Removal of organic magnesium in coccolithophore calcite

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Abstract

Coccolithophore calcite refers to the plates of calcium carbonate ($CaCO_3$) produced by the calcifying phytoplankton, coccolithophores. The empirical study of the elemental composition has a great potential in the development of paleoproxies. However, the difficulties to separate coccolithophore carbonates from organic phases hamper the investigation of coccoliths magnesium to calcium ratios (Mg/Ca) in biogeochemical studies. Magnesium (Mg) is found in organic molecules in the cells at concentrations up to 400 times higher than in inorganically precipitated calcite in present-day seawater. The aim of this study was to optimize a reliable procedure for organic Mg removal from coccolithophore samples to ensure reproducibility in measurements of inorganic Mg in calcite. Two baseline methods comprising organic matter oxidations with (1) bleach and (2) hydrogen peroxide (H_2O_2) were tested on synthetic pellets, prepared by mixing reagent grade $CaCO_3$ with organic matter from the non-calcifying marine algae *Chlorella autotrophica* and measured with an ICP-AES (inductively coupled plasma-atomic emission spectrometer). Our results show that treatments with a reductive solution [using hydroxylamine-hydrochloride ($NH_2OH{\cdot}HCl + NH_4OH$)] followed by three consecutive oxidations (using H_2O_2) yielded the best cleaning efficiencies, removing > 99% of organic Mg in 24 h. P/Ca and Fe/Ca were used as indicators for organic contamination in the treated material. The optimized protocol was tested in dried coccolithophore pellets from batch cultures of *Emiliania huxleyi*, *Calcidiscus leptoporus* and *Gephyrocapsa oceanica*. Mg/Ca of treated coccolithophores were 0.151 ±0.018, 0.220 ±0.040, and 0.064 ±0.023 mmol/mol, respectively. Comparison with Mg/Ca literature coccolith values, suggests a tight dependence on modern seawater Mg/Ca, which changes as a consequence of different seawater origins (< 10%). The reliable determination of Mg/Ca and Sr/Ca, and the low levels of organic contamination (Fe/Ca and P/Ca) make this protocol applicable to field and laboratory studies of trace elemental composition in coccolithophore calcite.

51 # 1. INTRODUCTION

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53 Coccolithophores are marine calcifying phytoplanktonic organisms that play a pivotal role by
54 contributing to the particulate matter production and export via the biological carbon pump
55 (Francois et al., 2002; Gehlen et al., 2007; Ridgwell et al., 2009). The export of inorganic carbon
56 takes place in the form of coccoliths, which are composed of calcium carbonate ($CaCO_3$), with
57 minor proportions of magnesium carbonate ($MgCO_3$) and strontium carbonate ($SrCO_3$) in bloom-
58 forming species such as *Emiliania huxleyi* and *Gephyrocapsa oceanica* (Siesser, 1977; Stoll et al.,
59 2001; Stoll et al. 2007; Ra et al., 2010; Müller et al., 2011). Mass accumulation of coccolithophore
60 carbonates has been taking place since coccolithophores first appeared in the sediment record of the
61 Permian/Triassic (P/T) boundary, ca. 250 million years ago (Bown et al., 2004; de Vargas et al.,
62 2004). Sedimentation of inorganic material that has not been dissolved during sinking and
63 accumulated on the seabed (Feely et al., 2004; Berelson et al., 2007) has thus formed an extensive
64 stratigraphical fossil record that is available for geochemical analysis in paleoceanographic studies.
65 The rate of trace elements incorporation (e.g. Mg, Sr and Ba) in coccolithophore calcite
66 depends largely on their concentration in seawater (Langer et al., 2006a; Ries, 2009; Langer et al.,
67 2009a), but it also follows thermodynamic, kinetic (Morse and Bender, 1990), and biological
68 discrimination imposed by the organisms (Stoll and Schrag, 2000), modulating calcite composition.
69 Experimental data (Stoll et al., 2001; Ra et al., 2010) suggest that temperature might also exert a
70 control on the Mg/Ca as in abiogenic calcites (Mucci and Morse, 1987; Tesoriero and Pankow,
71 1996), foraminifera (Barker et al., 2005) and echinoderms (Kroh and Nebelsick, 2010). Mg/Ca has,
72 therefore, been used as a paleothermometry proxy, although "cleaning issues" in removing organic
73 Mg have precluded a widespread implementation in coccolithophore carbonates (Stoll et al., 2001;
74 Stoll and Ziveri, 2004; Ra et al., 2010; Müller et al., 2011). An understanding of the Mg

contribution and composition in sinking carbonates also allows assessing susceptibilities to dissolution [e.g. the biomineral saturation state with respect to Mg phases (Andersson et al., 2008)]. However, this is more relevant in high magnesium (> 4 % $MgCO_3$) carbonates (e.g. Morse et al., 2006; Kuffner et al., 2007).

Magnesium is abundant in the organic fraction of coccolithophores. This element is present in biomolecules, such as chlorophyll, where it is a central ion in the porphyrin ring (e.g. Mg-protoporphyrin and Mg -2, 4-diviniyl pheoporphyrin a_5) (Stanier and Smith, 1959; Chereskin et al., 1982). Magnesium also binds to cellular polyphosphate compounds such as RNA and DNA (Lusk et al., 1968), and adenosine triphosphate (ATP), which is the main energetic molecule for cellular metabolism (Leroy, 1926). Furthermore, magnesium acts as a co-factor to activate multiple cell enzymes (Legong et al., 2001). Therefore, studies based on the Mg/Ca in biogenic calcite (e.g. laboratory incubations, sediment traps, and sediment cores) require removal of Mg associated to organic phases in order to prevent contamination of the inorganic phases (Stoll et al., 2001; Barker et al., 2003). The major present limitations in cleaning procedures are the small size of the individual coccoliths that complicate individual manipulation, and the low Mg content in calcite (< 0.1 mmol/mol) (Stoll et al., 2001, Stoll et al., 2007; Ra et al., 2010).

In this study we optimized cleaning methods using synthetic samples of non calcite-bearing marine organic matter and abiogenic reagent calcite whose Mg/Ca was independently measured. The effectiveness of the cleaning protocols and uncertainties can therefore be assessed. The optimization procedure focused on two baseline methods consisting in organic matter oxidations with (1) bleach, and (2) hydrogen peroxide. The protocol G, which yielded the highest cleaning efficiency with respect to reagent grade $CaCO_3$ (> 99%) and the lowest P and Fe contamination levels, requiring less time of incubation (~ 24 h), was applied to dry pellets of three widespread coccolithophore species (*Emiliania huxleyi*, *Gephyrocapsa oceanica*, and *Calcidiscus leptoporus*).

99 Calcite elemental ratios (Mg/Ca and Sr/Ca), organic phases and Fe oxides (Tang and Morel, 2006)
100 contamination indicators (P/Ca and Fe/Ca) of synthetic and coccolithophore pellets, were
101 determined via inductively coupled plasma-atomic emission spectrometry (ICP-AES). Additionally,
102 we report the culture media conditions (abiotic factors, seawater carbonate chemistry and Mg/Ca
103 ratios) as well as physiological parameters: particulate carbon production and organic C/N. The
104 protocol optimized and tested here considerably reduces the uncertainties in the study of Mg/Ca in
105 coccolithophore calcite and monitors organic matter contamination through P/Ca and Fe/Ca. This
106 will allow expanding the use of Mg/Ca as a proxy and also to measure/calibrate data from
107 laboratory experiments to assess responses of coccolith chemistry to different environmental
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123 # 2. METHODS

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125 ## 2.1. Culture methods

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127 Monoclonal cultures of two species of coccolithophore *Emiliania huxleyi* CAWPO6 and
128 *Calcidiscus leptoporus* RCC1169 and a green alga *Chlorella autotrophica* CCMP243 were grown
129 at the National Oceanography Center, Southampton (United Kingdom). Cultures were incubated at
130 19.3 ±0.8 ℃ in a light:dark cycle of 12:12 hours. The photosynthetically active radiation (PAR) was
131 125 ±10 μmol quanta m^{-2} s^{-1}, provided by cool-white fluorescent lamps (Osram LUMILUX), and
132 salinity was 35 ±1 (culture conditions are summarized in Table 1). The culture medium was
133 prepared using filter-sterilized (0.22 μm) seawater from the Celtic Sea, offshore Plymouth (UK),
134 and enriched with 100 μM sodium nitrate ($NaNO_3$) and 6.4 μM sodium di-hydrogen phosphate
135 (NaH_2PO_4), and trace metals and vitamins were added following the f/2 medium recipe (Guillard
136 and Ryther, 1962; Guillard, 1975).

137 *Chlorella autotrophica* was grown in triplicate using 12 L of culture medium in sterilized 20
138 L polycarbonate culture vessels under similar environmental conditions as the coccolithophores
139 (Table 1). *Emiliania huxleyi* and *C. leptoporus* were cultured in duplicate, using 3 L of culture
140 medium, in sterile 5 L borosilicate Erlenmeyer flasks. The carbonate chemistry system of the
141 medium reflected the original coastal water at present-day conditions (pH_{total} = 7.82 and 7.94,
142 respectively), and it was left equilibrating with the atmosphere in the chambers (see Table 1 for
143 initial and final values). At the start of all experiments the carbonate chemistry of the medium was
144 in the range of present-day observations (Key et al., 2004), but at the time of harvest (as a
145 consequence of high biomass) *C. leptoporus* had consumed 28.3% of the dissolved inorganic
146 carbon (DIC). Particulate organic and inorganic carbon measurements (cell quota and production

147 rates) were in agreement ($PIC/POC_{E. huxleyi}$ = 0.81, $PIC/POC_{C. leptoporus}$ = 2.18) with published data
148 sets at present-day carbonate chemistry conditions (e.g. Langer et al., 2006b; Iglesias-Rodriguez et
149 al., 2008) (see Table 1). *Gephyrocapsa oceanica* (RCC1303) was cultured at the Helmholtz Centre
150 for Ocean Research Kiel (GEOMAR, Germany) in a climate chamber at 20 ºC (Table 1) with a 16:8
151 hours light:dark cycle using a PAR of 150 μmol quanta m^{-2} s^{-1}. Cultures were grown in individual
152 2.5 L polycarbonate bottles (closed system) in artificial seawater (Kester et al., 1967) enriched with
153 64 μM of $NaNO_3$ and 4 μM of $NaH_2PO_4{\cdot}H_2O$ and trace metals and vitamins according to f/8
154 medium recipe (Guillard and Ryther, 1962; Guillard, 1975). Carbonate chemistry was adjusted to
155 present-day conditions (pH_{total} = 8.03) by combined additions of Na_2CO_3 and HCl as described in
156 the EPOCA Guide to best practices in ocean acidification research and data reporting (Gattuso et
157 al., 2010). Parameters of the carbonate chemistry system and determination for each culture are
158 summarized in Table 1. All experimental cultures (for the three species) were inoculated from
159 cultures pre-acclimated to experimental conditions for at least eight generations, in exponential
160 growth phase.
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162 **2.2. Pellet preparation**
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164 *2.2.1. Synthetic pellets*
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166 Synthetic pellets were prepared by mixing 5 mL of a suspension of 10 g L^{-1} of reagent grade
167 $CaCO_3$ powder, with 5 mL of a suspension of the non-calcifying marine microalgae *Chlorella*
168 *autotrophica* (~$1.5{\times}10^6$ cell mL^{-1}) (Electronic annex EA-1). The mixture was centrifuged with a
169 relative centrifuge force (RCF) of 1970 g for 20 minutes at 4 ºC in a Hettich ROTANTA 460RS
170 Centrifuge. After discarding the supernatant, the synthetic pellets were frozen at -80 ºC, freeze-

171 dried for 48 h in Falcon tubes (Harris, 1954), and kept at room temperature until analysis.

