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Capacity of the potentially toxic diatoms *Pseudo-nitzschia mannii* and *Pseudo-nitzschia hasleana* to tolerate polycyclic aromatic hydrocarbons

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ABSTRACT

This study investigates the effects of polycyclic aromatic hydrocarbons (PAHs) on two potentially toxic *Pseudo-nitzschia hasleana* and *P. mannii*, isolated from a PAH contaminated marine environment. Both species, maintained in non-axenic cultures, have been exposed during 144 h to increasing concentrations of a 15 PAHs mixture. Analysis of the domoic acid, showed very low concentrations. Dose–response curves for growth and photosynthesis inhibition were determined. Both species have maintained their growth until the end of incubation even at the highest concentration tested (120 μg l^{-1}), Nevertheless, *P mannii* showed faster growth and seemed to be more tolerant than *P. hasleana*. To reduce PAH toxicity, both species have enhanced their biovolume, with a higher increase for *P. mannii* relative to *P hasleana*. Both species were also capable of bioconcentrating PAHs and were able to degrade them probably in synergy with their associated bacteria. The highest biodegradation was observed for *P. mannii*, which could harbored more efficient hydrocarbon-degrading bacteria. This study provides the first evidence that PAHs can control the growth and physiology of potentially toxic diatoms. Future studies should investigate the bacterial community associated with *Pseudo-nitzschia* species, as responses to pollutants or to other environmental stressors could be strongly influence by associated bacteria.

1. Introduction

Chemical pollution of aquatic ecosystems is one of the major environmental problems because of its disastrous impacts on resources and services for these anthropized environments (Schwarzenbach et al., 2006). Polycyclic aromatic hydrocarbons (PAHs) are among the chemical contaminants that have required special attention because they are the most commonly encountered at high concentrations in marine environment and especially in coastal waters (Duran and Cravo-Laureau, 2016; Ben Othman et al., 2018). PAHs are also the most hazardous pollutants because of their strong toxicity, since some of them are known as mutagens and carcinogens (Sakari et al., 2008; Ben Othman et al., 2018). These pollutants have low water solubility, high hydrophobicity and low biodegradation and thus can be concentrated and bioaccumulated throughout the marine food webs and may seriously harm aquatic and human life (Joseph and Newman, 2010). The primary producers represent the entry route for PAHs into the marine food webs (Berrojalbiz et al., 2009). Therefore, the ability of phytoplankton to accumulate PAHs can control the fate of these pollutants and their magnification in higher trophic levels and consequently the risk for human health.

The high persistence and toxicity of PAHs has led to intensive research on the process of their elimination and degradation (Manviri et al., 2020). Environmental technology has employed microorganisms, such as bacteria and fungi, to remove PAHs from contaminated waters (Gutierrez, 2010; Gargouri et al., 2011). In recent years, more attention

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has been paid on the role of microalgae in the biodegradation of PAHs (Hong et al., 2008). Although PAHs can inhibit algal growth and decrease phytoplankton biomass, (Echeveste et al., 2010a, 2010b; Perez et al., 2010; Ben Othman et al., 2012), some microalgal species can resist to the toxic effects of these pollutants by accumulating and degrading them (Hong et al., 2008; Liu et al., 2019; Bretherton et al., 2020). However, the tolerance to PAHs varies among phytoplankton species and appears to be related to their cell biovolume (Ben Othman et al., 2012). In this vein, large algae, such as diatoms, are recognized as tolerating organisms, since they are able to maintain growth at high concentrations of PAHs. Hong et al. (2008) showed the capacity of two diatom species (*Skeletonema costatum* and *Nitzschia* sp.) in the accumulation and biodegradation of phenanthrene and fluoranthene, although with different efficiencies. In addition, field experiments conducted in two coastal Mediterranean lagoons (Bizerte and Thau lagoons) revealed the resistance of the diatoms *Skeletonema costatum* and *Chaetoceros sp*. to a cocktail of PAHs (Ben Othman et al., 2018).

Within diatoms, the genus *Pseudo-nitzschia* has received considerable scientific attention because of the increase of the number of species producing domoic acid (Teng et al., 2014; Sakka Hlaili et al., 2016). This neurotoxin is responsible for Amnesic Shellfish Poisoning (ASP) and thus blooms of *Pseudo-nitzschia* represent serious threats to the environment, aquaculture shellfish industries and human health. Unfortunately, blooms of the toxic diatoms are increasing in frequency and magnitude in several coastal environments (Loureiro et al., 2009; Giménez et al., 2013). For example, in Mediterranean coastal water (Bizerte lagoon, North of Tunisia), *Pseudo-nitzschia* spp. bloomed at high levels in different periods of the year ($> 10^6$ cells l^{-1}; Bouchouicha Smida et al., 2014; Sakka Hlaili et al., 2016). Since coastal waters can be severely contaminated by many substances, the impact of chemical pollutants on the occurrence of *Pseudo-nitzschia* species and their toxicity has been suspected (Trainer et al., 2012). Interestingly, a recent work showed that resuspension of contaminated sediment caused a shift in the phytoplankton community of the Thau lagoon, with dominance of *Pseudo--nitzschia* spp after contamination (Ben Othman et al., 2017). Moreover, toxic diatoms are known for their resistance to metal pollutants, such as copper and iron (Lelong et al., 2012). These species can use complexation strategy by increasing domoic acid production to bind metal out of the cell. The PAH response of several diatom species (*Nitzschia brevirostris Cyclotella caspia, Phaeodactylum tricornutum* and *Thalassiosira pseudonana*) were reported in field and laboratory studies (Hong et al., 2008; Croxton et al., 2015; Niehus et al., 2018). Nevertheless, there remains a considerable lack on data concerning potentially toxic diatoms, as *Pseudo nitzschia***.** Indeed, the majority of research has focused on trace metal pollution as a potential cause of domoic acid production by *Pseudo-nitzschia* species. This toxin plays the role of chelator for metal and thus can participate in increasing the bioavailability of a trace element (as Fe) or decreasing the availability of a toxic metal (as Cu) (Maldonado et al., 2002; Lelong et al., 2012; Trainer et al., 2012). As observed for trace metals, the eco-toxicology impact of organic pollutants on potentially toxic diatoms must be well understood and described in order to improve our knowledge of the driving factors regulating their blooms and their toxicity. In this context, our study aims investigating the responses to PAHs stress of the two species, *P. hasleana* and *P. mannii,* recently observed in SW Mediterranean Sea (Bizerte lagoon), where PAHs contamination has been reported (Lafabrie et al., 2013; Bancon-Montigny et al., 2019). In polluted marine environments, PAHs are commonly encountered in mixture and can act in an antagonistic or synergistic manner. Moreover, separate PAHs and PAHs in mixture do not usually induce the same effects and thresholds on algal species (Lei et al., 2007; Echeveste et al., 2010a, 2010b). Indeed, complex mixture of PAHs could induce more toxic impact on phytoplankton than single compound (Echeveste et al., 2010a, 2010b). Thus, the specific objectives of this study were (i) to assess the impact of increasing concentrations of a PAHs mixture on growth, photosynthetic parameters and biovolume of *Pseudo-nitzschia mannii* and *P. hasleana*, (ii) to assess the possible link between PAH contamination and the toxigenic potential of these harmful algae, and (iii) to examine their ability for biodegradation and bioaccumulation of these pollutants. For that purpose, we conduct laboratory experiments with cultures of *P. hasleana* and *P. mannii* exposed, during 144 h, to a PAHs mixture of 15 PAHs, which are included in the US Environmental Protection Agency's list of priority pollutants (US-EPA).

