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# Identifying the plant associated microbiome across aquatic and terrestrial environments: the effects of amplification method on taxa discovery.

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*Abstract* - Plants in terrestrial and aquatic environments contain a diverse microbiome. Yet, the chloroplast and mitochondria organelles of the plant eukaryotic cell originate from free-living cyanobacteria and Rickettsiales. This represents a challenge for sequencing the plant microbiome

with universal primers, as ~99% of 16S rRNA sequences may consist of chloroplast and mitochondrial sequences. Peptide nucleic acid clamps offer a potential solution by blocking amplification of host-associated sequences. We assessed the efficacy of chloroplast and mitochondria-blocking clamps against a range of microbial taxa from soil, freshwater, and marine environments. While we found that the mitochondrial blocking clamps appear to be a robust method for assessing animal-associated microbiota, Proteobacterial 16S rRNA binds to the chloroplast blocking clamp, resulting in a strong sequencing bias against this group. We attribute this bias to a conserved 14 base pair sequence in the Proteobacteria that matches the 17 base pair chloroplast blocking clamp sequence. By scanning the Greengenes Database, we provide a reference list of nearly 1500 taxa that contain this 14 base pair sequence, including 48 families such as the Rhodobacteraceae, Phyllobacteriaceae, Rhizobiaceae, Kiloniellaceae, and Caulobacteraceae. To determine where these taxa are found in nature, we mapped this taxa reference list against the Earth Microbiome Project database. These taxa are abundant in a variety of environments, particularly aquatic and semi-aquatic freshwater and marine habitats. To facilitate informed decisions on effective use of organelle-blocking clamps, we provide a searchable database of microbial taxa in the Greengenes and Silva databases matching various n-mer oligonucleotides of each PNA sequence.

*Key Words:* PNA clamps, plant microbiome, chloroplast, Earth Microbiome Project, Proteobacteria, aquatic environments

*Running Title:* Identifying the plant associated microbiome

Natural ecosystems contain an incredible diversity of microbiota, which remains largely undescribed (Locey and Lennon 2016). Recent advances in sequencing technologies have facilitated the description of this diversity throughout a range of terrestrial and aquatic biomes from the semi-natural environments of agricultural soils to the extreme environments of the deep sea (Caporaso et al. 2010, Gilbert et al. 2014). We are discovering the tremendous importance of

free-living and organismal-associated microbiota to both ecosystem and organismal health and functioning (Zak et al. 2003, Smith et al. 2015). Continued advancement in this field demands increasingly sophisticated studies that contrast the microbiomes across habitats and trace the source-sink dynamics of these microbial communities. Vital to this aim is use of a common methodology that enables comparisons across environments and microbial taxa. Ribosomal RNA genes are the typical targets for amplicon sequencing because they are conserved across microbial taxa, yet sufficiently polymorphic for taxonomic assignment.

Plant chloroplast and mitochondrial organelles are evolutionarily derived from free-living Cyanobacteria and Rickettsiales (Margulis 1981). Sequencing the internal or external plant microbiome thus represents a particular challenge because these organelles retain the microbial rRNA of their ancestors. Sequencing plant tissue typically yields upwards of 99% chloroplast and mitochondrial sequences (Lundberg et al. 2012, Zarraonaindia et al. 2015) (see published datasets in the Earth Microbiome Project Database for chloroplast content of leaf samples in Zarraonaindia et al.). Intensive sequencing, where only the remaining 1% of sequences is analyzed after filtering out chloroplast, is rarely an economically feasible option. Instead, a new method that blocks the amplification of these organelles using peptide nucleic acid PCR clamps, thus sequencing only the remaining microbes, has been proposed (Lundberg et al. 2013). These synthetic oligomers physically block amplification of a contaminant by binding tightly and specifically to the unique contaminant sequence (Egholm et al. 1993, Ørum et al. 1993, Ray and Nordén 2000, von Wintzingerode 2000, Karkare and Bhatnagar 2006). Although use of these organelle blockers may help reveal rare taxa of a microbiome in the presence of eukaryotic plant material, it might also bias discovery rates if applied across habitats, such as aquatic systems that often contain many free-living Cyanobacteria and Rickettsiales, by blocking amplification of nucleic acids of taxa closely related to organelles.

In our study, we aim to describe the benefits and drawback of using universal Earth Microbiome Project primers alone versus adding organelle-blocking clamps for studies across a range of environments and microbial taxa. By sequencing identical samples from terrestrial, marine and freshwater habitats we find that organelle-blocking clamps cause a strong bias against many taxa, particularly the Proteobacteria (including 48 families such as the Rhodobacteraceae, Phyllobaceriaceae, Rhizobiaceae, Kiloniellaceae, and Caulobacteraceae).

We trace this bias to a 14 base pair conserved region in bacteria that matches the chloroplast-blocking primer. We provide a scan of the Greengenes Database (http://greengenes.secondgenome.com/) for other taxa containing this conserved region, and using the Earth Microbiome Project Database (http://www.earthmicrobiome.org/ and https://qiita.ucsd.edu/), demonstrate that these particular taxa are abundant in many aquatic, terrestrial, and animal-associated environments. We conclude that use of these organelle blocking clamps poses a considerable bias for any studies aiming to eventually compare a plant-associated microbiome with a diversity of other environments.

## METHODS

*Field collections*:

Our field samples were collected for a number of different studies and are considered here only for comparing amplification methods. We summarize sample type and number in Table 1. The majority of samples were from an experiment designed to test for the direct versus indirect effects of individual variation within red alder tree leaf litter on microbial colonization in streams. The experiment was conducted in 2013 on the Hoko and Sekiu Rivers on the Olympic Peninsula of Washington (48° 15′29.58 N, 124° 21′8.59 W). We carried out a reciprocal transplant design in which fresh green leaves from individual trees growing along rivers were enclosed in mesh leaf packs and were either placed in the adjacent river or in a different river (4.5 km away). Our reciprocal transplant design is described in detail elsewhere (Jackrel and Wootton 2014, Jackrel et al. 2016). We sequenced the microbiome of a subset of these samples to compare sequencing results with EMP primers alone versus with EMP primers plus the organelle blocking PNA clamps. From each red alder tree, we constructed leaf packs containing 16 leaves each. Four leaves from each of these leaf packs were removed after 5, 10, 15, and 20 days of incubation, sealed in WhirlPak bags, and frozen.

