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Immunomodulation in eastern oysters, *Crassostrea virginica*, exposed to a PAH-contaminated, microphytobenthic diatom

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ABSTRACT

The trophic transfer of sediment-associated pollutants is a growing concern in shellfish harvesting areas. Previous studies have examined the role of phytoplankton in the transport of organic contaminants to bivalve species, but little information on microphytobenthic communities and their role as contaminant vectors exists. Polycyclic aromatic hydrocarbons (PAHs) are organic compounds formed during natural and industrial processes; they are termed "persistent organic pollutants" because they are only slowly degraded by natural processes. This study examined the transfer of PAH compounds (naphthalene, pyrene, and benzo(a)pyrene) by a microphytobenthic diatom to the eastern oyster, a commercially important shellfish species, to determine if dietary accumulation is a route of contaminant exposure capable of inducing physiological responses. PAH compounds were adsorbed to a diatom culture (*Nitzschia brevirostris*) in a range of concentrations (5, 125, 625, and 1000 $\mu g\,L^{-1}$), and eastern oysters were exposed experimentally to the contaminated diatom cultures to assess possible effects upon oyster hemocytes and selected immune-defense functions. A preliminary experiment was designed to identify individual effects of several PAH compounds (naphthalene, pyrene, and benzo(a)pyrene) on hemocyte viability and phagocytic activity. Results from this experiment revealed that the most-toxic compound, benzo(a)pyrene, at the highest concentration, stimulated an increase in agranular hemocyte counts. A follow-up study examined the effects of benzo(a)pyrene on hemocyte viability, adhesion, phagocytosis, and reactive oxygen species (ROS). These studies showed the ability of this benthic diatom to transport PAHs to the eastern oyster and to cause immunomodulation. Hemocyte responses to dietary PAH exposure included an increase in circulating hemocytes and increased production of reactive oxygen species by these cells.
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1. Introduction

Persistent organic pollutants are hydrophobic compounds found throughout the environment that are slow to degrade and have been found to alter the health of organisms. These environmental pollutants are characterized by persistence in the environment, ability to bioaccumulate in living organisms, and toxicity to exposed organisms (Kretchik, 2002). Polycyclic aromatic hydrocarbons (PAHs) constitute a group of persistent organic pollutants, formed through natural and industrial processes that are ubiquitous in the environment (Neff, 1979; Varanasi, 1989; Kennish, 1992). PAHs enter the aquatic ecosystem through atmospheric deposition, surface runoff, effluent discharge, and oil spills, and can persist in the environment for long periods of time.

In aquatic ecosystems, hydrophobic, organic pollutants are relatively insoluble and, thus, are mainly found attached to particulate matter (*e.g.*, sediment and biota) (Rubinstein et al., 1984). PAHs are hydrophobic and lipophilic, and therefore have a high affinity for biological compartments with high lipid content (Neff, 1979; Spacie and Hamelink, 1995). Microalgae are living particles to which hydrophobic pollutants can adsorb in an aquatic system. The adsorption of organic pollutants to suspended particles in the water column has been described as an essential transfer process influencing the bioavailability and toxicity of hydrophobic environmental pollutants (Lyman, 1995). PAHs are pollutants that have been found to cause alterations in the health status of exposed animals (Moore et al., 1996; Hwang et al., 2002; Oliver et al., 2003; Auffret et al., 2004; Mayrand et al., 2005).

Pollutant uptake from water, sediments, and food sources are three routes by which filter-feeding animals, including oysters, can be exposed to PAHs. In the natural environment these exposure routes occur simultaneously (Spacie et al., 1995). Physical and chemical properties of PAH compounds, such as molecular structure and steric properties, determine to what degree the compound will be accumulated by suspension feeders (Spacie and Hamelink, 1995; Capuzzo, 1996). Bioavailable PAH compounds in water are accumulated by passive diffusion over the gills of the oyster (Spacie

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and Hamelink, 1995). Lee et al. conducted an uptake experiment in which blue crabs (*Callinectes sapidus*) accumulated 10% of radiolabeled PAH compounds added to an aquarium (1976). Tissue analysis indicated that during the first two days of the experiment, the majority of the radioactivity was observed in the gills, the initial point of entry (Lee et al., 1976). Bivalves have the ability to accumulate PAHs following water-borne exposure, and these compounds have the potential to cause negative impacts on the health of bivalve species (Stegeman et al., 1973; Neff et al., 1976; Anderson et al., 1981; Lowe et al., 1981; Riley et al., 1981; Grundy et al., 1996a,b; Wooten et al., 2003; Siu et al., 2004; Boutet et al., 2004). More-recent studies include an experimental exposure of the marine mussel, *Mytilus edulis* (Coles et al., 1994), to fluoranthene. Mussels were exposed to the PAH compound in tanks for 7 days to determine effects upon immune functions of this bivalve species; findings indicated that total circulating hemocyte numbers increased with exposure to higher concentrations (200 $\mu g\,L^{-1}$ and 400 $\mu g\,L^{-1}$) of the compound, and a stimulation in the release of reactive oxygen metabolites was also observed. In many previous studies, PAH compounds added to seawater containing the animals were dissolved in small quantities of organic solvents, but the hydrophobic nature of the compounds would suggest that the PAHs could adhere very quickly to any particles in the seawater, thereby modifying the exposure route from dissolved to particulate vectors. Mussels, oysters, and other suspension feeders can be exposed to these pollutants as they feed on suspended particles. The use of particle-free seawater in exposure systems was not employed in any previous studies to block this route of exposure.

Evidence supporting the hypothesis that dietary accumulation is an important route of contaminant exposure in marine organisms is growing (Corner et al., 1976; Lee et al., 1976; Dillon, 1982; Rubinstein et al., 1984; Chu et al., 2000, 2003; Bekri and Pelletier, 2004). Corner et al. (1976) designed a laboratory study to determine if copepods accumulated aromatic hydrocarbons more readily through diet or direct uptake from water. The authors found that, not only did the copepod *Calanus helgolandicus* accumulate more naphthalene associated with food than through water accumulation, but depuration rates were much slower in copepods fed contaminated food sources than in those exposed to dissolved PAHs. Rubinstein et al. (1984) concluded that contaminated sediments and diets could serve as sources for contaminant uptake by the polychaete, *Nereis virens*, and subsequent transfer to higher trophic levels.

Phytoplankton have been the focus of two studies conducted to determine the efficiency of microalgae to transport organic contaminants to bivalve species (Kowalewska, 1999; Chu et al., 2003). Phytoplankton are rich in lipids and attract lipophillic, hydrophobic organic contaminants. Kowalewska found that phytoplankton in the Southern Baltic Sea absorbed PAHs and then transferred them to bottom sediments. Chlorophyll *a* concentrations in sediments were strongly correlated with sediment PAH concentrations (Kowalewska, 1999). Chu et al. (2003) fed eastern oysters PCB-contaminated algal paste to determine if subsequent effects could be seen in the offspring of these organisms. Oysters accumulated the organic compound, but reproductive success was not altered by experimental concentrations (Chu et al., 2003).

