**Spatial dispersion of environmental DNA in a nearshore marine habitat**

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**James L. O'Donnell\*1, Ryan P. Kelly1**, **Natalie C. Lowell2, Andrew O. Shelton3, Jameal F. Samhouri3, Gregory D. Williams3**

*1School of Marine and Environmental Affairs, University of Washington, 3707 Brooklyn Ave NE, Seattle, Washington 98105, USA*

*2School of Aquatic and Fishery Sciences, University of Washington, 1122 NE Boat St, Seattle, Washington 98105, USA*

*3NOAA Fisheries, Northwest Fisheries Science Center, 2725 Montlake Blvd E, Seattle, Washington 98112, USA*

%----------------------------------KEYWORDS-------------------------------------

metagenomics | metabarcoding | environmental monitoring | molecular ecology | marine | estuarine

%----------------------------------ABSTRACT-------------------------------------

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%---------------------------------INTRODUCTION----------------------------------

No existing biodiversity survey method completely censuses all of the organisms in a given area. For example, towed fishing nets can efficiently sample organisms larger than the mesh and slower than the boat; but overlook viruses and have undesirable effects on charismatic air-breathing species. From a boat or aircraft, scientists can visually quantify large whales, but not the krill on which they feed. However, DNA-based surveys show great promise as an efficient technique for detecting a previously unthinkable breadth of organisms from a single sample.

Microbiologists have used nucleic acid sequencing to quantify the composition and function of microbial communities in a wide variety of habitats (CITE VENTER, ARMBRUST). To do so, a sample of environmental medium (e.g. water) containing microorganisms is collected, their DNA is isolated and sequenced, and the identity and abundance of sequences is considered a direct measure of the identity and abundance of organisms contained in the sample, which indirectly estimates the quantity of organisms in an area.

Macroorganisms shed DNA-containing cells into the environment (environmental DNA or eDNA) that can be sampled in the same way. Such sampling is appealing because of its potentially high efficiency in capturing a broad swath of macroorganisms, but the accuracy and reliability of indirect estimates of macroorganismal abundance has been debated because the entire organisms are not contained within the sample. In part, concern surrounding eDNA methods for macroorganismal surveys is rooted in uncertainty about the scale of time and space that these methods sample. It is difficult to confidently infer local organismal presence from an environmental sample that a species is present locally without knowing how long DNA can persist in that environment and how far it can travel. As a result, estimating the spatial and temporal resolution of eDNA studies in the field is a key step in making these methods practical.

Ultimately, the spatial and temporal scope of an eDNA survey is determined by the rate of DNA degradation and the movement of the environmental medium (CITE recent Jerde paper with artificial streams; degradation over meters).The mechanisms driving DNA degradation include the presence of enzymes (CITE Klymus et al), the chemical environment of the water (CITE deepsea), and the intensity of UV light and heat (CITE Klymus), all of which are factors that vary in space and time. For example, DNA is likely to persist in cold environments for longer than it would in hot environments (CITE TEMPERATURE STUDY). However, on local scales, or within a single habitat type, environmental conditions are more likely to be consistent, and thus variance among samples is less likely to be attributable to environmental conditions impacting DNA persistence.

The relationship between local organismal abundance and DNA abundance is further complicated in habitats where the environmental medium itself may transport eDNA away from its source. We know that genetic material moves away from its source precisely because organisms can be detected indirectly without being present in the sample. eDNA is more likely to travel farther in a highly dynamic fluid such as the open ocean or flowing river than it would through the sediment at the bottom of a stagnant pond. (CITE DEINER STREAM EDNA CONVEYER BELTS, Shogren2016)

Yet even studies of extremely dynamic habitats such as coastlines with high wave energy have found remarkable evidence that eDNA transport is limited enough that DNA methods can detect community differences separated by less than 100 meters (CITE Port).

Several rigorous studies have investigated the specific effect of various environmental factors on eDNA persistence under controlled laboratory conditions (CITE klymus ENVIRONMENTAL FACTORS ~ DEGRADATION). Other studies have investigated the magnitude of environmental movement on the transport of eDNA in specific contexts, such as streams (CITE DEINER STREAM CONVEYER BELT). In theory, given enough data from manipulative experiments, it is possible that one could measure environmental variables--temperature, UV intensity, flow, etc--at the time of eDNA sample collection in order to more precisely estimate the origin of eDNA. In practice, this approach is unlikely to yield appreciable gains in accuracy because even the most advanced sensors ((thinking of ADCPs here)) and analytical techniques available cannot determine the origin of particles in highly dynamic fluid environments at scales relevant to most ecological questions (CITE PITERBARG2001, more recents??). % http://www.rsmas.miami.edu/LAPCOD/research/prediction/

Further, collecting and analyzing these data along with eDNA sampling efforts would severely limit the scalability of eDNA methods. A more practical approach is to compare the spatial distribution of communities of eDNA with expectations based on prior knowledge of the spatial distribution of communities of organisms.

