



# Co-culturing with bacteria modulates fatty acid composition in benthic diatom biofilms for lipid-based biotechnologies: A case study of *Amphora* sp.

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## ARTICLE INFO

### Keywords:

Bacterial-microalgae interactions  
Benthic diatoms  
Biomass production  
Biotic stress  
Co-culturing  
Lipid production  
Microalgal biofilms

## ABSTRACT

Despite being recognized as promising oleaginous microalgal resources, benthic diatom biofilms remain overlooked in microalgal biotechnology. To enhance their industrial potential, bacterial interactions can exploit to boost biomass, increase lipid yields and tailor lipid profiles. Given the complexity of natural biofilms, our study adopted a reductionist approach to investigate the impact of bacteria on the metabolism of a marine benthic diatom, *Amphora* sp., through binary co-cultures. Bacteria were isolated from non-axenic *Amphora* sp. biofilm cultures during the exponential phase in a lab-scale porous substrate biofilm photobioreactor. A bacterial biofilm assay was conducted to select biofilm-forming strains, followed by co-culturing them with *Amphora* sp. in bottle culture flasks, assuming these strains would persist and interact within the *Amphora* biofilm. All cultures were maintained for 6 days in F/2-enriched artificial seawater at 16 °C, under a 12:12 light:dark cycle (100 μmol photons.m<sup>-2</sup>.s<sup>-1</sup>). Biomass and lipid contents were quantified using the gravimetric method, while fatty acid profiles were analysed using GC-MS. Results showed that some bacterial strains reduced *Amphora* sp. biomass, while *Nitratireductor* sp. and *Sulfitobacter* sp. had no noticeable effect. However, significant shifts in fatty acid profile of *Amphora* sp. were observed in most co-cultures while none of the individual bacterial strains substantially affected lipid production compared to its axenic and non-axenic counterparts. Co-cultures with *Nitratireductor* sp. and *Sulfitobacter* sp. yielded 50–55 % saturated, 40–50 % monounsaturated, and 1–6 % polyunsaturated fatty acids, indicating favourable biodiesel properties. Thus, modifying the microbiome of microalgal biofilms could be an innovative strategy for tailoring fatty acid composition for lipid-based applications.

## 1. Introduction

The association of heterotrophic bacteria in microalgae production systems has garnered significant attention over the past three decades, as these interactions can positively influence the microalgal metabolism [1–3], modify the biochemical composition [4] and potentially lead to increased productivity and resilience of the culture systems [5]. The

interactions between these two inter-kingdom partners vary from mutualistic to antagonistic and are often considered species specific or strain specific [5,6]. From a biotechnological standpoint, mutualistic interactions are particularly valuable, as they enhance the fitness of both partners [7]. To leverage these interactions for biotechnological applications, co-culture systems have emerged as promising tools, offering simplified models to study the complex, multispecies relationships that

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<https://doi.org/10.1016/j.algal.2025.104449>

Received 26 June 2025; Received in revised form 10 November 2025; Accepted 1 December 2025

Available online 2 December 2025

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naturally occur between microalgae and bacteria [8]. Such approaches enable us to disentangle functional interactions and support the development of stable and scalable strategies, compared to multispecies co-cultures, to enhance biomass productivity and the biosynthesis of valuable microalgal metabolites for industrial applications [9,10].

Numerous studies have demonstrated that co-cultures with bacteria whether isolated from native environments [11], derived from long-term maintained stock cultures [12,13], or introduced as exogenous microorganisms [14] can not only increase microalgal biomass productivity but also enhance the production of economically valuable metabolites, such as lipids. For instance, bacterial strains such as *Bacillus*, *Rhizobium*, *Marinobacter*, and *Azospirillum* have been reported to increase biomass and lipid production in microalgae including *Chlorella vulgaris*, *Scenedesmus obliquus*, and *Phaeodactylum tricornutum* through metabolite exchange and nutrient cycling [15,16]. In contrast, certain bacteria like *Algoriphagus* and *Muricauda* exhibit algicidal activity or compete for nutrients, leading to detrimental effects [16]. Such variability depends on multiple factors including extracellular polymeric substances (EPS) composition in the phycosphere, microalgal and bacterial growth conditions, the nature of their association, and other environmental stimuli [2,17]. Therefore, a better understanding of the benefits and disadvantages of algal-bacterial associations is necessary for the microalgae industry to meet the expectations of bio-based economy through microbiome engineering [5,18].

Among co-cultivation strategies, biofilm-based microalgal-bacterial systems hold great potential in microalgal biotechnology [8], as bacteria can enhance microalgal growth, modify biochemical composition and stabilize biofilm structure [10]. These systems have been extensively explored in wastewater treatment, where complex interactions among multiple phototrophs and heterotrophs support nutrient removal [10,19]. However, due to their inherent complexity and the challenges associated with system management [8], the ecological and functional roles of these microbial interactions remain largely overlooked, and their utilization in broader biotechnological contexts remains underexploited [20]. On the other hand, effective development of such systems depends on selecting microalgal strains with appropriate biofilm-forming capacities, as this directly influences system design and performance [20,21].

Marine benthic diatoms are key photosynthetic microalgae in shallow marine environments, playing essential roles in sediment stabilization, nutrient cycling, and food web dynamics [22,23]. Many of these periphytic species naturally form biofilms, whose structure and productivity are influenced by environmental conditions and interactions with bacteria, fungi, and protozoa [17,24]. EPS secreted by diatoms maintain biofilm integrity, while heterotrophic partners aid in nutrient regeneration [25,26]. Thus, employing benthic microalgae in biofilm-based microalgal-bacterial systems can be economically advantageous, as it eliminates the need to induce biofilm formation using chemical agents such as flocculants or biopolymers [21]. Beyond their ecological importance, recent studies highlight that some benthic diatoms have significant potential as oil crops, making them valuable resources for lipid-based biotechnological applications, including food, cosmetics, pharmaceuticals, nutraceuticals, and biofuels [27–29]. Since the overall growth and health of biofilm-forming benthic diatoms are influenced by associated microorganisms, primarily bacteria, understanding these relationships and their impact on microalgal growth could provide an alternative strategy to eco-physiological modifications and technically complex genetic or metabolomic engineering for enhancing growth and the production of valuable metabolites in these diatoms [2,5,30].

Despite increasing interest in algal-bacterial co-cultures, most studies have focused on planktonic microalgae, with limited attention given to microalgal biofilms, particularly those involving benthic diatoms. Rather than introducing exotic bacteria, we hypothesised that long-term laboratory-maintained stock cultures of benthic diatom biofilms harbour naturally co-adapted bacteria that, through synergistic

interactions and metabolic complementarity, can serve as suitable candidates for microbiome engineering to enhance biomass and lipid productivity in microalgal biofilms. Therefore, this study represents a preliminary workflow using a reductionist approach to screen bacterial partners for our subsequent co-culture experiments. As not all native associates are necessarily beneficial [16], the main objective was to identify bacterial partners capable of enhancing the biomass and lipid production of the natural biofilm-forming marine benthic diatom *Amphora* sp. NCC169. This strain was selected because *Amphora* sp. NCC169 is recognized as an oleaginous benthic diatom, reportedly exceeding 20 % lipid content by dry biomass [27,28,31]. Bacteria were isolated from non-axenic *Amphora* sp. biofilm cultures during the exponential growth phase in a vertical, tubular, lab-scale biofilm porous substrate photobioreactor. To identify suitable bacterial candidates for co-culturing, a biofilm formation assay was conducted under the assumption that strains capable of forming biofilms would persist within the *Amphora* sp. biofilm and interact functionally. Subsequently, co-culture experiments were carried out to assess bacterial influence on *Amphora* sp. biomass and lipid production. To our knowledge, this is the first study to assess how binary co-cultures with bacterial partners affect the biomass and lipid composition of a benthic microalgal biofilm.

## 2. Materials and methods

This study comprises two distinct phases. In the first phase, non-axenic *Amphora* sp. was cultured in a vertical biofilm porous substrate photobioreactor (PSBR) (see Supplementary I) to isolate bacteria associated with the biofilm at different time points during its exponential phase (mid and late exponential phases). The second phase focused on identifying the role of these isolated bacteria in influencing the growth and lipid production of *Amphora* sp.

