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Effect of bacteria on growth and biochemical composition of two benthic diatoms

Halamphora coffeaeformis and *Entomoneis paludosa*.

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Abstract

Benthic diatoms are the dominant microalgae in intertidal mudflats and are in constant interaction with their surrounding bacteria. This study was designed to investigate the effect of bacteria on growth, biomass, elemental (C & N) and biochemical composition, and extracellular polymeric substances (EPS) excretion by two marine benthic diatoms, *Halamphora coffeaeformis* and *Entomoneis paludosa*. The experiments were conducted on diatom cultures previously exposed or not to antibiotics. The treatment with antibiotics caused a decrease of bacterial abundance from 24 to fewer than 1 bacteria per algal cell. In non-treated cultures of *E. paludosa* and *H. coffeaeformis*, the bacteria phylogenetic affiliation was equally distributed between Bacteroidetes (Flavobacteriia) and Proteobacteria (alpha- and gammaproteobacteria). After treatment with antibiotics, the residual bacterial community was ~37% Flavobacteriia (*Winogradskyella* genus), 34% for the alphaproteobacteria (mainly *Roseibacterium* sp. and *Antarctobacter* sp.) and 29% for the gammaproteobacteria (mainly *Methylophaga* sp. and *Stenotrophomonas* sp.). Growth of *H. coffeaeformis* and *E. paludosa* in non-treated cultures was enhanced by the abundance of the associated bacteria, with mean growth rate of 1 day⁻¹ compared to 0.7 for antibiotic treated cultures. In *E. paludosa*, maximal cell abundance was higher in the presence of bacteria while the final carbon biomass did not vary, but in *H. coffeaeformis* maximal cell abundance did not vary significantly while final carbon biomass was higher in the presence of bacteria. By contrast, for both diatoms, cellular content of protein and lipids decrease significantly, as did extracellular carbon (EPS fraction) in the presence of bacteria. However, only a minor effect was observed on cellular carbohydrates, C/N ratio, and pigments (Chl *a*). Diatoms carbon fluxes towards the main biochemical components were also modified, with the protein carbon fraction significantly lower relative to other carbon compounds in the presence of high bacterial biomass. These results showed the complex

interactions between diatoms and their associated bacteria. Promotion of diatoms growth by the presence of bacteria appears linked to change in microalgae biochemical composition that will modify the biofilm. Our results might help understanding the regulation of benthic biota in mudflat ecosystems.

Keywords

Diatom; Microphytobenthos; Bacteria; Lipids; Proteins; Carbohydrates.

Running title

Benthic diatoms and associated bacteria.

1. Introduction

The microphytobenthos (MPB) is an assemblage of benthic photosynthetic eukaryotic microalgae and cyanobacteria dominated by diatoms (MacIntyre et al., 1996). It is considered as a major contributor to mudflats primary production (Underwood and Kromkamp, 1999) and as an important energy source for secondary producers in intertidal ecosystems (Blanchard et al., 2001).

Microphytobenthic species are in a perpetual interaction with bacteria, both *in situ* and in culture conditions (Van Colen et al., 2014). These interactions are known to have positive (e.g. *Alteromonas* sp., *Muricauda* sp.) or negative effects (e.g. *Pseudomonas* sp., *Halomonas* sp.) on microalgal growth performance (Le Chevanton et al., 2013). Heterotrophic bacteria phyla commonly associated to diatom cultures are *Proteobacteria* and *Bacteroidetes* (Schäfer et al., 2002) and the main species belong to *Alteromonas*, *Flavobacterium*, *Roseobacter*, and *Sulfitobacter* genera (Grossart et al., 2005; Hunken et al., 2008; Kaczmarek et al., 2005; Sapp et al., 2007a; Sapp et al., 2007b; Sapp et al., 2007c). Some bacteria can improve microalgal growth through synergistic interactions (reviewed in Amin et al. (2012)). One of the most common interactions is the bacterial release of growth promoting factors such as vitamins that are needed by microalgae (e.g. cobalamin, thiamine and biotin) (Carlucci and Silberna, 1969; Croft et al., 2005; Haines and Guillard, 1974; Kazamia et al., 2012; Ryther and Guillard, 1962; Tang et al., 2010). By their role in the nitrogen cycle, bacteria (e.g. *Alteromonas* sp., *Muricauda* sp.) can also convert some nitrogen compounds into chemical forms subsequently assimilated by microalgae (Le Chevanton et al., 2013; Tai et al., 2009). Other improvement of the microalgal growth are made by hormones, such as indole-3-acetic acid by *Azospirillum* spp. (de-Bashan et al., 2008). *Sulfitobacter* species were recently found to promote *Pseudonitzschia multiseries* division via the production of this hormone by using the diatom's secreted and

endogenous tryptophan (Amin et al., 2015). Microalgae can also be protected by detoxifying bacteria from some of the products they produced during growth, such as hydrogen peroxide produced during photosynthesis, for which bacterial cleavage reduces the oxidative stress. This detoxification was shown for Proteobacteria affiliated to the alphaproteobacteria genus *Sulfitobacter*, the gammaproteobacteria genus *Colwellia*, and the genus *Pibocella* of the *Bacterioidetes* (Hunken et al., 2008). Furthermore, aerobic bacteria (*Pseudomonas diminuta* and *P. vesicularis*) can reduce photosynthetic oxygen tension within the phycosphere, thereby creating favourable conditions for photosynthetic algal growth (Mouget et al., 1995).

However, some bacterial species (e.g. *Pseudomonas* sp., *Halomonas* sp.) can have a negative impact on growth and development of microalgal species (Le Chevanton et al., 2013). A competition can occur between the two groups for the use of resources such as inorganic nutrients (nitrate, phosphate), especially under limiting conditions (Guerrini et al., 1998; Joint et al., 2002; Rhee, 1972). Bacteria seem more efficient than microalgae for phosphate assimilation under limiting P-conditions; whereas the inverse occurs when the two organisms are cultured with high concentration of phosphate (Guerrini et al., 1998).

Bacteria also have an effect on microalgal biochemical content and exudates. Addition of the bacteria *Azospirillum brasilense* significantly enhanced chlorophyll *a* and *b*, lutein, and violaxanthin pigment of two species of *Chlorella* (*C. vulgaris* and *C. sorokiniana*) as well as cellular lipids and fatty acids (de-Bashan et al., 2002). Similar results were found on carbohydrates and starch with the same bacteria and *Chlorella* species (Choix et al., 2012). Diatom-bacteria interaction in cultures also triggers the extracellular polymeric substances (EPS) excretion in some diatom species, as shown for *Phaeodactylum tricorutum* and *Escherichia coli* (Bruckner et al., 2011), or *Thalassiosira weissflogii* and *Marinobacter adhaerens* (Gardes et al., 2011; Gardes et al., 2012). This positive interaction is however

species-specific, and a complex relationship exists between diatom's growth and organic release (Grossart and Simon, 2007).

