Environmental DNA Reveals Patterns of Biological Invasion in a Fjord

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Abstract

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Introduced species have the potential to cause ecological and economic harm to coastal and estuarine ecosystems. Understanding which habitat types are most vulnerable to biological invasions, where invasions originate, and the vectors by which they occur can help direct limited resources to prevent or mitigate harm. Data on introduced species occurrence can help guide interventions at all stages of invasion, from first introduction, to naturalization and invasion. Monitoring at an ecosystem scale requires considerable investment of time, resources, and taxonomic expertise. Environmental DNA (eDNA) metabarcoding samples coastal ecosystems at broad spatial and temporal scales to augment established monitoring methods. I use eDNA sampling to identify the presence of introduced species across distinct habitats in the Salish Sea in Washington State, USA. Sampling sites cross a range of habitats, from estuarine mudflats to marine rocky shores. The introduced species captured by my assay include both well-documented invaders and species not previously reported in the Salish Sea. I find an introduced assemblage dominated by shellfish and algae with native ranges in the temperate western Pacific. My results reveal a far greater invasion level in estuarine habitats than on marine rocky shores. I find an increase in invasion level with higher water temperatures in spring and summer across habitat types. This analysis contributes to a growing understanding of the biotic and abiotic factors that influence invasion level. eDNA surveys provide an effective means to monitor ongoing biological invasions and to better understand the factors that drive these invasions.

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Abstract

Introduced species have the potential to cause ecological and economic harm to coastal and estuarine ecosystems. Understanding which habitat types are most vulnerable to biological invasions, where invasions originate, and the vectors by which they occur can help direct limited resources to prevent or mitigate harm. Data on introduced species occurrence can help guide interventions at all stages of invasion, from first introduction, to naturalization and invasion. Monitoring at an ecosystem scale requires considerable investment of time, resources, and taxonomic expertise. Environmental DNA (eDNA) metabarcoding samples coastal ecosystems at broad spatial and temporal scales to augment established monitoring methods. I use eDNA sampling to identify the presence of introduced species across distinct habitats in the Salish Sea in Washington State, USA. Sampling sites cross a range of habitats, from estuarine mudflats to marine rocky shores. The introduced species captured by my assay include both well-documented invaders and species not previously reported in the Salish Sea. I find an introduced assemblage dominated by shellfish and algae with native ranges in the temperate western Pacific. My results reveal a far greater invasion level in estuarine habitats than on marine rocky shores. I find an increase in invasion level with higher water temperatures in spring and summer across habitat types. This analysis contributes to a growing understanding of the biotic and abiotic factors that influence invasion level. eDNA surveys provide an effective means to monitor ongoing biological invasions and to better understand the factors that drive these invasions.

Introduction

Invasive marine species have increasingly high economic and ecological costs (Diagne et al., 2021; Molnar et al., 2008). They threaten fisheries, aquaculture, and marine recreation (Alemu I et al., 2019; Shiganova, 1998; Whitlow, 2010). They disrupt food-webs, alter habitat structure, and displace native species (Calizza et al., 2021; Irigoyen et al., 2011; Sullaway & Edwards, 2020). Climate change, coastal eutrophication, and increasing global trade all facilitate the spread and establishment of introduced species (Bailey, 2015; Chan et al., 2019; J. J. Stachowicz, Terwin, et al., 2002; van Tussenbroek et al., 2016). Consequently, there is a need to refine tools that may guide the deployment of limited resources to manage introduced species. Understanding the distribution of introduced species is an important component in the larger effort to determine when and how introduced species become established as invasive.

Prevention and early detection of biological invasions leads to the most successful economic and ecological outcomes (Davidson et al., 2015; Vander Zanden et al., 2010; Wimbush et al., 2009). Towards this goal, the sampling of environmental DNA (eDNA) has shown great promise when applied to the detection of introduced species (Woodell et al., 2021). Targeted Quantitative Polymerase Chain Reaction (qPCR) methods have successfully detected the presence of individual species of interest, including the terrestrial toad species *Bufo japonicus formosus* in Hokkaido Japan and invasive crayfish in Baden-Württemberg Germany (Chucholl et al., 2021; Mizumoto et al., 2022). Data gained from qPCR has motivated management and legal action on invasive carp in the Great Lakes region (Jerde, 2021). Sampling and analysis of eDNA data can also be used to complement established monitoring methods such as visual surveys. Data from eDNA sampling was paired with conventional trap data to better quantify the invasion front of European green crab in the Salish Sea, Washington (Keller et al., 2022).

Multiple species approaches show promise as well. In the United Kingdom and the Arctic Ocean marine eDNA metabarcoding has been used to identify the presence of multiple introduced species within water samples across a large spatial scale (Holman et al., 2019; Lacoursière-Roussel et al., 2018). This technique has also been employed to monitor the shifting ranges of harmful marine microalgae (Jacobs-Palmer et al., 2021).

A foundational question in marine invasion ecology and management is which biotic and abiotic factors make habitats vulnerable to invasion (Heger et al., 2013; Olyarnik et al., 2009). Significant progress has been made towards answering this question via visual sampling. Surveys in Northern Europe and San Francisco Bay have shown that habitats of mid-range salinity in estuaries have a greater invasion rate (introduced species richness / total species richness) than either tidal freshwater habitats or full salinity marine habitats (Paavola et al., 2005;

Preisler et al., 2009; Wasson et al., 2005; Wolff, 1998). A global literature review of invertebrate invasion data by Preisler et al. (2009) showed that with few exceptions, temperate estuaries are more invaded than adjacent open coasts. Proposed mechanisms for this phenomenon include: (1) most ports occupy mesohaline and polyhaline regions of estuaries; (2) species adapted to mid-range salinities are more likely to survive the variable temperature and salinity of transport by ballast or hull biofouling; (3) mid-salinity regions face propagule pressure from both salt tolerant freshwater species and freshwater tolerant estuarine species; and (4) mesohaline and polyhaline waters have lower native species richness than either fresh or euhaline waters (Nehring, 2006; Remane, 1934; Wolff, 1998).

Biotic resistance - the degree to which native species richness and abundance determines habitat invasibility – might also help inform the management and conservation of coastal ecosystems. Ecologists have hypothesized that biotic resistance encompasses multiple mechanisms including competition, disease, and predation (Jeschke et al., 2018). They have also hypothesized that resistance may result from a single key native species, or from the sum of resource utilization among the entire native assemblage (Olyarnik et al., 2009). Experimental, mesocosm, and observational studies have reached conflicting conclusions about the net effect of native species richness on habitat invasibility (Jeschke et al., 2012). The introduced zooplankton species *Daphnia lumholtzi* was more likely to be present in zooplankton mesocosms with higher native richness (Lennon et al., 2003). Conversely, in seagrass mesocosms, the biomass of introduced invertebrates was shown to have an inverse relationship with native invertebrate richness (France & Duffy, 2006). Observational field surveys have similarly reached mixed conclusions (J. J. Stachowicz et al., 2002). The net effect of native biotic resistance in marine systems remains unclear, as does its relative importance compared to abiotic factors, propagule pressure, and the characteristics of introduced species.

By allowing the amplification and measurement of eDNA from organisms across the tree of life, the Cytochrome Oxidase I (COI) primer set is a valuable tool in this ongoing line of inquiry into which biotic and abiotic factors make certain habitats more invasible than others. Amplicon sequencing reflects an essentially random sample of species present at a site (Kelly et al., 2017). There is unlikely to be systematic bias towards amplification of either introduced species or native species. As such, amplicon sequencing is a powerful way to investigate questions requiring simultaneous data on both native and introduced assemblage composition and species richness. The relative ease of collecting eDNA from water bottle samples allows for consistent sampling effort to be conducted at regional scales (Mize et al., 2019). If we consistently observe weaker eDNA signal from introduced species in samples with stronger native species signal across distinct habitats and sampling events, this would lend support to the biotic resistance hypothesis.

