Pseudo-nitzschia species distributions in Glacier Bay, AK as measured by automated ribosomal intergenic spacer analysis (ARISA)

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Abstract

Pseudo-nitzschia is a genus of ubiquitous marine diatoms that comprises of at least 30 known species. Eight species of *Pseudo-nitzschia* in the Pacific Northeast are known to produce the neurotoxin domoic acid (DA). *Pseudo-nitzschia* species distributions were measured in Glacier Bay, Alaska, 21-23 March 2008. The internal transcribed spacer 1 (ITS1) region of *Pseudo-nitzschia* genomes were amplified using *Pseudo-nitzschia* specific PnAll primers designed by Kate Hubbard. Automated ribosomal intergenic spacer analysis (ARISA) was used to generate peaks that corresponded to distinct ITS1 fragment lengths. Six distinct ARISA peaks were found in the waters in and around Glacier Bay. Five of the six peaks correspond to putative species matches in GenBank (*P. multiseries, P. delicatissima* type 10, *P. delicatissima* type 11, *P. granii, P. seriata* type 2 or *P. australis*). Species richness was high outside the bay (4 or 5 species) and generally decreased as one traveled into the inner regions of the Bay. Surface salinity and nutrients also generally decreased from inside to outside the Bay; this indicated melt water from glacial inputs was diluting marine water. It appears that as *Pseudo-nitzschia* species are advected into the Bay, they are exposed to unfavorable conditions that select for the most tolerant species that are able to persist in Glacier Bay's environment.

Introduction

Pseudo-nitzschia is a genus of marine diatoms found in all of the world's oceans and coasts. Although, there are about 30 described species (Lundholm et al. 2006), this is likely an underrepresentation of true species count due to cryptic diversity (Mann and Droop 1996). This pennate diatom has become the object of recent notoriety for three reasons. The first is due to its role in iron fertilization experiments. Almost two decades of experiments in high nitrate low chlorophyll (HNLC) waters has shown *Pseudo-nitzschia* species to be key responders; normally present in low numbers they quickly proliferate and can dominate large blooms (de Baar et al. 2005, Marchetti et al 2006). Secondly, one third of the described species (11) are known to produce domoic acid (DA; Moestrup and Lundholm 2007) a neurotoxin that can be harmful (and sometimes fatal) to humans, mammals, and birds when DA contaminated bivalves, shellfish, and fish are ingested (Bates et al. 1998). In the Northeast Pacific, eight species of *Pseudo-nitzschia* produce DA: *P. australis,*

P. multiseries, P. pseudodelicatissima, P. pungens, P. calliantha, P. cuspidate, P. fraudulenta and P. seriata (Moestrup and Lundholm 2007). Outbreaks of DA not only disrupt the food web, but also are responsible for large commercial losses when oysters and other bivalves become contaminated (Horner et al 1997). Finally, morphology sometimes cannot resolve *Pseudo-nitzschia* species identity even when examined by experts. A combination of genetic analysis and morphology has recently described two new species (Lundholm and Moestrup, 2006) and it is likely more species will be discovered when other *Pseudo-nitzschia* species are studied similarly.

Domoic acid production in *Pseudo-nitzschia* cells has been well studied in vitro (Bates et al. 1993, Pan et al. 2001, Maldonado et al. 2002) but it is unclear what natural conditions lead to HAB formation. The Washington coast typically exhibits higher levels of DA, with more frequent outbreaks, than Puget Sound, which has led to frequent closures of bivalve and shellfish harvesting on the coast (Horner et al. 1996). High salinity, nitrogen rich waters in the Juan de Fuca Eddy have been implicated in the seeding of toxic *Pseudo-nitzschia* cells along the Washington coast (Trainer et al 2002). Oceanic intrusions of *Pseudo-nitzschia* cells in high salinity, nitrogen rich water have been associated with DA events in Willapa Bay (Newton and Horner 2003). It remains unclear whether HABs formed in estuaries are seeded from oceanic waters such as these or whether HABs form from resident populations within estuaries.

The Washington State Department of Health routinely monitors DA levels year round in recreational and commercial beaches where shellfish are harvested (WSDOF website 2007). Unfortunately, DA levels are usually tested in bivalves and *Pseudo-nitzschia* species and their distributions are not measured. *Pseudo-nitzschia* species at sample sites are not collected or cultured for identification because cell culturing and preparation for transmission electron microscopy (TEM) or scanning electron microscopy (SEM) or genetic analysis because these processes are timely and expensive. Alaska also monitors domoic acid levels in bivalves, but only from samples sent in by volunteers.

