Metagenetic Analysis of 2017 Plankton Samples from Prince William Sound, Alaska.

Report to Prince William Sound Regional Citizens' Advisory Council (PWSRCAC)

From Molecular Ecology Laboratory

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Abstract

This report describes the methods and findings of the metagenetic analysis of plankton samples from the waters of Prince William Sound (PWS), Alaska. The study was done to identify zooplankton, in particular the larvae of invasive benthic species. Plankton samples, collected by the Prince William Sound Science Center (PWSSC), were analyzed by the Molecular Ecology Laboratory at the Moss Landing Marine Laboratories. The samples were taken from five stations in May of 2017 in Port Valdez and elsewhere in PWS. DNA was extracted from bulk plankton and a portion of the mitochondrial Cytochrome c oxidase subunit 1 gene, the most commonly used DNA barcode for animals, was amplified by polymerase chain reaction (PCR). Products of PCR were sequenced using Illumina reagents and MiSeq instrument. 211 operational taxonomic units (an approximation of biological species) were found and 52 were identified to species. Most species were crustaceans and molluscs, and none were non-native. We also compared PWSRCAC samples taken in 2016 to the current set of samples. Fewer species were identified in 2017 than in 2016, but sampling methods varied across years. Standardization of methods and a longer time series are necessary to investigate temporal trends.

Introduction

Monitoring marine habitat for species of concern, including invasive species, can be costly and time-consuming, which limits the information available to resource managers, scientists, and the public. Two of the reasons for the high cost of monitoring are labor-intensive sampling methods and the need for expert taxonomists to identify specimens. A genetic approach to species identification can reduce the reliance on taxonomic experts, as DNA sequences from all species are analyzed similarly (unlike morphological analysis). High throughput sequencing, particularly metagenetics or metabarcoding, is an increasingly popular tool for assessing aquatic biodiversity (Valentini et al. 2016, Borrell et al. 2017, Ransome et al. 2017). Metabarcoding, which allows for community level assessments of multispecies samples through the amplification of a single locus (Taberlet et al. 2012), is used to address questions in aquatic habitats such as community richness and composition (Ransome et al. 2017) and invasive species detection (Xiong et al. 2016, Borrell et al. 2017). Given the high sensitivity of the method for detecting low abundance or rare taxa (Zhan et al. 2013), this method has great appeal for early detection of aquatic invasive species (Xiong et al. 2016), an essential step to prevent the establishment of nuisance species.

For metabarcoding, sampling of benthic communities and processing (DNA analysis) costs can remain high if large volumes of benthic biomass are required. Plankton, in contrast, is relatively inexpensive to sample and process. The organisms in the benthos and plankton overlap because many benthic species have planktonic larval life stages, therefore plankton may be sufficient for discovery of both planktonic and benthic invasive species. Benthic larvae are seasonal and often patchy in the plankton, therefore a single plankton sample may not be as effective at surveilling benthic species as would direct observation of the benthos, yet the low cost can allow repeated sampling.

Methods

Field sampling

Plankton samples and metadata were collected at five sites in Port Valdez and Valdez Arm aboard the RV New Wave (PWSSC). Samples were taken on three sides of the Valdez Marine Terminal Security Zone, in Valdez Arm, and more remotely in the central PWS (Table 1, Figure 1). Multiple casts of a 30 centimeter (cm) wide, 80 micrometer (μm) mesh net were towed from a 5 meter (m) depth to the surface. Plankton was concentrated and preserved in DNE solution (20% DMSO, 500 mM EDTA, and NaCl at saturation). Plankton from an additional tow was preserved in formalin. A CTD cast (Seabird SBE25plus) with auxillary sensors (WETLabs FLNTU fluorometer, Seabird SBE43 oxygen sensor, and Turner Designs PhytoFlash active fluorometer) was also performed. Samples in DNE were shipped to Moss Landing Marine Laboratories. Further field notes are available from PWSSC (Rob Campbell).

Laboratory analysis

Laboratory procedures followed methods previously published (Lohan et al., 2019). The Cytochrome c oxidase subunit 1 gene (COI) was amplified, in triplicate, using primers mlCOIintF forward (5'- GGWACWGGWTGAACWGTWTAYCCYCC -3'; Leray et al. 2013) and jgHCO2198 reverse (5' - ACYTCIGGRTGICCRAARAAYCA -3'; Geller et al. 2013) with partial Nextera barcode indices added to the 5' ends (Illumina support 2013). The fragment amplified is at the 3' end of the commonly used COI barcode fragment (see Leray et al. 2013). The COI PCR product was sequenced on an Illumina MiSeq instrument.

