An improved protocol for flow cytometry analysis of phytoplankton cultures and natural samples

Dominique MARIE[(1,2)], Fabienne RIGAUT-JALABERT[(1,2)] and Daniel VAULOT[(1,2)]

[(1)]Sorbonne Universités UPMC (PARIS 06), UMR 7144 and FR2424, Station Biologique de Roscoff, Place Georges Teissier, 29688 Roscoff, FRANCE

[(2)] CNRS, UMR 7144 and FR2424, Station Biologique de Roscoff, Place Georges Teissier, 29688 Roscoff, FRANCE

ABSTRACT

Preservation of cells, choice of fixative, storage and thawing conditions are recurrent issues for the analysis of phytoplankton by flow cytometry. We examined the effects of addition of the surfactant Pluronic F68 to glutaraldehyde-fixed photosynthetic organisms in cultures and natural samples. In particular, we examined cell losses and modifications of side scatter (a proxy of cell size) and fluorescence of natural pigments. We found that different marine phytoplankton species react differently to the action of Pluronic F68. In particular, photosynthetic prokaryotes are less sensitive than eukaryotes. Observed cell losses may result from cell lysis or from cell adhesion to the walls of plastic tubes that are commonly used for flow cytometry analysis. The addition of the surfactant, Pluronic F68, has a positive effect on cells for long term storage. We recommend to modify current protocols for preservation of natural marine planktonic samples, by fixing them with glutaraldehyde 0.25% (final concentration) and adding Pluronic F68 at a final concentration of 0.01% in the samples before preservation. Pluronic F68 also appears effective for preserving samples without fixation for subsequent sorting, e.g. for molecular biology analyses.

INTRODUCTION

Enumeration of bacteria and phytoplankton both in cultures and natural samples has been one of the major applications of flow cytometry in oceanography during the last 3 decades, (Li et *al*., 1995; Veldhuis & Kraay, 2000; Gasol & del Giorgio, 2000). Photosynthetic plankton is composed by prokaryotes and eukaryotes that exhibit autofluorescence from pigments, in particular phycoerythrin and chlorophyll. Although flow cytometers can be used on cruise vessels and *in situ* systems have been developed (Campbell et *al*., 2010, Dubelaar et *al*., 1999, 2004, Olson et *al*. 2003), it is still often necessary to preserve samples for delayed analysis. For other applications, such as microscopy, lugol, formalin (that contains methanol), and alcohols are used for sample preservation. However, these chemicals drastically affect the fluorescence of natural pigments that is required to distinguish between phytoplanktonic and heterotrophic cells; therefore they cannot be used for flow cytometry (Marie et *al.*, 1999). Phytoplankton samples are commonly preserved using aldehydes such as glutaraldehyde or formaldehyde, because they are efficient at preserving both cellular structures and pigment autofluorescence (Vaulot et *al*., 1989; Lepesteur et *al*., 1993; Sato et *al*. 2006). Lepesteur et *al*. (1993) reported that there is no universal freezing process and they suggested that for mixed species populations, it is best to preserve aliquots by 3 methods: 1) rapid freezing with no addition of chemical to analyze fluorescence and size of algae, 2) slow freezing with glutaraldehyde fixation for cyanobacteria samples, and 3) same as the previous method but with addition of glycerol for cell counts (although they recorded large cell loss). However, they remarked that this is not always realistic.

Vaulot et *al.* (1989) have proposed the use of glutaraldehyde 1% following by deep-freezing to preserve phytoplanktonic cells, but some authors have preferred the use of formaldehyde because it minimizes cell losses (Sieracki et *al*., 1993) especially for long term preservation

(Trousselier et *al*., 1995, Charpy and Blanchot, 1998). Sato et *al*. (2006) suggested that the effects of fixation and preservation on phytoplankton for flow cytometry are different for cultures and for natural samples. Fixation of samples using aldehydes has been reported to result in cell losses (Vaulot et *al*., 1989; Lepesteur et *al*., 1993; Marie et *al*., 2000). Pan et *al*. (2005) reported up to 70% of *Prochlorococcus* cells loss in preserved samples from the East China Sea and Sato et *al*. (2006) suggested that cell losses are more critical in natural than in cultured samples, probably due to the different species composition or physiological status.

If the use of aldehydes for preserving microorganisms is commonly accepted (Eschbach et *al.*, 2001; Marie et *al*., 1999), the type of fixative, its final concentration, as well as the storage process have been debated. Because methanol is added to commercial solutions of formaldehyde, it should be prepared from its polymerized form, para-formaldehyde; however such para-formaldehyde solutions are not stable for more than one week. Because glutaraldehyde is less toxic than formaldehyde and because commercial solutions are available and stable over the time, it appears as the best option for fixation.

Pluronic is a non-ionic surfactant used as a culture media additive and as a non toxic cryoprotectant during cell freezing and thawing procedures (Lowe et *al*., 2001). It is also used as anti-foaming, wetting, dispersant, thickener or emulsifier agent, and as stabilizer of cell membranes against shearing during batch cultures (Papoutsakis, 1991). Hellung-Larsen et *al.* (2000) studied the effect of Pluronic F68 (P68) on the ciliate *Tetrahymena*. They found a marked protection against physical and chemical stresses, especially at low cell concentrations. P68 has also been used to minimize cells losses due to numerous steps of centrifugation required to couple *in situ* hybridization with flow cytometry (Biegala et *al*., 2003).

