/or degradation of BEHTP has occurred.

Four replicate oysters will be sampled at random (Battelle New England SOP No. 7-010-01) from the exposure chamber and the two control chambers on days 1, 3, 7, 10, 14, 18, 21 and 28 of the bioconcentration phase of the test and on days 1, 3, 7, 10 and 14 of the depuration phase of the test. If the bioconcentration phase of the test is terminated after 21 days, the 24 and 28-day water samples and 28-day oysters samples will not be taken. All oysters from the exposure tank will be processed as described below and analyzed for ^{14}C activity by liquid scintillation. The only control oysters which will be analyzed are those collected on the last day of the bioconcentration phase of the test. In addition, a pooled sample of 4 animals will be taken at the end of the bioconcentration phase and end of the depuration phase from the exposure tank and analyzed by thin-layer chromatographic techniques for BEHTP and metabolites and/or degradation products as described below. Values for exposure concentration and tissue residues will be corrected for metabolite/degradation product concentrations greater than ten percent of parent compound concentration.

4.4 Routine Monitoring Concentrations of BEHTP in Exposure Medium

Replicate 2-ml water samples will be taken with an Oxford Pipettor (Battelle New England SOP No. 3-061-01) and placed immediately in glass liquid scintillation vials. A different pipet tip will be used for the control and exposure water samples. One-tenth ml samples also will be taken from the phthalate stock solution at the times of water sample collection. All samples will be analyzed within 24 hours of collection by liquid scintillation counting (Battelle New England SOP No. 5-092-01).

In addition, replicate one-liter water samples will be collected from the exposure tank on day 1 and 28 of the bioconcentration phase and analyzed by gas chromatography for BEHTP to verify the accuracy of the liquid scintillation measurements (Section 5).

4.5 Monitoring Concentrations of BEHTP in Oyster Tissues

Shell height (greatest distance from umbo to ventral shell margin) of each oyster will be measured to the nearest 1.0 mm and recorded. Oysters will be shucked and the soft tissues will be blotted to remove excess moisture, minced, and weighed in a glass scintillation vial to the nearest 0.5 mg on a Mettler balance (Battelle New England SOP No. 3-007-01). If tissue weight is greater than 150 mg, the tissue must be homogenized in an all-glass tissue grinder and a 100-150 mg aliquot of the homogenate taken for further processing. 1.5 ml of PROTOSOL (New England Nuclear Corp.) tissue solubilizer will be added to each glass liquid scintillation vial and the vials will be sealed tightly with polyethylene-lined caps and heated at 55°C until the mixture is clear (6-12 hours). 100 µl of 30% hydrogen peroxide will be added to the samples and they will be heated again at 55°C for 30 minutes to remove any remaining color. Samples will be cooled and 12 ml ECONOFLUOR (New England Nuclear Corp.) will be added to each vial. The vials are sealed tightly and allowed to equilibrate in the dark (e.g., in the scintillation counter) for at least 60 minutes before counting.

Samples then will be counted on the Beckman LS-7500 Liquid Scintillation counter as described in Battelle New England SOP 5-092-01. To correct for counting efficiency and quench, a series of nine 100 mg unlabelled homogenized oyster tissue samples will be placed in liquid scintillation vials and different amounts of ¹⁴C-BEHTP added, each concentration twice the next lower concentration, to cover the expected (based on predicted BCF) concentration range in tissues of exposed oysters (e.g., sufficient to yield 1, 2, 4, 8, 16, 32, 64, 128, 256 mg/kg wet-weight phthalate). These samples will be processed and counted the same as the oyster samples and a calibration curve constructed of DPM vs. actual concentration of ¹⁴C-BEHTP.

To determine if the oysters have metabolized the BEHTP, a pooled sample of 4 oysters will be taken at the end of the bioconcentration phase and end of the depuration phase and analyzed for the presence of phthalate metabolites according to the method of Wofford et al. (1981).

