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IMPROVEMENT OF PHYTOPLANKTON CULTURE ISOLATION USING SINGLE CELL SORTING BY FLOW CYTOMETRY[1]

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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 in the culture medium into which cells were sorted drastically improved the success of initiation of pico- and nano-eukaryotic phytoplankton strains. Adding a mixture of antibiotics (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 cruise. We anticipate that these improvements will be useful to clone or purify existing cultures and to isolate novel cultures from oceanic samples.

Key index words: **antibiotics; BSA; flow cytometry; microalgal cultures; nanoeukaryotes; phytoplankton; picoeukaryotes; single cell sorting**

***Abbreviations*: AMT, Atlantic meridional transect; BSA, bovine serum albumin; DCM, deep chlorophyll maximum; PCR, polymerase chain reaction; PNS, Penicillin-Neomycin-Streptomycin; RCC, Roscoff culture collection**

In the context of the rapid development of metabarcoding and metagenomics approaches to characterize marine microbial communities (Sunagawa et al. 2015, de Vargas 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 data sets 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. (1987) did not find any physical damage or cell lysis due to fluidics or the laser beam, but reported a lag in growth for up to 48 h after sorting, suggesting physiological damage. Sieracki et al. (2005) found higher recoveries when sorting single cells into 24- rather than 96-well plates. However, while recovery of sorted *Isochrysis*, *Rhodomonas*, *Scrippsiella,* and *Thalassiosira* varied from 22% to 99%, single-cell sorting failed for the picoplanktonic green microalga *Micromonas*. Sinigalliano et al. (2010) found that the trauma due to automated cell sorting was not a limiting factor for obtaining unialgal dinoflagellate cultures compared to manual micropipetting. These reports emphasize the large taxon-specific variability in cell recovery after flow cytometry sorting.

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

In the present study we developed and tested a new protocol for strain isolation based on single cell sorting in culture medium supplemented with bovine serum albumin (BSA) followed by antibiotic addition after a few days. This protocol was developed on cultures from the RCC and then tested on a fresh sample from the English Channel off Roscoff and on enrichment cultures obtained during an Atlantic meridional transect (AMT) cruise. In the latter case, we compared flow cytometry sorting with more classical strain isolation approaches (serial dilution and enrichment).

MATERIALS AND METHODS

Cultures. Five strains from different taxonomic groups were obtained from the 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 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 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; 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, USA) 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 and Side Scatter with the minimum threshold value. Sorting was performed in "single cell" mode using 20 PSI as sheath pressure. An Accuri C6 (Becton Dickinson) 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 $\mu L \cdot min^{-1}$.

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

Effect of BSA concentration on cultures. RCC cultures were sorted into 1 mL of K medium either without BSA or with BSA (Ref A7030; Sigma-Aldrich, Saint-Quentin Fallavier, France) at concentrations of 0.01%, 0.1% or 0.5%. Typically one 48-well plate (CytoOne; Starlab, Orsay, France) was used for each culture: 1,000 cells (500 for *Scripsiella*) were sorted into 3 wells and 1 cell into each of the remaining 45 wells. Using the Accuri C6, cell concentration was monitored over 2 weeks for the wells containing 500 or 1,000 cells after sorting. Wells with 1 cell were monitored by epifluorescence microscopy between days 3 and 14 following sorting.

Effect of antibiotic addition. RCC cultures were sorted into 48-well plates containing 1 mL of K medium supplemented with 0.01% BSA. For each culture 500 (*Scripsiella*) or 1,000 (other strains) cells were sorted into 6 wells containing 1 mL of K medium. Three days after sorting, a mixture of Penicillin, Neomycin, and Streptomycin (PNS, Ref P4083; Sigma) was added at a final concentration of 0.1% to 3 of the wells. Cell concentration was subsequently monitored by flow cytometry for 2 weeks.