In the present work, we have tested the use of P68 either alone or in combination with glutaraldehyde to preserve cultured and natural populations of microalgae in order to improve the recovery of cells for subsequent analysis by flow cytometry.

Materials and methods

Cultures and samples: Cultures from the Roscoff Culture Collection (RCC, www.roscoff-culture-collection.org) have been used for the different experiments (Table 1). They were grown in 12:12 light:dark cycle and were collected in exponential growth phase. For the natural samples, surface seawater from the SOMLIT-Astan site (48°46’18’’N, 3°58’6’’W) off Roscoff (Brittany, France) was sampled during winter using a Niskin bottle.

Flow cytometry: Analyses of samples were performed using a FACSCanto II flow cytometer equipped with 488 and 633 nm lasers and standard filter setup. The flow rate was determined by measuring the difference of volume of filtered seawater in a plastic tube before and after run for a minimum of 10 minutes as described in Marie *et al.* (1999). Data acquisition was triggered on the red fluorescence signal and samples were run for two minutes for cultures at medium rate (~50 µL/min) and three minutes in duplicate for natural samples at high rate (~100 µL/min). To allow comparison between samples, 0.95 µm fluorescent beads were added to each sample as internal reference. Listmode files were computed with Cytowin (Vaulot 1989). Cell parameters (scatter, fluorescence) were then normalized by dividing the mean value for each cell population by the mean value for the beads. In the following, we consider that differences in cell numbers below 5% are not significant, since the coefficient of variation (CV) of 2 replicates of the same sample analyzed by FCM commonly ranges between 1 and 3%.

Epifluorescence microscopy: In order to assess whether cells could be trapped on the side walls of the tubes used for analysis, some tubes that contained eukaryotes were emptied and manually broken into pieces. These pieces were observed by epifluorescence microscopy using an Olympus BX 51 microscope.

Experiment 1. Effect of Pluronic concentration: Different concentrations of Pluronic F-68 (P68, Sigma-Aldrich P5556) from 10^{-6} to 10^{-1} % were tested on a mixture of *Prochlorococcus* RCC156, *Synechococcus* RCC263, *Micromonas pusilla* RCC114, *Pleurochrysis carterae* RCC190, and 0.95 µm fluorescent beads (Polysciences). After an initial flow cytometric analysis of the fresh sample, samples were fixed with glutaraldehyde (GLU, Sigma G5882, 0.25% final), incubated for 15 min and then flash-frozen in liquid nitrogen and stored at -80°C. After one day, frozen samples were thawed at room temperature just before a new analysis.

Experiment 2. Fixation of culture mixes: Two different mixes of cultures each containing, one *Prochloroccocus* strain (RCC156, RCC168), one *Synechococcus* strain (RCC263, RCC1085), one picoeukaryote strain (RCC114, RCC745) and one nanoeukaryote strain (RCC190, RCC400), and 0.95 µm fluorescent polyspheres (Polysciences) were divided into four 1 mL aliquots. Two of them were unfixed and two were fixed with GLU 0.25% (minimum incubation period of 15 minutes at room temperature). Then, P68 at a final concentration of 0.01% was added into one aliquot of each condition (unfixed and fixed). After a first analysis by flow cytometry, aliquots were deep frozen into liquid nitrogen and then stored at -80°C for one month before a new analysis.

Experiment 3. Fixation of individual cultures: Eukaryotic cultures from the RCC in exponential growth phase were used and diluted if necessary with 0.22 µm-filtered seawater to adapt cell concentrations for flow cytometry analysis. The same treatment than for Experiment 2 was performed.

Experiment 4. Natural samples. Four samples of 40 mL were prepared on board, two without fixative and two others fixed with GLU 0.25% final. P68 at a final concentration of 0.01% was added in one aliquot of each condition. Samples were stored in the dark and brought back to the laboratory. They were analyzed by flow cytometry and then aliquoted into 1.5 mL cryovials, deep frozen in liquid nitrogen and stored at -80°C. These aliquots were analyzed after different storage duration (1, 2, 3 etc... months, up to 15 months).

Results

Experiment 1. Effect of Pluronic concentration.

Our first step was to test the effect of addition of P68 at different concentrations on cell counts for 4 cultures (two cyanobacteria, one picoeukaryote, and one nanoeukaryote). For cyanobacteria, there was little effect of P68 concentrations (Figure 1). For eukaryotes, there was a clear decrease in cell concentration after fixation and freezing compared to fresh sample when no P68 was added. Increasing concentration of P68 had a beneficial effect allowing to obtain cell counts on fixed-frozen samples that were quite similar to the original counts (Figure 1). Overall, concentration of P68 ranging from 0.1 to to 0.001% seemed to be optimal for the 4 species tested and therefore 0.01% was chosen for subsequent analysis.

Effects of fixation and P68 addition

Experiment 2. Mix of cultures. First we tested two mixed cultures, each containing strains of *Prochlorococcus*, *Synechococcus*, picoeukaryotes and nanoeukaryotes (see Material and Methods).

For the two *Prochlorococcus* strains tested in the mixes, fixation had very little effect and we did not observe significant differences in cells counts with and without P68 (Figure 2). The Side Scatter (SSC) of *Prochlorococcus* was reduced 2.5 - 3 fold in unfixed samples after freezing, but was higher after fixation by GLU (Supplementary Figure 1). Chlorophyll fluorescence decreased in GLU-fixed samples and this was more marked for RCC168 (Supplementary Figure 2).

