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1 Biodegradation of *Emiliana huxleyi* Aggregates by a Natural 2 Mediterranean Prokaryotic Community under Increasing 3 Hydrostatic Pressure

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Abstract

In the deep ocean, fluxes of particulate organic carbon (POC) and calcium carbonate are positively correlated, suggesting that CaCO_3 could increase sinking particle densities and/or protect the organic matter from degradation by prokaryotes, the so called “ballast effect”. Here, we used the Particle Sinking Simulator system to investigate the effect of increasing pressure on the biodegradation of calcifying *Emiliana huxleyi* aggregates. A ten days incubation was performed, simulating the changes in temperature and pressure experienced *in situ* in the NW Mediterranean Sea. Aggregates sinking from 200m to 1700m depth (assuming an average sinking velocity of 150 m d^{-1}) were exposed to a fresh natural mesopelagic prokaryotic community, with limited influence of culture-associated prokaryotes. *E. huxleyi* aggregates lipid composition revealed more degradation under increasing hydrostatic pressure (relative to constant atmospheric pressure), suggesting increased cell lysis. In parallel, changes in particulate inorganic carbon and total alkalinity indicated CaCO_3 dissolution, potentially accelerated under pressure. However, increased hydrostatic pressure also had a positive effect on particle aggregation, which may compensate the effect of increased cell lysis. Our results imply that in coccolithophorid-dominated sinking aggregates, the ballasting and protection effects of coccoliths may collapse throughout the water column. The increase in aggregation potential with pressure observed in controlled conditions might balance the loss of mineral ballast to a certain extent, although this needs to be confirmed *in situ*.

Keyword: biological carbon pump; mesopelagic; mineral ballast; coccolithophorid; prokaryotes; biodegradation; hydrostatic pressure

38Introduction

39The ocean's biological carbon pump transports carbon into the deep ocean in the form of organic
40matter (Eppley and Peterson, 1979). Between 1-40% of the organic carbon newly formed from CO₂
41fixation at the sea surface is transported to the deep sea (Ducklow et al., 2001), away from direct
42contact with the atmosphere. This process absorbs a large portion of the anthropogenic CO₂
43emissions in the atmosphere, limiting greenhouse CO₂ gas build-up (Le Quéré et al., 2010). However,
44many climate-sensitive factors can influence organic carbon export and remineralization by
45mesopelagic heterotrophs, such as the mineral content of sinking particles (Armstrong et al., 2001;
46Kwon et al., 2009).

47Globally, approximately 5-20 Pg C yr⁻¹ leaves the euphotic zone (20-120m depth) in the form of
48sinking particulate organic carbon (POC) (Henson et al., 2011; Siegel et al., 2014). However, most of
49this POC is potentially remineralized to CO₂ in the upper mesopelagic zone (Buesseler and Boyd,
502009; Giering et al., 2014), where the activity of particle-attached prokaryotes and zooplankton play
51an important role in POC attenuation (Cho and Azam, 1988; Giering et al., 2014; Turley and Mackie,
521994, 1995). On one hand, sinking POC collected *in situ* can be rapidly remineralized by attached
53prokaryotes, as demonstrated by incubations done at atmospheric pressure (Goutx et al., 2007;
54Panagiotopoulos et al., 2002; Sempéré et al., 2000). On the other hand, the remineralisation of
55sinking particles (diatom aggregates or faecal pellets) and dissolution of biogenic minerals (opal) by
56fresh natural surface prokaryotic assemblages were observed to slow down under increasing pressure
57(Tamburini et al., 2006, 2009). Correlation between POC and mineral fluxes (calcium carbonate, opal,
58clays) in the deep ocean has lead to the interpretation that “ballast” minerals exert an important
59control over POC export (Armstrong et al., 2001; Francois et al., 2002; Klaas and Archer, 2002).

60Oceanic provinces in which calcifying plankton dominates display lower mesopelagic remineralisation
61fluxes than systems dominated by opal-producing diatoms (Cardinal et al., 2001, 2005; Jacquet et al.,
622005). In sinking particles collected *in situ*, the presence of minerals has been suggested to preserve

63organic matter from degradation (Hedges et al., 2001; Ingalls et al., 2003). These observations suggest
64that mineral and POC dissolutions in the water column may be inter-related. To understand these
65interactions, a few biodegradation studies have been conducted using aggregates obtained from
66cultures of opal-protected diatoms, which form important seasonal blooms (Treguer and Pondaven,
672000). Biologically-mediated biogenic opal dissolution has been observed to increase with organic
68matter degradation in surface waters (Bidle and Azam, 1999, 2001; Goutx et al., 2007) and to
69diminish with temperature decrease (Bidle et al., 2002) or hydrostatic pressure increase (Tamburini et
70al., 2006). Adding another ballast mineral (CaCO_3) to diatom aggregates diminished diatom aggregate
71remineralisation by zooplankton (Le Moigne et al., 2013). Regarding CaCO_3 , biologically-mediated
72dissolution has also been suggested (Antia et al., 2008; Harris, 1994; Jansen and Wolf-Gladrow, 2001;
73Milliman et al., 1999) to explain the fact that significant CaCO_3 dissolution is occurring in the upper
741000 m of the water column above the calcite or aragonite saturation horizon (Wollast and Chou,
751998, 2001). However, there is still no or contradictory evidence that this dissolution could be
76microbiologically-mediated (Bissett et al., 2011), and the link between CaCO_3 dissolution and POC
77degradation remains unclear.

78Ocean acidification experiments using diatom aggregates supplemented with CaCO_3 ballast indicate
79that CaCO_3 dissolution and POC degradation will potentially increase with increasing pCO_2 , a
80phenomenon that is exacerbated under increasing pressure (de Jesus Mendes and Thomsen, 2012).
81However, little is known about the biodegradation of calcifiers aggregates. Among the calcifiers,
82coccolithophorids contribute to air-sea CO_2 exchange, through their combined photosynthesis (sink of
83 CO_2) and calcification (source of CO_2) activities (Poulton et al., 2007). Coccolithophorid aggregates
84formation has been reported from many recent lab studies (Biermann and Engel, 2010; Chow et al.,
852015; Engel et al., 2009a, 2009b; Iversen and Ploug, 2010), but they are considered as being more
86sensitive to fragmentation than diatom aggregates (De La Rocha and Passow, 2007).

87Laboratory biodegradation experiments using aggregates from the coccolithophore *Emiliania huxleyi*
88showed that heavily calcified strains have higher settling velocities and are better preserved over time

89than poorly calcified strains (Engel et al., 2009a, 2009b). Oxygen consumption within *E. huxleyi*
90aggregates can be 4 to 7 times larger than that of marine snow and diatom aggregates, indicating that
91they may be more prone to degradation (Ploug et al., 2008). Nevertheless, the carbon-specific
92respiration rate per meter settled was found to be lower in *E. huxleyi* aggregates than in diatom
93aggregates of similar size (Iversen and Ploug, 2010). This potentially confers coccolithophorid
94aggregates a larger potential for transporting organic carbon deeper in the ocean relative to diatom
95aggregates. It is important to note that the biodegradation experiments cited above were conducted
96in the presence of culture-associated prokaryotes or natural prokaryotic assemblages stored for a
97long time before use and the effect of fresh natural prokaryotic assemblages remains to be assessed.
98In addition, the increase in pressure experienced by aggregates sinking through the water column has
99yet to be included in such experiments.

