Algal Research
June 2016, Volume 16, Pages 418-426
http://dx.doi.org/10.1016/j.algal.2016.04.011
http://archimer.ifremer.fr/doc/00326/43737/

Bacterial community structure of the marine diatom Haslea ostrearia

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Abstract :

Haslea ostrearia produces a water-soluble, blue-green pigment, called marennine, with proven economic benefits (as a bioactive compound used to green oysters, which improves their market value). Incomplete knowledge of the ecological features of this marine diatom complicates its cultivation. More specifically, the ecology of bacteria surrounding H. ostrearia in ponds is what remains unknown. The structure of this bacterial community was previously analyzed by means of PCR-TTGE before and after isolating H. ostrearia cells recovered from 4 localities in order to distinguish the relative parts of the biotope and biocenose and to describe the temporal dynamics of the bacterial community structure at two time scales (2 weeks vs. 9 months). The bacterial structure of the phycosphere differed strongly from that of bulk sediment. The level of similarity between bacteria recovered from the biofilm and suspended bacteria did not exceed 10%. On the other hand, similarities among the bacterial community structures in biofilms were above 90% regardless of the geographic origin of the algal isolates, while the percentages were lower for suspended bacteria. The differences in bacterial structures of two H. ostrearia isolates (HO-R and HO-BM) resulted in specific metabolomic profiles. The nontargeted metabolomic investigation revealed more distinct profiles in the case of this bacteria-alga association than for the H. ostrearia monoculture. At the culture cycle scale under laboratory conditions, the bacterial community depended on the growth stage. When H. ostrearia was subcultured for 9 months, a shift in the bacterial structure was observed as of 3 months, with the bacterial structure stabilizing afterwards (70%–86% similarities), in spite of the size reduction of the H. ostrearia frustule. Based on these results, an initial insight into the relationships between H. ostrearia and its surrounding bacteria could be drawn, leading to a better understanding of the ecological feature of this marine diatom.

Keywords : Biofilm, Ecology, Metabolic fingerprinting, Microalgae, Phycosphere, TTGE

1. Introduction

Haslea ostrearia is a cosmopolitan species of diatoms commonly found on the French Atlantic ~~cost~~coast, especially in oyster ponds of the Bay of Marennes-Oléron and Bay of Bourgneuf [1]. This diatom has long been the subject of curiosity [2] and became a topic of investigations due to its water-soluble, blue~~-~~-green~~-~~ pigment ~~named~~, called marennine, which is responsible for the greening of oysters. This blue~~-~~-green pigment, produced ~~during~~when *H. ostrearia* blooms, is released into the seawater, ~~and~~at which point the ponds turn green. At this stage, *H. ostrearia* is the dominant diatom species in ponds, and oyster farmers ~~use~~ take advantage of this phenomenon ~~in~~by immersing their oysters in these shallow waters for 'refinement' (fattening) and greening, ~~because~~since these last two ~~final~~ stages of ~~rearing are a~~ raising oysters guarantee ~~of the~~ product quality and improve ~~their~~a farm's profits. Beyond its ~~interest~~benefit in aquaculture for ~~the~~ greening ~~of~~ oysters [3], ~~it was demonstrated that~~ marennine ~~had~~has been shown to possess several biological functions with potential biotechnological applications, namely: i) antibacterial, anticoagulant and antiviral activities [4, 5~~],~~]; ii) antioxidant activity [6~~]~~]; and iii) antitumor and antiproliferative effects of the aqueous extract from *H. ostrearia* ~~against~~on solid tumors (lung and kidney carcinoma and melanoma cell ~~lines molecule). But up to date, the~~line molecules). Until now however, an incomplete knowledge of the ecological conditions under which this microalgae develops in its natural ecosystem ~~make~~has complicated controlling the cultivation of *H. ostrearia* ~~difficult to control although~~, even though dedicated photobioreactors adapted to the physiological specificity of this microalga ~~by using~~ through the use of artificially immobilized cells ~~-~~- were designed at the laboratory scale [7~~, 8, 9, -~~10]. ~~Indeed this~~This microalga indeed exhibits several ~~behaviours mainly~~types of behavior, primarily benthic, occasionally planktonic~~,~~ but also epiphytic [11, 12~~]~~], thus making immobilization a relevant~~. Ecophysiology~~ course of action. The ecophysiology of *H. ostrearia* is complex and moreover not yet completely

understood ~~as well~~. In oyster ponds, *H. ostrearia* can outcompete other microalgae ~~but~~yet is also being consumed by oysters [13~~, 14, ~~15]. The ~~biotope of~~ *H. ostrearia* ~~was~~biotope has also been studied~~. It~~; it was demonstrated that: i) ~~that~~ this diatom ~~has a high degree of tolerance~~is extremely tolerant to high irradiance~~, and~~ (thus offering an ecological advantage ~~on~~over the other main diatoms encountered in oyster~~-~~ ponds, ~~such as~~e.g. *Skeletonema costatum* [16, 17~~]~~]); and ii) ~~that~~ the greening phenomenon is controlled by the nutrient composition in oyster-~~ponds~~pond waters~~,~~ (see [18] for conditions ~~[18].~~). Recently, a more detailed genetic characterization of *H. ostrearia* was undertaken ~~with the development of~~by developing genetic molecular tools ~~and~~, which led to identifying new strains of *H. ostrearia* ~~were identified~~ [19] ~~and~~along with a second species of blue diatom ~~named~~called *Haslea karadagensis*.

~~Yet surprisingly,~~ Surprisingly, only a few studies have focused on bacteria-microalgae interactions, ~~while~~ in noting that some bacteria may increase the microalgal biomass ~~with consequently~~while offering potential applications in ~~aquacultures. Up to date~~aquaculture. For now, nothing is still known for the specific case of *H. ostrearia*, ~~while~~yet for ~~instance~~instances regarding bacteria and diatoms~~, “~~ overall, "they have co-occurred in common habitats throughout the oceans for more than 200 million years, fostering interactions between these two groups over evolutionary time scales~~”~~" [20]. The link between bacterioplankton and phytoplankton dynamics was recently demonstrated by Rooney-Varga ~~and colleagues~~*et al.* [21]. The habitat of phytoplankton-associated bacteria has been depicted by the ~~“~~concept of "phycosphere~~” concept,~~", i.e.~~,~~. the area around algal cells where bacteria feed on extracellular products of the algae [22]. The phycosphere is thus the aquatic analog of the rhizosphere in soil ecosystems and has direct implications for nutrient fluxes to and from algal cells. ~~The bacteria~~Bacteria-microalgae interactions ~~were~~have been studied for ~~few diatoms~~several diatom species ~~such as~~, including *Guinardia delicatula*, *Pseudonitizschia*

pugens, *Thalassiosira rotula*, *Skeletonema costatum* [23], *Ditylum sp.*, *Thalassiosira sp.*, *Asterionella sp.*, *Chaetoceros sp.*, *Leptocylindrus sp.*, *Coscinodiscus sp.* [24], *Pseudo-nitzschia multiseries* [25~~],~~] and *Nitzschia microcephala* [26]. Some of these common species are frequently encountered in oyster-pond waters and sediments [11, 18]. ~~Overall~~On the whole, the bacterial biodiversity of the phycosphere was shown to be ~~low when compared~~limited in comparison to the complexity of bacterial assemblages in bulk seawater [24]. The structure of the bacterial community ~~linked~~related to microalgae is ~~at least~~ specific to the microalgae species [20, 24, 27~~] except~~], though some bacterial phylotypes, such as bacteroides, are known to ~~have~~play a significant role in nutrient cycling ~~through degradation of~~by degrading algal macromolecules ~~and for its attached~~; moreover, such species attach to growth and are then recovered in most phycospheres [28]. To demonstrate this specific bacterial-algal interaction, Schäfer ~~and colleagues~~*et al.* [24] attempted, using two algal cultures, to associate each ~~one~~culture with the ~~“~~"satellite~~”~~" bacterial assemblage of the other ~~and showed that it was unfeasible~~culture and proved such an association infeasible.

Based on these considerations, i.e~~., the~~. an incomplete knowledge of the ecological features of *H. ostrearia*, ~~this~~the present work has been intended to: i) characterize the structure of the bacterial community by means of PCR-TTGE both before and after *H. ostrearia* isolation from oyster ponds ~~of~~in different localities~~,~~; ii) compare the bacterial community of the *H. ostrearia* phycosphere ~~of *H. ostrearia*~~ vs. free- cells ~~in~~within the culture medium~~,~~; iii) distinguish the relative ~~part~~portion of the biotope and ~~the~~ biocenose based on the ~~composition of the~~ bacterial structure composition; and iv) describe the temporal ~~dynamic of the structure~~ dynamics of the bacterial community structure at the time-~~scales~~ scale of ~~a~~one culture cycle ~~in~~under laboratory conditions and after several subculturing steps. A metabolic fingerprinting (untargeted approach) was aimed ~~to assess~~at assessing the global metabolic profile of *H.*

ostrearia cultures, whether or not associated ~~or not~~ with the ~~bacteria of the~~ phycosphere bacteria. Additional clarifications on the bacteria-*H. ostrearia* associations were provided ~~and~~, as well as on the role of the ~~geographical~~geographic origin of *H. ostrearia*. ~~Class(es) of~~The compound~~(s) being~~ class or classes potentially affected under ~~the studied~~these study conditions were not anticipated ~~and~~; moreover, specific compounds were not necessarily identified or quantified.

2. ~~Material~~Materials and methods

2.1. ~~-~~Sampling location

~~Samples~~The test samples were collected in oyster ponds ~~in~~from four localities ~~of~~along the French Atlantic ~~cost according to a North-South~~coast, along the following north-to-south gradient: Bouin (46.96°N; 2.04°W), La Barre-de-Monts (46.90 N; 2.11°W), Isle of Ré ~~island~~ (46.22 N; 1.45°W~~)~~), and La Tremblade (45.80 N; 1.15°W) (see Fig. 1). One liter of each sample was collected at the seawater-sediment interface~~, at~~ on the bottom of the oyster ponds in order to ~~have~~obtain both sediment and seawater. ~~Samples~~The samples were immediately stored at 4~~-~~°C. ~~At~~In the laboratory, the presence of *H. ostrearia* was ~~checked~~verified before isolation.

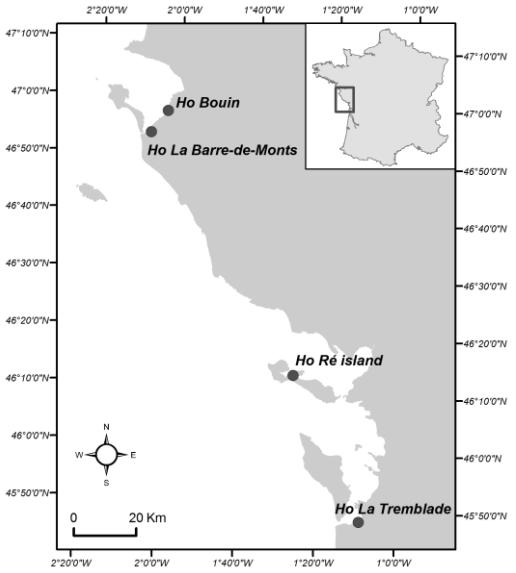

Fig. 1.: Map of the French Atlantic coastcoast showing oyster ponds localities-pond locations where samples were collected for isolation ofto isolate *Haslea ostrearia*: Bouin (46.96°N; 2.04°W), La Barre-de-Monts (46.90 N; 2.11°W), Isle of Ré island (46.22 N; 1.45° W)), and La Tremblade (45.80 N; 1.15°W).

2.2. Isolation of *H. ostrearia* from environmental samples and cultivation

Monospecific cultures of *H. ostrearia* were obtained by isolation ofisolating a single cell of *H. ostrearia* from the raw samples. ItThe specimen was recovered withusing a capillary pipette, using and an inverted microscope, and; it was then washed by successive cell subculturing in filtered seawater (0.22 µm) to remove contaminants (e.g.,, bacteria, other microalga, flagellate, larvae). Among the *H. ostrearia* isolates, six of them were selected for the following studystudies: HO1 and HO2 Bouin (HO1-B and HO2-B), HO La Barre-de-Monts (HO-BM), HO Isle of Ré island (HO-R), and HO1 and HO2 La Tremblade (HO1-T and HO2-T).

For the followingensuing experiments, the above mentionedaforementioned isolates were grown in 250 mL-Erlenmeyer flasks filled with 150 ml of the modified Provasoli [29] medium (ES1/3: [30]) to obtain enoughsufficient biomass. The monospecific isolates of *H.*

ostrearia were transferred ~~at~~during the exponential growth stage (every 7-10 days) ~~in~~into a fresh ES 1/3 medium. ~~Cultures~~The cultures were incubated in a culture chamber at 16~~-~~°C under 120 µmol photons m^{-2} s^{-1} and a 14:10 h light : dark regime.

2.3. ~~-~~Algal fingerprints and ~~structure of~~ the bacterial community structure

2.3.1. Sample preparation

Raw sediments: The seawater and ~~the~~ sediment of the raw samples recovered in the oyster ponds were separated by overnight sedimentation ~~overnight~~ in a culture chamber at 16~~-~~°C. ~~Samples~~The samples were then frozen at -20~~-~~°C. 0.5 g of ~~sediments~~sediment was used for DNA extraction~~-~~ purposes.

Supernatant and biofilm from ~~H. ostrearia~~ monospecific H. ostrearia cultures: To compare the ~~structure of the~~ bacterial community structure in the algal biofilm, ~~i.e.,~~ which entails comparing the bacteria ~~entrapped~~embedded in exopolysaccharides ~~making~~forming biofilm and epiphytic bacteria of *H. ostrearia*~~,~~ with that of the suspended cells in the culture medium, the biofilm and ~~the~~ supernatant from the culture of *H. ostrearia* were separated. From cultures ~~in~~during the exponential growth stage in 250~~-~~mL~~-~~ Erlenmeyer flasks filled with 150 mL of ES 1/3 medium, 100 mL of the liquid ~~-~~- attached at the bottom of the Erlenmeyer flasks ~~-~~- were carefully collected ~~carefully~~ to avoid ~~any~~ contact with the biofilm, and the few free alga (possibly associated with bacteria) were removed by centrifugation (SIGMA 3K30 Fisher Bioblock Scientific: 900 g, 90 s, 16~~-~~°C) to ~~recover~~ensure recovering in the supernatant ~~only~~just the bacteria in suspension ~~in~~within the culture medium. The supernatant was filtrated through a 0.22~~-~~- µm filter (cellulose nitrate membrane, Sartorius) so as to concentrate the bacteria on the filter, which was then frozen at -20~~-~~°C. Before DNA extraction, each filter was cut into small pieces of about 4 mm^2.

From ~~the~~this same culture, the remaining 50 mL were eliminated, and 30 mL of fresh ES 1/3 medium were added ~~in~~to the Erlenmeyer flask~~ and~~; the algal biofilm was recovered by ~~the mean~~means of the ~~mechanic~~mechanical action of a sterile bar magnet. Microalgae and bacteria (epiphytic and those embedded in the biofilm~~),~~) were both recovered by centrifugation (SIGMA 3K30 Fisher Bioblock Scientific: 6000 g, 5 min, 16~~-~~°C~~)~~), and the samples were frozen at -20~~-~~°C prior to DNA extraction.

Cultures of H. ostrearia at ~~different~~various growth stages and generations: ~~for~~For both experiments, ~~about~~approx. 1.5~~-~~·10^6 algal cells were collected ~~after~~once the culture ~~being homogeneizated,~~had been homogenized and then centrifuged (Universal 320 Hettich: 6000 g, 10 min, 16 °C~~), and~~); the pellets containing suspended cells and cells of the biofilm were frozen at -20~~-~~°C prior to DNA extraction.

The ~~structures of the~~ bacterial community structures of the HO-BM and HO-R isolates were studied at the time~~-~~ scale of ~~a~~one culture cycle. Samples were collected at the time of ~~the transfer of~~transferring *H. ostrearia* ~~in~~into the fresh ES 1/3 medium after being isolated and cultured in the laboratory ~~during~~for one year (~~day~~Day 0) and again after another 3, 7, 15 and 30 days.

The ~~structures of the~~ bacterial community structures of the HO-BM, HO-R, HO1-B, HO2-B, HO1-T and HO2-T isolates were also studied at the time~~-~~ scale of ~~different~~various subculturings of *H. ostrearia*: T0, T+3, T+6 and T+9 months ~~after~~following *H. ostrearia* isolation. At each ~~time~~of these times, a biometric ~~measure~~measurement (cell length) was ~~performed~~conducted on 90-200 algal cells ~~with~~using an Olympus AX70 PROVIS microscope ~~and was determined using to~~; final determination relied on the LUCIA G software.