2. Materials and methods

2.1. Isolation, identification and culture of phytoplankton species

Using a 20 μm mesh plankton net, strains of diatoms were collected from the Bizerte lagoon. The two *Pseudo-nitzschia* species were purified and cultured in f/2 medium (Guillard, 1975) under non-axenic conditions. The cultures were maintained in a thermostatic chamber at 22 °C illuminated with cool-white fluorescent tubes at light intensity of 100 μmol photons m^{-2} s^{-1} and under a photoperiod of 12 light:12 dark. The identification of species was done using a transmission electron microscopy (TEM). Details were reported in Melliti Ben Garali et al. (2016).

2.2. Preparation of PAH mixtures

A previous record showed the presence of 15 PAHs (anthracene, pyrene, benzo(a)pyrene, benz(a)anthracene, chrysene, fluoranthene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, benzo(b)fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, acenaphthene, fluorene and dibenz(a,h)anthracène) in the sediments of the Bizerte Lagoon. The specific in situ concentrations of each PAH were reported in Bancon-Montigny et al. (2019). The specific in situ concentration of each PAH were reported in Table 1. Based on these concentrations, stock solutions of each type of PAH were prepared by dissolving pure chemicals in reagent grade dimethylsulfoxide (DMSO, 0.05%) without exceeding the solubility threshold.

Then, the various stock solutions were mixed to obtain a first mixture solution containing 4% of every type of pure PAHs (dissolved concentration in wt/vol compare to the actual PAH concentrations in sediment in wt/wt). Finally, this solution (of 4% of PAHs mixture) was diluted with the DMSO to obtain three other cocktail solutions with 2%, 0.5% and 0.1% of PAHs. The choice of 4 percentages of present PAHs was arbitrary. The PAHs solutions were kept in dark and cold (4 °C) until the day of their uses during the contamination experiment. All chemicals (DMSO and PAHs; purity > 97%) were purchased from Sigma-Aldrich.

2.3. Experimental set-up

For each *Pseudo-nitzschia* species, 12 glass Erlenmeyer flasks, containing 2 L culture medium, were prepared and autoclaved. Before their use the flasks have been rinsed with 10% HCl solution. After sterilization, 3 μl of each PAH stock solution (see before) were added to three Erlenmeyer flasks to obtain four contaminated treatments in triplicates; C1, C2, C3 and C4 which received respectively 0.1%, 0.5%, 1% and 4% of PAH cocktails. Initial concentration of individual PAHs as well as properties of each one are reported in Table 1. Three flasks without PAHs were used as control treatment (C) and three others received only DMSO (at 0.05% vol/vol). This control treatment with DMSO (C_{DMSO}) was prepared to test its potential toxicity on the diatom species. *Pseudo-nitzschia* cells in exponential growth phase were then inoculated to each flask to get an initial concentration of 27 10^7 cells l^{-1}. The 36 Erlenmeyer flasks, prepared for both species, were finally incubated for 144 h in a thermostatic chamber under the same environmental conditions described above. The light intensity and the temperature within the incubator were checked throughout the experiment using a spherical quantum mini-recorder LI-250 A and Thermometer (Model TH-020), respectively. Sub-samples were taken from all treatments during incubation period for several measurements, as described below.

Table 1
Properties of PAHs used in experimentation, in situ concentrations in sediment and initial concentrations in deferent mixtures.

PAH name	Concentrations in sediment (mg kg^{-1})[a]	Initial concentrations (µg l^{-1})				Properties		
		4%	1%	0.5%	0.1%	MW[b]	SW[c] (mg l^{-1})	N[d]
Anthracene	22.2–85.5	1.180	0.295	0.147	0.029	178.24	0.434	3
Benzo(a)anthracene	151.2–616.4	5.441	1.361	0.680	0.136	228.30	0.0094	4
Benzo(a)pyrene	51.0–788.1	6.721	1.680	0.840	0.168	252.32	0.0016	5
Benzo(b)fluoranthene	191.3–1048.0	4.560	1.141	0.571	0.114	252.31	0.0008	5
Benzo(g,h,i)perylene	199.9–598.8	3.400	0.850	0.425	0.085	276.34	0.0003	6
Benzo(k)fluoranthene	33.2–165.2	3.308	0.827	0.413	0.082	252.32	0.0008	5
Chrysene	147.9–619.5	5.240	1.310	0.655	0.131	228.30	0.002	4
Fluoranthene	168.5–537.4	10.060	2.650	1.325	0.265	202.26	0.26	4
Indeno (1,2,3-cd)pyrene	185.6–558.7	4.160	1.040	0.520	0.104	276,33	0,0025	6
Naphtalene	47.7	0.377	0.094	0.047	0.009	128.18	31	2
Phenanthrene	234.6–342.7	4.400	1.100	0.550	0.110	178.24	1.15	3
Pyrene	141.1–492.6	8.200	2.050	1.025	0.205	202.26	0.135	4
Acenaphthyene	10.4–26.1	0.236	0.059	0.029	0.005	152.20	16.1	3
Fluorene	22.6−32.4	0.273	0.068	0.034	0.006	166.23	1.69	3
Dibenzo(a,h)anthracene	121.8–191.0	1.524	0.381	0.190	0.038	278.36	0.0002	5

[a] From Bancon-Montigny et al. (2019).
[b] Molecular weight.
[c] Water solubility.
[d] Number of benzene rings.

2.4. Sampling and analyses

2.4.1. PAH measurement

PAHs were analyzed in all treatments at the beginning, 72 h and 144 h and every day in treatment C3. Samples were filtered on glass fiber filters and the filtrates were used to analyze the dissolved phase PAHs in *Pseudo-nitzschia* culture. For this, each filtrate sample was gathered by solid-phase extraction (SPE) with a C18 column (preconditioned with 10 ml dichloromethane (DCM), 10 ml methanol and 10 ml ultrapure water two times) at a flow rate of 5 ml min^{-1}. Then, PAHs were eluted from the column with 12 ml DCM. The extraction was concentrated to 0.5 ml using Termovap Sample Concentrator in a water bath (45 ℃) and analyzed with GC-MS. To analyze particulate PAHs, the GF/F glass fiber filters were freeze-dried and weighed. The dried filters were extracted with 150 ml of the mixed solvent n-hexane and DCM (1:1, vol/vol) for 24 h. Extractions were purified by a self-packet silica-alumina column (from top to bottom: anhydrous sodium sulfate, 1 cm; the mixture of silicone and n-hexane, 10 cm; anhydrous sodium sulfate, 1 cm; a few glassfiber). Then, The PAHs eluent was concentrated to 0.5 ml for GC-MS analysis (Lei et al., 2007). The method detection limits (MDLs), defined as mean blank value + 3 × standard deviation (SD), ranged from 0.04 to 3 ng ml^{-1} for individual PAHs.