Bioinformatic analysis

The following analysis of sequences was performed with the software program USEARCH 10 (Edgar, 2010). To simplify initial analysis, the reads (a read is one raw DNA sequence) were dereplicated (replicates of identical reads removed) and sequences occurring once (singletons) were also removed. Singletons are often deemed unreliable variants potentially caused by sequencing error. Replicate reads were restored for analyses of read abundance. Only reads of the expected size were retained and reads that had quality less than Q20 Phred score (i.e., greater than 1% estimated error rate) were removed. Primers were also removed from sequences. Chimeric sequences (artifacts of PCR that contain sequence from >1 DNA template) were also detected and removed. Reads were then clustered at 95% similarity threshold. These 95% clusters are considered Operation Taxonomic Units (OTUs) and approximate biological species. All reads were then mapped to an in-house database of plankton OTUs (including many from California as well as all Valdez OTUs found herein) to produce a table of read-abundance for each OTU. Statistical analysis was performed with the software program PRIMER.

Because of variation in the rate of molecular evolution among taxa, a 95% cluster of COI sequences is not a perfect approximation of biological species. It is possible that some OTU contain more than one species that have little genetic separation. It is also possible that sequences from one biological species may cluster into more than one OTU if that species is unusually variable. This is the same problem faced by morphological taxonomists with highly polymorphic species on the one hand or morphologically similar cryptic species on the other.

The 95% clustered OTUs were compared using the software program BLAST (www.ncbi.nih.gov) to a proprietary in-house COI reference database of invasive species and to a curated database extracted from Genbank (Heller et al., 2018). OTUs matching database records at 95% or higher similarity were considered as provisionally identified, after correcting taxonomic error in Genbank known to us.

BLAST results were filtered by removing results that did not have full binomial names. Thus, genus-only or environmental samples, for example, are not listed here, as we cannot assign a species name. Without a species name, we cannot assess native or introduced status.

The possibility of non-native species among identifiable OTUs was evaluated by examining geographic distributions as reported in the World Register of Marine Species (WoRMS) or in published literature. The geographic source of reference sequences in Genbank was also examined, when such information was provided by depositors. For an identifiable OTU with multiple sequences in Genbank, phylogenetic trees were also made using FastTree, a program that uses a maximum likelihood algorithm to reconstruct evolutionary relationships based on molecular data, to investigate possible cryptic species complexes.

For statistical analysis of species composition and diversity at the different sites and to data from the previous year (2016), reads were mapped to all 95% OTU clusters (combining 2016 and 2017). Finally, the reads were rarefied to 107,278 reads per sample, which was the smallest number observed for any sample in this study. Rarefaction normalizes sequencing effort across samples for statistical purposes. The rarefied OTU abundance table was then used for multivariate statistical analysis in the software package Plymouth Routines in Multivariate Ecological Research (PRIMER 7) software (Clark and Gorley 2015). First, permutational multivariate analysis of variance (PERMANOVA), a non-parametric analysis of variation, was used to test for significant differences between years (2016 and 2017). Next, non-metric multidimensional scaling (nMDS) plots were generated to visually compare similarity between samples. In the nMDS, distance between samples, represented by dots plotted in two dimensions, indicates community similarity as estimated by a Bray-Curtis similarity index. The Bray-Curtis similarity index considers both taxonomic composition and abundance of taxa.

Results

Identification of OTU in 2017 samples

There were 211 OTUs from the Valdez 2017 plankton samples. The number of 95% OTUs found in all 5 samples, was 55.

Plankton samples collected from Valdez Marine Terminal Security Zone Station E and N (VT-E and VT-N) formed a cluster that is separated from the samples collected from Station W (VT-W), Valdez Arm (VA), and central PWS on a nMDS plot (Figure 2). The resulting separation on the nMDS plot indicates that community composition was similar between VT-E and VT-N but different from the rest of the stations.

Fifty two of the 211 OTUs could be taxonomically identified (Table 2). These 52 OTUs are dominated by crustaceans and molluscs. Among the crustaceans, Copepoda was the most prevalent taxon. Larval gastropods and, to a lesser extent, bivalves dominated the Mollusca in this study.