Effect of medium on sorting for a natural sample. Surface water from the English Channel (see above) was filtered through a 50 μm nylon mesh in order to remove larger cells that can clog the flow cell of the cytometer. Four media supplemented with BSA 0.01% were tested: K (Keller et al. 1987), f/2 (Ref G9903; Sigma, Guillard and Ryther 1962), water from the sampling site supplemented with K medium nutrients (SW+Nut) and not supplemented (SW). All media were filter-sterilized through a 0.2 μm filter just before sorting. Two 48-well plates containing 1 mL of medium per well were used per media tested, one for pico- and one for nano-phytoplankton. The first 3 wells were used to sort 1,000 cells for the pico-phytoplankton and 500 cells for the nano-phytoplankton, respectively. One cell was sorted in each of the remaining 45 wells. Three days after sorting, PNS 0.1% (final concentration) was added into all wells with 1,000 or 500 sorted cells and cell concentration was monitored over 2 weeks by flow cytometry (Fig. 2). Wells with 1 sorted cell were monitored by epifluorescence microscopy every 3 d for 2 weeks following sorting. When live cells were observed in a well, PNS 0.1% (final concentration) was added. Ten diatom strains isolated during this experiment were kept in culture and incorporated into the RCC.

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

For flow cytometry sorting, 24 AMT pre-cultures were sorted in 1 mL of K medium with 0.01% BSA into 48-well plates (Table 1). Half a plate was used for each population selected: 1,000 cells were sorted into the first two wells, 100 into the next two wells as controls and 1 cell into the remaining 20 wells. Plates were incubated in a 12:12 light:dark cycle for 10 d. Wells were then screened by inverted epifluorescence microscopy to detect the presence of photosynthetic cells. When live cells were observed in the wells with 1 sorted cell, they were transferred into a tube containing 5 mL of K medium supplemented with a final concentration of 0.1% PNS.

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

					Sorted wells (1 cell per well)				Dilution wells (10 cells per well)		
Precultures	Sampling date	Station	Depth	Enriched	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

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

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

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

All AMT strains and 10 strains isolated from the English Channel have been deposited in the RCC (RCC4548 to RCC4579 and RCC4657 to RCC4666, respectively) and their sequences deposited in GenBank under accession numbers KX014627–KX014660.

RESULTS

Effect of BSA addition. We first tested the effect of adding BSA at different concentrations (0%, 0.1%, 0.01% and 0.05%) to the culture medium into

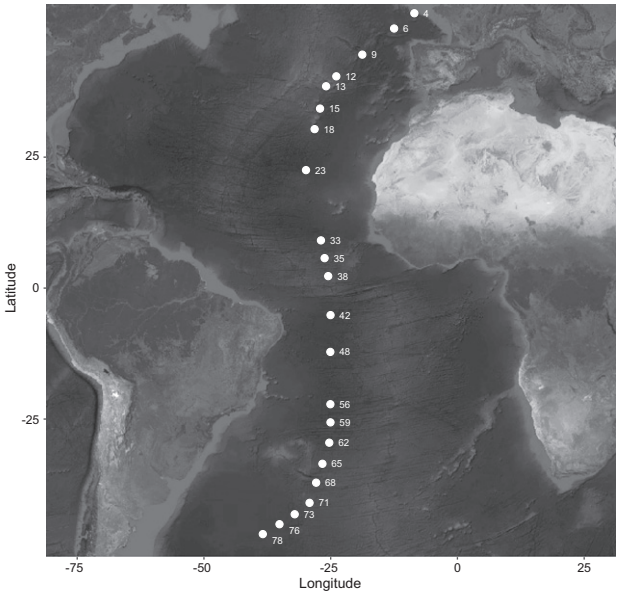

FIG. 1. Cruise track of Atlantic Meridional Transect # 24 in 2014 with the location of sampled stations.

which cells were sorted using 5 strains of eukaryotes (Fig. 2 and Table 2).

