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Removal and Biodegradation of Phenanthrene, Fluoranthene and Pyrene by the Marine Algae *Rhodomonas baltica* Enriched from North Atlantic Coasts

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Abstract This study is focused on the removal, accumulation and degradation of three environmental ubiquitous polycyclic aromatic hydrocarbons (PAHs), phenanthrene (PHE), fluoranthene (FLA) and pyrene (PYR), by the marine alga *Rhodomonas baltica* enriched from the English Channel. After separation, purification and culture in several phases, *R. baltica* was exposed to PAH concentrations that are frequently encountered in the field in several anthropized environments. The results showed that *R. baltica* can grow under PAH stress, efficiently remove up to 70% of these compounds from the medium by 216 h of culture and selectively bioaccumulate PAHs by their hydrophobicity. Between PHE, FLA and PYR, phenanthrene was the compound with higher degradation rates throughout incubation. The equilibrium partitioning theoretical approach showed that physico-chemical partitioning, rather than active bioconcentration, was the major factor governing the bioaccumulation, outlying a potential application in decontamination processes for this species.

Keywords Removal · Accumulation · *Rhodomonas* sp. · Phenanthrene · Fluoranthene · Pyrene

Polycyclic aromatic hydrocarbons (PAHs) are a widely distributed group of organic pollutants originating from petrogenic, pyrogenic and natural sources. As the interest in these compounds has increased rapidly during the last decades, they have been extensively studied regarding their origin and distribution in the environment (Yin et al. 2015). Polycyclic aromatic hydrocarbons are currently considered to be one of the major groups of environmental contaminants. However, unlike other harmful organic chemicals that have been banned or regulated, they continue to be released into the environment due to their widespread generation during the combustion process. They are emitted primarily by anthropogenic sources such as vehicle emissions, coal and fossil fuel powered generation, petroleum refining, straw and firewood burning, industrial processing, chemical manufacturing, oil spills and coal tars.

Marine and coastal areas are especially vulnerable zones to anthropogenic PAH introduction via urban runoff (McCready et al. 2006), industrial processes, vehicle exhaust (Stephanou 2005; Zhu and Wang 2005) and spillage of fossil fuels (Bajt 2014). Consequently, the concentration of these compounds in seawater and sediments undoubtedly carries toxicological significance to both benthic and pelagic marine organisms. As a general pathway, PAHs entering the water system can first accumulate in fine-grained sediments and suspended particles; they remobilise later in the seawater, become bioavailable to the local organisms (Wetzel and Van Vleet 2004) and

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finally accumulate in the biota. The literature dealing with the introduction of PAHs to the food web has focused on these routes; for example, mussels and other bivalves have been extensively investigated and successively used as sentinels for biomonitoring programs (the International Mussel Watch, Farrington et al. 1983; the European BIOMAR; Narbonne et al. 2005). Despite this, there is still a gap in the current knowledge about the bioaccumulation of PAHs at the lowest trophic levels. While algae, the primary producers in coastal and estuarine systems, play an important role in the fate of PAHs in aquatic ecosystems, the bioaccumulation of PAHs by algae is an issue which is currently still poorly understood. Moreover, even less attention has been paid to the biodegradation of PAHs by algae. Previous studies have dealt with the response of planktonic communities to petroleum hydrocarbons and various persistent organic pollutants, both in the field (Mackie et al. 1978; Middleditch et al. 1979; Serrazanetti et al. 1991; Kowaleska and Konat 1997; Cailleaud et al. 2007) and under experimental conditions (Skjoldal et al. 1982; Sibley et al. 2004; Zhang et al. 2011; Magnusson and Tiselius 2010; Stange and Swackhammer 1994; Gerofke et al. 2005). Concerning PAHs, Cailleaud et al. (2009) studied the uptake and elimination of some of these compounds by estuarine copepods in a laboratory study, while Lotufo (1998) investigated the uptake and elimination of fluoranthene in the field. Additionally, Chan et al. (2006) performed PAH removal studies using the freshwater alga *Selenastrum capricornutum*, while Hong et al. (2008) performed similar studies using the mangrove native marine diatoms *Skeletonema costatum* and *Nitzschia* sp. Therefore, the aim of this study was to investigate the ability of the marine algae *Rhodomonas baltica* to remove a mixture of these compounds, namely phenanthrene (PHE), fluoranthene (FLA) and pyrene (PYR), assessing their bioaccumulative capacity. Despite the technical challenges inherent in setting a marine algae culture, *R. baltica* was chosen because of its ubiquitous occurrence in the North Atlantic blooms and its potential use as a contaminant removal agent in decontamination processes.