Visual analysis of plankton samples by the PWSSC identified 14 taxa to the species level, of which eight were also identified by genetic analysis in 2017 (Table 2). Of the six species found only by visual examination in 2017 (see legend of Table 2), three have no representation in Genbank therefore we cannot say if their sequences were found here or not. Two copepod species, *Acartia longiramis* and *Oithona setigera* (listed by PWSSC by the synonym, *O. spinirostris*) do have Genbank records. These two were apparently not detected by sequencing, but it may be worthwhile to review these specimens and compare to the species of *Acartia* and *Oithona* that were found by sequencing. PWSSC also reported unidentified larval gastropods, bivalves, barnacles, bryozoan, krill, and nauplii (likely copepods or barnacles) which may have been from among the many genetically identified species in these groups.

Comparison between Valdez 2016 and 2017 samples

Combining 2016 and 2017 samples and rarefying to normalized read numbers, 99 individual OTUs were identified and 23 were found in both years (Table 2). Statistical analysis show that the community composition was significantly different between 2016 and 2017 samples (PERMANOVA, Pseudo-$F_{(1,13)}$=23.39, p(perm)=0.003; Table 3). Plankton samples collected in 2016 were similar within site and differed across sites (PERMANOVA, Pseudo-$F_{(2,8)}$=4.79, p(perm)=0.004). The Valdez Ferry Terminal and Alyeska samples were more similar to each (Figure 3).

Discussion

Sensitivity of analysis

Metagenetic analysis, also known as metabarcoding, is subject to sampling effects just as is any ecological survey: the more individuals per sample collected (or the more samples taken), the higher the probability of detection of rare species. For metagenetics, the probability of detection of a rare DNA sequence can also be increased by generating more DNA reads per sample. This is analogous to increasing the number of plankters visually identified from a single sample in a traditional study. In this study, we rarefied data to 107,278 reads per sample. This was necessary to compare samples with different numbers of reads (from variation inherent in Illumina DNA sequencing). In previous tests on plankton, we found that OTU accumulation never reached asymptote but that the increase in OTU

recovered decreases markedly after ~50,000 reads (Lohan et al., 2019). By increasing read number, additional rare OTU likely will be found, but at a cost to the number of samples that can be processed (given fixed monetary and time resources). Thus, one must balance the need for discovery of rare species in each sample against the number of samples and sites included in a study.

It should be remembered that relative abundance of sequences in a metagenetic PCR product may not be directly correlated to the abundance of the source species in the sample due to interspecific differences in body size (i.e., cell number and genome copies) or amplification bias. Ecologically rare species, if entrained in a plankton tow, might be over-represented or abundant species under-represented. When a species of interest is known a priori, or is discovered by sequencing, actual abundance is better estimated by quantitative PCR (qPCR), which can be employed retroactively on the same DNA extractions used for metagenetics.

Differences between sites and years

Samples from the two years, 2016 and 2017, were collected in substantially different ways. In 2016, samples were taken by vertical tows dockside and therefore in shallow water and in proximity to fouling communities. In 2017, samples were taken by boat in mid-channel and more distant to fouling communities. It is difficult, therefore, to interpret the substantial and statistically strong differences between years (Figure 4): while the difference between years suggests temporal variability, the sites chosen or methodological differences could impact the composition of the identified plankton community. Because this project is focused on detection of larvae of (primarily) benthic non-indigenous species (NIS), sampling closer to the source of larvae (fouling communities) is desirable. Additionally, larval abundance is seasonal and pulse-like within seasons. Therefore, repeated sampling during the reproductive season of adults should increase the potential for species detection.

Non-indigenous species (NIS)

No unambiguously NIS species were detected in 2017. Many species found here are considered circum-boreal, while others may not be listed as occurring in Alaska but are known from California to Washington. These are likely native in Alaska (rather than recent range expansions from the south), but simply understudied. One OTU was identified as the brown alga *Leathesia difformis*, which could be NIS but also appears to be part of poorly known species complex. We suspect this could be a local member of a widely distributed complex of species. Another OTU matched the brown alga, *Pylaiella littoralis*, which is not listed as present in Alaska, but we traced the matching Genbank record to a specimen of *P. washingtoniensis* from the USA in the Kobe University culture collection (GenBank: AB899179.1, http://ku-macc.nbrp.jp/strain/detail/9566). We surmise this is probably native to the Pacific Northwest coast.