2.4.2. Domoic acid measurement

A certificate calibration solution of DA (CRM-DA-f, 101.8 ± 2.1 µg ml^{-1}) was obtained from the Measurement Science and Standards, National Research Council of Canada. HPLC gradient grade and LC-MS hypergrade acetonitrile, methanol and formic acid were purchased from Merck (Darmstadt, Germany). Milli-Q water was obtained from a Millipore water purification system (Bedford, MA, USA).

For particulate domoic acid (pDA), samples (100 ml) were taken at the beginning and the end of incubation and filtered through GF/F filters (Whatman) and processed according to a rapid resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (de la Iglesia et al., 2008), with slight modifications (see Melliti Ben Garali et al., 2016). The instrumental limit of quantification (LQA) was 0.1 ng ml^{-1}.

2.4.3. Chlorophyll a analysis and cell counting

Samples (20 ml) were taken every day and filtered on Whatman GF/F filters. Chl *a* was extracted at 90% acetone (vol/vol) for 30 h in the dark at 4 °C. The pigment concentration was measured using the spectrophotometric method and following the procedure given by (Parsons et al., 1984).

Other samples (1 ml) were taken every day of the incubation and were fixed with Lugol's acid solution (3% final concentration). Cell counting was performed in triplicate, on aliquots of 5 µl, deposited between slide - coverslip and observed under light microscopy BX-102 A at ×40 magnification (Lundholm et al., 2004).

2.4.4. Photosynthetic efficiency of PSII

Samples (10 ml), collected throughout the experiment, were incubated for 20 min in the dark. The maximum quantum efficiency of PSII (Fv/Fm) was analyzed by Aqua-Pen C (manufactured by Photon System Instruments, www.psi.cz). A blue LED (450 nm) excites a sample of suspended cell by a saturating flash (2000 µE m^{-2} s^{-1}). The induction of chlorophyll fluorescence (680 nm) was then measured for 1 s at a time scale from 10 to 100 µs, depending on OJIP protocol of the manufacturer. This protocol allows the parameters F0 and Fm to be obtained, which represent, respectively, the minimum and maximum yield of Chl *a* fluorescence measured on dark-adapted samples. Then, both parameters were used to calculate the potential photosynthetic efficiency (Fv/Fm, with Fv = Fm – F0).

2.5. Calculation

2.5.1. Growth rate

For the various treatments, the net growth rates of *Pseudo-nitzschia* species were estimated using the exponential model of (Landry and Hassett, 1982),

$$N_t = N_0\, e^{\mu t} \quad (1)$$

$$\mathrm{Ln}\ (N_t / N_0) = \mu\, t \quad (2)$$

Where N_0 and N_t were the abundance of *Pseudo-nitzschia* at the beginning and at 72 h of incubation, respectively; µ (d^{-1}) is the net cell growth rate; and t (d) is the incubation time. µ is the slope of the line from the linear regression of ln (N_t/N_0) against time of the form y = a x.

2.5.2. Biovolume

The length (L, µm) and the width (W, µm) of diatoms (at least 50 cells for each species) were measured using a calibrated ocular micrometer and cell volumes (V, $µm^3$) were calculated by applying standard geometric formulae to each species, as proposed by Hillebrand et al. (1999) and modified by Lundholm et al. (2004). We assumed that *P. mannii* and *P. hasleana* have a shape of a prism on a parallelogram-based:

$V\ (\mu m^3) = (0.6 \times L \times W^2) + (0.4 \times 0.5 \times W^2)$

2.5.3. Percentages of PAHs accumulation, biodegradation and in the medium

The percentages of dissolved PAHs (i.e. in the medium, $\%PAH_{diss}$) and accumulated in the cells ($\%PAH_{acc}$) as well as degraded ($\%PAH_{deg}$) were calculated as follows (Chan et al., 2006),

$\%PAH_{diss}$ = (amount of PAHs remaining in the medium/amount of PAHs added) x 100%

PAH_{acc} = (amount of PAHs accumulated in the cells/amount of PAHs added) x 100%

PAH_{deg} = (amounts of PAHs added – amount of PAHs remaining in the medium - amount of PAHs accumulated in the cells)/amount of PAHs added x 100%

2.5.4. Ecotoxicological values

EC50s (concentrations inducing a response halfway between the control and the maximum toxicity effect) were determined using the REGTOX macro for Excel developed by E. Vindimian (Arzul et al., 2006). Optimal EC50s were selected and 95% confidence limits were given for each calculated value.

2.6. Statistical analysis

A two-factor parametric variance analysis (ANOVA) (treatment and time) was used to test the effect of PAH contaminations on all variables measured for both species. When the effect was significant, a multiple comparison posterior test (Tukey test) was performed to identify the significant contamination relative to control. A two-factor ANOVA (species and time) was also used to compare the decrease in biomass and cell abundance and percentage of PAH accumulation and degradation between the two species. A student test was used to compare the cellular biovolumes of species after 144 h relatively to the initial value in each treatment. When the normality of the data distribution (Kolmogorov-Smirnov test) and/or the homogeneity of the variances (Bartlett-Box test) were not verified, a non-parametric ANOVA analysis (Kruskal-Wallis) or a non-parametric student-test (test z) was used. All statistical analyzes were performed using SPSS 18.0 Software.

3. Results

3.1. Impact of PAHs on growth of Pseudo-nitzschia species

The cell abundances of *Pseudo-nitzschia* species and Chl *a* concentrations measured throughout the incubation did not vary significantly between DMSO treatment (C_{DMSO}) and control C (Fig. 1). Furthermore, both species showed similar growth rates (Table 2) between the two control treatments, their abundance increasing gradually throughout the experiment. For the four PAH concentrations tested, the diatoms were able to maintain continuous growth throughout the incubations, but with significant ($p < 0.05$) lower rates compared to the control (Table 2). For both species, the PAH-treated cells did not totally lose their viability as fluorescence was still observed. However, the diatom abundance was significantly reduced ($p < 0.05$) compared to that of the control (Fig. 1). A dose-dependent effect was observed, as the decrease of Chl *a* and cell abundance was inversely proportional to the level of contamination (Fig. 1). The percentages of inhibition increased with the incubation time. The treatment C1 induced a final reduction in Chl *a* of

