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Improvement of phytoplankton culture isolation using single cell sorting by flow cytometry

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► **To cite this version:**

Dominique Marie, Florence Le Gall, Roseline Edern, Priscillia Gourvil, Daniel Vaulot. Improvement of phytoplankton culture isolation using single cell sorting by flow cytometry. *Journal of Phycology*, 2017, 53 (2), pp.271-282 10.1111/jpy.12495 . hal-01408856

HAL Id: hal-01408856

<https://hal.sorbonne-universite.fr/hal-01408856>

Submitted on 5 Dec 2016

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12 **Abstract**

13 Flow cytometry provides a tool to physically sort single algal cells in order to obtain clonal
14 cultures. During sorting, cells are submitted to physical stress factors such as high fluidic
15 pressure, exposure to the laser beam, electrostatic charges, deflection through high voltage
16 fields and collisions with container surfaces. All of these can damage the cells of interest and
17 success rates for initiation of cultures from flow-sorted cells are generally very low. We found
18 that the addition of Bovine Serum Albumin (BSA) in the culture medium into which cells are
19 sorted drastically improved the success of initiation of pico- and nano-eukaryotic phytoplankton
20 strains. Adding a mixture of antibiotics (PNS: Penicillin, Neomycin, Streptomycin) to the
21 medium in order to slow down bacterial growth further improved culture development. This
22 approach was successfully used to isolate taxonomically diverse strains, including novel taxa,
23 from a fresh sample obtained in the English Channel and from enrichment cultures established
24 during an Atlantic Meridional Transect (AMT) cruise. We anticipate that these improvements
25 will be useful to clone or purify existing cultures and to isolate novel cultures from oceanic
26 samples.

27

28 **Keywords:** Flow cytometry, Single cell sorting, BSA, Picoeukaryotes, Nanoeukaryotes,
29 Antibiotics, Microalgal cultures, Phytoplankton

30 **Abbreviations :** BSA, Bovine Serum Albumin; AMT, Atlantic Meridional Transect; PNS,
31 Penicillin-Neomycin-Streptomycin; RCC, Roscoff Culture Collection; FSC, Forward Scatter;
32 SSC, Side Scatter; DCM, Deep Chlorophyll Maximum; PCR; polymerase chain reaction;

33

34 Introduction

35 In the context of the rapid development of metabarcoding and metagenomics approaches to
36 characterize marine microbial communities (de Vargas et al. 2015, Sunagawa et al. 2015), *ex*
37 *situ* laboratory cultures are absolutely critical to provide reference sequences for marker genes
38 such as 18S or 16S rRNA (Guillou et al. 2013, Decelle et al. 2015) or for genomes or
39 transcriptomes (Keeling et al. 2014), thereby facilitating annotation of the massive datasets
40 obtained. Many phytoplankton taxa are available in culture (del Campo et al. 2014), but culture
41 isolation relies mostly on traditional approaches such as serial dilution or single cell pipetting
42 (Andersen and Kawachi 2005). Novel methods are necessary to increase the diversity and
43 number of microalgal strains available in culture.

44 Flow cytometry provides a rapid method for analysis of planktonic cells in both cultures and
45 natural samples. Flow cytometry has been widely used to assess the abundance of autotrophic
46 and heterotrophic oceanic microorganisms for over three decades (Olson et al. 1985, Marie et
47 al. 1999, Zubkov et al. 2007). Some flow cytometry instruments are equipped with devices that
48 allow sorting of cells based on their physical and biological characteristics. Flow cytometry
49 sorting has been successfully applied to recover natural populations for genetic and genomic
50 characterization (Shi et al. 2009, Woyke et al. 2009, Rinke et al. 2013), as well as for
51 physiological measurements such as their capacity to fix carbon (Jardillier et al. 2010).
52 However, this technique has relatively rarely been used to bring novel microorganisms into
53 culture (Sensen et al. 1993, Sieracki et al. 2005, Sinigalliano et al. 2010, Cho et al. 2013), one
54 reason for this being the generally low level of recovery of viable cultures after sorting.

55 Using ¹⁴C-uptake, Rivkin et al. (1986) showed that the passage of cells through a flow
56 cytometer induces physiological damage to phytoplankton cells that results in a decrease in
57 growth rate during the first days following sorting. These authors associated cell damage to
58 high laser power, but found no effect of sheath fluid, droplet charging or sorting. Haugen et al.

59 (1987) did not find any physical damage or cell lysis due to fluidics or the laser beam, but
60 reported a lag in growth for up to 48 hours after sorting, suggesting physiological damage.
61 Sieracki et al. (2005) found higher recoveries when sorting single cells into 24- rather than 96-
62 well plates. However, while recovery of sorted *Isochrysis*, *Rhodomonas*, *Scrippsiella* and
63 *Thalassiosira* varied from 22 to 99%, single-cell sorting failed for the picoplanktonic green
64 microalga *Micromonas*. Sinigalliano et al. (2010) found that the trauma due to automated cell
65 sorting was not a limiting factor for obtaining unialgal dinoflagellate cultures compared to
66 manual micropipetting. These reports emphasize the large taxon-specific variability in cell
67 recovery after flow cytometry sorting.

68 The Roscoff Culture Collection (RCC) is one the largest service collections of marine
69 microalgal cultures (Vaulot et al. 2004). In the last decade, we have placed considerable focus
70 on using flow cytometry sorting to isolate unialgal cultures from natural samples (e.g. Le Gall
71 et al. 2008, Balzano et al. 2012), but cell recovery has always been very low, especially when
72 sorting single cells to obtain clonal cultures. In most cases, therefore, we have used flow
73 cytometry sorting for cell enrichment rather than for single cell isolation. We have tested a
74 range of solutions to try to obtain higher cell recovery such as lowering laser beam intensity,
75 changing sheath pressure or deflection voltage, or adding chemicals such as EDTA that trap
76 bivalent cations or Pluronic F68 that prevents adhesion of cells to the surfaces of plastic culture
77 vessels (Marie et al. 2014). None of these attempts improved cell recovery in a consistent way
78 (Marie, unpublished data).

79 In the present study we developed and tested a new protocol for strain isolation based on single
80 cell sorting in culture medium supplemented with Bovine Serum Albumin (BSA) followed by
81 antibiotic addition after a few days. This protocol was developed on cultures from the RCC
82 and then tested on a fresh sample from the English Channel off Roscoff and on enrichment
83 cultures obtained during an Atlantic Meridional Transect (AMT) cruise. In the latter case, we

84 compared flow cytometry sorting with more classical strain isolation approaches (serial dilution
85 and enrichment).

86

87 **Materials and methods**

88 **Cultures.** Five strains from different taxonomic groups were obtained from the Roscoff Culture
89 Collection (RCC, www.roscoff-culture-collection.org): *Micromonas pusilla* RCC299
90 (Mamiellophyceae), *Florenciella* sp. RCC1008 (Dictyochophyceae), *Isochrysis* sp. RCC90
91 (Prymnesiophyceae), *Rhodomonas baltica* RCC350 (Cryptophyceae) and *Scrippsiella* sp.
92 RCC4108 (Dinophyceae). Cultures were grown in K medium (Keller et al. 1987) in 12:12 hour
93 light:dark cycle and were sorted when in exponential growth phase.

94 **Oceanic samples.** Surface seawater from the English Channel was sampled in May 2015 at the
95 Estacade Station in Roscoff (Guilloux et al. 2013). A larger set of samples was obtained in 2014
96 during the Atlantic Meridional Transect (AMT, Rees et al. 2015) cruise # 24 at two depths
97 (surface and deep chlorophyll maximum, DCM). Twenty-two stations were sampled in the
98 Atlantic Ocean between the United Kingdom and the Falkland Islands (Table 1 and Fig. 1). Pre-
99 cultures were obtained by adding 1 mL of L1 culture medium to 25 mL of seawater sample.
100 Pre-cultures were maintained on board at 20°C in a L:D cycle and were transported promptly
101 back to Roscoff at the end of the cruise.