172 Additionally, control samples were prepared with a suspension of 10 g L^{-1} of reagent-grade $CaCO_3$

173 (without algal addition) following the same protocol as for the preparation of synthetic pellets (EA-

174 1). The pellets were produced in a single batch, with similar weights and $CaCO_3$/organic matter

175 ratios (EA-1), then freeze-dried for 48 h, and stored at room temperature for two weeks before

176 analysis. Thus, differences among the individual pellets as well as the bacterial oxidation effect

177 (Stoll et al., 2001) were minimized to assess the net organic Mg removal achieved purely by

178 chemical treatment (protocol). Stoll et al. (2001) reported that synthetic pellets of untreated *C.*

179 *autotrophica* + $CaCO_3$ had similar Mg/Ca as those from samples extracted from coccolithophore

180 cultures with high organic content (0.5-300 mmol/mol). Therefore, we assumed that our synthetic

181 pellets also reproduced well the properties of coccolithophore material to test the organic Mg

182 removal protocols.

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184 *2.2.2. Coccolithophore pellets*

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186 Cultures of coccolithophores were concentrated into cellular pellets (one pellet per replicate

187 bottle) by centrifugation. Since the culture experiments were conducted at different laboratories, the

188 facilities available conditioned the application of two different procedures of centrifugation (Fig. 1).

189 A Hettich ROTANTA 460RS Centrifuge was used at the National Oceanography Centre

190 Southampton (it only fits conic-bottom tubes), and a Beckman AVANTI^{TM} J-25 Centrifuge was

191 used at the GEOMAR (it fits only flat-bottom tubes). The two separation techniques used to harvest

192 coccolithophore pellets were as follows (Fig. 1): (1) *Gephyrocapsa oceanica* was centrifuged in

193 flat-bottom tubes, where the calcite forms a characteristic rim around the organic matter (free

194 coccoliths), and (2) *Emiliania huxleyi* and *Calcidiscus leptoporus* were centrifuged in conical-

195 bottom tubes, where all the material was mixed. In the first procedure, calcite was selected by
196 pipetting from the rim around the organic matter, which allows performing several initial manual
197 discrimination of organic matter. In the second one, this was not possible and all material remained
198 mixed until cleaning protocols were applied (Fig 1). Therefore a former method would be preferred.
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200 **2.3. Cleaning protocols**

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202 Two different oxidation procedures were applied during the protocol optimization. The first
203 one was a bleach-based method consisting of consecutive oxidations with a solution of sodium
204 hypochlorite (10% NaClO v/v) for 24 h at room temperature (Table 2). The second one involved an
205 oxidizing solution of alkaline hydrogen peroxide [0.33% (v/v) H_2O_2 + 0.98% (v/v) NaOH], based
206 on a method originally developed by Boyle (1983) and widely used to clean Mg/Ca samples in
207 foraminifera (Martin and Lea, 2002; Barker et al., 2003; Barker et al., 2005). In foraminifera
208 samples rich in organic phases from cultures (Russell et al., 2004) and sediment traps (Anand et al.,
209 2003; Pak et al., 2004), the oxidizing solution was applied in higher concentrations for longer time
210 periods. In the present study, the oxidative incubations started with pellet immersion in the alkaline
211 H_2O_2 solution (inside 15 mL tubes) during 10-15 min (Table 2) in an ultrasonic bath at room
212 temperature, which disrupts organic matter and enhances oxidation power. Afterwards, the
213 temperature was raised to ~100 ºC in a water-bath, to break down the residual H_2O_2, removing it
214 from the solution. Any associated impurities were brought into suspension, and then removed in
215 subsequent rinses with ultra pure water (UP-water) (Table 2). Several variations were introduced in
216 the original protocol to achieve the most effective and rapid treatment (Table 2): (1) Rinses with
217 UP-water and manual removal of organic matter by pipetting before the oxidative incubations
218 (treatments F-H), (2) reductive incubation using a solution of 4.76% (v/v) $NH_2OH{\cdot}HCl$ + 38% (v/v)

219 NH_4OH (Boyle, 1981) before oxidation (treatments C, E, G, H), (3) increase in the number of
220 oxidizing incubations (treatment B), and (4) modifications in the volume of reactive solution and
221 UP-water according to sample size. All the reagent solutions used were alkaline to avoid carbonate
222 dissolution. The efficiency in removing organic Mg phases was assessed by comparison with
223 elemental ratios measured on reagent-grade $CaCO_3$. Phosphorus and iron (P/Ca and Fe/Ca) were
224 used as indicators of contamination by organic matter and Fe-oxyhydroxides, respectively.
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226 **2.4. Measurements of elemental ratios *via* ICP-AES**

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228 Treated pellets were transferred to microfuge tubes (1.5 mL), dissolved in 250 µL of ultra-
229 pure 2% HNO_3 and diluted in 750 µL of UP-water. Elemental analysis was performed in an ICP-
230 AES, using the Thermo *i*CAP 6300 Series ICP Spectrometer (installed in the Department of
231 Geology, University of Oviedo, Spain). To improve precision by minimizing matrix effects, all
232 samples were diluted to similar Ca concentrations for final analysis of trace metal/Ca ratios. To this
233 end, an aliquot of 50 µL of dissolved material was analyzed for Ca concentration. Based on the
234 measured Ca, the reminder of the samples were diluted to a common Ca level, seeking the highest
235 possible Ca concentration within the range of standard calibration solutions (Ca = 15, 50, 100 ppm).
236 For trace elemental ratios, we measured in both radial and axial mode: P (177 nm axial), Fe (259
237 nm radial), Ca (315 nm radial) and Sr (407 nm radial). Calibrations were performed with multi-
238 element standards offline using the intensity ratio method described in de Villiers et al. (2002).
239 Elemental ratios of non-treated coccolithophore samples (only for *E. huxleyi* and *C.*
240 *leptoporus*) were obtained as a by-product from the measurements of Ca concentration for
241 determination of particulate inorganic carbon (PIC). These samples were obtained by filtering 200
242 mL of culture medium at harvesting time through a 0.22 µm Cyclopore polycarbonate membrane

243 and rinsed with buffered ultra-pure water (pH ~ 9). Samples were stored at -20 ºC until analysis.
244 Before analysis the samples were dried for 24 h at 60 ºC, dissolved in ultra-pure 2% HNO_3 and
245 analyzed using the ICP-AES, Thermo *i*CAP 6300 Series ICP Spectrometer.

246 Mg/Ca and Sr/Ca in seawater were determined separately by the method of standards addition
247 in culture medium samples (0.22 µm filtered) diluted to 1/200 and 1/10 respectively, and measured
248 with a Thermo *i*Cap 6300 Series ICP Spectrometer as described above. The partition coefficients of
249 Mg (D_{Mg}) and Sr (D_{Sr}) between coccoliths' calcite and seawater were calculated as elemental ratios
250 of coccolithophore calcite divided by the same elemental ratio obtained for the seawater [D_x =
251 $(x/Ca)_{calcite}/(x/Ca)_{seawater}$; where *x* is the trace element of interest].

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253 **2.5. Protocol assessment criteria**

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255 P/Ca and Fe/Ca were used as indicators of organic contamination and oxyhydroxides coating,
256 respectively (Fig. 3). The P/Ca was selected because phosphorus is an essential component of
257 biomolecules in the cell metabolism such as nucleotides [structural units of DNA and RNA, and
258 energetic molecules like ATP] and phospholipids (essential constituents of cellular membranes)
259 (Chu, 1946). Fe/Ca was selected because iron is generally a major compound in the trace metals
260 stock solution added to culture medium (e.g. Guillard and Ryther, 1962). It can be deposited on the
261 cell surface as Fe-oxides binding organic molecules (Ho et al., 2003; Tang and Morel, 2006), which
262 have high affinity to bind organic ligands (Wu and Luther, 1995; Rue and Bruland, 1997; Barker et
263 al., 2003).

264 The efficiency of the protocols was assessed by comparing different elemental ratios (Mg/Ca,
265 Fe/Ca) measured in treated synthetic pellets with the same elemental ratios measured in samples of
266 reagent-grade $CaCO_3$ in the same analysis (Fig. 2). In this study, elemental ratios of non-treated

267 synthetic pellets were not determined. Therefore, removal efficiency of organic Mg cannot be
268 accurately calculated with respect to synthetic pellets (*Chlorella* + $CaCO_3$), but it can be done with
269 respect to the original $CaCO_3$ sample. Since all the synthetic pellets were produced with similar
270 proportions of organic/inorganic material (see EA-1), the relative amount of organic Mg and Fe
271 removed after the treatment was estimated by comparison with the reagent-grade $CaCO_3$ following
272 the equation (Fig. 2): % organic contamination removed = $[(1 - ratio_{sample}) \times 100]/(1 - ratio_{reagent}$
273 $_{CaCO3})$.