Axenic and non-axenic H. ostrearia cultures for metabolomic profiling: HO-BM and HO-R were cultivated in 250~~-~~-mL~~-~~ Erlenmeyer flasks. ~~Cells making~~The cells forming a biofilm at the bottom of the vessel were recovered ~~in~~during the exponential growth stage~~,~~ after being

~~resuspended using~~re-suspended with a sterile bar magnet, and homogenized and counted using a Nageotte chamber. Microalgae were then inoculated at 3~~–~~·10^3 cells mL^{-1} (two replicates) in 100~~–~~mL~~-~~ Erlenmeyer flasks containing fresh ES 1/3 medium and an antibiotic antimycotic solution (10,000 units penicillin, 10 mg of streptomycin and 25 µg of amphotericin B mL^{-1}, BioReagent, A5955 SIGMA) diluted at 1:50 or 1:100. The cultures were incubated in a culture chamber at 16~~-~~°C under 120 µmol photons m^{-2} s^{-1} and a 14:~~ 10h~~10 h light : dark regime. ~~The treatment~~Treatment was ~~conducted during~~carried out for 7 days~~;~~ ~~then~~, subsequent to which the culture supernatant was removed~~;~~ and the algal biofilm ~~was~~ washed once with fresh ES 1/3 medium to eliminate the antibiotics. The cells were ~~resuspended~~re-suspended with a sterile bar magnet in 25-30 mL of fresh ES 1/3 medium, and 5 mL were transferred ~~in~~into 250~~–~~mL~~-~~ Erlenmeyer flasks filled with 150 mL of fresh ES 1/3 medium (triplicate). After 7~~-~~ days of culture without antibiotic treatment, a second 7-day treatment was conducted with the same antibiotic antimycotic solution diluted to 1:50, followed by 7 days of culture without treatment. After the second treatment, the bacterial concentration was drastically reduced compared to the non-axenic cultures. These cultures were thus called ~~“~~"axenic~~”~~". Cells were collected ~~in~~during the exponential growth stage: 200 µL of the supernatant of HO cultures were collected, filtered on 0.20~~–~~µm PTFE membrane filters (Interchim) and frozen at -80~~–~~°C prior to fingerprint acquisition. For non-axenic *H. ostrearia* cultures, from the stock cultures of HO-BM and HO-R ~~in~~during the exponential growth stage in 250~~—~~mL~~-~~ Erlenmeyer flasks, microalgae were inoculated in ~~triplicates~~triplicate in 24 ~~wells plate~~well plates at 3~~-~~·10^3 cells mL^{-1} to monitor ~~the~~ daily algal growth by measuring the fluorescence of ~~the~~ chlorophyll (BMG LabTech~~–~~: 440; 680 nm). Microalga were incubated ~~in~~under the same conditions as for the axenic *H. ostrearia* cultures. After 6 days of culture (during the exponential growth stage), 200 µL of the culture

supernatant ~~of culture~~ were collected, filtered on 0.20~~-~~µm PTFE membrane filters (Interchim) and frozen at -80~~-~~°C prior to fingerprint acquisition.

2.3.2. ~~Bacteria~~Bacterial DNA extractions

DNA from ~~i)~~ raw sediments, ~~ii)~~ as well as from supernatant derived from ~~*H. ostrearia*~~ monospecific *H. ostrearia* cultures and ~~iii)~~ *H. ostrearia* cultures at different growth stages and generations, was extracted using a NucleoSpin™ soil kit (Macherey-Nagel, GmbH & Co~~-~~., Germany). In the specific case of the supernatant of *H. ostrearia* cultures at different growth stages and generations, ~~first~~the initial steps of sample grinding were ~~realized~~performed with an MM400 Bead Beater (Retsch Germany) (3× 30 sec, 25 Hz). For biofilms, DNA was extracted using a NucleoSpin® Tissue kit (Macherey-Nagel, GmbH & Co~~-~~., Germany). ~~First step of~~ The first grinding step was ~~realized~~executed with the Bead Beater (~~1min, 25Hz)~~1 min, 25 Hz), then a pre-lysing step ~~of pre-lyzing including~~that included grinding (56~~-~~°C, 105 min; grinding, 10 sec, 25 Hz ~~each~~every 30 min) ~~is~~was applied according to ~~the manufacturer'srecommendations.~~manufacturer's recommendations; DNA concentrations were also measured (SPECTROstar Nano, BMG LABTECH LVi Plate, Germany).

2.3.3. PCR amplification

The V3 region of the 16S rRNA gene was amplified using the 357F-GC and 518R primers [31]. The PCR reaction mixture contained 0.6 ng.µL^{-1} of DNA template, 0.1 µmol.L^{-1} of each primer, 200 µmol.L^{-1} of dNTP, 0.012 unit.µL^{-1} of Taq polymerase 1× reaction buffer, 2.5 mmol.L^{-1} of $MgCl_2$, and 500 ng.µL^{-1} of ~~bovin~~bovine serum albumin and 5% (v/v) of dimethylsulfoxide ~~used in~~introduced into a 50-µL final reaction volume~~ of 50 µL~~. The following cycling conditions were used for ~~bacteria~~bacterial amplification: 1 cycle at 95~~-~~°C for 8 min, followed by 7 cycles of 95~~-~~°C for 30 s, 68~~-~~°C (-1~~-~~°C / cycle) for 30 s, 72~~-~~°C for 50

s, and 28 cycles at 95 °C for 30 s, 62 °C for 30 s, 72 °C for 50 s, and a final extension cycle at 72 °C for 30 min (CFX96 Touch™, Thermal Cycler, Bio-Rad, ~~US).~~U.S.).

2.3.4. Temporal temperature gradient electrophoresis (TTGE) and fingerprint acquisition

PCR products (from Section 2.3.3) were separated ~~according to~~by their GC% using a Temporal Temperature Gel Electrophoresis [32]. TTGE analyses were performed using a ~~DCode™System~~DCode™ System (Bio-Rad, ~~US).~~U.S.). The 9.5% polyacrylamide gel was composed of two parts: a top, urea-free~~, “ ~~"concentration~~”~~" part (stacking gel) ~~on about~~applied approx. 1 cm from the base of the wells upward~~,~~; and a bottom~~, “ ~~"denaturation~~”~~" part (resolving gel), at 8 mol.L^{-1} urea. Fifteen microliters of PCR products were deposited ~~in~~into each well. Migration ~~was performed~~took place in 1.25 × TAE for 750 min at 50 V, with a temperature gradient from 65 ~~°C~~° to 70 °C, i.e~~.,~~ +0.4 °C per hour. The gels were stained using GelRed™ (Biotium, ~~US)~~U.S.) and then imaged under UV light (Molecular Imager®Gel Doc™XRSystem, Bio-Rad, ~~US and~~U.S., along with Image Lab™ software).

2.4. ~~Untarget~~**Untargeted** metabolomic profiling

UHPLC-ESI-QToF~~ using~~, through implementing a non-targeted ~~analysis~~analytical strategy ~~by~~via high~~-~~-resolution mass spectrometry (HRMS) [33~~]~~], was used to detect small soluble extracellular target compounds produced by the bacteria and *H. ostrearia* recovered from the culture medium.

LC-TOF/MS analysis samples: ~~Aliquots~~aliquots (5 µL) of each sample from the ~~supernatant~~supernatant of *H. ostrearia* cultures (see ~~section~~Section 2.3.1.) were separated on a Kinetex, 1.7~~-~~-µm C18 100Å (Phenomenex) column (150 × 2.1 mm) maintained at 40 °C, using an Agilent 1290 Infinity LC system 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 present was as follows: 5% B from 0 to 2.4 min, raiseincreasing to 25% B from 2.4 to 4.5 min, then raiseraised to 30% B from 4.5 to 11 min, finally raise toreaching 100% B from 11 to 14 min and maintainheld there until 16.5 min, subsequentlyfollowed by a decrease to 5% B until 20 min have elapsed and maintainthen maintained at 5% B until 25 min. The eluent was directly introduced into the mass spectrometer by an electrospray. Mass spectrometry was performedconducted on a 6540 UHD Q-TOF mass spectrometer (Agilent Technologies, Waldbronn, Germany),) operating in positive ion mode. The capillary voltage, fragmentor voltage and skimmer were set toat 3,900, 150 and 60 V, respectively. The sheath gas was measured at 350 °C (12 mL min^{-1}) and the drying gas at 175 °C (5 mL min^{-1}) andwith a 43-psi nebulizer 43 psi. Nitrogen was used as the collision gas. Mass spectra were acquired in a full scan analysis over an m/z range of 50 - 1,700 using an extended dynamic range and storage ina centroid mode. Data of storage. The data station operating software was the MassHunter Workstation Software (versionBversion B.06).

2.5. Experimental replication and data exploitation processing

Experimental replication: For TTGE analysis, six samples, and then followed by six distinct DNA extracts, from the four studied localities were recovered from oyster ponds (see sectionSection 2.2.)), and their bacterial structures were compared (for raw sediments, biofilm, and water column). A genetic comparison of *H. ostrearia* isolates was also performed. For the monitoring ofcarried out. To monitor the temporal dynamic of the structure of the bacterial community on structure over the course of anone algal culture cycle, experiments were replicated two times and also for the monitoring of the temporal dynamic of thetwice and moreover used to monitor this structure of the bacterial community over for its

temporal dynamics during a 9-~~months~~month serial subculturing campaign. For the non-targeted metabolomic investigation, analyses were carried out in triplicate.

Data ~~exploitation~~processing: The 16S rRNA banding patterns on imaged TTGE gels were ~~analysed with~~analyzed using the Molecular Analyst Fingerprinting software: FPQuestTM (Bio-Rad, ~~US). Briefly~~U.S.). Put briefly, the software ~~performed~~constructed a density profile through each lane and calculated the relative contribution of each band to the total band signal in a lane, after applying a rolling disc to serve as background subtraction. The banding patterns of the samples in each lane were compared with ~~each other~~one another. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to draw a dendrogram from similarity coefficients. To ~~lower~~decrease the bias, we ~~chose~~opted to analyze band patterns by ~~using~~exercising the densitometric-curve option, as ~~already~~previously used by Kuntz ~~and colleagues~~*et al.* [34] and Seghers ~~and colleagues~~*et al.* [35~~],~~] instead of the band-detection option. For the metabolomic investigation, data were processed using the MassHunter Qualitative Analysis software (Agilent Technologies~~) where~~), with compounds ~~were~~being extracted from the raw data ~~using~~with the Molecular Feature Extraction (MFE) algorithm~~, and~~. Moreover, a principal component analysis ~~were performed~~ was conducted using the Mass Profiler Professional B12.05 statistical package (also from Agilent Technologies).

3. Results

3.1. Bacterial community structure of sediment samples from oyster ponds

The structure of the bacterial community from oyster ~~ponds~~-pond bulk sediment samples varied ~~according to the~~depending on sampling location: Isle of Ré ~~Island~~only showed ~~only~~

15% ~~of~~ similarity with other localities. ~~On the contrary~~In contrast, samples from the other localities ~~had~~displayed a similar bacterial community structure ~~(>~~(i.e. > 85~~%) (~~%, see Fig. 2).

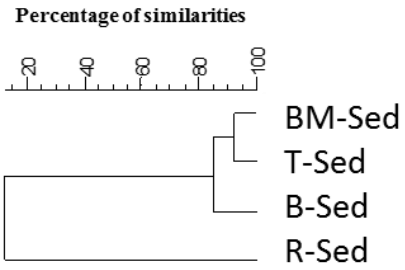

Fig. 2~~.~~: Bacterial community structure of sediment samples (Sed) collected in oyster ponds from ~~different~~various localities (B: Bouin, BM: La Barre-de-Monts, R: Isle of Ré ~~island~~, T: La Tremblade~~).~~)

3.2. ~~C~~Community structure of bacteria recovered from the biofilm after *Haslea ostrearia* isolation and from bacteria suspended ~~bacteria from~~in the water column

~~Firstly~~First of all, the structure of the bacterial community from the sediment compared to that of the biofilm after *H. ostrearia* isolation differed ~~strongly~~considerably (only 10% ~~of~~ similarity between sediment and biofilm~~) (~~, data not shown). This result demonstrates that the bacteria associated ~~to~~with *H. ostrearia* were specific to the microalga.

Furthermore, ~~the~~a comparison of ~~the~~ community structure of the bacteria recovered from the biofilm with those of the water column (WL), i.e~~.~~, suspended bacterial cells (Fig. 3~~) showed~~), revealed similarities that did not exceed 10%.

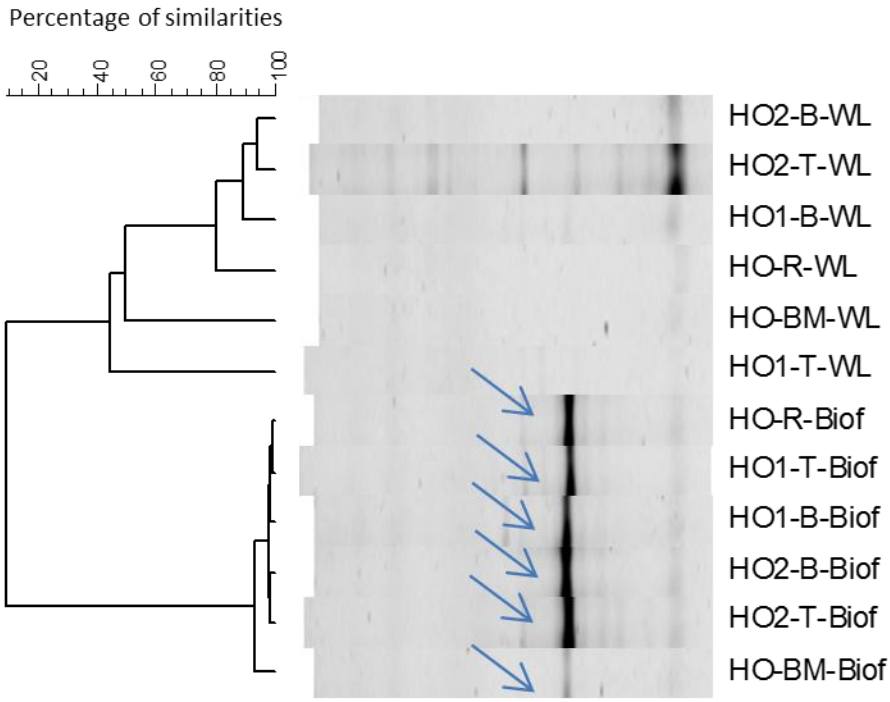

Fig. 3~~.~~: TTGE analysis of the bacterial community structure from biofilm (Biof) and water column (WL) samples after isolation of *H. ostrearia* from ~~different~~various localities and subculturing in ES 1/3 medium ~~in~~under laboratory conditions (B: Bouin, BM: La Barre-de-Monts, R: Isle of Ré ~~island~~, T: La Tremblade). Arrows indicate the position of the band corresponding to the suspected chloroplastic and/or mitochondrial DNA of *H. ostrearia*.

~~Regarding~~With respect to the biofilm, the observed similarities in bacterial community structures ~~were greater than~~exceeded 90% regardless of the geographic origin of the *H. ostrearia* isolates. The presence of a ~~band with~~ high ~~-~~intensity ~~(~~band (see arrows in Fig. 3) was ~~supposed~~assumed to ~~be~~represent chloroplastic and/or mitochondrial DNA from *H. ostrearia*. The resulting phylogenetic tree with and without this band ~~was~~remained unchanged.

For the water column, similarities ~~between~~among the ~~different~~various algal isolates were more variable and lower overall ~~lower~~ (e.g.~~,~~, only 40% between HO1-T and HO2-B ~~up~~, extending to 95% between HO2-B and HO2-T).

3.3. ~~-~~Metabolic fingerprinting differentiation of *Haslea ostrearia* isolates from La Barre de Monts and Isle of Ré ~~island~~

Two distinct *H. ostrearia* isolates were used for the metabolomic analysis, namely: HO-BM and HO-R. The number of total compounds was much lower in the so-~~-~~called "~~"~~axenic~~"~~" *H. ostrearia* cultures ~~accounting~~, which account for algal compounds (Fig. ~~4A)~~4a), compared to the non-axenic cultures encompassing both algal and bacterial compounds (Fig. ~~4B).~~ 4b).

