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Capacity of the potentially toxic diatoms *Pseudo-nitzschia mannii* and *Pseudo-nitzschia hasleana* to tolerate PAHs

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ABSTRACT

This study investigates the effects of polycyclic aromatic hydrocarbons (PAHs) on the potentially toxic *Pseudo-nitzschia hasleana* and *P. mannii* that, isolated from a PAH contaminated marine environment, the Bizerte Lagoon (Tunisia). Both species, maintained in non-axenic cultures, have been exposed during 144 h to increasing concentrations of a 15 PAH mixture. Analysis of the domoic acid, at the beginning and the end treatments, showed very low concentrations. PAH contaminations induced a dose-dependent reduction in growth and photosynthesis, but both species have maintained their growth until the end of incubation even at the highest concentration tested (120 µg L^{-1} PAH:), nevertheless, *P mannii* seemed to be more tolerant than *P. hasleana*. To reduce PAH toxicity, both species have increased their biovolume, with the highest increase for *P. mannii.* Both species were also capable of bio-concentrating and metabolizing PAHs and were able to degrade PAHs, with a higher biodegradation in *P. mannii* cultures than in *P. hasleana* cultures. This study provides the first evidence of the role that PAHs can play in controlling the growth and physiology of potentially toxic diatoms. It also describes for the first time the ecotoxicological parameters for *P. mannii* and *P. hasleanea* regarding PAHs.

Keywords: *P. mannii*; *P. hasleana*; PAH mixtures; Biodegradation; Eco-toxicology.

1. Introduction

Chemical pollution of aquatic ecosystems is one of the major environmental problems because of its disastrous impacts on these environments and their resources and services (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 the marine environment and especially in coastal waters (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. 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.

The high persistence and toxicity of PAHs has led to intensive research on the process of their elimination and degradation. Environmental technology has employed microorganisms, such as bacteria and fungi, to remove PAHs from contaminated waters (Gutierrez, 2010; Gargouri et al., 2011; Mansouri et al., 2017). In recent years, more attention 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 their biomass, through the process of photosynthesis and the enzyme activities of phytoplankton (Echeveste et al., 2010; Perez et al., 2010; Ben Othman et al., 2012), some microalgal species can resist the effects of these pollutants by accumulating and degrading them (Hong et al., 2008). However, the tolerance to PAHs varies among phytoplankton species and appears to be related to their cell biovolume (Ben Othman et al., 2012). So, large algae, such as diatoms, are recognized as tolerating

organisms, since they are able to maintain growth under contamination by PAHs. Therefore, 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 differing 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 the contamination with 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). 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. Interestingly, a recent work showed that resuspension of contaminated sediment caused a shift in the phytoplankton community of the Thau lagoon, with high tolerance 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 avoid metal contaminants. However, the responses of *Pseudo-nitzschia* to PAHs and persistent organic pollutants have never been addressed. 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. Therefore, the eco-toxicology impact of the organic pollutants on potentially toxic diatoms must be well understood and described in order to improve our knowledge of the initiating factors of their blooms and their toxicity.

In the lagoon of Bizerte (SW Mediterranean), diatoms of the genus *Pseudo-nitzschia* showed pronounced diversity and occurrence throughout the year (Sahraoui et al., 2011, 2012; Sakka Hlaili et al., 2016). In this coastal ecosystem, where PAHs contamination has been reported (Lafabrie et al., 2013), *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), suggesting that this genus could be resistant to PAHs. Furthermore, our previous work has described two species (*P. hasleana* and *P. mannii*) for the first time in the south Mediterranean Sea and gave the first report of their potential toxicity in culture (Melliti Ben Garali, 2016). Since the tolerance to PAHs contamination may be a factor contributing to the proliferation of *Pseudo-nitzschia* spp., our study's purpose was to investigate the responses of the two species recently observed in the Bizerte lagoon to PAHs stress. In polluted marine environment, PAHs are commonly encountered in a 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; 10 Echeveste et al., 2010). Thus, the specific objectives of the study are (i) to assess the impact of increasing concentrations of a PAHs mixture on the growth, the photosynthetic parameters and the biovolume of *Pseudo-nitzschia mannii* and *P. hasleana*, (ii) to determine if a link between the toxigenic potential of species and PAHs exists, and (iii) to examine the species ability for degradation and accumulation of these pollutants. The PAHs mixture used in this study was formed by 15 molecules, which were chosen based on the actual contamination of the Bizerte lagoon.

2. Materials and methods

2.1. Isolation, identification and culture of species

Using a 20 µm mesh plankton net, strains of diatoms were collected from the lagoon of Bizerte located in the north of Tunisia (SW Mediterranean Sea). 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^{o}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, benz(k)fluoranthene, acenaphthene, fluorene and dibenzo(a,h)anthracène) in the sediments of the Bizerte Lagoon. The specific *in situ* concentrations of each PAH were reported in Lafabrie et al., (2013). 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 w/v compare to the actual PAH concentrations in sediment in w/w) of every type of pure PAH. 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. These 4%, 2%,0.5% and 0.1% values correspond respectively to a 8/100, 2/100, 1/100 and 0.1/100 (mg/L) of the total PAHs present (mg/kg dry weight) in the Bizerte sediments. The choice of 4 percentages of present PAHs was arbitrary.

The theoretical concentrations, which correspond to every cocktail, were presented in Table 1. 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 one of the four PAH cocktails 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. Three flasks without PAHs were used as control treatment (C) and three others have received only the DMSO (at 0.05 % v/v). This control treatment with DMSO (C_{DMSO}) was prepared to test the toxicity of DMSO at 0.05 % on the diatom species. *Pseudo-nitzschia* cells reaching exponential growth phase were then inoculated to each flask to obtain 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 the 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.