To better understand the effect of bacteria on benthic diatoms, we performed experiments on two species in culture, *H. coffeaeformis* and *E. paludosa* to define the effects of their bacterial community on the growth, cellular biomass, biochemical composition and EPS excretion. These two species are usually found in biofilms on intertidal mudflat sediments (Meleder et al., 2007; Ribeiro et al., 2003; Ribeiro et al., 2013). In addition, this study assessed the efficiency of an antibiotic treatment on the bacterial community associated to benthic diatoms.

2. Materials and Methods

2.1. *Diatoms*: *Halamphora coffeaeformis* and *Entomoneis paludosa*

The benthic diatoms *H. coffeaeformis* (C. Agardh) Levkov (UTCC58) and *E. paludosa* (W. Smith) Reimer (NCC 18.2) have been maintained in 400 mL batch cultures, at a temperature of 17° C and a salinity of 35. A photoperiod of 14:10 light: dark was applied with a light intensity of 90 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ using cool light fluorescent lamp (Lumix day light, L30W/865, Osram). The microalgae stock-cultures were maintained in exponential growth phase by transferring weekly into fresh medium made of autoclaved artificial seawater (ASW) enriched with nutrients as described in Wolfstein and Stal (2002) but with a F/2 vitamins solution (Guillard, 1975; Guillard and Ryther, 1962).

2.2. *Experimental set up*

2.2.1. Antibiotic treatment

Five milliliters of a stock culture of each diatom species in exponential growth phase were inoculated in 50 mL of autoclaved ASW subsequently enriched in nutrient as above. Two milliliters of a commercial antibiotic-antimycotic mix composed of 10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per mL (Sigma-Aldrich, A5955) were added to the culture medium. The algae were exposed to this treatment for seven days, in triplicate and in the same environmental conditions as for the stock cultures. The cultures were carefully shaken twice a day to ensure a good homogenisation of the antibiotic mix. They were used as inoculum for the treated condition. In parallel, *H. coffeaeformis* and *E. paludosa* were cultured in triplicate

in the same conditions as above, but without addition of the antibiotic-antimycotic mix. These cultures were used as inoculum for the non-treated condition.

All cultures were sampled at the beginning and the end of the antibiotic treatment for microalgae and bacteria cell counts.

2.2.2. Growth experiment: effects of bacteria on diatoms in culture

To assess the effect of bacteria on diatom's growth and biochemical composition, an inoculum of 1 mL of the untreated and treated diatom cultures was transferred into 500 mL aerated Erlenmeyer flasks (Pyrex) filled with 400 mL of autoclaved enriched ASW and incubated as described above for the stock cultures. Diatom's growth was then followed until *H. coffeaeformis* and *E. paludosa* reached the beginning of the stationary phase of growth.

Samples for microalgae cell counts were done every two days and samples for bacteria count taken at the beginning and the end of the experiment (day 8, Fig. 1). Samples for biochemical characterization (C, N, proteins, lipids, carbohydrates, EPS and Chlorophyll *a*), nutrients (ammonium, nitrite, nitrate, phosphate and urea) and bacterial diversity were collected in all cultures in the stationary phase of growth at the end of the experiment (day 8, Fig. 1).

2.3. Microalgae cell count

Samples for microalgae enumeration were fixed with lugol. Cells were counted in triplicate using a Nageotte haemocytometer and an optical microscope (×400). To avoid microalgal aggregation, samples were homogenized with a vortex prior each enumeration.

Average growth rates (equation 1) were estimated during exponential phase of growth where C_0 and C_n were the cell concentration (cells mL⁻¹) at time t_0 and t_n (Guillard, 1973) :

$$\mu = \frac{\text{Ln}C_t - \text{Ln}C_0}{t} \quad (\text{equation 1})$$

Using the growth kinetic, a Gompertz model (equation 2) was also fitted to the data to assess the maximum growth rate (μ_{\max} in day⁻¹), the maximum cell concentration (α expressed in $\log(C_t/C_0)$ with C_t and C_0 in cell mL⁻¹) and the latency time (λ in day, if present) with a MatLab software:

$$f(t) = \alpha \times \exp\left(-\exp\left(\frac{\mu_{\max} \times \exp(1)}{\alpha} \times (\lambda - t) + 1\right)\right) \quad (\text{equation 2})$$

2.4. Bacteria cell count

To enumerate the attached and free-living bacteria, 2 mL of the two diatoms cultures were sampled for the three following culture conditions: the stock-culture, the culture at the end of the antibiotic treatment (after seven days of growth with or without antibiotics) and the culture at the end of the growth experiment (day 8, Fig.1). Samples were fixed in 0.2 μm filtered formaldehyde (final concentration 1%), and immediately stored at -80°C until enumeration.

Free living bacteria were counted using a BD FACS Canto II flow cytometer (BD Bioscience) equipped with an air-cooled blue laser (488nm, 20-mW solid state). Enumeration of bacteria was described by Marie et al. (1997). Briefly, 1 mL of sample was stained with SYBR Green I (1/10000 final concentration, Invitrogen) and incubated for 15 min in the dark. Green fluorescence was analysed in log mode for 1 min at low speed (16 $\mu\text{L min}^{-1}$) and medium speed (54 $\mu\text{L min}^{-1}$) for *E. paludosa* and *H. coffeaformis*, respectively. Results were analysed with the BD FACS Diva software.

For attached bacteria, diatoms (n = 30 per condition) were arbitrarily observed and their epiphytic bacteria and bacteria associated to large particles were counted using PicoGreen (Life Technologies) staining and fluorescence microscopy ($\times 500$, Olympus Ax70 with Olympus U-RFL-T).

2.5. Determination of the bacterial communities in diatom culture

Bacterial genomic DNA from untreated *E. paludosa* and *H. coffeaeformis* cultures and treated *E. paludosa* culture were sampled at the end of the growth experiment (day 8, Fig. 1). DNA was extracted on 0.2 μm filter (50 mL) using the Power Water DNA isolation kit (MoBio Laboratories) according to the manufacturer's instructions for maximum yields. DNA quality was checked by 1% (w/v) agarose gel electrophoresis and quantified using NanoDrop. The bacterial 16S rDNA full length gene were PCR-amplified using the primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Lane, 1991; Muyzer et al., 1993). The PCR mix (50 μL) contained 1X PCR buffer, 2.5 mM MgCl_2 , 200 mM of each dNTP, 25 pmol of each primer, 250 ng mL^{-1} of bovine serum albumin (BSA, Sigma), 1.5 units of HotStart Taq DNA polymerase (Qiagen), and 10 ng of DNA extract. PCR reaction was carried out in a Labcycler SensoQuest. The thermal PCR profile was as follows: initial denaturation at 94°C for 15 min followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and elongation at 72°C for 1 min. The final elongation step was 9 min at 72°C. The 16S rDNA products were analysed by electrophoresis in 1% agarose gels before cloning and sequencing. Sixteen S rDNA products were purified (QIAquick PCR Purification Kit, Qiagen) then cloned into a pGEM-T-Easy vector (Promega) to construct clone libraries. Because of the very low diversity in each sample, the normalisation of the libraries was done with 11 clones (sub. sample command from Mothur (Schloss et al., 2009)). Sequences were compared with 16S ribosomal RNA sequences in the GenBank database by using the BLAST (Basic Local Alignment Search Tool) service to determine their approximate taxonomic affiliations (Altschul et al., 1990). The nucleotide sequences reported have been

deposited in the GenBank database under accession no. KY077816 to KY077823 and KY094623 to KY094625.