At each of these four time points, we also sampled the freshwater microbiota immediately upstream of each leaf pack deployment location. Six liters of river water were pumped through Sterivex™ filters (EMD Millipore, Darmstadt, Germany) using a peristaltic pump. Immediately before and after the 20 day experiment, we collected both soil samples beneath each source tree and fresh leaves from each tree. All samples were kept cool and frozen at -20°C upon returning

from the field locations, and then stored at -80°C at Argonne National Laboratory until processing.

Seawater samples were collected using the same method described above for freshwater samples. Collections occurred on the outer coast of Washington State both immediately from the shore by standing on a rocky bench, Tatoosh Island, 48.39° N, 124.74° W and via shipboard collection offshore at 48.432 N, 124.738 W and 48.439 N, 124.831 W at approximately 70 m and 340 m total depth, respectively. The offshore samples were taken in July and August of 2011 and 2012 at both surface depths in the photic zone as well as depths below the photic zone (100, 125, 140, 300, 325 m) where 16S rRNA sequences from phototrophs would be minimal. Offshore samples were collected from the R/V Clifford Barnes with casts from a 12-sample CTD array (Seabird Electronics, Bellevue, Washington, USA) with 10-L Niskin bottles (General Oceanics, Miami, Florida, USA). Environmental variables associated with this collection are reported in Pfister et al. 2014 and online (http://www.bco-dmo.org/dataset/489045/data).

We extracted DNA from all samples using PowerSoil DNA Isolation Kits (MO BIO Laboratories, Carlsbad, California, USA). For water samples, Sterivex casings were cut with PVC cutters and half of the filter paper was removed, then ground and extracted as a solid sample. After extraction, we amplified the 253bp length V4 region using the Earth Microbiome Project universal primers (515F primer and 806 Golay-barcoded reverse primers) (Caporaso et al. 2012) with and without the mitochondrial and chloroplast blocking PNA clamps. We refer to this first method with PNA clamps as the EMP-PNA method, and the second method as the standard EMP method. The mPNA sequence to block mitochondria contamination is GGCAAGTGTTCTTCGGA and the pPNA sequence to block chloroplast contamination is GGCTCAACCCTGGACAG (PNABio, Thousand Oaks, California, USA). We pooled PCR products and cleaned products using an UltraClean®PCR Clean-Up Kit (MO BIO Laboratories, Carlsbad, California, USA). We sequenced DNA fragments in a MiSeq 2x151bp run at the Environmental Sample Preparation and Sequencing facility at Argonne National Laboratory following the procedures of Caparaso et al. (2012).

*Analysis:*

We performed all sequence quality analyses and microbial community difference metrics among samples using the QIIME pipeline (Caporaso et al. 2010). We classified operational

taxonomic units (OTUs) from the Illumina reads at the 97% similarity level using open reference-based clustering with uclust. For chimera detection, we used the mothur script chimera.uchime (Schloss et al. 2009), and found only 75 unique chimera sequences that constituted 0.25% of the total read pool. We assigned a taxonomy using the RDP taxonomic assignment comparing the OTUs sequences against the Greengenes database (version 13_8). We generated all rarefaction, alpha diversity, principal coordinate and Procrustes analyses following the QIIME pipeline (Caporaso et al. 2010). We used Procrustes analysis to statistically compare the shapes of two sets of corresponding points. To minimize the distance between the two sets of points, the second matrix is superimposed on the first matrix after translating, scaling and rotation (Gower 1975). In our study, our matrices are β-diversity outputs comparing samples amplified with EMP primers (i.e., EMP method) versus the same samples amplified with EMP primers plus PNA clamps (i.e., EMP-PNA method). We also identified the taxa significantly enriched and therefore responsible for the differences observed via paired t-tests and Wilcoxon sign-rank tests both before and after correction for multiple comparisons via Benjamini-Hochberg False Discovery Rate (R Development Core Team 2014, Benjamini and Hochberg 1995, Shogan et al. 2014, De Filippis et al. 2016). We then scanned each OTU sequence in our dataset for complete or partial matches (including all 12-mer, 13-mer, 14-mers, 15-mers, 16-mers, and 17-mers) to the mPNA and pPNA sequences (Geneious version 9.0.5). To search for other OTU matches not represented in our dataset, we scanned the entire Greengenes (version 13_8) and Silva (version 123) databases for all possible 12-mer – 17-mer oligonucleotide combinations of the mPNA and pPNA sequences. See Supplement F, Tables 1 and 2 for a list of the exact oligonucleotides that were scanned. We extracted all sequence matches for each oligonucleotide sequence and have appended this database of fasta files. In particular, we note that we found no complete matches, but we did find a subset of OTUs with a partial 14 of 17 base pairs match (***GGCTCAACCCTGGA***CAG) to the pPNA chloroplast-blocking sequence.

*Meta-analysis:*

Our new data described above draws comparisons across samples that were analyzed identically throughout OTU picking and all downstream analyses. In our meta-analyses, we instead drew comparisons using existing BIOM tables for all studies in the Earth Microbiome Project Database (we excluded studies from lab systems or the built environment) (QIITA,

https://qiita.ucsd.edu/) (Supplement E). Samples included in this database may have used varied OTU picking methods, while our new dataset controlled for these potential contributing sources of variation. For the datasets included in the meta-analysis, we removed all chloroplast and mitochondria sequences, and rarefied all samples to 5000 sequences. Some datasets were excluded because they contained only samples with less than 5000 sequences (see Supplement E). We scanned the remaining samples for all OTUs containing the 14 base pair match to the chloroplast pPNA clamp (see this reference list of OTUs in Supplement A). As we did not find bacterial OTU sequences that matched the mitochondrial mPNA clamp, our analysis focuses on the chloroplast-blocking clamp. Those samples containing at least 50 sequences of OTUs in this reference list (i.e., at least 1%) were assembled into Table 2, and we describe the environmental sample type using the metadata made available by the authors in the EMP Database.

