NUTRIENT ANALYSIS: STUDYING THE IMPACTS OF FREEZING AND THAWING PROCESSES ON SILICATE CONCENTRATIONS USING PROGRAMMABLE FLOW INJECTION

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ABSTRACT

Oceanographic nutrient analysis is important for understanding biogeochemical cycling. However, research cruises last days, weeks, or months, requiring oceanographers to preserve collected nutrient samples when shipboard analysis equipment is unavailable. Samples are often preserved by freezing because of the simplicity of the technique. The effects of freezing on nutrient concentrations has been studied for decades, with still no consensus for freezing/thawing method. Because silicate is known to polymerize (silicate molecules bond with each other), this study focused on the effect of the freezing step in silicate behavior by investigating differences in silicate concentrations between varied storage methods and durations. Samples were preserved at three different temperatures (refrigerated, -18°C freezer, and -80°C freezer), then thawed at room temperature overnight and measured at three intervals post-collection and compared to shipboard measurements. Cruise and post-cruise concentration measurements were determined using programmable flow injection, a novel spectrophotometric technique via the Mini-Sequential Injection Analysis (mini-SIA) platform. Autodilution, a recently developed technique that automatically dilutes high-concentration standard solutions via computer-controlled pumps, and the silicate chemical assay were optimized prior to sample measurements. In this study, results from the samples with silicate concentrations higher than 100 μM showed the most variability, and the data from the refrigerated samples showed the most stability over the entire twelve-week preservation period, indicating refrigeration is a reliable preservation method for silicate-containing samples.

Keywords: seawater, freezing, thawing, silicate, polymerization, preservation

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1.0 INTRODUCTION

1.1 OCEANOGRAPHIC NUTRIENT ANALYSIS

Open ocean nutrient analysis is essential for understanding air-sea-land and biological interactions, providing insight on ecosystem productivity and responses to changes (Sun et al. 2024). Oceanographic research vessels deploy rosettes to collect water samples from desired depths. Rosettes are equipped with sensors that measure parameters like temperature, dissolved oxygen, and pressure, providing valuable real-time data. However, for nutrient analysis, there are not sensors for directly measuring nutrient parameters. Therefore, nutrient samples are often collected from the rosette upon resurfacing and stored for land-based measurements at a later date. This is due to lack of lab space, equipment or nutrient analysts during shipboard sampling periods. Samples are stored multiple ways, including poisoning, acidification pasteurization, and freezing (Daniel et al. 2012). Freezing is simple, and therefore a widely accepted storage method, however, there is no standardized freezer temperature, duration, or thawing procedure. Additionally, research vessels do not all have the same types of freezers, mechanical problems cause temperatures to fluctuate while aboard, and there is risk of samples thawing during transport. Debate regarding nutrient sample preservation has plagued oceanographers for decades, and many experiments have been performed to study the effects of freezing and thawing on analysis accuracy (Table 1).

1.2 POLYMERIZATON OF SILICATES

Studying silicate concentrations in seawater samples after preservation is of particular interest because of the known polymerization of silicates during freezing. Polymerization is the bonding of (in this case) silicate monomers to form chains, or polymers (Hess 1970). As water around silicate molecules freezes, silicate molecules are driven towards each other and bond. The resulting polymers can alter the measured concentrations by spectrophotometric methods. Table 1 summarizes several effects of freezing and thawing processes on silicate concentrations from seven different papers. The specifics of the freezer temperature used and thawing and storage times are different, but all of the findings indicate a reduction in the frozen sample silicate concentrations in contrast to concentrations measured in the freshly obtained samples. Importantly, the deviation is larger at higher concentrations (more than 30 μM). This is expected because as silicate concentration increases, there are more silicate interactions and more polymerization is likely to occur (Hess 1970).

Table 1: An overview of published methods for the freezing, thawing, and duration specifics of silicate-related sample preservation studies and a summary of the key findings.

Paper	Storage method	Thawing method	Time frozen	Results
Burton 1970	Frozen at -20 degrees	N/A	2 days	No significant error at normal salinity levels
MacDonald and McLaughlin 1982	Frozen at -10 degrees	12 hours	2 weeks, 1,2,5 months, 1 year	Different results based on thaw time, losses compared to fresh, particularly for longer periods being frozen
Dore et al. 1986	Frozen at -20 degrees and refrigerated	4-12 hours at room temp or water bath	1 week and 1 year	Losses at greater than 120 µM
Macdonald 1986	Freezing, temperature not reported	8 hours	2 weeks, 1, 2, 5 months	Deviations are strongest at concentrations greater than 70µM
Chapman and Mostert 1990	Frozen at -20 degrees	24 hours	5 weeks	Deviations from fresh at about 30 µM
Segura-Noguera 2011	Frozen at -20 degrees	N/A	1 week	Freezing is valid if less than 60µM
Rho et al. 2022	-18, -23, -80 degrees	Under ambient light in 35 degree water bath	300-400 days	Differences between fresh and frozen samples for high concentrations

1.3 MINI-SIA PLATFORM

The mini-Sequential Injection Analysis (miniSIA-2) platform is a relatively newly developed analytical system, which uses spectrophotometric detection to determine the concentration of nutrients in seawater samples (Hatta et al. 2021). Continuous flow analysis (CFA) has been a primary, reliable method for analyzing nutrient samples aboard ships or days, weeks, or months after collection in shore-based labs (Becker et al.

2019). However, CFA requires large volumes of sample and reagents and analyzing samples later can skew results (Ma et al., 2014). In comparison, the cost and size of the novel mini-SIA instrument alone make it attractive. The instrument is easy to transport on and off ships and requires only 600 μL of sample per replicate, making it an extremely convenient option for in-situ nutrient analysis (Hatta et al. 2020). The instrument is also capable of automatically diluting a single high-concentration standard (a feature named autodilution) to produce a calibration curve, and then calculate the concentrations of unknown samples for a specific nutrient using the resulting curve. The entire setup requires less than 1 meter of lab bench space (Figure 1). Using the mini-SIA system, nutrient samples can be collected and analyzed at-sea on the same day they were collected, or at a later date in a shore-based laboratory. This reactivates the set of questions regarding the stability of samples during freezing and thawing.

Figure 1: The mini-SIA instrument setup in the shore-based laboratory: the instrument,

carrier solution and reagents on the left side, connected to a laptop and monitor running the Flo-ZF program.

1.4 GOALS AND APPLICATIONS OF THIS WORK

The goal of this work is to optimize and utilize the miniSIA-2 platform's autodilution feature to validify the platform's capabilities as an oceanographic research tool and establish a knowledge baseline of silicate sample preservation and the implications of storage on subsequent determination accuracy. This thesis is composed of three parts. First, autodilution accuracy was tested by generating calibration curves and varying factors such as standard dilution method (manual or autodilution), standard salinity, and standard aspiration time. Second, lab-made silicate standards were prepared and measured one week after being stored three different ways (refrigerated, frozen in a -18°C freezer, and frozen in a -80°C freezer). Third, seawater samples were collected and measured at different time intervals (at-sea, 1,6, and 12 weeks) after being stored in the same three ways as the lab standard experiments. The varied factors in the silicate experiments include salinity, presence of biological organisms, and storage temperature. Based on the literature, it was hypothesized that silicate concentrations would be lower after storage than they were prior to storage.

2.0 AUTODILUTION VERIFICATION

2.1 INSTRUMENTATION

The miniSIA-2 instrument utilizes a Lab-On-Valve (LOV) rotating system, where various reagents and standards are assigned to numbered ports (1-6) (Figure 2A). Using an LOV is advantageous because the carrier solution, reagents and sequences can be easily modified for different chemical assays. Figure 2B shows a schematic of the instrument, where two milliGAT pumps use a carrier solution, deionized (DI) water, to push reagents and standards within two temperature-adjustable (thermostat) holding coils. A flow cell is attached to port 2 on the LOV, where the final reacted product is dispensed from the holding coils to the flow cell and stored for 20 seconds to determine the target wavelength (20 cm).

