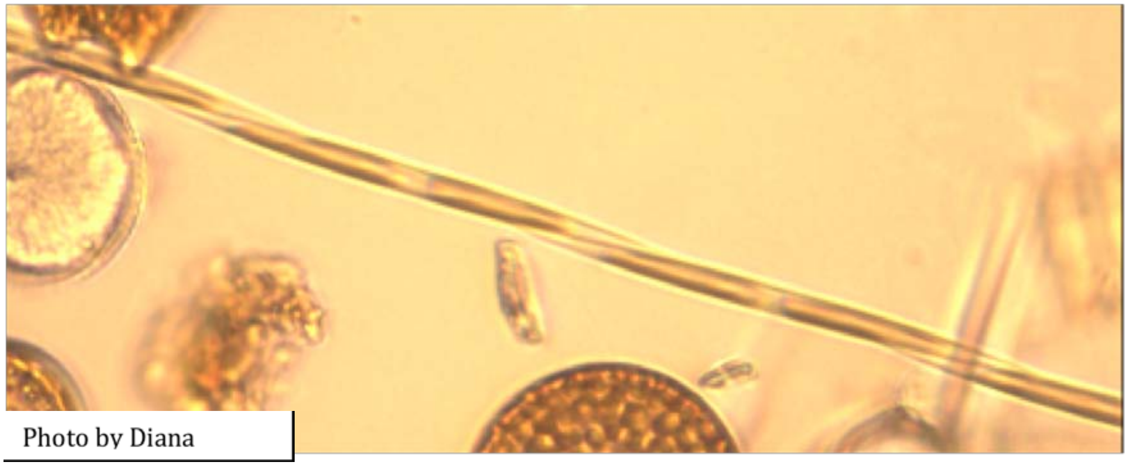
Pseudo-nitzschia species distribution in estuaries of the Pacific Northwest based on Automated Ribosomal Intergenic Spacer Analysis (ARISA)



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Non-technical Summary

Phytoplankton make up the base of the ocean's food web. Diatoms, a kind of phytoplankton, are essentially microscopic plants that live in the upper portion of the water column. *Pseudo-nitzschia* is a diatom that has been found all over the world that can produce a toxin called Domoic Acid (DA). Consuming too much DA can result in seizures, memory loss, and even death. Some species of *Pseudo-nitzschia* produce DA while others are not known to produce the toxin. Because the species look very similar, the only way to define to the species level is by looking at the organism using a very powerful microscope or at the molecular level. Automated Ribosomal Intergenic Spacer Analysis (ARISA) is a method that amplifies a piece of DNA called the Internal Transcribed Spacer 1 (ITS1) that all *Pseudo-nitzschia* have but in different lengths. ARISA was performed on samples from Puget Sound, WA USA and Barkley Sound, British Columbia, Canada in early spring 2010. The goals of this study were to compare the species in two regions and to see if the location within the sounds had an impact on which species were present. *Pseudo-nitzschia* was present at all stations. The dominating species was *P. delicatissima* in both sounds. Statistical analysis showed that location within the sound, rather than which region the stations were in, had more of an impact on which species of *Pseudo-nitzschia* were present. Although ARISA cannot be used to see how many *Pseudo-nitzschia* are present, it is essential in identifying species, some of which are threats to the health of many organisms because of their DA toxin production.

Acknowledgements

I would like to thank the UW School of Oceanography for funding my research. There have been many people who have helped me with my project. First and foremost is Kate Hubbard. Without her extensive knowledge on *Pseudo-nitzschia* and her willingness to share her time and methods, I would not have the thesis that I do today. I am very thankful to Gabrielle Rocap for allowing me to utilize her lab's resources. I would also like to thank Michele Guannel for helping me with lab work, statistical analysis, and draft editing. Thank you to the professors of Ocean 444 and Colleen Durkin for all of their encouragement and help. Zach Thomson, Claire Ellis, Samuel Monk, Carly Moreno, Kira Rombeau, Rachel Emswiler, and Franziska Lutz also helped me through out my project. Finally I would like to thank my mother, Catherine Coolidge, for giving me this opportunity to further my education.

