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1 **Functional diversity of microboring *Ostreobium* algae isolated from corals**

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18 nitrogen uptake, chlorophyll, fatty acids

19

20 **Running head:** Physiology of algal microborers of coral carbonate

21

22 **Abstract**

23 The filamentous chlorophyte *Ostreobium* sp. dominates shallow marine carbonate
24 microboring communities, and is one of the major agents of reef bioerosion. While its large
25 genetic diversity has emerged, its physiology remains little known, with unexplored
26 relationship between genotypes and phenotypes (endolithic *versus* free-living growth forms).
27 Here, we isolated 9 strains affiliated to 2 lineages of *Ostreobium* (>8% sequence divergence
28 of the plastid gene *rbcL*), one of which was assigned to the family Odoaceae, from the fast-
29 growing coral host *Pocillopora acuta* Lamarck 1816. Free-living isolates maintained their
30 bioerosive potential, colonizing pre-bleached coral carbonate skeletons. We compared
31 phenotypes, highlighting shifts in pigment and fatty acid compositions, carbon to nitrogen
32 ratios and stable isotope compositions ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$). Our data show a pattern of higher
33 chlorophyll *b* and lower arachidonic acid (20:4 ω 6) content in endolithic *versus* free-living
34 *Ostreobium*. Photosynthetic carbon fixation and nitrate uptake, quantified via 8h pulse-
35 labeling with ^{13}C -bicarbonate and ^{15}N -nitrate, showed lower isotopic enrichment in endolithic
36 compared to free-living filaments. Our results highlight the functional plasticity of
37 *Ostreobium* phenotypes. The isotope tracer approach opens the way to further study the
38 biogeochemical cycling and trophic ecology of these cryptic algae at coral holobiont and reef
39 scales.

40

41 **Introduction**

42 The microscopic algal biodiversity dissolving actively (or eroding) shallow-water
43 carbonates is a cryptic yet essential component of reef functioning, becoming dominant with
44 the general decline of coral reefs worldwide (Leggat *et al.*, 2019; Tribollet *et al.*, 2019).
45 Pioneer morphological observations showed that bioeroding filaments of the chlorophyte
46 *Ostreobium* are ubiquitous inside the skeleton of tropical coral reef-builders, both in dead and
47 actively growing colonies (Odum & Odum, 1955; Lukas, 1974; Le Campion-Alsumard *et al.*,
48 1995a; reviewed in Tribollet, 2008; Golubic *et al.*, 2019). Molecular data have recently
49 accumulated, based on amplicon sequencing of plastid encoded gene markers (*rbcL*, *tufA*,
50 UPA and 16S rRNA), revealing *Ostreobium* ubiquity in the core microbiome of tropical
51 corals and its high genetic diversity, delimiting an entire Ostreobineae suborder within the
52 Bryopsidales in the class Ulvophyceae (Gutner-Hoch & Fine, 2011; Marcelino & Verbruggen,
53 2016; Sauvage *et al.*, 2016; del Campo *et al.*, 2017; Marcelino *et al.*, 2017; Verbruggen *et al.*,

54 2017; Gonzalez-Zapata *et al.*, 2018; Marcelino *et al.*, 2018; Massé *et al.*, 2018). By chemical
55 means, *Ostreobium* filaments actively penetrate reef carbonates ranging from limestone rocks
56 to seashells and coral skeletons, creating galleries a few micrometers in diameter (Tribollet,
57 2008), thus living as true boring endoliths (i.e. euendoliths also called microborers; Golubic *et*
58 *al.*, 1981). Surprisingly, filaments of this photosynthetic chlorophyte can even be detected in
59 microboring communities down to 200 m depth in tropical ecosystems (Littler *et al.*, 1985;
60 Vogel *et al.*, 2000; reviewed in Tribollet *et al.*, 2011). Occasionally, *Ostreobium* filaments can
61 exit carbonate skeletons of coral holobionts or reef rubble to become epilithic (Kobluk &
62 Risk, 1977) and free-living filaments can be detected in the environment in seawater or
63 benthic biofilms (Massé *et al.*, 2018). Filaments can also be released from their calcium
64 carbonate substratum in culture (Kornman & Sahling, 1980; Sauvage *et al.*, 2016). In
65 declining reefs impacted by coral bleaching events and overfishing of algal grazers (Hughes *et*
66 *al.*, 2017; Roth *et al.*, 2018), the prevalence of free-living *Ostreobium* filaments is likely to
67 increase after detachment of epilithic filaments emerged from damaged coral colonies or reef
68 rubble (Leggat *et al.*, 2019), as a result of wave action during cyclone or storm events.

69 In massive adult coral colonies with a slow-growth, the endolithic layer dominated by
70 bioeroding *Ostreobium* filaments forms visible green bands just beneath the coral tissues
71 (Lukas, 1974; Le Campion *et al.*, 1995a). In contrast, in fast-growing branching corals those
72 green bands are absent, but *Ostreobium* filaments are still present, although at decreased
73 abundance in the skeleton towards branch tips (Godinot *et al.*, 2012; Massé *et al.*, 2018).
74 Recently, Massé *et al.* (2018) showed the horizontal transmission of *Ostreobium* from benthic
75 biofilms or propagules dispersed in seawater to a fast-growing *Pocillopora* coral host. These
76 microboring algae penetrate first into the skeleton of coral recruits, as soon as the primary
77 polyp is formed after larval metamorphosis (Massé *et al.*, 2018) and then follow the coral
78 vertical extension in order to access enough light to survive (Halldal, 1968; Shibata & Haxo,
79 1969; Shashar & Stambler, 1992). Several functional roles have been suggested for
80 *Ostreobium* (and other microborers) in living corals, such as nutrient recycling (Ferrer &
81 Szmant, 1988) and a possible ectosymbiotic relationship between microboring communities
82 dominated by *Ostreobium* and their coral host (Schlichter *et al.*, 1995; Fine & Loya, 2002;
83 Sangsawang *et al.*, 2017). However, assimilation of inorganic carbon and nitrogen has not
84 been quantified for individual, genetically referenced *Ostreobium* members of the skeleton
85 microbiome, and sites of putative active transfer of metabolites from algal filament to host
86 tissue are yet to be demonstrated in live reef corals. By contrast, in dead coral skeletons, the

87 ecological roles of *Ostreobium* dominated assemblages have been more intensively studied.
88 At complex community level, this microscopic alga is indeed, in synergy with bioeroding
89 sponges and the grazing macrofauna such as parrotfishes, one of the major agents of
90 bioerosion and calcium carbonate recycling (Tribollet & Golubic, 2005; Schönberg *et al.*,
91 2017). Together with other microboring phototrophs, it is also an important benthic primary
92 producer and thus, an important keystone in coral reef food web (Odum & Odum, 1955;
93 Vooren, 1981; Tribollet *et al.*, 2006; Clements *et al.*, 2016).

94 To date, very little is known about the functional diversity of *Ostreobium* algae.
95 Functional traits such as pigment and fatty acid compositions may vary in endolithic *versus*
96 free-living growth habit and between specific genetic lineages. Moreover, it is not clear to
97 which extent carbon (C) and nitrogen (N) sources and uptake rates may change to cover
98 contrasting *Ostreobium* energy needs as endolithic filaments within carbonate substrate *versus*
99 free-living filaments in seawater. Analyses of fatty acids and C and N stable isotopes could
100 provide information on autotroph and/or heterotroph sources of carbon and nitrogen for
101 *Ostreobium* filaments depending on their habitat. Pigment composition, and inorganic carbon
102 and nitrogen uptake may also indicate adaptation of metabolic activity. Phenotypic studies of
103 *Ostreobium* genetic lineages isolated from corals are thus crucial to better understand the role
104 of these carbonate microboring algae at coral holobiont and reef ecosystem scales, and how
105 they are impacted by environmental changes (Schönberg *et al.*, 2017; Pernice *et al.*, 2019;
106 Ricci *et al.*, 2019). This requires to compare in controlled laboratory settings the physiology
107 of contrasting growth forms (phenotypes) of *Ostreobium*, i.e. endolithic filaments colonizing
108 live coral colonies and reef carbonates *versus* free-living filaments.

109 Mono-algal cultures of *Ostreobium quekettii* Bornet and Flahaut 1889, which is the
110 type species of the genus *Ostreobium*, were initially isolated from shells of a temperate
111 mollusk in Brittany (France) (Bornet & Flahaut, 1889). Free-living strains 6.99 and B14.86
112 designated as *Ostreobium quekettii* were later used for further morphology and reproduction
113 studies (Kornmann & Sahling, 1980), and trophic potential and photoecology investigations
114 (Schlichter *et al.*, 1997). Other free-living *Ostreobium* strains have since been isolated from
115 reef-collected marine carbonates, genotyped with plastid encoded *tufA*, UPA and 16S rRNA
116 gene markers (Sauvage *et al.*, 2016; Marcelino & Verbruggen, 2016) and then used for
117 chloroplast genome sequencing and phylogenetic studies (Marcelino *et al.*, 2016; del Campo
118 *et al.*, 2017; Verbruggen *et al.*, 2017). Cultures of the endolithic form of *Ostreobium* have
119 however seldom been characterized physiologically, except for a very recent study on strain

120 6.99 of *Ostreobium quekettii* Bornet and Flahaut 1889, that showed filament-driven processes
121 of coral carbonate dissolution–reprecipitation and calcium transport (Krause *et al.*, 2019).

122 In this study we developed an *in vitro* approach to compare the physiological
123 characteristics of endolithic *versus* free-living *Ostreobium* filaments isolated from the fast
124 growing, small polyp coral model species *Pocillopora acuta* Lamarck 1816. We genotyped
125 nine strains of *Ostreobium* based on amplicon sequencing of the *rbcL* plastid gene marker,
126 and characterized successive subcultures of these strains in either endolithic (coral carbonate
127 eroding) or free-living growth habit to provide (see Table 1): (i) photosynthetic and accessory
128 pigment composition, (ii) fatty acid composition, (iii) bulk tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ stable isotope
129 values and (iv) inorganic C and N assimilation patterns, measured via uptake of ^{13}C -
130 bicarbonate and ^{15}N -nitrate stable isotope tracers.

131

132 **Results**

133 *Isolation and genotyping of Ostreobium strains to species-level rbcL clades*

134 A total of nine *Ostreobium* strains were isolated from branch tips of *Pocillopora acuta*
135 corals from long-term aquarium cultures (Aquarium Tropical, Palais de la Porte Dorée, Paris,
136 Fr, called ATPD-aquarium). After removal of coral tissues, branched filaments with typical
137 *Ostreobium* morphology emerged after ~3 weeks from the skeleton of 2 out of 3 colonies
138 (*Ostreobium* filaments did not emerge from the skeleton of one of the 3 colonies), forming
139 yellowish-green tufts of filaments which were pulled out or cut with a scalpel to initiate
140 cultures in free-living form. Siphonous filaments had a diameter varying between 5 and 12
141 μm (Fig. 1b), with small disc-shaped chloroplasts visible in the periphery of siphons, against
142 their inner sheath. Reproduction by spore formation was not observed. Mono-algal cultures,
143 established via serial sub-culturing (successive passages) of such *Ostreobium* filaments, have
144 been propagated *in vitro* in free-living and endolithic forms (see below) since September
145 2016, with vouchers deposited in the Museum national d'Histoire naturelle (Paris, France)
146 RBCell collection of microalgae and cyanobacteria (MNHN-ALCP-2019-873.1 to MNHN-
147 ALCP-2019-873.8; see Table 1).

148 The algal isolates were assigned to 3 species-level *Ostreobium* genotypes (clades)
149 defined by 99% sequence similarity thresholds of the chloroplast-encoded RuBisCo large

150 subunit *rbcL* gene (see Genbank Accession numbers in Table 1). The *rbcL* phylogeny showed
151 that the majority (7/9) of *Ostreobium* strains (obtained from 2 out of 3 host coral colonies)
152 clustered into one P1 clade (99% similarity over ~729 nt for 6 strains, and over 375 nt for
153 strain 022). Clade P1 is the dominant *Ostreobium* lineage detected in *Pocillopora acuta* corals
154 from long-term cultures at the ATPD-aquarium, and is also detected in *Pocillopora verrucosa*
155 from the Red Sea Eilat IUI reef (Massé *et al.*, 2018). The two other strains, named 018A and
156 06 (each isolated from a single coral colony) were assigned to distinct *rbcL* clades
157 (OTU>99%), named P12 and P14, respectively. Thus, although one coral colony did not
158 provide emerging *Ostreobium* filaments, each of the two other colonies harbored two co-
159 occurring *Ostreobium* lineages, with over-representation of one dominant (P1) genotype.

