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Evaluation of Triploid Oysters as a Tool to assess Short- and Long-term Seafood Contamination of Oil Spill-impacted Areas

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ABSTRACT 300222:

The objective of this field and laboratory study was to evaluate the use of triploid Eastern oysters, *Crassostrea virginica*, as a bioindicator of polynuclear aromatic hydrocarbon (PAH) contamination in oil spill-impacted areas. Bivalve mollusks have shown to be valuable tools for assessing the short-term (weeks to months) bioavailability and impact of hydrophobic contaminants following oil and chemical spills. Approximately 1-year after the initial Deepwater Horizon spill, PAH concentrations were measured in sediment and caged oysters at sites within the Northern Barataria Bay. Two (2) seven-week large-scale mesocosm studies were conducted with diploid and triploid oysters to assess the effects of multiple whole South Louisiana crude (SLC) oil concentrations and seasonal water temperature variation on the PAH bioaccumulation and depuration rates within the test populations. Tissue analyses from the mesocosm study showed that PAH concentrations were generally higher and less variable in triploids than diploids. The studies showed that triploid *Crassostrea virginica* can be an appropriate organism to serve as a bioindicator of PAH contamination as they are abundant, stationary filter-feeders that provide ample tissue for analysis, and accumulate PAHs in response to contamination. Although diploid oysters are more representative of ecological impacts, triploid oysters are the only ploidy to have the capability to accurately assess oil and chemical spill impacts during oyster breeding season.

INTRODUCTION:

Contamination of estuarine and navigable waterways by contaminants is a growing problem throughout the United States (O'Conner, 2002). Polycyclic aromatic hydrocarbons (PAHs) are oil-related hydrophobic contaminants found in our coastal waterways that arise from numerous anthropogenic sources. These sources range from bilge water discharge to large-scale deep sea releases, such as the *Deepwater Horizon* (DWH) spill in April 2010. Frequent sampling is required to accurately identify contaminant trends given the temporal and spatial variability of concentrations in water and sediment. As witnessed during the DWH spill, extensive and prolonged sampling is often very expensive and difficult to manage. A supplement to field sampling could be the use of bivalve organisms as bioindicators for determining short-term (weeks to months) exposure concentrations in the environment following oil and chemical spills.

Indigenous or caged bivalves can provide an assay of temporal and spatial concentrations of bioavailable contaminants in the aquatic ecosystem. Bivalves are widely accepted as an effective bioindicator species for monitoring hydrophobic contaminants in the aquatic environment (Gunther, et al., 1998). Bivalves have the ability to : (1) filter large volumes of seawater, incorporating dissolved and particulate-bound contaminants, (2) be easily transplanted and maintained over a long period of time, (3) tolerate a broad range of environmental conditions (e.g. temperature, salinity, and suspended solids loading), and (4) metabolize contaminants at various rates. Various studies have shown that PAH body burden within bivalves typically decline exponentially, with a biological half-life of approximately 16 days (Meador et al., 1995 and Hwang et al., 2014). The short half-life of PAHs within bivalve tissue limits their ability to serve as bioindicators to a maximum of 3-5 months after initial exposure, depending on length, frequency, and magnitude of exposure. One of the shortcomings of using natural oysters (diploid) for bioaccumulation studies is their high summer mortality rate, caused by high energy expenditure and decreased immunity during their reproductive cycle (Gueguen et al., 2012). In contrast to their diploid counterparts, the inhibition of reproduction in triploid oysters has shown them to have lower summer mortality rates than diploids due to a reduced energy expenditure, a limited protein metabolism, and a more effective immune system (Kesarcodi-Watson et al., 2001). In the summer months when diploid oysters are spawning, triploid oysters exert little energy into reproduction and remain firm, full and in excellent health. Triploids maintain their healthy conditioning during the late summer and early fall, when diploids are spawned-out, watery, and reduced in body mass. Triploids can occur naturally, but are typically manipulated through a process that causes the egg to contribute two sets of chromosomes and the sperm one set. This chromosomal configuration renders the triploid oyster essentially sterile and unable to spawn.

Seasonal trends in PAH concentrations within the bivalve population has been well documented (Piccardo et al 2001, Bruner et al 1994, Webster et al 1997, and McIntosh et al 2003). In general, the uptake of hydrophobic contaminants such as PAHS increases from the winter months to early spring, mainly due to gametogenesis and elevated uptake of nutrients. Spawning usually occurs in late spring to early summer, although this is highly dependent on environmental factors such as water temperature and salinity. In diploid oysters, spawning is typically associated with a decrease in PAH burden due to a significant decrease in body mass (i.e. glycogen levels) and disruption of normal feeding patterns.