Species and the communities they comprise are not homogeneously distributed in space; describing and predicting the spatial heterogeneity of ecosystems is of great interest to ecologists. One consistently observed pattern of community spatial heterogeneity is that communities close to one another tend to be more similar than those that are farther apart (CITE NEKOLA1999, BELL 2010, ). This decrease in community similarity with increasing spatial separation is called distance decay and has been reported from rainforests (CITE CONDIT2002, CHUST2006), ectomycorrhizal fungi (CITE bahram2013JECO), plant communities (CITE guo2015plosone), and marine microorganisms (CITE Martiny2011PNAS,Chust2013GLOBECOBIO, Wetzel2012plosone). (% coral reefs (CITE HUBBELL2001)). Typically, this relationship is assessed by regressing a measure of community similarity against a measure of spatial separation for a set of sites at which a set of species' abundances (or presences) is calculated. The underlying mechanism thought to drive the slope of this relationship is the rate of movement of individuals among sites. Because eDNA is shed and transported away from its source, we expect this increased movement of eDNA particles to homogenize community similarity, and thus erode the distance decay relationship of eDNA communities.

Shift to past tense in final paragraph. I think it is always easiest to write this paragraph in the present tense myself because it gives a sense of action and moving forward. Also, remember: you may no longer believe it but this is cool because you’ve been staring at it for forever, but the things you do / are trying to do are novel and hard and of general interest. Remind the readers why they are cool…. I wrote a little here but don’t feel strongly about the exact verbiage… just wanted to give examples.

Here, we explore the spatial dispersion of eDNA communities in a marine environment. We use PCR-based methods and massively parallel sequencing to survey 16s mtDNA from a suite of marine animals in water samples collected over a spatially regular grid extending from the shoreline out to 4 kilometers offshore in Puget Sound, Washington, USA. We then use our data to ask three general questions. First, we ask how the eDNA community similarity varies spatially and investigate the spatial patchiness of eDNA using distance-decay models. Second, to document the spatial scale for eDNA sampling in a field setting, we determine the minimum distance over which differences in community structure are detectable in this habitat. Finally, we use nonparametric classification methods to determine the number and identity of distinct eDNA communities in the spatial grid.

We evaluated 16s eDNA community similarity in space and tested for a distance decay relationship among sets of samples. We estimated the minimum distance over which differences in community structure are detectable in this habitat, in an effort to document the spatial scale over which eDNA sampling might be relevant in a field setting Finally, we used an unsupervised classification algorithm to determine the number and identity of distinct eDNA communities in the spatial grid.

<<< orphaned text but perhaps useful somewhere...>>>

Samples collected of ecological communities may vary in dissimilarity from 0 (completely identical) to 1 (completely different). For samples collected from multiple locations, the relationship between their spatial distance and community dissimilarity is of interest because it reflects the amount of community heterogeneity---changes in abundance and composition---over the spatial scale sampled. %In general, the relationship between community dissimilarity (0 = identical; 1 = completely different) and spatial distance is expected to be asymptotic, because communities nearer to each other tend to be more similar than those farther apart. The intercept is expected to be 0, because only within-sample comparisons can have 0 spatial separation, and communities have no dissimilarity within a sample if sampling method is repeatable. % technically impossible for samples to be taken at exactly 0; they are actually immediately adjacent. %Deviation from 0 indicates heterogeneous community composition/structure over fine scales. Likewise, dissimilarity cannot exceed 1, and reaches 1 only when multiple discrete community types are sampled. The trend is expected to be asymptotic if communities within a habitat are spatially structured, where the value of the asymptote and the rate of increase provide insight about community turnover. A flat relationship indicates that heterogeneity is not assorted spatially, and can be interpreted in different ways, depending on the asymptote. % ?or no relationship both

If the asymptote is close to 1, there is high spatial heterogeneity over the spatial scale of sampling. If the asymptote is close 0, all samples are similar, and we infer there is complete community homogeneity over the scale sampled. The rate at which community dissimilarity approaches the mean gives an indication of the rate of community turnover. <<< end orphan text >>>

%-----------------------------------METHODS-------------------------------------

Environmental Sampling

Starting from lower-intertidal patches of \textit{Zostera marina}, we collected 1L water samples at 1 meter depth from 8 points (0, 75, 125, 250, 500, 1000, 2000, and 4000 meters) along three parallel transects separated by 1000 meters (Figure \ref{site\_map}). To destroy residual DNA on equipment used for field sampling and filtration, we washed with a 1:10 solution of household bleach (8.25\% sodium hypochlorite; 7.25\% available chlorine) and deionized water, followed by thorough rinsing with deionized water. Each environmental sample was collected in a clean 1 liter high-density polyethylene bottle, the opening of which was covered with 500 micrometer nylon mesh to prevent entry of larger particles. Immediately after collecting the sample, the mesh was replaced with a clean lid and the sample was held on ice until filtering. 1 liter from each water sample was filtered in the lab on a clean polysulfone vacuum filter holder fitted with a 47 millimeter diameter cellulose acetate membrane with 0.45 micrometer pores. Filter membranes were moved into 0.9 milliliters of Longmire buffer (CITE LONGMIRE) using clean forceps and stored at room temperature (CITE Renshaw2015). To test for the extent of contamination attributable to laboratory procedures, we filtered three replicate 1 liter samples of deionized water. These samples were treated identically to the environmental samples throughout the remaining protocols.