Synechococcus cell concentrations decreased after freezing in unfixed samples, while fixation by GLU allowed to recover the original cell numbers (Figure 2). The SSC of *Synechococcus* increased after fixation by GLU, but was lower in presence of P68 (Supplementary Figure 1).

The phycoerythrin fluorescence of *Synechoccocus* cells increased up to 9 and 7 fold after freezing in absence of GLU for RCC263 and RCC1085, respectively, and this was strongly minimized on fixed cells with increases of 10 and 25%, respectively (data not shown). The same trend was observed for the chlorophyll fluorescence of *Synechococcus* that was increased by the fixation by GLU up to 2.5 and 3.7 fold after freezing in unfixed RCC263 and RCC1085, respectively (Supplementary Figure 2).

Freezing always resulted in cell losses for both picoeukaryotes tested with only 30 and 40% recovery in unfixed RCC114 and RCC745, respectively (Figure 2). The addition of P68 clearly minimized these losses. Fixation by GLU preserved well SSC of both *Micromonas* and *Ostreococcus* but it was strongly reduced by freezing, especially for *Ostreococcus* RCC745 (Supplementary Figue 1). Chlorophyll fluorescence per cell was little affected in both picoeukaryotes (Supplementary Figure 2).

For the nanoeukaryotes RCC190 and RCC400, the use of P68 minimized cell losses after freezing, especially in GLU-fixed samples (Figure 2). SSC was not affected by the fixation, but increased in *Pleurochrysis* RCC190 and decreased in *Chrysochromulina* RCC400 after freezing (Supplementary Figure 1). For both nanoeukaryotes, the chlorophyll content per cell was well preserved with and without P68 after freezing (Supplementary Figure 2).

Experiment 3. individual eukaryotic cultures: Based on the results reported in the previous experiment 2, different cultures seem to react differently to fixation. Therefore, we tested the effect of fixation with GLU and P68 addition on 11 strains of the RCC, belonging to a wide taxonomic range (Figure 3). Fixation and freezing could induce important cell losses as demonstrated with RCC174 (21%) or RCC91 (50%). Addition of P68 in the unfixed, unfrozen samples always resulted in higher cell counts that varied from 1 to 21%. The addition of P68 to fixed and frozen samples always allowed recovery of more cells compared to the original cell number obtained for not fixed and not frozen samples.

Cell recoveries higher than 100% have been observed for some strains and could be explained by the fact that cells were trapped on the side walls of the tubes used for analysis. In order to determine whether this was the case, tubes that contained unfixed and GLU-fixed *Chrysochromulina* RCC400 and *Scrippsiella* RCC91 were completely emptied and manually broken into pieces. These pieces were observed by epifluorescence microscopy. Photosynthetic cells were easily detected on the walls of the tubes for GLU-fixed samples, very few cells were detected for unfixed samples and no cells was detected on the walls of tubes that had contained P68.

Experiment 4. Natural samples: Seawater samples collected in winter off Roscoff showed typical flow cytometric signatures of cyanobacteria (Syn) with orange and red fluorescences, Cryptophyceae (Crypto) with strong orange and red fluorescences. Two populations of pico-

and nano-eukaryotes with only red fluorescence, are not clearly distinguishable from each other (Figure 4). As previously observed for cultures, P68 had little effect on cyanobacteria counts. Interestingly, cyanobacteria loss in unfixed sample was only 12% in presence of P68 after 12 months storage (Figure 5). The SSC of cyanobacteria was reduced by about 20% in unfixed and GLU-fixed samples after 1 day of preservation, and then it was stable over the time. As observed with cultures, the orange and red fluorescences increased after fixation with GLU.

Cell loss of total eukaryotes was low in unfixed samples in the absence of P68 reaching 14% after 2 months of storage (Figure 5). For the samples fixed with GLU or containing P68 we recorded up to 22% more cells after 15 months compared to the initial unfixed/unfrozen sample. Note that the percentage of cell loss was negligible in the unfixed sample containing P68, even after 12 months storage (Figure 5). For picoeukaryotes, we observed a slight increase in numbers over the time. The SSC of the picoeukaryotes decreased by about 40% immediately after fixation, but remained constant after that. For the nanoeukaryotes, after 5 months of preservation, we recorded 43% more cells in the GLU-fixed than in the original sample, and 23% more in the unfixed one. The SSC of nanoeukaryotes decreased by 20% immediately after fixation, and slowly decreased with time down to 50% after 15 months preservation. The fixation resulted in a decrease of the chlorophyll fluorescence for both pico- (20 to 24%) and nanoeukaryotes (12 to 21%), data are not shown for SSC and pigments analysis.

For the cryptophyte population, we observed 34% more cells in unfrozen GLU-fixed samples in presence of P68. After 1 month of preservation, 93 % of cryptophytes were lost in unfixed samples, and after 4 months storage, they almost disappeared in these samples (Figure 4). In contrast, the number of cryptophytes remained relatively constant in the fixed samples with or without P68 (Figure 5).

Discussion

Our results demonstrate a large variety of responses to fixation that are often species dependant, especially for eukaryotes. Kamiya et *al*. (2007) reported that fixation is not responsible of bacterial cell loss, that was attributed to flash freezing and storage at -80°C, with a rapid decrease of counts within the first 3 days, followed by a slower decline. We have not observed such tendency for the photosynthetic prokaryotes used in this study and fixed with GLU. Rapid fixation after sampling appears essential for preserving delicate cells such as cryptophytes. We regularly observed a rapid decline of this population, within a few hours, in unfixed samples. Conversely, abundances of *Synechococcus* and total eukaryotic cells remained relatively stable in unfixed samples. This can be of interest for some flow cytometry sorting applications for which samples cannot be fixed with aldehydes such as molecular biology analyses (barcoding or single cell genomics). Nevertheless care must be taken since certain types of eukaryotes present important cell losses after freezing (Figure 2).

