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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.

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				

abundance and the incubation time (in days). The percentage of cells with PAHs, in each sample, was obtained as the fraction of the cell abundance with PAHs detected in the sample relative to the total abundance of cells in each sample. We used *t*-test and regression analysis (JMP program) to analyze the significance of the differences observed in the results obtained using the different flow cytometer configurations, and between growth rates and percentages of cells with PAHs sorbed.

3. Results and discussion

3.1. Detection of PAHs sorbed to phytoplankton cells by epifluorescence-microscope

D. salina cells emitted red fluorescence from chlorophyll *a* when were illuminated by blue light (470–495 nm; Fig. 1a) under microscope, and this emission was not affected by the incubation with PAHs. Control cells without PAHs did not show blue fluorescence emission when illuminated with UV-light filters (Fig. 1b). *D. salina* cells growing with pyrene emitted fluorescence in the wavelength range of the filter set wavelengths of 420–460 nm of the microscope when were excited by UV-light (360–370 nm excitation microscope filter), and this emission was not seen in control cells, in the absence of PAHs (Fig. 1). Conversely, blue emission from UV-excitation, was not observed in anthracene treated cells due to rapid photo bleaching (Fan and Reinfelder, 2003; Lehto et al., 2000; Nagata and Kondo, 1977; Roper, 2006). Rapid emission fading and degradation of PAHs under UV light has previously been raised as an issue, which may limit the applicability of the epifluorescence microscope for the analysis of PAHs sorbed to cells, in research examining micro-scale spatial distribution of PAHs in different soil samples (Roper, 2006). Moreover, analysis by microscopy requires tedious and labor-intensive individual counting of phytoplankton cells, making impractical the analysis of high number of samples.

3.2. Cells with pyrene and anthracene detected by flow cytometry

The mean fluorescence intensity (MFI) of blue emission increased in samples of *T. suecica* treated with pyrene or anthracene with all excitation wavelengths tested (Fig. 2). The greatest increase in MFI was observed with 355 nm laser excitation, while the least increase of MFI was observed after the excitation with the 405 nm laser (Fig. 2). Two distinct peaks of MFI were observed with both [405] 450/50 nm and [375] 440/40 nm configurations. These likely represent cells of *T. suecica* with PAHs accumulated on cell surface (bright peak) versus *T. suecica* cells without PAHs (dim peak). In the samples excited by the [375] 440/40 nm configuration, the bright subset of cells showed a 4-fold increase in peak MFI for pyrene and a 6-fold increase in anthracene treated samples over controls (Fig. 2). This was sufficient in order to gate and quantify the number of cells with accumulated PAHs (Fig. S2). A similar result was observed in samples excited by the [405] 450/50 nm configuration (Fig. 2); with bright subset MFI increased 4 and 12 fold over the dim subset for pyrene and anthracene respectively (Fig. 2; Fig. S2). However, differentiation of bright-blue and dim blue fluorescent cells was less reproducible when the [355] 460/50 nm was used, because the blue fluorescence increased highly for all cells (Fig. 2). This large increase in blue fluorescence with respect to the cells in the control, impeded a clear gating to identify and quantify the number of cells with accumulated PAHs (Fig. 2). The different flow cytometer's configuration showed differences in the detection of bright-blue fluorescent cells. When *T. suecica* was exposed to high dosages of anthracene, the percentages of cells with bright-blue fluorescence emission were significantly lower when the [355] 460/50 nm BD Influx configuration was used for the analysis (Table 2); although there were no significant differences between configurations when cells were exposed to low anthracene doses (Table 2). When *T. suecica* cells were exposed to low dosages of pyrene, the configuration of the [375] 440/40 nm BD FACS Aria II was more efficient for the excitation, helping to identify a significant larger percentage of cells with pyrene sorbed than the other two configurations (Table 2). Therefore, as the [355] 460/50 nm BD Influx showed more variable data that the other two laser configurations (Table 2), further experiments were performed using the [405] 450/50 nm BD FACS Canto II and the [375] 440/40 nm BD FACS Aria II configurations. Probably, the detection filter set of 460 nm of the Influx configuration was less sensitive for the detection of cells with anthracene. The emission spectra (Fig. S1) indicated a strong fall in the fluorescence emission at 460 nm for cells with anthracene, including those cells excited at 355 nm.