Metabolites will be separated by thin layer chromatography on 0.25-mm silica gel plates (silica gel 60 PF-254) using a solvent system of chloroform: methanol (20:1 v/v) according to the methods described by Wofford et al., (1981). After development, bands 1-cm wide will be scraped directly into plastic scintillation vials and treated with hydrofluoric acid and water to insure solubilization of the sample and silica gel. Ten ml

of ECONOFLUOR scintillation cocktail will be added and vials will be counted on the Beckman LS-7500 liquid scintillation counter. If more than 10 percent of radioactivity is associated with metabolites, oyster body burdens will be corrected to true BEHTP and this will be documented in the project files.

4.6 Documentation

All records of test results related to biological monitoring, analytical measurements, statistical computations, and any additional miscellaneous information will be kept in appropriate forms. Examples of the data and information forms which will be used in this test have been included with this protocol.

5.0 Gas Chromatographic Analysis of BEHTP in Water

5.1 Technical Approach

Concentrations of BEHTP in test water will be measured using Battelle New England SOP No. 5-094-01 (Extraction and Analysis of Phthalate Esters in Water). In brief, a 1.0-liter water sample will be transferred to the laboratory and extracted with methylene chloride in a separatory funnel. If a sample is not extracted immediately, it will be stored at 4°C until extraction. All extractions will be completed within 24 hours of sample receipt. The methylene chloride extract is dried, concentrated, and transferred to hexane by repetitive addition and concentration. If necessary, additional sample preparation is performed on an alumina column. The extract is then analyzed by gas chromatography using the internal standard method of calibration (SOP No. 5-025-01).

Procedures used in this analytical support task will follow the SOPs listed in Table 5.1.

Each batch of 6 to 12 samples will include a quality control (QC) check sample consisting of authentic BEHTP at a nominal concentration of 50 µg/l which will be prepared and analyzed simultaneously with the batch, such that the total analytical program averages at least one QC check sample for every 6 to 12 test samples. The QC sample is used to verify precision and accuracy of the analytical method.

5.1.1 Accuracy. The determination of analytical accuracy will be validated prior to testing by the analysis of two sets of triplicate spiked samples using the test

TABLE 5.1. STANDARD OPERATING PROCEDURES WHICH WILL BE FOLLOWED IN THE ANALYTICAL SUPPORT TASK

Battelle New England SOP Number	Title
5-094-01	Extraction and Analysis of Phthalate Esters in Water
5-025-01	Gas Chromatography Protocols
6-007-01	Chemistry Laboratory Sample Custody and Laboratory Sample Identification
6-010-01	Chemistry Laboratory Sample Control
3-011-01	Mettler and Ohaus Top-Loading Balances
3-004-01	Use of CAHN Model 25 Electrobalance
3-009-01	Use and Maintenance of Deionized Water System in the Toxicology and the Biochemistry Laboratories
3-019-01	Chemistry Laboratory Glassware Cleaning
5-015-01	Solvent/Reagent Inventory and Contaminant Residue Checks
5-027-01	Stock Solution Preparation
6-009-01	Data Collection and Archival for Projects Conducted Under "Good Laboratory Practices"

dilution water and a spiking solution of BEHTP, prepared independently of the analytical standard and of the test dosing system, at an analyte concentration equal to the next to the lowest concentration used in the testing procedure. The analytically determined concentration for the recovery samples must be 80 to 119% of the actual analyte concentration. If any one analysis fails to meet the requirements, the recovery experiment would be repeated and the samples reanalyzed.

The analytically determined analyte concentration for any subsequent test samples analyzed must also fall within 80 to 119% of the actual analyte concentration.

A continuing check on accuracy shall consist of the analysis of procedural blank or blank spike samples at a frequency equal to one blank analysis equal to every 2 test samples analyzed. The requirement of this analytical test shall be that no analyte concentration in any blank analysis shall exceed the minimum detection limit (MDL) of the analyte. The requirements of the blank spike shall be that recovery shall be within the bounds established for the initial demonstration of accuracy.

