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Efficiency of benthic diatom-associated bacteria in the removal of benzo (*a*)pyrene and fluoranthene

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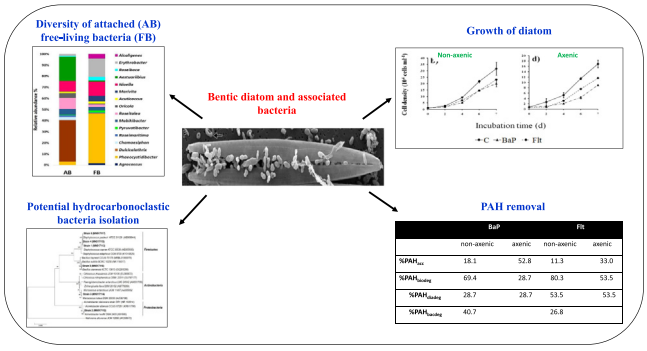
HIGHLIGHTS

- An indigenous benthic diatom accumulated BaP and Flt, but biodegradation played the main role in the removal of PAH.
- The biodegradation was enhanced by the presence of the diatom and its associated bacteria.
- The diatom harbored bacterial genera identified as potential PAH degraders.
- Strains isolated from the associated bacteria were able to grow in the presence of crude oil.

GRAPHICAL ABSTRACT



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ABSTRACT

We investigated the efficiency of a benthic diatom-associated bacteria in removing benzo(*a*)pyrene (BaP) and fluoranthene (Flt). The diatom, isolated from a PAH-contaminated sediment of the Bizerte Lagoon (Tunisia), was exposed in axenic and non-axenic cultures to PAHs over 7 days. The diversity of the associated bacteria, both attached (AB) and free-living bacteria (FB), was analyzed by the 16S rRNA amplicon sequencing. The diatom, which maintained continuous growth under PAH treatments, was able to accumulate BaP and Flt, with different efficiencies between axenic and non-axenic cultures. Biodegradation, which constituted the main process for PAH elimination, was enhanced in the presence of bacteria, indicating the co-metabolic synergy of microalgae and associated bacteria in removing BaP and Flt. Diatom and bacteria showed different capacities in the degradation of BaP and Flt. *Nitzschia* sp. harbored bacterial communities with a distinct composition between attached and free-living bacteria. The AB fraction exhibited higher diversity and abundance relative to FB, while the FB fraction contained genera with the known ability of PAH degradation, such as *Marivita*, *Erythrobacter*, and *Alcaligenes*. Moreover, strains of *Staphylococcus* and *Micrococcus*, isolated 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.

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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 et al., 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. Warshawsky et 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, in the phycosphere, could play a significant role in hydrocarbon degradation (Thompson et al., 2017, 2018). The phycosphere is a diffuse boundary layer that surrounds the phytoplankton cells, in which algal extracellular molecules stimulate the bacterial growth, representing the physical interface for close spatial interactions between phytoplankton and bacteria (Amin et al., 2012, 2015). Hence, the phycosphere has attracted considerable interest regarding its potential in pollutant degradation, and scientists have focused on its structure, ecology and interactions 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 their structure/molecular weight and hydrophobicity/water solubility, leading to potentially different degradation capacities by microalgae and bacteria. Flt ($C_{16}H_{10}$) is a four-ring, non alternant PAH consisting of a naphthalene and benzene unit connected by a five-membered ring. It is a structural isomer of the alternant PAH pyrene. BaP ($C_{20}H_{12}$) is a five-ring, alternant PAH consisting of five fused benzene rings (i.e., a benzene ring fused to pyrene). Flt and BaP (pyrogenic PAHs) are formed during the incomplete combustion of organic matter and fossil fuels. As PAHs containing more than three rings, they are hydrophobic, bioaccumulative, quite recalcitrant to microbial degradation and toxic for living organisms in all environments including marine medium (Cerniglia, 1992; Šepič et al., 2003; Ghosal et al., 2016). They are obviously part of the sixteen priority PAHs of the US-EPA. Due to its higher molecular weight and higher hydrophobicity BaP would be more recalcitrant than Flt. In addition, BaP would display a higher genotoxicity and carcinogenicity owing to its diol epoxide metabolites (known as BPDE) that react and bind to DNA to induce mutations and even cancers (Cerniglia, 1992; Frank et al., 2002; Ghosal et al., 2016; Honda and Suzuki, 2020). Subsequently, the structures and function diversity of bacteria associated with the diatom (attached or free-living) were also evaluated by Illumina MiSeq 16S rRNA sequencing and PICRUSt 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 of PAH-contaminated environments.