Given the amount and duration of South Louisiana crude (SLC) oil spilled in the northern Gulf of Mexico (GOM) during the DWH incident, the main goal of this study is to evaluate the triploid Eastern oyster, *Crassostrea virginica*, as an analytical tool to assess seafood contamination of oil and chemical spill impacted areas. Strong emphasis is placed on the bioaccumulation and depuration of oil-related PAHs by diploid and triploid Eastern oysters in a controlled laboratory exposure and depuration period (seven weeks). The information gathered from this study into the bioaccumulation and depuration of PAH contaminants will later be used to develop a generalized model of the environmental conditions.

MATERIALS AND METHODS:

Mesocosm experiments were conducted at the Louisiana State University (LSU) Department of Veterinary Science aquatic facilities (Baton Rouge, Louisiana) to investigate sediment-bound PAH uptake and depuration in the Eastern oyster (*Crassostrea virginica*). The complete study consists of two individual seasonal mesocosm studies. The first exposure study was conducted February 2012 (winter) at a water temperature of 19°C ± 1°C. The second study was conducted August 2012 (summer) at a water temperature of 26°C ± 1°C. The main objective was to reproduce field conditions (PAH and seasonal temperature) in the northern Barataria Bay estuary following the DWH oil spill and to monitor PAH concentrations in Eastern oysters (triploid and diploid) following a three week exposure to SLC oil and four week depuration period. The use of laboratory-based mesocosm studies allow the concentration of sediment-bound PAHs as well as the seasonal temperature (summer and winter) changes to be precisely controlled. In addition, the use of large-scale mesocosms will eliminate variability in a number of other environmental parameters such as pH, dissolved oxygen (DO), and salinity.

Experimental design and statistical analyses

In February 2012 and August 2012, 180 cultured triploid oysters (>100 mm) were obtained from the Louisiana Sea Grant Oyster Research and Demonstration Farm off Grand Isle, LA. Salinity recorded close to the farm by a U.S.G.S. continuous data recorder (# 073802516) averaged 21.2 ± 0.1 (mean ± SE, range: 5.0-33.6) between 2002 and 2013. The oysters were the progeny of oysters spawned in summer 2010. Approximately 220 diploid oysters (>75mm) were collected from a commercial lease located at Caillou Lake, Louisiana. Salinity recorded at the Caillou Lake (Sister Lake) site by a U.S.G.S. continuous data recorder (# 07381349) averaged 19.9 ± 0.3 (mean ± SE, range: 6.3-22.7) between 2002 and 2013. The diploid and triploid oysters were randomly assigned to five (5) dual recirculating tank systems (500 ppm, 5,000 ppm, 25,000 ppm, sediment only, and water only). Each treatment system consisted of two (2) 200-L tanks each filled with 175 L of aerated artificial seawater (ASW, Crystal Sea Marinemix, Aquatic Ecosystems, FL, USA) adjusted to 22 ‰ ± 2 ‰. An additional dual tank depuration system containing aerated ASW was used to hold the oysters during the designated depuration period. For each treatment system, the ASW was recirculated through a 0.5 ft^3 biological bead filtration device at a rate of 10 gallons per minute (gpm). Water temperature was maintained at 19°C ± 1° for the winter study and 26°C ± 1° for the summer study. The tanks were equipped with slotted polypropylene holding trays to allow stacking of the oysters and provide circulation of water between oysters. Approximately 35 triploid and 40 diploid oysters (75 adult oysters/m^2) were allotted to each treatment system and allowed to acclimate to the water for one (1) week. The study's population density is comparable to densities found in natural oyster reefs (60-150

adult oysters/m^2). Oysters were fed daily with 0.3 mL Pavlova 1800 (Reed Mariculture Inc., Campbell, CA, USA) per oyster.