5.1.2 Precision. The precision of the method shall be such that the standard deviation from the mean of any one analysis must be less than 20% of the mean determined by two sets of triplicate analyses performed in Section 5.1.1. If any one test analysis fails to meet requirements for precision, the test analysis shall be repeated until requirements are met.

5.1.3 Minimum Detection Limits. The minimum detection limit (MDL) of BEHTP will be determined in the laboratory as part of the initial method verification process. The method of standard dilutions will be used to determine instrumental detection limits, which, in turn, will be used to determine the MDL of BEHTP in seawater.

5.2 Sample Custody Procedures

All test samples and analytical standards received into the analytical laboratory as part of this project will be identified and tracked from the time of sample collection, through sample preparation and analysis, and up to final archival of sample extracts.

Samples will be collected by Chemistry Laboratory personnel. Samples will be logged in on the Sample Custody and Identification Form (Battelle New England SOP No. 6-007-01) by the Chemistry Laboratory Manager or Sample Custodian. At this

time, an in-house sample identification number will be assigned which will be used to identify the samples and data generated in the laboratory. The same ID number will be recorded on the bioconcentration test sampling data form. As a measure to track samples in the laboratory, a Sample Control Form will be initiated for each Sample (SOP No. 6-010-01). This form will accompany the sample through all phases of workup and analysis within the laboratory.

Upon receipt of the samples into the Chemistry Laboratory, the samples will be transferred to an analyst for sample preparation and analysis. If samples are not analyzed immediately, they will be placed into controlled access refrigerated storage with the proper preservative, if necessary. The receiving analyst is responsible for maintaining that the sample is in his/her possession or secured in the laboratory at all times. Upon completion of sample analyses, custody of sample extracts and data will be relinquished to the Toxicology Study Director for archival.

5.3 Documentation

All results of analytical measurements of concentrations of BEHTP in exposure water as determined by gas chromatography, as well as documentation of sample chain-of-custody, processing and analysis will be kept on appropriate forms. These, as well as the gas chromatograms, become part of the project file. Examples of the data and information forms which will be used in this project have been included with this protocol as part of the specific SOPs.

5.4 Data Reduction and Reporting

Data reduction and reporting for GC analyses will consist of any calculations of standard calibration factors (response factors) and final determinations of measured concentrations of TCP in test samples. These calculations are detailed in Battelle New England SOP Nos. 5-025-01 and 5-077-01. Peak areas are digitized, electronically integrated, and reported on the chromatographic data processor. The built-in software in the processor will reduce these data by the external standard method and report the reduced results. The chromatographic data report consisting of chromatograms, integration, and data reduction is the primary documentation of these data. Peak areas and calculated response factors and analyte concentrations will be transcribed to the Sample Preparation and Analysis Form.

6.0 Project Files

All data, including raw data, reduced data, and hard copies of computer data inputs and outputs generated during the performance of this project will be bound together in a unique project file. The project file will be archived for a minimum of ten years in the Battelle New England Quality Assurance Office (Battelle New England SOP No. 6-009-01). Access to the data archives is limited to the Battelle New England QA Office. Kodak will have access at any time, through the Battelle New England QA Office, to the unique project file generated during this study.

All data will be recorded in ink on standard data forms or print-outs from computers or automatic recording devices. Examples of data forms are appended to this protocol. The originals of these data types will be kept in the project file. In addition, the project file will contain all internal memoranda and external correspondence between Battelle New England and Kodak concerning this project.

The project file will contain a Table of Contents at the front identifying all sections of the project file. This will be followed by a copy of the study protocol and the major relevant Battelle New England SOPs. The major SOPs for this project include the following:

- 5-032-01 Oyster Shell Deposition Bioassay
- 5-083-01 Performance of Bioconcentration Test with Oysters
(Crassostrea virginica)
- 5-092-01 Analysis of Radiolabelled Test Material by Liquid
Scintillation
- 5-094-01 Extraction and Analysis of Phthalate Esters in Water

The next section will contain all correspondence, including summaries of telephone conversations, between Battelle New England and Kodak concerning technical aspects of this project.