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 nearshore 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 μmol 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

To obtain 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 further filtration of the 5.0-μm filtrate through the use of a 0.22-μm cellulose polyester filter. These filters (two filters of 5.0 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 (5.0 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 (600 cycles). 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 (2×) (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.

The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict metagenome functional content from 16S rRNA gene in attached and free-living bacteria associated with *Nitzschia* sp. (Langille et al., 2013). Metagenome function was predicted with NSTI (Nearest Sequenced Taxon Index) values followed by the metabolic pathway reconstruction using KEGG (Kyoto Encyclopedia of Genes and Genomes) database.

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× 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): $(NH_4)_2SO_4$, 1 g; 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., 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 82–101% and 96–113%. 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 analyses

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 (day^{-1}) is the net cell growth rate; t (days) 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 (Section 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 Section 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):

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

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

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

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

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

Sterile condition

$$\textbf{\textit{in the dark}}: \%PAH_{deg} = \%\mathbf{PAH_{vol}} + \%\mathbf{PAH_{ads}} \quad (1)$$

$$\textbf{\textit{in the light}}: \%PAH_{deg} = \%PAH_{vol} + \%PAH_{ads} + \%PAH_{pho} \quad (2)$$

So, Eq. (2) – Eq. (1) = $\mathbf{\%PAH_{pho}}$.

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

Axenic condition

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

Therefore, Eq. (3) – Eq. (2) = $\mathbf{\%PAH_{biodeg}}$.

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

Non-axenic condition

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

Therefore, Eq. (4) – Eq. (2) = $\mathbf{\%PAH_{biodeg}}$.

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

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 × 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 day^{-1} and 0.45 ± 0.0003 day^{-1}) were not significantly different from those of the control (0.50 ± 0.01 day^{-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-axenic control (37–177 μg Chl *a* L^{-1}; 1–31 10^4 cells mL^{-1}), concomitant with the lower growth rate (Table 1). Contamination with Flt caused a slight decrease in cell density (Fig. 1d), but the growth rate remained similar (0.41 ± 0.016 day^{-1}) to that of the control (0.43 ± 0.03 day^{-1}) (Table 1). In contrast, BaP addition provoked a significant decrease in Chl *a* and cell density (Fig. 1c, d), together with a decrease in growth rate (0.35 ± 0.002 day^{-1}) relative to the control (Table 1). When comparing the responses of the axenic and non-axenic diatom strains, the most pronounced decrease induced by PAHs (especially for BaP) on *Nitzschia* growth was observed in the axenic culture.