160 A phylogenetic tree was built from alignment of overlapping *rbcL* sequences (161 nt
161 length barcode) to assess the strains' diversity (Fig. S1). Congruent phylogenetic trees and
162 lineage affiliations were also obtained for longer *rbcL* sequence alignments, built over 729 nt
163 for strains 05, 010, 019, 018A, 018B, 018C, and 346 nt for strains 06 and 022 (data not
164 shown). Both clades P12 and P14 were putative sister species-level entities (OTU>99%),
165 related at genus level (>97% sequence similarity threshold) to a clade P3 previously reported
166 in aquarium-grown *Pocillopora acuta* colonies (Massé *et al.*, 2018; ATPD and Océanopolis
167 aquariums), and also detected in Pacific reef-collected *Pocillopora* sp. (from Gambier
168 Archipelago, Massé, pers. obs.). Indeed, sequence divergence between P12 and P3, and
169 between P14 and P3 was ~2%. Both P12 and P14 *rbcL* genotypes clustered together with
170 formerly detected clade P3 into a genus-level lineage (OTU>97%), while P1 *rbcL* genotype
171 was treated separately, as a distinct species-level lineage (OTU>99%). The overall *rbcL*
172 sequence divergence between those lineages was ~8-10%, with P1 genotype 91.9% similar to
173 P12 genotype and 90.6% similar to P14 genotype.

174 For comparison purposes with *tufA*-based *Ostreobium* classification, sequences of the
175 taxonomical marker *tufA* were also retrieved from available chloroplast genomes of the
176 reference strains *Ostreobium* sp. HV05042 (KY509314) and HV05007a (KY509315) isolated
177 from *Diploastrea* corals (Verbruggen *et al.*, 2017), which *rbcL* sequences best matched with
178 the sequences of our strains (Fig. S1). These *tufA* reference sequences aligned with 97.9%
179 similarity (over 484 nt) to the *tufA* sequence of *Ostreobium* sp. TS1408 (KU362015) which
180 was affiliated to the family Odoaceae (Sauvage *et al.*, 2016), awaiting formal description.
181 This result indicates that the *Ostreobium* strains in P12/P14 lineage were affiliated to the

182 (*tufA*) Odoaceae family (OTU>92%), while P1 strains represented a species-level genotype
183 belonging to a potentially different family.

184 *Bioerosive potential in culture conditions*

185 Free-living *Ostreobium* filaments (Fig. 1a, b) were exposed during several weeks to
186 coral skeletal chips measuring a few millimeters in thickness and prepared from pre-bleached
187 *Pocillopora acuta* (hypochlorite cleaned to remove external organic matter). The *Ostreobium*
188 filaments showed attachment to the skeleton surface (epilithic growth habit; Fig. 1a) and entry
189 of some algal filaments perpendicular to the substrate surface (endolithic growth habit). This
190 colonization by endolithic *Ostreobium* was observed by light microscopy on thin sections
191 (Fig. 1c). Toluidine blue-stained filaments penetrating the substrate formed a thick branching
192 network inside the carbonate (Fig. 1c). Galleries formed by this microboring alga
193 (microboring traces called *Ichnoreticulina elegans*; Radtke, 1991) were also observed by
194 scanning electron microscopy (Fig. 1d), below the skeletal surface, confirming *de novo*
195 colonization of the exposed carbonate by endolithic *Ostreobium*. Filaments penetrated
196 through all coral skeletal structures, i.e. growing across bundles of skeletal fibers and centers
197 of calcification without obvious preference for either microstructure. These observations
198 prove that the *Ostreobium* isolates kept their bioerosive potential *in vitro*, even after
199 propagation through several subcultures as free-living forms.

200 *Photosynthetic and accessory pigments*

201 Qualitatively, the pigment composition of *Ostreobium* strains belonging to lineage P1
202 (strains of less frequent lineage P12/P14 were not studied) was similar for endolithic and free-
203 living forms (3 and 5 individual strains, respectively, see Table 1). Indeed, similar
204 photosynthetic (chlorophyll) and accessory (carotenoid) pigments were detected for both
205 growth forms in high-performance liquid chromatography (HPLC) fingerprints of organic
206 extracts, although their relative abundance varied (Fig. S2b; with mean retention times
207 (\pm Standard Deviation) for the 58 peaks detected provided in Table S1). In contrast, profiles of
208 control bleached skeletons (uncolonized by endolithic *Ostreobium*) contained only three
209 peaks, rarely observed and at very low intensity in endolithic *Ostreobium* (Table S1).

210 Comparison with pure commercial standards allowed identification in endolithic and
211 free-living *Ostreobium* of chlorophyll *a* (chl *a*) in the profiles recorded at 664 nm at retention
212 time (RT) 37.2 ± 0.5 min (with chl *a* allomers at RT 34.6 ± 0.5 min, RT 36.6 ± 0.4 min and RT
213 38.8 ± 0.5 min) (Fig. S2a). Chlorophyll *b* (chl *b*) was also detected in the profiles recorded at

214 470 nm, at retention time 30.5 ± 0.5 min (with chl *b* allomers at RT 27.9 ± 0.4 and RT 32.3 ± 0.5
215 min) (Fig. S2a).

216 The non-metric multidimensional scaling (nMDS) analysis of profiles showed a great
217 variation of pigment content across strains and subcultures for each *Ostreobium* growth form.
218 Despite this variation, nMDS showed a separation between endolithic and free-living
219 *Ostreobium* phenotypes (except for the free-living subculture 018B¹ which was close to its
220 corresponding endolithic form) (Fig. 2a2, stress=0.06), with an average dissimilarity of 53%
221 (SIMPER analysis). This dissimilarity between phenotypes was attributed to differences in
222 their photosynthetic pigment proportions, i.e. chl *a*, chl *b*, an allomer of chl *b* (RT 27.9 ± 0.4
223 min) and two allomers of chl *a* (RT 34.6 ± 0.5 and 36.6 ± 0.4 min), with relative contribution of
224 14.6%, 13.8%, 10.9%, 7.6% and 5.6%, respectively. Pigment content of both *Ostreobium*
225 growth forms were distinct from the extracts of control bleached skeletons (Fig. 2a1,
226 stress=0.01). A PCA analysis of pigment relative content (not illustrated) showed congruent
227 data point distribution of strains, separating endolithic *versus* free-living phenotypes (except
228 for 018B¹), with chl *a* and chl *b* and their allomers as main drivers of the distribution pattern.

229 Quantitatively, a high inter-individual variability was noted for chlorophyll content
230 (chl *a* and chl *b*, and their respective allomers) especially across free-living strains and their
231 successive subcultures (illustrated in Fig. 2b and detailed in Table S2). Overall, despite
232 variable chl *a* content between subcultures of the free-living phenotype, chl *b* contents were
233 higher in endoliths (8.9 ± 1.2 µg/mg organic extract, respectively; SE, n=3) than free-living
234 forms (1.0 ± 0.3 µg/mg organic extract, respectively; SE, n=8). The ratio chl *b* : chl *a* was four
235 times higher in endolithic (0.84 ± 0.18 , SE, n=3) than in free-living (0.21 ± 0.03 , SE, n=8)
236 *Ostreobium* (Table S2).

237 *Fatty acid composition*

238 Qualitatively, the fatty acid (FA) composition of *Ostreobium* strains belonging to P1
239 and P12/P14 lineages was similar between endolithic and free-living forms (Table S3).
240 Representative fatty acid profiles obtained by gas chromatography (GC) are illustrated in
241 Figure S3. A total of 31 FAs were detected in *Ostreobium* strains, dominated by saturated
242 fatty acids (SFA) in endoliths *versus* polyunsaturated fatty acids (PUFA) in free-living forms
243 (Table S3). In contrast, only 5 saturated FAs (16:00, 18:00, 14:00, 15:0 and 12:0) were found
244 in control bleached skeletons (detailed in Table S3).

245 Quantitatively however, PCA and barplot analysis showed variability of FA content
246 (relative proportions) across *Ostreobium* strains and subcultures for each phenotype (Fig. 3).
247 Despite this variability, a general trend could be observed and showed a different FA content
248 in *Ostreobium* strains belonging to clade P1 (black color code, Fig. 3a) than in the majority of
249 their free-living counterparts (for all cultures but 010, 018B¹, 019, see also Fig. 3c). For clade
250 P12/P14, due to low number of available strains and limited available endolithic biomass, the
251 recorded similarity of FA content of endoliths and free-living forms is provisional because it
252 was based on a single determination for endolithic strain 06 (see Table S3).

253 The poly-unsaturated fatty acid content explained 86% of the variability between
254 endolithic and free-living phenotypes (PC1, Fig. 3a, 3b). Indeed a major difference was due to
255 arachidonic acid (20:4 ω 6) content increased by a factor ranging from 2.2 to 30 in P1 lineage,
256 from endolithic to free-living forms (illustrated in Fig. 3c, see also Table S3; a large range
257 attributed to variable physiological status across subcultures). Biosynthetic intermediates of
258 arachidonic acid, i.e. 16:3 ω 6, 18:2 ω 6 and 18:3 ω 6 were detected in endolithic *Ostreobium*,
259 like in free-living forms, but without longer-chain 20:4 ω 6 fatty acid. Among detected mono-
260 unsaturated fatty acids, 18:1 ω 7 was abundant in both *Ostreobium* forms, as well as 18:1 ω 9
261 which was simultaneously detected with 18:2 ω 6. Trace amounts (<0.5%) of methyl-branched
262 fatty acids (15:0iso, 15:0anteiso and 16:0iso) and long-chain PUFAs (20:5 ω 3, 22:5 ω 6 and
263 22:6 ω 3) were also detected in both *Ostreobium* forms, in variable amounts across strains and
264 their subcultures.

265 *C/N ratios and stable isotope values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$)*

266 The carbon to nitrogen (C/N) ratios in endolithic *Ostreobium* filaments removed from
267 their surrounding carbonate (by acid-decalcification), did not vary much among strains and
268 subcultures and between lineages, with mean C/N of 12.6 \pm 0.6 for lineage P1 (n=5, SD) and
269 11.9 \pm 0.3 for lineage P12/P14 (n=4, SD) (Table S4). These values are close to those measured
270 in the residual organic matrix from control acid-decalcified bleached skeletons (mean C/N
271 ratios of 11.1 \pm 1.1, n=4, SD). Conversely, in free-living forms, the C/N ratio varied greatly
272 among strains within both P1 and P12/P14 lineages (C/N of 16.3 \pm 2.5 n=9, SD for P1, and
273 14.3 \pm 1.4 n=4, SD for P12/P14), with more stable ratios between subcultures of each strain.
274 The overall pattern indicates that C/N ratios were higher in free-living than in endolithic
275 filaments of *Ostreobium*.

276 Stable isotope values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of endolithic (not decalcified, or acid-
277 decalcified) and free-living *Ostreobium* forms are presented in Figure 4 along with controls,
278 i.e. bleached coral skeleton substrate and its residual organic matrix. Detailed $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$
279 values of individual strains and growth forms are also presented in Table S5. Results showed
280 high $\delta^{13}\text{C}$ variability among strains and their subcultures, within and between each genetic
281 lineage (P1 and P12/14), for each growth form. Despite this variability, data of decalcified
282 endolithic *Ostreobium* were pooled as mean $\delta^{13}\text{C}$ values were not significantly different
283 between lineages ($p>0.05$). For the same reason, data of free-living *Ostreobium* were also
284 pooled (Fig. 4A). The non-decalcified endolithic *Ostreobium* belonging to P1 (non-
285 decalcified P12/P14 were not measured) were significantly ($p<0.05$) more enriched in ^{13}C (-
286 11.2 ± 0.8 ‰ SD, $n=4$) than the free-living phenotypes (-18.6 ± 2.6 ‰ SD, $n=16$ for all clades).
287 In contrast, decalcified endolithic *Ostreobium* were significantly ($p<0.05$) more depleted in
288 ^{13}C ($\delta^{13}\text{C}$ -23.8 ± 3.6 ‰ SD, $n=13$ for all clades) than the free-living forms. This depletion is
289 partly explained by the acid treatment of endolithic filaments to remove the carbonate. Indeed
290 a parallel control test of this acid treatment on free-living *Ostreobium* filaments showed a
291 significant ($p<0.05$) -6 ‰ amplitude depletion of $\delta^{13}\text{C}$ (i.e. -26.9 ± 2.6 ‰ SD, $n=3$ treated
292 *versus* -20.9 ± 2.6 ‰ SD, $n=3$ untreated, technical replicates of strain 010, Table S5). Thus,
293 acid treatment corrected $\delta^{13}\text{C}$ values of decalcified endolithic *Ostreobium* strains were on
294 average -17.8 ± 3.6 ‰ (SD, $n=13$ for all clades). However, when strain 010 was excluded from
295 the pool of data as it seems to behave differently from the other strains, decalcified endolithic
296 strains still appeared depleted in ^{13}C (corrected $\delta^{13}\text{C}$: -20.1 ± 2.3 ‰ SD, $n=8$, for all strains
297 except 010) compared to their corresponding free-living form (see Table S5).

