Flow cytometry application in marine phytoplankton study: a case study investigating effects of formalin preservation on phytoplankton count and cell integrity

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Flow cytometry has been used in oyster feeding studies at Delaware State University for counting and sorting of marine micro-algal cells to determine the filtration activities of American oyster (*Crasostrea virginica*). This study was conducted to develop a simple protocol for accurate quantification of several cultured algae, using a known quantity of fluorescent microspheres as an internal calibration standard. While using flow cytometry to analyze algal samples in filtration studies on the oysters (*C. virginica*), we observed shifts in algae populations in both preserved and unpreserved samples over time. Because of these shifts, we felt it was important to determine the most efficient way to quantify algae accurately used in the filtration study. Two algal species, *Isochrysis spp.* (TISO) and *Tetraselmis chui* (PLY-429) cultured in F/2 media under the laboratory conditions at 12/12 hour light/dark cycles at 24°C room temperature at a set light intensity (130 umol), were used in this study. In order to determine the effects of preservation, via formalin we examined formalin-preserved and unpreserved phytoplankton samples both with and without known concentrations of fluorescent counting beads. Cell counts of cultured algal species for each treatment were determined using flow cytometry. The technique for counting and calibrating equipment were discussed. Significant ($P < 0.05$) differences were noted in between formalin-preserved and unpreserved samples as well as over time in 50% of the experimental trials out of four trials conducted for each algae species. Unpreserved samples showed reduced cell counts after 6 days in 75% of trials. Formalin-preserved samples showed significant reductions ($P < 0.05$) in cell counts after 3 days. Samples preserved with formalin and samples having formalin and beads showed lower algae counts immediately after the first day samples analyzed. We observed a significant ($P < 0.05$) reduction of fluorescent intensity over time. This reduction was most visible in the formalin-preserved samples with and without beads on the sixth day of sampling. Fluorescent counting beads were also affected by seawater. This study is intended to provide information on the applications of flow cytometry in phytoplankton research, and basic protocols needed to perform measurements on formalin-preserved and unpreserved samples. The methods may need to be modified for other applications.

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Introduction

Flow cytometry is a technology that can measure and analyze multiple properties of single particles in a fluid stream through a laser light at a rate of thousands of cells per second. The technique was initially developed for the medical/clinical fields and applied to eukaryotic cells [1]. The properties measured include a cell's relative size, relative granularity or internal complexity, and relative fluorescence intensity at several wavelengths. Any cell from 0.2 to 150 µm in diameter is suitable for analysis. Techniques have also been developed

for the analysis of cell viability, metabolic state, and antigenic markers [2].

Flow cytometry has become increasingly popular among limnologists and marine biologists for laboratory and field studies. Since phytoplankton is typically remain in suspension, flow cytometry is an ideal way to identify, enumerate, and estimate cell densities and relative proportions of phytoplankton and picoplankton [2, 3]. Major advantages include rapid and accurate measurements of individual particles as well as the ability to discriminate between cells, detritus, and suspended sediments. Although there are a number of publications relating to the use of flow cytometry in phytoplankton studies, protocols have been omitted and the techniques are not readily adaptable to other samples and equipment. There is currently limited information and training available relating to the use of flow cytometry for marine phytoplankton samplings, making analysis challenging. Most training is oriented toward clinical uses.

The analysis of algal samples allows us to obtain information on the abundance, cell size and pigment content of the major photosynthetic phytoplankton groups. This type of analysis can be performed either on unpreserved or preserved samples [1, 4, 5]. Few studies have mentioned the effects of preservatives on the quantitative and qualitative accuracy of flow cytometry [4]. The accessory pigments in phytoplankton that are utilized in flow cytometry for their fluorescent properties are particularly sensitive to preservation methods. According to Vaulot *et al*. [4], a method to preserve picoplankton samples for flow cytometric analyses must meet several criteria including the preservation of the fluorescence properties of the pigments in picoplankton populations. Classical phytoplankton preservation methods, such as formalin or Lugol's fixation, do not generally meet these criteria, as the former modifies cell shape and the latter drastically affects fluorescence for analyses. We have observed similar effects of formalin on cultured plankton samples. This study shows our findings in relation to preservation and cell quantification. An article overview by Dubelaar and Jonker [6] presented on flow cytometry as a tool for counting, analysis and identification of phytoplankton species and groups suggested that even though formalin preservation has been used to preserve cell integrity and fluorescence properties for periods of months, fluorescence gradually decreases in many instances. They observed an initial short term (minutes to hours) boost in fluorescence by blocking the energy transfer mechanisms in the pigments in preserved samples.

Chlorophyll and phycoerythrin are easily distinguished through fluorescence examination and are commonly utilized to identify phytoplankton species with the flow cytometer. In preliminary studies examining formalin concentrations for algal preservation, we determined a concentration of 2% formalin that was ideal to preserve samples while minimizing initial reductions in fluorescence intensity [7]. In this study, we examined unpreserved and preserved phytoplankton samples for their fluorescence intensities against known concentration of 2 µm counting beads. We also examined the effects of the counting beads on the algae samples and the effects of seawater on the counting beads. The protocol presented here is aimed to provide information necessary to accurately quantify phytoplankton samples focusing on several cultured algae species. Our

primary hypothesis in this study that phytoplankton cells loose their fluorescence intensities and relatively lower cell counts monitored from the formalin preserved samples. It is also hypothesized that seawater negatively effects counting beads as an effective internal counting tool in both formalin preserved and unpreserved samples.

Materials and Methods

Sample Preparation

Isochrysis spp. (TISO) and *Tetraselmis chui* (PLY-429) were used independently in the first three trials. In the fourth trial, they were used independently and mixed. Strains were originally isolated at the NOAA Milford Laboratory, Milford, Connecticut and are currently being maintained at Delaware State University's Algae Culture Laboratory. For each trial, total of three replicates for each treatment were prepared. Three data sets were collected per replicate per tube by flow cytometry. Three 2 sets of tubes (one for *Isochrysis* and another for *T. chui*) were preserved using a total concentration of 2% formalin solution and two remained unpreserved. Sixty microliters of formalin provided total concentration of 2% formalin preservation in the test tube for 3,000 µl total solution. For each specific algal experimental set, four 5-ml test tubes were prepared with the designated algae species. Micrometer fluorescent counting beads (Polysciences, Inc.) were added to one preserved and one unpreserved sample, as well as to seawater alone (Table 1), concentration being approximately ≈ 380 x 10^4 beads / ml. The tubes were prepared in triplicates for each specific algal set in each trial. In the fourth trial, *Isochrysis* and *T. chui* were mixed equally and the same treatments were applied to these mixed tubes (algae mixed, formalin-preserved algae mixed, algae mixed with counting beads and formalin-preserved algae mixed with counting beads). This was done to accurately quantify the algal cells in solution [6] and to observe any changes in the counting beads independent from algae. Cell counts were performed with a hemocytometer prior to analysis using the flow cytometer. Samples used for microscopic examination were not stained for counting but initially preserved in formalin and stored at 4°C during a 6-day study period for most trials, except for the fourth trial which was studied over a 3-day period. This experiment was ceased after three days due to relocation of the flow cytometer. In order to examine the effects of both fluorescent counting beads and formalin on the algae and of seawater on the counting beads, a factorial design was developed to prepare the samples for analysis (Table 1). Initially, beads counts were considered to be equal or approximately the same as the cell numbers in a given volume. However, this was not the case for all samples or for different algal strains. Samples were analyzed immediately after preparation on the day of collection (day 1). After analysis, samples were stored in the dark at 4°C in order to retard mitosis in the unpreserved samples. Samples were then analyzed one day after collection, two days after collection, and five days after collection in order to track any reduction in cell counts or shifts in the algae population. There were three replicates of each treatment, for each algae species. Three data sets were collected per replicate in Cell Quest Pro software program (BD Biosciences Inc., CA, USA). Event rates or counts determined from the data sets were averaged and analyzed.

In a few instances where high algal densities (1 x 10^7cells/ml) were observed, samples were diluted 50% with 18 ppt 0.22 µm filtered

Table 1. Sample Tube Preparation: Samples stored at 4°C in the dark. Three separate trials and one combined trial were conducted for *T. chui* and *Isochrysis spp.* Three data sets were collected per replicate per tube by flow cytometry. Sixty microliters of formalin provided total concentration of 2% formalin preservation in the test tube for 3,000 µl total solution. Counting Beads used in all trials ranged between $328x10^4$ beads/ml to $746x10^4$ beads/ml depending on the trial and the sampling day. Samplings were performed at Days 1, 2, 3, and 6 in Trials 1, 2, and 3 when no sampling was done in Day 6 in Trial 4.