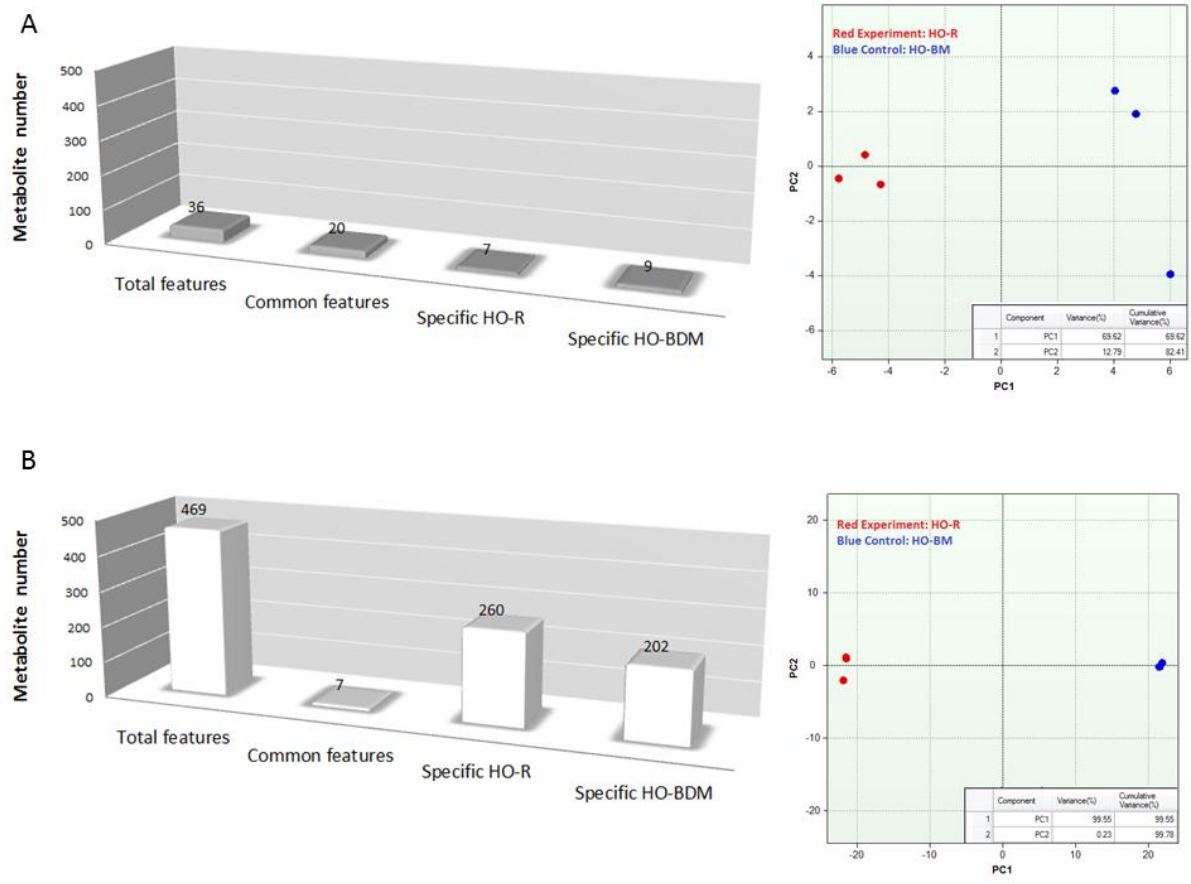

Fig. 4~~.~~: Number of compounds recovered from HO-BM and HO-R isolate cultures (left) and the related principal component analysis representations (right) (in considering $p < 0.05$ and fold-change > 1.2): ~~A~~a) axenic; and ~~B~~b) non-~~-~~axenic conditions. ~~"~~"Total~~"~~, ~~"~~", "common~~"~~" and ~~"~~"specific~~" means~~" refers to the total compounds recovered, compounds shared by both isolates and compounds specific to each ~~isolates~~isolate, respectively. ~~Analyses~~All analyses were performed in triplicate.

Axenic conditions ~~showed~~ were associated with a high number of common compounds (20 out of 36) and a ~~few numbers~~small number of compounds ~~were~~ specific to each isolate (7 and 9 for HO-R and HO-BM, respectively).

~~It~~The opposite was ~~shown the contrary~~exposed with non-axenic conditions, i.e~~.,~~, a low number of common compounds (7 out of 469~~) and~~), while 260 and 202 compounds were specific to HO-R and HO-BM, respectively.

~~Here~~In this instance, a Principal Component Analysis (PCA) approach ~~of~~to pattern recognition was used to distinguish ~~between~~the classes. ~~The data of~~Data from the control group (blue) and ~~the experiment~~experimental group (red) were processed~~,~~ and a data matrix ~~was gained. Figure 4 A, B showed~~generated. Figures 4a and b display the 2D PCA score plot of the control group and ~~the experiment~~experimental group ~~in~~under both axenic and non-axenic conditions. PCA analyses have confirmed that the differences in ~~the~~ metabolic profiling between HO-R and HO-BM ~~was~~were more significant ~~in~~under non-axenic conditions than ~~in~~ axenic conditions. The ~~points of~~ non-axenic model distribution points are indeed less dispersed (99% of the variance explained by ~~the~~ axis 1 of the PCA (PC1) ~~in~~under non-axenic conditions ~~against only~~vs. just 69% ~~in~~under axenic conditions).

3.4. ~~-~~Structure of the bacterial community from *Haslea ostrearia* isolates ~~at~~on two time~~-~~ scales

3.4.1. ~~On~~Over the course of ~~an~~one algal culture cycle

This experiment, performed in duplicate with two *H. ostrearia* isolates (HO-BM and HO-R~~)~~ ~~showed a~~), found perfect repeatability of the TTGE analysis (Fig. 5). At the ~~scale of a~~ culture cycle scale, a marked evolution ~~of~~in the bacterial community ~~was~~could be observed.

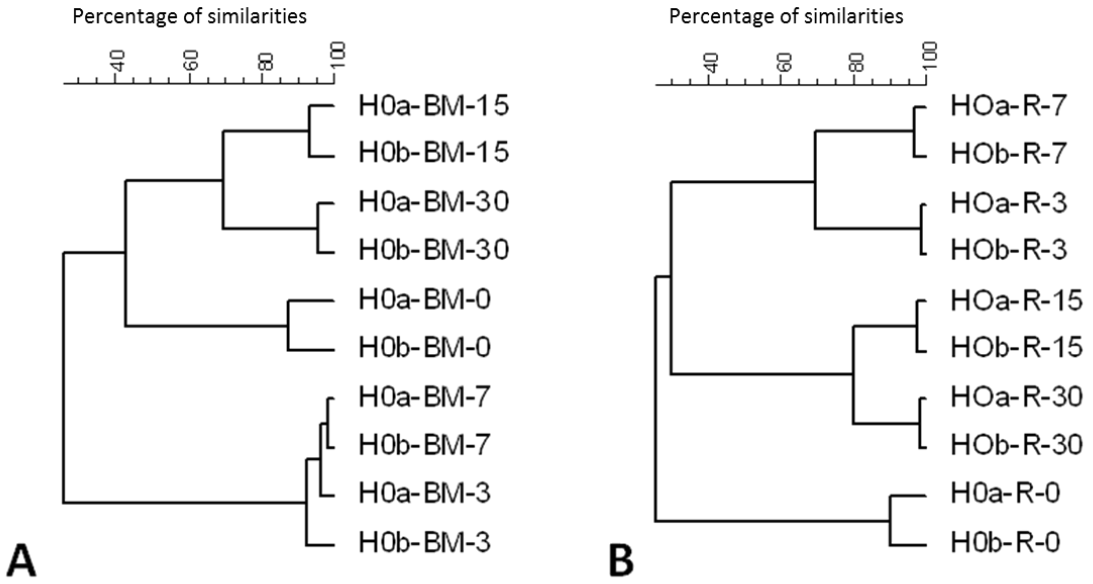

Fig. 5~~.~~: Structure of the global bacterial community (suspension cells and biofilm cells) during a growth cycle of two *Haslea ostrearia* isolates (at ~~day~~days 0, 3, 7, 15 and 30~~). A~~): a) HO-BM: La Barre-de-Monts, ~~B~~b) HO-R: Isle of Ré ~~island).~~. Letters a and b ~~are~~stand for the two experimental replicates ~~of the experiment~~.

Three ~~different~~distinct clusters ~~-~~emerged, all of which were related to the algal growth stage: day 0 (initial ~~structure of the~~ bacterial community structure), days 3 and 7 ~~days~~ (exponential phase), and days 15 and 30 ~~days~~ (stationary phase and decline phase).

3.4.2. Over a 9-~~months~~month serial subculturing campaign

Samples were recovered at the same growth stage, i.e~~.~~,. exponential ~~growth stage.~~. A ~~quite~~rather low similarity ~~of~~in the bacterial structure was observed between T0 and the subsequent subculturing, irrespective of the algal isolate (Fig. 6): 25% for HO-T, and 60% ~~of~~ similarity for HO-BM, HO-B and HO-R. ~~It shows~~This result demonstrates that once *H. ostrearia* was isolated and cultivated ~~in~~under laboratory conditions, the bacterial community structure ~~of the bacterial community has~~ evolved~~. But~~, but afterwards, i.e~~.~~,. from 3 ~~up~~ to 9

months, the bacterial community structure was fairly stable regardless of ~~the~~ isolate origin. Similarities ~~–~~in the bacterial community for each algal isolate during the 3- ~~up~~ to 9-month period of culture ~~were indeed greater than~~did in fact top 70%, with a maximum of 86% for HO-B.

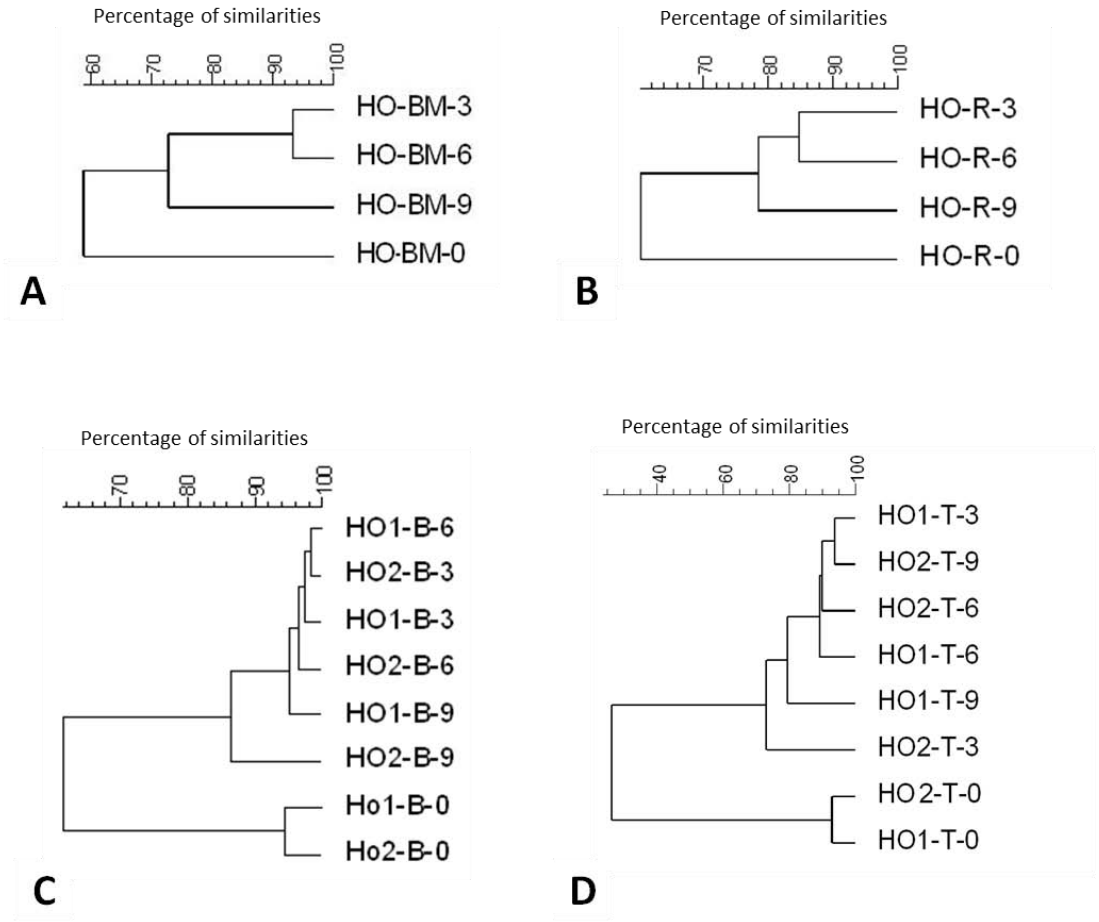

Fig. 6~~.~~: TTGE analysis of the global bacterial community structure of *Haslea ostrearia* at the time of isolation (T-0) and after 3, 6 and 9 months of cultivation (T-3, T-6~~,~~ and T-9, respectively). ~~A~~a) HO-BM: La Barre-de-Monts, ~~B~~b) HO-R: Isle of Ré ~~island, C~~, c) HO1-B and HO2-B: Bouin, ~~D~~d) HO1-T ~~et~~and HO2-T: La Tremblade.

For this pennate diatom, ~~at~~upon each cell division, the new valve is always formed within the parental theca, thus causing the average size of ~~the~~ frustules in the population to slowly decrease. The size reduction ~~of~~in the *H. ostrearia* frustule, measured over the 9-month

subculturing~~,~~ campaign (i.e~~.~~, about 28.6 ~~-~~% ± 2.6 ~~% (~~%, data not shown) ~~had~~exerted no influence on the bacterial community structure.

4. Discussion

4.1. ~~-~~Bacterial community structure of sediment samples from oyster ponds

Except for the HO-R isolate ~~displaying only~~, which displayed just a 15% similarity with other isolates, ~~high~~strong similarities ~~of the~~in bacterial ~~structure~~structures were ~~shown~~observed between isolates (> 85~~%) (~~%, Fig. 2~~)~~), while the sampling locations are separated by up to ~~a hundred kilometres apart~~100 kilometers (Fig. 1~~) but with similar~~), although their climatic conditions are similar. For HO-R, the ~~low similarity~~relative dissimilarity is most likely due to the ~~depth of the~~ sampling depth, which was higher ~~as~~ compared to the sampling conditions for other localities~~—~~: not only was the sediment that deposited on the bottom of oyster ~~pond was~~ponds collected, but ~~also~~so was some underlying sediment ~~making~~constituting the bed of the oyster pond (specific ~~physic-chemical~~physicochemical characteristics). We ~~then~~have thus postulated that the bacterial assemblage of the sediment deposited on the bottom ~~on~~of the ~~oyster pond~~ponds and the underlying ~~one~~sediment were distinct.

For other localities showing high levels of similarities, the specific ~~characteristic~~characteristics and functioning of the oyster ponds could explain these results. These ecosystems are indeed ~~ecosystems~~ managed by ~~humans~~human beings for ~~an~~ optimal oyster production. Nutrients (quality, amount) for oysters ~~but also~~, as well as for all living ~~being of the~~ oyster ~~ponds~~pond organisms including algae and bacteria, were most ~~probably~~likely to be similar from one pond to another, as ~~shown~~proven by Turpin ~~and~~

colleagueset al. [18] for oyster ponds ofin the same areas as those studied in the present study. herein.

Whether or not the oysters were present at the time of sampling or not (no oysters were found in the ponds of La Barre-de-Monts and Bouin-; oysters were present in the pondponds of La Tremblade and Isle of Ré Island –) did not seem to influence on the structure of the bacterial community. The structure. Since the pond management history beingis an unknown, it canmay be assumed that empty ponds were filled with oysters shortly before the samplingssampling campaign and moreover that the composition of the seawater composition in these ponds remained unchanged in the meantime.-.

4.2. -Community structure of bacteria recovered from the biofilm and suspended bacteria from the water column after *Haslea ostrearia* isolation

Once *H. ostrearia* washad been isolated from the sedimentssediment samples of the differentvarious localities, it iswas reasonable to assume that the specific biotope of each locality no longer hadexerted an effect. Indeed, *H. ostrearia* isolates were further cultivated inunder the same controlled conditions (i.e. culture medium, light, temperature).