298 The mean $\delta^{13}\text{C}$ of endolithic *Ostreobium* was compared to that of their habitat (coral
299 skeleton substrate) to track shifts indicative of potential C sources. Results showed that the
300 $\delta^{13}\text{C}$ values in undecalcified endolithic *Ostreobium* were higher (-11.2 ± 0.8 ‰ SD, $n=4$ clade
301 P1 strain 010) than those of control skeleton substrates (-13.9 ± 0.03 ‰ SD, $n=3$). Such ^{13}C
302 enrichment ($+2.7$ ‰) was also observed and was significant ($p<0.05$) using another sample
303 preparation method, the automated carbonate preparation device Kiel IV (endolithic
304 *Ostreobium* $\delta^{13}\text{C}$ -10.6 ± 0.4 ‰ SD, $n=8$ clade P1 strain 010 *versus* carbonate substrate $\delta^{13}\text{C}$ -
305 13.3 ± 0.4 ‰ SD, $n=8$). Regarding decalcified endolithic *Ostreobium*, their $\delta^{13}\text{C}$ values were
306 higher (-23.8 ± 3.6 ‰ SD, $n=13$) than those of their control decalcified skeleton substrate, i.e.
307 the coral skeletal organic matrix (-29.4 ± 1.3 ‰ SD, $n=4$).

308 For $\delta^{15}\text{N}$ (Fig. 4B), similarly to $\delta^{13}\text{C}$, high inter-individual variability was observed for
309 each growth form, between strains and their subcultures and within each genetic lineage (P1
310 *versus* P12/14). However, this variability was much higher for the decalcified endoliths (+4.3
311 ‰ min, +17.9 ‰ max), than for the free-living forms (+1.4 ‰ min, +9.0 ‰ max). The
312 lineage had a significant effect on mean $\delta^{15}\text{N}$ of free-living *Ostreobium* ($p>0.05$) but not on
313 that of decalcified endoliths (due to high variability within lineage). In order to compare N
314 sources between growth forms, data for both genetic lineages were pooled. Results showed
315 that the $\delta^{15}\text{N}$ values of most decalcified endolithic *Ostreobium* strains were significantly
316 enriched (14.3 ± 3.1 ‰ SD, $n=8$ for all clades, except strain 010) compared to the $\delta^{15}\text{N}$ values
317 of their corresponding free-living forms (5.3 ± 2.1 ‰ SD, $n=10$ for all clades, except strain
318 010). Only for strain 010 within P1 (dotted circles in Fig. 4B), the recorded $\delta^{15}\text{N}$ values were
319 low and similar for both the non-decalcified and decalcified endolithic form (4.2 ± 0.6 ‰ SD,
320 $n=3$ and 4.9 ± 0.4 ‰, SD, $n=5$, respectively), but still enriched compared to the $\delta^{15}\text{N}$ of their
321 free-living counterparts (2.7 ± 0.7 ‰ SD, $n=6$). There was no effect of the acid treatment as
322 $\delta^{15}\text{N}$ values of control treated or untreated free-living forms were not significantly different
323 (2.2 ± 1.2 ‰ SD, $n=3$ treated *versus* 2.3 ± 0.5 ‰ SD, $n=3$ untreated, technical replicates of
324 strain 010; Table S5). Compared to $\delta^{15}\text{N}$ of the coral skeletal organic matrix (5.5 ± 0.5 ‰ SD,
325 $n=4$), most values of the decalcified endolithic *Ostreobium* strains (except strain 010) were
326 enriched (14.3 ± 3.1 ‰ SD, $n=8$) corresponding to a +9‰ average shift.

327 *Photosynthetic C and N assimilation*

328 Bicarbonate and nitrate uptake by endolithic *Ostreobium* and their corresponding free-
329 living forms ($n=3$ strains of P1 lineage, and $n=2$ strains of P12/P14 lineage) are illustrated in
330 Figure 5, in relation to pH changes during the 8h dual labeling pulse with ^{13}C -bicarbonate and
331 ^{15}N -nitrate, under light or dark conditions. Different patterns of C and N assimilation were
332 recorded between endolithic *versus* free-living phenotypes, despite strain-related variations.
333 Indeed a high metabolic variability was observed among strains within each lineage (P1 or
334 P12/P14). Due to low replication, the lineage effect could not be tested (experimental
335 culturing difficulties limited to 2 the number of available P12/P14 strains and strain 010 in P1
336 was analyzed only in light conditions).

337 Fixation of ^{13}C -bicarbonate (Fig. 5a) in light condition was lower in endolithic forms
338 (973 ± 616 ‰ SD, $n=3$) than in free-living forms (2392 ± 405 ‰ SD, $n=3$) for three strains: 05
339 (P1) and 06, 018A (P12/P14). However, strains 022 and 010 (both in P1) displayed an

340 opposite pattern. In dark conditions, very little ^{13}C enrichment was recorded for both
341 endolithic (14.9 ± 10.2 ‰ SD, $n=4$) and free-living *Ostreobium* (30.5 ± 11.6 ‰ SD, $n=4$) for all
342 strains, indicating that ^{13}C enrichment resulted from photosynthesis. Indeed, in light
343 conditions photosynthetic activity was confirmed by the rise in pH during the 8h labeling
344 pulse, for both endolithic ($+0.11\pm 0.03$ SD, $n=5$) and free-living *Ostreobium* ($+0.26\pm 0.15$ SD,
345 $n=5$). For endolithic phenotypes, seawater alkalization may also result from carbonate
346 dissolution activity, in addition to photosynthesis. Although the pH increase was much lower
347 in endolithic than free-living forms (consistent with lower biomass of filaments: 0.9 ± 0.4 mg
348 vs 5.9 ± 2.6 mg dry weight, $n= 5$, respectively), large pH increase corresponded to high ^{13}C -
349 enrichment, except for the free-living form of strain 010 (clade P1). In dark conditions, pH
350 did not vary for the free-living forms while it increased for the endolithic forms ($+0.1\pm 0.01$
351 SD, $n=4$), indicative of carbonate dissolution processes.

352 Assimilation of ^{15}N -nitrate (Fig. 5b) was recorded at the end of the 8h labeling pulse in
353 both endolithic and free-living *Ostreobium* in light and also in dark conditions. Uptake of ^{15}N -
354 nitrate was however 40% reduced in dark compared to light conditions. In light, assimilation
355 of ^{15}N -nitrate was lower in endolithic forms (1253 ± 556 ‰ SD, $n=4$) than in free-living forms
356 (2346 ± 442 ‰ SD, $n=4$) for four strains (06, 018A, 022, 05). Strain 010 displayed an opposite
357 pattern. In dark conditions, ^{15}N -assimilation was also lower in endolithic forms (567 ± 384 ‰
358 SD, $n=4$) than in free-living forms (1552 ± 81 ‰ SD, $n=4$).

359 We calculated the turnover of exogenous inorganic ^{13}C and ^{15}N (i.e. the rate of
360 inorganic C and N used for cell growth) in endolithic *versus* free-living filaments for a 12h
361 photoperiod, by extrapolation of the uptake data recorded at the end of the 8h pulse of ^{13}C -
362 bicarbonate and ^{15}N -nitrate (equation in Material & Methods and data for each strain in Table
363 S6). In daylight, C and N turnover rates varied among strains for both phenotypes. Although
364 variability between technical replicates within a same strain was low (SD<5% of the mean for
365 free-living strain 010), differences were observed between genetic lineages (Table S6).
366 Indeed, C and N turnover increased by a factor ~ 3 in P12/P14 compared to P1 when
367 *Ostreobium* filaments lived free in the culture medium, whereas P12/P14 was less active than
368 P1 when filaments lived inside carbonate substrate (with C and N turnover ~ 60 and 50 %
369 weaker, respectively). Regarding phenotypes, C and N turnover in P1 were relatively similar
370 between growth forms, whereas in P12/P14 it decreased by a factor 3.6 for C, and a factor 2.7
371 for N in endoliths compared to free-living *Ostreobium* forms. In dark conditions, C turnover
372 for both phenotypes was very low (<0.1 % / 12h period, Table S6). In contrast, N turnover

373 was detected for both phenotypes but at reduced level compared to light conditions. For free-
374 living *Ostreobium*, N turnover was similar among strains and between genetic lineages (Table
375 S6). For endolithic *Ostreobium*, N turnover greatly varied among strains within lineage P1,
376 whereas lower variability was observed within P12/P14 (Table S6). Regarding phenotypes, N
377 turnover decreased in endolithic compared to free-living *Ostreobium*, by a factor 2.2 for
378 lineage P1 and 3.3 for lineage P12/P14. Combined together, these C and N turnover results
379 indicated a lower efficiency of dissolved inorganic carbon and nitrogen (DIC & DIN) uptake
380 from seawater by endolithic *Ostreobium* than by their free-living counterparts.

381 **Discussion**

382 *Diversity of Ostreobium strains from Pocillopora corals*

383 Despite rapidly growing evidence of the high genetic diversity of the microboring
384 Chlorophyta *Ostreobium* in living corals in reef ecosystems (Marcelino & Verbruggen, 2016;
385 Sauvage *et al.*, 2016; Marcelino *et al.*, 2017; del Campo *et al.*, 2017; Gonzalez-Zapata *et al.*,
386 2018), phenotypic characterization of specific *Ostreobium* genotypes has lagged behind.
387 Indeed, phenotype studies have so far focused only on strains 6.99 and B14.86 designated as
388 *Ostreobium quekettii* Bornet & Flahaut 1889 (Kornmann & Sahling, 1980; Schlichter *et al.*,
389 1997; Krause *et al.*, 2019). Phenotypic comparisons of *Ostreobium* strains belonging to
390 distinct genetic lineages are key to better understand the functional diversity of this
391 microboring alga in reef ecosystems. Here, the nine *Ostreobium* strains obtained from the
392 fast-growing branching coral *Pocillopora acuta* Lamarck 1816 add to the diversity of the few
393 coral-isolated strains (mostly from massive, slow-growing coral hosts) that were so far used
394 only for phylogenetic studies (Verbruggen *et al.*, 2017; Sauvage *et al.*, 2016). Although
395 isolated from aquarium-grown, long-term propagated coral colonies, these *Ostreobium* strains
396 are representative of 2 lineages from reef settings, including a lineage provisionally assigned
397 to the family Odoaceae (*sensu* Sauvage *et al.*, 2016), which are also detected in natural
398 communities of *Pocillopora* corals from Northern Red Sea and South Pacific reefs (Massé *et*
399 *al.*, 2018; Verbruggen *et al.*, 2017).

400 Fast-growing branching corals are known to harbor less abundant and diverse
401 *Ostreobium* than slow-growing massive corals, with patchy spatial distribution along the
402 branch growth axis (Godinot *et al.*, 2012; Massé *et al.*, 2018; Marcelino *et al.*, 2017). Culture
403 bias likely allowed only a fraction of endolithic microbes to be isolated and potential multiple

404 isolation of the same *Ostreobium* lineage due to filament fragmentation, with one dominant
405 (P1) and one minor (P12/P14) lineage detected per branch. These reasons explain the uneven
406 size of datasets generated in this study, largest for strains affiliated to the P1 lineage (7
407 strains), and reduced to 2 strains for the P12/P14 lineage. Isolation and characterization of
408 more strains from P12/P14 would be necessary to further test metabolic differences between
409 lineages. Nevertheless, this collection of *Ostreobium* strains that kept their bioerosive
410 potential after isolation from a branching *Pocillopora* coral species, provides novel tools to
411 study the nature of *Ostreobium* interactions with branching corals and their endosymbiotic
412 *Symbiodiniaceae*, sensitive to bleaching events in a changing environment.

