The Use of Environmental DNA for Understanding Changing Ecosystems: Monitoring Harmful Algal Blooms

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Abstract

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Environmental DNA is a rapidly developing tool that offers a unique opportunity to sample the communities of an ecosystem in greater depth than other sampling methods. When analyzing environmental samples using general primers and a metabarcoding method, researchers are able to detect a multitude of taxa, such as plankton, invertebrates, fish, and mammals, even at low concentrations. Given the changing environment in Puget Sound, biological responses such as the increasing frequency of harmful algal blooms, are a concern. Environmental DNA can help detect and track harmful algal species, as it has been used for other harmful or invasive species. In this study, I use environmental DNA to detect a potentially toxic dinoflagellate in the family Kareniaceae, which is not well reported in Puget Sound, and assess the environmental conditions with which it is associated. In the present dataset, the species occurs only at depth, and in particular, in low-pH, low-dissolved-oxygen, and high-salinity conditions. I then use logistic regression to predict the presence of the dinoflagellate given the data in hand; the resulting model suggests that a change in water pH from 7.8 to 7.4 increases the probability of Kareniaceae *sp.* presence by from 29% to 95%. With decreasing pH levels within

Puget Sound, the continuing progression of ocean acidification could lead to bloom conditions for the taxon. This method could become a first-step monitoring tool for species such as Kareniaceae *sp.* and to focus investigative efforts to prevent HABs.

1. INTRODUCTION

Environmental DNA (eDNA) is rapidly growing in popularity due to its versatility in ecology and environmental science. Organisms shed genetic material into their environments; this genetic material can come in the form of whole cell intracellular DNA or extracellular DNA if degradation has occurred (Taberlet, Bonin, Zinger, & Coissac, 2018), and it can then be recovered from samples of the environmental media. These eDNA samples are easy to collect and require no specialized permits or equipment, making this technique an excellent way to collect samples opportunistically, whether it be from water, air, or soil (Kelly, Gallego, & Jacobs-Palmer, 2018; Kelly, Port, Yamahara, & Crowder, 2014; Shelton et al., 2016; Turner, Uy, & Everhart, 2015).

Although eDNA has been used by microbiologists for many years to study microbial communities in water and soil, the technique has more recently been applied to broader ecological studies (Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012). This technique has been applied to several environmental media, including permafrost (Epp et al., 2012), snow (Dalén, Götherström, Meijer, & Shapiro, 2007), soil (Andersen et al., 2012), air (Folloni et al., 2012), and even honey (Schnell, Fraser, Willerslev, & Gilbert, 2010), but most applications are seen in marine and freshwater environments, including ponds, rivers, and streams (Bohmann et al., 2014). Studies using eDNA in aquatic ecosystems have been used to monitor the presence of both aquatic and terrestrial organisms (Balasingham, Walter, Mandrak, & Heath, 2018; Ishige et al., 2017; Qu & Stewart, 2019; Ushio et al., 2017; Williams, Huyvaert, Vercauteren, Davis, & Piaggio, 2018)

There are two methods of eDNA analysis: quantitative Polymerase Chain Reaction (qPCR) and metabarcoding, used to look at one or many species, respectively (Taberlet et al., 2018). These methods select a set of molecular primers that will isolate a specific but common portion

of the organismal genome and amplify that section for sequencing (Shelton et al., 2016). The species-specific approach uses primers that are tailored to the genome of a specific organism of interest, and qPCR can be used to assess the abundance of small quantities of amplified DNA. By contrast, the use of generalistic primers in a standard PCR reaction, will provide less quantitative—perhaps only presence/absence— data for that organism. Metabarcoding analyzes environmental samples using more generalistic primers, such as ones that target a gene found in the mitochondrial genome of eukaryotes (cytochrome c oxidase subunit 1; CO1). Analytical methods for metabarcoding reflect a multitude of taxa, such as plankton, invertebrates, fish, and mammals, even at low concentrations (Kelly et al., 2014, p. 201).

Using eDNA leads to a new understanding of the environment, in part because of the ability to examine samples for multiple species at once and in turn identify species that have not been the primary focus of past research efforts. The versatility of eDNA and ecological characterization makes this method a candidate for a first-step monitoring tool in habitats and ecosystems where conservation is vital; for example, the technique can aid in monitoring and tracking endangered species (Thomsen et al. 2012), evaluating the presence of invasive species (Balasingham et al., 2018; Bohmann et al., 2014; Kelly et al., 2014; Williams et al., 2018), and detecting species that may be new to the area or not previously recorded.

Given the changing environment in Puget Sound with warmer surface water temperatures, low oxygen levels, increased acidification, and eutrophication, (Busch, Harvey, & McElhany, 2013; Feely et al., 2010; Snover, Mote, Whitely Binder, Hamlet, & Mantua, 2005) researchers are concerned about the impact to organisms in the area. Surface temperatures of the Puget Sound region have risen 1.0°C since the 1950s, dissolved oxygen levels are below 5 mg/L in deeper sections of the Sound, creating potential stress on organisms, areas such as the Hood Canal are more susceptible to the effects of eutrophication, and pH has dropped by 0.05 - 0.15

units since pre-industrial era (~1750) (Busch et al., 2013; Feely et al., 2010; Mauger et al., 2015; Snover et al., 2005). Of particular concern are harmful algal blooms (HABs), which appear to be increasing in frequency and severity (Fu, Tatters, & Hutchins, 2012; Mauger et al., 2015), and some of which are associated with threats to human health (Mauger et al., 2015; Snover et al., 2005).

HABs have become increasingly common along the western coast of North America, and more damaging within the last decades. The diatoms from the genus *Pseudo-nitzschia*, which produce domoic acid are common, along with the dinoflagellates *Alexandrium*, *Gymnodinium*, and *Pyrodinium*, which produce an array of toxins collectively known as paralytic shellfish toxins (PSTs) (Lewitus et al., 2012). Changing environmental conditions have direct effects of *A. catenella* growth, as projections show increases in growth as conditions such as temperature and salinity become more favorable for the species (Mauger et al., 2015). Increased water temperatures and lengthened warm conditions are also expected create larger windows of growth for HABs, extending the number of ideal condition days by nearly 13 days and allowing blooms to begin earlier in the year and persist later (Mauger et al., 2015; Moore et al., 2008). Estimates show that ocean pH could drop by as much as 0.32 by 2100 further increasing the toxicity and growth of HAB species (Fu et al., 2012; IPCC, 2013; Mauger et al., 2015)