100The objective of the present study was therefore to investigate the effect of hydrostatic pressure on
101the lipid composition (an indicator of cell degradation) and CaCO_3 dissolution within *E. huxleyi*
102aggregates in the presence of fresh free-living natural prokaryotic assemblages collected at 200 m
103depth in the NW Mediterranean Sea. Incubations were performed under controlled pressure
104conditions simulating a sinking rate of 150 m d^{-1} , in which we monitored the structure of the
105prokaryotic communities as well as the degradation of *E. huxleyi* lipids over 10 days following the
106experimental design presented in Tamburini et al. (2009). Total alkalinity, particulate inorganic and
107organic carbon (PIC and POC) and total organic carbon (TOC) concentrations were also monitored as
108indicators of CaCO_3 dissolution and organic carbon remineralization.

109**Results**

110Two sets of incubations (Exp A and Exp B) were performed at 13°C , with two pressure conditions
111applied in parallel: constant atmospheric pressure (ATM) and increasing hydrostatic pressure
112corresponding to a sinking simulation from 200 to 1700 m depth (HP). The *E. huxleyi* aggregate
113dilutions in either sterile-filtered (Exp A) or fresh GF/F-filtered (Exp B) seawater were sampled at t0

and after 10 days in the incubation vessels (t10). Exp A was performed with sterile-filtered seawater to monitor the development of prokaryotes associated with the *E. huxleyi* culture, while Exp B was designed to observe the development on *E. huxleyi* aggregates of a natural freshly collected seawater free-living prokaryotic community, and its effect on the organic and inorganic carbon contents of the aggregates.

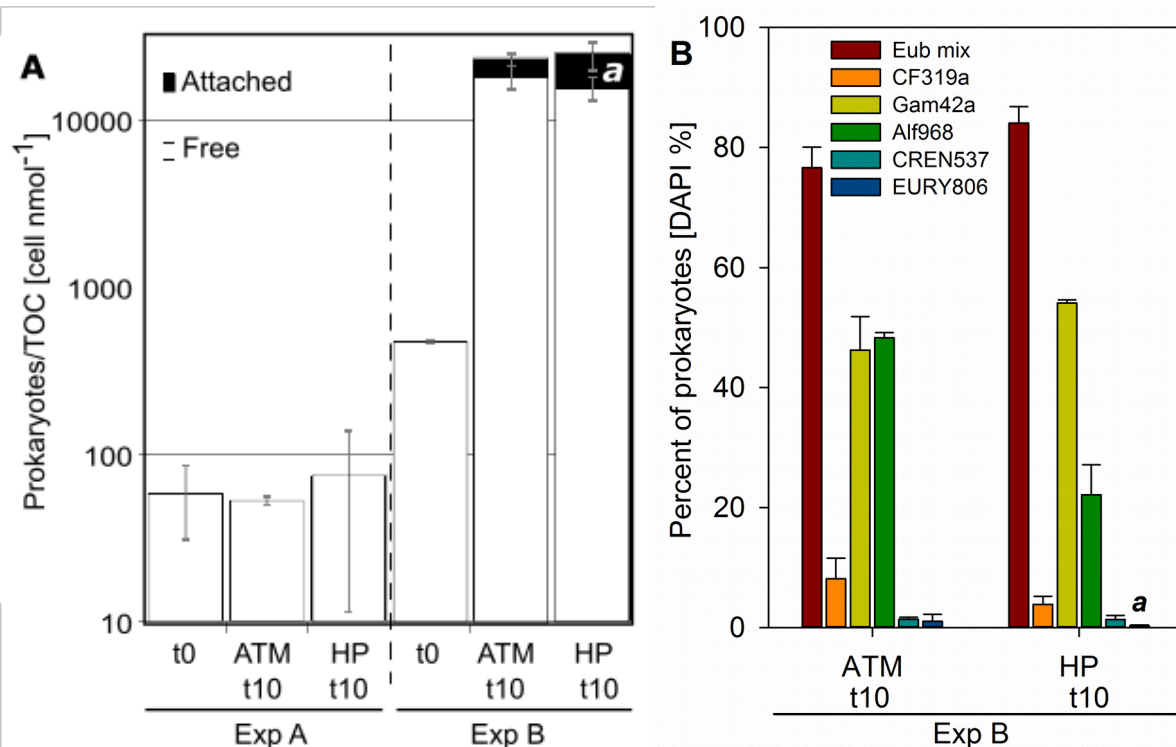
Prokaryotic development

The prokaryotic abundance associated with *E. huxleyi* culture aggregates (Exp A) did not change significantly between the start (t0: $7.7 \pm 1.8 \times 10^3$ cells mL⁻¹, average \pm SD) and end of the incubation (ATMt10: $6.5 \pm 0.2 \times 10^3$ cells mL⁻¹; HPt10: $9.4 \pm 3.92 \times 10^3$ cells mL⁻¹). Normalizing cell concentrations by the total organic carbon (TOC) concentration measured in each replicate to account for potential variations in the initial amount of aggregates added to each bottle, no significant difference was observed between both pressure conditions (Fig. 1A).

In Exp B, the total prokaryotic abundance increased by two orders of magnitude after 10 days (ATMt10: $2.2 \pm 0.4 \times 10^6$ cells mL⁻¹, HPt10: $2.7 \pm 0.0 \times 10^6$ cells mL⁻¹), with no significant difference between pressure treatments, when normalised by TOC (Fig. 1A). The growth of prokaryotes attached to *E. huxleyi* aggregates was stimulated under increasing pressure incubation (HP), where large aggregates were observed at the end (Suppl. Fig. 1). In this condition, attached prokaryotes ($> 2 \mu\text{m}$) were significantly more abundant (35.9 ± 14.7 % of total DAPI positive cells) than under constant atmospheric pressure (20.9 ± 4.7 %, Mann-Whitney-MW $p < 0.05$).

Under both atmospheric and increasing pressure conditions, abundances of *Archaea* and *Bacteria* were two and three orders of magnitude higher than at t0, respectively. Although the proportion of *Euryarchaea* was 4.1 times lower under pressure (Fig. 1B, significant, MW $p < 0.05$), increasing pressure had no effect on the proportion and abundance of *Crenarchaea* (ATMt10: $2.3 \pm 0.2 \times 10^4$ cell mL⁻¹, HPt10: $2.6 \pm 0.4 \times 10^4$ cell mL⁻¹, MW $p = 0.51$) and *Bacteria* (ATMt10: $1.3 \pm 0.2 \times 10^6$ cell mL⁻¹, HPt10: $1.4 \pm 0.2 \times 10^6$ cell mL⁻¹, MW $p = 0.83$). Among the *Bacteria*, Alpha- and Gamma-proteobacteria cell

139 concentrations increased by three orders of magnitude during the incubations, and the fraction of
 140 Alphaproteobacteria was twice as low in conditions of increasing HP than at ATM (not significant,
 141 MW $p = 0.13$). The proportion of Bacteroidetes also tended to be on average 1.9 times lower in HP
 142 than in ATM conditions (not significant, MW $p = 0.83$), but their concentrations increased only by one
 143 to two orders of magnitude.