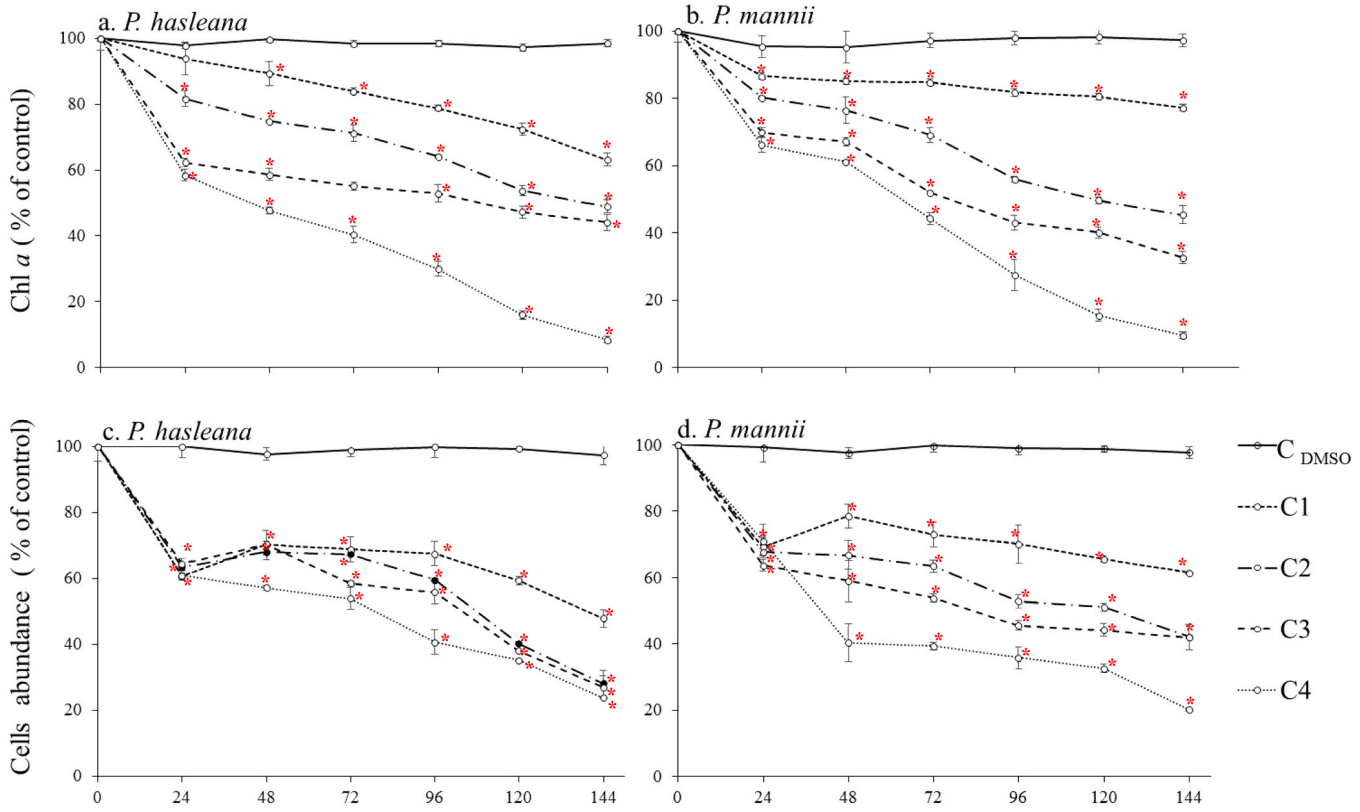

Fig. 1. Evolution of Chl *a* concentrations (a, b) and cell abundances (c, d) in contaminated treatments [C1: 0.1% (3 µg l^{-1}); C2: 0.5% (15 µg l^{-1}); C3: 2% (30 µg l^{-1}); C4: 4% (120 µg l^{-1})] relatively to the control (C) during the experiments performed for *P. hasleana* and *P. mannii* (Mean ± SD). *significantly ($p < 0.05$) lower than the control (C) based on Tukey test.

Table 2
Exponential growth rate (d^{-1}) of *P. hasleana* and *P. mannii* in the controls (C and C_{DMSO}) and contaminated treatments with PAH cocktails [C1: 0.1% (3 μg l^{-1}); C2: 0.5% (15 μg l^{-1}); C3: 2% (30 μg l^{-1}); C4: 4% (120 μg l^{-1})] (Mean ± SD, n = 3). *Significantly lower ($p < 0.05$) than control (C) based on Tukey test.

	C	C_{DMSO}	C1	C2	C3	C4
P. hasleana	2.04 ± 0.01	1.80 ± 0.04	0.83 ± 0.02*	0.53 ± 0.00*	0.55 ± 0.09*	0.39 ± 0.02*
P. mannii	1.83 ± 0.01	1.85 ± 0.03	1.08 ± 0.02*	0.82 ± 0.05*	0.66 ± 0.09*	0.44 ± 0.02*

40% and 23% for *P. hasleana* and *P. mannii*, respectively. In the other treatments, Chl *a* decreased rapidly, reaching 50–90% less than control at the end of incubation (Fig. 1a, b). The lowest PAH contamination (i.e. C1) was followed by 35% final decrease of *P mannii* abundance, but > 50% for *P. hasleana* (Fig. 1c, d). Similarly, the C2 and C3 treatments induced more reduction in final cell concentrations ($p < 0.05$) for *P. hasleana* (75%) than *P. mannii* (55%). At the end of the C4 contaminations, cell reductions of 79% was noted for *P. hasleana* and *P. mannii*, which proliferated four and three times less than control (Table 1).

3.2. Impact of PAHs on the physiology of Pseudo-nitzschia species

As observed for growth, 0.05% DMSO did not have a significant effect on the efficiency of the PSII, since similar values of Fv/Fm were measured in control with or without DMSO throughout the incubation (Fig. 2). When *P. hasleana* was exposed to C1 and C2 contaminations, the Fv/Fm did not show a significant difference ($p > 0.05$) compared to control. On the contrary, under higher contamination (C3, C4), the Fv/Fm dropped abruptly during the first 24 h, with a 100% reduction compared to the control (Fig. 2a). For *P. mannii*, only the highest contamination (C4) induced a negative effect on the PSII efficiency. During the first 48 h of this treatment, the Fv/Fm was reduced by 24% relatively to the control, but by 100% after 72 h (Fig. 2b). The EC50s measured for this photosynthesis parameter after 72 h of contamination were 135 and 115 μg PAHs l^{-1} for *P. mannii* and *P. hasleana*, respectively.