## RESULTS

Our plant dataset generated using the EMP method generally contained greater percentages of chloroplast sequences than the dataset generated from the identical samples amplified using the EMP-PNA method. For example, after rarefaction to even sampling depth, the proportion of remaining sequences in our fresh red alder leaf samples that were of chloroplast and mitochondrial origin was reduced from 77.4 ± 17.0% (mean ± 1 S.D.) chloroplast and 1.25 ± 0.47% mitochondria of all sequences using the EMP method to 4.84 ± 3.17% chloroplast and 4.29 ± 6.06% mitochondria using the EMP-PNA method. Similarly, red alder leaves decomposing in river water contained greater chloroplast content with the EMP method versus EMP-PNA method, while seawater, freshwater, and soils contained similar percentages of chloroplast and mitochondria regardless of method (see Table 1).

Beyond this targeted reduction in chloroplast and mitochondrial amplification, sequencing identical samples across a range of aquatic and terrestrial environments demonstrated that the EMP versus EMP-PNA methods yielded substantial discontinuities. The Proteobacteria phylum contained a number of taxa amplified at significantly different relative abundances in the EMP versus EMP-PNA sequence data. We illustrate that samples particularly enriched in Alphaproteobacteria, such as seawater, show sharp discrepancies when amplified with EMP primers versus EMP primers plus PNA clamps (Supplement D, Table 4; Figure 1A). In particular, the Rhodobacterales (including Octadecabacter, Pseudoruegeria, Loktanella, and

Sulfitobacter species), Rhizobiales (include the Phyllobacteriaceae and Hyphomicrobiacea families), and Kiloniellales (family Kiloniellaceae) were all lower in relative abundance in seawater when amplified with the EMP-PNA method (all $p < 0.01$ with false discovery rate correction, Supplement D, Table 4). Pairwise differences for all freshwater, submerged alder leaves, fresh alder leaves, and soil samples are illustrated in Supplement D. In addition to these results in seawater, we again found particular taxa to be of lower abundance in most of these samples when amplified using the EMP-PNA method (Supplement D, Figures 1 – 3). In submerged alder leaf samples, Alphaproteobacteria (including Rhodobacterales and Caulobacterales), Deltaproteobacteria (Bdellovibrionales), Spartobacteria (Chthoniobacterales), and other taxa were amplified at lower abundances using the EMP-PNA method (Supplement D, Table 3, all $p < 0.05$ with false discovery rate correction). Further, while our freshwater and soil results were not significant after false discovery rate correction, the same patterns were observed. In freshwater samples, Alphaproteobacteria (including Rhodobacterales, Rhizobiales, and Rickettsiales), Betaproteobacteria (including Methylophilales and Burkholderiales), Deltaproteobacteria (Myxococcales), Flavobacteria, Actinobacteria, and other taxa (Supplement D, Table 1) were amplified at lower abundances with the EMP-PNA method (all $p < 0.05$ prior to correction for false discovery rate, Supplement D, Table 1). In soil samples, we found the EMP-PNA method amplified a number of rare taxa at lower abundances, including the Alphaproteobacteria (Rhodobacterales, Caulobacterales, and Sphingomonadales), Betaproteobacteria (Burkholderiales), Deltaproteobacteria (Myxococcales), Spartobacteria (Chthoniobacterales) and other taxa (Supplement D, Table 2, all $p < 0.02$ prior to correction for false discovery rate). Lastly, our fresh alder leaf samples were highly variable, and although we did not find significant trends in this group, those samples containing a high abundance of Actinobacteria and Alphaproteobacteria when amplified with the standard EMP method showed sharp declines in these groups when amplified with the EMP-PNA method.

We found that nearly all of these taxa at lower abundances across these samples have a common conserved 14 base pair sequence that matches most of the 17 base pair pPNA chloroplast blocking clamp (***GGCTCAACCCTGGA***CAG). We provide a full list of OTUs that contain this conserved 14 base pair sequence in the database of fasta files in Supplement A (pPNA14merD.fna file). Additionally, we provide a list of OTUs matching this 14-mer sequence, as well as all possible 12-mer through 17-mer oligonucleotides of the mPNA and pPNA

sequences, in both the Greengenes and Silva databases (see summary Tables 1 and 2 in Supplement F, and fasta files in Supplement A). We found that 1,405 OTUs in the Greengenes Database (1.41% of the 99,322 total OTUs) match this 14 base pair sequence, and therefore likely bind to the pPNA clamp (see comparable results for the Silva Database in Supplement F, Table 2). Proteobacteria comprised 76% of these Greengenes OTUs. Our dataset also contains OTUs not yet included in the database, and 6,391 of these OTUs unique to our dataset match this 14 base pair sequence as well. When we filtered out this 7,796 OTU list and repeated our pairwise comparisons across seawater, freshwater, leaf and soil samples, we found greater community similarity between replicate samples amplified with the two methods via weighted UniFrac distances (seawater comparisons: paired t-test, $t_8 = 4.01$, $p < 0.01$, Figure 1B; and Supplement D for other sample comparisons). Many other OTUs in the Greengenes Database contained subsets of the 14-mer described above. A total of 1,887 OTUs contained the 13-mer (***GGCTCAACCCTGG***ACAG) and 2,381 OTUs contained the 12-mer section (***GGCTCAACCCTG***GACAG). The discrepancies between our replicate samples that remain even after filtering out taxa listed in the pPNA14merD.fna file of Supplement A may be due to such taxa with similar sequences that may also bind to the pPNA clamp, however evidence that removing all taxa containing the 12-mer section improves this discrepancy is mixed (see Supplement D, Table 5). In contrast, when we scanned the Greengenes and Silva Databases for all 12-mer subsections of the mPNA clamp, we found no matches and therefore conclude that this clamp likely remains broadly useful for eukaryotes, including animal-associated studies.

We next aimed to compare these amplification methods by specifically contrasting communities where the abundance of photosynthetic organisms differed. Using our Tatoosh seawater samples that were collected at varying depths, we compare these two amplification methods for surface samples (which should contain phototrophic communities) versus samples 100m and deeper (which in contrast should be dominated by chemolithotrophic communities). Weighted UniFrac distances between replicated samples were used to quantify community similarity (see Figure 1 for the distance metric for each pairwise comparison). Amplification method bias was significantly stronger among phototrophic communities than deeper water assemblages that are likely chemolithotrophic (t-test: $t_7 = 5.66$, $p < 0.001$). This increased bias was likely due to the greater natural abundance in these phototrophic communities of the Rhodobacterales, which contain the 14-mer conserved region that likely binds to the pPNA

clamp. After filtering out all OTUs containing this 14-mer (i.e., OTUs listed in Supplement A), phototrophic and chemolithotrophic communities showed a similar degree of bias by amplification method (t-test: $t_7 = 1.07$, $p = 0.32$).