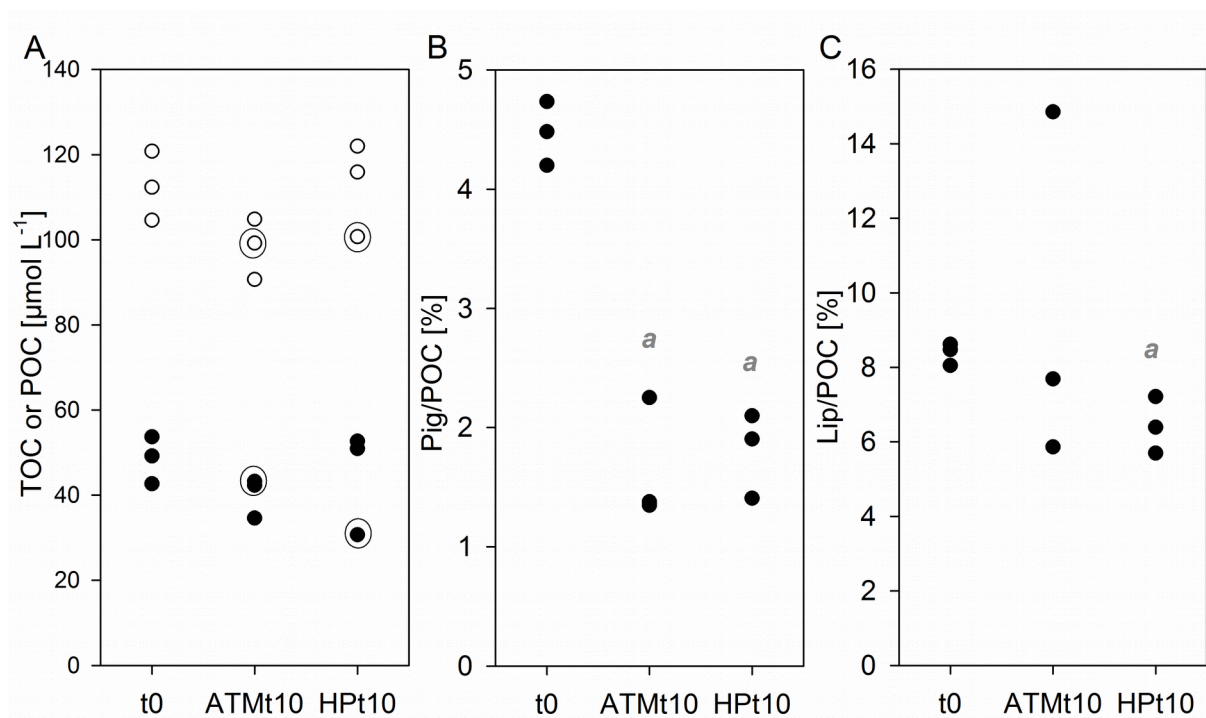


144
 145 **Figure 1: (A)** Total prokaryotic abundance (DAPI-stained cells) normalized by sample TOC concentration in
 146 *Emiliania huxleyi* aggregates biodegradation Exp A (no natural prokaryotes added) and Exp B (GF/F-filtered
 147 natural prokaryotic community added) initially (t0) and after 10 days under constant atmospheric pressure
 148 (ATMt10) or increasing hydrostatic pressure (HPt10). White bars: free-living prokaryotes; Black bars: >2μm
 149 particle-attached prokaryotes; (B) Percent of total prokaryotes (DAPI-stained cells) at the end of Exp B,
 150 detected by catalyzed reporter deposition coupled with fluorescence *in situ* hybridization (CARD-FISH). Eub
 151 mix (Eub338, Eub-II and Eub-III): *Bacteria*; CF319a: *Bacteroidetes*; Alf968, Gam42a: alpha- and gamma-
 152 subclasses of *Proteobacteria*; Cren537: *Crenarchaea*; Eury806: *Euryarchaea*. Average ± SD. Significant differences
 153 between ATM and HP are indicated as **a** (Mann-Whitney $p < 0.05$) for particle-attached (A) or total prokaryotes
 154 (B).

155 **E. huxleyi detritus organic carbon content and lipid degradation**

156 During the Exp B, TOC and particulate organic carbon (POC) concentrations tended to decrease over
 157 the 10 days incubation under atmospheric pressure only (Fig. 2A, ATMt10, not significant, $p = 0.13$).
 158 The POC pigment content decreased significantly after 10 days of exposure to natural prokaryotic

159assemblages, with no effect of pressure (Fig. 2B). In contrast, the lipid content (excluding pigments)
 160decreased significantly in HPt10 but not in ATMt10 (Fig. 2C).

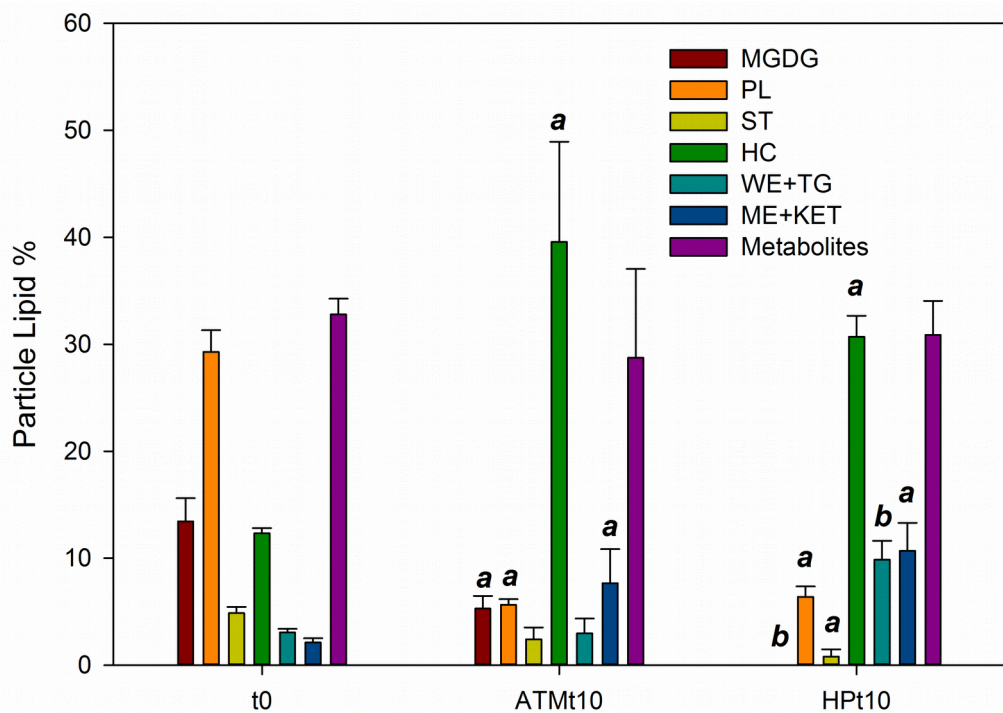


161
 162**Figure 2: Variation in organic carbon composition in Exp B: (A) total or particulate organic carbon (TOC-empty**
 163**circles, or POC-filled circles, $\mu\text{mol L}^{-1}$), (B) POC pigment fraction (%), (C) POC lipid fraction (pigments excluded,**
 164**%).** Significant differences (Mann-Whitney $p < 0.05$) are indicated as: **a** between t0 and t10. Circled data points
 165belong to the same ATM and HP replicates circled in Fig. 4.

166At the start of Exp B, the lipid composition in *E. huxleyi* aggregates was similar to the one described in
 167an earlier study with the same strain of *E. huxleyi* in exponential growth (Bell & Pond, 1996; see
 168suppl. Table 1). Overall, chloroplast lipids (including pigments and membrane monogalactosyl-
 169diacylglycerol-MGDG) and cell membrane lipids (phospholipids) dominated the lipid composition
 170(61.9-63.1 %). Sterols, hydrocarbons, neutral lipids (wax esters, triglycerides, methyl esters, ketones,
 171free fatty acids and alcohols) and metabolites (degradation lipids: mono- and di-acylglycerols)
 172accounted for 35.1-37.2 % of the particle lipids.