While previous marine and aquatic metabarcoding surveys have cataloged invasion fronts and introduced species distribution, none have investigated invasion level across a range of coastal habitats. Visual surveys have cataloged introduced species assemblages in the Salish Sea but an eDNA survey of introduced species in this region has yet to be conducted (Cohen et al., 1998). In order to investigate variation in invasion level across the Salish Sea, water samples were collected from sites across a range of salinities, temperatures, and substrates. My results reveal that higher introduced species richness is associated with higher water temperature and lower native species richness across multiple sites. I find estuarine habitats to have far higher introduced species richness and invasion rate than marine habitats.

Methods

Water Sampling

In order to characterize eDNA assemblages I analyzed data originally generated for Gallego et al. (2020). Water samples were collected in the intertidal zone of state and county parks along the Hood Canal fjord and the shore of San Juan Island, Washington, USA **(Figure 1)**. Abiotic conditions at these sites fall roughly along a gradient of salinity, temperature, wave energy, and substrate - with the San Juan sites experiencing higher salinity, lower temperature, and greater wave energy. The three southernmost sites along the Hood Canal consist of mudflat habitat. The two northern sites on the Hood Canal are cobble beaches. The San Juan Island sites are primarily rocky bench (Gallego et al., 2020).

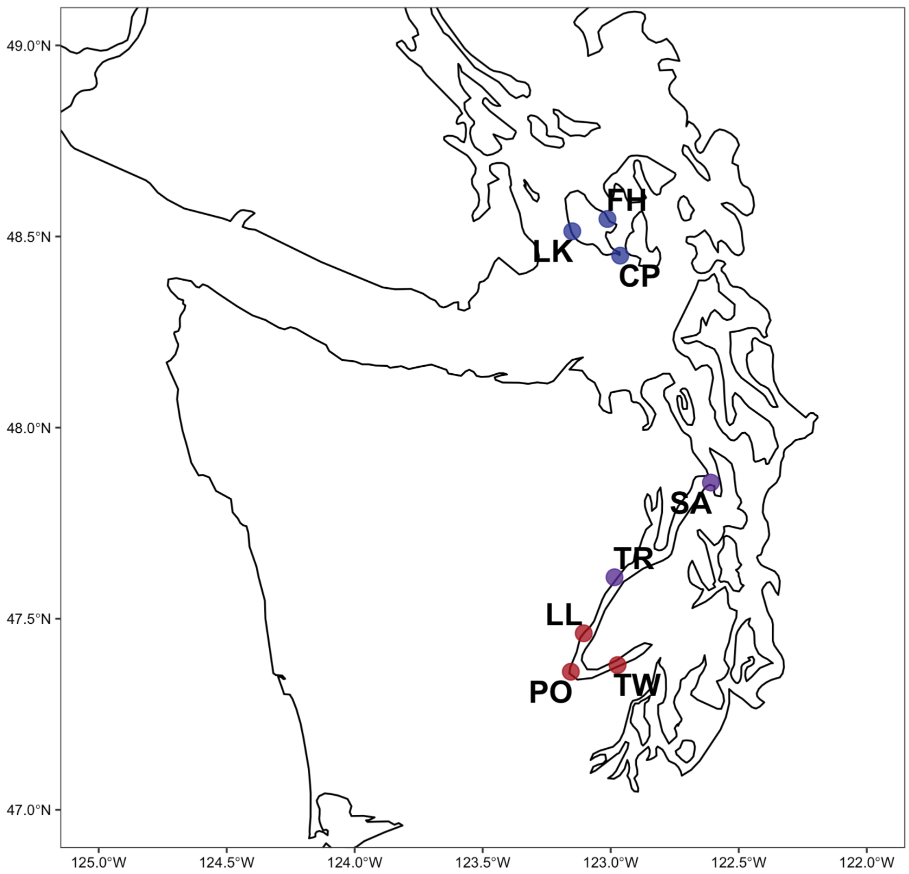


Figure 1. The southern portion of the Salish Sea, a fjord system of the Northwest Pacific located in Washington State, USA. Sampling sites are coded by habitat type, red = estuarine mudflat, purple = estuarine cobble beach, blue = rocky bench.

Three one-liter replicate bottle samples were collected at each of the eight sites monthly between March 2017 and August 2018. Samples were filtered through 0.45 µm cellulose and these filters were preserved in Longmire buffer according to Renshaw et al. 2015 prior to DNA extraction. Water temperature, salinity, and dissolved oxygen were measured during each sampling event with a multiprobe (Hannah Instruments, USA) and a salinity refractometer.

Sequencing and Bioinformatics

The sampled DNA was purified with a phenol-chloroform-isoamyl alcohol extraction following procedures in Renshaw et al. (2015). Extracted DNA served as a template for polymerase chain reaction (PCR), amplifying 313 base pairs of the COI gene region (Leray et al., 2013). PCR was conducted using protocols from Kelly et al. (2018). Three subsamples of the template DNA served as technical process replicates. These technical replicates were amplified separately and sequenced separately to assess the variability of the PCR process itself. Secondary indexing tags were introduced with a two-step PCR protocol following O'Donnell et al. (2016) in order to avoid index amplification bias. I sequenced multiplexed sampling events using MiSeq v2-500 and v3-600 sequencing kits via manufacturer specifications. Each sequencing run included three samples of terrestrial species DNA not present in the study region – red kangaroo *Macropus rufus* and ostrich *Struthio camelus* - as a positive control to avoid misassignment of sequences to samples, and to measure tag-jumping (Gallego et al., 2020; Schnell et al., 2015).

Sequencing quality control and taxon assignment to Amplicon Sequence Variants (ASVs) was conducted using custom Bash and R scripts. A Github repository containing these scripts, and access to the corresponding FASTA sequence data is linked in the supplementary material **(Supplemental 1)**.

First, I executed Unix scripts to run the open-access programs Cutadapt and DADA2 for primer-trimming and removal of PCR artifacts (Callahan et al., 2016; Martin, 2011) DADA2 was used to estimate the ASV composition of each sample. Second, I conducted further quality control of samples using the R pipeline outlined in Kelly et al. (2018) to remove technical replicates containing PCR anomalies.

Lastly, I used two bioinformatic tools to assign ASVs to taxa. *Insect* v1.1 was run to assign taxa via informatic sequence classification trees (Wilkinson et al., 2018). Additional ASVs were assigned to taxa using a custom COI database with *anacapa* and *Bowtie2* (Curd et al., 2019; Langmead & Salzberg, 2012). For the purpose of quantifying the species richness of the eDNA sampled, I only retained ASVs identified to the species level. A species was considered present in a sample if an ASV assigned to that species appeared in at least 1 out of 3 replicates for a given site and date.

Identification of Native, Cryptogenic, and Introduced Species

Conservatively, I performed a secondary BLASTn search on all ASVs assigned to the species level. Any ASV with a percent sequence identity (pident) < 95% with its best-match reference sequence was considered unclassified. The retained ASVs with species-level assignments were referenced against published literature and peer-reviewed online databases to classify each species as either native to the Salish Sea, introduced to the Salish Sea, or cryptogenic. If World Register of Marine Species (WORMS), Algaebase, Biodiversity of the Central Coast, or National Estuarine and Marine Exotic Species Information System (NEMESIS) listed a species as having a distribution containing the Salish Sea; then the species was classified as native (Starzomski et al., 2022; Horton et al., 2022; M.D. Guiry & G.M. Guiry, 2022; PW Fofonoff et al., 2018). If no peer-reviewed source contained the species distribution, or if there were less than three published detections of the species, then the species was classified as cryptogenic.

