First insight on interactions between bacteria and the marine diatom *Haslea ostrearia* : Algal growth and metabolomic fingerprinting

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Abstract :

A first insight on the interactions between bacteria isolated from monospecific cultures of the marine diatom *Haslea ostrearia* and this microalgae is described in this work. From 28 bacterial isolates, the maximal growth rate and the maximal cell concentration were on average 58% and 27% higher (max. 67% for isolate B22). Isolates B5, B10 (both *Saccharospirillum* sp.) and B22 (*Rhodobacter* sp.) were the most efficient. The geographical origin of *H. ostrearia* from which bacteria were isolated had no impact on the algal growth performances, and a given bacterial isolate led to the same algal growth irrespective of the geographical origin of *H. ostrearia*. To explain the results obtained, a comprehensive integrative strategy was conducted that analyzed the variations in the thousands of metabolites present in our culture extracts. Beyond a holistic view in which all organic substances of natural origin derived from the metabolism of a living organism were analyzed, we focussed on metabolites specific to each partner of the bacteria-*H. ostrearia* co-cultures, and relating to a particular biological function. To this end, a high throughput analytical technique, mass spectrometry was used, along with uni- and multivariate association statistical methods. Metabolic fingerprinting (untargeted approach) enabled compounds specific to each association to be identified, especially those concerning the positive bacterial effect on the growth of *H. ostrearia*.

Highlights

► *Haslea ostrearia* and bacteria isolated from this marine diatom were co-cultivated. ► *Saccharospirillum* sp. and *Rhodobacter* sp. increased the most the algal growth. ► The maximal cell concentration of *H. ostrearia* was on average 27% (max. 67%) higher. ► A given bacteria led to the same algal growth irrespective of the *H. ostrearia* origin. ► Metabolic fingerprinting enabled compounds specific to each association.

1. Introduction

Microalgae are recognized for their high productivity, for their ability to grow in wastewater, non-arable land, etc. under well controlled conditions in photobioreactors as well as in open ponds, and with a wide commercial applications (fuel industries, wastewater treatment, and also high value-added molecules) as reviewed for diatoms by Lebeau and Robert [1]. Although the habitat of phytoplankton-associated bacteria has long been depicted by the concept of the "phycosphere", *i.e.*, the area around algal cells where bacteria feed on the extracellular products of the algae [2], which is still a matter of research [3–5]. Indeed, "algae have received much attention for industrial exploitation but their interaction with bacteria is often considered as a contamination during commercialization", as reported by Ramanan et al. [6], probably because research in bioengineering considers that axenic microalgae, i.e., a single population, is easier to control than several microbial populations and/or it is less expensive to recover the final product (algal biomass, molecules). Yet potential applications of bacteria-algae cocultures exist in aquaculture [7]. The nature of the bacterial chemical compounds involved in the supplementary algal growth (indole-3-acetic acid. Vitamin B_{12}, siderophores, antibiotics: [8], [6]) is central in the bacteria-microalgae interactions but have been poorly studied to date in comparison with higher plant-microbe interactions. Culture-independent methods like metagenomics and metabolomics can be employed to investigate the complexity of such interactions [9]. Some bacteria-microalgae interactions deal with diatom species (see review by Amin et al. [10]), including *Guinardia delicatula*, *Pseudonitizschia pugens*, *Thalassiosira rotula*, *Skeletonema costatum* [11], *Ditylum sp.*, *Thalassiosira sp.*, *Asterionella sp.*, *Chaetoceros sp.*, *Leptocylindrus sp.*, *Coscinodiscus sp.* [12], *Pseudo-nitzschia multiseries* [13] and *Nitzschia microcephala* [14]. It has been shown

that some bacteria (or yeasts) may increase the biomass of various microalgal genera including diatoms [15–21].

As yet, nothing is known about the interactions between the marine diatom *Haslea ostrearia* and its surrounding bacteria in the natural environment. This microalga is a cosmopolitan diatom species commonly found on the French Atlantic coast, especially in oyster ponds of Marennes-Oléron Bay and Bourgneuf Bay. It became a subject of investigation since 1927 [22] due to its water-soluble, blue-green pigment, called marennine, which is responsible for the greening of oysters and because the conditions of its synthesis, as the result of a peculiar biological cycle (successively planktonic and benthic periods) as described by Robert et al. [23], was specific of this blue diatom (for more details, see the review of Gastineau et al. [24]). It is noteworthy that some other marennine applications have been reported in this overview: i) antibacterial, anticoagulant and antiviral activities; ii) antioxidant activity, and iii) antitumor and antiproliferative effects of the aqueous extract from *H. ostrearia* on solid tumors (lung and kidney carcinoma and melanoma cell line molecules) and whose photoprotective role of this pigment can act as complexing ligands leading to decreased bio-availability of metals [25–27].

Yet, nothing was known about the bacteria surrounding *H. ostrearia* until our recently published work regarding the analysis of the bacterial structure of the *H. ostrearia* phycosphere [28]. This work notably showed that the level of similarity between the bacteria recovered from the microalgal biofilm and the suspended bacteria did not exceed 10%. On the contrary, similarities between the bacterial community structures in biofilms were above 90% regardless of the geographical origin of the *H. ostrearia* isolates. This finding shows that the bacteria of the phycosphere are specific to *H. ostrearia* without yet supplying any data on the potential interactions.

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In the continuity to this preliminary study, the present work aimed to test i) the impact of bacteria isolated from monospecific cultures of *H. ostrearia* on the growth of this microalga and ii) to provide an insight into these interactions based on the analysis of metabolites resulting from the bacteria-*H. ostrearia* interactions. Firstly bacteria were isolated from either the water column (free cells) or biofilms surrounding *H. ostrearia* cells and tested in co-culture with an *H. ostrearia* isolate. Thereafter, some bacteria co-cultivated with this algal isolate were also co-cultivated with another algal isolate harboring the most distinct bacterial structure in its natural environment to test the specificity of the bacteria-*H. ostrearia* interactions. Eventually, to assess the overall metabolic profile of the extracellular compounds synthesized by both *H. ostrearia* isolates associated with some of the isolated bacteria, metabolic fingerprinting (untargeted approach) was performed, along with the identification of some compounds, by liquid chromatography high resolution mass spectrometry (LC-HRMS). Then an attempt was made to relate the different profiles obtained with the ability (or not) of bacteria to stimulate microalgal growth.

2. Material and methods

2.1. Isolation of bacteria from *Haslea ostrearia* cultures

Bacteria were isolated from *Haslea ostrearia* isolates cultivated in ES 1/3 medium [29] in laboratory conditions over a 3-month period. These microalgal isolates were recovered from four sites on the French Atlantic coast: Bouin (46.96°N; 2.04°W), La Barre-de-Monts (46.90 N; 2.11°W), Isle de Ré (46.22 N; 1.45°W), and La Tremblade (45.80 N; 1.15°W) [28] and were non-axenic, *i.e.*, they harbored autochthonous bacteria (*H. ostrearia* only axenic for metabolomic analysis: see section 2.2.).

Suspended bacterial cells in the culture medium and cells trapped in the microalgal biofilm surrounding *H. ostrearia* cells (named hereafter "free cells" and "biofilm cells", respectively) were isolated. For "free cells", 100 mL of the *H. ostrearia* culture in the exponential growth stage was carefully collected to avoid any contact with the algal biofilm (wholly attached at the bottom of the Erlenmeyer flasks at this stage), filtered at 4 µm to eliminate suspended microalgae and centrifuged (SIGMA 3K30 Fisher Bioblock Scientific, 6000 *g*, 10 min, room temperature). The bacterial pellet was re-suspended in fresh ES 1/3 medium from which serial dilutions in NaCl 28 g L^{-1} were made and plated on Petri dishes filled with Marine Agar (Difco™ Marine Broth 2216 supplemented with agar 15 g L^{-1}). For the "biofilm cells", all the culture medium was removed. Thirty milliliters of fresh ES 1/3 medium was added and the biofilm was set in suspension (by the mechanical action of a sterile bar magnet) from which serial dilutions and plating were carried out as mentioned above for free cells.

After a 4-week incubation at 16°C and under 120 µmol photon m^{-2} s^{-1} and a 14:10 h (light: dark) regime in Marine Agar, different bacterial morphotypes were selected (color, appearance). Each bacterial isolate was re-suspended in NaCl 28 g L^{-1} and purified by streaking 3 times on Petri dishes filled with Marine Agar. Next, the isolates were cultured in liquid Marine Broth (Difco™ Marine Broth 2216) and stored at -80°C after glycerol addition (0.3 mL glycerol for 1.2 mL bacterial culture). Before each bacteria-*H. ostrearia* co-culture, isolated bacteria were thawed and cultivated in liquid Marine Broth for 72 h at 16°C.

In addition to the bacterial collection, *Azospirillum brasilense* was used as a control bacterium, known for its ability to stimulate the growth of terrestrial plants and microalgae [30]. It was cultivated, stored and thawed in the same conditions as all isolated bacteria.

2.2. Axenic culture of *H. ostrearia*

Only *H. ostrearia* isolates from La Barre-de-Monts (Ho-BM) and Isle de Ré (Ho-R) were used for axenization and used for experiments dedicated to the metabolomic fingerprinting. The protocol was described extensively in [28]. Briefly, non-axenic cultures of *H. ostrearia* in the exponential growth stage were treated twice with an antibiotic antimycotic solution (10,000 units of penicillin, 10 mg of streptomycin and 25 μg mL^{-1} of amphotericin B, BioReagent, A5955 SIGMA) for 7 days. After the second treatment, cultures were called "axenic" if no turbidity was observed after 10 days of culture in obscurity on two specific marine bacterial liquid medium (FAG and FG tests).