The most interesting aspect of this study concerns the use of the surfactant Pluronic F68. The effect of P68 is not clear for prokaryotes, but clearly minimizes cell losses for eukaryotes. P68 has a short term effect, as demonstrated on eukaryotes from cultures or from natural assemblages. More cells were recovered in tubes containing the surfactant when analyzing unfrozen samples (Figures 2 and 5). These differences can be explained by cell lysis or by a tendency of such organisms to attach to the plastic walls of the tubes. Epifluorescence microscopy observations of the cell walls of tubes that had contained RCC400 (*Chrysochromulina sp*) or RCC91 (*Scrippsiella trochoidea*), suggest that, in the absence of P68, GLU-fixed cells have a very strong tendency to attach to the tube walls. These interactions are minimized in presence of P68. Electrostatic effects of plastic tubes can also be observed with the 0.95 µm beads added in natural sample from Roscoff, 20 %

more beads were obtained in the unfrozen unfixed samples containing P68. In unfixed sample, we observed a regular increase in beads numbers over the time, up to 85% more after 5 months preservation (data not shown).

In conclusion, we strongly recommend either, to run all marine plankton (cultured and natural) with the addition of Pluronic F68 at a final concentration of 0.01%, or alternatively to fix them with glutaraldehyde (0.25% final concentration) in presence of P68 (0.01% final concentration), to freeze the tubes in liquid nitrogen, and to keep them at -80°C for delayed analysis.

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Figure legends

Figure 1: Experiment 1. Effect of Pluronic (P68) concentration on the percentage of cell recovery (defined as the ratio of the cell concentration after treatment to that of initial untreated sample), obtained for a mixture of *Prochlorococcus* RCC156, *Synechococcus* RCC263, picoeukaryote RCC114 and nanoeukaryote RCC190, not fixed and non frozen (blue), fixed with GLU 0.25% and frozen for one day (red). Dashed line corresponds to 100% of recovery.

Figure 2: Experiment 2. Effect of freezing and fixation by GLU (0.25%) on the percentage of cell recovery obtained for 2 mixtures each containing one *Prochloroccocus* strain (RCC156, RCC168), one *Synechococcus* strain (RCC263, RCC1085), one picoeukaryote strain (RCC114, RCC745) and one nanoeukaryote strain (RCC190, RCC400), in presence (P) or absence of P68 at concentration of 0.01% analyzed by FCM, unfrozen (blue), and frozen for 1 month (red).

Figure 3: **Experiment 3.** Percentages of recovery of eukaryotes from the RCC unfixed and non frozen (blue), unfixed and non frozen with addition of P68 (dark blue), GLU-fixed and non frozen (red), GLU-fixed and frozen with addition of P68 (dark red).

Figure 4: Experiment 4. Phycoerythrin vs chlorophyll fluorescence of a natural sample from the English Channel (Station Astan) analyzed after 5 months preservation with no fixative (A and C) or fixed with GLU 0.25% (B and D) in absence (A and B) or in presence (C and D) of P68 (0.01%). Sub-populations are: 0.95 µm beads (Beads), *Synechococcus* (Syn), Picoeukaryotes (Pico), Nanoeukaryotes (Nano) and Cryptophyceae (Crypto). Typicall

regions used to discriminate the different populations are drawn on figure B. The unidentified events correspond to non-living particles.

Figure 5: Experiment 4. Percentages of recovery of *Synechococcus*, Cryptophyceae, and total eukaryotic cells per mL obtained for a natural sample taken off Roscoff, not fixed (blue), not fixed but with P68 (dark blue), fixed with GLU 0.25% (red), fixed with GLU with P68 (dark red). Analyses were performed immediately on non frozen samples (T0). They were then frozen and stored at -80°C for 1 to 15 months.

Supplementary Figures:

Supplementary Figure 1: Side scatter normalized to that of 0.95 µm beads obtained for 2 mixtures each containing one *Prochloroccocus* strain (RCC156, RCC168), one *Synechococcus* strain (RCC263, RCC1085), one picoeukaryote strain (RCC114, RCC745) and one nanoeukaryote strain (RCC190, RCC400), in presence (P) or absence of P68 at concentration of 10^{-2} analyzed by FCM, unfrozen (blue), and frozen for 1 month (red).

Supplementary Figure 2: Chlorophyll fluorescence normalized to the red fluorescence of 0.95 µm beads obtained for 2 mixtures containing each, one *Prochloroccocus* strain (RCC156, RCC168), one *Synechococcus* strain (RCC263, RCC1085), one picoeukaryote strain (RCC114, RCC745) and one nanoeukaryote strain (RCC190, RCC400), in presence (P) or absence of P68 at concentration of 10^{-2} analyzed by FCM, unfrozen (blue), and frozen for 1 month (red).

Table 1: Cultures used in this study. The RCC column corresponds to the strain number in the Roscoff Culture Collection (http://roscoff-culture-collection.org/).