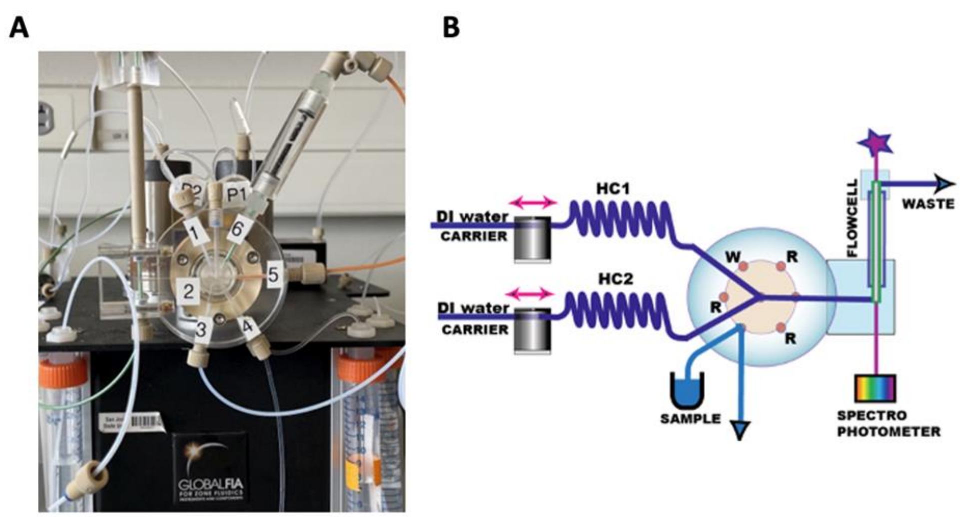

Figure 2: A: mini-SIA LOV system, with ports labeled 1-6. B: Schematic of a rotating LOV (center) system attached to and controlled by two bi-directional computer controlled milli-GAT pumps attached to each holding coil (HC1 and HC2). Completed chemical assays are transferred to the flow cell, where the chemical mixture is paused and the target wavelength is measured by the spectrophotometer. From Hatta et al., 2021.

Calibration curves plotting absorbance units vs. concentration must be made daily prior to measuring unknown samples, and are made with the absorbances of 5 different concentrations. A linear equation is generated, which is then used to calculate the concentration of an unknown sample after the absorbance of that sample is measured. Calibration curves can be made two ways: using manual dilution or autodilution. Manual dilution means making a calibration curve by manually preparing standards and measuring the absorbance of each standard individually. Autodilution is a technique that uses the difference in bidirectional flow volume and flowrates (aspirate and dispense) to dilute a sample or standard solution. As one pump is aspirating a standard solution, the second pump is dispensing a diluent (carrier solution, DI water in this study); when the dispensing flow rate and volume is lower than the aspiration, the result is low pressure that causes the standard solution to fill volume void. This phenomenon is called natural aspiration (Figure 3). By adjusting the ratio of flow rates and volumes between aspiration and dispensing, the dilution factor can be precisely controlled, allowing the solution to be diluted accurately through sequence programming in FloZF software.

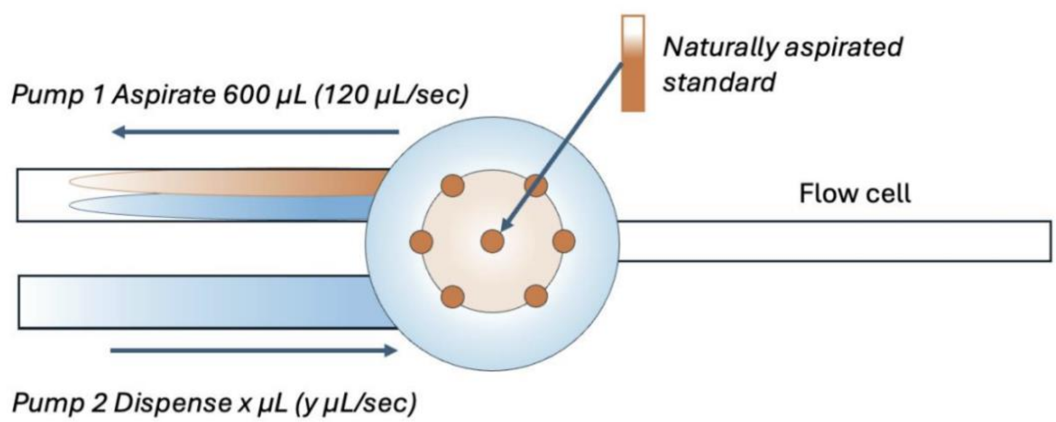

Figure 3: A schematic of natural aspiration by dispensing a volume, x, at a flow rate, y. From Davis, 2024.

Standard dilution is automated by autocalibration by using the program's counter system (Figure 4, Box 1). Several dilutions are predetermined by changing the ratio of the dispensing and aspirating flow rates to create a set of standard concentrations from a single, concentrated standard solution. These standards are measured in order from highest to lowest concentrations and are then automatically plotted as absorbance units (AU) vs. nutrient concentration (µmol/L), resulting in a calibration curve that can be used to calculate the concentrations of unknown samples. The number of dilutions desired per table, and the 'DilVol' and 'DilFR' commands establish the specific volume and flow rates, respectively, needed to create the desired standard concentrations (Table 2). There is always 600 µL of sample aspirated, and there must always be a volume of diluent dispensed (thus, the highest standard must always be diluted a small amount). Calibration curves can also be made using a manual sequence, which uses the same method, but excludes certain steps such as the automatic counter system highlighted by Box 1 in Figure 4 (Hatta et al., 2024). Manually made standards are introduced individually, and the software does not dilute them. The absorbances are plotted to generate a standard curve.

The benefits of programmable flow injection (pFI) are multifold. One benefit is that the final solution is stopped in the flow cell for measurement (Figure 4, Box 3). This means that the schlieren effect (changes in the refractive index of a flowing sample) is eliminated and the spectrophotometer signal can be corrected by a previously determined baseline (Hatta et al., 2021).