413 *Habitat-driven changes in morpho-functional traits (pigments and fatty acids)*

414 Pigments of *Ostreobium* algae have so far been mainly studied in complex natural
415 microboring communities, i.e. green bands of dead or live corals, using spectrophotometry
416 and Jeffrey and Humphrey equations for chlorophylls, or HPLC technique (Fork & Larkum,
417 1989; Fine & Loya 2002; Fine *et al.*, 2006; Tribollet *et al.*, 2006; Sangsawang *et al.*, 2017)
418 with a few pioneer studies focusing on individual strains of *Ostreobium* sp. (called *O.*
419 *quekettii* Bornet & Flahaut 1889) using HPLC methods (Jeffrey, 1968; Schlichter *et al.*, 1997;
420 Koehne *et al.*, 1999). For the first time, we provide pigment datasets (HPLC profiles) for
421 several *Ostreobium* strains of a referenced genotype (*rbcL* P1 lineage) and show differences
422 in endolithic *versus* free-living forms, supporting adaptation to habitat-driven lower light
423 intensities in the dense coral biomineral *versus* seawater. Indeed, although pigment
424 composition was qualitatively similar among strains, chlorophyll *b* content and chl *b* : chl *a*
425 ratio were higher in endolithic forms compared to free-living forms. These results obtained
426 for genetically identified *Ostreobium* strains are in agreement with those reported for natural
427 microboring communities, highlighting the major contribution of *Ostreobium* to the
428 photobiology of complex coral skeletal microbiomes. Indeed, in the green layer of massive
429 (*Favia*) corals, chlorophyll *b* reached up to 60-75% of the chlorophyll *a* content (Jeffrey,
430 1968). In the endolithic algae colonizing the coral *Leptoseris frugilix*, the chl *b* : chl *a* ratio
431 was also higher in deep colonies than shallow ones (Schlichter *et al.*, 1997). Additionally, in
432 free-living cultures of *Ostreobium* sp. strain B14.86, Schlichter *et al.* (1997) experimentally
433 showed that chlorophyll *a* and *b* concentrations increased with decreasing light (photon flux
434 from 60 to 0.5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), suggesting adaptation of the light harvesting system to low light
435 conditions. Our experimental results indicate that endolithic *Ostreobium* strains adapted their
436 photosynthetic apparatus (chl *b* content) to the low light microenvironment inside the dense

437 coral carbonate substrate (Enriquez *et al.*, 2017), which here was the bleached skeleton (bared
438 of tissue) of a fast-growing, branched coral. Contrary to other studies on (unidentified)
439 *Ostreobium* sp. strain from Chilean coral (Koehne *et al.*, 1999; Wilhelm & Jakob, 2006), or
440 complex endolithic community from coralline algae (Behrendt *et al.*, 2011), the far-red
441 absorbing chlorophyll *d* was not detected (i.e. no peak with a maxima absorption around 700
442 nm) in our endolithic *Ostreobium* strains. Red-shifted chlorophyll *d* is mainly associated to
443 photoadaptation of *Ostreobium* algae to life in low light microenvironment inside the coral
444 carbonate skeleton of living colonies, shaded by intact coral tissue cover (Haldall, 1968). Our
445 experimental settings, i.e. illumination with $\sim 30 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light and absence
446 of light-absorbing coral tissue cover, may have prevented the full expression of *Ostreobium*
447 pigment repertoire. Future studies may involve manipulation of light quality and intensity,
448 and use of high spatial resolution sensors (Magnusson *et al.*, 2007; Wangpraseurt *et al.*,
449 2012), to better resolve the optical microniche environments inside coral skeletons *versus*
450 inside free-living tufts of filaments, and compare the photobiology and photoadaptation of
451 diverse genotypes of *Ostreobium*.

452 Fatty acids are important for physiological adaptation of algae to environmental stress
453 such as salinity and temperature (Zhila *et al.*, 2010, de Carvalho & Caramujo, 2018). They
454 may also be used to understand the nutritional value of *Ostreobium* to the coral host
455 (suggested by Fine & Loya, 2002) and to grazing organisms such as parrotfishes (Clements *et*
456 *al.*, 2016), and more generally, as trophic markers to trace food sources in reef organisms.
457 Here we provide first detailed records of *Ostreobium* fatty acid profiles, highlighting their
458 variability across growth forms, genotypes, strains and subcultures.

459 Fatty acid composition of *Ostreobium* was typical of chlorophytes but varied
460 quantitatively between the endolithic growth forms and their free-living counterparts. Palmitic
461 acid (16:0), abundant in both endolithic and free-living *Ostreobium*, is indeed a saturated FA
462 reported in several green macroalgae (Kumari *et al.*, 2010; Pereira *et al.*, 2012). Both
463 phenotypes displayed high contents of C18 polyunsaturated FA (PUFA) which are
464 characteristic of chlorophytes (Jamieson & Reid, 1972; Khotimchenko & Svetashev, 1987).
465 But some FAs commonly found in other algae of the order Bryopsidales were not detected in
466 our *Ostreobium* strains. Indeed, a comparison of Ulvales and Cladophorales with the order
467 Bryopsidales (the latter listed by Aknin *et al.* (1992) as Siphonales, a previous affiliation of
468 members of this order) indicates that these algae are characterized by high quantity of 16:3 ω 3
469 combined with low quantities of 16:4 ω 3 and 18:4 ω 3 (Aknin *et al.*, 1992). The absence of

470 these $\omega 3$ fatty acids from all analyzed *Ostreobium* strains suggests their absence in the
471 suborder Ostreobinae, at least in the two lineages to which our strains were classified.

472 Simultaneous detection in our *Ostreobium* cultures of 18:2 $\omega 6$ and 18:1 $\omega 9$ is indicative
473 of fungal presence (Frostegård *et al.*, 1996; Mikola & Setälä, 1998; Chen *et al.*, 2001;
474 Meziane *et al.*, 2006), and confirms that cultures were not axenic. Fungi from the microbiome
475 of the coral skeleton may have been co-isolated. Structures morphologically similar to fungal
476 hyphae are indeed known to colonize *Ostreobium* filaments in various carbonate substrates
477 (Le Campion-Alsumard *et al.*, 1995b; Tribollet & Payri, 2001; Golubic *et al.*, 2005).
478 Likewise, trace amounts of methyl-branched saturated fatty acids were detected, indicative of
479 bacterial presence (Daalsgard *et al.*, 2003). Endophytic bacteria have indeed been reported in
480 the genus *Bryopsis* (Hollants *et al.*, 2011) and some bacteria may be associated with
481 *Ostreobium* filaments. Long-chain PUFAs (20:5 $\omega 3$, 22:5 $\omega 6$ and 22:6 $\omega 3$) typical of
482 dinoflagellates (Mansour *et al.*, 1999) were also sometimes detected at low levels, especially
483 in some of the free-living forms.

484 An important feature of the fatty acid composition of *Ostreobium* strains was the high
485 abundance of arachidonic acid (20:4 $\omega 6$) in free-living forms, consistently dropping in
486 endolithic forms (observation valid for lineage P1, as low replication of endolithic forms in
487 the less represented P12/P14 prevented comparisons). This essential FA for living organisms
488 is a constituent of phospholipids in biological membranes, involved in fluidity, permeability
489 and cellular signalization (Maulucci *et al.*, 2016). The shift recorded in *Ostreobium* membrane
490 composition suggests important adjustments of fluidity and permeability, possibly for
491 adaptation to the lifestyle constraint experienced by the siphons. Endolithic *Ostreobium*
492 siphons are indeed supported by the rigid environment provided by the coral carbonate
493 mineral. C/N ratios were also lower in endolithic *versus* free-living *Ostreobium* suggesting
494 lower production inside the carbonate habitat of C-rich compounds such as lipids and
495 polysaccharides exudates. In contrast, free-living filaments may benefit from more fluid and
496 flexible membranes that offer more mechanical resistance to shear forces and local seawater
497 turbulence. In addition high PUFA content may provide more protection from temperature
498 changes (de Carvalho & Caramujo, 2018). Decreased degree of fatty acid unsaturation in
499 endoliths could also help the alga to acclimate to salinity stress (Zhila *et al.*, 2010). Overall a
500 shifting arachidonic acid content between both phenotypes may reflect differential
501 signalization activity (de Carvalho & Caramujo, 2018), and a shift in communication with

502 associated microbes. Such potential differences could occur within the same filament, with
503 one end as free-living *Ostreobium* emerging as epilith from carbonate substrates and the other
504 end as endolith. This remains to be investigated in light of the important nutritional role of
505 epilithic and endolithic biofilms in reef trophic food chain (Kobluk & Risk, 1977; Adey,
506 1998; Clements *et al.*, 2016).

507 *Nutritional (C and N) sources for Ostreobium phenotypes*

508 The sources of carbon (C) and nitrogen (N) used by endolithic *Ostreobium* for
509 photosynthesis and growth inside a coral carbonate habitat remain little known. A few authors
510 showed that microboring communities dominated by *Ostreobium* sp. in dead reef carbonate
511 substrates are stimulated by elevated seawater $p\text{CO}_2$, suggesting that those microboring algae
512 are limited in DIC and use mainly seawater DIC (Tribollet *et al.*, 2009; Reyes-Nivia *et al.*,
513 2013; Tribollet *et al.*, 2019). A similar trend was also observed for another reef microboring
514 community dominated by the chlorophyte *Phaeophila* sp. in dead carbonate substrates
515 (Enochs *et al.*, 2016). In light of a recent study by Guida *et al.* (2017), endolithic microalgae
516 such as *Ostreobium*, could also use bicarbonate ions (HCO_3^-) released during carbonate
517 dissolution for photosynthesis. Indeed, these authors showed that the microboring
518 cyanobacterium, *Mastigocoleus testarum* (in cultured strain BC008 and under natural
519 conditions), is able to fix carbon derived from mineral calcite substrate when seawater DIC is
520 limiting. Here, the stable isotope analysis of *Ostreobium* strains provides new information on
521 their sources of carbon and nitrogen for photosynthesis and nutrition.

522 Regarding carbon, the only possible source of carbon for free-living *Ostreobium* was
523 DIC from seawater, which is confirmed by the uptake of ^{13}C -bicarbonate in our experiment
524 (Fig. 5a). Their measured $\delta^{13}\text{C}$ values (ranging between -24 and -15 ‰) were in agreement
525 with those recorded for green fleshy macroalgae (Raven *et al.*, 2002), especially in the class
526 Ulvophyceae (Wang & Yeh, 2003). For endolithic *Ostreobium* boring through the coral
527 biomineral, multiple C sources are possible: (i) DIC from seawater and diffusing to the
528 interstitial space at the interface between filament and skeleton; (ii) DIC released from the
529 CaCO_3 biomineral by active carbonate dissolution and (iii) organic C released from the
530 skeletal organic matrix by carbonate dissolution and/or the activity of microbial associates.
531 Here, we show that non-decalcified endolithic *Ostreobium* filaments ($\delta^{13}\text{C}$ -11.2 ± 0.8 ‰) were
532 depleted in ^{13}C compared to reference seawater DIC (in the range of -5 to 2 ‰; see Patterson
533 & Walter, 1994 for $\delta^{13}\text{C}$ values of tropical seawater in carbonate reefs), and more enriched

534 compared to their coral carbonate substrates. Indeed, the mean $\delta^{13}\text{C}$ value of bleached
535 *Pocillopora acuta* coral skeletons was $-13.9\pm 0.03\text{‰}$, which corresponds to the low range of
536 values recorded for hermatypic, symbiotic coral skeletons (Linsley *et al.*, 2019), likely due to
537 aquarium coral growth restrictions and maybe reduced photosynthesis, increased respiration
538 and oxidation of organic matter. This result, combined with the ^{13}C -bicarbonate uptake
539 experiment, strongly supports the hypothesis that endolithic *Ostreobium* used mainly DIC
540 from seawater.

541 After correction for acidification treatment (+6 ‰), most decalcified endolithic
542 *Ostreobium* strains had lower $\delta^{13}\text{C}$ compositions compared to that of their corresponding free-
543 living forms (Table S5). The uptake of ^{13}C -labeled bicarbonate and exogenous C turnover
544 were however reduced (Fig. 5a and Table S6, respectively) in endolithic compared to free-
545 living *Ostreobium*, indicating lower photosynthetic activity inside the light-limited carbonate.
546 This supports the hypothesis that most endolithic *Ostreobium* had access to the same source
547 of seawater carbon as their free-living counterparts. Indeed, low photosynthetic rates are
548 expected to reduce $\delta^{13}\text{C}$ values of macroalgae (O'Leary, 1988; Wiencke & Fischer, 1990) due
549 to preferential uptake of dissolved CO_2 if not limiting, and carbon isotope fractionation during
550 photosynthetic ^{12}C - CO_2 fixation by the RuBisCO enzyme, which discriminates against ^{13}C -
551 CO_2 (Farquhar *et al.*, 1989). Moreover, the presence of contaminant residual skeletal organic
552 matrix with very low $\delta^{13}\text{C}$ values ($-29.4\pm 1.3\text{‰}$) also likely contributed to depletion of ^{13}C in
553 decalcified endolithic *Ostreobium*.