The secondary BLASTn search also determined if ASVs were classified as introduced. ASVs with percent sequence identity > 98% and bitscore > 480 against an NCBI reference entry were classified as introduced if multiple peer-reviewed sources listed the species as introduced to the Salish Sea or introduced to other temperate estuaries (n = 17). If there was no such record, the ASV was classified as introduced only if all native species within the same genus were present in the NCBI COI database, and dissimilar to the queried ASV (n = 2). ASVs with percent sequence identity > 95% and bitscore > 460 against a reference entry were classified as introduced only if multiple peer-reviewed sources documented the species as introduced to the Salish Sea or introduced to other temperate estuaries, and if all native species within the same genus were present in the reference database and dissimilar to the queried sequence (n = 2). Lastly, an amino acid translated BLASTx search was performed to confirm ASV dissimilarity from any native relatives. Where peer-reviewed literature contained contradictory information regarding native distribution, species were classified as introduced if at least three distinct sources supported introduced status in the Salish Sea. See supplementary materials for complete BLAST results and classification flowchart **(Supplemental 1 & 2)**.

Analysis

To determine which abiotic and biotic conditions of the sampled habitats best predict introduced species richness, I used R (version 4.1.3) and the rstanarm package to compare poisson regression models with introduced species richness as the outcome variable - and salinity, temperature, and native species richness as candidate predictor variables. This pool of predictive variables was selected because I was able to measure a range of these factors in the field and because invasion ecology literature suggests introduced species richness varies across ranges of salinity, temperature, and native species richness. I tested models with combinations of these three factors. The best predictive model was selected using leave-one-out cross validation.

To determine if introduced species richness and invasion rate varied across sampling sites and sampling months, I used R and the vegan package to perform permutational multivariate analyses of variance (PERMANOVA) with site and month as the grouping factors.

Results

In total, sequencing and bioinformatic analysis yielded 50.8 million sequences across 86 unique sampling events. Quality control retained 45.0 million of these sequences made up of 4,848 unique ASVs. Of these ASVs 1,364 could be annotated to a taxonomic level of family, genus, or species, representing 22.6 million reads.

Composition

I was able to detect 405 species from 41 phyla across the eight sampling sites. Of these 405 species, I identified 384 as native or cryptogenic, and 21 as introduced **(Table 1)**. Eight phyla were represented among these introduced species: Florideophyceae, Mollusca, Arthropoda, Dictyochophyceae, Bangiophyceae, Chordata (Tunicata), Cnidaria, and Annelida. Introduced species included 9 algal species, 5 bivalve species, 3 copepod species, 1 ascidian species, 1 hydrozoan species, 1 polychaete species, and 1 amphipod species.

Two introduced species - Manilla clam (*Ruditapes philippinarum*) and Pacific oyster (*Crassostrea gigas*) - are commonly cultivated in commercial aquaculture operations along the Hood Canal. These aquaculture species were detected in 25 of 86 and 13 of 86 samples respectively.

species	n_detections	BLASTn pident	Bitscore
Pseudochattonella farcimen	59	100	579
Gracilaria vermiculophylla	34	100	579
Ruditapes philippinarum	25	100	579
Caulacathus okamurae	23	100	579
Nuttallia olivacea	17	100	579
Crassostrea gigas	13	100	562
Callithamnion corymbosum	13	100	579
Neoporphyra haitanensis	12	99.68	566
Monocorophium acherusicum	9	100	579
Gelidiophycus freshwateri	9	100	579
Botrylloides violaceus	6	100	579
Bougainvillia muscus	3	100	579
Lomentaria hakodatensis	3	100	579
Mya arenaria	3	100	584
Gelidium vagum	2	100	579
Musculista senhousia	2	96.16	512
Nitokra spinipes	2	98.91	490
Hediste diadroma	1	99.68	569
Melanothamnus harveyi	1	98.72	556
Mytilicola orientalis	1	97.77	464
Stenhelia pubescens	1	100	579

Table 1. Introduced species recorded at least once across all 86 sampling events. Also displayed is the number of times each species was detected and the highest BLASTn percent identity for the best-match ASV.

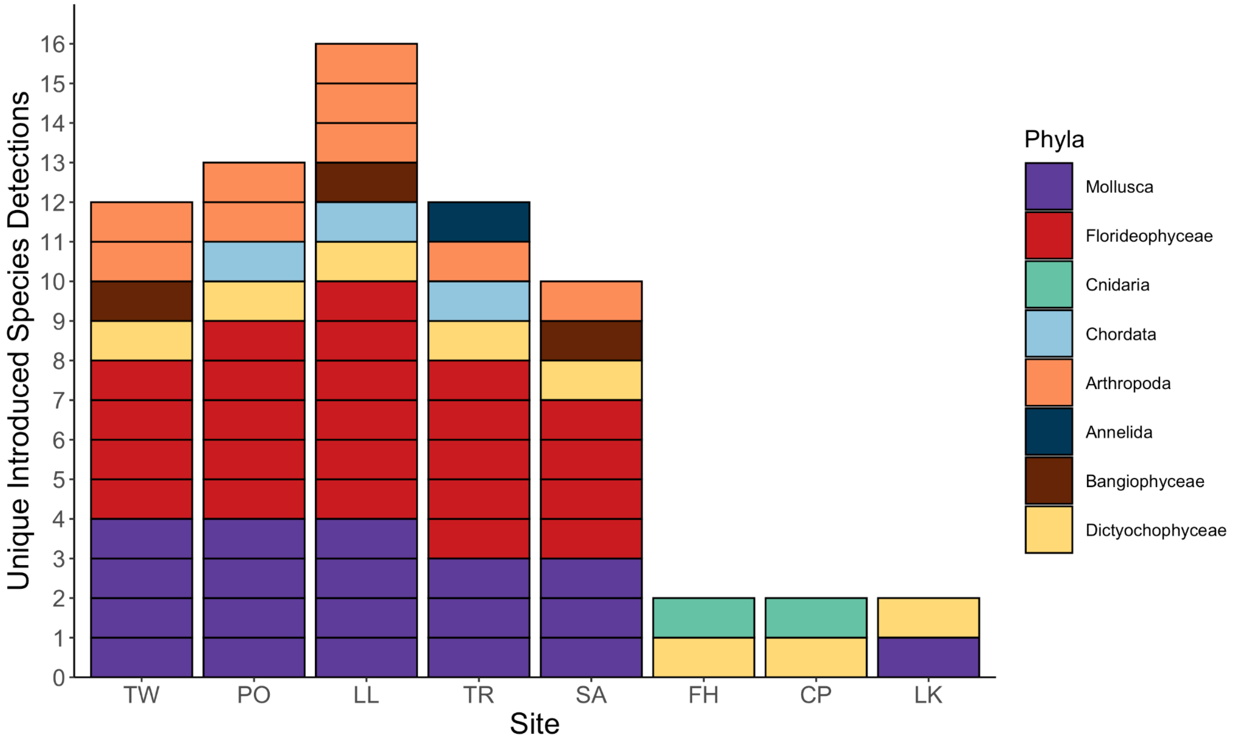


Figure 2. Number and phyla of unique detections of introduced species at the eight sampling sites. For each site, if a species was detected at least once during the 18-month sampling period it is displayed as a unique detection.