Compared to the structure of the bacterial community structure in the bulk sediment, the structure relatedrelative to the phycosphere of *H. ostrearia*, i.e.-,, epiphytic bacteria and those entrappedembedded in the biofilm, was very distinct with cross-sample similarities between samples that did not exceedexceeding 10% (data not shown). This result is usuallytypically observed in higher plants whenwhere the bacteria of the rhizosphere are compared to those of the bulk soil [36]. Regarding the TTGE pattern band numbers of TTGE patterns, it was surprising to note that they were higher for the biofilm samples than for the bulk sediment samples. On the contrary, Schäfer and colleagueset al. [24] observed on the contrary that the bacterial biodiversity of the phycosphere was lowlimited when compared to the complexity of

bacterial assemblages in bulk seawater. Nonetheless ~~different~~, various results were shown when ~~bacteria of~~ the rhizosphere bacteria were compared to ~~those of~~ bulk soil bacteria [36]. In our case, it can be ~~supposed~~assumed that the extracellular compounds of *H. ostrearia* are sufficiently diversified to support the growth of various bacterial populations. At least 36 (~~up to~~out of 469) compounds were indeed detected (Fig. 4) depending on whether *H. ostrearia* was axenic or not.

Irrespective of the geographic origin of the *H. ostrearia* samples, the bacterial structure of the biofilm samples was found to be similar (Fig. 3), including the HO-R isolate, while the bacterial structure of the sediment samples for this locality ~~strongly~~ differed markedly (Fig. 2). This result shows that the bacterial structure of the phycosphere is specific to *H. ostrearia*. ~~It was~~; it has indeed been demonstrated that the structure of the bacterial community ~~linked to~~associated with the microalgal strain was specific to the microalgae [20, 24, 27]. For HO-R, ~~we~~it could then be concluded that the particular bacterial structure of the sample recovered from the sediment was ~~due~~ solely ~~to the characteristic of~~influenced by the biotope characteristics, i.e. ~~,~~, the specific ~~characteristics of the~~ sediment ~~.~~ characteristics.

The significant differences in the ~~structures of the~~ bacterial community structures between ~~the bacteria of the~~ phycosphere bacteria and ~~those from the~~ bulk water column bacteria (15% similarity between the two groups, Fig. 3) ~~confirms~~confirm the ~~major~~significant effect of *H ostrearia* in ~~the establishment of~~establishing a specific community hosted by the phycosphere. The highest level of bacterial ~~structures similarities~~structure similarity observed between biofilm samples (> 90~~%)~~%), as compared to 40% ~~up to~~ 90% for the suspended bacteria of the same samples ~~showed~~, has also revealed the predominant ~~part~~role of *H. ostrearia* in ~~the establishment of~~establishing a bacterial structure specific to this species. ~~Indeed the~~The nutrient composition of *H. ostrearia* biofilms was ~~most probably~~, in all likelihood, very different ~~to~~from that of seawater, as the result of biofilm enrichment by algal exudates. The

specificity of the bacterial community, with respect to ~~the~~ algae in the environment where bacteria live, was ~~already shown~~previously presented by Eigemann ~~and colleagues~~*et al.* [37~~]~~], who demonstrated that the influence of the algal host ~~overruled~~dominated the effect of changes in environmental conditions. Liu ~~and colleagues~~*et al.* [38] ~~demonstrated~~showed that the phytoplankton community succession ~~influenced~~influences changes in bacterial community composition. In the specific case of toxin production ~~of toxins such as~~(like domoic acid) by algae, ~~it has been shown by~~ Sison-Mangus ~~and colleagues~~*et al.* [39] reported that the three tested ~~species of~~ *Pseudo-nitzschia* species, which vary in toxin production, had phylogenetically distinct bacterial communities~~,~~ and, moreover, that toxic *Pseudo-nitzschia* ~~had lower~~exhibited less microbial diversity than non-toxic *Pseudo-nitzschia*. Bruckner ~~and colleagues~~*et al.* [40] ~~indicated~~suggested an adaptation of Proteobacteria and Bacteroidetes ~~to~~in the microenvironment created by the diatom biofilm. For the suspended bacteria in the water column, the ~~various~~varying geographic origins of the ~~samplings, most~~samples, which very probably ~~showing~~reveal differences~~,~~ - even slight~~,~~ - in biotope characteristics, ~~most~~ likely ~~explained~~explains the differences in ~~the~~ bacterial structures ~~as~~given that they were not under the direct influence of *H. ostrearia*.

Regarding the two isolates of La Tremblade (HO1-T and HO2-T), the ~~low~~limited similarity of the suspended bacterial ~~cells~~cell structures (40%, Fig. 3) ~~could~~might be the result of initially different bacterial structures ~~which~~that did not converge after *H. ostrearia* isolation ~~contrary to~~, in contrast with what was observed for the biofilm (> 95%) ~~as the result of~~due to the considerable influence of exudation.

4.3. ~~-~~Metabolic profiles of HO-R and HO-BM

One of the ~~highest~~greatest differences in the ~~structure of the~~ bacterial community structure was shown for HO-BM and HO-R isolates ~~for~~in both the biofilm and ~~the~~ water column (Fig.

3~~) and can~~); this finding may be explained by ~~the~~ differences in the metabolic profiles (Fig. 4~~)~~ ~~. Indeed~~). The axenic cultures of *H. ostrearia* ~~displayed~~indeed display distinguishable metabolomes (Fig. ~~4A), although not very different~~ 4a), though their differences remain minimal: 20 compounds in common out of 36~~—~~, which is not surprising since the biometric analysis of the isolates by scanning electron microscopy (SEM), based on the density of transapical and longitudinal striations of the frustule ~~showed~~, indicates that they belong to the *H. ostrearia* species.

~~.~~—To access the bacterial metabolom, it ~~could~~might have been tempting to subtract the number of compounds of the axenic treatment (Fig. ~~4A~~4a) from the number of the xenic compounds (Fig. ~~4B). But one should~~4b). Extreme care must be ~~very careful~~exercised however since some bacteria-microalgae interactions cannot be excluded. Bacteria and microalgae ~~indeed do not have~~ probably ~~same~~exhibit different metabolic profiles depending on whether *H. ostrearia* is cultivated as a monoculture or in association with the ~~bacteria of the~~ phycosphere~~. Yet it~~ bacteria. It should still be noted that the metabolic profile of the bacteria-*H. ostrearia* association is very specific ~~of~~to HO-R and HO-BM, with ~~only~~just 7 common compounds out of 469.

It cannot be ~~excluded~~overlooked that a few compounds were not those produced by the alga and/or ~~the~~ bacteria, but ~~those~~instead compounds of other organisms since the culture medium (ES 1/3) consists of filtered (to 0.2 µm) seawater enriched with minerals. We have assumed that ~~the~~medium sterilization ~~of the medium~~ degraded ~~almost~~nearly all of the remaining compounds. The seawater used ~~for the preparation of~~to prepare the culture medium was the same for ~~the cultivation of~~cultivating the two isolates, ~~then~~thus avoiding any bias resulting ~~of~~from possible ~~geographical~~geographic differences.

4.4. ~~-~~Structures of the bacterial community from *Haslea ostrearia* isolates ~~at~~on two time~~-~~ scales

At the ~~scale of a~~ culture cycle ~~(ea~~scale (i.e. one ~~up~~ to two weeks), the changes in ~~the structure of the~~ bacterial community structure (Fig. 5) were related to the ~~different~~ various growth stages of ~~the growth of~~ *H. ostrearia*, i.e~~,~~ lag time, exponential growth stage~~,~~ and stationary stage, most ~~probably~~likely as a result of exudates in ~~various amounts~~varying quantities and compositions. This phenomenon is well documented for bacteria growing in the rhizosphere of plants [41]. At the seasonal time~~-~~ scale~~ of the season~~, Liu ~~and colleagues~~*et al.* [38] demonstrated that phytoplankton community succession ~~influenced the composition of~~influences the bacterial community~~.~~ composition.

The change in ~~the structure of the~~ bacterial community structure is ~~cyclic~~cyclical. When *H. ostrearia* was sampled at the same (exponential) growth stage ~~(exponential stage) during~~over a nine-month period (~~*c.a.*~~,i.e. 30 ~~subculturing), the structure of~~subculturings), the bacterial community structure remained ~~rather~~quite stable (75% ~~up~~ to 85% similarity between 3 and 9 months~~) (~~, see Fig. 6). It can be assumed that the ~~whole~~entire bacterial community associated ~~to~~with *H. ostrearia* (~~entrapped~~embedded, epiphytic and free bacteria) was transmitted at each subculturing. The decrease in the frustule size of diatoms, already ~~shown~~witnessed for *H. ostrearia* [42], did not ~~impact~~alter the bacterial structure ~~although~~even though the algal size of some isolates at the end of the experiment (between 52 and 82 µm, with an average ~~at~~of 61~~,~~2 µm) was in the range for auxosporulation (i.e. between 50 and ~~68µm~~68 µm [43, 44]).

Between the first subculturing (T-0) and the third month, the bacterial structure changed significantly (25% ~~up~~ to 60% ~~of~~ similarity) as the result of the ~~adaptation of the~~ bacterial community adapting to the maintenance culture conditions with ~~a~~ stabilization after 3 months due to the well-controlled conditions for the culture of ~~the~~ *H. ostrearia* isolates. This finding suggests that the laboratory conditions ~~of~~for *H. ostrearia* (culture medium composition,

temperature, light) also influence the composition of the bacterial assemblage, as demonstrated by Sapp ~~and colleagues~~*et al.* [45~~]. These~~]; these authors ~~showed~~found a shift in the bacterial populations associated with diatoms between isolation and cultivation of algal cells, with an increase in the number of phylotypes belonging to ~~members of~~ *Gammaproteobacteria* members.

5. Conclusion

For the first time, this study has analyzed the bacterial ecosystem surrounding the marine diatom *H. ostrearia* and showed that ~~the~~this bacterial structure is specific to the geographic origin of the microalgal isolate. ~~In~~Under laboratory conditions, ~~after~~once *H. ostrearia* ~~isolation~~has been isolated from oyster ponds, the ~~structure of the~~ bacterial community structure was shown to be resilient over a 9-month subculturing ~~although the structure of the bacterial community~~despite structural changes at the culture time scale ~~of a culture depending on~~according to the growth stage. ~~Likewise~~Similarly, the differences in ~~the~~ bacterial structures of two *H. ostrearia* isolates (HO-R and HO-BM) ~~resulted in~~gave rise to specific ~~metabolomics~~metabolomic profiles. These profiles were more distinct with non-axenic microalgae, i.e~~., including~~ . with inclusion of their ~~associating~~associated bacteria, than with axenic ~~ones,~~microalgae, thus suggesting reciprocal relationships between bacteria and *H. ostrearia* cells.

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Acknowledgments

This study was carried out under the COSELMAR project supported by IFREMER and Nantes University and co-funded by the Regional Council of the “Pays de la Loire”. The authors thank la Roche-sur-Yon Agglomération for its participation to finance bead beater. We thank Thomas Glauner, Maxime Grives, John Lee and Thierry Faye of Agilent Technologies for their collaboration on the Agilent Q-ToF technology and software. We are also grateful to Romain Gastineau for COI1 and COI6 primers, Clovis Genuy and Thierry Jauffrais for sampling from La Barre-de-Monts and Ré island, and Astrid Lerouxel for the map with *H. ostrearia* localities.

Figure

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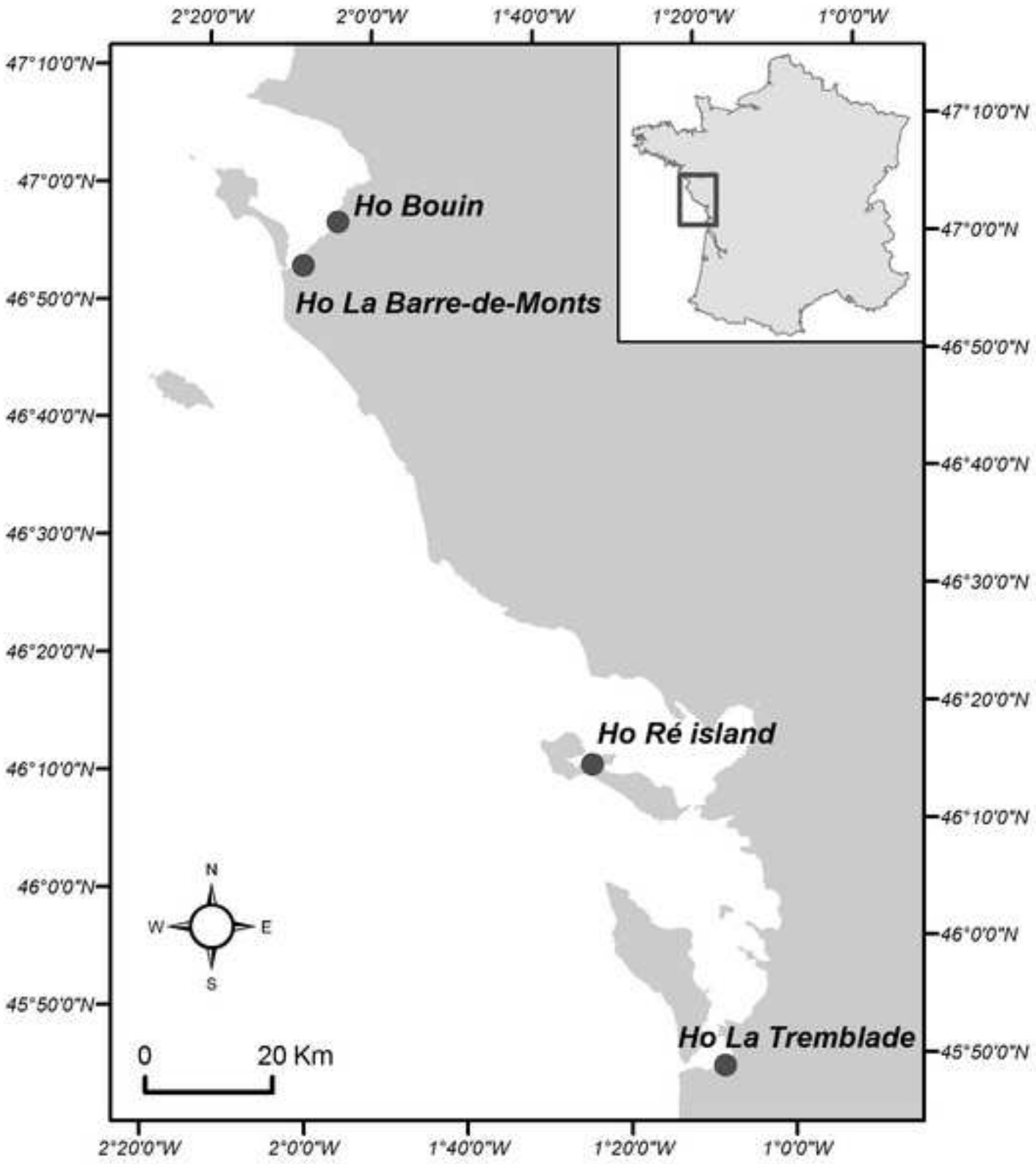

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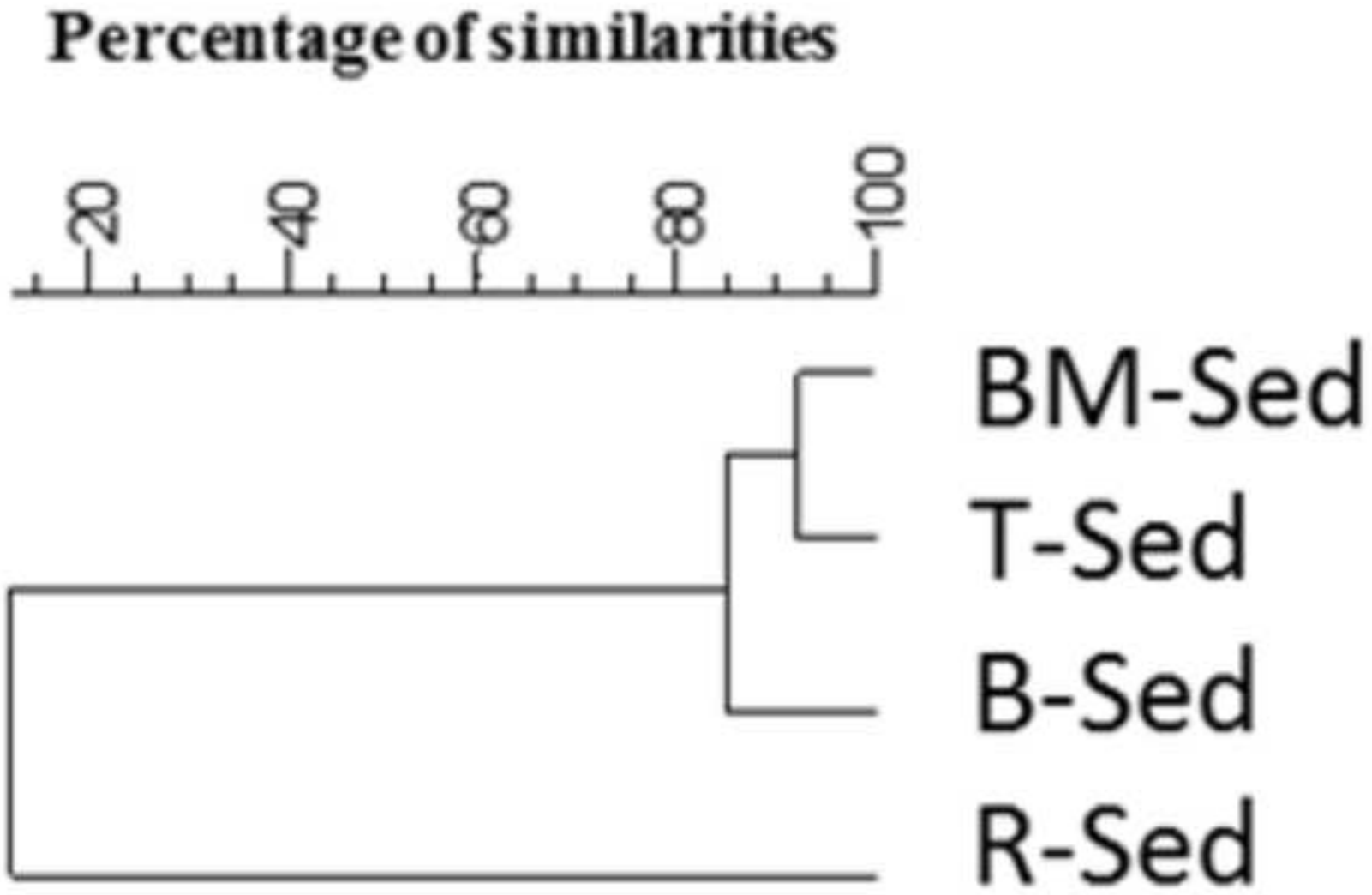