102 **Flow cytometry.** A FACS Aria flow cytometer (Becton Dickinson, San Jose CA) equipped with
103 488 and 633 nm lasers and a standard filter setup was used for cell sorting. In order to minimize
104 the impact of the laser, only the 488 nm laser was employed. Acquisition was triggered on red
105 fluorescence, Forward Scatter (FSC) and Side Scatter (SSC) with the minimum threshold value.
106 Sorting was performed in “single cell” mode using 20 PSI as sheath pressure. An Accuri C6
107 (Becton Dickinson, San Jose CA) equipped with a CSampler was used to monitor cell
108 concentration after flow cytometry sorting. Acquisition was triggered on red fluorescence and
109 performed for 1 min at a rate of 65 μ L/min.

110 **Microscopy.** An inverted microscope (Olympus IX71) equipped with epifluorescence (blue
111 light excitation, red light emission) was used to detect the presence of photosynthetic cells in
112 wells into which 1 cell was sorted. When cells with chlorophyll fluorescence were observed in
113 a well, it was considered as positive for culture growth.

114 **Effect of BSA concentration on cultures.** RCC cultures were sorted into 1 mL of K medium
115 either without BSA or with BSA (Ref A7030, Sigma) at concentrations of 0.01, 0.1 or 0.5%.
116 Typically one 48-well plate (CytoOne, Starlab) was used for each culture: 1000 cells (500 for
117 *Scropsiella*) were sorted into 3 wells and 1 cell into each of the remaining 45 wells. Using the
118 Accuri C6, cell concentration was monitored over 2 weeks for the wells containing 500 or 1000
119 cells after sorting. Wells with 1 cell were monitored by epifluorescence microscopy between
120 days 3 and 14 following sorting.

121 **Effect of antibiotic addition.** RCC cultures were sorted into 48-well plates (CytoOne, Starlab)
122 containing 1 mL of K medium supplemented with 0.01% BSA. For each culture 500
123 (*Scropsiella*) or 1000 (other strains) cells were sorted into 6 wells containing 1 mL of K medium.
124 Three days after sorting, a mixture of Penicillin, Neomycin and Streptomycin (PNS, Ref P4083,
125 Sigma) was added at a final concentration of 0.1% to 3 of the wells. Cell concentration was
126 subsequently monitored by flow cytometry for 2 weeks.

127 **Effect of medium on sorting for a natural sample.** Surface water from the English Channel
128 (see above) was filtered through a 50 µm nylon mesh in order to remove larger cells that can
129 clog the flow cell of the cytometer. Four media supplemented with BSA 0.01% were tested: K
130 (Keller et al. 1987), f/2 (Ref G9903, Sigma, Guillard and Ryther 1962), water from the sampling
131 site supplemented with K medium nutrients (SW+Nut) and not supplemented (SW). All media
132 were filter-sterilized through a 0.2 µm filter just before sorting. Two 48-well plates containing
133 1 mL of medium per well were used per media tested, one for pico- and one for nano-

134 phytoplankton. The first 3 wells were used to sort 1000 cells for the pico-phytoplankton and
135 500 cells for the nano-phytoplankton, respectively. One cell was sorted in each of the remaining
136 45 wells. Three days after sorting, PNS 0.1% (final concentration) was added into all wells with
137 1000 or 500 sorted cells and cell concentration was monitored over 2 weeks by flow cytometry
138 (Fig. 2). Wells with 1 sorted cell were monitored by epifluorescence microscopy every three
139 days for 2 weeks following sorting. When live cells were observed in a well, PNS 0.1% (final
140 concentration) was added. Ten diatom strains isolated during this experiment were kept in
141 culture and incorporated into the RCC.

142 ***Oceanic strain isolation.*** Three different isolation approaches were compared using pre-
143 cultures obtained during the AMT 2014 cruise (see above): flow cytometry sorting, enrichment
144 and serial dilution.

145 For flow cytometry sorting, 24 AMT pre-cultures were sorted in 1 mL of K medium with 0.01%
146 BSA into 48-well plates (Table 1). Half a plate was used for each population selected: 1000
147 cells were sorted into the first two wells, 100 into the next two wells as controls and 1 cell into
148 the remaining 20 wells. Plates were incubated in a 12:12 light:dark cycle for 10 days. Wells
149 were then screened by inverted epifluorescence microscopy to detect the presence of
150 photosynthetic cells. When live cells were observed in the wells with 1 sorted cell, they were
151 transferred into a tube containing 5 mL of K medium supplemented with a final concentration
152 of 0.1% PNS.

153 For enrichment, AMT pre-cultures were screened and counted by flow cytometry. When the
154 pre-culture seemed to contain only one population of cells, the isolation technique used was an
155 enrichment in the appropriate culture medium. This technique was used for 19 pre-cultures from
156 AMT (Table 1). We used K medium for eukaryotes and PCRS11 medium (Rippka et al. 2000)
157 for cyanobacteria.

158 For dilution, AMT pre-cultures were screened and counted by flow cytometry. Fifteen pre-
159 cultures that contained several cell populations with different sizes and chlorophyll signals were
160 diluted into K medium in 48-well plates. Eight wells were used for each pre-culture with a final
161 target density of 10 cells per well (Table 1). Plates were incubated in a 12:12 light:dark cycle
162 for 3 weeks, after which an inverted epifluorescence microscope was used to check for the
163 presence of cells.

164

165 ***Strain characterization.*** Strains were characterized by sequencing partially the 18S rRNA
166 gene. The cells were heated for 5 min at 95°C and cooled to 4°C. The 18S rRNA gene was
167 amplified by polymerase chain reaction (PCR) using the eukaryote specific primers 63F/1818R
168 (Lepère et al. 2011) and the Phusion Master Mix (Thermo Fisher Scientific, France). For PCR,
169 a 5 minute initial denaturation step at 95°C was followed by 35 cycles including 1 min of
170 denaturation at 95 °C, 90 sec of annealing at 57°C and 90 sec extension at 72°C. The PCR
171 program was finished by a final extension of 10 min at 72°C followed by cooling at 4°C. PCR
172 products were purified using Exosap (USB products, Santa Clara, USA). Partial 18S rRNA
173 gene sequences were determined by using Big Dye Terminator V3.1 (Applied Biosystems,
174 Foster city, CA, USA) and the internal primer Euk 528f (Elwood et al. 1985). Sequencing was
175 carried out on an ABI prism 3100 sequencer (Applied Biosystems). Partial 18S rRNA
176 sequences were compared to those available in public databases with NCBI BLAST
177 (blast.ncbi.nlm.nih.gov/Blast.cgi).

178 All AMT strains and 10 strains isolated from the English Channel have been deposited in the
179 Roscoff Culture Collection (RCC4548 to RCC4579 and RCC4657 to RCC4666, respectively)
180 and their sequences deposited in GenBank under accession numbers KX014627-KX014660.

181

182

183 **Results**184 **Effect of BSA addition.**

185 We first tested the effect of adding BSA at different concentrations (0, 0.1, 0.01 and 0.05 %) to
186 the culture medium into which cells were sorted using 5 strains of eukaryotes (Fig. 2 and Table
187 2).

188 For the picoplanktonic strain *Micromonas pusilla* (Mamiellophyceae) RCC299, we observed a
189 rapid decline in cell concentration for the 3 wells with 1000 sorted cells in the absence of BSA
190 and no live cells were detected by flow cytometry after 5 days of incubation (Fig. 2A). After 8
191 days, cells were detected in 2 of the 3 wells. In presence of BSA, no lag phase or decline in cell
192 numbers were observed. Initial growth rates were identical for the three BSA concentrations
193 tested, but after 5 days of incubation better growth was obtained at the lowest BSA
194 concentration (0.01%). Epifluorescence microscope observations of wells into which 1 cell was
195 sorted (Table 2) revealed the absence of cells in the absence of BSA, 100% recovery with 0.01%
196 BSA, and 50 to 60% recovery with 0.1 and 0.5%, respectively.