For the picoplanktonic strain *M. pusilla* (Mamiellophyceae) RCC299, we observed a rapid decline in cell concentration for the 3 wells with 1,000 sorted cells in the absence of BSA and no live cells were detected by flow cytometry after 5 d of incubation (Fig. 2A). After 8 d, cells were detected in 2 of the 3 wells. In presence of BSA, no lag phase or decline in cell numbers were observed. Initial growth rates were identical for the three BSA concentrations tested, but after 5 d of incubation better growth was obtained at the lowest BSA concentration (0.01%). Epifluorescence microscope observations of wells into which 1 cell was sorted (Table 2) revealed the absence of cells in the absence of BSA, 100% recovery with 0.01% BSA, and 50%–60% recovery with 0.1% and 0.5%, respectively.

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

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

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

Effect of antibiotic treatment. For cultures sorted into medium with 0.01% BSA, growth stopped after 4 d for *Scrippsiella* and 7 d for *Micromonas* and *Rhodomonas* in the absence of the antibiotic cocktail (PNS), while cultures continued to grow when PNS was added (Fig. 3). *Isochrysis* had a higher growth rate in the absence of PNS reaching stationary phase earlier than with PNS, although after 2 weeks cell yield was similar. For *Florenciella*, addition of PNS resulted in significant cell loss, but cultures recovered 1 week after sorting to reach a higher final density than in the absence of PNS (Fig. 3).

Recovery and isolation of phytoplankton sorted from a fresh seawater sample. Wells into which 1,000 picoeukaryote cells were sorted had similar growth rates irrespective of the medium, but final cell concentration was lower in f/2 and unamended seawater (Fig. 4A). Single picoeukaryote sorted cells began to grow in some wells after 3 d and the percentage of wells with detectable growth after 2 weeks was highest using f/2 medium, reaching almost 100%, followed by amended SW, SW and K medium for which recovery was only 50% (Table 3). After 9–15 d, ~70% of wells containing f/2 or SW with nutrients and only 30% for SW or K medium were dense enough to allow transfer to tubes containing 4 mL of K medium. All strains obtained from single cell sorting (Table 4) were Chlorophyta representing two classes, the Mamiellophyceae (*Micromonas*, *Ostreococcus,* and *Bathycoccus*) and the Trebouxiophyceae (*Picochlorum*). The highest number of different genera was obtained using f/2 medium and the lowest in unamended seawater.

No difference in growth rate was observed after sorting 500 nanoeukaryote cells in different media, but after 6 d, cell numbers were significantly higher in wells with f/2 and SW+Nut (Fig. 4B). Heterogeneity in cell numbers was observed among the three wells in all media. As for picoeukaryotes, cells were detected in some single-cell wells after only 3 d (Table 3) and the number of wells with detectable