2.6. Biochemical analysis

The elemental composition in carbon and nitrogen was determined using a CHNS Analyzer (Thermo Scientific FLASH 2000). Ten milliliters of culture were filtered on a pre-combusted glass filter (Whatman GF/C), subsequently dried (70°C, 48 h) and encapsulated for analysis. Particulate nitrogen (PN) and carbon (PC) were determined and computed on the basis of the mean algal cell concentration. Protein extraction was adapted from previous protocols (Barbarino and Lourenco, 2005; Marchetti et al., 2013). Samples (10 mL culture) were centrifuged (2500 g, 5°C, 30 min), the algal pellet extracted and precipitated in an iced bath (30 min) using 100% acetone (0.5 mL). Proteins were subsequently centrifuged and rinsed twice with 70 % acetone (2.5:1, v/v) and finally solubilised in 0.5 mL of ultra-pure water. An aliquot was quantified using a BCA protein assay kit (Pierce, with bovine serum albumin (BSA) protein standard) based on Lowry et al. (1951). Total lipids were weighed from 50 mL culture samples. Samples were extracted using the Bligh and Dyer's (1959) method as described in Marchetti et al. (2013). Total particulate carbohydrates and the colloidal EPS fraction were determined using 10 mL of culture. After centrifugation (2500 g, 5°C, 30 min), pellet was separated from the supernatant. Both fractions were then analyzed according to the sulfuric acid colorimetric method of Dubois et al. (1956), based on phenolphthalein absorbance at 490 nm. Chlorophyll *a* (chl *a*) was determined using 10 mL of culture subsequently centrifuged (2500 g, 5°C, 30 min). The pellet was extracted using 5 mL of 90 % acetone, kept 24 h in the dark at 4°C to ensure complete pigment extraction. Quantification was performed using the Lorenzen (1967) equations based on spectrophotometric methods.

2.7. Nutrient analysis

The standard analytical methods of Strickland and Parsons (1972) were applied for nitrite, nitrate and phosphate after filtration of the culture medium on Nuclepore polycarbonate filter (2 μ m). Ammonium and urea were determined in samples of freshly filtered (Nuclepore polycarbonate filter 2 μ m)) culture medium, using respectively the method of Koroleff (1970) and Goeyens et al. (1998).

2.8. Data analysis

All data are expressed as mean \pm standard deviation (SD). T-tests were performed after testing normality (Shapiro-Wilks test) and equality of variances (Levene test). The analysis were carried out with the software Statgraphics Centurion (StatPoint Technologies, Inc.).

3. Results

3.1. Bacterial abundance and diversity

Antibiotic treatment was efficient in all treated cultures. For both diatoms after seven day of exposure to the antibiotic-mix, free living and attached bacteria were almost undetectable by flow cytometry or fluorescence microscopy. In *H. coffeaeformis* treated cultures 0.09 ± 0.02 bacteria per algal cell (free + attached bacteria) were counted, whereas a bacterial population of 15 ± 2 bacteria per algal cell was measured in the untreated cultures. Similar results were obtained with *E. paludosa*, the bacterial biomass drastically decreased from 24 ± 2 (free +

attached bacteria) bacteria per algal cell in the untreated culture to 0.3 ± 0.1 bacteria per algal cell when treated with the antibiotic mixture.

At the end of the growth experiment (Fig. 1), the diatoms previously exposed to the antibiotics were still bacteria “free” (or very clean, $<0.4 \pm 0.1$ bacteria per algal cell) (Fig. 2), whereas the untreated cultures had concentrations close to 25 bacteria per algal cell (free living + attached bacteria). These values were similar to what was found at the end of the antibiotic treatment. Furthermore, composition of the bacteria assemblage was close to similar in the cultures previously treated or not with the antibiotic mix (Table 1). In the untreated cultures the most abundant Operational taxonomic Units (OTUs) of the satellite bacteria were similar for both diatoms, and all of them are affiliated to marine species. They were quite equally distributed between the Flavobacteriia class (OTU 1, affiliated to *Winogragskyella* sp.) and the Proteobacteria (*Antarctobacter* sp., Alphaproteobacteria, OTU 3; *Methylophaga* sp., Gammaproteobacteria: OTU 2). Similar distribution between the bacterial phylotypes was found in the *E. paludosa* treated culture, but with a slight decrease of the Flavobacteriia class (OTU 1, 37% *Winogragskyella* sp.) and a higher diversity within the Proteobacteria phylum: Alphaproteobacteria, OTUs 3,4, 6 and 8 (*Antarctobacter* sp., *Roseibacterium* sp., *Sulfitobacter* sp., and *Brevindimonas* sp.) for 34% of the total, and Gammaproteobacteria, OTUs 2, 5 and 7 (*Methylophaga* sp., *Stenotrophomonas* sp., and *Thioalkalivibrio* sp.) for 29% of the total (Table 1)

3.2. Diatom growth and biochemistry

Growth performances of both diatoms (Fig. 1 and Table 2) and final composition of the dissolved nutrients (Table 3) varied significantly between treated and untreated. For *H. coffeaeformis*, significant differences were found for the maximal ($p = 0.03$) and mean growth

rate ($p = 0.002$) and the lag phase ($p = 0.009$) but not for the maximal cell abundance ($p = 0.7$) and the final carbon biomass ($p = 0.173$) (Table 2). In untreated cultures with the highest bacteria abundance, both the maximum and the mean growth rates were improved by an increase of $+0.4$ and $+0.3 \text{ day}^{-1}$ respectively, while the lag phase was reduced from 2.05 to 1.34 day. Maximum and mean growth rate of *E. paludosa* also increased in the untreated cultures ($+0.5$ and $+0.3 \text{ day}^{-1}$, respectively). However, no differences were observed between the lag phases in the two culture conditions ($p = 0.212$). A significant enhancement of the maximal cell abundance ($p = 0.012$) was observed in untreated cultures whereas, final carbon biomass did not vary between the two conditions ($p = 0.831$, Table 2).