The percentage of the bright subsets increased with the increasing pyrene and anthracene dose ($p < 0.0001$; Fig. 3), ranging from 3% to 10% respectively of total cells, to values of 100% for the highest PAHs dosages tested (Fig. 3). For dosages of pyrene lower than 50 μg L^{-1} nominal concentration, the percentage of the bright subset was lower when excited by the 405 nm laser than the 375 nm laser ($p < 0.001$). Conversely, when the pyrene dose exceeded 50 μg L^{-1}, there were not differences in the bright subset between two lasers. No such difference in the bright subset between lasers was observed with anthracene, regardless of dose. This reflects differences in the emission spectra of pyrene as was demonstrated by spectrofluorometry (Fig. S1), being differences in the fluorescence emission detected as a result of the laser used for the excitation. The fluorescence of cells with pollutant adsorbed, after the excitation by the 405 nm laser, showed lower values than those obtained when samples were excited by the 375 nm laser; these differences were statistically significant ($p < 0.001$).

A population with the characteristic bright blue fluorescence, representing accumulated PAHs, was already discernable at 1 h (Fig.

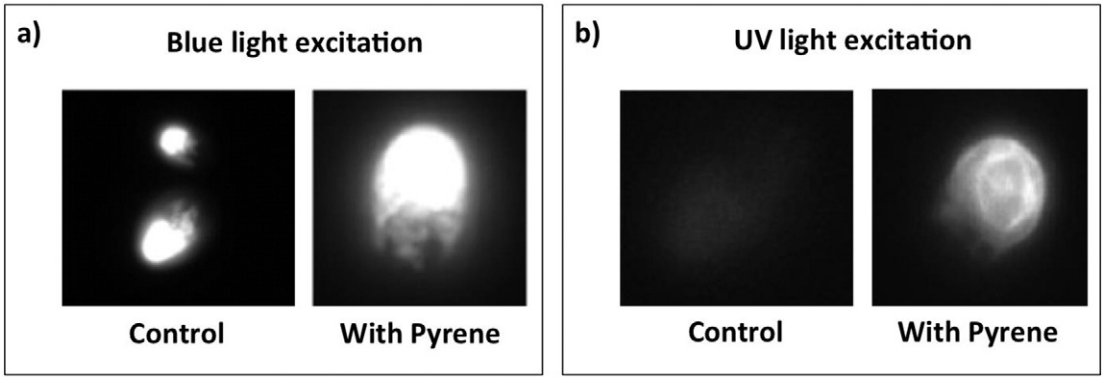

Fig. 1. Two different cultures of *Dunaliela salina* observed by inverted epifluorescence microscope (Olympus IX8; ×600 magnification Olympus PlanApo 60× NA1.4 oil immersion objective). Control sample correspond to a culture without PAHs, and the treatment was a culture of *Dunaliela salina* growing with pyrene. (a) Samples excited by blue light (470–495 nm), observed by FITC filter (red emission). (b) Samples excited by UV-light (360–370 nm), observed by blue filter (emission: 420–460 nm). Images were taken by a digital monochrome camera.