Abstract

The cosmopolitan diatom genus *Pseudo-nitzschia* comprises approximately thirty species, at least twelve of which are known to produce a neurotoxin called domoic acid (DA). DA is responsible for causing Amnesic Shellfish Poisoning which can result in seizures, memory loss, and even death in upper trophic level consumers. The presence of DA and harmful algal blooms (HABs) has been reported in the coastal regions of the Pacific Northwest. Due to the similar morphologies of each *Pseudo-nitzschia* species, identification beyond genus is not possible using light microscopy; only electron microscopy or molecular methods can accurately identify to the species level. This study used a genetic fingerprinting method to determine species distribution in areas of Barkley Sound and Puget Sound in late March, early April 2010. *Pseudo-nitzschia*-specific primers were used for Automated Ribosomal Intergenic Spacer Analysis (ARISA) to amplify the polymorphic region of the ITS1. The goal of this research was to determine which species of *Pseudo-nitzschia* were present in Barkley Sound and Puget Sound and to compare the similarity of *Pseudo-nitzschia* communities spatially. *P. delicatissima* represented approximately 50% or more of the communities in both sounds. Four different species of toxin-producing *Pseudo-nitzschia* were identified, two in each sound. Two open ocean species (*P. heimii* and *P. granii)* made up more than 10% of the community composition in each sound. This research investigates the speculative movement and varying distribution of the toxin-producing diatom *Pseudo-nitzschia* in estuaries of the Pacific Northwest.

Introduction

Pseudo-nitzschia is a cosmopolitan diatom genus, some species of which produce a neurotoxin called domoic acid (DA) (Bates 1998). DA can cause health risks to consumers in different trophic levels as well as have large economic impacts when shellfish beds need to be closed as the toxin accumulates (Dyson and Huppert 2010). *Pseudo-nitzschia* species have been found in estuarine, coastal, and oligotrophic waters. Oligotrophic waters found in the open ocean are characterized by low phytoplankton biomass and are dominated by cells smaller than 5 μm. Diatoms that are larger than 5 μm, like *Pseudo-nitzschia,* generally have low concentrations due to the limiting of essential nutrients in the water column. *Pseudo-nitzschia* were initially believed to be only found in coastal waters, but it is now known that their wide range of tolerance for different marine environments allows them to survive in the open ocean as well (Marchetti et al. 2008).

The seasonally present Juan de Fuca eddy forms off the Northwestern corner of Washington State and is known to contain high concentrations of *Pseudo-nitzschia*, including some toxin-producing species (Trainer et al. 2009). These cells can be transported out of the eddy and to the coast during certain wind conditions, potentially threatening nearby shellfish beds. Waters within the eddy have a slightly lower salinity than offshore waters because of the input of fresh water from the Fraser River (MacFadyen et al. 2008). Comparing temperature and salinity from different water masses can illustrate the origins of the water. By knowing the origin of the water masses, a clearer picture will be available of the *Pseudo-nitzschia* community composition. Due to the fact that certain species produce DA, understanding the distribution of *Pseudo-nitzschia* and the controlling factors of their distribution is important.

Identification of *Pseudo-nitzschia* to the species level is not possible with light microscopy so molecular methods or scanning electron microscopy (SEM) are needed. Automated Ribosomal Intergenic Spacer Analysis (ARISA) uses fluorescently labeled *Pseudo-nitzschia*-specific primers to amplify a polymorphic region of the internal transcribed spacer 1 (ITS1) (Hubbard et al. 2008). The different lengths of the ITS1 fragment are associated with certain species when this genetic fingerprinting method is used in conjunction with SEM. Fifteen different fragment lengths have been sequenced and identified to the species level from the open ocean and estuaries in the Pacific Northwest (Hubbard et al. 2008, Marchetti et al. 2008).

Only coastal species of *Pseudo-nitzschia* are known to produce DA, although low levels of DA production by open ocean species have been reported (Trick et al. 2010). The five different species of *Pseudo-nitzschia* that have been identified as open ocean species are: *P. dolorosa, P. granii, P. heimii, P.* cf. *heimii,* and *P.* cf. *turgidula* (Marchetti et al 2008). Two species that are associated with producing high levels of DA are *P. multiseries* and *P. australis* (Thessen et al. 2009). On the west coast of the United States of America (USA) *P. delicatissima* is found to be most abundant in the spring (Fryxell 1997). Three species that have been seen in Barkley Sound, British Columbia, Canada are *P. australis, P. delicatissima*, and *P. multiseries* (Taylor and Haigh 1996, Hay et al. 2002). In Puget Sound, Washington State, USA, *P. australis* and *P. pseudodelicatissima* have been responsible for shellfish harvesting closures due to high DA levels (Bill et al.