2.4. Sampling and analyses

2.4.1. PAH measurement

PAHs were analyzed in all treatments at the beginning, 72 h and 144 h of the experiment and every day in the treatment C3. Samples were filtered by 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 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, v/v) for 24 hrs. Extractions were purified by a self-packet silica-alumina column (from top to bottom: anhydrous sodium sulphate, 1 cm; the mixture of silicone and n-hexane, 10 cm; anhydrous sodium sulphate, 1 cm; a few glassfiber). Then, The PAHs eluent was concentrated to 0.5 ml for GC-MS analysis.

2.4.2. Domoic acid measurement

According to the analysis of DA in shellfish and seawater (particulate), 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).

2.4.3. Chl *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 (v/v) for 30 h in the dark at 4^{o}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 one second 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 dimensions of diatoms (at least 50 cells for each species) were measured using a calibrated ocular micrometer and biovolume (V, μm^3) were estimated by applying standard geometric formulae to each species, as proposed by Hillebrand et al. (1999). We assumed that *P. mannii* and *P. hasleana* have a shape of a prismatic geometric shape:

$$V\ (\mu m^3) = [(\text{length of the cell}) / 3 * \pi * (\text{width} /2)] * 2$$

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 used to compare available data on the toxicity of PAHs mixture to microalgae. They 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 contamination with the PAH cocktails on all variables measured for both species. When the effect was significant, a multiple comparison posterior test (Tukey test) was performed to determine which contamination had a significant difference from the

control. 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 (Kuskal-Wallis) was used. All statistical analyzes were performed using SPSS18.0 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 the two controls (ANOVA test) [negative control without DMSO (treatment C) and positive control with DMSO (treatment C_{DMSO})]. Furthermore, both species showed similar proliferation and growth rates (1.80 - 2.04 d^{-1}, Table 2) between the two control treatments (C and C_{DMSO}), their abundance increasing gradually throughout the experiment (Fig. 1). This indicates that the 0.05% v/v of DMSO, used to prepare the PAH cocktails, had no toxic effect on the diatoms. Under the various contaminations, the diatoms were able to maintain continuous growth throughout the incubations, but with lower rates compared to the control (Table 2).

For each species, the PAH-treated cells did not totally lose their viability as fluorescence was still observed. However, their biomasses were significantly reduced compared to that of the control (Fig. 1A). In the *P. hasleana* culture, treatment C1 induced a significant ($P < 0.05$) decrease in the Chl *a* compared to control after 48 h (reduction of 10.72%). At the end of the incubation, a decrease of 37% was observed (Fig.1A). The other treatments resulted in a rapid decrease of Chl *a* (during the first 24 hours), with a reduction of 20 % for C2 and 40% for the contaminations C3 and C4 (Fig. 1A). After 144 h, Chl *a* concentration in treatments C2, C3 and C4 were 50, 56 and 90% lower than those of the controls, respectively (Fig. 1). A dose-dependent effect was also detected for the *P. mannii* culture. Indeed, the decrease of Chl *a* was inversely proportional to the level of contamination

(Fig. 1B). At the end of treatments, reductions of 23%, 55%, 67% and 90% were noted in treatments C1, C2, C3 and C4, respectively (Fig.1B).

For *P. hasleana*, all PAH cocktails induced reduction (≅40% of control) of the cell concentrations at the beginning of the experiment. At the end of incubation, the number of cells was reduced by about 53% in the C1 treatment and by about 75% in the other contaminated treatments (Fig. 1C).This species exhibited growth rates 2, 3 and 4 times lower than the control in treatments C1, C2 and C3/C4, respectively (Table 2). The same trend was observed for *P. mannii*, with ≅31% reduction in first day of incubation. After 144 h, the PAH contaminations caused decreases of 38, 57, 58 and 79% in the C1, C2, C3 and C4 treatments, respectively (Fig.1D). Under C1 and C2 treatments, this species proliferated two times as slow as untreated cells, whereas under heavy contamination (C3 and C4), its growth was three times lower than that of the control (Table 2).

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 (on average 0.4) were measured in control with or without DMSO throughout both experiments (Fig. 2). When *P. hasleana* was exposed to C1 and C2 contaminations, the Fv/Fm (mean value 0.7) 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 hours, 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 (≈ 210μm^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 biovolume has increased proportionally with the level of contamination.

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

3.3. Fate of PAHs during incubation

The concentrations of PAHs measured in each treatment at the beginning of the experiment were compared with those used theoretically for contamination (Table 1). The results showed that the measured levels were close to 95% of the theoretical concentrations. In addition, the measured and theoretical concentrations were linear for the concentration ranges of all the samples (n = 12). Therefore, the measured concentrations were used in the remainder of the results.

The daily monitoring of PAHs in the C3 treatment (contaminated with 2% of the PAH cocktail), carried out for both species, 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 decrease, the final levels of dissolved PAHs were 2.3 to 5.1 μg l^{-1}, indicating that both *Pseudo-nitzschia* species were maintained in contaminated media until the end of the experiments. In contrast, 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).

In each treatment, the percentage of dissolved PAHs ($\%PAH_{diss}$), accumulated PAHs and degraded PAHs varied significantly throughout the incubation time for both species (Figs. 5 and 6). 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 (Figs. 5B, 6B). In all treatments, there were more PAHs accumulated in the cells at 144 h (30 – 57% and 25- 59%) than at 72 h (12-26%and 10-35%) (Figs. 5A, 6A).

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 (Figs. 5C, 6C).

4. Discussion

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

In control microcosms (C and C_{DMSO}), both species proliferated well with fairly similar growth rates (1.83 - 2.04 d^{-1}, Table 2). Their biomasses and abundances gradually increased from the beginning to end of the experiment (Fig. 1). At 144 h, the concentrations reached by these two species were 10 times higher than the initial densities. This indicates that all the physico-chemical conditions (temperature, light, nutrients, etc.) under which the experiments were conducted were adequate for an optimal growth of diatoms.