3.2. Fate of BaP and Flt

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

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

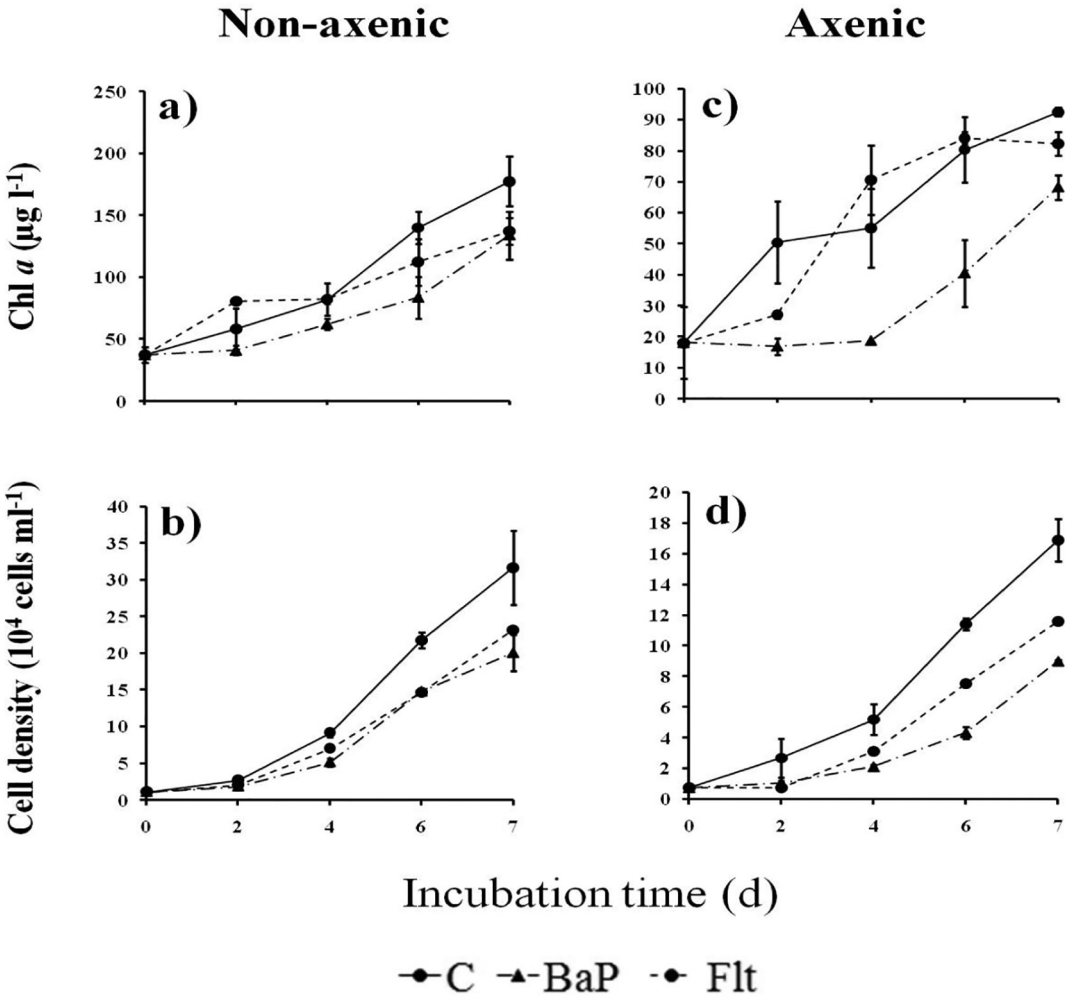

Fig. 1. Temporal evolution of Chl *a* concentration and growth kinetics of *Nitzschia* sp. under non-axenic (a and b) and axenic (c and d) conditions in control (C) and contaminated treatments by BaP and Flt. Values are means ± standard deviations.

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 AB ($2.1 \pm 0.42 \times 10^5$ DNA copies mL^{-1}).

AB community was dominated by four phyla: Proteobacteria (5×.X %), Cyanobacteria (35.5%), Bacteroidetes (3.4%), and Planctomycetes (2.7%) (Fig. 2a). Regarding Archaea, Euryarchaeota was exclusively observed at a low abundance (0.25%) in the AB fraction (Fig. 2a). The FB were also dominated by Proteobacteria (60%), followed by Bacteroidetes (36.6%), then Betaproteobacteria (4.8%), Firmicutes (2.0%), and Planctomycetes (1.2%). The classes Alphaproteobacteria (representing more than half of the prokaryotic communities) and Planctomycetia (found in lower proportion) were observed in both AB and FB fractions (Fig. 2b). However, the classes Actinobacteria, Betaproteobacteria, and Firmicutes were typically found in the FB fraction, while Cyanophyceae and Gammaproteobacteria were exclusively found in the AB. Moreover, Flavobacteriia (Bacteroidetes) were more abundant in the FB than AB sample.

A Venn diagram demonstrated that OTUs differed among AB and FB fractions (Fig. 2d). In total, 217 OTUs were shared between both samples. A larger number of specific OTUs were 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. 2c. The most abundant genera in both AB and FB fractions were: *Phaeocystidibacter*, *Mabikibacter*, *Roseitalea*, *Nioella*, and *Erythrobacter*. The genera *Dulcicalothrix*, *Chamaesiphon*, *Roseimaritima*, *Oricola*, and *Aestuariibius*

Table 1
Exponential growth rate (day^{-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.

Measured concentrations	Theoretical concentration	
	Flt (256 µg L^{-1})	BaP (3 µg L^{-1})
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

Table 3
The % of PAHs (BaP and Flt) accumulated in cells ($\%PAH_{acc}$), remaining in the medium ($\%PAH_{dis}$) and degraded by all processes ($\%PAH_{deg}$), including abiotic process [photo-oxidation ($\%PAH_{pho}$), volatilization ($\%PAH_{vol}$) and adsorption ($\%PAH_{ads}$)] and biodegradation by microorganisms ($\%PAH_{biodeg}$) [by diatom ($\%PAH_{diadeg}$) or by bacteria ($\%PAH_{bacdeg}$)], in the *Nitzschia* sp. culture under non axenic, axenic an sterile conditions. Values are mean ± standard deviation.