197 In the absence of BSA and at the two lowest BSA concentrations (0.01 and 0.1%), the other
198 picoplanktonic strain *Florenciella* sp. RCC1008 (Dictyochophyceae) decreased in abundance
199 during the first week and no cells were detected 10 days after sorting (Fig. 2B). Between days
200 10 and 14, cell abundance recovered slowly. In wells with the highest BSA concentration
201 (0.5%), cell abundance did not decrease significantly and we observed exponential growth after
202 day 7 (Fig. 2B). For single sorted cells of this strain, no recovery was detected at the lowest
203 BSA concentration (0.01%). In the absence of BSA and at 0.1% BSA, 1 and 2 wells,
204 respectively, exhibited growth after 2 weeks. In wells with the highest BSA concentration
205 (0.5%), growth was detected in 7 out of 9 wells at day 14 (Table 2).

206 BSA did not affect growth of the nanoplanktonic strains *Isochrysis* sp. RCC90
207 (Prymnesiophyceae) and *Rhodomonas baltica* RCC350 (Cryptophyceae) (Fig. 2C and D). For
208 *Isochrysis*, growth was observed after day 5 in some wells with 1 cell and all wells had growing
209 cultures by the end of the experiment for all BSA concentrations (Table 2). For *Rhodomonas*,
210 growth was observed in almost all wells at day 3 (Table 2).

211 For *Scrippsiella* sp. RCC4108 (Dinophyceae), a small decrease in cell number was observed
212 immediately after sorting of 500 cells in the absence of BSA (Fig. 2E). However, growth rate
213 was very similar in all four conditions. After day 7, cell number was highest with BSA 0.01%
214 and lowest with BSA 0.1%. No effect of BSA was observed for single sorted cells of this strain.
215 The maximum number of wells with detectable growth was obtained on day 3 in the absence
216 of BSA (5/9) and there was no evolution in the number of positive wells past day 7 (Table 2).

217 Effect of antibiotic treatment.

218 For cultures sorted into medium with 0.01% BSA, growth stopped after 4 days for *Scrippsiella*
219 and 7 days for *Micromonas* and *Rhodomonas* in the absence of the antibiotic cocktail (PNS),
220 while cultures continued to grow when PNS was added (Fig. 3). *Isochrysis* had a higher growth
221 rate in the absence of PNS reaching stationary phase earlier than with PNS, although after 2
222 weeks cell yield was similar. For *Florenciella*, addition of PNS resulted in significant cell loss,
223 but cultures recovered one week after sorting to reach a higher final density than in the absence
224 of PNS (Fig. 3).

225 Recovery and isolation of phytoplankton sorted from a fresh seawater sample

226 Wells into which 1000 picoeukaryote cells were sorted had similar growth rates irrespective of
227 the medium, but final cell concentration was lower in f/2 and unamended seawater (Fig. 4A).
228 Single picoeukaryote sorted cells began to grow in some wells after 3 days and the percentage
229 of wells with detectable growth after 2 weeks was highest using f/2 medium, reaching almost

230 100%, followed by amended SW, SW and K medium for which recovery was only 50% (Table
231 3). After 9 to 15 days, approximately 70% of wells containing f/2 or SW with nutrients and
232 only 30% for SW or K medium were dense enough to allow transfer to tubes containing 4 mL
233 of K medium. All strains obtained from single cell sorting (Table 4) were Chlorophyta
234 representing two classes, the Mamiellophyceae (*Micromonas*, *Ostreococcus* and *Bathycoccus*)
235 and the Trebouxiophyceae (*Picochlorum*). The highest number of different genera was obtained
236 using f/2 medium and the lowest in unamended seawater.

237 No difference in growth rate was observed after sorting 500 nanoeukaryote cells in different
238 media, but after 6 days, cell numbers were significantly higher in wells with f/2 and SW+Nut
239 (Fig. 4B). Heterogeneity in cell numbers was observed among the 3 wells in all media. As for
240 picoeukaryotes, cells were detected in some single-cell wells after only 3 days (Table 3) and
241 the number of wells with detectable growth did not increase beyond day 9. More than half of
242 the wells yielded growth and this proportion was highest with SW (71%) and lowest with K, as
243 was the case for picoeukaryotes. Strains obtained from single cell sorting were mostly small-
244 (*Minidiscus*, *Minutocellus*) or medium-sized (*Thalassiosira*, *Skeletonema*, *Cylindrotheca*)
245 diatoms, or the common prymnesiophyte *Phaeocystis* (Table 4). The largest variety of genera
246 was obtained using amended SW and the lowest using K, as was the case for picoeukaryotes.

247 Isolation of phytoplankton strains from oceanic enrichments

248 During AMT cruise #24, from the UK to the Falkland Islands, two depths (surface and DCM)
249 were sampled at a range of stations in the Atlantic Ocean from temperate to equatorial
250 environments. During the cruise, pre-cultures were obtained by adding L1 medium to natural
251 samples and these pre-cultures were promptly brought back to the laboratory at the end of the
252 cruise. Single-cell flow cytometry sorting was applied to 24 pre-cultures using K medium with
253 0.01% BSA, yielding a total of 700 wells (Table 1). After 10 days of incubation, phytoplankton
254 growth was observed in 278 (40%) wells. The percentage of wells with growth varied with the

255 pre-culture, ranging from 0% to 100% (Table 1). Positive wells were transferred into tubes
256 containing 5 mL of K medium supplemented with a final concentration of 0.01% PNS.

257 In parallel, we prepared 120 wells by serial dilution from 15 pre-cultures (8 wells per pre-
258 culture) with a target concentration of 10 cells per well. After 3 weeks of incubation, 39 (33%)
259 wells showed positive growth (Table 1). As for sorting, the success rate was variable, again
260 ranging from 0 to 100% depending on the pre-culture (Table 1). Cells from positive wells were
261 transferred into tubes using K medium for eukaryotes and PCR-S11 for cyanobacteria. Finally,
262 a simple enrichment approach was performed on nineteen pre-cultures using different media
263 and sixteen cultures were isolated in this manner.

264 Genetic characterization of the AMT strains using the 18S rRNA gene revealed that many
265 cultures belonged to the same genus. For cultures obtained either by flow cytometry sorting or
266 dilution, all tubes originating from the same pre-culture and obtained with the same technique
267 yielded identical sequences. For example, the 36 positive wells originating from pre-culture 04
268 (Table 1) were all affiliated to *Phaeocystis*. When we obtained the same affiliation with the 3
269 techniques of isolation, we decided to prioritize maintenance of the cultures isolated by flow
270 cytometry sorting because these strains were more likely to be clonal. In total, 31 representative
271 cultures were deposited to the RCC, corresponding to 11 different genera (Table 5). The highest
272 diversity was obtained with cell sorting, but some taxa were only obtained by dilution or
273 enrichment (Table 6). The genera isolated from the largest number (8) of pre-cultures (Table
274 6) belonged to Pelagophyceae (*Pelagomonas*) and Cyanophyceae (*Synechococcus*), although
275 the latter were only isolated by dilution or enrichment since only eukaryotes were targeted by
276 sorting. Strains isolated from more than one pre-culture belonged to the Chlorophyta
277 (*Micromonas*, prasinophyte clade VII), Prymnesiophyceae (*Phaeocystis*, *Emiliana*, *Isochrysis*)
278 and Pelagophyceae. Finally some strains were only isolated from a single pre-culture. This

279 was in particular the case for RCC4570, the 18S rRNA sequence of which was affiliated to the
280 Heterokontophyta.

281 Discussion

282 Isolation of phytoplankton strains from oceanic waters is a difficult task because many taxa
283 have unknown nutritional requirements or are quickly outcompeted by fast growing species.
284 For small cells (pico- and nano-phytoplankton), the most common approach involves serial
285 dilution (Andersen and Kawachi 2005) using media such as K which have been specifically
286 designed for oceanic strains (Keller et al. 1987). However, this strategy is blind and provides
287 random results since no specific group is targeted. For example, during the BIOSOPE cruise
288 in the South East Pacific, serial dilution resulted in isolation of 34 strains of *Pelagomonas*
289 *calceolata* (Le Gall et al. 2008), but did not recover any of the dominant taxa in this region that
290 belong to the haptophyte, chrysophyte and prasinophyte clade IX lineages (Shi et al. 2009).
291 The theoretical advantages of flow cytometry include the possibility of targeting specific groups
292 based on their size and chlorophyll content, as well as the capacity to isolate single cells,
293 resulting in clonal cultures. The former factor should allow elimination of fast growing species
294 that tend to be over-represented when using other techniques. However, as outlined above,
295 success rates for culture recovery have been very low for single cell flow cytometric sorting.