The two species showed similar cell biovolume (≈ 210 μm^3) in both control treatments (C and C_{DMSO}) at the beginning (0 h) and at the end (144 h) of the incubation. After 144 h of PAH exposure, the biovolumes of both species were significantly enhanced (Fig. 3). This increase was more pronounced in *P. mannii* (16–35% of the initial volume) than in *P. hasleana* (11–17% of initial volume). Note that for each species, the

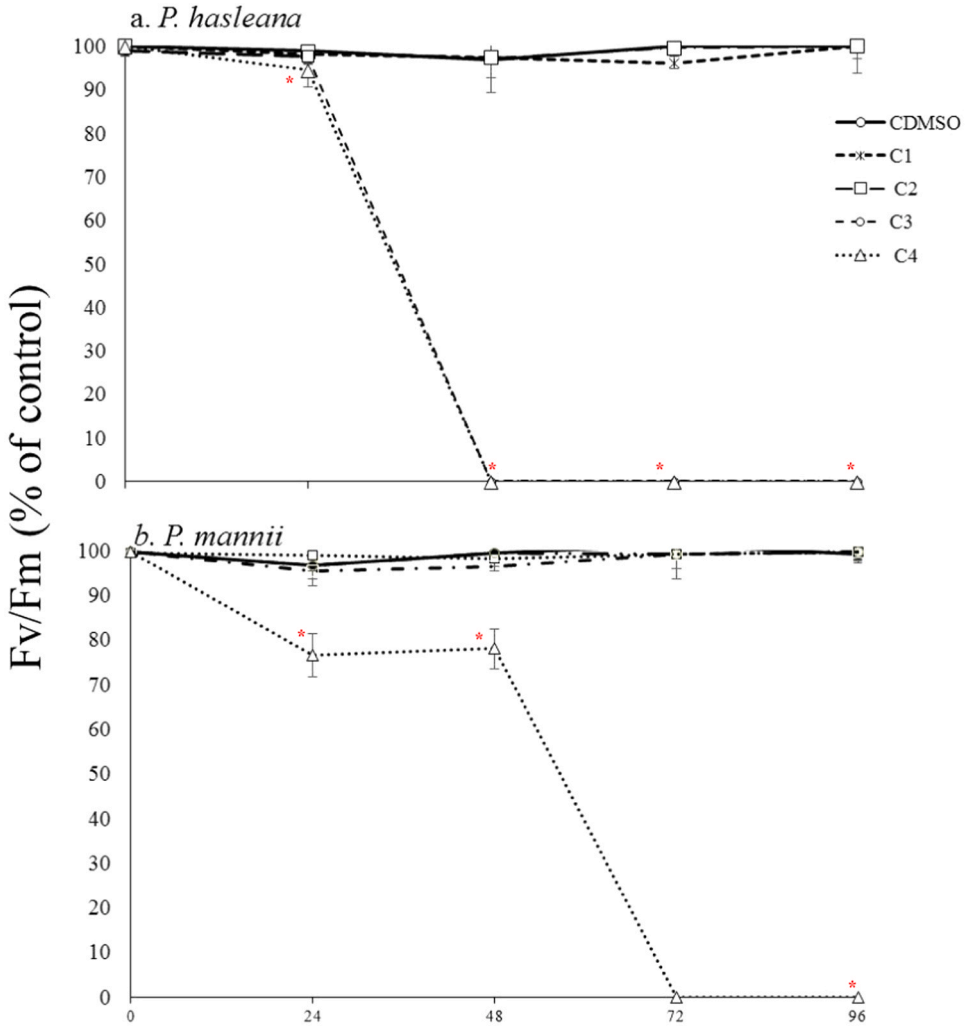

Fig. 2. Evolution of the efficiency of photosystem II (Fv/Fm) in contaminated treatments [C1: 0.1% (3 μg l^{-1}); C2: 0.5% (15 μg l^{-1}); C3: 2% (30 μg l^{-1}); C4: 4% (120 μg l^{-1})] relatively to the control (C) during the experiments performed for *P. hasleana* and *P. mannii* (Mean ± SD). *significantly ($p < 0.05$) lower than the control (C) based on Tukey test.

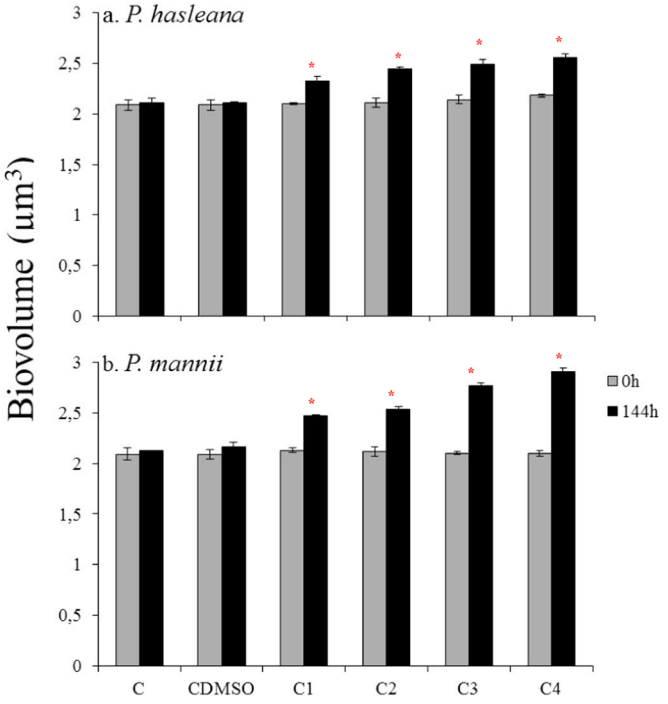

Fig. 3. Effects of PAH cocktails on cellular biovolume of *P. hasleana* (a) and *P. mannii* (b) in different treatments [C; C_{DMSO}; C1: 0.1% (3 µg l^{-1}); C2: 0.5% (15 µg l^{-1}); C3: 2% (30 µg l^{-1}); C4: 4% (120 µg l^{-1})] (Mean ± SD). *significant increase ($p < 0.05$) after 144 h based on Student test.

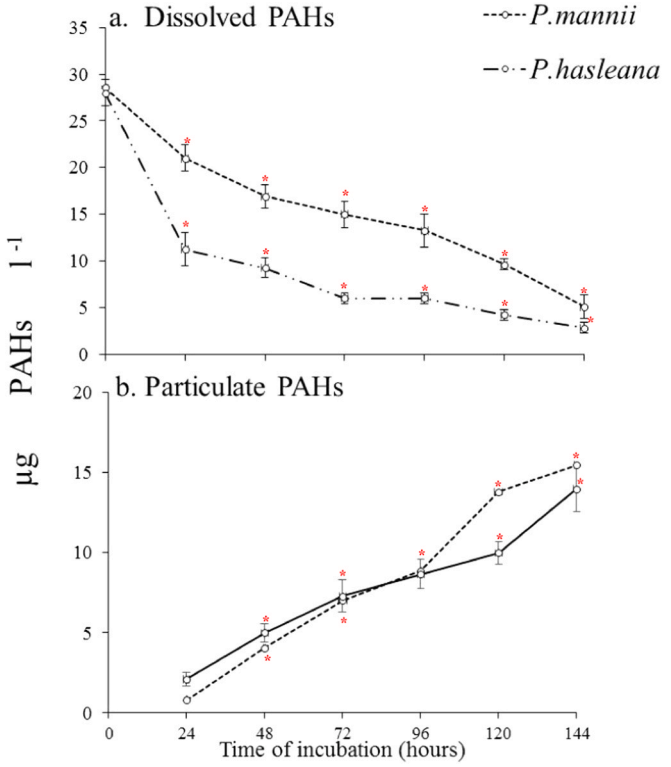

Fig. 4. Concentration of dissolved (a) and particulate PAHs (b) in the treatment C3 contaminated by 2% of PAHs cocktail (means ± 91% confidence intervals, n = 3). *significant decrease/increase ($p < 0.05$) from the initial value based on two-factor ANOVA.

biovolume has increased proportionally with the level of contamination.