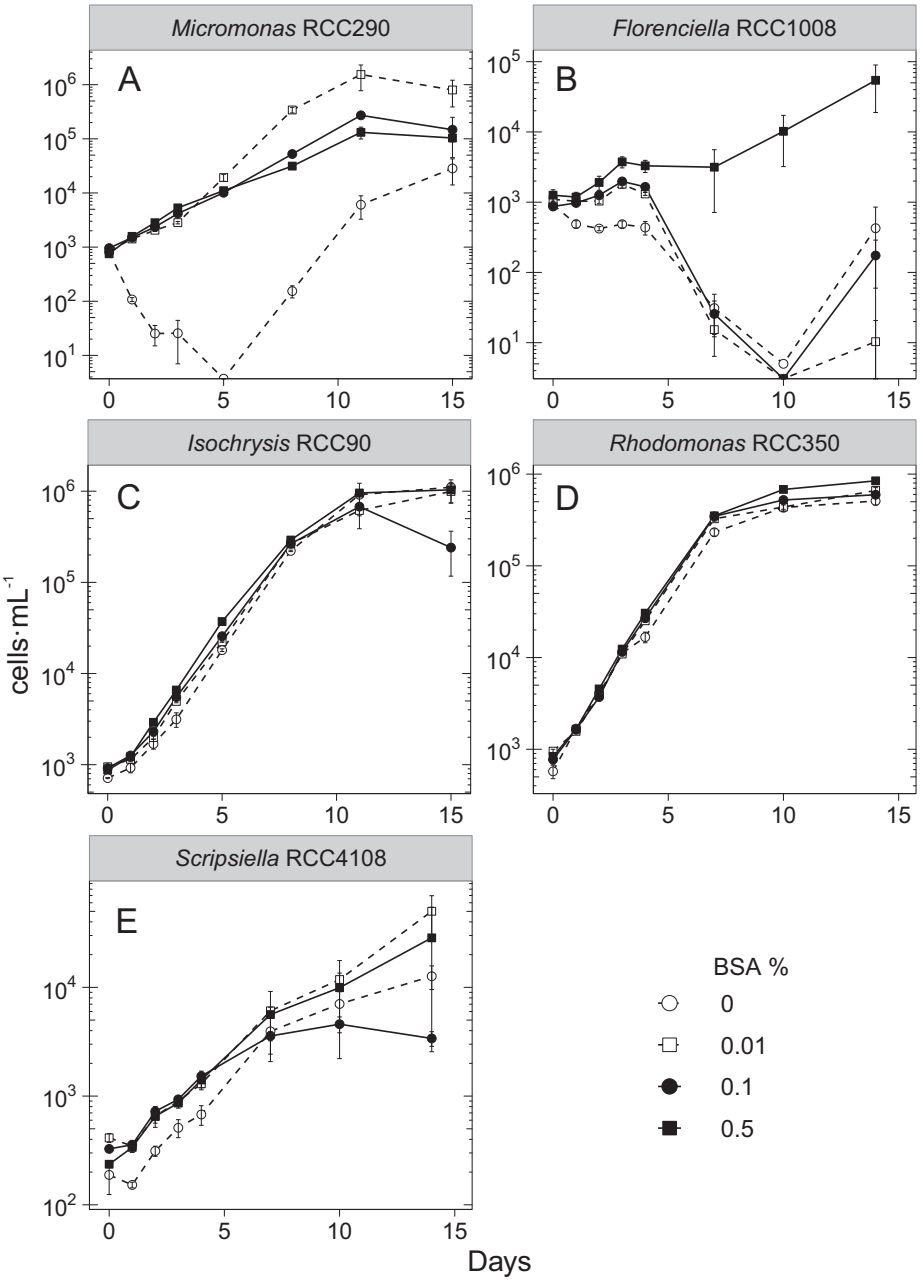

FIG. 2. Effect of the concentration of BSA on the recovery of RCC cultures after sorting of 1,000 cells of *Micromonas pusilla* RCC299 (A), *Isochrysis* sp. RCC90 (B), *Rhodomonas baltica* RCC350 (C), and *Florenciella* sp. RCC1008 (D), and 500 cells of *Scrippsiella* sp. RCC4108 (E) into 1 mL of K medium. Cell concentration was followed by flow cytometry. Error bars correspond to the standard error from three replicates.

growth did not increase beyond day 9. More than half of the wells yielded growth and this proportion was highest with SW (71%) and lowest with K, as was the case for picoeukaryotes. Strains obtained from single cell sorting were mostly small- (*Minidiscus*, *Minutocellus*) or medium-sized (*Thalassiosira*, *Skeletonema*, *Cylindrotheca*) diatoms, or the common prymnesiophyte *Phaeocystis* (Table 4). The largest variety of genera was obtained using amended SW and the lowest using K, as was the case for picoeukaryotes.

Isolation of phytoplankton strains from oceanic enrichments. During AMT cruise #24, from the UK to the Falkland Islands, two depths (surface and DCM) were sampled at a range of stations in the Atlantic Ocean from temperate to equatorial environments. During the cruise, pre-cultures were obtained by adding L1 medium to natural samples and these pre-cultures were promptly brought back to the laboratory at the end of the cruise. Single-cell flow cytometry sorting was applied to 24 pre-cultures using K medium with 0.01% BSA, yielding a total of 700 wells (Table 1). After 10 d of incubation, phytoplankton growth was observed in 278 (40%) wells. The percentage of wells with growth varied with the pre-culture, ranging from 0% to 100% (Table 1). Positive wells were transferred into tubes containing 5 mL of K medium supplemented with a final concentration of 0.01% PNS.