At the end of the growth the lowest nitrate concentration in the culture media of both diatoms was $287 \mu\text{M-N}$ (Table 3) indicating that nitrate was never a limiting factor. Nitrate concentrations were however significantly lower in cultures with the largest number of bacteria (NT: $p = 0.025$ and T: $p = 0.037$, Table 3). The three other nitrogen sources, ammonium, nitrite and urea were detected at a much lower concentration than nitrate ($< 5 \mu\text{M-N}$). Both final nitrite and urea concentrations did not show significant differences between culture conditions (Table 3). The concentration of NH_4 in the two culture media of *H. coffeaeformis* was significantly different ($p = 0.001$; untreated culture = $0.81 \pm 0.11 \mu\text{M-N}$ and treated culture = $1.35 \pm 0.11 \mu\text{M-N}$), whereas this was not significant in *E. paludosa* cultures ($p = 0.212$), with values of 0.73 ± 0.55 and $0.27 \pm 0.02 \mu\text{M-N}$ in untreated and treated cultures, respectively.

Diatom biochemical composition also varied, depending on the cultures and bacterial abundance. *E. paludosa* carbon and nitrogen contents were significantly different (PN, $p = 0.032$ and PC, $p = 0.035$) with a decrease of 2 pg N cell^{-1} and $16 \text{ pg C cell}^{-1}$ in the cultures with a higher bacterial abundance (Table 4). Nonetheless, the C/N ratio did not vary significantly ($p = 0.86$, Table 4). Chlorophyll *a* content of *E. paludosa* was also similar between both culture conditions, either in, per-cell unit ($3.6\text{-}3.9 \text{ pg cell}^{-1}$, Fig 3A) or on carbon basis ($0.05 \pm 0.01 \text{ g}$

g⁻¹ C, Table 5). In contrast no significant differences were observed in *H. coffeaeformis* cultures on both nitrogen (PN, p = 0.188) and carbon (PC, p = 0.234) contents and on their ratio (C/N, p = 0.11). However, chlorophyll *a* content (Fig.3A) was significantly higher (p = 0.017) in untreated culture (3.54 ± 0.16 pg cell⁻¹) than in the other one (2.82 ± 0.27 pg cell⁻¹) but this difference was not observed for Chl *a* content standardised in per-carbon unit (0.04 ± 0.01 g g⁻¹ C and p = 0.77, Table 5).

Lipid content in per-cell unit basis significantly decreased in both diatom species in conditions with high abundance of bacteria (*H. coffeaeformis* p = 0.01 and *E. paludosa* p = 0.021) (Fig. 3B). This decrease was still observed in per-carbon unit for *H. coffeaeformis* (p= 0.028; Table 5), but not for *E. paludosa* (p = 0.734). For both diatoms significant differences were found in carbohydrates in per-cell unit (Fig. 3C). However, *H. coffeaeformis* carbohydrate content was higher in untreated culture (75 ± 10 pg cell⁻¹) than in culture with low bacterial abundance (56 ± 3 pg cell⁻¹, p = 0.008) whereas, the inverse was observed in *E. paludosa* (81 ± 7 pg cell⁻¹ and 124 ± 7 pg cell⁻¹ p = 0.001, respectively). Nonetheless, for both algae, no significant difference was found in per-carbon unit (p = 0.96 and p = 0.09, Table 5). The protein content of *H. coffeaeformis* grown under both conditions was not significantly different (p = 0.223, Table 5) whereas protein content of *E. paludosa* was significantly lower (p = 0.017, Table 5) in untreated culture (Fig. 3D and Table 5). In both diatoms a similar pattern of the dissolved extracellular polymeric substances (EPS), was observed, a lower amount per cell was measured in the presence of bacteria high biomass (Fig. 3E, *H. coffeaeformis* p = 0.01 and *E. paludosa* p = 0.05); although, difference was not significant when EPS were standardized in per-carbon unit (0.05 < p-value < 0.1, Table 5):

4. Discussion

4.1. Antibiotic treatment efficiency

A simple and efficient method was applied to obtain clean and almost axenic diatom culture. The microalgae inoculum was diluted in an axenic medium to decrease the initial bacteria concentration, then exposed to an antibiotic mixture during seven days. As shown by other authors the response of microalgae to an antibiotic treatment is often species-specific (D'Costa and Anil, 2011) and long exposure period and low initial cell concentration are needed (Cho et al., 2002). All parameters of the treatment have thus to be optimized for each species. For the present study, the initial algal concentration, the nature and concentration of antibiotics and the length of the treatment have been optimized (unpublished data) for an efficient bacteria removal without preventing diatom growth. The treatment solution was a mix of two antibiotics (penicillin and streptomycin) and an antimycotic (amphotericin B). Penicillin does not affect directly diatom's growth (D'Costa and Anil, 2014) whereas streptomycin inhibits protein synthesis in diatom organelles (Cullen and Lesser, 1991). In the present study the antibiotic mixture and the length of exposure were sufficient to suppress bacterial growth in the microalgal cultures and were found to be non-toxic for the two diatoms for which further inoculum grew normally in antibiotic-free medium after the treatment (Fig. 1). Moreover, the small volume of inoculum treated with antibiotics (1/400 dilution of algal culture in new medium) prevented important antibiotic traces and particularly of streptomycin. The streptomycin concentration was below $1\mu\text{g mL}^{-1}$, a non-toxic concentration for diatoms (Berland and Maestrini, 1969). Clean microalgal cultures of *H. coffeaeiformis* and *E. paludosa* were thus obtained with very low bacterial biomass, as shown in figure 2. This allowed us to perform the experiment to assess the effect of bacteria on growth and biochemical composition of the two diatoms.

As discussed above, the antibiotic treatment affected the bacterial abundance with a drastic decrease of cell number > 98 %. This however did not highly modify the proportion of the two bacteria phyla, Bacteroidetes (Flavobacteriia) and Proteobacteria (alpha- and gammaproteobacteria) were found at the end of the microalgal growth in both treated and untreated cultures (Table 1). The composition of this bacterial community corresponds to the phylotypes generally found in microalgal cultures, and in particular in association with diatoms (Amin et al. 2012). Satellite populations are mostly members of the Cytophaga-Flavobacterium-Bacteroides (CFB) phylum or belong to alphaproteobacteria (Shäfer et al. 2002). Bacteria belonging to the CFB (Bacteroidetes) group are mainly attached on particles (Grossart et al 2005) and Flavobacteriaceae is a family often attached to the surfaces of a wide range of marine algae (Hanzawa et al., 1998; Nedashkovskaya et al., 2005). The Proteobacteria determined in the cultures are also from marine origins, and the alpha- and gammaproteobacteria are mainly found as free-living bacteria (Grossart et al 2005). Among them *Methylophaga* spp. (gammaproteobacteria) and *Roseobacter* spp. (alphaproteobacteria) are found in marine sediment (Doghri et al., 2015; Janvier et al., 2003) and *Roseobacter* spp. is part of the bacteria forming biofilms in mud flat environments (Doghri et al., 2015). The presence of *Methylophaga* spp. and Rhodobacteraceae (*Roseibacterium*) in *E. paludosa* cultures thus indicates the conservation in the cultures of natural *in-situ* bacterial genus generally associated to benthic diatoms.