Although the focus of previous studies has been on phytoplankton, benthic microalgae also provide food to both deposit- and suspension-feeding, benthic organisms. Microphytobenthic communities consist of microscopic, photosynthetic, eukaryotic algae and cyanobacteria that inhabit surface sediments (Pickney and Zingmark, 1993; MacIntyre et al., 1996). In the environment, sediments are a major sink for hydrophobic organic compounds (Neff, 1979; Elroy et al., 1989; Kennish, 1992). Hydrophobic PAHs become associated with particles in the water column and ultimately settle to the sea floor. Sessile bivalves are exposed to these contaminated sediments during physical disturbances, such as wind turbulence or tidal currents (Elroy et al., 1989). Studies using contaminated sediments to measure pollutant uptake rates have confirmed that sediments are able to transfer contaminants to marine organisms, impacting overall health (Bender et al., 1988; Sami et al., 1993). These studies were able to show positive correlations between sediment concentrations of pollutants and concentrations found in tissues.

Microphytobenthic algae provide food for suspension-feeders when resuspended into the water column in areas where phytoplankton are not abundant (Miller et al., 1996). Because of the close association with sediments, microphytobenthic algae have a particularly strong potential to transfer organic contaminants to bivalve species.

The present study applied a controlled, experimental approach to examine the potential for a microphytobenthic diatom to transfer PAH compounds to the eastern oyster with subsequent physiological impacts, specifically on hemocytes and their immune functions. The immune defense system of oysters is attributed to hemocytes that circulate freely within all tissues. Hemocytes have nutrient-transport, waste-removal, detoxification, and defense functions. Hemocytes are the principle defense cells for the eastern oyster and are found in two morphological forms: granular and agranular (Cheng, 1996; Oliver and Fisher, 1999). These cells are responsible for protecting the organism from living pathogens and parasites and also for detoxification of pollutants (Fisher, 1986; Cheng, 1996; Oliver and Fisher, 1999). Hemocyte defense functions include adhesion, followed by phagocytosis of the foreign particle, and internal degradation by reactive oxygen species (Fisher, 1986; Cheng, 1996). Immuno-compromised oysters may become more susceptible to pathogens and diseases, with consequences to fisheries production.

This experimental study simulated the processes described above – adsorption of PAHs to microphytobenthic algae and subsequent trophic transfer to feeding oysters – to test under controlled conditions the potential for this contaminant-exposure route to cause immunomodulation in oysters. Adsorption of PAHs to a cultured diatom was quantified as was trophic transfer to feeding oysters. Hemocyte characteristics and immune functions of PAH-exposed oysters were compared with those of oysters feeding on the same diatom with no PAH exposure to confirm a subsequent physiological effect following trophic transfer.

2. Materials and methods

Two experiments were conducted in which oysters were exposed to PAH-contaminated microalgae. The first experiment, Experiment I, was designed as a "range-finding" investigation into the relative responses of oysters to a cultured diatom contaminated with several PAH compounds at wide ranges of concentrations. A finding of no significant effect in Experiment I would have precluded further experiments; however, significant effects were found. Thus, a second experiment, Experiment II, was focused on one PAH compound, benzo(a)pyrene (b(a)p), and tested a larger number of oyster hemocyte responses than the first experiment. Experimental concentrations chosen for this study were based upon concentrations reported in the field (Fisher et al., 2000, 2003; Oliver et al., 2001).

2.1. Experimental organisms

Crassostrea virginica (30–35-mm shell height) used in this study were obtained from populations of un-selected, local oysters maintained at the Milford Laboratory. Oysters for both

experiments were originally housed in a flow-through system at 10 °C prior to placement in the experimental set-up. For Experiment II, oysters were removed from the original flow-through system a week prior to the experiment and placed in another flow-through system with water temperatures similar to the experimental water temperature of 17 °C and a salinity of 23 ppt. The additional, acclimation flow-through system was included to minimize temperature responses of experimental oysters when transferred to the experimental set-up.

The benthic diatom *Nitzschia brevirostris* (strain 0–1), obtained from the Milford Laboratory Microalgal Culture Collection, was used as the microphytobenthic contaminant vector in this experiment. Algal cultures were grown in enriched seawater medium with silicate (ESI) and incubated in a 20 °C-controlled room with 24-h illumination of 300 $\mu E\,m^{-2}\,s^{-1}$ photosynthetically active radiation (PAR), provided by banks of cool-white, fluorescent bulbs (Ukeles, 1973).

2.2. Test contaminants

2.2.1. Experiment I

PAH compounds naphthalene, pyrene, and b(a)p were tested. Naphthalene (99+%), pyrene (98%), and b(a)p (98%) were purchased from Fisher Scientific. Reagent grade acetone (Fisher Scientific) was used as the solvent vehicle into which the water-insoluble compounds were dissolved before addition to the aqueous diatom culture. Acetone-dissolved PAHs were pipetted volumetrically into microalgal-cultures to achieve final PAH concentrations of 5, 125, 625, and 1000 $\mu g\,L^{-1}$.

2.2.2. Experiment II

Benzo(a)pyrene (97%) was purchased from Sigma, and reagent-grade acetone, purchased from Fisher Scientific, was used as the solvent vehicle into which the water-insoluble compound was dissolved. Benzo(a)pyrene concentrations added to the diatom culture in this experiment were 100 and 1000 $\mu g\,L^{-1}$, based upon results of Experiment I and field studies.

2.3. Microalgal exposure

2.3.1. Experiment I

Twelve, 1-L sub-samples of *N. brevirostris* were taken from a semi-continuous culture during the exponential growth phase. The cell density in the 1-L aliquots was adjusted to $4.04 \times 10^4\,mL^{-1}$ with ultrafiltered (0.2-μm) seawater. Naphthalene, pyrene, and benzo(a)pyrene were added to the 1-L diatom cultures at the respective concentrations. An acetone control was tested instead of a naphthalene 5 $\mu g\,L^{-1}$ treatment because of equipment limitations for maintaining safety and environmental compliance while working with these highly toxic pollutants. This acetone treatment served as the solvent-control treatment for the experiment. The PAH-contaminated cultures and acetone control were stirred continuously on stirplates for 12 h to allow for adsorption of PAHs to the diatom culture (Chu et al., 2003).

2.3.2. Experiment II

Twelve, 1-L sub-samples of the benthic diatom were taken from a semi-continuous culture during the exponential growth phase. The final cell density in the 1-L aliquots was adjusted with ultrafiltered seawater to $1 \times 10^4\,mL^{-1}$. Benzo(a)pyrene and acetone were added to the 1 L diatom cultures to achieve the desired concentrations. Acetone- and PAH-contaminated cultures were stirred continuously as above.