274 For the coccolith samples, subsequently cleaned with the optimized protocol, we cannot
275 calculate the cleaning efficiency since we did not independently determine the trace elemental ratios
276 in the pure coccolithophore calcite. For these samples we estimate the percentage of organic Mg
277 removed during the cleaning treatment comparing with the elemental ratios determined in non-
278 treated samples (*Emiliania huxleyi* and *Calcidiscus leptoporus*) (Table 3).
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291 # 3. RESULTS

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293 ## 3.1. Protocol optimization on synthetic pellets

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295 Mg/Ca measured in synthetic pellets treated with bleach [treatments A-C (Table 2, Fig. 2)]
296 ranged from 0.335 to 0.545 mmol/mol, which were higher than 0.136 ± 0.008 mmol/mol measured
297 in the samples of certified $CaCO_3$ used as a control (EA-2). The estimated percentage of organic
298 Mg removed was < 80% in all the bleach-based treatments (A-C) (Fig. 2a). These protocols were
299 effective in removing P (average P/Ca = 0.072 mmol/mol) (Fig. 3b). However, Fe/Ca was still high,
300 > 2 mmol/mol (Fig. 3a). The Sr/Ca was 0.043 ± 0.002 mmol/mol in the bleach-based treatments (A-
301 C) and the values measured in the reagent grade $CaCO_3$ samples were 0.042 ± 0.002 mmol/mol.
302 The introduction of an additional oxidation step in treatment B (Table 2), after four oxidations with
303 bleach (24 h each incubation), did not decrease the Mg/Ca (0.415 mmol/mol), even though the P/Ca
304 decreased from 0.042 to 0.012 mmol/mol. In treatment C, the introduction of an initial reductive
305 incubation (Table 2) decreased the Fe/Ca from 4.808 mmol/mol (treatment A) to 0.157 mmol/mol.
306 However, the Mg/Ca was similar to previous treatments (0.429 mmol/mol). Total time of
307 incubation required in the bleach-based treatments was 96 h for treatment A, and 120 h for
308 treatments B and C. Treatments D-H were based on oxidations with H_2O_2 and were in general more
309 efficient in reducing the Fe/Ca, although Mg/Ca and P/Ca did not behave equally (Fig. 3).
310 Treatment D, consisting in four consecutive oxidations with H_2O_2, retrieved 0.954 ± 0.056
311 mmol/mol of Mg/Ca and the P/Ca was still high (0.171 ± 0.043 mmol/mol) in comparison to the
312 values measured in the reagent grade $CaCO_3$ (0.123 ± 0.007 and 0.005 ± 0.001 mmol/mol,
313 respectively). However, it was more effective in decreasing the Fe/Ca, requiring only ~1 h of
314 incubation (Fig. 2). Treatment E, which introduced a reductive incubation before the oxidations,

315 retrieved a lower Mg/Ca (0.230 ± 0.068 mmol/mol) and P/Ca (0.098 ± 0.033 mmol/mol). The
316 percentage of Mg removed rose up to 87.72% and the P/Ca decreased a further 43%. The
317 application of UP-water rinses before the oxidation steps (treatment F) decreases the P/Ca and
318 Fe/Ca in 40% and 66%, in comparison with treatment E, and the Mg/Ca decreases to 0.188 ± 0.021
319 mmol/mol. Therefore, a combination of initial UP-water rinses, reductive and oxidative incubations
320 was applied in treatment G (Table 2) and the Mg/Ca decreased to 0.158 ± 0.0003 mmol/mol as well
321 as the P/Ca and Fe/Ca (Fig. 2, 3). Before applying this protocol to the coccolithophore samples,
322 minor adjustments in the number of UP-water rinses, oxidation steps, and volumes used were
323 introduced in treatment H to prevent carbonate loss during samples cleaning (previously observed
324 in other treatments, with more reactive volume and more UP-water rinses and oxidations). In a short
325 time [24.5 h of incubation (Table 2)], treatment H delivered the best results in removing organic Mg
326 (> 99.9%) and was selected to apply to coccolithophore samples. The measurements of P/Ca and
327 Fe/Ca (0.043 and 0.029 mmol/mol, respectively) after application of treatment H were still above
328 measurements in reagent grade $CaCO_3$ samples (0.028 and 0.001 mmol/mol, respectively) (Fig. 2).
329 The large standard deviation registered in the P/Ca in synthetic pellets may be attributed to the
330 variability introduced by the cleaning protocol. When samples of reagent grade $CaCO_3$ were treated
331 with the optimized protocol H, the Mg/Ca and Sr/Ca did not vary from those measured in non-
332 treated $CaCO_3$ (Fig. 4).
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334 **3.2. Application of the optimized protocol to coccolithophore samples**

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336 The Mg/Ca determined in untreated culture samples of *Emiliania huxleyi* was 48 ± 4
337 mmol/mol (Table 3). After the implementation of protocol H, the Mg/Ca was 0.15 ± 0.02 mmol/mol
338 (Fig. 4), and for treated *Gephyrocapsa oceanica* pellets it was 0.06 ± 0.02 mmol/mol (Fig. 4). The

339 Mg/Ca determined for treated samples of *Calcidiscus leptoporus* was 0.22 ± 0.04 mmol/mol (Fig.
340 4), while for non-treated samples this ratio was 4.2 ± 0.4 mmol/mol (Table 3). In samples of *E.*
341 *huxleyi* the cleaning treatment was estimated to remove 99.7% of Mg, 99.3% of P, and 98.1% of Fe
342 associated with organic phases (Table 3). Just a 22.6% of the Sr was removed, which indicates that
343 the contribution of organic phases to inorganic Sr was small. In samples of *Calcidiscus leptoporus*,
344 the estimated removal of Mg during cleaning was 94.8%, estimated Sr removal was 6.9%, and
345 estimated phosphorus and iron removal were 93.4% and 79.6% respectively. The Fe/Ca determined
346 in *E. huxleyi* and *C. leptoporus* (7.6 and 5.6 mmol/mol) was much higher than that in *G. oceanica*
347 (0.001 mmol/mol) (Fig. 4). The P/Ca was overall higher in *E. huxleyi* and *G. oceanica* (0.75 and
348 0.79 mmol/mol, respectively) compared to *C. leptoporus* (0.40 mmol/mol). Sr/Ca varied among the
349 different species; the lowest ratio was observed in *E. huxleyi* (2.73 ± 0.22 mmol/mol), followed by
350 *C. leptoporus* (3.05 ± 0.010 mmol/mol) and then *G. oceanica* (3.41 ± 0.10 mmol/mol). Since we
351 used, artificial (laboratory) and natural seawater (coastal), the medium Mg/Ca varied (5.67 mol/mol
352 in *E. huxleyi*, 5.83 mol/mol in *C. leptoporus* and 5.63 mol/mol in *G. oceanica* cultures at harvesting
353 time). The variation in Mg/Ca of coccoliths from different species, all grown at similar temperatures
354 (19.8 ± 0.3 ºC), was correlated with the seawater Mg/Ca ($R^2 = 0.84$; $F = 140.40$, $P < 0.0001$) (Fig.
355 5). Partition coefficients for Mg also varied among the different species from $1.1\times10^{-5} \pm 0.4\times10^{-5}$ in
356 *G. oceanica*, and $2.7\times10^{-5} \pm 0.3\times10^{-5}$ in *E. huxleyi*, to $3.8\times10^{-5} \pm 0.7\times10^{-5}$ in C. *leptoporus*.
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363 # 4. DISCUSSION

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365 ## 4.1. The cleaning protocol

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367 In this study, bleach-based cleaning treatments removed a very low percentage of organic Mg
368 and took a long incubation time. Therefore, the optimization efforts focused on H_2O_2-based
369 treatments. Even though the decrease in Fe/Ca obtained with treatment C (Table 2) indicated that
370 further optimization tests including initial rinses with UP-water might improve the efficiency in
371 removing organic phases, the experimental matrix of this study was not completed. Pak et al. (2004)
372 also implemented bleach- and H_2O_2-based protocols on sediment trap foraminifera material (rich in
373 organic phases). Their results concluded that Mg/Ca on samples treated with bleach were
374 consistently higher and reproducibility was significantly lower than the samples treated with H_2O_2.
375 Oxidizing reagents are effective in decomposing organic compounds into more hydrophilic groups,
376 which was reflected in the removal of phosphorus (Fig. 2). However, in coccolithophore culture
377 samples, a portion of the iron added to the culture medium (in the trace metals stock solution) forms
378 ferric oxyhydroxides, and oxide (FeO_x) precipitates, which become associated with cell surfaces,
379 and might adsorb other trace elements interfering with the elemental analysis (Ho et al., 2003; Tang
380 and Morel, 2006). Therefore, a reductive incubation with a solution of hydroxylamine-
381 hydrochloride (Boyle, 1981) was introduced as a previous step before the oxidative incubations to
382 remove organic phases associated with metal oxides. The decrease in the Fe/Ca in coccolithophore
383 samples was large, but a complete removal of iron was not achieved (Fig. 2). The values of Fe/Ca
384 (0.0006 mmol/mol) in pellets of *G. oceanica* were lower than the Fe/Ca measured in *E. huxleyi* and
385 *C. leptoporus* (Fig. 4). This could be attributed to the smaller addition of Fe in the culture medium
386 ($2.93 \cdot 10^{-3}$ μM of $FeCl_3 \cdot 6H_2O$ for *G. oceanica*, compared to $11.7 \cdot 10^{-3}$ μM of $FeCl_3 \cdot 6H_2O$ added