4.2. Effect of bacteria on diatom's growth and biochemical composition

It is currently accepted that bacteria can positively or negatively affect microalgal growth performance (Cole, 1982; Le Chevanton et al., 2013; Natrah et al., 2014; Park et al., 2008). In the present study, both higher diatoms growth rates and cell division were related to the highest bacterial abundance, a positive interaction already observed for *Thalassiosira rotula* (Grossart

and Simon, 2007) and *Skeletonema costatum* (Grossart et al., 2006b) in culture. As no limitation by the major mineral nutrients (nitrogen (Table 3) and phosphate (data not shown) were detected during the experiments, the improved diatoms growth could be due to some positive nutritional relationships between microalgae and bacteria (Sullivan and Palmisano, 1984). For example, the *Roseobacter* – algae interaction is well known in marine ecosystems (Geng and Belas, 2010). Members of the Rhodobacteraceae (alphaproteobacteria) play an important role in carbon and sulphur biogeochemical cycle and are known to have a mutualistic interaction with different species of microalgae (Geng and Belas, 2010; González et al., 2000; Ramanan et al., 2016). This positive effect driven by the high bacterial biomass might also be due to the release of trace elements, such as a growth promoting factor, the hormone indole-3-acetic acid (de-Bashan et al., 2008), or by production of vitamins (Amin et al., 2012). This was already demonstrated for another diatom (*Pseudonitzschia multiseriis*) where *Sulfitobacter* species promoted cell division (Amin et al. 2015).

Bacteria can also have a detoxification role and help to the disappearance of toxic compounds derived from the algal metabolism (Hunken et al., 2008; Mouget et al., 1995). In particular the reduction of the oxidative stress linked to the photosynthetic activity of the diatom *Amphiprora kufferathii* was observed with bacteria affiliated to the alphaproteobacteria (*Sulfitobacter*), gammaproteobacteria (*Colwellia*) and bacteroidetes (*Pibocella*) (Hunken et al., 2008). Commensal bacteria identified in *E. paludosa* cultures might have a similar positive role on the diatom's growth in the untreated cultures of the present study.

On the other hand, some of the bacterial genus detected in *E. paludosa* cultures could have negative effect by inhibiting microalgal growth or competing for a nutritional resource. This could be the case for *Methylophaga* found in our cultures. The gammaproteobacteria *Methylophaga* was suggested to rely on phytoplankton for carbon and energy sources and was identified to compete with diatoms for cobalamin (Bertrand et al., 2015). However in our study,

vitamins were given in excess in the culture media and *Methylophaga* should probably not really compete with *E. paludosa* for cobalamin. This could explain why this bacteria did not affect the diatom's growth despite a higher percentage in the untreated cultures (28%) than in the treated one's (9%). Some species of Rhodobacteraceae also have been described with negative effects on microalgae. They decreased both growth and maximal biomass of *Dunaliella* in culture (Le Chevanton et al, 2013), but in this later case, bacterial strains were distinct from those of *Dunaliella*'s assemblage, isolated from some other microalgae (*E. huxleyi* and diatoms) culture media. Their action could be thus considered as exogenous factor on the microalgae biology, a situation that does not correspond to the present study where bacterial strains have been harboured for a long time with the two diatoms in culture.

Despite the lack of information on the other bacterial clones identified with *E. paludosa* either in non-treated cultures (*Winogragskyella* sp., and *Antarctobacter* sp.) or in treated cultures (*Stenotrophomonas* sp. and other minor clones, see Table 1), and whatever the complexity of diatom-bacteria interactions (Amin et al., 2012) it seems that in the present study the two diatoms benefitted from the bacterial community for their growth. Algicidal bacteria (Mayali and Azam, 2004) such as antibiotic producers (Cole, 1982) were thus absent or not predominant in the diatoms cultures. This might be the consequences of the preservation of our two diatom strains during many generations in xenic cultures with associated bacteria that would then have kept a synergetic effect for growth. This was the inverse in Le Chevanton et al. (2013) where individual bacterial strain had negative or insignificant effect, as the result of their exogenous origin from the microalgae tested. In our case, results with the antibiotic treatment suggest that within the satellite bacteria of the untreated culture, the probiotic bacteria have been reduced, decreasing the beneficial effect of the bacterial assemblage for the diatom growth.

Moreover, it does not seem that change induced by the antibiotic treatment in the distribution of the satellite bacteria promoted a shift toward bacterial toxicity, as the overall metabolism of the diatoms was not highly disrupted. Differences due to the two bacterial conditions was not reflected in the C/N ratio for the two diatoms, and correspond to good physiological status of the cells (Table 4). Values obtained for *E. paludosa* (C/N = 8.6) and *H. coffeaeformis* (C/N = 8.2 to 8.5) were similar to ratios measured in culture or *in situ* diatoms species (*E. paludosa*, *T. fluvialis*, *T. pseudonana*) in sufficient nitrogen conditions (Jauffrais et al., 2015, 2016; Marshall Darley, 1977; Rios et al., 1998). The photosynthetic pigments also were not affected by the treatment and chlorophyll *a* content as well as chlorophyll *a* /C remained similar in both culture conditions. Even the main cell components were higher for both diatoms in the cultures treated with antibiotics, at the exception of carbohydrates and total carbon for *H. coffeaeformis* (Fig 3, Table 4). When biochemical components were standardized to the cellular carbon, lipid/C, protein/C, EPS/C and total carbohydrate (including EPS/C) were still higher in the treated cultures and cells increased lipid/C in *H. coffeaeformis* by a factor 2.5 and protein/C in *E. paludosa* by a factor 4 (Table 5). Only carbohydrate/C did not vary significantly between the two cultures conditions (Table 5). Lipids are known to increase and at the inverse proteins to decrease under N limitation (Taguchi et al., 1987; Wainman and Smith, 1997). In our experiments both compounds increased in the cultures treated with antibiotics. Moreover dissolved nitrogen was never exhausted in the culture media (Table 3). Nitrogen availability thus probably did not affect the biochemical pattern observed in the treated and non-treated cultures.

The metabolic regulation between the main cellular components was nonetheless modified in the two diatoms. It is likely that microalgae carbon metabolic pathways and/or bacterial interferences for carbon products were differently regulated. In untreated cultures, despite the decrease of both protein (g Prot/gC: Table 5) and carbon compounds (lipids+total carbohydrates

g/gC; *H. coffeaeformis*: NT = 1.3 and T = 2.4; *E. paludosa*: NT = 2.9 and T = 3.6), the carbon compounds/protein ratio increased for both diatoms (*H. coffeaeformis*: NT = 4.3 and T = 3.1; *E. paludosa*: NT = 8.9 and T = 2.7). This higher ratio in the untreated cultures was also found for all specific carbon compounds: carbohydrate/protein (*H. coffeaeformis*: NT = 2.4 and T = 0.9; *E. paludosa*: NT = 3.8 and T = 1.1); (carbohydrate+EPS)/protein (*H. coffeaeformis*: NT = 3.0 and T = 1.8; *E. paludosa*: NT=5.9 and T=1.9); and lipid/protein (*E. paludosa*: NT = 3.0 and T = 0.7). Only *H. coffeaeformis* lipid/protein ratio did not vary between the two treatments (NT = 1.3 and T = 1.4). All these results suggest a relative higher accumulation of carbon reserves in cells growing with high bacterial biomass, despite a good general physiological status in both culture conditions and similar pigment concentration.