DNA Purification

DNA was purified from the membrane following a phenol:chloroform:isoamyl alcohol protocol following Renshaw (CITE RENSHAW). Preserved membranes were incubated at 65C for 30 minutes before adding 900 microliters of phenol:chloroform:isoamyl alcohol and shaking vigorously for 60 seconds. We conducted two consecutive chloroform washes by centrifuging at 14,000 rpm for 5 minutes, transferring the aqueous layer to 700 microliters chloroform, and shaking vigorously for 60 seconds. After a third centrifugation, 500 microliters of the aqueous layer was transferred to tubes containing 20 microliters 5 molar NaCl and 500 microliters 100\% isopropanol, and frozen at -20C for approximately 15 hours. Finally, all liquid was removed by centrifuging at 14000 rpm for 10 minutes, pouring off or pipetting out any remaining liquid, and drying in a vacuum centrifuge at 45C for 15 minutes. DNA was resuspended in 200 microliters of ultrapure water. Genomic DNA extracted from tissue of a species absent from the sampled environment (Oreochromis niloticus) served as positive control for the remaining protocols.

PCR Amplification

From each DNA sample, we amplified an approximately 115 base pair (bp) region of the mitochondrial gene encoding 16S RNA using a two-step polymerase chain reaction (PCR) protocol described by ODONNELL (CITE ODONNELL2016). In the first set of reactions, primers were identical in every reaction (forward: AGTTACYYTAGGGATAACAGCG; reverse: CCGGTCTGAACTCAGATCAYGT); primers in the second set of reactions included these same sequences but with 3 variable nucleotides ('NNN') and an index sequence on the 5PRIME end (see CITE PRIMER TABLE/METADATA). We used the program OligoTag \cite{Coissac2012} to generate 30 unique 6-nucleotide index sequences differing by a minimum Hamming distance of 3 (CITE primer\\_table). %\nameref{primer\_table}). Indexed primers were assigned to samples randomly, with the identical index sequence on the forward and reverse primer to avoid errors associated with dual-indexed multiplexing (CITE Schnell2015). In a UV-sterilized hood, we prepared 25 microliter reactions containing 18.375 microliters ultrapure water, 2.5 microliters 10x buffer, 0.625 microliters deoxynucleotide solution (8 millimolar), 1 microliter each forward and reverse primer (10 micromolar, obtained lyophilized from Integrated DNA Technologies (Coralville, IA, USA)), 0.25 microliters Qiagen HotStar Taq polymerase, and 1.25 microliter genomic eDNA template at 1:100 dilution in ultrapure water. PCR thermal profiles began with an initialization step (95C; 15 min) followed by cycles (40 and 20 for the first and second reaction, respectively) of denaturation (95C; 15 sec), annealing (61C; 30 sec), and extension (72C; 30 sec). 20 identical PCRs were conducted from each DNA extract using non-indexed primers; these were pooled into 4 groups of 5 in order to ensure ample template for the subsequent PCR with indexed primers. In order to isolate the fragment of interest from primer dimer and other spurious fragments generated in the first PCR, we used the AxyPrep Mag FragmentSelect-I kit with solid-phase reversible immobilization (SPRI) paramagnetic beads at 2.5x the volume of PCR product (Axygen BioSciences, Corning, NY, USA). A 1:5 dilution in ultrapure water of the product was used as template for the second reaction. PCR products of the second reaction were purified using the Qiagen MinElute PCR Purification Kit (Qiagen, Hilden, Germany). Ultrapure water was used in place of template DNA and run along with each batch of PCRs to serve as a negative control for PCR; none of these produced visible bands on an agarose gel. In total, four separate replicates from each of 31 DNA samples were carried through the two-step PCR process for a total of 124 sequenced PCR products. These were combined with additional samples from other projects, totaling 345 samples for sequencing.

PCR paragraph edited by Ole [Does this loose too much info?]

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DNA Sequencing

PCR products were pooled according to their primer index in equal concentration, and 150 ng was prepared for library sequencing using the KAPA high-throughput library prep kit with real-time library amplification protocol (KAPA Biosystems, Wilmington, MA, USA). Each of these ligated sequencing adapter including an additional 6 base pair index sequence (NEXTflex DNA barcodes; BIOO Scientific, Austin, TX, USA). Thus, each PCR product was identifiable via its unique combination of index sequences in the sequencing adapters and primers. Fragment size distribution and concentration of each library was quantified using an Agilent 2100 BioAnalyzer. Libraries were pooled in equal concentrations and sequenced for 150 base pairs in both directions (PE150) using an Illumina NextSeq at the Stanford Functional Genomics Facility (machine NS500615, run 115, flowcell H3LFLAFXX), where 20\% PhiX Control v3 was added to act as a sequencing control and to enhance sequencing depth. Raw sequence data in fastq format is publicly available (see Data Availability).