Overall α-diversity measured as phylogenetic diversity, was greater in samples amplified with the EMP than EMP-PNA method (Figure 2A, paired t-test: $t_{45} = 3.24$, $p < 0.01$) (see Supplement C for similar results using OTU #, Chao's α-diversity, and rarefaction curves). Even after filtering out taxa that contain the 14-mer conserved region there remained greater diversity in the EMP amplified samples (Figure 2B, $t_{45} = 3.74$, $p < 0.01$). While we observed significant amplification differences when using these two methods that resulted in different α-diversity levels and relative abundances of particular taxa, we found that each method still generated the same general trends across sample types. Each environmental sample type is depicted in distinct clusters regardless of method (Procrustes Analysis, $p < 0.001$, $M^2 = 0.091$, Figure 3A when filtering out only chloroplast and mitochondria, and Figure 3B when filtering for chloroplast, mitochondria, and OTUs in Supplement A). Generally, analysis on each environmental sample type independently also showed similar trends regardless of amplification method (such as a geographic gradient with soil samples, freshwater samples, and aquatic leaf samples, as well as a depth gradient within seawater samples; see Supplement B, Figure 1 – 5).

Lastly, in our survey of the Earth Microbiome Project database, we found that the OTUs containing the conserved 14 base pair sequence were abundant throughout a diversity of environments. All except two of the 113 datasets that we surveyed contained taxa listed in Supplement A. Ninety-five of these datasets contained at least one sample that was comprised of at least 1% of these taxa (Table 2). Seaweeds, seawater, freshwater, and aquatic sediments contained the highest abundance of these taxa (Table 2). Fish, reptile, amphibian, mammal, and avian-associated samples also contained high abundances of these taxa. These percentages are also likely conservative estimates because in our dataset, over 90% of the OTUs that matched this conserved sequence were from our open reference clustering of environmental samples. The percentages we report in our meta-analysis only scan for those taxa remaining in the closed reference sequences that map to an OTU in the Greengenes database.

## DISCUSSION

Comparative microbial ecology studies across environments are becoming increasingly common. A significant part of the discovery of microbes across ecosystems is the demonstration that microbes live in association with animals (Muegge et al. 2011, Sullam et al. 2012, Bolnick et al. 2014, Kwong and Moran 2016) and phototrophs including seaweeds (Egan et al. 2013, Campbell et al. 2015, Singh and Reddy 2015), terrestrial angiosperms (Berendsen et al. 2012, Badri et al. 2013) and more. These plant and animal-associated microbial communities are proving essential for elucidating the dynamic ecology of both the organisms and the ecosystems in which they reside (Zak et al. 2003, Kardol et al. 2007). As plants dominate many global environments, unbiased comparative analytical tools to characterize the associated microbial ecology require a degree of universality that until now has not been assessed.

We found that the use of PNA chloroplast-blocking clamps can strongly bias the characterization of nearly 1,500 microbial OTUs inhabiting a diversity of environments, particularly in aquatic samples containing high relative abundances of Alphaproteobacteria. Chloroplast-blocking pPNA clamp appear to adhere to similar sequences, including those containing 14 of the 17 base pairs. Many of the discrepancies between our replicate samples that remain even after filtering out taxa listed in Supplement A could be due to other taxa with similar sequences, such as those 2,381 OTUs containing a 12-mer subsection of the 14-mer, binding to the pPNA clamp. However, the evidence for these less conserved sequences playing a major role is weak (see Supplement D, Table 5).

We found that these taxa are abundant in a diversity of ecosystems, and would likely be under sampled with a pPNA clamp. Our meta-analysis showing the ubiquity of these taxa illustrates the potential biases of studies contrasting the microbiome of multiple ecosystems. For example, studies that could use the chloroplast pPNA clamps to assess microbes associated with agricultural crops may mask the presence of certain taxa that are relatively abundant in agricultural soils. In contrast, mitochondrial mPNA clamps did not appear to result in bias, and so these clamps remain useful for animal-only studies. We note that studies comparing animal and plant microbiomes, such as diet studies, should use these clamps with caution. Given that we found a number of herbivorous reptiles, birds, and mammals contained these taxa in their gut and feces, use of pPNA clamps to assess the plant microbiome and compare that with an herbivorous animal microbiome may yield biased results. However, aquatic plants themselves pose one of the largest biases for using the pPNA clamps due to the clear utility of chloroplast-blocking clamps

and the abundance of particular taxa, such as the typically surface-associated Rhodobacterales that are abundant in seawater and on the surface of seaweeds (Gilbert et al. 2012, Fu et al. 2013, Taylor et al. 2014).

We highlighted our results from such marine systems by comparing surface phototrophic against deeper chemolithotrophic communities, which contrast strongly in community membership. We found that phototrophic communities tend to contain a far greater proportion of taxa containing the 14-mer oligonucleotide. Due to these natural differences in community membership, the EMP-PNA amplification method yielded substantially more biased results in the photic zone, where indeed the use of these pPNA clamps would otherwise be particularly useful for studying plant-associated microbiomes. While the EMP-PNA amplification method may remain a technically viable option below the photic zone because of the apparent lack of taxa containing the 14-mer oligonucleotide, we do not expect these methods to be particularly useful in such ecosystems with few photosynthetic organisms and therefore minimal contaminating chloroplast.

Further, we used our marine samples to ask whether these amplification methods are biased in the detection of cyanobacteria. As the free-living predecessors to chloroplast, we tested whether a chloroplast blocking technique would inhibit their amplification. We found that both methods yield quite robust results for cyanobacteria. Of the 774 non-chloroplast cyanobacteria OTUs in our dataset and the 1389 non-chloroplast cyanobacteria OTUs in Greengenes, only 7 OTUs in our dataset and 21 OTUs in Greengenes contain the 14-mer oligonucleotide that matches the pPNA clamp. None of these OTUs, or indeed any cyanobacteria, were amplified at significantly different levels with the two methods. With suitable sequencing depth, either method should yield satisfactory results for studying cyanobacteria. However, using the EMP method and simply screening out chloroplast reads will give equivalent results for cyanobacteria without the issue of reduced Alphaproteobacteria and similar taxa (listed in Supplement A).