554 Only endolithic filaments of strain 010 had enriched $\delta^{13}\text{C}$ values compared to their
555 free-living filaments (Fig. 4, Table S5). This could be explained by the relatively low $\delta^{13}\text{C}$
556 values of its free-living phenotype due to low photosynthetic activity, supported by very low
557 chlorophyll content (Table S2) and reduced exogenous C turn-over (Table S6). It might also
558 point to an uptake by the endolithic phenotype of strain 010 of non-isotopically labeled DIC,
559 released by dissolution of the coral CaCO_3 substrate, and carbonic anhydrase conversion of
560 bicarbonate ions (Shashar & Stambler, 1992) which would increase its $\delta^{13}\text{C}$ values. Indeed in
561 dark conditions, we recorded a significant pH increase in endolithic cultures (Fig. 5)
562 suggesting *Ostreobium*-driven carbonate dissolution. Moreover, a preliminary experiment
563 with *Ostreobium* strain 018B also indicated *in vitro* production of alkalinity over a 24h
564 day/night cycle (measured by colorimetry according to Sarazin *et al.*, 1999). Combined
565 together, these results suggest that the endolithic *Ostreobium* strains were dissolving the coral
566 carbonate during our experiments. This dissolution is consistent with recent studies that

567 showed for *Ostreobium quekettii* strain (Krause *et al.*, 2019) or microboring communities
568 dominated by *Ostreobium* in dead coral skeletons (Tribollet *et al.*, 2019) that endolithic
569 filaments are actively dissolving carbonates, increasing seawater alkalinity and thus, the
570 concentration of bicarbonate ions in their environmental vicinity. Although in our
571 experimental settings most endolithic strains were using seawater DIC, the relative
572 contribution of each carbon source for endolithic *Ostreobium* growth forms needs to be
573 further investigated, testing a range of seawater DIC. The possible use by endolithic
574 *Ostreobium* of organic C released from the skeletal organic matrix and the activity of
575 associated microbes should also be investigated. This would allow to better understand
576 *Ostreobium* nutritional plasticity and their efficiency at dissolving carbonates under different
577 environmental conditions.

578 Regarding nitrogen, the positive shift of $\delta^{15}\text{N}$ values recorded in this study for
579 endolithic compared to free-living *Ostreobium* suggests a higher trophic level and different
580 nitrogen sources. The increased $\delta^{15}\text{N}$ values may also reflect nutrient limitation and complete
581 utilization by endoliths of the nitrate source pool in the small interstitial spaces between
582 *Ostreobium* filaments and their carbonate substrate (Torres *et al.*, 2012). For free-living
583 forms, the only N source was dissolved inorganic nitrogen (DIN) in the form of nitrate (NO_3^-)
584 from seawater medium, confirmed by uptake of ^{15}N -nitrate in our experiment (Fig. 5b). For
585 endolithic forms, two N sources are possible: (i) DIN from seawater and diffusing to the
586 interstitial space at the interface between filament and skeleton and (ii) organic N released
587 from the skeletal organic matrix by carbonate dissolution and/or from the activity of microbial
588 associates. Similarly to the free-living forms, nitrate provided in the incubation seawater was
589 assimilated by *Ostreobium* endoliths, as shown by their ^{15}N -enrichment at the end of the 8h
590 pulse of labeling with ^{15}N -nitrate. The nitrate uptake was however higher in light than dark
591 conditions. This reduction (40%) is likely explained by the contribution of photosynthesis to
592 supply energy and organic carbon skeletons (C-backbones) for nitrogen assimilation into
593 amino-acids (Kopp *et al.*, 2013) necessary for protein synthesis and algal filament growth.
594 However, the $\delta^{15}\text{N}$ values of most of the decalcified endolithic strains were higher than those
595 of the residual skeletal organic matrix after carbonate dissolution (Fig. 4; not an effect of the
596 acid-treatment used for decalcification, see results). Thus, the skeletal organic matrix, known
597 to be rich in amino-acids and glycoproteins (Marin *et al.*, 2016) could be an additional
598 heterotrophic nitrogen source for *Ostreobium* endoliths. Other microbial processes might be at
599 play, and contribute to nitrogen isotope fractionation, linked to N_2 fixation, nitrate reduction,

600 and cycling activities of associated endolithic bacterial/fungal microorganisms in the coral
601 skeleton microbiome (Ferrer & Szmant, 1988; Wegley *et al.*, 2007; our results showing the
602 presence of fungal and bacterial fatty acid markers in cultures of *Ostreobium*). The
603 demonstrated uptake of nitrate by *Ostreobium* strains, especially high for the free-living
604 phenotype but also significant for the endolithic phenotype, highlights an important role of
605 *Ostreobium* in reef nitrogen cycling, as suggested in early works on nutrient regeneration in
606 living corals (Risk & Müller, 1983; Ferrer & Szmant, 1988).

607 *Conceptual model of C and N sources for Ostreobium*

608 In light of our results, we propose a model of nutritional sources for *Ostreobium* algae,
609 illustrated in Figure 6. In this conceptual model, an endolithic filament emerges from the coral
610 carbonate surface as an epilith, which can then be detached and grown in seawater as a free-
611 living filament. Dissolved CO₂ and bicarbonate ions (HCO₃⁻) from seawater are in direct
612 contact with epilithic *Ostreobium* filaments, and diffuse from gallery opening to the
613 interstitial space between endolithic filaments and skeleton. Dissolved CO₂ is also provided
614 by respiration. Bicarbonate ions are converted to CO₂ via the activity of carbonic anhydrase
615 enzymes (CA) (Shashar & Stambler, 1992). Thus, CO₂ (from seawater or formed after
616 conversion of HCO₃⁻ by the CA) diffuse inside the *Ostreobium* filament and is fixed in
617 chloroplasts by the ribulose-1,5-bisphosphate Carboxylase/Oxygenase (RuBisCO) enzyme for
618 photosynthetic production of C-rich organic compounds (i.e. glucose, fatty acids) used for
619 growth. At the dissolution front of endolithic *Ostreobium*, inorganic C might be taken up
620 directly by the filament for photosynthesis, or after conversion of organic C from dissolved
621 organic matter (DOM) released by the breakdown of the skeletal organic matrix and recycled
622 by associated endolithic bacteria and fungi. Dissolved inorganic nitrogen (DIN, in this study
623 NO₃⁻) is provided by the incubation seawater and diffuses to the interstitial space between
624 endolithic filament and coral skeleton. Assimilation of DIN may be combined to organic N
625 from other microbial processes (hypothesized by Risk & Müller, 1983; Ferrer & Szmant,
626 1988) and the mobilization of DOM from biogenic coral carbonate dissolution. The exact
627 metabolic processes of organic and inorganic N acquisition, i.e. their diffusion or active
628 transport into *Ostreobium* filaments, remain to be investigated.

629 We suggest that endolithic *Ostreobium* may have mixed inorganic and organic C and
630 N sources, compared to free-living forms which fix inorganic C and assimilate N through
631 photosynthesis-dependent processes. This nutritional plasticity hypothesis proposes that

632 *Ostreobium* filaments may change their metabolism to adapt to their habitat and/or
633 environmental conditions (i.e. both seawater chemistry and thickness/composition of
634 associated living coral tissues or epilithic biofilms), shifting from photoautotrophy in
635 epilithic/free-living growth form to mixotrophy in endolithic growth form. This could explain
636 the large bathymetrical distribution of endolithic *Ostreobium*, including depths below the
637 photic zone (Vogel *et al.*, 2000), and the rapid colonization of freshly killed corals by initially
638 present endolithic *Ostreobium* filaments (Leggat *et al.*, 2019). Genotype-driven differences in
639 C and N acquisition strategies should be further investigated, in light of our results showing
640 highly variable $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values and DIC and DIN uptake rates among strains within and
641 between genetic lineages. Further isolation and cultivation of strains from diverse genetic
642 lineages under diverse nutrient conditions will definitely help to better understand *Ostreobium*
643 eco-physiology and the responses of microboring communities to global changes (e.g.
644 Carreiro-Silva *et al.*, 2005 for eutrophication effects, and Tribollet *et al.*, 2009 for ocean
645 acidification effects).

646 *Conclusion*

647 This study highlights habitat-driven changes in chlorophylls, fatty acids and stable
648 isotope values ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) of coral-associated *Ostreobium* strains from two genetic lineages,
649 providing novel information on their nutritional value as free-living/epilithic and endolithic
650 filaments. Lower photosynthetic assimilation and nitrate uptake from seawater by endolithic
651 *versus* free-living phenotypes may be combined for some *Ostreobium* strains with
652 heterotrophic mobilization of organic matter from microbial associates and recycled from the
653 skeletal organic matrix after active dissolution of the carbonate biomineral. We propose that
654 nutritional plasticity may depend on habitat and environmental conditions, with *Ostreobium*
655 filaments shifting from photoautotrophic to mixotrophic lifestyle when free-living filaments
656 (or propagules in seawater) colonize reef carbonates as endoliths. The dual isotope tracer
657 approach used here opens the way to further study the biogeochemical cycling and trophic
658 ecology of these cryptic algae inhabiting coral holobionts and reef carbonates, to help
659 understand coral reef resilience to global changes.

660 **Materials and Methods**

661 *Isolation of Ostreobium strains*

662 Strains of *Ostreobium* were isolated from healthy *Pocillopora acuta* Lamarck 1816
663 coral colonies (genetically identified with mtORF markers after Johnston *et al.*, 2017, also
664 called *Pocillopora damicornis* type *beta* coral), growing in closed-circuit at the Aquarium
665 Tropical du Palais de la Porte Dorée (Paris, France originally from Indonesia). One apical
666 branch fragment ('apex', ~10 mm length) was sampled from each of three distinct *P. acuta*
667 colonies. Skeleton was cleaned off coral tissues containing *Symbiodinium* and other microbes
668 using a blast of pressurized filtered (0.2 μm) seawater (WaterPik[®] method; Johannes &
669 Wiebe, 1970), then crushed into big pieces using autoclaved mortar and pestle. Fragments
670 were separated in 3 fractions, each deposited in an individual microplate well filled with ~5
671 mL of Provasoli Enriched Seawater medium (PES; Provasoli, 1968) supplemented with
672 penicillin G sodium (100 U mL⁻¹) and streptomycin sulfate (100 μg mL⁻¹). Cultures were
673 incubated at 25°C with 30-40 rpm orbital shaking and a 12h light / 12h dark cycle of
674 illumination with white fluorescent light tubes at photosynthetic photon flux density of
675 $31 \pm 5.5 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ (measured with a spherical quantum sensor Li-Cor, USA). Culture
676 medium was changed every 3-4 weeks. After ~1 month, epilithic filaments emerged from the
677 skeletal carbonate chips, identified as *Ostreobium* by their typical morphology in inverted
678 light microscopy (Olympus CK40-SLP). Other green and red algae could sometimes be
679 observed. Epilithic *Ostreobium* filaments were pulled out or cut with a scalpel from the
680 skeleton surface and then serially transferred to fresh PES medium for propagation into
681 monoalgal cultures of free-living filaments. The obtained *Ostreobium* cultures were not
682 axenic as they also contained bacteria and sometimes dinoflagellates, which population
683 densities were controlled by regular subculturing (passage) of isolated algal filaments. For
684 each strain, the number of successive subcultures since initial sampling from skeletal piece
685 was recorded. Endolithic cultures were obtained from the free-living cultures, by colonization
686 during several months of pre-bleached native coral carbonate chip substrates (see below).
687 Strain vouchers have been deposited in the Collection of microalgae and cyanobacteria of the
688 Muséum national d'Histoire naturelle in Paris, France (Table 1).