### 2.1. Culture maintenance

#### 2.1.1. Stock culture of marine benthic diatom, *Amphora* sp. NCC169

*Amphora* sp. NCC169 is a strain collected from the Northwest French Atlantic coast (47°22'06" N / 02°32'52" W) in 2005, previously hosted by the Nantes Culture Collection (NCC) at Nantes University, France, under the reference NCC169, and currently hosted by Roscoff Culture collection (RCC) under the reference RCC5813. The non-axenic *Amphora* sp. culture was maintained in artificial seawater (see Supplementary II, salinity - 28 ‰, pH - 7.8) enriched with F/2 medium at 16 °C under a 12:12 light-dark cycle and a photon flux density of 100  $\mu\text{mol.photons.m}^{-2}.\text{s}^{-1}$ , without agitation.

#### 2.1.2. Preparing axenic culture of *Amphora* sp.

Axenic *Amphora* sp. cultures were prepared from non-axenic cultures using two separate antibiotic treatments, administered at one-week intervals, each involving an antibiotic-antimycotic solution at a concentration of 10 mL per liter (10,000 units of penicillin, 10 mg of streptomycin, and 25  $\mu\text{g}$  of amphotericin B per mL; BioReagent, A5955 SIGMA) [32]. At the end of each treatment, the absence of bacteria was verified by plating the culture media on marine agar. If bacteria were still present, an additional antibiotic treatment was performed. Culture conditions were the same as described above.

### 2.2. Phase I: Bacteria isolation from non-axenic *Amphora* sp. cultivated in the biofilm PSBR

#### 2.2.1. Culturing non-axenic *Amphora* sp. in the biofilm PSBR for bacteria isolation

Non-axenic *Amphora* sp. NCC169 was cultivated in a vertical tubular biofilm PSBR based on Arnaldo's PSBR implementation [31] with a few minor modifications. In brief, pre-combusted (at 400 °C for 4 h) and pre-weighed microfiber glass filter discs ( $\varnothing$  25 mm, 1.2  $\mu\text{m}$  porosity, Whatman™ GF/C, China) were inoculated at a concentration of  $2 \times 10^6$

cells per 2 mL in tissue culture 6-well plates (Dutscher, China) and maintained for 4 days under the culture conditions described in section 2.1, with continuous white light, to obtain a stabilized biofilm on the filter discs before introducing them into the biofilm PSBR. In total, 30 discs were introduced into the PSBR and culturing *Amphora* sp. NCC169 was then carried out at 18–20 °C with continuous light at a photon flux density of 100  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  of photosynthetically active radiation (PAR). To identify the exponential and stationary phases, the non-axenic *Amphora* sp. was first cultivated for 15 days in the biofilm PSBR (in three trials; see Supplementary III). Once these phases were identified, *Amphora* sp. was cultivated again under the same conditions for 6 days to reach the late exponential phase, allowing for bacterial isolation at different time points.

### 2.2.2. Bacteria isolation and purification

Biofilm PSBR culturing of non-axenic *Amphora* sp. for bacterial isolation was conducted in two trials, during which three discs with well-established biofilms, based on visual inspection, were collected at mid (Day 3) and late exponential phase (Day 6). Day 0 was represented by discs collected at the end of the inoculation period, just before introduction to the PSBR (see section 2.2.1). Collected discs were then placed in 2 mL microtubes for bacterial isolation, filled by 1.5 mL of sterilized artificial seawater culture medium, followed by vigorous pipetting (up and down) for about 1 min. The resulting suspension was then serially diluted from  $10^{-1}$  to  $10^{-7}$ , and 100  $\mu\text{L}$  from each dilution was plated on marine agar medium and incubated in the dark at 20 °C for 2–3 weeks. All handling was performed in an aseptic environment, and marine agar plates were prepared with cycloheximide ( $\text{C}_6\text{H}_{12}\text{N}_2\text{O}_4$ ) at a concentration of 20  $\text{mg}\cdot\text{L}^{-1}$  to inhibit fungal and diatom interference [33]. During the incubation period, the emergence of colonies was recorded. Discrete colonies with different bacterial morphotypes were identified based on colony size, color, margin, texture, opacity, and shape. After the incubation period, bacterial isolates displaying similar morphological characteristics from each harvesting day of each trial were further purified by streaking three times on marine agar plates. Finally, purified bacteria were cultivated in liquid marine broth and stored at –80 °C with the addition of 30 % glycerol [11].

## 2.3. Phase II: Co-culturing bacteria and *Amphora* sp. for assessing the lipid production

### 2.3.1. Selection of bacteria based on their biofilm forming ability

The biofilm production of isolated bacteria was quantified in triplicate using microplates to identify the most effective biofilm-forming bacterial strains according to the method described by Joublin-Delavat et al. [34]. Briefly, purified bacteria (see section 2.2.2) were cultured in 5 mL marine broth for 48 h at 18 °C with shaking. In order to increase the biofilm formation, culture strains were diluted with a fresh sterile marine broth medium until a final  $\text{OD}_{600\text{nm}}$  of 0.01. Then, 200  $\mu\text{L}$  of this OD adjusted suspension was inoculated in quadruplicate in a 96-polystyrene-well microplate and placed in a microplate reader (Varioskan Lux, Thermo Fisher Scientific™, France) for 48 h at ambient temperature (19–21 °C) without shaking, and  $\text{OD}_{600\text{nm}}$  was measured every 30 min. After 48 h, cell suspensions were removed and about 220  $\mu\text{L}$  of crystal violet (0.1 %) was added into the wells to stain the biofilm. After 10 min incubation, wells were washed twice with distilled water to remove excess crystal violet. Microplates were dried for 24–48 h at room temperature, and crystal violet was dissolved with 30 % acetic acid to solubilize the stained biofilm for 10 min before  $\text{OD}_{550\text{nm}}$  measurement. Biofilm production was estimated via the Normalized Biofilm Formation (NBF) calculation (Eq. 1), by dividing the  $\text{OD}_{550\text{nm}}$  measured after crystal violet staining by the maximal  $\text{OD}_{600\text{nm}}$  measured during the growing time of the corresponding well to mitigate biases arising from variations in cell numbers among different bacterial strains. Accordingly, NBF value was used to characterise the biofilm production:  $< 4$  = weak biofilm-forming bacteria, 4–12 = moderate biofilm-forming

bacteria, and  $> 12$  = strong biofilm-forming bacteria. Moderate to strong biofilm-forming bacteria were then selected for co-culturing with axenic *Amphora* sp. in bottle culture flasks.

$$\text{NBF} = \frac{\text{OD}_{550\text{nm}}}{\text{OD}_{600\text{nm}}} \quad (1)$$

### 2.3.2. Bacteria identification and phylogenetic analysis

Approximately 2 mL of individual bacterial cultures in marine broth were centrifuged at 10,000g for 5 min. Total DNA was extracted using the NucleoSpin Microbial DNA Mini Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. DNA quantity and quality ( $\text{OD}_{260\text{nm}}/\text{OD}_{280\text{nm}} = 1.8$ –2.2 and  $\text{OD}_{260\text{nm}}/\text{OD}_{230\text{nm}} = 2.0$ –2.2) were measured using SkanIt microplate reader software (version 7.0.2.5) and a Varioskan Lux spectrophotometer with a  $\mu\text{Drop}$  plate (Thermo Fisher Scientific™, France) based on spectral scanning data. The 16S rRNA genes were PCR-amplified using the primers, 357f-GC (5'-CCTACGG-GAGGCAGCAG-3') and 907rM (5'-CCGTCAATTCMTTGTAGTTT-3'). The PCR products were analysed by electrophoresis on 2 % agarose gels and purified PCR amplicons were sent to the sequencing facility (Eurofins, France) for Sanger sequencing. All sequences were compared with known sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) from NCBI (<http://www.ncbi.nlm.nih.gov/>) to identify the closest relatives, using a  $> 99$  % similarity value as the cutoff.

### 2.3.3. Cell enumeration

**2.3.3.1. *Amphora* sp. NCC169.** For *Amphora* sp. cell enumeration, starter cultures or bottle co-cultures were gently agitated by magnetic stir (Fisherbrand®, France) for 2 min to allow cells homogenization and aggregate destruction. Cell concentration was then performed using a Neubauer haemocytometer (MARIENFELD, Germany) and an optical microscope (OLYMPUS CH40, Japan, objective Olympus  $\times 20$ ).

**2.3.3.2. Bacteria.** Bacterial cell enumeration for co-cultures with *Amphora* sp. was performed using the colony-forming unit (CFU) method. A small aliquot of the stock culture was incubated in marine broth at 18–19 °C for 48 h. Subsequently, the marine broth culture was serially diluted ( $10^{-1}$  to  $10^{-7}$ ), and a known volume of a small aliquot of each diluted culture were plated onto marine agar medium. After incubation at 20 °C for one-week, bacterial colony-forming units (CFUs) were counted from plates where the count ranged between 30 and 300 CFUs and expressed as CFU per millilitre.

