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Abstract

A benthic diatom *Nitzschia sp*., isolated from a PAH-contaminated sediment, was exposed in axenic and non-axenic cultures to BaP and Flt over 7 days. The diversity of the associated bacteria, attached (AB) and free-living bacteria (FB), was analyzed by the MiSeq-derived 16S rRNA. The diatom was able to grow under BaP and Flt treatments and to accumulate both PAHs. Biodegradation, which constituted the main process for PAH elimination, was enhanced in the presence of bacteria. Diatom and bacteria showed different capacities in the degradation of PAHs. The attached bacteria exhibited higher diversity and abundance relative to free-living bacteria, while the FB fraction contained genera with the known ability of PAH degradation. Isolated strains from the FB community, showed the capacity to grow in the presence of crude oil. These results suggest that a "benthic *Nitzschia sp.*-associated hydrocarbon-degrading bacteria" consortium can be applied in the bioremediation of PAH-contaminated sites.

Key words

Accumulation, biodegradation, Bizerte Lagoon, *Nitzschia sp*. phycosphere, hydrocarbon-degrading bacteria

1. Introduction

Marine pollution through chemical contaminants has become a matter of great concern because of its deleterious impacts on marine ecosystems. Polycyclic aromatic hydrocarbons (PAHs) are among the most hazardous pollutants, with potential harmful effects on biota and ecosystems (Hylland, 2006). They are hydrophobic and ubiquitous pollutants detected in all ecosystems, from polar regions to the tropics (Wilcke, 2007; Duran and Cravo-Laureau, 2016).

Sixteen PAHs have been included in the US Environmental Protection Agency's list of priority pollutants (US-EPA). Because of their strong toxicity, persistence, and accumulation in marine animals and plants, a great deal of attention has been paid to the elimination and degradation of PAHs (Duran and Cravo-Laureau, 2016; González-Gaya et al., 2019). Physicochemical and thermal techniques are used in several processes of soil or sediment treatments, but biological remediation has been recognized as the most efficient method for decontaminating environments polluted by PAHs. Indeed, PAHs are biodegraded by a wide range of microorganisms (McKew et al., 2007, Gutierrez, 2013, Thompson et al., 2017). Among them, bacteria are considered as the dominant agents in hydrocarbon biodegradation, and several marine hydrocarbon-degrading isolates have been described worldwide (Ben Said et al., 2008; Haritash and Kaushik, 2009; Jiménez et al., 2011). Thus, "marine bacterial consortia" have been widely used in efforts to depollute environments contaminated by PAH mixtures and oil, such as harbors and wastewater effluents, or during oil spills (Arulazhagan and Vasudevan, 2009, Jiménez et al., 2011). Microalgae can also play a significant role in the removal of PAHs. Warshaskyet al. (1988) have shown that the freshwater microalgae *Selenastrum capricornitum* was able to metabolize benzo(a)pyrene (BaP) to cis-dihydrodiols through a system of enzyme dioxygenases such as that found in bacteria. Two diatom species, *Nitzschia* sp. and *Skeletonema costatum*, can accumulate and degrade phenanthrene (Phe) and fluoranthene (Flt) (Hong et al., 2008). Diatoms of the genus *Pseudo-nitzschia* have recently been reported to accumulate and degrade a mixture of PAHs (Melliti Ben Garali, 2016). Other studies have reported that Phe and pyrene (Pyr) could be converted by photosynthetic microorganisms into soluble diols, phenols, lactones, naphthoic acid, and phthalic acid, which may be excreted in the overlying water column (Seo et al., 2007).

Other works have demonstrated that a "microalgae-bacteria consortium" could be successfully used in the treatment of aromatic pollutants ((Borde et al., 2003; Muñoz et al., 2003). There is a synergetic relationship between algae and bacteria, in which algae provide oxygen

(through photosynthesis) required by aerobic bacteria in the aerobic biodegradation of PAHs. In turn, bacteria produce carbon dioxide for photosynthetic metabolism. Borde et al. (2003) have shown that the removal of Flt and Pyr was better (up to 85%) when the green microalga *Chlorella sorokiniana* was cultivated with bacteria. Other studies have also reported that the use of microalgae is an efficient bioremediation strategy for PAH removal, since phototrophs can also co-metabolize PAHs together with bacteria (Haritash and Kaushik, 2009). Indigenous microalgae-bacteria consortia have effectively been applied in the biodegradation of crude oil and naphthenic acids water (Tang et al., 2010; Mahdavi et al., 2015). More recently, studies have shown that bacteria associated with microalgae, the phycosphere, could play a significant role in hydrocarbon degradation (Thompson et al., 2017; 2018). Hence, the phycosphere has attracted considerable interest regarding its potential in pollutant degradation, and scientists have focused on its structure, ecology and interaction with algae. Studies have shown that the bacteria associated with algae, which comprise both algae-attached and free-living bacteria, are specific to algal species, as algae can release strain-dependent organic compounds that are used by specific bacteria (Bagatini et al., 2014). The presence of oil- and hydrocarbon-degrading bacteria within the phycosphere has been reported for dinoflagellates and diatoms (Gutierrez et al., 2012; Mishamandani et al., 2016; Severin et al., 2016; Thompson et al., 2017, 2018). These associated bacteria can influence the phytoplankton taxon-specific response to hydrocarbon pollution and oil spills (Severin and Erdner, 2019). Most studies have concerned planktonic microalgae and their associated bacteria, but little is known about the PAH biodegradation potential of microbenthic organisms. Benthic diatoms, thriving in contaminated sediments, can acquire physiological abilities to tolerate and metabolize pollutants (Kottuparambil and Agusti, 2018).

Considering these findings, the present study assessed the role of an indigenous benthic diatom and associated bacteria (co-culture, non-axenic) or benthic diatom alone (axenic culture) in the removal of two PAHs of interest, i.e. BaP and Flt. These two PAHs differ in

structure/molecular weight and hydrophobicity/water solubility, leading to potentially different degradation capacities by microalgae and bacteria. Subsequently, the structures of bacteria associated with the diatom (attached or free-living) were also evaluated by Illumina MiSeq 16S rRNA sequencing analysis. Finally, isolated strains from associated bacteria were screened for their potential use of crude oil for growth. The diatom was isolated from a sediment of the Bizerte Lagoon (Tunisia), where high levels of PAHs have been recorded, with Flt and BaP as dominant compounds (265 and 168 µg kg^{-1} dry wt., respectively) (Lafabrie et al., 2013; Pringault et al., 2016). Furthermore, previous works have highlighted that the indigenous microorganisms of the lagoon (bacteria or diatoms) can potentially biodegrade PAHs (Ben Said et al., 2008; Melliti Ben Garali, 2016). Therefore, it seems relevant to evaluate their co-metabolic synergy for a more efficient and faster bioremediation.

2. Material and methods

2.1. Microalgae isolation, culture, and identification

An indigenous diatom was isolated from sediment of the Bizerte Lagoon (north of Tunisia) and then cultured according to the protocol described by Lundholm et al. (2011) and slightly modified by Melliti Ben Garali et al. (2016). Sediments were collected using a Van Veen grab (Hydrobios) at a station (37°15'40.22''N and 9°51'30.49''E) located in front of a cement manufactory, which is highly contaminated by PAHs (Lafabrie et al., 2013; Barhoumi et al., 2014). The sediment was sieved through a 2-mm mesh to remove coarse debris. In the laboratory, 3 g of fresh sediment were mixed with 90 mL of a sterilized f/2 hydroponic culture medium (Guillard and Ryther, 1962) and incubated for 6 days in a thermostatic chamber at 22°C, illuminated with cool-white fluorescent tubes at a light intensity of 100 µmole photons m^{-2} s^{-1} and under a photoperiod of 12 light: 12 dark. During incubation, the mixture was gently stirred to allow the release of microalgae from the sediments and their recovery to the surface. The supernatant,

containing the cells, was taken and served to isolate the dominant diatom. Samples with live cells were examined under an inverted microscope (CETI, Versus, Belgium), and single cells were isolated using a glass Pasteur pipette into a tissue culture plate containing f/2 medium. All cultures were kept under the conditions described previously. The strain was re-inoculated into fresh medium at 2-week intervals. Cells from the culture were observed under a light microscope BX-102. The diatom was identified as a species of *Nitzschia*, based on the morphometric characteristic described by Bouchouicha Smida et al. (2014) (width, length, and form of the valves; number and form of the chloroplasts; presence or absence of the central interspace).