173Within the non-pigment lipids, a significant decrease in the proportion of chloroplast membrane and
 174whole cell membrane lipids (MGDG and PL, t0 9.8-18.2 % and 25.4-33.5 %, respectively) was
 175observed in both ATMt10 (2.5-6.8 % and 4.8-6.8 %) and HPt10 samples (0.0 % and 4.7-8.5 %).
 176Meanwhile, the proportion of hydrocarbons, methyl esters and ketones increased significantly in both

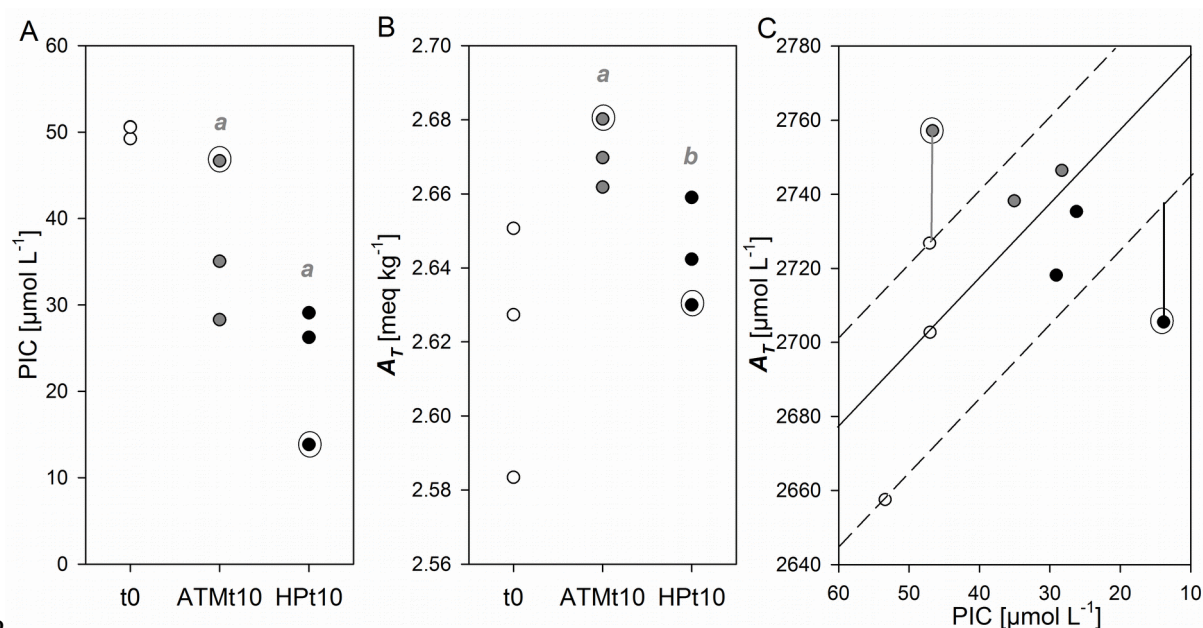
177conditions (Fig. 3). HPt10 samples experienced significant losses of membrane sterols and MGDG
 178relative to ATMt10 samples. At the same time, the relative proportion of reserve wax esters and
 179triglycerides were significantly higher in HP (HP: 5.9-12.5 %; ATM: 0.0-5.5 %), mainly due to the
 180appearance of triacylglycerides.



181
 182Figure 3: Variation in the lipid composition of *E. huxleyi* detritus in Exp B: Proportions of the different lipids
 183(pigments are excluded, % L_{tot}). MGDG: monogalactosyldiacylglycerol, PL: phospholipids, ST: sterols, HC:
 184Hydrocarbons, WE+TG: wax esters and triglycerides, ME+KET: methyl esters and ketones, ; Average \pm SD.
 185Significant differences (Mann-Whitney $p < 0.05$) are indicated as: **a** between t0 and t10, **b** between ATM and HP.

186Inorganic carbon content variations

187A significant drop of particulate inorganic carbon (PIC, Fig. 4A) was observed in both ATM and HP
 188conditions after 10 d ($p < 0.05$). It was larger under HP than at ATM (not significant, $p = 0.13$). Total
 189alkalinity (A_T) increased significantly between t0 and ATMt10 (Fig. 4B, $p < 0.05$) but the increase was
 190significantly lower under HP. The increase in A_T observed between t0 and HPt10 was indeed not
 191statistically significant ($p = 0.27$).



192

193 **Figure 4: Variation in (A) particulate inorganic carbon (PIC, $\mu\text{mol L}^{-1}$), (B) total alkalinity (A_T , meq kg^{-1}), and (C)**
 194 **A_T converted to $\mu\text{mol L}^{-1}$ (using a seawater density of 1028.7 L kg^{-1}) vs PIC in Exp B. Full and dashed lines on**
 195 **panel C model the expected co-evolution of A_T and PIC during calcite dissolution. Significant differences (Mann-**
 196 **Whitney $p < 0.05$) are marked with an “a” between t0 and t10, “b” between ATM and HP. Circled data points**
 197 **point at the ATM2 and HP3 replicate incubations displaying values out of the expected range for calcite**
 198 **dissolution from the measured t0 values shown in panel C.**

199 Discussion

200 *Effect of hydrostatic pressure increase on the prokaryotic community*

201 During the present *E. huxleyi* aggregate biodegradation experiment, initial natural mesopelagic
 202 Mediterranean Sea prokaryotic concentrations increased by up to two orders after 10 days in
 203 controlled temperature and hydrostatic pressure (HP) conditions simulating particle sinking from
 204 200m to 1700m depth at a rate of 150 m d^{-1} . Such a high prokaryotic development has recently been
 205 observed with deep Mediterranean seawater natural prokaryotic communities amended with labile
 206 marine broth, both under atmospheric (ATM) and HP conditions (Wannicke et al., 2015). This might
 207 therefore indicate that *E. huxleyi* aggregates constitute a relatively labile substrate for mesopelagic
 208 Mediterranean prokaryotic communities to grow on.

209 The natural prokaryotic diversity was dominated by *Bacteria*, with a lower proportion of *Archaea* at
 210 the end of the incubation, as previously observed in similar experiments (Tamburini et al., 2006,

2112009), despite a significant increase in absolute abundance. The initial fraction of *Bacteria*,
212*Crenarchaea* and *Euryarchaea* were lower in the present experiment (30%, 7% and 4%, respectively)
213than in previous experiments with 200m depth seawater sampled in different areas of the
214Mediterranean Sea (Tamburini et al., 2006, 2009). Different sea surface mixing conditions at the time
215of the natural prokaryotic community sampling for the different experiments might have resulted in
216different initial community compositions (Pinazo, pers. Com). When focusing on the classes of
217*Bacteria* generally found associated to marine aggregates (Acinas et al., 1999; Delong et al., 1993;
218Moeseneder et al., 2001; Ploug and Grossart, 1999; Simon et al., 2002; Thiele et al., 2015), only
219Bacteroidetes and Alphaproteobacteria tended to be sensitive to increasing hydrostatic pressure
220conditions (HP). Similar trends were observed during previous particle sinking simulation experiments
221with natural faecal pellets (Tamburini et al., 2009). The latter authors proposed that the sensitivity of
222Bacteroidetes to pressure may have reduced the amount of particulate carbohydrate hydrolysed. This
223could result in a lower production of dissolved carbohydrates as substrate for Alphaproteobacteria
224(Tamburini et al., 2009). A similar mechanism might be at play in the present experiment with *E.*
225*huxleyi* detritus, which is supported by the observation of large sticky particles and aggregates under
226HP condition only, as in Tamburini et al. (2009)(Suppl. Fig. 1). These large sticky aggregates might
227indeed be the result of less degradation of transparent exopolymeric particles (TEP) under HP relative
228to ATM (Tamburini et al., 2009), associated with the fact that more dissolved carbohydrates may have
229been released under HP by *E. huxleyi*, possibly resisting prokaryotic degradation (Nanninga and
230Tyrrell, 1996) and scavenged by TEP (Engel et al., 2004).