### 2.3.4. Co-culturing bacteria with axenic *Amphora* sp. NCC 169 in bottle culture flasks

Based on the results of the biofilm assay, moderate to strong biofilm-forming bacteria were used for co-culturing with axenic *Amphora* sp. For rapid screening of their effects on *Amphora* sp. lipid production, 100 mL (175  $\text{cm}^2$ ) bottle cell culture flasks were preferred, with no stirring to avoid any resuspension and also to mimic horizontal microalgal biofilm system. This setup allowed multiple co-cultures to be conducted in triplicate and in parallel, which would not have been possible with a single biofilm PSBR. The axenic culture served as a reference for pure *Amphora* sp. growth, while the non-axenic culture was used to simulate more natural environment. Therefore, both axenic and non-axenic cultures of *Amphora* sp. were used as control groups to provide a baseline for comparison. For co-cultures, axenic *Amphora* sp. and bacteria were inoculated in the flasks at a 10:1 ratio (30,000 *Amphora* sp. cells. $\text{mL}^{-1}$  to 3000 bacteria cells. $\text{mL}^{-1}$ ) and cultured for 6 days, reaching the late exponential phase as determined for controls in the bottle culture flasks (see Supplementary IV). This ratio was selected to support the establishment of a diatom-dominated biofilm, taking into account literature that has reported algae-to-bacteria ratios of 1:1 [11], 3:1 [45], and 10:1 [42,61], although these ratios are known to be species-specific. For this preliminary work, we adopted a 10:1 ratio as a starting point to favour

diatom dominance during the exponential phase while avoiding extremes of bacterial concentration that could lead to nutrient and space competition. Thereafter, the communities were allowed to equilibrate naturally. The cultures were maintained under the same conditions described in Section 2.1. The relative growth rates,  $\mu$ , of *Amphora* sp. in axenic, non-axenic and co-cultures were calculated using the following equation (Eq. 2).

$$\mu = \frac{(\ln N_{t2} - \ln N_{t1})}{T} \quad (2)$$

where,  $\mu$  - the relative growth rate ( $\text{day}^{-1}$ ),  $N_{t1}$  - cell concentration (cells.  $\text{mL}^{-1}$ ) at  $t_1$ ,  $N_{t2}$  - cell concentration (cells.  $\text{mL}^{-1}$ ) at the  $t_2$ ,  $T$  - number of days between  $t_1$  and  $t_2$ .

### 2.3.5. Biomass estimation

The total volume of each sample was filtered through pre-combusted ( $400^\circ\text{C}$  for 4 h) and pre-weighed glass microfiber filters ( $\varnothing$  47 mm, 0.7  $\mu\text{m}$  porosity, Whatman™ GF/F, China) after homogenizing the biofilm using a magnetic stirrer. Filters with cells were washed using  $\sim 10$  mL of isotonic solution ( $\text{NH}_4\text{HCO}_2$  at  $68 \text{ g.L}^{-1}$ ) to prevent cell lysis during the washing step, while removing salt residues. Then, wet filters were frozen at  $-80^\circ\text{C}$ , freeze-dried at  $-50^\circ\text{C}$  and weighed (Explorer EX125, Ohaus Explorer® semi-micro analytical balance, France). These filtration and washing steps were assumed to remove a substantial proportion of bacteria, whereas bacteria tightly attached to diatom cells were not intentionally removed, as their biomass contribution was considered negligible. The biomass production (Eq. 3) and biomass productivity (Eq. 4) were estimated as follows,

$$\text{Biomass production} = \frac{\text{Total dry biomass}}{\text{Total culture volume}} \quad (3)$$

$$\text{Biomass productivity} = \text{Biomass production} \times \mu \quad (4)$$

where, biomass production = expressed in  $\text{g.L}^{-1}$ , Biomass productivity = expressed in  $\text{g.L}^{-1}.\text{day}^{-1}$ ,  $\mu$  = the relative growth rate ( $\text{day}^{-1}$ ).

### 2.3.6. Lipid analysis

**2.3.6.1. Quantification of the total lipid content of *Amphora* sp. NCC169 co-cultures.** The total lipid extraction and lipid quantification were conducted as described by Cointet et al. [27], based on the modified Bligh and Dyer method [35]. Lyophilized biomass was soaked in 10 mL of solvent mixture of dichloromethane / methanol ( $\text{CH}_2\text{Cl}_2$  /  $\text{CH}_3\text{OH}$ ) at 1:1 (v/v) ratio for 24 h. Then the mixture was filtered using pleated filters, 100 mm diameter, 10  $\mu\text{m}$  pore size, to remove cell debris including silica. Thereafter, the filtrate was washed by adding 50 % of volume of distilled water and allowed to separate the organic phase. The lipid fraction (organic phase) was retrieved and dried over with anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ). The dried organic phase was filtered and evaporated under  $\text{N}_2$  and weighed to determine the amount of crude lipid extract. The total lipid rate was expressed in the percentage of the total extracted dry biomass (DW) (Eq. 5). The lipid production and lipid productivity were calculated using eq. 6 and 7 respectively. All lipid extracts were stored at  $-20^\circ\text{C}$  after adding 1 mL of  $\text{CH}_2\text{Cl}_2$  until fatty acid profile analysis.

$$\text{Total lipid rate} = \frac{\text{Crude lipid content}}{\text{DW}} \times 100 \quad (5)$$

$$\text{Lipid production} = \frac{\text{Crude lipid content}}{\text{Total culture volume}} \quad (6)$$

$$\text{Lipid productivity} = \text{Lipid production} \times \mu \quad (7)$$

where, crude lipid = expressed in g, DW = dry weight of biomass (g), lipid production = expressed in  $\text{g.L}^{-1}$ , lipid productivity = expressed in

$\text{g.L}^{-1}.\text{day}^{-1}$ ,  $\mu$  = the relative growth rate ( $\text{day}^{-1}$ ).

**2.3.6.2. Fatty acids profile analysis via gas chromatography- mass spectrometry analyses (GC - MS).** A mixture of 500  $\mu\text{L}$  of 3  $\text{mol.L}^{-1}$  hydrochloric methanol ( $\text{CH}_3\text{OH}/\text{HCl}$ ), 300  $\mu\text{L}$  methanol and 100  $\mu\text{L}$  chloroform ( $\text{CHCl}_3$ ) was added on  $\sim 1$  mg of crude lipid into a dry bath tube and refluxed at  $80^\circ\text{C}$  for 5 h. After cooling, 500  $\mu\text{L}$  hexane ( $\text{C}_6\text{H}_{14}$ ) was added and centrifuged at 1236g for 5 min. The hexane phase was collected and a small amount of  $\text{Na}_2\text{SO}_4$  was added. Finally, the fatty methyl esters (FAME) weight was then obtained after evaporation under  $\text{N}_2$  into a pre-weighed vial. N-Acyl pyrrolidides (NAP) was prepared from FAME to determine the positions of double bonds in unsaturated fatty acids. For this, 500  $\mu\text{L}$  of pyrrolidine and 100  $\mu\text{L}$  of acetic acid were added on the dried FAME and refluxed at  $85^\circ\text{C}$  for 1 h. After cooling, 4 mL of  $\text{CH}_2\text{Cl}_2$  and 500  $\mu\text{L}$  of distilled water were added. The mixture was shaken well and centrifuged at 1236g for 5 min. The aqueous phase was removed, a small amount of  $\text{Na}_2\text{SO}_4$  was added and centrifuged under the same conditions. Supernatant was collected to a pre-weighed vial and evaporated under  $\text{N}_2$  at  $45^\circ\text{C}$ . Finally, 1 mL of  $\text{CH}_2\text{Cl}_2$  per 1 mg of each FAME or 1 mg of NAP was added to analyse the fatty acid profile using gas chromatography coupled with mass spectrometry (GC-MS), following the conditions outlined by Cointet et al. (2021). Chromatogram peaks were evaluated, and peak areas were quantified using OpenChrom lablicate edition 1.5.0 (McLafferty) software. Fatty acids identification was confirmed by comparing mass spectra and retention time data with a library build from previous analyses, available on LipidMaps (<https://www.lipidmaps.org/>).