2.2. *Sampling of attached and free-living bacteria*

Tbtain attached bacteria (AB), 200 mL of the *Nitzschia sp.* culture were filtered through a 5.0-µm Nuclepore polycarbonate membrane. The free-living bacteria (FB) were recovered by collecting 5.0 µm of the filtrate onto a 0.22-µm cellulose polyester filter. These filters (two filters of 0.5 µm and two filters of 0.22 µm) were used for microbiological and molecular experiments.

2.3. DNA extraction, qPCR quantification, and Illumina Miseq sequencing and analysis

Total DNA was extracted from AB and FB filters (0.5 and 0.22 µm, respectively) using the Ultra Clean Water DNA kit (MO BIO), following the instructions of the manufacturer. The quantification of the DNA obtained and assessment of its purity were performed using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

The abundance of total bacteria in AB and FB DNAs was estimated by real-time PCR targeting 16S rRNA genes, using the primer sets 331F/797R. The qPCR was made in triplicate in a Bio-Rad CFX-96 real-time system (Bio-Rad). The reaction components and qPCR protocol conditions have been described previously (Ben Abdallah et al., 2018). The abundance of total bacteria was reported as DNA copy numbers of the corresponding gene per mL, using the standard curves.

The AB and FB DNAs samples were subjected to sequencing on an Illumina Miseq platform (CBS, SFAX, TUNISIA), using a paired-end 300-bp sequence read running with the Miseq Reagent Kit V3 (600cycles). The V3-V4 regions of prokaryotic 16S rRNA genes were PCR-amplified with primers Pro341/Pro805R (Takahashi et al., 2014), using KAPA HiFi HotStart ReadyMix (2X) (KAPA Biosystems, Kit Code KK2602). The DNA samples were independently amplified in triplicated 25 µL reactions containing 0.1-10 ng target DNA, 1× Taq PCR Master Mix, and 400 nM of each primer. The PCR program was 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C for 30 cycles, followed by 10 min of final primer extension. The products of PCR products were purified using AMPure XP beads (Beckman Coulter, USA) following the manufacturer's recommendations and analyzed with a BioAnalyzer DNA 1000 Chip Kit (Agilent Technologies) and the Qubit ® ds DNA HS Assay Kit (Life Technologies). Taxonomic analyses of sequence reads were performed with the QIIME version 1.9.1 software package (Caporaso et al., 2010). Chimera detection was performed with UCHIME, and chimeric sequences were filtered and discarded prior to further analysis (Edgar et al., 2011). Operational taxonomic units (OTUs) were assigned at 97% similarity threshold with the UCLUST algorithm (Edgar, 2010). Taxonomic assignments were performed with the Greengenes 13.8 database. Sequences from selected dominant OTUs (> 1% of all sequences) were compared with related sequences retrieved from NCBI databases using BLAST. The 16S rRNA gene sequences determined in this study were deposited in the GenBank database under accession numbers MT229144 to MT229165.

For AB and FB communities, alpha diversity indices including Chao1 richness estimator, observed species and diversity indices (Shannon and Weaver, 1949; Simpson, 1949), as well as the phylogenetic diversity (PD whole-tree) index (Lozupone and Knight, 2008) were calculated with the Qiime software. The VENN DIAGRAM PLOTTER program (http://omics.pnl.gov/software/ VennDiagramPlotter.php) was used to generate the Venn diagram.

2.4. Isolation, identification, and screening for bacteria growing on crude oil

2.4.1. Isolation

In an attempt to isolate strains of attached and free-living bacteria, the filters (5.0 and 0.22 µm, respectively) were transferred to sterile tubes containing physiological water (NaCl 9‰). Aliquots (100 mL) of serial dilutions were plated onto solid Luria-Bertani as rich medium, containing (per liter): 10 g (w/v) tryptone (Bio Basic, Canada); 5 g (w/v) yeast extract (Panreac, Espagne); 5 g (w/v) NaCl (Bio Basic, Canada), and 20 g (w/v) agar (Bio Basic, Canada). The pH was adjusted to 7 with 10 M KOH before autoclaving. After 1-3 days of incubation at room temperature (approximately 27°C), yellowish, cream, and white colonies were obtained. Different colonies were picked and re-streaked several times to obtain pure cultures and then stored at -80°C in the isolation medium supplemented with 30% glycerol. Cell morphology was observed at 100 x magnification under oil immersion (Nikon Optiphot, Tokyo, Japan).

2.4.2. Molecular identification

Genomic DNA of both FB and AB isolates was extracted using a GF1-vivantis Nucleic acid extraction kit according to the manufacturer's protocol. The 16S rRNA genes were amplified using primer sets FD1 (5'- AGAGTTTGATCCTGGCTCAG-3') (Weisburg et al., 1991)/1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991). The PCR cycling conditions were the same as previously described (Karray et al., 2018). The PCR products were sequenced using the Big Dye® Terminator cycle Sequencing kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with FD1 and 1492r primers. Phylogenetic analysis of 16S rRNA gene sequences was performed as previously described (Ben Abdallah et al., 2018). The 16S rRNA gene sequences of the bacterial isolates were deposited in GenBank under accession numbers MN517112 to MN517117.

2.4.3. Screening for bacteria growing on crude oil

The isolated strains were grown aerobically at room temperature in mineral salt medium (MSM) containing (per liter): $(NH_4)_2SO_4$, 1g; NaH_2PO_4, 0.8; K_2HPO_4, 0.2; $MgSO_4$, 0.2; $CaCl_2$, 0.1; $FeCl_3$, 0.005; $MnSO_4$, 0.0002; $ZnSO_4$, 0.0001; $CuSO_4$, 0.00002; yeast extract, 0.5 g (pH = 7)

(Yirui et al., modified 2009). Cells were cultured in 250-mL flasks containing 100 mL of liquid medium with 1 g of crude oil (BAL 150 type Arabian light) for approximately 1 month. A control (without inoculation) was included. Strain evolution was followed by microscopic observation, and their capacity to grow in the presence of crude oil was detected by visual changes in the culture (i.e., clear appearance indicated use of crude oil).

2.5. PAHs contamination experiment

2.5.1. Chemicals and preparation of PAH solution

Two concentrated solutions of BaP and Flt were prepared with HPLC-grade dimethylsulfoxide (DMSO, Sigma Aldrich D4540), and both PAH solutions were kept in the dark at 4°C prior to use. The PAHs (purity > 98%) were purchased from Sigma-Aldrich. Stock solutions of BaP and Flt (0.12 and 2.4 g L^{-1}, respectively) were prepared by dissolving pure chemicals in reagent-grade dimethylsulfoxide (DMSO, 0.05%) without exceeding the solubility threshold.