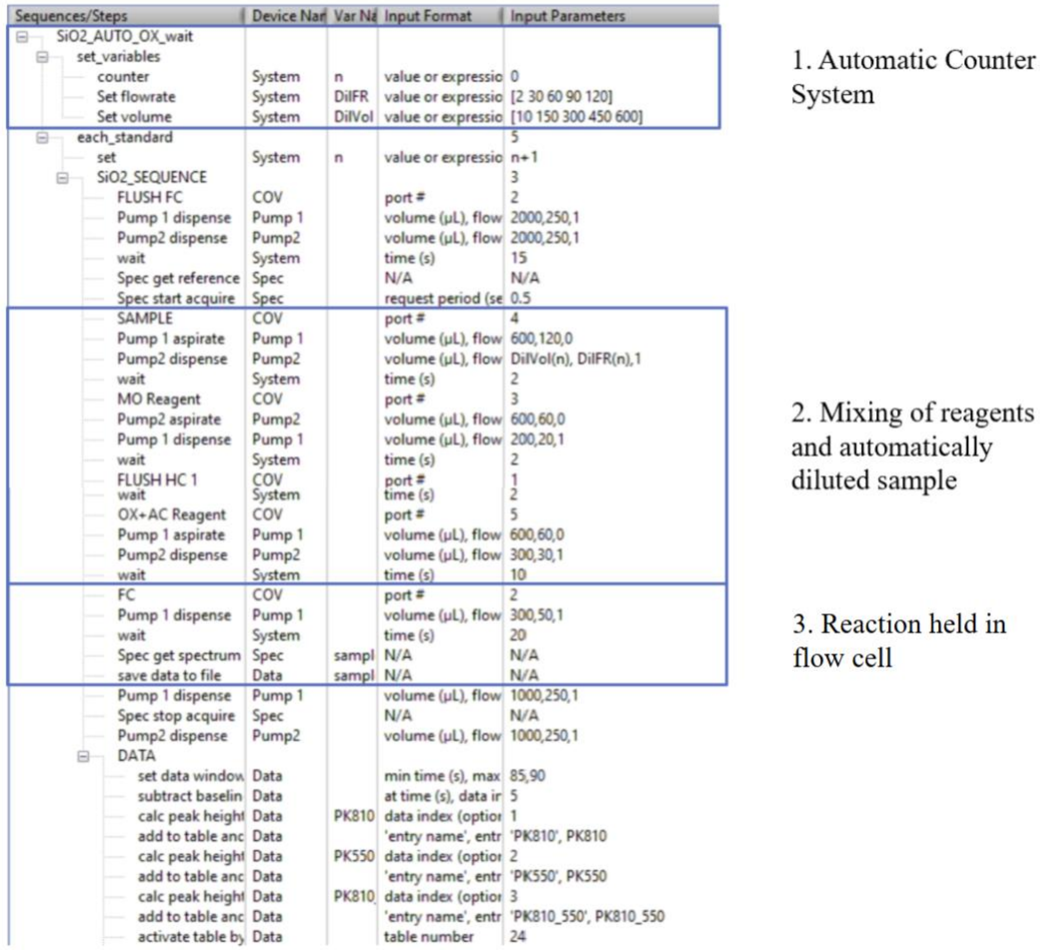

Figure 4: The sequence begins by assigning volumes and flow rates for a set number of dilutions indicated by the counter. For each determination, reagents are dispensed and mixed with the diluted sample. The spectrometer gets a baseline reference. The reacted sample is pushed into the detector and its spectrum is then measured and saved.

Table 2: Concentration of a 20 µM silicate standard after autodilution at specified volumes and flowrates.

Aspirated Sample Volume (µL)	Diluent Volume 'DilVol' (µL)	Diluent Flowrate 'DilFR' (µL/s)	Approximate resulting concentration using a 20 µM standard
600	10	2	19.7
600	150	30	15
600	300	60	10
600	450	90	5
600	600	120	0

2.2 SILICATE ASSAY

All experiments were conducted using established pFI protocol (Hatta et al. 2021) using the miniSIA-2 platform. The platform was manufactured by Global FIA (Seattle, WA). Equipped milliGAT pumps are manufactured by Valco Instruments. The external light source was a tungsten Ocean Insight HL-2000 (Orlando, FL). The Spectrophotometer is an Ocean Optics FLAME-T with a near infrared (NIR) range (343 nm – 1033 nm). The instrument uses a 20 cm length flow cell with green polyetheretherketone (PEEK) tubing (0.8mm I.D.). The two thermostatic holding coils consist of a 1 m coil of polytetrafluoroethylene (PTFE) tubing (0.8 mm I.D.) with an internal volume of 1mL. The holding coils are held at 50°C for all experiments.

In addition to a silicate standard or sample, the silicate method requires 2 reagents: an optimized molybdate solution (reagent 1) and a mixture of 2% oxalic acid (OA)/ascorbic acid (AA)/sodium dodecyl sulfate (SDS) solution (reagent 2). The mixing of these 3 solutions is shown in Figure 5. To prepare 200 mL of the molybdate reagent, 2.0 grams of ammonium molybdate tetrahydrate (A674-500, CAS12054-85-2, certified ACS, Fisher Scientific) was added to 100 mL of DI water. Once the ammonium molybdate tetrahydrate was dissolved, 1 mL of concentrated sulfuric acid sulfuric acid (A300-500, CAS7664-93-9, certified ACS, Fisher Scientific) was added to the solution. The solution was then topped up to 200 mL with DI water. This solution is stable for one month (Hatta et al. 2021). To produce a 2% OA (75688-50G, CAS144-62-7, Sigma-Aldrich)/AA (A15613, CAS50-81-7, Alfa Aesar)/SDS (J75819-22, CAS151-21-3, Thermo Scientific) 50 mL solution, 1 gram of each chemical was sequentially added to 50 mL of DI water. This solution is only stable for a few days.

For the silicate stock solution, 0.5 M hydrochloric acid (HCl) solution was used to neutralize the intermediate silica standard. A 1000 µM silicate standard (15747-100ML, Fluka) was used as the intermediate, and working standards were diluted form that with DI water. To use the autodilution calibration curves, a working standard solution with a 20 µM silicate concentration was prepared. When the manual calibration curve was being made, 5, 10, and 15 µM standards were manually diluted from the stock standard solution with DI water. Working standards were made fresh daily and all solutions were made gravimetrically for accuracy.

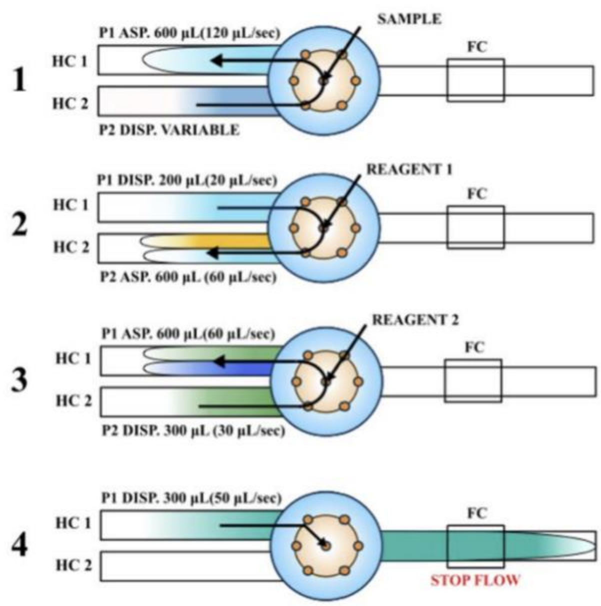

Figure 5: A schematic of holding coil mixing for the mini-SIA silicate assay, from Davis 2024.

2.3 AUTODILUTION VERFIFCATION EXPERIMENTS

2.3.1 COMPARING MANUAL AND AUTODILUTION

Differences between manual and autodilution calibration curves were tested to verify the accuracy of the autodilution feature of the instrument prior to any freezing/thawing experiments. This was done by making manual and autodilution calibration curves on the same day with the same standards and reagents to make note of any differences between the two methods.

Figure 6 shows the overlay of two calibration curves for the silicate assay made on the same day. The blue line was produced using the autodilution feature, while the orange line was produced with manually-made standards. The ratio between the slope values on one day was 0.9937. The difference between manual and auto dilution was deemed negligible based on the capabilities of the instrument.

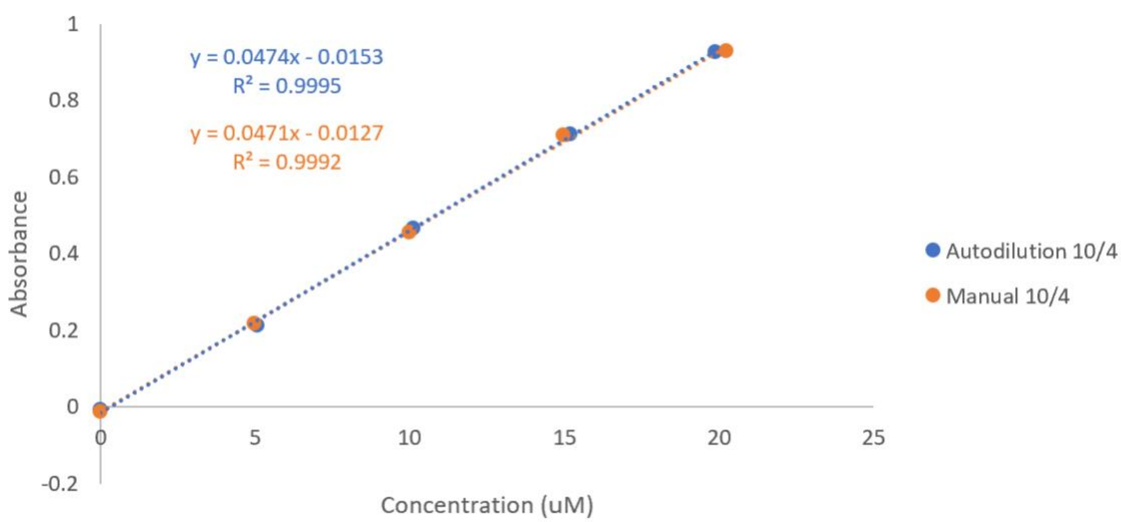

Figure 6: Calibration curves for the manual and auto dilution silicate sequences produced on the same day and overlaid.