RCC	Class	Taxon	Size (µm)	Flagellate	Outer layer	Growth medium
156	Cyanophyceae	*Prochlorococcus marinus*	0.6	No		PCRS11- Red sea
168	Cyanophyceae	*Prochlorococcus sp*	0.6	No		PCRS11- Red sea
263	Cyanophyceae	*Synechococcus sp*	1	No		PCRS11- Red sea
1085	Cyanophyceae	*Synechococcus sp*	1	No		PCRS11- Red sea
365	Chlorarachniophyceae	*Partenskyella glossopodia*	3	No		K
480	Chrysophyceae	*Ochromonas sp*	5	Yes		K
446	Dictyochophyceae	*Florenciella parvula*	4	Yes		K
91	Dinophyceae	*Scrippsiella trochoidea*	20	Yes	Theca	f/2
504	Eustigmatophyceae	*Nannochloropsis gaditana*	3	No		f/2
114	Mamiellophyceae	*Micromonas pusilla*	2	Yes		K
745	Mamiellophyceae	*Ostreococcus tauri*	0.8	No		K
180	Pavlovophyceae	*Pavlova lutheri*	7	Yes		Keller
100	Pelagophyceae	*Pelagomonas calceolata*	3	Yes		K
432	Prasinophyceae	*Pycnococcus sp*	2.5	No		K
174	Prymnesiophyceae	*Emiliania huxleyi*	4	No		K
400	Prymnesiophyceae	*Chrysochromulina sp*	4	Yes	Scales	K
190	Prymnesiophyceae	*Pleurochrysis carterae*	3	No	Coccoliths	K
475	Trebouxiophyceae	*Nannochloris sp*	2	No		K

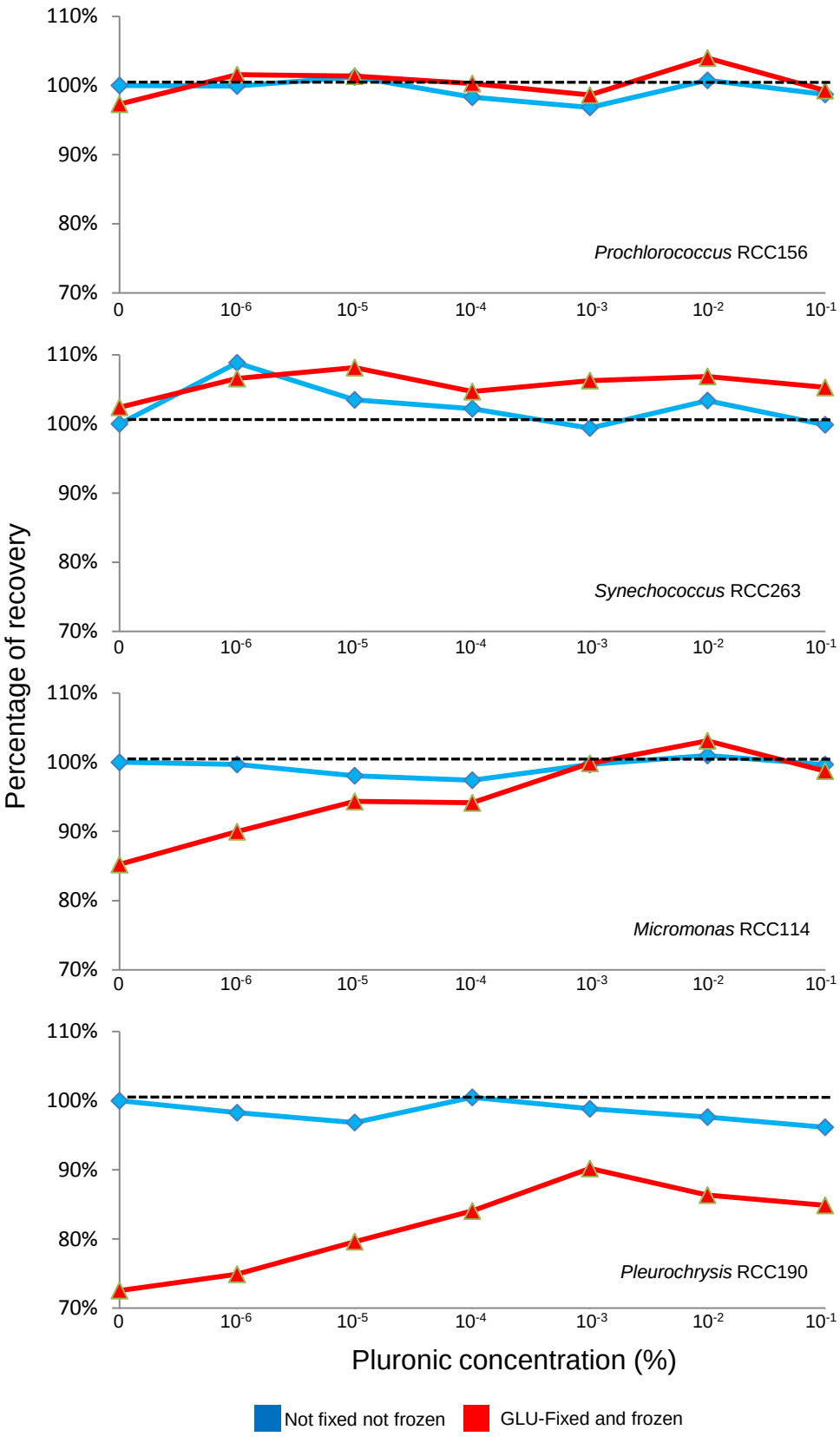
110%
100%
90%
80%
70%
Percentage of recovery
Prochlorococcus RCC156
Synechococcus RCC263
Micromonas RCC114
Pleurochrysis RCC190
0
10-6
10-5
10-4
10-3
10-2
10-1
Pluronic concentration (%)
Not fixed not frozen
GLU-Fixed and frozen