Each *Pseudo-nitzschia* strain was tested negative for the production of domoic acid. The concentrations of this neuro-toxin measured at the end of the incubation in the different contaminations including the control were below the instrumental limit of quantification (LQA = 0.1 ng ml^{-1}).

3.3. Fate of PAHs during incubation

The measured concentrations of the sum of PAHs in each treatment at the beginning of the experiment were not significantly different to the nominal expected concentrations (Table 1). Concentration were close to 95% of the theoretical concentrations. In addition, the measured and the theoretical concentrations were proportional with a slope close to 1 (0.983 and 0.996 for *P. hasleana* and *P. mannii,* respectively) for the concentration range used (n = 12). Therefore, the measured concentrations were used in the remainder of the results.

The daily monitoring of PAHs in the C3 treatment showed a gradual drop in dissolved PAH concentrations from the beginning (28–28.7 µg l^{-1}) to the end of incubations (Fig. 4a). Despite this gradual decrease, the final concentrations of dissolved PAHs were 2.3–5.1 µg l^{-1}, indicating that both *Pseudo-nitzschia* species were maintained in contaminated media until the end of the experiments. Concomitantly, concentrations of particulate PAHs increased throughout the experiments (from 2.1 to 14 µg l^{-1} for *P. hasleana* and from 0.8 to 15.5 µg l^{-1} for *P. mannii*) (Fig. 4b). Significance difference ($p < 0.05$) was observed between both species for the gradual decrease of PAH in the dissolved phase, with a higher rate for *P hasleana* (Table 3).

In each treatment, the percentage of dissolved PAHs (%PAH_{diss}), accumulated PAHs (%PAH_{acc}) and degraded PAHs (%PAH_{deg}) varied significantly throughout the incubation time for both species (Fig. 5). The % PAH_{diss} dropped significantly ($p < 0.05$) from the start to the end of incubation, in all treatments. It decreased from 100% to 21–45% and to 17−29% for *P. hasleana* and *P. mannii*, respectively (Fig. 5b). In all treatments, there were more PAHs accumulated in the cells at 144 h

Table 3
Theoretical and measured concentrations (µg l^{-1}) of PAHs mixtures in *Pseudo-nitzschia* cultures (n = 3).

	Theoretical concentrations			
	C1	C2	C3	C4
	3	15	30	120
Measured concentrations				
Culture of *P. hasleana*	2.1 ± 0.1	13.8 ± 0.7	28.0 ± 1.2	117.0 ± 3.5
Culture of *P. mannii*	2.4 ± 0.2	13.7 ± 0.5	28.7 ± 2.3	118.6 ± 4.4

(30–57% and 25–59%) than at 72 h (12–26%and 10–35%) (Fig. 5a). The PAH degradation generally increased over time in most treatments and for both species. The highest %PAH_{deg} (51%) was observed for *P. mannii* contaminated by C4 cocktail at the end of incubation (Fig. 5c).

4. Discussion

4.1. Impact of PAHs on the growth of Pseudo-nitzschia species

The DMSO concentration (0.05%) used for the dissolution of PAH did not affect cell growth and physiology, since specific growth rates and Fv/Fm values in C and C_{DMSO} treatments were not significantly different (Table 2). This was already observed in previous ecotoxicological studies with phytoplankton culture exposed to PAH contamination (Ben Othman et al., 2018).

The contamination of the two species by the different concentrations of the PAH cocktails induced a decrease in their growth (Table 2) resulting in a significant reduction in their biomasses and abundances (Fig. 1). A previous work on the toxicity of PAHs mixtures on natural phytoplankton communities from Bizerte and Thau Lagoons has shown pronounced changes in algal composition and significant decrease in