Lundberg et al. (2013) found that both amplification methods yielded similar relative abundances of all tested microbial OTUs (including 75 OTUs in plant roots and 1,010 OTUs in soil samples). They found when amplifying replicate soil samples, their PNA method excluded 31 OTUs compared to the EMP method (Lundberg et al. 2013). Although in our scan of the Greengenes and Silva databases, we found a 14-mer match to 1405 OTUs to the pPNA clamp, Lundberg et al. scanned 9-mer through 13-mer oligonucleotides of the their pPNA and mPNA

sequences against the Greengenes database and did not find matches. The reason for this discrepancy is unclear.

Despite the constraints of organelle-blocking clamps, this amplification method did not obscure general trends in our datasets. We were able to clearly observe differences across soil, freshwater, seawater and plant samples. Geographic gradients within each of these sample categories remained consistent regardless of amplification method. These methods may therefore remain suitable for more targeted studies focusing on particular taxa that do not contain the conserved region. We did not find any taxa that matched either the entire pPNA or mPNA clamp sequence. Future studies could aim to optimize these organelle clamps by modifying the PCR technique to select for higher specificity, such as through modifying the temperature protocol or perhaps lengthening the clamp sequence (Mullis et al. 1989). The standard pPNA clamp sequences that we used in our study was designed by considering the chloroplast sequences from a diverse group of 35 plant species (Lundberg et al. 2013). Now having identified certain biases that result from using these standard chloroplast-blocking pPNA sequences, particularly in aquatic environments, future research could design new targets. Custom species-specific pPNA clamps could be tested for improved effectiveness in aquatic systems, however such an approach would not generate a common methodology that could be used for cross-ecosystem studies and larger-scale data syntheses. Additional analytical tools could also be investigated, such as alternative OTU clustering algorithms, to attempt to improve the utility of these clamps. Other methods using different primers entirely (including modified 799F primers) have been used with success. However, this approach typically involves tailoring primers to species specific contaminating sequences, and while proven effective in limiting chloroplast contamination in plants and folivorous arthropods (Chelius and Triplett 2001, Hanshew et al. 2013), such approaches restrict possibilities for comparisons across studies. When particular biases are known, the bases of universal primers can be modified to optimize amplification of taxa of interest, however such methods also limit comparisons across studies (Sim et al. 2012). Given the current limitations of these other methods, studies in ecosystems likely to contain many taxa shown to be biased by pPNA clamps may obtain best results by continuing to use universal primers at sufficiently high sequencing depth to obtain sizable bacterial sequences remaining after filtering chloroplast contaminating sequencing.

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CONFLICT OF INTEREST

All the authors declare no conflict of interest.

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## DATA ACCESSIBILITY

All microbial sequences and associated metadata have been uploaded to the Earth Microbiome Project database, project number 10773. Analysis scripts and fasta files for Supplement A are available at: https://github.com/sjackrel/Identifying-the-plant-associated-microbiome-across-aquatic-and-terrestrial-environments.

## AUTHOR CONTRIBUTIONS

SLJ collected, prepared, and analyzed microbial data, performed metaanalysis, and wrote the paper; SMO prepared, sequenced, and analyzed microbial data; JAG recommended and assisted with data analyses and edited the paper; CAP collected, prepared and analyzed seawater data, recommended and assisted with data analyses and edited the paper.

Figure 1. Seawater samples from Tatoosh Island, Washington, including onshore surface (#20, #22), offshore surface (#21, #23), 100 m deep (#11), 125 m deep (#24) 140 m deep (#10), 300 m deep (#13) and 325 m deep (#12). Relative abundance of microbial taxa at the family level depicted via color. (A) Includes all OTUs after filtering out chloroplast and mitochondria, and (B) excludes all chloroplast, mitochondria and OTUs listed in Supplement A (pPNA14merD.fna

548 file). Weighted UniFrac distances listed adjacent to each sample number quantify the similarity
549 of the microbial community amplified with the EMP vs EMP-PNA method. [See supplemental
550 materials for all habitat results].
551
552 Figure 2. Alpha diversity is consistently greater with the EMP versus EMP-PNA method both
553 when (A) filtering out chloroplast and mitochondrial sequences and when (B) filtering out
554 chloroplast, mitochondrial sequences, and OTUs in Supplement A (pPNA14merD.fna file).
555
556 Figure 3. Larger scale trends remain evident regardless of the EMP versus EMP-PNA method,
557 illustrated as a Procrustes analysis. (A) Samples are shown after filtering out chloroplast and
558 mitochondria, and (B) chloroplast, mitochondria, and OTUs in Supplement A (pPNA14merD.fna
559 file). White lines point to the EMP sample and red lines point to the corresponding PNA sample.