Previously noted NIS in 2016 (the clam *Mya arenaria* and the sea squirt *Botrylloides leachii*) were not detected in 2017. We suspect this reflects variation in abundance and distribution of the larval pool in these years. *Botrylloides*, especially, has poorly dispersing, short lived larva and only sampling near adults (i.e., near docks) is likely to capture any.

Conclusions

Results of 2017 plankton sampling showed communities highly dominated by copepods and larval molluscs, at least among OTUs that could be identified. As before, the majority of OTUs could not

be identified because reference databases lack completeness. An effort to barcode Alaskan shallow water and planktonic invertebrates would address this deficiency. 2016 and 2017 samples were very different, but were also from different locations. No NIS were discovered in 2017, however the location of the plankton tows may not have been ideal for this purpose. Sampling concentrated in the vicinity of fouling communities (e.g. inside the Valdez Small Boat Harbor) would likely capture more of the NIS species present in those assemblages. Future studies might include comparison to biomass collected directly from fouling communities, analyzed by metagenetics or traditional methods. Additionally, eDNA (DNA free of live organisms) collected directly from seawater or from concentrated baths of fouling material may be a simpler method for repeated studies if it proved equally or more effective than plankton tows or benthic sampling.

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Table 1. Sampling locations for vertical (from 5 m depth to the surface) plankton tows. Stations VT-E, VT-N, and VT-W are Valdez Marine Terminal Safety Zone east, north, and west, respectively. Station VA is in the Valdez Arm and station PWS is in central PWS. See Figure 1. (Data from report to PWSRCAC from PWSSC.)

Date	Time	Station	Bottom Depth (m)	Latitude	Longitude
5/16/2017	18:44	VT-E	61.5	61.089ºN	146.346 ºW
5/16/2017	19:20	VT-N	59	61.112 ºN	146.392 ºW
5/16/2017	19:52	VT-W	108	61.089 ºN	146.450 ºW
5/16/2017	20:03	VA	315	61.037 ºN	146.934 ºW
5/17/2017	9:12	PWS	445	60.583 ºN	146.934 ºW

Table 2. Species identified from OTUs recovered as 95% clusters of COI reads from metagenetic sequencing of plankton DNA from Valdez, PWS, Alaska. A representative sequence from each OTU was compared to Genbank and a proprietary database of invasive species found in California using BLAST. A positive match was when unknown and reference sequences had greater than 95% similarity with >98% sequence overlap. 2017 (PWSSC) are results from visual analysis of samples by the PWSSC. "?" indicates the analysis included only a genus identification. Also found and identified visually were the copepods *Acartia longiremis*, *Microsetella norvegica*, *Oithona setigera*, and *Oncaea borealis;* the larvacean *Oikopleura dioica*; and the chaetognath *Parasagita elegans*,

Phylum	Species	2016	2017	2017 (PWSSC)
Annelida	*Harmothoe rarispina*		x	
	Micronereis nanaimoensis	x		
	Nereis vexillosa	x	x	
	Pholoides asperus	x		
	Prionospio steenstrupi		x	
Bryozoa	*Membranipora membranacea*	x	x	
Chordata	*Limanda aspera*	x		
	Microstomus pacificus		x	
	Oncorhynchus kisutch	x		
Cnidaria	*Aurelia labiata*	x		
	Clytia gregaria		x	x
	Clytia hemisphaerica	x		
	Proboscidactyla flavicirrata	x	x	
	Sarsia bella		x	
Crustacaea	*Metacarcinus gracilis*	x		
	Metridia lucens	x		
	Acartia californiensis	x		
	Acartia hudsonica	x		
	Balanus crenatus	x		
	Balanus glandula	x		
	Bomolochus cuneatus	x		
	Calanus marshallae	x	x	x
	Calanus pacificus	x		
	Centropages abdominalis	x		x
	Chthamalus dalli		x	
	Ctenocalanus vanus		x	
	Ectinosoma melaniceps	x		
	Epilabidocera amphitrites	x	x	
	Eucalanus bungii		x	
	Euphausia pacifica		x	
	Ismaila belciki	x		
	Lepeophtheirus salmonis	x		
	Neocalanus flemingeri	x	x	x
	Neocalanus plumchrus		x	x