689 *Taxonomic assignation to rbcL clade (phylotype)*

690 For each algal strain, free-living filaments were subsampled for DNA extraction (with
691 DNeasy PowerSoil[™] Kit, Qiagen Laboratories Inc., CA) and genotyping based on sequences
692 of the chloroplast-encoded *rbcL* gene marker coding for the large subunit of the ribulose-1,5-
693 bisphosphate carboxylase -RuBisCo- enzyme, according to our previous classification scheme
694 (Massé *et al.*, 2018). A partial fragment (~1134 nt) of the 1428 nt chloroplast-encoded

695 *Ostreobium rbcL* gene was amplified with the following oligonucleotide primer pair, specific
696 to the Bryopsidales order within the Ulvophyceae: *rbcLF250* [5'
697 GATATTGARCCTGTTGTTG GTGAAGA 3'] modified from Gutner-Hoch & Fine (2011)
698 and *rbcL1391R* [5'TCTTTCCA AACTTCACAAGC 3'] (Verbruggen *et al.*, 2009). A smaller
699 ~390-440 nt fragment of the *rbcL* gene was obtained for two cultures which DNA was more
700 difficult to amplify, using the primer pair: *rbcLF250* and *rbcLR670* [5'
701 CCAGTTTCAGCTTGWGCTTTATAAA 3'] modified from Gutner-Hoch & Fine (2011).
702 Amplification reactions were performed in 25 µL volume containing 1 µL DNA extract
703 template, 0.5 µL of each primer (10 µM final concentration), 2 µL MgCl₂ (25 mM), 0.5 µL
704 dNTP (10 mM), 5 µL GoTaq 5X Buffer, 0.125 µL enzyme GoTaq® G2 Flexi DNA
705 Polymerase (Promega, France) in sterile water. Cycling conditions were 4 min at 94°C, 40
706 cycles of [30 s at 94°C, 45 s at 55°C, 90 s at 72°C], and 5 min terminal extension at 72°C.
707 Amplified fragments were visualized on 1 % agarose gel with SYBRGold or SYBRSafe
708 (Invitrogen). Purified *rbcL* amplicons (NucleoSpin® gel and PCR clean-up kit, Macherey-
709 Nagel, France) were either Sanger-sequenced directly or cloned into pGEM-T easy vector
710 plasmids using competent *Escherichia coli* JM109 cells (Invitrogen, France). Plasmid DNA of
711 insert-containing colonies was extracted (Wizard Plus SV Minipreps, Promega, France) and
712 Sanger-sequenced in both directions by Eurofins Genomics (Germany). Forward and reverse
713 sequences were assembled and checked manually with BioEdit software. Taxonomic
714 assignation of *Ostreobium* strains was determined by BLASTn against GenBank database. All
715 sequences generated during this study have been deposited in Genbank under accession
716 numbers MK095212 to MK095220 (Table 1).

717 *Bio-erosive potential in culture conditions*

718 Pieces of *Pocillopora acuta* coral skeletons (bared of coral tissue) were bleached by
719 immersion during one to three days at room temperature in sodium hypochlorite (NaClO
720 48%, commercial bleach) to remove potential residual algae, coral tissues and surface organic
721 matter. They were rinsed thoroughly with tap water then deionized water and finally 70%
722 ethanol then air-dried under laminar flow hood. For each *Ostreobium* strain, bleached
723 skeletons were put in contact with free-living filaments to test whether these filaments kept
724 their erosive activity and would be able to colonize their native carbonate substrate, as
725 indicated by (i) filament attachment to skeletal surfaces and (ii) progressive color change of
726 the white, bleached skeletal chip to green *Ostreobium*-invaded chip. These visible signs of
727 colonization were confirmed microscopically : a bleached coral skeleton exposed for about 3

728 months to filaments of *Ostreobium* 010 was fixed in 4% paraformaldehyde in sucrose
729 containing phosphate-buffer, dehydrated in ethanol series (50%, 70%, 96%, 100%), gradually
730 infiltrated with resin (1:2; 1:1; 3:1; v:v ethanol- Spürr resin) then resin-embedded (100%
731 Spürr). Sections cut with a circular diamond saw were polished down to ~20-30 μm , slightly
732 decalcified with formic acid 5% and rinsed with deionized water. These thin sections were
733 either stained with 5% toluidine blue and coverslipped in Spürr for light microscopy
734 observations (Olympus CK40-SLP) or gold-coated and mounted for scanning electron
735 microscopy observations (Hitachi SU 3500, MNHN PtME platform for electron microscopy)
736 of endolithic filaments.

737 Thus, for each free-living *Ostreobium* strain, we also obtained its corresponding
738 endolithic growth form. Before all biochemical and physiological analyses, coral skeletal
739 chips colonized by endolithic *Ostreobium* were scraped with a sterile toothbrush, or blasted
740 with a jet of pressurized filtered seawater (Waterpik[®] method) and transferred to 0.2 μm
741 filtered seawater for 24h to 3 days, in order to remove most outgrowing epilithic filaments
742 and select the targeted microboring endolithic growth form.

743 *Photosynthetic and accessory pigment analysis*

744 Free-living *Ostreobium* isolates (5 strains) and their corresponding endolithic growth
745 forms (3 strains, due to limited available biomass) belonging to lineage P1 (see Table 1, less
746 frequent strains P12/P14 were not studied) were washed three times with large volumes (1:25)
747 of autoclaved deionized water to mechanically remove most surface bacteria and
748 dinoflagellate contaminants and decrease the salt concentration before organic extractions. To
749 evaluate variability in pigment profile across subcultures within same strain, two successive
750 passages (i.e. subcultures) of 3 free-living *Ostreobium* strains were analyzed (see Table 1).
751 Furthermore, bleached *Pocillopora acuta* coral skeletons (uncolonized substrate, n=3) were
752 analyzed as controls for potential residual pigments. The samples were lyophilized and
753 ground to powder. Final dry weights were of 20.6 \pm 12.2 mg for free-living forms, 2253 \pm 1058
754 mg for endolithic forms (without carbonate dissolution), and 1256 \pm 361 mg for control
755 bleached coral skeletons (without carbonate dissolution). Samples were extracted twice in
756 dichloromethane/methanol ($\text{CH}_2\text{Cl}_2/\text{MeOH}$; 1:1 v:v), sonicated (10-15 minutes) in ice in the
757 dark to prevent pigment degradation, and then filtered. After evaporation, organic extracts
758 were re-solubilized in a mixture of $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (1:1 v:v) to a final concentration of 10 mg
759 mL^{-1} . Using high-performance liquid chromatography (HPLC, Agilent 1220 infinity)

760 equipped with a diode array detector (DAD), each extract was analyzed on a reverse phase
761 column (C18 Capcell Pak, Shiseido, 4.6 x 250 mm). Elution solvents were those used by
762 Frigaard *et al.* (1996), i.e. solvent A (methanol:acetonitrile:water, 42:33:25) and solvent B
763 (methanol:acetonitrile:ethyl acetate, 39:31:30) starting with the gradient elution of solvent B
764 from 40 to 100% during 60 minutes (flow 1 mL min⁻¹). The wavelengths of 664 nm and 470
765 nm were used to visualize chlorophyll *a* and its allomers, as well as other
766 chlorophylls/carotenoids, respectively. All peaks were characterized by their retention time
767 (RT) and maxima absorption spectra. The peaks corresponding to chlorophyll *a* (chl *a*) and *b*
768 (chl *b*) were identified (RT and maxima absorption spectra) by direct injection of chl *a* and
769 chl *b* standards (Sigma-Aldrich C-5753 and 00538, respectively) under the same HPLC
770 conditions. Moreover, one *Ostreobium* free-living strain extract was supplemented with both
771 standards as internal controls in order to confirm their respective localization within the
772 HPLC profiles (Fig. S2a). Calibration curves were established for chl *a* and chl *b* standards to
773 quantify chl *a* and chl *b* within the different HPLC profiles of *Ostreobium* cultures (relative
774 proportion and chl *b* : chl *a* ratio).

775 *Fatty acid analysis*

776 Endolithic and free-living *Ostreobium* strains (total 8 strains, from P1 and P12/P14,
777 see Table 1), as well as control bleached skeletons (n=4), were lyophilized and ground rapidly
778 (a few seconds) to powder. Two to three subcultures were analyzed for each of 1 endolithic
779 and 3 free-living *Ostreobium* strains, to evaluate variability in fatty acid composition across
780 subcultures within same strain. Final dry weights were 709±378 mg for endolithic forms
781 (without carbonate dissolution), 2.44±0.3 mg for free-living forms, and 504±100 mg for
782 control skeletons (without carbonate dissolution). Crushed samples were frozen at -20°C until
783 lipid extraction, according to a method modified from Bligh & Dyer (1959). Briefly, after
784 sonication for 20 min in water/methanol/chloroform (H₂O/MeOH/CHCl₃; 1:2:1, v:v:v),
785 H₂O/CHCl₃ (1:1 v:v) was added to form an aqueous-organic two-layer system. The lipid-
786 containing lower chloroform phase was recovered after centrifugation (3000 rpm, 5 min). The
787 aqueous phase was re-extracted a second time. Combined chloroform phases were evaporated
788 under a nitrogen stream. Saponification of extracts was performed at 90°C for 90 min with a
789 solution of NaOH(2M):MeOH (1:2, v:v), followed by acidification with ultra-pure HCl
790 solution (35%). Lipids were recovered by centrifugation (3000 rpm, 5 min) after adding
791 chloroform (2 x 1.5 mL). Chloroform phases were again combined and evaporated under a
792 nitrogen stream. Transmethylation of total lipids was conducted using Boron trifluoride-

793 methanol (BF₃) at 90°C for 10 min. Lipids were finally re-extracted and washed in
794 H₂O:CHCl₃ (1:1, v:v). Chloroform phases recovered were evaporated under a nitrogen stream
795 before being solubilized in hexane and stored at -20°C for analysis by gas chromatography
796 (Varian 450-GC and VF-WAXms column 30 m x 0.25 mm; 0.25 µm film thickness). Peaks of
797 fatty acids were identified using GC-mass spectrometry (Varian 220-MS) and comparison
798 with retention times of commercial standards (Supelco 37, Supelco PUFA N°1 and Supelco
799 PUFA N°3).

800 *Stable isotope values and measurements of photosynthetic C, N assimilation*

801 Isotope dual labeling experiments were conducted for endolithic and their free-living
802 *Ostreobium* counterparts (n=4 pairs, 2 per each genetic lineage P1 and P12/P14; see Table 1).
803 Skeletons colonized by endolithic forms were brushed to remove epilithic filaments as
804 described above, and all culture samples (free-living and endolithic growth forms) were
805 transferred for 3 days before the labeling experiment in Artificial SeaWater (ASW- adapted
806 from Harrison *et al.*, 1980) in order to rinse the nutrient rich PES medium. The isotope
807 labeling pulse was conducted during 8h in white light at 30.5±2.5 µmol photons.m⁻².s⁻¹
808 Photosynthetically Active Radiation (PAR) measured with a LI-250A (LI-COR) quantum-
809 meter equipped with a submersible spherical Micro Quantum Sensor US-SQS/L (WALZ) or
810 in control darkness (inside aluminium foil-wrapped box), starting at the beginning of the light
811 period. Samples of *Ostreobium* cultures (n=4 pairs) or control bleached skeletons
812 (uncolonized, n=3) were incubated at 25°C, under gentle orbital shaking (30-40 rpm, Infors
813 Minitron incubator, Fr) in glass jars with plastic lids, filled to ¾ of their volume with 25 mL
814 of ASW (initially free of bicarbonate and nitrate) supplemented with 2 mM ¹³C-bicarbonate
815 (NaH¹³CO₃, 99 atom% [Sigma-Aldrich]) and 5 µM ¹⁵N-nitrate (K¹⁵NO₃, 98 atom% [Sigma-
816 Aldrich]) or in ‘control unlabeled ASW’ supplemented with 2 mM natural abundance sodium
817 bicarbonate [Sigma-Aldrich] and 5 µM natural abundance potassium nitrate [Sigma-Aldrich].
818 The labeling experiment was repeated later in a separate experiment for an additional strain
819 010 (lineage P1), to increase strain and technical replication under similar light conditions.

820 Seawater pH was measured in glass jar with samples at beginning and end of the 8h
821 experiment (NBS scale; Electrode pH Mettler Toledo Inlab 413 with a pHmeter Mettler
822 Toledo MP220). Control bleached skeletons (uncolonized by endoliths) showed a slight
823 decrease of pH (-0.04) at the end of the labeling pulse in light conditions. Assuming a similar

824 trend in dark conditions, we added this correction value from those pH values obtained for all
825 endolithic strains.

826 At the end of the 8h labeling pulse, samples were rinsed three times with ASW (free of
827 bicarbonate and nitrate). Endolithic *Ostreobium* and control bleached skeletons were
828 decalcified with formic acid 5% and rinsed with deionized water. To assess the effect of the
829 acid-decalcification treatment on ^{13}C and ^{15}N isotope enrichments (labeling intensities), strain
830 010 labeled in free-living form was divided in two parts: one part was lyophilized directly and
831 the other part was treated with formic acid 5%, similar to the carbonate removal protocol to
832 analyze decalcified endolithic forms and control skeletons. The effect of the acid-
833 decalcification treatment was also tested on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ stable isotope values of free-living
834 strain 010 untreated or treated with formic acid 5%.

835 To increase replication of measurements of C and N stable isotope values across
836 subcultures, strains, and genetic lineages, additional samples were analyzed, of endolithic
837 *Ostreobium* strains (not decalcified, or decalcified with formic acid 5%) and their
838 corresponding free-living forms, and control bleached skeleton substrate (see Table1).