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). This result is consistent with other ongoing work on the adverse effect of PAHs in mixture on phytoplankton growth (Djomo et al., 2004; Vieira and Guilhermino, 2012) and more on that of diatoms (Nayar et al., 2005). It seems that the

silicate present in the frustules of the diatoms allows the adsorption and the retention of the PAHs, which 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 2). In addition, decrease in Chl *a* and cell abundance was proportional to the PAH concentrations (Fig. 1). However, the dose-dependent impact of PAHs on the algal growth was different between both species. In the short term (i.e. 24 h after contamination), the treatments C2, C3 and C4 induced more pronounced decrease in Chl *a* and cell densities for *P. hasleana* (20 -42% and 37 – 40%, respectively) than for *P. mannii* (20 – 30% and 31-35%, respecttively). Moreover, at 144 h, *P. hasleana* showed more reduced final cell concentration under all contaminations (53 – 77%) than *P. mannii* (38 – 75%). The dose-dependent impact of PAHs on microalgae has been previously reported by several authors (Echeveste et al., 2010; 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 (Fig. 1), suggesting that *P.mannii* and P. *hasleana* can tolerate these organic pollutants. However, the first species seemed to be more tolerant than the second. Under the highest contaminations, *P mannii* proliferated a little more quickly than *P. hasleana* (Table 2) and as mentioned above, the decreases in final cell concentrations were less pronounced for *P. mannii* than for *P. hasleana* (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 biomass and growth, our results revealed that PAHs also had an impact on the physiology of both species, as the effectiveness of their photosystem II was also negatively affected by these contaminants. However, the effect 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 higher 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., 2010; Ben Othman et al., 2018). Hydrocarbons, known by their hydrophobic character, tend to accumulate in chloroplasts whose structure is rich in lipids. These pollutants can be introduced by altering the membrane of chloroplasts (Baker, 1970), 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. Thus, 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).

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}). These results prove that *P. mannii* was physiologically better adapted to high levels of PAHs, which explains its higher tolerance to PAHs than *P. hasleana*, confirming the better proliferation of the first species. The measured EC50 exceed the concentrations found in the water of the Bizerte lagoon. 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.

In addition to physiological adaptations, cell biovolume has also been identified as a factor in the resistance or sensitivity of species to PAHs (Echeveste et al., 2010). Small cells, with a high surface to volume ratio, absorb the dissolved elements more and are therefore more exposed and more affected by the contaminants. By opposition, large microalgae (such as diatoms and dinoflagellates) are more resistant to pollutants, such as PAHs (Ben Othman et al., 2012). Both *Pseudo-nitzschia* species had at the beginning of the experiment the same biovolume (≅210 μm^3) which kept it almost until the end in the control treatments (Fig. 3). However, both diatoms have increased their biovolumes after 144 hours' exposure to all concentrations of the PAH cocktails (Fig. 3). Moreover, this increase was all the more important as the level of contamination increased. Thus, it seems that these diatoms have adopted this strategy of increasing size, for example by accumulating certain substances like lipid bodies, to reduce the toxicity of PAHs. In fact, exposing diatoms to abiotic stresses can boost lipid synthesis and yield (Zulu et al., 2018). In addition, the biovolume of *P. mannii* increased from 16 to 35% compared to the baseline, whereas the increase in *P. hasleana* was lower (11 – 17%). This could therefore reinforce the resistance of *P. mannii* to PAHs compared to that of the other species. So, this strategy allows diatoms to maintain their growth under high contamination.

Several studies have shown that 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 pollutants did not stimulate it and so the release of domoic acid 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

Our results clearly showed that the growth and the physiology of both species of *Pseudo-nitzschia* were affected by the PAH cocktails, with *P. mannii* being less affected than *P. haslena*. Both species can reduce the PAH toxicity by increasing their biovolume, but this increase was more pronounced for *P. mannii* that exhibited a better tolerance to PAHs. The resistance of both diatoms to PAHs could be also related to their ability to eliminate these pollutants, which would be expected to be more important for *P. mannii*.

The *Pseudo-nitzschia* species were effectively capable to uptake the PAHs from the medium and accumulating them. Indeed, over time (0 h to 72 h and 72 h to 144 h), the percentages of PAHs remaining in each treatment decreased in parallel with an increase in accumulation percentages (Figs. 5 and 6). Furthermore, in cultures contaminated with the C3 cocktail, 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 microalagal species,

including diatoms to accumulate several individual PAHs) or in mixture (Hong et al., 2008; Melliti Ben Garali, 2016). After 144 h exposure to C2 contamination, both species were able to accumulate ≈ 60% of PAHs (Figs. 5A, 6A), a percentage exceeding value found for other diatoms, such as *Skeletonema costatum* (45%; Hong et al., 2008). This proves the excellent potential of the two species of *Pseudo-nitzschia* for PAH accumulation. However, the percentages of accumulation were slightly different between the species (Figs. 5A, 6A). It is known that the magnitude of bioaccumulation of organic contaminants by microalgae is species-dependent, since for the same PAH, some species may accumulate it more than others (Lei et al., 2007). The size and shape of species have an important role in the adsorption and accumulation of these molecules. So, *P. mannii,* by increasing much more its biovolume in all contaminations treatments (Fig. 3), could have a lower area:volume ratio and subsequently lower PAH absorbance, resulting in less accumulated PAHs. The bioaccumulation of PAHs by phytoplankton can also directly be related to the initial cell concentration used in the inoculum. Chan et al. (2006) found that the uptake of three PAHs in a mixture (phenanthrene, fluoranthene and pyrene) by *Selenastrum capricornutum* increased as its initial concentration increased from 5 10^4 to 10^7 cells ml^{-1}. Nevertheless, in the present study, the cell densities of the two species were the same (5 10^6 cells ml^{-1}) at the beginning of incubations, so the concentration of inocula could not contribute to the observed differences in PAH accumulation between *P. mannii* and *P. hasleana*.