Materials and Methods

A PAH solution was prepared under laboratory conditions simulating frequent PAH levels described for the Seine estuary (Cailleaud et al. 2007), including three major PAHs found in the Atlantic Ocean: phenanthrene, fluoranthene and pyrene (Sigma Aldrich, Maidstone, UK). The PAHs were dissolved in acetone, which was allowed to evaporate for 30 min in the experimental bottles before adding the seawater. Deuterated internal standards, Phenanthrene-d_{10} and Pyrene-d_{10}, were purchased from Aptochem (Montreal, Canada). HPLC-grade solvents (hexane, dichloromethane, methanol and acetonitrile) were purchased from Dislab (Lens, France). No significant amount of target compounds could be detected in procedural blanks. Ultrapure water (Milli-Q) was produced by a Millipore apparatus (Merck, Darmstadt, Germany) with 18.2 MΩ/cm resistivity. Merck silica gel 60 (70–230 mesh ASTM) activated at 450°C was heated at 120°C for 12 h prior to use. Glassware was washed with detergent (Decon Lab, Philadelphia, USA), rinsed with ultrapure water and acetone and dried at 120°C prior to use.

Cultures of *R. baltica* were originally obtained from Roscoff culture collection (Roscoff, France), and cultured in several phases at the Marine Station of Wimereux (Laboratory of Oceanology and Geosciences, LOG, France) in order to obtain PAH-free algal culture. The axenic stock cultures were routinely maintained at 20°C, under a 12:12 h light and dark (L:D) cycle in a light- and temperature-controlled incubator for more than three dilutions in a semi-continuous culture mode during the log-phase of growth in filtered, aged seawater. The strain of *R. baltica* was maintained in the laboratory in 250 mL glass Erlenmeyer flasks and were shaken by hand daily. The culture was kept in an incubator (SANYO model MLR, Osaka, Japan) at 18°C and a photoperiod of 12L:12D under a fluorescent light with an intensity of 2500 lx Batch cultures in 2–6 L flasks were used to grow the microalgae for the experiments. The culture flasks were filled with autoclaved seawater of salinity 33 psu and enriched with Conway medium following the protocol described in Sadovskaya et al. (2014) and Tlili et al. (2016). The composition of Conway medium was: each liter of autoclaved seawater contained 100 mg $NaNO_3$, 20 mg NaH_2PO_4, 45 mg Na_2EDTA, 33.6 mg H_3BO_3, 0.36 mg $MnCl_2$, 1.3 mg $FeCl_3$, 0.021 mg $ZnCl_2$, 0.02 mg $CoCl_2{\cdot}6H_2O$, 0.02 mg $CuSO_4{\cdot}5H_2O$, 0.09 mg $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.2 mg thiamine HCl (vitamin B1) and 0.01 mg cyanocobalamin (vitamin B12). Cultures were aerated with sterile air and incubated in the same condition as the strain inoculums.