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

Culture	BSA%	Days 3–5	Days 7–8	Days 10–11	Day 14
Micromonas pusilla	0%	0	0	0	0
RCC299	0.01%	9	9	9	9
	0.10%	4	4	4	4
	0.50%	5	5	5	5
Florenciella sp.	0%	0	0	0	1
RCC1008	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	0%	8	9	9	9
RCC350	0.01%	9	9	9	9
	0.10%	8	8	8	8
	0.50%	9	9	9	9
Scrippsiella sp.	0%	5	5	5	5
RCC4108	0.01%	0	2	4	4
	0.10%	3	3	3	3
	0.50%	2	3	3	3

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

Genetic characterization of the AMT strains using the 18S rRNA gene revealed that many cultures belonged to the same genus. For cultures obtained either by flow cytometry sorting or dilution, all tubes originating from the same pre-culture and obtained with the same technique yielded identical sequences. For example, the 36 positive wells originating from pre-culture 04 (Table 1) were all affiliated to *Phaeocystis*. When we obtained the same affiliation with the 3 techniques of isolation, we decided to prioritize maintenance of the cultures isolated by flow cytometry sorting because these strains were more likely to be clonal. In total, 31 representative cultures were deposited to the RCC, corresponding to 11 different genera (Table 5). The highest diversity was obtained with cell sorting, but some taxa were only obtained by dilution or enrichment (Table 6). The genera isolated from the largest number (8) of pre-cultures (Table 6) belonged to Pelagophyceae (*Pelagomonas*) and Cyanophyceae (*Synechococcus*), although the latter were only isolated by dilution or enrichment since only eukaryotes were targeted by sorting. Strains isolated from more than one pre-culture belonged to the Chlorophyta (*Micromonas*, prasinophyte clade VII), Prymnesiophyceae (*Phaeocystis*, *Emiliania*, *Isochrysis*) and Pelagophyceae. Finally some strains were only isolated from a single pre-culture. This was in particular the case for RCC4570, the 18S rRNA sequence of which was affiliated to the Heterokontophyta.

DISCUSSION

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

While trying different additions to sorting medium, we discovered that BSA had a spectacular impact on cell recovery. The reason for the positive effect of BSA is unclear. Albumin is a major protein from bovine or human serum, with multifunctional properties and is widely used for mammalian cell cultures (Francis 2010). It is well-known as a strong antioxidant and has also been proved to avoid attachment of cells onto plastic walls (Fletcher 1976). BSA is also often added when preparing protoplasts from macroalgae (e.g., Bodian et al. 2013). Unfortunately, no detailed study on its effect or role on cell culture has been published. Of the five strains tested in the present study belonging to a range of phylogenetic groups, the nanoplanktonic strains *Isochrysis*, *Rhodomonas,* and *Scrippsiella* recovered well after sorting and BSA did not seem to have a significant effect except in the case of *Scrippsiella* for which the lag phase was reduced with BSA addition (Fig. 2, C–E; Table 2). In contrast, the picoplanktonic species *Micromonas* and *Florenciella* could not recover after sorting without the addition of BSA (Fig. 2, A and B;