Sequence Data Handling (Bioinformatics)

<<Consider: Detailed bioinformatic methods are provided in the supplemental material, and scripts used from raw sequencer output onward can be found in the public project directory (see Data Availability).>>

Reads passing the preliminary Illumina quality filter were demultiplexed on the basis of the adapter index sequence by the sequencing facility. We used fastqc to assess the fastq files output from the sequencer for low-quality indications of a problematic run. Forward and reverse reads were merged using PEAR v0.9.6 \cite{Zhang2014} and discarded if more than 0.01 of the bases were uncalled. If a read contained two consecutive base calls with quality scores less than 15 (i.e. probability of incorrect base call = 0.0316), these bases and all subsequent bases were removed from the read. Paired reads for which the probability of matching by chance alone exceeded 0.01 were not assembled and omitted from the analysis. Assembled reads were discarded if assembled sequences were not between 50 and 168 bp long, or if reads did not overlap by at least 100 bp.

We used vsearch VSEARCHVERSION (CITE VSEARCH) to discard any merged reads for which the sum of the per-base error probabilities was greater than 0.5 ("expected errors") \cite{Edgar2010}. Sequences were demultiplexed on the basis of the primer index sequence at base positions 4-9 at both ends using the programming language AWK. Primer sequences were removed using cutadapt v1.7.1 \cite{Martin2011}, allowing for 2 mismatches in the primer sequence. Identical duplicate sequences were identified, counted, and removed in python to speed up subsequent steps by eliminating redundancy, and sequences occurring only once were removed. We checked for and removed any sequence likely to be a PCR artifact due to incomplete extension and subsequent mis-priming using a method described by Edgar (CITE UCHIME\cite{Edgar2010}) and implemented in vsearch VSEARCHVERSION. Sequences were clustered into operational taxonomic units (OTUs) using the single-linkage clustering method implemented by swarm version SWARMVERSION with a local clustering threshold (d) of 1 and fastidious processing (CITE SWARM).

Reads that did not contain primer index sequences (or combinations thereof) in the expected position result from processes that leave the same signature as contamination. For example, errors during oligonucleotide synthesis or sequencing of the indexed primers could cause reads to be erroneously assigned to samples. We calculated the maximum rate Similarly, we calculated rates of cross-library contamination by counting occurrences of primer sequences from 12S amplicons prepared separately but pooled and sequenced alongside our samples. These occurrences indicate an error in the preparation or sequencing procedures. This represents a general minimum rate at which we can expect that sequences from one environmental sample could be erroneously assigned, and so we considered for further analysis only those reads occurring with greater frequency than this across the entire dataset.

We checked for experimental error by evaluating the Bray-Curtis dissimilarity of proportional read abundance among replicate PCRs from the same DNA sample (0.033$\pm$0.063), and excluded one PCR replicate for which the dissimilarities between itself and the other replicates exceeded 1 SD.% (lib\\_B\\_tag\\_GCGCTC)

To account for variation in the number of sequencing reads (sequencing depth) recovered per sample, we multiplied the within-sample proportional abundance of each OTU by the minimum sequencing depth, and rounded to the nearest integer.

Because each step in this workflow is sensitive to contamination, it is possible that some sequences are not truly derived from the environmental sample, and instead represent contamination during field sampling, filtration, DNA extraction, PCR, fragment size selection, quantitation, sequencing adapter ligation, or the sequencing process itself. Some authors have argued that these risks could bias sequence abundance, making those data meaningless and prohibiting quantitative estimates; instead these authors advocate for converting count data to binary presence absence data on the basis of sequence abundance greater than some arbitrary threshold. Recent work indicates that this binary treatment of data can overestimate taxon richness and falsely elevate the estimate of taxon turnover among samples (CITE LERAY FORTHCOMING). We take the view that contaminants are unlikely to manifest as sequences in the final dataset in consistent abundance across replicates; indeed, our data show that the process from PCR onward is remarkably consistent. Thus, we calculated from our data the maximum number of sequence counts (after scaling to correct for sequencing depth variation) for which there is turnover in presence-absence among PCR replicates within an environmental sample. We use this number to determine a conservative minimum threshold above which we can be confident that counts are consistent among replicates and not of spurious origin, and exclude from further analysis observations where the mean abundance across PCR replicates within samples does not reach this threshold. % the number is turnover\_threshold=178