The oil-contaminated sediments used for dosing treatment systems were prepared by adding 175 mg, 1.75 g, and 8.75 g of British Petroleum (BP) South Louisiana crude (SLC) surrogate oil (Figure 1) to 350 g of wet sediment (50% moisture) into a 1-gallon glass mixing container. The sediment only container received 350 g of wet sediment. The sediment sample was collected near the Louisiana Sea grant and Research Demonstration Farm in Grand Isle, Louisiana. Approximately 2-L of ASW was added to each mixing container and placed on an orbital shaker for 48 hours. The BP SLC surrogate oil used in these studies is currently being distributed by BP as a surrogate research oil for the oil spilled during the DWH incident. Gas chromatography/mass spectrometry (GC/MS) analysis showed the BP SLC surrogate oil contained a total PAH (tPAH) concentration of 13,200 mg/Kg. After mixing on the shaker a small sediment sample from the 500 ppm, 5000 ppm, 25,000 ppm, and sediment only mixing containers were analyzed and average tPAH concentrations were determined to be 10.7 mg/Kg, 114 mg/Kg, 469 mg/Kg, and 0.671 mg/Kg, respectively. Prior to dosing with spiked sediments, five diploid and five triploid oysters were collected from each treatment system to determine baseline PAH concentrations within the oysters. Immediately after baseline sampling, designated treatment systems were dosed with the two (2) liters of the 500 ppm, 5000 ppm and 25,000 ppm oil-contaminated sediments (wet weight basis) and unoiled sediment (sediment control) for a final sediment suspension load of 500 mg (dry weight) per liter. Water in the tanks was mixed daily to re-suspend sediments settled overnight. At the end of week one (1), four diploid and four triploid oysters were collected from each treatment system for PAH and condition index (CI) determination. Whole oysters were carefully rinsed with deionized (DI) water, opened, and meat removed and weighed (g) prior to storage in a -80 °C freezer for future PAH analysis. Following week one sampling, the designated treatment systems were spiked with a second dose of a two (2) liter volume of 500 ppm, 5000 ppm and 25,000 ppm oil-contaminated sediments (wet weight basis) and unoiled sediment (sediment control) for an estimated total sediment suspension load of 1000 mg (dry weight) per liter for each treatment system. At the end of weeks two and three oyster (4 diploid and 4 triploid) were collected from the five treatment systems and processed as described for week one sampling. Following week three sampling, a total of 115 oysters (59 diploid and 57 triploid) were collected from the three (3) oil-sediment treatment systems and gently rinsed with ASW to remove any sediment residual from the shell. The oil-contaminated diploid and triploid oysters were transferred to designated holding trays and placed in the depuration tank system. A total of 43 oysters (23 diploid and 20 triploid) from the sediment control treatment system were transferred into designated holding trays and placed into the water control treatment system. The oysters were later sampled (4 per ploidy) on a weekly basis from week four to the end of the 7-week study. Oyster mortality was checked daily and cumulative weekly mortality was calculated correcting for sampling according to Ragone Calvo et al. (2003). Five diploid and five triploids were collected at the end of each study for total lipid and glycogen determination. Methods to determine CI, total lipid, glycogen, and PAH concentrations in oysters are described below.

Cumulative mortality of oysters exposed to oil-contaminated sediment, sediment control, and water control were compared with a Chi-squared analysis at one, three, and seven weeks. Oyster responses to oil and total PAHs concentration were analyzed with two-way analysis of variance (ANOVA) using SPSS statistical software. In this design the first factor was ploidy and had two

levels, diploid and triploid. The second factor within the design was sediment treatment concentration and had three levels: 500 ppm, 5000 ppm, and 25,000 ppm. Physiological determinations (i.e. CI, lipid, and glycogen) were analyzed with a one-way ANOVA. Least-square means with a Tukey adjustment were used following significant ANOVA results ($P<0.05$) to examine the differences among treatments. Prior to statistical analysis data was tested for significant outliers, normality, and homogeneity of variance. All data are presented as mean ± standard deviation.

Total polycyclic aromatic hydrocarbons (PAH) concentrations

PAH concentrations of oyster tissues were determined using a modified QuEChERS-based (AOAC Official Method 2007.01) tissue preparation/chromatographic cleanup procedure and GC/MS analysis. The procedure involves homogenizing the sample and extracting/partitioning the contaminants using an organic solvent (65% methyl ethyl ketone/ 35% hexane mixture) and salt solution. Lipids are then cleaned from the supernatant using dispersive solid phase extraction (dSPE). Multiple validation studies have been performed using this procedure for the determination of PAHs in edible seafood (Norli et al. 2011, Zhang et al. 2007, and Barker 2007). GC/MS analyses of the extracts were performed on an Agilent 7890A GC system configured with a 5% diphenyl/95% dimethyl polysiloxane high resolution capillary column directly interfaced to an Agilent 5975 inert XL MS detector system. The extracts were injected in splitless mode at an injection temperature of 280°C. The transfer line and ion source were at 280°C and 200°C, respectively. The column temperature was initially held at 70°C for 2 min, raised to 280°C at the rate of 6°C/min. and held for 3 min, and finally to 300°C at the rate of 3°C/min. Helium was used as a carrier gas at a constant flow rate of 1.1 ml/min. Mass spectrometry was acquired using the electron ionization (EI) and selective ion monitoring (SIM) modes.

Condition Index

Condition index was calculated as the ratio of dry tissue weight to shell cavity volume multiplied by 100 (Lawrence and Scott 1982). For each oyster, a 10-mL aliquot of tissue homogenate in ASW was dried at 65 ˚C for 48 h and dry weight determined by subtracting the dry weight of ASW only. The dry weight for the whole oyster was calculated based on the total volume of homogenized tissue in ASW. Shell cavity volume was determined by subtracting the weight of the oyster shells from the weight of the intact oyster.

Glycogen Content

The glycogen content was determined with the method described by Li (2000). Briefly, freeze –dried oyster samples were suspended in a 60 volumes of 30% KOH solution, and saponified by heating to 100°C for approximately 30 minutes. After cooling, an aliquot of the saponified solution was treated with the cold 0.2% anthrone-sulfuric acid solution for 10 min, and then the absorbance of the resulting colored complex was measured at the wavelength of 620 nm.