2.5.2. Experimental set up

The contamination experiment was conducted under the following conditions: non-axenic, axenic (without bacteria), and sterile (without microorganisms). For the non-axenic condition, nine Erlenmeyer flasks (2 L, Schott-Duran glass), containing 150 mL of exponential diatom culture (initial concentration of 1.1 10^4 cells mL^{-1}) and fresh f/2 medium, were used to carry out three treatments in triplicate: control treatment without any PAH (treatment C); treatment contaminated with Flt and treatment contaminated with BaP. The Flt and BaP were added to achieve final concentrations of 265 and 3 µg L^{-1}, respectively. These levels were very close to their respective solubility. For the axenic condition, the culture medium was autoclaved and sterilized with a mixture of two antibiotics (10,000 penicillin units and 10 mg of streptomycin per mL, Sigma Aldrich, PO781,) according to the protocol of Réveillon et al. (2016). Then, the three treatments (C, Flt, and BaP) were subjected to the same conditions as the non-axenic treatment. The axenic and non-axenic treatments (C, Flt, and BaP) were incubated for 7 days in a thermostatic chamber

under the same conditions used for diatom cultures. The light intensity and the temperature within the incubator were checked throughout the experiment, using a spherical quantum mini-recorder LI-250 A and a thermometer (Model TH-020), respectively. Sterile treatments were performed in triplicates, in which the Erlenmeyer flask contained only f/2 culture medium (for treatment C) and f/2 medium with the PAHs for the treatments Flt and BaP. The sterile treatments were incubated for 7 days in light or in dark and settled to determine PAH removal by abiotic processes, i.e., photooxidation, volatilization, and adsorption on glass walls (see below).

2.5.3. PAH analysis

The PAHs were analyzed at the beginning and the end of the incubation in axenic and non-axenic cultures as well as in sterile Erlenmeyer flasks in the light and the dark. Sub-samples (100 mL) were filtered through precombusted Whatman GF/F filters, and the filtrate and suspended particulate matter (SPM) adsorbed to the filters were used to extract dissolved and particulate PAHs, respectively.

Extraction of the SPM-loaded filters was conducted as previously described (Liu et al., 2016), with some modifications. Briefly, after freeze drying and weighing, the filters were spiked with surrogate standard (1-methylpyrene) and ultrasonicated for 1 h at 30°C with 30 mL of a mixture of *n*-hexane:acetone (1:1, v/v). Subsequently, the obtained extract was concentrated to about 5 mL in a rotary evaporator. Additional 10 mL of *n*-hexane were added to the pear-shaped flask and evaporated down to few hundreds of µL. This concentrated extract was purified through a glass column packed with 1 g anhydrous sodium sulfate in the upper part and 2 g of silica gel (previously activated by heating at 150°C overnight before use) in the lower part. After conditioning with 8 mL of *n*-hexane, the fraction containing PAHs (Flt and BaP) was eluted with 10 mL of the mixture of n-hexane and dichloromethane (1:1, v/v). The eluents were concentrated to 1-2 mL, subjected to solvent exchange to acetonitrile, and concentrated to 1.0 mL by a rotary

evaporator prior to HPLC analysis. Extraction of dissolved PAHs was performed by Liquid–Liquid extraction (LLE) with 15 mL of dichloromethane (DCM) for 5 min. The extraction was repeated three times, and the obtained extracts were combined. The subsequent steps were identical as those described for SPM-loaded filter pretreatment.

Quantification of Flt and BaP was conducted using an analytical HPLC unit (JASCO, Japan) equipped with a JASCO PU-2089 HPLC pump, a type 7125 Rheodyne injector (with a 20 μL loop), and a fluorescence detector (FP-2020) with excitation and emission wavelengths that could be varied throughout the analysis (Ex/Em: 288/462 nm (Flt), Ex/Em: 290/430 nm (BaP). Separation was carried out using a SUPELCOSIL LC-PAH (Supelco, Inc. Bellefonte, PA) reverse-phase C18 column (4.6×250 mm, 5 μm particle size) specific for PAH analysis. Acetonitrile was used as mobile phase (isocratic elution mode), operated at a flow rate of 1 mL min^{-1}; injection volume was 20 μL. We identified Flt and BaP via comparison of their retention time with those of the authentic standards and quantified them using the internal calibration method.

All data were subjected to strict quality control procedures. A series of solvent blanks (to determine any background contamination), spiked blanks, and a spiked matrix (to monitor recovery efficiency) were analyzed during the treatment and analysis procedures. Solvent blanks showed that Flt and BaP were detected in small amounts (< 5% of sample values). The spiked recoveries of Flt and BaP in water and filters were set in the range 82.3-100.6% and 95.7-113.3%. Method detection limits (MDLs), defined as mean blank value + 3 × standard deviation (SD), ranged from 0.02 to 0.10 ng mL^{-1} in water and filters. The concentrations of Flt and BaP were blank corrected, but not corrected with recoveries. All solvents used for sample processing and analyses (DCM, hexane, acetone, and acetonitrile) were of high-performance liquid chromatography (HPLC) grade and were purchased from Fisher (UK). All glassware was intensively cleaned before using.

2.5.4. Chl a analyies

Subsamples (50 mL) from axenic and non-axenic cultures were taken daily and filtered through glass microfiber filters (GF/F, Whatman). The Chl *a* was extracted with 90% acetone (v/v) for 30 h in the dark at 5°C. The pigment concentration was measured using the spectrophotometric method provided by Lorenzen and Jefferey (1980), following the procedure described in Parsons et al. (1984).

2.5.5. Growth kinetics

To determine the diatom growth kinetics, subsamples (1 mL), taken daily from replicates of axenic and non-axenic treatments, 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 a light microscope BX-102 A at × 40 magnification (Lundholm et al., 2004). The exponential growth rate was calculated by considering three successive counts made in the exponential growth phase, according to the formula $N_t = N_0\ e^{\mu t}$ and $\ln (N_t / N_0) = \mu\ t$, where N_0 and N_t are the initial and final abundance values of the diatom, respectively; µ (d^{-1}) is the net cell growth rate; 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.

2.5.6. Bacterial monitoring in axenic cultures

To ensure the success of the axenization method, total bacteria were enumerated via real-time PCR targeting 16S rRNA genes, using the primer sets 331F/797R as described above (Paragraph 2.3). Subsamples were collected from axenic cultures at the beginning of the experiments and after 3 and 7 days. Subsamples (200 mL) were filtered (0.5 and 0.22 µm), and total DNA was extracted from filters as described in Paragraph 2.3.

2.6. Determination of the relative contribution of the different PAH removal processes

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

$$\%PAH_{dis} = C_{PAHdis} / C_{PAHinitial} \times 100$$

305 $\%PAH_{acc} = C_{PAHpart} / C_{PAHinitial} \times 100$

306 $\%PAH_{deg} = (C_{PAHinitial} - C_{PAHdis} - C_{PAHpart}) \times 100$,

307 where $C_{PAHinitial}$ represents the amount of PAHs added at the beginning of the incubation and
308 C_{PAHdis} and $C_{PAHpart}$ represent the amounts of PAHs measured at the end in the dissolved and
309 particular fractions, respectively.

310 Many processes could contribute to the degradation of PAHs during the incubation. The
311 main abiotic process is photodegradation (PAH_{pho}), but volatilization (PAH_{vol}) and adsorption on
312 the walls of the glass bottles (PAH_{ads}) may also lead to PAH decrease and were therefore
313 considered in the calculation. The biotic processes were assigned to the biodegradation by
314 microorganisms (PAH_{biodeg}), including diatoms (PAH_{diadeg}) and total bacteria (PAH_{bacdeg}). The
315 importance of these processes is expected to differ among the experimental conditions, and hence,
316 the percentage of degraded PAHs is likely to vary:

317

318 ***Sterile condition***

319 ***in the dark***: $\%PAH_{deg} = \%\mathbf{PAH_{vol}} + \mathbf{\%PAH_{ads}}$ (Eq. 1)

320 ***in the light***: $\%PAH_{deg} = \%PAH_{vol} + \%PAH_{ads} + \%PAH_{pho}$ (Eq. 2)

321 So, Eq2 - Eq1 = $\mathbf{\%PAH_{pho}}$

322 The $\%PAH_{vol} + \%PAH_{ads}$ and $\%PAH_{pho}$ were considered as equivalent for all treatments and conditions.

323

324 ***Axenic condition***

325 $\%PAH_{deg} = \%PAH_{vol} + \%PAH_{ads} + \%PAH_{pho} + \%PAH_{biodeg}$ (Eq. 3)

326 Therefore, Eq3 – Eq2 = $\mathbf{\%PAH_{biodeg}}$

327 In axenic cultures, biodegradation was due to the diatom, and therefore, the $\mathbf{\%PAH_{biodeg}}$ was equal to
328 the $\mathbf{\%PAH_{diadeg}}$, which would not differ considerably from that of the non-axenic culture.