The spatial diversity of *Pseudo-nitzschia* species is relatively unknown. There is little historical data on 'typical' *Pseudo-nitzschia* distributions along coasts or in estuaries, which compounds the difficulty in determining why harmful algal blooms (HABs) occur more frequently in certain areas and which specific species are producing DA. Recent studies suggest salinity is a controlling factor in the growth and geographic distribution of *Pseudo-nitzschia* (Newton and Horner 2003, Thessen et al 2005). Members of this euryhaline genus adapt well to large changes in salinity (Thessen et al 2005) but each species usually has a preferred salinity in which growth is not limited.

Cryptic diversity in *Pseudo-nitzschia* makes species and strain identification difficult because light microscopy often cannot resolve species identity. *Pseudo-nitzschia* cells must be isolated and cultured to proliferate cells for TEM or SEM in order to determine species identity (Lundholm et al 2006). Even this is sometimes not enough; some species have similar morphologies but are genetically distinct. Hubbard et al developed a molecular fingerprinting technique that can identify at least 11 currently known species of *Pseudo-nitzschia* (Hubbard et al 2008) using an automated ribosomal intergenic spacer analysis (ARISA). This technique uses primers specific to the *Pseudo-nitzschia* internal transcribed spacer 1 region (ITS1) and can accurately identify six of the eight toxic species typically found in Northeast Pacific waters.

Like many coastal estuaries in the Northeast Pacific, the geographical distribution of *Pseudo-nitzschia* is unknown within Glacier Bay. Glacier Bay is a fjord in Southeast Alaska 83 kilometers from Juneau that potentially har-

bors many of these toxic species. Receding glaciers input large amounts of fresh water into Glacier Bay each year (Hooge and Hooge 2002). This results in high sediment loads, regionally high turbidity, and a large salinity gradient in Glacier Bay's 100km long estuary. Salinity can range anywhere from 0 to 31psu in Glacier Bay depending on sample site and time of year.

Methods

Field Methods

Stations in Glacier Bay were sampled March 21-23, 2008 aboard the R/V Thomas G. Thompson. Eighteen USGS stations (Hooge and Hooge 2002; Table 1) were sampled.

Niskin bottles were used to collect water from the surface and chlorophyll maximum as determined by the CTD fluorometer. When a chlorophyll maximum was absent, a depth of 10 meters was sampled. For each sample, 500mL of water was filtered onto 0.45μm pore size mixed cellulose 20mm diameter Millipore filters (Millipore, Billerica, MA, USA). Filters were immediately frozen at -80℃ until analysis in the Armbrust Lab at the University of Washington, Seattle, Washington.

Dissolved nutrients (nitrate, nitrite, ammonia, phosphate, and silica) were taken at each station at the surface or within the first three meters and analyzed in at the University of Washington, Seattle, Washington.

Laboratory Methods

DNA from filters was extracted with Qiagen Dneasy Plant Mini Kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's instructions. Filters were cut with sterile scissors prior to DNA extraction to improve DNA yield. A NanoDrop 1000 was used to determine the amount of environmental DNA in each sample (Thermo Fischer Scientific, Wilmington, Delaware, USA). After extraction, environmental DNA was stored at -20℃ when not in use.

Quantitative polymerase chain reaction (qPCR) was performed to determine the optimal cycle number (lower cycle threshold (Ct)) for PCR. Each 20μL reaction consisted of 4.8μL sterile MilliQ water, 10μL of iQ SYBR Green Supermix (Bio-Rad Laboratories), 1.6μL each of PnAll forward and reverse primers (PnAll F/R; Kate Hubbard et al. in press), and 2μL environmental DNA or 2μL sterile water. Samples were run in triplicate, along with negative controls of sterile water. Quantitative PCR was performed by an iCycler iQ Real-Time PCR (Bio-Rad Laboratories, Hercules, CA, USA).

Internal transcribed spacer 1 (ITS1) DNA was amplified using PnAll F/R primers (Hubbard et al 2008). Polymerase chain reactions were ran in triplicate, with negative controls, in 20μL volumes. Reactions consisted of 2μL MgCl2, 2μL buffer, 1μL dNTPs, 0.8μL PnAll F, 0.8μL PnAll R Qiagen 5' fluorescent FAM label, 11.25μL sterile MilliQ water, 0.15μL Promega taq polymerase, and either 2μL environmental DNA or 2μL MilliQ water. Once Ct was found using qPCR, 4 additional cycles were added to Ct for PCR reactions. This ensured that sufficient DNA product was amplified to meet minimum levels for ARISA detection.