Solution per Sample (µl) (n = 3)					
Treatment	**Culture**	**Seawater**	**Bead Solution**	**Formalin Solution**	**Total Sample Volume**
Algae	3,000	0	0	0	3,000
Formalin in Algae	2,900	0	100	0	3,000
Beads in Algae	2,940	0	0	60	3,000
Formalin & Beads in Algae	2,840	0	100	60	3,000
Beads in Seawater	0	2,900	100	0	3,000

seawater and similar preparation was prepared for diluted samples. The stock-counting bead solution was enumerated using a hemocytometer prior to each trial to assure values given by the manufacturer's counts (6,622,561,000 beads/ml).

We did not use any fluorochromes/stains on our samples. The natural autofluorescence of algae was used to define our algae populations. We were looking at autofluorescent properties because as cells age, the fluorescence intensity is expected to be reduced.

Instrument and Instrument Settings

A four color BD FACSCalibur (BD Biosciences Inc, CA, USA) automated bench top flow cytometer/sorter was used to analyze algae samples. A 488 nm, argon laser was used for detecting Forward Scatter (FSC) which determines cell size and shape and detects ~ 635 nm wavelength, Side Scatter (SSC) which determines internal complexity and detects 478-498 nm wavelengths range, FL1 which detects green fluorescence at 500-560 nm wavelengths range, FL2 which detects orange fluorescence at 543-627 nm wavelengths range, and FL3 which detects dark red fluorescence at 670 nm wavelength while FL4 detects red fluorescence at 645-677 nm wavelengths range. Fluorescence derived from each particle is split by a 595 nm dichromic mirror and is received by photomultiplier tubes located at 90° to the intersection of the laser beam and sample stream. In order to reduce the effects of osmotic stresses and to limit possible contaminants, 0.22 µm filtered seawater was used rather than standard sheath fluid. CellQuest Pro software program (BD Biosciences Inc., CA, USA) was used to acquire and analyze data collected from samples.

Due to the relatively small size of our algae species (*Isochrysis spp.* 3 - 6 µm and *T. chui* 14 – 23 µm), we chose to focus on fluorescence intensity of algal cells to determine populations. The fluorescent range of the species of interest determines which channels should be used for optimal visibility. In preliminary studies, FL1 (green fluorescence) and FL3 (red fluorescence) appeared to be optimal parameters for isolating both *Isochrysis spp.* and *T. chui*. Density and dot

plots were used, since they clearly showed algae populations, counting beads, and background noise. In order to reduce background noise, a threshold of 52 was set on the FL3 channel. Threshold allows us to set a channel number below which data will not be processed. Threshold can be set for only one parameter (FSC, SSC, FL1, FL2, or FL3) at a time if we have a single laser system [2]. This setting was determined to minimize background noise while showing the clearest views and most accurate counts of both algae samples and counting beads. Because the threshold can only be set on one channel, it is important to determine what works best for all of the aspects you need to take into consideration. Anything producing a signal below your threshold setting will not be processed by Cell Quest Pro software program.

Compensation settings were very critical in this study to locate the population of interest. Compensation is utilized when there is an overlap of multiple signals. The brighter the signals the more overlap occurs. Since we did not work with multiple populations with similar fluorescent signals, we did not utilize compensation.

Detectors and amplifiers are very important settings in flow cytometry. The detectors are the photodiode and photomultiplier tubes. Forward Scatter (FSC) is the photodiode. It is used for high intensity signals like light-scatter. Side Scatter (SSC), FL1, FL2, FL3, and FL4 are the photomultiplier tubes (PMTs). They receive photons and convert them to electrical signals. Adjusting the voltages alters the sensitivity of the photomultipliers. We provided detectors and amplifiers settings in Table 2 in our result section.

The amplification mode is either linear or logarithmic. Because of the sizes of our cells, we use a logarithmic scale. When using a logarithmic scale, you cannot adjust the amplification gain (Amp Gain). Amp Gain adjusts the sensitivity of measurements, but can only be used with linear signals.

Threshold allowed us to separate our algae cell population better by eliminating unwanted background noise appearing on the plots. The dots are caused by setting the threshold for only one parameter and data above that threshold is processed for analysis. All settings were determined prior to the start of the experiment and were used for both *T. chui* and *Isochrysis spp.* (Table 2).

After settings were optimized, region gates, where the populations of interests are isolated with lines, were created for the algae populations and the counting beads, independently. The gates made it possible to determine algal cell numbers proportionally to number of beads observed on the plots.

Data Acquisition

Samples were vortexed for 10 – 20 sec before being placed on the sample injection port in order to assure that cells and beads were in suspension. Data was acquired for 1 min. Three datasets were acquired for each sample and averaged to obtain a mean event rate (count) in order to account for any anomalies, like cells or beads clumping.

The FACSCalibur has three sampling speeds: 1) low: 12 μl/min, 2) medium: 35 μl/min, and 3) high: 60 μl/min of sample through the sample flow cell. Based on preliminary trials we determined that sampling at medium (35

Table 2. Flow cytometer setting for *T. chui* and *Isochrysis spp.* with and without counting beads. Threshold is set for FL3 at 52. FSC is set on E00 voltage on the log mode. To reduce background noise, a threshold of 52 was set on the FL3 channel. Threshold allows setting a channel number below which data will not be processed. Threshold set for only one parameter (FSC, SSC, FL1, FL2, or FL3) at a time if a single laser system is used.

FACS Calibur Cytometer Setting				
Detectors / Amplifiers:				
Parameter	*Detector*	*Voltage*	*Amp Gain*	*Amp Mode*
P1	FSC	E00	1.00	Log
P2	SSC	287	1.00	Log
P3	FL1	448	1.00	Log
P4	FL2	427	1.00	Log
P5	FL3	338	1.00	Log
P6	FL2-A		1.00	Lin
P7	FL2-W		1.00	Lin
Threshold:				
Primary Parameter :		FL3		
Value:		52		

µl/min) would allow for enough sampling with limited doublet and triplet cell clusters passing through the laser. Sampling at medium speed also permitted the initial samples to last over a six day period. In order to reduce cross-contamination, 0.22 µm seawater was run in between samples until the lines were flushed and no particles were observed for about 90 sec. This process flushed out any remaining particles from the previous sample and from the sample injection port that could potentially contaminate other samples. This process is very important when you are running samples with multiple species or varying cell concentrations.

Calculation and Statistics

Cell concentration, Cc (cells/ml), for each sample at each sampling period was calculated according to Campbell [1] using the equation Cc = N / (T x R) x CF x 1,000 µl/ml. Where: N equals the number of events acquired (cells), T equals the duration of analysis (60 sec), R equals the sample delivery rate (µl/min), and CF equals the correction factor.

A correction factor was used to offset the dilution from adding beads and formalin into the samples. Once sample volume was corrected for 3,000 µl in each tube, the event number obtained per 35 µl over 60 sec was transferred to 1,000 µl/min.

A mixed-model repeated-measures analysis for ANOVA ($\alpha = 0.05$) was used to analyze cell counts over time [8]. A compound symmetry covariance structure best fit the experimental data. Replicates and interactions with replicates were considered random effects while days were considered fixed effects. Treatment means of algal event rates were separated using planned orthogonal contrasts. Planned comparisons were also determined during analysis to find the treatment differences for each sampling time. Regression analyses were used to determine the relationship among flow cytometric analysis and microscopic counts. For all statistical analyses, a confidence interval of 95% and alpha level (α) of 0.05 was used.

Results

In all four trials for each algae species used, there were four treatment options studied. These treatment options were; i. sample only

(either *Tetraselmis chui* or *Isochrysis spp.*); ii. sample with counting beads; iii. 2% formalin preserved sample; and iv. 2% formalin preserved sample with counting beads. In the fourth trial, besides these four treatment options, algal species were mixed and the same treatment options were applied to the mixed samples.

***Tetraselmis chui* (PLY-429)**

Four trials used in this study had four different treatments to compare and do contrasts among these treatments. The treatments were *Tetraselmis chui (PLY-429)* only, *T. chui* with counting beads, 2% formalin preserved *T. chui*, and 2% formalin preserved *T. chui* with counting beads.