Total Lipid Content

The total lipid content was determined using the Smeded method (1999). A 5.0-10 g portion of oyster tissue was homogenized in a Waring blender and weighed into a 50-ml centrifuge tube. Approximately 18 ml of isopropanol and 20 ml of cyclohexane is then added to centrifuge tube

and sample homogenized. Approximately 17 ml of deionized water is added and the sample is homogenized again. A centrifuge is used to separate the organic extract from the particulate material. The organic extract is removed and placed in a pre-weighed round-bottomed flask. A second extraction is carried out using 25 ml of a 13% (v/v) isopropanol in cyclohexane solution. Again, a centrifuge is used to separate solvent and particulate material. The organic solvent from second extraction is combined with the first extract in the flask. The sample is dried with a rotovap and then flask is placed in an oven (80°C ± 5°C) for 1 hour. The residual is weighed and the lipid content calculated.

RESULTS:

Cumulative Mortality and Condition Index

No significant difference in oyster mortality could be found between oil treatments after one, three, and seven weeks. There was a statistically significant difference in CI between ploidy (diploid and triploid) as determined by one-way ANOVA (F (3, 16) = 53.439, $p < 0.0005$). A Tukey post-hoc test revealed that the condition index was significantly lower for the summer diploids (6.96 ± 0.7) when compared to the summer triploids (10.0 ± 0.5, $p < 0.0005$), winter diploids (10.4 ± 0.53, $p = < 0.0005$), and winter triploids (11.3 ± 0.55, $p < 0.0005$). There were no statistically significant CI differences between the winter diploids and the winter triploids ($p = 0.092$) and summer triploids ($p = 0.814$). Results for the condition index determinations are shown in Figure 2.

Total Lipid Content

There was a statistically significant difference in total lipid content between ploidy as determined by one-way ANOVA (F (3, 16) = 32.936, $p < 0.0005$). A Tukey post-hoc test revealed that the % total lipid content was significantly higher for the summer diploids (14.9 ± 0.86) when compared to the summer triploids (10.6 ± 0.58, $p < 0.0005$), winter diploids (9.6 ± 0.92), and winter triploids (10.6 ± 1.23, $p < 0.0005$). There were no significant differences between the winter triploids and the summer triploids ($p = 1.000$) and winter diploids ($p = 0.337$). Also, there were no significant differences between the winter diploids and the summer triploids ($p = 0.337$). Results for the total lipid content determinations are shown in Figure 3.

Glycogen Content

There was a statistically significant difference in glycogen content between ploidy as determined by one-way ANOVA (F (3, 16) = 349.284, $p < 0.0005$). A Tukey post-hoc test revealed that the % glycogen content was significantly lower for the summer diploids (3.91 ± 0.81) when compared to the summer triploids (24.3 ± 1.62, $p < 0.0005$), winter diploids (27.7 ± 1.87, $p = < 0.0005$), and winter triploids (28.5 ± 1.10, $p < 0.0005$). Additionally, the statistical analysis showed that the glycogen content was significantly lower for the summer triploids when compared to the winter triploids ($p < 0.001$) and winter diploids ($p < 0.007$). There were no statistically significant glycogen differences between the winter diploids and the winter triploids ($p = 0.649$). Results for the glycogen content determinations are shown in Figure 4.

PAH Concentration in Oysters

A two-way ANOVA of the diploid and triploid oyster results from the summer study indicated there were statistically significant between the effects of ploidy and treatment levels on

the PAH concentration ($p < 0.0005$). The summer triploid oysters had maximum average total PAH concentrations of 564, 2005, and 2912 ng/g for the 500, 5000, and 25000 ppm sediment treatments, respectively. The summer diploid oysters had maximum average total PAH concentrations of 403, 1380, and 1956 ng/g for the 500, 5000, and 25000 ppm sediment treatments, respectively. The winter triploid oysters had maximum average total PAH concentrations of 598, 2099, and 3094 ng/g for the 500, 5000, and 25000 ppm sediment treatments, respectively. The winter diploid oysters had maximum average total PAH concentrations of 531, 1812, and 2781 ng/g for the 500, 5000, and 25000 ppm sediment treatments, respectively. A comparison of the summer and winter triploids showed there was no significant interaction between the effects of ploidy and treatment levels on the PAH concentration in triploid oysters ($p=0.814$). A statistical analysis of the winter diploid and triploid study indicated there was no significant interaction between the effects of ploidy and treatment levels on the PAH concentration in triploid oysters ($p=0.742$). A comparison of the winter and summer diploid oyster concentrations showed there was a statistically significant interaction between the effects of ploidy and treatment levels on PAH concentrations in diploid oysters ($p= 0.001$). The tPAH burden in the spiked diploid and triploid oysters reached their maximum levels approximately two weeks after exposure and decreased exponentially till the end of the 7-week studies. During the summer study the average tPAH half-life for diploid and triploid oysters were 4.7 and 4.4 weeks, respectively. The average tPAH half-life for both the diploid and triploid oysters during the winter study was 4.2 days. Statistical analysis of the tPAH half-lives of the diploid and triploid oysters indicated there was no significance difference between the summer and winter seasons. Results for the PAH determinations are shown in Figures 5 and 6.

