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

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Abstract

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

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

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

Introduction

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

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

Using ^{14}C-uptake, Rivkin et al. (1986) showed that the passage of cells through a flow cytometer induces physiological damage to phytoplankton cells that results in a decrease in growth rate during the first days following sorting. These authors associated cell damage to 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

Materials and methods

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

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

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

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 *Florenciella* 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 *Florenciella* (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, *Florenciella*, 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 *Florenciella* 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 Vaulot 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.

Acknowledgments

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
Micromonas pusilla RCC299	0%	0	0	0	0
	0.01%	9	9	9	9
	0.10%	4	4	4	4
	0.50%	5	5	5	5
Florenciella 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
Isochrysis 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
Rhodomonas baltica RCC350	0%	8	9	9	9
	0.01%	9	9	9	9
	0.10%	8	8	8	8
	0.50%	*9*	*9*	*9*	*9*
Scrippsiella 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	*Ostreococcus*	■	■	■	■
Chlorophyta	Mamiellophyceae	*Micromonas*		■	■	■
Chlorophyta	Mamiellophyceae	*Bathycoccus*			■	
Chlorophyta	Trebouxiophyceae	*Picochlorum*			■	
			Nanoeukaryotes			
Heterokontophyta	Coscinodiscophyceae	*Minidiscus*	■	■	■	■
Heterokontophyta	Coscinodiscophyceae	*Thalassiosira*	■	■	■	■
Haptophyta	Prymnesiophyceae	*Phaeocystis*	■	■	■	■
Heterokontophyta	Mediophyceae	*Minutocellus*	■	■		
Heterokontophyta	Mediophyceae	*Unknown*		■	■	
Heterokontophyta	Bacillariophyceae	*Cylindrotheca*	■			■

556

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	*Phaeocystis*	1	LOST
AMT-06	Enrichment	Cyanophyta	Cyanophyceae	Synechococcales	*Synechococcus*	1	LOST
AMT-08	Dilution	Heterokontophyta	Pelagophyceae	Pelagomonadales	*Pelagomonas*	1	4548
AMT-08	Cell sorting	Haptophyta	Prymnesiophyceae	Isochrysidales	*Emiliania*	1	4549
AMT-09	Dilution	Haptophyta	Prymnesiophyceae	Phaeocystales	*Phaeocystis*	1	LOST
AMT-11	Enrichment	Cyanophyta	Cyanophyceae	Synechococcales	*Synechococcus*	1	4550
AMT-12	Dilution	Heterokontophyta	Pelagophyceae	Pelagomonadales	*Pelagomonas*	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	*Synechococcus*	1	4554