2.3.2 ALTERING FLOW RATE

It had been observed in prior experiments that when using the autodilution sequence, the highest and lowest points on the calibration curve result in the most variability between curves (Figure 7). Thus, one of the goals of these experiments was to determine the limits to the detectable range of the pFI autodilution sequence. Autodilution was tested by making three different calibration curves using different natural aspiration flow rates to determine if flow rate was affecting the lowest and highest points of the calibration curves.

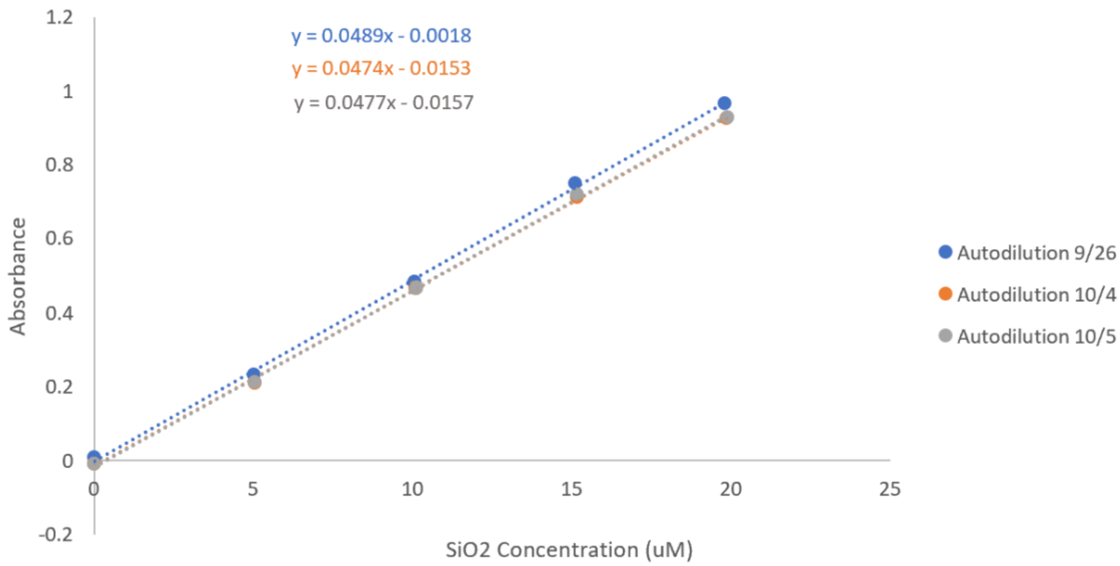

Figure 7: Calibration curves for the silicate assay made using autodilution on three different days, overlaid.

At first, to visually see the physical mixing within the steps, a manual calibration curve was created using manually made bromothymol blue (BTB) standard solutions of 132 ppb, 264 ppb, 396 ppb, and 528 ppb. The indicator dye was used to avoid any chemical effects and matrix influences inherent in a reaction sequence. Then, the autodilution sequence was used to create a calibration curve with the same concentration range. The volume of diluent was set to 150µL, 300µL, and 450µL. The flow rate was originally set to 30µL/s, 60µL/s, and 90µL/s. These flow rates resulted in the BTB solution being aspirated in 5 seconds. The above process was then repeated, but with slower flow rates (15µL/s, 30µL/s, and 45µL/s). These flow rates resulted in the BTB solution being aspirated in ten seconds. Lastly, flow rates were changed to 6µL/s, 12µL/s, and 18µL/s (solution aspirated in 25 seconds). It was hypothesized that these two experiments would provide information on detection limits associated with flow rate.

The linear equations for the calibration curves made using each aspiration time are plotted in Figure 8. The ratios of the 5 second, 10 second, and 25 second autodilution slopes are all ~1 in relation to each other, and within the error of the method (3%).

Altering the aspiration time for the sample did not result in any calibration curve differences, even at the ends of the curve, indicating that flow rate does not affect the autodilution capabilities of the instrument, disproving the pressure-vacuum hypothesis. For the remainder of the experiments, the 5 second aspiration time was used.

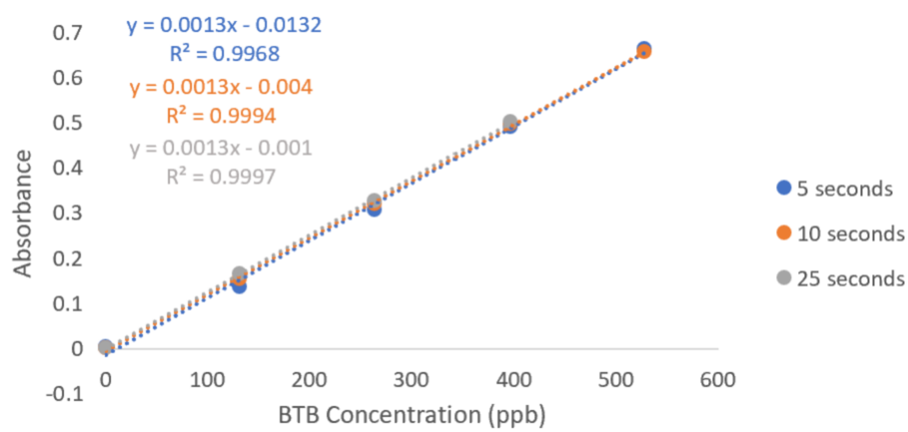

Figure 8: Calibration curves and their equations produced using the instrument's BTB auto dilution sequence with different sample aspiration times.

2.3.3 INTRODUCTION OF SALT TO THE SYSTEM

In order to use autodilution on silicate-containing seawater samples, it is important to confirm that there is no difference between different sample matrices. A series of lab tests were done testing the influence of salinity on the instrument's auto dilution capabilities for the silicate assay. Calibration curves were made using auto dilution for DI-based standards and DI-water carrier solution, saline standards and DI-water carrier solution, and saline standards and saline carrier solution. Saline solutions were made by adding 3.5% w/w NaCl to DI-water to approximate a salinity of 35 psu.

Lab tests introducing salt to the system were performed to determine the effects before measuring silicate concentrations in seawater samples. The salinity of standards and carrier solution were altered in 3 different ways. Figure 9 shows that upon addition of salt, slope values decreased. The ratio between the slope of the curve made with DI-based standards/DI-based carrier solution and the slope of the curve made with saline standards/DI-based carrier is 0.943, indicating a ~6 percent decrease (larger than the methodological error). However, the ratio between the slope of the curve made with saline standards/DI-based carrier and the slope of the curve made with saline standards /saline carrier is 0.976, which is a difference within 3%, the methodological error.

Introducing salt to the system (via the standard solution) caused a statistically significant decrease in the slope of the calibration curve. However, once salt was present in the standard, additional salt (via carrier solution) did not cause a further statistically significant change. Notably, the two highest concentrations (corresponding to the lowest dilution) show the greatest difference between DI/saline matrix combination. For seawater sample analysis in this study, autocalibration was performed using a DI-based standard was used for autocalibration, and samples were requiring dilution were diluted with DI water manually.