DISCUSSION:

The results of the mesocosm studies have shown uptake and bioaccumulation of petroleum-based PAHs into all treatment tank systems. It can be seen that uptake and bioaccumulation of PAHs can be a complex process that is more than simply dietary ingestion of contaminated sediment. The bioaccumulation and depuration of hydrophobic contaminants by marine bivalves can vary significantly depending on a wide range of variables including seasonal effect (temporal changes in both physiological response and feeding patterns), behavioral patterns, sexual condition, water temperature, and length of contaminant exposure (Duchemin et al. 2007).

This study provides useful information on the relationship between oyster ploidy and the seasonal uptake and depuration rates of the Eastern oysters exposed to SLC oil under laboratory conditions. The study will ultimately provide information on the rates of bioaccumulation and depuration but was not presented in this publication. The relationship between seasonal temperature variations and oyster ploidy suggest that triploid oysters may be a more suitable bioindicator of PAH contamination during the natural oyster spawning season, typically between the months of May through August. The lack of reliability in diploid oysters is due to the physiological changes encountered during spawning season and change in nutrient/feeding patterns associated with the summer months. Seasonal trends in PAH concentrations have been well documented during in-situ studies (Namiesnk et al. 2008, Porte and Albaiges 1993, and Yesudhason et al. 2013) and are clearly seen in this mesocosm study. The significant increase in lipid and decrease in glycogen content (Leon et al. 2013) within the diploid oysters during the

summer study clearly indicates that the oysters were in the process or had recently undergone spawning. As a consequence of biochemical replacement during reproduction, the lipid content of diploid oysters varied from 13.5 to 15.8 % of total tissue dry weight during the spawning season. The results from the study indicated there was no seasonal variation in the total PAH a concentration within the triploid oysters. The total PAH concentrations within the winter diploid oysters were not significantly different ($p = 0.038$) than the concentrations recorded for both the summer and winter triploid oysters. Many studies have shown how caged diploid bivalves can be used to demonstrate short-term contaminant trends, temporal and spatial variability, and dose-response relationships (Short and Harris 1996, Salazar and Salazar 1997, and Green et al. 1985). However, this study has demonstrated the usefulness and versatility of cage triploid oysters as an effective tool for assessing exposure and effects in a variety of marine environments.

CONCLUSION:

The bioaccumulation of oil-related PAHs was characterized in diploid and triploid Eastern oyster (*Crassostrea virginica*) in a laboratory mesocosm study and their response to seasonal temperature variations was determined. The bioaccumulation and depuration of PAHs is similar for all triploid oysters (winter and summer) and winter diploid oysters. However, PAH concentrations within the summer diploids were significantly lower in comparison to the triploid and winter diploid oysters. The decrease in PAH concentrations can be attributed to physiological and behavioral changes induced by the spawning process. This study has shown the triploid Eastern oyster to be a suitable bioindicator organism for short- and long-term monitoring of oil and chemical spills within the marine environment.