Phylum	Species	2016	2017	2017 (PWSSC)
	Oithona similis	x	x	x
	Oregonia gracilis		x	
	Pagurus hirsutiusculus		x	
	Pareucalanus attenuatus		x	
	Pleopis polyphemoides	x		
	Pseudocalanus acuspes	x	x	?
	Pseudocalanus mimus	x	x	?
	Pseudocalanus minutus	x	x	?
	Pseudocalanus newmani	x	x	?
	Semibalanus cariosus		x	
	Themisto pacifica	x		x
	Thysanoessa spinifera		x	
Diatom	*Ditylum brightwelli*	x		
	Melosira nummuloides	x		
	Thalassionema nitzschioides	x		
Dinoflagellata	*Azadinium dalianense*	x		
Echinodermata	*Ophiopholis kennerlyi*	x		
	Strongylocentrotus droebachiensis		x	
Fungi	*Penicillium digitatum*		x	
Mollusca	*Acanthodoris atrogriseata*	x		
	Acanthodoris nanaimoensis	x		
	Aglaja ocelligera	x		
	Alia gausapata	x		
	Angulus nuculoides	x		
	Aplysiopsis enteromorphae	x		
	Clione limacina		x	
	Compsomyax subdiaphana	x		
	Corambe steinbergae	x	x	
	Crepipatella lingulata	x		
	Cryptonatica aleutica		x	
	Dendronotus albus	x	x	
	Dendronotus venustus	x		
	Elysia hedgpethi		x	
	Fusitriton oregonensis	x		
	Haminoea virescens	x		
	Hermissenda crassicornis	x	x	
	Humilaria kennerleyi	x		
	Keenocardium californiense		x	
	Kellia suborbicularis	x		
	Knoutsodonta jannae	x	x	
	Lacuna vincta	x	x	
	Limacina helicina	x		x

Phylum	Species	2016	2017	2017 (PWSSC)
	Macoma balthica	x	x	
	Margarites pupillus		x	
	Melanochlamys diomedea	x	x	
	Modiolus modiolus	x		
	Mytilus trossulus	x	x	
	Nassarius mendicus	x	x	
	Odostomia tenuisculpta	x		
	Olea hansineensis	x	x	
	Onchidoris bilamellata	x	x	
	Onchidoris muricata		x	
	Pandora bilirata	x		
	Rostanga pulchra	x		
	Saxidomus gigantea		x	
	Stiliger fuscovittatus	x		
Nemertea	*Carcinonemertes errans*	x		
	Gurjanovella littoralis		x	
	Paranemertes californica	x		
	Poseidonemertes collaris		x	
	Procephalotrix spiralis		x	
Phaeophyceae	*Leathesia difformis*		x	
	Pylaiella littoralis	x	x	
Rhodophyta	*Ectocarpus siliculosus*		x	
Sipunculida	*Phascolosoma agassizii*	x		
	Total	**70**	**52**	**8**
	Found in both years	**23**		

Table 3. PERMANOVA test comparing plankton community composition between Valdez samples collected in 2016 and 2017. Data from two years were combined and processed through a COI data processing pipeline in USEARCH (version 10). The combined data were mapped to a 95% similarity threshold clustered MLML master OTU list. The data were rarefied to 107,278 reads per sample. (*df*, degrees of freedom; *SS*, sum of squares; *MS*, mean square; *F* statisitic; *P* (*perm*), *P*-value after permutation procedure)

Factor	df	SS	MS	Pseudo-F	P(perm)
Year	1	13,070	13,070	23.39	0.003
Residue	12	6,706.2	558.85		
Total	13	19,776			