2.4. Oyster exposure to a PAH-contaminated microphytobenthic diatom

2.4.1. Experiment I

Six to seven oysters, depending upon size, were placed in Pyrex glass baking dishes (22 cm × 11 cm × 6 cm, containing 1.5 L of ultrafiltered seawater per contaminant treatment). Each dish was placed in a nalgene container to collect over-flow effluent, which was then connected, by rubber tubing, to a 120-L drum for disposal as hazardous waste. Oyster treatments were fed continuously a $10^4\,mL^{-1}$ cell density of contaminated algae at a flow rate of 5 mL min^{-1} for 3 days. Actual concentrations to which oysters were exposed over the duration of the experiment ranged from 1 $\mu g\,L^{-1}$ to 2100 $\mu g\,L^{-1}$.

2.4.2. Experiment II

Six oysters were placed in each glass baking dish per contaminant-treatment replicate (control, acetone, and b(a)p). Each dish was then placed in a nalgene container to collect effluent that flowed into a 120-L drum for hazardous-waste disposal. Oyster treatments were fed continuously contaminated algae ($10^4\,mL^{-1}$ cell density) at a flow rate of 2 mL min^{-1} for 7 days. Flow rates were reduced in Experiment II as a result of the longer duration of the study. Each exposure treatment was carried out in triplicate. Actual concentrations to which oysters were exposed over the duration of the experiment were 201 $\mu g\,L^{-1}$ and 2010 $\mu g\,L^{-1}$.

2.5. Hemocyte analyses

After removing oysters from the experimental conditions, a notch was made with pliers on the dorsal shell edge which allowed access to the adductor muscle. Hemolymph was extracted from the muscle using a 1-mL tuberculin syringe with needle, and light microscopy was used to ensure that hemocytes relatively free of contaminating particles were collected. Hemolymph from each oyster was placed in an eppendorf tube on ice, to retard hemocyte aggregation. Hemolymph samples from individual oysters in each dish were pooled, and composite samples were distributed into 5-mL, Fisherbrand polycarbonate tubes for hemocyte analysis. Hemocyte characteristics and defense functions were analyzed from one pool from each baking dish using protocols described by Hegaret et al. (2003a,b) and analyzed on a BD Biosciences FACScan flow cytometer (San Jose, CA).

2.5.1. Characterization and viability of hemocytes using Sybr green and propidium iodide (Experiments I and II)

Hemocytes were identified on flow cytometer plots using the DNA probe Sybr Green (Invitrogen) and the FL1 detector (530 ± 30 nm). To identify dead hemocytes, we used propidium iodide (Sigma), which is permeable in only dead cells and emits fluorescence in the cytometer FL2 channel. Briefly, 100 μL of pooled hemolymph was mixed in a polycarbonate test tube with 300 μL of 0.2-μm-filtered seawater, 4 μL of Sybr green at a final dilution of 10^{-3}, and 4 μL of propidium iodide at a final concentration of 20 $\mu g\,mL^{-1}$. Hemolymph and reagents were allowed to incubate in the dark for 1 h prior to flow-cytometer analysis. In addition to hemocyte-type percentages (granular and agranular), total hemocyte counts were collected for 30-sec intervals in Experiment II. Methodologies for Experiment I were exploratory and were not standardized as in the final experiment; hemocyte-type percentages in Experiment I; therefore, were not converted to hemocyte cell densities.

2.5.2. Phagocytosis of fluorescent microbeads

2.5.2.1. Experiments I and II. A 150-μL sample of pooled hemolymph was added to a 5-mL Fisherbrand test tube containing 150 μL of ultrafiltered seawater and 30 μL of fluoresbrite

bead suspension (Fluoresbrite YG Microspheres, 2.00 μm; Polysciences). Test tubes were incubated for 2 h in the dark, and analyzed on the flow-cytometer. Bead fluorescence was measured using the FL1 detector.

2.5.3. Aggregation of hemocytes

2.5.3.1. Experiment II only. A 100-μL sample of hemolymph was placed in each well of a 24-well microplate along with 100 μL of ultrafiltered seawater. The microplate was incubated in the dark for 3 h. Following the 3 h incubation, settled, but not attached hemocytes were resuspended in the well by drawing 150 μL into and out of a pipet several times. The resuspended, non-adhering cells then were pipetted into a test tube with 200 μL of 6% formalin and 4 μL of Sybr green. Test tubes were incubated in the dark for 1 h. Percentages of hemocytes adhering to wells were calculated as the difference between hemocyte counts in aggregation tubes and characterization tubes.

2.5.4. Unstimulated reactive oxygen species content of hemocytes

2.5.4.1. Experiment II only. A test tube containing 100 μL of pooled hemolymph, 300 μL of filtered seawater, and 4 μL of dichlorofluorescin diacetate (DCFH-DA, Invitrogen) was incubated in the dark for 2 h. Oxidation of non-fluorescent DCFH-DA to fluorescent product DCFH was used to quantify the production of reactive oxygen species (ROS) by hemocytes in the absence of chemical or particulate stimulants.

2.6. PAH analysis

PAH analysis was conducted on oyster gill and mantle tissues excised from experimental oysters after hemolymph sampling, and on algal cultures following incubations with PAHs from Experiment II to quantify accumulated concentrations. Oyster gill and mantle tissues were stored in a −80 °C freezer until PAH analysis. Contaminated algal cells were centrifuged, the supernatant was decanted, and cells were stored in a −80 °C freezer for PAH analysis. PAH analyses of gill/mantle and algal samples were conducted by Texas A&M's Geochemical and Environmental Research Group (GERG). Tissue samples were homogenized using stainless steel blenders with titanium blades. A 15-g subsample of wet tissue was dried, and homogenized tissues were extracted with 100% dichloromethane using an Accelerated Solvent Extractor. Extracted volumes were reduced in volume and purified using column chromatography. Purified extracts were reduced to 1 mL and analyzed by gas chromatography/mass spectrophotometry. Quality control (QC) samples were included in sample batches for validity of data. Methods for the extraction of PAHs followed protocols detailed in the NOAA Technical Memorandum NOS NCCOS 30, used by the NOAA Status and Trend's Program (Kimbrough et al., 2006). Detection limits for PAH compounds by this method are also listed in this document.

2.7. Statistical analysis

The un-replicated factorial design of Experiment I permitted the statistical analyses of the effects of one independent variable (compound or concentration used), upon dependent variables. This design was chosen to maximize the ability to detect effects of PAH structure and concentration in a single experiment limited to 12 exposure containers. The intention was to provide only "yes" or "no" answers to the questions: "Are hemocyte responses affected by PAH structure?" and "Are hemocytes affected by PAH concentration?" in a single experiment. "No" answers to both questions would have indicated that further studies were not warranted, as there was no response within reasonable environmental ranges of concentration.