Species Richness and Invasion Rate

The richness of introduced and native species varied significantly between sampling sites and sampling months (PERMANOVA: site $R^2 = 0.51$, $p < 0.001$; month $R^2 = 0.17$, $p < 0.001$). The majority of introduced species (18 of 21) were only ever detected at the Hood Canal sample sites (Twanoh, Potlatch, Lilliwaup, Triton Cove, Salisbury) (TW, PO, LL, TR, SA). Only 3 of 21 species were detected at the San Juan Island sample sites (Friday Harbor, Cattle Point, Lime Kiln) (FH, CP, LK). Of these, the hydrozoan *Bougainvillia mucus* was the only species detected exclusively at the San Juan sites. The clam *Nuttallia olivacea* was detected both on San Juan Island and throughout the Hood Canal. The harmful algae *Pseudochattonella farcimen* was likewise detected both on San Juan Island and throughout the Hood Canal. *P. farcimen* was present in the greatest number of samples of all recorded introduced species.

The mean richness of native species was greater at the San Juan Sites (Friday Harbor = 67, Cattle Point = 78, Lime Kiln = 85) than it was in the Hood Canal Sites (Twanoh = 48, Potlatch = 51, Lilliwaup = 46 , Triton Cove = 53, Salisbury = 56) **(Figure 3)**.

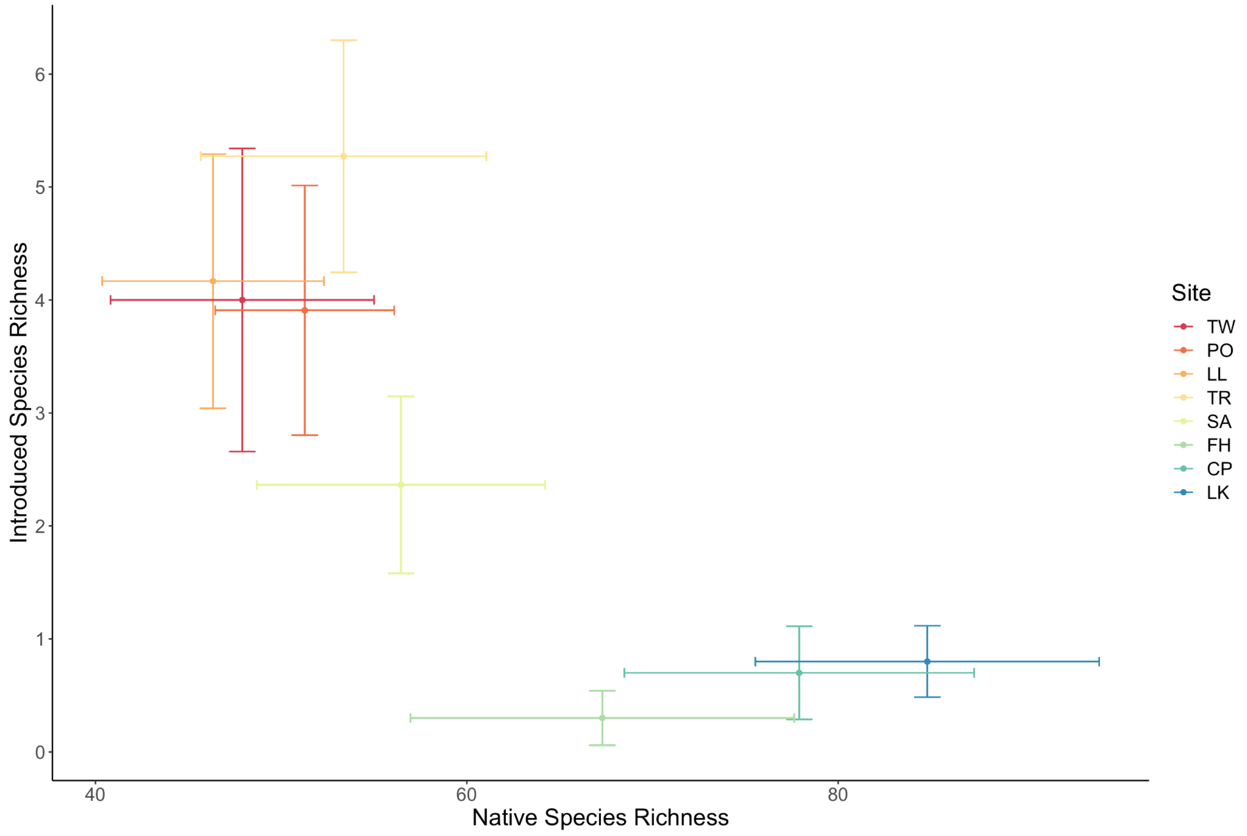


Figure 3. The mean introduced and native species richness at the eight sampling sites. Error bars correspond to +/- 1 Standard Deviation. The color gradient reflects the rough gradient from warmer, low wave energy estuarine mudflats (red) to cooler, higher wave energy marine rocky shores (blue).

Invasion rates were calculated as *introduced species richness / native species richness*. Invasion rates varied significantly between sampling sites and sampling months (PERMANOVA: site $R^2 = 0.55$, $p < 0.001$; month $R^2 = 0.17$, $p < 0.001$). At the San Juan sites, invasion rates were negligible across the months sampled. At the Hood Canal sampling sites, invasion rates were higher during the productive spring and summer months (May-September), and lower during the cooler months (October-March) **(Figure 6)**.

Median invasion rates for the Hood Canal generally increased from south to north (Twanoh = 0.04, Potlatch = 0.05, Lilliwaup = 0.07, Triton Cove = 0.09). However, the median invasion rate was lower at Salisbury at the mouth of the Hood Canal (Salisbury = 0.02). I found substantial variation in invasion rate at each of the Hood Canal sites across sampling events. **(Figure 7)**.

Introduced Species Richness across Biotic and Abiotic Conditions

Sampling revealed a broad range of water temperatures (min = 7.17 C, max = 22.60 C), salinity (min = 10.00 ppt, max = 30.31 ppt) and native species richness (min = 19, max = 106). Candidate poisson regression models of introduced species richness included combinations of salinity, temperature, and native species richness as predictive variables. The best model - as selected by leave-one-out cross validation - included just temperature and native species richness. **(Figure 4 & Figure 9).** Within the selected model, the point estimate for the temperature coefficient was 0.141 (0.116, 0.167), with a 10% - 90% credibility interval. The point estimate for the native species richness coefficient was -0.010 (-0.016, -0.004) with a 10% - 90% credibility interval. **(Figure 5 & Figure 9).** An increase in mean sea surface temperature of 2.5 C is possible in temperate regions under the IPCC’s Relative Concentration Pathway 4.5 - stabilization scenario (Meier et al., 2019). Based on the selected model we would expect to observe, on average, one additional introduced species with an increase in water temperature from 15 C to 17.5 C, under conditions similar to those sampled here. This is an increase in introduced species richness of at minimum 12% in the context of these samples.

Model	**Δ ELPD**	**Δ SE**
temp, native richness	0	0
temp, salinity	-0.8	3.2
temp	-0.9	2.6
temp, salinity, native richness	-1.1	1.6
native richness	-27.0	11.2

Figure 4. Leave-one-out model selection including parameters, difference in expected log pointwise predictive density (ELPD) and difference in Standard Error (SE).

	mean	std dev	10%	50%	90%
(Intercept)	-0.762	0.491	-1.399	-0.760	-0.118
Temperature	0.141	0.020	0.116	0.141	0.167
Native Richness	-0.010	0.005	-0.016	-0.010	-0.004

Figure 5. Description of the best-fit model of introduced species richness. Table contains details of the credibility intervals for the posterior distributions of the model's coefficients.