The data packets for each test will be separate and self-contained (oyster shell deposition test first, oyster bioconcentration test second). Data packets will contain the following information:

1. Records associated with test procedures and equipment or facilities use, including oyster procurement and acclimation records; algal culture and feeding records; dosing system operation records; water quality monitoring records; records of equipment maintenance and calibration.
2. Biological monitoring records, including daily observation of oyster condition and behavior; records of oyster shell growth (shell deposition test) and shell size and tissue wet weight (bioconcentration test);
3. Analytical and sample tracking records, including records of water and oyster sample collection; sample tracking, processing, and analysis; records of analytical raw and reduced data; and records of all appropriate quality control checks and results;
4. Records of data reduction and analysis, including descriptions and records of all mathematical and statistical manipulations of the data; copies of final data products (e.g., EC₅₀, BCF, time to steady state, etc.) and data presentations (e.g., graphs, tables, etc.).
5. A description of any deviations from the protocol with an explanation of why the deviation occurred and its effect on the quality and validity of the test.

The project file also will contain a copy of the final draft of the final report of the project. Any correspondence, critiques, or audit reports by the client, internal and external QA auditors, Study Director responses to audits, and Battelle technical reviewers of the tests and draft report will be included.

The data file will be organized and cross-indexed in such a way that all data can be tracked easily from initial recorded observations to final data products and interpretation.

7.0 Reporting

A record of the results of the oyster shell deposition and bioconcentration tests will include the following information either directly or by reference to existing publications.

1. Names of test(s) and investigators, name and location of laboratory, and dates of initiation and termination of each test;
2. Source of test material, its lot number, composition, known physical and chemical properties, identity and concentration of the carrier solvent used;
3. For radiolabelled compounds, the identity of the isotope, its location(s) on the test molecule, specific activity, amount of compound by mass and radioactivity received, purity of radiolabelled compound;
4. Source of dilution water, the mean and standard deviation and range of salinity, pH, temperature and dissolved oxygen during test period, and a description of any pretreatment;
5. Detailed information about oysters used, including source, shell size (mean and range), acquisition and acclimation history, method of confirmation of prespawn condition, supplemental feeding used;
6. Description of experimental design, including test chambers, flow-through dosing system, water flow rate to each chamber number of replicates per treatment, number of treatments, number of oysters per treatment, aeration, lighting, heating (cooling) systems mean measured values, rate of supplemental feeding (if used), and waste treatment system;
7. Methods for preparation of stock and test solutions, and test chemical and solvent carrier concentrations used;
8. The number of dead and live test oysters, percentage of oysters that died, and number that showed any abnormal effects in the control and in each test chamber at each observation period;
9. Methods and data records of all chemical analyses of water quality parameters and test substance concentrations, including method validations and reagent blanks;
10. Any events during the course of the test which might have affected the results;

11. A statement that the test was carried out in agreement with the study protocol and all SOP's referenced therein; otherwise a description of any deviations from the protocol with an explanation of how the deviation might have affected the test results;

For oyster shell deposition test

12. The 96-hr shell growth measurements of each oyster; the mean, standard deviation and range of the measured shell growth at 96 hours; and the number and percent of oysters which showed no measureable growth, in each concentration of test compound and control;
13. The calculated 96-hr EC₅₀ value and its 95 percent confidence limits, and the statistical methods used to calculate these values;
14. When observed, the highest test concentration at which no statistically significant effect of exposure for 96 hours on shell growth and percent oysters exhibiting shell growth was measured;
15. A graph of the concentration-response curve based on the 96 hour mean measured test chemical concentration and shell growth measurements upon which the EC₅₀ value was calculated;

For oyster bioconcentration test

16. Description of sampling times and methods of sample storage (if required) and analytical methods for water and tissue analyses for the test chemical;
17. The mean, standard deviation and range of the concentration of test chemical in the test solution and oyster tissue at each sampling period;
18. Graph of log tissue concentration versus exposure time to include both the bioconcentration and depuration phases of the test;
19. The time to steady-state, and how calculated;
20. The steady-state or maximum BCF, its 95 percent confidence limits, and how calculated;
21. The time to 95 percent elimination of accumulated residues of the test chemical from test oysters and a description of the type of depuration model the depuration data fit;

22. Documentation of the presence and relative concentration of any metabolites or degradation products of the test material in oyster tissues at the end of the bioconcentration and depuration phases of the test.