Figure 1

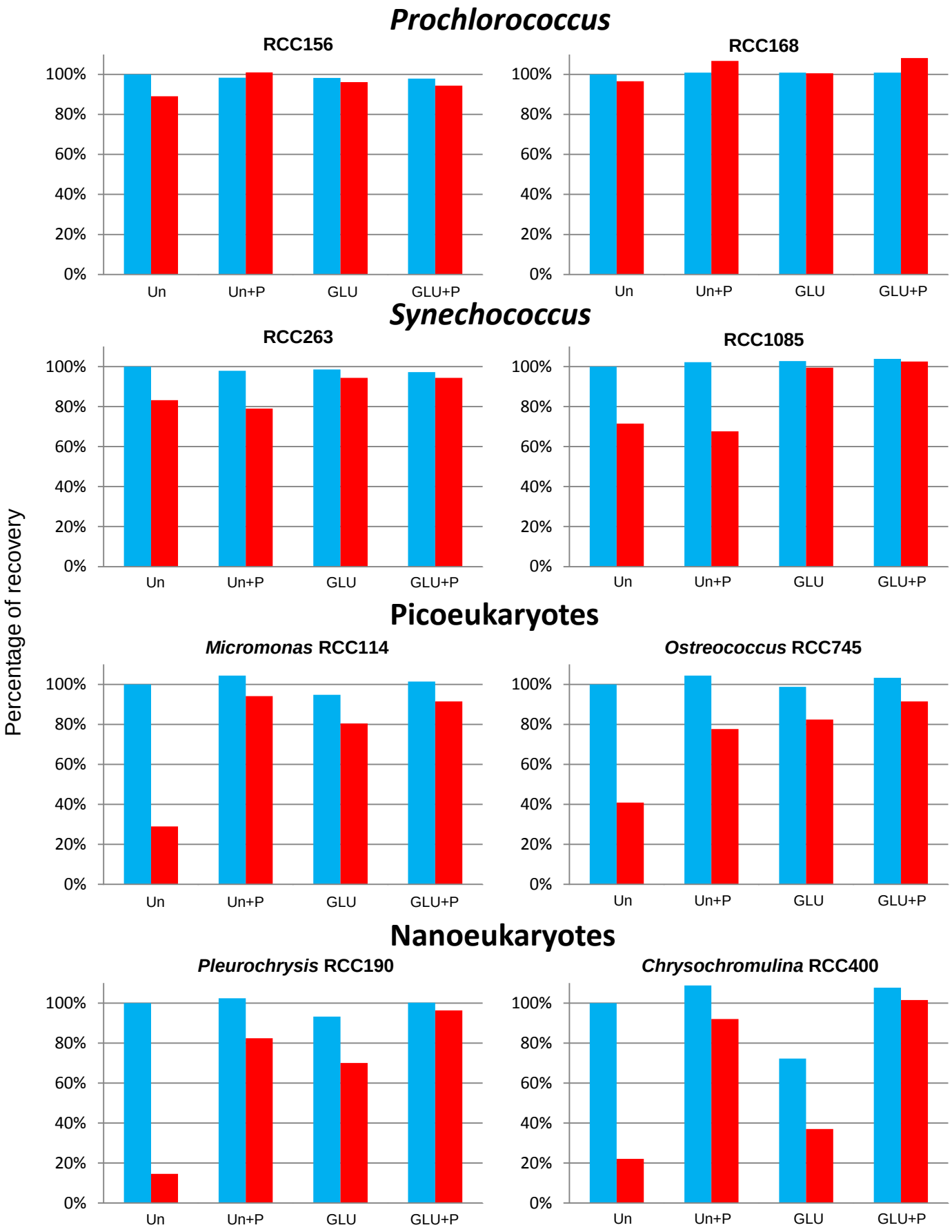
Prochlorococcus
RCC156
RCC168
Synechococcus
RCC263
RCC1085
Picoeukaryotes
Micromonas RCC114
Ostreococcus RCC745
Nanoeukaryotes
Pleurochrysis RCC190
Chrysochromulina RCC400
Percentage of recovery
100%
80%
60%
40%
20%
0%
Un
Un+P
GLU
GLU+P
Not frozen
Frozen for 1 month