	BaP			Flt		
	Non-axenic	Axenic	Sterile	Non-axenic	Axenic	Sterile
$\%PAH_{acc}$	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	10.0 ± 1.8	7.9 ± 1.1	7.9 ± 1.1	7.9 ± 1.1
$\%HAP_{vol}$ + $\%PAH_{ads}$	0	0	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		

were greatly related to the AB fraction. On the contrary, the FB fraction was particularly dominated by a member of the genus *Phaeocystidibacter* (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. Prediction of bacterial functions by PICRUSt

The PICRUSt predicted metabolic analysis revealed similar functional profiles between the free-living (FB) and attached bacterial (AB) communities associated with *Nitzschia* sp. culture. AB and FB showed NSTI values of 0.13 and 0.06, respectively. PICRUSt predictions suggested a number of potential pathways associated with membrane transport, replication and repair, amino acid metabolism, carbohydrate metabolism, and energy metabolisms present at the significant relative abundance (>4%) in both AB and FB fractions (Fig. 3a). Interestingly, KEGG pathways involved in xenobiotics biodegradation and metabolism were also found in AB (4.1%) and FB (4.6%) fractions. Among xenobiotic degradation, genes related to benzoate, aminobenzoate, caprolactam, naphthalene, toluene, chloroalkane, chloroalkene, and polycyclic aromatic hydrocarbon degradation were observed with slightly difference in relative abundance in both fractions (Fig. 3b). Cytochrome P450 enzymes implicated into the degradation of xenobiotic compounds were also detected. Overall, PICRUSt analysis highlighted the degradation potential of a wide range of aliphatic and aromatic hydrocarbons by bacterial communities in AB and FB fractions.

3.5. 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 × 0.1 μm wide) or coccoid (0.2–0.5 μm size), occurring as single cells or in pairs.

Table 4
Richness and diversity of attached and free-living bacteria communities associated to *Nitzschia* sp.

	Attached bacteria (AB)	Free-living bacteria (FB)
Seqs/sample	109.908	43.479
Chao1	584.45	315.66
Observed OTUs	562.00	303.00
Shannon index	5.03	4.50
Simpson index	0.91	0.84
PD_whole_tree	26.48	11.12

3.6. 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. 4). 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.

3.7. 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 be 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