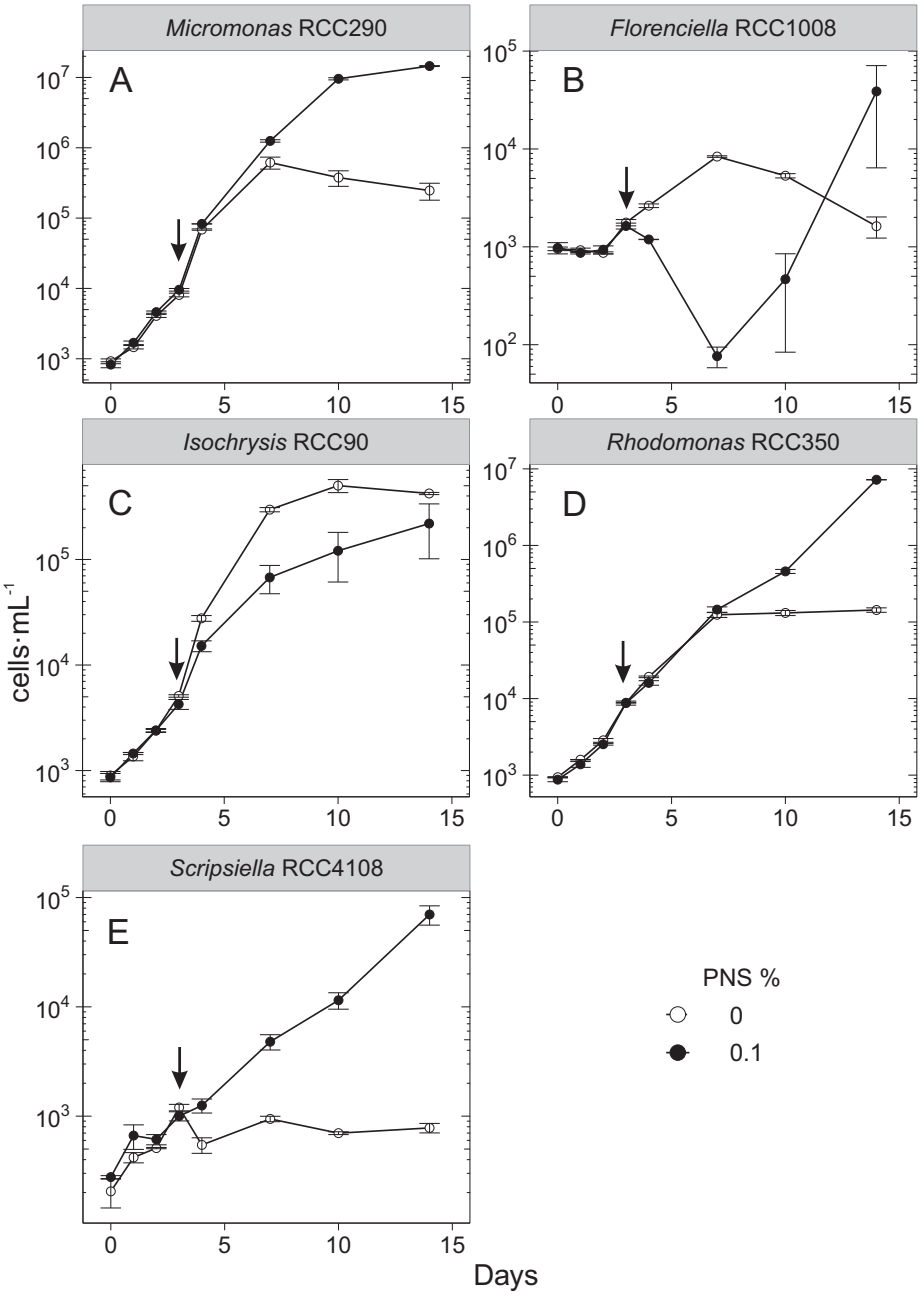

FIG. 3. Evolution of cell concentration for 1,000 cells of *Micromonas pusilla* RCC299 (A), *Isochrysis* sp. RCC90 (B), *Rhodomonas baltica* RCC350 (C), *Florenciella* sp. RCC1008 (D), and 500 cells of *Scrippsiella* sp. RCC4108 (E) sorted into 1 mL K medium containing 0.01% of BSA with and without addition of PNS 3 d after flow cytometric cell sorting (arrow indicates PNS addition). Error bars correspond to the standard error from three replicates.