There is recent evidence, however, than an additional dinoflagellate, *Karenia mikimotoi* (family Kareniaceae), is present along the West Coast, specifically off of Alaska and California (Djurhuus et al., *In Revision*; "Working with State to Document First Occurrence of Harmful Karenia mikimotoi Algae in Alaskan Waters," n.d.). This family of dinoflagellates includes several species that have formed notable toxic blooms around the globe. These blooms include *Karenia brevis* in North America on the Atlantic and Gulf coasts, *K. mikimotoi* in Japan, Europe, Australia, and New Zealand, *K. selliformis* in Tunisia and Chile, *K. brevisulcata* in New Zealand,

and *Karlodinium veneficum* in North America, Australia, and Europe (Nézan et al., 2014). Many species within the genus *Karenia* produce toxins, although these toxins exist in different forms and toxicity levels. The best-known toxic species, *K. brevis*, produces neurotoxins known as brevetoxins which interfere with sodium channels in the body, interfering with nerve transmission in the affected animal (Holland et al., 2012; Solter & Beasley, 2013). Beyond immediate damage, brevetoxins can also put individuals at greater risk of infections by suppressing immunity functions (Solter & Beasley, 2013). Other species, such as *K. mikimotoi,* produce hemolytic substances that cause gill damage in finfish, respiratory distress, bacterial infections, and death. The species is also linked to the death of benthic filter feeding organisms, such as shellfish (Haywood et al., 2004; Shi et al., 2012; Yang et al., 2011). Similarly to *K. mikimotoi*, *Karlodinium micrum*, *K. veneficum,* and *K. armiger* produce hemolytic and ichthyotoxic substances resulting in fish kills (Deeds, 2003; Fernández-Tejedor, Soubrier-Pedreño, & Furones, 2007).

Environmental DNA can help detect and track harmful algal species, as it has been used for other harmful or invasive species. For example, eDNA has been used successfully to detect invasive species such as the round goby (*Neogobius melanostomus*) in two Great Lakes (Balasingham et al., 2018), the bigheaded Asian carp (*Hypophthalmichthys spp.)*, in Indiana and Kansas (Turner et al., 2015), as well as the endangered Bornean orangutan (*Pongo pygmaeus*), Asian elephant (*Elephas maximus*), and Sunda pangolin (*Manis javanica*) from water on salt licks in Borneo (Ishige et al., 2017). Most relevantly, the technique was used to detect an invasive diatom, *Didymosphenia geminate,* in the Rocky Mountains (Olson, 2016) and *Psuedo-nitzschia seriata* and *P. delicatissima* in salmonid aquaculture experiments (Peters et al., 2018). These studies suggest that eDNA might be especially useful for tracking harmful algal bloom-forming species in Puget Sound. This study therefore specifically investigates the presence of a

toxic taxon of phytoplankton, Kareniaceae *sp*., that is possibly novel to Puget Sound, and assess the environmental conditions associated with its presence.

2. METHODS

2.1. Field Sampling

I sampled at a selection of stations surveyed by the Washington Ocean Acidification Center during triannual cruises throughout Puget Sound, Washington, USA (Fig. 1). I sampled seven stations in September 2017, eight stations in April 2018, and four stations in September 2018 (Fig. 1). A CTD cast with twelve Niskin bottles was deployed at each station, collecting data on environmental variables such as temperature, salinity, pH, dissolved oxygen, etc. at several depths along the cast. I collected three 1-L water samples at each station; one at the deepest point in the cast, one at 5-10 m depth, and one at the surface. Once collected, 500 mL of water was immediately filtered through cellulose acetate filters (47 mm diameter; 0.45 μm pore size) using vacuum filtration. The cellulose filters were then placed in Longmire's buffer (Renshaw, Olds, Jerde, McVeigh, & Lodge, 2015) and stored at room temperature until further processing.

2.2. DNA Extraction, Amplification, and Sequencing

To improve DNA yields and reduce the co-extraction of PCR inhibitors, we developed the following extraction protocol, modifying the one described in Renshaw et al. (2015). At least 24 hours before extraction, the cellulose filters in Longmire's buffer were placed in a -80°C freezer to reduce the strength of the filter. Approximately 0.5 g of glass beads were added to each sample tube and agitated on a Bio Spec Products Mini Bead Beater (Bio Spec Products, Bartlesville, OK, USA) at the highest speed for approximately 90 seconds to shred the filter and dislodge the filtered cells. The samples were then incubated at 56°C for 30 minutes. The samples

were agitated again at the highest speed for approximately 45 seconds, before 20 μL of Proteinase K was added for protein digestion. The samples were then incubated at 56°C for another 2 hours. In preparation for the final steps, 1.5 mL tubes containing 700 μL of isopropanol and 20 μL of 5 M sodium chloride were stored in a -20°C freezer until needed.

Once incubation was complete, 900 μL of buffered phenol:chloroform:isoamyl (25:24:1) was added to each tube. Samples were then vortexed, rocked to mix, and then centrifuged at 14,000 rpm for 5 minutes (referred to as V, R, C throughout this protocol). The supernatant was transferred to a new 1.5 mL tube and 800 μL of chloroform was added, followed by V, R, C. This supernatant was again transferred to a new 1.5 mL tube, where 700 mL of chloroform was added, followed by V, R, C. The supernatant from this step was then transferred to the previously prepared isopropanol sodium chloride solution. The samples were then placed in a -20°C freezer for a least 2 hours or overnight. After freezing, the samples were centrifuged at 14,000 rpm for 15 minutes, resulting in a small pellet at the bottom of the tube. The supernatant was carefully discarded as to not dislodge the pellet. A volume of 700 μL of 70% ethanol was added to the sample, which was then centrifuged at 14,000 rpm for 5 minutes. The supernatant was once again carefully discarded. The samples were placed in a Thermo Scientific Savant DNA 120 Speed Vac Concentrator (Thermo Fisher Scientific, Waltham, MA, USA) for approximately 20 minutes, or until no liquid remained in the tube. Once dry, the pellet was resuspended in 200 μL of deionized H_2O. Samples were also diluted to 1:10 or 1:100 with deionized water to reduce inhibitors that may have interfered with PCR success. If initial PCR reactions, as outlined below, were unsuccessful, additional cleanup using a Zymo OneStep PCR Inhibitory Removal Kit (Zymo Research, Irvine, CA, USA) was done and the appropriate 1:10 or 1:100 dilutions were made until the sample was successful.