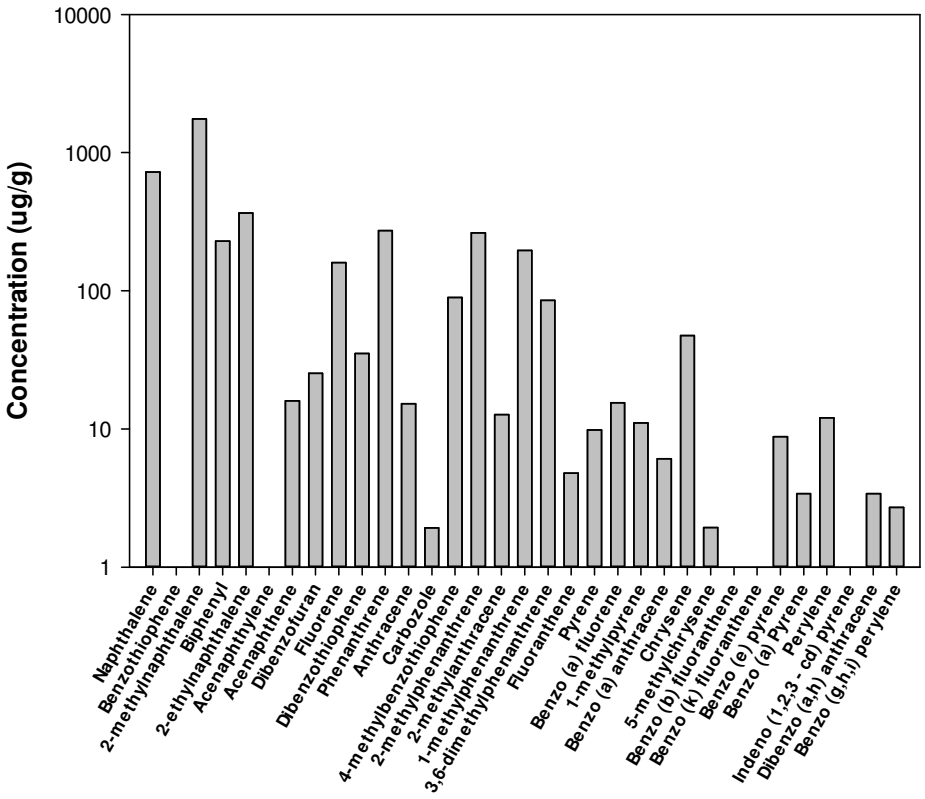

Figure 1. Individual PAH concentrations in MC252 surrogate oil.

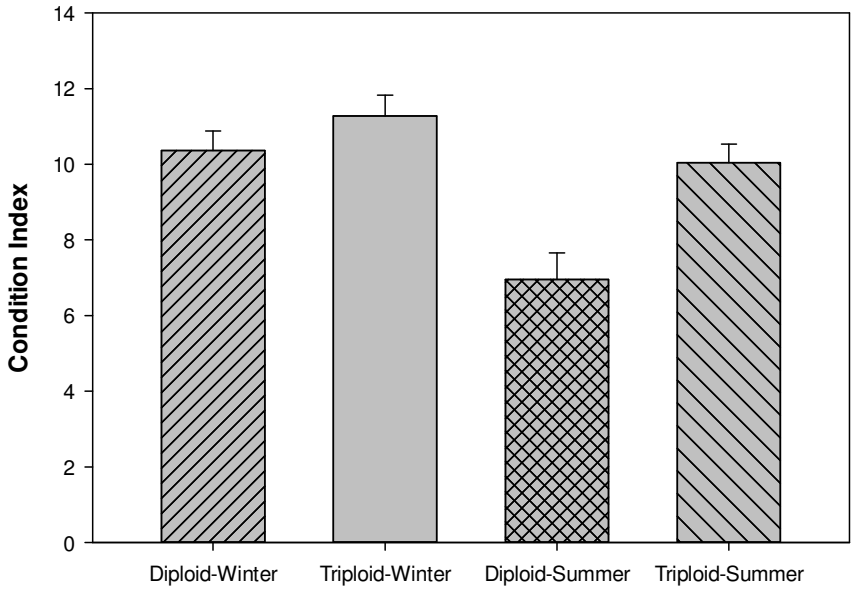

Figure 2. Condition index for diploid and triploid oysters at end of studies.

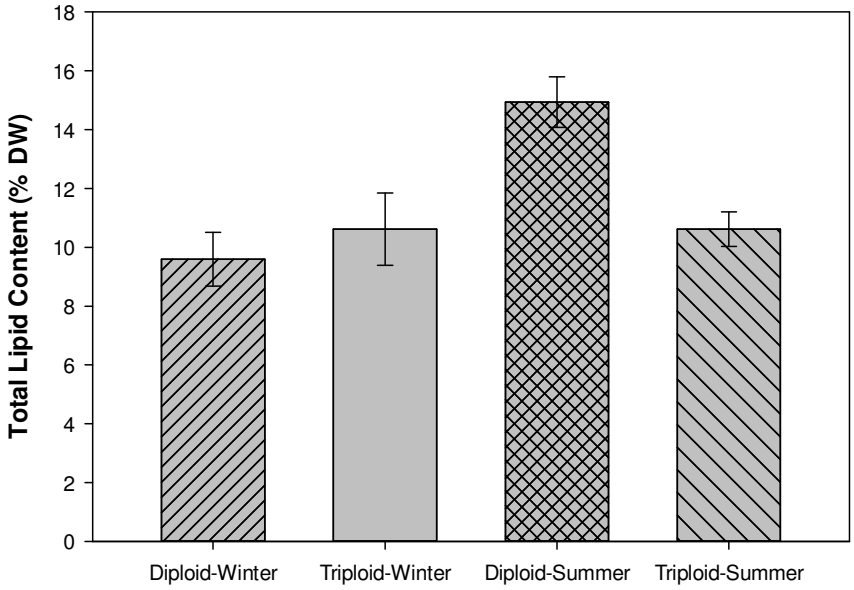

Figure 3. Total lipid content for diploid and triploid oysters at end of studies.