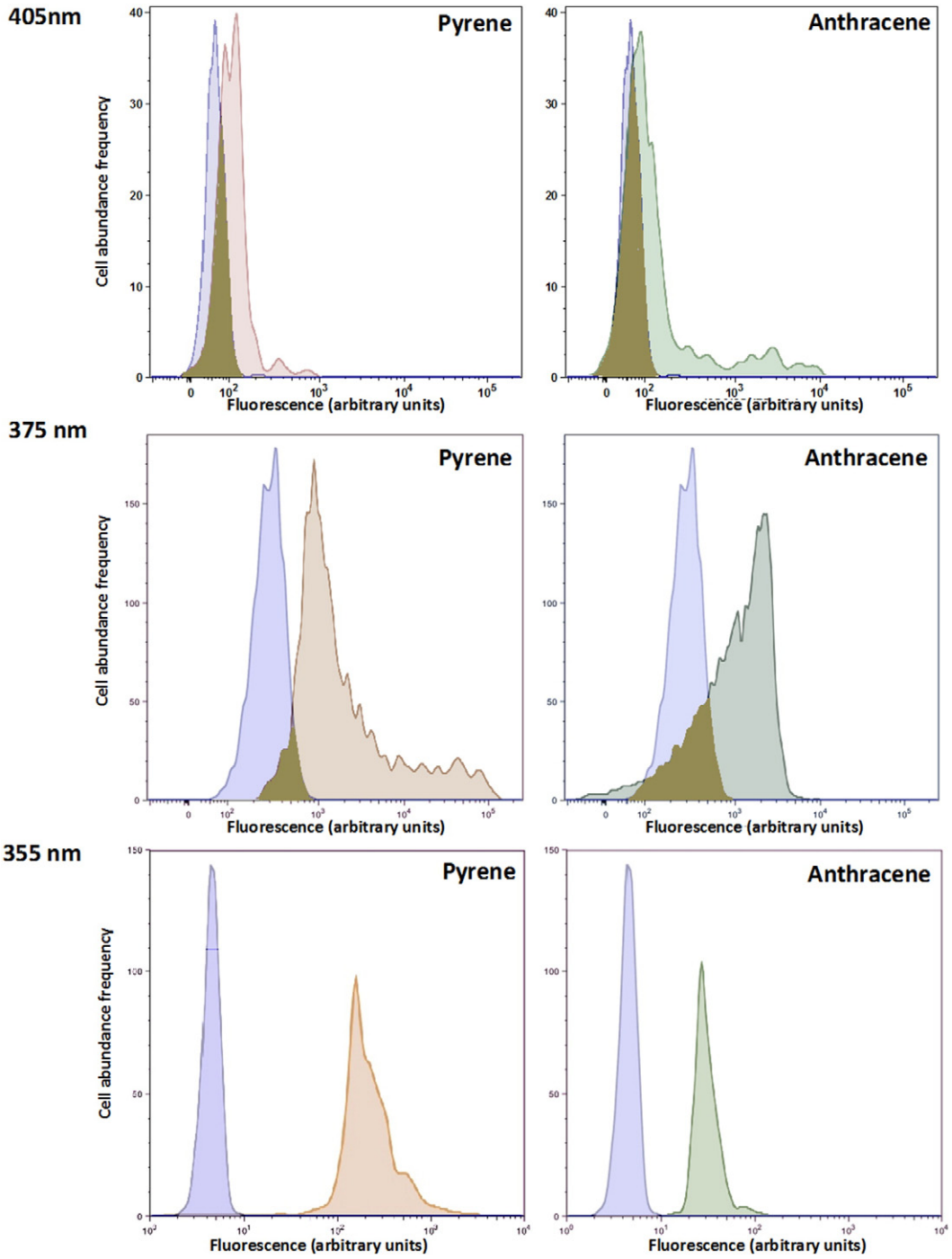

Fig. 2. Flow cytometer frequency histograms of *T. suecica* cell's blue florescence emission after excitation at the different configuration lasers of a) 405 nm, b) 375 nm and c) 355 nm. The blue histograms encompassed the dim-blue fluorescence emission range of cells without PAHs sorbed growing in controls without PAHs; pink and green histograms delimits the region of the dim and bright-fluorescence emission signals of cells growing with pyrene and anthracene, respectively. The darker areas in the last histograms, represent the overlap with the dim-blue fluorescence emission range of cells without PAHs, and help to identify the bright-fluorescence emission signals of cells with PAHs sorbed. The Y-axes represent the frequency of cells and the X-axes represent the intensity of cell blue fluorescence signals (arbitrary units).

3c and d). This is consistent with the rapid kinetics of PAHs surface accumulation described by Fan and Reinfelder (2003) in diatoms (10–20 min after pollutant exposure). Other studies have shown that, in addition to rapid membrane accumulation or organic pollutants, the incorporation into cell by diffusion process is much slower (Swackhamer and Skoglund, 1993; Wallberg et al., 2001; Wallberg and Andersson, 1999). In our study, the proportion of cells with bright PAHs staining increased with longer exposure time (Fig. 3c and d; $p < 0.001$). However, at low dosages the percentage of PAHs-bright cells decreased with increasing cell abundance (Fig. 4), suggesting clearance of sorbed pollutants. This corresponds to the previously described dilution of PAHs with cell growth (Fan and Reinfelder, 2003; Skoglund et al., 1996), which corresponds with a decrease of PAHs concentration at which cells are exposed, as a result of the increase in the number of cells present in the medium with a constant concentration of PAHs. Alternatively, intracellular incorporation by diffusion may reduce the quantity of membrane accumulation at the cell surface (Skoglund et al., 1996), but would still contribute to cell MFI by flow cytometry. When samples were observed