2.3. Bacteria-*H. ostrearia* co-cultures

Experiment 1: A non-axenic culture of *H. ostrearia* from La-Barre-de-Monts (Ho-BM) was co-cultivated with each of the 28 marine bacteria isolated from the *H. ostrearia* isolates of the four localities and with *Azospirillum brasilense.* All the co-cultures were compared to a control, *i.e.*, a non-axenic culture of *H. ostrearia* without addition of bacterial isolates (called a "monoculture"). *H. ostrearia* cells from a 250-mL culture in the exponential growth stage were inoculated in a 24-well plate (CytoOne, StarLab) at 3.10^3 cells mL^{-1}. Bacterial strains were pre-cultivated in Marine Broth for 72 h at 16°C, then washed twice with ES 1/3 medium, harvested by centrifugation at 5000 *g* for 6 min at 16°C (SIGMA 3K30 Fisher Bioblock Scientific) and inoculated in each well at 3.10^6 cells mL^{-1}. The co-culture experiments were performed in triplicate. The bacteria-microalgae co-cultures were incubated in a culture chamber equipped with LED panels at 16°C under 120 μmol photon m^2 s^{-1} and a 14:10 h (light:dark) regime (i.e., conditions adapted to *H. ostrearia*).

Experiment 2: Bacterial isolates B5, B10, B26 and B36 selected based on the results of experiment 1 and *A. brasilense* were co-cultivated with *H. ostrearia* isolates from two different geographical origins: Isle de Ré (Ho-R) and La-Barre-de-Monts (Ho-BM). All the co-culture conditions were the same as for Experiment 1 and were compared to non-axenic monocultures of Ho-R and Ho-BM used as controls.

2.4. Analysis

2.4.1. Microalgal cell counting

Algal growth was monitored by measuring the fluorescence of chlorophyll *a* in each well (wavelength: excitation 440 nm, emission 680 nm, BMG LabTech) twice a day for ten days. Parameters and specifications were as follows: WellScan mode, matrices 4×4, diameter 15, 30 flashes per well, 0.1 sec to wait in between each well. To avoid any contamination during the measurements, an adhesive film was used to cover the plate. The microalgal density was determined as a function of the fluorescence intensity based on a calibration curve (for each counting). Monocultures were realized at the same time as the co-cultures and in the same conditions (temperature, medium, light intensity) to be able to take into account the potential changes of the chlorophyll content of the cells. Moreover, bacterial interference was taken into account: i) bacterial densities in the range 5×10^7-9.10^8 cells mL^{-1} were added to two microalgal suspensions set at 3×10^3 cells mL^{-1} and 3×10^4 cells mL^{-1}; ii) a fixed bacterial density, *i.e.*, 10 times that used for the inoculation of plate wells, was added to the microalgal suspensions at a density ranging from 3×10^3 to 9×10^4 cells mL^{-1}.

2.4.2. Non-targeted metabolomic analysis

Currently, a major challenge in the field of metabolomics is interpreting untargeted profiling results in the context of biological studies. LC/MS-based untargeted metabolomics is now an established technology that is routinely used to study biological systems. Here ultra-high performance liquid chromatography quadrupole/time of flight-mass spectrometry (UHPLC-QTOF-MS): see below for details) was used with multivariate statistical analysis to detect the differences in compound patterns between Ho-R co-cultures with bacteria B10 and B26, and Ho-BM co-cultures with bacteria B10 and *A. brasilense*. Axenic and non-axenic *H. ostrearia* cultures were compared. The different *H. ostrearia* culture extracts were profiled to find out if this unbiased approach, without preconceptions, confirmed the biological hypothesis put forward, *i.e.*, highlighting growth-stimulating compounds specific to a microalgal-bacterial association.

Sample preparation: 200 µL of the supernatant of a 6-day *H. ostrearia* culture (in the exponential growth stage) was collected, filtered at 0.20 µm (PTFE membrane filters, Interchim) and frozen at -80°C prior to fingerprint acquisition. Analyses were performed on thawed samples by UHPLC-QTOF-MS, implementing a non-targeted analytical strategy [31] to detect small soluble extracellular compounds produced by the bacteria and *H. ostrearia* recovered from the culture medium. The experimental design was carried out on the basis of 24 complex matrices (n = 3) equivalent aliquots (5 µL) of each sample from the supernatant of *H. ostrearia* cultures , a simple matrix (culture medium, n = 3) serving as a control, and a certified reference materials of seven equimolar concentrations phycotoxins [32], including okadaic acid (OA), dinophysistoxin-1 (DTX1), yessotoxin (YTX), azaspiracid-1 (AZA1), 13-desmethyl spirolide C (desMeC), gymnodimine (GYM), pectenotoxin-2 (PTX2) purchased from National Research Council Canada) were prepared as a quality control (QC) sample, as

well as a blank (water injection) that were analyzed periodically throughout the random sequence of samples.

LC-TOF/MS analysis: An Agilent 1290 Infinity II LC system coupled to an Agilent 6540 UHD Accurate-Mass QTOF hybrid mass spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with a dual electrospray ionization (ESI) source for simultaneous spraying of a mass reference solution to calibrate detected m/z ratios continuously was used. The samples were, thus, monitored in high resolution mode. The Agilent Mass Hunter Workstation software (version B.07) was used to process the raw MS data, including extraction of molecular features (MFs), generation of molecular formulas, library searching and database searching. Without additional preparation, aliquots (5 µL) of each sample from the supernatant of *H. ostrearia* cultures were separated on a Kinetex 1.7 µm C18 100 Å (Phenomenex) column (150 × 2.1 mm) maintained at 40°C, with a gradient mobile phase (0.5 mL min^{-1}) comprising 0.1% aqueous acetic acid (A) and acetonitrile containing 0.1% acetic acid (B). The gradient was as follows: 5% B from 0 to 2.4 min, rising to 25% B from 2.4 to 4.5 min, to 30% B from 4.5 to 11 min, and to 100% B from 11 to 14 min, maintained until 16.5 min, subsequently decreasing to 5% B until 20 min and maintained at 5% B until 25 min. This HPLC system was connected to the ultra-high definition quadrupole time-of-flight mass spectrometer Model 6540 Agilent equipped with electrospray Jet Stream Technology, operating in positive or negative ion mode, using the following operation parameters: The capillary voltage, fragmentor voltage and skimmer were set at 3,900, 150 and 60 V, respectively. The sheath gas was at 350 °C (12 mL min^{-1}), the drying gas at 175 °C (5 mL min^{-1}) and the nebulizer at 43 psi. Nitrogen was used as the collision gas. Mass spectra were acquired in full scan analysis over an m/z range of 50-1,700 using the extended dynamic range and storage in centroid mode. Our instrument gave a resolution of 15,000 FWHM (Full Width at Half Maximum) at m/z 118.0862 and 30,000 FWHM at m/z 922.0098. To ensure the

desired mass resolution, continuous internal calibration (Tuning Mix) was performed during analyses by using the signals at m/z 121.0509 (protonated purine) and m/z 922.0098 [protonated hexakis (1H,1H,3H-tetrafluoropropoxy) phosphazine (HP-921)] in the positive ion mode, while in the negative ion mode ions with m/z 119.0362 (proton abstracted purine) and m/z 1033.9881 (trifluoroacetic acid adduct of HP-921) were used. For all samples analyzed, three replicates of each analytical sample were injected in the same batch and the order of sample injection was randomized to avoid sample bias. Besides, methanol injections were included every five samples as a blank run to avoid carry-over effects.

Data processing and statistical analysis: all the metabolic profiles are publicly available on the online infrastructure Ifremer Sextant (catalogue of referential data from marine environments; DOI: http://dx.doi.org/10.12770/046e1e6a-864e-48a6-944b-d8613d67de0f).

In this study, several pre-processing steps (feature finding, alignment, filtration and normalization) were used to reduce the large number of data to an operational size and to obtain an adequate set of compounds before applying multivariate analysis. To process all the data obtained by QTOF-MS in full scan MS mode, potential molecular features (MFs) were first extracted from the raw data files using the appropriate algorithm included in the software. The Molecular Feature Extraction (MFE) algorithm was optimized to improve the data extraction of metabolite information with the following parameters: (i) the peak filters were set to centroid height and the minimum threshold absolute abundance of ions was set at 300 counts for negative and positive ionization modes with all charge states allowed for the chromatograms obtained. These cut-off values were established taking into account the low intracellular medium chromatographic background noise samples; (ii) the isotopic distribution to consider MFs valid was defined by two or more ions (with a peak spacing tolerance of 0.002 m/z, plus 5.0 ppm in mass accuracy); ion and adduct formation in the positive (+H, +Na) and negative ionization (-H, -2H,) modes, as well as neutral loss by dehydration, were

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included to identify features corresponding to the same potential metabolite; thus, ions with identical elution profiles and related m/z values (representing different adducts or isotopes of the same compound) were extracted as entities characterized by their retention time (RT), intensity in the apex of the chromatographic peaks and accurate mass. In this way, raw data files were created in compound exchange format files (cef files) for each sample and exported into the Mass Profiler Professional software package (MPP version 13.1.1, Agilent Technologies, Santa Clara, CA, USA) for further processing. The parameters of Mass Profiler Professional were set as follows: Created an experiment type: Combined (Identified + Unidentified); Organism: None; Workflow type: Data Import Wizard; Minimum absolute abundance: 500 counts; Compound alignment: RT window = 0.5% + 0.5 min, Mass window = 15.00 ppm +2.0 mDa; Baseline Option: None.

Identification of the most relevant entities was supported by MS information and searches in the METLIN database (http://metlin.scripps.edu) and the Dictionary of Marine Natural Products (DMNP) library [33,34].

2.4.3. Identification of bacterial isolates

Bacterial isolates B5, B10 and B22 were isolated on Marine Agar medium (Difco™ Marine Broth 2216 supplemented with agar 15 g.L^{-1}). The 16S rDNA genes of these isolates were amplified, purified and sequenced by Eurofins (Eurofins Medigenomix GmbH). The 1400-bp sequences were submitted to the National Center for Biotechnology Information (NCBI) data bank and compared using BLASTN.