231Interestingly, we observed that prokaryotes grew significantly more on > 2 µm aggregates under
232increasing pressure (HP) than at atmospheric pressure (ATM). This pattern was not observed in a
233previous similar controlled experiment studying the biodegradation of natural aggregates collected in
234the Mediterranean Sea (mainly faecal pellets) during 6.5 days, although the formation of visible sticky
235aggregates had also been observed under HP (Tamburini et al., 2009). The fact that the present
236simulation lasted longer and reached higher pressure levels could explain that the pattern observed

237here may not have been detected in the previous study. Finally, it also is also possible that
238coccolithophorid cells are more sensitive to pressure than faecal pellets or diatom aggregates
239(Tamburini et al., 2006, 2009). Hence, more labile intracellular organic matter would be released in
240the aggregates surroundings, favouring higher prokaryotic growth. To verify the latter hypothesis, we
241analysed the aggregate lipid contents and calculated a lipolysis index.

242**Degradation of *E. huxleyi* aggregates organic matter**

243Assuming a prokaryotic cellular carbon content of 12.4-53.0 fg C cell⁻¹ (Fukuda et al., 1998; Kogure
244and Koike, 1987), prokaryotes made up for 0.05-0.21% of the TOC at t₀, which increased to 1.84-
24511.44 % at ATMt₁₀ and 2.32-11.94 % at HPt₁₀. Heterotrophic growth could have been supported by
246the dissolved organic matter present at the start of the incubation, and/or by the degradation of *E.*
247*huxleyi* aggregates. A lipolysis index defined as the ratio of metabolites resulting from acyl-lipid
248degradation to the sum of acyl lipids can be used to characterize the degradation stage of labile
249organic matter (Goutx et al., 2000, 2003). Although the lipid index in *E. huxleyi* aggregates increased
250significantly between the beginning (t₀: 0.72±0.06) and the end of the experiment (ATMt₁₀:
2512.07±1.22, HPt₁₀: 1.93±0.32), there was no significant pressure effect (MW $p = 0.51$). However, the
252lipid fraction of *E. huxleyi* detritus organic carbon decreased significantly after ten days under HP,
253contrasting with previous observations using natural aggregates, mainly consisting in faecal pellets
254(Tamburini et al., 2009). In addition, compared to the lipid composition at ATM, membrane lipids
255(phospholipids, sterols, MGDG) disappeared from the particulate fraction significantly more under HP
256than at ATM, which could indicate higher *E. huxleyi* cell lysis under pressure (Baldi et al., 1997). *E.*
257*huxleyi* aggregates might therefore be more vulnerable to degradation under pressure than faecal
258pellets.

259 ***Changes in mineral content***

260 Particulate inorganic carbon (PIC) decreased more under HP than at ATM, suggesting that more
261 calcite dissolution may have occurred under increased hydrostatic pressure (Fig. 4). Enhanced PIC
262 dissolution under HP has been observed in a previous study with diatom-mineral (carbonate-
263 kaolinite-smectite) aggregates, together with a decreased POC decay rate (de Jesus Mendes and
264 Thomsen, 2012). We here show that increased CaCO_3 dissolution can occur in calcite-sheathed cell
265 aggregates sinking through mesopelagic waters in the presence of natural prokaryotic communities.

266 According to the stoichiometry of calcification, two moles of total alkalinity are produced per mole of
267 CaCO_3 dissolved (approximated as moles of PIC in Fig. 4C). However, although there was more calcite
268 dissolution under pressure, the increase in total alkalinity was larger at atmospheric pressure.
269 Nonetheless, values were within the range expected for calcite dissolution, considering the starting
270 total alkalinity and PIC conditions (Fig. 4C), except for the two outliers circled in Fig. 4 (namely ATM2
271 and HP3 incubations, also circled in Fig. 2). Processes other than CaCO_3 dissolution may explain the
272 outlying PIC- A_T pairs measured in replicates ATM2 and HP3, which respectively also displayed the
273 lowest prokaryotic abundance ($1.77 \cdot 10^6 \text{ cell mL}^{-1}$) and POC concentration ($30.7 \mu\text{mol L}^{-1}$).

274 Total alkalinity may indeed be affected by microbial aerobic and anaerobic organic matter
275 remineralization/transformation through the consumption or release of ammonium, nitrate,
276 phosphate and sulphate (Gattuso et al., 1999). The processes of respiration, nitrification and sulphate
277 reduction have adverse effects on protons release/uptake. However, the difference of $+31 \mu\text{mol L}^{-1} A_T$
278 between the value measured in ATM2 and the value expected from the triplicate t0 PIC
279 concentrations cannot be solely explained by dissimilation of ammonium by organic matter
280 respiration. This process would result in a A_T increase of 0.17 moles per mole of TOC respired (Gattuso
281 et al., 1999), when the difference in TOC between t0 replicates and ATM2 was only 5.4 to $21.6 \mu\text{mol L}^{-1}$
282¹ (which was comparable to the other 2 ATM replicates). Since the t0 replicates consisted in
283 independent splits of the initial aggregate-seawater mixture that was further split into ATM and HP
284 replicate incubations, another explanation for this outlying pair could be that the ATM2 incubation

25

285may have started with a higher PIC concentration around 65 μM (higher concentration of detached
286coccoliths).

287Similarly, the $-31 \mu\text{mol L}^{-1}$ difference in A_T between the value measured in HP3 and the expected value
288cannot be explained solely by the dissimilation of nitrate by organic matter respiration. Such a
289process would lead to a decrease in A_T of 0.13 moles per mol of CO_2 released (Gattuso et al., 1999),
290whereas the difference in TOC concentration between t0 replicates and HP3 comparable to that with
291ATM2 was observed (Fig. 2). Given the large A_T variability observed between the independent splits
292sampled at t0, it is more likely that the HP3 incubation started with a much lower A_T than the other
293HP replicates (around $2635 \mu\text{mol L}^{-1}$, or $2561 \mu\text{mol kg}^{-1}$ at $50 \mu\text{M PIC}$).

294***Implications for the carbon pump and the ballast hypothesis***

295In the Mediterranean Sea, CaCO_3 -sheathed coccolithophores can dominate the phytoplankton
296community (Ignatiades et al., 2009). They are highly diverse, including large numbers of the
297ubiquitous species *E. huxleyi* (Cros and Fortuño, 2002; Knappertsbusch, 1993). Several studies have
298stressed the need for controlled experiments to validate the hypothesis that CaCO_3 may be a more
299efficient POC “carrier” than biogenic silica (De La Rocha and Passow, 2007; Passow and De La Rocha,
3002006; Passow, 2004). Our experiment contributes to a better understanding of this issue. In contrast
301with previous experiments using natural marine snow/faecal pellets (Tamburini et al., 2009; Turley,
3021993), organo-mineral aggregates (de Jesus Mendes et al., 2007) or diatom culture aggregates (de
303Jesus Mendes and Thomsen, 2012; Tamburini et al., 2006), CaCO_3 -containing *E. huxleyi* culture
304aggregates appear more sensitive to the increase in hydrostatic pressure. Our results suggest that
305increased pressure results in higher cell lysis and CaCO_3 dissolution relative to ATM. The protecting
306effect of coccoliths may therefore not remain effective throughout the water column, since coccoliths
307might detach from cells and dissolve more readily under pressure. Calcium carbonate may therefore
308not be an efficient POC carrier to the deep ocean in the form of intact coccolithophorid cells.