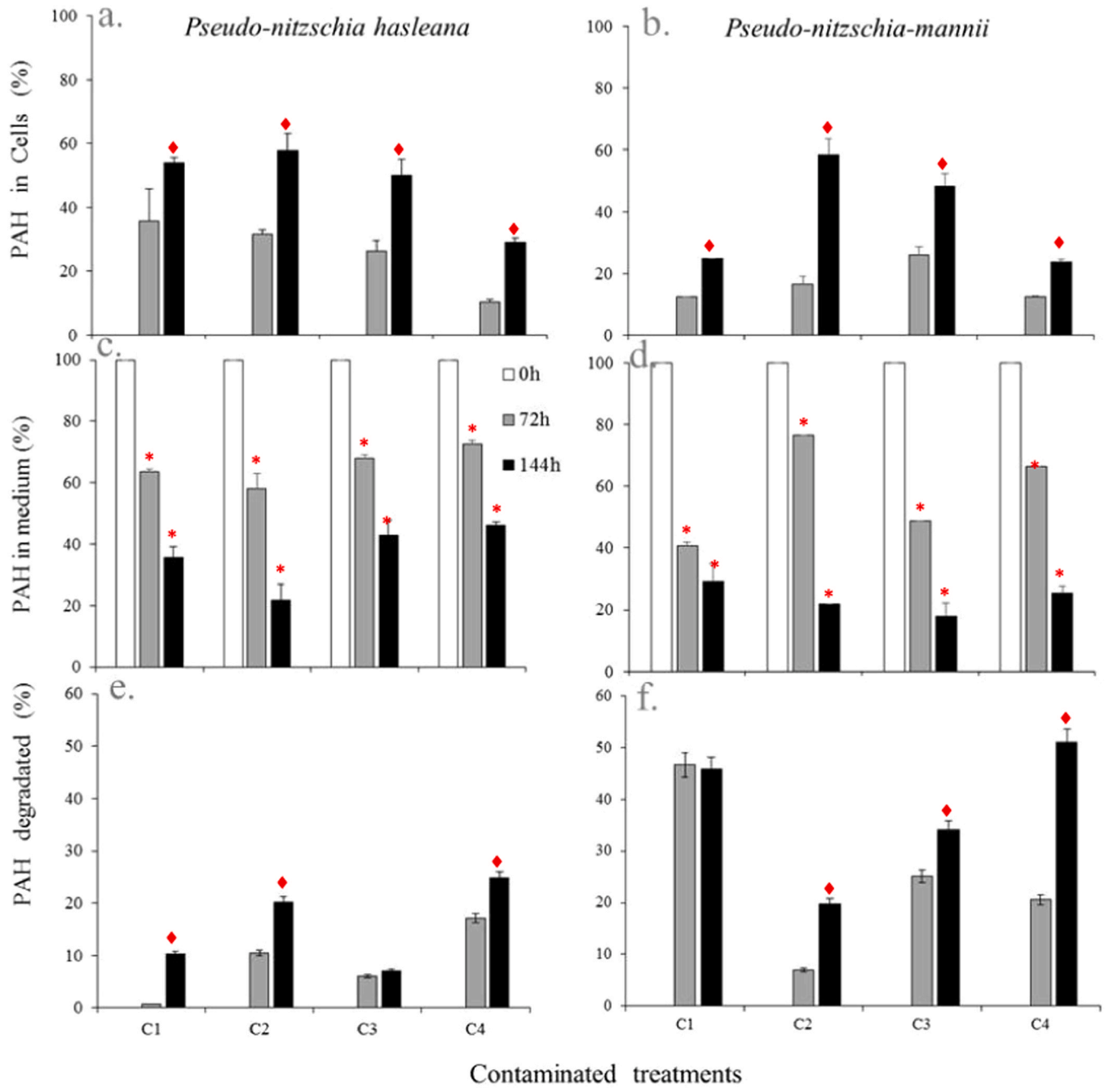

Fig. 5. Percentages of PAHs accumulated in the cells (PAH_{acc}, a), remaining dissolved in the culture medium (PAH_{diss}, b) and degraded (PAH_{deg}, c) in the *P. hasleana* and *P. mannii* cultures over time in the different contaminated treatments [C1: 0.1% (3 μg l^{-1}); C2: 0.5% (15 μg l^{-1}); C3: 2% (30 μg l^{-1}); C4: 4% (120 μg l^{-1})] (Mean ± SD). *significant decrease ($p < 0.05$) from the initial value (at 0 h), ◆significant increase ($p < 0.05$) from the value at 72 h based on two-factor ANOVA.

phytoplankton abundance (Ben Othman et al., 2018). Our results are also consistent with other ongoing work on the adverse effects of PAHs in mixture on phytoplankton in general (Djomo et al., 2004; Vieira and Guilhermino, 2012) and more particularly on diatoms (Nayar et al., 2005). It seems that single PAHs or in mixture can strongly repress genes of diatoms involved in the transport of silicon and the formation of silica shell (Bopp and Lettieri, 2007; Carvalho et al., 2011). This can provoke the alteration of the cell membrane by these molecules and therefore their penetration into the cytoplasm, resulting in a decrease in cell growth.

The negative effect of PAHs was dose-dependent, as both species proliferated with rates that were lower as the level of contamination increased (Table 1). In addition, decrease in Chl *a* and cell abundance was proportional to the PAH concentrations (Fig. 1). The dose-dependent impact of PAHs on microalgae has been previously reported by several authors (Echeveste et al., 2010a, 2010b; Ben Othman et al., 2018). Previous work that examined the impact of increasing concentrations of PAH cocktails on different microalgal species, often reported complete inhibition of phytoplankton growth and even lethal status under high levels of contamination (Ben Othman et al., 2018). This was not observed during our experiment, indeed, both species-maintained growth even under the highest concentration of PAHs tested (i.e. C4). Moreover, after 144 h, there were approximately 50 10^6 ml^{-1} cells of each species in the C4 microcosms, suggesting that *P. mannii* and *P. hasleana* can tolerate these organic pollutants. Moreover, the PAH cocktails used in the experiment were mainly dominated by

fluoranthene and pyrene, which could be less toxic than others PAHs. However, the first species seemed to be a little more tolerant than the second. *P mannii* had lower initial growth rate but proliferated a little more quickly under contaminations than *P. hasleana.* The *P. mannii* growth was less reduced by the high contaminations (three times relatively to control) than that of *P. hasleana* (four times relatively to control) (Table 2). Furthermore, the C1, C2 and C3 treatments, induced significant lower decrease in cell abundance for *P. mannii* than *P. hasleana* ($p < 0.05$; Fig. 1). The tolerance of both *Pseudo-nitzschia* species to various PAH cocktails and the difference in the degree of this tolerance between them may be related to physiological adaptations that allowed the microalgae to overcome the toxic effects of PAHs.

4.2. Impact of PAHs on physiology of Pseudo-nitzschia species

In addition to significant toxic effects on biomass and growth, our results revealed that PAHs had also an impact on the physiology of both species, as the effectiveness of their photosystem II was negatively affected by PAH exposure. However, the effects was variable among PAH cocktails and between species. The treatments C1 and C2 did not elicit by any significant effect ($p > 0.05$) on the Fv/Fm variable, suggesting that both species were physiologically unaffected (Fig. 2). In contrast, the highest contaminations (i.e. treatments C3 and C4) resulted in a sharp drop in the maximum quantum yield of the PSII during the first 48 h (Fig. 2). These results are consistent with those of other studies showing that pronounced PAH cocktail concentrations (i.e. 14–75 μg l^{-1}) strongly reduce the photosynthetic efficiency of phytoplankton (Echeveste et al., 2010a, 2010b; Ben Othman et al., 2018). Hydrocarbons, known by their hydrophobic character, tend to accumulate in chloroplasts whose structure is rich in lipids (Sorigué Damien et al., 2016). These pollutants can be introduced by altering the membrane of chloroplasts (Patrick et al., 2007), then that of thylakoids, where the PSII is located (Sargian et al., 2005). By subsequently interfering with photosynthetic electron transport (Duxbury et al., 1997), PAHs can affect the efficiency of PSII. Nevertheless, diatom cells are physiologically able to tolerate low PAH concentration, by increasing, for example, the PSII absorption cross-section (increase in the size of the antennas) in order to maintain an efficient photosynthetic function (Moore et al., 2006). This mechanism of adaptation can be envisaged to explain the lack of significant impact observed for the low concentration (C1 and C2) in both species.

For *P. mannii*, the photosynthetic efficiency was reduced only by the highest PAH level (C4 contamination) and was totally deteriorated after 48 h, whereas for *P. hasleana*, even the C3 treatment provoked a marked fall and total deterioration in the first 24 h (Fig. 2). In addition, the measured EC50 for Fv/Fm was higher for *P. mannii* (135 μg PAHs l^{-1}) than for *P. hasleana* (115 μg PAHs l^{-1}). This suggests that *P. mannii* was physiologically better adapted to PAHs, which may explain the less inhibition of final abundance and growth observed for *P. mannii* (Table 2, Fig. 1). The measured EC50 exceed the concentrations found in the water of the Bizerte lagoon (Bancon-Montigny et al., 2019). However, high amounts of PAHs could be released from sediment resuspension (Cornelissen et al., 2008), or deposited by atmospheric aerosol (Barhoumi et al., 2018) or loaded by oil spill (Wang et al., 2014). All these phenomena could lead to the contamination of water column by PAHs, which would be toxic for algal species.