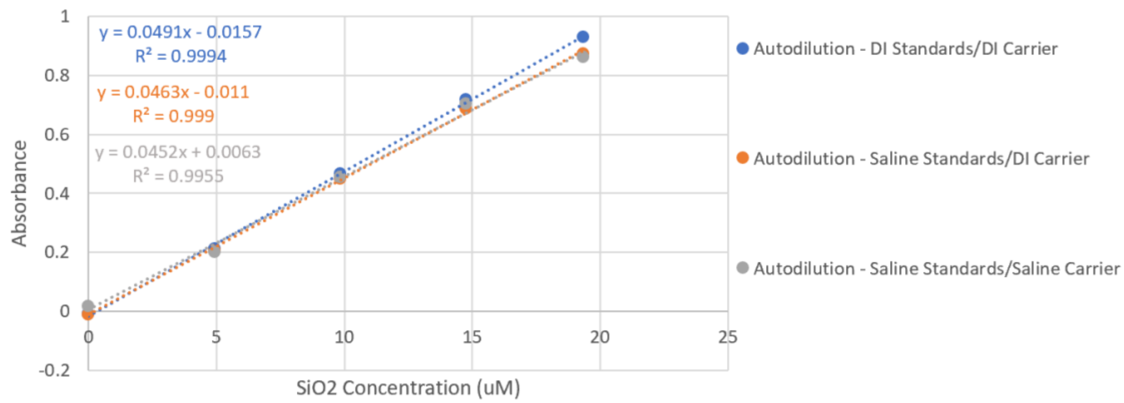

Figure 9: Calibration curves and their equations for auto dilution silicate sequence used where salinity of standards and carrier solution was varied.

2.3.4 AUTODILUTION ACCURACY BASED ON DILUTION FACTOR

The range of oceanic silicate concentrations (1 to ~150 µM) is greater than can be accommodated in a single standard curve. However, the autodilution feature can also be used to dilute high concentration samples into a more limited calibration range. To establish the validity of this instrument's ability to dilute an unknown sample by varying dilution factors was also tested with KANSO Certified Reference Material (CRMs). A calibration curve was made, and reference material was measured at different dilution factors: 2x, 3x, 4x, 6x, and 8x. It was hypothesized that diluting more times would result in more deviation from the consensus values of the reference material.

Four different CRMs ranging from 14 to 100 µM silicate were tested. The calibration curve that the unknown sample concentration calculations are based on were in the range from 0 to 20 µM silicate, keeping absorbance below 1 AU above which nonlinearity is observed. Thus, samples with concentrations outside of that range must be diluted to fall within the range of the calibration curve. It was observed that diluting to a concentration at the higher end of the calibration curve resulted in more error than towards the concentrations on the middle of the calibration curve. When diluted to a concentration towards the middle of the curve, the error is within 3%, even at higher dilution factors.

Testing autodilution of an “unknown” KANSO standard revealed that diluting high-concentration standards to a concentration in the middle of a calibration curve is optimal to reduce error. This knowledge was used in the later experiments when diluting the DI-based standards and seawater samples.

Table 3: Instrument calculated SiO_2 values at different dilution factors using KANSO certified reference material (DI standards and carrier).

KANSO CRM Code:	CR	CO	CM	CM
Consensus Value (µM SiO):	14.0 ± 0.3	34.72 ± 0.16	100.5 ± 0.5	100.5 ± 0.5
Dilution factor:	1x	2x	6x	8x
Average calculated concentration (µM SiO):	13.81 ± 0.12	34.70 ± 0.35	89.97 ± 1.5	96.63 ± 1.36
% Error (averaged value compared with the consensus value):	1.36	0.06	10.47	3.85

3.0 FREEZING AND THAWING EXPERIMENTS WITH LAB-MADE, DI-BASED STANDARDS

3.1 EXPERIMENTAL DESIGN

To test the impacts of freezing and thawing on silicate concentrations without the added factors of salinity and biology, 10 standards with different concentrations were made by weight using DI water. Concentrations ranged from 1 to 150 µM silicate, to mimic the concentrations of a full depth silicate profile at Station ALOHA (45.0'N, 158 00.0'W). The samples were measured using the instrument's Unknown sequence (Figure 10), and were diluted by hand to fall towards the middle of the calibration curve (about 10 µM SiO_2 and 0.5 AU) prior to being measured. The samples were measured the day they were made (Day 0), and divided into three groups to be preserved in three different ways: a refrigerator (4℃), -18 ℃ freezer (-18℃), and -80 ℃ freezer (-80℃). All three groups were taken out after six days and left standing at room temperature in a dark room and thawed for 18-24 hours. The standards were then measured one week (Day 7) after being made to study stability after being frozen one week.

Sequences/Steps	Device Nar	Var Na	Input Format	Input Parameters
UNKNOWN SiO2				
name sample	Data		'sample name'	'Shallow 4 Fridge'
each_standard				1
SiO2_SEQUENCE				3
FLUSH FC	COV		port #	2
Pump 1 dispense	Pump 1		volume (µL), flow	2000,250,1
Pump2 dispense	Pump2		volume (µL), flow	2000,250,1
wait	System		time (s)	15
Spec get reference	Spec		N/A	N/A
Spec start acquire	Spec		request period (se	0.5
SAMPLE	COV		port #	4
Pump 1 aspirate	Pump 1		volume (µL), flow	600,120,1
wait	System		time (s)	2
MO Reagent	COV		port #	3
Pump2 aspirate	Pump2		volume (µL), flow	600,60,0
Pump 1 dispense	Pump 1		volume (µL), flow	200,20,1
wait	System		time (s)	2
FLUSH HC 1	COV		port #	1
Pump 1 dispense	Pump 1		volume (µL), flow	1000,250,1
wait	System		time (s)	2
OX+AC Reagent	COV		port #	5
Pump 1 aspirate	Pump 1		volume (µL), flow	600,60,0
Pump2 dispense	Pump2		volume (µL), flow	300,30,1
wait	System		time (s)	10
FC	COV		port #	2
Pump 1 dispense	Pump 1		volume (µL), flow	300,50,1
wait	System		time (s)	20
Spec get spectrum	Spec	sampl	N/A	N/A
save data to file	Data	sampl	N/A	N/A
Pump 1 dispense	Pump 1		volume (µL), flow	1000,250,1
Spec stop acquire	Spec		N/A	N/A
Pump2 dispense	Pump2		volume (µL), flow	1000,250,1
DATA				
set data window	Data		min time (s), max	85,90
subtract baselin	Data		at time (s), data ir	5
calc peak height	Data	PK810	data index (optior	1
add to table anc	Data		'entry name', entr	'PK810', PK810
calc peak height	Data	PK550	data index (optior	2
add to table anc	Data		'entry name', entr	'PK550', PK550
calc peak height	Data	PK810	data index (optior	3
add to table anc	Data		'entry name', entr	'PK810_550', PK810_550
activate table by	Data		table number	5
calc value	Data	Unknc	dilution factor	0.1
add to table anc	Data		'entry name', entr	'Unknown', Unknown

Figure 10: FloZF sequence for measuring silicate concentrations in unknown samples.

3.2 MEASURED SILICATE CONCENTRATIONS AFTER ONE WEEK IN STORAGE

The obtained results in Figure 11 shows the effects of the preservation method in silicate concentrations on silicate standards by using a “perfect” 1:1 line with the Day 0 measured values. The majority of the refrigerated samples fall along the 1:1 line (aside from the point at 130 µM). For each freezer temperature, the one-week-storage measurements are lower than the Day 0 measurements. For each freezer method, the deviation from Day 0 measurements increases as the concentration of the standard increases, which could be attributable to dilution because higher concentrations required higher dilution factors. The refrigerated samples are the closest to Day 0 values. The -18 °C freezer samples are not accurate at all, remaining near 0 and never exceeding 20 µM. The -80 °C freezer samples fall between the refrigerated and -18 °C freezer samples, but are still only a fraction of what the Day 0 concentration was.