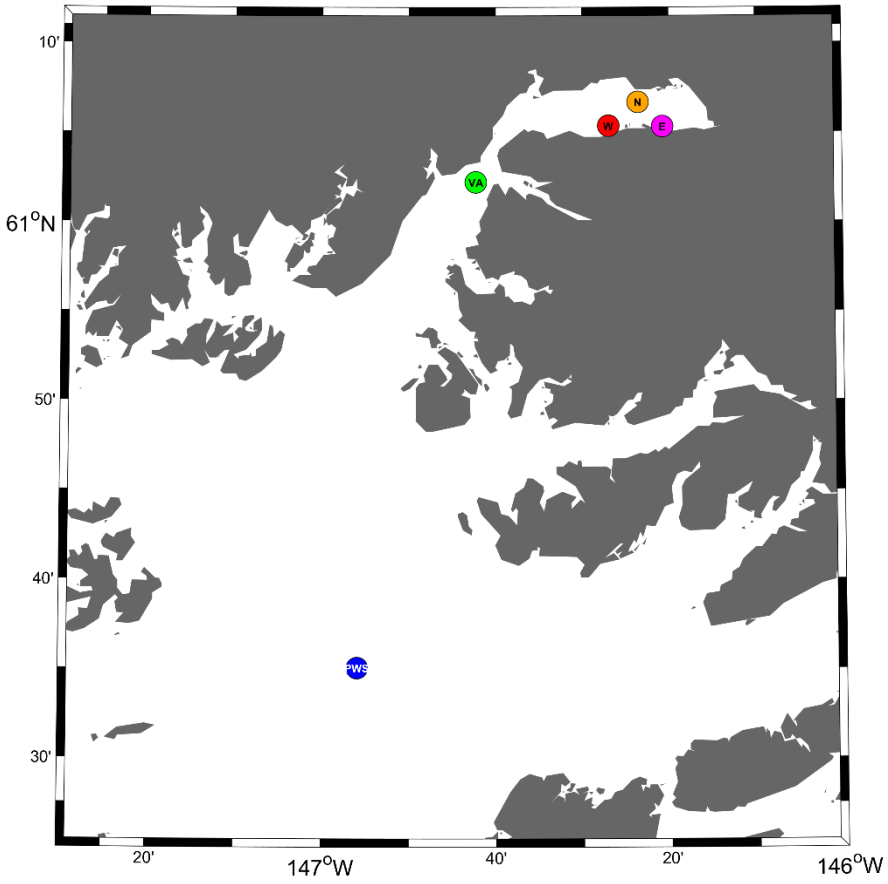

Figure 1. Map of 2017 station locations in Port Valdez (VT-E, VT-N, VT-W), Valdez Arm (VA), and central PWS. See Table 1. (Figure from report to PWSRCAC from PWSSC.)