ANOVA results indicated significant differences between the treatments, sampling days, and interaction effects between the treatments and sampling days for 50% of the trials (Table 3). The first *T. chui* trial resulted in no significant differences ($P \geq 0.05$) regarding treatments, sampling days, and interaction effects between the treatments and sampling days (Table 1). Furthermore, Planned Comparison and Orthogonal Contrasts study resulted in no significant differences ($P \geq 0.05$) between any of the treatments in Trial 1. There were significant differences ($P < 0.05$) for sampling days and interaction effects for the Trial 2; however, there were no significant differences ($P \geq 0.05$) in regards to treatments. In addition, results from Planned Comparisons and Orthogonal Contrasts analysis showed no significant differences ($P < 0.05$) between treatment types. Trial 3 showed significant differences ($P < 0.05$) between the treatments, sampling days, and treatments vs. sampling days for *T. chui* samples with ANOVA. Significant differences ($P < 0.05$) were observed in five of seven contrasts in Trial 3; there were no significant differences ($P \geq 0.05$) between the algae sample only and algae sample with counting beads. Furthermore, 2% formalin preserved algae sample and 2% formalin preserved algae sample with counting beads resulted in no significant differences ($P < 0.05$).

In Trial 4, the following eight treatments were used: i. *T. chui* only; ii. *T. chui* with counting beads; iii. 2% formalin preserved *T. chui*; iv. 2% formalin preserved *T. chui* with counting beads; v. *T. chui* and *Isochrysis spp.* mixed; vi. *T. chui* and *Isochrysis spp.* mixed with counting beads; vii. 2% formalin preserved *T. chui* and *Isochrysis spp.* mixed; and viii. 2% formalin preserved *T. chui* and *Isochrysis spp.* mixed with counting beads. Significant differences ($P < 0.05$) were observed in the ANOVA results for treatments; however, no significances were observed for sampling days and interaction effects between treatments and sampling days. In Trial 4, we used additional treatments with algae species, *T. chui* and *Isochrysis spp.* mixed. Results from the Orthogonal Contrasts study showed significant differences ($P < 0.05$) in seven out of eighteen treatment options. Significant differences ($P < 0.05$) were monitored between the two mixed algae species and 2% formalin preserved mixed algal samples compared to *T. chui* sample only and 2% formalin preserved *T. chui* sample treatments. Significant differences ($P < 0.05$) in algal counts occurred when 2% formalin preserved mixed algae sample, mixed algae with counting beads, and 2% formalin preserved mixed algae with counting beads were compared to mixed algae treatment only. In addition, algal counts were significantly different ($P < 0.05$) between *T. chui* sample only and mixed *T. chui* and *Isochrysis spp.* Significant differences ($P < 0.05$) were reported between mixed algae only and 2% formalin preserved

Table 3. ANOVA tables for treatments, sampling days and interaction between treatments and sampling days for *T. chui* (α = 0.05). The treatments were; i. *T. chui* only; ii. *T. chui* with counting beads; iii. 2% formalin preserved *T. chui*; and iv. 2% formalin preserved *T. chui* with counting beads. In the fourth trial, besides these four treatments, algal species (***T. chui*** and ***Isochrysis spp***.) were mixed and the same treatment options were applied to the mixed samples. Samplings were performed at Days 1, 2, 3, and 6 in Trials 1, 2, and 3 when no sampling was done in Day 6 in Trial 4.

	Effect	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F Value	Pr > F
Trial 1	Treatment	3	1	1.09	0.5916*
	Day	2	1	2.18	0.4321
	Treatment * Day	6	1	0.82	0.6880
Trial 2	Treatment	4	40	0.57	0.6868
	Day	3	40	6.27	0.0014†
	Treatment * Day	12	40	4.18	0.0003
Trial 3	Treatment	3	32	17.19	< 0.0001
	Day	3	32	4.23	0.0126
	Treatment * Day	9	32	4.98	0.0003
Trial 4	Treatment	7	23	4.59	0.0025
	Day	2	1	10.54	0.2128
	Treatment * Day	14	1	1.48	0.5758

* not significant (P ≥ 0.05)
† significant (P < 0.05)

mixed algae sample. Algae counts were significantly different ($P < 0.05$) only between the mixed algae sample and preserved mixed algae sample with counting beads. Significant differences ($P < 0.05$) were only observed when the preserved mixed algae and preserved mixed algae with counting beads were compared to the mixed algae sample with counting beads.

Isochrysis spp.

Four trials were conducted with four different treatments to compare and do contrasts studies. The treatments were *Isochrysis spp.* only, *Isochrysis spp.* with counting beads, 2% formalin preserved *Isochrysis spp.,* and 2% formalin preserved *Isochrysis spp.* with counting beads.

ANOVA results indicated significant differences ($P < 0.05$) between the treatments for 50% of the trials, 75% of sampling days, and 50% for interaction effects between the treatments and sampling days for *Isochrysis spp.* trials (Table 4). In the first *Isochrysis spp.* trial, there were no significant differences ($P \geq 0.05$) observed between the treatments, sampling days, and treatment vs. sampling days. In Trial 1, there were significant differences ($P < 0.05$) observed in all but one contrasts in the Orthogonal Contrast Analysis; no significant differences were observed between the 2% formalin preserved *Isochrysis spp.* and 2% formalin preserved *Isochrysis spp.* with counting beads. The second *Isochrysis spp.* trial resulted in significant differences ($P < 0.05$) between the treatments and sampling days; however, interaction effects between the treatments and sampling days were not significant ($P \geq 0.05$). Significant differences ($P < 0.05$) were observed in all contrast studies for the second *Isochrysis spp.* trial.

The ANOVA output in *Isochrysis spp.* Trial 3 showed significant differences ($P < 0.05$)

Table 4. ANOVA tables for treatments, sampling days and interaction between treatments and sampling days for *Isochrysis spp.*(α = 0.05). The treatments were; i. *Isochrysis spp.*; ii. *Isochrysis spp.* with counting beads; iii. 2% formalin preserved *Isochrysis spp.*; and iv. 2% formalin preserved *Isochrysis spp.* with counting beads. In the fourth trial, besides these four treatments, algal species (***T. chui*** and ***Isochrysis spp***.) were mixed and the same treatment options were applied to the mixed samples. Samplings were performed at Days 1, 2, 3, and 6 in Trials 1, 2, and 3 when no sampling was done in Day 6 in Trial 4.

	Effect	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F Value	Pr > F
Trial 1	Treatment	3	1	130.20	0.0643*
	Day	3	1	35.79	0.1221
	Treatment * Day	9	1	49.66	0.1097
Trial 2	Treatment	3	1	215.64	0.0500
	Day	3	1	3011.17	0.0134†
	Treatment * Day	9	1	66.29	0.0951
Trial 3	Treatment	3	32	15.95	<0.0001
	Day	3	32	65.62	<0.0001
	Treatment * Day	9	32	2.19	0.0497
Trial 4	Treatment	7	1	1.85	0.5138
	Day	2	24	11.65	0.0003
	Treatment * Day	14	24	2.78	0.0135

* not significant ($P \geq 0.05$)
† significant ($P < 0.05$)

between the treatments, sampling days, and interaction effects between the treatments and sampling days. In addition, the contrasts study returned significant differences ($P < 0.05$) for all but one test; there were no significant differences ($P = 0.7483$) observed between *Isochrysis spp.* and *Isochrysis spp.* with counting beads.

In *Isochrysis spp.* Trial 4, eight treatments were: i. *Isochrysis spp.* only; ii. *Isochrysis spp.* with counting beads; iii. 2% formalin preserved *Isochrysis spp.*; iv. 2% formalin preserved *Isochrysis spp.* with counting beads; v. *T. chui* and *Isochrysis spp.* mixed*;* vi. *T. chui* and *Isochrysis spp.* mixed with counting beads; vii. 2% formalin preserved *T. chui* and *Isochrysis spp.* mixed; and viii. 2% formalin preserved *T. chui* and *Isochrysis spp.* mixed with counting beads. The fixed effects study, ANOVA, resulted in no significant differences ($P \geq 0.05$) for treatments; however, there were significant differences ($P < 0.05$) were observed for sampling days and interaction effects between the treatments and sampling days, P = 0.0003 and 0.0135 in Trial 4. Three out of eighteen contrasts returned significantly different ($P < 0.05$). Significant differences ($P < 0.05$) were observed between *Isochrysis spp.* and *T. chui* mixed when compared to the *Isochrysis spp.* sample only and 2% formalin preserved *Isochrysis spp.* sample. In addition, there were significant differences ($P < 0.05$) between *Isochrysis spp*. sample with counting beads and 2% formalin preserved *Isochrysis spp.* sample with counting beads. The difference was significant ($P < 0.05$) between the preserved *Isochrysis spp.* sample and preserved *Isochrysis spp.* sample with counting beads when compared to the *Isochrysis spp.* sample with counting beads.