2.4.4. Statistical analysis

Growth curves were established for each triplicate of control (*H. ostrearia* monoculture) and bacteria-*H. ostrearia* co-cultures. The data obtained were used in MATLAB R2016b software for fitting to the Gompertz model (Eq. 1), used as a good model to describe growth kinetic data [35].

$$f(x) = \alpha \exp\left(-\exp\left(\frac{\mu_{max} \exp(1)}{\alpha}(\lambda - t) + 1\right)\right) \qquad (Eq.1)$$

Then, in the Gompertz model, f(x) is expressed in ln (cell mL^{-1}) and t correspond to time (in days) to obtain maximal concentration in culture. The three fitted parameters, maximum specific growth rate (μ_{max} in day^{-1}), maximum cell concentration (α in ln cell mL^{-1}), and lag time (λ in day) were estimated for each growth curve. Data were expressed as mean of triplicate (n=3) ± 95% confidence interval. Kruskal-Wallis one-way analysis of variance on ranks was carried out for μ_{max}, α and λ. Differences were considered significant at p < 0.05. To isolate the group or groups that differed from the others, a multiple comparison procedure was used *vs*. control (*i.e.*, monoculture) with Dunn's method. Sigmastat software (Systat Software, Inc.) was used for all these statistical analyses.

In a second phase, Agglomerative Hierarchical Clustering (AHC) using XLSTAT software (Addinsoft, Inc.) was carried out with mean parameters to detect the cultures that had similar growth kinetic patterns.

3. Results and discussion

3.1. Isolation of bacteria from *Haslea ostrearia* cultures

Bacterial isolates (a total of 28) were chosen for the co-culture tests with *H. ostrearia* based on visual differences: color and appearance of the colonies grown on Petri dishes, and state of the bacteria at the time of their isolation from the algal cultures, *i.e.*, free cells *vs*. cells associated with *H. ostrearia* in a biofilm form (Table 1). Bacteria were isolated from *H. ostrearia* isolates sampled from three localities out of the 4 (2 from La Tremblade, 21 from Bouin and 5 from Isle de Ré). No cultivable isolate was obtained from La Barre-de-Monts. About half the isolates were recovered from *H. ostrearia* biofilms and the remaining half were from the water column. The differences in bacterial morphotypes from one locality to another were most probably due to the differences in the structure of the bacterial community, as shown in a previous study [28]. *Azospirillum brasilense*, a plant growth-promoting rhizobacteria (PGPR), known for its ability to stimulate the growth of terrestrial plants and microalgae [36], was included in the set of bacteria to be tested in co-culture with *H. ostrearia* as a potential positive control.

3.2. Bacterial isolates co-cultivated with *H. ostrearia* of La Barre-de-Monts

3.2.1. Growth performances

Experiments were performed in plate wells whose advantages have already been reviewed by Bramucci et al.[37]. This non-invasive method, based on chlorophyll fluorescence for measuring algal growth of multiple parallel cultures, has already been used by Le Chevanton et al. [7] and Zecher et al. [38] for the co-culture of the bacterium *Rheinheimera sp.* strain Tn16 with *Thalassiosira pseudonana* or *Phaeodactylum tricornutum*.

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Each bacterial isolate (out of the 28 isolated from the *H. ostrearia* isolates) and *A. brasilense* was co-cultivated with *H. ostrearia* from La Barre-de-Monts (Ho-BM) (Table 1).

Table 1. Bacteria isolated from *H. ostrearia* clones: i) geographical origin of the microalga; ii) bacterial state when collected (biofilm *vs.* water column); iii) *H. ostrearia* isolates with which bacteria were co-cultivated (Ho-BM: La Barre-de-Monts; Ho-R: Isle de Ré).

Id° bacterial strain	Geographical origin of *H. ostrearia*	Free cells /biofilms	*H. ostrearia* isolates tested in co-culture	Id° bacterial strain	Geographical origin of *H. ostrearia*	Free cells /biofilms	*H. ostrearia* isolates tested in co-culture
B1	Ho La Tremblade	Free cells	Ho-BM	B21	Ho Bouin	Biofilms	Ho-BM
B2	Ho La Tremblade	Free cells	Ho-BM	B22	Ho Bouin	Biofilms	Ho-BM
B5	Ho Bouin	Biofilms	Ho-BM/Ho-R	B23	Ho Bouin	Biofilms	Ho-BM
B6	Ho Bouin	Biofilms	Ho-BM	B24	Ho Bouin	Biofilms	Ho-BM
B7	Ho Bouin	Biofilms	Ho-BM	B25	Ho Bouin	Biofilms	Ho-BM
B9	Ho Isle de Ré	Biofilms	Ho-BM	B26	Ho Bouin	Biofilms	Ho-BM/Ho-R
B10	Ho Isle de Ré	Biofilms	Ho-BM/Ho-R	B27	Ho Bouin	Biofilms	Ho-BM
B11	Ho Isle de Ré	Biofilms	Ho-BM	B29	Ho Bouin	Free cells	Ho-BM

B13	Ho Isle de Ré	Free cells	Ho-BM	B30	Ho Bouin	Free cells	Ho-BM
B14	Ho Isle de Ré	Free cells	Ho-BM	B31	Ho Bouin	Free cells	Ho-BM
B17	Ho Bouin	Free cells	Ho-BM	B33	Ho Bouin	Free cells	Ho-BM
B18	Ho Bouin	Free cells	Ho-BM	B34	Ho Bouin	Free cells	Ho-BM
B19	Ho Bouin	Free cells	Ho-BM	B35	Ho Bouin	Free cells	Ho-BM
B20	Ho Bouin	Free cells	Ho-BM	B36	Ho Bouin	Free cells	Ho-BM/Ho-R

Three typical growth features were observed with the different co-cultures, as shown with the example of the bacterial isolates B5, B10 and B25 (Fig. 1a): i) higher λ, lower μ_{max} and lower α than the control for B25. In this case, these bacteria clearly had a negative effect on the microalgal growth; ii) lower λ, higher μ_{max} and higher α than the control for B10; and iii) higher λ, higher μ_{max} and higher α than the control for B5. A competition for nutrients between bacteria and microalgae most probably established to the benefit of bacteria as shown by Qu et al. [40]. In their study, growth of *C. vulgaris* was promoted at low concentrations of bacteria, whereas opposite trend was observed for treatments with high bacteria density.
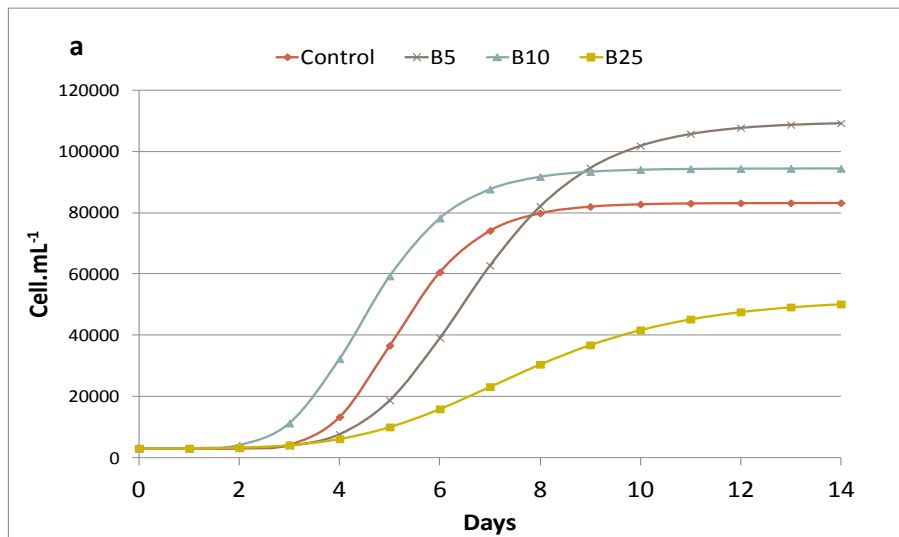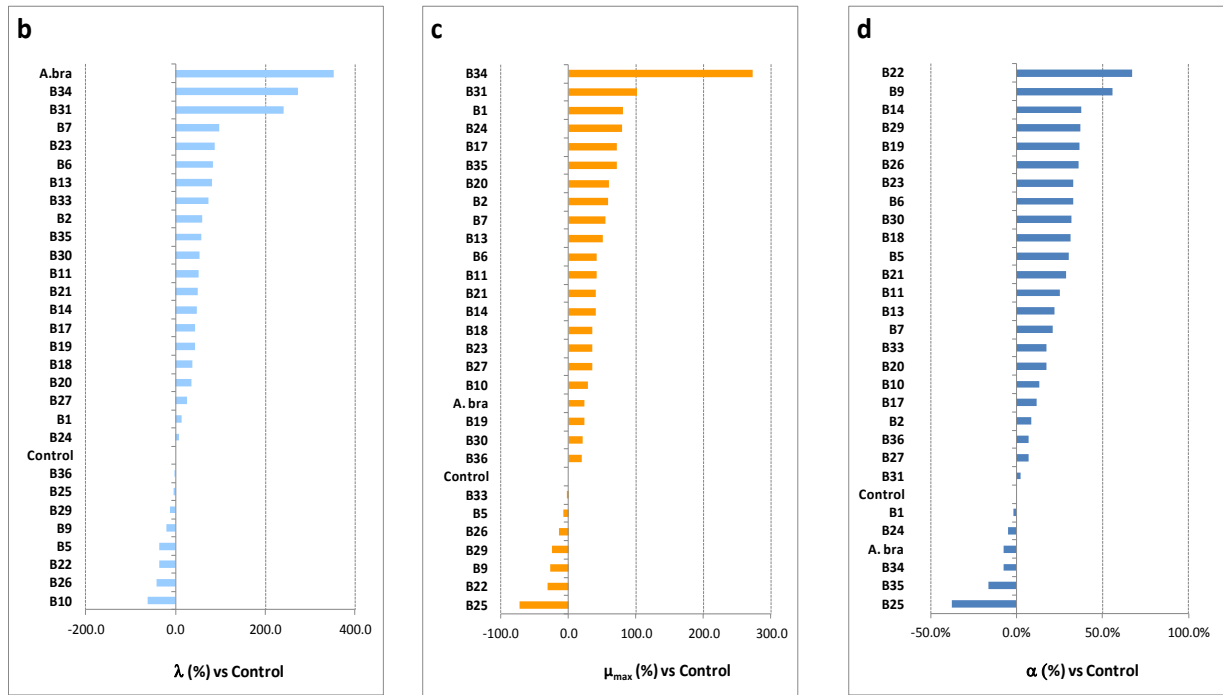
Fig. 1. (a) Typical growth kinetics model of *H. ostrearia* from La Barre-de-Monts co-cultivated with bacteria isolates B5, B10 or B25 and compared to the control (algal monoculture), (b) relative lag time (λ), (c) relative maximum specific growth rate (μ_{max}), and (d) relative maximal biomass at the stationary growth stage (α) of *H. ostrearia* (La Barre-de-Monts) co-cultivated with each bacterial isolate or *A. brasilense* (*A.bra*). Values are expressed as mean of triplicate (n=3) ± 95% confidence interval and are given as a percentage of the control (*H. ostrearia* monoculture).