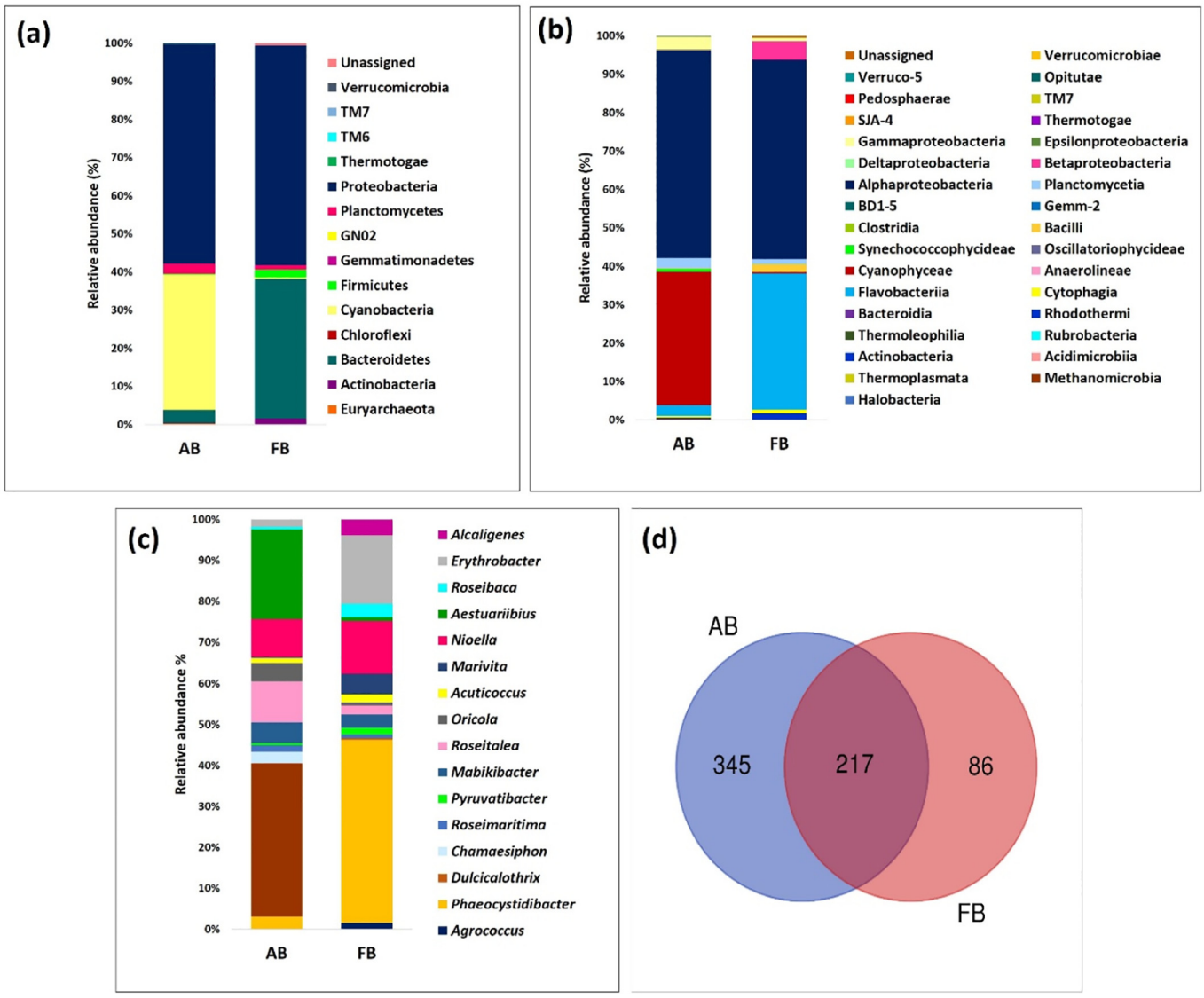

Fig. 2. Relative abundance of microbial communities in attached (AB) and free-living bacteria (FB) from *Nitzschia* sp. culture. Relative phylogenetic abundance was based on frequencies of 16S rRNA gene sequences affiliated with archaea and bacterial phyla (a) and classes (b) in AB and FB fractions.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

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 PAHs. The growth of another species of *Nitzschia* (*N. brevirostris*) was shown to be unaffected by high concentration of naphthalene (1000 μg L^{-1}) (Croxton et al., 2015). 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 Othman 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 associated bacteria could protect the microalgae by reducing PAH toxicity, as it has been observed for metals and pesticides (Fouilland et al., 2018) or tannic acid (Bauer et al., 2010).