387 besides the natural values for *E. huxleyi* and *C. leptoporus*) (Boye and van der Berg, 2000; Ho et al.,
388 2003; Tang and Morel, 2006). Results from Bian and Martin (2010) on foraminifera $CaCO_3$
389 samples indicate that the use of reductive treatments may be acceptable for the Mg/Ca analysis even
390 though there is a potential risk of sample partial dissolution, lowering the Mg/Ca (Barker et al.,
391 2003; Yu et al., 2007). In the present study, Mg/Ca measured in treatments F and G [essentially
392 identical except in the initial reductive cleaning in G (see Table 2)] indicated that the
393 hydroxylamine-hydrochloride solution applied decreased the Mg/Ca by 0.030 mmol/mol (Fig. 2,
394 EA-2). Even though, the potential partial dissolution of carbonate phases was not directly assessed
395 (no SEM images available), we suggest that this reduction is associated with removal of organic Mg
396 rather than partial dissolution of the $CaCO_3$ (Barker et al., 2003; Yu et al., 2007; Bian and Martin,
397 2010). This is because the Mg/Ca determined in samples of reagent grade $CaCO_3$ treated with the
398 optimized protocol (treatment H) was equal to the Mg/Ca in non-treated reagent-grade $CaCO_3$
399 (0.149 mmol/mol in both cases). Identical results were obtained for the Sr/Ca determined in treated
400 and non-treated reagent-grade $CaCO_3$ (0.039 mmol/mol) (Fig. 4). Thus, the optimized protocol does
401 not alter Mg/Ca and Sr/Ca of reagent grade $CaCO_3$, and we assumed the same occurred in
402 coccolithophore calcite. However, distribution of Mg in coccolithophore plates is unknown, thus
403 potential effects on partial dissolution remain open. Anand et al. (2003), Pak et al. (2004) and
404 Russell et al. (2004) applied stronger concentrations of H_2O_2-based oxidizing solutions during
405 longer time periods in organically enriched foraminifera samples, which suggests that optimization
406 tests based on variations in reactive concentration and time of incubation should be performed to
407 improve the organic removal efficiency. However, foraminifers' tests are about 10 times thicker
408 than coccoliths (Eggins et al., 2003; Young et al 2003). Therefore, potential higher dissolution
409 susceptibilities of coccolithophore calcite should be kept in mind. In addition to the chemical
410 treatment, previous manual separation and removal of organic material represents an important

411 improvement in the final efficiency when removing organic Mg as demonstrated in treatment F
412 (Fig. 1). Initial rinses with UP-water (Boyle, 1981) combined with ultrasonic bath, and removal of
413 visible organic phases by pipetting decreased the P/Ca, Fe/Ca, and Mg/Ca. Determination of Mg/Ca
414 and Sr/Ca in this study were supported by the use of P/Ca and Fe/Ca (obtained simultaneously in
415 the elemental analysis via ICP-AES for the same sample) as indicators of organic matter
416 contamination. In addition, since all the ratios are normalized to Ca, the sample concentration
417 should be openly provided in future studies to determine the reliability of the elemental ratios. Its
418 implementation does not require additional steps and we can consider it an indirect method to assess
419 the reliability of cleaning procedures in different laboratories. Fe/Ca and P/Ca should also be openly
420 provided in future geochemical studies as indicators of organic contamination to allow an accurate
421 results interpretation. They should be carefully considered as indicators of organic matter, although,
422 they may not have a unique relation with cleaning efficiency. For example, iron concentration,
423 which is not strictly associated with organic matter, greatly depends on the sample origin (cultures,
424 sediment traps, and natural community or sediment samples).

425 Sample characteristics such as the dry-weight of material and the species used are two
426 important factors that *a priori* might compromise the cleaning efficiency of organic Mg phases. The
427 volume of reagent applied should be proportional to the sample size to avoid sample loss associated
428 with pipetting during the intermediate rinses. Moreover, pellet weight should be kept within a small
429 range of variation when the volume of reagents is constant, otherwise this would compromise
430 reproducibility between samples. Additionally, the species-specific calcite/organic matter ratio
431 (PIC/POC) of coccolithophores (e.g. Langer et al., 2009b) may also be affecting the efficiency of
432 this protocol. For example, *Emiliania huxleyi* (PIC/POC ~ 0.8), unlike *C. leptoporus* (PIC/POC >
433 2), requires the removal of larger proportions of organic matter. The later has lower initial
434 contribution of organic phases (relative to calcite), therefore, the fraction of elements removed

435 during oxidative cleaning of organic phases was lower than in *E. huxleyi* (Table 3). The P/Ca
436 measured in *C. leptoporus* pellets (0.40 mmol/mol) was smaller that in *E. huxleyi* (0.75 mmol/mol),
437 reflecting the greater ease to effectively clean this species with higher ratio of calcite/organics.
438 Amongst the samples used in this study, *E. huxleyi*, with the lowest PIC/POC, and the smallest and
439 most structurally complex coccoliths, requires a more efficient removal of organic Mg than *C.*
440 *leptoporus* and *G. oceanica*.

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442 **4.2. The elemental composition of coccolithophores**

443

444 The Mg/Ca of *Emiliania huxleyi* and *Gephyrocapsa oceanica* (Fig. 4) obtained in this study
445 were within the range of variation of previous data from batch cultures of the same species (Stoll et
446 al., 2001), with data from coccoliths obtained from sediment traps (Stoll et al., 2007), and with
447 cultured coccoliths cleaned with acetone and H_2O_2 (Ra et al., 2010). The much higher Mg/Ca
448 (2.710 mmol/mol) measured in living cultures of *E. huxleyi* at present seawater conditions cleaned
449 with a bleach-based protocol (Müller et al., 2011), or in coccoliths of other cultured species for
450 which no cleaning is reported (Stanley et al., 2005) may be due to incomplete removal of
451 organically sourced Mg. The lack of a robust and common cleaning protocol hampers inter-
452 laboratory comparisons to bring together different data sets, and thus advance the use of Mg/Ca in
453 coccoliths.

454 We suggest that the recorded variation in coccolithophore Mg/Ca in cleaned samples within
455 the species concept can be attributed to natural seawater variability. Ra et al. (2010) report, for
456 coccoliths treated with acetone/H_2O_2-based protocol, Mg/Ca between 0.029 and 0.051 mmol/mol in
457 *E. huxleyi*, and between 0.011 and 0.025 mmol/mol in *G. oceanica*, grown in seawater with a
458 Mg/Ca of 5.18 mol/mol. These data points fit on the regression implied by the Mg/Ca of our

459 coccoliths cultured in coastal seawater with Mg/Ca of 5.670-5.827 mol/mol (Fig. 5). Wild samples
460 of *E. huxleyi* obtained from the Bermuda Oceanic Flux Program (OFP) sediment traps, pre-treated
461 with H_2O_2 and analyzed with ion probe, yield comparable low values, although they are subjected
462 to higher uncertainty depending on the Mg blank in the epoxy mounting resin (Stoll et al., 2007).
463 Nonetheless, assuming oceanic waters in the North Atlantic Ocean have a Mg/Ca of 5.162 mol/mol
464 (Fabricand et al., 1967), the sediment trap samples off Bermuda fit the regression well (Fig. 5). It
465 reflects the variability of coccolithophore Mg/Ca as a function of modern seawater Mg/Ca of
466 different origins (e.g. coastal *versus* oceanic). This has been already observed for seawater Mg/Ca
467 (Fabricand et al., 1967; Zang et al., 2003) and Sr/Ca (de Villiers, 1999) showing latitudinal and
468 biogeographical variability, which we propose could drive the natural coccoliths composition and
469 may have implications for the sinking carbonates and the dissolution at depth. Further investigation
470 of deviations from the constant elemental proportions in seawater [Marcet's principle (1918)]
471 should aim to understand natural variability as a control of Mg and Sr in coccolithophores. The
472 number of coccolithophore samples and Mg/Ca seawater ranges examined is relatively small;
473 therefore, a broader comparison is required to fully test this relationship, expanding from de Villiers
474 (1999) study on seawater Sr/Ca to Mg/Ca across large latitudinal gradients.

475 Interpretations of the Mg/Ca variability based on published data are currently limited by: (1)
476 uncertainty in different organic Mg removal treatments applied, (2) variable medium conditions
477 (carbonate chemistry, seawater Mg/Ca), and (3) biological effect on elemental partitioning imposed
478 by the physiological fingerprint of species and strains used (Stanley et al., 2005; Müller et al.,
479 2011). For Sr/Ca the situation is simpler because the organic contamination is minimal and Sr/Ca
480 measured in the three species (Fig. 4) were in agreement with values measured in culture samples
481 and sediment traps (Stoll et al., 2002; Stoll et al., 2007).
482

483 ## 5. CONCLUSIONS

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485 The Mg/Ca retrieved after organic phases removal were in accordance with bibliographic data
486 and culture conditions. However, this study cannot guarantee that the removal of organic Mg was
487 truly complete. Further optimization work is needed, especially to work out the minimum amount of
488 sample required, whether the proportion of organic phases to calcite (PIC/POC) should be
489 considered when optimizing the protocols and SEM analyses to assess partial dissolution. The
490 protocol matrix could be extended by testing other organic removal methods such as combustion,
491 and the combination of acetone treatments (Ra et al., 2010) with H_2O_2-based protocols. In order to
492 improve the yield of our protocol, we recommend introducing a manual separation of calcite (Fig.
493 1). The mechanical pre-selection before the chemical treatment enables to remove big
494 agglomerations of organic matter and concentrate efforts on removing organic material adhered to
495 calcite. While this technique prior to reduction and oxidation was not used before, it helps targeting
496 selectively the calcite fraction from the beginning of the protocol. In addition, the trace metal
497 concentration of the culture medium is an important factor in the formation of metal-oxides in
498 samples from living cultures. Thus, it is recommended to adjust the amount of trace metals added to
499 the minimum amount required without compromising phytoplankton growth rates (Boye and van
500 der Berg, 2000; Ho et al., 2003; Tang and Morel, 2006), to increase the efficiency of oxidizing
501 reagents. Additionally, in paleoceanographic applications it is better to target culture efforts on
502 species with high PIC/POC such as *Calcidiscus* sp., which also can be individually extracted from
503 sediments. Finally, to routinely measure Mg/Ca in living coccolithophore material from laboratory
504 experiments and field samples, it is necessary to establish a series of baseline measures (quality
505 control) to make datasets comparable. Environmental conditions of growth and carbonate chemistry
506 in the culture media should always be provided because elemental partition (e.g. D_{Mg}) is affected by

507 the carbonate chemistry (Ries, 2011) and seawater elemental composition (Ries, 2010; Müller et al.,
508 2011). Values of Mg removal efficiency, associated to organic phases, and the P/Ca, Fe/Ca and Ca
509 concentration should be provided along with the Mg/Ca results to allow independent assessment
510 and comparison of datasets. In the short term, we should aim to calibrate the coccolithophore
511 Mg/Ca as a proxy for temperature and study relationships to carbonate chemistry parameters (e.g.
512 CO_3^{2-}), contributing to the development of a coccolithophore multi-proxy approach. This will ease
513 more accurate estimations by reducing biases originating in different habitats, ecophysiology and
514 productivity regimes. We should also be able to understand coccolithophore Mg/Ca and Sr/Ca
515 responses to environmental perturbations such as $p\text{CO}_2$ and temperature variability, and investigate
516 other trace elements incorporated in the calcite and their potential biogeochemical applications.
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532

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TABLES

Table 1. Culture conditions, medium chemistry and sample parameters of coccolithophores in experimental cultures.