The comparative incubation experiments were designed to study the variability in the growth rate in relation to PAH occurrence in the surrounding media. Duplicate incubation bottles were prepared using 2 L glass bottles and three conditions were set-up: control, solvent condition (acetone, 100 µL) and PAHs (the three PAHs were combined and added to the "Conway" medium: PHE 130 ng/mL, FLA 148 ng/mL, and PYR 113 ng/mL, each one representing the final concentration in the medium). Experimental incubation was conducted by transferring cells during the log-phase into the incubation bottles to ensure subsequent exponential growth. Cell density was maintained by shaking daily to ensure that all cells of the population are equally exposed to the light and nutrients. Samples were taken daily at a fixed time during the light period to avoid

the diel cycle effect. Subsamples for cell counts were fixed using a 5% formaldehyde/lugol solution and stored in the dark at 4°C until further analysis (Iwasawa et al. 2009). Cell counting was performed in triplicate under an inverted microscope. Cell density was determined using a haemocytometer (Erma, depth 0.1 mm, Tokyo, Japan) under a compound microscope (Olympus, IX71, Tokyo, Japan) at 100-fold magnification using the method that was previously described by Guillard and Sieracki (2005). The growth rate (μ/day) was estimated using the following exponential growth equation (Guillard and Siercki 2005):

$$\mu = \ln\left(\frac{N_1}{N_0}\right)/\left(T_1 - T_0\right) \quad (1)$$

where N_1 and N_0 are the cell densities in cells/mL at the beginning of (T_0) and at 72 h after the exposure to an L:D cycle (T_1), respectively.

These sets of experiments were designed to study the PAH partition between the media and algal cells during the normal exponential stage of *R. baltica*. The main purpose of the design was to test the ability of the alga to efficiently remove PAHs and elucidate the hypothesis by which *R. baltica* can actively bioaccumulate PAHs from the surrounding medium (either through assimilation or adsorption). Duplicates of incubation bottles were prepared using 5 L glass bottles, and two conditions were set-up: control and PAH treatments. At the beginning of the experiment, a fresh algal culture were collected from axenic exponential phase cultures for each experiment the same day as the exposures started. Initial cell densities were around 1.1×10^5 cells/mL (range 0.98–1.20 cells/mL). Polycyclic aromatic hydrocarbons were added to the medium at the start of the incubation period leading to the following initial concentrations after equilibrium: PHE 223 ng/mL, FLA 255 ng/mL, and PYR 194 ng/mL. Every 2 days, beakers were enriched with Conway medium while cell counting was performed daily. Water subsamples were collected from each bottle immediately before the addition of *R. baltica* to investigate both DOC and the initial (To) PAH concentrations. Subsequently, water and algal subsamples were obtained, and from each bottle, an average of 750 mL of water containing the alga was filtered through a precombusted GF/F filter (cutoff size 0.7 μm). Alternatively, algae (filters) and filtered media were spiked with the PAH surrogate standards, extracted (ASE and L/L extraction) and analysed for PAHs using Gas Chromatography/Mass Spectrometry.

The PAH percentages in the medium and cells were calculated following Chan et al. (2006):

$$\%\ \text{of dissolved PAHs} = \left(\frac{\text{amounts of PAHs added} - \text{amounts remaining in the medium}}{\text{amounts of PAHs added}}\right) \times 100 \quad (2)$$

$$\%\ \text{of PAHs uptaked adsorbed} = \left(\frac{\text{amounts of PAHs accumulated in cells}}{\text{amounts of PAHs added}}\right) \times 100 \quad (3)$$

$$\%\ \text{of degraded PAHs} = \left(\frac{\text{amounts of PAHs added} - \text{amounts remaining in the medium} - \text{amounts accumulated in the cells}}{\text{amounts of PAHs added}}\right) \times 100 \quad (4)$$

After the experimental phase, each sample was kept in clean glass bottles capped with Teflon-lined lids. Samples were rapidly filtered using 0.7 μm GF/C glass microfiber filters (Whatman, Maidstone, UK) and both particulate and dissolved phases were kept. While the filtered water was extracted using liquid–liquid extraction (LLE, Tanacredi 1977), the algae retained in the filters were extracted using accelerated solvent extraction (ASE 200, Dionex Corp., CA, USA).