2006, Trainer et al. 2007). Additional species found in Puget Sound are *P. pungens, P. multiseries,* and *P. seriata* (Hubbard et al. 2008).

The goals of this research were to determine which species of *Pseudo-nitzschia* were present in Barkley Sound and Puget Sound and to compare the similarity of *Pseudo-nitzschia* communities spatially within each sound. Specifically, it was hypothesized that locations close to the entrances of the sounds would have similar *Pseudo-nitzschia* community compositions, and those farthest away from the entrances would have similar communities. This could be due to an impact from the Juan de Fuca eddy or from waters leaving the Juan de Fuca Strait. *Pseudo-nitzschia* populations were analyzed using ARISA in two regions in the Pacific Northwest (Fig. 1). Samples in Puget Sound were collected in early April 2010 and samples in Barkley Sound were collected in late March 2010. This study helped elucidate distributions and identify possible toxin-producing species of *Pseudo-nitzschia* in both sounds.

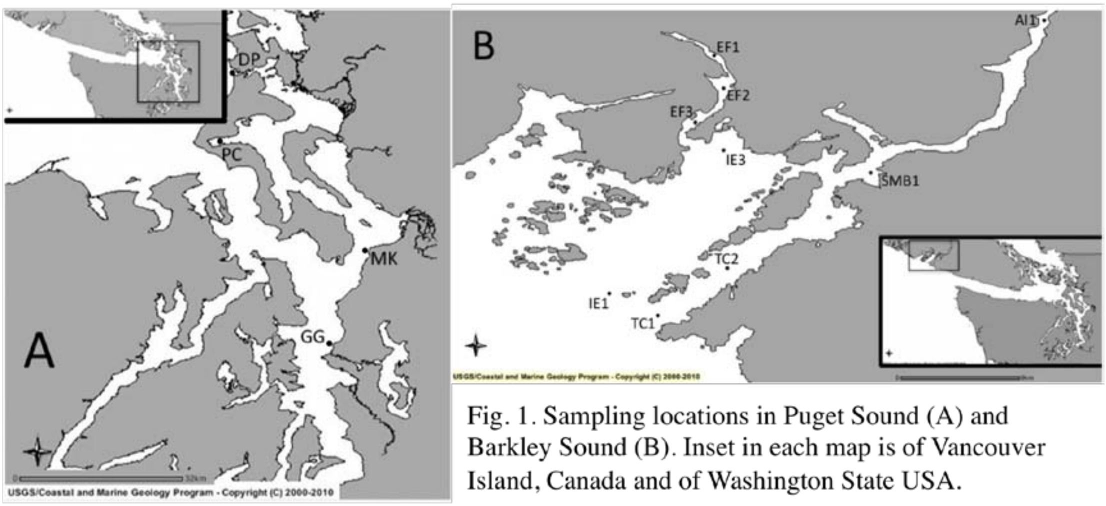

Fig. 1. Sampling locations in Puget Sound (A) and Barkley Sound (B). Inset in each map is of Vancouver Island, Canada and of Washington State USA.

Methods

Water sites and collection

Water was collected in Barkley Sound on the southwestern coast of Vancouver Island, Canada from March 24-25th, 2010 and in Puget Sound, Washington on April 6th 2010 (Fig 1). In Barkley Sound, sampling stations were located in Effingham Inlet (EF), Trevor Channel (TC), Imperial Eagle Channel (IE), and Alberni Inlet (AI). In Puget Sound, stations were chosen based on their location within the sound. Deception Pass (DP) was sampled at high tide to represent Juan de Fuca Strait waters; Penn Cove (PC) was sampled to represent Whidbey Basin; Mukilteo (MK) was sampled to show the mixture of Whidbey Basin and Main Basin; and Golden Gardens (GG) was sampled to represent Main Basin waters. In Puget Sound 500 mL of water were collected by hand past the wave break where less sediment was mixed in the water column. Temperature and salinity were measured with a HI 9828 Multiparameter Meter with GPS (Hanna Instruments). Net tows were collected with a 20 µm mesh tow to determine presence or absence of *Pseudo-nitzschia* by light microscopy. In Barkley Sound, 500 mL of seawater were collected with a hand held Niskin bottle at the chlorophyll maximum, which was determined by the fluorometer on the Seabird SBE-19 at each station. A 10 µm mesh net tow was used to collect concentrated surface samples of the phytoplankton community at all but one station. In both Barkley Sound and Puget Sound, whole water samples were stored in dark Nalgene bottles until filtered on 0.45 µm 47 mm diameter mixed cellulose filters (Millipore) and then frozen at -80° C for later analysis. Each filter was analyzed using ARISA (see below) for the presence and relative abundance of *Pseudo-nitzschia* species.