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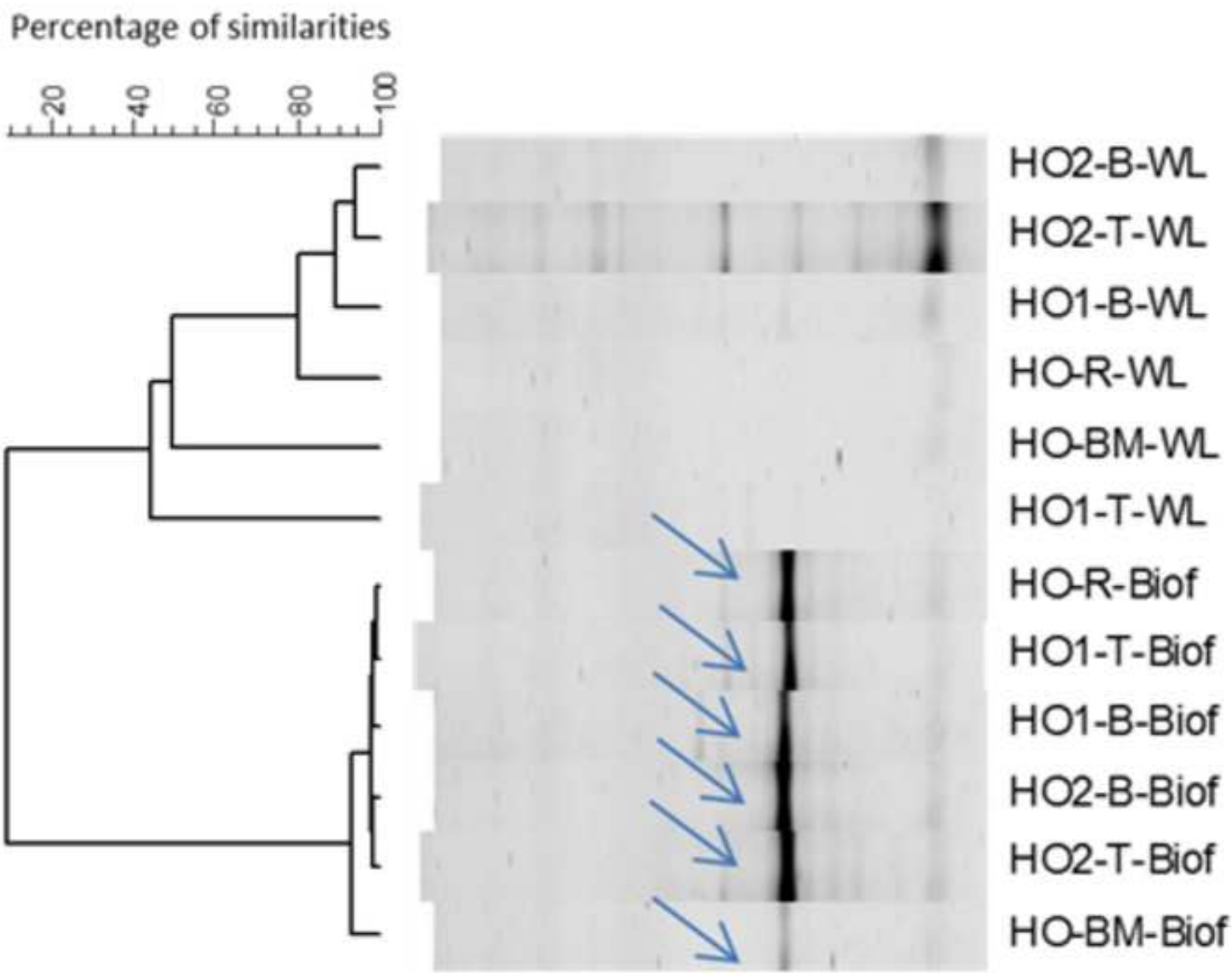

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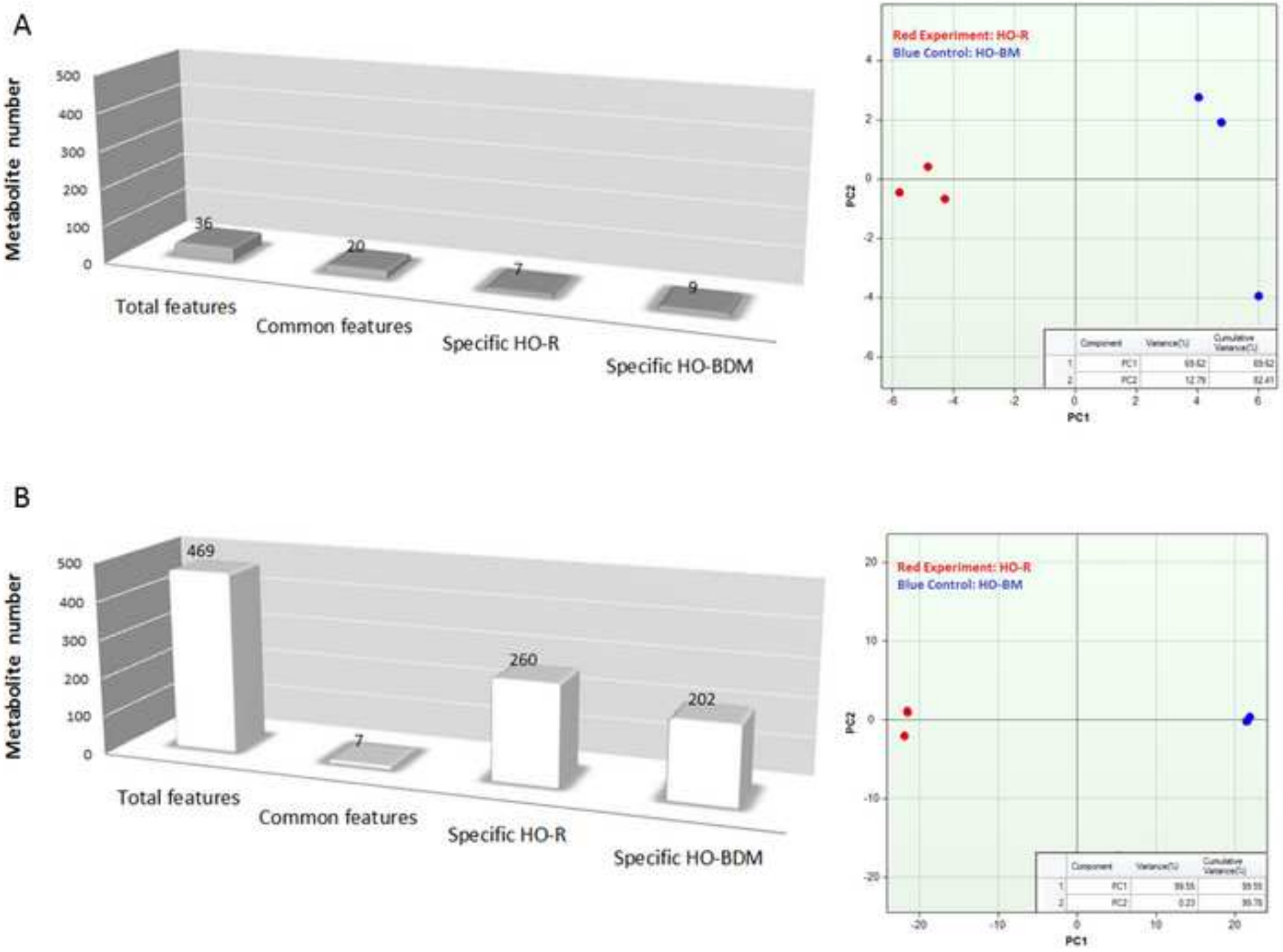

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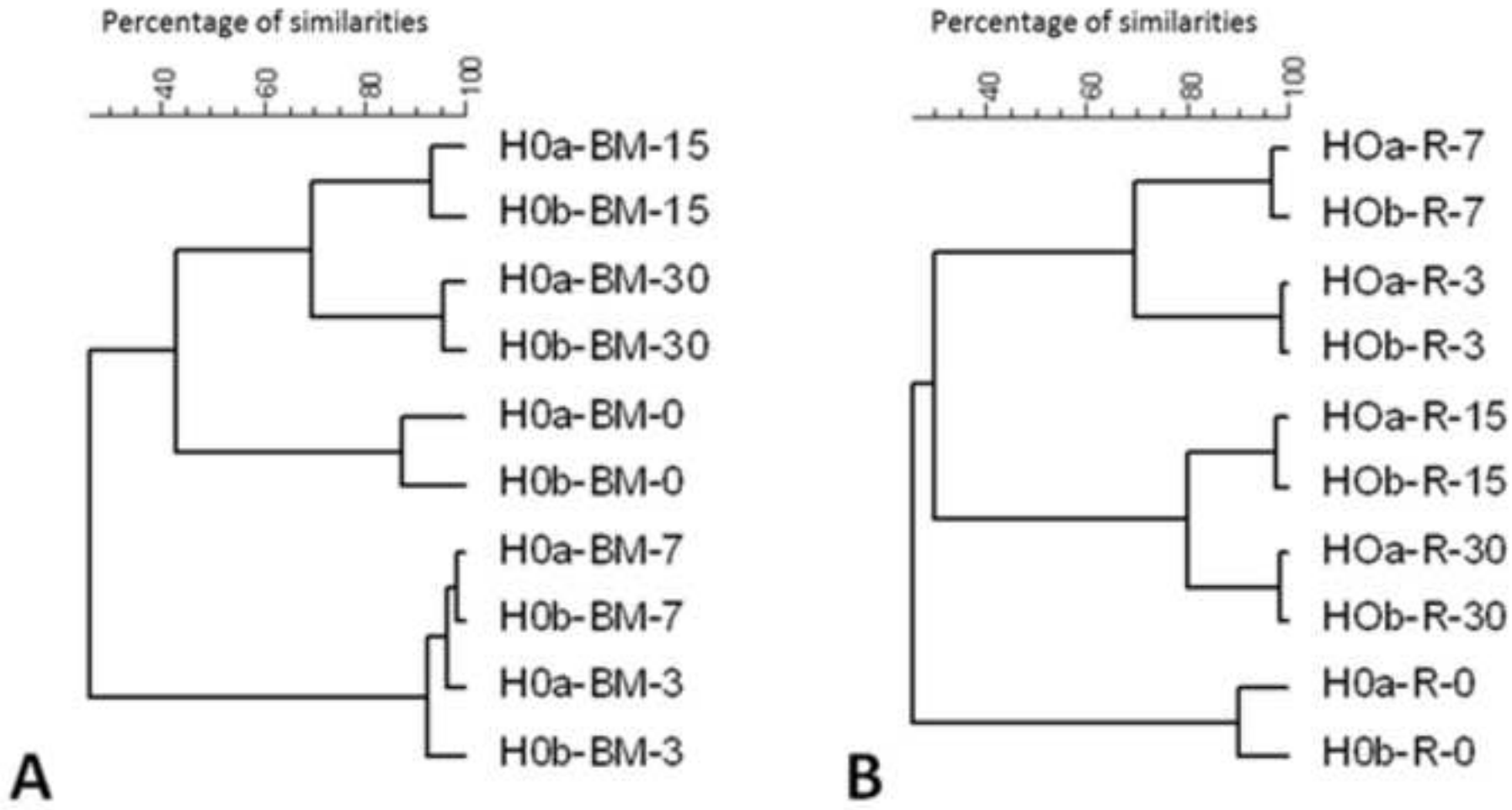

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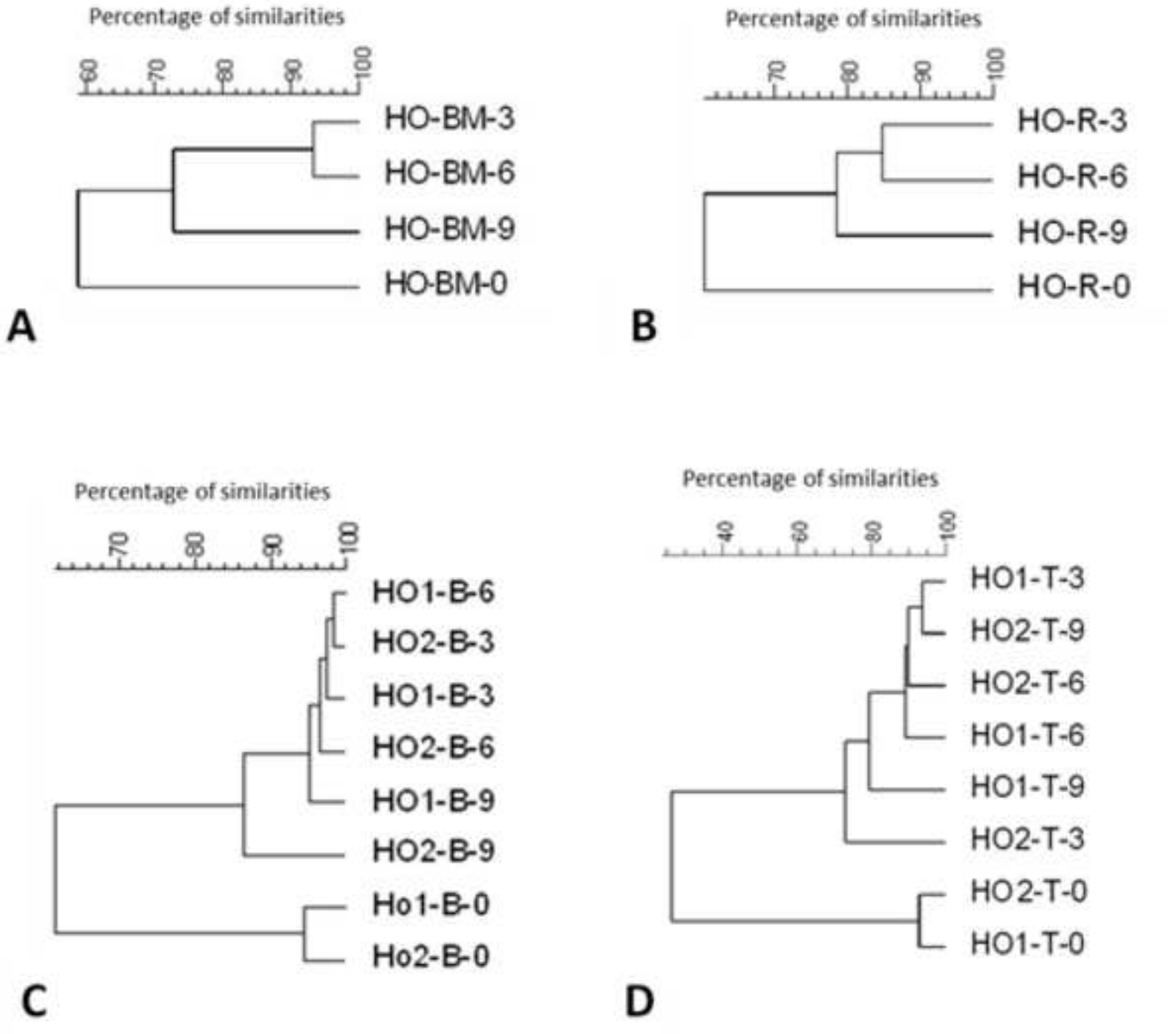

Highlights:

The bacterial structures (TTGE) of *H. ostrearia* biofilm and bulk sediment are distinct.