In the second experiment, a correlation matrix of dependent, hemocyte variables was generated to determine associations between the hemocyte variables. Principle components analysis (PCA) was performed to identify relationships between hemocyte characteristics and immune-defense functions. PCA profiles can be more-sensitive indicators of physiological effects in that aggregated responses in oysters with high variability between individuals reveal consistent changes following experimental treatment. ANOVA ($P < 0.05$) was performed to determine the statistical significance of PAH effects upon PCA profiles, and individual ANOVA tests were done for individual hemocyte variables. Percentage data were transformed (arcsin of square root) to normalize variance to mean. All statistical analysis was performed using *Statgraphics Plus* (Manugistics, Rockville, MD) software (Tables 1 and 2).

3. Results

3.1. PAH analysis

3.1.1. Diatom assimilation of PAH compounds

3.1.1.1. Experiment II. PAH analysis of diatom cultures contaminated with benzo(a)pyrene revealed detectable concentrations of the test compound in exposed cultures (Table 3). Benzo(a)pyrene concentrations were 515.4 ng g^{-1} in cultures exposed to 100 μg L^{-1} treatments and 10,730 ng g^{-1} in the 1000 μg L^{-1} culture treatments. Control test cultures did not contain detectable concentrations of benzo(a)pyrene.

Results from this analysis also identified additional PAH compounds in all algal cultures. The additional compounds were believed to be background environmental levels, or impurities in the test material, or metabolites of the original compound produced by the diatom (Table 3). Total PAH concentrations in the control culture were 12.3 ng g^{-1}, 524.8 ng g^{-1} in cultures exposed to 100 μg L^{-1} of benzo(a)pyrene, and 10,750 ng g^{-1} in cultures exposed to 1000 μg L^{-1} of benzo(a)pyrene. Both low-molecular-weight and-high-molecular-weight PAHs were detected at low concentrations in benzo(a)pyrene-exposed cultures. Several low-molecular-weight PAH compounds were found in the control algal culture. The presence of PAH trace amounts may be attributable to algal degradation or photo-oxidation of parent compounds or background levels in seawater used to make culture medium.

3.1.2. Accumulation of PAHs in oysters

3.1.2.1. Experiment II. PAH analysis of pooled, excised gill and mantle tissues from experimental oysters yielded benzo(a)pyrene concentrations of 68.9 ng g^{-1} in oysters exposed to algae treated with 1000 μg L^{-1} of benzo(a)pyrene (Table 2). Benzo(a)pyrene concentrations in both control oysters and oysters exposed to algae treated with 100 μg L^{-1} were below method detection limits. Similar to the algal cultures, additional PAHs were found in the combined gill and mantle tissues of each treatment. Both low-molecular-weight and high-molecular-weight PAH compounds were detected in low concentrations in all treatments. Mean total PAH concentrations were 160 ng g^{-1} in pooled tissues from control treatment, 83 ng g^{-1} in pooled tissues from the lower benzo(a)pyrene treatment, and 125.2 ng g^{-1} in pooled tissues from the higher benzo(a)pyrene exposure.

3.2. Hemocyte analyses

3.2.1. Characterization of hemocytes

Exposure to naphthalene, pyrene, and benzo(a)pyrene-treated diatoms induced significant responses in hemocytes of the eastern oyster in both experiments. Granular and agranular hemocyte populations were represented in all treatments at varying percentages.

Table 1
Summary of PAH concentrations (ng g^{-1} wet weight) in experimental algal cultures. ND = not detected, <MDL = below method detection limit.

PAH compounds	Algal culture control	Algal culture 100 μg L^{-1} benzo(a)pyrene	Algal culture 1000 μg L^{-1} benzo(a)pyrene
Naphthalene	<MDL	<MDL	<MDL
C1-naphthalenes	<MDL	<MDL	<MDL
C2-naphthalenes	7.4	6.7	6.4
C3-naphthalenes	ND	ND	ND
C4-naphthalenes	ND	ND	ND
Biphenyl	<MDL	<MDL	<MDL
Acenaphthylene	ND	ND	ND
Acenaphthene	ND	ND	ND
Fluorene	ND	<MDL	<MDL
C1-fluorenes	ND	ND	11.8
C2-fluorenes	ND	ND	4.8
C3-fluorenes	ND	ND	ND
Phenanthrene	<MDL	ND	5.3
Anthracene	ND	ND	ND
C1-phenanthrenes/anthracenes	ND	ND	5.8
C2-phenanthrenes/anthracenes	ND	4.6	5.2
C3-phenanthrenes/anthracenes	ND	ND	ND
C4-phenanthrenes/anthracenes	ND	ND	ND
Dibenzothiophene	ND	ND	ND
C1-dibenzothiophenes	ND	ND	ND
C2-dibenzothiophenes	ND	ND	ND
C3-dibenzothiophenes	ND	ND	ND
Fluoranthene	ND	ND	<MDL
Pyrene	ND	ND	<MDL
C1-fluoranthenes/pyrenes	ND	ND	ND
C2-fluoranthenes/pyrenes	ND	ND	ND
C3-fluoranthenes/pyrenes	ND	ND	ND
Benzo(a)anthracenes	ND	ND	<MDL
Chrysene	ND	ND	<MDL
C1-chrysenes	ND	ND	ND
C2-chrysenes	ND	ND	14.7
C3-chrysenes	ND	ND	ND
C4-chrysenes	ND	ND	ND
Benzo(b)fluoranthene	ND	ND	<MDL
Benzo(k)fluoranthene	ND	ND	<MDL
Benzo(e)pyrene	ND	ND	ND
Benzo(a)pyrene	ND	515.4	10,730
Perylene	ND	ND	ND
Indeno(1,2,3-c,d)pyrene	ND	ND	ND
Dibenzo(a,h)anthracene	ND	ND	ND
Benzo(g,h,i)perylene	ND	ND	ND
2-Methylnaphthalene	<MDL	<MDL	<MDL
1-Methylnaphthalene	<MDL	<MDL	<MDL
2,6-Dimethylnaphthalene	<MDL	<MDL	<MDL
1,6,7-Trimethylnaphthalene	ND	ND	ND
1-Methylphenanthrene	ND	ND	ND

In several naphthalene and pyrene treatments, experimental oysters were in poor health and hemolymph could not be extracted from the adductor muscle.

3.2.1.1. Experiment I. Overall, hemocyte percentages were not significantly ($P > 0.05$) altered by experimental concentrations used in this preliminary exposure study. Although not significant, trends in granular hemocyte counts were observed at several concentrations used in this study (Fig. 1). Granular hemocyte percentages appeared to increase slightly in oysters exposed to naphthalene at concentrations of 125 and 625 μg L^{-1}. A decrease in granular hemocyte percentages was observed in pyrene-exposed oysters at higher concentrations. The type of PAH compound used in the experiment was more important in altering granular hemocyte percentages than experimental exposure concentrations. There was a highly significant ($P \leq 0.05$) reduction in granular hemocyte percentages in oysters exposed to benzo(a)pyrene when compared to naphthalene and pyrene-exposed oysters, regardless of concentration (Fig. 1).