Analysis for Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Deoxyribonucleic acid (DNA) was extracted from each filter using a DNeasy Plant Mini Kit (Qiagen) according to manufacturer instructions. The DNA was then amplified for ARISA with polymerase chain reactions (PCRs) using *Pseudo-nitzschia*-specific primers, PnallF and the FAM-labeled PnallR (Operon, Huntsville, AL, USA) as described in Hubbard et al. (2008). Briefly, 20 μl PCRs were done in triplicate for each sample. PCRs contained 0.8 mM dNTP's, 0.8 nmol of both primers, 0.75 U taq polymerase (Genechoice, Apex™ Bioresearch Products, San Diego, CA), 2.5 mM of $MgCl_2$, 1x standard buffer (Genechoice), and a standardized concentration of DNA. Four negative controls, using water instead of DNA, were generated. The PCR cycling parameters were referenced from Hubbard et al. (2008). PCR products were purified with MultiScreen PCRμ96 filter plates (Millipore). Quantification of the amplified DNA was done with PicoGreen® (Invitrogen, Carlsbad, CA, USA) on a SpectraMax M2 microplate reader (Danaher Medical Technologies, Washington D.C., USA). A standardized concentration of PCR product was purified for ARISA with an ethanol precipitation. A mix of 0.078 μL 10% Tween 20, 9.77 μL sterile water, and 0.15 μL fluorescent size standard Et-ROX 400 (GE Healthcare) was added to resuspend precipitated samples, which were then analyzed on a MegaBace 1000 capillary sequencer (GE Healthcare). Full descriptions of the methods will be available in Hubbard's next publication.

ARISA profiles were analyzed with the Data Acquisition and Analysis Software, DAx (van Mierlo Software Consultancy, The Netherlands). Peak heights were measured in relative fluorescent units (RFUs) and used to estimate the contribution of each species to the *Pseudo-nitzschia* community. Peaks less than 3% of total RFUs per individual ARISA profile were excluded. Percentages of each species contribution in each sound were calculated by averaging three triplicate runs on the MegaBace. The triplicates tested for the MegaBace's ability to replicate data. The average was then divided by the total peak height for a particular species in each sample to get a percentage. Fragment lengths identified by Hubbard et al. (2008) and Marchetti et al. (2008) were used to compare to the fragment lengths found in Barkley Sound and Puget Sound.

Statistical analysis of *Pseudo-nitzschia* species diversity was performed using the statistical software PRIMER (Clarke and Warwick 2001). *Pseudo-nitzschia* communities were compared using cluster analysis in a dendrogram based on the Bray-Curtis similarity coefficient. One-way and two-way analysis of similarity (ANOSIM) tests calculated significance values comparing locations and regions. Similarity percentages using species contributions (SIMPER) was used with region and location as factors. Region was defined as either Puget Sound or Barkley Sound. Location was defined as either within an inlet (AI1, EF1, EF2, EF3) or in a channel (all other stations). Heat maps of *Pseudo-nitzschia* community composition at each station were created using a matrix2ping interface through www.bioinformatics.ubc.ca.

Analysis of sea surface temperature and wind speeds

Goddard Earth Sciences Data and Information Services Center Interactive Online Visualization and Analysis Infrastructure (GIOVANNI) was utilized to create sea surface temperature maps to verify the presence of the Juan de Fuca eddy. The ocean color radiometry instance was chosen to compile monthly data from MODIS-Terra images for February and March 2010.

Average wind speeds and directions were calculated for the week of sampling in Barkley Sound based on data taken in duplicates from Fisheries and Oceans Canada. The closest buoy to Barkley Sound is C46206 which is located southwest of the entrance to the sound. Average wind speeds and directions were calculated for the week of sampling in Puget Sound based on data from National Oceanic and Atmospheric Administration (NOAA) buoys. Two stations, 46088 and SISW1, were chosen that are located within the Juan de Fuca Strait. Station 46087, which is located at the entrance of the Juan de Fuca Strait, could not be used because it stopped transmitting and will not be repaired until July 2010. General NOAA Operational Modeling Environment (GNOME) was used to analyze models of surface water movement based on wind speeds and directions over a three-day period (April 5-7, 2010). The parameters chosen for the models were the smallest volumes available (one gallon of non weathering pollutant) to simulate the movement of *Pseudo-nitzschia* cells.