The samples were first spiked with the internal standard (Phe-d_{10} and Pyr-d_{10}) and then extracted using LLE extraction. Each water subsample (1 L) was extracted with 80 mL of dichloromethane and repeated three times. The extracts were then pooled and dried using Na_2SO_4. Finally, the extract was concentrated using a rotary evaporator followed by a slight stream of nitrogen before GC-MS analysis.

Algae biomass was spiked with deuterated internal standards (Phe-d_{10}, Pyr-d_{10}). After a delay of equilibration, the algal culture was extracted using accelerated solvent extraction technique (Thermo Scientific, CA, USA; Schantz 2006). The extraction conditions were preheat, 0 min; heat, 5 min; static solvent extraction time, 5 min (n = 2) at 100°C; purge 3 min, 115% flush, 1500 *psi*. Dichloromethane was used as the extraction solvent. High purity nitrogen was employed as the purge gas. The extraction procedure afforded a total extract volume of 40 mL. The extracts were concentrated, solvent-exchanged to hexane, and then purified and fractioned by liquid chromatography on a silica column to eliminate organic interferences. The elution was performed using hexane and then with the mixture of hexane/

dichloromethane (3/1 and 1/1 v/v). The sample was concentrated using a rotary evaporator followed by a slight stream of high purity nitrogen before GC-MS analysis.

The extracts were analysed using a Varian 3900 gas chromatograph (Varian, CA, USA) equipped with a deactivated fused-silica guard column (5 m, 0.53 mm i.d.) and a fused-silica capillary Phenomenex XLB (60 m length, 0.25 mm i.d., 0.25 µm film thickness; California, USA), coupled with a Varian Ion Trap Saturn 2000 Mass Spectrometer (MS). The carrier gas was helium held at a constant flow rate of 1 mL/min. Samples were injected in the splitless mode at 280°C and the injector was purged with helium after 1 min. The transfer line and the ion trap were respectively held at 260 and 220°C. Each contaminant was identified based on the retention time and the mass spectrum from chromatogram of standard solutions acquired in full scan mode. Quantification was then performed in the single ion storage (SIS) mode for better selectivity. Response factors were determined relative to the deuterated internal standards response and to standard mixtures. Deuterated standards were chosen in order to better fit to the properties of each group of contaminants. Quality Assurance Procedural blanks for water, filters, glass materials and solvents were conducted throughout all of the experiments. The effectiveness of the different analytical procedures was evaluated by analysing NIST Reference Material (SRM 1944 and 2978 for PAHs; Maryland, USA). The mean recoveries for PAHs compared with the certified concentrations were in the range of 90%–110%. The limits of detection for PHE, FLA, and PYR were 0.86, 1.23 and 1.12 µg/L, respectively; whereas the corresponding limits of quantification were 2.87, 4.08 and 3.72 µg/L, respectively.

The approach of assuming an equilibrium or near-equilibrium of PAH concentrations between the aqueous and the particulate phase is generally known as equilibrium partitioning (EqP) theory (Mackay and Boethling 2000; Burgess et al. 2003). The concept of EqP can be quantified in the following way

$$Kp = \frac{C_p}{C_d} \tag{5}$$

where K_p is the partition coefficient (L/Kg) of an organic compound between particulate (C_p) and dissolved phases (the data used in these calculations are expressed on a dry weight basis). The relationship between Cp and Cd is assumed to be linear, which allows for the generic normalisation by f_{oc}—fraction organic carbon—in Eq. 6, leading to the partition coefficient between organic carbon and water (K_{OC}, Eq. 6).

$$K_{oc} = \frac{K_p}{f_{oc}} \tag{6}$$

Finally, bioconcentration factors (BCFs) were defined as:

$$BCF = \frac{C_t}{C_d} \tag{7}$$

where C_t was the concentration in the algal biomass and C_d was the dissolved concentration. Units for BCF are L/Kg tissue and all concentrations were calculated in dry weight.