839 All organic samples were lyophilized, weighted (0.5-2 mg) into tin capsules and sent
840 for bulk isotope dual ^{13}C and ^{15}N analyses on an elemental analyzer interfaced to a continuous
841 flow isotope ratio mass spectrometer (EA-IRMS) at UC Davis Stable Isotope Facility
842 (California, USA) using bovine liver, glutamic acid, and ^{15}N -enriched alanine as internal
843 standards. Alternately, the series of *Ostreobium* 010 samples and additional unlabeled
844 replicate strains were analyzed in an automated combustion system (EA Flash 2000 Thermo)
845 interfaced with a DeltaV Advantage Thermo continuous flow IRMS at the MNHN SSMIM
846 facility (Paris, France) using normal abundance alanine (0.3 mg) as internal standard. The
847 analytical uncertainties within the SSMIM run estimated from 14 repeated analyses of the
848 alanine laboratory standard was lower than 0.13 ‰ (1SD) for $\delta^{15}\text{N}$ values and lower than
849 0.10 ‰ (1SD) for $\delta^{13}\text{C}$ values.

850 The Carbon to Nitrogen (C/N) ratio was calculated for each sample following the
851 equation: $\text{C/N ratio} = (\text{C Amount}/12.01) / (\text{N Amount}/14.006)$

852 Natural isotope abundances were expressed according to the delta notation:

853
$$\delta^{13}\text{C}_{\text{nat}} \text{‰} = ((^{13}\text{C}/^{12}\text{C})_{\text{sample}} / (^{13}\text{C}/^{12}\text{C})_{\text{standard}} - 1) * 1000$$

854
$$\delta^{15}\text{N}_{\text{nat}} \text{‰} = ((^{15}\text{N}/^{14}\text{N})_{\text{sample}} / (^{15}\text{N}/^{14}\text{N})_{\text{standard}} - 1) * 1000$$

855 with $R_{\text{standard}} = 0.01123$ for $^{13}\text{C}/^{12}\text{C}$ (Vienna Peedee Belemnite calcite standard) and 0.00367
856 for $^{15}\text{N}/^{14}\text{N}$ (atmospheric N_2 standard).

857 For the carbonate samples, i.e. the undecalcified coral carbonate substrate controls
858 (uncolonized) or endolithic growth forms (coral skeletal chips colonized by *Ostreobium*), an
859 alternative sample preparation method was used, the Kiel IV (Thermo) automated carbonate
860 preparation device interfaced to a Delta V Advantage IR-MS (Thermo), at the MNHN
861 SSMIM facility. Sample powders (40-60 μg) were analyzed individually. The $\delta^{13}\text{C}$ values
862 were expressed versus the Vienna-Pee Dee Belemnite calcite standard, and corrected by
863 comparison with a laboratory standard (*Marbre LM*) normalized to the NBS 19 international
864 standard ($\delta^{18}\text{O}$ values are not presented here). The mean analytical precision within the run
865 was calculated from 8 measurements of the *Marbre LM* averaging 0.038 ‰ (1SD) for $\delta^{13}\text{C}$
866 values.

867 For isotopically labeled samples (free-living and decalcified endolithic *Ostreobium*,
868 and control decalcified skeletons, i.e. skeletal organic matrix), all ^{13}C and ^{15}N -enrichment
869 levels were expressed according to the delta notation: $\delta X (\text{‰}) = ((R_{\text{sample}}/R_{\text{control}})-1)*1000$.
870 R_{sample} is the ratio $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ of labelled samples, and R_{control} is the measured ratio of
871 corresponding control non-labeled samples (i.e. natural isotope abundance ratio).

872 Labeling values for decalcified endolithic forms were corrected by subtracting the
873 average of 3 replicate values obtained for control decalcified skeletons, corresponding to non-
874 specific adsorption of the isotopic label to the skeletal organic matrix (means of 0.4 ± 2 ‰ for
875 enriched $\delta^{13}\text{C}$ and 0.9 ± 0.2 ‰ for enriched $\delta^{15}\text{N}$). We also tested the effect of formic acid
876 treatment and showed that it introduced variability but did not change average ^{13}C and ^{15}N
877 enrichments in free-living *Ostreobium* (^{13}C -enrichment of 585 ± 106 versus 553 ± 19.7 ‰ and
878 ^{15}N -enrichment of 384 ± 222 versus 377 ± 42 ‰, with or without formic acid respectively; $n=3$
879 technical sub-replicates of strain 010). We thus assumed that the acid-formic treatment did not
880 significantly affect enrichment results obtained for decalcified endolithic *Ostreobium*.

881 *Data analysis*

882 Multivariate analysis of pigment compositions was performed using PRIMER 5
883 software. A triangular similarity matrix was created using Bray-Curtis similarity coefficient,
884 followed by non-metric multidimensional scaling (nMDS) to spatially visualize samples of
885 endolithic, free-living *Ostreobium* strains and control skeletons with similar composition.
886 Similarity and dissimilarity percentages obtained by SIMPER analysis allowed to determine

887 which pigments (or RT of peaks in case of non-identified pigment) drove the observed
888 differences between datasets (i.e. endolithic *versus* free-living *Ostreobium* *versus* control
889 skeletons).

890 Principal component analysis (PCA) was performed on % level of fatty acids using R
891 software (version 3.6.2), to reveal spatial variability among strains, genetic lineage, and
892 growth forms of *Ostreobium*, and identify FAs that explain most the variance in our datasets.

893 For $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ stable isotope values, parametric Student-test were performed with
894 R software (version 3.6.2) to determine if the genetic lineage (P1 *versus* P12/P14) influenced
895 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for both *Ostreobium* growth form. Then, an analysis of variances
896 (ANOVA) was performed on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ datasets, with post hoc Student-Newman-Keuls
897 (SNK) tests, highlighted differences between *Ostreobium* growth forms and with their habitat
898 (coral skeleton substrate). Significance threshold were set to p-value <0.05.

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910 **Authors Contributions**

911
912 A.M, I.D.C, and A.T conceived the research and designed the experimental protocols,
913 A.M performed the experiments with help from C.Y. for strain isolation, C.S. for stable
914 isotope experiments, A.L. for pigment extraction and N.T. for fatty acid analyses. T.M, M-
915 L.B-K and A.C. helped analyze and discuss the data. A.M., A.T. and I.D-C. wrote the
916 manuscript, which was commented by all authors. A.M. A.T. and I.D.C. revised the
917 manuscript.

918 **Conflict of Interest**

919

920 The authors declare no conflict of interest.

921

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1143

1144 **Tables and Figures**

Table 1: Analyses carried out per *Ostreobium* strain in endolithic versus free-living growth form. Controls are either bleached coral carbonate skeleton or residual skeletal organic matrix (after carbonate decalcification) of *Pocillopora acuta* host. (Strain 022 was lost since the analyses); subcult.: replicate subculture, techn. replicates: technical replicates.

<i>Ostreobium</i> strain code	Phylogenetic affiliation (<i>rbcL</i> gene # Genbank)	MNHN RBCell voucher #	Growth form	Photosynthetic and accessory pigments	Fatty acids	Stable isotope values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$)	^{13}C -bicarbonate and ^{15}N -nitrate assimilation	
							Light condition	Dark condition
010	Clade P1 (MK095212)	MNHN-ALCP-2019-873.3	Free-living	+	+	+ (2 subcult., each with 3 techn. replicates)	+ (3 techn. replicates)	
			Endolithic	+	+	+ (2 subcult., each with 1-4 techn. replicates)	+	
017	Clade P1 (MK095214)	MNHN-ALCP-2019-873.4	Free-living		+			
			Endolithic		+			
018B	Clade P1 (MK095215)	MNHN-ALCP-2019-873.6	Free-living	+ (2 subcult.)	+ (3 subcult.)	+		
			Endolithic	+	+ (2 subcult.)			
019	Clade P1 (MK095213)	MNHN-ALCP-2019-873.8	Free-living	+ (2 subcult.)	+	+		
			Endolithic	+	+			
05	Clade P1 (MK095217)	MNHN-ALCP-2019-873.1	Free-living	+	+ (2 subcult.)	+ (2 subcult.)	+	+
			Endolithic		+	+ (2 subcult.)	+	+
018C	Clade P1 (MK095216)	MNHN-ALCP-2019-873.7	Free-living	+ (2 subcult.)				
			Endolithic		+			
022	Clade P1 (MK095219)	NA	Free-living			+ (2 subcult.)	+	+
			Endolithic			+ (2 subcult.)	+	+
018A	Clade P12 (MK095218)	MNHN-ALCP-2019-873.5	Free-living		+ (3 techn. replicates)	+ (2 subcult.)	+	+
			Endolithic			+ (2 subcult.)	+	+
06	Clade P14 (MK095220)	MNHN-ALCP-2019-873.2	Free-living		+ (2 subcult.)	+ (2 subcult.)	+	+
			Endolithic		+	+ (2 subcult.)	+	+
Substrate controls for endolithic growth forms				Bleached coral skeleton (n=3)	Bleached coral skeleton (n=4)	Bleached coral skeleton (n=3) Skeletal organic matrix (n=4)	Skeletal organic matrix (n=3)	
Corresponding data (Figures, Tables and Supplementary information)				Fig. 2; Fig. S2; Table S1 & S2	Fig. 3; Fig. S3; Table S3	Fig. 4; Table S4& S5	Fig. 5; Table S6	

Figure 1: Morphology of *Ostreobium* cultured as free-living or endolithic filaments. Light (a, b, c) and scanning electron (d) microscopy observations of (a) free-living tuft of algal filaments attaching to a fragment of coral carbonate skeleton (outlined by black dotted lines) as epilithic growth form (black arrow) and colonizing this carbonate substrate as endolithic growth form (white arrow). (b) Isolated free-living filaments with visible green chloroplasts inside the branched siphons. (c) Endolithic (bioeroding) filaments (white arrow) stained with toluidine blue in skeleton thin section. (d) Galleries formed by endolithic filaments of *Ostreobium* (white arrow) after re-colonization by free-living filaments of a pre-bleached coral skeleton substrate.

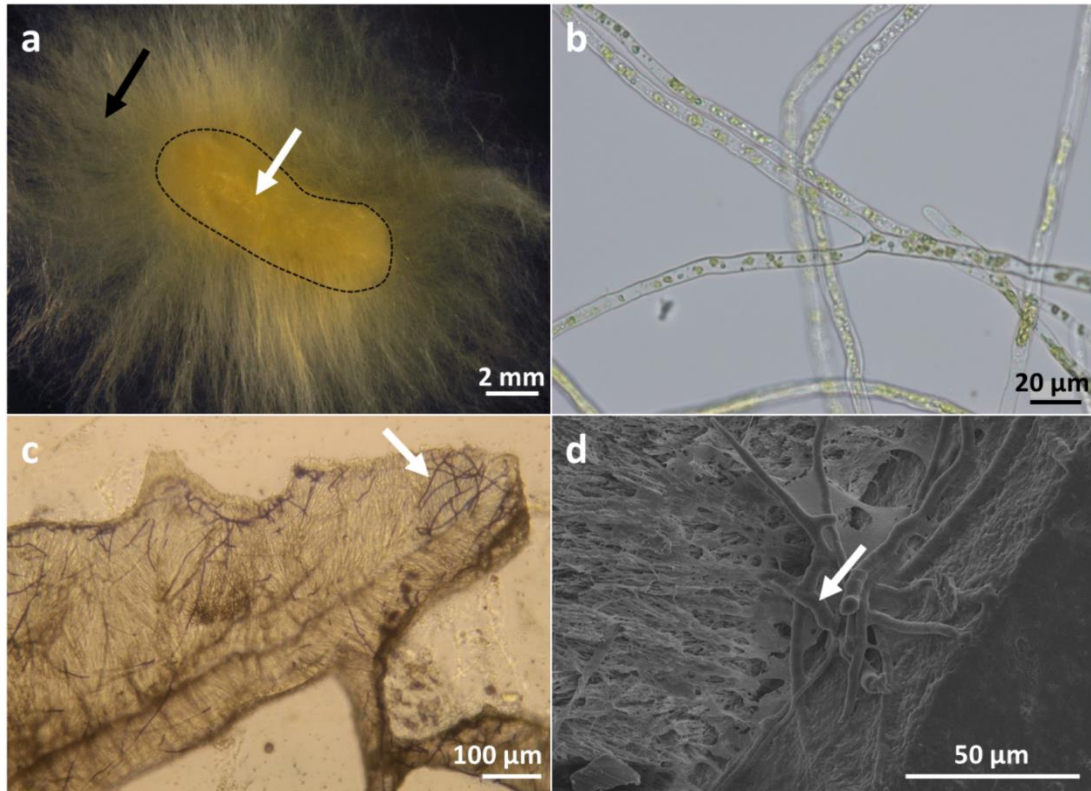


Figure 2: Compared pigment composition of endolithic versus free-living *Ostreobium* strains. (2a) Non-metric Multidimensional Scaling (nMDS) plot of Bray-Curtis similarities of pigment composition in endolithic (circles) versus free-living (squares) *Ostreobium* strains (named 0xx) belonging to lineage P1 (in black), and control bleached coral carbonate skeleton (triangle in white). ¹ and ² indicate successive subcultures of strains. (2b) Barplot of relative proportions of pigments in individual strains (P1) and growth forms.