AMT-14	Enrichment	Cyanophyta	Cyanophyceae	Synechococcales	*Synechococcus*	1	4555
AMT-16	Enrichment	Cyanophyta	Cyanophyceae	Synechococcales	*Synechococcus*	1	LOST
AMT-20	Dilution	Heterokontophyta	Pelagophyceae	Pelagomonadales	*Pelagomonas*	2	LOST
AMT-25	Enrichment	Cyanophyta	Cyanophyceae	Synechococcales	*Synechococcus*	1	4556
AMT-26	Dilution	Cyanophyta	Cyanophyceae	Synechococcales	*Synechococcus*	1	4557
AMT-27	Enrichment	Heterokontophyta	Chrysophyceae	Chromulinales	*Spumella*	1	4558
AMT-27	Dilution	Heterokontophyta	Pelagophyceae	Pelagomonadales	*Pelagomonas*	1	4559
AMT-28	Dilution	Heterokontophyta	Pelagophyceae	Pelagomonadales	*Pelagomonas*	1	LOST
AMT-30	Dilution	Bacillariophyta	Bacillariophyceae	Bacillariales	*Pseudo-nitzschia*	1	LOST
AMT-30	Cell sorting	Haptophyta	Prymnesiophyceae	Isochrysidales	*Emiliania*	1	4560
AMT-30	Cell sorting	Heterokontophyta	Pelagophyceae	Pelagomonadales	*Pelagomonas*	1	4561
AMT-32	Cell sorting	Heterokontophyta	Pelagophyceae	Pelagomonadales	New species 2	2	4562, 4563
AMT-32	Cell sorting	Heterokontophyta	Pelagophyceae	Pelagomonadales	*Pelagomonas*	1	4564
AMT-32	Dilution	Chlorophyta	Prasinophyceae	Clade VII	Unknown	1	4565
AMT-32	Cell sorting	Haptophyta	Prymnesiophyceae	Phaeocystales	*Phaeocystis*	1	LOST
AMT-36	Dilution	Heterokontophyta	Pelagophyceae	Pelagomonadales	*Pelagomonas*	1	4566
AMT-36	Cell sorting	Haptophyta	Prymnesiophyceae	Isochrysidales	*Emiliania*	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	*Synechococcus*	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	*Micromonas*	1	4574
AMT-49	Cell sorting	Chlorophyta	Mamiellophyceae	Mamiellales	*Micromonas*	2	LOST
AMT-50	Cell sorting	Haptophyta	Prymnesiophyceae	Prymnesiales	*Imantonia*	2	4575, 4576
AMT-52	Enrichment	Chlorophyta	Mamiellophyceae	Mamiellales	*Micromonas*	1	LOST
AMT-52	Cell sorting	Chlorophyta	Trebouxiophyceae	Unknown	Unknown	3	LOST
AMT-53	Enrichment	Haptophyta	Prymnesiophyceae	Phaeocystales	*Phaeocystis*	1	LOST
AMT-54	Cell sorting	Haptophyta	Prymnesiophyceae	Prymnesiales	*Imantonia*	1	4577
AMT-55	Cell sorting	Chlorophyta	Mamiellophyceae	Mamiellales	*Micromonas*	2	LOST
AMT-56	Cell sorting	Chlorophyta	Mamiellophyceae	Mamiellales	*Micromonas*	2	LOST
AMT-57	Enrichment	Haptophyta	Prymnesiophyceae	Prymnesiales	*Imantonia*	1	4578
AMT-58	Cell sorting	Haptophyta	Prymnesiophyceae	Phaeocystales	*Phaeocystis*	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	*Synechococcus*	8		■	■
Heterokontophyta	Pelagophyceae	*Pelagomonas*	8	■	■	
Chlorophyta	Mamiellophyceae	*Micromonas*	5	■		■
Haptophyta	Prymnesiophyceae	*Phaeocystis*	5	■		■
Chlorophyta	Prasinophyceae	clade VII	4	■	■	
Haptophyta	Prymnesiophyceae	*Emiliania*	3	■		
Haptophyta	Prymnesiophyceae	*Imantonia*	3	■		■
Heterokontophyta	Pelagophyceae	Undescribed	2	■		
Chlorophyta	Trebouxiophyceae	Unknown	1	■		
Heterokontophyta	Bacillariophyceae	*Pseudo-nitzschia*	1		■	
Heterokontophyta	Chrysophyceae	*Spumella*	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