PCR products were purified and precipitated with a QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen).

Internal transcribed spacer 1 lengths were measured by a MegaBACE 1000 automated sequencer in genotyping mode (Amersham Biosciences, Piscataway, New Jersey, USA) and analyzed using DAx software (van Mierlo Software, Eindhoven, Netherlands). Fragment lengths were determined using DAx protocols developed by Kate Hubbard.

Results

In Glacier Bay, 6 distinct ITS1 fragment lengths (ARISA peaks) were identified (Table 1). Five of the six peaks corresponded to predicted ITS1

fragment lengths of known *Pseudo-nitzschia* species found in GenBank. An ARISA peak of 168 base pairs was detected at every station. One ARISA peak of 246 base pairs was detected and does not match any known ITS1 fragment in GenBank.

Species diversity was highest in stations outside Glacier Bay and overall decreased as one traveled into the mouth of Glacier Bay and up into the West and East Arms. There was a strong correlation (0.837 Pearson R value) and significance ($P < 0.0001$) between species richness and distance from Icy Straight (Fig 1). Overall, surface salinity and surface nutrients decreased as one traveled away from Icy Straight and into the interior regions of Glacier Bay (Fig 2; Appendix 1).

The spatial variability of ITS1 variants is high over Glacier Bay and surrounding waters (Fig 3). Several variants were present throughout the Bay (ITS1 168, 144, and 246) while others were only present in Icy Straight stations and mostly main and lower basin stations (ITS1 180, and 207). ITS1 variant 180 was only detected at station 05.

Surface chlorophyll increased linearly from Icy Straight to stations in the Main Basin and decreased from station 07 in the West Arm to station 21 at the head of the West Arm (Fig 4). Station 16 is in the East Arm has low surface chlorophyll like stations at the head of the West Arm. Station 22 is in Gieke Inlet and had surface chlorophyll similar to station 06. Chlorophyll in surface waters was generally low and corresponded to pre-bloom chlorophyll levels.

High species richness was found at stations that were in Icy Straight and Glacier Bay stations that had a depth less than 300 meters (Fig 5). Icy Straight all have species richness of 5 ITS1 variants. Glacier Bay stations with depths less than 300 meters all have high and intermediate species richness. Stations in Glacier Bay that are deeper than 300 meters have intermediate to low species richness.

Lower Bay stations (00-03) were well mixed and stations in the Main Basin (04-05) and lower West Arm (06-08) were stratified (Fig 6). Stratification was greatest around stations 06, 07, and 08. Stations in the upper West Arm (9/10b to 21) were less stratified than waters in the Main Basin and lower West Arm.

Discussion

All ITS1 fragment lengths generated from PnAll primers and ARISA analysis should be considered variants and not true species matches. Sequencing of the ITS1 region is needed to determine true species identity; this was not monetarily possible for this study.

Species richness appears to be closely linked to water origin in Glacier Bay. All of the six peaks identified throughout the Bay were found outside the Bay (except ITS1 150 variant) indicating that all species within the bay originated from outside the bay. I hypothesize that as *Pseudo-nitzschia* species are advected into the Bay they encounter unfavorable conditions (such as decreased light levels, grazing, or competition for nutrients) which leads to selection pressure for the most tolerant species. The ITS1 168 variant appears to be well adapted to conditions within the estuary because it was found at every station. The ITS1 180 and 207 variant were only found in stations in Icy Straight and Lower Bay stations. If the ITS1 180 variant is *Pseudo-nitzschia granii* (its putative species match) this could explain its distribution within Glacier Bay; *P. granii* is an open ocean species (Marchetti et al 2008) and is likely not to be adapted to conditions within an estuary.

ITS1 fragment length (base pairs)						
	144	150	168	180	207	246
	Putative Species match					
USGS Stations	*P. multiseries*	*P. austalis/ P. seriata* type 2	*P. delicatissima* type 11	*P. granii*	*P. delicatissima* type 10	unknown
A	x		x	x	x	x
AA	x		x	x	x	x
0	x		x	x	x	x
1	x		x	x	x	x
2			x	x	x	x
3	x		x		x	x
4	x		x	x		x
5	x	x	x			x
6			x			x
7			x	x		
8			x			x
9/10b			x			
10			x			
11	x		x			x
12			x			
16			x			
21	x		x			x
22	x		x	x		x

Table 1: ITS1 fragment lengths are listed at each station they were detected (denoted by an 'x'). The putative species match denotes *Pseudo-nitzschia* species in GenBank that have an ITS1 fragment length of the same size.