Table 2), confirming the report of Sieracki et al. (2005) who did not obtain any growth of *Micromonas* when sorting 1 cell per well. With addition of BSA, we obtained 100% regrowth of *Micromonas* (Table 2). Interestingly, the optimum BSA concentration varied with the species. While concentrations below 0.5% had no effect on *Florenciella* (Fig. 2; Table 2), 0.01% was optimum for *Micromonas* (Fig. 2; Table 2). This suggests that it will be necessary to test different BSA concentrations when dealing with specific cultures or natural populations. In particular, *Florenciella*, which apparently requires a high BSA concentration to facilitate regrowth after single cell sorting, has rarely been isolated into culture: there are only 11 strains isolated from 3 cruises available in culture collections (all in the RCC).

When using flow cytometry to sort phytoplankton cells from natural samples, heterotrophic bacteria

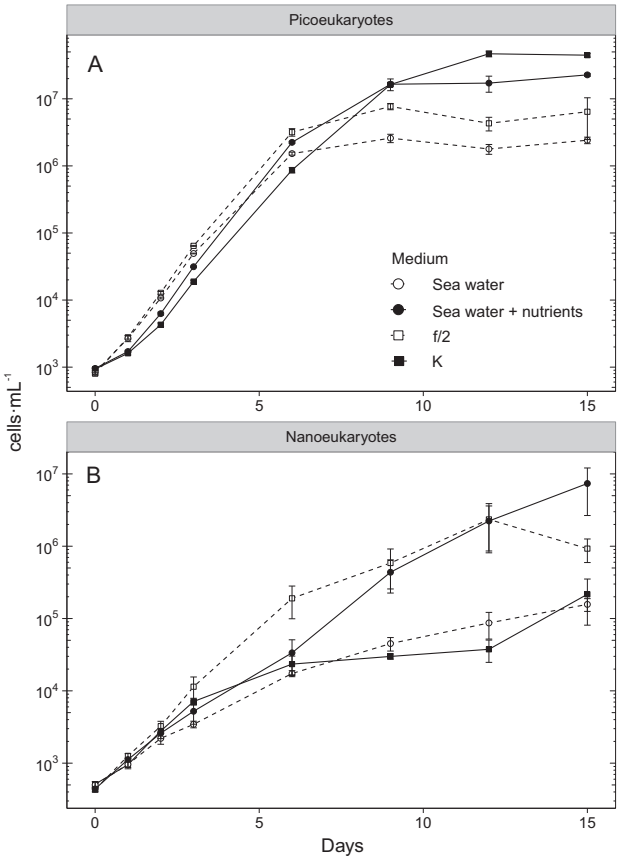

FIG. 4. Concentration of pico- and nano-eukaryotes (A and B, respectively) from a natural sample from the English Channel after flow cytometry sorting into four different media: seawater from sampling site (SW), seawater from sampling site supplemented by nutrients from K medium (SW+Nut), f/2 and K medium.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

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

		Day				
Population	Medium	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%

are also likely to be sorted since they often outnumber phototrophic cells by an order of magnitude. Although flow cytometry has been used to produce axenic cultures (Sensen et al. 1993), it is difficult to completely remove associated bacteria, especially those that adhere to the surface of microalgal cells (Surek and Melkonian 2004), and antibiotics are often necessary to obtain axenic cultures (Cottrell and Suttle 1993). Even when axenic cultures are not targeted, antibiotic treatment may be critical to reduce competition for nutrients from bacteria. This was apparently the case for *Micromonas*, *Rhodomonas,* and *Scrippsiella* which stopped growing after a few days in the absence of antibiotics, while growth continued when PNS was added (Fig. 3, A, D, E). The case of *Florenciella* was paradoxical. Addition of PNS led to an initial decrease in cell concentration which then recovered after day 7, while in the absence of PNS initial growth was continuous but final cell density was ten times lower. This could be explained by *Florenciella* being mixotrophic, as reported for field populations (Frias-Lopez et al. 2009). The initial decrease in the population when antibiotics were added could therefore be due to the decrease in bacterial prey density, followed by a switch to autotrophic nutrition inducing regrowth.