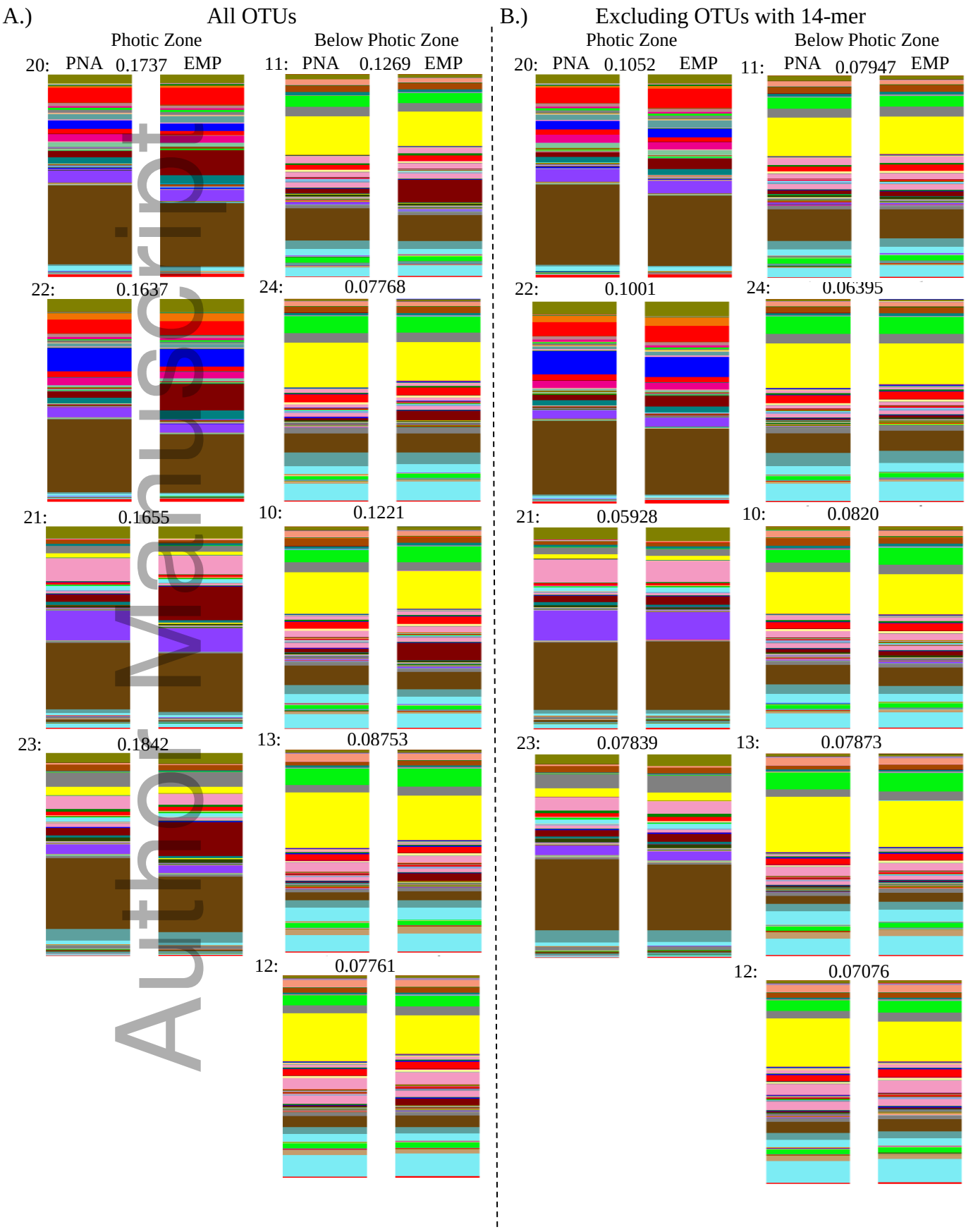
Table 1. Summary of organelle contamination in different sample types when using the EMP versus EMP-PNA method. See data accessibility section to access sequencing data.

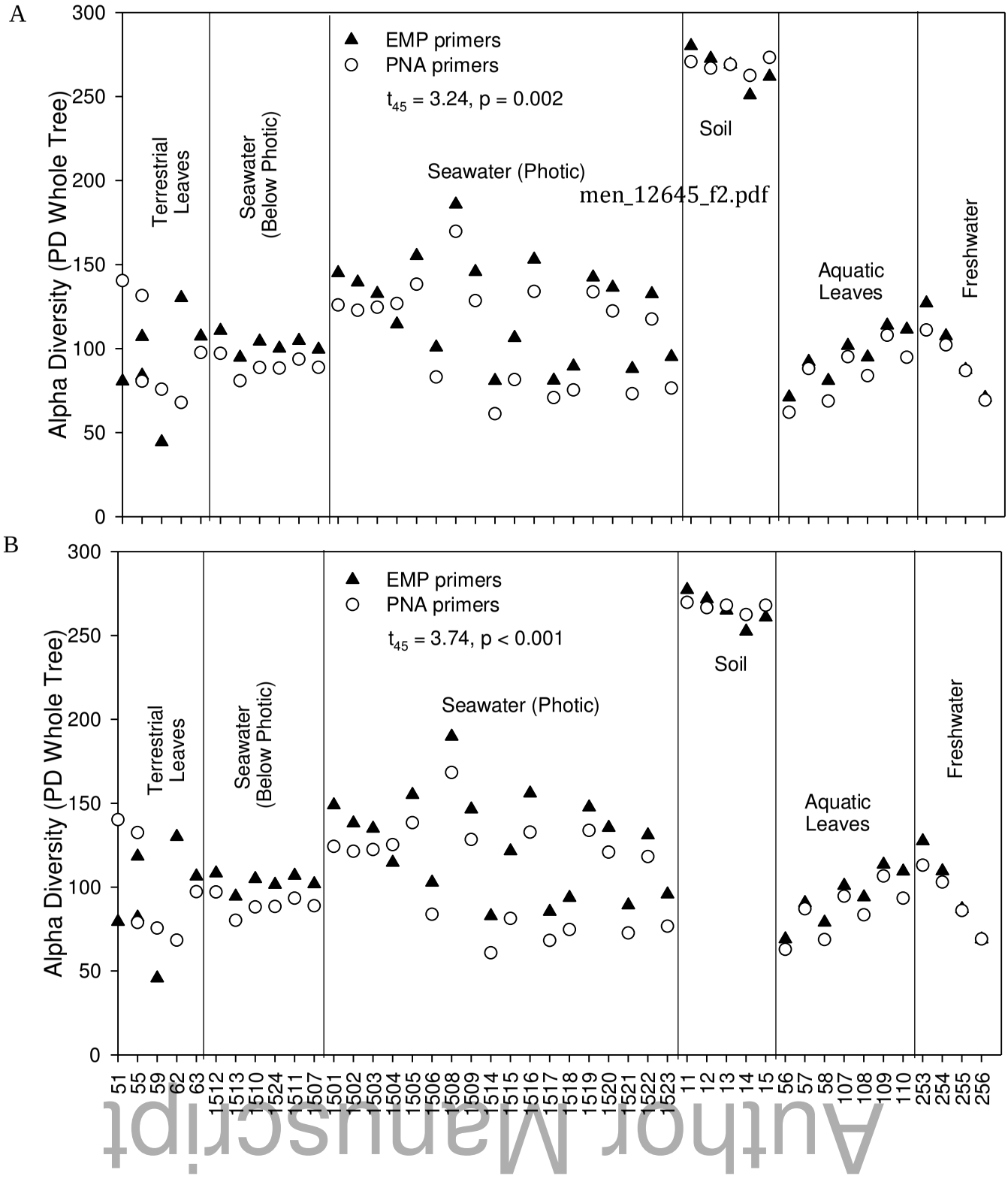
| Sample Type | Sample # (x 2) | Chloroplast content EMP vs. EMP –PNA (mean ± SD %) | | | Mitochondrial content EMP vs. EMP-PNA (mean ± SD) | | | Sequencing Runs |
|---|---|---|---|---|---|---|---|---|
| Seawater | 24 | 5.54 ± 11.7 | vs. | 6.38 ± 12.5 | 0.02 ± 0.058 | vs. | 0.045 ± 0.13 | # 2 (EMP-PNA), # 3 (EMP) |
| Freshwater | 4 | 0.208 ± 0.229 | vs. | 0.189 ± 0.14 | 0.0056 ± 0.01 | vs. | 0.0132 ± 0.012 | # 2 (EMP-PNA), # 4 (EMP) |
| Terrestrial leaves | 4 | 77.4 ± 17.0 | vs. | 4.84 ± 3.17 | 1.25 ± 0.47 | vs. | 4.29 ± 6.06 | # 1 (EMP-PNA and EMP) |
| Aquatic leaves | 8 | 11.6 ± 7.03 | vs. | 0.21 ± 0.33 | 1.05 ± 0.51 | vs. | 1.25 ± 0.67 | # 2 (EMP-PNA), # 4 (EMP) |
| Riparian Soil | 5 | 0.236 ± 0.20 | vs. | 0.498 ± 0.23 | 0.0165 ± 0.016 | vs. | 0.043 ± 0.036 | # 1 (EMP-PNA and EMP) |