Counting Beads

In this study, separate ANOVA analysis were conducted In order to obtain differences between the sample tubes having unpreserved

Table 5. ANOVA tables for treatments, sampling days and interaction between treatments and sampling days for counting beads in *T. chui* and 2% formalin preserved *T. chui* (α = 0.05). The treatments in Trials 1 and 2 were; i. counting beads in *T. chui* and ii. counting beads in 2% formalin preserved *T. chui.* The third treatment was included in Trial 3 as iii. counting beads in seawater; and iv. counting beads in 2% formalin preserved seawater. Samplings were performed at Days 1, 2, 3, and 6 in Trials 1, 2, and 3 when no sampling was done in Day 6 in Trial 4.

	Effect	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F Value	Pr > F
Trial 1	Treatment	1	1	0.48	0.6156*
	Day	2	1	1.51	0.4987
	Treatment*Day	2	1	0.46	0.7212
Trial 2	Treatment	1	17	2.15	0.1605
	Day	3	17	2.95	0.0621
	Treatment*Day	3	17	2.76	0.0737
Trial 3	Treatment	2	1	44.14	0.1058
	Day	3	1	4.46	0.3319
	Treatment*Day	6	1	4.57	0.3436

* not significant (P ≥ 0.05)

algae with counting beads and 2% formalin preserved algae with counting beads, also between the sample tubes having distilled water with counting beads with or without 2% formalin preservation and the sample tubes having seawater with counting beads with or without 2% formalin preservation.

Results from the four trials conducted for *T. chui* sampling with counting beads were compared and reported in this section. In the first two trials only two treatment options were analyzed; beads in *T. chui* and 2% formalin preserved *T. chui*. According to the ANOVA results, no significant differences ($P \geq 0.05$) were monitored between the treatments and no interaction effects between the treatments and sampling days. There were only significant differences ($P < 0.05$) between the sampling days for 25% of the trials (Table 5). For counting beads in *T. chui* Trials 1, 2, and 3 there were no significant differences ($P \geq 0.05$) between the treatments, sampling days, and interaction effects between the treatments and sampling days (Table 5). Furthermore, there were no significant differences ($P \geq 0.05$) were observed in Planned Comparison Analysis between *T. chui* samples with counting beads and preserved *T. chui* sample with counting beads. A significant difference ($P < 0.05$) was observed in counting beads in *T. chui* Trial 4 between the sampling days.

Separate statistical analysis were conducted to monitor any differences in counting beads used in *Isochrysis spp.* trials where differences between counting beads in unpreserved *Isochrysis spp.* and counting beads in 2% formalin preserved *Isochrysis spp.* for all four *Isochrysis spp.* trials. Moreover, differences between counting beads in seawater with and without 2% formalin preservation monitored in *Isochrysis spp.* Trial 3. ANOVA results indicated significant differences ($P < 0.05$) between the treatments for 50% of the trials for counting beads in *Isochrysis spp.* and 2% formalin preserved *Isochrysis spp.* trials (Table 6). However no differences ($P \geq 0.05$) were monitored between the sampling days and interaction effects between the treatments and

Table 6. ANOVA tables for treatments, sampling days and interaction between treatments and sampling days for counting beads in *Isochrysis spp.* and 2% formalin preserved *Isochrysis spp.* (α = 0.05). The treatments in Trials 1 and 2 were; i. counting beads in *Isochrysis spp. and* ii. counting beads in 2% formalin preserved *Isochrysis spp.* The third treatment was included in Trial 3 as iii. counting beads in seawater; and iv. counting beads in 2% formalin preserved seawater. In Trial 4, 10 treatments were: i. counting beads in *Isochrysis spp.;* ii. counting beads in 2% formalin preserved *Isochrysis spp.* iii. counting beads in *T. chui* ; iv. counting beads in 2% formalin preserved *T. chui*; v. counting beads in mixed *T. chui* and *Isochrysis spp.;* vi; counting beads in 2% formalin preserved *T. chui* and *Isochrysis spp.;* vii. counting beads in seawater; viii. counting beads in 2% formalin preserved seawater; ix. counting beads in distilled water; and x. counting beads in 2% formalin preserved seawater. Samplings were performed at Days 1, 2, 3, and 6 in Trials 1, 2, and 3 when no sampling was done in Day 6 in Trial 4.

	Effect	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F Value	Pr > F
	Treatment	1	16	12.73	0.0026*
Trial 1	Day	3	1	6.77	0.2736*
	Treatment*Day	3	1	4.94	0.3168
	Treatment	1	16	3.48	0.0805
Trial 2	Day	3	1	75.37	0.0844
	Treatment*Day	3	1	18.95	0.1669
	Treatment	2	1	44.14	0.1058
Trial 3	Day	3	1	4.46	0.3319
	Treatment*Day	6	1	4.57	0.3436
	Treatment	9	31	2.47	0.0298
Trial 4	Day	2	31	0.3	0.7459
	Treatment*Day	18	31	0.6	0.875

* significant ($P < 0.05$)

sampling days. Significant differences ($P < 0.05$) were observed between the treatments in regards to the first trial using counting beads in *Isochrysis spp.* and counting beads in 2% formalin preserved *Isochrysis spp*. (Table 6). There were no significant differences between sampling days and interaction effects between the sampling days in *Isochrysis spp.* Trial 1. The contrasts for beads in *Isochrysis spp.* Trial 1 resulted in significant differences ($P < 0.05$) between the sample with counting beads and preserved sample with counting beads. The results of beads counts in *Isochrysis spp.* Trial 2 from the ANOVA showed no significant differences ($P \geq 0.05$) regarding treatments, sampling days, and interaction effects. The contrast study for beads in *Isochrysis spp.* Trial 2 showed no significant differences ($P \geq 0.05$) in algal counts between *Isochrysis spp.* samples with counting beads and preserved *Isochrysis spp.* sample with counting beads. It should be noted that the counting beads in *Isochrysis spp*. Trial 3, showed no significant differences ($P \geq 0.05$) between the treatments, sampling days, and interaction effects. It should also be noted that the counting beads in *Isochrysis spp.* Trial 3 used seawater to observe the effects of seawater on the beads. There were no significant differences ($P \geq 0.05$) in regards to beads counts for the contrast studies, including those using seawater.

For beads in *Isochrysis spp*. Trial 4 we used a mixture of *Isochrysis spp*. and *T. chui*; the fixed effects test, ANOVA, resulted in observed significances ($P < 0.05$) between the treatments. There were no significant differences ($P \geq 0.05$) observed between the sampling days and