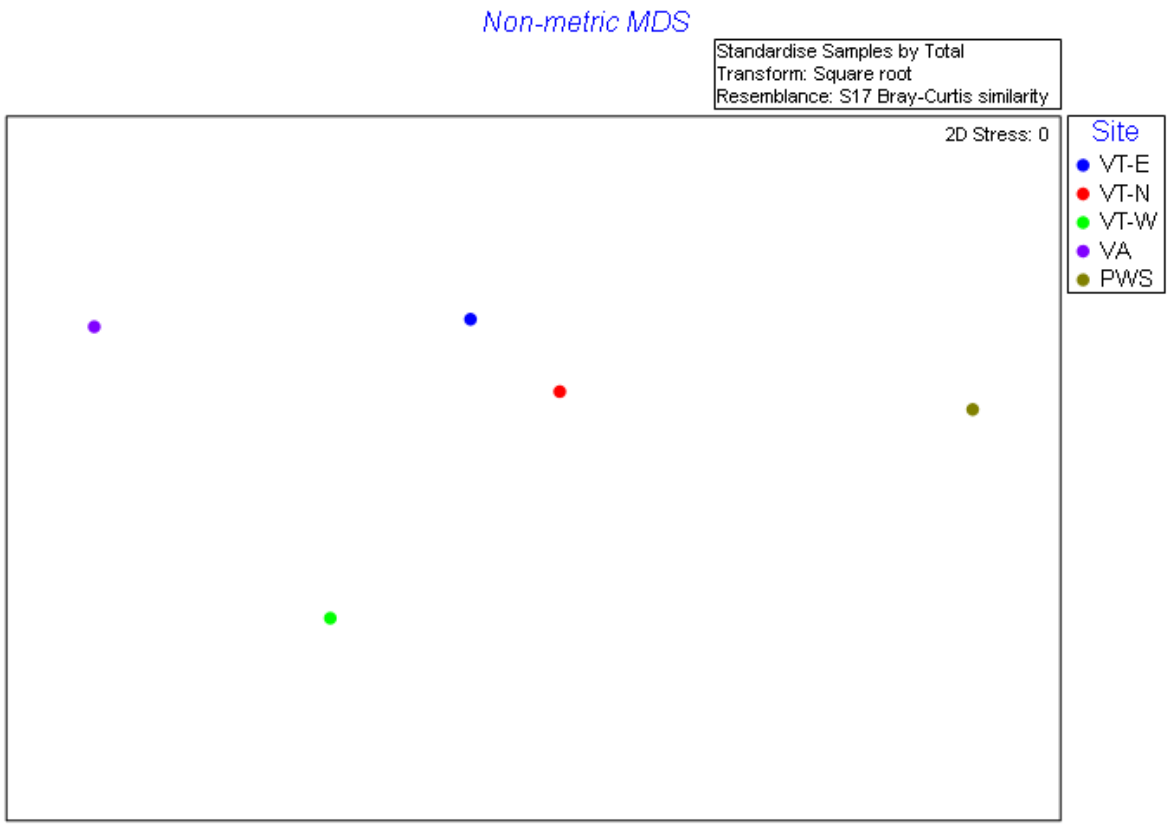

Figure 2. nMDS plot showing relationships of plankton samples from Valdez Marine Terminal Security Zone Station E (blue), Station N (red), Station W (green), Valdez Arm (purple), and central PWS (yellow) in May 2017. Data from two Illumina sequencing runs were combined and processed through a COI data processing pipeline in USEARCH (version 10). Reads rarefied to 107,278 (the number in the lowest yielding sample) and were mapped to a master OTU list to generate frequency distributions for each OTU in each sample. Distance between samples, represented by the colored dots, reflects community similarity as estimated by a Bray-Curtis similarity index. The Bray-Curtis similarity index considers both taxonomic composition and abundance of taxa.