### 2.4. Statistical analysis

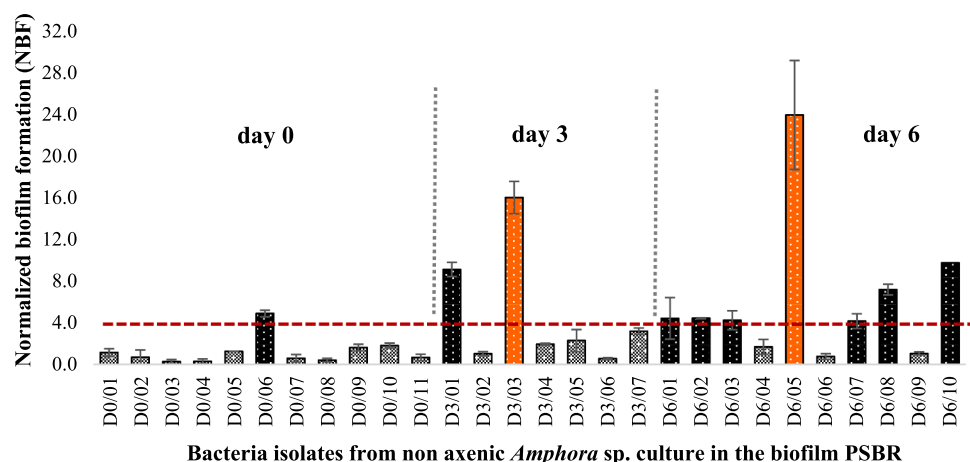
All experiments were conducted in triplicate, and the results are presented as means  $\pm$  standard deviation. The normality of the growth, biomass, and lipid parameters of *Amphora* sp. in axenic, non-axenic, and co-cultures was assessed using the Shapiro-Wilk test to determine the appropriate statistical analyses. For normally distributed data, parametric tests were applied, while non-normally distributed data were analysed using non-parametric tests. Significant differences ( $p$ -value  $\leq 0.05$ ) in the growth, biomass, and lipid-related parameters of *Amphora* sp. under each culture condition were identified using one-way ANOVA (or the Kruskal-Wallis test), followed by Tukey's post hoc test at a 95 % confidence interval (or Dunn test post hoc test). The abundance of fatty acid profiles in each culture condition was visualized using a heatmap generated with the 'ggplot2' package, while data manipulation and reshaping were facilitated by the 'tidyr' package. Principal Component Analysis (PCA) was used to visualize the distribution of all growth, biomass, and lipid-related parameters of *Amphora* sp. in axenic, non-axenic, and co-cultures. PCA was performed using the 'prcomp()' function from the 'stats' package and visualized with the 'factoextra' package. All statistical tests, graphics, and tables were generated using RStudio (version 4.1.2) and Microsoft Excel 365.

## 3. Results

### 3.1. Bacteria associated with the *Amphora* sp. NCC169 in the biofilm PSBR and their biofilm-forming ability

A total of 28 bacterial isolates were obtained during the exponential phase of non-axenic *Amphora* sp. culturing (see Supplementary V). Eleven isolates were obtained from inoculated discs at day 0, while seven isolates were obtained at day 3 (i.e. mid-exponential phase), and ten at day 6 (i.e. late exponential phase). Among these, 18 of the isolated bacteria showed NBF values  $< 4$ , confirming their weak biofilm-forming ability and suggesting a lower likelihood of retention in the biofilm of *Amphora* sp. (Fig. 1). Further, bacterial isolates recovered at day 0 exhibited relatively lower biofilm-forming abilities compared to those





**Fig. 1.** Biofilm-forming ability of bacterial isolates recovered from the non-axenic culture of *Amphora* sp. NCC169 in the biofilm PSBR by crystal violet assay at day 0 (before inoculation in the PSBR), day 3 (mid-exponential phase of *Amphora* sp.) and day 6 (late-exponential phase of *Amphora* sp.). Each bar consists of the mean value of NBF ( $n = 3$  biological replicates, 4 wells per replicate)  $\pm$  standard deviation; orange color bars indicate the strongest biofilm forming bacterial isolates (NBF  $> 12$ ), black color bars indicate the moderate biofilm forming bacterial isolates ( $4 < \text{NBF} < 12$ ), grey color bars indicate the weak biofilm forming bacterial isolates (NBF  $< 4$ ). the red dotted line: NBF = 4. The code refers to the isolates, but for the corresponding Genus name, see Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

recovered at day 3 and day 6. In parallel, the number of bacterial isolates with higher biofilm-forming ability increased with the age of the culture of *Amphora* sp. Consequently, the top 10 biofilm forming bacterial isolates, with NBF values  $> 4$ , comprising 8 moderate and 2 strong biofilm-forming isolates, were selected for the co-culturing experiment. This included 1 isolate from day 0, along with 2 and 7 isolates associated with *Amphora* sp. during day 3 and day 6 respectively.

### 3.2. Bacteria identification

Results showed that the ten bacterial isolates used for co-culturing with axenic *Amphora* sp. originated from three different phyla: Proteobacteria, Bacteroidetes, and Actinobacteria. At the class level, these isolates belonged to  $\alpha$ -Proteobacteria (7 isolates),  $\gamma$ -Proteobacteria (1 isolate), Flavobacteria (1 isolate), and Actinobacteria (1 isolate). Furthermore, among these ten bacterial isolates, *Nitratireductor* sp. isolated on day 0 and *Sulfitobacter* sp. isolated on day 3 were also present on day 6. They were morphologically similar (see Supplementary V), and their impact on the biomass and lipid production of *Amphora* sp. did not differ significantly from each other. Therefore, the results of these two genera are presented as a group hereafter (Table 1).

### 3.3. Growth and biomass analysis of *Amphora* sp. in co-cultures

Even though no significant differences ( $p > 0.05$ , Tukey's HSD) were observed between the axenic and non-axenic cultures, the trends indicated a higher growth rate ( $0.55 \pm 0.02 \text{ day}^{-1}$ ) and cell density ( $83 \pm 10 \times 10^4 \text{ cells.mL}^{-1}$ ) for non-axenic *Amphora* sp., whereas axenic *Amphora* sp. exhibited higher biomass productivity ( $0.08 \pm 0.01 \text{ g.L}^{-1} \text{ day}^{-1}$ ) and biomass production ( $0.16 \pm 0.01 \text{ g.L}^{-1}$ ) (Table 2). None of the co-cultures exhibited significantly higher growth or biomass parameters compared to the controls. However, most co-cultures, including those with *Maribacter* sp., *Pseudomonas* sp., *Janibacter* sp., *Brevundimonas* sp., *Aureimonas* sp., and *Erythrobacter* sp., exhibited significantly lower ( $p < 0.05$ , Tukey's HSD) growth and biomass parameters compared to at least one of the controls. The co-cultures with *Nitratireductor* spp. and *Sulfitobacter* spp., demonstrated no significant differences ( $p > 0.05$ , Tukey's HSD) in all biomass parameters, except for cell density in co-cultures with *Sulfitobacter* spp.

**Table 1**

Identification of moderate to strong biofilm-forming bacteria associated with the biofilm of *Amphora* sp. NCC169.

Code	Genus name/ Grouping		Class	Phylum
D0/ 06 and D6/ 01	<i>Nitratireductor</i> sp.	<i>Nitratireductor</i> spp.	$\alpha$ -Proteobacteria	Proteobacteria
D3/ 01 and D6/ 07	<i>Sulfitobacter</i> sp.	<i>Sulfitobacter</i> spp.	$\alpha$ -Proteobacteria	Proteobacteria
D3/ 03	<i>Maribacter</i> sp.	<i>Maribacter</i> sp.	Flavobacteria	Bacteroidetes
D6/ 02	<i>Pseudomonas</i> sp.	<i>Pseudomonas</i> sp.	$\gamma$ -Proteobacteria	Proteobacteria
D6/ 03	<i>Janibacter</i> sp.	<i>Janibacter</i> sp.	Actinobacteria	Actinobacteria
D6/ 05	<i>Brevundimonas</i> sp.	<i>Brevundimonas</i> sp.	$\alpha$ -Proteobacteria	Proteobacteria
D6/ 08	<i>Aureimonas</i> sp.	<i>Aureimonas</i> sp.	$\alpha$ -Proteobacteria	Proteobacteria
D6/ 10	<i>Erythrobacter</i> sp.	<i>Erythrobacter</i> sp.	$\alpha$ -Proteobacteria	Proteobacteria

### 3.4. Lipid production of *Amphora* sp. in co-cultures

The lipid production, lipid percentage of dry biomass, and lipid productivity of all co-cultures showed no significant differences compared to both axenic and non-axenic controls ( $p > 0.05$ , Tukey's HSD) (Table 3). However, the general trend suggested that axenic *Amphora* sp. exhibited slightly higher volumetric lipid production ( $0.22 \pm 0.00 \text{ g.L}^{-1}$ ) and lipid productivity ( $0.011 \pm 0.002 \text{ g.L}^{-1} \text{ day}^{-1}$ ) compared to non-axenic *Amphora* sp. and all co-cultures. However, all co-cultures exhibited an average lipid-to-dry biomass weight of more than 15 % compared to the controls. Notably, the co-cultures with *Maribacter* sp. and *Janibacter* sp. had lipid percentages ranging from 20 to 25 % of their dry biomass, while the co-culture with *Brevundimonas* sp. ranged from 19 to 32 %.