Similar to granular hemocytes, agranular hemocyte percentages were not significantly affected by PAH concentration. Although not significant, a decrease in agranular percentages was observed

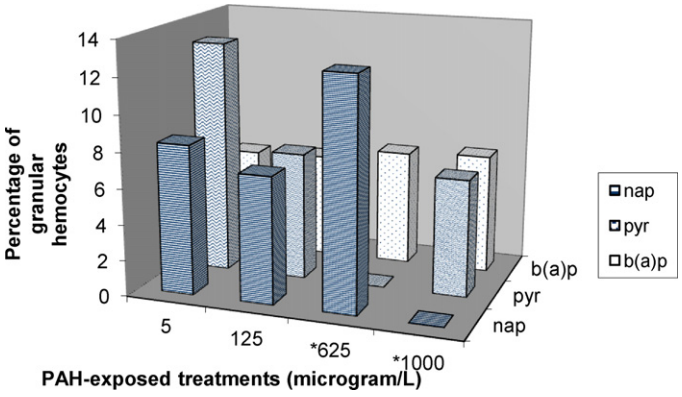

Fig. 1. Percentage of granular hemocytes in PAH-exposed treatments. Granular hemocyte counts were significantly reduced in oysters exposed to benzo(a)pyrene. *Note: an insufficient quantity of hemolymph was extracted in naphthalene 1000 μg L^{-1} and pyrene 625 μg L^{-1} treatments to complete all assays.

Table 2
Summary of PAH concentrations (ng g^{-1} wet weight.) in gill and mantle tissues of experimental oysters after feeding on benzo(a)pyrene-contaminated microalgae. Standard error of mean concentration values is listed in parentheses. ND = not detected. <MDL = below method detection limit.

PAH compound	Gill/mantle control	Gill/mantle 100 μg L^{-1}	Gill/mantle 1000 μg L^{-1}
Naphthalene	45.7 (2.7)	28.9 (0.1)	15.8 (8.0)
C1-naphthalenes	<MDL	<MDL	<MDL
C2-naphthalenes	39.9 (25.5)	25.4 (9.3)	30.0 (10.7)
C3-naphthalenes	ND	75.0 (0.1)	73.0 (8.6)
C4-naphthalenes	ND	ND	104.4 (10.8)
Biphenyl	33.5 (2.0)	10.8 (5.1)	11.6 (5.3)
Acenaphthylene	ND	ND	ND
Acenaphthene	ND	ND	ND
Fluorene	<MDL	<MDL	<MDL
C1-fluorenes	110.1 (21.3)	58.9 (25.5)	58.8 (23.1)
C2-fluorenes	169.4 (23.7)	142.0 (6.6)	70.3 (23.6)
C3-fluorenes	581.3 (94.7)	283.0 (132.6)	226.2 (70.4)
Phenanthrene	31.2 (6.1)	<MDL	13.0 (6.5)
Anthracene	<MDL	ND	ND
C1-phenanthrenes/anthracenes	50.5 (16.0)	16.7 (0.6)	12.6 (6.4)
C2-phenanthrenes/anthracenes	39.2 (7.4)	25.3 (5.7)	19.5 (8.6)
C3-phenanthrenes/anthracenes	110.2 (18.4)	32.2 (0.1)	12.7 (4.2)
C4-phenanthrenes/anthracenes	144.6 (27.4)	36.0 (1.7)	21.8 (8.9)
Dibenzothiophene	ND	ND	ND
C1-dibenziothiophenes	28.7 (2.8)	17.7 (2.2)	13.8 (4.9)
C2-dibenziothiophenes	49.7 (5.8)	24.4 (0.7)	12.7 (5.7)
C3-dibenziothiophenes	37.8 (4.5)	35.9 (0.1)	21.1 (0.1)
Fluoranthene	21.8 (0.1)	<MDL	<MDL
Pyrene	28.4 (0.1)	<MDL	<MDL
C1-fluoranthenes/pyrenes	85.4 (5.5)	<MDL	<MDL
C2-fluoranthenes/pyrenes	ND	ND	ND
C3-fluoranthenes/pyrenes	ND	ND	ND
Benzo(a)anthracene	ND	ND	ND
Chrysene	18.7 (0.2)	<MDL	2.9 (0.1)
C1-chrysenes	37.6 (0.1)	<MDL	<MDL
C2-chrysenes	56.2 (6.0)	65.4 (1.2)	53.0 (16.8)
C3-chrysenes	ND	ND	ND
C4-chrysenes	ND	ND	ND
Benzo(b)fluoranthene	ND	ND	ND
Benzo(k)fluoranthene	ND	ND	ND
Benzo(e)pyrene	ND	ND	ND
Benzo(a)pyrene	<MDL	<MDL	68.9 (25.6)
Perylene	ND	ND	ND
Indeno(1,23-c,d)pyrene	ND	ND	ND
Dibenzo(a,h)anthracene	ND	ND	ND
Benzo(g,h,i)perylene	ND	ND	ND
2-Methylnaphthalene	25.5 (0.1)	16.3 (0.1)	<MDL
1-Methylnaphthalene	<MDL	<MDL	<MDL
2,6-Dimethylnaphthalene	ND	ND	ND
1,6,7-Trimethylnaphthalene	ND	ND	ND
1-Methylphenanthrene	<MDL	ND	ND

between concentrations 125 and 625 μg L^{-1} in oysters exposed to naphthalene; and an increase in agranular hemocyte percentages in oysters exposed to pyrene at concentrations greater than 5 μg L^{-1} was also observed (Fig. 2). A significant increase ($P \leq 0.05$) in the percentage of agranular hemocytes was observed in pyrene and benzo(a)pyrene treatments, compared to naphthalene-exposed oysters.

3.2.2. Hemocyte mortality

3.2.2.1. Experiment I. A significant increase ($P \leq 0.05$) in the percentage of dead, granular hemocytes was observed in oysters exposed to PAH concentrations between 5 μg L^{-1} and 1000 μg L^{-1}, in addition to 125 μg L^{-1} and 1000 μg L^{-1} (Fig. 3). A significant ($P \leq 0.05$) increase in dead granular hemocytes was identified between naphthalene and pyrene treatments, and naphthalene

Table 3
Immune status of oysters exposed to b(a)p concentrations in Experiment II. Standard error of mean values is listed in parentheses. Asterisks denote statistical significance ($P \leq 0.05$).