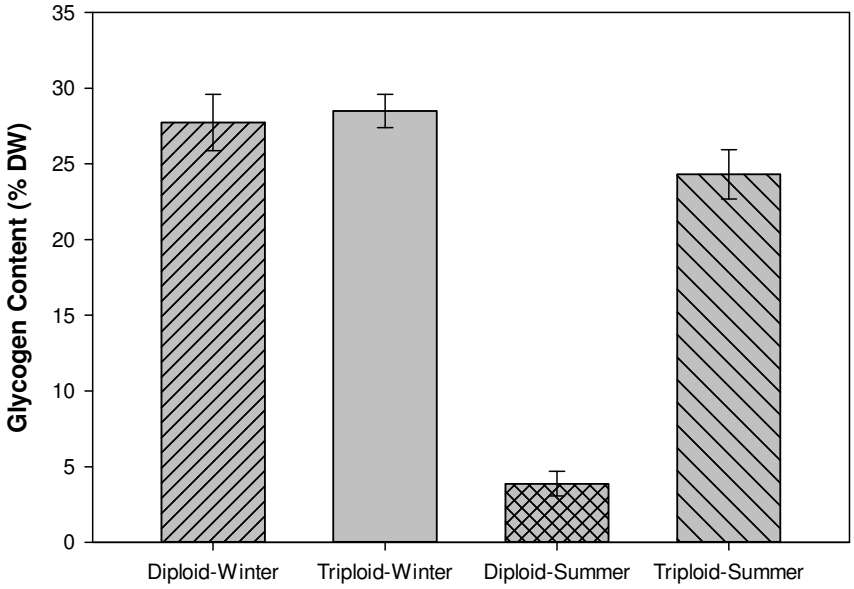

Figure 4. Glycogen content of diploid and triploid oysters at end of studies.