Fig. 1b-d focuses on the growth parameters (λ, μ_{max}, α) for all bacterial isolates. Compared to the control, the relative lag time (λ) decreased up to a maximum of 63% (Fig. 1b) for the isolates B36, B25, B29, B9, B5, B22, B26 and B10, while it increased for all the others (up to 353% for *A. brasilense*). When λ was higher, it could be hypothesized that the bacteria outcompeted the microalgae (e.g., for nutrients) in the early stage of the culture, as shown by Murphy et al. [39] and Windler et al. [40]. In our study, the higher λ values for most algae-

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bacteria associations could also be explained by the simultaneous introduction of bacterial isolates and Ho-BM in the plate wells. To allow the balanced growth of both bacteria and microalgae, bacteria should be added when enough microalgal exudates have been produced to support the bacterial growth itself and also to avoid any bacterial stress towards *H. ostrearia*. In previous studies on the positive effect of bacteria on microalgal growth, no standard condition for the co-cultures was reported. In the study of Suminto and Hirayama [19], the microalgae and the bacteria were inoculated at the same time as in our study. Bi et al. [41] showed that a certain cell density of the bacteria *Sphingomonas paucimobilis* should be reached to promote the growth of *Chlorella vulgaris*. On the contrary, Qu et al. [42] showed that the growth of *C. vulgaris* was promoted at low concentrations of bacteria (5×10^6 cells mL^{-1}) while an opposite trend was observed for treatments with higher bacterial density (10×10^6 cells mL^{-1} and 20×10^6 cells mL^{-1}). Wang et al. [20] calculated an optimal bacteria to microalgae ratio for optimal algal growth.

For all bacteria, except B33, B5, B26, B29, B9, B22, and B25, μ_{max} was on average 58% higher and up to a maximum of 272.9% for B34 (Fig. 1c).

The maximal biomass (α; expressed in ln (cell concentration)) enables the maximal cell concentration in the stationary growth phase to be estimated. For each co-culture, an increase in α (versus the control) of 27% on average was observed for 23 out of the 29 bacteria) (Fig. 1d). Although this increase did not exceed 70% (with B22), it is economically far from negligible. Most studies on co-cultures of bacteria-diatoms showed a positive effect of bacteria on microalgal growth with an increase in the microalgal biomass in the range of what was obtained with *H. ostrearia* [6,7,19,43]. It is worth noting that *A. brasilense* did not enhance the microalgal growth, contrary to what was reported by Gonzalez and Bashan [30] when this bacterium was co-cultivated with *Chlorella vulgaris*. Compared to the algal monoculture, the stationary growth phase (α) of the co-cultures lasted longer for the bacterial

isolates B1, B5, B7, B9, B22, B24, B25, B31, B33 and B34 (data not shown), as already observed by Suminto and Hirayama [19] for *Chaetoceos gracilis* and two phytoflagellates (*Isochrysis galbana* and *Pavlova lutheri*) co-cultivated with *Flavobacterium sp.* In contrast, Grossart et al. [44] showed a rapid decline with *Thalassiosira rotula* associated with a bacterial isolate from the Roseobacter group.

Based on *H. ostrearia* growth criteria (λ, μ_{max} and α), with a greater weight being given to α (maximal biomass) as the major parameter for algal producers in aquaculture, the best bacteria were B5, B10 and B22. Compared to *H. ostrearia* monoculture, λ, μ_{max} and α were -36%, -8%, +30.6% for B5, -62%, +28%, +13.4% for B10 and -37%, -31%, +67.1% for B22, respectively. B5 and B10 were identified as *Saccharospirillum* sp. and B22 as *Rhodobacter* sp. The *Saccharospirillum* genus belongs to the order Oceanospirillum [45,46]. Currently, this genus includes three species: *S. impatientis* [45] and *S. salsuginis* [47] were isolated from hypersaline environments, and *S. aesturii* was isolated from a tidal flat sediment [48]. Rhodobacter is a purple non-sulfur bacterium (PNSB). PNSB are known to produce plant growth-promoting substances (PGPSs) [49].

Growth performances of Ho-BM in co-culture with the different bacterial isolates were not correlated to the geographical origin of *H. ostrearia* from which bacteria were isolated nor to the bacterial state (free cells *vs.* biofilm) at the time of their isolation (see Table 1, Fig. 1 & 2). We can assume that the isolated bacteria were not specific to the geographical origin of *H. ostrearia* or to the bacterial state. Although the structures of the bacterial communities between the different *H. ostrearia* isolates studied were different [28], all bacteria were considered in that case, while only cultivable bacteria were used here for the selection of the bacterial isolates to be co-cultivated with *H. ostrearia*.

Taking into consideration the three above-mentioned criteria together, *i.e.*, λ, μ_{max} and α (means of triplicate), homogenous groups were distinguished from the 29 bacteria by

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applying an Ascending Hierarchical Classification (AHC) (Fig. 2). This clearly distinguished

two groups of bacteria with a similarity close to zero. Within each group, the similarity

between bacteria was *ca.* 65%, and some bacteria had a similarity close to 100%. The isolates

B5 and B10 were logically in the same group (both *Saccharospirillum* sp.) with a similarity

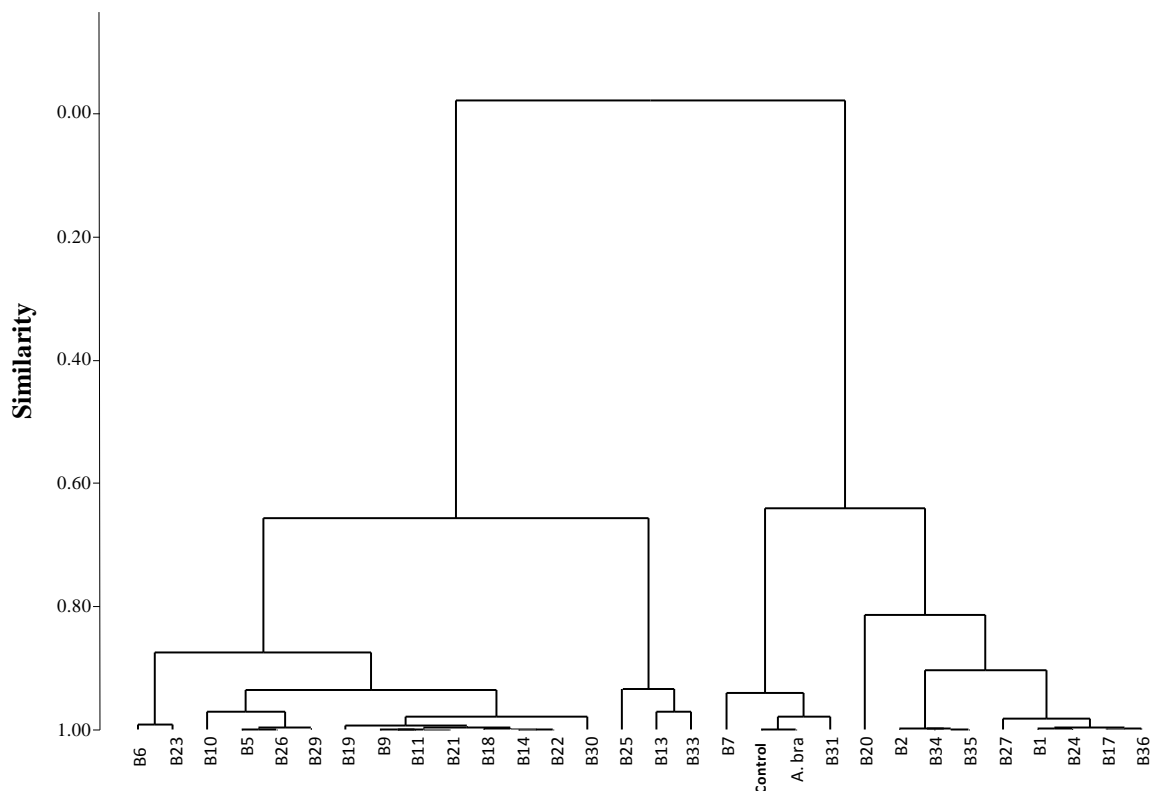close to 100% and B22 was in another group (*Rhodobacter* sp.).

Fig. 2. Ascending Hierarchical Classification (AHC) based on the three parameters of *H.*

ostrearia growth (i.e., lag time (λ), maximal growth rate (μ_{max}), and maximal algal biomass

(α)) taken into consideration together. AHC was applied to the 29 bacteria co-cultivated with

H. ostrearia from La Barre-de-Monts. Control: Ho-BM monoculture. Experiments were

performed in triplicate.