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EASTMAN KODAK COMPANY

Oyster Bioconcentration Test

N-0950-2203

APPENDIX 2

Deviations From Test Protocols

APPENDIX 2

Deviations From The Test Protocols During The Oyster Bioconcentration Test For ¹⁴C-BEHTP

GLP Regulations (Part 792, Section 792.120)

The amendments to Study Protocol "Oyster Shell Deposition and Bioconcentration Test with BEHTP," dated 19 September 1985, were not formally signed by the Study Director. This is a deviation from the recognized GLP regulations. The amendments to protocol were made to upgrade the existing test procedures and were relatively self-explanatory. The cover letter dated 10 December 1985, which was attached to the amendment package and was submitted by the Study Director to Kodak, indicated the Study Director's approval of the amendments. This deviation does not have any negative impact in the conduct of the test. A copy of the protocol amendment has been signed and dated by the Study Director and is included in the final report.

1. Deviations From The Test Protocol Dated 19 September 1985¹.

Amendment to Protocol

Page 1 of 3: Test initiation did not occur until 12/17/85, not on 12/2/85 as stated in the amendment to test protocol. A weight determination of the test material received at BNEMRL was necessary in order to ascertain the amount of ¹⁴C-material to be used in the stock preparation procedures. The reason for the delay

Page 3 of 3: Approximately 41% of the test oyster population fell below the 40-mm to 60-mm size specified in the amendment to test protocol. EPA guidelines suggest oysters to be 25 to 50 mm in size for the bioconcentration test. Because the test oysters ranged from 35 to 56 mm, this deviation did not affect the results of this test.

¹ Study Protocol "Oyster Shell Deposition and Bioconcentration Test with Bis(2-ethylhexyl) Terephthalate," September 19, 1985.

The size of the test aquaria used during the bioconcentration study was 20-gallon, not 30-gallon as specified in the amendment/test protocol. The miscalculation was made in the original test protocol and not verified by the biological task leader for inclusion into the amendments to protocol. This deviation, however, did not affect the results of this test.

The stand-pipe drainage system used in the 20-gallon aquaria was at a depth of 15 cm, not at 16 cm as stated in the amendment to test protocol. This was a miscalculation on the part of the biological task leader and should not have been changed from the original 15 cm as stated in the test protocol. This deviation did not affect the results of this test.

Oysters were measured to the nearest 1.0 mm as stated in the test protocol and not to the nearest 0.1 mm as stated in the amendment to the protocol. The procedure for measuring oyster shell growth to the nearest 0.1 mm is used during an oyster shell deposition test. Because the correct procedure was stated in the original document and followed throughout the testing period, this deviation did not affect the results of this test.

Study Protocol

Section 4.3 Sampling Strategy

Samples were collected during the study period on Days 6 and 17 of the exposure period and Day 11 of the depuration period instead of Days 7 and 18 of exposure and Day 10 of depuration. The sampling schedule was modified to conform with normal working days to avoid holidays and weekends when additional support personnel would not be available. Because EPA guidelines refer to sampling intervals being no longer than four days apart, this deviation did not affect the results of this test.

Additional analyses were performed on solvent control oysters and not only at the end of the bioconcentration phase as stated in the test protocol. Day 1 analyses were performed in order to document the background radiation in oyster tissues which was used as the correction factor in all analyses performed on oysters for Days 1 to 21 from the exposed system. Analyses performed on Day 14 of depuration to verify any change in background radiation in oyster tissues exposed to toxicant-free flowing water. This deviation had positive effects on the results of this test.