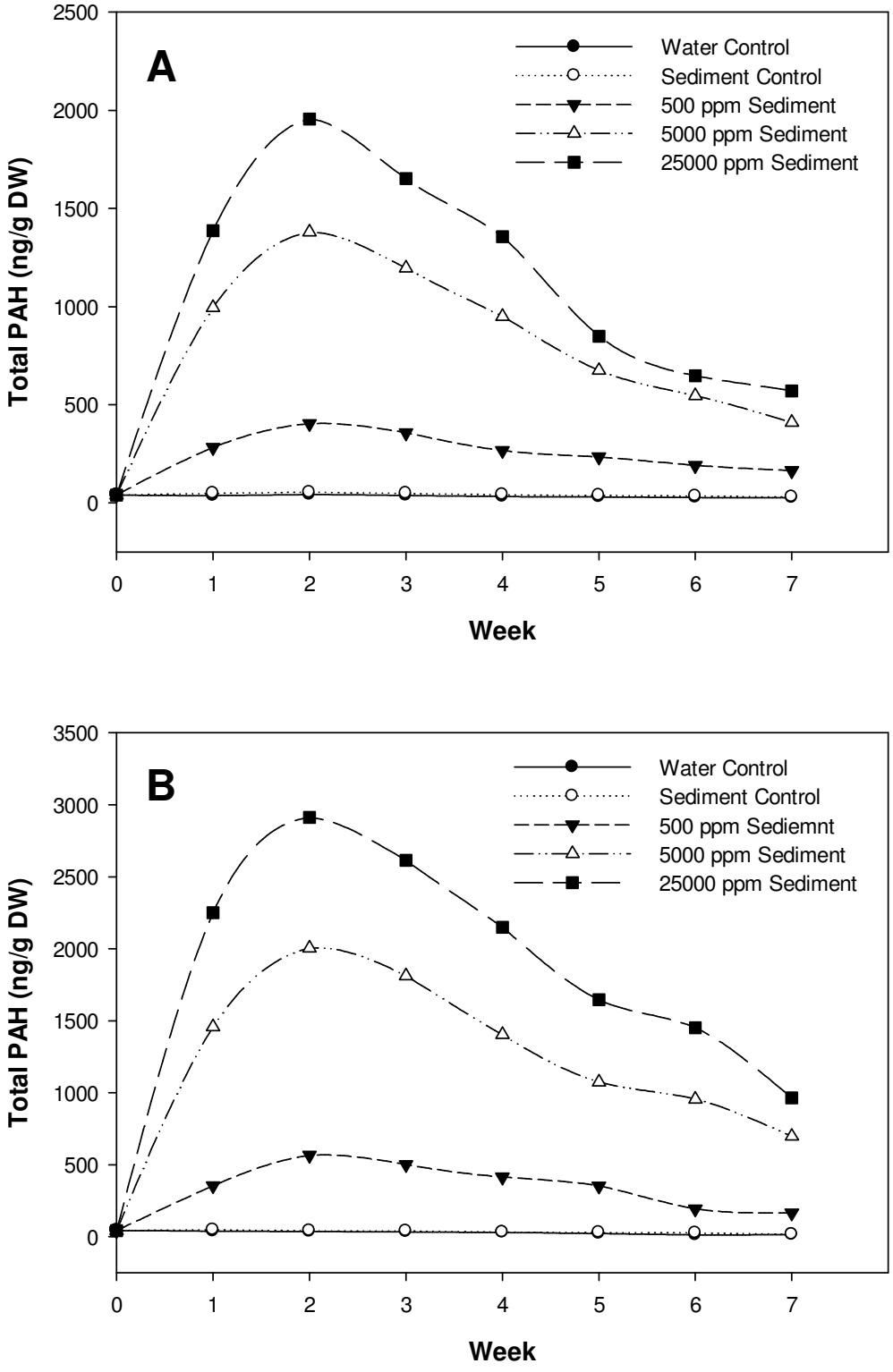

Figure 5. Total PAH concentrations in (A) diploid and (B) triploid oysters for individual treatments during summer study.

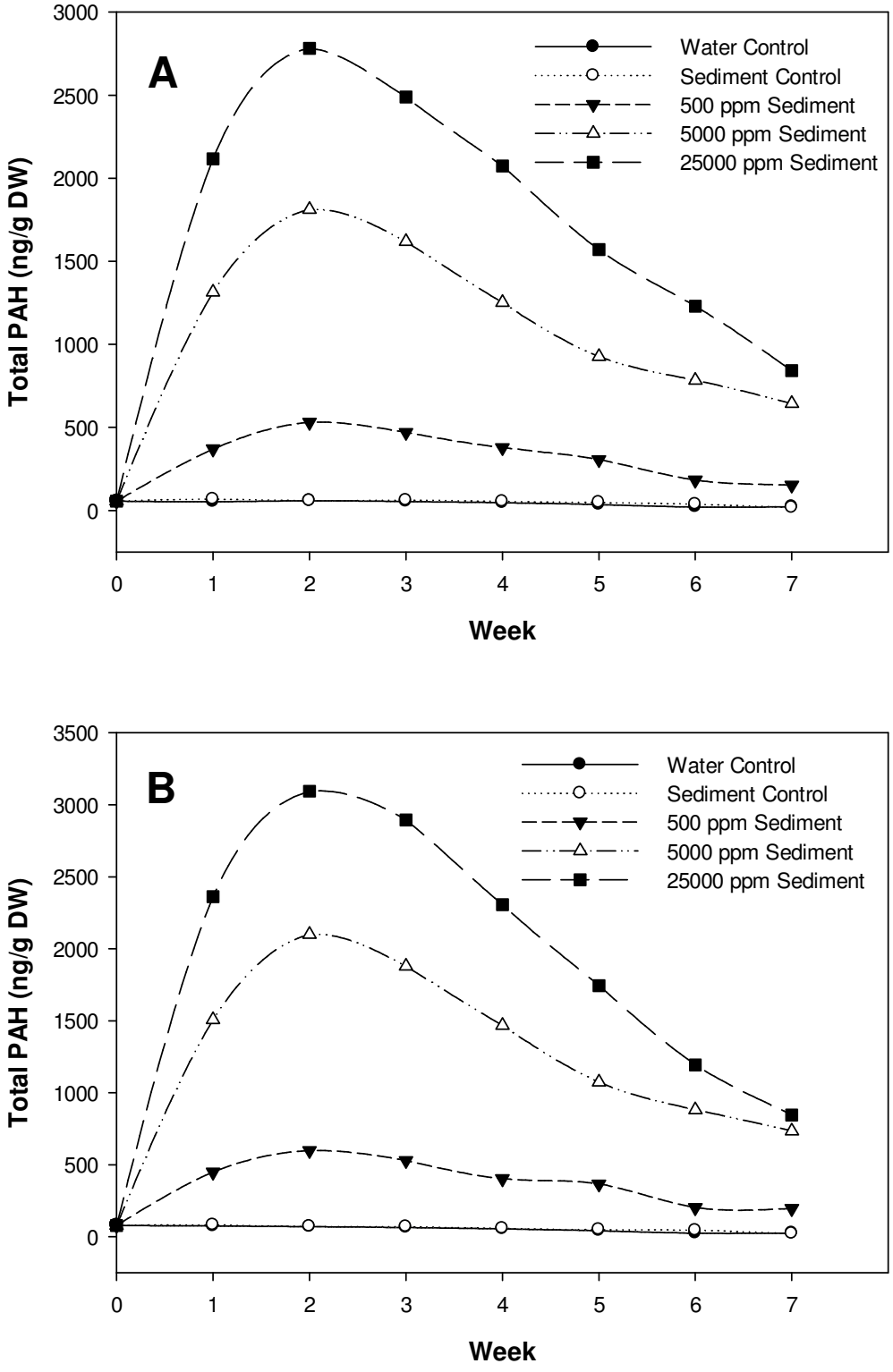

Figure 6. Total PAH concentrations in (A) diploid and (B) triploid oysters for individual treatments during winter study.

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