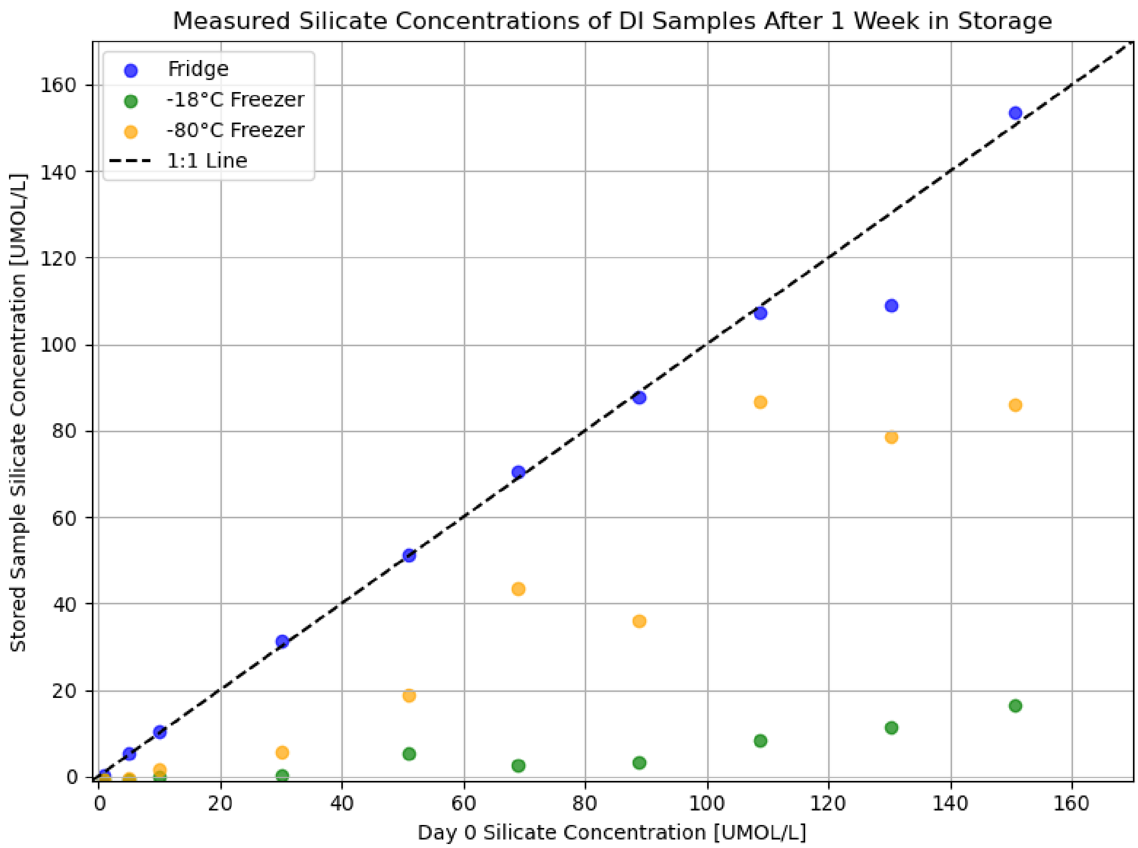

Figure 11: Measured silicate concentrations for each storage method overlaid, with Day 0 data (see Table 3 for Day 0 concentrations).

Table 4 shows percent recovery for refrigerated samples is above 95% for all samples aside from 9, while percent recovery for the -18 °C freezer samples is never above 11%. Percent recovery for -80 °C samples is scattered.

Table 4: Percent recovery of measured silicate concentrations for each storage method based on calculated concentrations from when samples were made on Day 0.

Sample	Day 0 Measured Value	Fridge % Recovery	-18 Freezer % Recovery	-80 Freezer % Recovery
2	4.60	112.05	*-14.41	*-6.54
3	9.52	107.65	*-1.55	15.86
4	34.73	89.72	0.62	16.47
5	65.36	78.57	7.99	28.59
6	82.38	85.49	3.16	52.78
7	112.25	78.20	2.83	32.03
8	124.35	86.28	6.63	69.71
9	108.13	100.81	10.58	72.57
10	148.10	103.61	11.03	58.07

*Measured value was close to the instrument's limit of detection and/or below zero.

3.3 DISCUSSION

A shown in Section 3.2, frozen DI-based standards showed dramatic changes over a one-week period. Unfortunately, there was a silicate water contamination in the lab's DI water system during the analysis of the samples for the 6-week and 12-week groups that resulted in inconsistent reproducibility and calibration curves. Thus, the obtained data for the six-week and twelve-week measurements cannot be used and is not shown. Refrigerated samples had the closest values to the Day 0 values, which corresponds with the literature indicating polymerization occurs during the freezing process. Notably, the -80 ℃ samples displayed significantly higher percent recovery than the -18 ℃ samples. This is most likely because the freezing in a -80 ℃ freezer happens so rapidly that there is not enough time for polymers to form (Rho et al. 2022).

4.0 FREEZING AND THAWING EXPERIMENTS WITH SEAWATER SAMPLES

4.1 EXPERIMENTAL DESIGN

A full depth profile of silicate concentrations was made using samples collected from a shallow and deep water cast aboard the Hawaii Ocean Time-series (HOT) Cruise 347. The 20 non-filtered seawater samples were collected in 50 mL Falcon tubes that were rinsed with the seawater three times, and then divided into three different groups to be stored the same way as the lab-made standards described in Section 2.4 were: in a refrigerator, -18°C freezer and -80°C freezer (Figure 12). About 12 mL of nonfiltered sample from each depth was poured into new, 15 mL centrifuge tubes. Some of each sample depth was set aside and measured within hours of collection. The three other sets of samples were measured one week, six weeks, and 12 weeks post-collection. It is important to note all samples were in different containers, and not refrozen. The day before measurement, all three groups were taken out of storage and left standing at room temperature in a dark room for 18-24 hours. When measured, samples were diluted by hand to fit the calibration curve. Based on the literature review, it was hypothesized that silicate concentrations would decrease over time.

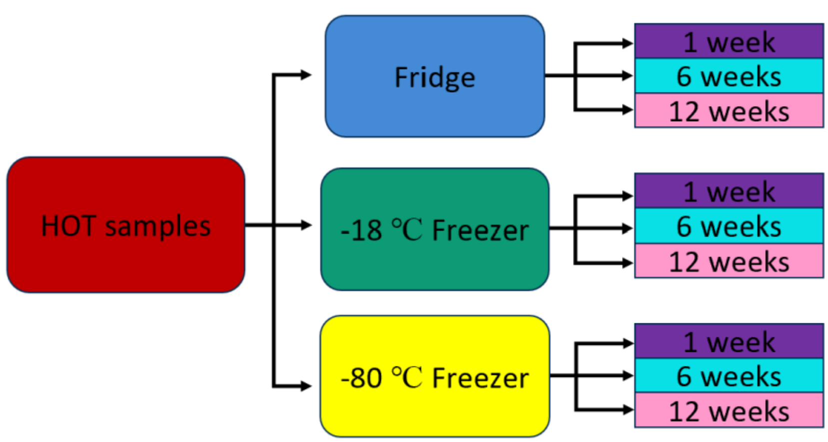

Figure 12: Flow chart showing the experimental setup for cruise samples

4.2 RESULTS WITH RESPECT TO TIME

Figure 13 shows the vertical profiles of samples and their silicate concentrations from the depths 0 to 4800 meters. In general, measured silicate concentrations were higher than cruise measurements, which is contrary to the hypothesis made based on the literature review (discussed in section 4.4). Samples with concentrations below 100 µM SiO_2 (depths above 1000 meters) are consistent between preservation method and time interval. In the deeper waters, the differences between time intervals were more scattered, especially the bottom 6 samples from the -80 °C freezer at the 6-week interval. Those points are very far apart from each other and the -18 °C freezer/refrigerator samples. Possible causes are discussed in section 4.4. In addition to the outliers at 6 weeks, there is notable scatter in the 6-week data. There is hardly any scatter in the 1-week measurements. The 12-week measurements also show more scatter than the 1-week measurements. However, at 12 weeks, the -80 °C freezer points did not show the same issues as 6 weeks did. The samples at 12 weeks came from the same rosette as the 6-week and 1-week samples, indicating that the outlier data at 6 weeks was not due to a recurring problem.