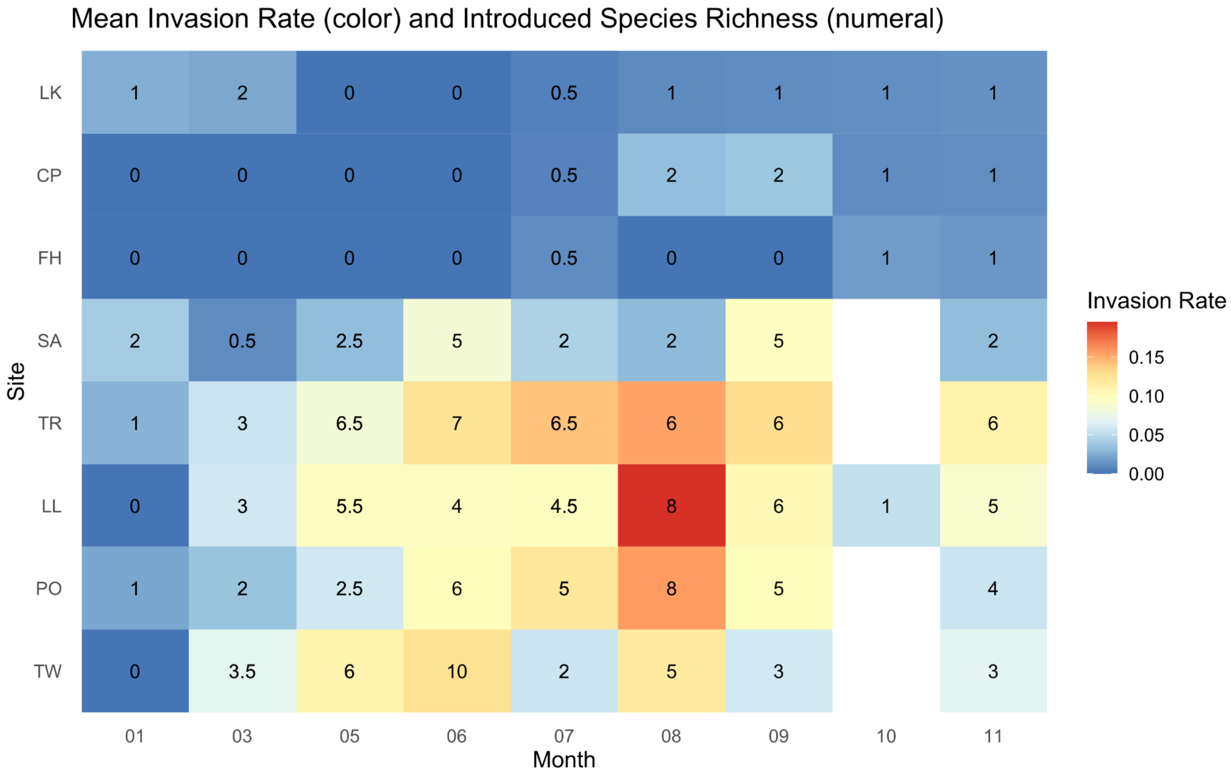


Figure 6. Invasion rates (introduced species richness / total species richness) across months and sampling location. Numbers in black are the mean introduced species richness detected at a site if it was sampled in both 2017 and 2018, or the introduced species richness if it was only sampled in 2017.

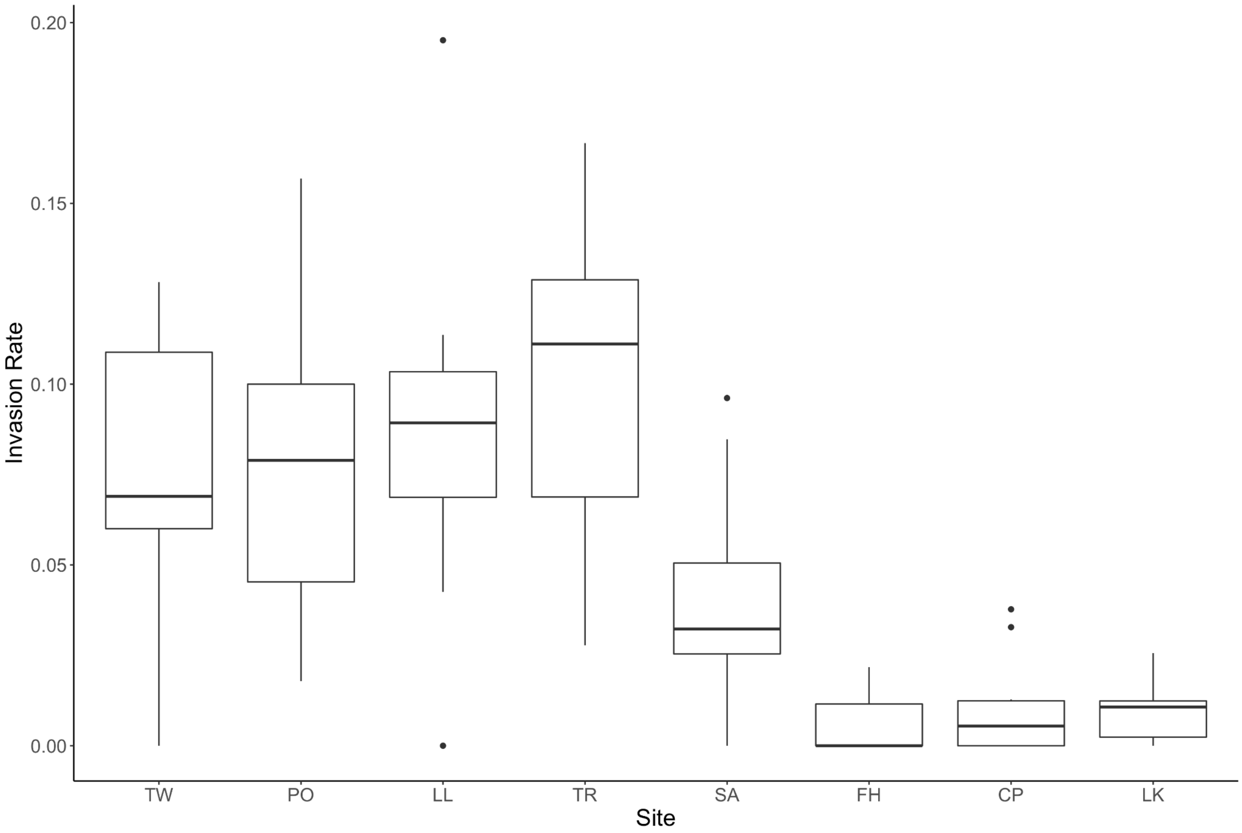


Figure 7. Boxplots of invasion rate distribution (introduced species richness / total species richness) across the eight sampling sites. Sites are ordered from the southernmost soft-bottom estuarine sites on the left, to the northernmost rocky bench sites on the right. Horizontal black lines mark the median invasion rate, boxes mark the interquartile range.