296 While trying different additions to sorting medium, we discovered that BSA had a spectacular
297 impact on cell recovery. The reason for the positive effect of BSA is unclear. Albumin is a
298 major protein from bovine or human serum, with multifunctional properties and is widely used
299 for mammalian cell cultures (Francis 2010). It is well known as a strong antioxidant and has
300 also been proved to avoid attachment of cells onto plastic walls (Fletcher 1976). BSA is also
301 often added when preparing protoplasts from macroalgae (e.g. Bodian et al. 2013).
302 Unfortunately, no detailed study on its effect or role on cell culture has been published. Of the
303 five strains tested in the present study belonging to a range of phylogenetic groups, the

304 nanoplanktonic strains *Isochrysis*, *Rhodomonas* and *Scrippsiella* recovered well after sorting
305 and BSA did not seem to have a significant effect except in the case of *Scrippsiella* for which
306 the lag phase was reduced with BSA addition (Fig. 2C-E and Table 2). In contrast, the
307 picoplanktonic species *Micromonas* and *Florenziella* could not recover after sorting without
308 the addition of BSA (Fig. 2A-B and Table 2), confirming the report of Sieracki et al. (2005)
309 who did not obtain any growth of *Micromonas* when sorting 1 cell per well. With addition of
310 BSA, we obtained 100% regrowth of *Micromonas* (Table 2). Interestingly, the optimum BSA
311 concentration varied with the species. While concentrations below 0.5% had no effect on
312 *Florenziella* (Fig. 2 and Table 2), 0.01% was optimum for *Micromonas* (Fig. 2 and Table 2).
313 This suggests that it will be necessary to test different BSA concentrations when dealing with
314 specific cultures or natural populations. In particular, *Florenziella*, which apparently requires
315 a high BSA concentration to facilitate regrowth after single cell sorting, has rarely been isolated
316 into culture: there are only 11 strains isolated from 3 cruises available in culture collections (all
317 in the RCC).

318 When using flow cytometry to sort phytoplankton cells from natural samples, heterotrophic
319 bacteria are also likely to be sorted since they often outnumber phototrophic cells by an order
320 of magnitude. Although flow cytometry has been used to produce axenic cultures (Sensen et al.
321 1993), it is difficult to completely remove associated bacteria, especially those that adhere to
322 the surface of microalgal cells (Surek and Melkonian 2004), and antibiotics are often necessary
323 to obtain axenic cultures (Cottrell and Suttle 1993). Even when axenic cultures are not targeted,
324 antibiotic treatment may be critical to reduce competition for nutrients from bacteria. This was
325 apparently the case for *Micromonas*, *Rhodomonas* and *Scrippsiella* which stopped growing
326 after a few days in the absence of antibiotics, while growth continued when PNS was added
327 (Fig. 3A, D, E). The case of *Florenziella* was paradoxical. Addition of PNS led to an initial
328 decrease of cell concentration which then recovered after day 7, while in the absence of PNS

329 initial growth was continuous but final cell density was ten times lower. This could be
330 explained by *Florenciella* being mixotrophic, as reported for field populations (Frias-Lopez et
331 al. 2009). The initial decrease of the population when antibiotics were added could therefore
332 be due to the decrease in bacterial prey density, followed by a switch to autotrophic nutrition
333 inducing regrowth.

334 Another factor to take into account during isolation of phytoplankton strains is the composition
335 of the culture medium. Very few studies have assessed the effect of different media on the
336 success of culture isolation or even on culture growth. Harrison and Berges (2005) quoted
337 McLachlan (1973): “Numerous enriched and synthetic media have been formulated, which
338 together with generally trivial modifications, almost equal the number of investigators”. The
339 two most commonly used media for marine microalgae are f/2 (Guillard and Ryther 1962) and
340 K (Keller et al. 1987). The effect of these two media along with unamended and amended sea
341 water was tested using a fresh sample from the English Channel (Tables 3 and 4) . For
342 picoeukaryotes, the use of f/2 resulted in the widest diversity of strains, while for
343 nanoeukaryotes differences were less marked. This could be explained by the fact that f/2 is
344 more appropriate for coastal species (Keller et al. 1987). However, some species, such as
345 *Ostreococcus*, *Phaeocystis*, *Minidiscus* and *Thalassiosira*, were always isolated irrespective of
346 the medium used. Our approach allowed isolation from a single sample of three picoplanktonic
347 genera, *Micromonas*, *Ostreococcus* and *Bathycoccus*, that are dominant in English Channel
348 coastal waters as demonstrated previously both by fluorescent *in situ* hybridization (FISH) and
349 18S rRNA clone libraries (Not et al. 2004, Romari and Vaultot 2004, Marie et al. 2010). The
350 nanoplanktonic species isolated were mostly diatoms reflecting the fact that the sample was
351 taken during the spring diatom bloom (Sournia et al. 1987).

352 Our protocol proved to be efficient for obtaining clonal strains from pre-cultures established
353 during an Atlantic cruise. In particular, we repeatedly obtained strains of *Pelagomonas* and

354 prasinophyte clade VII, both of which are typically isolated from oceanic tropical and sub-
355 tropical waters (Le Gall et al. 2008). We obtained strains of several taxa that, to our knowledge,
356 have not previously been cultured. Within prasinophyte clade VII, we obtained for the first
357 time a representative of clade VIIB3 (Lopes dos Santos et al., submitted). In addition, three
358 Pelagophyceae cultures corresponded to undescribed taxa. The 18S rRNA sequences of two
359 Pelagophyceae strains from the tropical Atlantic (RCC4562 and RCC4563) matched with 100%
360 identity the sequence of RCC1024, previously isolated from the South East Pacific (Le Gall et
361 al. 2008). These strains are also related to the endosymbiont of the tropical dinoflagellate
362 *Amphisolenia bidentata* (Daugbjerg et al. 2013). Strain RCC4552, isolated from the temperate
363 North Atlantic, had a sequence only 98.7% similar to any other GenBank sequence and
364 probably corresponds to a novel species. Its sequence was most closely related to RCC2505,
365 isolated from polar waters (Balzano et al. 2012). The 18S rRNA sequence of RCC4570, a
366 stramenopile strain obtained by dilution, was related to two strains (RCC853 and RCC862) that
367 had been previously isolated from the South East Pacific (Le Gall et al. 2008), but unfortunately
368 lost since. This group of sequences suggests the existence of a novel algal class, called MOCH-
369 5 by Massana et al. (2014). As evident from Table 1, the success of the three approaches we
370 used (sorting, dilution, enrichment) was highly variable. In several cases only one technique
371 was successful for a given pre-culture: for example, strains were obtained by dilution but not
372 sorting for pre-cultures 26 , 27 and 42, whereas the opposite result was obtained for pre-cultures
373 54 and 55.

374 The novel protocol proposed for culture isolation using single-cell flow cytometry sorting has
375 several advantages compared to other techniques for culture isolation. It is much more rapid
376 than serial dilution. A 48-well plate can be sorted in less than 1 minute. Results are obtained
377 in less than 10 days, compared to at least three weeks for serial dilution. More importantly,
378 specific populations can be targeted. For example, many of our AMT serial dilution wells

379 produced untargeted *Synechococcus* cultures, while sorting targeted only eukaryotes. Flow
380 cytometry sorting is best done on fresh samples since the diversity of the taxa recovered is high
381 and more representative of the initial diversity, as demonstrated for an English Channel sample
382 obtained near our laboratory from which we isolated at least 10 different taxa. However, this
383 requires the availability of a bulky sorting flow cytometer near the sampling site or on the cruise.
384 An alternative strategy for large scale cruises is to enrich samples to obtain pre-cultures. This
385 can be done many different ways, without or with filtration in order to select specific size
386 classes, without or with tangential flow filtration which allows concentration of samples, or
387 with a range of different media and additives, such as germanium dioxide to prevent diatom
388 growth (Vaulot et al. 2004, Le Gall et al. 2008, Balzano et al. 2012). In such cases, since there
389 can be a long delay before the pre-cultures can be sorted, each pre-culture usually becomes
390 dominated by one or two species. Single-cell sorting allows rapid purification and cloning of
391 these pre-cultures as demonstrated for the AMT cruise. Since diversity is typically rather low
392 for a given pre-culture, a relevant strategy to obtain different taxa in culture is to apply sorting
393 to a large number of pre-cultures originating from different stations and depths (Table 5).