Some measurable ^{14}C -residue concentrations were detected in the homogenized tissue of solvent control oysters (KOBT No. 114-115) sampled on Day 24 of exposure. These measurable ^{14}C -residues were attributed to contamination of the samples during preparation for analysis rather than to any ^{14}C -residues actually present in the animals prior to sampling. Because no measurable ^{14}C -residues were present in the two remaining oysters from that sample set, this deviation had little, if any impact on the results of this test.

Four additional samples, not required by the test protocol, were prepared and analyzed six days after test initiation. The procedural blank prepared on Day 0 was found to be contaminated. The procedural blank was repeated, duplicate 20-ml samples of test water were reanalyzed, and a QA/QC recovery spike was added to check method recovery. This deviation does not appear to have affected the results of this test.

Section 4.4 Routine Monitoring Concentrations of BEHTP in Exposure Medium

Replicate 2-ml water samples of test water were not taken as specified in the test protocol. Instead, duplicate 1-ml samples were taken throughout the study period. This change in sample routine was documented in Section 4.3, Sample Strategy but, due to an oversight, was not amended in Section 4.4. This deviation did not affect the results of this test because a 1-ml sample provided sufficient material to be analyzed by liquid scintillation counting techniques.

The Oxford Pipettor described in SOP No. 3-061-01 was not used to collect water samples during this study as specified in the test protocol. This was an error in terminology and should have read "with an adjustable single volume Oxford Pipet SOP No. 3-010-01." This deviation did not affect the results of this test because the biological task leader was certified in the use of both instruments.

Stock solution samples were not routinely taken at a volume of 0.1 ml as specified in the test protocol. Because more accurate measurements of the stock solution were made with the use of a Hamilton microliter syringe at a volume of 5 μ l, this deviation did not affect the results of this test.

Stock solution samples (14 December 1985) and depuration water samples (11 January 1986) were not analyzed within 24 hours after collection as specified in the test protocol. Samples were collected and put into 15 ml of counting solution at the time of collection to stabilize overnight according to Section 3.1 of SOP 5-092-01, Sample Preparation. Because the preparation of ^{14}C -samples was performed according to liquid scintillation counting techniques, this deviation did not affect the results of this test.

Section 4.5 Monitoring Concentrations of BEHTP in Oyster Tissues

Mettler Balance No. 224228 (SOP No. 3-006-01) was used instead of Mettler Balance No. 334722 (SOP No. 3-007-01) as specified in the test protocol. Balance No. 224228 was used because of its accessibility to the study area. Because both balances are of comparable sensitivity and accuracy, this deviation did not affect the results of this test.

No formal SOP had been written for the homogenization of oyster tissue at the time of testing. This was an oversight on the part of the biological task leader. Because procedures for tissue homogenization were relatively simple and straightforward, this deviation had relatively little impact on the results of this test.

A Protosol volume of 1 ml was used to solubilize the oyster tissues prior to analyses instead of 1.5 ml as specified in the test protocol. Based on the instruction pamphlet, "the lower ratios of Protosol to sample lead ultimately to higher counting efficiencies." The amount of 1.5 ml should have been amended to 1 ml. This deviation did not affect the results of this test.

A series of nine 100-mg oyster tissue samples were not spiked with known amounts of ^{14}C -BEHTP to correct for counting efficiency and quench as stated in the test protocol. Because the Beckman 7500 Liquid Scintillation Counter is equipped with automatic quench compensation (AQC) which adjusts the instrument to varying conditions of quench, this deviation did not affect the results of this test.

No formal SOP had been written for the analysis of oyster tissues by thin layer chromatography at the time this study was initiated. This was an oversight on the part of the task leaders and a methods development was conducted prior to the analyses of oyster tissues. The method used for the detection and quantification of phthalate metabolites was a modification of Wofford et al. (1981) cited in the test protocol. Since the modifications did not differ significantly from the original method, this deviation did not affect the results of this test. The method used is included in Appendix 4 as SOP No. 5-110-01 (draft).