In order to determine the most likely taxon from which each sequence originated, the representative sequence from each OTU was then queried against the NCBI nucleotide database (GenBank; version October 2015) using the blastn command line utility (CITE BLAST). In order to maximize the accuracy of this computationally intensive step, we implemented a nested approach whereby each sequence was first queried using strict parameters (e-value = 5e-52), and if no match was found, the query was repeated with decreasingly strict e-values (5e-48 5e-44 5e-40 5e-36 5e-33 5e-29 5e-25 5e-21 5e-17 5e-13). Other parameters were unchanged among repetitions (word size: 7; maximum matches: 1000; culling limit: 100; minimum percent identity: 0). Each query sequence can be an equally good match to multiple taxa either because of invariability among taxa or errors in the database (e.g. human sequences are commonly attributed to other organisms when they in fact represent lab contamination). In order to guard against these spurious results, we used an algorithm to find the lowest common taxon for at least 80\% of the matched taxa, implemented in the R package taxize (CITE TAXIZE VERSION). Similarly, we repeated analyses using the dataset consolidated at the same taxonomic rank across all queries, for the rank of both family and order.

The data for input to further analyses are thus a contingency table of counts of unique sequences, OTUs, or taxa present in each PCR.

Ecological Analyses

AOS: Need to take a step back and remind the reader what you are doing and why you are doing it. Something like: After gathering the eDNA data, we use the eDNA community observed at each location to make inference about the spatial patterning of eDNA communities. We use statistical tools from community ecology to assess the spatial structure of eDNA communities.

[also, I would put the various “what I did” parts before the section on subsetting, which I think is least important]

Due to the huge number of observations in each sample, we conducted each analysis on multiple subset of the data to expose different attributes of the data. We report the results for one subset for each analysis, and report results from alternative subsets in the supplemental material. We subset the data in eight ways: (1) exclude rare taxa (i.e., low abundance threshold), (2) exclude abundant taxa (i.e., high abundance threshold), (3) subsample taxa randomly, (4) subsample of taxa proportional to their abundance, (5) subsample taxa inversely proportional to their abundance, (6) exclude taxa found in only one environmental sample (7) exclude non-marine taxa, (8) exclude taxa whose known individual range (including gametes and larvae) exceeds the spatial scale of our study. Comparing results among different subsets of the data allow us to assess the sensitivity of our results to particular components of the eDNA community. We also tested a variety of transformations of the mean scaled abundance data, including (1) log ($log\_{e}x$), and (2) binary ($1 = x > 1$; $0 = x < 1$).

To ask how eDNA communities vary in space we first used multivariate clustering algorithms.

We simultaneously assessed the existence of distinct community types and the membership of samples to those community types using an unsupervised classification alogrithm known as partitioning around medoids (CITE PAM, sometimes referred to as k-medoids clustering), as implemented in the R package fpc (CITE fpc). The classification of samples to communities was made on the basis of their pairwise Bray-Curtis dissimilarity, calculated using the function vegdist in the R package vegan (CITE VEGAN). % consider Gower distance

To ask if spatially proximate samples were more similar than spatially distant samples, we asked if there was a decay of community similarity with distance. We assessed community similarity in space using pairwise Bray-Curtis dissimilarity between samples and the great circle distance between points using the Haversine method as implemented by the R package geosphere (CITE geosphere). Given an efficient sampling technique, replicate samples taken at the same position in space should be identical, and thus the intercept of the regression of dissimilarity against distance should be zero, and deviation from zero is an indicator of the efficiency of the sampling method. % NOTE: We did not take multiple environmental samples from the same position in space, but conducted multiple PCRs from each environmental sample.

Topic sentence with something about community similarity with reference to a know environmental gradient? I don’t know what the motivation for this analysis is To estimate the maximum dissimilarity and the rate of community turnover in space (distance decay), we modeled community dissimilarity as a function of distance from shore following a Michaelis-Menten relationship, which has an intercept at zero:

\begin{equation}\label{MichaelisMenten}

com \sim V\_{max}[d]/K\_{m}[d]

\end{equation}

where $com$ is community dissimilarity, $d$ is spatial distance, and where the asymptote is given by $V\_{max}$, and the distance at which half the asymptote has been reached is given by $K\_{m}$. Model fit was assessed using the function nls in R (CITE R). % I also assessed fit to a generalized additive model, I can add this if needed or replace the Michaelis-Menten with it.

% Calculate Moran's I on ?(relative abundance | raw count) of ?(taxa | OTU | uniq\_sequences) to report dispersion of DNA in space? There is a multiple comparison issue and the results are generally not informative to my eye.

One more easy analysis: calculate dominance (evenness) of observed eDNA samples in each sample.

Organismal Life History Data

We collated coarse-scale data on life history characteristic for each of the major taxonomic groups recovered, including dispersal range of the gametes, larvae, and adults, adult habitat type and selectivity, and adult body size. Dispersal range was given as an order-of-magnitude approximation of the scale of dispersal: for example, internally fertilized species were assigned a gamete range of 0 km, while broadcast spawners were assigned a gamete range of 10 km. Similarly, adult range size was approximated as 0 km (sessile), 1 km (motile but not pelagic), or 10 km (highly mobile, pelagic). Variables were specified as 'multiple' for groups known to span more than 1 magnitude of range size. For groups to which sequences were annotated with high confidence, but for which life history strategy is diverse or poorly known (e.g. families in the phylum Nemertea), we used conservative, coarse approximations at a higher taxonomic rank.