In general, ARISA peaks were several orders of magnitude smaller than ladder peaks. The ITS1 150 variant was only detected at station 05. The molecular ladder used in ARISA has a ladder peak at 150 base pairs; this likely masked the presence of more ITS1 150 variants at other stations. However at station 05, the ARISA peak at 150 was very large and it was able to overcome the ladder peak signal. More work with optimization of PCR cycles and ladder volumes is needed in order to determine if the ITS1 150 variant was present at more stations.

Surface salinity and surface nutrients decreased from Icy Straight as one traveled into the inner regions of the Bay. This indicates that water from glacial input (rivers and glacier calving) was diluting marine waters. This lensing of fresher water in the West Arm, Gieke Inlet, and East Arm would create a barrier to the transport of *Pseudo-nitzschia* cells from the surface waters of the Main Bay. Any cells present in the Main Bay would likely be advected into these arms

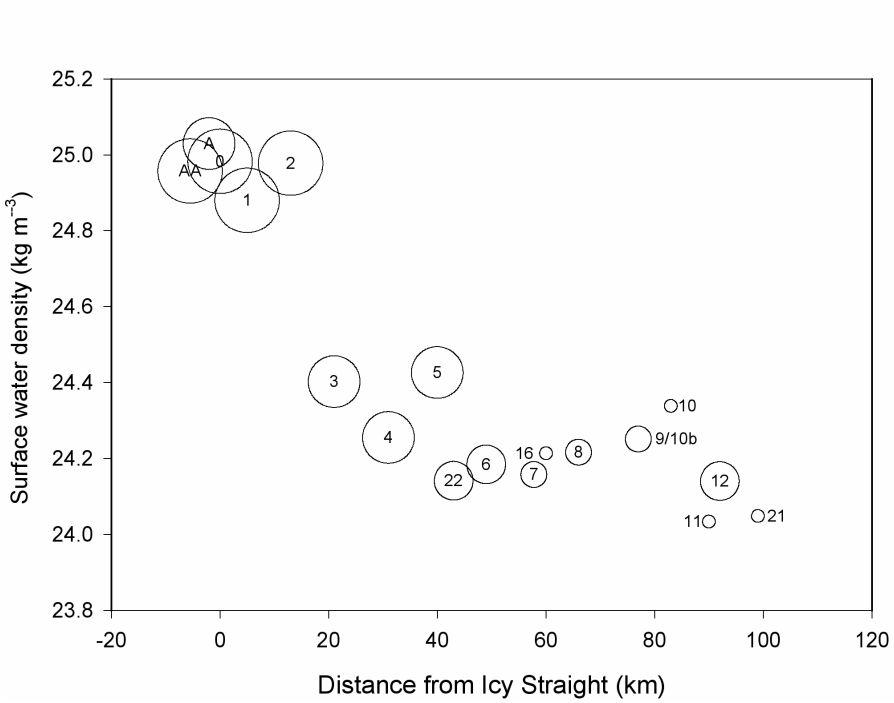

Figure 1: USGS stations that are farther removed from Icy Straight generally have lower density surface waters and less species richness. The size of the bubbles reflects species richness at each station with 5 species having the biggest bubble and 1 species the smallest bubble.

at depth, perhaps out of the photic zone. As cells traveled at depth, low light levels would select for species that are more shade tolerant. Another possibility is that as cells were advected into the Bay they encountered predators such as copepods that selectively grazed *Pseudo-nitzschia*. This is unlikely as one study in the Pacific Northeast found copepod grazing to be negligible on *Pseudo-nitzschia* (Olson et al 2006). Another possibility is that *Pseudo-nitzschia* species were out competed by other phytoplankton more adapted to estuarine conditions.

Nutrients and salinity did not vary greatly from inside to outside the Bay. *Pseudo-nitzschia* species are well known for their adaption to great ranges in salinity, 15-40 PSU for optimal growth in some species (Thessen et al 2005) and the salinity ranged from 30.17-31.55 PSU in Glacier Bay. Nutrients were also high with phosphate being in the 1.57-2.30μM which is unlikely to cause nutrient limitation. Therefore, it is unlikely that changes in salinity or changes in nutrients caused *Pseudo-nitzschia* species to die off when they were advected into Glacier Bay.