In the C3 treatment (contaminated with 2% of the PAH cocktail), a gradual drop in dissolved PAH concentrations was observed over time in cultures of both species (Fig. 4A). Several studies have shown that the decrease in concentrations of dissolved PAHs during contamination experiments may be due to several processes, including volatilization, photooxidation, absorption and biodegradation (Lekunberri et al., 2010). Besides photochemical decomposition, the activity of *P. mannii* and *P. hasleana* could also contribute

to PAH degradation. Effectively, bio-degradation of individual or mixed PAHs has been largely demonstrated for some diatoms (*Nitzschia* and *Skeletonema*) (Hong et al., 2008; Echeveste et al., 2010). In addition to phototrophs, bacteria also have the ability to degrade PAHs (Arulazhagan and Vasudevan, 2009; Jiménez et al., 2011). Since the cultures of the two *Pseudo-nitzschia* species were not axenic, it is very likely that the heterotrophic activity of bacteria present in the diatom cultures contribute to the biodegradation of PAH during the incubation. Recent studies have shown the presence of hydrocarbonoclastic bacteria intimately associated with diatoms (Thompson et al., 2018, Severin et al., 2019). It appears that *P. mannii* maintained in culture was able to degrade PAHs, with a higher percentage of degradation (72 h: 7 - 47%, 144 h: 20-51%) than those found in culture of *P. hasleana*, (72 h: 0.7 - 17%, 144 h: 10 - 25%) (Figs. 5C, 6C). It has been reported that the solubility and molar mass of PAHs significantly affect their bio-degradation. In general, low molecular weight PAHs having 2 to 3 aromatic nuclei rings (such as naphthalene and phenanthrene) are more degraded than those with more than 3 aromatic nuclei rings(such as phenanthrene, anthracene and fluoranthene) (Haritash and Kaushik, 2009). In addition, the presence of one PAH can stimulate the bio-degradation of another. This synergistic effect has been observed for some PAHs (such as pyrene, phenanthrene, fluoranthene and naphtalene) degraded by phytoplankton species (Chan et al., 2006). In our experiments, 15 different PAH compounds, with different molar masses, were present in mixture. Some of these molecules may be more susceptible or resistant to degradation than others. Moreover, the interactions between them can contribute to a simultaneous degradation of the mixture of the PAHs used.

5. Conclusion

The contamination of two species of *Pseudo-nitzschia* (*P. mannii* and *P. hasleana*) by increased concentrations of PAH mixture in culture induced a reduction in cell growth, Chl *a* concentration and cell density of the two species. This effect was dose-dependent, with a

more pronounced decrease as the level of contamination increased. PAH contamination also had a negative effect on the physiology of both diatoms, since their photosynthetic efficiency was altered. Both species of *Pseudo-nitzschia* did not produce domoic acid in the experimental conditions imposed, irrespective of the PAH concentration added in the culture medium. Therefore, unlike metals, domoic acid does not seem to be involved in their defense mechanisms to face organic pollutants such as PAHs and thus PAH pollution might not be one of the factors controlling the toxicity of *Pseudo-nitzschia* species. On the other hand, *P. mannii* and *P. hasleana* have a morphological capacity to tolerate PAHs, by increasing their biovolume and subsequently their surface:volume ratio decreases which makes them less sensitive to the presence of contaminants outside the cells. The biovolume augmentation can be an effective strategy adopted by microalgae that allows them, maintaining their proliferation in coastal environments contaminated by PAHs. *P. mannii* showed more biovolume increase and appeared to be more tolerant than *P. hasleana*. The percentage of accumulation of PAHs by both species, which increased over time, was inversely proportional to PAH cocktail concentrations. This suggests that *P. mannii* and *P. hasleana* were able to bio-concentrate and metabolize PAHs with probably the help of the heterotrophic bacteria present in the non-axenic cultures, but the second species appeared to have greater accumulating potency. Both diatoms with associated bacteria in cultures were also able to degrade PAHs, with percentages in *P. mannii* cultures greater than those found in *P. hasleana* cultures. The tolerance of *Pseudo-nitzschia* species to PAHs, related to their morphological defense strategy and their ability to bioaccumulate and degrade PAHs, could help to understand their increasing occurrence and their intense blooms observed in highly PAH contaminated coastal waters, like the Bizerte lagoon.

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Figure legends

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).

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).

Fig. 3. Effects of PAH cocktails on cellular biovolume of *P. hasleana*(a) and*P. mannii* (b) in different treatments [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).

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).

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* 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).

Fig. 6. 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. 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).