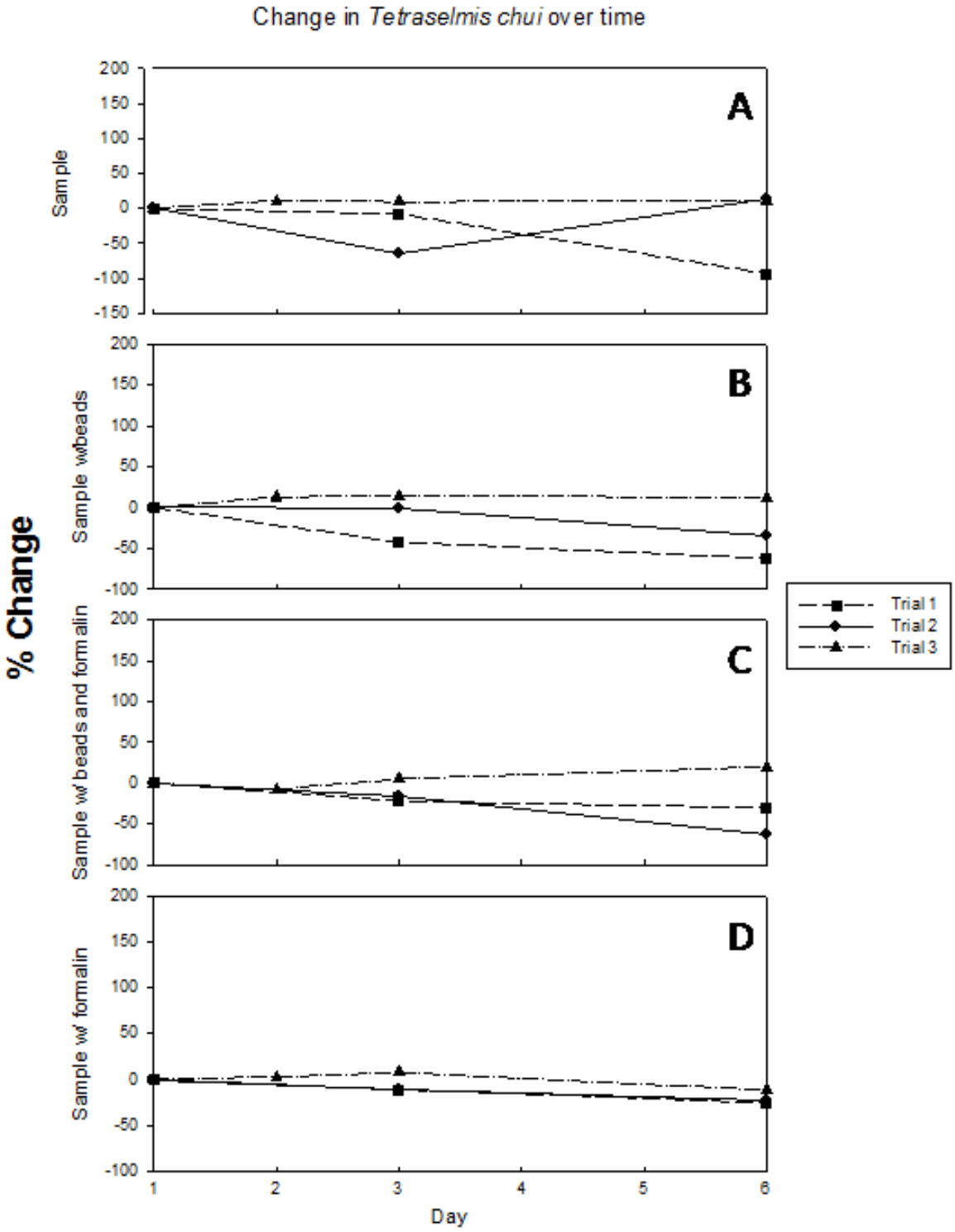


Figure 1. Percentage change in *T. chui* counts over 6 days study period. Four different treatment options are: A) algae sample only, B) algae sample with counting beads, C) formalin preserved algae sample and D) formalin preserved algae sample with counting beads.

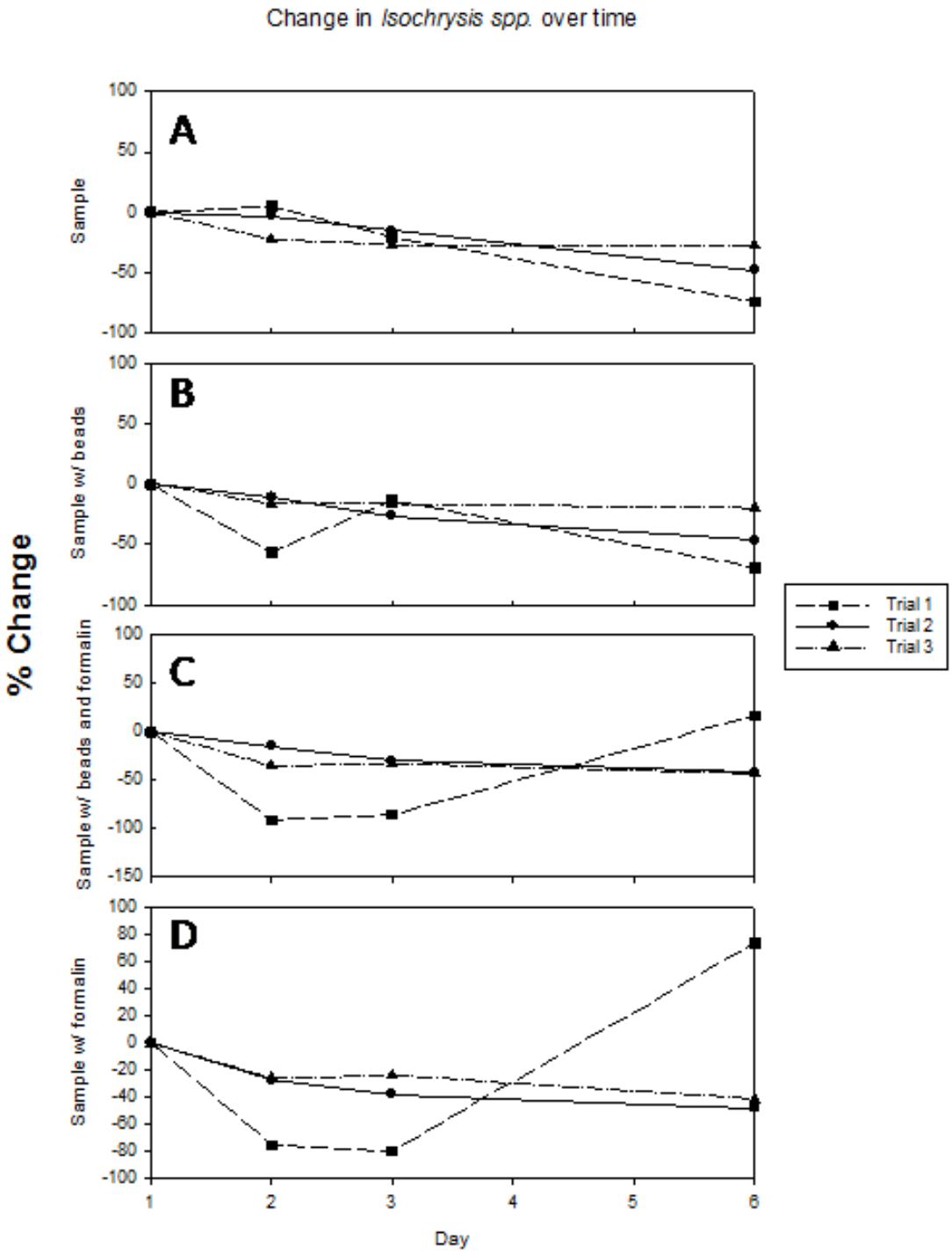


Figure 2. Percentage change in *Isochrysis sp*.counts over 6 days study period. Four different treatment options are: A) algae sample only, B) algae sample with counting beads, C) formalin preserved algae sample and D) formalin preserved algae sample with counting beads.

interaction effects between the treatments and sampling days in *Isochrysis spp.* Trial 4. Furthermore, there was one significant difference ($P < 0.05$) observed in the twelve contrasts analysis. The significant difference ($P < 0.05$) was observed when the counting beads

in distilled water, beads in mixed sample, beads in *T. chui* sample, beads in seawater, and beads in *Isochrysis spp.* where compared to the counting beads in 2% formalin preserved distilled water, beads in 2% formalin preserved mixed sample, counting beads in 2% preserved *T. chui*, beads in 2% formalin preserved seawater, and beads 2% formalin preserved *Isochyrysis spp.* samples.

Our observations have been equally important in the uses of flow cytometry and understanding any event occurring during sample preparation and analysis. Without having a detail observation log book, we would not have clues about what might go wrong during analysis in this study. Therefore, we included an observation section in our results to provide a better outlook about the procedure and analysis to our readers and flow cytometer users.