394 Another useful application of this protocol would be to make existing cultures clonal. We
395 recently applied this approach to clone RCC strains of *Micromonas*, *Ostreococcus*, *Bathycoccus*
396 and *Triparma* (PG, unpublished data). It can also be used to purify mixed cultures, in which
397 case there is no need to perform single cell sorting; sorting of several hundred cells for each
398 target population should provide good results.

399 **Acknowledgments**

400 We thank Monica Moniz and Dave Scanlan from Warwick University for providing samples
401 from the AMT 2014 cruise #24 as well as Ian Probert for his help improving the English
402 language. Financial support for this work was provided by the European Union's 7th
403 Framework Programme MaCuMBA project (FP7-KBBE-2012-6-311975) and the Betty and

404 Gordon Moore Foundation (MMI EMS grant #4972). The publication reflects the views only
405 of the author, and the European Union cannot be held responsible for any use which may be
406 made of the information contained therein.

407

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- 532

533 **List of Tables**

534 Table 1 : List of pre-cultures obtained during the Atlantic Meridional Transect (AMT) cruise
 535 #24 in 2014 with station number, date and depth. Columns indicate whether enrichments were
 536 established from pre-cultures (see Material and Methods), the number of single-cell flow
 537 cytometry sorted wells and the number of dilution wells. For flow cytometry sorting, the type
 538 of population sorted is indicated (P for picoeukaryotes and N for nanoeukaryotes). The number
 539 and percentage of positive wells obtained by the two techniques is also indicated. The position
 540 of the stations is provided in Fig. 1.

Precultures	Sampling date	Station	Depth	Enriched	Sorted wells (1 cell per well)				Dilution wells (10 cells per well)		
					Pico/Nano	total	positive	% positive	total	positive	% positive
AMT-04	27/09/2014	4	Surface	+	N	40	36	90%			
AMT-06	28/09/2014	6	Surface	+							
AMT-08	30/10/2014	9	Surface		P/N	40	28	70%	8	2	25%
AMT-09	01/10/2014	12	60		N	20	15	75%	8	8	100%
AMT-11	02/10/2014	13	78	+							
AMT-12	02/10/2014	13	Surface		P/N	40	13	33%	8	3	38%
AMT-13	03/10/2014	15	100	+							
AMT-14	03/10/2014	15	Surface	+							
AMT-16	04/10/2014	18	Surface	+							
AMT-20	06/10/2014	23	Surface		N	20	1	5%	8	4	50%
AMT-25	10/10/2014	33	45	+							
AMT-26	10/10/2014	33	Surface		N	20	0	0%	8	1	13%
AMT-27	11/10/2014	35	75	+	P	20	0	0%	8	1	13%
AMT-28	11/10/2014	35	Surface		N	20	15	75%	8	2	25%
AMT-30	12/10/2014	38	Surface		P/N	40	5	13%	8	8	100%
AMT-32	14/10/2014	42	Surface	+	P/N	80	49	61%			
AMT-36	16/10/2014	48	Surface		P/N	60	16	27%	8	1	13%
AMT-42	20/10/2014	56	Surface		P	20	0	0%	8	6	75%
AMT-43	21/10/2014	59	135	+	P	20	1	5%			
AMT-44	21/10/2014	59	Surface	+	N	20	2	10%	8	0	0%
AMT-46	22/10/2014	62	Surface		P	20	6	30%	8	3	38%
AMT-47	23/10/2014	65	87								
AMT-48	23/10/2014	65	Surface	+	P	20	13	65%			
AMT-49	24/10/2014	68	44	+	P	20	12	60%			
AMT-50	24/10/2014	68	Surface	+	N	20	17	85%			
AMT-51	25/10/2014	71	50	+							
AMT-52	25/10/2014	71	Surface	+	P	20	20	100%			
AMT-53	26/10/2014	73	40	+	N	20	3	15%			
AMT-54	26/10/2014	73	Surface		P/N	40	5	13%	8	0	0%
AMT-55	27/10/2014	76	40		P/N	40	14	35%	8	0	0%
AMT-56	27/10/2014	76	Surface	+	P	20	5	25%			
AMT-57	28/10/2014	78	46	+							
AMT-58	28/10/2014	78	Surface		N	20	2	10%	8	0	0%
Total	Number of pre-cultures treated			19		24			15		
	Number of wells					700	278	40%	120	39	33%

541

542

543 Table 2 : Evolution over two weeks of the numbers of positive wells (out of a total of 9 wells)
 544 observed by epifluorescence on an inverted microscope after single-cell flow cytometry sorting
 545 for 5 RCC cultures. Cells were sorted into 1 mL of K medium with BSA at different
 546 concentrations.

Culture	BSA	Days 3 - 5	Days 7 - 8	Days 10 - 11	Day 14
<i>Micromonas pusilla</i> RCC299	0%	0	0	0	0
	0.01%	9	9	9	9
	0.10%	4	4	4	4
	0.50%	5	5	5	5
<i>Florenciella</i> sp. RCC1008	0%	0	0	0	1
	0.01%	0	0	0	0
	0.10%	0	1	2	2
	0.50%	0	2	5	7
<i>Isochrysis</i> sp. RCC90	0%	7	8	8	8
	0.01%	4	9	9	9
	0.10%	5	9	9	9
	0.50%	5	6	7	7
<i>Rhodomonas baltica</i> RCC350	0%	8	9	9	9
	0.01%	9	9	9	9
	0.10%	8	8	8	8
	0.50%	9	9	9	9
<i>Scrippsiella</i> sp. RCC4108	0%	5	5	5	5
	0.01%	0	2	4	4
	0.10%	3	3	3	3
	0.50%	2	3	3	3

547

548 Table 3 : Percentage of positive wells (out of 45 wells) obtained from single cell sorting by flow
 549 cytometry of picoeukaryotes and nanoeukaryotes into four different media from a natural
 550 sample from the English Channel off Roscoff taken in May 2015.

Population	Medium	Day				
		3	6	9	12	15
Picoeukaryotes	SW	33%	47%	51%	62%	69%
	SW+Nut	33%	56%	71%	78%	80%
	f/2	24%	84%	98%	98%	98%
	K	7%	22%	36%	49%	51%
Nanoeukaryotes	SW	27%	60%	69%	69%	71%
	SW+Nut	18%	60%	67%	67%	67%
	f/2	20%	51%	60%	60%	60%
	K	16%	33%	47%	49%	49%

551

552

553 Table 4 : Comparison of the taxonomic identity of cultures isolated by single cell flow
 554 cytometry sorting of picoeukaryotes and nanoeukaryotes into four different media of a natural
 555 sample from the English Channel off Roscoff taken in May 2015.

Division	Class	Genus	Medium			
			SW	SW+Nut	f/2	K
Picoeukaryotes						
Chlorophyta	Mamiellophyceae	<i>Ostreococcus</i>				
Chlorophyta	Mamiellophyceae	<i>Micromonas</i>				
Chlorophyta	Mamiellophyceae	<i>Bathycoccus</i>				
Chlorophyta	Trebouxiophyceae	<i>Picochlorum</i>				
Nanoeukaryotes						
Heterokontophyta	Coscinodiscophyceae	<i>Minidiscus</i>				
Heterokontophyta	Coscinodiscophyceae	<i>Thalassiosira</i>				
Haptophyta	Prymnesiophyceae	<i>Phaeocystis</i>				
Heterokontophyta	Mediophyceae	<i>Minutocellus</i>				
Heterokontophyta	Mediophyceae	<i>Unknown</i>				
556 Heterokontophyta	Bacillariophyceae	<i>Cylindrotheca</i>				

557

558 Table 5 : List of cultures isolated from the AMT transect by single-cell flow cytometry sorting,
 559 serial dilution and enrichment.