Results and Discussion

Figure 1 shows the effects of PAHs, solvent (acetone) and incubation time on cell densities of *R. baltica* cultures. Results showed differences between the "control" (including solvent control) and the "PAHs" condition; in fact, *R. baltica* cultures under PAH treatment showed lower cell counts. Further, ANOVA multivariate tests of significance showed significant differences between the different conditions. Subsequent *t* tests for independent samples showed differences between controls (control 1: no addition; control 2: solvent addition) and PAH treatments, with *p* values averaging 0.02. As a general result, *R. baltica* growth was significantly inhibited by the PAH mix compared to that of the control. The PAHs condition showed on average smaller slopes and lower biomass densities. Growth rate values were consistent with these results, averaging 1.25 for control conditions vs. 0.45 for the PAHs condition (per day).

Figure 2 shows the percentages of PAHs taken up by cells, remaining in the medium and degraded in the *R. baltica* culture system at different incubation times. The amounts of PAHs remaining in the media decreased significantly ($p < 0.01$) as the incubation time increased. Simultaneously, as shown in Fig. 2b, the percentage of PHE, FLA and PYR taken up by cells increased in a continuous trend up to 168 h of incubation and then, in the following 48 h, followed a decreasing trend. Consistently, the percentage of

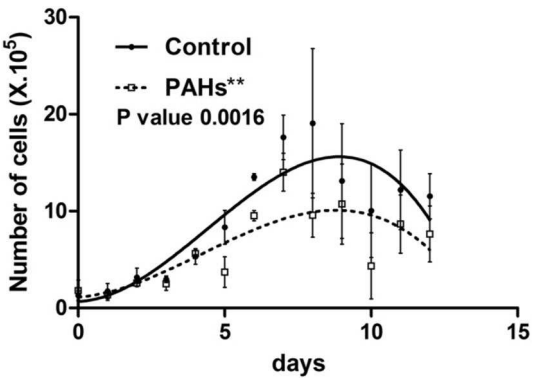

Fig. 1 *Rhodomonas baltica* growth curves for each experimental condition. Each day represents triplicate counting while bars indicate the standard deviation. The PAH exposed algae growth was highly statistically significant ($p < 0.01$) compared to the control

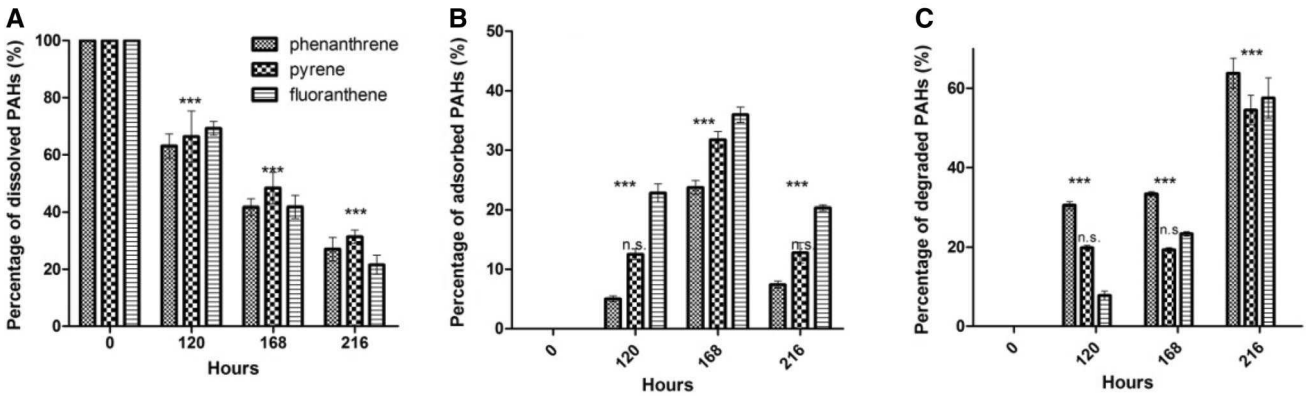

Fig. 2 The percentage of PAHs **a** dissolved, **b** uptake/adsorbed and **c** degraded in the *Rhodomonas baltica* culture system in relation to incubation time (hours, conducted at the exponential phase of algae growth). Following Bonferroni post tests, for graph **a** "all percentages grouped by time were highly significantly different from each other"; graph **b** "all percentages were highly significant ($p < 0.001$; ***) compared to the initial time and each other, with the exception of pyrene at 120 h vs. 216 h"; and graph **c** "all percentages were highly significant ($p < 0.001$; ***) compared to the initial time and each other with the exception of pyrene at 120 h vs. 168 h". Each bar includes mean ± 95% CI