Species	***Chlorella autotrophica*** [a]	***Emiliania huxleyi*** [b]		***Calcidiscus leptoporus*** [b]		***Gephyrocapsa oceanica*** [c]
Strain	CCMP243	CAWPO6 (NZEH)		RCC1169		RCC1303
Location	North Atlantic	New Zealand		Mediterranean Sea		North Atlantic
Latitude	43.41 °N	46.58 °S		43.41 °N		44.60 °N
Longitude	73.10 °W	168.05 °E		7.19 °E		1.5 °W
Culture conditions [d]						
Temperature (ºC)	19.90 ± 0.18	18.49 ± 0.31		20.09 ± 0.10		20.00 ± 0.00
PAR (μmol quanta m^{-2} s^{-1})	130.57 ± 2.71	123 ± 4		133 ± 5		150 ± 0.00
Salinity	35 ± 0.00	35.2 ± 0.1		35.0± 0.1		35.0 ± 0.1
Nitrate (μM)	100 ± 0.00	95.25 ± 0.00		94.33 ± 0.00		58.80 ± 3.09
Phosphate (μM)	6.40 ± 0.00	4.88 ± 0.00		2.82 ± 0.00		6.28 ± 0.21
Medium carbonate chemistry [d]						
		t_0	t_n	t_0	t_n	t_0
TA (μmol kg^{-1})	-	2272.7[e]	2242.7 ± 1.7	2234.5[e]	1555.3 ± 817.8	2343.5 ± 1.9[f]
DIC (μmol kg^{-1})	-	2131.1[e]	2046.5 ± 3.7	2031.6[e]	1456.1 ± 58.5	2081.7 ± 10.8[g]
pH_{total}	8.13 ± 0.04	7.82	7.95 ± 0.01	7.94	7.75± 0.03	8.05 ± 0.02
pCO_2 (μatm)	-	722.0	506.3 ± 8.4	512.4	585.6 ± 41.3	408.4 ± 26.8
HCO_3^- (μmol kg^{-1})	-	1994.2	1886.0 ± 5.0	1866.7	1367.3 ± 3.9	1880.1 ± 18.6
CO_3^{2-} (μmol kg^{-1})	-	112.5	143.5 ± 1.6	148.4	69.9 ± 5.3	188.4 ± 8.6
CO_2 (μmol kg^{-1})	-	24.3	17.0 ± 0.3	16.5	18.9 ± 1.3	13.2 ± 0.9
Ω Calcite	-	2.68	3.43 ± 0.03	3.55	1.67 ± 0.13	4.51 ± 0.21
Seawater Mg/Ca (mol/mol)[h]		5.67 ± 0.03	5.67 ± 0.03	5.40 ± 0.03	5.83 ± 0.04	5.63 ± 0.02[i]
Seawater Sr/Ca (mmol/mol)[h]		8.59 ± 0.04	8.61 ± 0.07	8.72 ± 0.06	8.87 ± 0.05	7.95 ± 0.08[i]
Sample parameters [d]						
Cell density (cell ml^{-1})	$1.51 \cdot 10^6 \pm 5.08 \cdot 10^5$	33890 ± 2009		9066 ± 945		25000 – 50000[k]
Growth rate (μ)	0.73 ± 0.042	1.45 ± 0.015		0.37 ± 0.009		0.91 ± 0.120
PIC quota (pg C $cell^{-1}$)	-	6.42 ± 0.89[j]		433.87 ± 49.48[j]		28.00 ± 2.80[l]
PIC prod. (pg C $cell^{-1}$ d^{-1})	-	9.35 ± 1.25j		162.56 ± 14.58[j]		25.31 ± 1.02[l]
POC quota (pg C $cell^{-1}$)	-	7.96 ± 1.04		198.71 ± 1.77		26.85 ± 0.49
POC prod. (pg C $cell^{-1}$ d^{-1})	-	11.59 ± 1.41		74.57 ± 1.31		11.14 ± 0.34
PIC:POC (wt:wt)	-	0.81 ± 0.07		2.18 ± 0.23		1.92 ± 0.14
C:N (mol:mol)	-	6.58 ± 0.04		17.45 ± 0.12		11.14 ± 0.01

(a) Non-calcifying algae used for synthetic pellet preparation along with pure calcite. Medium carbonate chemistry only recorded as pH_{total}.

(b) Strains cultured in natural seawater.

(c) Strain cultured in artificial seawater (see main text for details). Sampling for physiological parameters at final time (t_n) was performed 2-16 hours before the cell harvesting for pellet production. At the time of cells harvesting the culture are expected being in exponential growth phase, therefore the lag between sampling and cell harvesting should not affect the physiological parameters.

(d) Medium carbonate chemistry was determined for time zero of the experiment (t_0) and at harvesting time (t_n) with the software CO2SYS (Pierrot et al., 2006) using TA and DIC as imput data. The constants used were: K_1, K_2 from Mehrbach et al., 1973 refit by Dickson and Millero, 1987 and K_{HSO4} from Dickson (1990b). Data of *C. autotrophica*, *E. huxleyi*, *C. leptoporus* cultures at t_n are calculated form duplicates. *Gephyrocapsa oceanica* values are the average from two CO_2 conditions (381 and 496 µatm) sampled one day before harvesting the pellets. Initial and final conditions are presented as an average of both replicates.

(e) Measured with VINDTA instrument (Mintrop 2006). TA and DIC at harvesting (final) were extremely low probably as a consequence of high densities of *C. leptoporus* consuming DIC and lowering TA due to calcification. To confirm measured samples, DIC was re-calculated using TPC build-up: $DIC_{final} = DIC_{initial} - TPC$, and then TA re-calculated using measured pH_{total} values. The re-calculated conditions were: $TA_{final} = 1666.40 \pm 111.21$, $DIC_{final} = 1500.76 \pm 100.72$. These conditions do not affect the work on the cleaning of organic Mg and the subsequent ICP-AES measurements.

(f) TA measured in a Metrohm Basic Titrino 794 titration device.

(g) DIC measured photometrically in a QUAATRO analyzer (Stoll et al., 2001).

(h) Standard error calculated form duplicate measurements of the same sample analysed with ICP-AES.

(i) Sample was collected 2-16 hours before the cell harvesting.

(j) PIC measured as calcite with an ICP-AES and obtained values where corrected for contribution of seawater salts (see main text for details).

(k) Cell density was not measured at harvesting time. These values indicate the range of variation estimated.

(l) PIC measured from: PIC = TPC - POC, with an elemental analyzer Euro EA (Sharp, 1974).

Table 2. A summary of protocols tested, elemental ratios, and cleaning efficiencies on *Chlorella autotrophica* and calcite pellets.

Protocol code	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**[d]
Cleaning protocol	Bleach[a]	Bleach	Red.[b] Bleach	Oxid.[c]	Red. Oxid.	Oxid.	Red. Oxid.	Red. Oxid.
Pellet n°	(1-6)	(1-6)	(7-8)	(9-11)	(12-14)	(12-14)	(15)	(16)
Pre-treatment								
Rinses UP[e]	-	-	-	-	-	x6	x5	x3
Volume (ml)	-	-	-	-	-	2	2	2
Reduction + Oxidation								
Reduction	-	-	Red.	-	Red.	-	Red.	Red.
Volume (ml)	-	-	1	-	0.350	-	0.750	0.750
Sonication (min.)	-	-	15	-	15	-	20	20
Incubation (h)	-	-	24	-	24	-	24	24
Temperature (°C)	-	-	22	-	22	-	22	22
Rinse UP	-	-	x2	-	x4	-	x4	x4
Volume (ml)	-	-	2	-	2	-	2	2
1st Oxidation	Bleach	Bleach	Bleach	Oxid.	Oxid.	Oxid.	Oxid.	Oxid.
Volume (ml)	2	2	2	3	3	3	2	2
Sonication (min)	15	15	15	10	10	10	10	10
Incubation	24 h	24 h	24 h	10 min	10 min	10 min	10 min	10 min
Temperature (°C)	22	22	22	100	100	100	100	100
Rinse UP	x2	x2	x2	x3	x3	x3	x2	x2
Volume (ml)	2	2	2	2	2	2	2	1
2nd Oxidation	Bleach	Bleach	Bleach	Oxid.	Oxid.	Oxid.	Oxid.	Oxid.
Volume (ml)	2	2	2	3	3	3	1	1
Sonication (min)	15	15	15	10	10	10	10	10
Incubation	24 h	24 h	24 h	10 min	10 min	10 min	10 min	10 min
Temperature (°C)	22	22	22	100	100	100	100	100
Rinse UP	x2	x2	x2	x3	x3	x3	x2	x2
Volume (ml)	2	2	2	2	2	2	2	1
3rd Oxidation	Bleach	Bleach	Bleach	Oxid.	Oxid.	Oxid.	Oxid.	Oxid.
Volume (ml)	2	2	2	1.5	1.5	1.5	1	1
Sonication (min)	15	15	15	10	10	10	10	10
Incubation	24 h	24 h	24 h	10 min	10 min	10 min	10 min	10 min
Temperature (°C)	22	22	22	100	100	100	100	100
Rinse UP	x2	x2	x2	x3	x3	x3	x2	x2
Volume (ml)	2	2	2	2	2	2	2	1
4th Oxidation	Bleach	Bleach	Bleach	Oxid.	Oxid.	Oxid.	Oxid.	-
Volume (ml)	2	2	2	0.5	0.5	0.5	1	-
Sonication (min)	15	15	15	10	10	10	10	-
Incubation	24 h	24 h	24 h	10 min	10 min	10 min	10 min	-
Temperature (°C)	22	22	22	100	100	100	100	-
Rinse UP	x4	x2	x4	x4	x4	x4	x4	-
Volume (ml)	2	2	2	1	1	1	1	-
5th Oxidation	-	Bleach	-	-	-	-	-	-
Volume (ml)	-	2	-	-	-	-	-	-
Sonication (min)	-	15	-	-	-	-	-	-
Incubation	-	24 h	-	-	-	-	-	-
Temperature (°C)	-	22	-	-	-	-	-	-
Rinse UP	-	x4	-	-	-	-	-	-
Volume (ml)	-	2	-	-	-	-	-	-
Protocol time[f]	96 h	120 h	120 h	40 min	24.6 h	40 min	24.6 h	24.5 h

(a) Oxidation solution: 10% NaClO (v/v).