**Table 2**  
Bacterial co-culturing effect on the growth and biomass of *Amphora* sp. in bottle culture flasks at the end of 6-day culture period, with comparisons to axenic and non-axenic *Amphora* sp.

Culture condition	Growth rate (day <sup>-1</sup> )	Cell density (Cells×10 <sup>4</sup> .mL <sup>-1</sup> )	Biomass productivity (g.L <sup>-1</sup> .day <sup>-1</sup> )	Biomass production (g.L <sup>-1</sup> )
Axenic	0.50 ± 0.02	61 ± 06	0.08 ± 0.01	0.16 ± 0.01
Non axenic	0.55 ± 0.02	83 ± 10	0.07 ± 0.00	0.13 ± 0.01
Axenic + <i>Nitratireductor</i> spp.	0.51 ± 0.02	63 ± 09	0.07 ± 0.01	0.14 ± 0.02
Axenic + <i>Sulfitobacter</i> spp.	0.50 ± 0.06	46 ± 14●	0.04 ± 0.02	0.09 ± 0.03
Axenic + <i>Maribacter</i> sp.	0.31 ± 0.15▲	33 ± 05●	0.03 ± 0.00	0.07 ± 0.01
Axenic + <i>Pseudomonas</i> sp.	0.43 ± 0.04	39 ± 10●	0.03 ± 0.00▲	0.06 ± 0.00▲
Axenic + <i>Janibacter</i> sp.	0.30 ± 0.04▲	19 ± 04▲	0.02 ± 0.01▲	0.07 ± 0.01*
Axenic + <i>Brevundimonas</i> sp.	0.41 ± 0.01	35 ± 03●	0.03 ± 0.00*	0.07 ± 0.00*
Axenic + <i>Aureimonas</i> sp.	0.40 ± 0.08	35 ± 16●	0.02 ± 0.00▲	0.06 ± 0.01*
Axenic + <i>Erythrobacter</i> sp.	0.48 ± 0.04	54 ± 13	0.03 ± 0.00*	0.05 ± 0.00▲

Note: Values represent the mean ± standard deviation of minimum three biological replicates, \* significant different to axenic *Amphora* sp., ● significant different to non-axenic *Amphora* sp., ▲ significant different to both axenic and non-axenic *Amphora* sp.

**Table 3**  
Lipids production, lipid % to dry biomass (DW) and lipid productivity of *Amphora* sp. in axenic, non-axenic, and co-culture conditions based on the modified Bligh and Dyer gravimetric method in bottle culture flasks after a 6-day culture period. Lipid parameters were not significantly different across the groups.

Culture condition	Lipid production (g.L <sup>-1</sup> )	Lipid % DW	Lipid productivity (g.L <sup>-1</sup> .day <sup>-1</sup> )
Axenic	0.022 ± 0.00 <sup>ns</sup>	14.73 ± 3.31 <sup>ns</sup>	0.011 ± 0.002 <sup>ns</sup>
Non axenic	0.018 ± 0.01 <sup>ns</sup>	14.28 ± 5.39 <sup>ns</sup>	0.010 ± 0.004 <sup>ns</sup>
Axenic + <i>Nitratireductor</i> spp.	0.018 ± 0.01 <sup>ns</sup>	19.43 ± 6.94 <sup>ns</sup>	0.009 ± 0.004 <sup>ns</sup>
Axenic + <i>Sulfitobacter</i> spp.	0.014 ± 0.01 <sup>ns</sup>	17.92 ± 12.41 <sup>ns</sup>	0.007 ± 0.004 <sup>ns</sup>
Axenic + <i>Maribacter</i> sp.	0.015 ± 0.00 <sup>ns</sup>	21.85 ± 2.27 <sup>ns</sup>	0.006 ± 0.000 <sup>ns</sup>
Axenic + <i>Pseudomonas</i> sp.	0.017 ± 0.01 <sup>ns</sup>	26.14 ± 11.68 <sup>ns</sup>	0.007 ± 0.003 <sup>ns</sup>
Axenic + <i>Janibacter</i> sp.	0.016 ± 0.00 <sup>ns</sup>	24.05 ± 0.67 <sup>ns</sup>	0.006 ± 0.002 <sup>ns</sup>
Axenic + <i>Brevundimonas</i> sp.	0.017 ± 0.01 <sup>ns</sup>	25.33 ± 6.29 <sup>ns</sup>	0.007 ± 0.002 <sup>ns</sup>
Axenic + <i>Aureimonas</i> sp.	0.011 ± 0.00 <sup>ns</sup>	17.03 ± 1.96 <sup>ns</sup>	0.004 ± 0.001 <sup>ns</sup>
Axenic + <i>Erythrobacter</i> sp.	0.008 ± 0.00 <sup>ns</sup>	15.51 ± 3.01 <sup>ns</sup>	0.004 ± 0.000 <sup>ns</sup>

Note: Values represent the mean ± standard deviation of minimum three replicates. ns-not significantly different.

3.4.1. Fatty acid profile of axenic, non-axenic and *Amphora* sp. NCC169 in co-cultures

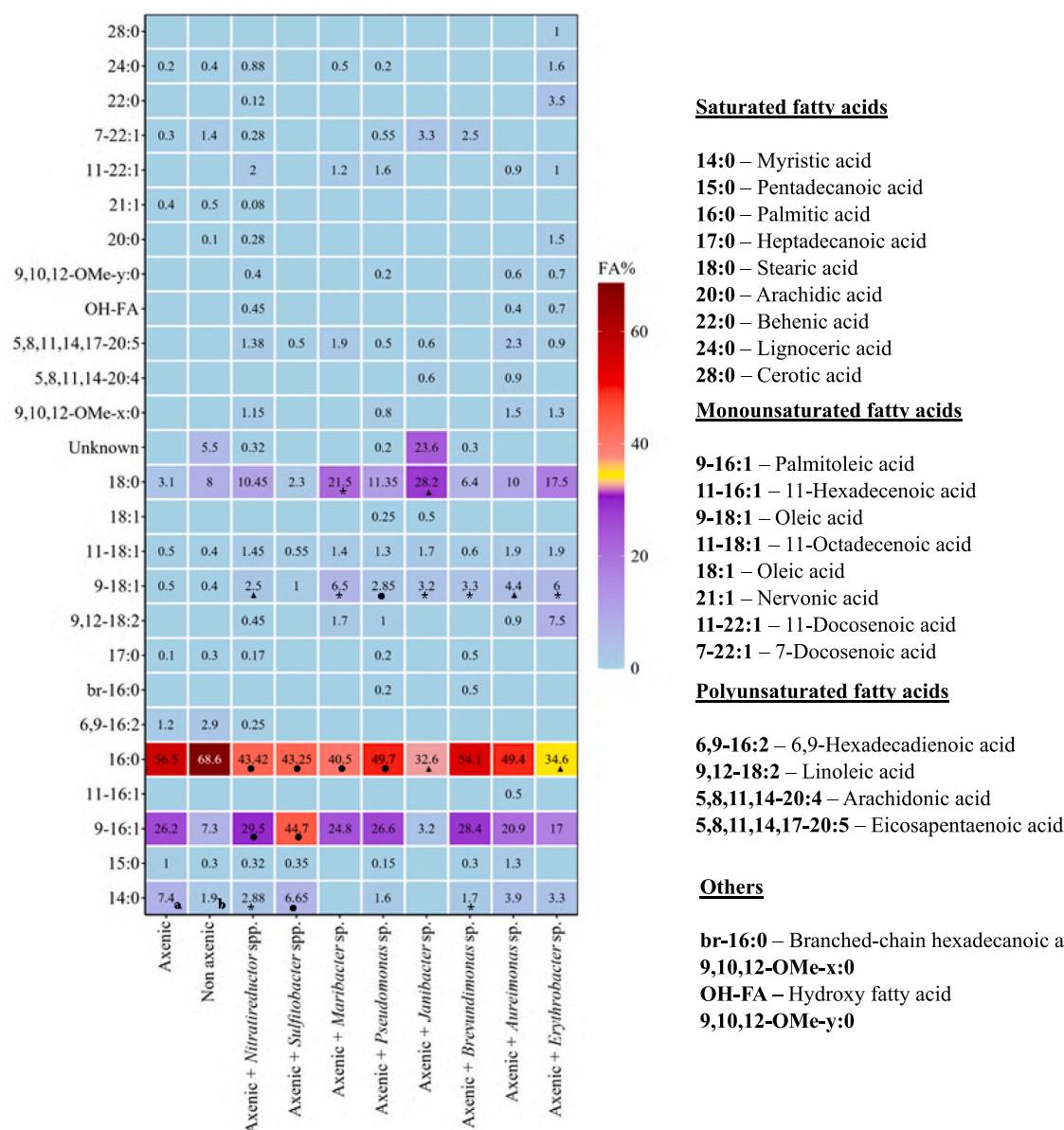
In our experiment, a total of 25 fatty acids (FAs) were identified across axenic *Amphora* sp., non-axenic *Amphora* sp., and co-cultures with eight bacterial strains. This profile included 9 saturated fatty acids (SFAs), 8 monounsaturated fatty acids (MUFAs), 4 polyunsaturated fatty acids (PUFAs), 3 branched FAs and 1 hydroxyl FA (Fig. 2). In general, the fatty acids of axenic, non-axenic, and eight co-cultures primarily consisted of palmitic acid (16:0), palmitoleic acid (9–16:1), stearic acid (18:0), myristic acid (14:0) and oleic acid (9–18:1), while all other fatty acids were present in minor amounts. Among these, palmitic acid and palmitoleic acid together accounted for over 70 % of the total FAs content in most cultures, except in co-cultures with *Maribacter* sp., *Janibacter* sp., and *Erythrobacter* sp. The palmitic acid (16:0) content in non-axenic *Amphora* sp. was the highest, ranging from 65 to 75 % of the total fatty acid content. Among co-cultures, significantly lower palmitic acid levels in *Amphora* sp. were observed in co-cultures with *Nitratireductor* spp., *Sulfitobacter* spp., *Maribacter* sp., and *Pseudomonas* sp. compared to the non-axenic control, whereas co-cultures with *Janibacter* sp. and *Erythrobacter* sp. exhibited significantly lower palmitic acid