A bacterial structure varies with the geographic origin of *H. ostrearia*.

Metabolic profiles are more distinct for bacteria-algae than for algal monocultures.

The bacterial structure of the phycosphere is specific to the algal growth stage.

The bacterial structure of the phycosphere remains unchanged over a 9-month subculturing.

Bacterial community structure of the marine diatom *Haslea ostrearia*

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Keywords: biofilm, ecology, metabolic fingerprinting, microalgae, phycosphere, TTGE.

Abstract

Haslea ostrearia produces a water-soluble, blue-green pigment, called marennine, with proven economic benefits (as a bioactive compound used to green oysters, which improves their market value). Incomplete knowledge of the ecological features of this marine diatom complicates its cultivation. More specifically, the ecology of bacteria surrounding *H. ostrearia* in ponds is what remains unknown. The structure of this bacterial community was previously analyzed by means of PCR-TTGE before and after isolating *H. ostrearia* cells recovered from

4 localities in order to distinguish the relative parts of the biotope and biocenose and to describe the temporal dynamics of the bacterial community structure at two time scales (2 weeks vs. 9 months). The bacterial structure of the phycosphere differed strongly from that of bulk sediment. The level of similarity between bacteria recovered from the biofilm and suspended bacteria did not exceed 10%. On the other hand, similarities among the bacterial community structures in biofilms were above 90% regardless of the geographic origin of the algal isolates, while the percentages were lower for suspended bacteria. The differences in bacterial structures of two *H. ostrearia* isolates (HO-R and HO-BM) resulted in specific metabolomic profiles. The non-targeted metabolomic investigation revealed more distinct profiles in the case of this bacteria-alga association than for the *H. ostrearia* monoculture. At the culture cycle scale under laboratory conditions, the bacterial community depended on the growth stage. When *H. ostrearia* was subcultured for 9 months, a shift in the bacterial structure was observed as of 3 months, with the bacterial structure stabilizing afterwards (70%-86% similarities), in spite of the size reduction of the *H. ostrearia* frustule. Based on these results, an initial insight into the relationships between *H. ostrearia* and its surrounding bacteria could be drawn, leading to a better understanding of the ecological feature of this marine diatom.

1. Introduction

Haslea ostrearia is a cosmopolitan species of diatoms commonly found on the French Atlantic coast, especially in oyster ponds of the Bay of Marennes-Oléron and Bay of Bourgneuf [1]. This diatom has long been the subject of curiosity [2] and became a topic of investigations due to its water-soluble, blue-green pigment, called marennine, which is responsible for the greening of oysters. This blue-green pigment, produced when *H. ostrearia*

blooms, is released into the seawater, at which point the ponds turn green. At this stage, *H. ostrearia* is the dominant diatom species in ponds, and oyster farmers take advantage of this phenomenon by immersing their oysters in these shallow waters for 'refinement' (fattening) and greening, since these last two stages of raising oysters guarantee product quality and improve a farm's profits. Beyond its benefit in aquaculture for greening oysters [3], marennine has been shown to possess several biological functions with potential biotechnological applications, namely: i) antibacterial, anticoagulant and antiviral activities [4, 5]; ii) antioxidant activity [6]; 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). Until now however, an incomplete knowledge of the ecological conditions under which this microalgae develops in its natural ecosystem has complicated controlling the cultivation of *H. ostrearia*, even though dedicated photobioreactors adapted to the physiological specificity of this microalga - through the use of artificially immobilized cells - were designed at the laboratory scale [7-10]. This microalga indeed exhibits several types of behavior, primarily benthic, occasionally planktonic but also epiphytic [11, 12], thus making immobilization a relevant course of action. The ecophysiology of *H. ostrearia* is complex and moreover not yet completely understood. In oyster ponds, *H. ostrearia* can outcompete other microalgae yet is also being consumed by oysters [13-15]. The *H. ostrearia* biotope has also been studied; it was demonstrated that: i) this diatom is extremely tolerant to high irradiance (thus offering an ecological advantage over the other main diatoms encountered in oyster ponds, e.g. *Skeletonema costatum* [16, 17]); and ii) the greening phenomenon is controlled by the nutrient composition in oyster-pond waters (see [18] for conditions). Recently, a more detailed genetic characterization of *H. ostrearia* was undertaken by developing genetic molecular tools, which led to identifying new strains of *H. ostrearia* [19] along with a second species of blue diatom called *Haslea karadagensis*.

Surprisingly, only a few studies have focused on bacteria-microalgae interactions, in noting that some bacteria may increase the microalgal biomass while offering potential applications in aquaculture. For now, nothing is still known for the specific case of *H. ostrearia*, yet for instances regarding bacteria and diatoms overall, "they have co-occurred in common habitats throughout the oceans for more than 200 million years, fostering interactions between these two groups over evolutionary time scales" [20]. The link between bacterioplankton and phytoplankton dynamics was recently demonstrated by Rooney-Varga *et al.* [21]. The habitat of phytoplankton-associated bacteria has been depicted by the concept of "phycosphere", i.e. the area around algal cells where bacteria feed on extracellular products of the algae [22]. The phycosphere is thus the aquatic analog of the rhizosphere in soil ecosystems and has direct implications for nutrient fluxes to and from algal cells. Bacteria-microalgae interactions have been studied for several diatom species, including *Guinardia delicatula*, *Pseudonitizschia pugens*, *Thalassiosira rotula*, *Skeletonema costatum* [23], *Ditylum sp.*, *Thalassiosira sp.*, *Asterionella sp., Chaetoceros sp., Leptocylindrus sp., Coscinodiscus sp.* [24], *Pseudo-nitzschia multiseries* [25] and *Nitzschia microcephala* [26]. Some of these common species are frequently encountered in oyster-pond waters and sediments [11, 18]. On the whole, the bacterial biodiversity of the phycosphere was shown to be limited in comparison to the complexity of bacterial assemblages in bulk seawater [24]. The structure of the bacterial community related to microalgae is specific to the microalgae species [20, 24, 27], though some bacterial phylotypes, such as bacteroides, are known to play a significant role in nutrient cycling by degrading algal macromolecules; moreover, such species attach to growth and are then recovered in most phycospheres [28]. To demonstrate this specific bacterial-algal interaction, Schäfer *et al.* [24] attempted, using two algal cultures, to associate each culture with the "satellite" bacterial assemblage of the other culture and proved such an association infeasible.

Based on these considerations, i.e. an incomplete knowledge of the ecological features of *H. ostrearia*, the present work has been intended to: i) characterize the structure of the bacterial community by means of PCR-TTGE both before and after *H. ostrearia* isolation from oyster ponds in different localities; ii) compare the bacterial community of the *H. ostrearia* phycosphere vs. free cells within the culture medium; iii) distinguish the relative portion of the biotope and biocenose based on the bacterial structure composition; and iv) describe the temporal dynamics of the bacterial community structure at the time scale of one culture cycle under laboratory conditions and after several subculturing steps. A metabolic fingerprinting (untargeted approach) was aimed at assessing the global metabolic profile of *H. ostrearia* cultures, whether or not associated with the phycosphere bacteria. Additional clarifications on the bacteria-*H. ostrearia* associations were provided, as well as on the role of the geographic origin of *H. ostrearia*. The compound class or classes potentially affected under these study conditions were not anticipated; moreover, specific compounds were not necessarily identified or quantified.

2. Materials and methods

2.1. Sampling location

The test samples were collected in oyster ponds from four localities along the French Atlantic coast, along the following north-to-south gradient: Bouin (46.96°N; 2.04°W), La Barre-de-Monts (46.90 N; 2.11°W), Isle of Ré (46.22 N; 1.45°W), and La Tremblade (45.80 N; 1.15°W) (see Fig. 1). One liter of each sample was collected at the seawater-sediment interface on the bottom of the oyster ponds in order to obtain both sediment and seawater. The samples were immediately stored at 4°C. In the laboratory, the presence of *H. ostrearia* was verified before isolation.

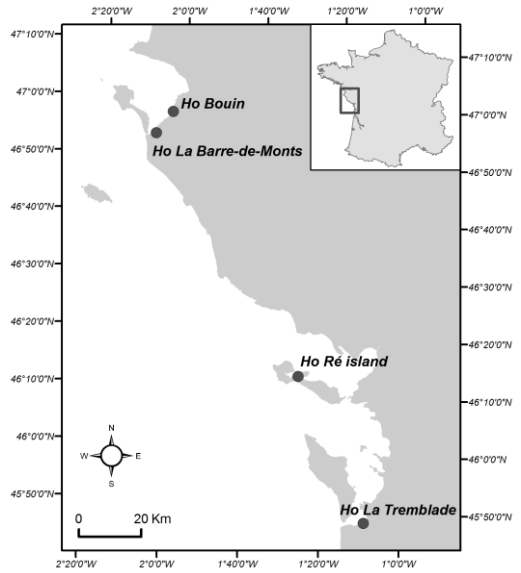


Fig. 1: Map of the French Atlantic coast showing oyster-pond locations where samples were collected to isolate *Haslea ostrearia*: Bouin (46.96°N; 2.04°W), La Barre-de-Monts (46.90 N; 2.11°W), Isle of Ré (46.22 N; 1.45° W), and La Tremblade (45.80 N; 1.15°W).

2.2. Isolation of *H. ostrearia* from environmental samples and cultivation

Monospecific cultures of *H. ostrearia* were obtained by isolating a single cell of *H. ostrearia* from the raw samples. The specimen was recovered using a capillary pipette and an inverted microscope; it was then washed by successive cell subculturing in filtered seawater (0.22 µm) to remove contaminants (e.g. bacteria, other microalga, flagellate, larvae). Among the *H. ostrearia* isolates, six were selected for the following studies: HO1 and HO2 Bouin (HO1-B and HO2-B), HO La Barre-de-Monts (HO-BM), HO Isle of Ré (HO-R), and HO1 and HO2 La Tremblade (HO1-T and HO2-T).

For the ensuing experiments, the aforementioned isolates were grown in 250 mL-Erlenmeyer flasks filled with 150 ml of the modified Provasoli [29] medium (ES1/3: [30]) to obtain sufficient biomass. The monospecific isolates of *H. ostrearia* were transferred during the

144 exponential growth stage (every 7-10 days) into a fresh ES 1/3 medium. The cultures were
145 incubated in a culture chamber at 16°C under 120 µmol photons m^{-2} s^{-1} and a 14:10 h light :
146 dark regime.
147
148 **2.3. Algal fingerprints and the bacterial community structure**
149 **2.3.1. Sample preparation**
150 ***Raw sediments:*** The seawater and sediment of the raw samples recovered in the oyster ponds
151 were separated by overnight sedimentation in a culture chamber at 16°C. The samples were
152 then frozen at -20°C. 0.5 g of sediment was used for DNA extraction purposes.
153 ***Supernatant and biofilm from monospecific H. ostrearia cultures:*** To compare the bacterial
154 community structure in the algal biofilm, which entails comparing the bacteria embedded in
155 exopolysaccharides forming biofilm and epiphytic bacteria of *H. ostrearia* with that of the
156 suspended cells in the culture medium, the biofilm and supernatant from the culture of *H.*
157 *ostrearia* were separated. From cultures during the exponential growth stage in 250-mL
158 Erlenmeyer flasks filled with 150 mL of ES 1/3 medium, 100 mL of the liquid - attached at
159 the bottom of the Erlenmeyer flasks - were carefully collected to avoid contact with the
160 biofilm, and the few free alga (possibly associated with bacteria) were removed by
161 centrifugation (SIGMA 3K30 Fisher Bioblock Scientific: 900 g, 90 s, 16°C) to ensure
162 recovering in the supernatant just the bacteria in suspension within the culture medium. The
163 supernatant was filtrated through a 0.22-µm filter (cellulose nitrate membrane, Sartorius) so
164 as to concentrate the bacteria on the filter, which was then frozen at -20°C. Before DNA
165 extraction, each filter was cut into small pieces of about 4 mm^2.
166 From this same culture, the remaining 50 mL were eliminated, and 30 mL of fresh ES 1/3
167 medium were added to the Erlenmeyer flask; the algal biofilm was recovered by means of the
168 mechanical action of a sterile bar magnet. Microalgae and bacteria (epiphytic and those

169 embedded in the biofilm) were both recovered by centrifugation (SIGMA 3K30 Fisher
170 Bioblock Scientific: 6000 g, 5 min, 16°C), and the samples were frozen at -20°C prior to
171 DNA extraction.
172 ***Cultures of H. ostrearia at various growth stages and generations:*** For both experiments,
173 approx. $1.5 \cdot 10^6$ algal cells were collected once the culture had been homogenized and then
174 centrifuged (Universal 320 Hettich: 6000 g, 10 min, 16 °C); the pellets containing suspended
175 cells and cells of the biofilm were frozen at -20°C prior to DNA extraction.
176 The bacterial community structures of the HO-BM and HO-R isolates were studied at the time
177 scale of one culture cycle. Samples were collected at the time of transferring *H. ostrearia* into
178 the fresh ES 1/3 medium after being isolated and cultured in the laboratory for one year (Day
179 0) and again after another 3, 7, 15 and 30 days.
180 The bacterial community structures of the HO-BM, HO-R, HO1-B, HO2-B, HO1-T and HO2-
181 T isolates were also studied at the time scale of various subculturings of *H. ostrearia*: T0,
182 T+3, T+6 and T+9 months following *H. ostrearia* isolation. At each of these times, a
183 biometric measurement (cell length) was conducted on 90-200 algal cells using an Olympus
184 AX70 PROVIS microscope; final determination relied on the LUCIA G software.
185 ***Axenic and non-axenic H. ostrearia cultures for metabolomic profiling:*** HO-BM and HO-R
186 were cultivated in 250-mL Erlenmeyer flasks. The cells forming a biofilm at the bottom of the
187 vessel were recovered during the exponential growth stage after being re-suspended with a
188 sterile bar magnet, and homogenized and counted using a Nageotte chamber. Microalgae were
189 then inoculated at $3 \cdot 10^3$ cells mL^{-1} (two replicates) in 100-mL Erlenmeyer flasks containing
190 fresh ES 1/3 medium and an antibiotic antimycotic solution (10,000 units penicillin, 10 mg of
191 streptomycin and 25 µg of amphotericin B mL^{-1}, BioReagent, A5955 SIGMA) diluted at 1:50
192 or 1:100. The cultures were incubated in a culture chamber at 16°C under 120 µmol photons
193 m^{-2} s^{-1} and a 14:10 h light : dark regime. Treatment was carried out for 7 days, subsequent to

which the culture supernatant was removed and the algal biofilm washed once with fresh ES 1/3 medium to eliminate the antibiotics. The cells were re-suspended with a sterile bar magnet in 25-30 mL of fresh ES 1/3 medium, and 5 mL were transferred into 250-mL Erlenmeyer flasks filled with 150 mL of fresh ES 1/3 medium (triplicate). After 7 days of culture without antibiotic treatment, a second 7-day treatment was conducted with the same antibiotic antimycotic solution diluted to 1:50, followed by 7 days of culture without treatment. After the second treatment, the bacterial concentration was drastically reduced compared to the non-axenic cultures. These cultures were thus called "axenic". Cells were collected during the exponential growth stage: 200 µL of the supernatant of HO cultures were collected, filtered on 0.20-µm PTFE membrane filters (Interchim) and frozen at -80°C prior to fingerprint acquisition. For non-axenic *H. ostrearia* cultures, from the stock cultures of HO-BM and HO-R during the exponential growth stage in 250-mL Erlenmeyer flasks, microalgae were inoculated in triplicate in 24 well plates at $3 \cdot 10^3$ cells mL^{-1} to monitor daily algal growth by measuring the fluorescence of chlorophyll (BMG LabTech: 440; 680 nm). Microalga were incubated under the same conditions as for the axenic *H. ostrearia* cultures. After 6 days of culture (during the exponential growth stage), 200 µL of the culture supernatant were collected, filtered on 0.20-µm PTFE membrane filters (Interchim) and frozen at -80°C prior to fingerprint acquisition.