High species richness was associated with stations that had well mixed less stratified waters. There was a strong correlation between high species richness and Icy Straight stations and Glacier Bay stations that had a depth of less than 300 meters. This is interesting because stations in the West Arm that had high species richness (stations 12 and 21) were furthest from the Main Bay. The ITS1 144 variant was present at shallow stations in the West Arm and Gieke Inlet but was not present in stations in between the Main Bay and West Arm. It remains unclear what mechanisms are controlling the spatial distribution of *Pseudo-nitzschia* species in Glacier Bay.

The ITS1 246 variant could be a new *Pseudo-*

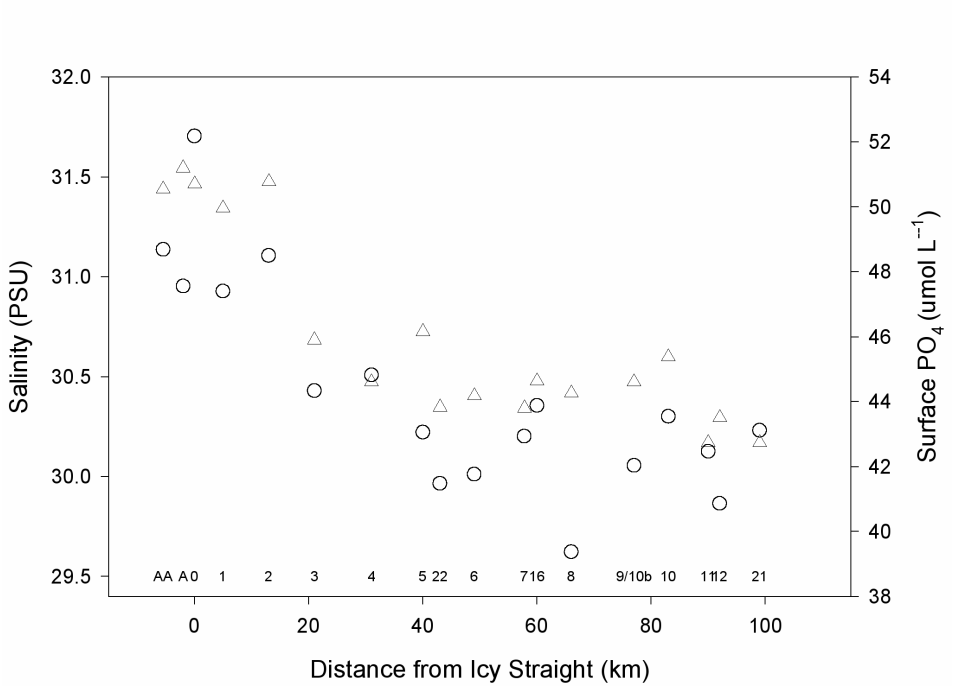

Figure 2: Surface salinity and surface phosphate both generally decrease as one travels from Icy Straight into the inner regions of Glacier Bay. Triangles denote salinity values and circles denote phosphate values. Stations are listed just above the x-axis and below each stations salinity and phosphate symbols.

nitzschia species, a closely related diatom, or an artifact from PCR reactions. Further work such as sequencing of the ITS1 fragment would help elucidate the origin of this peak.

If the putative species matches are correct then it is interesting to note that Glacier Bay samples have a mixture of species found in Puget Sound and in Northeast Pacific open ocean waters. *P. multiseries* (144 bp), *P. delicatissima* type 11 (168 bp), P. delicatissima type 10 (207 bp), and P. seriata type 2 or P. australis (150 bp) have been found in waters off the coast of Vancouver Island and in Puget Sound Washington (Hubbard et al 2008). *P. granii* (180 bp) and P. delicatissima type 10 (207 bp) were found in oceanic waters at Station Papa (Marchetti et al 2008). This suggests there exists specific geographic communities of P. species over the Northeast Pacific.

Conclusions

- 6 distinct ITS1 variants were detected in Glacier Bay
- 5 of the 6 variants have putative matches in GenBank
- Stations in Icy Straight and in the Lower Bay had high species richness (4-5 species) and stations in the Central Bay, West Arm, and East Arm, had lower species richness. Gieke Inlet had 4 species present.
- There was a strong correlation between distance from Icy Straight and species richness.
- Stations that were well mixed, less stratified, and had shallow depths had high species richness (4-5 species).
- Salinity and nutrients generally decreased in surface waters from Icy Straight to the