(b) Reduction solution: 4.76% (v/v) Hydroxylamine-hydrochloride $NH_2OH{\cdot}HCl$ + 38% (v/v) NH_4OH.

(c) Oxidation solution: 0.33% (v/v) H_2O_2 + 0.98% (v/v) NaOH.

(d) Protocol H was used to treat all coccolith samples.

(e) UP stands for alkaline ultra pure water rinses, which pH_{total} was adjusted between 9 and 10 with NH_4OH to avoid carbonate dissolution. After the rinses all pellets were centrifuged at 3000 rpm for 10 minutes and the supernatant was removed. Time for incubations only. This excludes handling and preparation for ICP-AES.

(f) Time of incubations only. This excludes handling and preparation for ICP-AES analyzes.

Table 3. Target elemental ratios measured in non-treated samples and the pellets treated with the optimized cleaning protocol H, and estimation of sample recovery.

	Non-treated samples[a]	**Cleaned pellets**	**Elem. removal**[b] (%)	**Ca recovery**[c] (%)
Emiliania huxleyi[d]				
Mg/Ca (mmol/mol)	48 ± 4	0.15 ± 0.02	99.69	-
Sr/Ca (mmol/mol)	3.5 ± 0.02	2.73 ± 0.22	22.63	-
P/Ca (mmol/mol)	99 ± 5	0.75 ± 0.24	99.25	-
Fe/Ca (mmol/mol)	392 ± 31	7.61 ± 6.25	98.06	-
Ca (ppm)	48 ± 7	18.9 ± 0.66	-	3.94
Calcidiscus leptoporus[d]				
Mg/Ca (mmol/mol)	4.2 ± 0.4	0.22 ± 0.04	94.79	-
Sr/Ca (mmol/mol)	3.3 ± 0.1	3.05 ± 0.01	6.87	-
P/Ca (mmol/mol)	6 ± 1	0.40 ± 0.31	93.35	-
Fe/Ca (mmol/mol)	27 ± 3	5.63 ± 5.42	79.62	-
Ca (ppm)	911 ± 44	162 ± 14	-	1.78

(a) Elemental ratios obtained as a by-product in measurements of calcite (PIC) samples (from the same strain) where no cleaning procedure is applied. Measurements include elements in salt, organic, and carbonate phases. PIC data is shown in Table 1.

(b) The percentage of element removal during the cleaning process. Calculated as: [(non-treated samples – treated pellets) × 100] / non-treated samples.

(c) Ca concentration was not measured in the pellet previously to cleaning treatment. Therefore it was estimated based on the concentration measured in subsamples and the volume of culture used for the pellet production.

(d) Elemental ratios from PIC samples (non-treated) measured via ICP-AES are only available for these two strains and not for *G. oceanica*, where PIC was measured via Elemental Analyzer (see Table 1 for details)].

1

FIGURE CAPTIONS

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4 Fig.1: Schematic representation of two procedures to harvest coccolithophore pellets using
5 different centrifugation devices: (a) Using a Hettich ROTANTA 460RS Centrifuge and flat-bottom
6 tubes allowing the manual pre-selection of $CaCO_3$ in samples before applying cleaning protocols
7 and measuring in the ICP-AES. (b) Using a Beckman AVANTI^{TM} J-25 Centrifuge and conical-
8 bottom tubes; the initial separation between calcite and organic matter is not obvious as in
9 procedure (a). Images taken by S. B. A. and M. L., and courtesy of the "Integration & Application
10 Network" (http://ian.umces.edu/).

11

12 Fig.2: Elemental ratios measured in synthetic pellets with reagent grade $CaCO_3$. Grey
13 circles (●) are synthetic pellets treated with different protocols applied to coccolithophore samples
14 from monoclonal cultures: A (Bleach oxidation), B (Bleach oxidation), C (Reductive incubation and
15 bleach oxidation), D (H_2O_2 oxidation), E (Reductive incubation and H_2O_2 oxidation), F (H_2O_2
16 oxidation), G (Reductive incubation and H_2O_2 oxidation) and H (Reductive incubation and H_2O_2
17 oxidation). White diamonds (◊) are the reagent grade $CaCO_3$ measured in the same ICP-AES run.
18 (a) Mg/Ca measured in reagent grade $CaCO_3$ and the synthetic pellets treated following the different
19 protocols. (b) Sr/Ca. (c) Fe/ca. (d) P/Ca. Details about each protocol are given in Table 2 and raw
20 data of the measurements are given in EA-2.

21

22 Fig.3: Mg/Ca of synthetic pellets treated with individual protocols plotted against the
23 corresponding Fe/Ca (a), P/Ca (b), used as indicators of organic phases contamination in biogenic
24 calcite, and calcium concentration (c).

25

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27 Fig.4: Mg/Ca and Sr/Ca in *Emiliania huxleyi*, *Gephyrocapsa oceanica*, and *Calcidiscus*
28 *leptoporus* cleaned with protocol H (UP + Red. + Oxid.). The relationship to the P/Ca is shown in
29 the x-axis in (a) and (c), and to the Fe/Ca in (b) and (d). We also determined the same ratios in
30 samples of treated and non-treated certified $CaCO_3$ to remove organic Mg following protocol H.
31 Bidirectional error bars (standard deviation) from repeated measurements of each pellet represent
32 the individual error of the analysis. In panel (b), the values indicate calcium concentration (ppm)
33 measured in each coccolithophore sample. (*) Denotes average values.
34
35 Fig.5: Relationship of coccoliths Mg/Ca and seawater Mg/Ca in coccolithophores in this
36 study and the literature. Coccolithophore data from the literature were selected including samples
37 grown at ~20 ºC. Average North Atlantic Ocean seawater Mg/Ca used for the data set from Stoll et
38 al., 2007 were taken from Fabricand et al., 1967. Bidirectional error bars represent the standard
39 deviation from repeated measurements of each sample. Solid line represents the regression plot
40 which linear equation is: $y = 0.2894 \pm 0.0012\, x - 1.4851 \pm 0.0009$; $R^2 = 0.838$; $F = 140.398$ and $P <$
41 0.0001. Dotted lines represent 95% confidence bands.
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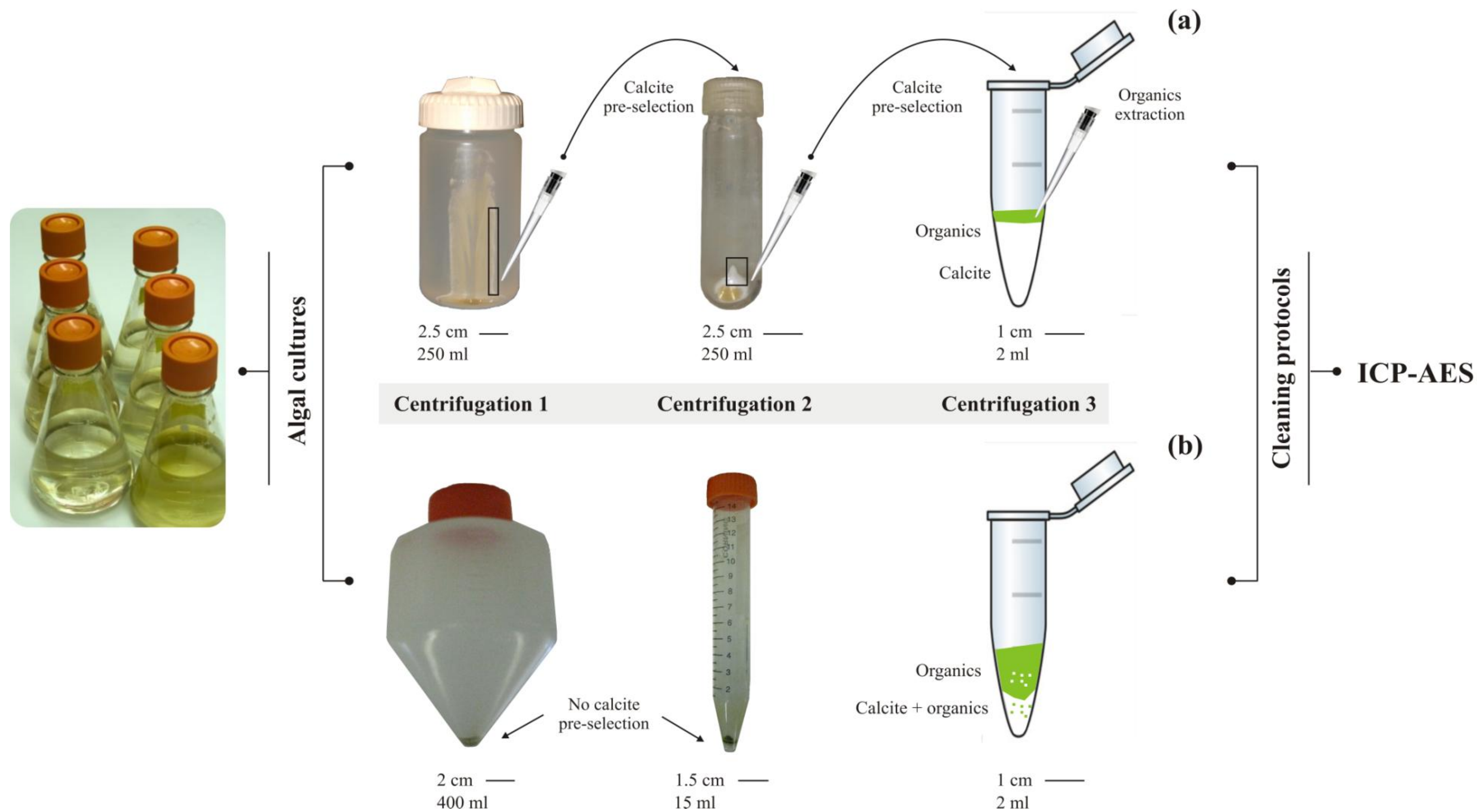
FIGURES