Results

Nine different *Pseudo-nitzschia* species types were identified within two regions of the Pacific Northwest. ITS1 lengths corresponding to eight specific *Pseudo-nitzschia* species were identified, in addition to one unidentified fragment at 155 base pairs (bp) long (Fig. 2). Both sounds had five different types of *Pseudo-nitzschia* present. *P. delicatissima* and the ITS1 length that corresponds to either *P. australis* or *P. seriata* was found in both sounds. *P. delicatissima* was present at every station in both sounds, and

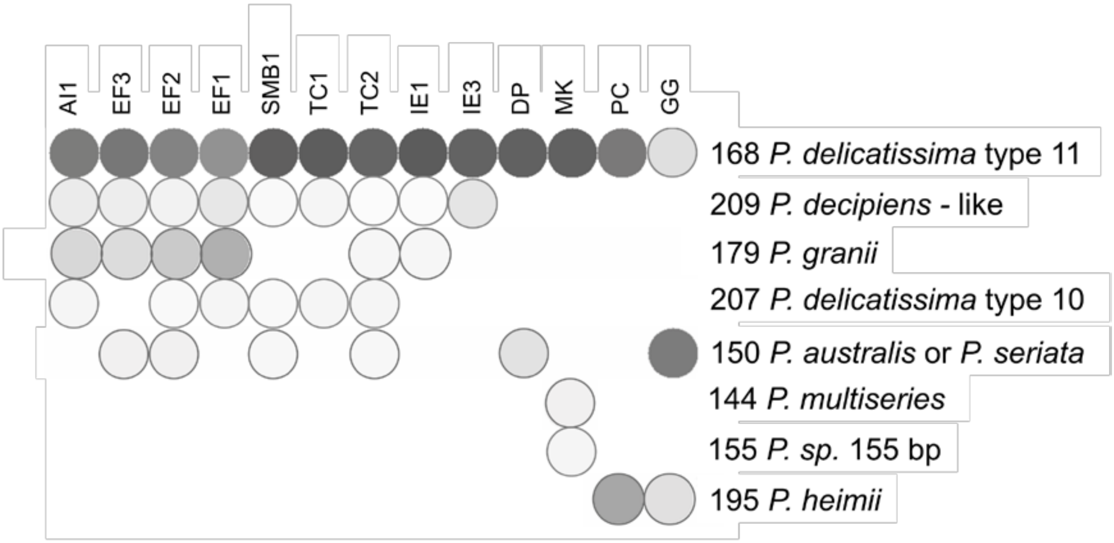

Fig. 2. A heat map showing base pair fragment length and corresponding species for each station. Grey scale is based on percentage abundance per station where darker spheres represent a higher percentage.

made up the largest percentage of both *Pseudo-nitzschia* communities (Fig. 3). Fragment length 209 was defined as *P. decipiens*-like based on total nucleotide divergence among Pacific Northwest clones (Hubbard et al. 2008).

The *Pseudo-nitzschia* community in Puget Sound was dominated by two species. *P. delicatissima* type 11 made up 48% of the *Pseudo-nitzschia* community (Fig 3A). *P. australis/P. seriata* represented 29% of the community. *P. heimii*, an open ocean species, was only present in Penn Cove (PC) (Fig. 2). Mukilteo (MK) and Golden Gardens (GG) both had three different species present, while Deception Pass (DP) and Penn Cove had two different species present. A fragment length of 155 bp that did not correspond with a known ITS1 fragment for *Pseudo-nitzschia* was only present at Mukilteo.

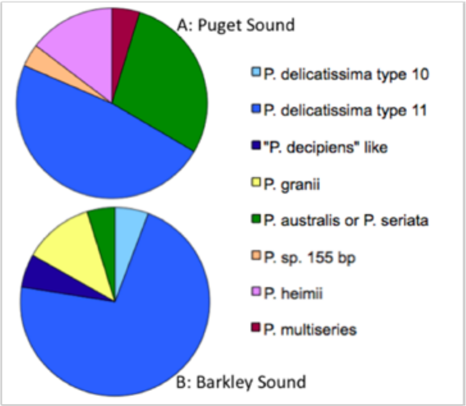

Fig. 3. Percentage of *Pseudo-nitzschia* species in Puget Sound (A) and Barkley Sound (B) based on ARISA fragment lengths.