To prepare the PCR mix, 1 µL of the diluted DNA (1:10 or 1:100) was used as template DNA. A 313 base pair (bp) segment of the eukaryotic COI gene was used to assess the biodiversity in the samples, amplified using a primer set developed by Leray et al. (2013). A two-step PCR protocol was used to amplify and index the samples for sequencing (O'Donnell et al. 2016). The PCR mix includes 1X HotStar Buffer, 25 mM $MgCl_2$, 0.5 mM dNTP, 0.3 µM of each primer and 0.5 units of the HotStar Taq (Qiagen Corp. Valencia, CA, USA) in a 20 µL reaction. The initial 40 cycle PCR includes a touchdown annealing step that reduces the annealing temperature from 62°C to 46°C (-1°C per cycle) in 16 cycles, then keeps the annealing temperature at 46°C for 25 cycles (Kelly et al., 2018; Leray et al., 2013).

Three PCR replicates were produced for each water sample for a total of 165 separate PCR products, 162 of which were successful. Each PCR replicate was sequenced individually to understand the variation in signals collected through environmental DNA. This variation is a product of stochastic amplification during PCR cycles. Positive control replicates of Kangaroo (*Macropus rufus* and *M. fuliginosus*) were also sequenced, chosen because they are not present in the study area. Negative controls were used for PCR amplification steps but were not pooled into libraries and subsequently sequenced after it was determined that negative controls were clean of DNA through gel electrophoresis (see Kelly et al. 2018 for discussion of the merits of sequencing positive and not negative controls).

Library preparation was carried out according to KAPA Biosystems protocols (KAPA Biosystems, Wilmington, MA, USA; NEXTflex DNA barcodes; BIOO Scientific, Austin, TX, USA). Sequencing was completed on an Illumina MiSeq (250 bp, paired-end) platform using a MiSeq V.3 run.

2.3. Bioinformatics and BLAST

Raw sequence reads were quality filtered and assigned to each sample with a custom bioinformatics pipeline (https://github.com/ramongallego/demultiplexer for DADA2), and the original sequence composition was inferred using the R package DADA2, after following the general bioinformatics protocol described in Kelly et al. (2018). Contamination in the samples was controlled in three ways. First, site-occupancy modeling was used to estimate occurrence and data with less than 80% estimated probability of occurrence was eliminated. Second, cross-contamination among samples was estimated by calculating the number of sequences from the positive controls and that proportion was subtracted from the respective data. Lastly, PCR replicates that showed high dissimilarity amongst PCR replicates were not included in further analysis. The resulting sequences from the data were compared with the NCBI database, following Kelly et al. (2018), returning up to 50 matches from the database and identifying the last common ancestor with 75% or greater identity of equally good matches to return the most likely taxon associated with each sequence. The abundance of amplicon reads for each individual taxon was first normalized (into within-sample proportions) and then scaled (within-taxon, across samples) to create an index of abundance that varies from zero to one, as described in Kelly et al. (*In Review*). The annotated sequences were surveyed for known dinoflagellate taxa to ensure this technique could successfully identify documented species within Puget Sound, providing confidence in the method. Previously documented taxa present in the dataset include Dinophyceae, *Akashiwo sanguinea*, *Protoperidinium,* and *Hematodinium*.

The sequenced 313 bp DNA fragment matches several species of *Karenia* identically and was therefore annotated in our bioinformatics pipeline as their common taxonomic family, Kareniaceae. We cannot resolve species-level identity with the data in hand, however, given the alignment of the sequences of this study against other known Kareniaceae sequences in the

NCBI database, the dinoflagellate identified is most likely of the genus *Karlodinium*, though the genus *Karenia* is tightly associated. *Karlodinium* and *Karenia* are not easily distinguishable at the CO1 fragment in hand, therefore genus and species level identification cannot be confirmed with this data. I hereafter refer to this taxon as Kareniaceae *sp.*, understanding that taxonomic assignment is limited. The genus *Karenia* is the primary focus of this analysis, given that no species of *Karenia* has previously been reported, to my knowledge, from Puget Sound.

2.4. Statistical Analysis & Modeling

The environmental context of Kareniaceae *sp.* detections was evaluated by using a principal components analysis (PCA) to derive a synthetic environmental variable, making use of several correlated co-variates: temperature, salinity, oxygen, depth, and pH. I carried out a logistic regression to predict the presence or absence of Kareniaceae *sp*. using the first principal component (PC) of the PCA. I then repeated the logistic regression using only pH as a predictor variable. The variable pH was used because of the suspected relationship between pH and HAB growth and toxicity; in addition, pH was a significant predictor of Kareniaceae *sp.* incidence in the datasets. Models were compared using AIC values and likelihood ratio tests.

3. RESULTS

An Illumina MiSeq V.3 sequencing run resulted 4,974,110 sequence reads after clean up protocols. A total of 1242 amplicon sequence variants (ASVs) were also identified. Blast annotation of the sequence reads confirmed the presence of 81 unique taxa ranging in specificity from general phyla placement to species-specific identification (21 family annotations, 45 family-genus annotations, and 24 family-genus-species annotations).

Kareniaceae *sp.* was found in 6 of 55 samples (10.9%) within Puget Sound; all six detections were during the two 2018 cruises. The highest concentrations of Kareniaceae *sp.* eDNA were found at stations in the lower bend of the Hood Canal (April; P12 & P402, September; P11, P12, & P402) (Fig. 2). Other stations that show evidence of Kareniaceae *sp.* were located at Whidbey Island, the Central Basin, and the Hood Canal respectively (April; P4 & P28, September; P14) (Fig. 2). All samples in which Kareniaceae *sp.* was identified were taken below the surface at depths greater than 40 m (Fig. 3) and where photosynthetically active radiation (PAR; i.e., light) was zero. These same waters invariably also featured low dissolved oxygen, low pH, and high salinity waters (Fig. 4). Most reads occurred below a pH of 7.8 and a dissolved oxygen concentration of 5 mg/L. Salinity measurements for these samples were above approximately 30.0 ppt (Fig. 4).

Because these environmental parameters were strongly correlated, I developed a synthetic environmental variable using PCA, as described in the Methods, using depth, pH, salinity, oxygen, and temperature variables. The first PC accounted for 50.51% of environmental variation in the dataset (Table 1). A logistic regression using this first PC (Model 1) significantly correlates to the presence of Kareniaceae *sp.* eDNA ($p < 0.05$). A logistic regression using pH as the only predictor variable (Model 2) also significantly correlated to the presence of Kareniaceae *sp.* eDNA ($p < 0.05$). Model 1 resulted in a smaller residual deviance and AIC value suggesting that a model using a combination of variables better explains variation in the dataset. A likelihood ratio test lead to the same conclusion showing a high likelihood for Model 1 (Table 2).

Model 2 was plotted with the original presence/absence data to show the increased likelihood of Kareniaceae *sp.* presence at pH below 8.0 (Fig. 5).