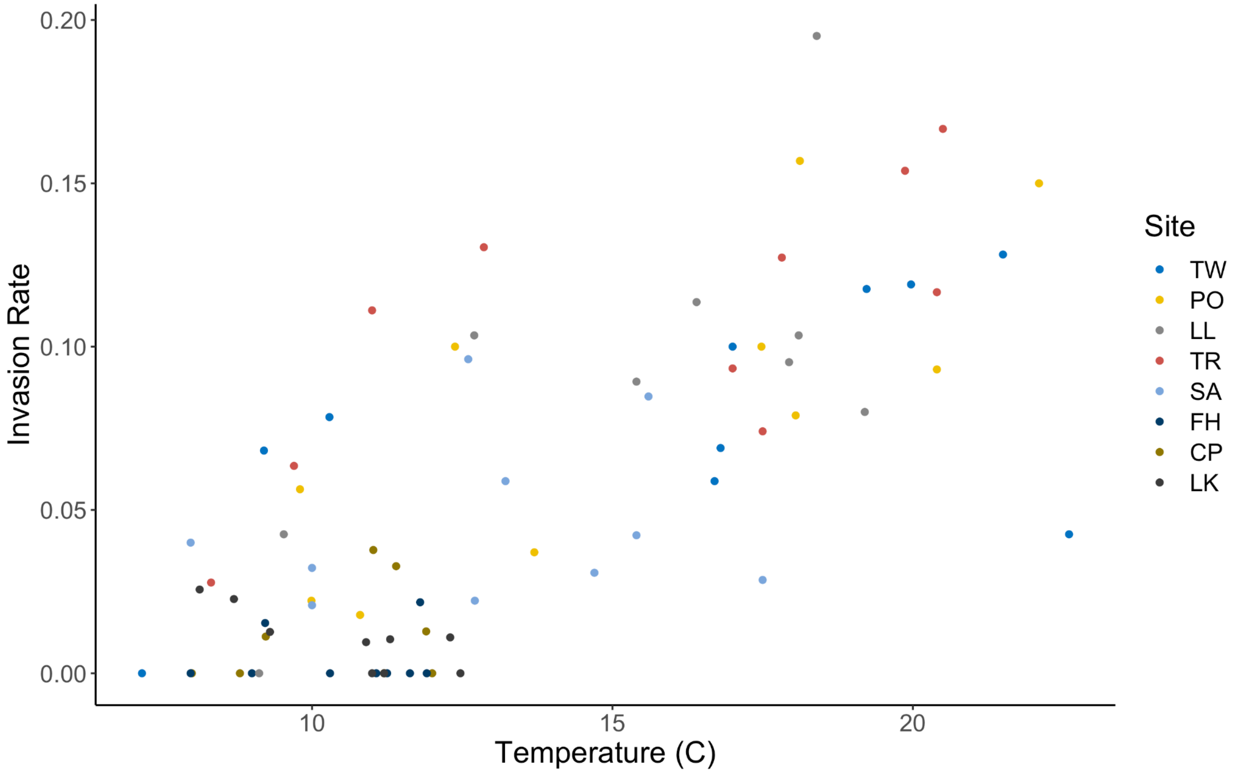


Figure 8. Each point represents a sampling event. Sampling sites are differentiated by color. Invasion rate (introduced species richness / total species richness) appears on the y axis, temperature appears on the x axis.

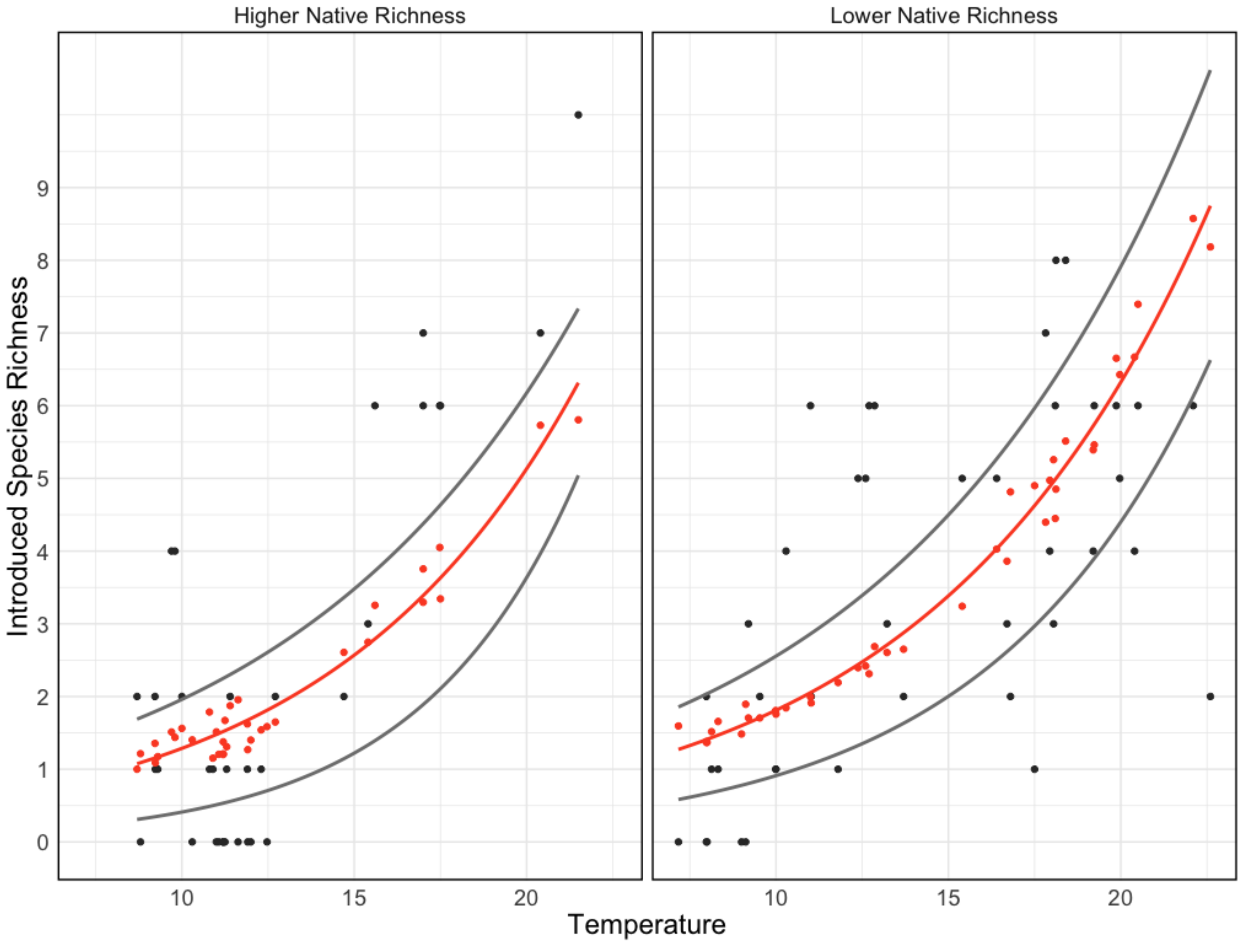


Figure 9. Predictions from the selected model - with temperature and native species richness as parameters **(Figure 2).** Predictions are plotted as a function of temperature, the dominant effect. Data is subdivided into "Higher Native Richness" (n = 37) and "Lower Native Richness" (n = 39), by the 50th percentile among all sampling events.