PAH degradation (calculated from Eq. 4) was relatively low and not significantly different during the first days of exposure (10%–30%, 168 h), increasing up to 50%–60% during the following 48 h. These results demonstrated that part of the PAHs had been metabolised by *R. baltica* by a constant rate during the first 168 h of incubation. From this point, there was a significant increase in the percentage of PAH degradation. Significance in percentage of PAH removal, bioaccumulation and degradation was tested and noted by (*) in Fig. 2.

General results are listed in Table 1. For each tested PAH, both BCF and Log K_{oc} were calculated sequentially during the alga's exponential growth phase (from day 5 to day 9, Fig. 1) according to Equations (5–7). To convert the number of *R. baltica* cells to biomass (g) the equivalence 1 cell = 1.210^{-7} and the literature value of organic carbon content for phytoplankton cells (20 fg/cell) were used (Fukuda et al. 1998).

The results obtained for the *R. baltica* growth experiment outline the toxicological effect of the spiked PAHs on *R. baltica*. These results are in agreement with experiments performed elsewhere, where different PAHs exhibited a potential for bioaccumulation and had negative effects on different algal species. For example, Othman et al. (2012) demonstrated that FLA was toxic to marine phytoplankton because it reduced the photosynthetic efficiency after 24 h of exposure and decreased the algal biomass after 3 days, while Hong et al. (2008) showed a synergic effect of PHE and FLA for algal inhibition. Consistently, in the present study, the PHE, FLA and PYR mix was shown to inhibit cell division of *R. baltica*, reducing its growth rate in comparison to reference cultures. Although there are no specific effective-concentration data for *R. baltica* (EC_{50}), it can be hypothesised that the initial PAH concentrations were over the specific EC_{50} for this species.

As stated elsewhere, the mechanism involved in the removal of PAHs by microalgae is similar to that of heavy metals and other organic contaminants (Soto et al. 1975; Hong et al. 2008; Tam et al. 2002). This generally involves two stages, biosorption (or adsorption) and absorption. The first refers to the rapid adsorption at the cell surface level, driven by physico-chemical forces independently of

Table 1 Literature and experimental values of organic carbon (Koc) normalised partition coefficients for the three PAHs tested in this study including the calculated BCFs

PAH	Mass weight	Aqueous solubility log S, (mol/L)[a]	Log K_{ow}[b]	Experimental BCF[d]	Log K_{oc}[c]	Experimental log K_{oc}[d]
Phenanthrene	178	−5.21	4.57	7.51	4.2	4.45
Fluoranthene	202	−5.93	5.08	12.89	4.8	4.73
Pyrene	202	−6.19	4.92	16.51	4.8	4.78

[a]Di Toro et al. (2000)

[b]Gustafsson and Gschwend (1997) and Karickhoff (1981)

[c]Gustafsson and Gschwend (1997)

[d]Averaged experimental values from this study

metabolism. The second one refers to a slow active absorption, accumulation and degradation, which is species-specific. In all cases, and even though the methodological design could not differentiate between these two pathways, the obtained results represent the first data on the bioaccumulation kinetics of *R. baltica*. Maximum yields of bioaccumulation were achieved at 168 h of culture incubation which were followed by a shift in the biodegradation processes. The bioaccumulation process was compound-sensitive: the different PAHs showed different bioaccumulation yields and followed the pattern FLA > PYR > PHE in a time-independent fashion. The amounts of passive/active accumulation were up to 35%, 33% and 25% at 168 h for FLA, PYR and PHE, respectively (Fig. 2).