% INCLUDE OR OMIT? JO: I think we can omit this. %We assessed whether or not marine invertebrate taxa are thought to be present in Puget Sound by checking for their presence in a comprehensive checklist of invertebrates of Puget Sound CITE KOZLOFF (RYAN HAS OTHER DATA IF NEEDED <EOPS?>). These data are available as part of the REFERENCE SUPPLEMENTAL DATA.

%-----------------------------------RESULTS-------------------------------------

Data Quality (Bioinformatics)

All value ranges are reported as (mean plus and minus one standard deviation). A total of 371,576,190 reads passing filter were generated in each direction, with XXX\% of base calls having a 0.001 or lower probability of incorrect base call (Phred q-score of 30 or higher). Each environmental sample in the present study was represented by no fewer than 130402 reads. There was a very low frequency of cross-contamination from other libraries into those reported here (5e-05$\pm$8e-05; max proportion 0.00034)

Sequence abundances among PCR replicates (i.e., within water samples) were remarkably consistent: 92 of the 96 environmental samples had a mean Bray-Curtis dissimilarity $\leq$ 0.052 among PCR replicates. A single environmental sample had high dissimilarity (0.341) among PCR replicates before removing a faulty PCR replicate; after removal of this PCR replicate, the highest mean Bray-Curtis dissimilarity among replicates within an environmental sample was 0.034. OTUs with abundance greater than 178 reads experienced no turnover among PCR replicates within a sample.

Community Analysis

AOS: OK. Here is how I think about the results I see in the figures. Need to note which subset of the data each analysis is on… Also, I’d present results in the same order they were described in the Methods for ease in following.

First, re-emphasize the bioinformatics result from the first section – Virtually no variability in PCRs… therefore all of these results are the consequence of real spatial variation.

Second, warm up the audience with a couple single species winner plots of the grids (e.g. shore-associated: Embiotocidea; shore and pelagic, found everywhere: Mytilus; variable but generally offshore: Thysanoessa). Point is that some of the species identified make some sense but there is variability… the world is not uniform.

Third, I see Fig. 2 (diss\_by\_dist.pdf) as saying things that are further away being very different from things that are close. This is expected. What is not expected is the slope of that line being so shallow (or alternately, the intercept being so large). I’d bring in alternate subsets of the data here – do all subsets show the same pattern? Is this driven by common (or rare?) species? If diff subsets look different, that seems important. If they are the same, that also seems important. I’d think you could write the whole paper around just different subsets of the data with this since analysis if you wanted.

Next there is the PAM analysis which is a little confusing. I like “pam\_in\_space.pdf” but am wondering about the value of “pam\_plot.pdf.” It makes me wonder about the distance between the center of the multi-variate mediods (i.e. are they numbered coherently so that 1 is closer to 2 than it is to 3? Or not?). From just the pam\_plot it looks like #1,2,5 are pretty close together and #3 is sorta by itself, 6 and 7 are in the ballpark or each other and 4 and 8 are outliers. But then this is just the first two components which may (or may not) be misleading depending on how much of the variation is absorbed by these first two components. A second option for doing this analysis might be to pre-specify the numbers of groups (2,3,4,5,etc) and then make a plot like pam\_in\_space that shows a series of increasing group numbers and see if the patterns go from biologically coherent to biologically nonsensical. Again, at least mention the PAM results for different subsets (same or different?)

Community dissimilarity rapidly increased with spatial distance, flattening within several hundred meters, such that samples [1km apart were as distinct from one another as samples 4km apart] (FIGURE 2). The asymptotic curve fit through the origin reflects the theoretical expectation that samples at zero distance from one another are necessarily identical. [In a model test, this is the best fit curve… etc.]. The estimated asymptote of community dissimilarity as a function of spatial distance ($V\_m$) was 0.72 ($p <<< 0.05$), and the distance at which half this dissimilarity was accumulated ($K\_m$) is 23.8 kilometers ($p = 0.006$). Residual standard error of the fit of the model is 0.1563 on 274 degrees of freedom.

[if including PAM analysis, put those results here and refer to the figure with an overarching sentence like “PAM analysis identified 8 distinct communities, but these were not spatially coherent.]

Excluding spatially-invariant taxa ?(taxa which occur in only one spatial location) had no discernible effect on the outcome of the PAM analysis (number of clusters, assignment to clusters) (CITE FIGUREPAM).

Taxonomic Annotation and Natural History

The vast majority (97.6\%) of sequences and OTUs (96.9\%) were matched to organisms that have high potential for dispersal at either the gamete, larval, or adult stage. Of the 6 OTUs annotated to taxa known to have very limited dispersal [LIST these],, only 2 occurred in more than two samples. These were assigned to \textit{Cymatogaster aggregata}, a viviparous nearshore fish with internal fertilization, and \textit{Cupolaconcha meroclista}, a sessile marine gastropod with internal fertilization and short larval dispersal (CITE???). Thus, we are unable to compare the patterns of dispersion for varying life history categories.