Table 1. Importance of principal components created from depth, pH, salinity, oxygen, and temperature variables.

	PC1	PC2	PC3	PC4	PC5
Standard Deviation	1.5892	1.16	0.79	0.69	0.17
Proportion of Variance	0.5051	0.27	0.13	0.10	0.01
Cumulative Proportion	0.5051	0.77	0.90	0.99	1.00

Table 2. Comparison of logistic regression models using the first principal component of a principal component analysis combining depth, pH, salinity, oxygen, and temperature variable (Model 1) and a logistic regression using only pH as the predictor variable (Model 2). The lower the value of these three indicators gives evidence that it is a better model fit for the data.

Model	Residual Deviance	AIC	Likelihood
Model 1: PC 1	40.74	44.74	-20.37
Model 2: pH	57.28	61.28	-28.64

4. DISCUSSION

4.1. Presence of Kareniaceae in relation to environmental conditions

The identification of Kareniaceae *sp.* illustrates a use of environmental DNA that has so far been only hypothetical: detecting species in the process of colonizing new habitat. Here, the likely invasive Kareniaceae *sp.* may also be toxic, given its lineage, and so eDNA monitoring for this species has additional policy relevance. Other HAB monitoring policies and programs currently exist to assess domoic acid presence and diarrhetic shellfish toxins in Puget Sound and along the outer Washington Coast, run by the Olympic Region Harmful Algal Bloom (ORHAB) Partnership, the Washington Departments of Health (WDOH), Fish and Wildlife (WDFW) , and the Northwest Fisheries Science Center (NWFSC) (Chadsey, Trainer, & Leschine, 2012; Trainer et al., 2013). Furthermore, citizen-science initiatives like SoundToxins recruit volunteers to aid

in its monitoring (Chadsey et al., 2012; Trainer et al., 2013). Similar efforts could be extended to toxins produced by species within Kareniaceae.

Kareniaceae *sp.* was found at six stations within Puget sound (P4, P11, P12, P14, P28, P402), most of which are located in the southern section of the Hood Canal, which is poorly flushed by tides (Khangaonkar, Nugraha, Xu, & Balaguru, 2019) and has experienced water-quality problems in the past, particularly with low dissolved oxygen levels and eutrophication due to nitrogen input (Snover et al., 2005). Overall the documentation of the presence of this family of dinoflagellates is poor. There is no published literature that reports the presence of the genus *Karenia* and little record of the genus *Karlodinium* within Puget Sound (S.K. Moore et al., 2018; Zhang, Litaker, Vandersea, Tester, & Lin, 2008).

The data suggest that this taxon occurs only in lower-pH waters of the region, in combination with other environmental factors, which is consistent with studies showing that Kareniaceae *sp.* thrives under high pCO_2 (low pH) conditions (Hu et al., 2017). Although growth of the dinoflagellate does not significantly increase according to in vitro studies (Hu et al., 2017), the prediction model presented in this study suggests that a change in water pH from 7.8 to 7.4 increases the probability of Kareniaceae *sp.* presence by from 29% to 95% (Fig. 5). Average surface ocean pH values are projected to drop by as much as 0.32 from 8.2 to 7.8 by 2100, and Puget Sound model estimates for 2095 suggests that the average bottom pH in the Hood Canal will be 7.2-7.4 depending on the time of year (Khangaonkar et al., 2019). Therefore a prediction of 7.4 is not unlikely for the naturally more acidic Puget Sound area (IPCC, 2013; Murray et al., 2015). With the already decreasing pH levels within Puget Sound and a decreased ability to buffer further pH drops, the continuing progression of ocean acidification (OA) could lead to bloom conditions for the taxon (Busch et al., 2013). High CO_2 is found in the Hood Canal, which

corresponds to the low pH values recorded in relation to Kareniaceae *sp.* in 2018 (S.K. Moore et al., 2018).

Kareniaceae *sp.* was found mainly in the lower section of the Hood Canal exclusively in subsurface samples in 2018. These samples also corresponded to low pH values, low dissolved oxygen concentrations, and high salinities. Low oxygen concentrations and low pH values are related, therefore the relationship of Kareniaceae *sp.* to both variables is unsurprising. Oxygen concentrations are also decreasing in Puget Sound, with sections of the sound reaching low levels of 5 mg/L (Snover et al., 2005). The combined effect of low dissolved oxygen and low pH could mean the presence of Kareniaceae *sp.* will likely be sustained. However, Barnes et al. (2015) only found the species *K. mikimotoi* in significant abundances in low salinity conditions..

The depths and low light conditions in which Kareniaceae *sp.* was found are unusual, as this taxon is photosynthetic and thus requires light for cell processes. This result may indicate that this analysis may have identified a benthic cyst form of the taxon, of which there are two kinds, temporary and resting. Temporary benthic cysts are typically formed by dinoflagellates when environmental conditions are not ideal for growth or when there are adverse nutrient conditions (Fistarol, Legrand, Rengefors, & Granéli, 2004; Zhao, Tang, Zhao, & Wang, 2017). Similarly, resting cysts can be produced when conditions are not ideal, and remain in a dormant state for days, months, or years (McMinn & Martin, 2013). When in a dormant state, the cysts are inactive and do not require photosynthesis, meaning they can survive in the sediment with little to no light exposure. Given the depths and light conditions in which Kareniaceae *sp.* was found in this dataset, it is possible that signals were produced from inactive resting cysts, though this type of cyst is not yet documented in any *Karenia* species (Cuadrado, Bustos, & Figueroa, 2019).

There was no evidence of this taxon in the September 2017 cruise. In Puget Sound, concentrations of dinoflagellates have been low for several years when compared to the

concentrations of diatoms in the same area (Moore et al., 2018). The year 2017 also saw lower than normal nitrate levels in areas deeper than 75 m during the September cruise (Moore et al., 2018). The low nitrate levels combined with minimal hypoxia recording could explain the lack of presence of Kareniaceae *sp.* during this cruise. Further investigation into the exact environmental conditions during the 2017 and 2018 cruises is needed to determine the reason for presence of the taxon or lack thereof.

The growth of populations of Kareniaceae *sp.* may not be causally related to eutrophication but is affected by nutrient input and limitation. In vitro studies showed growth of *K. mikimotoi* was stunted when nitrogen concentrations were reduced, and growth continued in replenished nitrogen conditions (Lei & Lü, 2011; Zhao et al., 2017). During the nitrogen-limited conditions, the cells could produce temporary cysts (Zhao et al., 2017). These cysts are easily re-established into their motile state when conditions improve (Fistarol et al., 2004). This suggests that the species can survive adverse environmental condition and revive once nitrogen is supplied.