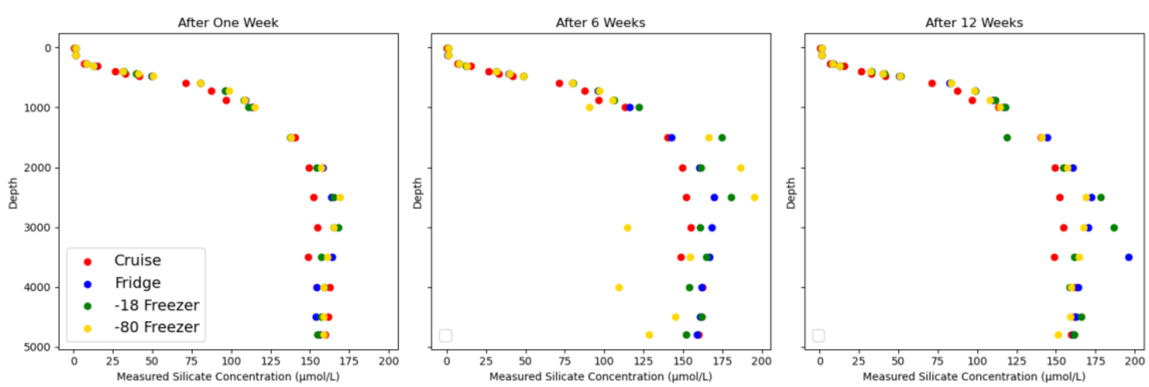

Figure 13: Measured silicate concentrations for each storage method at each time interval, overlaid.

4.3 RESULTS WITH RESPECT TO STORAGE METHOD

Figure 14 shows that samples in the upper 1000 m of the water column show no difference between preservation methods. The refrigerated samples show the most consistency across the 12-week measurement period. Excluding the outliers at six weeks, the -80 °C freezer also shows consistency. The -18 °C freezer samples show the most scatter.

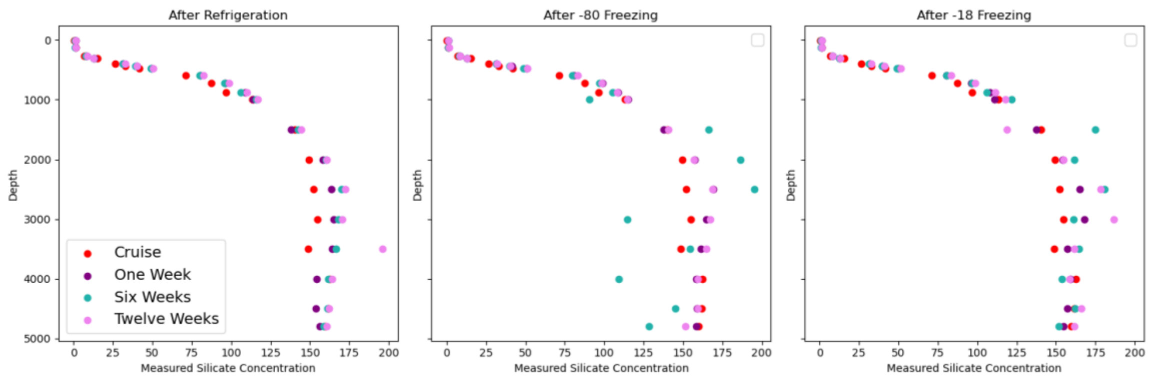

Figure 14: Measured silicate concentrations at each time interval for each storage method, overlaid.

Using a 1:1 line (Figure 15) for each combination against the cruise measurements shows the same results: consistency for each storage method and time interval up to 100 µM silicate. Above concentrations of 100 µM, there is deviation from the line. For each combination, points lie both above and below the line, so declaring trends in negative or positive bias is not possible with the current data.

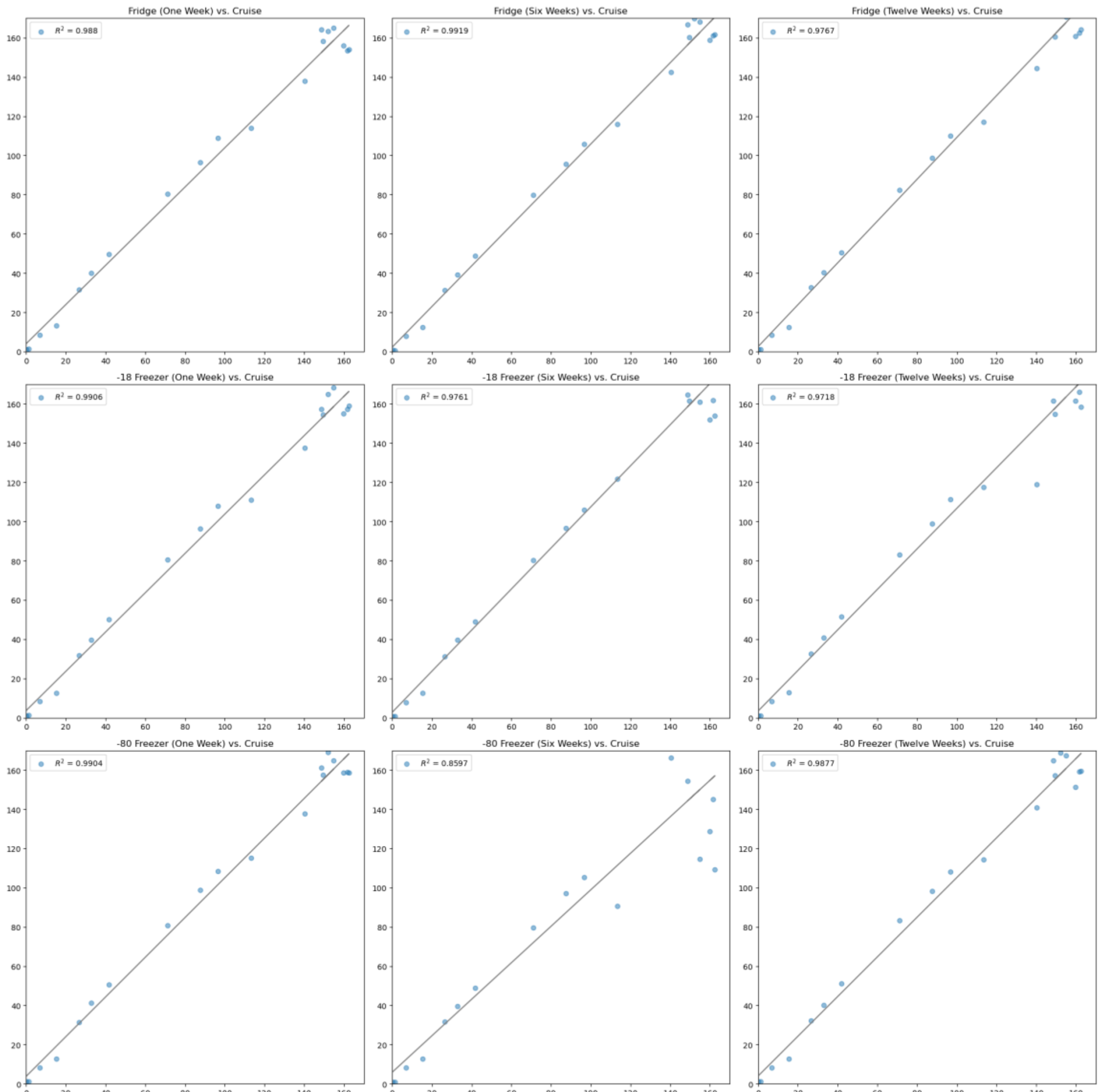

Figure 15: Each storage method and time interval compared to cruise measurements on a 1:1 line. The x-axis is the measured silicate value on the cruise, and the y-axis is the measured silicate concentration for the changing preservation method/duration.