2.3.2. Bacterial DNA extractions

DNA from raw sediments, as well as from supernatant derived from monospecific *H. ostrearia* cultures and *H. ostrearia* cultures at different growth stages and generations, was extracted using a NucleoSpin™ soil kit (Macherey-Nagel, GmbH & Co., Germany). In the specific case of the supernatant of *H. ostrearia* cultures at different growth stages and generations, the initial steps of sample grinding were performed with an MM400 Bead Beater

(Retsch Germany) (3× 30 sec, 25 Hz). For biofilms, DNA was extracted using a NucleoSpin® Tissue kit (Macherey-Nagel, GmbH & Co., Germany). The first grinding step was executed with the Bead Beater (1 min, 25 Hz), then a pre-lysing step that included grinding (56°C, 105 min; grinding, 10 sec, 25 Hz every 30 min) was applied according to manufacturer's recommendations; DNA concentrations were also measured (SPECTROstar Nano, BMG LABTECH LVi Plate, Germany).

2.3.3. PCR amplification

The V3 region of the 16S rRNA gene was amplified using the 357F-GC and 518R primers [31]. The PCR reaction mixture contained 0.6 $ng.\mu L^{-1}$ of DNA template, 0.1 $\mu mol.L^{-1}$ of each primer, 200 $\mu mol.L^{-1}$ of dNTP, 0.012 $unit.\mu L^{-1}$ of Taq polymerase 1× reaction buffer, 2.5 $mmol.L^{-1}$ of $MgCl_2$, and 500 $ng.\mu L^{-1}$ of bovine serum albumin and 5% (v/v) of dimethylsulfoxide introduced into a 50-µL final reaction volume. The following cycling conditions were used for bacterial amplification: 1 cycle at 95°C for 8 min, followed by 7 cycles of 95°C for 30 s, 68°C (-1°C / cycle) for 30 s, 72°C for 50 s, and 28 cycles at 95°C for 30 s, 62°C for 30 s, 72°C for 50 s, and a final extension cycle at 72°C for 30 min (CFX96 Touch™, Thermal Cycler, Bio-Rad, U.S.).

2.3.4. Temporal temperature gradient electrophoresis (TTGE) and fingerprint acquisition

PCR products (from Section 2.3.3) were separated by their GC% using a Temporal Temperature Gel Electrophoresis [32]. TTGE analyses were performed using a DCode™ System (Bio-Rad, U.S.). The 9.5% polyacrylamide gel was composed of two parts: a top, urea-free "concentration" part (stacking gel) applied approx. 1 cm from the base of the wells upward; and a bottom "denaturation" part (resolving gel), at 8 $mol.L^{-1}$ urea. Fifteen

244 microliters of PCR products were deposited into each well. Migration took place in 1.25 ×
245 TAE for 750 min at 50 V, with a temperature gradient from 65° to 70°C, i.e. +0.4°C per hour.
246 The gels were stained using GelRed™ (Biotium, U.S.) and then imaged under UV light
247 (Molecular Imager®Gel Doc™XRSystem, Bio-Rad, U.S., along with Image Lab™ software).
248

249 **2.4. Untargeted metabolomic profiling**

250 UHPLC-ESI-QToF, through implementing a non-targeted analytical strategy via high-
251 resolution mass spectrometry (HRMS) [33], was used to detect small soluble extracellular
252 target compounds produced by the bacteria and *H. ostrearia* recovered from the culture
253 medium.
254 LC-TOF/MS analysis samples: aliquots (5 µL) of each sample from the supernatant of *H.*
255 *ostrearia* cultures (see Section 2.3.1.) were separated on a Kinetex, 1.7-µm C18 100Å
256 (Phenomenex) column (150 × 2.1 mm) maintained at 40°C, using an Agilent 1290 Infinity LC
257 system with a gradient mobile phase (0.5 mL min^{-1}) comprising 0.1% aqueous acetic acid (A)
258 and acetonitrile containing 0.1% acetic acid (B). The gradient present was as follows: 5% B
259 from 0 to 2.4 min, increasing to 25% B from 2.4 to 4.5 min, then raised to 30% B from 4.5 to
260 11 min, finally reaching 100% B from 11 to 14 min and held there until 16.5 min, followed by
261 a decrease to 5% B until 20 min have elapsed and then maintained at 5% B until 25 min. The
262 eluent was directly introduced into the mass spectrometer by an electrospray. Mass
263 spectrometry was conducted on a 6540 UHD Q-TOF mass spectrometer (Agilent
264 Technologies, Waldbronn, Germany) operating in positive ion mode. The capillary voltage,
265 fragmentor voltage and skimmer were set at 3,900, 150 and 60 V, respectively. The sheath gas
266 was measured at 350°C (12 mL min^{-1}) and the drying gas at 175°C (5 mL min^{-1}) with a 43-psi
267 nebulizer. Nitrogen was used as the collision gas. Mass spectra were acquired in a full scan
268 analysis over an m/z range of 50 - 1,700 using an extended dynamic range and a centroid

mode of storage. The data station operating software was the MassHunter Workstation Software (version B.06).

2.5. Experimental replication and data processing

Experimental replication: For TTGE analysis, six samples followed by six distinct DNA extracts from the four studied localities were recovered from oyster ponds (Section 2.2), and their bacterial structures were compared (for raw sediments, biofilm and water column). A genetic comparison of *H. ostrearia* isolates was also carried out. To monitor the temporal dynamic of the bacterial community structure over the course of one algal culture cycle, experiments were replicated twice and moreover used to monitor this structure for its temporal dynamics during a 9-month serial subculturing campaign. For the non-targeted metabolomic investigation, analyses were carried out in triplicate.

Data processing: The 16S rRNA banding patterns on imaged TTGE gels were analyzed using the Molecular Analyst Fingerprinting software: FPQuestTM (Bio-Rad, U.S.). Put briefly, the software constructed a density profile through each lane and calculated the relative contribution of each band to the total band signal in a lane, after applying a rolling disc to serve as background subtraction. The banding patterns of the samples in each lane were compared with one another. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to draw a dendrogram from similarity coefficients. To decrease the bias, we opted to analyze band patterns by exercising the densitometric-curve option, as previously used by Kuntz *et al.* [34] and Seghers *et al.* [35] instead of the band-detection option. For the metabolomic investigation, data were processed using the MassHunter Qualitative Analysis software (Agilent Technologies), with compounds being extracted from the raw data with the Molecular Feature Extraction (MFE) algorithm. Moreover, a principal component analysis

was conducted using the Mass Profiler Professional B12.05 statistical package (also from Agilent Technologies).

3. Results

3.1. Bacterial community structure of sediment samples from oyster ponds

The structure of the bacterial community from oyster-pond bulk sediment samples varied depending on sampling location: Isle of Ré only showed 15% similarity with other localities. In contrast, samples from the other localities displayed a similar bacterial community structure (i.e. > 85%, see Fig. 2).

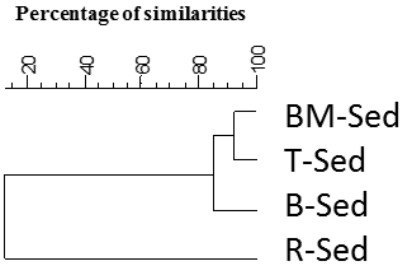


Fig. 2: Bacterial community structure of sediment samples (Sed) collected in oyster ponds from various localities (B: Bouin, BM: La Barre-de-Monts, R: Isle of Ré, T: La Tremblade)

3.2. Community structure of bacteria recovered from the biofilm after *Haslea ostrearia* isolation and from bacteria suspended in the water column

First of all, the structure of the bacterial community from the sediment compared to that of the biofilm after *H. ostrearia* isolation differed considerably (only 10% similarity between sediment and biofilm, data not shown). This result demonstrates that the bacteria associated with *H. ostrearia* were specific to the microalga.

Furthermore, a comparison of community structure of the bacteria recovered from the biofilm with those of the water column (WL), i.e. suspended bacterial cells (Fig. 3), revealed similarities that did not exceed 10%.

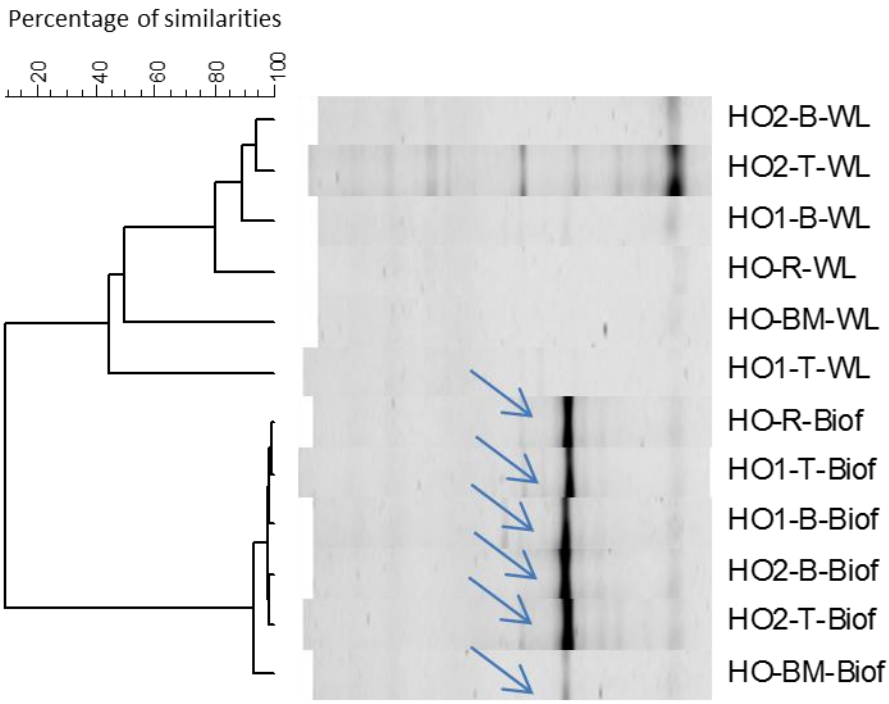


Fig. 3: TTGE analysis of the bacterial community structure from biofilm (Biof) and water column (WL) samples after isolation of *H. ostrearia* from various localities and subculturing in ES 1/3 medium under laboratory conditions (B: Bouin, BM: La Barre-de-Monts, R: Isle of Ré, T: La Tremblade). Arrows indicate the position of the band corresponding to the suspected chloroplastic and/or mitochondrial DNA of *H. ostrearia*.

With respect to the biofilm, the observed similarities in bacterial community structures exceeded 90% regardless of the geographic origin of the *H. ostrearia* isolates. The presence of a high-intensity band (see arrows in Fig. 3) was assumed to represent chloroplastic and/or mitochondrial DNA from *H. ostrearia*. The resulting phylogenetic tree with and without this band remained unchanged.

For the water column, similarities among the various algal isolates were more variable and lower overall (e.g. only 40% between HO1-T and HO2-B, extending to 95% between HO2-B and HO2-T).

3.3. Metabolic fingerprinting differentiation of *Haslea ostrearia* isolates from La Barre de Monts and Isle of Ré

Two distinct *H. ostrearia* isolates were used for the metabolomic analysis, namely: HO-BM and HO-R. The number of total compounds was much lower in the so-called "axenic" *H. ostrearia* cultures, which account for algal compounds (Fig. 4a), compared to the non-axenic cultures encompassing both algal and bacterial compounds (Fig. 4b).

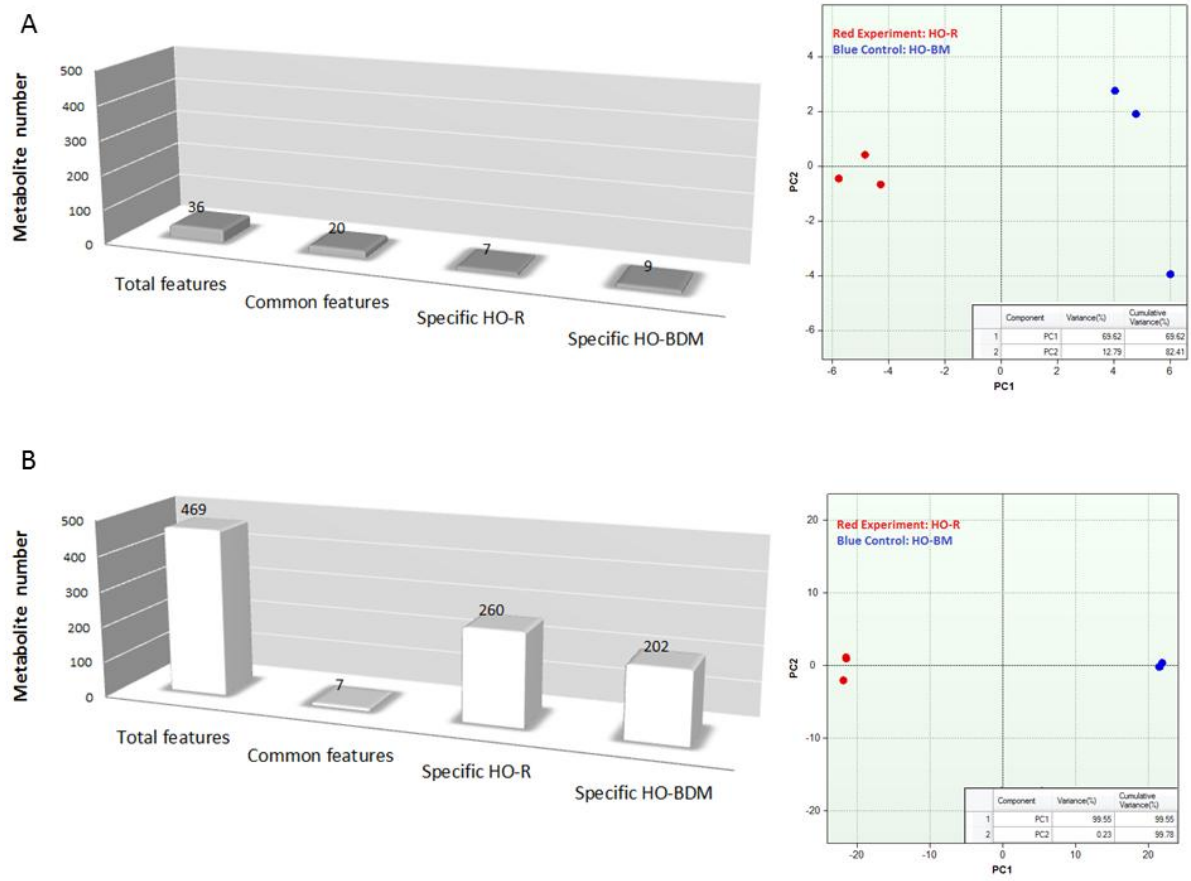


Fig. 4: Number of compounds recovered from HO-BM and HO-R isolate cultures (left) and the related principal component analysis representations (right) (in considering $p < 0.05$ and fold-change > 1.2): a) axenic; and b) non-axenic conditions. "Total", "common" and "specific" refers to the total compounds recovered, compounds shared by both isolates and compounds specific to each isolate, respectively. All analyses were performed in triplicate.

Axenic conditions were associated with a high number of common compounds (20 out of 36) and a small number of compounds specific to each isolate (7 and 9 for HO-R and HO-BM, respectively).

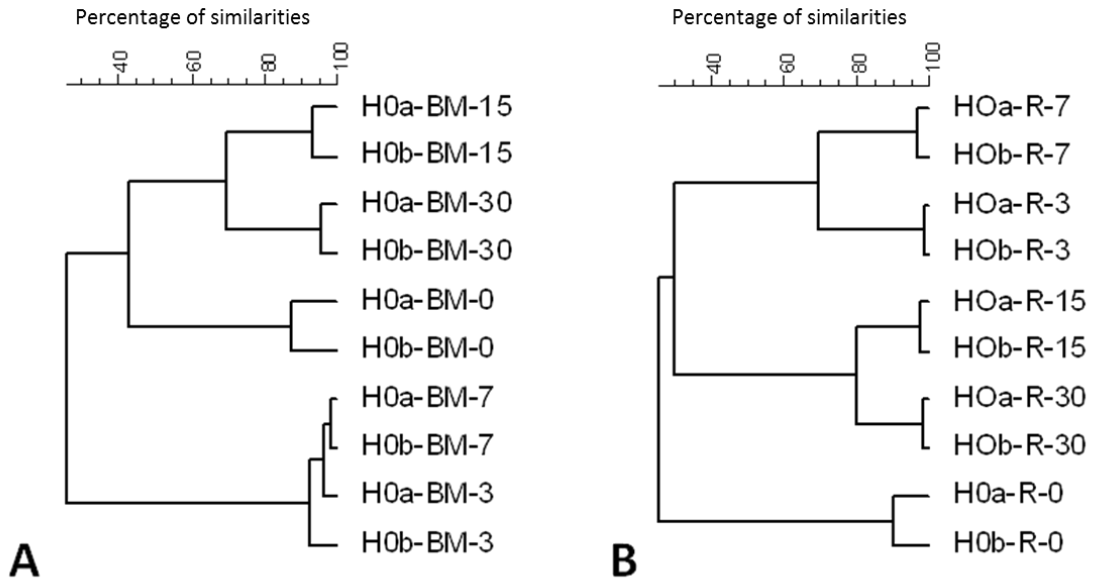
The opposite was exposed with non-axenic conditions, i.e. a low number of common compounds (7 out of 469), while 260 and 202 compounds were specific to HO-R and HO-BM, respectively.