Research Observation

We observed decline in algae counts after the first day among the treatments and over time for both algae studied; *T. chui* and *Isochrysis spp.* both in our preliminary studies and all four trials used in this study. This decline is more pronounceable for samples preserved with 2% formalin and samples with counting beads. We observed a small short term increase in fluorescence of not only preserved, but also other treatment groups analyzed in this study (Figs. 1 and 2). This hike may be due to the blockage in energy transfer mechanisms in the pigments in preserved samples as stated by [6]. After three days, microscopic examination was very difficult and fluorescence intensities were reduced making it difficult to distinguish cells under compound microscope. We also observed higher algal counts in few samples (approximately 30% samples) due to detritus and background noise after six days, mostly in *T. chui* study on the flow cytometer (Fig. 4). This is mainly due to the clumping we observed with *T. chui* in formalin preserved samples and formalin preserved sample with counting beads. However, we only reported the results we obtained during the first week of this experiment. According to Wikfors (personal communication), *T. chui* is a lot stickier and clump more during analysis. This may cause the event reading low in the flow cytometer due to the fact that this clumping is counted as one during acquisition. In the fourth trial, *T. chui* was much harder to flush from the instrument than *Isochrysis spp.* Moreover, *Isochrysis spp.* seemed to be affected by formalin much more and quicker than *T. chui*. There was a further shift in *Isochrysis spp.* due to the formalin preservation (Fig. 5).

Fig. 4 shows the results from the fourth trial for *T. chui* over a 6-day period. Notice small changes in algae counts attributed to a short term boost in fluorescence intensities of cells, especially in 2% formalin preserved samples with counting beads. Fig. 3 shows the decline in algae counts obtained in flow cytometry from the fourth trial for *Isochrysis spp.* over a 6-day study period. Both 2% formalin preserved *Isochrysis spp.* and 2% formalin preserved *Isochrysis spp.* with counting beads declined more than other treatment groups after a 6-day study period.

Generally, we observed some shifts in both preserved treatments and preserved treatments with counting beads. After the first day, we established separate gates (regions on the graph) for those samples and obtained our algae counts on these new gates (Figs. 3 and 4). We monitored a greater shift in event rates in 2% formalin preserved *T. chui* compared to 2%

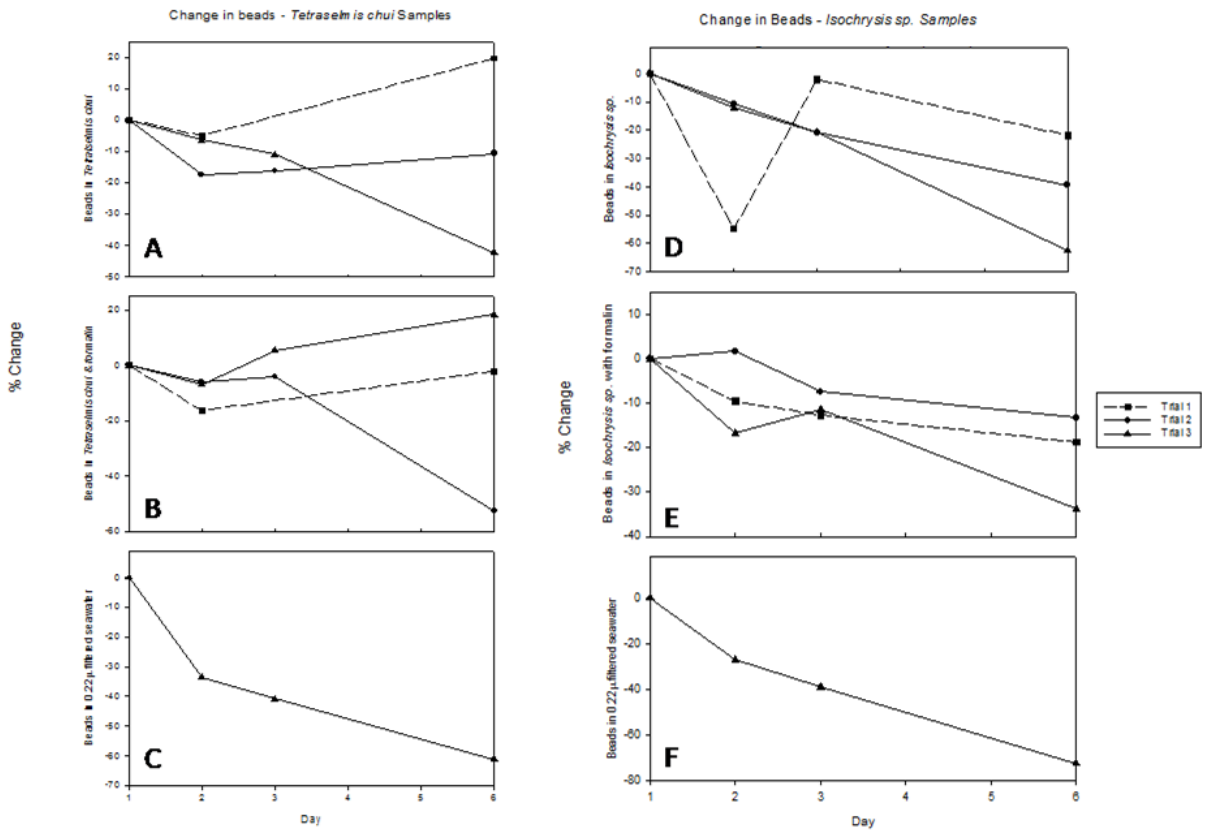


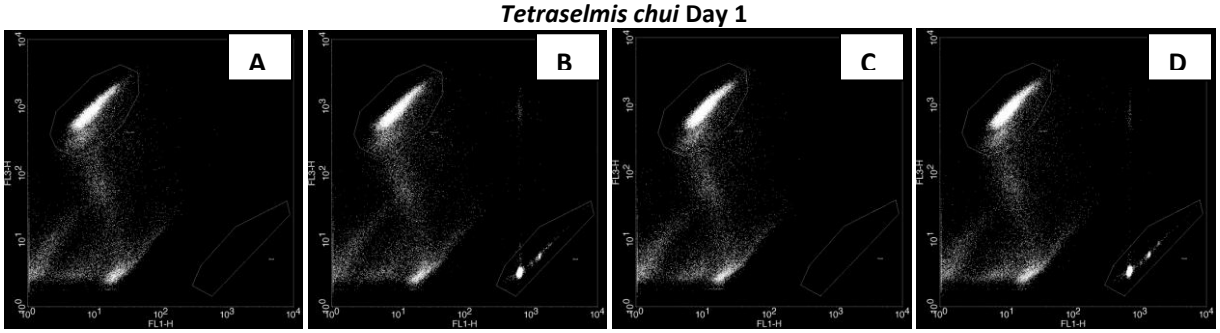
Figure 3. Percentage change in counting beads counts over 6 days study period. Six different treatment options are: A) counting beads in *T. chui* sample, B) counting beads in formalin preserved *T. chui* sample, C) counting beads in seawater, D) counting beads in *Isochrysis spp*. sample, E) counting beads in formalin preserved *Isochrysis spp*. sample, F) counting beads in seawater.

formalin preserved *Isochrysis spp.* However, hemocytometer readings were much more difficult to acquire for preserved *Isochrysis spp.* compared to preserved *T. chui* because of its smaller cell sizes (5-6 µm) and the faster reduction of fluorescent intensity of pigment with formalin. Fortunately, we did not observe any clumping with *Isochrysis spp.* making easier to count cells with the flow cytometry.

Formalin preservation and additional counting beads in samples caused an increase in fluorescence intensities of *Isochrysis spp.* cells in the third day and the fluorescence intensity declined after a six-day period in some samples. However, we observed decline in fluorescence intensity of *T. chui* after three days while fluorescence intensities seemed to increase again after six days. Especially preserved samples with counting beads shifted upward on the analysis plots for both species (Figs. 1 and 2). This may be mainly due to interaction between algae and counting beads causing equipment to pick them as a separate population during analysis.

We monitored shifts in bead counts and observed some seawater effects on the beads. In a separate experiment, we examined beads mixed in seawater and beads mixed distilled water. We also observed declined in bead counts in seawater after the first two days.