Cell biovolume has been identified as a factor in the resistance or sensitivity of species to PAHs (Echeveste et al., 2010a, 2010b; Bretherton et al., 2020). The *Pseudo-nitzschia* species have increased their biovolumes after 144 h' exposure to all PAH cocktails. This increase was all the more important as the level of contamination increased (Fig. 3). The exposure of diatoms to abiotic stresses (like PAH contaminations) could boots lipid synthesis and yield (Zulu et al., 2018), leading to an increase in cell size to reduce the toxicity of PAHs. The size enhancement strategy allows the large microalgae to better tolerate PAHs (Ben Othman et al., 2012) and hence to maintain their growth under contamination by toxic molecules. The biovolume of *P. mannii* increased from 16% to 35% compared to the baseline, whereas the increase in *P. hasleana* was significantly lower (11–17%). This could therefore reinforce the good tolerance of *P. mannii* to PAHs compared to that of the other species.

The production of domoic acid by *Pseudo-nitzschia* species can be involved in their resistance against metallic contaminants (Lelong et al., 2011). This amino acid can chelate ETMs and subsequently decrease their bioavailability for diatoms, thereby reducing metal toxicity to cells. Unlike ETMs, *Pseudo-nitzschia* defense strategies against organic pollutants are not known and the role that DA could play in this resistance is still unknown. Strains of *P. mannii* and *P. hasleana*, used in our experiments, were tested negative for the production of DA. The monitoring of this toxin during the incubation showed that the species remained non-toxic throughout the experiment under the different contaminant concentrations. This indicates that either the two species were physiologically and genetically incapable of producing DA, or that even though they had the toxic potential, PAH contamination did not stimulate DA production. Consequently, the release of DA by the diatoms can't be envisaged as a potential strategy to face PAH contamination.

4.3. Accumulation and degradation of PAHs by Pseudo-nitzschia species

In addition to their physiological adaptation, the *Pseudo-nitzschia* species could tolerate PAHs thanks to their ability to remove these toxic compounds. Effectively, both species were capable to uptake the PAHs from the culture medium and accumulating them, as the percentages of PAHs remaining in each treatment decreased in parallel with the increase in accumulation percentages (Fig. 5). Furthermore, under C3 contamination, concentrations of particulate PAHs increased over days in a linear pattern (Fig. 4b). This is in agreement with previous reports of the ability of several microalgal species, including diatoms to accumulate several individual PAHs or in mixture (Hong et al., 2008; González-Gaya et al., 2019). *P. mannii* showed a slight lower accumulation than *P. hasleana* (Fig. 5a). The bioaccumulation of organic contaminants by microalgae can be related to the initial cell concentration used in the inoculum (Chan et al., 2006). In our experiment, the same cell density was used for both species at the beginning of incubation. The bioaccumulation of PAHs is however species-dependent (Lei et al., 2007), since the size and shape of cells could affect the adsorption and accumulation of these molecules. So, *P. mannii,* by increasing much more its biovolume in all contaminations treatments (Fig. 3) relative to *P hasleana*, could limit the PAH absorbance, resulting in less accumulated PAHs.

In the C3 treatment, a gradual drop in dissolved PAH concentrations was observed over time in cultures of both species (Fig. 4a). Several abiotic processes, including volatilization, photooxidation and adsorption could contribute to PAH elimination (Lekunberri et al., 2010; Ghosal et al., 2016), but the biodegradation may be the main factor of PAHs removal in microalgae culture (Hong et al., 2008; Kumari et al., 2016; García de Llasera et al., 2017). Phytoplankton, included diatoms, had the ability to degrade individual and mixed PAHs (Hong et al., 2008; Echeveste et al., 2010a, 2010b; González-Gaya et al., 2019; Liu et al., 2019) and bacteria are the dominant biodegraders of PAHs (Jiménez et al., 2011). Moreover, hydrocarbonoclastic bacteria were commonly found associated with diatoms in culture (attached or free-living) (Thompson et al., 2018, Severin et al., 2019). Thus, there may be a metabolic synergy between the Pseudo-nitzschia and their associated bacteria in the degradation of PAHs (Kumari et al., 2016), as the cultures used in the present study were not axenic. Moreover, our previous work showed that a diatom and associated bacteria, isolated from the Bizerte lagoon, were efficient in the degradation of benzo(a)pyrene and fluoranthene (Kahla et al., 2021), both PAHs are among the most molecules present in the cocktails used in this study (Table 1). The biodegradation was significantly higher for *P. mannii* than *P hasleana*, ($p < 0.05$,

Fig. 5c). This difference may be linked to the change in the composition of associated bacteria between the two *Pseudo-nitzschia* species, as the bacterial community structure associated to microalgae is known to be species-dependent (Bagatini et al., 2014; Severin and Erdner, 2019).

5. Conclusion

The study provided new data on PAH toxicity on potentially toxic diatoms. Exposure of non-axenic cultures of *P. mannii* and *P. hasleana* during 144 h to increased concentrations of PAH mixture induced significant decrease in cell growth, biomass and cell abundance of both species and an alteration of their photosynthetic efficiency. These effects were dose-dependent, with a more pronounced decrease as the level of contamination increased. However, the two diatoms were able to tolerate these pollutants, by adopting strategy based on the increase of their biovolume, to limit the absorption of PAHs and consequently reducing their toxicity. The PAH tolerance of the diatoms has been strengthened by their ability to accumulate PAHs and degrade them probably in synergy with their associated bacteria. High degradation was found in culture of *P. mannii*, which showed also the highest biovolume increase and then appeared to be more tolerant to PAHs than *P. hasleana*. These findings could help to understand the increase of *Pseudo-nitzschia* spp. blooms in contaminated PAHs coastal waters. The diversity of associated bacteria of potentially toxic diatoms and the interaction between both components should be investigated in the future studies, since associated bacteria could strongly influence the pollutants response of these species and hence their occurrence and dynamics.

CRediT authorship contribution statement

Sondes Melliti Ben Garali: Conceptualization, Methodology, Investigation, Writing - original draft. **Inès Sahraoui**: Conceptualization, Methodology. **Hiba Ben Othman**: Investigation. **Abdessalem Kouki**: Investigation. **Pablo De La Iglesia**: Investigation. **Jorge Diogene**: Investigation. **Céline Lafabrie**: Investigation. **Karl B. Andree**: Writing - review. **Margarita Fernández-Tejedor**: Investigation. **Kaouther Mejri**: Investigation. **Marouan Meddeb**: Investigation. **Olivier Pringault**: Conceptualization, Methodology, Writing - original draft. **Asma Sakka Hlaili**: Conceptualization, Methodology, Investigation, Writing - original draft, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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