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Flow cytometry detection of planktonic cells with polycyclic aromatic hydrocarbons sorbed to cell surfaces

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ABSTRACT

Polycyclic aromatic hydrocarbons are very important components of oil pollution. These pollutants tend to sorb to cell surfaces, exerting toxic effects on organisms. Our study developed a flow cytometric method for the detection of PAHs sorbed to phytoplankton by exploiting their spectral characteristics. We discriminated between cells with PAHs from cells free of PAHs. Clear discrimination was observed with flow cytometer provided with 375 or 405 nm lasers in addition to the standard 488 nm laser necessary to identify phytoplankton. Using this method, we measured the relationship between the percentages of phytoplankton organisms with PAHs, with the decrease in the growth rate. Moreover, the development of this method could be extended to facilitate the study of PAHs impact on cell cultures from a large variety of organisms.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a subset of persistent organic pollutants in crude oil, refining oil and coal (McGrath et al., 2007). Oil spills, ship traffic and atmospheric transport are the major sources of PAHs pollution to the oceans (Neff, 1985). PAHs are toxic for organisms and show a strong hydrophobic sorption affinity for particulate surfaces (Gelboin, 1980; Lehr and Jerina, 1977; Meador et al., 1995). PAHs have low water solubility and tend to accumulate in planktonic organisms when deposited in oceans and lakes (Dachs et al., 2002; Fordham et al., 1985). When are sorbed and accumulated on the cell surface of phytoplanktonic organisms, PAHs enter slowly into the cells by a diffusive process (Fan and Reinfelder, 2003). The intracellular concentration of pollutant increases with time while the concentration in the cell membrane decreases (Fan and Reinfelder, 2003). Once sorbed to cells, PAHs interfere with plasma membranes (Neff, 1979), being able to seriously damage the DNA (Gelboin, 1980), and alter cell processes such as growth or photosynthesis (Singh and Gaur, 1988). These pollutants emit fluorescence when are excited by ultraviolet (UV) light (Dartnell et al., 2012; Suzuki et al., 2009). Pyrene and phenanthrene have peak excitation wavelengths between 355 and 405 nm, and emission wavelengths from 400 to 500 nm (Fig. S1). This property has been used to detect PAHs in environmental samples by different techniques, such as high-performance liquid chromatography (HPLC) and fluorescence spectrometry. This method allows the determination of PAHs concentrations in waters samples with good sensitivity, which detection limit is between 0.34 and 14.11 ng mL^{-1} (Habibi and Hadjmohammadi, 2008).

Due to their fluorescent properties, we hypothesized that PAHs could be detected when are sorbed to particles and cell surfaces using a microscope or in a flow cytometer with a UV laser source. Epifluorescence microscopy, with UV illumination, was recently used to visualize crude oil droplets inside the digestive tract of different marine organisms such as copepods (Almeda et al., 2014a), heterotrophic dinoflagellates (Almeda et al., 2014b), *Barnacle nauplii* and *Tornaria larvae* (Almeda et al., 2014c). Also, the same technique was successful to observe the presence of crude oil in the fecal pellets of copepods (Almeda et al., 2014a). Fluorescent microscopy and flow cytometry techniques, are now broadly used in aquatic sciences to identify and quantify the cell abundance of phytoplankton and bacteria. Both techniques provide a single-cell analysis, but fluorescence microscopy is well suited to the resolution of morphological analysis (Muratori et al., 2008), and to follow kinetic and trophic responses in single cells (Godfrey et al., 2005). Flow cytometry allows fast automated cell counting as well as simultaneous multiparametric analysis of different cellular properties such as cell size and pigments auto-fluorescence (Marie et al., 2000), cell viability (Agustí and Sánchez, 2002), DNA content and cell cycle (Marie et al., 1997), and enzymatic and

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immunological responses, among a large variety of cellular properties after the appropriate staining of cells (Shapiro, 2005). Moreover, the flow cytometers allow the identification the tiny *Prochlorococcus* sp. populations, the most abundant photosynthetic organism in the open ocean (Chisholm et al., 1998), which cell fluorescence is too dim to be discriminated by epifluorescence microcopy. Flow cytometry techniques are used for a variety of studies, including toxicological tests (Czechowska and Van der Meer, 2011), and the analysis of the pollutants effects in aquatic environments (Echeveste et al., 2010, 2011; Hjorth et al., 2007). New generation multi-laser flow cytometers include a variety of excitation wavelengths, including those in the blue, violet and ultraviolet band, and a variety of wavelengths for emission filters increasing the detection of different cellular properties by multiplying the simultaneous fluorescent signals. Multi-laser flow cytometers are therefore a useful tool to discriminate planktonic cells from other particles. Phytoplankton has been very well identified by natural red fluorescence (emission > 610 nm when excited with blue light), due to the presence of chlorophyll *a* (Chll*a*) (Yentsch and Yentsch, 1979). In this study we propose that flow cytometers, implemented with a combination of blue and UV lasers, could allow the detection of PAHs sorbed to phytoplankton or other planktonic organisms.

The goal of our study was to assess whether phytoplanktonic cells with PAHs sorbed can be discriminated from unbound phytoplankton cells by flow cytometry, and determine the corresponding effect of PAHs sorption on the growth rate. For this purpose, we exposed phytoplankton cultures to different concentrations of PAHs, and compared the efficiency of three cytometers with different excitation lasers (UV, near UV and violet) to detect phytoplankton cells with sorbed PAHs. The development of this technic could be very useful in field studies, and may be relevant to monitor the evolution of oil spills or petrol inputs, and the dispersion in addition to its consequences for the marine environment.