<u>Discussion</u>

The results of this survey of native and introduced species richness demonstrate varying levels of invasion between habitat types in the Salish Sea. The more marine San Juan Island habitats have negligible rates of invasion. The estuarine mudflats of the Hood Canal have greater rates of invasion driven primarily by introduced bivalves and algae. The transitional habitat at Salisbury at the mouth of the Hood Canal has an intermediate invasion rate. These results reinforce known dynamics in invasion ecology: that estuarine habitats exhibit higher invasion rates than adjacent open coasts and that increasing temperatures make marine and estuarine habitats more vulnerable to invasion (Lee et al., 2003; Preisler et al., 2009; Sorte et al., 2010; J. Stachowicz & Byrnes, 2006; J. J. Stachowicz et al., 2002). This survey also provides evidence in support of the biotic resistance hypothesis - that habitats with greater native species richness may resist invasion. Lastly, it contributes new data to monitoring known introduced and invasive species in the Salish Sea, and detects introduced species previously unreported within the Salish Sea.

Monitoring Introduced Species to the Salish Sea

Gracilaria vermiculophylla

G. vermiculophylla is a red alga whose native range encompasses the Northwest Pacific. It is a known invader of temperate estuaries around the world and has been documented in the eastern Pacific from Mexico to Alaska, as well as in estuaries throughout the temperate Atlantic (Krueger-Hadfield, 2018). The ecological impacts of *Gracilaria vermiculophylla* are mixed. It has been demonstrated to out-compete native algae, but also to increase habitat complexity (Weinberger et al., 2008). It has been previously identified in the Salish Sea by both visual and molecular methods. Possible vectors of introduction are hull fouling of commercial vessels, transport by the aquaculture oyster *Crassostrea gigas*, and ballast water (Krueger-Hadfield, 2018; Saunders, 2009). *Gracilaria vermiculophylla* is known to thrive in mesohaline conditions. This is reflected in my data where it was present in 34/86 samples, exclusively in the Hood Canal.

Caulacanthus okamurae

C. okamurae is a red alga whose native range encompasses the western Pacific. It is a known invader of the Eastern Pacific, detected in Baja California, Mexico in 1944 and in Prince William Sound, Alaska in 1989. It has been documented on San Juan Island and the nearby Strait of Georgia (PW Fofonoff et al., 2018). I detected *C. okamurae* at all 5 Hood Canal sites, and in 23 of 64 samples. It was not detected on San Juan Island. In California, *C. okamurae* has increased total turf cover and overall algal and invertebrate diversity in intertidal systems. It was found to displace macroinvertebrates while favoring copepods, ostracods and other algae including *Ulva spp.*, *Chondracanthus spp.*, and *Gelidium spp.* (Smith et al., 2014, NEMESIS).

Pyropia haitanensis

P. haitanensis is a red alga native to the western Pacific where it is widely cultivated. It makes up the majority of all Chinese cultivation of *Pyropia spp.* (Wang et al., 2020). The taxonomy of the family of seaweeds that includes *Pyropia spp.*, *Porphyra spp.*, and *Neoporphyra spp.* remains an active area of research. There are no prior visual records or herbarium specimens of *P. haitanensis* or its historical synonyms in the Eastern Pacific or the Salish Sea. A metabarcoding survey of seaweed communities in the Northern Gulf of Mexico detected *P. haitanensis* using an 23S rDNA assay, distinct from COI. Little is known about the ecological impacts of dispersal of introduced *Pyropia spp.*

Callithamnion corymbosum

C. corymbosum is a red alga native to the temperate Atlantic. There are no prior visual records or herbarium specimens of *C. corymbosum* in the Salish Sea. *C. corymbosum* was documented by visual survey at Willapa Bay on the outer coast of Washington State by Hansen (2008). There is no published literature on the impacts of *C. corymbosum* on native communities.

Botrylloides violaceus

B. violaceus is a colonial ascidian native to the western Pacific. It is a well-documented invader of the Eastern Pacific. It was documented by visual survey in the southern Salish Sea in 1998 (Cohen et al., 1998). *B. violaceus* outcompetes both native and introduced fouling species including native ascidians and mussels (Rajbanshi & Pederson, 2007). It fouls aquaculture equipment and impacts recruitment of aquaculture species (Carman, 2009).

Gelidiophycus freshwateri* and *Gelidium vagum

G. freshwateri is a red algae which was differentiated from the genus *Gelidium* by Boo et al. (2013). I detected *G. freshwateri* in 9 of 86 samples and *Gelidium vagum* in 2 of 86. There are no prior visual records or herbarium specimens of *G. freshwateri* in the Eastern Pacific or the Salish sea. Boo et al. describe the range of *G. freshwateri* as encompassing Korea, China and Japan. There is no published literature on the ecological impacts of introduced *Gelidophycus and Gelidium* algae.

Biotic and Abiotic Factors Influencing Invasion Level

Temperature

Previous mesocosm and observational studies have found introduced fouling species to be more tolerant of high temperatures than their native counterparts. Field studies have found increased recruitment of introduced ascidians - including *B. violaceus* - to be positively correlated with higher water temperatures, and native recruitment to be negatively correlated with higher temperatures (Sorte et al., 2010; J. J. Stachowicz, Terwin, et al., 2002). In Long Island Sound, USA the invasive alga *Gracilaria vermiculophylla* was found to have greater growth rates at higher temperatures relative to the native *Gracilaria tikvahiae* (Gorman et al., 2017). Among the poisson regression models tested, I found the model including temperature and native species richness to be the best predictor of introduced species richness. Temperature was the dominant factor **(Figure 9)**. This result provides additional evidence, at an ecosystem scale, for the success of introduced species relative to native species under higher temperature conditions. While it is difficult to completely distinguish temperature from other potential drivers of invasion such as propagule pressure, water flow, wave energy, and geography, I observed an increase in invasion rate with increases in temperature across the range of habitat types and conditions represented in my longitudinal samples.

Protected Estuary vs. Coastal Rocky Bench

Globally, temperate estuaries experience higher rates of invasion than adjacent coastal habitats (Preisler et al., 2009; Wasson et al., 2005; Wolff, 1998). This is the first survey to compare rates of invasion in an estuary with adjacent marine habitat using eDNA methods. My findings - higher invasion rates in the estuarine Hood Canal, intermediate rates at the transitional habitat at Salisbury, and negligible invasion rates in the more marine San Juan sites - are consistent with the findings of conventional surveys. Multiple mechanisms proposed by previous investigators may account for these results.

Globally, ports are most often located in protected estuaries. It follows that estuarine species are more often transported, and therefore estuarine habitats are more vulnerable to invasion by the assemblage of transported species (Nehring, 2006; Ruiz et al., 1997). It has also been suggested that the variable conditions of ballast tanks more closely mirror the variable environmental conditions of estuaries, making estuarine species more likely to survive transport (Wolff, 1998).

Oceanographic factors may also play a role. Limited circulation in estuaries may increase the chance that newly introduced species will successfully reproduce (Wasson et al., 2005). Our study sites are consistent with these circulation dynamics. Circulation is most limited in the south of the Hood Canal and less limited at Salisbury in the north. Water circulation is greatest around San Juan Island (MacCready et al., 2021).

Aquaculture

My findings align with the historical context of the study region. It is probable that the Hood Canal sites experienced greater introduced propagule pressure in the form of historical transport of shellfish for aquaculture, along with introduced algae and parasites accompanying these shellfish. The escape and establishment of introduced species from aquaculture operations is a well documented phenomena globally (Haupt et al., 2010; Ju et al., 2020; Mckindsey et al., 2007). The high number of Manilla clam and Pacific oyster detections, as well as the high proportion of species native to the temperate western Pacific, are consistent with this mechanism. Of all species detected 15 of 21 have native ranges in Japan and the western Pacific. Among the algae, 7 of 9 have native ranges including Japan. *Mytilicola orientalis* - native to the western Pacific - is a copepod parasite of *Crassostrea gigas*. Dabob Bay is an inlet in the Hood Canal situated between the Salisbury and Triton Cove sites **(Figure 1)**. Between 1919 and 1935 Dabob Bay was a destination for unregulated shipments of *Crassostrea gigas* oyster spat on discarded shells originating from Japan (Nims, 2020; White et al., 2009).