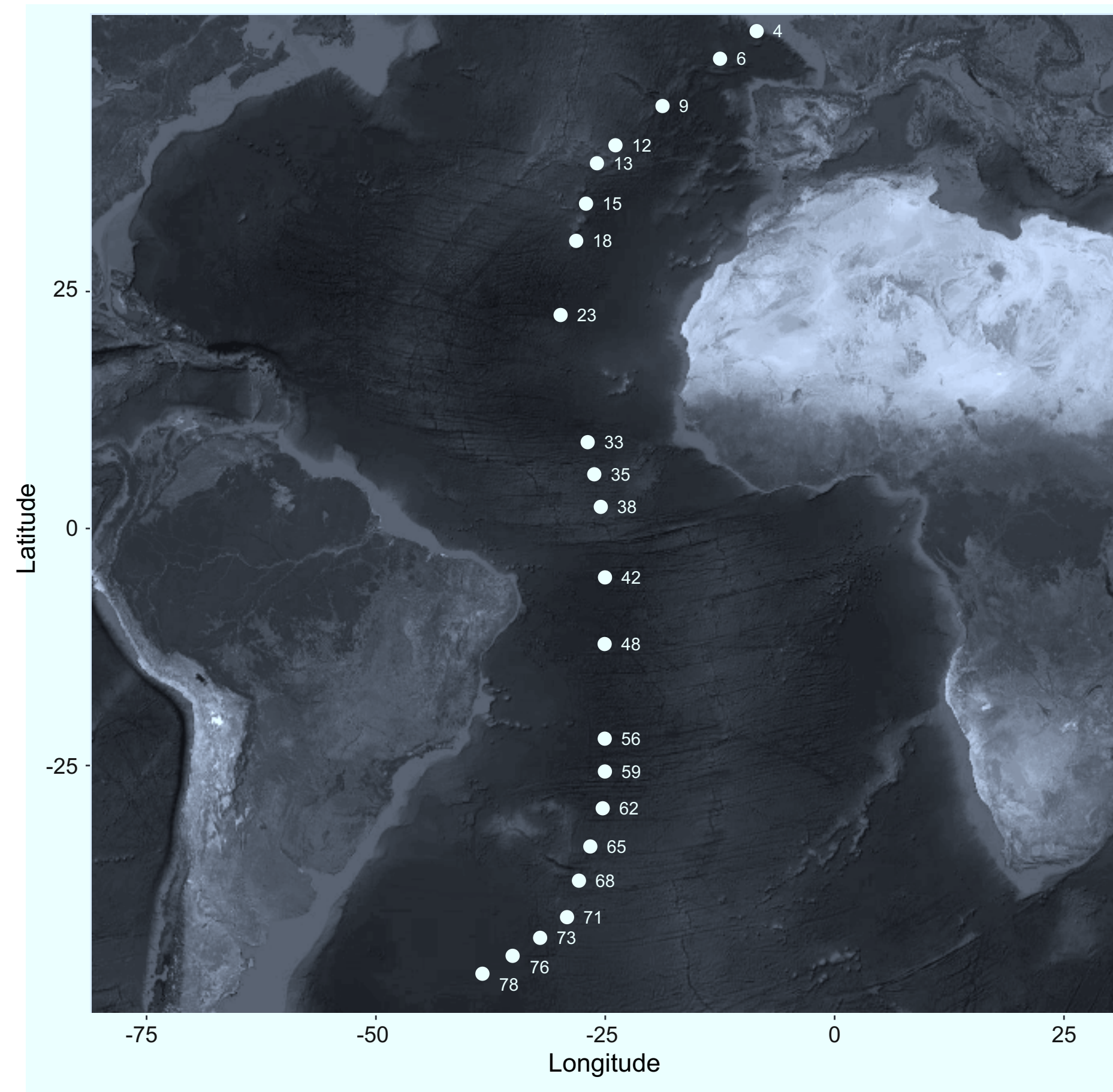583 replicates.
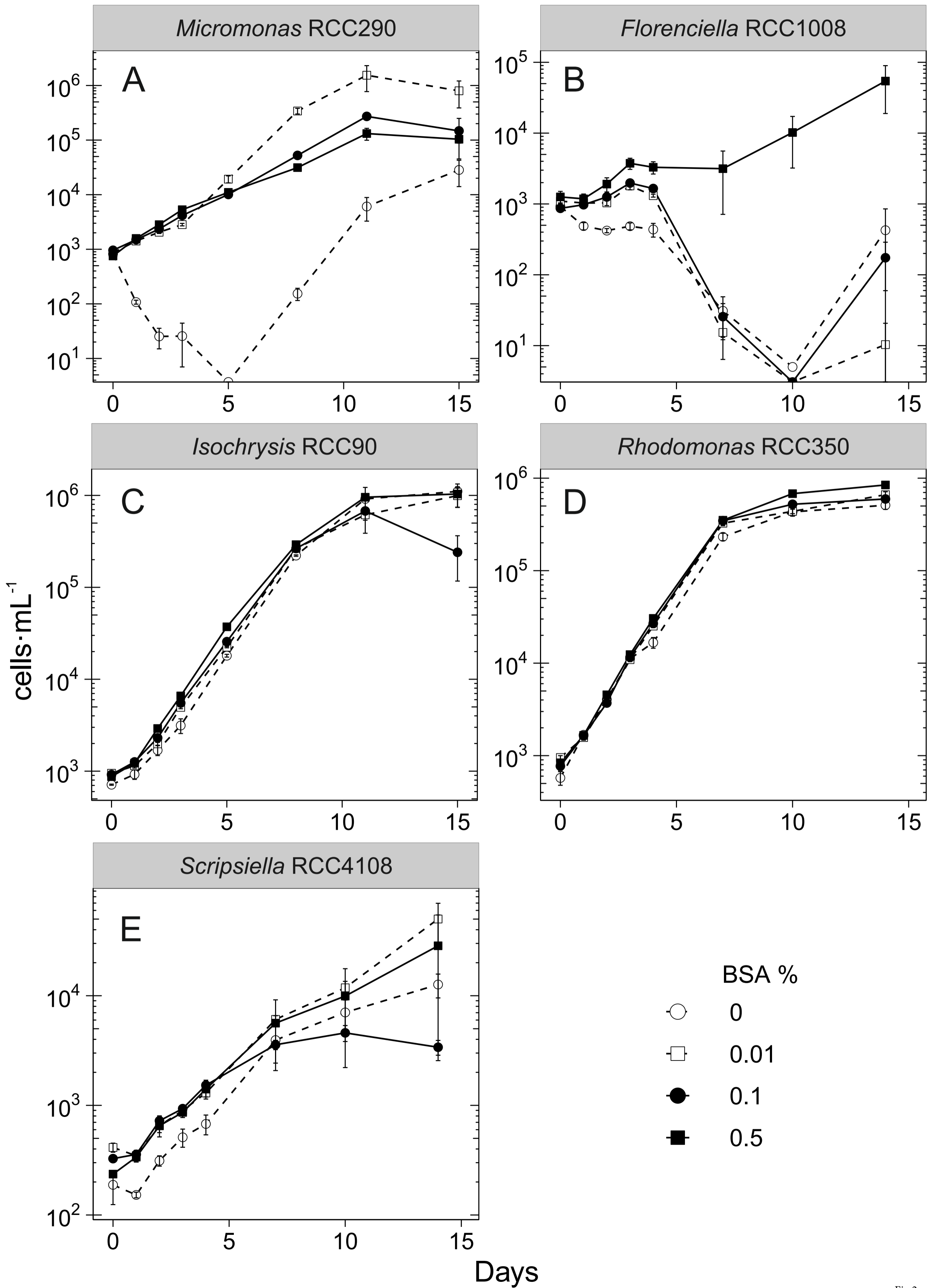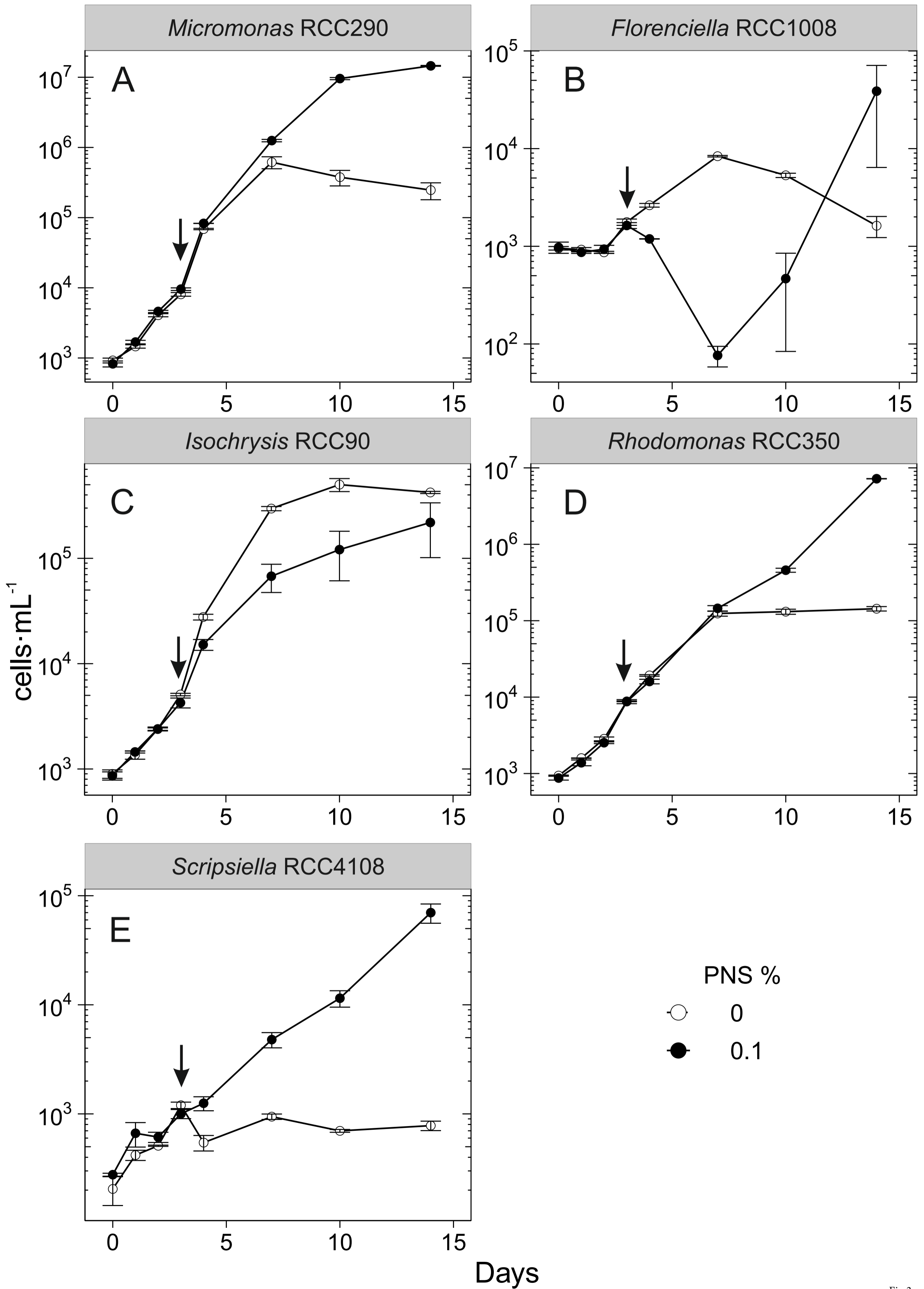