In Barkley Sound, one species of *Pseudo-nitzschia* was dominant. The species *P. delicatissima* was represented by two different types, which together represented 78% of the *Pseudo-nitzschia* community (Fig 3B). *P. granii*, another open ocean species, was the next most abundant at 12%. An ITS1 length that corresponds to either *P. australis* or *P. seriata* was present at four out of nine stations. One fragment length (209 bp) that has been identified as *P. decipiens*-like made up 6% of the *Pseudo-nitzschia* community and was found in Barkley Sound stations EF3, IE3, and TC1.

Statistical analysis using the Bray-Curtis similarity coefficient in PRIMER revealed how similar *Pseudo-nitzschia* communities were to one another based on location and region. The locations were defined as either in an inlet or in a channel. Cluster analysis showed the presence of four distinct groups (Fig. 4). *Pseudo-nitzschia* community composition at Golden Gardens (GG) was approximately 20% similar to all of the other areas sampled. Penn Cove also had a community composition that was significantly different, but it was approximately 70% similar to communities in Barkley

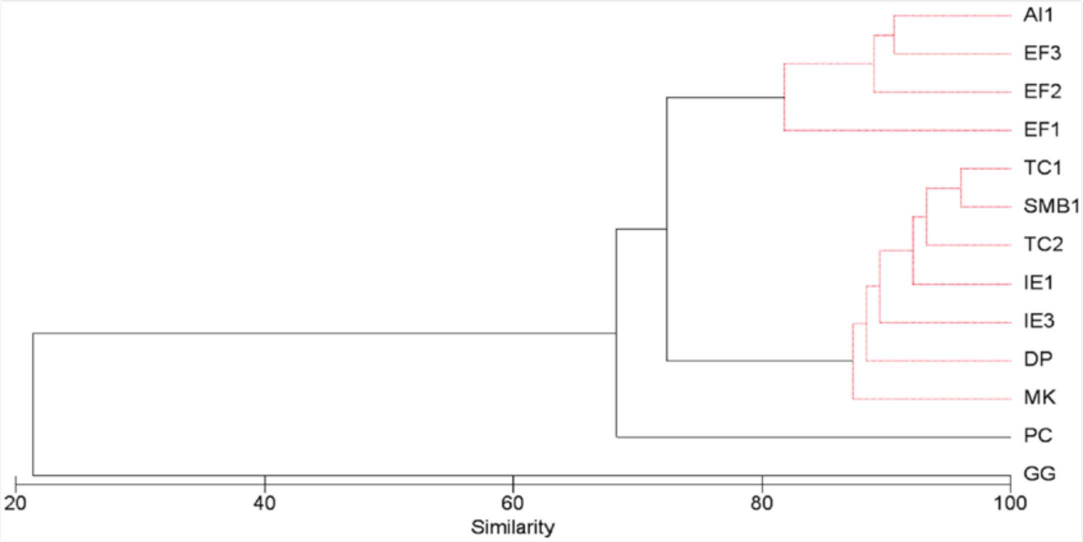

Fig. 4. Dendrogram showing station similarities based on *Pseudo-nitzschia* community composition. Red lines show the grouping of those stations are not significantly different from one another (p>5%).

Sound. The two final groupings separated all the stations within the inlets of Barkley Sound (AI1, EF3, EF2, EF1) from the stations in the channels of Barkley Sound and the rest of the Puget Sound stations (DP, MK).

An ANOSIM two-way crossed analysis calculated an R-value of 0.434 describing the difference between species composition in the two sounds (p=0.8%). An R-value equal to one indicates that stations within a group are more similar to each other than from any other grouping (Clarke 1993). P-values show statistical significance and are used to interpret whether the data found could occur by chance. Values less than 5% were interpreted as significant. There was a significant difference in *Pseudo-nitzschia* community composition between inlets and channels (R=0.944, p=0.8%).

A two-way SIMPER analysis explored the species responsible for the similarities between community compositions in both regions. *P. delicatissima* contributed between 71-94% to the similarities of the community composition in both regions and all locations. *P. granii* had the next highest contribution at 16%. *P. heimii* was responsible for a 26% contribution to the dissimilarities between the sounds. *P. delicatissima* percentage abundance caused most of the dissimilarity between the sounds. The difference in species abundance percentages caused 32% of the variation. The *P. australis/P. seriata* group was also present in both sounds and caused 26% of the differences.