309Coccolithophores are the main producers of the pelagic CaCO_3 biogenically produced in the global
310ocean ($0.8\text{--}1.4 \text{ Gt year}^{-1}$) (Feely et al., 2004) and may contribute to about 50% of pelagic CaCO_3
311deposition in sediments (Broecker and Clark, 2009; Poulton et al., 2007). Yet, the processes and rates
312of coccolith CaCO_3 dissolution in the upper layers of the ocean are poorly identified and quantified.
313Since calcification decreases the ocean CO_2 uptake capacity by reducing total alkalinity, the depth of
314calcite dissolution likely has important implications on the net gradient in pCO_2 between the ocean
315and atmosphere (Antia et al., 2001; Barrett et al., 2014).

316The dissolution of pelagic CaCO_3 particles has long been assumed to occur primarily at great depths
317below the calcite and aragonite saturation depths (Broecker, 1977). In fact, up to 80% of the CaCO_3
318that is exported out of the surface ocean might actually dissolve in the upper 1000 m, well above the
319lysocline (Chung et al., 2003; Feely et al., 2004; Iglesias-Rodriguez et al., 2002; Jansen and Wolf-
320Gladrow, 2001; Milliman et al., 1999; Wollast and Chou, 1998, 2001). Grazing by microzooplankton
321has been shown to play an important role in coccolithophorid calcite dissolution (Antia et al., 2008;
322Harris, 1994; Jansen and Wolf-Gladrow, 2001; Milliman et al., 1999). In contrast, carbonate
323dissolution on individual calcifying cells by microbes was deemed negligible (Bissett et al., 2011), but
324the presence of chemical gradients within sinking aggregates (Ploug and Bergkvist, 2015) may create
325micro-environments with lower pH and calcite saturation state that could facilitate (a)biotic
326dissolution. We show here that calcium carbonate dissolution in the presence of natural prokaryotic
327communities might be higher under increasing hydrostatic pressure. Pressure was recently suggested
328to affect dissolution kinetics over and above its influence on the saturation constants (Dong et al.,
3292016). The combined effect of pressure and microbial activities therefore remains to be assessed in a
330more mechanistic manner.

331On the other hand, we show that increasing hydrostatic pressure potentially triggers the
332development of Mediterranean mesopelagic prokaryotic communities on *E. huxleyi* aggregates (as
333opposed to diatom detritus or natural aggregates, Tamburini et al., 2006, 2009), as well as
334aggregation. The study by Tamburini et al. (2009) had already shown that particulate

335carbohydrates:POC and Transparent Exopolymer Particles (TEP):POC ratios could be significantly
336higher in conditions of increasing hydrostatic pressure. The prokaryotes themselves may promote
337aggregation, through the production of sticky extracellular muco-polysaccharides (Biddanda and
338Pomeroy, 1988; Biddanda, 1986; Turley, 1992). Moreover, coccoliths have been proposed as
339aggregation nuclei for colloidal DOM, therefore offering organic matter protection from
340solubilization/remineralization (Engel et al., 2009a). The increased aggregation observed under
341pressure in our reaction vessels could therefore compensate the loss of mineral ballast at depth, by
342increasing aggregate sizes and/or mineral scavenging potential. To what extent pressure-induced
343aggregation balances calcite dissolution *in situ* remains to be observed and assessed, by including
344model calculations, for instance.

345Methods

346*Production of Emiliana huxleyi aggregates and experimental design*

347The TW1 *E. huxleyi* strain (AlgoBank-Caen) was maintained in exponential growth phase in K/2
348medium (Keller et al., 2007) at 21°C, under a 12-12h light-dark cycle (intensity: 4300 Lux). The culture
349was supplemented with Provasoli's antibiotic concentrate (3% final concentration, Sigma) until the
350bacterial numbers reached a minimal proportion of one to two prokaryote per phytoplankton cell.
351The antibiotic solution was then removed for two culture passagings before *E. huxleyi* cells in
352exponential phase were concentrated by centrifugation (3000 rpm, 15 min) (Houdan et al., 2005;
353Sawada and Shiraiwa, 2004). Fresh detritus was obtained according to a procedure modified from
354(Tamburini et al., 2006) by exposing cell culture to 55 °C for 10 min only, which avoided cell disruption
355observed during freeze-thaw cycles. The detritus aliquots were immediately diluted in sterile-filtered
356or GF/F-filtered Mediterranean Seawater (for Exp A and Exp B, respectively, see details below) to
357reach a final concentration of about 600 µg POC L⁻¹ (cell density of 5000 cell mL⁻¹) close to natural
358coccolithophore bloom (Holligan et al., 1993). Aliquots were sampled at the starting point of the
359incubations (t₀) for the various microbiological and chemical parameters described below.

360 ***Incubation in the PArticle Sinking Simulator (PASS)***

361 The two experiments (Exp A and Exp B) were conducted in March 2007 using Mediterranean
362 seawater (13°C) collected at 200 m depth, above the Marseilles Trench (43°01'48 N-05°11'158 E,
363 600m bottom depth). In Exp A, seawater was left in the dark at 4 °C for 15 d and gently filtered
364 through 0.2 µm-porosity polycarbonate filters to subtract the prokaryotic community. In Exp B, fresh
365 natural prokaryotic assemblages <0.7 µm were recovered in the filtrate of a gentle filtration onto pre-
366 combusted GF/F filters of the freshly collected seawater sample.

367 Exp A and Exp B were incubated for 10 d at *in situ* temperature (13°C). The PASS system was used
368 following a procedure described in (Tamburini et al., 2009), except a peristaltic liquid dispenser
369 (Jencons Scientific Ltd.) was used to obtain homogeneous replicate aliquots. Aliquots were incubated
370 in 500 mL high-pressure bottles (HPBs) where pressure increased linearly using a piloted pressure
371 generator in order to reproduce the increase in pressure that occurs when particles sink.
372 Coccolithophores detritus was kept in suspension by regular half-revolutions of the HPBs in water
373 baths kept at *in situ* temperature (Tamburini et al., 2006). For Exp A, two duplicate HPBs were kept at
374 atmospheric pressure and two duplicate HPBs were exposed to increasing pressure (1.5 MPa d⁻¹
375 corresponding to a sinking rate of 150 m d⁻¹). This is applicable to mid-density particles, as discussed
376 in (Tamburini et al., 2006) and corresponds to sinking rates observed in previous *E. huxleyi* aggregates
377 studies (Engel et al., 2009b; Iversen and Ploug, 2010; Ploug et al., 2008). Exp B was performed with
378 triplicate HPBs. After 10 days of incubation (HPT10, corresponding to a depth of 1700 m), the
379 pressurised coccolithophores detritus dilutions were decompressed and sampled as described in
380 (Tamburini et al., 2009) for the parameters described below.

381 ***Microbiological analyses***

382 Samples for prokaryotic abundances (50-135 mL) were fixed using 0.2-µm filtered formaldehyde (2%
383 final concentration), kept at room temperature for 15 min, and subsequently stored at 4°C in the dark
384 for around 12h. For total (>0.2 µm) and free-living (0.2-2.0 µm) prokaryotic abundances, samples

385were filtered onto 0.2- μ m-pore-size polycarbonate filters supported by 0.45- μ m-pore-size cellulose
386nitrate filters directly, or after filtration by gravitation onto 2.0- μ m-pore-size polycarbonate filters, as
387in (Tamburini et al., 2009). Cells were stained with diamidinophenylindole (DAPI) according to (Porter
388and Feig, 1980). The fraction of “attached” or large prokaryotes >2 μ m was calculated by subtracting
389values of the 0.2-2.0 μ m fraction from the total fraction.