Hmmm. Need to think about this some more.

%----------------------------------DISCUSSION-----------------------------------

Outline of the big picture things we want to emphasize. Ole’s list includes

1. as in most community level analyses, expectations for simple patterns are not born out by the data.
2. Individual species patterns do make some sense. Point out that these patterns only make sense if you do not only think about their adult forms (e.g. Mussels) which are typically the only thing macroscopic.
3. Despite some complexity, eDNA patterns still show distance decay relationships in similarity.
   1. Major point of our paper is that samples that are relatively close together can be considerably different.
   2. Supports the careful use of eDNA for surveys over relatively small areas. Just like looking for patchy communities (think of maybe sand patches and coral patches in the tropics).
   3. This is not the final word in the spatial aspects of eDNA.
4. PAM analyses show…. Some confusion but potentially some coherence.
5. Discussion of different subsets giving different answers.
6. Something about the life-history info…. Not sure what.

Indirect surveys of organismal presence are a key development in ecosystem monitoring in the face of increased anthropogenic pressure and dwindling resources for ecological research. Detection of organisms using environmental DNA is an especially promising method, given the rapid pace of advancement in technological innovation and cost efficiency in the field of DNA sequencing and quantification.

We demonstrate that a distance-decay relationship exists for eDNA communities in this dynamic environment despite some expected homogenization of signal compared to known community distributions, and that the effective scale of individual water samples for eDNA analysis is likely to be quite small (<100m) in this nearshore environment.

The distribution of community types did/does not fit our expectations based on known distributions of benthic communities. In this area (as in much of Puget Sound), a narrow band of shallow, soft bottom hugs the shoreline and supports eelgrass meadows which harbor diverse epifaunal and infaunal communities, and attract transient species which use the meadows for shelter and to feed on epifaunal biota. This shallow habitat quickly gives way to a central depth of approximately 200 meters. Intertidal shoreline varies between soft, fine sediment to cobble and boulder rubble. Hard intertidal surfaces support a well-documented ?(biota/fauna/community) ?(including/dominated by)? barnacles (Sessilia), mussels (Bivalvia:Mytilidae), anemones (Actinaria), sea stars (Asteroidea), urchins (Echinoidea), Bryzoans (Ectoprocta), and crustaceans (Decapoda). Soft intertidal sediments are inhabited by burrowing bivalves (Bivalvia:various families), segmented worms (Annelida), acorn worms (Enteropneusta), and in the lower intertidal and high subtidal ranges by eelgrass ?(beds | meadows). Hard bottoms: lower intertidal and high subtidal = macroalgae such as nereocystis etc (check M. Foley's list) which provides habitat for a diverse community of ?(fish and invertebrates)

Our samples were collected at the surface over depths ranging from approximately 2 to 200 meters. The benthic habitats directly below each sample point vary from eelgrass meadow to soft-bottom ?( sub-photic? ) habitats. At the sampling points over the ?(deepest water/ greatest depth), samples were closer to the bottom than they were to the next closest sampling point. However, we found no distinct pattern (/signal) of deep-water communities, indicating (1) the a greater biomass of nearshore (/shallow) organisms results in ?(more recoverable genetic material | greater concentrations of eDNA)? even over long distances.

{ Material from these deep water communities is not transported to the surface in ?(quantities / amounts )? sufficient for detection in sequencing based approaches using general primers, as in the present study.

Contrary to our ?(perhaps naive) expectations based on well-documented patterns of benthic community distribution, we found no discernible pattern of communitiy turnover as a function of distance from shore, nor did the composition of those communities change as expected. The most abundant taxa at sites furthest from shore are taxa whose adult stages are restricted to intertidal hard substrates. However, the larvae and gametes of these taxa are pelagic and can be transported long distances by water movement (CITE).

Of the XXXX taxa ?(for which we recovered data | detected by our methods), we were able to confirm only one which lacks any microscopic and potentially dispersive life stage, the Shiner Surfperch (Embiotocidae). Adults and juveniles of this species are strictly associated with shallow, near-shore habitats. Like all members of the family, they are internal fertilizers that give birth to live young, meaning there are no dispersive gametes or larvae which may travel far from juveniles and adults and be detected by our sampling methods. C. aggregata DNA was abundant at sites nearest to shore, (MXXXXX\%+-SD of all sequences,), rare or absent elsewhere (CITE FIG). We interpret this as evidence that the chaotic spatial distribution of eDNA communities (CITE FIG PAM) results from our primers' affinity for many species which at some point exist as microscopic pelagic gametes or larvae. Communities of microscopic organisms can be exceptionally variable in space over small scales due to oceanographic features such as eddies. Such fine-scale variation would be missed by our sampling design; denser sampling in concert with current profiling would ?(XXXX) the role of ?(ocean movement???) on eDNA dispersal.