Anthropogenic Disturbance

Anthropogenic disturbances are known to facilitate biological invasions in marine environments. Such disruptions may make habitat less suitable for native species while also creating space and freeing up resources for introduced species. Shoreline armoring may facilitate the spread of introduced seaweeds (Bulleri & Airoldi, 2005). *Musculista senhousia* is more successful in disturbed eelgrass beds than pristine beds (Allen & Williams, 2003). Additionally, the disturbance caused by the introduction of a single fouling species can facilitate the establishment of additional introduced species, and result in an invasional cycle. Such a cycle has been documented in the Gulf of Maine facilitated by the Japanese algae *Codium fragile spp.* (Grosholz, 2005; Levin et al., 2002).

The Hood Canal has undergone more development and has a far greater percentage of shoreline armoring than San Juan Island. The historical aquaculture trade may have served as the catalyst for an invasional cycle in addition to supplying propagule pressure. In sum, the relatively high frequency and intensity of anthropogenic disturbances in the Hood Canal could contribute to the higher rate of invasion recorded there.

Biotic Resistance

Consistent with numerous previous investigations, I found lower native species richness at soft-bottom mesohaline and polyhaline habitats and greater native species richness at more saline rocky bench habitats (Dethier & Schoch, 2005; Remane, 1934). My best-fit model of introduced species richness includes both temperature and native species richness. This model outperforms the model that includes temperature alone. This finding aligns with previous investigations in which native species diversity has been found to offer some resistance to invasion, but is eclipsed by other factors (Olyarnik et al., 2009; J. Stachowicz & Byrnes, 2006). Previous field studies have reached conflicting conclusions on the degree to which native species richness offers resistance to biological invasion. Arenas et al. (2006) found that native algal functional group richness did not influence invasion success. An observational study of fouling communities by J. Stachowicz & Byrnes (2006) suggested that introduced richness is negatively correlated with native richness only if space is limited and species that facilitate introduced species settlement are rare.

Detection and Annotation with eDNA

As with other methods of introduced species monitoring, eDNA surveys are subject to false positives, false negatives, and biases (Cristescu & Hebert, 2018). eDNA shedding and decay rates vary across species, life-stages, and environmental conditions (Andruszkiewicz Allan et al., 2021). Primer bias may lead to inconsistent probabilities of detection across taxa (Elbrecht &

Leese, 2017). For tracking on-going biological invasions, open source sequence data offers an advantage over visual data in that it can be amended as genetic reference databases improve. However, the degree to which eDNA assemblages reflect the underlying community depends on the present quality of these databases (Stoeckle et al., 2020). These are important considerations when interpreting eDNA data. For example, the invasive western Pacific alga *Sargassum muticum* can be visually identified at many of my sample sites, but *Sargassum muticum* DNA was never identified in the water samples. However, given the diversity of life history and morphology represented among both native and introduced species in our study region - there is no reason to assume a systemic bias towards detection of either native or introduced species. Therefore, inferences about invasion level and introduced species assemblages are as likely to reflect underlying ecological processes as inferences drawn from visual survey data.

Conclusion

These results show that within the Salish Sea protected estuarine habitats with mid-range salinity exhibit higher levels of biological invasion. Invasion levels of marine rocky bench habitats are comparatively low. Across habitat and season, higher water temperatures and lower native species richness are associated with higher introduced species richness. The species level data generated by this survey may guide ongoing monitoring efforts, especially for species that were previously undetected in the Salish Sea or are difficult to differentiate from native relatives by visual means, such as the algae *Gelidiophycus freshwateri* and *Pyropia haitanensis*. These species data provide a rough "when and where" for future monitoring and control efforts. Invasion rate, as calculated by eDNA assemblage, provides an additional metric for the thorny management choice of where it is most efficient to employ prevention measures vs. containment measures.

The significant positive relationship observed between temperature and invasion level suggests that habitats may be most vulnerable to invasion during seasonals windows in the spring and summer. Salish Sea habitats may become increasingly vulnerable as anthropogenic climate change expands seasonal windows and causes waters to warm. This result highlights the need to incorporate climate change considerations into biological invasion monitoring and control.

eDNA surveys can consistently sample both introduced and native species at an ecosystem scale. This tool offers a promising new stream of data for illuminating regional invasions, and for investigating foundational questions in invasion ecology.

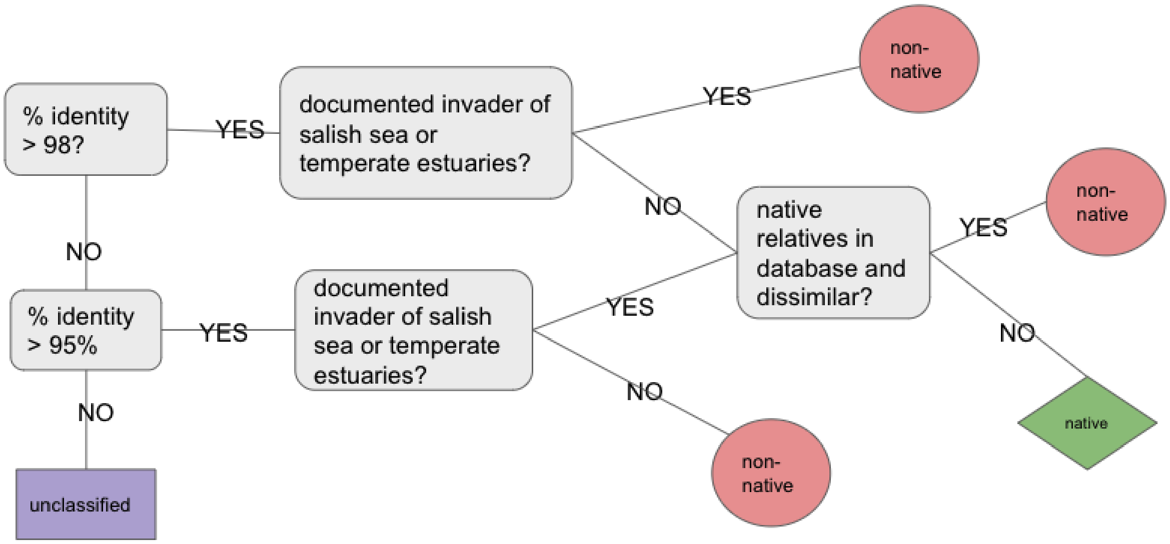
Supplemental

Supplemental 1.

https://github.com/ramongallego/eDNA.and.Ocean.Acidification.Gallego.et.al.2020
Github repository containing links to raw FASTA sequence data, as well as sequencing quality control and taxon assignment to ASVs.

https://github.com/jdduprey/patterns_of_invasion
Github repository containing the introduced species data and analysis described in this manuscript.

https://github.com/jdduprey/patterns_of_invasion/blob/main/data/introduced_species_BLASTn.csv
CSV file containing BLASTn output for all candidate introduced species sequences including top 5 sequence matches. query accesion, sequence ID, percent identity, bitscore, scientific name and other relevant data.



Supplemental 2. Flowchart of the decision process for classification of an ASV as either native, introduced or unclassified based on BLAST metrics.

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