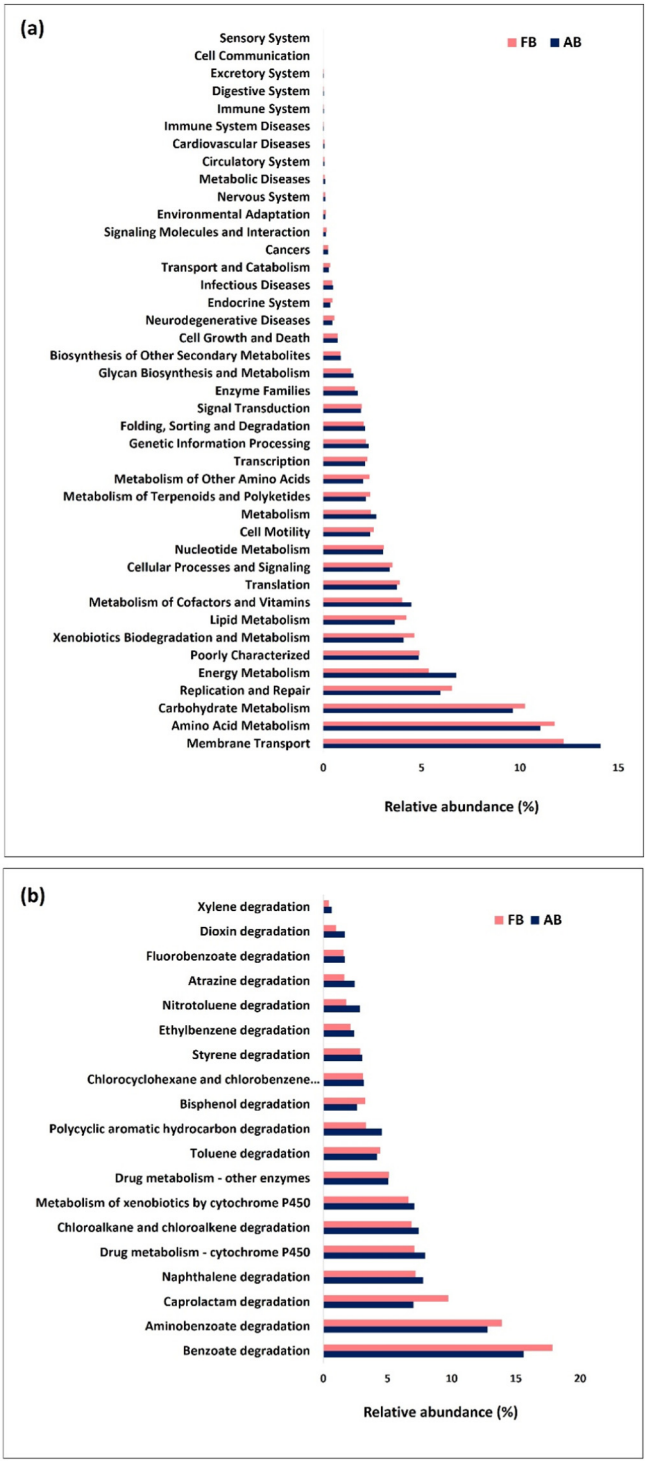

Fig. 3. (a) Predicted KEGG pathways in attached (AB) and free-living bacteria communities (FB) at level 2. (b) Predicted xenobiotic degradation pathways in KEGG in attached (AB) and free-living bacteria (FB) communities.

4.3. PAH accumulation and biodegradation

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

The amounts of PAHs biodegraded by *Nitzschia* sp. alone (29–54%) were 1.5–2.4 times lower than when bacteria were present (69–80%) (Table 3). This suggests co-metabolic synergy between microalgae and bacteria in the removal of PAHs, as previously reported (Borde et al., 2003; Coulon et al., 2012; Kumari et al., 2016). The known hydrocarbon-degrading genera *Marivita*, *Erythrobacter*, and *Alcaligenes* (Fig. 2c) and strains growing on crude oil (Table 3) were observed in the bacterial community associated to *Nitzschia*. This suggests that these associated hydrocarbonoclastic bacteria may enhance biodegradation of PAHs in non-axenic cultures, as previously highlighted (Mishamandani et al., 2016; Thompson et al., 2017, 2018; Severin and Erdner, 2019). The degradation of PAHs by microorganisms depends on their molecular weight, hydrophobicity, and water solubility (Haritash and Kaushik, 2009). In general, PAHs with lower molecular weights, lower octanol-water partitioning coefficients (K_{ow}), and higher water solubility are more susceptible to biodegradation. The Flt (four-ring compound) displays a lower molecular weight, a lower log K_{ow}, and a higher water solubility compared to the five-ring BaP (154 vs. 252 g mol^{-1}, 5.2 vs. 6, and 240 vs. 1.5 μg L^{-1}, respectively). Biodegradation of Flt by *Nitzschia* sp. (54%) was higher than that of BaP (29%), whereas a higher proportion of BaP remained accumulated in the diatom, as estimated by the PAH concentration measured in the particular phase (Table 3). This suggests that Flt was more easily metabolized by *Nitzschia* sp. than BaP, which appeared to be more stable and more difficult to degrade. However, in the non-axenic culture, bacterial degradation of BaP (41%) was greater than that of Flt (27%). Indeed, a bacterial consortium has previously been shown to prefer degrading five-to-six-ring PAHs (Moscoso et al., 2012; Pugazhendi et al., 2016). Bacteria utilize dioxygenase enzyme to incorporate two atoms of molecular oxygen into the aromatic nucleus to form *cis-dihydrodiols*. The substitution on the PAH molecule by two hydroxyl groups can be done in *orth* or *para* position. There is then fission of the aromatic rings in *orth* of the hydroxyl groups (cleavage between the carbons carrying the hydroxyl) or in *meta* (cleavage between the two carbons adjacent to the diol) by the action of a second dioxygenase. Other oxidation reactions lead to dihydroxylated metabolic intermediates of catechol or protocatechol type. These molecules are then converted into intermediates in the tricarboxylic acid cycle (Cerniglia, 1992; Gibson and Parales, 2000). Although bacteria utilize a common enzyme to degrade PAHs, the microalgae have several enzymes involved in PAH oxidation (Lei et al., 2007). The formation of *cis-dihydrodiols* suggests a dioxygenase similar to that found in bacteria (Warshawsky et al., 1995), but other enzymes, such as *o*-diphenol oxidase, peroxidase and glutathione *S*-transferase can play a role in the metabolism of PAHs by microalgae, and their activities varied from species to species (Lei et al., 2003, 2007).