4.2. Establishment of a species

There are recent indications of the species *K. mikimotoi* in Kachemak Bay, Alaska ("Working with State to Document First Occurrence of Harmful Karenia mikimotoi Algae in Alaskan Waters," n.d.) and in Monterey Bay, California (Djurhuus et al., *In Revision*). Given the presence of the species *K. mikimotoi* in these nearby locations, it is possible that the Kareniaceae *sp.* detection in Puget Sound is also *K. mikimotoi*, although more specific molecular and morphological identification is needed to confirm this.

The appearance of a previously absent species within a region can be a result of several possible circumstances. One mechanism of dispersal of *K. mikimotoi* into the Puget Sound area could be international shipping routes and shellfish transport (Hégaret, Shumway, Wikfors, Pate,

& Burkholder, 2008; Kang, Hyun, & Shin, 2010; Laabir et al., 2007; Seebens, Gastner, & Blasius, 2013). In order for phytoplankton to successfully be established in a new location, conditions would have to be optimum for growth at the receiving location and is not common (Hégaret et al., 2008; Kang et al., 2010). The transportation of shellfish has potential to spread species of phytoplankton due to the filter feeding method of the organism. The threat of parasite and pathogen spread is recognized by Interstate Shellfish Sanitation Committee (ISSC), the National Shellfish Sanitation Program (NSSP), but little is done by these organizations to regulate the spread of phytoplankton (Hégaret et al., 2008). It has been shown that phytoplankton cells can be recovered from bio-deposits of transplanted shellfish after 24 hours. Many combinations of shellfish and phytoplankton were tested by Hégaret et al. (2008), and it was confirmed that motile cells from *K. mikimotoi* were present 24 hours after deposition from the blue mussel and manila clam, but were no longer detected at 48 hours. This evidence shows that transport through shellfish is a viable hypothesis for the spread of HAB species.

Although Kareniaceae *sp.* is not well documented within Puget Sound, it is possible that this group has been present in the area but has not been detected due to the lack of inspection. Prior to 2011, there was no evidence of toxicity associated with *Dinophysis*, a dinoflagellate which produces toxins that cause diarrhetic shellfish poisoning (DSP) when ingested (Trainer et al., 2013). Though not fatal to humans, DSP causes diarrhea, nausea, and vomiting shortly after consumption and symptoms may last for several days. Washington state had no monitoring protocols for DSP in place prior to 2011, when several people fell ill after consuming contaminated mussels, despite the fact that there was evidence of this species prior to this event (Trainer et al., 2013). A similar story could be unfolding with Kareniaceae *sp.* It is possible that this family of dinoflagellates has been in the Puget Sound area, but without the techniques to look at whole phytoplankton communities, the pertinent species may have been overlooked.

The samples identified as Kareniaceae *sp.* in this study were all located at depth, despite the need for light to undergo photosynthesis. As mentioned previously, it is possible that the signal could be from benthic cysts produced during adverse conditions. This cannot be a complete explanation, however, because of the lack of detection during September 2017 at any depth. It would be expected that Kareniaceae *sp.* would be detected at or near the surface before a benthic form is detected. For this reason, it is also possible that this study is detecting a new benthic species of *Karenia* or *Karlodinium* that has not yet been identified. The species, which would also be able to thrive is a full range of salinities given the data, may be heterotrophic, thus not requiring the light that is absent at depth.

4.3. Risks associated with HAB formation

The recorded outbreak of *K. mikimotoi* in Alaska was the first for the area. This species is known for causing massive fish kills and mortality in benthic organisms in New Zealand, Japan, China, Ireland, and France. The mechanism by which the toxins become lethal to organisms is still under investigation, as there are likely many. It is thought that one threat is the clogging of the fish gills, allowing the direct deliverance of the toxin to the fish (Lei & Lü, 2011; O'Boyle, McDermott, Silke, & Cusack, 2016). The size of the blooms may also cause oxygen depletion in the water, after decomposition, posing a threat to benthic organisms (O'Boyle et al., 2016). These massive die-offs create problems for the ecosystem, but also lead to economic losses for the affected region (Lu et al., 2016; Yang et al., 2011).

It is unclear whether humans can be affected by the consumption of shellfish contaminated with *K. mikimotoi*, however other toxic species within the genus are harmful to humans. The toxins produced by *K. mikimotoi* are not the extremely hazardous brevetoxins produced by *K. brevis* (Chen, Yan, Yu, & Zhou, 2011). Work has been done to determine the extent of damage

K. mikimotoi toxins could have on mammalian cells when concentrations are high. The dinoflagellates showed production of compounds that inhibited cell proliferation and cause oxidative degeneration of lipids in mammalian cells, which suggests that there are potential human health impacts, though the extent to which this is true is unclear (Chen et al., 2011). The presence of small amounts of this species does not necessarily indicate toxicity. The potential impact of Kareniaceae *sp*. on Puget Sound would depend on the cell concentration, which in turn indicates toxicity of the bloom (Hardison, Sunda, Tester, Shea, & Litaker, 2014). The blooms that reached harmful levels had cell concentrations of 10^7 cell L^{-1} in China (Chen et al., 2011), 10^6 cell L^{-1} in Ireland (O'Boyle et al., 2016), 10^4 cell L^{-1} in France (Gentien, Lunven, Lazure, Youenou, & Crassous, 2007), and 10^6 cell L^{-1} in New Zealand (Shi et al., 2012).

If a bloom of *K. mikimotoi* were to occur in Puget Sound, there is evidence that a bacteria-algal interaction with *Thalassospira* could be a biological control method to prevent red tide damage. The benzoic acid produced by *Thalassospira* successfully killed *K. mikimotoi* in a laboratory setting (Lu et al., 2016).

Similar effects would occur if there was a bloom of *Karlodinium veneficum*. A notable bloom occurred in Maryland, USA in September of 2005, causing mortalities of gizzard shad (*Dorosoma cepedianum*), hickory shad (*Alosa mediocris*), sunfish (*Lepomis spp.),* white perch (*Morone americana*), yellow perch (*Perca flavescens*), and carp (*Cyprinus carpio*) (Place et al., 2012). Other species such as *K. micrum* have more recently been characterized for their toxicity, but have shown to be toxic in Chesapeake Bay, USA and in aquaculture facilities (Deeds, 2003).