Differences between the two treatments were also illustrated by extracellular carbon (EPS). This cannot be only related to diatoms activity but to the overall community of microalgae and associated bacteria. Complex relationships exist between these two organisms for both growth and carbon flux, bacteria can enhance EPS excretion by microalgae and/or fed on the excreted carbon (Amin et al, 2012; Ramanan et al 2016). Previous studies on the diatom *T. weissflogii* found variable results on transparent exopolymer particles (TEP) secretion related to the bacterial community associated to the algae (Crocker and Passow, 1995; Passow, 2002). In particular addition of specific bacterial strains (e.g. *Marinobacter adhaerens*) enhanced the TEP secretion by this species (Gardes et al., 2011; Gardes et al., 2012). Rhodobacteraceae, known to be part of the bacterial biofilms in mud flat environments (Doghri et al., 2015), might also have similar effect on the EPS concentration. Conversely, diatom EPS were found to promote growth of bacteria (e.g. *Acinetobacter*-related bacteria) in estuarine sediments (Haynes et al., 2007). In the present study, the increase of the assimilated carbon flux toward carbohydrates at the expense of proteins for the two diatoms growing with high bacterial biomass was paralleled by a lower EPS concentration, both as EPS/cell (Figure 3) and relative EPS fraction (g EPS/g

C, Table 5). This results in a lower EPS/cellular carbohydrate ratio in the untreated culture for *H. coffeaeformis* (NT = 0.23 and T = 1) and *E. paludosa* (NT = 0.55 and T = 0.74) and suggests that part of the extracellular carbon released by the diatoms was used by bacteria of the untreated community. Based on the fraction of EPS/cellular carbohydrate measured in treated cultures (Table 5) if the relative carbon excretion by diatoms was similar in the untreated cultures, bacteria would have taken around 39 % of the EPS produced by the diatoms in this condition. That could have stimulated the growth of the two major genus determined in *E. paludosa* untreated cultures, *Winogradskyella* sp. and *Methylophaga* sp., as these bacteria are known to rely on phytoplankton for carbon and energy sources (Bertrand et al., 2015). It is however difficult to evaluate the bacterial part in this carbon balance, as bacteria also excrete and/or transform exopolysaccharides of the biofilm (Grossart et al., 2006a, 2006b; Amin et al., 2012). Nevertheless, results of this study confirmed the close interactions between diatoms physiology and bacteria that are known to characterize biota in benthic environment.

5. Conclusion

The antibiotic treatment was successfully applied against satellite bacteria of the two benthic diatoms *H. coffeaeformis* and *E. paludosa*, belonging to Bacteroidetes (Flavobacteriia) and Proteobacteria (alpha- and gamma-proteobacteria). This allowed to obtain diatom cultures with very low bacterial biomass but without significant modification of the percentage of the three bacterial groups. In untreated cultures, high bacterial abundance promoted cell division, increased growth rate and cellular abundance of *H. coffeaeformis* and *E. paludosa*. Bacteria however did not affect *E. paludosa* final carbon biomass. No major change was observed in both diatoms for cellular carbohydrates, C /N ratio, and pigments (Chl *a*). By contrast, high bacteria biomass in the culture induced lower diatoms cellular proteins and lipids, and extracellular carbon (EPS fraction). This also modified the metabolic fluxes and decreased the

carbon proteic fraction relative to other carbon compounds. Differences in EPS concentrations between bacteria- and bacteria-free cultures suggest a significant use by bacteria of the diatom's excreted carbon. Results of the present study show the mutual beneficial effects of satellite bacteria and benthic diatoms that promoted microalgae growth and maintain the bacterial community.

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Figure 1. A. Growth curves as a function of time of *H. coffeaeformis* and *E. paludosa* in culture with the inocula previously treated (T) or not (NT) with antibiotics (mean \pm SD, n=3). B. Gompertz model fitted to cell concentration as a function of time with the adjusted R^2 for the different conditions.

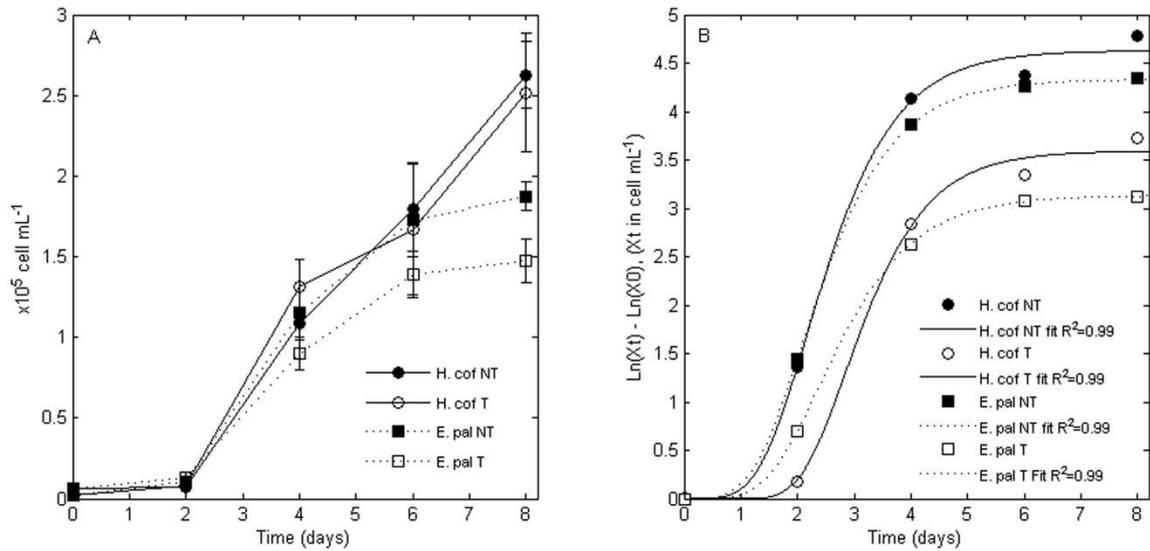


Figure 2. Free living (A) and attached bacteria (B) per cell of *H. coffeaeformis* and *E. paludosa* at the end of the growth experiment (day 8, Fig. 1) in culture with the inocula previously treated (T) or not (NT) with antibiotics. Values with * are significantly different ($p < 0.05$), (mean \pm SD, n=3).