Section 5.1 Technical Approach

SOP 5-094-02 "Extraction and Analysis of Phthalate Esters in Water" had not been approved at the time of test initiation. Because this SOP (approved on 12/19/85) accurately reflects the work as it was performed, this deviation did not affect the test.

Section 5.1.1 Accuracy

A second set of two triplicate recovery experiments was not performed prior to test initiation as stated in the SOP. This was an oversight on the part of the analytical task leader. Because the analytical method was the same as that used during the oyster shell deposition test, this deviation did not affect the results of this test.

Section 7.0 Reporting

The 95 percent confidence limits were not calculated for the maximum BCF or the steady-state BCF as stated in the test protocol. The 95% confidence limit was not calculated because the steady state condition was obvious and confirmed by ANOVA and no additional useful information would be gained from the confidence limit calculation. This deviation did not have any adverse affects on the results of this test.

The time to 95% elimination of accumulated ^{14}C -residues from exposed oysters was not calculated as stated in the test protocol. The half-life or 50% elimination of accumulated ^{14}C -residues was calculated for oysters previously exposed to ^{14}C -BEHTP because of its more general acceptance and usage in toxicological testing. This deviation did not affect on the results of this test.

2. Deviations From The Test Protocol SOP No. 5-083-01².

Section 2.1 Procurement

The histological examination of oysters was performed on a batch of oysters received earlier, not on the actual batch used for testing. Because both batches of oysters were from the same source, spawn and water conditions, and because there were no differences observed between the response of chemical-exposed and control oysters, this deviation did not affect the quality of the test.

Section 2.2 Acclimation and Initial Observations

Algal density was not measured during the acclimation of test oysters, nor was supplemental feeding provided during this time. Oysters also received filtered seawater as opposed to unfiltered seawater as proposed in the SOP. Since oysters were observed to be filtering normally during the acclimation period, it was more essential to maintain the culture within the water quality parameters of testing conditions. This deviation did not appear to affect the quality of the test.

Section 2.4 Characteristics of BNEML Flowing Seawater System

Oysters received filtered seawater during the testing period as opposed to unfiltered seawater as indicated in the SOP. Because supplemental feeding of Isochrysis galbana was supplied to the oysters during testing and oysters exhibited normal growth during the testing period, this deviation did not affect the quality of the test results.

Section 2.5.3 Environmental Conditions

The photoperiod during the testing period was 14 hours light/10 hours dark instead of 16/8 as specified in the test protocol. This study was run concurrently with a test that had a more stringent requirement for the testing photoperiod. EPA guidelines

² Battelle New England Marine Research Laboratory SOP No. 5-083-01 "Performance of Bioconcentration Test with Oysters (Crassostrea virginica)."

for the oyster test acknowledge that light has little effect on oyster growth, and growth was not the primary objective during the bioconcentration test; therefore, this deviation did not affect the results of this test.

3. Deviations From The Test Protocol SOP No. 5-092-01³.

Section 3.1 Sample Preparation

During the conduct of testing, samples of test solutions were prepared in Aquasol-2(New England Nuclear), a general purpose liquid scintillation cocktail, and not

Atomlight (New England Nuclear) as stipulated in the SOP. Atomlight is a high-efficiency counting solution used at Battelle for test chemicals that may cause interference with Aquasol-2. This was not the case with ^{14}C -BEHTP. This deviation did not affect the results of this test and the SOP will be amended to include all the liquid scintillation fluids used for testing.

Water and stock solution samples were analyzed within one hour from sample collection to assess the proper function of the physical system. Samples were then allowed to sit overnight to stabilize prior to analysis as specified in the SOP. Only recounted samples were used to calculate the concentrations used in the report. Although not a true deviation, the procedure for stabilization of samples is unnecessary and the SOP will be revised to specify an adequate time period for samples to stabilize prior to analyses.