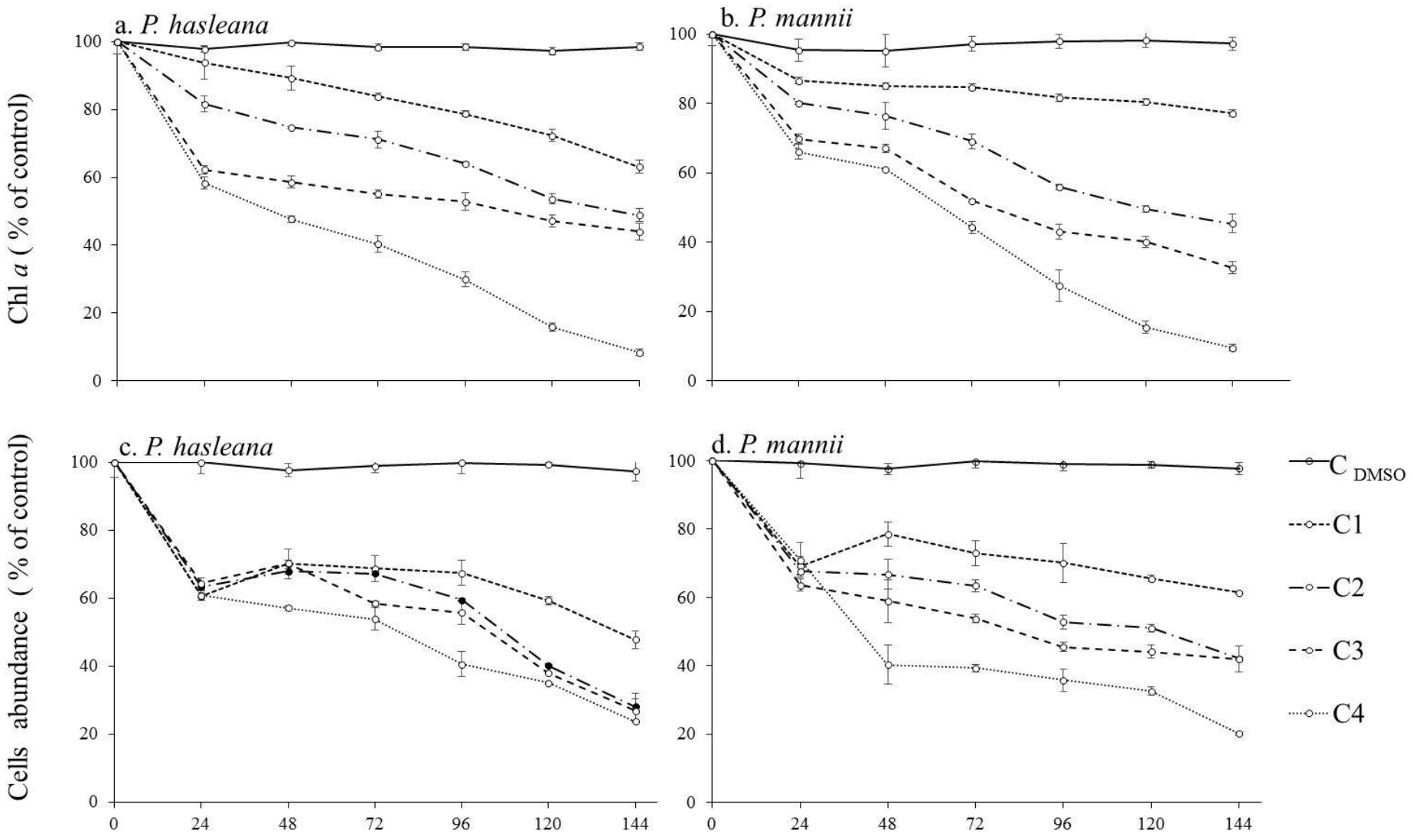
a. P. hasleana
b. P. mannii
c. P. hasleana
d. P. mannii
Chl a (% of control)
Cells abundance (% of control)
0
20
40
60
80
100
0
24
48
72
96
120
144
C DMSO
C1
C2
C3
C4

Fig. 1

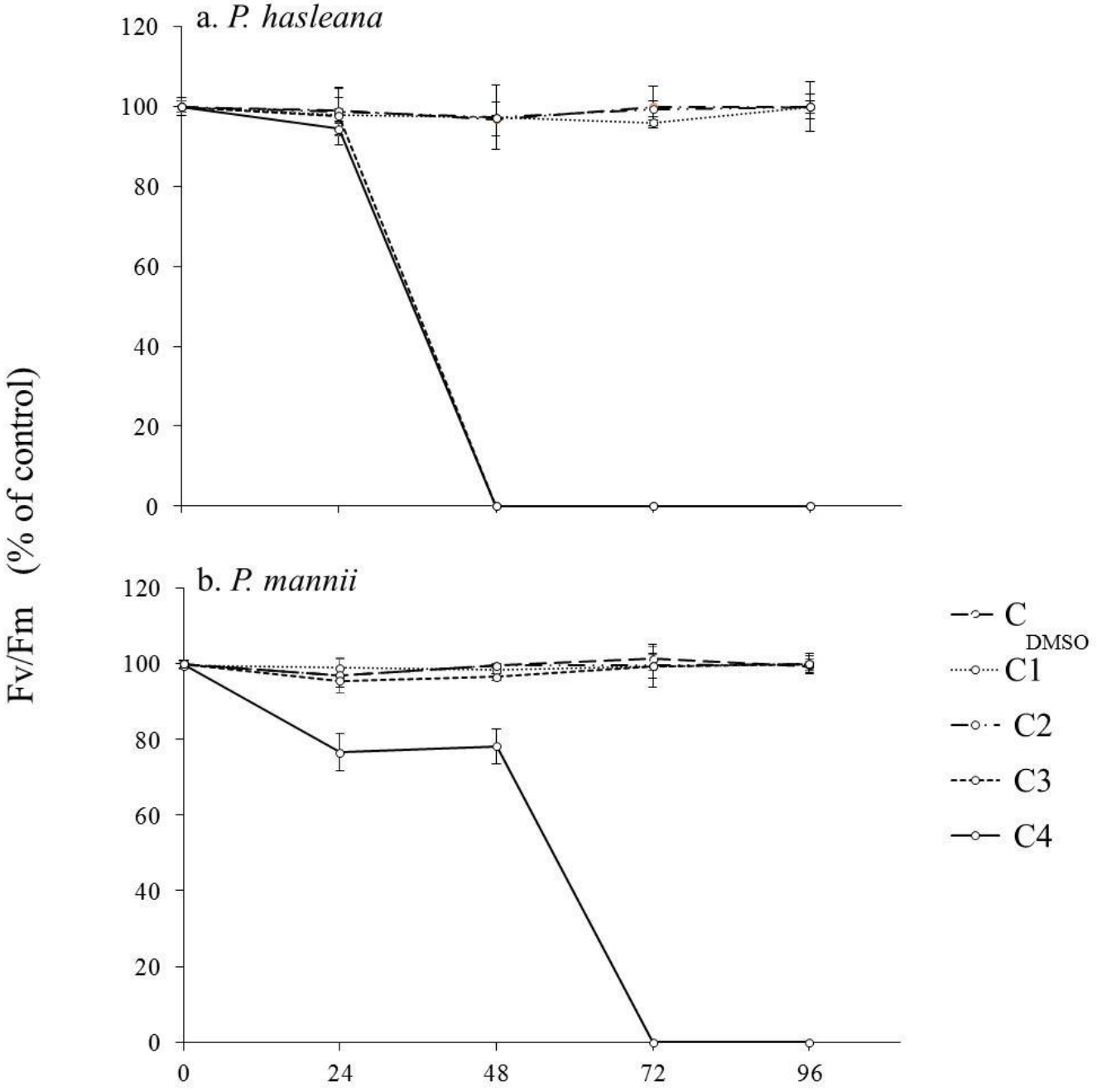
a. P. hasleana
b. P. mannii
Fv/Fm (% of control)
120
100
80
60
40
20
0
0
24
48
72
96
C DMSO
C1
C2
C3
C4

Fig. 2.