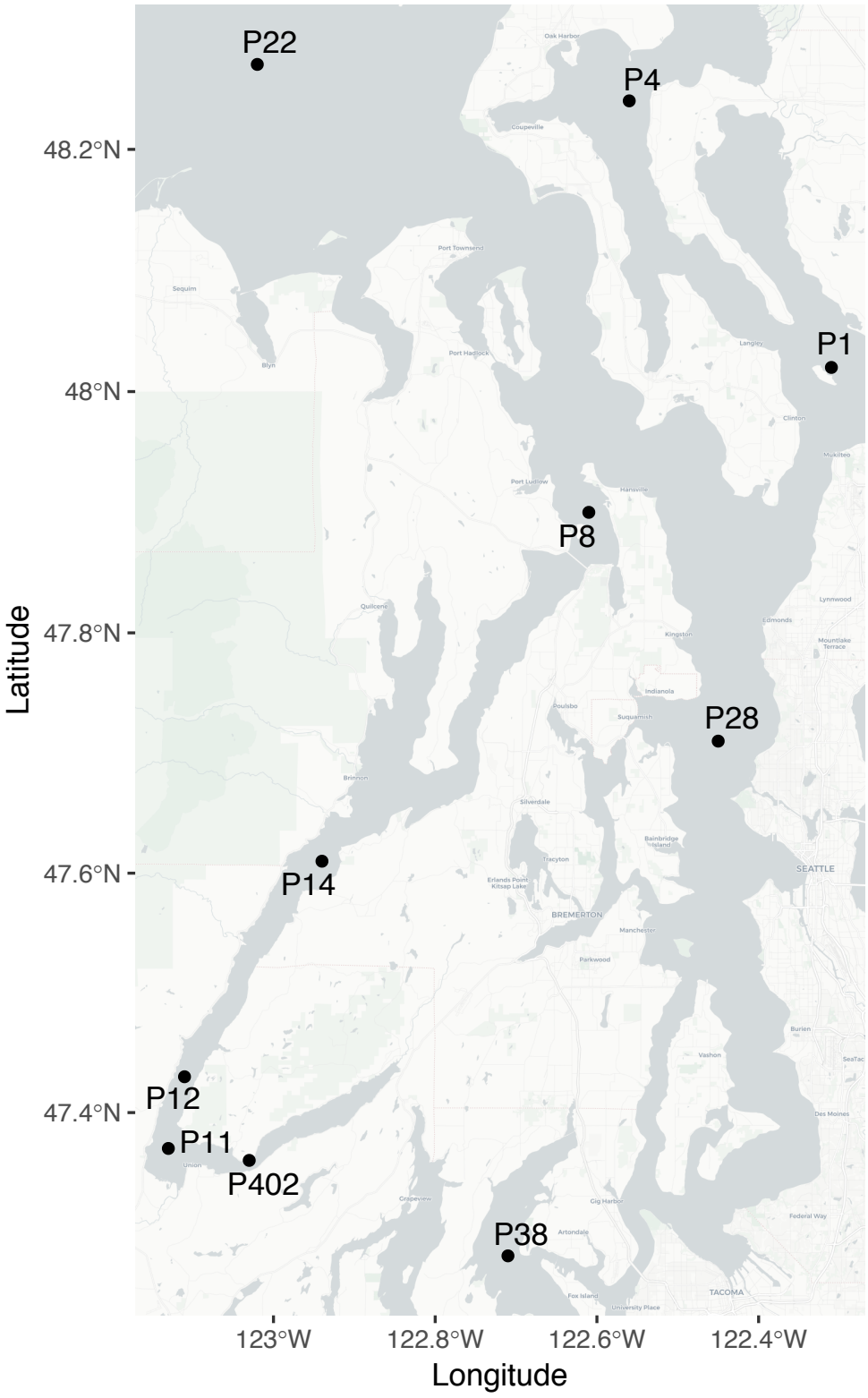
4.4. Next steps

This study shows the successful identification of a taxon that has the potential to form a HAB in Puget Sound using eDNA. Though eDNA surveys allow for a larger look at the ecosystem,

bias is still present, as with any other survey method. Environmental DNA analysis does not detect every single species that may be present in the study area, mainly due to bias introduced by the primers used for analysis (Kelly et al., 2017). The DNA primers interact with the sample such that the primer may selectively amplify DNA fragment from some organisms more than others, despite environmental factors (Kelly et al., 2017). There is also limitation to the level of classification that can be achieved with general primers, a task that is especially difficult for phytoplankton (Peters et al., 2018). Further analysis of the samples used for this study could be done with more phytoplankton specific primers, though, the more general primers allow identification of taxa without intentional observation of its presence. The environmental relationships associated with Kareniaceae *sp.* which are outlined in this study are rudimentary, given the conditions in which the taxon was found. Additional samples containing Kareniaceae *sp.* are needed to create a clearer picture of conditions in which the taxon is found and generate predictions. This work is currently underway.

This technique offers a unique opportunity to investigate the presence of many species at once, however, is particularly useful for a first step monitoring tool for taxa such as Kareniaceae *sp.* This method could provide some indication that species of interest are present in the Puget Sound region and aid in the decision-making process as to where further investigative efforts should be focused to prevent a HAB.

6. FIGURES



Figures. 1. Map denoting sampling locations in Puget Sound, WA.