In this instance, a Principal Component Analysis (PCA) approach to pattern recognition was used to distinguish the classes. Data from the control group (blue) and experimental group (red) were processed and a data matrix generated. Figures 4a and b display the 2D PCA score plot of the control group and experimental group under both axenic and non-axenic conditions. PCA analyses have confirmed that the differences in metabolic profiling between HO-R and HO-BM were more significant under non-axenic conditions than axenic conditions. The non-axenic model distribution points are indeed less dispersed (99% of the variance explained by axis 1 of the PCA (PC1) under non-axenic conditions vs. just 69% under axenic conditions).

3.4. Structure of the bacterial community from *Haslea ostrearia* isolates on two time scales

3.4.1. Over the course of one algal culture cycle

This experiment, performed in duplicate with two *H. ostrearia* isolates (HO-BM and HO-R), found perfect repeatability of the TTGE analysis (Fig. 5). At the culture cycle scale, a marked evolution in the bacterial community could be observed.



372 _

373 **Fig. 5:** Structure of the global bacterial community (suspension cells and biofilm cells) during a
374 growth cycle of two *Haslea ostrearia* isolates (at days 0, 3, 7, 15 and 30): a) HO-BM: La Barre-de-
375 Monts, b) HO-R: Isle of Ré. Letters a and b stand for the two experimental replicates.

376

377 Three distinct clusters emerged, all of which were related to the algal growth stage: day 0
378 (initial bacterial community structure), days 3 and 7 (exponential phase), and days 15 and 30
379 (stationary phase and decline phase).

380

381 ### 3.4.2. Over a 9-month serial subculturing campaign

382 Samples were recovered at the same growth stage, i.e. exponential. A rather low similarity in
383 the bacterial structure was observed between T0 and the subsequent subculturing, irrespective
384 of the algal isolate (Fig. 6): 25% for HO-T, and 60% similarity for HO-BM, HO-B and HO-R.
385 This result demonstrates that once *H. ostrearia* was isolated and cultivated under laboratory
386 conditions, the bacterial community structure evolved, but afterwards, i.e. from 3 to 9 months,
387 the bacterial community structure was fairly stable regardless of isolate origin. Similarities in
388 the bacterial community for each algal isolate during the 3- to 9-month period of culture did
389 in fact top 70%, with a maximum of 86% for HO-B.

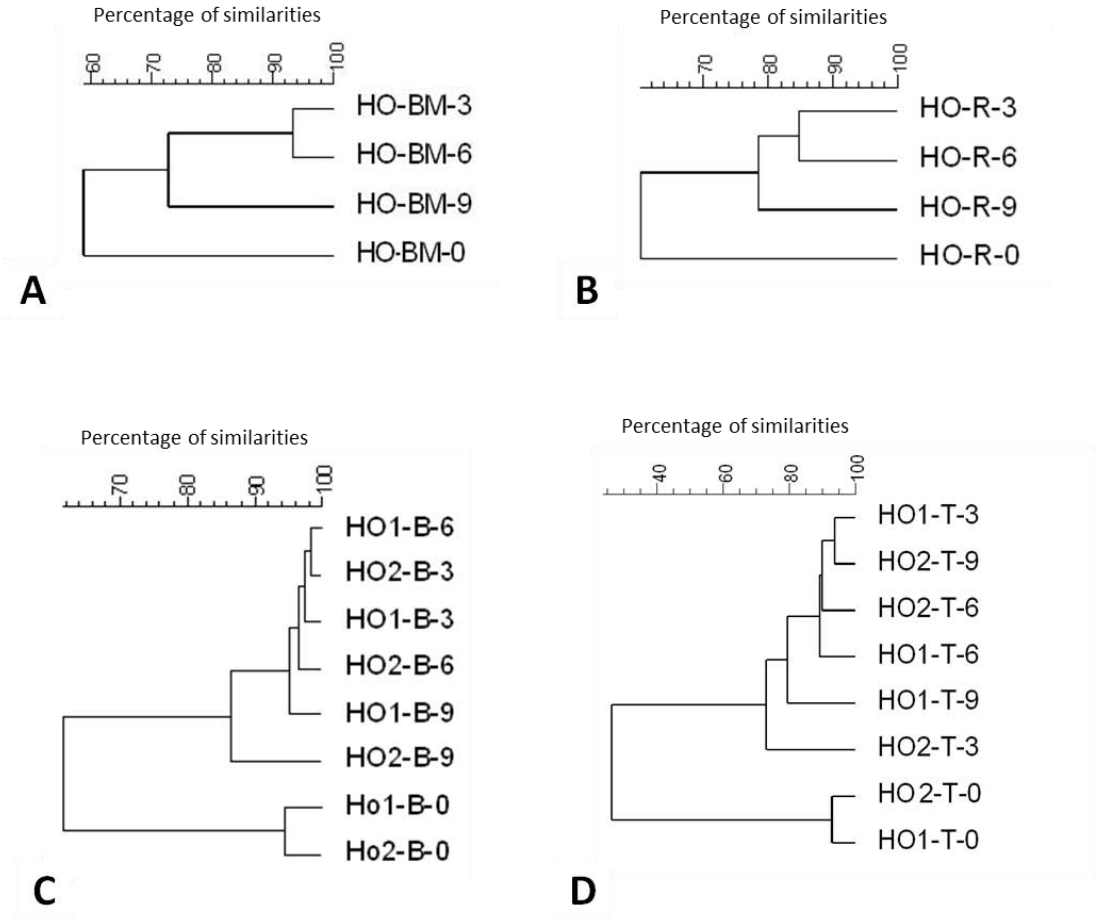


Fig. 6: TTGE analysis of the global bacterial community structure of *Haslea ostrearia* at the time of isolation (T-0) and after 3, 6 and 9 months of cultivation (T-3, T-6 and T-9, respectively). a) HO-BM: La Barre-de-Monts, b) HO-R: Isle of Ré, c) HO1-B and HO2-B: Bouin, d) HO1-T and HO2-T: La Tremblade.

For this pennate diatom, upon each cell division, the new valve is always formed within the parental theca, thus causing the average size of frustules in the population to slowly decrease. The size reduction in the *H. ostrearia* frustule, measured over the 9-month subculturing campaign (i.e. about 28.6% ± 2.6%, data not shown) exerted no influence on the bacterial community structure.

4. Discussion

4.1. Bacterial community structure of sediment samples from oyster ponds

Except for the HO-R isolate, which displayed just a 15% similarity with other isolates, strong similarities in bacterial structures were observed between isolates (> 85%, Fig. 2), while the sampling locations are separated by up to 100 kilometers (Fig. 1), although their climatic conditions are similar. For HO-R, the relative dissimilarity is most likely due to the sampling depth, which was higher compared to the sampling conditions for other localities: not only was the sediment that deposited on the bottom of oyster ponds collected, but so was some underlying sediment constituting the bed of the oyster pond (specific physicochemical characteristics). We have thus postulated that the bacterial assemblage of the sediment deposited on the bottom of the ponds and the underlying sediment were distinct.

For other localities showing high levels of similarities, the specific characteristics and functioning of the oyster ponds could explain these results. These ecosystems are indeed managed by human beings for optimal oyster production. Nutrients (quality, amount) for oysters, as well as for all living oyster pond organisms including algae and bacteria, were most likely to be similar from one pond to another, as proven by Turpin *et al.* [18] for oyster ponds in the same areas as those studied herein.

Whether or not the oysters were present at the time of sampling (no oysters were found in the ponds of La Barre-de-Monts and Bouin; oysters were present in the ponds of La Tremblade and Isle of Ré) did not seem to influence the bacterial community structure. Since the pond management history is an unknown, it may be assumed that empty ponds were filled with oysters shortly before the sampling campaign and moreover that the seawater composition in these ponds remained unchanged in the meantime.

4.2. Community structure of bacteria recovered from the biofilm and suspended bacteria from the water column after *Haslea ostrearia* isolation

Once *H. ostrearia* had been isolated from the sediment samples of the various localities, it was reasonable to assume that the specific biotope of each locality no longer exerted an effect. Indeed, *H. ostrearia* isolates were further cultivated under the same controlled conditions (i.e. culture medium, light, temperature).

Compared to the bacterial community structure in the bulk sediment, the structure relative to the phycosphere of *H. ostrearia*, i.e. epiphytic bacteria and those embedded in the biofilm, was very distinct with cross-sample similarities not exceeding 10% (data not shown). This result is typically observed in higher plants where the bacteria of the rhizosphere are compared to those of the bulk soil [36]. Regarding the TTGE pattern band numbers, it was surprising to note that they were higher for the biofilm samples than the bulk sediment samples. On the contrary, Schäfer *et al.* [24] observed that the bacterial biodiversity of the phycosphere was limited when compared to the complexity of bacterial assemblages in bulk seawater. Nonetheless, various results were shown when the rhizosphere bacteria were compared to bulk soil bacteria [36]. In our case, it can be assumed that the extracellular compounds of *H. ostrearia* are sufficiently diversified to support the growth of various bacterial populations. At least 36 (out of 469) compounds were indeed detected (Fig. 4) depending on whether *H. ostrearia* was axenic or not.

Irrespective of the geographic origin of the *H. ostrearia* samples, the bacterial structure of the biofilm samples was found to be similar (Fig. 3), including the HO-R isolate, while the bacterial structure of the sediment samples for this locality differed markedly (Fig. 2). This result shows that the bacterial structure of the phycosphere is specific to *H. ostrearia*; it has indeed been demonstrated that the structure of the bacterial community associated with the microalgal strain was specific to the microalgae [20, 24, 27]. For HO-R, it could then be

453 concluded that the particular bacterial structure of the sample recovered from the sediment
454 was solely influenced by the biotope characteristics, i.e. the specific sediment characteristics.
455 The significant differences in the bacterial community structures between phycosphere
456 bacteria and bulk water column bacteria (15% similarity between the two groups, Fig. 3)
457 confirm the significant effect of *H ostrearia* in establishing a specific community hosted by
458 the phycosphere. The highest level of bacterial structure similarity observed between biofilm
459 samples (> 90%), as compared to 40% - 90% for the suspended bacteria of the same samples,
460 has also revealed the predominant role of *H. ostrearia* in establishing a bacterial structure
461 specific to this species. The nutrient composition of *H. ostrearia* biofilms was, in all
462 likelihood, very different from that of seawater, as the result of biofilm enrichment by algal
463 exudates. The specificity of the bacterial community, with respect to algae in the environment
464 where bacteria live, was previously presented by Eigemann *et al.* [37], who demonstrated that
465 the influence of the algal host dominated the effect of changes in environmental conditions.
466 Liu *et al.* [38] showed that the phytoplankton community succession influences changes in
467 bacterial community composition. In the specific case of toxin production (like domoic acid)
468 by algae, Sison-Mangus *et al.* [39] reported that the three tested *Pseudo-nitzschia* species,
469 which vary in toxin production, had phylogenetically distinct bacterial communities and,
470 moreover, that toxic *Pseudo-nitzschia* exhibited less microbial diversity than non-toxic
471 *Pseudo-nitzschia*. Bruckner *et al.* [40] suggested an adaptation of Proteobacteria and
472 Bacteroidetes in the microenvironment created by the diatom biofilm. For the suspended
473 bacteria in the water column, the varying geographic origins of the samples, which very
474 probably reveal differences - even slight - in biotope characteristics, likely explains the
475 differences in bacterial structures given that they were not under the direct influence of *H.*
476 *ostrearia*.

Regarding the two isolates of La Tremblade (HO1-T and HO2-T), the limited similarity of the suspended bacterial cell structures (40%, Fig. 3) might be the result of initially different bacterial structures that did not converge after *H. ostrearia* isolation, in contrast with what was observed for the biofilm (> 95%) due to the considerable influence of exudation.

4.3. Metabolic profiles of HO-R and HO-BM

One of the greatest differences in the bacterial community structure was shown for HO-BM and HO-R isolates in both the biofilm and water column (Fig. 3); this finding may be explained by differences in the metabolic profiles (Fig. 4). The axenic cultures of *H. ostrearia* indeed display distinguishable metabolomes (Fig. 4a), though their differences remain minimal: 20 compounds in common out of 36, which is not surprising since the biometric analysis of the isolates by scanning electron microscopy (SEM), based on the density of transapical and longitudinal striations of the frustule, indicates that they belong to the *H. ostrearia* species.

To access the bacterial metabolom, it might have been tempting to subtract the number of compounds of the axenic treatment (Fig. 4a) from the number of the xenic compounds (Fig. 4b). Extreme care must be exercised however since some bacteria-microalgae interactions cannot be excluded. Bacteria and microalgae probably exhibit different metabolic profiles depending on whether *H. ostrearia* is cultivated as a monoculture or in association with the phycosphere bacteria. It should still be noted that the metabolic profile of the bacteria-*H. ostrearia* association is very specific to HO-R and HO-BM, with just 7 common compounds out of 469.

It cannot be overlooked that a few compounds were not those produced by the alga and/or bacteria, but instead compounds of other organisms since the culture medium (ES 1/3) consists of filtered (to 0.2 µm) seawater enriched with minerals. We have assumed that

medium sterilization degraded nearly all of the remaining compounds. The seawater used to prepare the culture medium was the same for cultivating the two isolates, thus avoiding any bias resulting from possible geographic differences.

4.4. Structures of the bacterial community from *Haslea ostrearia* isolates on two time scales

At the culture cycle scale (i.e. one to two weeks), the changes in bacterial community structure (Fig. 5) were related to the various growth stages of *H. ostrearia*, i.e. lag time, exponential growth stage and stationary stage, most likely as a result of exudates in varying quantities and compositions. This phenomenon is well documented for bacteria growing in the rhizosphere of plants [41]. At the seasonal time scale, Liu *et al.* [38] demonstrated that phytoplankton community succession influences the bacterial community composition.

The change in bacterial community structure is cyclical. When *H. ostrearia* was sampled at the same (exponential) growth stage over a nine-month period (i.e. 30 subculturings), the bacterial community structure remained quite stable (75% to 85% similarity between 3 and 9 months, see Fig. 6). It can be assumed that the entire bacterial community associated with *H. ostrearia* (embedded, epiphytic and free bacteria) was transmitted at each subculturing. The decrease in the frustule size of diatoms, already witnessed for *H. ostrearia* [42], did not alter the bacterial structure even though the algal size of some isolates at the end of the experiment (between 52 and 82 µm, with an average of 61.2 µm) was in the range for auxosporulation (i.e. between 50 and 68 µm [43, 44]).

Between the first subculturing (T-0) and the third month, the bacterial structure changed significantly (25% to 60% similarity) as the result of the bacterial community adapting to the maintenance culture conditions with stabilization after 3 months due to the well-controlled conditions for the culture of *H. ostrearia* isolates. This finding suggests that the laboratory

conditions for *H. ostrearia* (culture medium composition, temperature, light) also influence the composition of the bacterial assemblage, as demonstrated by Sapp *et al.* [45]; these authors found a shift in the bacterial populations associated with diatoms between isolation and cultivation of algal cells, with an increase in the number of phylotypes belonging to *Gammaproteobacteria* members.

5. Conclusion

For the first time, this study has analyzed the bacterial ecosystem surrounding the marine diatom *H. ostrearia* and showed that this bacterial structure is specific to the geographic origin of the microalgal isolate. Under laboratory conditions, once *H. ostrearia* has been isolated from oyster ponds, the bacterial community structure was shown to be resilient over a 9-month subculturing despite structural changes at the culture time scale according to the growth stage. Similarly, the differences in bacterial structures of two *H. ostrearia* isolates (HO-R and HO-BM) gave rise to specific metabolomic profiles. These profiles were more distinct with non-axenic microalgae, i.e. with inclusion of their associated bacteria, than with axenic microalgae, thus suggesting reciprocal relationships between bacteria and *H. ostrearia* cells.

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