Precultures	Isolation	Division	Class	Order	Genus	Number of cultures	RCC id
AMT-04	Cell sorting	Haptophyta	Prymnesiophyceae	Phaeocystales	<i>Phaeocystis</i>	1	LOST
AMT-06	Enrichment	Cyanophyta	Cyanophyceae	Synechococcales	<i>Synechococcus</i>	1	LOST
AMT-08	Dilution	Heterokontophyta	Pelagophyceae	Pelagomonadales	<i>Pelagomonas</i>	1	4548
AMT-08	Cell sorting	Haptophyta	Prymnesiophyceae	Isochrysidales	<i>Emiliana</i>	1	4549
AMT-09	Dilution	Haptophyta	Prymnesiophyceae	Phaeocystales	<i>Phaeocystis</i>	1	LOST
AMT-11	Enrichment	Cyanophyta	Cyanophyceae	Synechococcales	<i>Synechococcus</i>	1	4550
AMT-12	Dilution	Heterokontophyta	Pelagophyceae	Pelagomonadales	<i>Pelagomonas</i>	1	4551
AMT-12	Cell sorting	Heterokontophyta	Pelagophyceae	Pelagomonadales	New species 1	1	4552
AMT-12	Enrichment	Chlorophyta	Prasinophyceae	Clade VIIA3	Undescribed	1	4553
AMT-13	Enrichment	Cyanophyta	Cyanophyceae	Synechococcales	<i>Synechococcus</i>	1	4554
AMT-14	Enrichment	Cyanophyta	Cyanophyceae	Synechococcales	<i>Synechococcus</i>	1	4555
AMT-16	Enrichment	Cyanophyta	Cyanophyceae	Synechococcales	<i>Synechococcus</i>	1	LOST
AMT-20	Dilution	Heterokontophyta	Pelagophyceae	Pelagomonadales	<i>Pelagomonas</i>	2	LOST
AMT-25	Enrichment	Cyanophyta	Cyanophyceae	Synechococcales	<i>Synechococcus</i>	1	4556
AMT-26	Dilution	Cyanophyta	Cyanophyceae	Synechococcales	<i>Synechococcus</i>	1	4557
AMT-27	Enrichment	Heterokontophyta	Chrysophyceae	Chromulinales	<i>Spumella</i>	1	4558
AMT-27	Dilution	Heterokontophyta	Pelagophyceae	Pelagomonadales	<i>Pelagomonas</i>	1	4559
AMT-28	Dilution	Heterokontophyta	Pelagophyceae	Pelagomonadales	<i>Pelagomonas</i>	1	LOST
AMT-30	Dilution	Bacillariophyta	Bacillariophyceae	Bacillariales	<i>Pseudo-nitzschia</i>	1	LOST
AMT-30	Cell sorting	Haptophyta	Prymnesiophyceae	Isochrysidales	<i>Emiliana</i>	1	4560
AMT-30	Cell sorting	Heterokontophyta	Pelagophyceae	Pelagomonadales	<i>Pelagomonas</i>	1	4561
AMT-32	Cell sorting	Heterokontophyta	Pelagophyceae	Pelagomonadales	New species 2	2	4562, 4563
AMT-32	Cell sorting	Heterokontophyta	Pelagophyceae	Pelagomonadales	<i>Pelagomonas</i>	1	4564
AMT-32	Dilution	Chlorophyta	Prasinophyceae	Clade VII	Unknown	1	4565
AMT-32	Cell sorting	Haptophyta	Prymnesiophyceae	Phaeocystales	<i>Phaeocystis</i>	1	LOST
AMT-36	Dilution	Heterokontophyta	Pelagophyceae	Pelagomonadales	<i>Pelagomonas</i>	1	4566
AMT-36	Cell sorting	Haptophyta	Prymnesiophyceae	Isochrysidales	<i>Emiliana</i>	1	4567
AMT-42	Dilution	Chlorophyta	Prasinophyceae	Clade VII	Undescribed	1	4568
AMT-42	Dilution	Chlorophyta	Prasinophyceae	Clade VIIA3	Undescribed	1	4569
AMT-43	Enrichment	Heterokontophyta	Novel class RCC853	Undescribed	Undescribed	1	4570
AMT-44	Enrichment	Cyanophyta	Cyanophyceae	Synechococcales	<i>Synechococcus</i>	1	4571
AMT-46	Dilution	Chlorophyta	Prasinophyceae	clade VIIB3	Undescribed	1	4572
AMT-46	Dilution	Chlorophyta	Prasinophyceae	clade VIIA4	Undescribed	1	4573
AMT-48	Cell sorting	Chlorophyta	Mamiellophyceae	Mamiellales	<i>Micromonas</i>	1	4574
AMT-49	Cell sorting	Chlorophyta	Mamiellophyceae	Mamiellales	<i>Micromonas</i>	2	LOST
AMT-50	Cell sorting	Haptophyta	Prymnesiophyceae	Prymnesiales	<i>Imantonia</i>	2	4575, 4576
AMT-52	Enrichment	Chlorophyta	Mamiellophyceae	Mamiellales	<i>Micromonas</i>	1	LOST
AMT-52	Cell sorting	Chlorophyta	Trebouxiophyceae	Unknown	Unknown	3	LOST
AMT-53	Enrichment	Haptophyta	Prymnesiophyceae	Phaeocystales	<i>Phaeocystis</i>	1	LOST
AMT-54	Cell sorting	Haptophyta	Prymnesiophyceae	Prymnesiales	<i>Imantonia</i>	1	4577
AMT-55	Cell sorting	Chlorophyta	Mamiellophyceae	Mamiellales	<i>Micromonas</i>	2	LOST
AMT-56	Cell sorting	Chlorophyta	Mamiellophyceae	Mamiellales	<i>Micromonas</i>	2	LOST
AMT-57	Enrichment	Haptophyta	Prymnesiophyceae	Prymnesiales	<i>Imantonia</i>	1	4578
AMT-58	Cell sorting	Haptophyta	Prymnesiophyceae	Phaeocystales	<i>Phaeocystis</i>	1	4579

560

561

562 Table 6 : Comparison of the taxonomic identity of the cultures obtained by single-cell flow
 563 cytometry sorting vs. dilution or enrichment.

Division	Class	Genus	Number of pre-cultures where genus isolated	Cell Sorting	Dilution	Enrichment
Bacteria	Cyanophyceae	<i>Synechococcus</i>	8			
Heterokontophyta	Pelagophyceae	<i>Pelagomonas</i>	8			
Chlorophyta	Mamiellophyceae	<i>Micromonas</i>	5			
Haptophyta	Prymnesiophyceae	<i>Phaeocystis</i>	5			
Chlorophyta	Prasinophyceae	clade VII	4			
Haptophyta	Prymnesiophyceae	<i>Emiliana</i>	3			
Haptophyta	Prymnesiophyceae	<i>Imantonia</i>	3			
Heterokontophyta	Pelagophyceae	Undescribed	2			
Chlorophyta	Trebouxiophyceae	Unknown	1			
Heterokontophyta	Bacillariophyceae	<i>Pseudo-nitzschia</i>	1			
Heterokontophyta	Chrysophyceae	<i>Spumella</i>	1			
Heterokontophyta	Unknown RCC853	Unknown	1			
Total number of genera isolated				8	5	6

564

565

566 **List of figures**

567 Figure 1 : Cruise track of Atlantic Meridional Transect # 24 in 2014 with the location of sampled
568 stations.

569 Figure 2: Effect of the concentration of BSA on the recovery of RCC cultures after sorting of
570 1000 cells of *Micromonas pusilla* RCC299 (A), *Isochrysis* sp. RCC90 (B), *Rhodomonas baltica*
571 RCC350 (C), and *Florenciella* sp. RCC1008 (D), and 500 cells of *Scrippsiella* sp. RCC4108
572 (E) into 1 mL of K medium. Cell concentration was followed by flow cytometry. Error bars
573 correspond to the standard error from three replicates.