MODIS-Terra images of sea surface temperature show that during the month of March 2010 the seasonal eddy had begun to form (Fig. 5). A monthly average of sea surface temperature in February 2010 showed that the eddy had not yet formed (data not shown). Data compiled by fellow classmates included *Pseudo-nitzschia* cells per liter concentrations (EF1: 4.4×10^3, EF2: 2.0×10^4, EF3: 3.5×10^4) (Rombeau 2010, Moreno 2010), as well as drifter speeds within Barkley Sound (0.1 ms^{-1}) (Monk 2010) and the age of surface water in Effingham inlet (~8 days) (Emswiler 2010). In Puget Sound the average wind speed and direction during the week of sampling was approximately 9 m/s N. In Barkley Sound the average wind speed and direction was 7 m/s NNW. A GNOME model of the Puget Sound using daily averaged wind speeds and directions showed movement of surface waters by Deception Pass (DP) towards the north and towards the west at the mouth of the Strait of Juan de Fuca (data not shown).

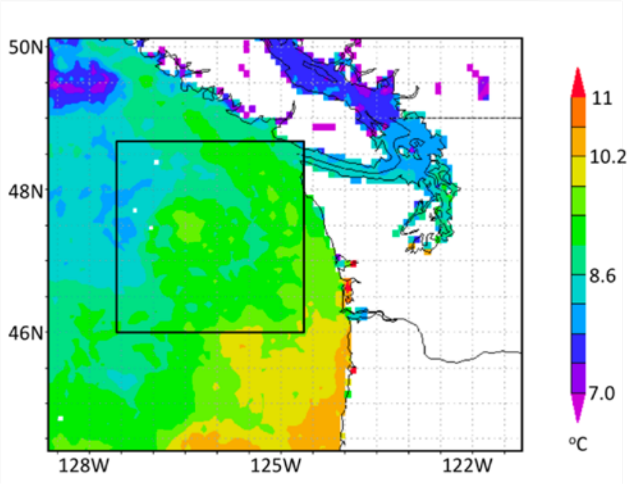

Fig. 5. Average sea surface temperature for March 2010 from MODIS-terra satellite. Color scale is in degrees Celsius. Juan de Fuca eddy highlighted in box.

Discussion

The Juan de Fuca eddy generally develops near the spring transition and is fully developed by late summer (MacFadyen 2008). MODIS-Terra images of sea surface temperature show that during the month of March 2010 the eddy had begun to form (Fig. 5). The eddy can contain *Pseudo-nitzschia* in high concentrations of millions of cells per liter (Trainer et al. 2009). These cells can then be transported out of the eddy and to the coast where toxins can accumulate in shellfish beds and other filter feeders. Storms and upwelling favorable winds have been cited as factors that can create filaments off the eddy and advect cells southward. Models based on drifter movement show that during downwelling conditions drifters move north of the eddy, towards the entrance of Barkley Sound (MacFadyen et al. 2005). Northward moving, downwelling favorable winds were present throughout the sampling period in Barkley Sound (Monk 2010). Downwelling favorable winds occur though out the winter and are most likely responsible for the presence of open ocean species of *Pseudo-nitzschia* far into the inlets of Barkley Sound.

P. granii is an open ocean species that was found at five different stations (Fig. 2), four of which were in the inlets of Barkley Sound. Because the sampling was done near the winter and spring transition it is possible that the downwelling favorable winds advected the cells from the oligotrophic waters into the sound. The distance from the entrance of Barkley Sound to the head of Effingham Inlet is 34 km and 47 km to the location sampled in Alberni Inlet. Based on the average speed of surface drifters of 0.1 ms^{-1}, it would take approximately 4 days for water from the entrance of Barkley Sound to reach these inlet stations (Monk 2010). This timeframe is on the same order of magnitude as the age of water in Effingham Inlet compiled by Emswiler (2010). Therefore these species would have been advected into Barkley Sound within the week that they were sampled. This calculation does not include information on tides. It has been shown that *P. granii* will produce low levels of DA with the addition of iron, which is generally the limiting nutrient in oligotrophic waters (Trick et al. 2010). Iron is not a limiting nutrient in estuaries due to the proximity to anthropogenic sources and run off from land. Modeling done with GNOME could not include Barkley Sound and ended at the mouth of the Juan de Fuca Strait. The model did show that surface waters from the mouth of the Strait would move north and along the coast, which could result in species of *Pseudo-nitzschia* to move from Puget Sound into Barkley Sound.