390Samples for catalyzed reporter deposition coupled with fluorescence *in situ* hybridization (CARD-FISH)
391were treated as in (Tamburini et al., 2009): briefly, samples were filtered onto 0.2- μ m-pore-size
392polycarbonate filters as for the total prokaryotic abundances. Filters were washed twice with 0.2- μ m
393filtered MilliQ water, air-dried, and stored in scintillation tubes at -20°C until analysis. Cells were
394embedded in low-gelling point 0.1% agarose (Sigma), dried at 37°C for 10min, and dehydrated with
39595% ethanol. Bacterial cell walls were permeabilized by subsequent treatments with lysozyme (10 mg
396ml⁻¹, Sigma)(Amann et al., 1995) and with achromopeptidase (60 U, Sigma)(Sekar et al., 2003).
397Archaeal cell walls were permeabilized with proteinase K (0.2 ml ml⁻¹, Fluka)(Teira et al., 2004). Acid
398treatment in 0.01M HCl was performed to denature endogenous peroxydase before hybridization
399with the horseradishperoxydase (HRP)-labeled oligonucleotide probes listed in Table1: for a detailed
400protocol see (Tamburini et al., 2009). The filter portions hydridized with each probe were mounted in
401a Citifluor:Vectashield:DAPI mix, and DAPI and CARD-FISH-stained cells were quantified under an
402Olympus BX61 microscope equipped with a 100-WHg-lamp and appropriate filter sets according to
403(Tamburini et al., 2009).

404**Table 1:** 16S rRNA-targeted oligonucleotide probes used in this study.

Probe	Sequence(5'-3')	Target organisms	% Formamide	References
Eub338/I	GCT GCCTCCCGTAGGAGT	Domain ofbacteria	55	(Amann et al., 1990)
EubII	GCA GCCACCCGTAGGTGT	Domain ofbacteria	55	(Daims et al., 1999)
EubIII	GCT GCCACCCGTAGGTGT	Domain ofbacteria	55	(Daims et al., 1999)
ALF968	GGTAAGGTTCTGCGCGTT	Most of Alfa of Proteobacteria	55	(Manz et al., 1992)
GAM42a ^a	GCC TTCCCACATCGTTT	Gamma-Subclass of Proteobacteria	55	(Manz et al., 1992)
CF319a	TGGTCCGTGTCTCAGTAC	Cytophaga- Flavobacter cluster	55	(Manz et al., 1996)

Cren537	TGACCACTTGAGGTGCTG	Crenarchaea	20	(Teira et al., 2004)
Eury806	CACAGCGTTTACACCTAG	Euryarchaea	20	(Teira et al., 2004)
NegControl	TAGTGACGCGCTCGA	For non-specific probe binding	55	(Karner and Fuhrman, 1997)

405^a Including an unlabeled competitor probe BET42a (5'-GCCTTCCCACTTCGTTT-3'), see (Manz et al., 1992) for
406details.

407

408**Chemical analyses**

409All glassware was pre-combusted before use. Additionally, glassware was rinsed with 1 N HCl and
410Milli-Q water after each sample. All plastic ware was rinsed with 1 N HCl and Milli-Q water and
411changed for each sample. Sample aliquots for particulate organic carbon (POC, 50-104 mL) and lipids
412(P-lips, 140-500mL) were filtered onto pre-combusted 0.7 µm GF/F filters (25 mm filter diameter)
413under a low vacuum (<50mm Hg) and preserved at -20°C or in liquid nitrogen, respectively.

414Filters for particulate total carbon (P_{tot}C) and POC were divided in two and analyzed as in Raimbault
415et al. (2008). The filter portions used for P_{tot}C were analyzed directly, while filter portions for POC
416measurement were acidified with 100 µL H₂SO₄ (0.5N) and subsequently analyzed using high
417combustion (1000°C) on a mass spectrometer (CN-Integra tracer-mass). The concentration of
418particulate inorganic carbon (PIC) was calculated from the difference between P_{tot}C and POC.

419Lipids were extracted from filters using a Bligh and Dyer (1959) protocol, and lipid classes were
420separated on chromarods and quantified on a thin layer chromatography/flame ionization detection
421(TLC/FID) Iatroscan apparatus model MK-6s (Iatron,Tokyo) coupled to a PC equipped with a Chromstar
4226.1 integration system (Bionis, Paris), as in Goutx et al. (2007). The separation of the 16 following lipid
423classes was obtained: neutral lipids including hydrocarbons (HC), sterol esters co-eluting with wax
424esters (WE), ketones (KET), triacylglycerols (TG), free fatty acids (FFA), alcohols (ALC), sterols and
425diglycerides (DG); chloroplast lipids containing pigments (Pig), glycolipids and
426monogalactosyldiacylglycerol (MGDG); monoacylglycerols (MG); and phospholipids (PL) including
427diphosphatidylglycerides co-eluting with phosphatidylglycerides (DPG+PG), phosphatidylethanol-
428amine (PE) and phosphatidylcholine (PC). A lipolysis index was calculated as in Goutx et al. (2000,

4292003), defined as the ratio of the sum of the metabolites (DG, MG, FFA, ALC) to the sum of the intact
430acyl-lipids (TG, PE, PI, DPG+PG, PC, WE, MGDG).

431TOC concentrations were obtained by adding POC to DOC values. Samples for DOC were obtained and
432preserved as in Tamburini *et al.* (2009). The DOC measurements were carried out in the laboratory at
433the Pisa section of the Institute of Biophysics (IBF), CNR, with a Shimadzu TOC-VCSN. Samples were
434acidified with 2N HCl and sparged for 3 minutes with CO₂-free ultra-high purity air in order to remove
435inorganic carbon. From 3 to 5 replicate injections were performed until the analytical precision was
436lower than 1%. The system blank was measured every day at the beginning and the end of analyses
437using low-carbon water (2-3 $\mu\text{mol L}^{-1}$ C) produced by a Milli-Q system. Measurement reliability was
438assessed twice a day by comparison of data with DOC Consensus Reference Waters (CRM; Hansell,
4392005) (measured concentration = 42.7 $\mu\text{mol L}^{-1}$, nominal concentration: 41-44 $\mu\text{mol L}^{-1}$). For further
440analytical details refer to Santinelli *et al.* (2013)

441Samples for total alkalinity (A_T) were filtered on GF/F membranes (duplicate 50 mL filtrate aliquots
442from the P_{tot}C and POC GF/F filtrations), immediately poisoned with 100 μL of a 50% saturated HgCl₂
443solution (37 g HgCl₂ in 1 L distilled water) and stored at 4°C in the dark pending analysis. A_T was
444determined on 20 mL subsamples using a home-made titration system composed of a 20 mL open
445temperature-controlled titration cell (kept at 25.0°C), an Orion 8103SC pH electrode calibrated on the
446National Bureau of Standards scale and a computer-driven Metrohm 665 Dosimat titrator,
447Switzerland. The seawater samples and the acid titrant (0.1 N HCl) were kept at a constant
448temperature of 25°C. Samples were weighted with a precision of 0.01 g before the titration to
449determine their exact volume from temperature and salinity. A_T was calculated from a Gran function
450applied to pH variations from 4.2 to 3.0 as a function of added volume of HCl. A_T measurements had a
451reproducibility of 3 $\mu\text{mol kg}^{-1}$.