Table 2
Percentages of *T. suecica* cells showing bright-blue fluorescence emission (indicative of PAHs sorbed) identified by flow cytometers configured with different excitation lasers of 405 nm, 375 nm and 355 nm wavelengths. Cells were exposed to different dosages of pyrene and anthracene.

	Pyrene (500 μg L^{-1})			Anthracene (300 μg L^{-1})		
	405 nm	375 nm	355 nm	405 nm	375 nm	355 nm
Mean	95.09	94.43	91.76	62.31	74.88	20.80*
SE	1.77	2.10	2.19	11.31	7.16	6.48
	Pyrene (25 μg L^{-1})			Anthracene (10 μg L^{-1})		
	405 nm	375 nm	355 nm	405 nm	375 nm	355 nm
Mean	37.28	87.80*	49.35	20.57	27.08	26.07
SE	10.66	7.01	14.55	6.38	7.18	7.29

*The asterisks indicate significant differences (*t*-test Anthracene laser 555 nm: laser 375 nm t = 54, df = 10, $p < 0.0005$; laser 405 nm t = 41, df = 10, $p < 0.005$. *t*-test pyrene laser 375 nm: laser 355 nm t = 39, df = 10, $p < 0.05$; laser 405 nm t = 49, df = 10, $p < 0.01$).

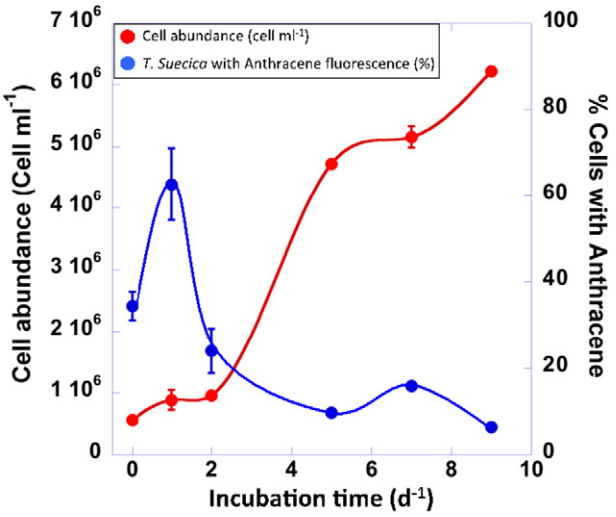

Fig. 4. Changes in the cell abundance (cell mL^{-1}) (red dots), and in the percentage of anthracene bright-blue fluorescent *T. suecica* cells (blue dots) during the incubation time (hours) with 1.5 μg L^{-1} anthracene. The error bars represent the SE values.

by epifluorescence microscopy, blue fluorescence of *D. Salina* treated with PAHs, was not co-localized with any cellular structure. At high dosages of PAHs, most cells of *Tetraselmis suecica*, analyzed by flow cytometry, showed PAHs fluorescence, increasing the fluorescence with duration of PAHs treatment (Fig. 3c and d). These findings correspond with previous studies, showing that the exposure time, the pollutant dosages and cell growth are mechanisms that influence the accumulation of organic pollutants in phytoplankton organisms. The lipidic composition and the chemicals hydrophobicity were also identified as relevant mechanisms contributing to their accumulation in phytoplankton, that must help to explain the differences detected among species and chemicals (Koelmans, 2014; Skoglund et al., 1996). The lipidic content and composition of phytoplankton varies with taxa, and is influenced