329

330 ***Non-axenic condition***

331 $\%PAH_{deg} = \%PAH_{vol} + \%PAH_{ads} + \%PAH_{pho} + \%PAH_{biodeg}$ (Eq. 4)

332 Therefore, Eq4 – Eq2 = $\mathbf{\%PAH_{biodeg}}$

333 In non-axenic cultures, biodegradation was due to both diatom biodegradation and bacterial
334 biodegradation; therefore, the $\mathbf{\%PAH_{biodeg}}$ minus the $\mathbf{\%PAH_{diadeg}}$ equaled to $\mathbf{\%PAH_{bacdeg}}$.

335

336

337 ***2.7. Statistical analysis***

Statistical analyses were performed using the SPSS software version 14.0 for Windows. Analysis of variance (ANOVA) was used to test the effects of BaP and Flt on Chl *a*, cell density, and growth rate. When the effect was significant, a multiple comparison posterior test (Tukey´s test) was performed to compare treatments in a 2 x 2 design. When the normality of data distribution (test of Kolmogorov-Smirnov) and/or the homogeneity of the variances (Bartlett-Box test) could not be verified, a non-parametric ANOVA (Kuskal-Wallis) was used. A student test was performed to compare the fractions of PAH remaining, accumulated, and degraded between axenic and non-axenic conditions and the fraction of PAHs degraded by diatoms and bacteria.

3. Results

3.1. Responses of Nitzschia *sp. to BaP and Flt*

The diatom maintained in the non-axenic control showed a pronounced growth, as the Chl *a* concentration increased significantly from an initial value of 37 ± 2.6 to 177 ± 19.5 µg L^{-1} at the end of the experiment (Fig. 1a). In BaP and Flt treatments, Chl *a* also showed an increase throughout the incubation, with slightly lower concentrations at the end of the experimental period (134 -137 ± 10.5 – 19.5 µg L^{-1}) relative to the control (Fig. 1a). In non-axenic cultures, the overall effect of the two PAHs on biomass was not significant ($P > 0.05$). In the control, *Nitzschia sp.* exhibited a growth kinetics characterized by a 2-day latency, followed by exponential growth until the end of the incubation period, where it reached extremely high densities (31 ± 5.2 10^4 cells mL^{-1}) (Fig. 1b). In both PAH treatments, the diatom showed the same pattern of proliferation; growth rates (0.44 ± 0.009 d^{-1} and 0.45 ± 0.0003 d^{-1}) were not significantly different from those of the control (0.50 ± 0.01 d^{-1}) ($P > 0.05$, Table 1).

The axenization of the diatom was successfully maintained during the experiment, as no bacterial abundance was detected by qPCR throughout the incubation period. The axenic strain of *Nitzschia sp.* showed continuous proliferation in the control, with Chl *a* concentrations (18-92 µg L^{-1}) and cell density levels (0.75-17 10^4 cells mL^{-1}) significantly lower than those in the non-

363 axenic control (37-177 µg Chl *a* L^{-1}; 1-31 10^4 cells mL^{-1}), concomitant with the lower growth rate
364 (Table 1). Contamination with Flt caused a slight decrease in cell density (Fig. 2d), but the growth
365 rate remained similar (0.41 ± 0.016 d^{-1}) to that of the control (0.43 ± 0.03 d^{-1}) (Table 1). In contrast,
366 BaP addition provoked a significant decrease in Chl *a* and cell density (Fig. 1c, d), together with
367 a decrease in growth rate (0.35 ± 0.002 d^{-1}) relative to the control (Table 1). When comparing the
368 responses of the axenic and non-axenic diatom strains, the most pronounced decrease induced by
369 PAHs (especially for BaP) on *Nitzschia* growth was observed in the axenic culture.

370 ***3.2. Fate of BaP and Flt***

371 The concentrations of Flt and BaP, measured in each treatment at the beginning of the
372 experiment, were compared with those used theoretically for contamination (Table 2). The results
373 show that the measured levels were close to 95% of the theoretical concentrations. Therefore, the
374 measured concentrations were used in the remainder of the results.

375 For all treatments and conditions, PAH levels remaining at the end of the experiment were
376 low (maximum 8.5 ± 1.4% for BaP). In contrast, PAH degradation (%PAHdeg) and PAH
377 accumulation in the cells ($\%PAH_{acc}$) were the dominant processes involved in PAH removal
378 (Table 3). However, the $\%PAH_{acc}$ and $\%PAH_{deg}$ were significantly different between non-axenic
379 and axenic cultures ($P < 0.05$, Table 3). There was more PAH accumulated in the axenic culture
380 (52.8% ± 3.0% for BaP and 33% ± 2.0% for Flt) than in the non-axenic culture (18.1 ± 1.6% for
381 BaP and 11.3 ± 1.5% for Flt). Conversely, the highest degradation was observed in non-axenic
382 cultures for both BaP and Flt (79.4 ± 2.3% and 88.2 ± 4.2%, respectively). Interestingly, the abiotic
383 removal of PAHs, mainly due to the photooxidation, did not account for more than 10 ± 1.8% for
384 BaP and 7.9 ± 1.1% for Flt. Hence, biodegradation was generally the main process of PAH
385 removal. Biodegradation of Flt was higher than that of BaP under both conditions, while more BaP
386 was accumulated. Interestingly, significant differences ($P < 0.05$) were observed for bacterial and
387 diatom degradation between both PAHs. Bacteria were able to degrade 40.7 ± 2.5% of the initial

BaP concentration, whereas the diatom showed a lower ability to degrade this PAH (28.7 ± 2.6%). In contrast, the microalgae exhibited a high degradation of Flt (53.5 ± 3.2%) compared to the bacteria (26.8 ± 2.2%). Moreover, the degradation level of Flt by the diatom was significantly ($P < 0.05$) larger than that of BaP.

3.3. Diversity and abundance of attached and free-living bacteria communities associated to Nitzschia *sp.*

The diversity of attached (AB) and free-living bacteria (FB) associated to *Nitzschia* sp. was evaluated using the 16S rRNA gene high-throughput sequencing approach. To identify and compare both AB and FB diversity, sequences were analyzed under the same conditions. Overall, 865 different OTUs were detected in the prokaryotic communities associated to the *Nitzschia sp.* culture. However, the AB community displayed higher species richness (observed OTUs) (562 OTUs) and species diversity (Simpson, Shannon indices) than the FB community (303 OTUs) (Table 4). In addition, the average abundance of free-living cells was lower ($0.34 \pm 0.06 \times 10^5$ DNA copies ml^{-1}) than that of attached bacteria ($2.1 \pm 0.42 \times 10^5$ DNA copies mL^{-1}).

Analysis of AB community composition showed that Alphaproteobacteria (54.0%), Cyanobacteria (35.5%), Bacteroidetes (3.4%), Gammaproteobacteria (3.3%), and Planctomycetes (2.7%) dominated the bacterial communities (Fig. 2a). The FB were greatly dominated by Alphaproteobacteria (51.9%), followed by Bacteroidetes (36.6%), Betaproteobacteria (4.8%), Firmicutes (2.0%), and Planctomycetes (1.2%). Within the archaea domain, Euryarchaeota was exclusively presented at low abundance (0.25%) in the AB fraction (Fig. 2a). Alphaproteobacteria (representing more than half of the prokaryote communities) and Planctomyctes (found in lower proportion) were observed in both AB and FB fractions. Actinobacteria, Betaproteobacteria, and Firmicutes were typically found among the FB fraction, while Cyanobacteria and Gammaproteobacteria were exclusively found among the attached bacteria, while Bacteroidetes were more abundant in the FB fraction than in the AB sample.