3.2.2. Effect of the origin of the *H. ostrearia* isolate (La Barre-de-Monts *vs*. Isle de Ré) on the microalgal growth in co-culture with bacteria

The effect of the geographical origin of *H. ostrearia* on the algal growth when co-cultivated with a given bacterial isolate was studied. From the different Ho-BM-bacterial associations (Fig. 3), four bacterial isolates with contrasting results on algal growth were co-cultivated with Ho-R (B5, B10, B26 and B36). The two algal isolates were chosen because they harbored the most distinct structure of the bacterial communities [28].
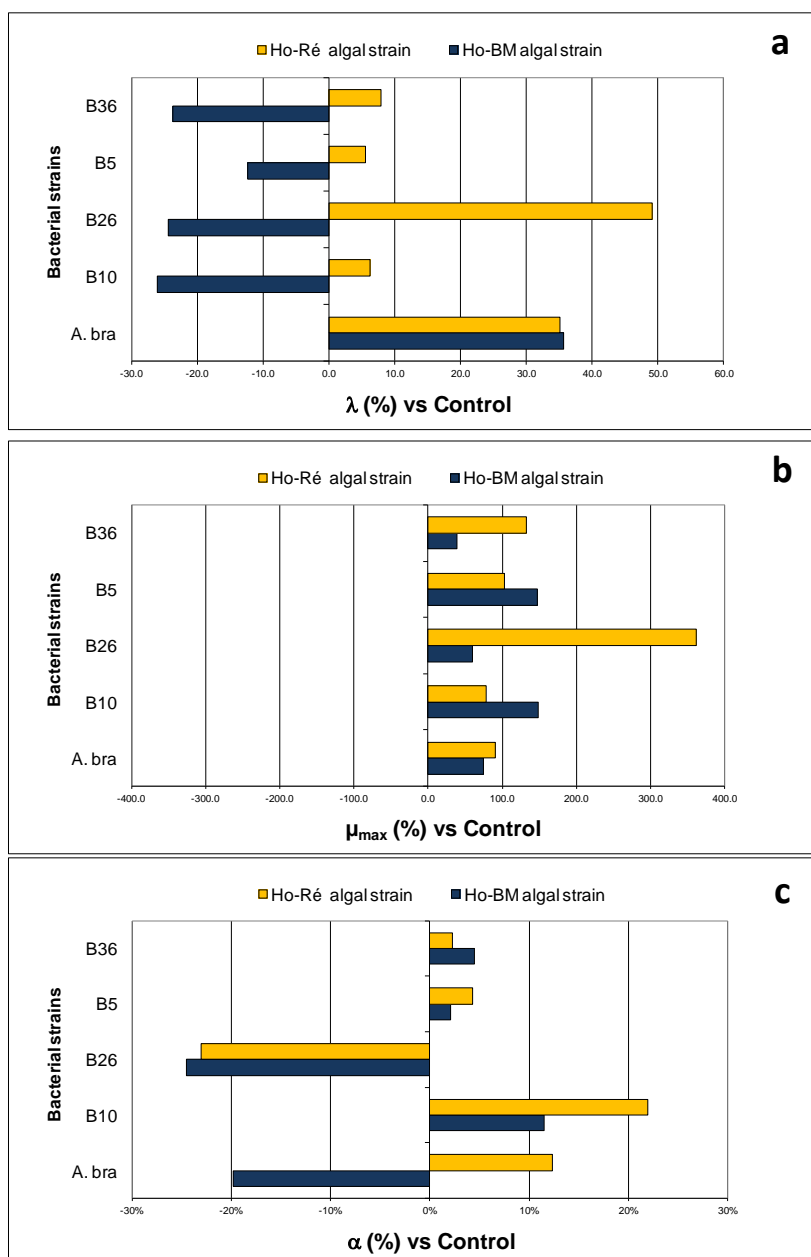
Fig. 3. (a) Lag time (λ), (b) maximum specific growth rate (μ_{max}), and (c) maximal biomass at the stationary growth stage (α) of five bacterial strains in co-culture with Ho-BM (*H. ostrearia* La Barre de Monts) and Ho-R (*H. ostrearia* Isle de Ré). Values are given as a percentage of the control (*H. ostrearia* monoculture) from average values.

Opposite values of λ (Fig. 3a) were obtained (except for *A. brasilense*) with Ho-BM and Ho-R in co-culture with the bacterial isolates (lower and higher than the control, respectively).

The higher λ with Ho-R could be the result of a competition for nutrients between the bacteria and the microalgae while the lower λ with Ho-BM could show a stimulating effect of bacteria. In contrast, the μ_{max} (Fig. 3b) of *H. ostrearia* in co-culture with bacteria was higher than the control irrespective of both the geographical origin of *H. ostrearia* (Ho-BM *vs*. Ho-R) and the bacterial isolate. The conclusion was similar for α (Fig. 3c) except with *A. brasilense* (lower than the control for Ho-BM and higher for Ho-R). Overall, it can be concluded from these results that different bacteria first isolated from *H. ostrearia* culture and co-cultivated with different clones of *H. ostrearia* will lead to a relatively similar algal growth. This is not surprising since we showed in a previous study [28] that the structure of the bacterial community in the phycosphere of *H. ostrearia* had 90% similarity irrespective of the locality where microalgae were recovered, most probably demonstrating the close composition of the microalgal exudates. This finding is obviously an important outcome of this work for aquaculturists. It should be noted that among the 4 bacterial isolates, only B26 shows variable results when Fig. 1 and Fig. 3 were compared. We have no explanation except that the two experiments were not performed at the same time. As compared with most microalgae, *H. ostrearia* is very sensitive to very small changes in the culture conditions. The composition of the culture medium (seawater used as a base for the culture medium) might have been slightly different. Yet, co-cultures with Ho-Ré and Ho-BM algal strains can be reasonably compared as they were performed at the same time and under the same conditions. Although the bacterial isolates (except *A. brasilense*) acted in the same manner on the growth of Ho-BM and Ho-R (regarding μ_{max} and α), this effect was somewhat modulated, depending on the bacterial isolate. These differences were most probably the result of: i) the difference in size between the two isolates (75.2 µm for Ho-BM; 109.3 µm for Ho-R) with a probable repercussion on microalgal physiology; ii) the effect of epiphytic bacteria closely associated with the microalgal cells whose community structure was specific to the microalgal isolate

[28]. In fact, non-axenic cultures of *H. ostrearia* were deliberately used to simulate culture conditions in aquaculture. Then, the supply of the same bacterial inoculum (called bioaugmentation) to Ho-BM and Ho-R had the most significant impact on algal growth but the epiphytic bacteria, whose community structure was quite different, most probably modulated the effect on algal growth.

3.3. Metabolic fingerprinting differentiation of *Haslea ostrearia* in combination with bacterial isolates

Metabolomic profiling methods have recently been shown to be a powerful technology for depicting the intrinsic similarities and differences of biological samples by exhaustive profiling and comparison of metabolites in an organism [50,51]. Bacterial isolates B10 and B26 co-cultivated with Ho-R were chosen for their positive *vs*. negative effect, respectively, on the growth of *H. ostrearia* in the conditions of this study. For the co-culture with Ho-BM, B10 was chosen again for its positive effect and B26 was replaced by *A. brasilense* for its more negative effect on Ho-BM growth. To study the specific relationship between bacteria used in co-culture with *H. ostrearia* isolates in more depth, two *H. ostrearia* controls were used, *i.e.*, axenic and non-axenic cultures.

Considering the complexity of the data generated by the "*H. ostrearia*" matrix, the same molecular feature extraction (MFE) algorithm method was used to extract the molecular features (MFs) of all samples [52]. Using this chromatographic deconvolution, 4639 peaks were extracted for all samples including co-eluting compounds. The number of these MFs was influenced by the intensity threshold used during the process (300 counts in this study). A preliminary evaluation of data was developed with the four different sample conditions (axenic *vs*. non axenic *H. ostrearia* cultures, positive *vs*. negative effect of bacteria on

microalgal growth) to check the quality of the samples, to find tendencies of grouping without any *a priori* hypothesis, and to study the impact of the two considered factors: positive or negative effect of bacteria on *H. ostrearia* growth. Based on mass accuracy and retention time, 2188 compounds out of the 4639 were obtained after applying a filter (frequency analysis) and constituted the compounds (entities) always present (100%) in at least one condition. These molecular features were subjected to unsupervised analysis approaches, and a Principal Component Analysis (PCA) was first applied as an exploratory analytical method (Fig. 4). This is performed via the transformation of measured variables into uncorrelated principal components, each one being a linear combination of the original variables [53].
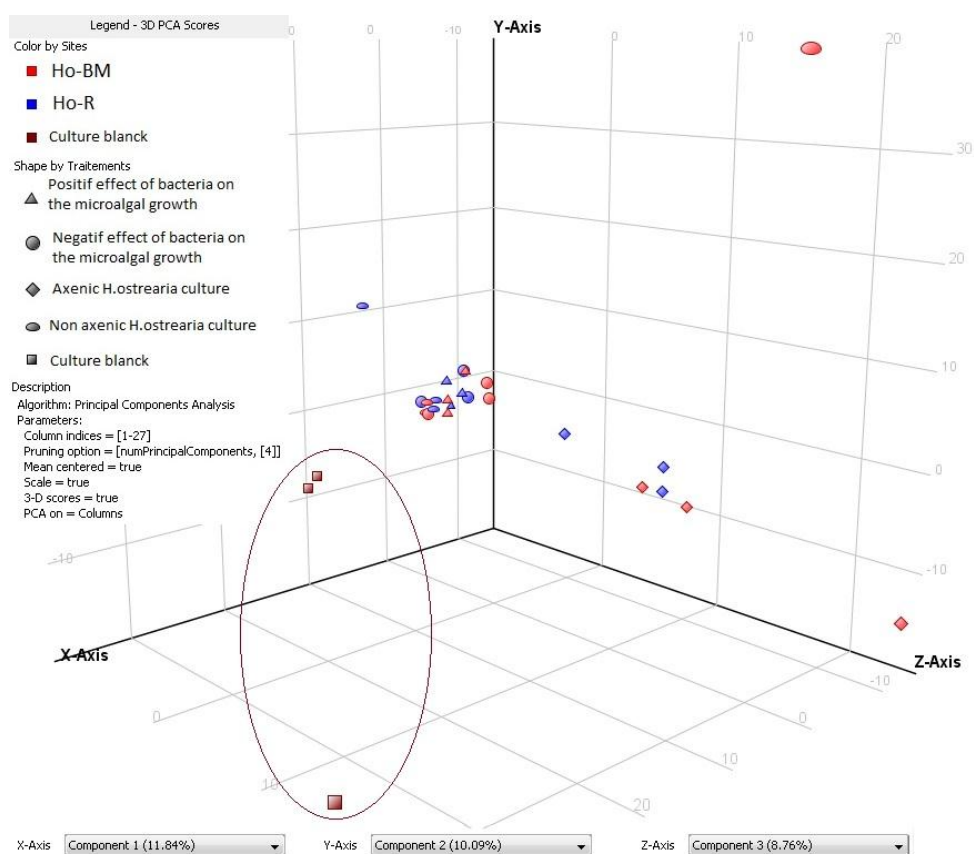

Fig. 4. 3-D principal component analysis (PCA) score plot based on samples (n = 3 per modality) from Isle de Ré (Ho-R) and La Barre-de-Monts (Ho-BM) cultures associated or not

with bacteria showing positive (B10) or negative (B26 for Ho-R and *A. brasilense* for Ho-BM) effects on algal growth and compared to axenic and non-axenic algal cultures.