	Control treatment	Acetone treatment	B(a)p 100 μg L^{-1} treatment	B(a)p 1000 μg L^{-1} treatment
Hemocyte assays				
Hemocyte counts	8397.33 (2437.95)	13,869.11 (9698.26)	8350.67 (5272.61)	9483.06 (2990.48)
Percent dead hemocytes				
Granular	6.20 (0.96)	7.19 (2.72)	0.47* (0.42)	0.17* (0.10)
Agranular	8.49 (2.49)	9.80 (3.13)	16.71 (9.13)	17.25 (10.52)
Percent phagocytic hemocytes	8.24 (0.84)	7.74 (0.61)	9.88 (0.52)	10.11 (2.47)
Hemocyte production of reactive oxygen species (cytometer FL1 units)				
Granular	39.21 (9.08)	65.73 (25.11)	57.66 (39.37)	100.85 (9.65)
Agranular	16.68 (4.04)	21.83 (2.98)	24.27 (8.61)	37.58 (5.63)
Percent adhering hemocytes	99 (0.97)	99 (0.97)	99 (0.97)	99 (0.98)

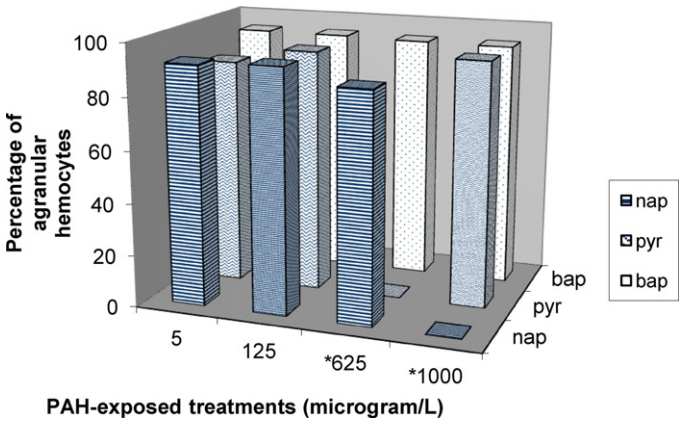

Fig. 2. Percentage of agranular hemocytes in PAH-exposed treatments. A significant increase in agranular hemocyte percentages was observed in pyrene and benzo(a)pyrene-exposed oysters. *Note: an insufficient quantity of hemolymph was extracted in naphthalene 1000 $\mu g\,L^{-1}$ and pyrene 625 $\mu g\,L^{-1}$ treatments to complete all assays.

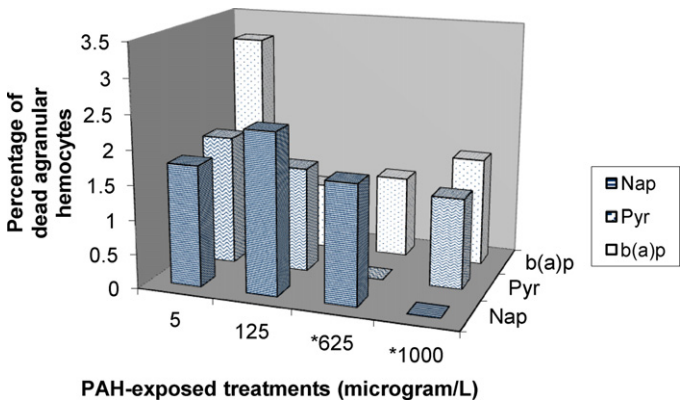

Fig. 4. Percentage of dead agranular hemocytes in PAH-exposed treatments Mean values of dead agranular hemocytes exposed to 5 $\mu g\,L^{-1}$ were significantly higher than remaining treatment concentrations. *Note: an insufficient quantity of hemolymph was extracted in naphthalene 1000 $\mu g\,L^{-1}$ and pyrene 625 $\mu g\,L^{-1}$ treatments to complete all assays.

and benzo(a)pyrene exposure treatments, regardless of concentration. Oysters exposed to increasing benzo(a)pyrene concentrations exhibited an apparent slight, but statistically insignificant increase in the percentage of dead granular hemocytes (Fig. 3).

Also noted in this study were significantly ($P \leq 0.05$) reduced percentages of dead agranular hemocytes measured in oysters exposed to concentrations between 5 and 1000 $\mu g\,L^{-1}$ (Fig. 4). Mean values of 5 $\mu g\,L^{-1}$ treatments had significantly ($P \leq 0.05$) more dead agranular hemocytes than all other treatment concentrations. Nevertheless, there were no significant effects on agranular hemocyte mortality by individual PAH compounds.

3.2.3. Phagocytosis of fluorescent microbeads

3.2.3.1. Experiment I. Hemocyte phagocytosis of the fluoresbrite beads was not significantly ($P > 0.05$) affected by PAH compounds or concentrations tested. Although not significant, a trend of increasing numbers of highly phagocytic hemocytes was observed in naphthalene treatments as concentration increased (Fig. 5). Pyrene-exposed oysters demonstrated a statistically unresolved increase in phagocytic activity between concentrations of 5 and 125 $\mu g\,L^{-1}$, but at the highest concentration, this activity was lowered. The most-toxic PAH, benzo(a)pyrene, caused a decrease in hemocyte phagocytic activity between concentrations of 5 $\mu g\,L^{-1}$ and 125 $\mu g\,L^{-1}$, but an increase in hemocyte phagocytosis was also observed at the highest concentration.

3.2.4. Characterization of hemocytes

3.2.4.1. Experiment II. Hemocyte variables, including total hemocyte counts, individual hemocyte-type counts, hemocyte mortality, ROS, and phagocytosis, were entered into a correlation matrix to determine associations among the variables tested. Principle components analysis was then used as a tool to visually identify positive and negative relationships between the hemocyte variables tested in this experiment. These PCA profiles then were subjected to ANOVA to determine possible effects of b(a)p at the two concentrations tested. Results from ANOVA of principle component analysis suggest that there are no statistically significant differences ($P > 0.05$) between the various contaminant treatments. The large amount of variability associated with the benzo(a)pyrene 100 $\mu g\,L^{-1}$ treatment may have masked the significance of contrasts between the treatments; therefore, the decision was made to remove this concentration from the analysis to contrast control and 1000 $\mu g\,L^{-1}$ treatments statistically.

Although not significant, an increase in granular hemocyte counts between control and benzo(a)pyrene 1000 $\mu g\,L^{-1}$ treatments was observed (Table 3). A biologically meaningful increase was also observed in total hemocyte counts in the acetone treatment when compared to control and benzo(a)pyrene treatments. The large variance in the acetone treatment indicates that there was a varied response between oysters to this solvent.

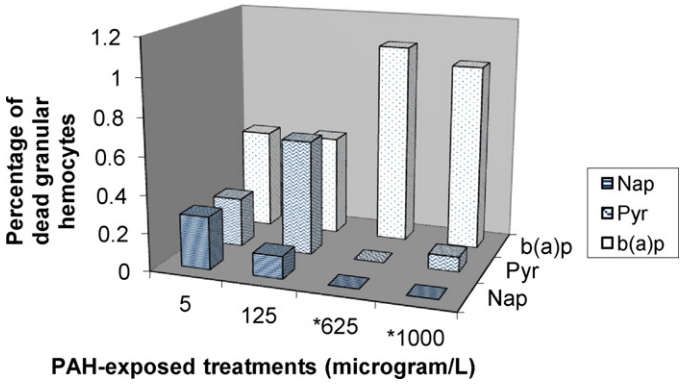

Fig. 3. Percentage of dead granular hemocytes in PAH-exposed treatments. Percentages of dead granular hemocytes increased as experimental concentrations increased. *Note: an insufficient quantity of hemolymph was extracted in naphthalene 1000 $\mu g\,L^{-1}$ and pyrene 625 $\mu g\,L^{-1}$ treatments to complete all assays.