levels than both control conditions. Palmitoleic acid (9–16:1) was the second most abundant fatty acid in all cultures, except in the non-axenic culture and the co-culture with *Janibacter* sp. Co-cultures with *Nitratireductor* spp. and *Sulfitobacter* spp. exhibited significantly higher palmitoleic acid levels than non-axenic *Amphora* sp., ranging from 30 to 47 % ( $p < 0.05$ , Kruskal-Wallis Dunn test). Stearic acid (18:0) was significantly abundant (18–30 %) in co-cultures with *Maribacter* sp. and *Janibacter* sp. while significant variations in myristic acid (14:0) content were observed between axenic, non-axenic conditions ( $p < 0.05$ , Tukey’s HSD) and three co-cultures, with *Nitratireductor* spp., *Sulfitobacter* spp., and *Brevundimonas* sp. However, myristic acid was not detected in co-cultures with *Maribacter* sp. and *Janibacter* sp. Regarding oleic acid (9–18:1), most co-cultures exhibited significantly higher levels than at least one control ( $p < 0.05$ , Kruskal-Wallis Dunn test), ranging from 3 to 11 % of the total fatty acid content, except for *Sulfitobacter* spp.

At the scale of FAs classes, even though there was no significant difference between axenic and non-axenic conditions, SFAs and PUFAs contents were comparatively higher in non-axenic conditions, at approximately 80 % and 3 %, respectively, while MUFAs content was higher in axenic *Amphora* sp., around 28 % (Fig. 3). Among the co-cultures, the fatty acid classes; SFAs, MUFAs, and PUFAs of the two co-cultures with *Aureimonas* sp. and *Erythrobacter* sp. showed no significant differences compared to either control. The SFAs content in co-cultures with *Nitratireductor* spp. and *Sulfitobacter* spp. was significantly lower compared to the non-axenic *Amphora* sp. culture ( $p < 0.05$ , Kruskal-Wallis Dunn test), while other co-cultures showed no significant differences compared to either control. MUFA levels in five co-cultures (*Nitratireductor* spp., *Maribacter* sp., *Pseudomonas* sp., *Janibacter* sp., and *Brevundimonas* sp.) were significantly higher ( $p < 0.05$ , Tukey’s HSD) than in the non-axenic *Amphora* sp. culture, while the co-culture with *Sulfitobacter* spp. showed significantly higher MUFA ( $p < 0.05$ , Tukey’s HSD) than both controls. Although the PUFA content in each co-culture was not significantly different from either control, the co-culture with *Brevundimonas* sp., reported no PUFA at all. Overall, the PUFA content was relatively low across controls and co-cultures, ranging from 0 % to 6 % of the total fatty acids.

3.5. Overall perspective of biofilm-forming bacteria on the biomass and lipid production of *Amphora* sp.

Considering the overall impact of individual bacteria on the growth, biomass and lipid production of *Amphora* sp., the PCA plot reveals three distinct clusters: (1) axenic *Amphora* sp., non-axenic *Amphora* sp., and the co-culture with *Nitratireductor* spp. cluster together, (2) the co-culture with *Sulfitobacter* spp. forms a separate cluster near the controls, and (3) all other co-cultures group together, distinct from the controls (Fig. 4). This indicates that axenic and non-axenic *Amphora* sp. share similar biomass and lipid parameters, suggesting that the presence



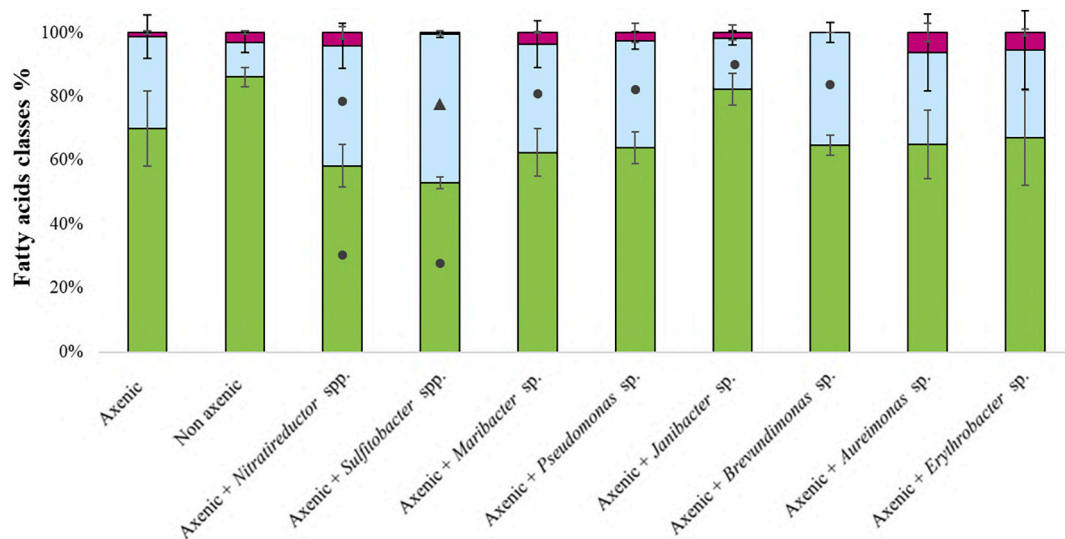
**Fig. 2.** Heat map of fatty acids (FAs) abundance of axenic *Amphora* sp., non-axenic *Amphora* sp. and co-cultures with 8 bacterial genera. FA % denotes the mean relative percentage of each fatty acid out of total fatty acids across a minimum of three biological replicates. \* indicates significant differences compared to axenic *Amphora* sp., • indicates significant differences compared to non-axenic *Amphora* sp., ▲ Significant difference to both axenic and non-axenic *Amphora* sp., letters indicate the significance difference between axenic and non-axenic condition, Significance symbols and letters are placed below the FA% value within each relevant grid cell. Only non-zero FA% values are annotated.

or absence of the bacterial pool in these controls does not significantly alter the fundamental characteristics of *Amphora* sp. Regarding the co-cultures, the clustering of *Nitratireductor* spp. with the axenic and non-axenic controls and the close proximity of the *Sulfitobacter* spp. co-culture to the controls implies that this bacterial strain may have a neutral or mutualistic relationship, where its presence does not impact the growth and lipid production of *Amphora* sp. In contrast, the cluster positioned farther from the controls suggests that the other bacteria may influence the growth kinetics and metabolic pathways of *Amphora* sp., either positively or negatively.