Section 5.1 Accuracy

The SOP states that with each sample run, triplicate samples will be analyzed in the next to lowest concentration, and repeated if the results are not within the precision of the instrument. This procedure is unnecessary and was not followed during the sample analysis portion of this test. At a minimum of once a week during testing, a sealed standard of ^3H (tritium) is counted to check the precision and accuracy of the calibrated memory of the Beckman 7500 Liquid Scintillation Counter. During the course

³ Battelle New England Marine Research Laboratory SOP No. 5-092-01 "Analysis of Radiolabelled Test Materials by Liquid Scintillation."

of the past year, the task leader in charge of LSC determinations has not observed any reduction in the precision and accuracy of the instrument. This deviation did not affect the results of this test and the SOP will be revised to specify an adequate procedure for checking the precision of the instrument.

Section 5.2 Precision

The SOP states that a sealed standard of ^{14}C -labeled toluene will be counted with each group of samples to check the precision and accuracy of the counter. This procedure is unnecessary for the reasons stated in the aforementioned Section 5.1, Accuracy, and the procedure was not followed during the course of this study. This deviation did not affect the results of this test and the SOP will be revised to specify the appropriate procedures for checking the precision and accuracy of the instrument.

Section 5.4 Calibration Curve

The SOP states that the consistency of the instrument and sample preparation technique will be checked weekly by preparing three concentrations from the stock solution and comparing the fit to the original curve. It is an unnecessary procedure because the Beckman 7500 is equipped with automatic quench compensation (AQC) that adjusts the instrument to variable conditions. The AQC is directly related to the precision and accuracy of the instrument, which has been discussed in Section 5.1. This deviation did not affect the results of this test and the SOP will be revised to specify the appropriate procedure for checking the calibration of the instrument.

4. Deviations From the Test Protocol SOP No. 5-094-02⁴

Section 3.3 Sample Chain-of-Custody and Data Transfer

Sample control forms were not used for this study as stated in the SOP. The use of sample control forms was not necessary since all samples for chemical analysis remained in the chemistry laboratory from time of collection to analysis. This deviation did not affect the results of this test.

⁴ Battelle New England Marine Research Laboratory SOP No. 5-094-02 "Extraction and Analysis of Phthalate Esters in Water."

Section 3.5 Extract Concentration

One sample extract (Day 0) was inadvertently blown to dryness under nitrogen. Because BEHTP is a semi-volatile compound, some analyte may have been lost through volatilization. This error could be the reason why the reported concentration for that sample was 40% of the nominal concentration of 50 µg/L.

Section 3.7.2.4 RF Calibration

The first continuing calibration did not meet $\pm 15\%$ RSD quality criteria established in the SOP. Calibration was then continued by repeating the initial three-point calibration. The mean calibration was within $\pm 15\%$ of the initial procedure. Because the final calibration indicated that the instrument was still within the quality criteria established around the initial calibration, this deviation did not appear to affect the results of this test.

The continuing calibration is $\pm 20\%$ of the initial calibration, not $\pm 15\%$ as stated in the SOP. This deviation resulted from an error in relating $\pm 15\%$ RSD to be the same as 30% quality criteria. The impact of this deviation is that analysis performed on 1/17/86 may be actually 5% greater than that allowed in the SOP.

The low concentration calibration solution was used instead of the mid-range calibration solution for continuing calibration as specified in the SOP. Since these solutions are interchangeable for the purpose of continuing calibration and the calibration solution used must be one of those used for initial calibration, this deviation did not affect the results of this test.

5. Deviation From The Test Protocol SOP 6-007-01⁵

The samples and standards investigated during the TLC analysis were referred to by their descriptive names and/or original sample ID numbers designated by the toxicology section rather than by the ID labelling procedure described in the SOP. This deviation did not affect the results of this test.

⁵ Battelle New England Marine Research Laboratory SOP No. 6-007-01 "Chemistry Laboratory Sample Custody and Laboratory Sample Identification."