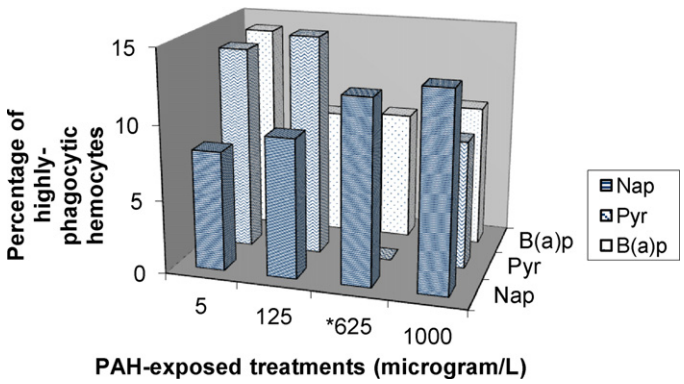

Fig. 5. Percentage of highly phagocytic hemocytes in PAH-exposed treatments. Although not significant, phagocytosis activity in naphthalene treatments appeared to increase as experimental concentrations increased. Asterisks denote concentrations in which oyster muscles were too watery to extract hemolymph.

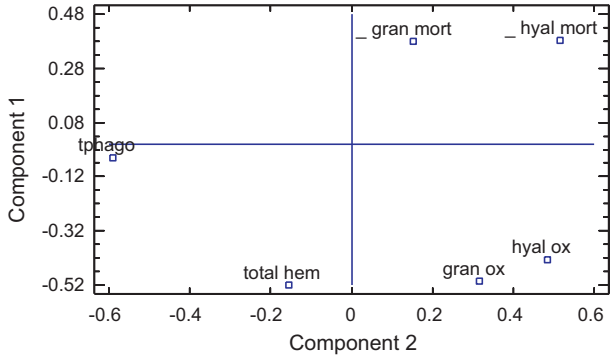

Fig. 6. Principle components analysis of benzo(a)pyrene-exposed oyster hemocyte variables. Positive and negative correlations were identified between hemocyte immune variables. _gran mort = granular hemocyte mortality; _hyal mort = agranular hemocyte mortality; tot hem = hemocyte count; gran ox = granular hemocyte ROS; hyal ox = agranular hemocyte ROS; phago = hemocyte phagocytosis.

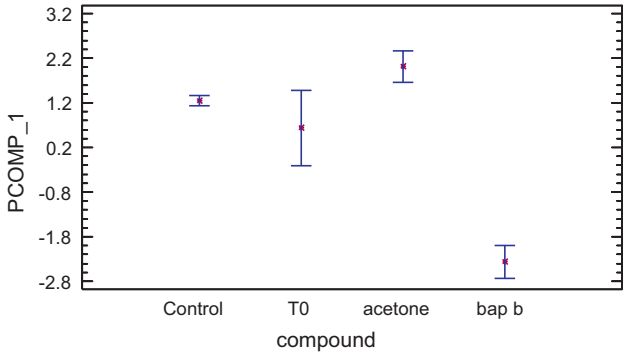

Fig. 7. Analysis of variance on principle component analysis oyster profile. Oysters exposed to benzo(a)pyrene at 1000 μg L^{-1} had higher oxidative responses, more hemocytes, and few dead hemocytes.

3.2.5. Percentage of dead hemocytes

3.2.5.1. Experiment II. There was a significant decrease ($P \leq 0.05$) in the percentages of dead granular hemocytes in oysters exposed to benzo(a)pyrene-contaminated microalgae; percentages were 0.02 and 0.05 for benzo(a)pyrene 100 and benzo(a)pyrene 1000 treatments, respectively (Table 3). Statistically, there were no differences between the experimental treatments in agranular hemocyte mortality. A biologically relevant increasing trend in percentages of dead agranular hemocyte was observed in the benzo(a)pyrene-exposed oysters (Table 3).

3.2.6. Phagocytosis of fluorescent microbeads

3.2.6.1. Experiment II. Phagocytosis of fluorescent microbeads by hemocytes was not significantly ($P > 0.05$) altered by any of the experimental treatments (Table 3). There was a slight, but insignificant increase in the percentage of hemocytes incorporating beads in the benzo(a) pyrene treatment.

3.2.7. Unstimulated reactive oxygen species

3.2.7.1. Experiment II. Granular hemocyte reactive oxygen species production was not significantly ($P > 0.05$) altered by the experimental treatments (Table 3).

ROS activity in agranular hemocytes was not significantly affected by experimental treatments, but an increasing trend in mean values with benzo(a)pyrene concentration was observed (Table 3). Mean values for agranular hemocyte ROS were almost doubled in the highest concentration of benzo(a)pyrene as compared to control values.

3.2.8. Hemocyte aggregation

3.2.8.1. Experiment II. The experimental treatments used in this study did not have an observable effect upon adhesion of hemocytes; adhesion was essentially 100% in all cases (Table 3).

3.2.9. ANOVA and principle components analysis

Results from the PCA indicated that oysters with a higher percentage of hemocyte mortality had reduced ROS production and fewer agranular hemocytes (Fig. 6). In addition, positive correlations were identified between granular and agranular hemocyte mortality percentages; while negative correlations were observed between granular and agranular hemocyte counts, oxidative burst response, and phagocytosis. In case there were non-linear (*e.g.*, threshold) associations between the variables used in PCA, a non-metric multidimensional scaling (MDS) analysis, which does not depend upon linear relationships, was run. Essentially identical biplots were obtained by this method, confirming that PCA was appropriate for this data set. To determine if the combination of associated hemocyte characteristics was related to compound or concentration, ANOVA was run on PCA Component 1.

ANOVA on PCA profiles indicated that there was a statistically significant difference ($P \leq 0.05$) between the benzo(a)pyrene 1000 μg L^{-1} treatment and the remaining experimental treatments (Fig. 7). ANOVA on the PCA profile indicated that oysters exposed to benzo(a)pyrene had higher ROS production, more hemocytes, and fewer dead hemocytes.

4. Discussion

The present study provided new evidence supporting the expectation that PAH-contaminated, microalgal food can transfer these pollutants to oysters, resulting in physiological impacts, specifically on hemocytes and their immune functions. As PAH compounds are ubiquitous, improved understanding of the routes of transport and physiological effects upon marine organisms will improve environmental management efforts related to seafood production in impacted ecosystems. Several previous studies have examined the effects of persistent organic pollutants upon the immune systems of bivalves (Riley et al., 1981; Grundy et al., 1996a,b; Gomez-Mendikute et al., 2002; Lyons et al., 2002; Boutet et al., 2004), but the importance of microphytobenthic communities as possible contaminant-transfer vectors has received little research attention. This study revealed that hydrophobic PAHs do become associated with benthic diatoms, that PAHs can be transferred to oysters from diatoms during feeding, and that this trophic transfer of pollutants can alter oyster physiology, specifically hemocyte characteristics and immune functions.