Moreover, if entire multicellular individuals (such as larvae) were directly collected in our water samples, community profiles would be further distorted. Our sampling bottles excluded particles larger than 500 micrometers, but gametes and very small larvae could have gained entry. It is possible that even a single small individual, containing many thousand mitochondria, would overwhelm the signal of another species from which hundreds of cells had been sloughed from many, larger individuals. Data on larval size distribution at the time of sampling from each species in our data set would allow us to estimate the frequency of such events. Nevertheless, it is precisely the sensitivity to small particles that makes the eDNA approach powerful, so we are reluctant to recommend that aquatic eDNA sampling use finer pre-filtering. Instead, we emphasize the importance of designing and selecting primer sets that selectively amplify target organisms. In the case of the present study, in order to recover patterns matching our expectations, this would be non-transient, benthic marine organisms lacking any pelagic life stage. Likewise, our results highlight the need for curated life-history databases. As technological advances increase the speed and throughput of biodiversity surveys, making sense of these data in a timely manner requires that natural history data be stored in standard formats in centralized repositories. << The rate at which we can make sense of high-throughput biodiversity survey methods will be limited by our ability to incoporate external data. Some databases like EOL, GBIF, Fishbase, that house records of taxonomy, occurrence, and other basic data types are growing in number of users, support, and maintenance, but there is no centralized, standardized repository for natural history data. As NCBI's nucleotide and protein sequence database (GenBank) has facilitated a multitude of transformative studies in diverse fields, an ecological analog would ?(be a huge boon for | revolutionize ) biodiversity science.

%-------------------------------ACKNOWLEDGEMENTS--------------------------------

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%-----------------------------------FUNDING-------------------------------------

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%--------------------------------CONTRIBUTIONS----------------------------------

Conceived and designed the experiments: James L. O'Donnell, Ryan P. Kelly, A. Olaf Shelton. Collected the data: James L. O'Donnell, Greg Williams, Natalie C. Lowell, Ryan P. Kelly, A. Olaf Shelton, Jameal F. Samhouri. Conducted the analyses: . Wrote the first draft: . Edited the manuscript: .

%-------------------------------------DATA-------------------------------------%

% TODO SUBMIT DATA AND METADATA; ADD URL

All sequence files and metadata are available from EMBL:\\ \verb!http://www.ebi.ac.uk/ena/data/view/XXXXXXXXX! \\

All analyses were performed using scripts available from the project repository on GitHub:\\ \verb!https://github.com/jimmyodonnell/Carkeek\_eDNA\_grid!\\

%----------------------------------REFERENCES-----------------------------------

%-----------------------------------FIGURES-------------------------------------

/Figures/site\_map.pdf}

Caption: TODO: Plot with GEBCO 30-second data or remove grid coloring and color by isobath. Looking into filling by contour. Geographic position of collected samples. Lines give XXX meter isobaths.}

/Figures/diss\_by\_dist.pdf}

Caption: Pairwise Bray-Curtis dissimilarity of eDNA communities plotted against pairwise spatial distance. Line represents prediction of the Non-linear Least Squares regression to a Michaelis-Menten model ($V\_m = 0.72$, $p <<< 0.05$; $K\_m = 23.8$ kilometers, $p = 0.006$; RSE = 0.1563; df = 274.). Restricting comparison to within-transect has no qualitative difference in the outcome (see 'diss\\_by\\_dist\\_by\\_transect.pdf').

/Figures/dissimilarity\_from\_shore.pdf}

Caption: Pairwise dissimilarity (Bray-Curtis) across transects plotted against distance from shore. A linear model determined no effect of distance from shore on dissimilarity.}

/Figures/variance\_from\_shore.pdf}

Caption: Within-OTU variance across transects plotted against distance from shore.}

/Figures/heatmap\_sites.pdf}

Caption: Heatmap of pairwise site dissimilarity (Bray-Curtis).}

/Figures/pam\_plot.pdf

Caption: TODO figure out color of ellipses; I can't even plot them gray without Plot of partitioning around medoids (PAM) analysis of OTU sequence abundance from 4 replicate PCRs at each of 24 sampling points. Points represent communities of OTUs; color and shape indicate cluster membership as determined by PAM analysis. Ellipses indicate the smallest area of a cluster that contains all of its members.

/Figures/pam\_in\_space.pdf

Caption: Geographic position of collected samples, colored by membership to clusters identified by partitioning around medoids algorithm.

/Figures/otu\_in\_space.pdf

Caption: Example of a DNA sequence's spatial distribution. This sequence is annotated to SPECIES X, which is found only in shallow, structured habitats such as patches of \textit{Zostera marina}. Point size and color transparency indicates abundance relative to other DNA sequences from that sample, scaled to the maximum value for this sequence (no fill = 0, full fill = 1). Samples from which this sequence was not recovered are indicated by an "x".