Marine Pollution Bulletin xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

The use of flow cytometric applications to measure the effects of PAHs on growth, membrane integrity, and relative lipid content of the benthic diatom, *Nitzschia brevirostris*

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Oil pollution
Fluorescent probes
Membrane permeability
Microphytobenthos
Algal cellular physiology
Rapid method

ABSTRACT

This laboratory study measured the direct effects of three polycyclic aromatic hydrocarbon (PAH) compounds (naphthalene, pyrene, and benzo(a)pyrene) upon cell growth, membrane integrity, and BODIPY-stained lipid fluorescence intensity of the benthic diatom *Nitzschia brevirostris* using flow cytometry as an analysis tool. Previous field and laboratory studies have reported reductions in algal populations following PAH exposure, but specific, functional responses of the microalgae to these pollutants could not be revealed by cell numbers alone. Using flow-cytometric measurements, we confirmed that maximal cell densities in PAH-exposed diatom cultures were significantly lower compared to controls; however, we also discovered increases in lipids and cells with compromised membranes in PAH-exposed cultures. These results highlight new tools for measuring the direct effects of organic pollutants upon the physiology of taxa comprising microphytobenthic communities important in estuarine food webs.

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1. Introduction

Originally used in clinical applications for immunology, hematology, and oncology, flow-cytometry has been used by researchers for over three decades as a technique to analyze phytoplankton (Berglund and Eversman, 1988; Olson et al., 1993; Mandy et al., 1995; Franqueira et al., 2000; Collier, 2004; Yentsch and Yentsch, 2008). Flow-cytometry provides rapid measurements of optical properties of single cells, allowing researchers to quantify phytoplankton populations and estimate growth rates and comparative chlorophyll *a* concentrations (Collier, 2000, 2004; Yentsch and Yentsch, 2008), as well as differentiate phytoplankton groups based upon accessory pigment fluorescence (Hofstraat et al., 1994).

Microalgae, both planktonic and benthic, are a major component of aquatic ecosystems and are responsible for primary production and cycling nutrients throughout these environments (Christensen and Nyholm, 1984; Skoglund and Swackhamer, 1994; Lewis, 1995). Microphytobenthic communities are composed of a group of photosynthetic, eukaryotic algae and cyanobacteria that inhabit surface sediments (MacIntyre et al., 1996; Aberle-Malzahn, 2004). The microphytobenthic community is rarely acknowledged fully for its role in primary production in areas where phytoplankton abundance is low (Underwood and Kromkamp, 1999). These organisms also serve as sediment stabilizers, promoters of the transfer of nutrients between sediments and the water column, and food sources for deposit and suspension feeders, including bivalve species (MacIntyre et al., 1996; Aberle-Malzahn, 2004). The close proximity of microphytobenthic communities and sediments exposes this algal community to hydrophobic pollutants that may alter their physiological processes, thereby modifying their critical roles as primary producers and sediment stabilizers in the ecosystem. An example of a large event during which benthic algal communities were exposed to organic pollutants occurred in April 2012 when the Deepwater Horizon oil spill released several million barrels of oil into the productive waters of the Gulf of Mexico for nearly three months (Soniat et al., 2011).

Polycyclic aromatic hydrocarbons (PAHs) are a group of hydrophobic organic compounds commonly found in aquatic ecosystems. These compounds are formed during industrial processes and can persist in the environment over a long period of time. Petroleum spills and improper disposal of used oil products are two sources of acute PAH contamination in aquatic environments (Holland et al., 2006). In aquatic ecosystems, hydrophobic

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http://dx.doi.org/10.1016/j.marpolbul.2014.12.010
0025-326X/Published by Elsevier Ltd.

compounds are attracted to suspended particulate matter and lipid-rich biological membranes (Rubinstein et al., 1984). Particles with sorbed, hydrophobic substances settle to the seafloor where they are deposited onto the sediment surface. Aquatic surface sediments act as contaminant reservoirs for many environmental pollutants, including PAHs (Elroy et al., 2000). Microalgae associated with sediments are exposed to these organic pollutants and can potentially serve as vectors in the trophic transfer of these compounds as they are resuspended and filtered by bivalve species (Croxton et al., 2012).

Extensive research has been conducted on the physiology of marine organisms exposed to hydrophobic pollutants (Anderson et al., 1981; Grundy et al., 1996; Fisher et al., 2000; Chu et al., 2002), but little data exist concerning the toxic effects of hydrophobic pollutants upon microphytobenthic communities located in these marine environments (Hook and Osborn, 2012). Several authors have studied the effects of specific PAH compounds and petroleum products on growth rates of both planktonic and benthic algae (Winters et al., 1976; Mahoney and Haskin, 1980; Nyholm, 1990; Moreno-Garrido et al., 2007). Findings from these studies indicate that hydrocarbon exposure can inhibit algal growth, but the degree of this inhibition varies between algal species. In contrast, Dunstan et al. (1975) observed stimulation of growth of phytoplankton species exposed to low-molecular-weight hydrocarbons. Dunstan and colleagues suggested that the volatile fraction of low-molecular-weight hydrocarbons included the most biologically-active hydrocarbons, and this characteristic could explain the varied responses among algal species in various studies. The PAH compounds chosen for this study represent increasing molecular weights, with naphthalene having the lowest and benzo(a)pyrene having the highest molecular weight (Neff, 1979; Varanasi, 1989).