Results from the algal-adsorption experiment supported the hypothesis that organic compounds are attracted to lipid membranes of algal cells. Corner et al. (1976), Harding and Phillips (1978), and Lynn et al. (2007) also observed accumulation of organic contaminants by algal species. Corner et al. (1976) exposed diatom cells to 300 μg L^{-1} of radioactively labeled naphthalene and found concentrations of 89.8 ng L^{-1} in cells after three days of exposure.

Oyster gill and mantle tissue (Table 2) contained low concentrations of the transferred contaminants. These low concentrations of benzo(a)pyrene could be explained by the choice of tissues selected for organic analysis, accumulation and retention by the oyster, or the experimental set-up. Oyster gill and mantle tissues were selected for PAH analysis in this experiment because previous studies have shown that these tissues have lipid profiles similar to hemocytes (Delaporte et al., 2003). Because gills are the first point of contact for dissolved contaminant exposure, it is hypothesized that initial gill concentrations would be greatest when adsorption is from the dissolved phase, followed by a decline as the pollutant is transferred to other tissue compartments (Lee et al., 1976; Riley

et al., 1981). Therefore, it is possible that benzo(a)pyrene concentrations used in this study had been transferred to other body parts before the gill and mantle tissues were excised, such as the digestive gland, where food-borne compounds would be released.

The gills of the eastern oyster are responsible for capturing and sorting particles from the water column. Captured particles are transported by cilia, located on the gills, to the mouth for ingestion or to the mantle for rejection as pseudofeces. Feces from experimental oysters were analyzed microscopically, and the presence of partially digested cells was observed. These observations provided confidence that the oysters were filtering, ingesting, and digesting the contaminated diatom. Likewise, the flow-through design of the experimental set-up had the potential to impact the amount of algal cells accumulated by the oysters. Despite low concentrations in oyster gill and mantle tissues, hemocytes and their immune defense functions in the experimental treatments were altered as a result of exposure to the contaminated algae. In the first experiment, PAHs thought to be less toxic were less bioactive and in both Experiments I and II, only high benzo(a)pyrene concentrations were very bioactive. Concentrations used in the initial experiment did not cause any significant effects on the percentage of hemocytes; however, the type of compound (benzo(a)pyrene) did cause a significant decrease in both granular and agranular hemocytes when compared to naphthalene and pyrene treatments. Wooten et al. (2003) also observed a lack of significant changes in total hemocyte counts of bivalves exposed to phenanthrene. In the second experiment, granular and agranular hemocyte populations were significantly altered by the highest experimental benzo(a)pyrene exposure concentration. A decrease in granular hemocyte mortality was observed at the 1000 $\mu g\,L^{-1}$ concentration. In contrast, no change in agranular cell mortality was observed at this concentration. These results are similar to an *in vitro* benzo(a)pyrene exposure experiment which also showed a decrease in cell viability in mussel hemocytes at a high concentration of 40 $\mu g\,mL^{-1}$ (Gomez-Mendikute et al., 2002).

Adhesion, the first step in phagocytosis, as well as a response to tissue damage (Cheng, 1996), was not altered by experimental concentrations or compounds. Phagocytosis of microbeads by hemocytes was not significantly modified, although naphthalene appeared to increase in phagocytic activity as concentrations increased during the initial experiment. Both pyrene and benzo(a)pyrene treatments seemed to induce phagocytosis slightly at the lowest concentration (5 $\mu g\,L^{-1}$), but as concentrations increased, a reduction in phagocytic activity was observed. A laboratory study conducted by Grundy et al. (1996b) showed a significant reduction in hemocyte phagocytosis in mussels exposed to a PAH cocktail at a concentration of 500 $\mu g\,L^{-1}$ for two weeks. An additional study described in Grundy et al. (1996a) examined the *in vitro* and *in vivo* effects of individual and combined PAH compounds in marine mussels. Phagocytic activity of hemocytes in the *in vitro* experiment was significantly inhibited by fluoranthene and anthracene at 50 ppm.

The degradation and killing of foreign particles by hemocytes is an important step in the defense of the eastern oyster. Following adherence and endocytosis of the foreign material, reactive oxygen species are released for degradation. Reactive oxygen species production in neither granular nor agranular hemocyte populations was significantly impacted by the experimental conditions; however, an increasing trend was observed in the second experiment (Table 3). Likewise, the generation of reactive oxygen species was stimulated in mussel hemocytes when exposed to concentrations of PAHs (Coles et al., 1994).

Initial ANOVA on PCA analysis showed that the variance between oysters in the b(a)p 100 $\mu g\,L^{-1}$ (Treatment A) was so great that the statistical significance of differences between the other treatments were masked. When Treatment A was removed from the analysis (Fig. 7) results showed that b(a)p at the 1000 $\mu g\,L^{-1}$ concentration stimulated circulating hemocytes and reactive oxygen species production, while possibly inhibiting phagocytic activity.

Variance between individual organisms is commonly observed in molluscan hemocyte research. Environmental and internal factors including temperature, food availability, food quality, salinity, and reproductive status are all variables thought to have effects upon the variability of hemocyte responses. Several groups of investigators (Ashton-Alcox and Ford, 1998; Ford and Paillard, 2009; Flye-Sainte-Marie et al., 2009) have attempted to explain such variance in hemocyte populations. Ashton-Alcox and Ford (1998) used both microscopy and flow-cytometric analyses to determine the variance among hemocyte populations in eastern oysters and found that temperature and food availability explained only a small amount of their variability, and that most of the variation measured was attributable to individual differences and not a specific environmental or internal variable. The authors also noted that the amount of variance in hemocytes among individual oysters was similar to measurements described in vertebrates (Ashton-Alcox and Ford, 1998). Similarly, Flye-Sainte-Marie et al. (2009) also concluded that, although some variation in hemocyte population responses could be attributed to environmental and internal factors, these contributions did not constitute a large amount in a clam species sampled for several months.

Overall, the results of this study indicate that dietary accumulation of PAH compounds by bivalves is a necessary route of trophic transfer to consider when studying the effects of these compounds on the health of bivalve species. Although ANOVA on individual immune defense assays did not consistently reflect significant perturbations, the use of a multi-variate PCA profile provided a more comprehensive analysis of the overall effects of PAH compounds upon immune defense functions of this bivalve species. This study the confirmed the transfer of PAH compounds from a microphytobenthic diatom to the eastern oyster, and demonstrated subsequent effects of these compounds upon the immune defense functions of hemocytes.

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