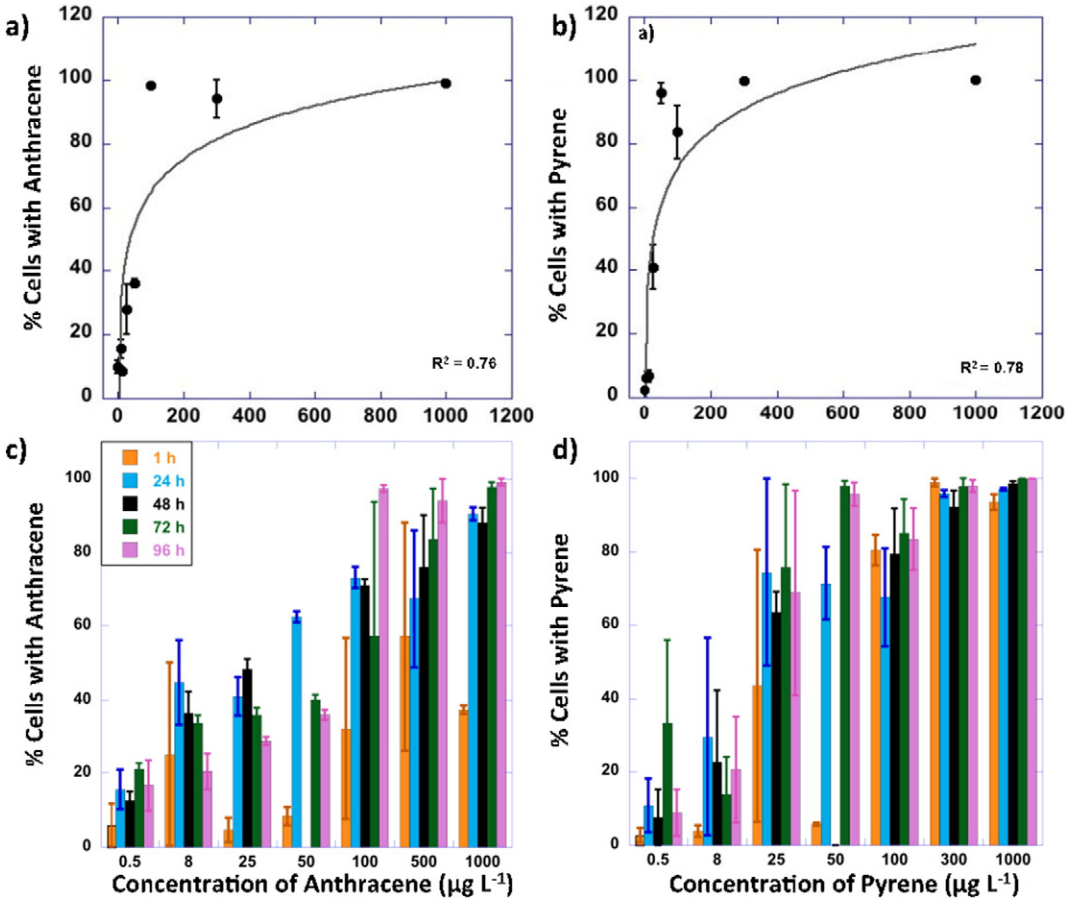

Fig. 3. Relationship between the percentages of cells detected with anthracene (a) and pyrene (b) fluorescence with the concentration of PAHs tested (μg L^{-1}), 96 h after the dosage of pollutants. The line represents the logarithmic fit. Bar graphs represent the cell percentages with anthracene (c) and pyrene (d), in function of the theoretical concentration of pollutants dosed to the cultures (μg L^{-1}). For each concentration is represented the percentages of cells with pollutants attached, detected after the samples excitation by 375 nm and 405 nm, during the incubation time after the addition of the pollutants: firth hour (orange-bars), 24 h (blue-bars), 48 h (black-bars), 72 h (green- bars) and 96 h (pink-bars). The error bars represent the SE values.