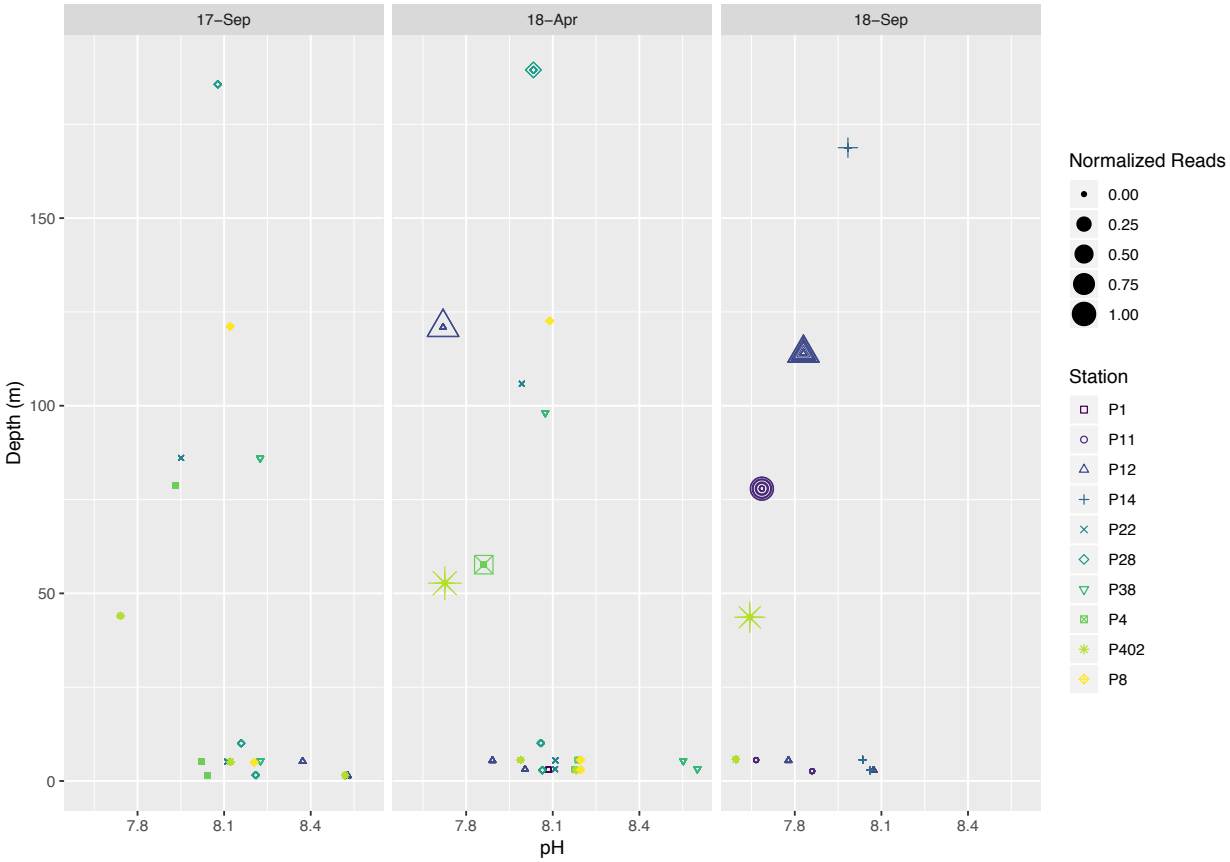


Figure 2. Presence of Kareniaceae *sp.* as represented by normalized sequence reads plotted against depth and pH according to cruise and station.

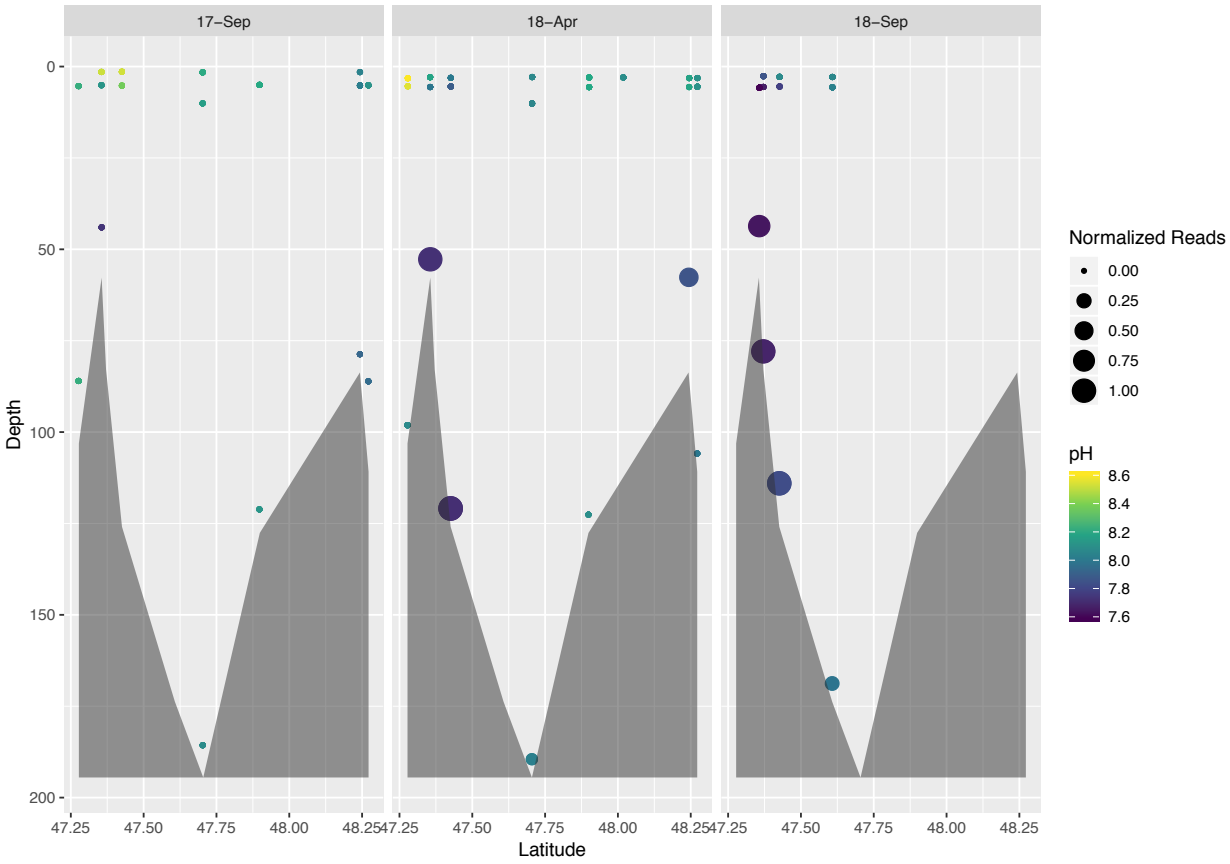


Figure 3. Presence of Kareniaceae *sp*. as represented by normalized sequence reads plotted against depth and latitude according to cruise. Shading denotes bottom contour at sampled locations; color denotes the pH of each sample.