A Venn diagram demonstrated that OTUs differed among AB and FB fractions (Fig. 2c). In total, 217 OTUs were shared between both samples. A larger number of specific OTUs was detected in the AB fraction (345 OTUs) than in the FB fraction (86 OTUs). However, AB and FB were only dominated by 12 and 14 OTUs, respectively (Table S1). To further analyze the microbial community composition and structure, dominant genera (> 1% of all sequences) are presented in Fig. 2b. The most abundant genera, namely *Phaeocystidibacter*, *Mabikibacter*, *Roseitalea*, *Nioella*, and *Erythrobacter*, were identified in both AB and FL fractions. The genera *Dulcicalothrix*, *Chamaesiphon*, *Roseimaritima*, *Oricola*, and *Aestuariibius* were greatly related to the AB fraction. The FB fraction was particularly dominated by a member of the family Cryomorphaceae, previously isolated from marine algae (35.5% of all sequences), followed by the genera *Agrococcus*, *Pyruvatibacter*, *Acuticoccus*, *Marivita*, *Roseibaca*, and *Alcaligenes*.

3.4. Morphological characterization of bacterial isolates

Based on the morphological characteristics of the colonies, six bacterial strains from diatom *Nitzschia sp.* pure cultures were isolated and tested for their growth on crude oil (Table 5). Five strains (2, 3, 4, 5, and 6) belonged to the FB fraction, while Strain 1 was obtained from the AB fraction. Colonies were small, large, circular, round, smooth, oval, and approximately between 0.05 and 5 µm of diameter. Among these colonies, three strains (1, 2, 6) forming cream-colored colonies were obtained. Other colonies were yellow (Strain 3), white (Strain 4), and transparent (Strain 5). Cells of aerobic isolates were long rods (3-6 µm long x 0.1 µm wide) or coccoid (0.2-0.5 µm size), occurring as single cells or in pairs.

3.5. Phylogenetic analysis of attached and free-living bacterial isolates

The 16S rRNA gene sequences from the six strains were generated to determine their taxonomic group. Phylogenetic analysis revealed that those isolates belonged to the three phyla: *Proteobacteria* (*Gammaproteobacteria* class), *Firmicutes*, and *Actinobacteria* (Fig. 3). Strains 1 (attached bacteria), 4, and 6 (free-living bacteria) were related to the genus *Staphylococcus.* The

free-living bacterial Strains 2 and 3 belonged to *Acinetobacter* and *Micrococcus*, respectively. Strain 5 was affiliated to the species *Bacillus amyloliquefaciens*. These bacterial taxa showed low abundances (< 0.5% of all sequences) in both AB and FB fractions.

3.6. Screening for bacteria growing on crude oil

Following isolation and purification, all selected strains were screened for their potential growth in the presence of crude oil. After 15 days of incubation, there was a visual change in the aspect of oil crude with Strains 3, 4, and 6. After 30 days, microscopic observation showed that cultures of these strains had a clear appearance and a turbid aspect, suggesting that Strains 3, 4, and 6 (belonging to the genera *Micrococcus* and *Staphylococcus*) could maintain their growth in the presence of crude oil under aerobic conditions (Table 5).

4. Discussion

4.1. Influence of the associated bacteria on Nitzschia *growth*

In comparison to the axenic condition, *Nitzschia sp.* exhibited higher growth in non-axenic cultures (Table 1), with higher biomass and cell density (Fig. 1). This result agrees with previous findings that the presence of bacteria can promote the proliferation of microalgae (Kazamia et al., 2012; Amin et al., 2015). A commonly studied interaction between microalgae and their associated bacteria is the bacterial production of vitamins (cobalamin, thiamine, and biotin)) required by algal species for their growth (Kazamia et al., 2012). Diatom growth can also stimulated by bacteria (mainly *Proteobacteria*) through the excretion of extracellular polysaccharides (EPS) by phototrophs. These EPS, after bacterial remineralization, constitute an important nutrient source (Bruckner et al., 2008). Other studies also reported that the bacterial presence is beneficial for diatoms through the secretion of extracellular indole acetic acid (IAA), which is an auxin that optimizes algal growth (Seyedsayamdost et al., 2011; Lépinay et al., 2018). Amin et al. (2015)

showed that *Sulfitobacter* species could favor the division of the diatom *Pseudonitzschia multiseries via* the production of IAA by using the diatom's secreted and endogenous tryptophan.

4.2. Tolerance of Nitzschia *sp. to PAHs*

In this study, *Nitzschia sp*. was exposed to high levels of PAHs (256 µg Flt L^{-1} and 3 µg BaP L^{-1}), which exceeded the EC50 of growth inhibition reported for diatoms (18-200 µg L^{-1} for Flt and 1.18 µg L^{-1} for BaP) (Liu et al., 2006; Niehus et al., 2018). Despite this, the diatom kept a continuous proliferation until the end of the incubation in both axenic and non-axenic conditions (Fig. 1). In some case, biomass and cell density in contaminated cultures were lower than in the control, but growth rates were not significantly different, except for BaP in the axenic culture (Table 1; Fig. 1). This indicates that *Nitzschia sp*. can tolerate both HAPs; this is in agreement with the study of Croxton et al. (2015), who showed that the growth of *Nitzschia brevirostris* was unaffected by high a concentration of naphthalene (1,000 µg L^{-1}). Nevertheless, other studies have observed a significant decrease in diatom growth after PAH exposition (Bopp and Lettieri, 2007; Niehus et al., 2018). Obviously, the degree of tolerance/sensitivity to pollutants varies among algal species and depends greatly on the environment in which microalgae evolve (Ben Othmen et al., 2018; Pikula et al., 2019). According to Kottuparambil and Agusti (2018), microalgae, via natural evolution, can gain higher resistance to pollutants, allowing the survival of their populations in highly polluted environments. Consequently, *Nitzschia sp.*, isolated from a PAH-contaminated sediment of the Bizerte Lagoon (LaFabrie et al., 2013; Pringault et al., 2016), seems to be accustomed to high levels of PAHs and has acquired physiological abilities to tolerate and even metabolize these pollutants. This is in line with the idea that several diatom species were identified as good indicators of high PAH levels in surface sediments (Potapova et al., 2016). Interestingly, in PAH treatments, especially for BaP, *Nitzschia sp*. growth rates were lower in axenic conditions compared to non-axenic ones (Table 1, Fig. 1). This suggests that the presence of bacteria was beneficial for the diatom by improving its capacity to tolerate PAHs, which leads us to infer that

487 associated bacteria could protect the microalgae by reducing PAH toxicity, as has been observed
488 for metals and pesticides (Fouilland et al., 2018) or tannic acid (Bauer et al., 2010).

489 ***4.3. PAH accumulation and biodegradation***

490 The PAH concentration in the dissolved fraction was extremely low at the end of the
491 incubation, as the amounts of BaP and Flt remaining in the medium did not exceed 2.5-8.5 and
492 0.5-5.6%, respectively (Table 3). Several processes, including abiotic and biotic factors, can
493 contribute to PAH elimination (Ghosal et al., 2016). Abiotic losses, mainly due to photooxidation,
494 did not account for more than 10%. This indicates the high capacity of microorganisms to eliminate
495 PAHs, which is in agreement with previous studies reporting that a large fraction of PAH removal
496 in microalgae culture was attributed to biotic degradation (Hong et al., 2008; Diaz et al., 2015;
497 Kumari et al., 2016; García et al., 2017).