574 Figure 3: Evolution of cell concentration for 1000 cells of *Micromonas pusilla* RCC299 (A),
575 *Isochrysis* sp. RCC90 (B), *Rhodomonas baltica* RCC350 (C), *Florenciella* sp. RCC1008 (D),
576 and 500 cells of *Scrippsiella* sp. RCC4108 (E) sorted into 1 mL K medium containing 0.01%
577 of BSA with and without addition of PNS three days after flow cytometric cell sorting (arrow
578 indicates PNS addition). Error bars correspond to the standard error from three replicates.

579 Figure 4: Concentration of pico- and nano-eukaryotes (A and B, respectively) from a natural
580 sample from the English Channel after flow cytometry sorting into four different media:
581 seawater from sampling site (SW), seawater from sampling site supplemented by nutrients from
582 K medium (SW+Nut), f/2 and K medium. Error bars correspond to the standard error from three
583 replicates.

584

585 [Supplementary material](#)

586

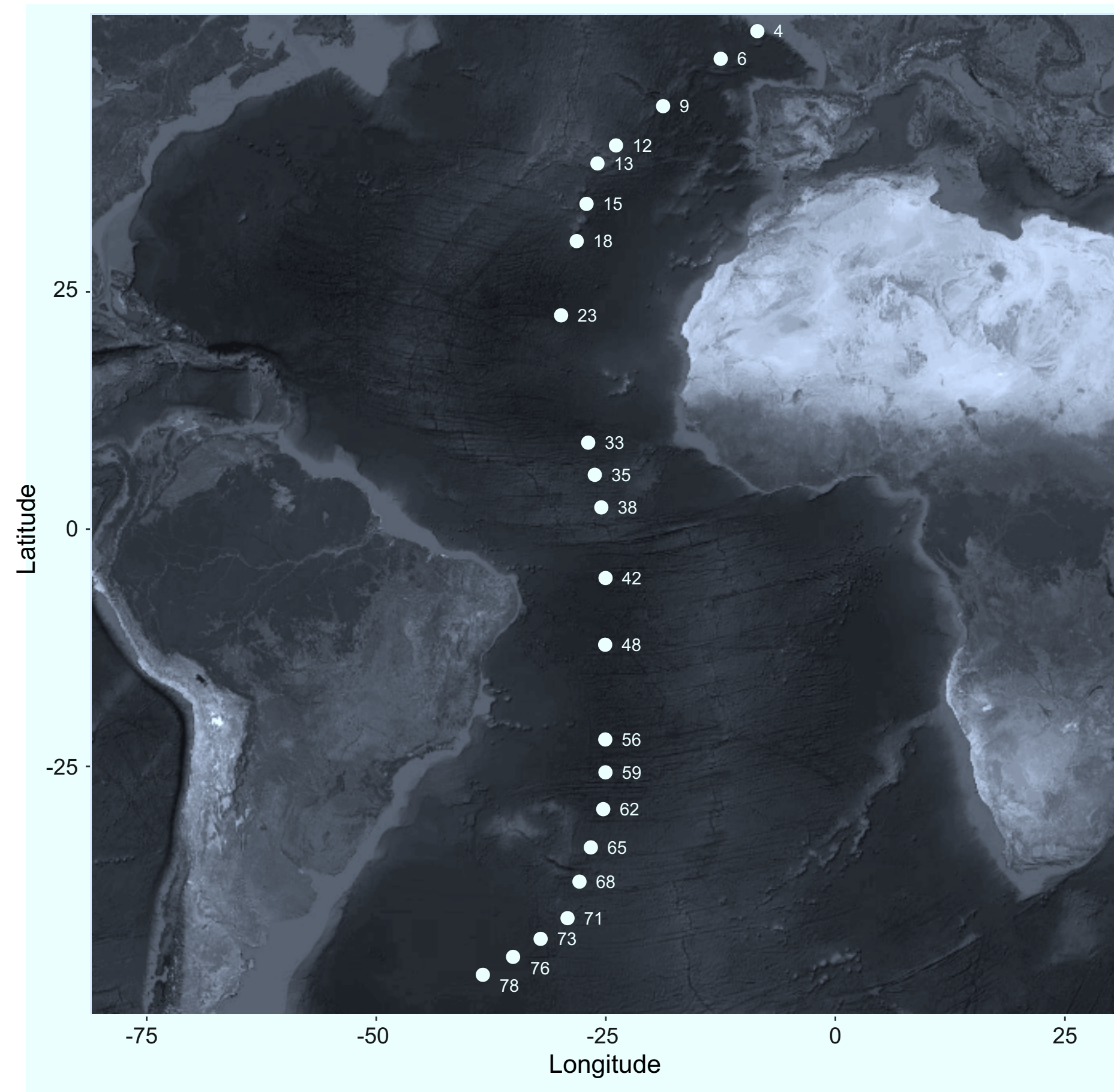


Fig. 1

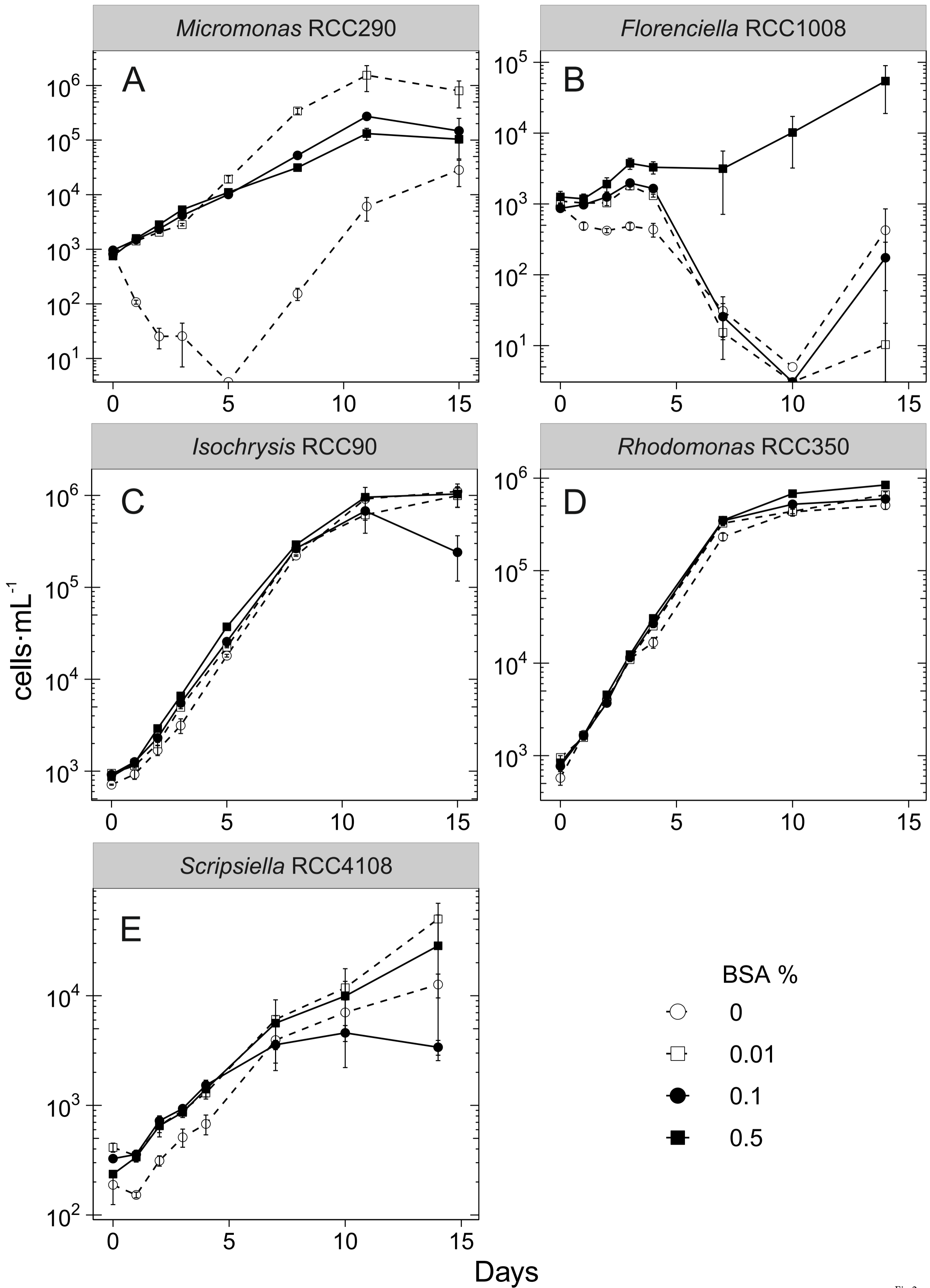


Fig 2

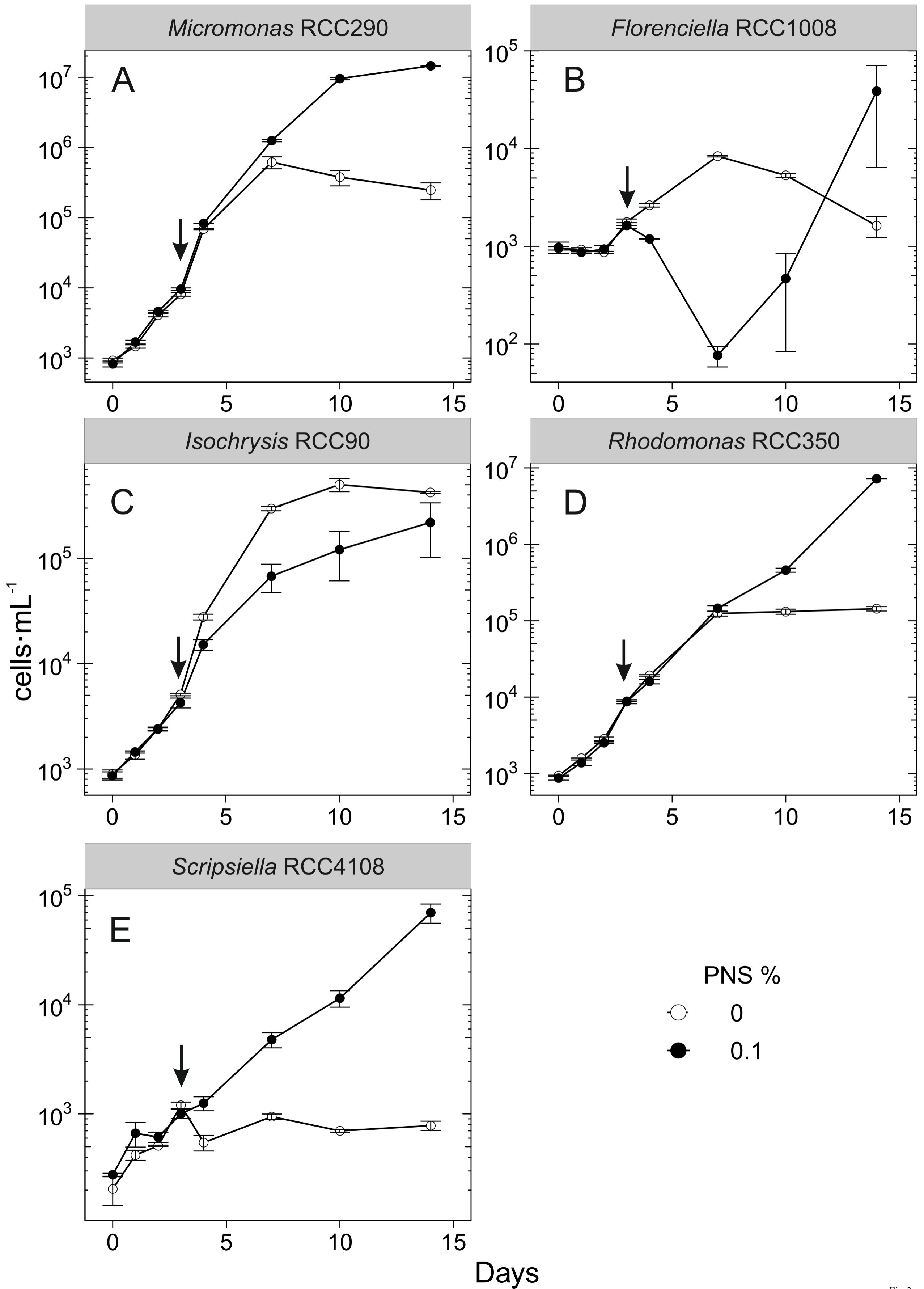
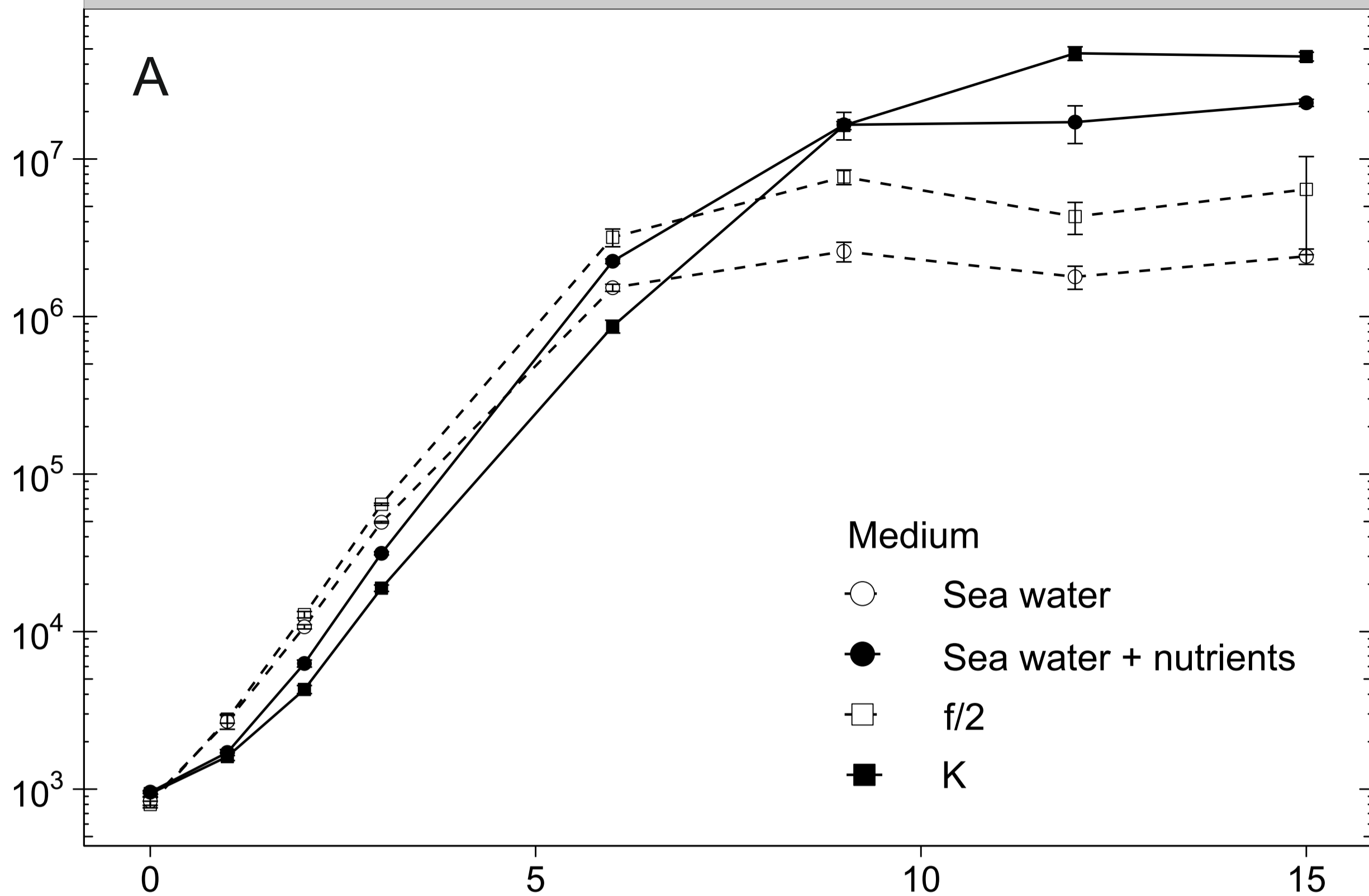


Fig 3

Picoeukaryotes

A



Nanoeukaryotes

B