The accumulation of PAHs by the diatom also contributed to their removal, with $\%PAH_{acc}$ varying from 11 to 53 (Table 3). This is in agreement with previous reports for PAH accumulation by microorganisms, including diatoms (such as *Skeletonema costatum*, *Nitzschia* sp., *Pseudonitzschia* spp.) (Lei et al., 2007; Hong et al., 2008). The $\%PAH_{acc}$ values were higher in axenic (53% for BaP and 33% for Flt) than in non-axenic cultures (18% for BaP and 11% for Flt) (Table 3). The bioaccumulation of PAHs by microalgae is directly related to the size and the morphology of cells, as well as the initial cell inoculum (Chan et al., 2006). In our experiment, the same isolate of *Nitzschia* sp. was used in both axenic and non-axenic cultures with similar inoculum densities. However, in the non-axenic culture, bacteria were able to degrade BaP and Flt during the incubation, thereby decreasing the amount of PAH that might be accumulated in the diatom cells (Table 3). Besides their degradation, associated bacteria

Table 5
Morphological characteristics of the aerobic bacterial isolates from *Nitzschia* sp. culture.

Isolate	Classification	Pigmentation	Colony size (μm)	Colony form	Cell morphology	Cell size (μm) Width × length or diameter	Mobility	Growth on crude oil	Genus
1	Attached bacteria	Cream	0.05	Small, round	Coccoid single or in pairs	0.2	−	−	*Staphylococcus*
2		Cream	1	Big, round	Coccoid single or in pairs	0.3	+	−	*Acinetobacter*
3		Yellow	0.05	Small, spherical	Geometrical form	0.5	−	+	*Micrococcus*
4	Free-living bacteria	White	0.05	Small, round	Coccoid single or in pairs	0.2	−	+	*Staphylococcus*
5		Transparent surrounded by a white layer	5	Big, oval	Long rods	0.1 × 3–6	−	−	*Bacillus*
6		Cream	0.05	Small, round	Coccoid single or in pairs	0.2	−	+	*Staphylococcus*

might also influence the availability of BaP and Flt for the diatom through the production of refractory/hydrophobic dissolved organic matter (DOM). Heterotrophic bacteria are known to produce refractory DOM via the assimilation of freshly produced/labile phytoplankton DOM (Jiao et al., 2010; Moran et al., 2016). This bacteria-derived DOM, more aromatic/hydrophobic, could in turn bind with Flt and BaP through hydrophobic interactions. These DOM-PAHs complexes are larger and more polar than the free dissolved PAHs, thereby decreasing their availability for the diatom (Mei et al., 2009; 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