4.4 DISCUSSION

In general, the post-cruise concentration measurements at depths deeper than 1000 meters were higher than the cruise measurements, for each time interval and storage method. This was contradictory to the reports in the literature, and requires further investigation. When compared to the official HOT silicate profile from the same cruise

(Figure 16), the miniSIA-2 measurements align with the data until a depth of 2500 m, where points then fall both above and below the line. The inconsistency might be due to increased error with manually diluting high concentration samples to concentrations that fall towards the middle of the 20 µM calibration curve. Thus, it is still unknown why the post-cruise measurements for samples below 1000 meters are higher than the cruise measurements, but one potential cause is calibration curve error on the day of cruise measurements for the deep samples. Another potential cause is the contamination of lab DI water with higher silicate concentrations, which is what limited the DI-based experiments discussed in section 3. Blank samples showed silicate concentrations above 0 µM, indicating there was silicate in the lab's DI water. If there was a similar contamination during the seawater experiments, it would have shifted the 1 week, 6 week and 12-week concentrations to the right in comparison to cruise measurements, which is shown by figures 13 and 14.

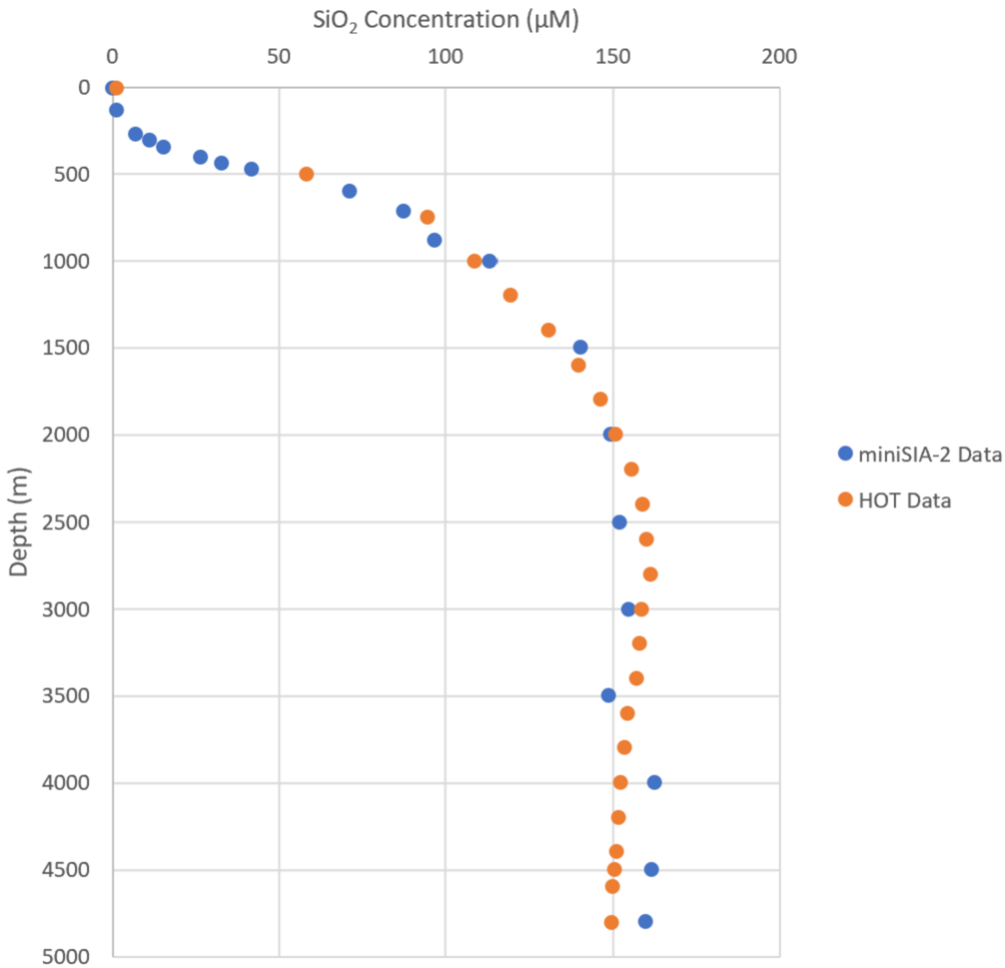

Figure 16: The ship-based silicate measurements from the miniSIA-2 system overlaid with the official HOT lab-based measurements from the same cruise.

It is hypothesized that something happened to the deep cast samples for the -80 °C 6-week group due to the large variability between the points that does not exist at the 12-week interval. It was not due to a calibration curve problem, and is hypothesized to be because of contamination.

Scatter between preservation method becomes prominent at about 100 µM SiO_2. When looking at each preservation method over time, consistency is shown in both the refrigerated and -80 °C frozen samples (with the exception of six weeks for the latter). The -18 °C freezer samples show a lot of variability. This aligns with the findings from the DI-based standard experiments. It is hypothesized that the formation of brine and polymers during the slower freezing process in the -18 °C freezer is altering concentrations in each sample tube. All samples were diluted manually with the same 1000 μL pipette, and the samples that show the most variability between each other (>100 μM SiO_2) were all diluted at a dilution factor between 10 and 14 times to fall in the middle of the calibration curve. Because all samples were diluted the same way, using the same pipette, scattering associated with dilution error is unlikely. In the future, diluting the high concentration standards to lower concentrations prior to freezing might also show different results.

Lastly, none of the seawater samples were filtered, meaning the differences between the DI-based standards and the seawater samples are salinity, pH, presence of biology and other suspended particles. Because the frozen DI-based standards showed significant silicate losses while the seawater samples did not, it is hypothesized that salinity, pH and/or biology is limiting polymerization in the seawater samples. Saltwater is slightly basic, with a pH around 8.2. The DI-based standards were all slightly acidic. Focusing a future experiment on pH might reveal new information. Additionally, filtered/nonfiltered experiments could be done to eliminate the effects of biology and other particles that might be interfering with absorbance and polymerization. It is not possible to identify the reason the silicate concentrations in the seawater samples were not as affected as the DI-based standards without further experiments.

5.0 CONCLUSION

The results of the lab experiments allow for comment on the capabilities of the instrument's auto dilution sequence, the impacts of freezing/thawing on lab-made, DI-water based silicate standards, and the impacts of freezing/thawing on nonfiltered seawater samples collected at Station ALOHA.

These experiments proved the accuracy and reliability of the mini-SIA's autodilution feature and optimized the capabilities for measuring silicate concentrations using the autodilution and unknown samples sequences. However, the highest points on the calibration curve still cause problems for unknown sample measurements, which is why samples must be diluted towards the middle of the calibration curves to fall within the methodological error. This is likely because the absorbance values are too close to 1, where measurements begin to deviate from Beer's Law. More work needs to be done to investigate this.

After completing the validation process, silicate concentrations in DI-based standards and seawater samples were measured and stored three different ways. Of the three preservation methods tested (refrigeration, -18 °C freezing, and -80 °C freezing), refrigerated samples showed the most consistency over time. Samples stored in the -18 °C freezer showed the most variability throughout the twelve-week measurement period. Thus, refrigeration was determined to be the most reliable preservation method if samples cannot be measured in-situ and must be stored. The -80 °C freezer is the next best option, and a -18 °C freezer should not be used. With the present data, it is not possible to determine a negative bias in frozen seawater samples because all post-cruise measurements show higher concentrations than cruise measurements, for reasons still being investigated but likely due to a water contamination (silicate present in blank samples).

The knowledge obtained from these experiments can be applied in areas such as the polar regions, where silicate concentrations are generally high, with silica-using diatoms being the prominent primary producers in the area. Because the polar regions are remote and cruises are months long, an understanding of the inaccuracies associated with long-term storage of silicate samples is important for drawing accurate conclusions regarding silicate cycling in the polar regions.

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