The three PCA plotted on the X-, Y-, and Z-axis accounted for only 30.69% of the total variability in the data set. All the blanks (*i.e.*, culture medium without microalgae or bacteria) and axenic microalgal cultures were clearly separated between themselves and the rest of the samples (*i.e.*, non-axenic algal culture, bacteria-microalgae co-cultures). On the contrary for non-axenic algal culture and bacteria-microalgae co-cultures, PCA analysis was unable to show differences between modalities in which bacteria had a positive effect on *H. ostrearia* growth and those with a negative effect. Moreover PCA did not reveal differences according to the geographical origin of *H. ostrearia* isolates (Ho-R *vs*. Ho-BM).

To attempt to highlight differences between the different co-culture modalities, only the compounds that were detected in all replicates for each modality were considered and a Venn diagram was produced (Fig. 5). Venn diagrams are visual representations of mathematical sets [54] and were computed with the Mass Profiler Professional software (MPP) solution. These diagrams show both the compounds shared by all the modalities and the compounds specific to each modality. Firstly, 99 compounds were common to all samples, which explains why the PCA was unable to differentiate sample groups. For the Ho-R isolate (Fig. 5a), overall 160 compounds were identified when bacteria isolate B10 with a positive effect on algal growth was used, and 155 compounds for bacteria isolate B26 with a negative effect on algal growth. Once the compounds affiliated to the axenic and non-axenic cultures were removed, *i.e.*, 128 and 165 compounds, respectively, 6 specific compounds remained for the bacteria B10-Ho-R co-culture and 7 for the bacteria B26-Ho-R co-culture with 3 compounds in common. It is noteworthy that 5 compounds were specific to the axenic algal cultures and 8 to the non-axenic ones.

The same analysis was performed for Ho-BM (Fig. 5b). The distribution of the compounds between the modalities was similar with, however, 2 more specific metabolites for Ho-BM (16) compared to Ho-R (8) without any repercussion on the number of specific metabolites relative to the co-cultures (3 and 4 specific metabolites for positive and negative bacterial effects on *H. ostrearia* growth).
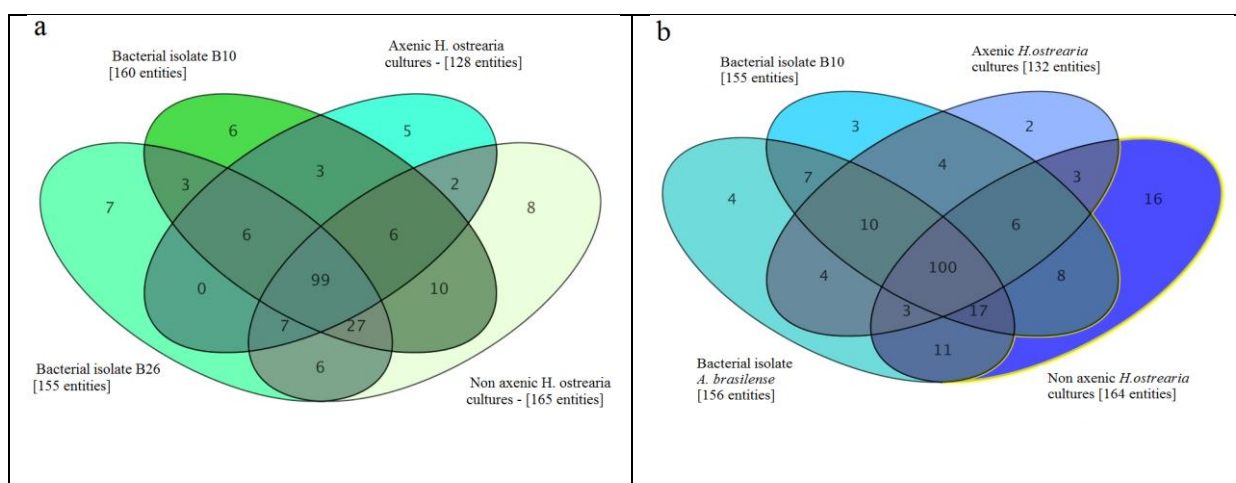

Fig. 5. Venn diagrams showing the common and specific compounds based on the samples (n = 3 per modality, non-averaged, Filtered by Frequency with cut-off percentage: 100) from: a) Isle de Ré (Ho-R) cultures associated or not with bacteria showing positive (B10) or negative (B26) effects on algal growth and compared to axenic and non-axenic algal cultures; b) La Barre-de-Monts (Ho-BM) cultures associated or not with bacteria showing positive (B10) or negative (*A. brasilense*) effects on algal growth and compared to axenic and non-axenic algal cultures.

The literature has given evidence of extracellular indole acetic acid (IAA) production in two axenic cultures and recently, the identification of IAA as well as another metabolite, phenylacetic acid (PAA), also known to be an auxin that improves algal growth [55], have been detected by Segev et al. [56] in instrumental conditions sufficiently close to our

innovative metabolomic approach to allow us a retrospective detection of nitrogen-containing IAA [M+H] (Fig. 6). One might have expected IAA production in bacteria-*H. ostrearia* co-cultures more than with axenic microalgae. Yet it was shown that axenic green microalgae (*Scenedesmus armatus* and *Chlorella pyrenoidosa*) were able to produce and release IAA to the culture medium [57]. In the case of co-cultures, one can assume that IAA produced by bacteria was immediately metabolized by *H. ostrearia*.
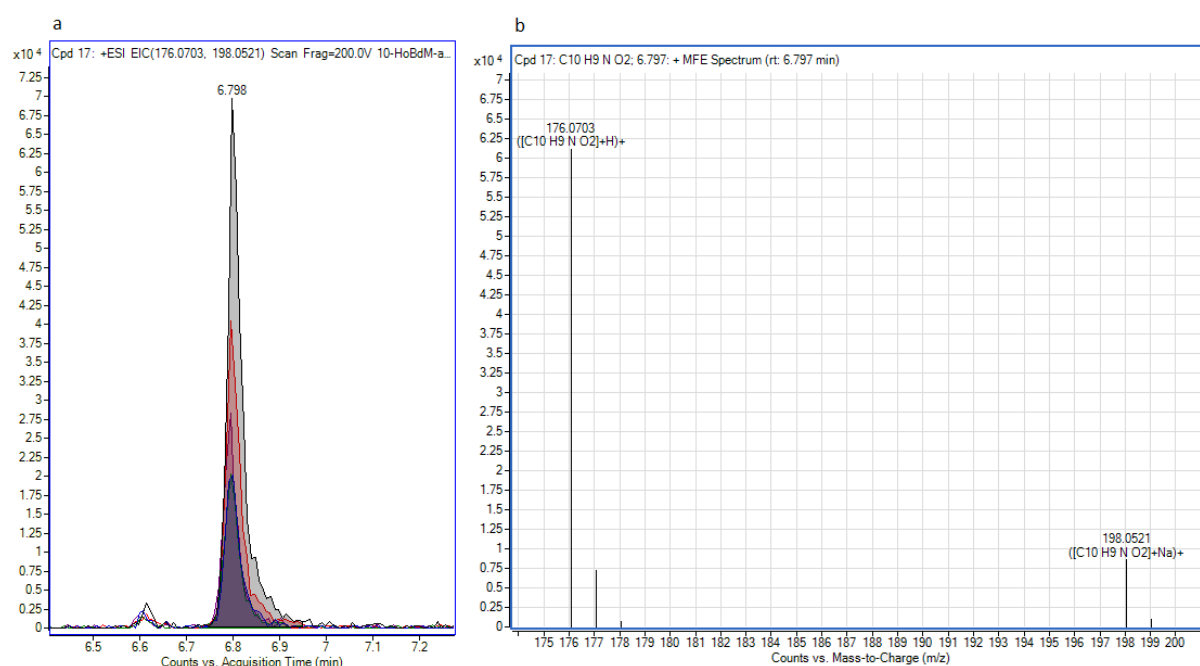

Fig. 6. a) First window is abundances of extracted ion (EIC, m/z 176.0706) overlays of an indole acetic acid (IAA) metabolite detected in triplicate in axenic La Barre-de-Monts (Ho-BM) (grey) and Isle de Ré (Ho-R) (red) cultures; b) spectrum for sample Ho-BM with the IAA monoisotopic mass, its isotopic pattern and its sodium adducts. Data were processed using Agilent Profinder Software (B.08.00).

In addition, IAA is only and strictly found as 1 of the 5 metabolites specific group to the axenic condition of Ho-R (Fig. 5a) while it is part of 1 of 2 metabolites isolated as specific to

the Ho- BM. As expected in Fig. 5a,b, represented respectively by the compartment with 6 metabolites for Ho-R (Fig. 5a) and the compartment with 3 metabolites for Ho-BM (Fig.5b) the IAA is not retained as a potential candidate to associate with the positive effect of the bacterial presence to improve algal growth. Nevertheless, it is necessary to weight this observation with the fact that, within the various biosynthetic pathways [58] of IAA, this compound is very labile. On the contrary, can be considered very interesting the presence of IAA without a priori signals only in the axenic form of the microalgae, but nevertheless present in the isolates from two different geographical zones (La Barre-de-Mont and Isle de Ré).