498 The amounts of PAHs biodegraded by *Nitzschia sp*. alone (28.7-53.7%) were 1.5-2.4 times
499 lower than when bacteria were present (69.4-80.3%) (Table 3). This suggests co-metabolic
500 synergy between microalgae and bacteria in the removal of PAHs, as previously reported (Borde
501 et al., 2003; Coulon et al., 2012, Kumari et al., 2016). The known hydrocarbon-degrading genera
502 *Marivita, Erythrobacter*, and *Alcaligenes* (Fig. 2b) and strains growing on crude oil (Table 3) were
503 observed in the bacterial community associated to *Nitzschia*. This suggests that these associated
504 hydrocarbonoclastic bacteria may enhance biodegradation of PAHs in non-axenic cultures, as
505 previously highlighted (Mishamandani et al., 2016; Thompson et al., 2017, 2018; Severin and
506 Erdner, 2019). The degradation of PAHs by microorganisms depends on their molecular weight,
507 hydrophobicity, and water solubility (Haritash and Kaushik, 2009). In general, PAHs with lower
508 molecular weights, lower octanol-water partitioning coefficients (K_{ow}), and higher water solubility
509 are more susceptible to biodegradation. The Flt (four-ring compound) displays a lower molecular
510 weight, a lower log K_{ow}, and a higher water solubility compared to the five-ring BaP (154 *vs.*
511 252 g mol^{-1}, 5.2 *vs.* 6, and 240 *vs.* 1.5 µg L^{-1}, respectively). Biodegradation of Flt by *Nitzschia sp.*

512 (53.5%) was higher than that of BaP (28.7%), whereas a higher proportion of BaP remained
513 accumulated in the diatom, as estimated by the PAH concentration measured in the particular phase
514 (Table 3). This suggests that Flt was more easily metabolized by *Nitzschia sp*. than BaP, which
515 appeared to be more stable and more difficult to degrade. However, in the non-axenic culture,
516 bacterial degradation of BaP (40.7%) was greater than that of Flt (26.8%). Indeed, a bacterial
517 consortium has previously been shown to prefer degrading five-to-six-ring PAHs (Moscoso et al.,
518 2012; Pugazhendi et al., 2016).

519 The accumulation of PAHs by the diatom also contributed to their removal, with $\%PAH_{acc}$
520 varying from 11.3 to 52.8 (Table 3). This is in agreement with previous reports for PAH
521 accumulation by microorganisms, including diatoms (such as *Skeletonema costatum*, *Nitzschia sp.*,
522 *Pseudonitzschia spp.*) (Lei et al., 2007; Hong et al., 2008). The $\%PAH_{acc}$ values were higher in
523 axenic (52.8% for BaP and 33% for Flt) than in non-axenic cultures (18.1% for BaP and 11.3%
524 for Flt) (Table 3). The bioaccumulation of PAHs by microalgae is directly related to the size and
525 the morphology of cells as well as the initial cell inoculum (Chan et al., 2006). In our experiment,
526 the same isolate of *Nitzschia sp*. was used in both axenic and non-axenic cultures with similar
527 inoculum densities. However, in the non-axenic culture, bacteria were able to degrade BaP and Flt
528 during the incubation, thereby decreasing the amount of PAH that might be accumulated in the
529 diatom cells (Table 3). Besides their degradation, associated bacteria might also influence the
530 availability of BaP and Flt for the diatom through the production of refractory/hydrophobic
531 dissolved organic matter (DOM). Heterotrophic bacteria are known to produce refractory DOM
532 via the assimilation of freshly produced/labile phytoplankton DOM (Jiao et al., 2010; Moran et al.,
533 2016). This bacteria-derived DOM, more aromatic/hydrophobic, could in turn bind with Flat and
534 BaP through hydrophobic interactions. These DOM-PAHs complexes are larger and more polar
535 than the free dissolved PAHs, thereby decreasing their availability for the diatom (Mei et al., 2009;
536 Yang et al., 2016). The capacity of bacteria associated to *Nitzschia sp.* in reducing PAH

accumulation in the diatom and their potential effect on microalgal metabolism may enable the diatom to cope with PAHs, resulting in better growth in non-axenic cultures compared to axenic ones (Table 3, Fig. 1).

4.4. Composition of associated bacterial communities and hydrocarbonoclastic potential of bacterial strains

There was a difference in community structure between attached (AB) and free-living (FB) bacteria associated to *Nitzschia sp*; the AB community exhibited a higher diversity and abundance than the FB community. Additionally, a significant variation in the relative abundance between attached and free-living communities was observed (Table 4, Fig. 2). Many studies indicated prominent differences in the community composition of particle-attached and free-living bacteria (Grossart et al., 2005; Bagatini et al., 2014; Rieck et al., 2015). The structure of the bacterial community in the phycosphere, both attached and free-living, is specific to algal species, since algae can release strain-dependent organic compounds that are used by specific bacteria (Bagatini et al., 2014). In this sense, the difference between *Nitzschia*-AB and FB could be explained by the consistent associations between specific bacterial taxa and the *Nitzschia sp*. host (Amin et al., 2012; Sison-Mangus et al., 2014; Behringer et al., 2018). Our results revealed that Nitzschia-attached bacteria mainly belonged to Proteobacteria (i.e., Alphaproteobacteria) and Cyanobacteria (Fig. 2a). Previous studies have indicated that Proteobacteria are commonly associated to benthic diatom species (Jauffrais et al., 2017; Keodoodor et al., 2019), and autotrophic nitrogen-fixing bacteria (*Cyanobacteria*) are known to interact with diatoms (Amin et al., 2012). Alphaproteobacteria also predominated in the FB community of *Nitzschia sp.*, as reported previously (Grossart et al., 2005; Buchan et al., 2014). Bacteroidetes, known as a common bacterial phylum associated to diatoms (Grossart et al., 2005; Amin et al., 2012; Jauffrais et al., 2017; Keodoodor et al., 2019), formed a large fraction of the FB of *Nitzschia sp.* (Fig. 2a). Attached and free-living communities were dominated by 16 genera (Fig. 2b), many of which are commonly

being observed in algal cultures or as epibionts in several phycospheres of dinoflagellates, diatoms, and coccolithophores (Foster et al., 2011; Wang et al., 2016).

The genera *Marivita*, *Erythrobacter*, and *Alcaligenes*, mainly found in the FB community (Fig. 2b), degrade a wide range of xenobiotics, including aliphatic and polyaromatic hydrocarbons (Yuan et al., 2015; Durán et al., 2019; Severin and Erdner, 2019). Furthermore, Strains 3, 4, and 6, isolated from the associated bacterial community, were able to grow in the presence of crude oil (Table 5), suggesting the development of oil degraders in the bacterial community associated to the diatom. Our result corroborated with previous reports on the presence of oil-degrading bacteria in some microalgae phycospheres (Mishamandani et al., 2015; Severin et al., 2016; Thompson et al., 2017, 2018). Strains 3, 4, and 6 were related to the genera *Staphylococcus* and *Micrococcus*, which have previously been identified as PAH degraders (Dong et al., 2015; Ghosal et al., 2016; Alegbeleye et al., 2017). Furthermore, species of *Micrococcus* and *Staphylococcus*, isolated from contaminated marine sediments, have been identified as naphthalene-degraders (Melcher et al., 2002; Zhuang et al., 2003) and as biosurfactant producers (Ibrahim et al., 2013; Bao et al., 2014; San et al., 2015). Although associated bacterial communities are generally species-dependent, they can also be influenced by environmental conditions. The benthic *Nitzschia sp*., used in our study, thrives in PAH-contaminated sediments (LaFabrie et al., 2013; Pringault et al., 2016), conditions that favor the presence of hydrocarbonoclastic bacteria (Ben Said et al., 2008) that might be in close interactions with the benthic diatom. The phycosphere of *Nitzchia sp.* could have contained hydrocarbonoclastic bacteria (mainly in the FB fraction), which could play an important role in the strong biodegradation of Flt and BaP under non-axenic conditions (Table 3).

5. Conclusions

The biodegradation, the most significant process of PAH removal, was significantly higher when bacteria were present in the culture of the benthis diatom (*Nitzschia sp.*). This highlights a possible co-metabolic synergy between microalgae and bacteria in PAH biodegradation, allowing

potential bioremediation applications for the removal of PAHs from marine environments. Investigation of the structure of bacterial communities associated to *Nitzschia sp.* (the phycosphere) revealed the possible presence of hydrocarbon-degrading bacteria belonging to the genera *Marivita, Erythrobacter,* and *Alcaligenes*. Furthermore, the associated bacteria harbored some strains, assigned to *Staphylococcus* and *Micrococcus,* which were able to grow on crude oil. This indigenous diatom, with its associated hydrocarbonoclastic bacteria, could find an interesting application in environmental biotechnology, enhancing the bioremediation of PAH-contaminated sites, or in depollution strategies following oil spills.