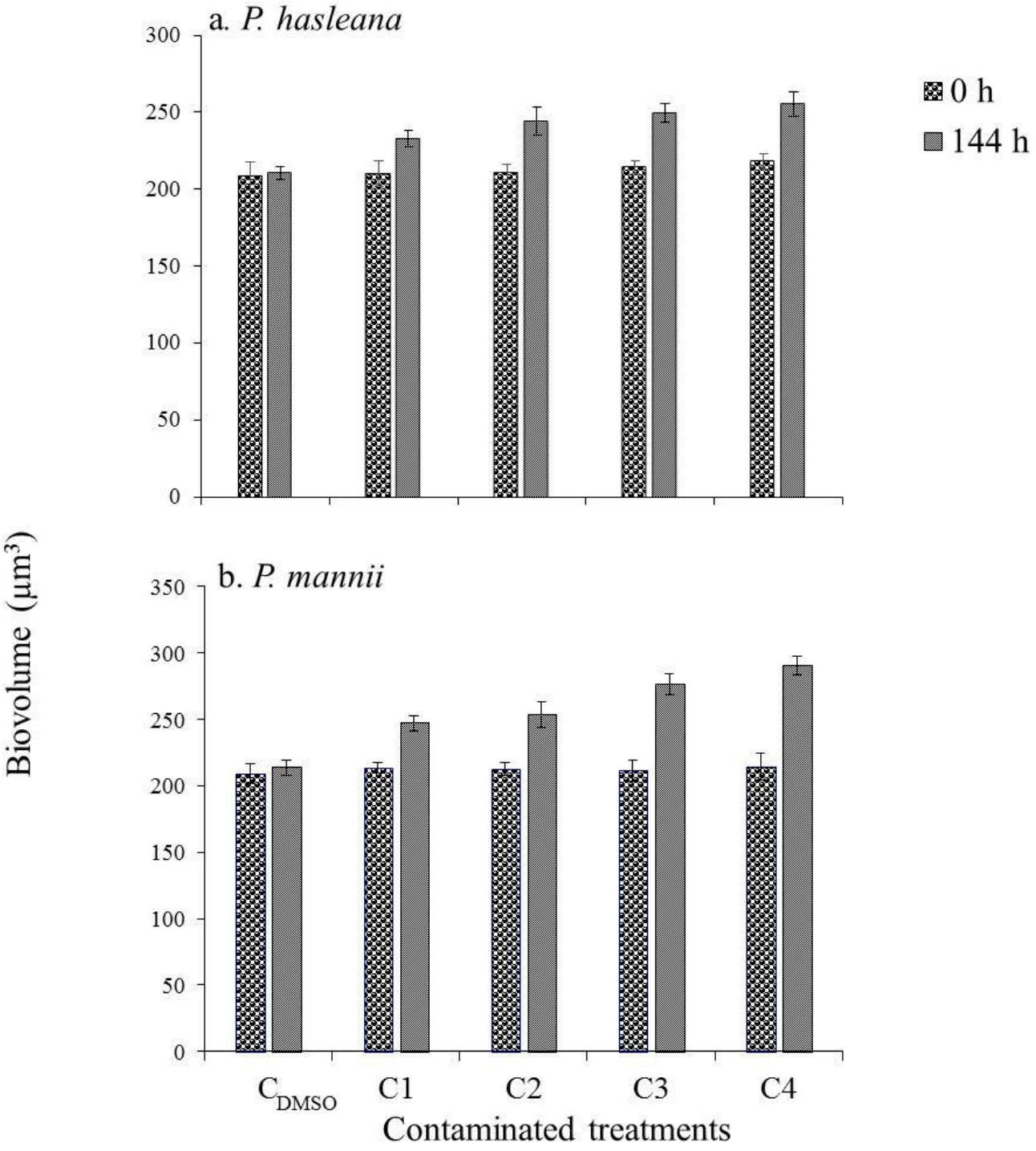
a. P. hasleana
b. P. mannii
0 h
144 h
Biovolume (µm³)
300
250
200
150
100
50
0
350
C_DMSO
C1
C2
C3
C4
Contaminated treatments

Fig. 3.