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55 Fig. 1

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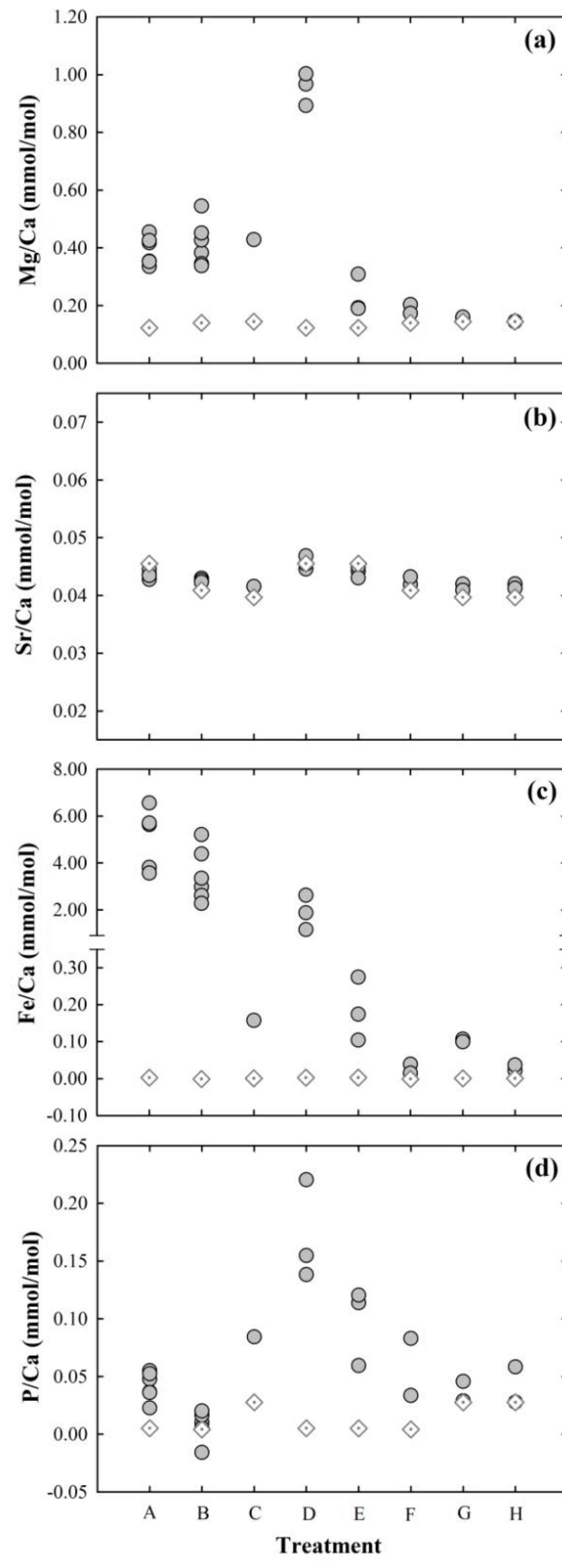

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59 Fig. 2

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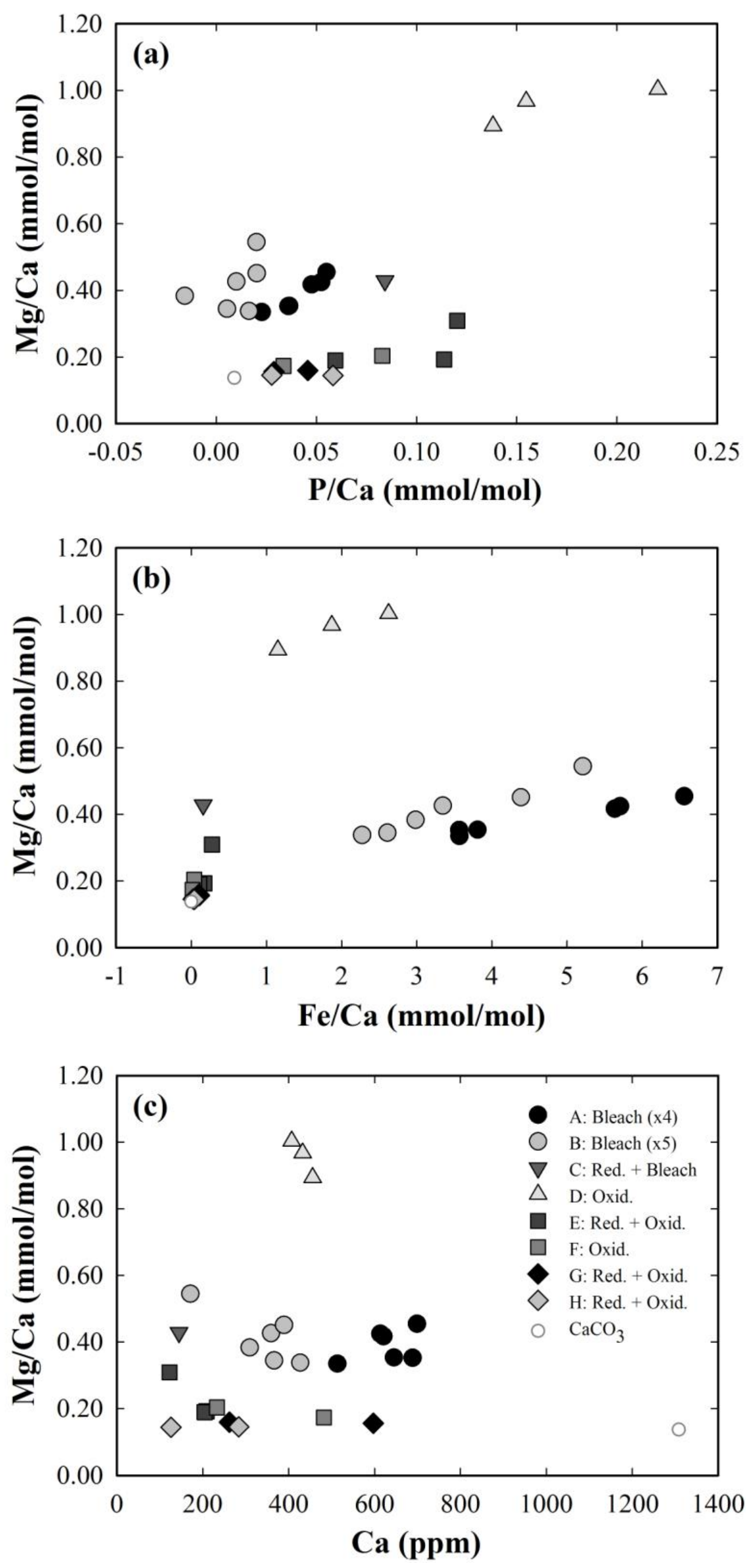

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63 Fig. 3

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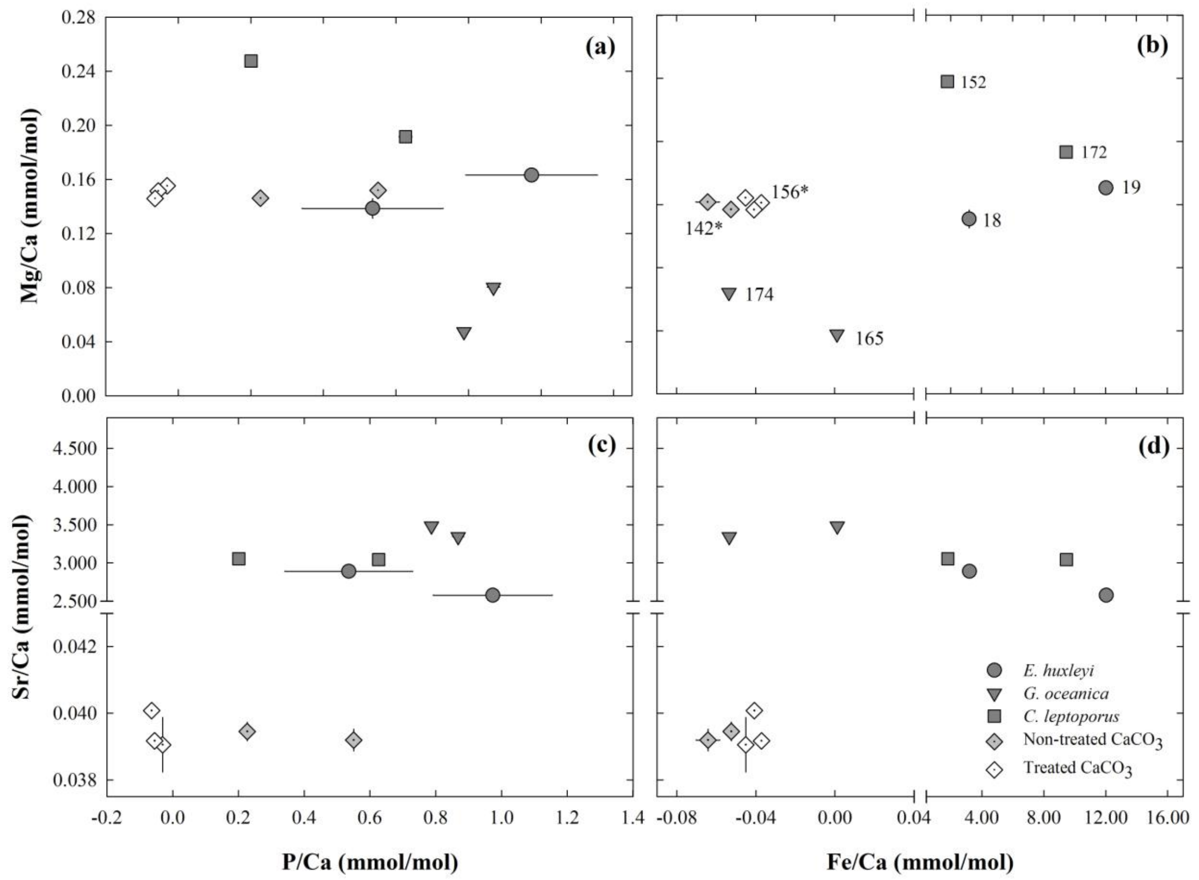