Previous studies of microphytobenthic communities and petroleum products tend to highlight the effects of these compounds upon abundance, community structure, and grazing (Bennett et al., 1999, 2000; Piehler et al., 2003; Suderman and Thistle, 2004), but not physiology. These studies focused on trophic interactions between grazers and benthic communities located in polluted environments. Although these interactions are critical in understanding the overall effects of PAHs in an ecosystem, it is equally important to understand the direct effects of PAHs upon microphytobenthic algae. Chung et al. (2007) investigated the responses of the benthic microalga *Chlorococcum meneghini* to PAH-spiked sand and found that this exposure diminished cell densities and chlorophyll *a* concentrations. Similar studies also examined chlorophyll *a* concentrations and photosynthetic rates of PAH-exposed benthic algae (Bennett et al., 1999; Piehler et al., 2003).

Earlier studies conducted by plant physiologists suggest that PAH compounds target plasma membranes and change permeability (Currier, 1951; Dallyn and Sweet, 1951; Currier and Peoples, 1954; Dunstan et al., 1975). Interactions between petroleum products, freshwater algae, and bacteria also have been investigated by several authors (Kauss and Hutchinson, 1977; Sikkema et al., 1994; McCann and Solomon, 2000; Hook and Osborn, 2012). Findings from these studies suggest that hydrocarbons are adsorbed onto biological membranes where they target lipid-containing structures (Kauss and Hutchinson, 1977; Weimburg et al., 1981; McCann and Solomon, 2000). The disruption of these structures alters lipid arrangement in membranes, causing the formation of pores and increasing the permeability of cells (McCann and Solomon, 2000; Chung et al., 2007).

The aim of this study was to explore new methods for determining physiological responses of a representative microbenthic diatom to purified PAH compounds – to use flow-cytometric applications coupled with fluorescent probes to measure cell growth, membrane integrity, and lipid storage of a pure, microphytobenthic diatom culture exposed to naphthalene, pyrene, and benzo(a)pyrene (b(a)p) at experimentally-varied concentrations.

2. Materials and methods

2.1. Microphytobenthic diatom culture

The benthic diatom, *Nitzschia brevirostris* (strain 0–1), obtained from the Milford Laboratory Microalgal Culture Collection, was used as the test alga to determine the effects of PAH compounds on growth, membrane integrity, and lipid storage.

2.2. Test compounds

The PAH compounds naphthalene, pyrene, and benzo(a)pyrene were the environmental pollutants used to investigate possible effects upon the diatom. Naphthalene (99+%) and pyrene (98%) were purchased from Fisher Scientific; benzo(a)pyrene (97%) was purchased from Sigma. Reagent-grade acetone (Fisher Scientific) was used as the solvent carrier into which the water-insoluble compounds were dissolved before addition to the aqueous diatom culture. Acetone-dissolved PAHs were pipetted volumetrically into microalgal-culture media to achieve final concentrations of 10, 100, and 1000 $\mu g\ L^{-1}$ for each PAH compound. The PAH concentrations selected represent a range of toxicity (e.g. low to high concentrations) found in natural settings (Fisher et al., 2000).

2.3. Experimental conditions

N. brevirostris was transferred aseptically into 250-mL flasks containing Enriched Seawater Medium with Silicate (ESi; Ukeles, 1973) to achieve an initial cell density of 10^4 cells mL^{-1} in each flask. Control culture flasks (not exposed to PAH compounds), and acetone culture flasks (contained 1% of the solvent carrier) were prepared in addition to the PAH concentrations described above. All treatments were carried out in triplicate. Experimental flask cultures were incubated at 18 ± 1 °C with a light intensity of 300 $\mu E\ m^{-2}\ s^{-1}$ PAR from cool-white fluorescent bulbs on a 12:12 h. light:dark cycle. Flask cultures were allowed to grow for 21 days and sub-sampled on days 0, 2, 5, 7, 9, 12, 14, 16, 19, and 21.

2.3.1. Fluorescent dyes

Viability of diatom cells was measured using the fluorescent probe SYTOX Green (30 μM initial concentration, Invitrogen; Carlsbad, CA), which stains the nucleic acids of cells with compromised membranes. SYTOX-positive cells generally are considered to be dead. SYTOX emits fluorescence in the flow-cytometer FL1 channel (530 nm wavelength) wherein natural chlorophyll fluorescence in the 650-nm range does not interfere. Lipid storage in diatom cells was measured using the fluorescent stain BODIPY 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene) (10 mg initial concentration, Invitrogen; Carlsbad, CA). BODIPY is a lipophilic fluorophore that emits fluorescence in the FL1 cytometer channel (530 nm wavelength). Without calibration using an independent method (e.g., chromatography), BODIPY fluorescence is recorded in arbitrary fluorescence values that allow contrasts between experimental treatments to be determined, but not quantitative measurements of lipid per cell.

2.3.2. Experimental procedures

A 600-μL sample was taken from each triplicate culture flask immediately following the addition of PAH compounds for initial sampling. Algal cells and fluorescent probes were distributed into 5-mL polycarbonate tubes. A 200-μL sub-sample of cells and

Please cite this article in press as: Croxton, A.N., et al. The use of flow cytometric applications to measure the effects of PAHs on growth, membrane integrity, and relative lipid content of the benthic diatom, *Nitzschia brevirostris*. Mar. Pollut. Bull. (2014), http://dx.doi.org/10.1016/j.marpolbul.2014.12.010