Figure 2

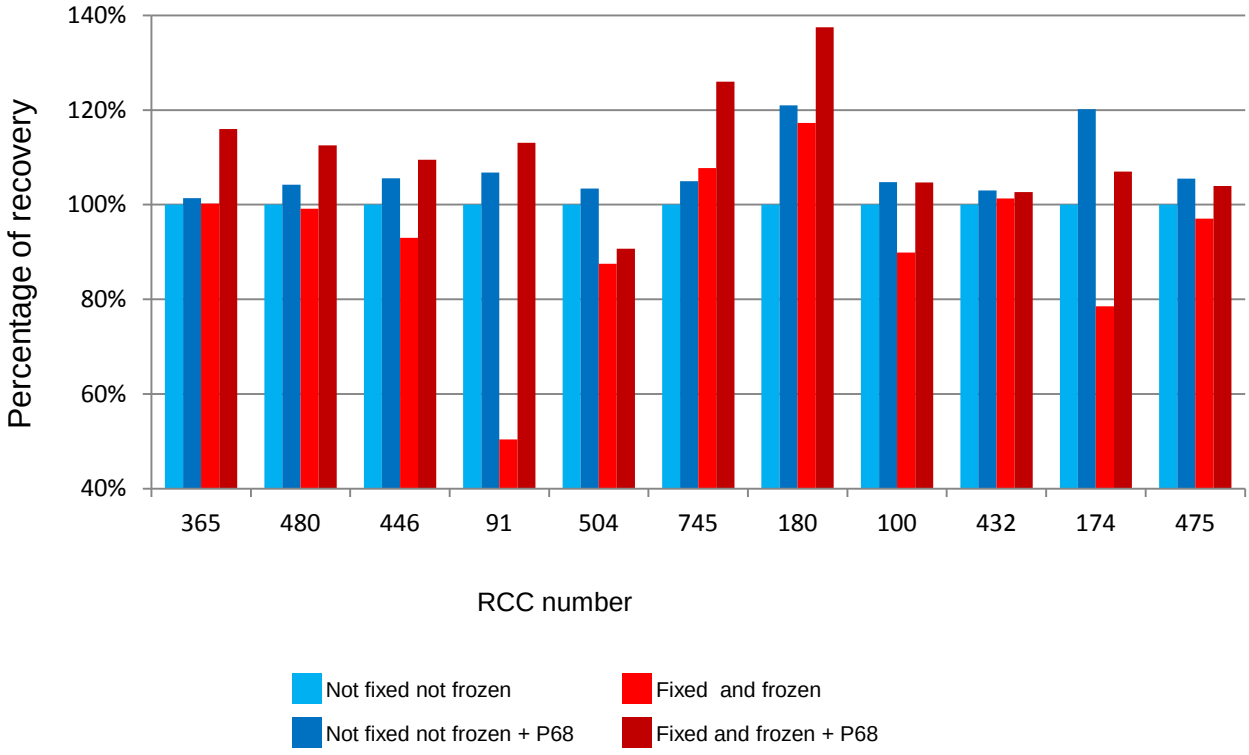
140%
120%
100%
80%
60%
40%
Percentage of recovery
365
480
446
91
504
745
180
100
432
174
475
RCC number
Not fixed not frozen
Fixed and frozen
Not fixed not frozen + P68
Fixed and frozen + P68

Figure 3

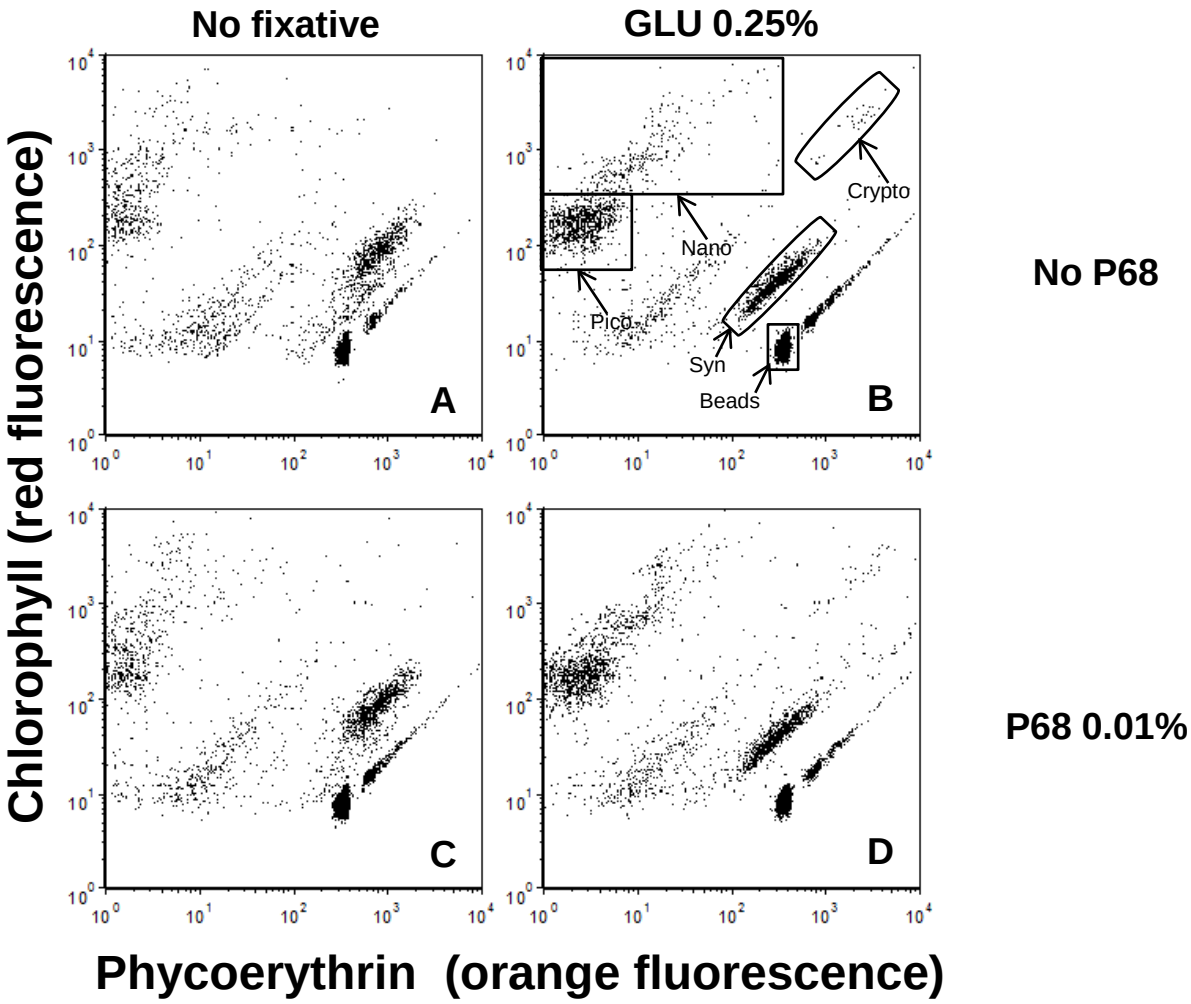
No fixative
GLU 0.25%
Crypto
Nano
Pico
Syn
Beads
A
B
C
D
No P68
P68 0.01%
Chlorophyll (red fluorescence)
Phycoerythrin (orange fluorescence)