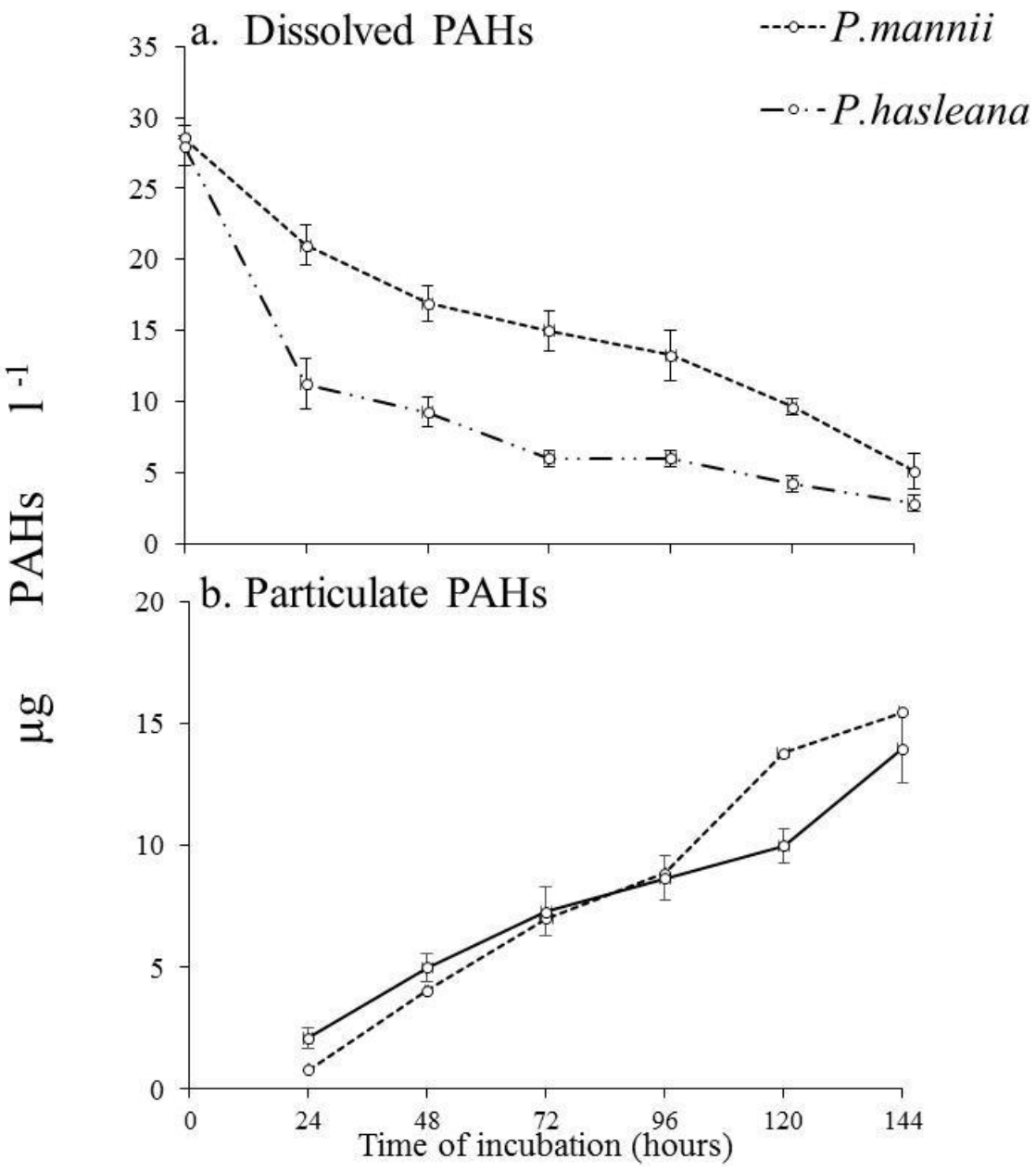
a. Dissolved PAHs
P.mannii
P.hasleana
µg PAHs l-1
35
30
25
20
15
10
5
0
b. Particulate PAHs
20
15
10
5
0
0
24
48
72
96
120
144
Time of incubation (hours)

Fig. 4.

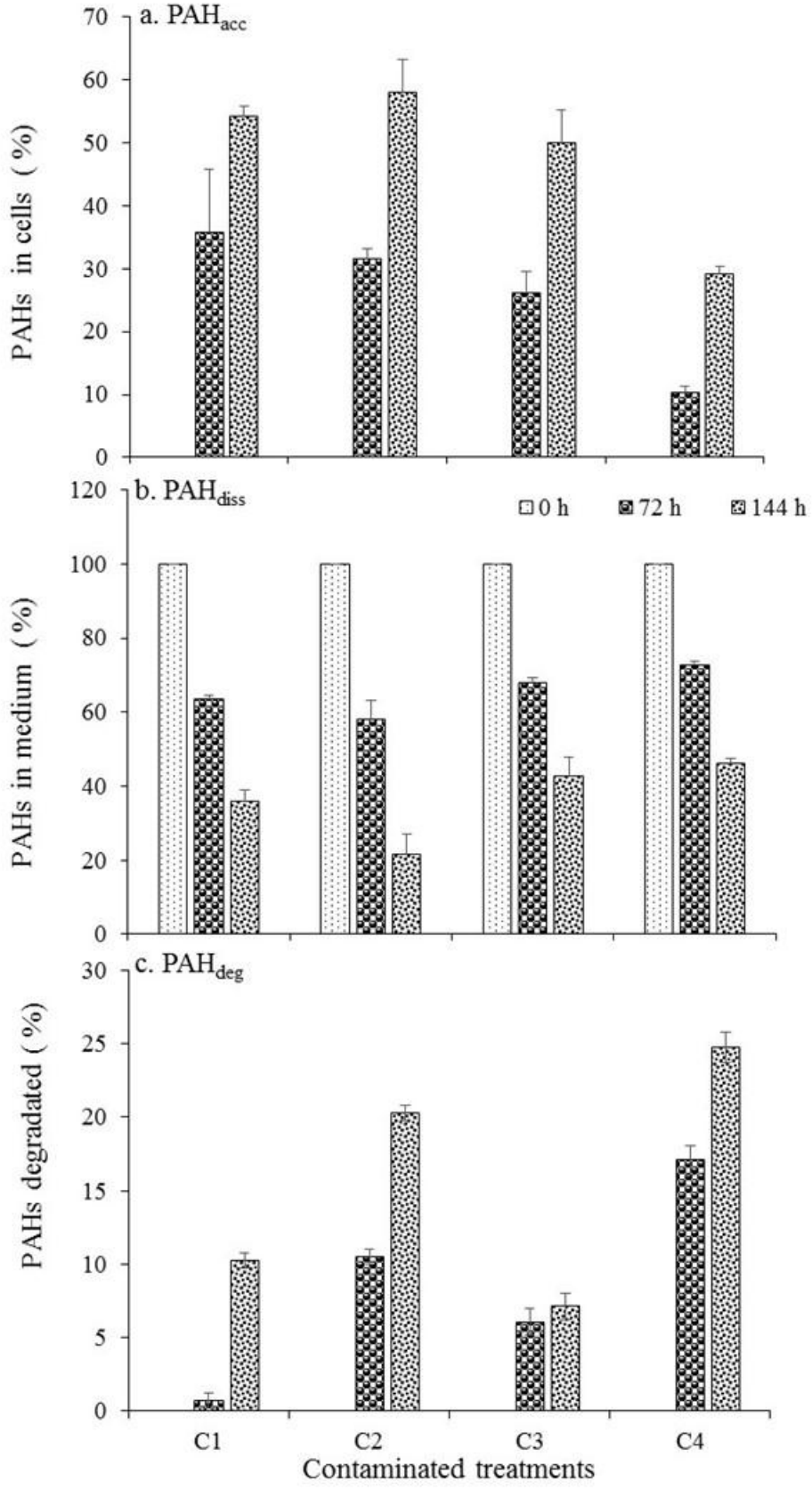
a. PAH$_{acc}$
PAHs in cells (%)
70
60
50
40
30
20
10
0
b. PAH$_{diss}$
0 h
72 h
144 h
PAHs in medium (%)
120
100
80
60
40
20
0
c. PAH$_{deg}$
PAHs degradated (%)
30
25
20
15
10
5
0
C1
C2
C3
C4
Contaminated treatments