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Table 1

Exponential growth rate (d^{-1}) of *Nitzschia sp.* grown under non-axenic and axenic conditions in control (C) and contaminated treatments by two PAHs (BaP and Flt). Values are means ± standard deviations, letters indicate homogenous group, r^2 is coefficient of determination

Treatment	Non-axenic	Axenic
C	0.50 ± 0.01 [c] (r^2 = 0.94)	0.43± 0.03 [b] (r^2 = 0.85)
BaP	0.44 ± 0.009 [b] (r^2 = 0.96)	0.35 ± 0.002 [a] (r^2 = 0.90)
Flt	0.45 ± 0.0003 [b] (r^2 = 0.93)	0.41 ± 0.016 [b] (r^2 = 0.91)

Table 2

Theoretical and measured concentrations of Flt and BaP in *Nitzschia sp.* cultures under different conditions (axenic, non-axenic and sterile)

Values are means ± standard deviations

	Theoretical concentration	
	Flt (256 µg L^{-1})	**BaP (3 µg L^{-1})**
Measured concentrations		
Axenic	238.11 ± 4.68	2.53 ± 0.42
Non-axenic	250.79 ± 2.65	2.36 ± 0.27
Sterile, light	255.68 ± 3.09	2.59 ± 0.43
Sterile, dark	242.39 ± 4.10	2.65 ± 0.41

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929 **Table 3**
930 The % of PAHs (BaP and Flt) accumulated in cells ($\%PAH_{acc}$), remaining in the medium
931 ($\%PAH_{dis}$) and degarded by all processes ($\%PAH_{deg}$), including abiotic process [photo-oxidation
932 ($\%PAH_{pho}$), volatilization ($\%PAH_{vol}$) and adsorption ($\%PAH_{ads}$)] and biodegradation by
933 microorganisms ($\%PAH_{biodeg}$) [by diatom ($\%PAH_{diadeg}$) or by bacteria ($\%PAH_{bacdeg}$)], in the
934 *Nitzschia sp.* culture under non axenic and axenic conditions. Values are mean ± standard
935 deviation.
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	BaP		**Flt**	
	non-axenic	**axenic**	**non-axenic**	**axenic**
$\%PAH_{acc}$	18.1 ± 1.6	52.8± 3.0	11.3 ± 1.5	33.0 ± 2.0
$\%PAH_{dis}$	2.5 ± 0.6	8.5± 1.4	0.5 ± 0.0	5.6 ± 1.3
$\%PAH_{deg}$	79.4 ± 2.3	38.7± 2.0	88.2 ± 4.2	61.4 ± 4.1
$\%PAH_{pho}$	10.0 ± 1.8	10.0± 1.8	7.9 ± 1.1	7.9 ± 1.1
$\%HAP_{vol}$+ $\%PAH_{ads}$	0	0	0	0
$\%PAH_{biodeg}$	69.4 ± 2.6	28.7 ± 2.6	80.3 ± 5.2	53.5 ± 3.2
$\%PAH_{diadeg}$	28.7 ± 2.6	28.7 ± 2.6	53.5 ± 3.2	53.5 ± 3.2
$\%PAH_{bacdeg}$	40.7 ± 2.5		26.8 ± 2.2	

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Figure captions

Fig. 1. Temporal evolution of Chl *a* concentration and growth kinetics of *Nitchizia sp.* under non-axenic (a and b) and axenic (c and d) conditions in control (C) and contaminated treatments by BaP and Flt. Values are means ± standard deviations.

Fig. 2. Relative abundance of microbial communities in attached (AB) and free-living bacteria (FB) from *Nitzschia sp.* Culture. **A.** Relative phylogenetic abundance was based on frequencies of 16S rRNA gene sequences affiliated with archaea and bacterial phyla or proteobacterial classes in AB and FB fractions. **B**. Major genera from dominant OTU (>1% of all sequences) in AB and FB fractions. **C**. Venn diagram showing the distribution of prokaryotic measurable OTUs in AB and FB samples.

Fig. 3. Phylogenetic tree based on similarities of 16S rRNA sequences of bacterial isolates and its relatives. The tree was based on the Juke-Cantor model and the Neighbor-Joining method with bootstrap values for 1000 replicates. The *scale bar* represents 5% estimated sequence divergence. The archaeal sequence of *Natrinema altunense* was used as the outgroup

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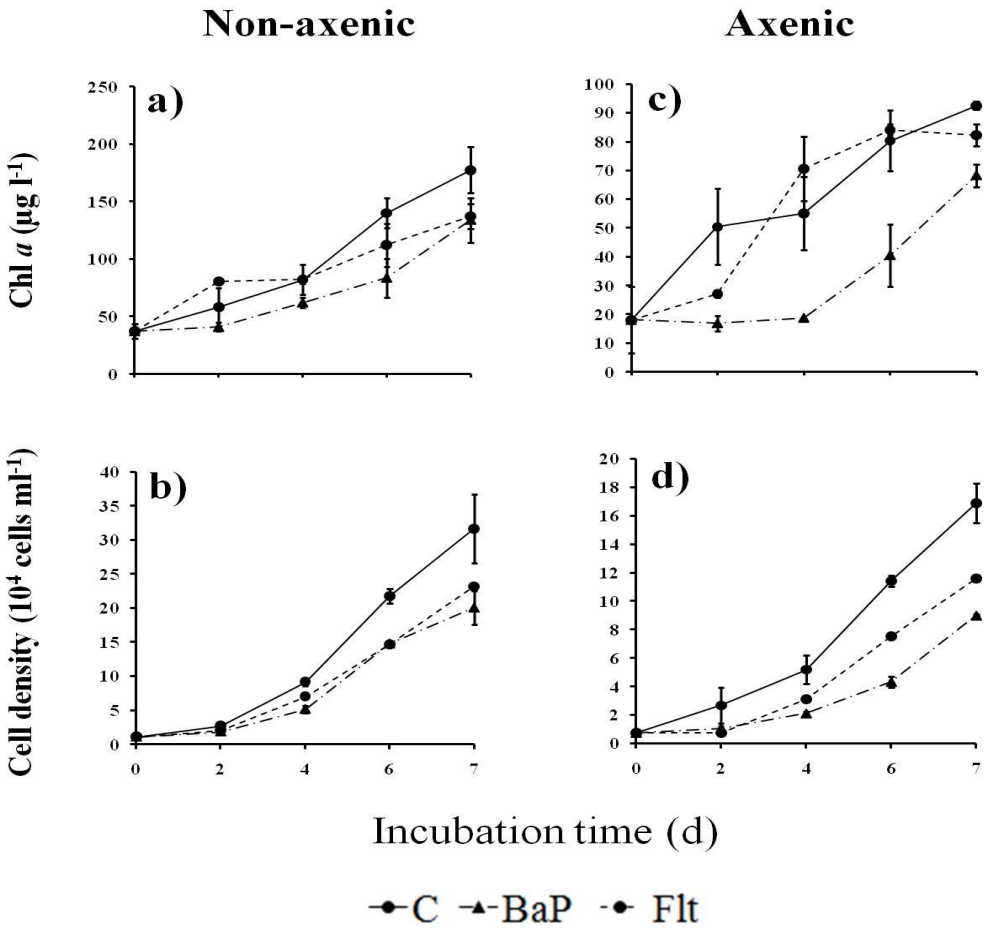