2. Materials and methods

2.1. Experimental organisms and PAHs

Cultures of two marine phytoplankton species: *Tetraselmis suecica* (CSIRO CS-187) and *Dunaliella salina* (CSIRO CS-353) were used. *D. salina* was used in microscopy experiments, while *T. suecica* was used in the flow cytometry analysis. Cultures were grown in sterile polycarbonate bottles at 20 °C under continuous 140 nMol Photons m^{-2} s^{-1} light and nutrient-rich medium (f/2).

Concentrated solutions of pyrene and anthracene (Sigma Aldrich purity > 98%), of 2×10^4 μg mL^{-1} for pyrene and 10^4 μg mL^{-1} for anthracene, were prepared in acetone (ACS) as solvent. Both solutions were kept at 4 °C to avoid acetone evaporation.

2.2. Approaches/techniques

We used flow cytometry and epifluorescence microscopy to detect PAHs sorbed to phytoplankton.

2.2.1. Epifluorescence microscopy

An Olympus IX81 epifluorescence microscope, with a blue light filter (excitation wavelength 470–495, emission wavelength 510–550 nm, dichromatic 505 nm), was used for the excitation of chlorophyll *a* in *D. Salina* cultures. Hydrocarbons sorbed to cell surface in cultures treated with PAHs (pyrene and anthracene), or in untreated cultures, were analyzed using a UV filter cube (excitation wavelength 360–370 nm, emission wavelength 420–460 nm, dichromatic 400 nm). Samples were observed at ×600 magnification (Olympus PlanApo 60× NA1.4 oil immersion objective). A digital monochrome camera and the ImageJ 1.45 s software were used for the image analysis.

2.2.2. Flow cytometry with UV

Flow cytometry analyses were performed on three different instruments (BD Influx, BD FACSAria II, and BD FACSCanto II) in order to compare the different optical configurations for the measurement of cells with PAHs. All instruments were equipped with a 488 nm laser and phytoplankton was identified by characteristic 488 nm laser scatter and fluorescence emission in the 585/42 nm (accessory pigments) and 690/40 nm (Chll*a*) ranges. The appropriate excitation wavelength for pyrene and anthracene has been described between 320 and 340 nm and 320–360 nm, respectively (Basu et al., 2006; Dartnell et al., 2012; Suzuki et al., 2009), with emission spectra in the violet-blue range 360–430 nm (Dartnell et al., 2012; Suzuki et al., 2009). We therefore compared a common optical configuration for each cytometer with excitation between the UV and violet range. Analysis with the BD Influx ([355] 460/50 nm configuration: excitation laser of 355 nm and emission filter of 460/50 nm emission) and BD FACS Canto II ([405] 450/50 nm configuration: excitation laser of 405 nm and emission filter of 450/50 nm) were performed at the Centre for Microscopy, Characterization and Analysis (CMCA; University of Western Australia, Perth), while analysis with the BD FACS Aria II ([375] 440/40 nm: excitation laser of 375 nm and emission filter of 440/40 nm) were performed at the Mediterranean Institute of Advanced Studies (IMEDEA, CSIC, Spain).

Replicated samples (0.5 mL) from the different culture treatments, and from the controls, were analyzed. For the quantification of cell concentrations, an aliquot of a calibrated solution of fluorescent beads with 1 μm diameter (Lot: 453,837; Polysciences Inc.) was included in each sample as an internal standard. Blank samples of filtered medium were measured after removing the cells using a 0.2 μm pore size syringe filter system. Cultures growing with and without pyrene and anthracene were also analyzed to control residual fluorescence from the growth medium. The tests indicated no fluorescence noise signals from the medium that could have interfered with PAHs signals of phytoplankton cultures. The analysis of the cytograms acquired by flow cytometry was performed using FlowJo 9.4.4. and FACSdiva (Becton Dickinson) software.

2.2.3. Experimental sets

For conducting the experiments, the cultures were grown in triplicated bottles (2 L volume) until the start of the exponential growth phase. 200 mL of the cultures were aliquoted into sterile bottles containing pyrene or anthracene, to achieve final concentrations in the range from 0.5 to 10^3 μg L^{-1} (as outlined in Table 1), and were incubated between 5 and 14 days. Before add the 200 mL of cultures into the treatment bottle, the acetone from pyrene and anthracene solutions was allowed to evaporate during 60 min. Samples were driven for microscopic analysis every 48 h, and for flow cytometer every 24 h.

2.2.4. Calculations

The cultures growth rates (μ d^{-1}) were calculated as the slope from the linear relationship between the natural logarithm of cells

Table 1
The different concentration (μg L^{-1}) of pyrene and anthracene dosed to cultures of *T. suecica* during the flow cytometry experiments. The wavelengths correspond to the different lasers used for PAHs excitation, installed in the different flow cytometers used.

405 nm		375 nm		355 nm	
Pyrene	Anthracene	Pyrene	Anthracene	Pyrene	Anthracene
0.5	0.5	2	0.5	0.5	10
3.9	1.5	6	8	25	25
7.8	3.1	25	25	50	50
11.6	10	300	300	100	100
25	25			300	500
50	50			1000	1000
100	100				
300	500				
1000	1000				

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