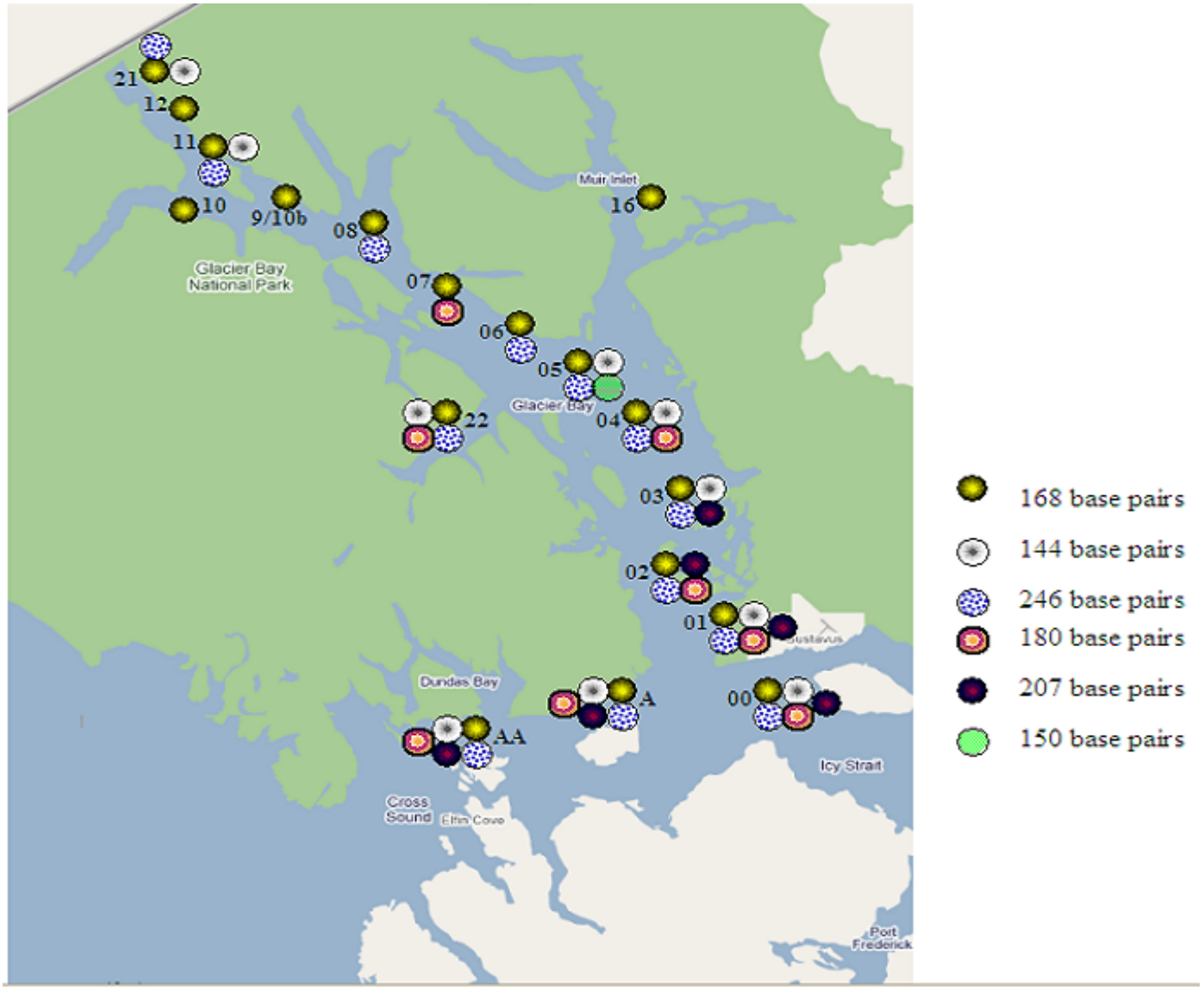

Figure 3: ITS1 variants are shown at each station they were detected. Map adapted from Google Earth ©.

inner regions of Glacier Bay. This was most likely due to fresh water diluting marine water.

- Surface chlorophyll levels were generally low (winter conditions) but did increase as one traveled from Icy Straight into the Main Basin. At station 07 the surface chlorophyll reached a maximum and then decreased as one traveled into the inner regions of the West Arm.

- Pseudo-nitzschia cells could not freely circulate in surface waters in Glacier Bay and were likely subducted when advected into the West Arm and East Arm. This probably caused selection pressure on more tolerant species.