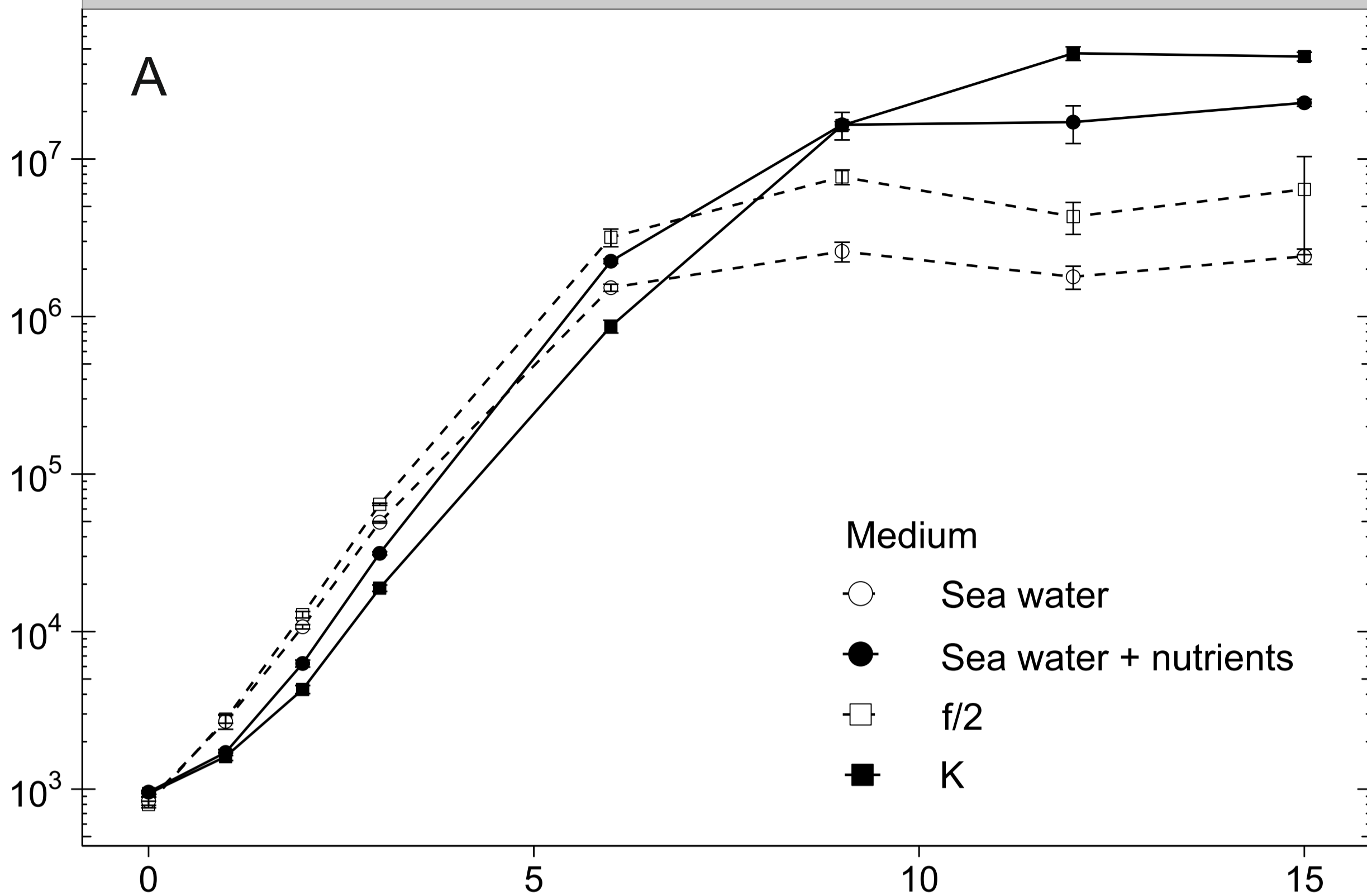
584

585   **Supplementary material**

586

Fig. 1

Fig 2

Fig 3

Picoeukaryotes

Nanoeukaryotes

cells·mL^{-1}

Days

Medium
- ◯ Sea water
- ● Sea water + nutrients
- ☐ f/2
- ■ K

A

B

Fig 4