P. heimii is the open ocean species that was present in Puget Sound. It was found in both Penn Cove (PC) and Deception Pass (DP). The average wind direction in the Juan de Fuca Strait for the week of sampling was towards the north, which would result in surface waters moving north as well. Due to this, the *P. heimii* cells were most likely advected into Deception Pass and Penn Cove long before sampling occurred in the area. This leaves two possibilities for how *P. heimii* were advected into the sound. One is that the cells could have been advected at depth due to estuarine circulation and survived long enough until they reached the photic zone where the necessary light is for their growth. Water in Puget Sound moves through Main Basin at depth, is then mixed with surface waters and advected into Whidbey Basin (Babson et al. 2006). Another possibility is that the cells reached Deception Pass (DP) and Penn Cove (PC) when winds were blowing towards the east. NOAA buoy data for 2010 shows that the wind was generally blowing towards the northwest. Buoy data for 2009 shows that summer months had the most days with winds blowing towards the northeast. To have surface waters move from the mouth of the Strait of Juan de Fuca into Puget Sound winds would need to blow towards the northeast (Hickey and Banas 2003). This means that the *P. heimii* would have been advected into Whidbey Basin around 9 months before they were sampled. None of these estimates take tides into consideration. *P. heimii* was responsible for a 26% contribution to the dissimilarities between the sounds due to the fact is was only present in Puget Sound.

A SIMPER analysis on the two regions highlighted the *Pseudo-nitzschia* species responsible for creating differences in the community compositions. *P. delicatissima* percentage abundance caused most of the dissimilarity between the sounds even though it was present in both regions. The difference in species abundance percentages caused 32% of the variation. The dominating presence of *P. delicatissima* in both sounds agrees with previous data on the abundance of *Pseudo-nitzschia* species in the spring (Fryxell 1997). The *P. australis/P. seriata* group was also present in both sounds and caused 26% of the differences. The community at Golden Gardens (GG) was dominated by 71% *P. australis/P. seriata* group, whereas it made up only 7% in Barkley Sound. *P. australis* has been associated with shellfish bed closures due to accumulation of DA past regulatory levels (Trainer et al. 2007). Based on net tow data (not shown) and cell counts by Rombeau and Moreno (2010), *Pseudo-nitzschia* was not present in bloom concentrations at any station sampled.

The ARISA method is based on PCRs producing qualitative, not quantitative results. Additional cell counts would have to accompany ARISA data for abundance approximations. Cell counts were only done in Effingham Inlet and *Pseudo-nitzschia* was identified at the genus level. Further analysis to define species into morphological groups can be done on preserved net tow samples from these samples. Although ARISA is limited in describing abundance, it is essential in differentiating the multiple morphocryptic species of *Pseudo-nitzschia,* some of which are threats to the health of many organisms because of their DA toxin production.

Conclusions

- Both region and location were significant factors in the composition of the *Pseudo-nitzschia* communities.
- Nine different *Pseudo-nitzschia* species types were identified in Barkley Sound and Puget Sound. Only one of the nine fragments found did not have a corresponding species identification.

- *Pseudo-nitzschia* community composition within the inlets of Barkley Sound was significantly different from all other stations. Golden Gardens and Penn Cove in Puget Sound had community structures that differed from all the other stations sampled.

- *P. delicatissima* made up the largest percentage of the community in both sounds. Two types of *P. delicatissima* made up over 75% of the *Pseudo-nitzschia* in Barkley Sound.

- Open ocean *Pseudo-nitzschia* species were present in coastal waters in both Barkley Sound and Puget Sound. *P. granii* was likely advected into Barkley Sound at the surface as a result of the prevailing wind conditions. *P. heimii* could have been transported into Puget Sound at depth.

- High toxin-producing species were found in both sounds, but made up a larger percentage of the Puget Sound communities.

- The Juan de Fuca eddy was present during sampling, along with downwelling favorable winds which could have brought *Pseudo-nitzschia* towards Barkley Sound.

- ARISA is essential in differentiating the multiple morphocryptic species, but needs to be supplemented by cell counts to obtain abundance levels.

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