Of the 2188 metabolites that make up our dataset, we observed that one hundred compounds defined the different groups and that the number of compounds specific to each group was in the order of about ten units. This is typical for plankton studies, where low abundance signals are encountered in a complex matrix [59,60]. Irrespective of the *H. ostrearia* origin, the common compounds solely specific to a bacterial positive effect on *H. ostrearia* growth, *i.e.*, 6 for Ho-R and 3 for Ho-BM (Fig. 7a), were clustered. No common compound were identified in this step. The two compartments (green and blue) were then compared to the compartment (yellow) which represents the 235 compounds detected in the culture medium (Fig. 7b) composed of seawater enriched with mineral salts (see "Material and Methods" section). By subtracting the compounds belonging to the algal growth medium, this operation allowed to conclude that only 3 and 1 compounds were specific Ho-R and Ho-BM respectively.
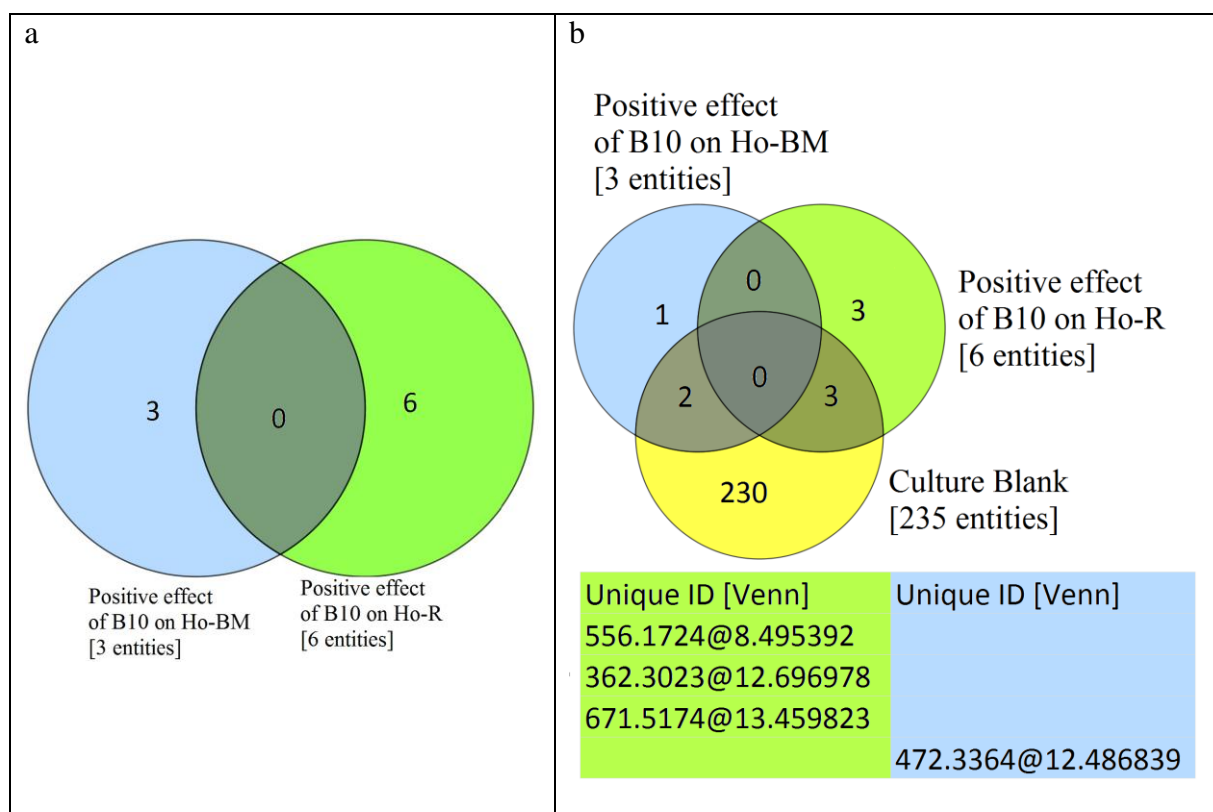
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Fig. 7. Venn diagrams showing the compound distribution between La Barre-de-Monts (Ho-BM) and Isle de Ré (Ho-R) isolates in co-culture with the bacteria isolate B10 (positive effect on algal growth): a) comparison of common compounds in growth activation for Ho-BM and Ho-R; b) metabolites specific (mass@ Retention time) to growth activation for Ho-BM and Ho-R after subtracting the compounds specific to the culture medium.

The most specific compounds with a significance threshold of p-value $\leq$ 0.05 and a fold change $\geq$ 2.0 (*i.e.*, 1 compound for Ho-BM and 3 for Ho-R: see Fig. 7b) were tentatively elucidated by METLIN and DMNP databases. The most probable elemental composition was calculated for each metabolite, based on the accurate mass and isotope distribution pattern (Table 3).

Table 3. Putative elemental composition (METLIN or DMNP database) for 4 compounds involved in the growth activation process.

Compound number (positive polarity)	Elemental composition	Score	RT	Height	Mass	Ions*	Mass (MFG)**	Mass difference (ppm)***	Spectrum
1	$C_{24}H_{46}N_3O_6$	89.06	12.487	22784	472.3364	3	472.3387	4.7	see Suppl. Fig 1
2	$C_{30}H_{26}N_3O_8$	98.05	8.495	274698	556.1723	8	556.172	-0.49	see Suppl. Fig 2
3	$C_{18}H_{40}N_3O_4$	98.28	12.697	43222	362.3023	5	362.3019	-1.26	see Suppl. Fig 3
4	$C_{44}H_{67}N_2O_3$	96.88	13.46	10100	671.5158	3	671.5152	-0.41	see Suppl. Fig 4

* Number of ions identified as belonging to a compound

** Mass of "M" calculated from the proposed formula

*** Difference between the observed mass and the mass of the best generated for the formula

.

For metabolomics research, especially when applied to non-sequenced organisms, very many peaks remain unidentified [61]. Complementary MS and NMR methods need to be developed for proper matching metabolite identification. Unsurprisingly, our compounds were identified as unknown; nevertheless the high score (>95) obtained is an encouraging result.

4. Conclusion

Irrespective of their state (as suspended cells or in a biofilm form), when recovered from monospecific cultures of *H. ostrearia*, most bacteria isolated co-cultivated with this microalgae increased the microalgal biomass, which may create opportunities for aquaculturists who produce green oysters. Nonetheless, this result should be validated at full-scale. This study also highlighted, from the metabolic profiles of the extracellular compounds synthesized by both *H. ostrearia* and added bacteria, a few not fully identified compounds shown to be synthesized specifically by bacteria that stimulate microalgal growth. These data give new original insights in the understanding of the relationships between bacteria and diatoms, and more especially *H. ostrearia*.

Author contributions

The following authors have participated sufficiently in the work described in the manuscript to take public responsibility for appropriate parts of the content. Each author's contributions relating to sections (1), (2) & (3) are mentioned below:

- Régis BARON: conception and design of the study, revising it critically, final approval of the version

- Hervé CAPIAUX: acquisition of data, drafting the article, final approval of the version

- Quentin GRANDET-MARCHANT; acquisition of data, analysis and interpretation of the data, final approval of the version

- Thierry LEBEAU: conception and design of the study, drafting the article, final approval of the version & responsible for the integrity of the work as a whole, from inception to finished article (Thierry.lebeau@univ-nantes.fr)

- Alexandra LEPINAY: conception and design of the study, drafting the article, final approval of the version

- Florence MONDEGUER: experimental design study, acquisition of data, drafting the article, final approval of the version

- Vincent TURPIN: conception and design of the study, drafting the article, final approval of the version

Conflict of interest

The authors declare that there are no conflicts of interest.
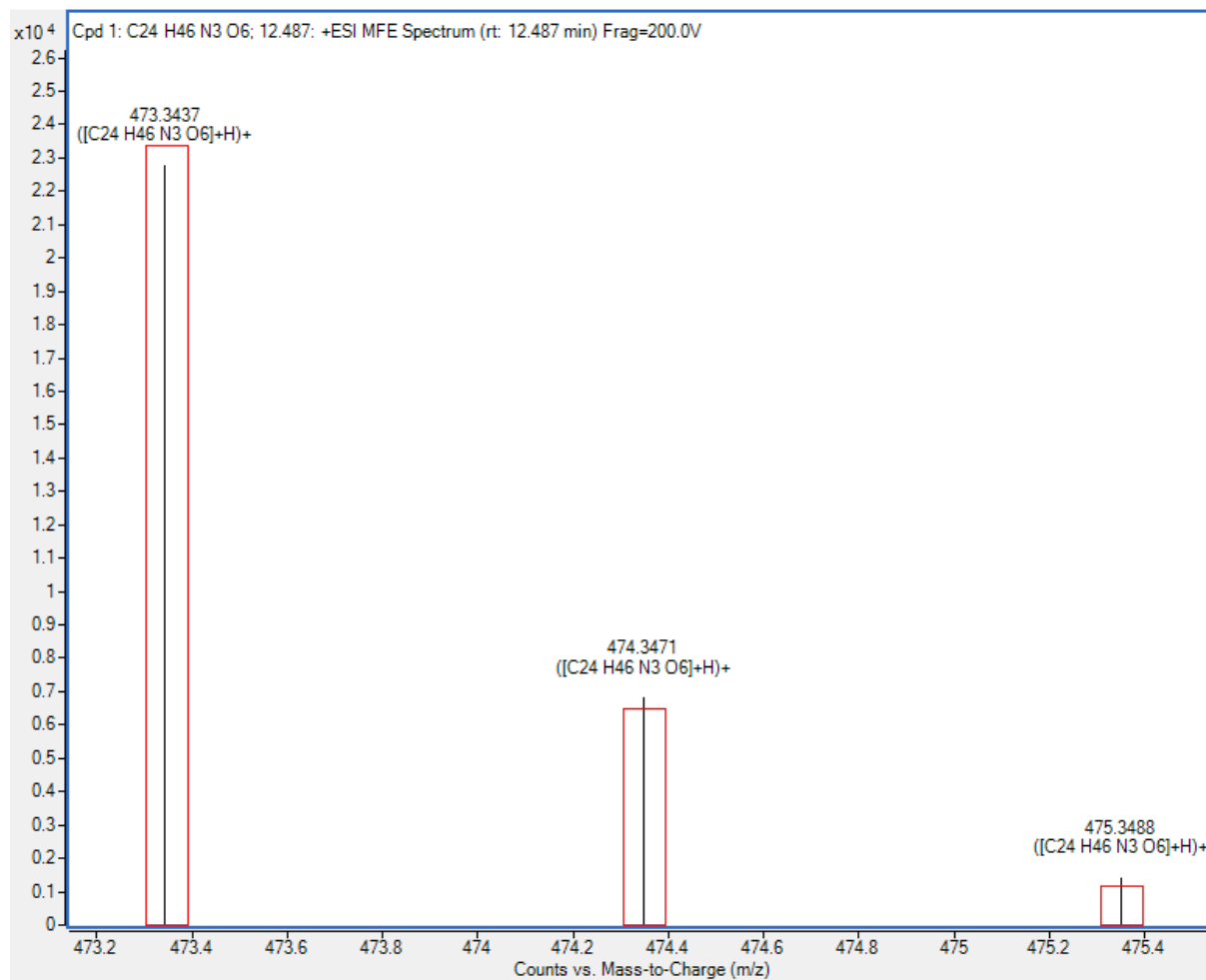
Animal use