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

Our protocol proved to be efficient for obtaining clonal strains from pre-cultures established during an Atlantic cruise. In particular, we repeatedly obtained strains of *Pelagomonas* and prasinophyte clade VII, both of which are typically isolated from oceanic tropical and sub-tropical waters (Le Gall et al. 2008). We obtained strains of several taxa that,

TABLE 4. Comparison of the taxonomic identity of cultures isolated by single cell flow cytometry sorting of picoeukaryotes and nanoeukaryotes into four different media of a natural sample from the English Channel off Roscoff taken in May 2015. Black rectangles correspond to the conditions that allowed the isolation of a given genus.

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*	■			■

TABLE 5. List of cultures isolated from the AMT transect by single-cell flow cytometry sorting, 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	Undescribed	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

TABLE 6. Comparison of the taxonomic identity of the cultures obtained by single-cell flow cytometry sorting versus dilution or enrichment. Black rectangles correspond to the conditions that allowed the isolation of a given genus.

Division	Class	Genus	Number of precultures 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

to our knowledge, have not previously been cultured. Within prasinophyte clade VII, we obtained for the first time a representative of clade VIIB3 (Lopes dos Santos et al., 2016). In addition, three Pelagophyceae cultures corresponded to undescribed taxa. The 18S rRNA sequences of two Pelagophyceae strains from the tropical Atlantic (RCC4562 and RCC4563) matched with 100% identity the sequence of RCC1024, previously isolated from the South East Pacific (Le Gall et al. 2008). These strains are also related to the endosymbiont of the tropical dinoflagellate *Amphisolenia bidentata* (Daugbjerg et al. 2013). Strain RCC4552, isolated from the temperate North Atlantic, had a sequence only 98.7% similar to any other GenBank sequence and probably corresponds to a novel species. Its sequence was most closely related to RCC2505, isolated from polar waters (Balzano et al. 2012). The 18S rRNA sequence of RCC4570, a stramenopile strain obtained by dilution, was related to two strains (RCC853 and RCC862) that had been previously isolated from the South East Pacific (Le Gall et al. 2008), but unfortunately lost since. This group of sequences suggests the existence of a novel algal class, called MOCH-5 by Massana et al. (2014). As evident from Table 1, the success of the three approaches we used (sorting, dilution, enrichment) was highly variable. In several cases only one technique was successful for a given pre-culture: for example, strains were obtained by dilution but not sorting for pre-cultures 26, 27 and 42, whereas the opposite result was obtained for pre-cultures 54 and 55.

The novel protocol proposed for culture isolation using single-cell flow cytometry sorting has several advantages compared to other techniques for culture isolation. It is much more rapid than serial dilution. A 48-well plate can be sorted in less than 1 min. Results are obtained in less than 10 d, compared to at least 3 weeks for serial dilution. More importantly, specific populations can be targeted. For example, many of our AMT serial dilution wells produced untargeted *Synechococcus* cultures, while sorting targeted only eukaryotes. Flow cytometry sorting is best done on fresh samples since the diversity of the taxa recovered is high and more representative of the initial diversity, as demonstrated for an English Channel sample obtained near our laboratory from which we isolated at least 10 different taxa. However, this requires the availability of a bulky sorting flow cytometer near the sampling site or on the cruise. An alternative strategy for large scale cruises is to enrich samples to obtain pre-cultures. This can be done many different ways, without or with filtration in order to select specific size classes, without or with tangential flow filtration which allows concentration of samples, or with a range of different media and additives, such as germanium dioxide to prevent diatom growth (Vaulot et al. 2004, Le Gall et al. 2008, Balzano et al. 2012). In such cases, since there can be a long delay before the pre-cultures can be sorted, each pre-culture usually becomes dominated by one or two species. Single-cell sorting allows rapid purification and cloning of these pre-cultures as demonstrated for the AMT cruise. Since diversity is typically rather low for a given pre-culture, a relevant strategy to obtain different taxa in culture is to apply sorting to a large number of pre-cultures originating from different stations and depths (Table 5).

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

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