***Tetraselmis chui* Day 1**



***Tetraselmis chui* Day 6**

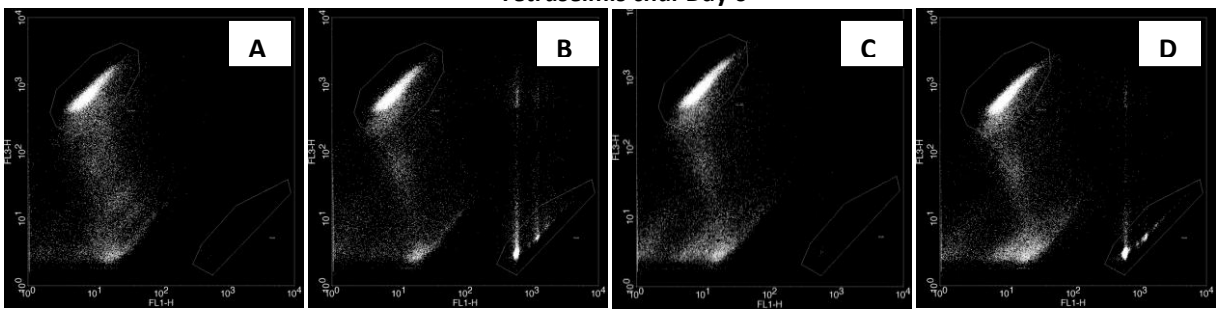
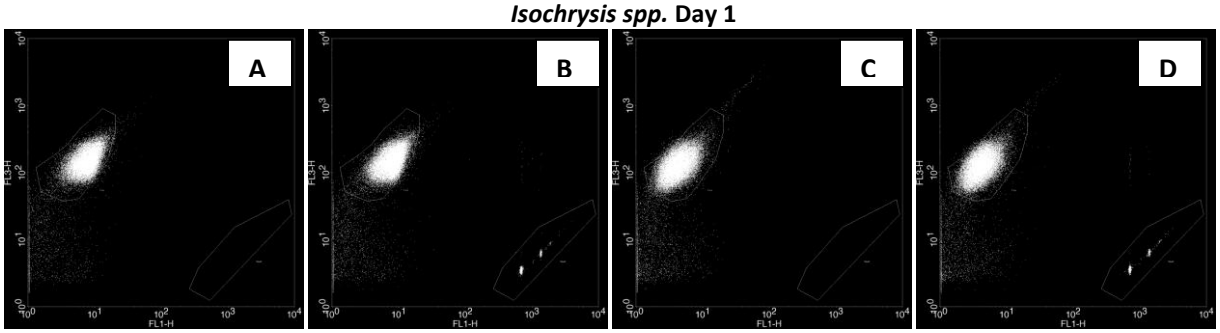


Figure 4. Dot plots showing event rates for *T. chui* in sampling days 1 and 6 in this study for four different treatments: A) algae sample only, B) algae sample with counting beads, C) formalin preserved algae sample and D) formalin preserved algae sample with counting beads. *T. chui* sample interfered with beads showing a separate population in the last sampling days on the plot, see arrow.

***Isochrysis spp.* Day 1**



***Isochrysis spp.* Day 6**

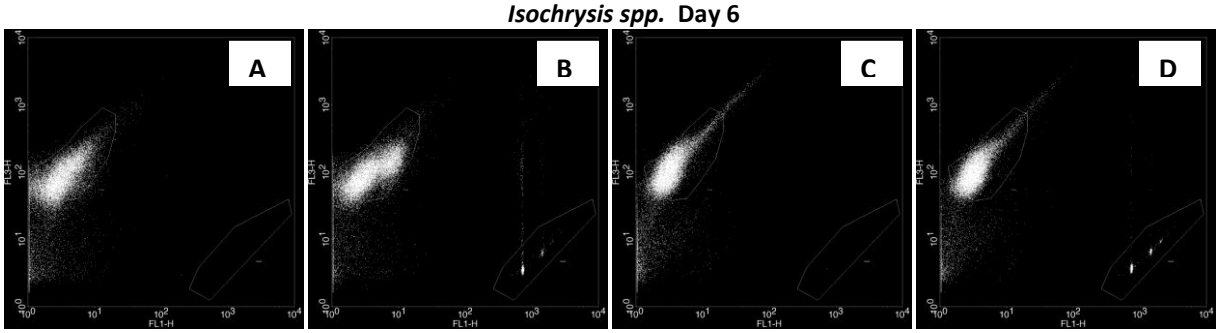


Figure 5. Dot plots showing event rates for *Isochrysis spp.* in sampling days 1 and 6 in this study for four different treatments: A) algae sample only, B) algae sample with counting beads, C) formalin preserved algae sample and D) formalin preserved algae sample with counting beads. *Isochrysis spp.* sample interfered with beads showing a separate population in the last sampling days on the plot, see arrow.

Confirming our thoughts that sea water was affecting the counting beads in some way (Fig. 3). No differences were observed between the first and the sixth days in counting beads counts in distilled water. During the fourth trial, we observed shorter flushing time of beads in the SIP line with distilled water than seawater (2 min vs. 5 min). Seawater seemed to affect the beads and beads seemed to stick up or clump in the tubing.

In these cases, beads and samples interfered with each other and created separate population that did not appear in the defined gates. It was possible to analyze our samples based on cell size and granularity and this might have resulted different responses we obtained in our analysis, but preservation cause burst in cell integrity. Results from this setting might have appeared shifted even more than what we observed in our experiment. Another reason we chose to use FL1 (green fluorescence) and FL3 (dark red fluorescence) fluorescence was the relatively small size of our algae. We had to use a logarithmic scale and the fluorescent intensity of cells better represented the algae populations of interest than the size and granularity which could be distorted because of the scale.

Discussion

Sampling marine phytoplankton with flow cytometry can be fairly easy if the protocol developed can be applied to your samples. There are a number of studies published on marine phytoplankton analysis, however further details on the instrument settings and operation, and sample preservation have been necessary to perform flow cytometry analysis without a long and frustrating training period. Generally, instrument settings play a major role for an accurate quantification of algae cells. Simple methods of preserving phytoplankton samples have also major effects on quantification, but these effects may not be as significant as others caused by analytical differences (instrument settings; threshold, plot determination…etc.) and human error. We used formalin preservation in this study because of its common use in algae sampling and phytoplankton preservation.

One would expect to find slight differences in the fluorescence properties of cells in formalin preserved samples as we obtained, however this shift in algae samples with beads was unexpected. A possible reason for this shift may be a higher number of counting beads possibly interfering with the fluorescence properties of the cells or both cell and bead integrity were broken from preservation and/or long storage. For instance, T. *chui* samples were extensively examined on flow cytometry, and were then examined under the microscope (Nikon TE200 Inverted Microscope) for comparisons of their cell properties. Microscopic examination showed fading of bright yellow fluorescence of preserved phytoplankton cells from their natural colors in unpreserved samples.

Generally, samples analyzed without formalin showed decreased in algal counts compared to those preserved with formalin after the first day of sampling. A few samples showed increased algae cells after three days in formalin preserved samples, supporting the observation of Dubelaar and Jonker [6]. Beside the blockage in energy transfer mechanisms in the pigments in preserved samples, decline in some algae counts may have been due to clumping.

In preliminary studies, we observed shifts in algae populations over time as the cells'

fluorescence intensity decreased. We also noted that using formalin to preserve samples caused shifts in the populations due to changing fluorescent intensity. The results from this study also suggested that the algae samples kept in the dark with and without formalin lose its fluorescence intensities after the first day of sampling. Samples preserved with formalin may not maintain their fluorescence intensities enough to be counted accurately on flow cytometry after the first day if one wants to obtain an accurate quantification of a population of an interest.

In this study with 2% formalin preservation, we established a separate gate for preserved samples and preserved samples with counting beads in all trials with *T. chui* and *Isochrysis spp.* (Figs. 4 and 5). We monitored shifts in both algae and beads population from a sample having counting beads in most of these trials. Interestingly, formalin preservation of *Isochrysis spp.* population shifted algal population faster as compared to *T. chui*. In most cases, this shift was towards less fluorescence areas on the plots (less FL1 and/or FL3). During analysis of *T. chui*, we observed separate population at high fluorescence intensity region on the plots (Fig. 4). Based on our microscopic examination, we observed interaction (clumping) between formalin preserved algal samples and counting beads after the first day of the study. According to Wikfors (personal communication) formalin added in the samples with counting beads may act together as surfactant and may foam bead solution. Surfactants may need to be there to prevent from coagulation of beads.

Besides effects of preservation on algae quantification, the uses of the proper settings and thresholds for the algae/beads that are being analyzed are important for flow cytometry technique. Settings changes are based on not only sample ages and how it is preserved, but also various algal species you may use, and if using more than one species, or if using algae and counting beads on the same plot, sometimes one may compromise on the settings to obtain both populations of interest. There may be an ideal setting for each component separately, but in order to get the total picture you may lose some of the quality, since the more complex the sample and the more detectors in use, the more one needs to compromise individual components (Wikfors 2004 – personal communication).