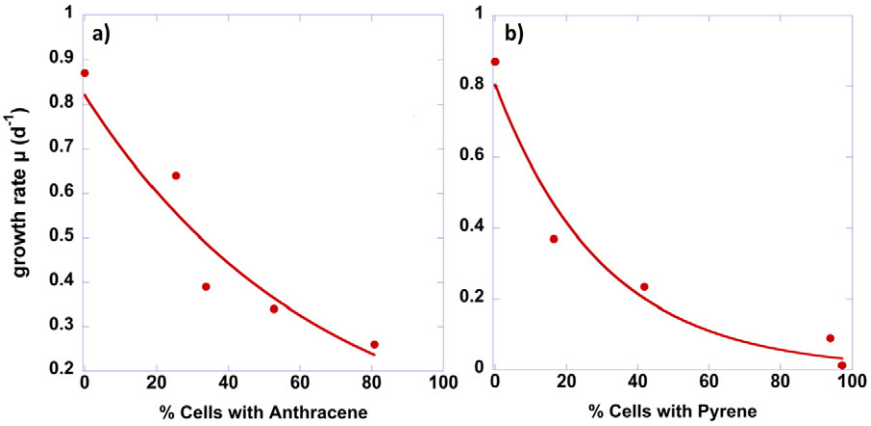

Fig. 5. Relationship between the growth rates (μd^{-1}) of *Tetraselmis suecica* and the percentages of bright-blue fluorescent cells with anthracene (a) and pyrene (b) sorbed, as measured by flow cytometry. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

by growth conditions as limitation by nutrients and solar radiation (Shifrin and Chisholm, 1981; Wang and Chai, 1994).

Limitations of our novel flow cytometry method include the inability to differentiate membrane associated and intracellular accumulations of PAHs over time. The dilution process observed at low PAHs dosages has been described previously in several studies (Fan and Reinfelder, 2003; Skoglund et al., 1996). Berrojalbiz et al. (2011) observed that the concentration of PAHs per cell decrease exponentially with the increase in biomass. Thus analysis by flow cytometry may only be appropriate in assessment of planktonic communities exposed to pollution from oil inputs during the first days of exposure, or in marine areas where the concentration of pollutants is high, like harbors.

Exposure of the cell cultures to the PAHs had a clear negative effect on growth. Growth decreased significantly as the percentage of PAHs-bright cells increased for anthracene ($p < 0.03$) and pyrene ($p < 0.04$; Fig. 5). Pyrene was between 2 and 20 times more effective at inhibiting growth than anthracene ($p < 0.001$; Fig. 5). This may reflect increased relative toxicity of pyrene, resulting from its highly aromatic structure (Millemann et al., 1984). These results demonstrate that accumulation of pollutants on cells, as demonstrated by flow cytometry, reduced the growth rate in culture. At 100% PAHs-bright cells, the culture collapsed, with negative growth rate i.e. cell death. Previous studies have described the reduction of motility and absorption of nutrients and substances by exchange membrane as a result PAHs sorption to the cell membrane (Jiang et al., 2010; Skoglund et al., 1996). Once internalized, PAHs continue having negative effects on cell growth and viability (Jackson et al., 1989), decreasing protein synthesis and photosynthesis efficiency (Echeveste et al., 2011; Jiang et al., 2010).

4. Conclusion

In our study we have identified and quantified phytoplankton cells with PAHs (pyrene and anthracene) accumulated on cells by relatively simple and accessible flow cytometric techniques, using a configuration of UV lasers and optical filters in addition to the standard 488 nm laser necessary to identify phytoplanktonic organisms. We applied the technique in an ecotoxicological study under laboratory conditions, demonstrating growth inhibition with the accumulation of PAHs to cells in culture. In addition to applications in similar ecotoxicology research with cultured cells, flow cytometry allows the rapid counting of plankton abundance and identification of subpopulations within communities and is routinely used in oceanographic studies. Traditional techniques to measure PAHs in biological matrixes require the chemical analysis (Berrojalbiz et al., 2009; Skoglund et al., 1996) that in the case of marine phytoplankton implies a previous concentration of samples. The flow cytometer technique described here could allow the cell-by-cell analysis of a variety of properties simultaneously within minutes of sampling, such as photosynthetic pigments auto-fluorescence and cell size (Marie et al., 2000), cell viability (Agustí and Sánchez, 2002), DNA content (Marie et al., 1997), rRNA hybridization (Simon et al., 1995), and so on. Cell-by-cell analysis may be more relevant than the averaged concentration from the whole populations achieved by traditional techniques. Further development of our seminal flow cytometry technique may extend applicability for a variety of approaches improving capabilities to study PAHs toxicity in other planktonic organisms at their natural environment affected by pollution. It is important to monitor marine oil pollution as PAHs compounds have important and well-described negative effects in the marine community (Echeveste et al., 2010; González et al., 2009; Jiang et al., 2010; Skoglund et al., 1996).

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. The three authors contributed equally.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2017.02.006.

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