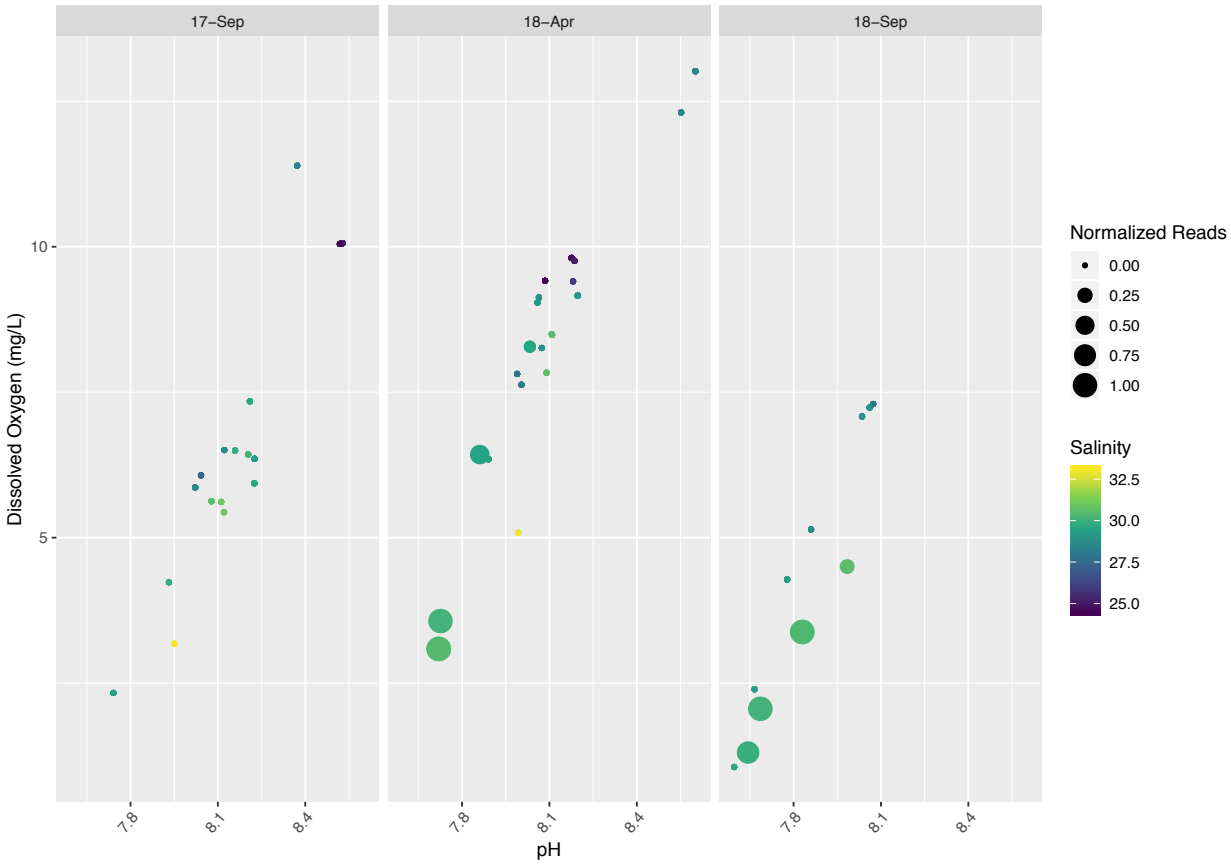


Figure 4. Presence of Kareniaceae *sp.* as represented by normalized sequence reads plotted against dissolved oxygen and pH according to cruise. Color denotes the salinity (ppt) at that sample location.

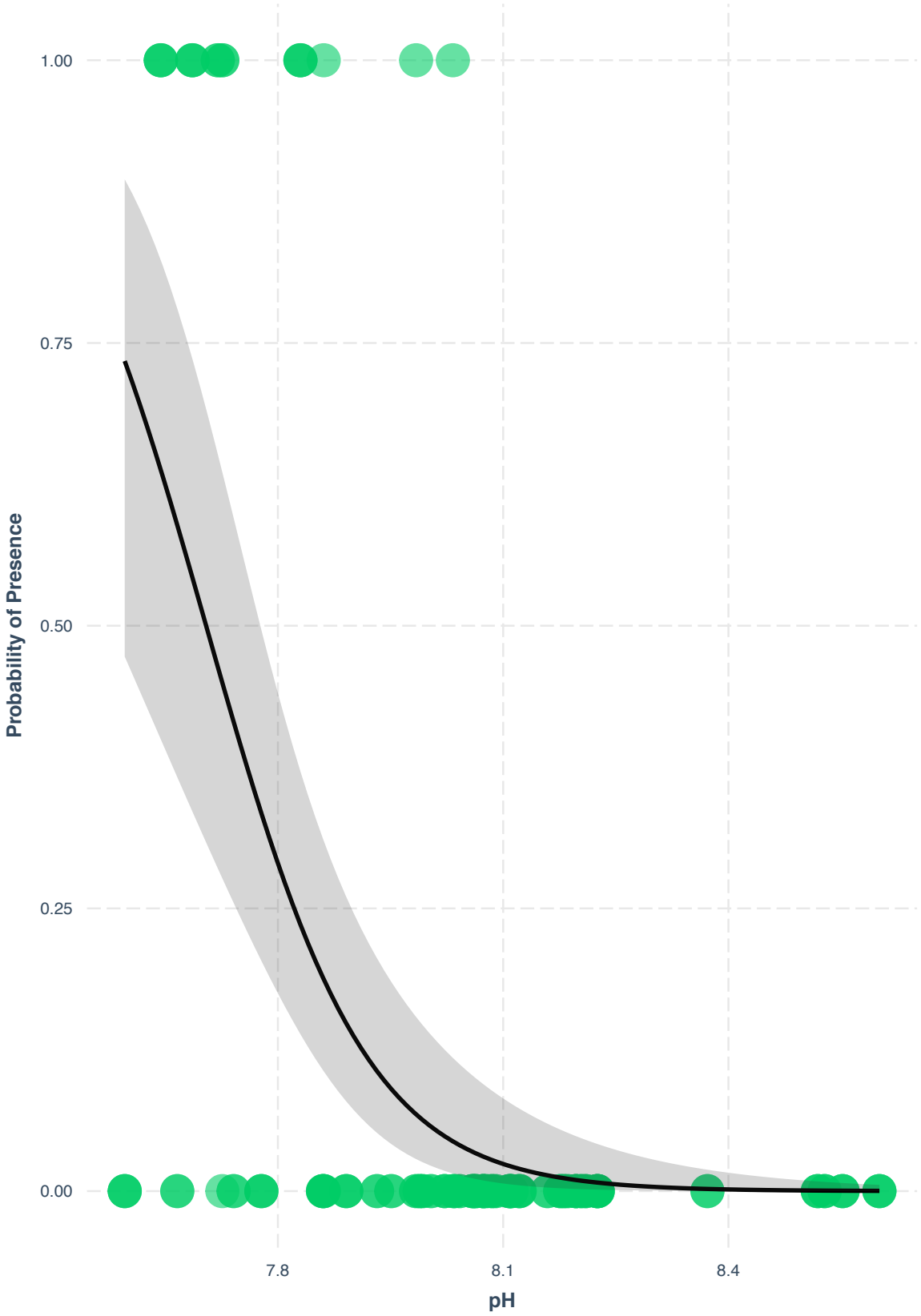


Figure 5. Logistic regression of Kareniaceae *sp.* as a function of pH ($p = 35 \times 10^{-6}$) .

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