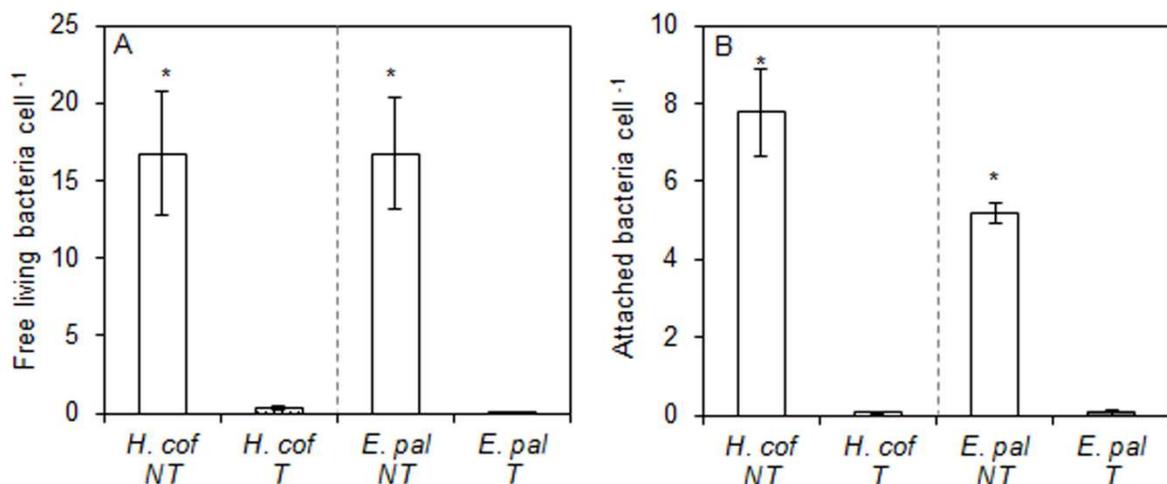


Figure 3. Cellular content at the end of the growth (day 8, Fig.1) in chlorophyll *a* (A), lipids (B), carbohydrates (C) and proteins (D), and dissolved extracellular polymeric substances (EPS) (E) produced by *H. coffeaeformis* and *E. paludosa* in culture with the inocula previously treated (T) or not (NT) with antibiotics. Data are expressed in pg cell^{-1} . Values with * are significantly different ($p < 0.05$), (mean \pm SD, $n=3$).

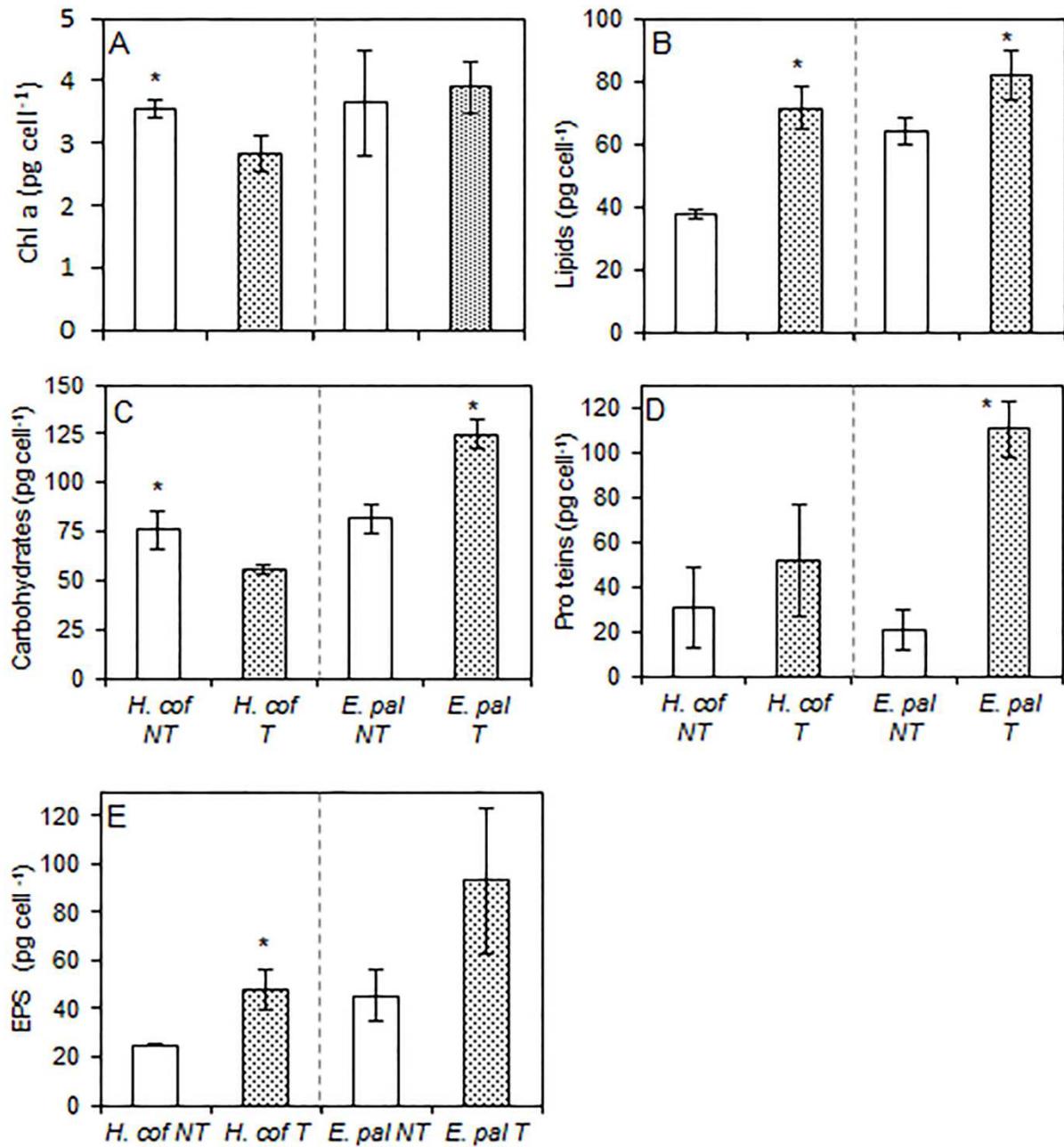


Table 1. Bacterial diversity associated to *E. paludosa* in cultures previously treated (T) or not (NT) with antibiotics (day 8, Fig.1).