452 **Statistical analyses**

453 Differences in prokaryotic abundances (N = 3 replicate incubation bottles per condition) were
454 analyzed by the non-parametric pairwise Mann-Whitney test on raw data (i.e. individual replicate
455 values), due to the low number of replicates, using the Statistica 6 software. Unless otherwise stated,
456 data are reported as averages and standard deviation (SD) and statistical difference was accepted at p
457 ≤ 0.05 .

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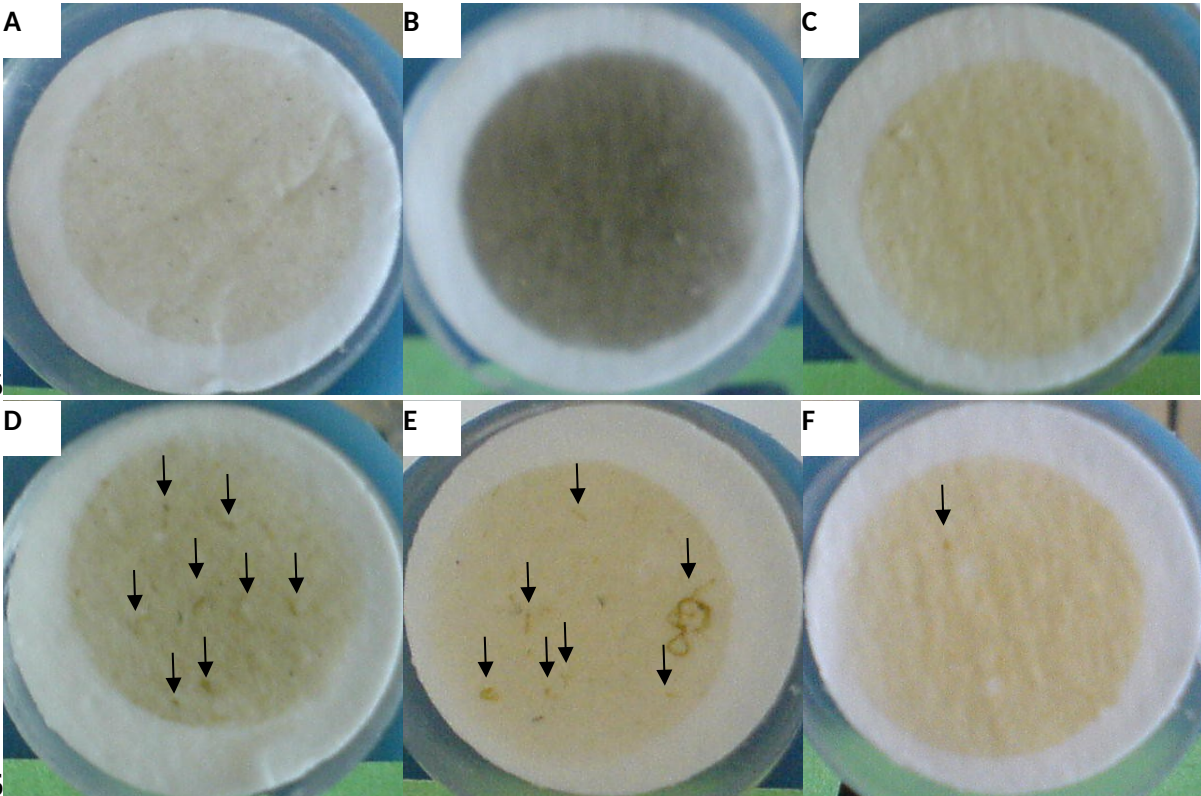
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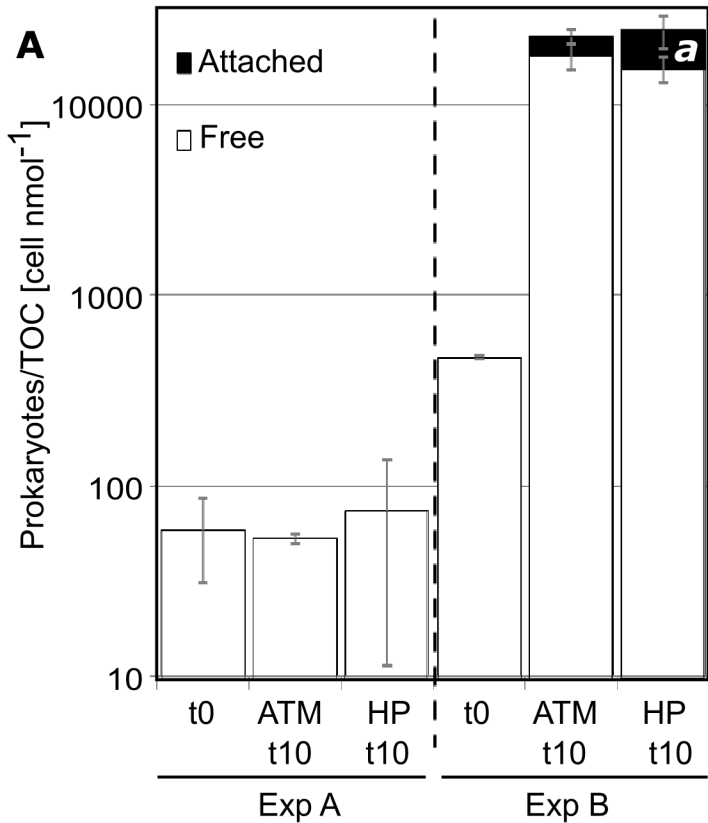
735**Supplementary data**

736**Supplementary Table 1:** Lipid proportions (% Total Particulate Lipids) in the *E. huxleyi* culture at day 4
737(Bell and Pond, 1996) and during Exp B. Numbers 1, 2 and 3 stand for replicate incubations.
738Chloroplastic: pigments + monogalactosyldiacylglycerol, Phospholipids: diphosphatidylglycerides +
739phosphatidylglycerides + phosphatidylethanolamine + phosphoinositol + phosphatidylcholine, ST:
740sterols, Neutral: wax esters + methyl esters + ketones + triglycerides + Free Fatty Acids + fatty
741alcohols, Metab: Monoglycerides + DiGlycerides, HC: Hydrocarbons.

	Chloroplastic	Phospholipids	Sterols	Neutral	Metab	HC
<i>E. huxleyi</i> 4 days (Bell and Pond, 1996)	41.3 ± 0.8	21.5 ± 0.4	3.6 ± 0.4	23.3 ± 1.3	ND	9.4 ± 0.3
t0-1	39.3	22.5	2.4	23.2	4.0	7.6
t0-2	44.8	18.2	3.3	17.9	5.5	8.4
t0-3	46.5	16.6	3.8	19.2	4.4	8.1
ATMt10-1	10.8	5.0	0.0	50.3	0.0	31.2
ATMt10-2	32.5	3.4	3.1	13.6	0.0	43.6
ATMt10-3	20.7	5.8	2.5	37.7	0.0	20.6
HPt10-1	22.5	3.6	1.9	37.2	5.2	25.6
HPt10-2	19.8	6.8	0.0	43.1	0.0	21.0
HPt10-3	23.0	4.6	0.0	35.3	0.0	25.3

742**Supplementary Figure 1:** Photographs of the GF/F filters from Exp B at ATMt10 (A, B, C) and HPt10 (D,
743E, F). A-C, and B-F are the replicate incubations. Arrows point at some of the large visible aggregates
744in HPt10 incubations.





B