Fig. 5.

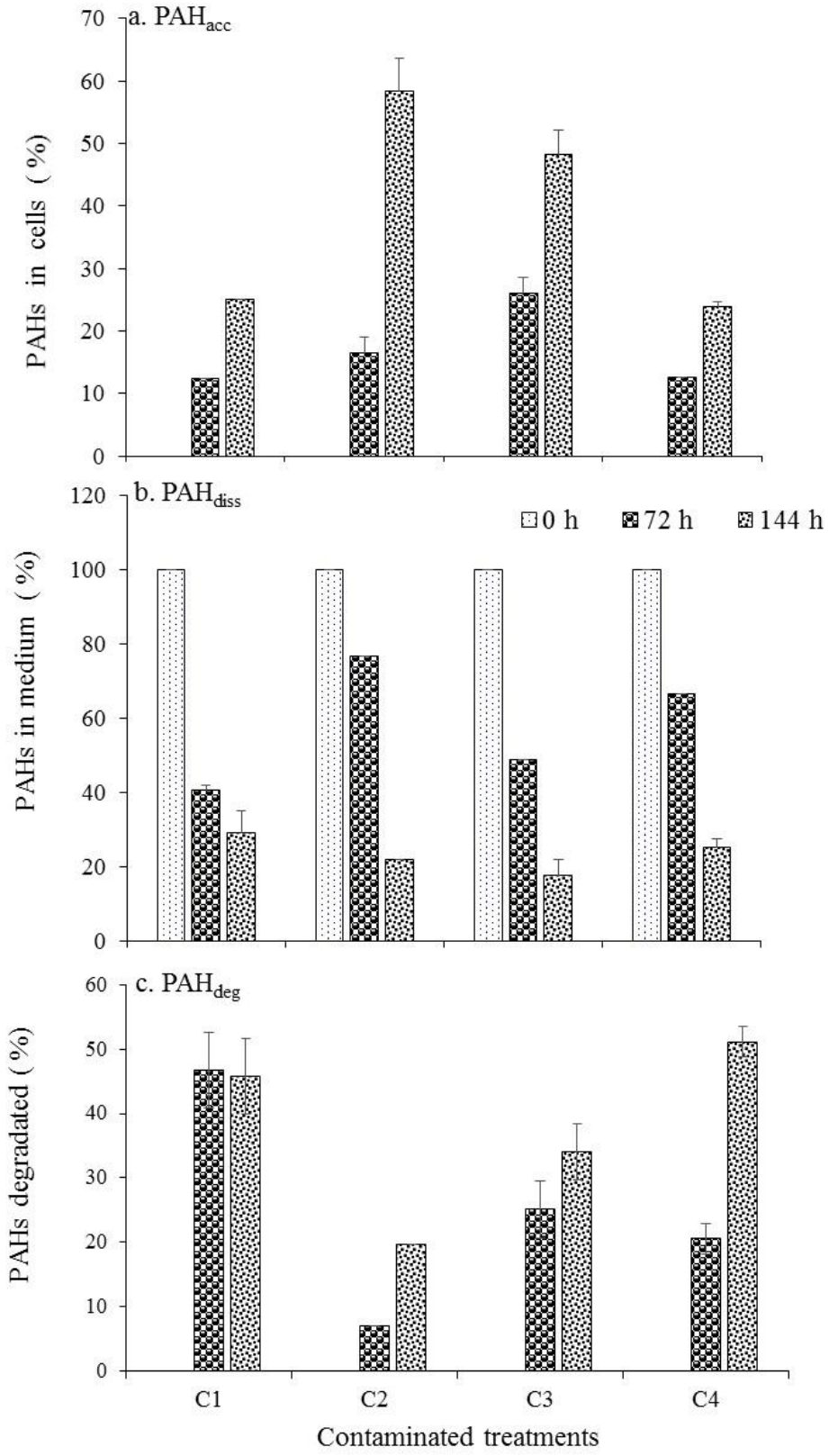
a. PAH$_{acc}$
PAHs in cells (%)
70
60
50
40
30
20
10
0
b. PAH$_{diss}$
0 h
72 h
144 h
PAHs in medium (%)
120
100
80
60
40
20
0
c. PAH$_{deg}$
PAHs degradated (%)
60
50
40
30
20
10
0
C1
C2
C3
C4
Contaminated treatments

Fig. 6

Fig. 6

Table 1

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

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)

	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*