OTU	Representative clone	Accession number	% clones	Closest relative strain/type strain	Class	% sequence similarity	Accession number of closest relative
<i>E. pal</i> /NT							
OTU1	Clone 721	KY077818	54 %	<i>Winogradskyella litoriviva</i> (strain KMM6491)	Flavobacteria	95%	NR_137338.1
OTU2	Clone 704	KY077820	28 %	<i>Methylophaga frappieri</i> (strain JAM7)	Gammaproteobacteria	95%	NR_121698.1
OTU3	Clone 708	KY077822	18%	<i>Antarctobacter heliothermus</i> (strain DSM 11445)	Alphaproteobacteria	98%	NR_115889.1
<i>E. pal</i> /T							
OTU1	Clone 805	KY077817	37%	<i>Winogradskyella lutea</i> (strain A73)	Flavobacteria	97%	NR_116855.1
OTU2	Clone 802	KY077819	9%	<i>Methylophaga frappieri</i> (strain JAM7)	Gammaproteobacteria	94%	NR_121698.1
OTU3	Clone 807	KY077821	15%	<i>Antarctobacter heliothermus</i> (strain DSM 11445)	Alphaproteobacteria	98%	NR_115889.1
OTU4	Clone 831	KY077823	9%	<i>Roseibacterium elongatum</i>	Alphaproteobacteria	96%	NR_121734.1
OTU5	Clone 812	KY077816	15%	<i>Stenotrophomonas maltophilia</i> (strain ATCC 19861)	Gammaproteobacteria	99%	NR_040804.1
OTU6	Clone 816	KY094625	5%	<i>Sulfobacter guttiformis</i> (strain Ekho lake-38)	Alphaproteobacteria	99%	NR_029347.1
OTU7	Clone 829	KY094623	5%	<i>Thioalkalivibrio sulfidophilus</i> strain HL-EbGR7	Gammaproteobacteria	88%	NR_074692.1
OTU8	Clone 834	KY094624	5%	<i>Brevundimonas variabilis</i> (strain CB17)	Alphaproteobacteria	98%	NR_037106.1

Table 2. General growth characteristics (mean \pm SD) of *H. coffeaeformis* and *E. paludosa* previously treated (T) or not (NT) with antibiotics.

	Maximum growth rate μ_{\max} (day ⁻¹)*	Lag phase λ (day ⁻¹)*	Mean growth rate μ (day ⁻¹)*	Maximal biomass (10 ⁵ cell mL ⁻¹)	Final carbon biomass (μ g C mL ⁻¹)
<i>H. cof</i> NT	2.01 \pm 0.16	1.34 \pm 25	1.04 \pm 0.07	2.51 \pm 0.36	23.77 \pm 0.73
<i>H. cof</i> T	1.64 \pm 0.10	2.05 \pm 0.08	0.71 \pm 0.03	2.63 \pm 0.21	19.40 \pm 4.51
p-value**	0.029	0.009	0.002	0.672	0.173
<i>E. pal</i> NT	1.80 \pm 0.10	1.20 \pm 0.09	0.97 \pm 0.05	1.88 \pm 0.13	12.49 \pm 0.41
<i>E. pal</i> T	1.28 \pm 0.13	1.48 \pm 0.32	0.66 \pm 0.05	1.47 \pm 0.14	12.27 \pm 1.63
p-value**	0.006	0.212	0.001	0.012	0.831

* μ_{\max} and λ are calculated using the Gompertz model (equation 2), and μ using equation 1

**The p-value is considered significant when $p < 0.05$, $n=3$

Table 3. Concentrations of nitrogen compounds ($\mu\text{M-N}$, mean \pm SD) at the end of the growth (day 8, Fig.1), in the cultures with the two conditions (NT and T).

	NO_3 ($\mu\text{M-N}$)	NH_4 ($\mu\text{M-N}$)	NO_2 ($\mu\text{M-N}$)	Urea ($\mu\text{M-N}$)
<i>H. cof</i> NT	318 \pm 5	0.81 \pm 0.11	4.68 \pm 0.41	2.47 \pm 0.71
<i>H. cof</i> T	346 \pm 13	1.35 \pm 0.11	4.17 \pm 0.50	3.12 \pm 1.20
p-value*	0.025	0.001	0.238	0.461
<i>E. pal</i> NT	287 \pm 75	0.73 \pm 0.55	4.22 \pm 0.87	3.80 \pm 0.86
<i>E. pal</i> T	392 \pm 25	0.27 \pm 0.02	3.24 \pm 0.24	3.06 \pm 0.24
p-value*	0.037	0.212	0.114	0.117

*The p-value is considered significant when $p < 0.05$, $n=3$

Table 4. Cellular content (mean \pm SD) of *H. coffeaeformis* and *E. paludosa* previously treated (T) or not (NT) with antibiotics at the end of the growth experiment (day 8, Fig. 1): nitrogen (PN), carbon (PC), C/N (PC/PN mol/mol).

	PN (pg N cell ⁻¹)	PC (pg C cell ⁻¹)	C/N
<i>H. cof</i> NT	13.61 \pm 2.14	96.01 \pm 15.62	8.23 \pm 0.22
<i>H. cof</i> T	10.25 \pm 2.98	74.60 \pm 21.43	8.49 \pm 0.07
p-value*	0.188	0.234	0.110
<i>E. pal</i> NT	9.07 \pm 0.75	66.77 \pm 7.70	8.57 \pm 0.28
<i>E. pal</i> T	11.30 \pm 1.06	83.29 \pm 7.29	8.61 \pm 0.22
p-value*	0.032	0.035	0.863

*The p-value is considered significant when $p < 0.05$, $n=3$

Table 5. Final cellular content (mean \pm SD) of *H. coffeaeformis* and *E. paludosa* previously treated (T) or not (NT) with antibiotics at the end of the growth experiment (day 8, Fig.1) in chlorophyll *a*, lipids, proteins and carbohydrates, and also in dissolved extracellular polymeric substances (EPS) produced. Data are expressed in per carbon unit (i.e., compound weight/ particulate carbon weight, g g⁻¹).

	Chl a (g Chl a /g C)	Lipids (g Lip /g C)	Proteins (g Prot /g C)	Carbohydrates (g Carbo / g C)	EPS (g EPS /g C)	Total carbohydrates (g Carbo+g EPS) / g C)
<i>H. cof</i> NT	0.04 \pm 0.01	0.39 \pm 0.07	0.30 \pm 0.14	0.74 \pm 0.02	0.17 \pm 0.06	0.91 \pm 0.13
<i>H. cof</i> T	0.04 \pm 0.01	1.01 \pm 0.31	0.76 \pm 0.53	0.69 \pm 0.20	0.70 \pm 0.31	1.39 \pm 0.52
p-value*	0.77	0.028	0.223	0.963	0.073	0.240
<i>E. pal</i> NT	0.05 \pm 0.01	0.96 \pm 0.04	0.32 \pm 0.17	1.23 \pm 0.25	0.68 \pm 0.08	1.91 \pm 0.12
<i>E. pal</i> T	0.05 \pm 0.01	0.99 \pm 0.12	1.31 \pm 0.23	1.50 \pm 0.10	1.11 \pm 0.33	2.61 \pm 0.24
p-value*	0.126	0.734	0.017	0.085	0.09	0.068

*The p-value is considered significant when $p < 0.05$, $n=3$