Threshold settings had significant impacts on the sample count during preliminary studies. SSC52 threshold settings showed lower algal concentrations than SSC 421 and FL3 52 in most cases. The FL3 threshold setting of 52 appeared to show the greatest amount of algae and beads (Table 7). A possible explanation for this is that SSC could not accurately represent *Isochrysis spp.* and *T. chui* cells in a logarithmic scale. The fluorescent intensity of a cell is not altered by the use of the logarithmic scale and is a more accurate representation of the cell. This is why we prefer it for our acquisition plots as well as our threshold settings [7]. Because, very low FL3 threshold is not eliminating anything but electronic noise, essentially all particles are being included as we expected. The beads show up above the threshold because the green fluorescence is so strong it is being detected in FL3. SSC 52 threshold settings showed lower algal concentrations than SSC 421 and FL3 52 in most cases (Fig. 6).

There a number of artifacts which we tried to minimize in this study which were important in order to obtain accurate information. These are the common errors that were caused by having

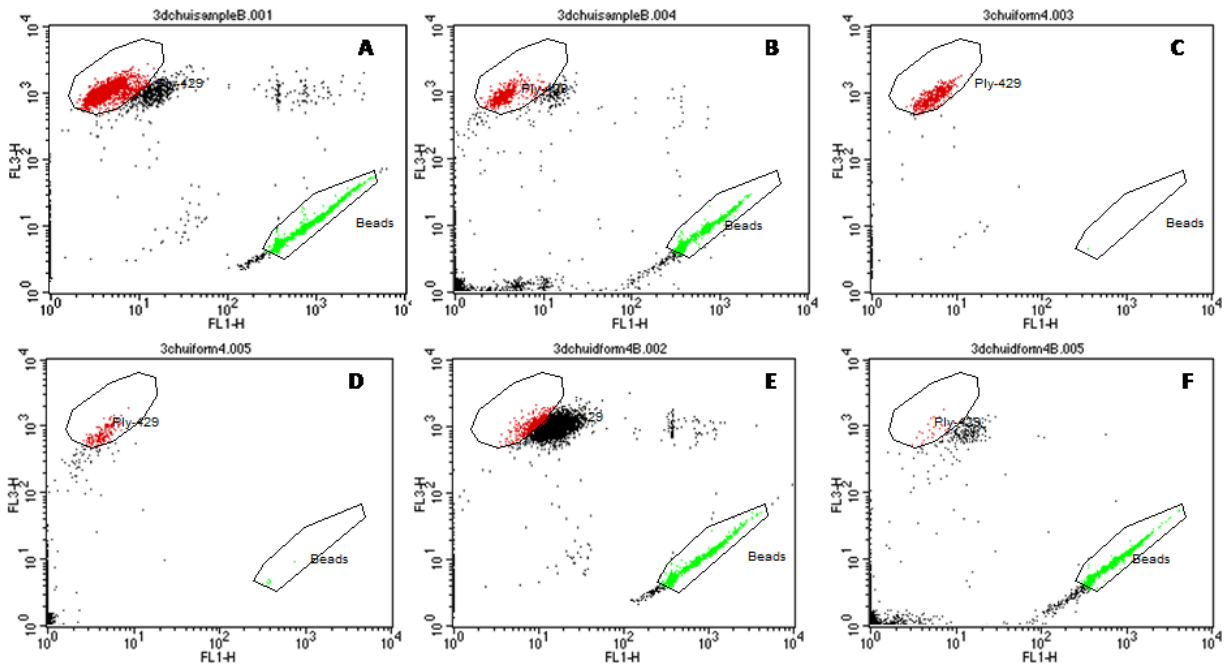


Figure 6. *T. chui* sample analysis on flow cytometry A) Sample with beads at FL3 52 threshold setting, B) Sample with beads at SSC 52 threshold setting, C) Sample with 4% formalin at FL3 52 threshold setting, D) Sample with 4% formalin at SSC 52 threshold setting, E) Sample with 4% formalin and beads at FL3 52 threshold setting and, F) Sample with 4% formalin and beads at SSC 52 threshold setting.

separate analysts working on the samples, leaving samples on the sample injection port too long, not vortexing samples enough, keeping samples in the light during analysis, analyzing algae in poor or good quality initially, not rinsing the sample injection port lines with 0.22 µm filtered seawater well between samples, using wrong acquisition template to run statistics on, clogging in the sample injection port line, and having separate persons to do algae counts in microscopic counts. This list probably goes further on and these are few major causes for an error during sample analysis in flow cytometry technique one may introduce.

Because phytoplanktonic cells are discriminated on the basis of scatter and pigment fluorescence, Marie et al. [9] suggested cryopreservation and short term freezing of fixed samples at -80°C provides better protection of phytoplankton cells for flow cytometry or microscopic examination. However, liquid nitrogen might not be available so Marie et al. [9] suggested that the use of aliquoted formaldehyde solutions may be used to preserve samples for a limited time of less than one week. They also noted that physical treatments such as centrifugation and classic or tangential filtration must be avoided because such techniques induce variable losses. Therefore, the development of alternate, practical, and economical preservation techniques is an important area for investigation and long term data collection.

This study, as well as preliminary experiments, was conducted to develop guidelines and a protocol to reduce the number of artifacts when preserving samples with commonly used levels of formalin solution; including considerations for the interactions between

Table 7. Event numbers of *T. chui* sample preserved with 2% formalin with counting beads at three threshold settings and on two different gates. Gates were established to isolate the population of an interest in the graph and reading and statistics were performed for the samples in the gates.

Threshold Setting	*Tetraselmis chui* (strain PLY-429) with 2% formalin & 2 µm beads Events at Low Run (12 µl/min)		
		Adjusted Gate	Unadjusted Gate
FL3 52	**Sample**	4299	1827
	Bead	20480	20480
	Sample	3786	1628
	Bead	21865	21865
	Sample	3197	1360
	Bead	21693	21693
SSC 52	**Sample**	1262	456
	Bead	16995	16995
	Sample	1350	531
	Bead	17333	17333
	Sample	1503	569
	Bead	18104	18104
SSC 421	**Sample**	3034	1380
	Bead	21340	21340
	Sample	3015	1322
	Bead	21076	21076
	Sample	3078	1335
	Bead	20516	20516

algal species and for instrument settings. During our preliminary study, we monitored algal counts for one week from samples kept in dark at cool temperature of 4°C with and without formalin preservation. We repeated this preliminary work twice to determine the optimum time frame in which to count cells with the flow cytometer. After three days, about 75% of the formalin-preserved (treated) samples were clustered and counting could not be done accurately resulting in lower cell counts. The remaining 25% of samples could not be counted with flow cytometry. After 6 days, we observed a sharp decline in cell counts from

unpreserved (untreated) samples. If fluorescent stain had been used during our preliminary studies, we might have observed better cell counting, but staining may not work for some of the phytoplankton cells studied or may not be available.

If samples are going to be preserved, a fluorescent stain may be useful. Fluorescent intensity degrades over time and may need to be compensated for. When looking at a single algal population, this is a less of a concern. Even if the algal population of an interest has shifted due to their sizes and complexity on the flow cytometer plots, it may still be isolated by using fluorescent stains. However, when fluorescent intensity is being used to isolate multiple species a reduction of fluorescence may result in an apparent overlap of species that would otherwise be distinctive. Since formalin caused a major shift in *Isochrysis spp.,* it does not seem worthwhile to preserve *Isochrysis spp.* with formalin because of the rapid degradation.

This study as well as preliminary experiments was conducted to account for any discrepancies in samples preserved with commonly used levels of formalin solution, interactions between algal species and instrument settings. The intention of this study was to create some guidelines and protocol development methods for flow cytometry users as it relates to phytoplankton analyses. Further studies may need to be done to examine these interactions with other individual cultured and natural algae species, and instrument settings. Although we observed shifts in cell counts over time as well as differences in cell counts between the treatments for both cultured algae species in these trials, we would expect to obtain different responses from other species that may be analyzed with flow cytometer. Formalin preservation may have minimal effects on natural algae during longer storage for flow cytometric analyses. However, we strongly suggest analyzing samples as soon as possible for greater accuracy.

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