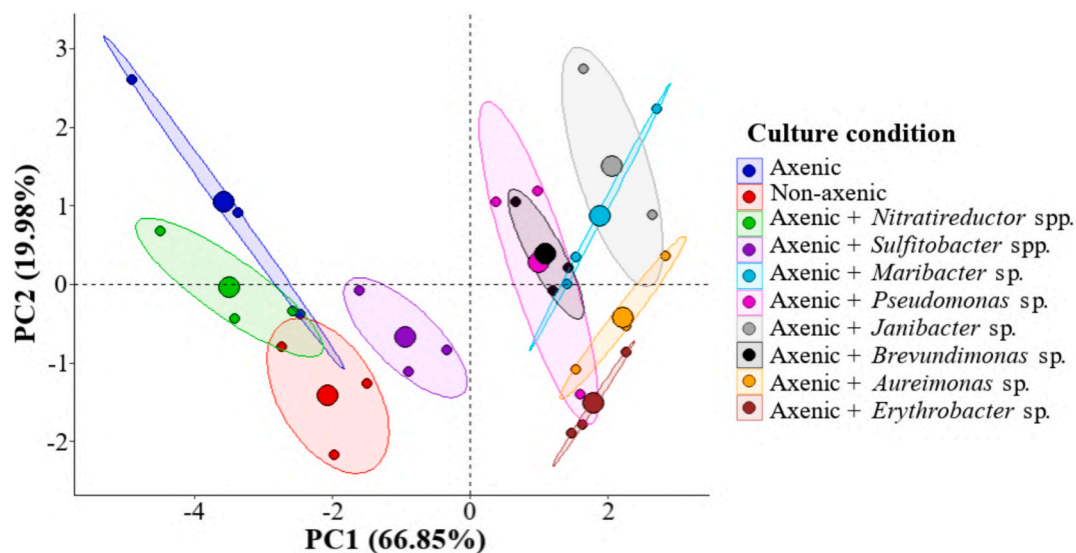
#### 4. Discussion

In a biofilm microalgal culture, bacteria can exist either as free-living cells in the culture medium or as biofilm-associated bacteria [11]. The latter are closely associated with microalgal cells, either as epiphytic

bacteria attached to the algal surface or as embedded bacteria within the extracellular matrix of algae-derived exopolymers, which could together form a joint algal–bacterial matrix [36] and can form strong biofilms [40]. On the other hand, given the growing interest in the design of vertical biofilm photobioreactors, which emphasize space efficiency and light distribution capacity [57,60], only bacteria capable of stable association are likely to exert significant effects on microalgae in microalgal biofilm, without bacterial washout where nutrients and medium are periodically replenished. For this reason, the relative strength of bacterial biofilm-forming ability was used as the initial bacteria selection criterion for co-culturing with axenic *Amphora* sp., as it could indicate the potential for bacterial persistence within the *Amphora* biofilm and functional interactions, while also aligning with our future work related to microbiome engineering in vertical PSBRs with *Amphora* sp. Consistent with our findings, Proteobacteria dominated bacterial isolates, along with other phyla such as Bacteroidetes and



**Fig. 3.** Fatty acids classes; saturated fatty acids (■), monounsaturated fatty acids (□), polyunsaturated fatty acids (■) of axenic *Amphora* sp., non-axenic *Amphora* sp., and co-cultures with 8 bacterial genera. Values are mean  $\pm$  standard deviation (minimum 3 biological replicates), except for the *Amphora*-*Erythrobacter* co-culture where one outlier was excluded ( $n = 2$ ). ● indicates significant differences compared to non-axenic *Amphora* sp., ▲ significantly different to both axenic and non-axenic *Amphora* sp. Significance symbols are positioned within the middle of the corresponding saturated fatty acids and monounsaturated fatty acids sections of the bars. No significant differences were observed between polyunsaturated fatty acids content of *Amphora* sp. in co-cultures with axenic and non-axenic controls.



**Fig. 4.** Principal Component Analysis (PCA) of axenic *Amphora* sp., non-axenic *Amphora* sp., and co-cultures with 8 bacterial genera based on biomass and lipid parameters ( $n \geq 3$  biological replicates).

Actinobacteria, have been reported in microalgal, marine, and freshwater biofilms [17,36–39]. Additionally, these bacterial taxa are known as predominant biofilm formers in microalgal phycospheres, capable of enhancing the overall biofilm stability [39,40]. Despite variations in biofilm formation among bacterial isolates from the biofilm of non-axenic *Amphora* sp., the abundance of biofilm-forming bacteria increased during its later growth phase. This trend could be attributed to the rising EPS production by *Amphora* sp. over time, as demonstrated by Lipsman et al. [36], who showed that bacterial biofilm formation strengthens with increasing algal exudates. In line with our findings, the two strongest biofilm formers, *Maribacter* sp. and *Brevundimonas* sp., which were isolated from the mid- and late-exponential phases of *Amphora* sp., respectively, exerted a negative impact on its biomass under our culture conditions. Among the moderate biofilm-forming bacteria isolated throughout the exponential phase, only *Nitratireductor* sp. and

*Sulfitobacter* sp. exhibited neutral effects on both biomass and lipid production of *Amphora* sp. while all other bacteria (*Maribacter* sp., *Pseudomonas* sp., *Janibacter* sp., *Brevundimonas* sp., *Aureimonas* sp., and *Erythrobacter* sp.) decreased the biomass. These findings suggest that the strength of bacterial biofilm formation may not necessarily correspond to positive interactions or improved yields of microalgal biofilms.

Regardless of the neutral or negative impacts of individual bacteria on growth and biomass production, non-axenic *Amphora* sp. with native microbiota exhibited better overall growth and biomass, indicating that the mixed bacterial community in the biofilm is more inclined toward cooperation than competition. For instance, some bacteria in the consortium may neutralize harmful effects from potentially pathogenic species by producing antimicrobial compounds, thereby fostering a more balanced microbial community that supports *Amphora* sp. growth [30,41,42]. These cooperative dynamics may contribute to create a



more stable environment, where functional guilds emerge, with species performing complementary roles and engaging in synergistic interactions that outweigh potential negative effects [7,30,38]. Similarly, Bruckner et al. [17] reported higher biomass (using chlorophyll content as a proxy) in the freshwater biofilm-forming diatom *Cymbella microcephala* when cultured with a bacterial consortium, compared to axenic and co-culture conditions. Co-cultures with *Nitratireductor* sp. and *Sulfitobacter* sp., showed neutral impact on the growth parameters of *Amphora* sp. compared to controls. This outcome may be attributed to the insufficient initial bacterial concentration to significantly enhance the growth and biomass of *Amphora* sp. In contrast, all other bacterial strains (*Maribacter* sp., *Pseudomonas* sp., *Janibacter* sp., *Brevundimonas* sp., *Aureimonas* sp., and *Erythrobacter* sp.) negatively affected the growth and biomass production of *Amphora* sp. NCC169, even at the lower inoculum concentration applied. These findings contradict previous studies reporting a significant microalgal biomass enhancement effects of some of these bacterial genera on different algal species, such as *Nannochloropsis oceanica* with *Nitratireductor* sp. [43], *Pseudo-nitzschia multiseriata* with *Sulfitobacter* sp. [44], *Scenedesmus obliquus* with *Pseudomonas* sp. [45], *Chlorella ellipsoidea* with *Brevundimonas* sp. [12] and *Marinichlorella kaistiae* KAS603 with *Erythrobacter* sp. [46]. These discrepancies could be due to the nature of microalgae-bacteria interactions (e.g., antagonistic), strain-level differences for both the alga and the bacterium, variations in culture conditions, competition for nutrients, and the production of harmful metabolites [5,6,16,47]. Although the mechanisms underlying the influence of heterotrophic bacteria on microalgal growth are not fully understood, mutualistic interactions have been shown to enhance algal growth substantially through the exchange of nutrients and metabolites, including siderophores, vitamin B<sub>12</sub>, small peptides (e.g., Diketopiperazines), phytohormones (e.g., indole-3-acetic acid, cytokinins and gibberellins) and polysaccharide-degrading enzymes [1,11,16,45,47–49].