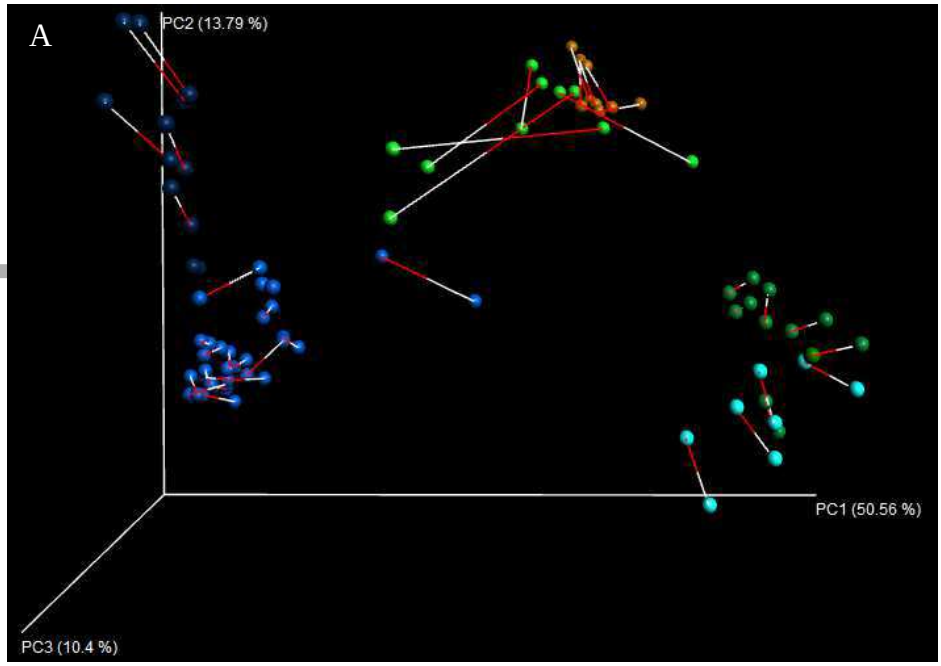
Table 2. Subset of datasets from the EMP Database containing samples with 1% or more of their sequences matching taxa containing the conserved 14 base pair sequence, listed in Supplement A (pPNA14merD.fna).