Figure 4

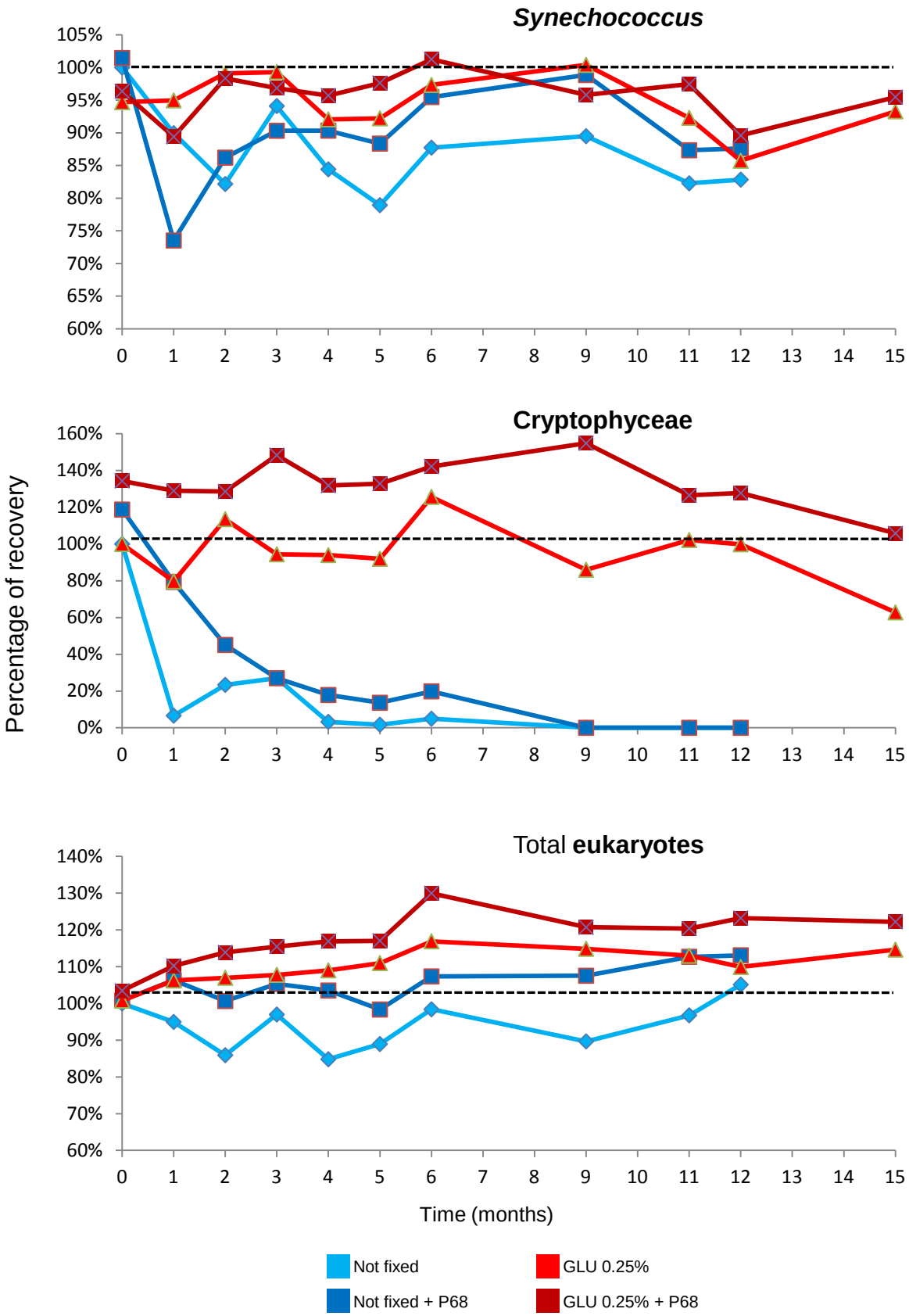
Synechococcus
105%
100%
95%
90%
85%
80%
75%
70%
65%
60%
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Cryptophyceae
160%
140%
120%
100%
80%
60%
40%
20%
0%
Percentage of recovery
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Total eukaryotes
140%
130%
120%
110%
100%
90%
80%
70%
60%
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (months)
Not fixed
GLU 0.25%
Not fixed + P68
GLU 0.25% + P68

Figure 5