Lipid production in *Amphora* sp. across axenic, non-axenic, and co-culture conditions ranged from 8 % to 35 % of dry biomass, consistent with the findings of Cointet et al. [27] and Arnaldo et al. [31] for the same strain, as well as other studies on *Amphora* strains under diverse culture conditions and modes [29,50–52]. However, the lipid percentage relative to dry biomass was notably higher in all co-cultures, exceeding 15 % and reaching over 25 % in some co-cultures, surpassing the 20 % threshold for biotechnological applications [53]. Although the differences in lipid parameters between co-cultures and controls were not statistically significant, noticeable variation in lipid responses was evident among treatments. The variability observed among biological replicates likely reflects the inherent heterogeneity of microbial interactions and metabolic responses in co-cultures. Although some co-cultures exhibited higher lipid percentages exceeding 25 % of dry biomass (e.g., axenic *Amphora* + *Pseudomonas* sp.), these co-cultures showed significantly lower *Amphora* biomass, which could have led to the higher lipid % DW values. This pattern suggests that bacterial interactions may have imposed physiological stress or altered the metabolic regulation of the diatom, leading to a redirection of carbon flux from growth toward storage lipid synthesis [42]. Consequently, the proportion of lipids within the remaining biomass increased, while overall lipid parameters remained comparable to those of the controls. In contrast, several studies have reported significantly increased lipid content and productivity in microalgae-bacteria co-cultures [4,16,45]. This could be attributed to the stimulation of lipid synthesis pathways in microalgae by bacteria under mutualistic or antagonistic interactions, nutrient depletion, stress responses, metabolite exchange, and, more broadly, culture conditions, including light regime, pH, and temperature [42,45,47,54]. The fatty acid composition of microalgae can vary considerably depending on the strains and culture conditions, including various biotic and abiotic stressors [4]. Similar to our findings, several studies have reported alterations in the fatty acid profiles of microalgae in response to various bacterial interactions (beneficial and negative) [42,43,45]. In contrast, Nascimento et al. [13] observed that while lipid

production increased under mutualistic interaction, the fatty acid composition remained unchanged. Interestingly, co-cultures with *Nitratireductor* sp. and *Sulfitobacter* sp. exhibit SFA levels of 50–55 %, MUFA levels of 40–50 %, and lower PUFA content (1–6 %), closely resembling the FA profile of palm oil (SFA 47 %, MUFA 44 %, PUFA 9 %), a widely used commercial biodiesel feedstock [55].

Taken together, biofilm-forming microalgal cultures could offer a competitive advantage over suspension cultures by considerably reducing water requirements and harvesting costs, providing a more efficient and cost-effective approach for biomass recovery and valuable metabolite production [20,56,57]. From an economic standpoint, naturally biofilm-forming microalgal strains could be of industrial interest, as they eliminate the need for active immobilization techniques such as flocculants, chemical attachment, or gel encapsulation [21]. However, the co-occurrence of bacteria and other microorganisms (originating from air, water, or photobioreactors) in microalgal biofilm cultures is inevitable due to increased EPS production, which acts as a chemoattractant [58]. Therefore, understanding the interactions among these diverse species and their functional roles is crucial for enhancing the growth kinetics and metabolic yield of microalgal biofilms on an industrial scale. In agreement with our study, the use of non-axenic *Amphora* sp. presents industrial advantages, particularly by eliminating the resource-intensive and impractical maintenance of axenic microalgal cultures. Furthermore, the high proportion of palmitic acid (16:0), constituting two-thirds of its total fatty acid composition compared to other cultures, makes it suitable for various industrial applications such as personal and household products. On the other hand, co-culturing microalgal biofilms with bacteria could be strategically leveraged to tailor fatty acid profiles for specific industrial applications, such as pharmaceutical, nutraceutical and biofuel production.

Given the limited knowledge of microbiome engineering in microalgal biofilms, our study has several limitations. In line with the main objective, this preliminary work focused on identifying bacterial partners that could enhance the biomass and lipid production of *Amphora* sp. biofilms for subsequent studies. Accordingly, nutrient analysis and bacterial growth monitoring were not included, as the emphasis was primarily on functional impacts. Based on our previous experiment with this strain in Fernbach culture flasks [31], we assumed that nutrients were non-limiting during the short culture period of this study, as it was conducted in nutrient-rich F/2-enriched artificial seawater. The inoculum ratio is another critical factor in co-culture studies, as biofilm productivity has been shown to depend on the initial inoculum concentration even in mono-algal biofilm systems [62]. Since we did not optimize it and began with a lower bacterial concentration, the impact on *Amphora* sp. biomass and lipid production was limited, particularly in *Nitratireductor* and *Sulfitobacter* co-cultures, which were identified as having a neutral influence under the conditions tested. Thus, future research should refine the algae-to-bacteria ratio to improve biomass and lipid productivity, while also monitoring bacterial dynamics. Despite these limitations, our binary co-culture approach offered fundamental insights into how individual bacterial strains affect the biomass and lipid production of *Amphora* sp., allowing their interactions to be classified as neutral, beneficial, or negative. Building on these findings, future research should explore co-cultivation with multispecies bacterial consortia with *Amphora* sp. biofilm, including neutral strains such as *Nitratireductor* sp. and *Sulfitobacter* sp., under optimized conditions. Such tailored consortia may better replicate natural ecosystems while improving the stability of microalgal biofilms and enhancing metabolic outputs, offering potential for industrial application. Similarly, it is important to study the behavioural shifts of bacteria over the course of a biofilm-based microalgal culture, as bacteria that are initially mutualistic may later become commensalistic or even antagonistic due to changing interactions, environmental factors and competitive dynamic [59]. Investigating these behavioural changes will help design more robust and predictable consortia. Taking the limitations of this study into account, our next study will focus on binary co-culturing

*Nitratireductor* and *Sulfitobacter* with *Amphora* sp. in a vertical biofilm PSBR, incorporating nutrient analysis, microbial growth monitoring and metabolomics. We anticipate that this system will facilitate scale-up by better reflecting industrial cultivation conditions and may support improvements in biomass and lipid yields. Additionally, incorporating nutrient starvation (e.g., nitrogen, phosphorus, and silicon) into co-culturing approaches may lower the dependence on nutrient-rich media, as bacteria within the biofilm can recycle essential nutrients by remineralizing algal exudates. However, these developments should be followed by a techno-economic analysis to assess scalability and industrial feasibility.

## 5. Conclusion

To our knowledge, this is the first study to assess how bacterial co-cultures influence the biomass and lipid composition of a benthic microalgal biofilm. Although the biofilm-forming ability of bacteria supports their persistence within the microalgal biofilm, its strength does not necessarily reflect mutualistic interactions or lead to enhanced microalgal biomass or lipid output. Our results further showed that some bacterial strains significantly reduced *Amphora* sp. biomass, whereas *Nitratireductor* sp. and *Sulfitobacter* sp. had no discernible effect under the provided conditions. Interestingly, the native bacterial community in non-axenic conditions appeared to buffer these negative impacts, suggesting that bacterial consortia may offer greater benefits than single-strain associations. While none of the individual bacterial strains substantially affected lipid production compared to axenic and non-axenic controls, significant shifts in fatty acid profiles were observed across most co-cultures. This highlights the potential of microalgal biofilm–bacteria co-cultures for the targeted modulation of fatty acid composition in lipid-based industrial applications such as pharmaceuticals, nutraceuticals, and biofuels. Future research should focus on scaling up these systems using different culture modes, such as photobioreactors, and integrating OMICS approaches (e.g., transcriptomics, metabolomics) to fully realize their industrial potential.

## CRediT authorship contribution statement

**Nadeeshani Dehel Gamage:** Writing – original draft, Methodology, Investigation, Conceptualization. **Aurélié Mossion:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Paul Délérès:** Writing – review & editing, Methodology, Investigation. **François Delavat:** Writing – review & editing, Methodology. **Leïla Tirichine:** Writing – review & editing, Resources. **Vony Rabesaotra:** Methodology, Investigation. **Thierry Lebeau:** Writing – review & editing, Supervision, Conceptualization. **Gaëtane Wielgosz-Collin:** Writing – review & editing, Supervision, Conceptualization. **Vona Méléder:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

## 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.

## Acknowledgement

We gratefully acknowledge the financial support from the Région Pays de la Loire (France) through the SMIDAP grant BIOFILM (Agreement No. 2021\_04302), the European Union via the REWRITE project (Grant Agreement-101081357), and the French Ministry of Higher Education, Research and Innovation. We sincerely thank Murtaza Khan (M. Sc. in Nutrition, Health and Mobility - M2, University Clermont Auvergne, France) for his additional contribution to this research during his master's thesis, and Océane Guillot (B.Sc. in Pharmacy, Nantes

University, France) for selecting our team to enhance her research skills through a short-term internship. We also gratefully acknowledge the technical support provided by Philippe Rosa and Alexandra Petit at the ISOMer lab for assistance with autoclaving.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2025.104449>.

## Data availability

Data will be made available on request.

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