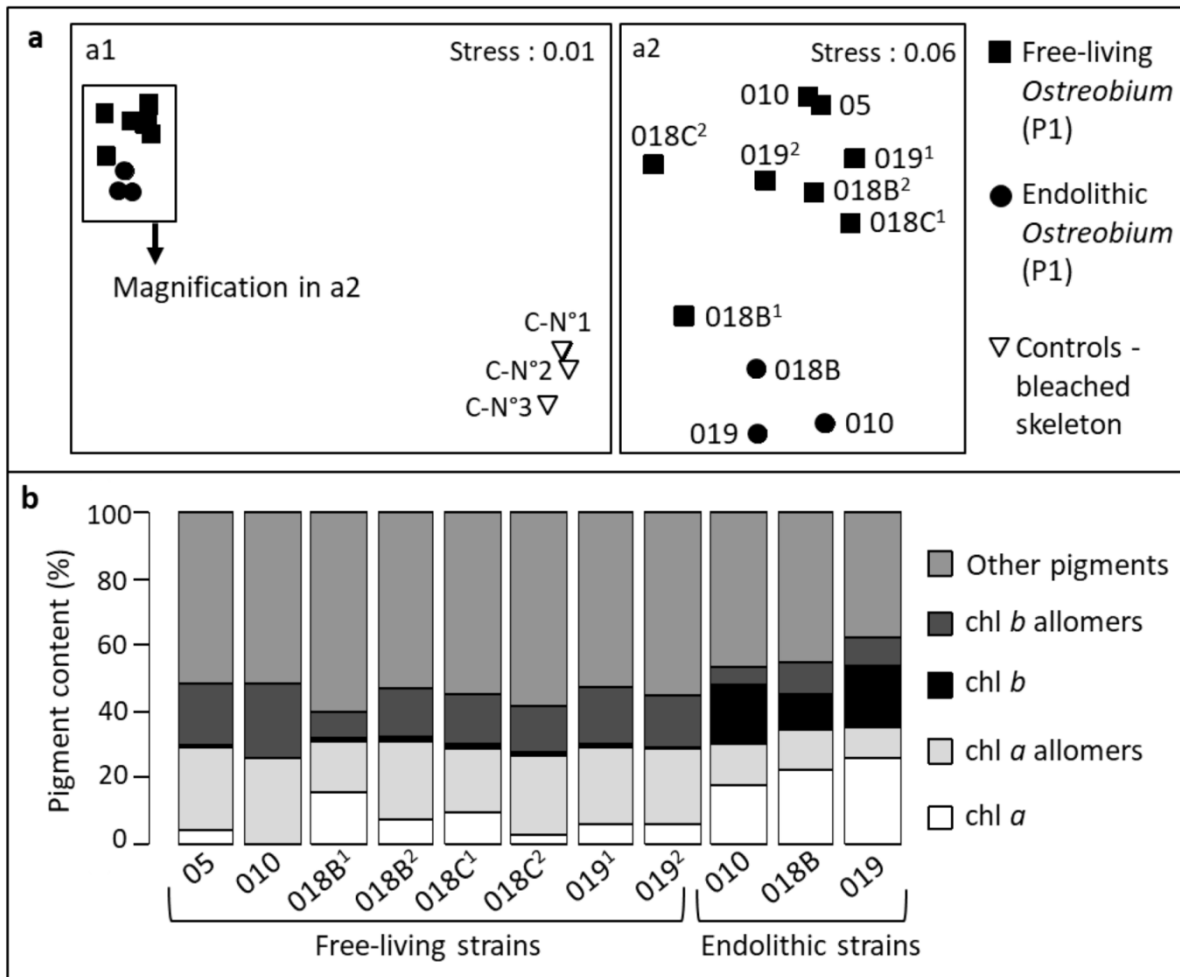


Figure 3: Fatty acid composition of endolithic versus free-living *Ostreobium* growth forms. (a) Scores plot and (b) loading plot for PCA analysis of relative fatty acid methyl ester (FAME) proportions measured in GC profiles after GC-MS annotation of endolithic (circle) and free-living (square) strains (named 0xx). Genetic lineages are color coded (black: P1; grey: P12/P14). (c) Barplot of relative proportions of fatty acids in individual strains and growth forms. Means and Standard Error (SE) of % total fatty acids are detailed in Table S3, along with polyunsaturated and saturated fatty acid values per growth form and lineage.

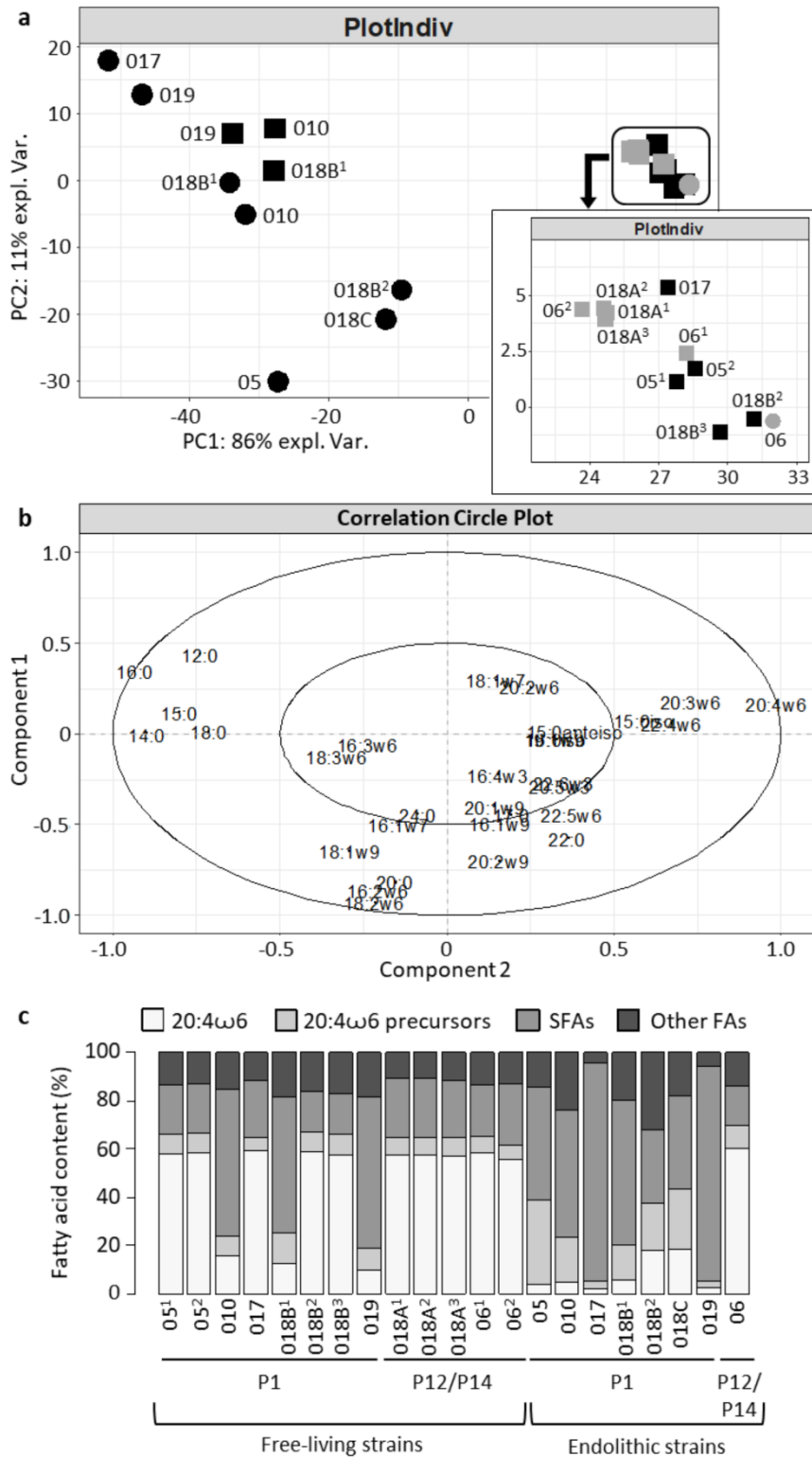


Figure 4: Stable isotope values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of endolithic versus free-living *Ostreobium*. Endolithic filaments were analyzed by EA-IRMS either within their carbonate substrate (endolithic *Ostreobium*, filled circles) or after decalcification with formic acid 5% (decalcified endolithic *Ostreobium*, empty circles). The acid treatment depletes $\delta^{13}\text{C}$ values of decalcified endolithic *Ostreobium* by -6 ‰ (here data are presented before correction). Control substrate of endoliths was either bleached coral carbonate skeleton (composed of CaCO_3 and organic matrix, filled triangle) or skeletal (acid-insoluble) organic matrix (empty triangles). Corresponding free-living filaments (squares) were also analyzed. Genetic lineages are color coded (black: P1; grey: P12/P14). Intra-carbonate $\delta^{15}\text{N}$ of coral skeleton was below EA-IRMS detection limit. For graph clarity, strain code names are not indicated, except for dotted circled $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of strain 010 which plot separately from other P1 strains. Grey bars indicate means. Different letter indicate significant differences (ANOVA and student test $p < 0.05$). $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of individual strains and growth forms are provided in Table S5.

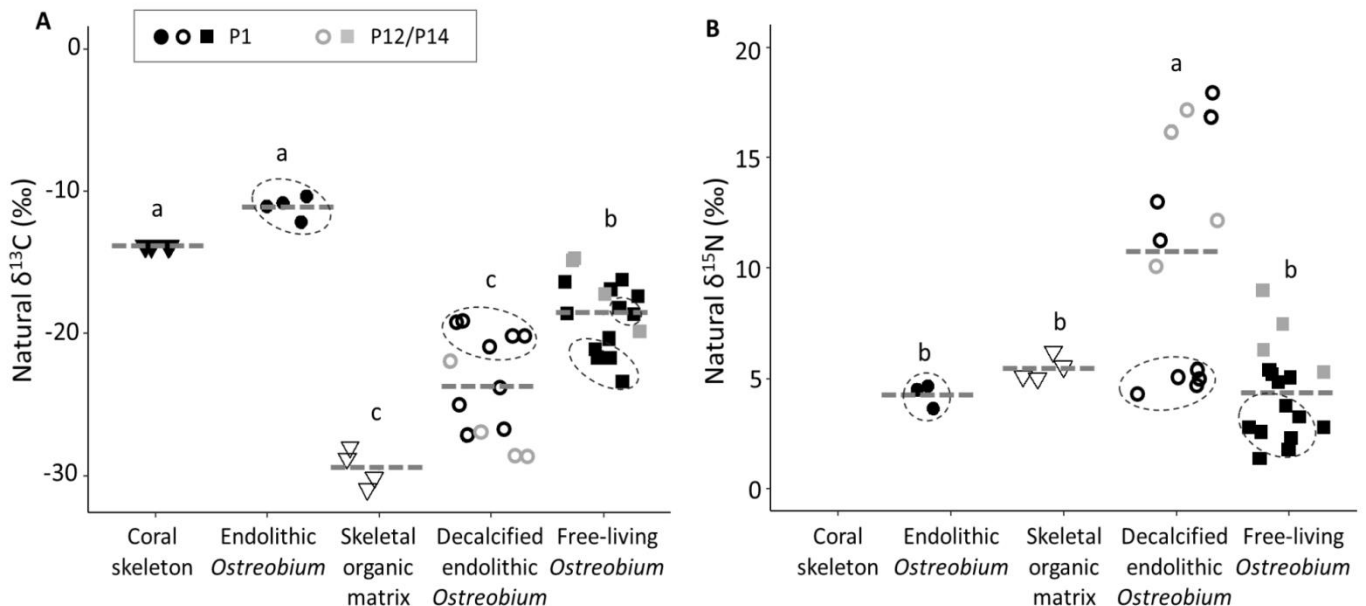


Figure 6: Conceptual model of C and N sources for *Ostreobium* filaments as endolithic and epilithic/free-living growth form. BioCaCO₃: biogenic carbonate. ?: putative ion transporter. Dotted line indicates hypothetical use of DIC from carbonate dissolution, and organic N by endoliths.