| Dataset | # of Samples | Range (%) | Description of Samples (at or near max.) |
|---|---|---|---|
| 659 | 7 | 1.02 – 1.64 | Agricultural Soils, New Zealand |
| 1721 | 174 | 1 - 38.52 | Agricultural Soils, Australia |
| 1642 | 25 | 1 - 1.64 | Rice Agricultural Soil sand Rhizosphere, Japan |
| 1717 | 47 | 1.06 - 3.14 | Agricultural Soils, Kenya |
| 1711 | 51 | 1 – 3.54 | Agricultural and Forest Soils, Kenya |
| 846 | 13 | 1.2 – 3.84 | Agricultural Soil, Italy |
| 805 | 8 | 1 – 2.3 | Agricultural Soils, Scotland |
| 1001 | 20 | 1.04 – 3.66 | Agricultural soils, Cannabis, USA |
| 1792 | 63 | 1.02 – 10.8 | Agricultural soil, maize, USA |
| 1674 | 135 | 1.04 - 5.78 | Rooftop Soils, New York City |
| 2104 | 632 | 1 - 7.54 | Soils, Central Park, New York City |
| 10180 | 36 | 1 – 1.84 | Agricultural soil, sugarcane, Brazil |
| 1715 | 18 | 1 – 1.4 | Agricultural Soils, coffee, Nicaragua |
| 829 | 2 | 2.30 – 2.58 | Semi-arid soil, Thar Desert, India |
| 864 | 48 | 1 – 2.38 | Montane Grassland Soils, Mongolia |
| 990 | 29 | 1 – 2.62 | Grassland soils, USA |
| 1043 | 6 | 1 – 1.24 | Grassland soils, USA |
| 1526 | 82 | 1.02 – 7.3 | Soils, Glens Canyon, USA |
| 1579 | 43 | 1 – 4.38 | Volcanic Soil, Hawaii |
| 10278 | 29 | 1 – 2.92 | Peat bog soils, Whales |
| 1713 | 10 | 1.28 – 2.8 | Forest Soils, Malaysia |
| 1714 | 10 | 1 – 2.14 | Forest Soils, Malaysia |
| 1716 | 4 | 1 – 1.54 | Forest Soils, Panama |
| 808 | 11 | 1.00 – 1.70 | Forest soils, Florida |
| 1031 | 3 | 1.06 – 1.60 | Forest soils, USA |
| 1038 | 14 | 1 – 3.72 | Forest soils, USA |
| 10363 | 55 | 1.16 – 4.40 | Coniferous Forest Soils, USA |
| 1030 | 123 | 1 – 4.44 | Soils, Boreal Forest, Alaska |
| 1036 | 14 | 1 – 3.74 | Permafrost soils, USA |
| 1530 | 85 | 1.14 – 13.12 | Soils, Alaska |
| 1578 | 7 | 1.04 – 3.08 | Soils, Alaska |
| 10246 | 58 | 1.02 - 9.02 | Tundra Soils, Alaska |
| 1692 | 26 | 1.04 – 6.67 | Soils and Biofilms, Alaska |
| 1037 | 2 | 1.02 – 3.90 | Soils, Canada |
| 632 | 3 | 1.10 – 1.34 | Soils, Canada |
| 1034 | 9 | 1 – 4.32 | Soils, Arctic |
| 1702 | 17 | 1.02 – 2.74 | Montane Shrub land Soils, China |
| 1035 | 9 | 1 - 13.82 | Sand, Antarctic |
| 1033 | 3 | 1.06 – 10.32 | Soils, Antarctic |
| 776 | 2 | 1.46 – 1.58 | Soil, Antarctica |
| 10245 | 7 | 1 – 2.22 | Leaf litter, Peru |
| 807 | 43 | 1.02 – 2.96 | Riverbed Sediments, USA |
| 809 | 13 | 1.14 - 3.92 | Lakebed Sediments, Canada |
| 925 | 9 | 1 – 5.18 | Hot springs Microbial Mats, Yellowstone |
| 1622 | 35 | 1 - 15.88 | Freshwater Pond Sediment, USA |
| 1627 | 6 | 1.28 – 5.74 | Freshwater Sediment, Tibetan Plateau |
| 10156 | 47 | 1 – 4.8 | Wetland Soils, USA |
| 638 | 58 | 1.10 - 64.56 | Freshwater Lakes, Antarctic |
| 945 | 320 | 1 – 68.4 | Freshwater Lakes, Germany |
| 1041 | 43 | 1.04 - 5.14 | Freshwater, Great Lakes, USA |
| 1242 | 11 | 1 – 5.68 | Freshwater, Lake Mendota, USA |
| 1288 | 397 | 1 – 15.82 | Freshwater, Temperate Bog, USA |
| 1818 | 52 | 1 – 16.96 | Wastewater, Florida |
| 1883 | 794 | 1 – 16.52 | Lake water, Seawater, Lake Epithilion, Alaska |
| 861 | 8 | 1.86 - 24.78 | Karst Sinkholes, Mexico |
| 940 | 32 | 1 – 5.6 | Freshwater Fish (Fecal, and Surface Mucus), USA |
| 2259 | 5 | 1.12 - 3.94 | Stickleback gut, USA |

| | | | |
|---|---|---|---|
| 10308 | 172 | 1 – 36.34 | Freshwater Fish (Mucosal Surface), USA |
| 10272 | 31 | 1.24 – 10.92 | Amphibian Skin Swabs, USA |
| 10196 | 2 | 1.82 – 2.04 | Panamanian Golden Frog, captive, skin swab |
| 1064 | 4 | 1.06 – 2.02 | Bee, Puerto Rico |
| 10324 | 1 | 1.68 | Lone Star Tick, USA |
| 1845 | 8 | 1.1 – 5.24 | Deer Tick, USA |
| 1632 | 37 | 1 – 6.98 | Bird Eggshells, Spain |
| 1694 | 114 | 1 - 97.62 | Starling Eggshells |
| 1773 | 76 | 1.04 - 19.16 | Passerine Bird (Intestine), Venezuela |
| 963 | 6 | 1 – 2.28 | Iguana feces |
| 1747 | 22 | 1.1 – 6.48 | Komodo Dragon saliva, captive, USA |
| 2338 | 6 | 1.08 – 4.56 | Frugivorous bat feces, Costa Rica |
| 1734 | 8 | 1.12 – 58.76 | Phyllostomid bat feces, Belize |
| 1056 | 14 | 1.06 – 7.72 | Fecal, Ant-eating Mammals |
| 1736 | 1 | 1.12 | Cape Buffalo feces, South Africa |
| 894 | 85 | 1 – 24.92 | Marsupial Feces, Australia |
| 1665 | 30 | 1.16 – 17.14 | Skin Surface, Marine Mammals |
| 910 | 1 | 1.54 | Coral/algae tissue, Curacao Island |
| 804 | 56 | 1.06 – 32.2 | Hydrothermal Vent Chimney Biofilms |
| 10273 | 23 | 1.2 – 10.26 | Coral Mucus Swabs, USA |
| 10346 | 285 | 1 – 41.96 | Seawater and Sponges, Spain, Madagascar |
| 1740 | 282 | 1 – 42.22 | Seawater and Sponges, Australia, Spain, Madagascar |
| 2229 | 1271 | 1 – 74.18 | Seaweeds (Surface Swab), Australia |
| 933 | 321 | 1.36 - 51.38 | Kelp Forest, Australia |
| 1197 | 101 | 1.12 – 36.14 | Contaminated Ocean Sediment, Deepwater Horizon, USA |
| 1198 | 57 | 1.94 - 15.92 | Marine Sediment, Argentina and Antarctica |
| 678 | 204 | 1 – 5.34 | Marine Sediments, England |
| 905 | 38 | 1.04 – 11.86 | Marine Sediments, Scandinavia |
| 1039 | 8 | 1.76 – 9.2 | Marine Sediment and Seawater, Brazil |
| 1580 | 8 | 1.18 – 5.94 | Saline Freshwater and Seawater, USA |
| 2080 | 26 | 1.08 – 9.66 | Seawater, North Atlantic Ocean |
| 10145 | 86 | 2.4 – 28.76 | Seawater, British Columbia |
| 1222 | 71 | 18.02 - 58.26 | Seawater, Scandinavia |
| 1235 | 256 | 1.02 – 18.88 | Seawater, Scandinavia |
| 1240 | 140 | 1.02 – 53.76 | Seawater, English Channel |
| 662 | 42 | 1.04 - 54.1 | Seawater, Pacific Northwest |
| 723 | 64 | 1.02 – 9.12 | Seawater, Arctic |
| 889 | 7 | 1.04 – 1.74 | Seawater, Italy |








Seawater (Below Photic) Terrestrial Leaves Aquatic Leaves
Seawater (Photic) Soil Freshwater