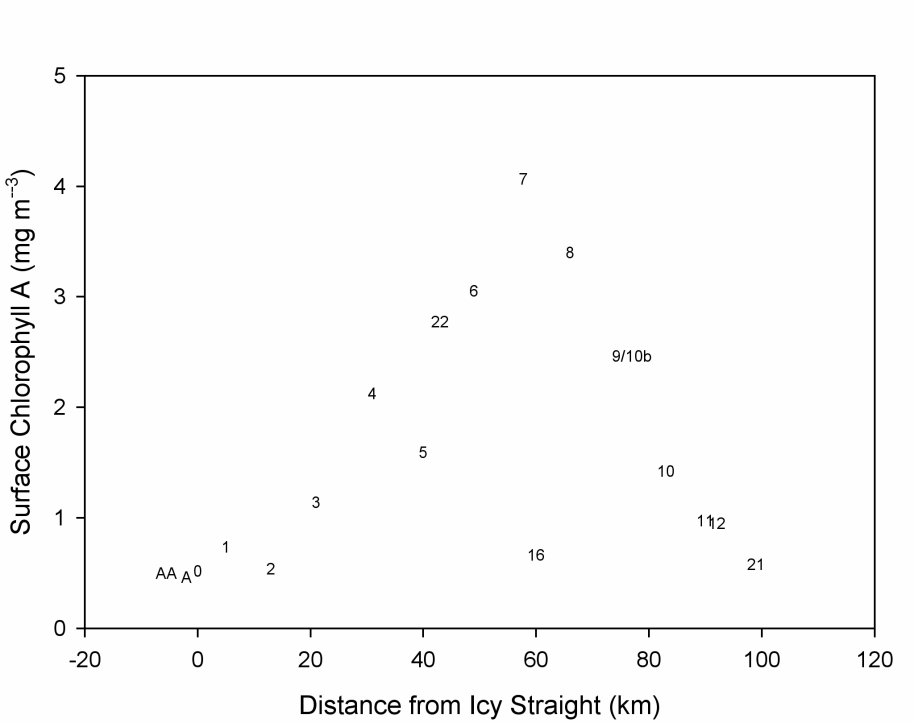

Figure 4: Station distance from Icy Straight and that station's surface chlorophyll.

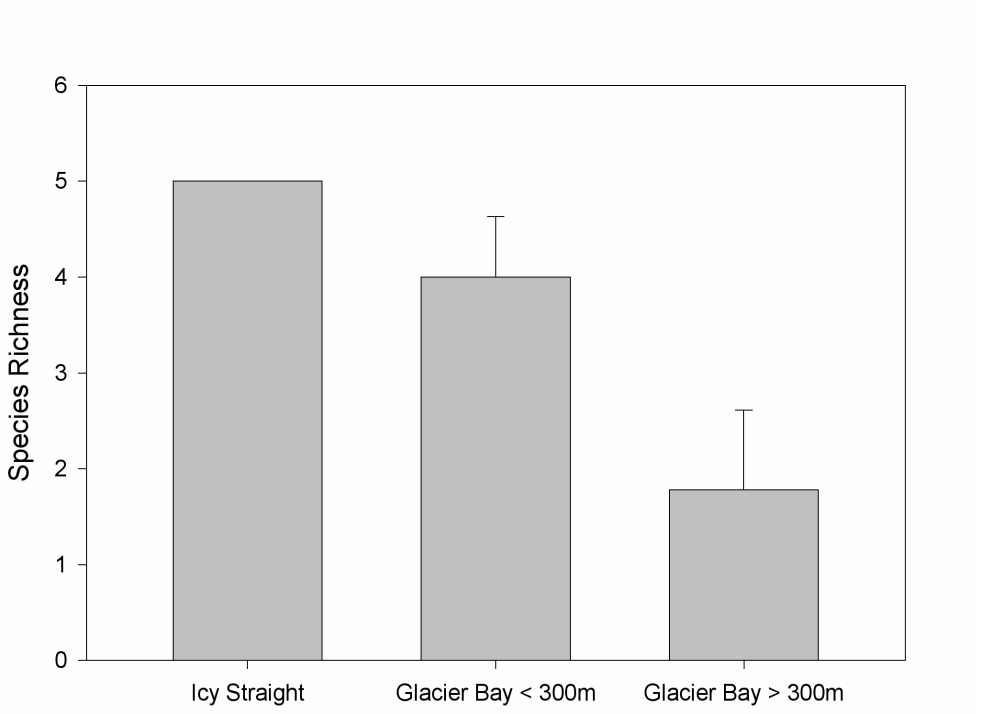

Figure 5: Stations were put together that have similar characteristics such as station depth and whether the stations are inside Glacier Bay or in Icy Straight.