No animal use.

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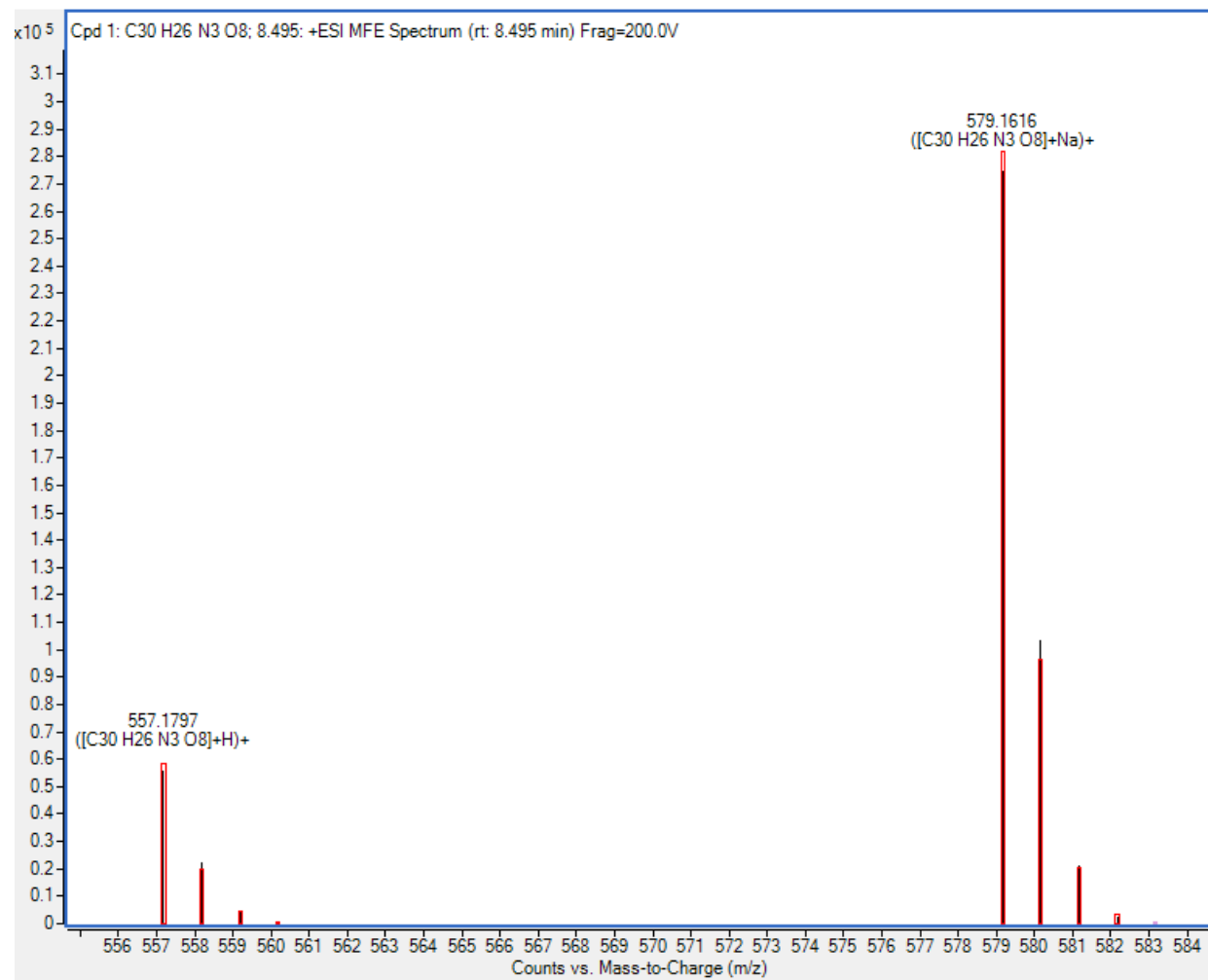
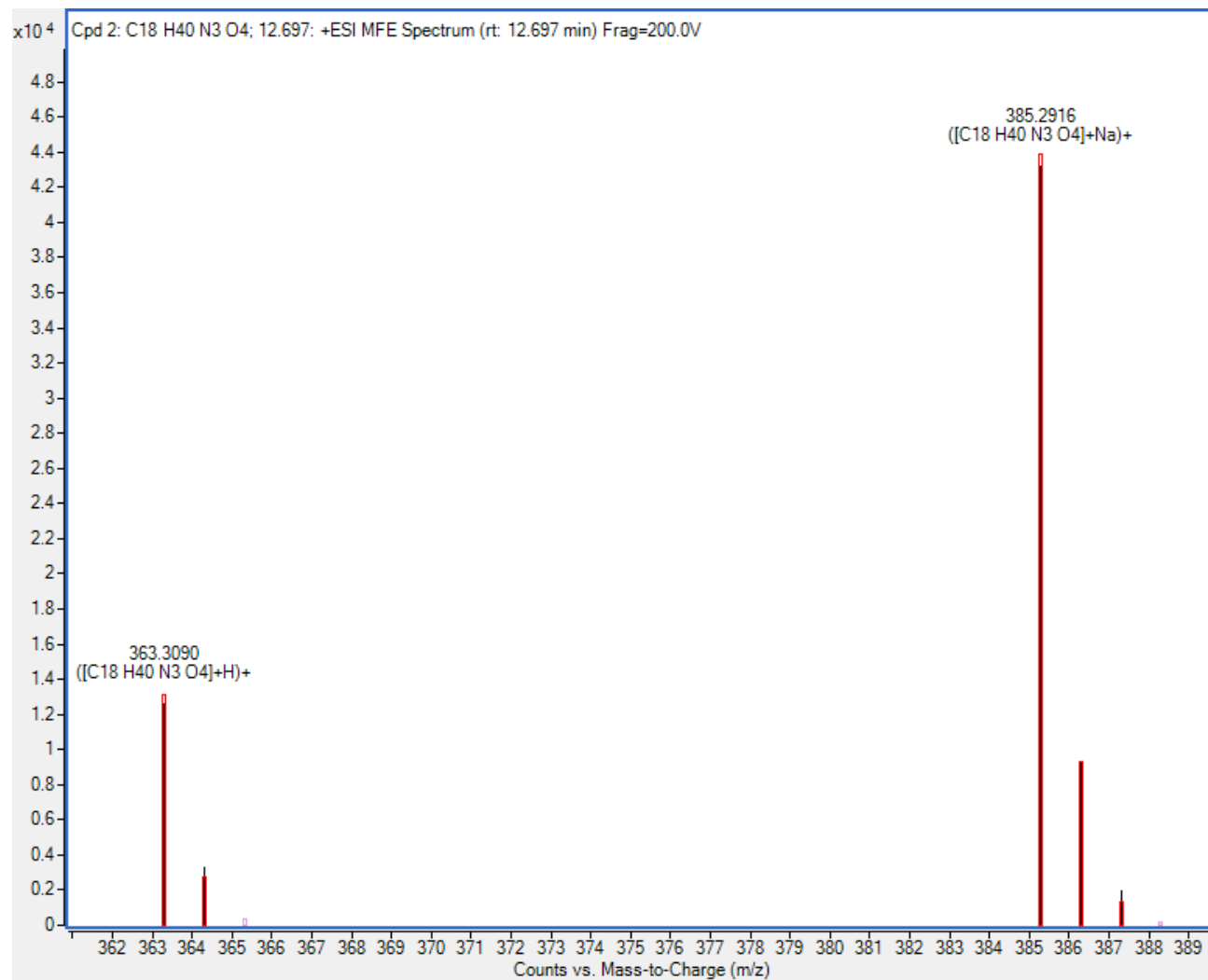1   **Supplementary Figure 1**

2

3

4

5   **Supplementary Figure 2**

Cpd 1: C30 H26 N3 O8; 8.495: +ESI MFE Spectrum (rt: 8.495 min) Frag=200.0V

579.1616
([C30 H26 N3 O8]+Na)+

557.1797
([C30 H26 N3 O8]+H)+

Counts vs. Mass-to-Charge (m/z)

6

7

Supplementary Figure 3

Cpd 2: C18 H40 N3 O4; 12.697: +ESI MFE Spectrum (rt: 12.697 min) Frag=200.0V

11   **Supplementary Figure 4**

Cpd 3: C44 H67 N2 O3; 13.460: +ESI MFE Spectrum (rt: 13.460 min) Frag=200.0V

12