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67 Fig. 4

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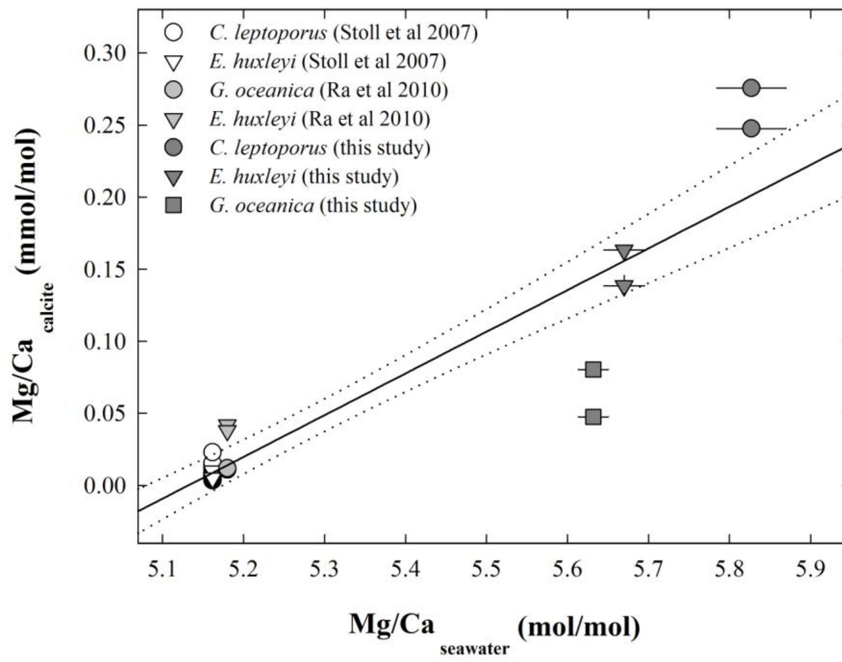

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81 Fig. 5

ELECTRONIC ANNEXES

EA-1. Characterization of the synthetic pellets produced by mixing the non-calcifying alga *Chlorella autotrophica* + reagent-grade $CaCO_3$, and the treatments for what they ave been used.

Pellet n° [a]	Label [b]	Cell density (cell ml^{-1})	Total cells (in 50 ml) [c]	Pellet weight (mg) [d]	$CaCO_3$/organic (wt/wt) [e]	Protocol key [f]
1	a	1.93×10^6	2.316×10^9	64.50	0.775	A - B
2	a	1.93×10^6	2.316×10^9	64.20	0.778	A - B
3	b	1.66×10^6	1.992×10^9	65.50	0.763	A - B
4	b	1.66×10^6	1.992×10^9	68.30	0.732	A - B
5	c	9.45×10^5	1.134×10^9	62.40	0.801	A - B
6	c	9.45×10^5	1.134×10^9	62.00	0.806	A - B
7	a	1.93×10^6	2.316×10^9	64.60	0.773	C
8	a	1.93×10^6	2.316×10^9	65.30	0.765	C
9	a	1.93×10^6	2.316×10^9	70.40	0.710	D
10	b	1.66×10^6	1.992×10^9	64.70	0.772	D
11	c	9.45×10^5	1.134×10^9	61.00	0.819	D
12	a	1.93×10^6	2.316×10^9	65.20	0.778	E - F
13	b	1.66×10^6	1.992×10^9	64.70	0.772	E - F
14	c	9.45×10^5	1.134×10^9	62.40	0.801	E - F
15	b	1.66×10^6	1.992×10^9	64.50	0.775	G
16	b	1.66×10^6	1.992×10^9	65.50	0.763	H
17	- [g]	0	0	192.90	Pure calcite [h]	-
18	-	0	0	197.30	Pure calcite [h]	-

(a) Pellet code given for identification purposes for the different protocols.

(b) The label indicates the origin of the seawater batch with a different cell density for a, b, and c. *Chlorella autotrophica* were grown in each batch (12 L).

(c) Cell density in 50 ml aliquots. 5 ml were transferred from the re-suspended material to the final pellets.

(d) Dry weight only from the organic material.

(e) The $CaCO_3$ dry weight is 50 mg for all treatments. The ratio (by weight) varies depending on the amount of organic pellet centrifuged. It resembles the proportions found in pellets made from living *E. huxleyi* cells with a high calcite content.

(f) The code is used to identify the protocols applied to each pellet in Table 2.

(g) - indicates "not applicable" or "not given" in all tables.

(h) Only calcite re-suspended and centrifuged in the treatments.

EA-2. Carbonate P/Ca and Fe/Ca (contamination proxies), Mg/Ca, Sr/Ca, Ca concentration and percentage of sample recovery determined in the synthetic pellets (*Chlorella autotrophica* + $CaCO_3$) pellets treated with different protocols and the reference $CaCO_3$ material used.

Protocol key	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Cleaning protocol	Bleach	Bleach	Red. Bleach	Oxid.	Red. Oxid.	Oxid.	Red. Oxid.	Red. Oxid.
Pellet nº	(1-6)	(1-6)	(8)	(9-11)	(12-14)	(12-13)	(15)	(16)
P/Ca (mmol/mol)	0.037	0.016	-	-	-	-	-	-
	0.048	0.01	-	-	-	-	-	-
	0.023	0.005	-	-	-	-	-	-
	0.036	0.016	-	0.138	0.114	-	-	-
	0.055	0.02	-	0.155	0.12	0.083	0.029	0.028
	0.052	0.02	0.084	0.22	0.059	0.034	0.046	0.058
Average	***0.042***	***0.009***	***0.084***	***0.171***	***0.098***	***0.058***	***0.037***	***0.043***
SD	*0.012*	*0.014*	*0*	*0.043*	*0.033*	*0.035*	*0.012*	*0.022*
Reagent $CaCO_3$	**0.005**	**0.004**	**0.028**	**0.005**	**0.005**	**0.004**	**0.028**	**0.028**
SD	0.001	0.004	0.008	0.001	0.001	0.004	0.008	0.008
Fe/Ca (mmol/mol)	3.811	2.987	-	-	-	-	-	-
	5.636	3.349	-	-	-	-	-	-
	3.568	2.609	-	-	-	-	-	-
	3.566	2.272	-	1.150	0.174	-	-	-
	6.559	4.388	-	1.867	0.275	0.039	0.107	0.022
	5.707	5.209	0.157	2.626	0.104	0.015	0.099	0.037
Average	***4.808***	***3.469***	***0.157***	***1.881***	***0.184***	***0.027***	***0.103***	***0.029***
SD	*1.314*	*1.122*	*0*	*0.738*	*0.086*	*0.017*	*0.005*	*0.01*
Reagent $CaCO_3$	**0.003**	**0**	**0.001**	**0.003**	**0.003**	**0**	**0.001**	**0.001**
SD	0.002	0.001	0.001	0.002	0.002	0.001	0.001	0.001
Mg/Ca (mmol/mol)	0.354	0.383	-	-	-	-	-	-
	0.417	0.427	-	-	-	-	-	-
	0.335	0.345	-	-	-	-	-	-
	0.353	0.338	-	0.893	0.193	-	-	-
	0.455	0.452	-	0.967	0.309	0.204	0.156	0.145
	0.425	0.545	0.429	1.003	0.190	0.173	0.16	0.144
Average	***0.39***	***0.415***	***0.429***	***0.954***	***0.230***	***0.188***	***0.158***	***0.144***
SD	*0.049*	*0.078*	*0*	*0.056*	*0.068*	*0.021*	*0.003*	*0.001*
Reagent $CaCO_3$	**0.123**	**0.14**	**0.144**	**0.123**	**0.123**	**0.14**	**0.144**	**0.144**
SD	0.007	0.001	0	0.007	0.007	0.001	0	0
Sr/Ca (mmol/mol)	0.044	0.043	-	-	-	-	-	-
	0.044	0.043	-	-	-	-	-	-
	0.043	0.043	-	-	-	-	-	-
	0.044	0.043	-	0.045	0.044	-	-	-
	0.045	0.043	-	0.045	0.045	0.042	0.042	0.042
	0.043	0.042	0.042	0.047	0.043	0.043	0.041	0.041
Average	***0.044***	***0.043***	***0.042***	***0.046***	***0.044***	***0.043***	***0.041***	***0.042***
SD	*0.001*	*0*	*0*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*
Reagent $CaCO_3$	**0.046**	**0.041**	**0.04**	**0.046**	**0.046**	**0.041**	**0.04**	**0.04**
SD	0.001	0.003	0	0.001	0.001	0.003	0	0
Ca (ppm)	645.3	309.1	-	-	-	-	-	-
	621.0	358.8	-	-	-	-	-	-
	512.9	365.9	-	-	-	-	-	-
	688.7	426.7	-	455.6	208.8	-	-	-
	698.8	388.7	-	432.1	122.5	232.5	597.1	283.3

	613.7	170.9	144.7	406.6	204.3	481.6	262.2	125.6
Average	***630.1***	***336.7***	***144.7***	***431.5***	***178.5***	***357.1***	***429.6***	***204.4***
SD	*67.0*	*89.9*	*0.0*	*24.5*	*48.5*	*176.2*	*236.8*	*111.5*
Reagent CaCO3	**2849.3**	**635.4**	**215.7**	**2849.3**	**2849.3**	**635.4**	**215.7**	**215.7**
SD	756.5	72.7	89.6	756.5	756.5	72.7	89.6	89.6
Sample recovery (%)	0.21	0.10	-	-	-	-	-	-
	0.20	0.11	-	-	-	-	-	-
	0.17	0.12	-	-	-	-	-	-
	0.23	0.15	-	0.16	0.07	-	-	-
	0.22	0.12	-	0.14	0.04	0.08	0.19	0.09
	0.19	0.05	0.05	0.12	0.06	0.15	0.08	0.04
Average	***0.20***	***0.11***	***0.05***	***0.14***	***0.06***	***0.11***	***0.14***	***0.07***
SD	*0.02*	*0.03*	*0.00*	*0.02*	*0.02*	*0.05*	*0.08*	*0.04*
Reagent $CaCO_3$	**2.75**	**0.57**	**0.18**	**2.75**	**2.75**	**0.57**	**0.18**	**0.18**
SD	0.73	0.07	0.07	0.73	0.73	0.07	0.07	0.07

(a) Values of reference $CaCO_3$ powder sample used changed according to protocol used (also in different dates).