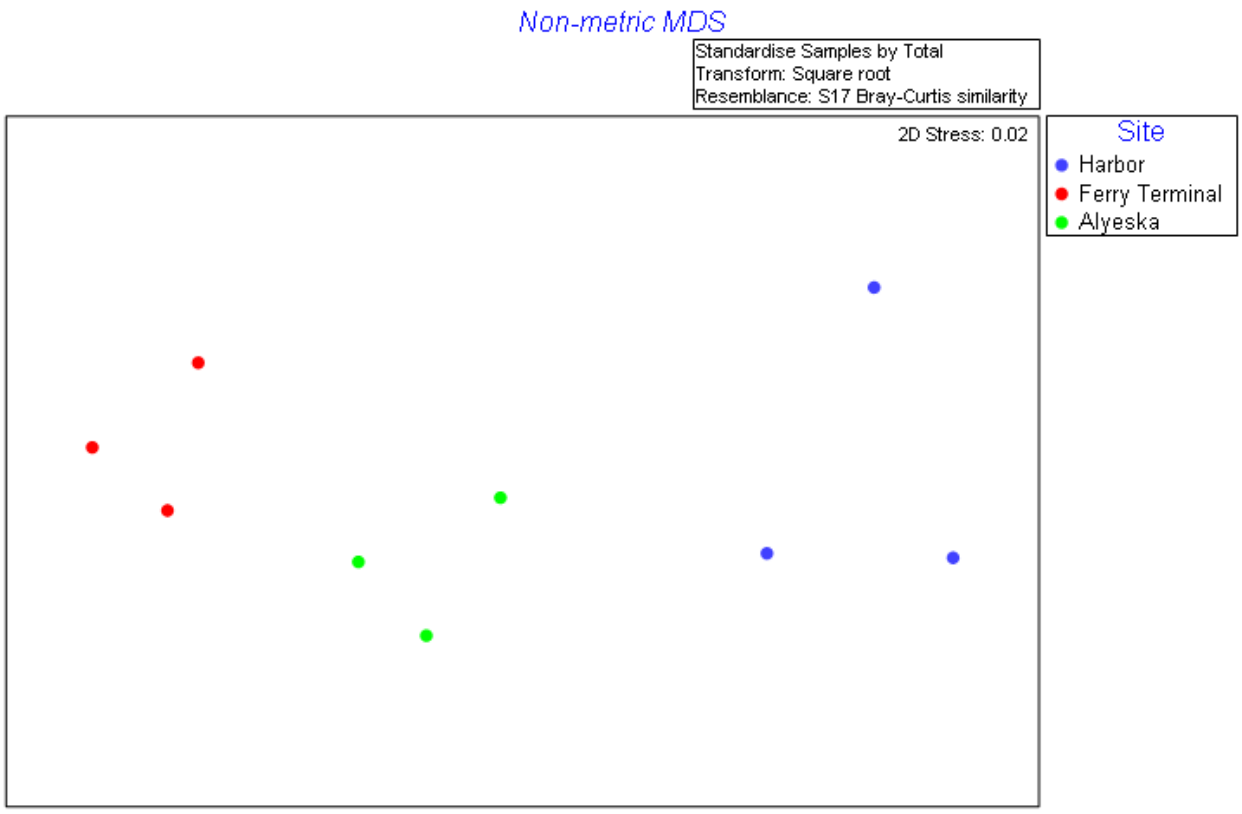

Figure 3. nMDS plot showing relationships of plankton samples from the Valdez Small Boat Harbor (blue), Valdez Ferry Terminal (red), and the Alyeska Marine Terminal (green) in 2016. Data were processed as noted in Figure 2. See Figure 2 for explanation of nMDS plots.

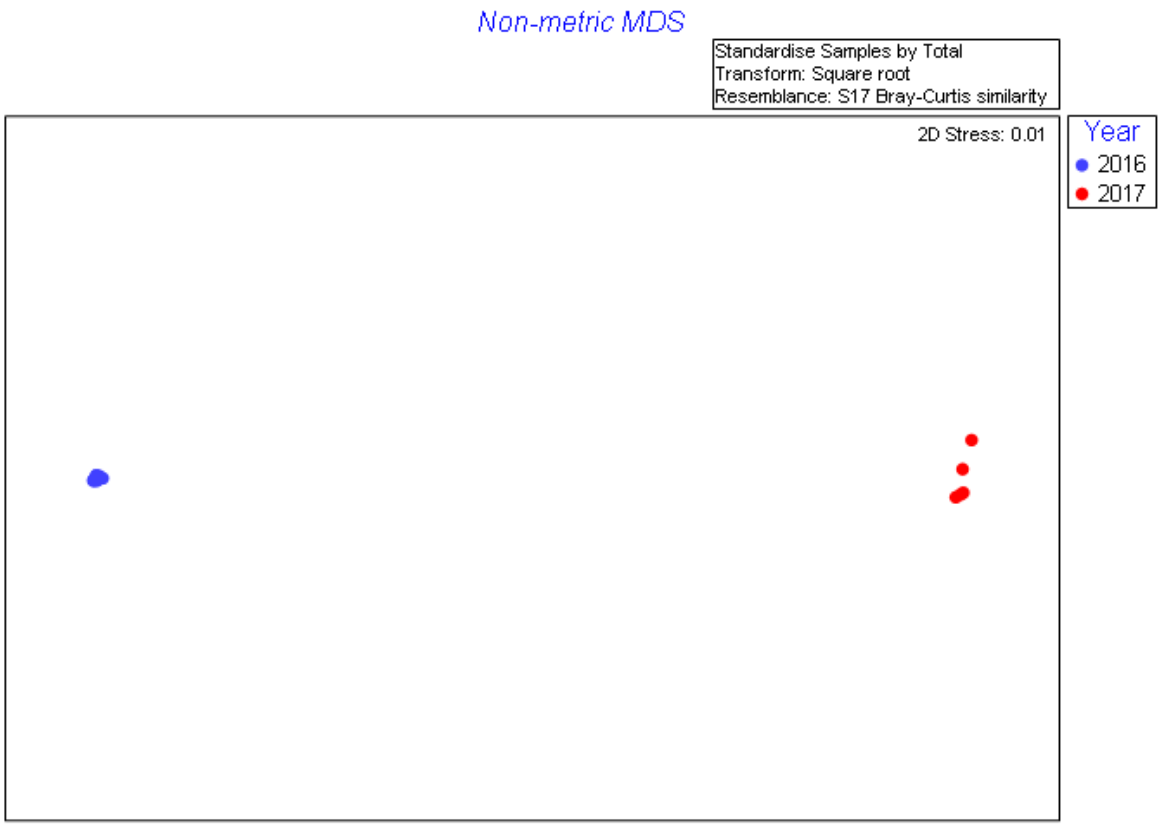

Figure 4. nMDS plot showing relationships of plankton samples from 2016 and 2017. Tight overlap within and large distance between 2016 and 2017 reflect the great difference between these sets of samples. Sites were actually statistically distinct in both years (see Figure 2 and 3).