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993 **Fig. 1**

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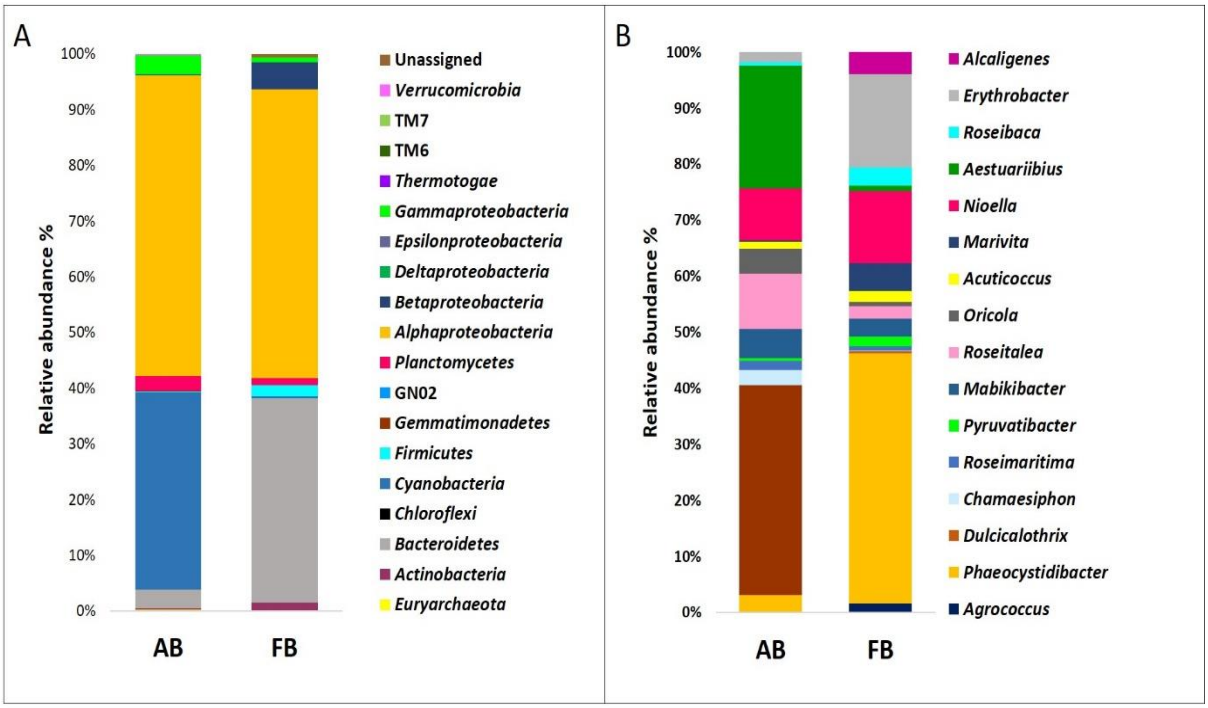
a) **b)**


A
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TM7
TM6
Thermotogae
Gammaproteobacteria
Epsilonproteobacteria
Deltaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Planctomycetes
GN02
Gemmatimonadetes
Firmicutes
Cyanobacteria
Chloroflexi
Bacteroidetes
Actinobacteria
Euryarchaeota
B
Alcaligenes
Erythrobacter
Roseibaca
Aestuariibius
Nioella
Marivita
Acuticoccus
Oricola
Roseitalea
Mabikibacter
Pyruvatibacter
Roseimaritima
Chamaesiphon
Dulcicalothrix
Phaeocystidibacter
Agrococcus

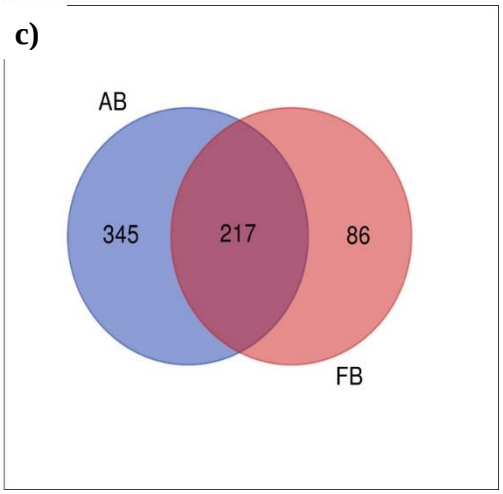
c)
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Fig. 2

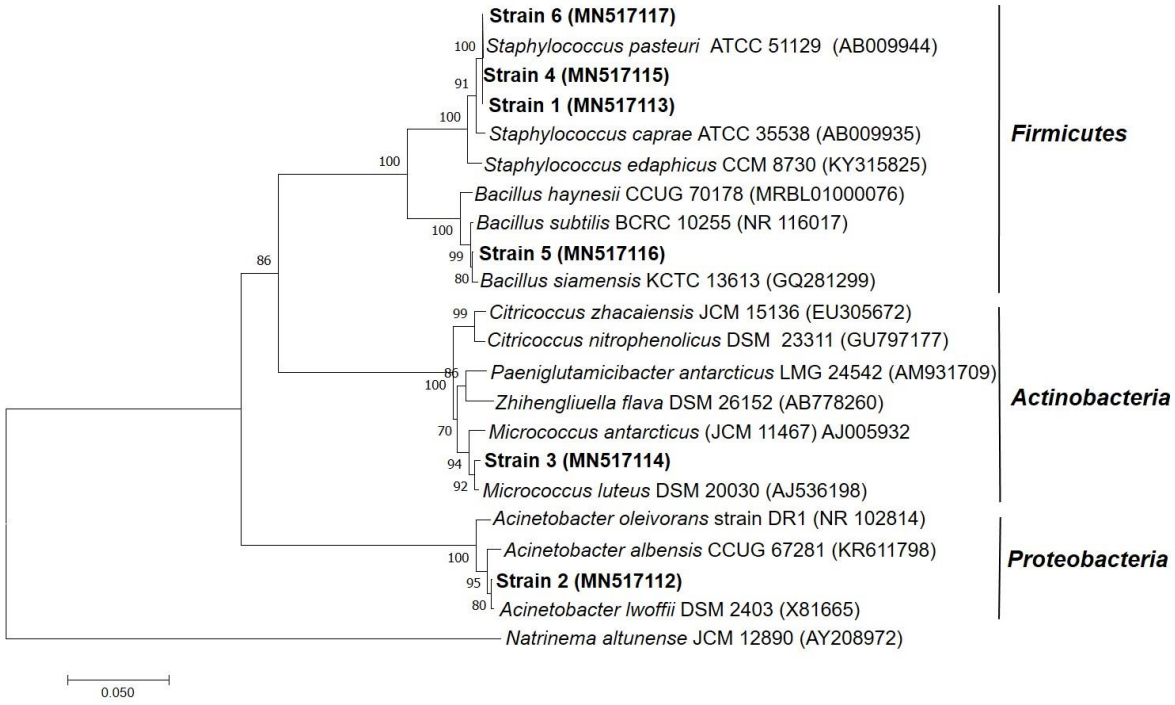
Strain 6 (MN517117)
Staphylococcus pasteuri ATCC 51129 (AB009944)
Strain 4 (MN517115)
Strain 1 (MN517113)
Staphylococcus caprae ATCC 35538 (AB009935)
Staphylococcus edaphicus CCM 8730 (KY315825)
Bacillus haynesii CCUG 70178 (MRBL01000076)
Bacillus subtilis BCRC 10255 (NR 116017)
Strain 5 (MN517116)
Bacillus siamensis KCTC 13613 (GQ281299)
Citricoccus zhacaiensis JCM 15136 (EU305672)
Citricoccus nitrophenolicus DSM 23311 (GU797177)
Paeniglutamicibacter antarcticus LMG 24542 (AM931709)
Zhihengliuella flava DSM 26152 (AB778260)
Micrococcus antarcticus (JCM 11467) AJ005932
Strain 3 (MN517114)
Micrococcus luteus DSM 20030 (AJ536198)
Acinetobacter oleivorans strain DR1 (NR 102814)
Acinetobacter albensis CCUG 67281 (KR611798)
Strain 2 (MN517112)
Acinetobacter lwoffii DSM 2403 (X81665)
Natrinema altunense JCM 12890 (AY208972)
Firmicutes
Actinobacteria
Proteobacteria
0.050

Fig. 3

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