It is known that low molecular weight PAHs up to five benzene rings can be rapidly degraded through organism activity in both aerobic (Harms and Bosma 1997) and anaerobic conditions (Haritash and Kaushik 2009); therefore, is possible to expect an amount of PAH biodegradation activity from *R. baltica*. In fact, results showed PAH degradation with a significant difference as the incubation time varied (216 h vs. rest, $p < 0.05$). Degradation yields were highest at 216 h of culture, with the maximum rate for PHE, followed by FLA and PYR. Consequently, PHE was more easily degraded by *R. baltica*, either in an active or passive fashion. Other studies showed distinct biodegradation yields for this compound. For example, *Nitzschia* sp. showed maximum rates for PHE degradation (Hong et al. 2008) while *S. capricornutum* showed minimum rates for PHE compared to higher molecular weight PAHs (Chan et al. 2006).

The equilibrium partitioning theoretical approach was applied to the experimental data. As shown in Table 1, the averaged experimental K_{oc} (±confidence interval, $\alpha = 0.05$) were 4.45 ± 0.44, 4.73 ± 0.38 and 4.79 ± 0.38 for PHE, FLA and PYR, respectively. In general, these results were in agreement with K_{oc} values obtained by Gustafsson and Gschwend (1997) for the partition between PAHs and particulate sediment. As described elsewhere, the biosorption process refers to the rapid physico-chemical adsorption and ion exchange processes that occur at the cell surface, and is metabolism independent (Tobin and Cooney 1999). Therefore, biosorption may occur in abiotic particles, and living and dead cells. Therefore, as the present results parallel the literature partition coefficients between PAHs and abiotic sediment particles, it can be hypothesised that the mechanisms governing the PAH sorption to *R. baltica* could be passive and metabolism independent. It has been pointed out that dead cells might be more effective than living ones in adsorbing toxic pollutants. Further, the use of dead microalgal cells could be advantageous because they are not affected by the toxic pollutant and are easy to handle (Tam et al. 2002; Tsezos and Bell 1989). Apart from not needing nutrients or special conditions, non-living biomass can be re-used for many cycles (Aksu 2005), and in some cases, it has been demonstrated that non-viable biomass of algae could absorb contaminants to the same or greater extent as living cells (Aksu and Kutsal 1990; Aksu 2005).

Average BCF values (7.51, 12.90 and 15.81 for PHE, FLA and PYR, respectively) followed the general rule by which BCF tends to increase with hydrophobicity, reflecting a greater affinity of more hydrophobic compounds for organism tissue relative to water (Meador et al. 1995). Therefore, *R. baltica* was able to bioaccumulate PAHs according to their increasing hydrophobicity. Despite this, the PAH BCFs obtained for *R. baltica* in this study were much lower than other similar species such as *Rhodomonas salina* (Berrojalbiz et al. 2009) and *Nitzschia seriata* (Lukitaningsih and Sudarmanto 2010), emphasising the existence of better algal biomass alternatives as contaminant adsorbents.

In this study, for the first time, simultaneous removal, bioaccumulation and degradation of a mixture of PHE, FLA and PYR by the marine algae *R. baltica* was observed. While PAHs appeared to exert negative effects on the algal growth, *R. baltica* demonstrated the ability to grow under that stress. *R. baltica* efficiently removed up to 70% of PAHs from the medium at 9 days of culture. While PAH bioaccumulation yields followed the hydrophobicity of each compound, PHE was the compound with higher degradation rates throughout the time of incubation. In regards to the PAH accumulation mechanism, results suggested that PAHs (log K_{oc} 4.57–5.08) were at near-equilibrium with the water concentrations and that physico-chemical partitioning, rather than active bioconcentration, was the major factor governing the bioaccumulation. Finally, since only three PAHs with similar concentrations were investigated, more detailed research must be conducted in order to obtain a deeper understanding of the interactions and to test the potential application of this species in decontamination processes. In light of these results, we recommend extending this research to the upper levels of the food chain, starting with marine copepods.

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