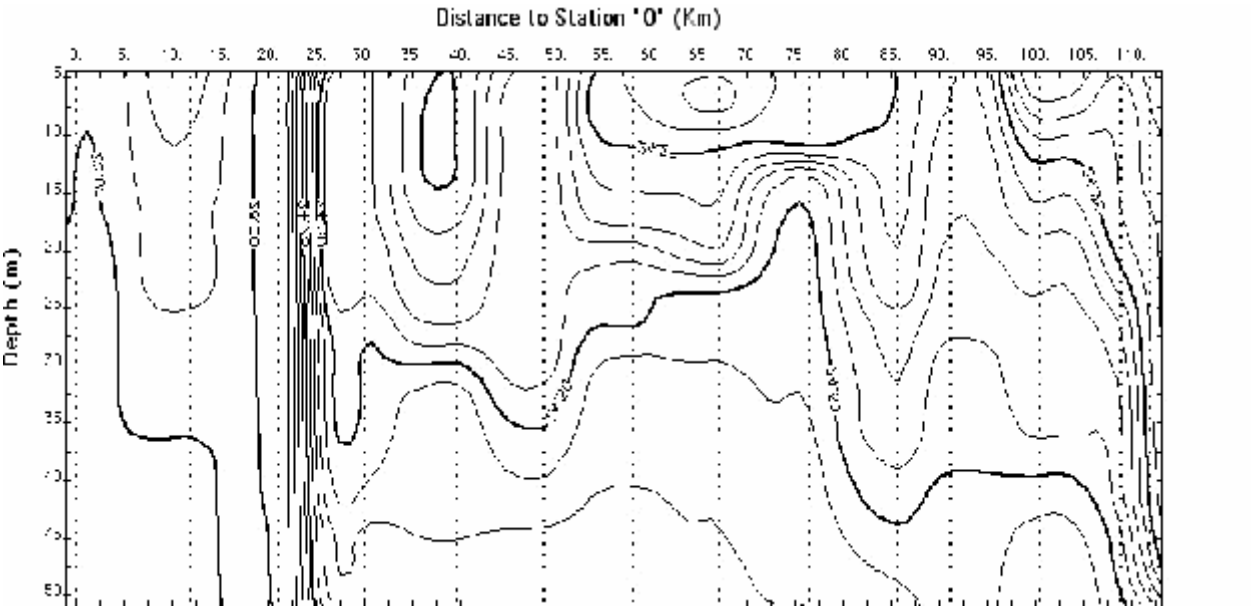

Figure 6: Density profile of the upper 50 meters of the water column from station 00 to station 21. Station 00 in the Icy Straight and stations in the Main Basin and West Arm were only included in this profile. Other stations in Icy Straight, Giekie Inlet, and the East Arm were excluded.

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Appendix 1

Station	$[PO_4]$ (mM)	$[Si(OH)_4]$ (mM)	$[NO_3]$ (mM)	$[NO_2]$ (mM)	$[NH_4]$ (mM)	Chl. a (°C)	Temp. (PSU)	Salinity
A	1.98	47.57	23.83	0.18	0.08	0.46	4.09	31.55
AA	2.31	48.69	25.90	0.22	0.29	0.49	3.97	31.44
00	2.10	52.19	26.88	0.20	0.76	0.52	3.93	31.47
01	2.04	47.41	25.09	0.19	0.25	0.73	3.99	31.34
02	2.07	48.51	25.54	0.19	0.13	0.54	4.07	31.48
03	1.94	44.34	23.99	0.12	0.09	1.14	3.47	30.69
04	1.63	44.83	21.56	0.19	2.16	2.12	3.24	30.48
05	1.83	43.06	22.41	0.12	0.10	1.59	3.57	30.73
06	1.77	41.77	20.70	0.20	0.46	3.05	3.42	30.41
07	1.75	42.94	21.21	0.13	0.26	4.06	3.15	30.34
08	1.55	39.38	20.12	0.19	0.63	3.40	3.17	30.42
9/10b	1.60	42.04	20.14	0.18	0.62	2.46	3.28	30.47
10	1.90	43.55	23.68	0.15	0.31	1.42	3.43	30.60
11	1.70	42.47	21.76	0.18	0.28	0.97	2.97	30.17
12	1.75	40.87	19.73	0.11	0.10	0.95	2.89	30.30
16	1.93	43.89	24.27	0.08	0.03	0.66	3.76	30.48
21	1.79	43.12	23.26	0.15	0.23	0.58	2.79	30.17
22	1.57	41.49	18.33	0.21	0.58	2.77	3.39	30.35

Table 2: Surface Nutrients, Temperature and Salinity