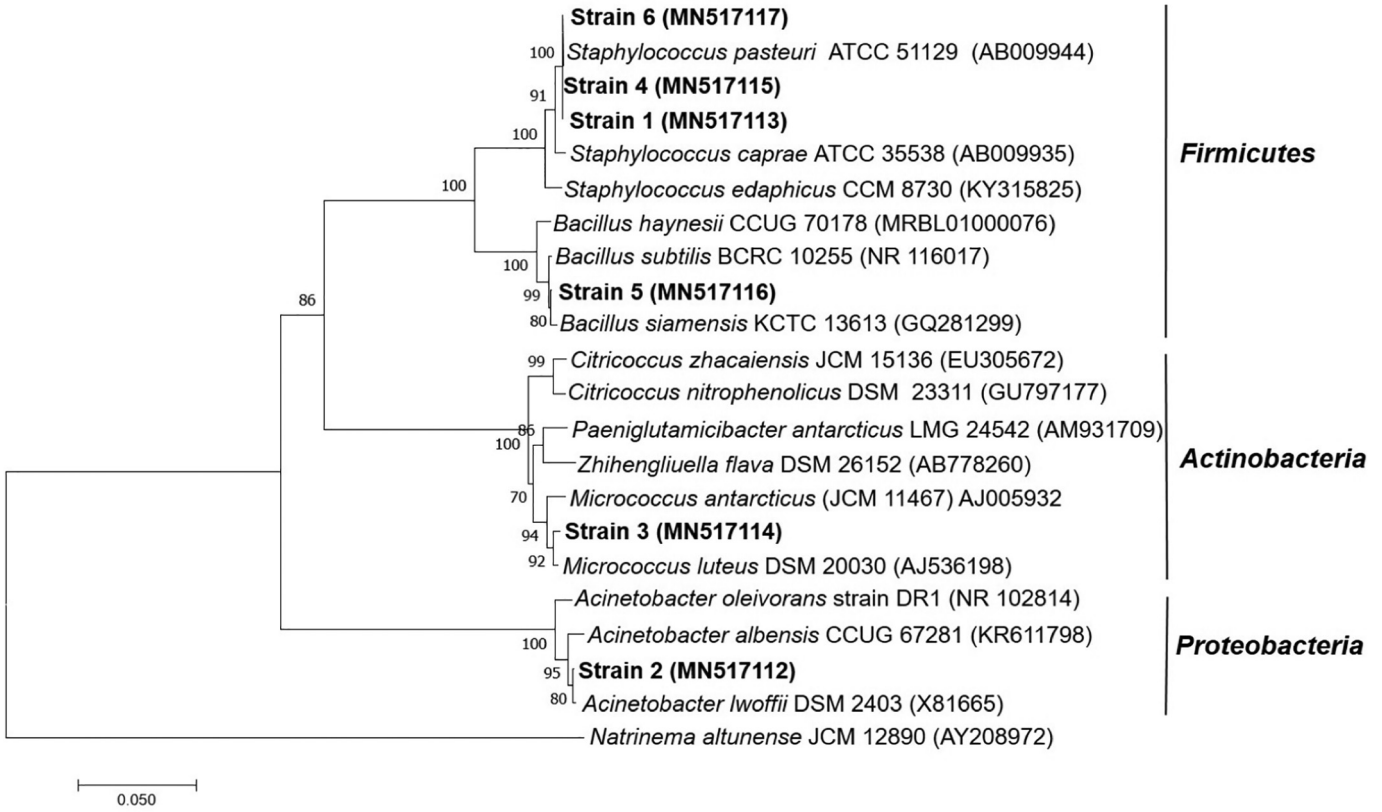

Fig. 4. Phylogenetic tree based on similarities of 16S rRNA sequences of bacterial isolates and its relatives. The tree was based on the Juke-Cantor model and the Neighbor-Joining method with bootstrap values for 1000 replicates. The scale bar represents 5% estimated sequence divergence. The archaeal sequence of *Natrinema altunense* was used as the outgroup.

(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 and b). Previous studies have indicated that Proteobacteria are commonly associated to benthic diatom species (Jauffrais et al., 2017; Koedooder 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; Koedooder et al., 2019), formed a large fraction of the FB of *Nitzschia* sp. (Fig. 2a).

Similar bacterial community functional patterns were obtained in associated bacteria and free-living bacteria. In particular, the abundant xenobiotics biodegradation and metabolism in both fractions could be explained by the occurrence of genes that could involve in the aromatic hydrocarbon degradation by bacterial communities associated with algal culture.

Attached and free-living communities were dominated by 16 genera (Fig. 2c), 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. 2c), 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 (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 *Nitzschia* 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 benthic 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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CRediT authorship contribution statement

Oumayma Kahla: Methodology, Investigation, Writing - original draft. **Sondes Melliti Ben Garali:** Methodology, Formal analysis, Investigation. **Fatma Karray:** Conceptualization, Investigation, Writing - original draft, Writing - review & editing. **Manel Ben Abdallah:** Methodology, Writing - original draft. **Najwa Kallel:** Methodology, Formal analysis. **Najla Mhiri:** Methodology, Formal analysis. **Hatem Zaghden:** Formal analysis. **Badreddine Barhoumi:** Methodology. **Olivier Pringault:** Funding acquisition, Writing - review & editing. **Marianne Quéméneur:** Writing - review & editing. **Marc Tedetti:** Methodology, Writing - review & editing. **Sami Sayadi:** Funding acquisition, Resources. **Asma Sakka Hlaili:** Conceptualization, Supervision, Writing - review & editing.

Declaration of competing interest

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

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