

## **Analysis of Membrane Proteins in Metagenomics: Networks of correlated environmental features and protein families**

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## Abstract

Recent metagenomics studies have begun to sample the genomic diversity among disparate habitats and relate this variation to features of the environment. Membrane proteins are an intuitive, but thus far overlooked, choice in this type of analysis as they directly interact with the environment, receiving signals from the outside and transporting nutrients. Using Global Ocean Sampling data, we found nearly ~900K membrane proteins in large scale metagenomic sequencing, approximately a fifth of which are completely novel, suggesting a large space of hitherto unexplored protein diversity. Using GPS coordinates for the GOS sites, we extracted additional environmental features via interpolation from the World Ocean Database, the National Center for Ecological Analysis and Synthesis, and empirical models of dust occurrence. This allowed us to study membrane protein variation in terms of natural features, such as phosphate and nitrate concentrations, and also in terms of human impacts, such as pollution and climate change. We show that there is widespread variation in membrane protein content across marine sites, which is correlated with changes in both oceanographic variables and human factors. Further, using these data, we developed an approach, *Protein Families and Environment Features Network (PEN)*, to quantify and visualize the correlations. *PEN* identifies small groups of co-varying environmental features and membrane protein families, which we call “bimodules”. Using this approach, we find that the affinity of phosphate transporters is related to the concentration of phosphate and that the occurrence of iron transporters is connected to the amount of shipping, pollution, and iron-containing dust.

## Introduction

Integral membrane proteins play a fundamental role in sensing and interacting with the environment, allowing the influx and efflux of ions and molecules and relaying information about environmental conditions to the cell. Thus, the abundance and types of membrane protein families in a microbial community may give information about functional capabilities and nutritional requirements. In marine microorganisms, especially those inhabiting the oligotrophic (nutrient-poor) surface waters of the oceans, membrane protein content might provide insight into types of nutrients and conditions in the waters in which the organisms were isolated. For example, the recent discovery of spectral tuning of the light-driven proton pump proteorhodopsin reveals a relationship between a single amino acid mutation and dominant light wavelengths in the microbes surroundings (Rusch et al. 2007).

A number of recent studies have begun to relate functional attributes of microbial communities, such as central metabolism or broad functional classes (e.g. protein synthesis), to specific habitats (Dinsdale et al. 2008; Tringe et al. 2005) or environmental features (DeLong et al. 2006; Gianoulis et al. 2009; Kunin et al. 2008). In addition, new methods are allowing the integration of quantitative features of the environment alongside microbial function (DeLong et al. 2006; Gianoulis et al. 2009).

Given their important role in environmental sensing and transport, membrane proteins may serve as an even more sensitive barometer of environmental conditions than broad functional classes or central metabolism. In addition, integration of many different environmental conditions is needed to develop a comprehensive understanding of the complex interplay between environmental conditions and microbial communities. In particular, new techniques are needed to investigate the relationship between natural

processes such as nutrient fluxes and the impact of humans on the environment (anthropogenic effects), such as pollution. Given the nutrient fluctuations and anthropogenic effects observed in the world's oceans, understanding the relationship between such factors and microbial adaptations is particularly timely. Indeed, Halpern et al. (Halpern et al. 2008) estimated that 40% of the world's oceans are substantially affected by human activity by computing indices for pollution, shipping, ultraviolet radiation, and climate change, among others.

To gain a better understanding of the relationship between environmental conditions and membrane protein content and abundance, we used 29 samples of the Global Ocean Sampling (GOS) Expedition (Rusch et al. 2007). This survey provided metagenomic sequence and environmental data (chlorophyll, water depth, sample depth, salinity, temperature), as well as GPS coordinates of the sampled sites. We used the GPS coordinates to extract additional environmental features from several disparate sources, providing both natural features, such as nutrient concentrations, and anthropogenic features, such as pollution. Integration of these quantitative measurements allowed us to investigate the relationship between microbial communities, nutrient dynamics, and anthropogenic effects, and in particular the relative importance of the various classes of membrane proteins in microbial adaptations.

## **Results**

### **Integration of Environmental Features**

The GPS coordinates provided for the sampled sites were essential to cross reference different sources of information, mainly provided as annotated maps of the ocean. We integrated this data by interpolation of the map projections onto the GOS geographic coordinates (latitude and longitude information). To select the sites for further analysis, we used Google Earth to compare locations of GOS sites to locations of available data (some maps were sparse). We were able to extract an additional 11 environmental features for 29 sites: phosphate, nitrate, silicate, dissolved oxygen, and apparent oxygen utilization information from the World Ocean Database (Antonov et al. 2006; Garcia et al. 2006; Locarnini et al. 2006), pollution, shipping routes, ultraviolet radiation, ocean acidification, and climate change information from National Center for Ecological Analysis and Synthesis (NCEAS) (Halpern et al. 2008), and dust levels, which serve as a proxy for oceanic iron concentrations from Jickells et al. (Jickells et al. 2005) (see supplement for additional information on environmental features). We have placed the features data for each of the sites on an interactive GoogleEarth map: <http://metagenomics.gersteinlab.org/membrane/>.

### **Membrane Protein Prediction/Variation**

Using PRODIV-TMHMM (Viklund and Elofsson 2004), we identified ~1.3 million proteins of the 6 million proteins in the GOS protein dataset (Yooseph et al. 2007) as having at least one membrane spanning region. We filtered this set to include only high confidence peptides (see supplement and methods for more details on protein filtering) which resulted in 873,718 predicted membrane proteins. Due to the nature of the prediction, there is likely a bias against membrane proteins with a small number of transmembrane helices. Further, as our selection method is quite stringent, we are likely underestimating total membrane protein content, however, the relative proportions between the sites should remain consistent. Membrane protein content ranged from

12.2% (Gulf of Maine) to 15.0% (Off Key West, Fl and Roca Redonda) with an average of 14.2% (Supplementary Table 1). For comparison, in the known heterotrophic/photosynthetic microbial genomes, the predicted transmembrane helical protein content ranges from 21% (*Acinetobacter baumannii*) to 33% (*Chloroflexus aurantiacus*) with a median of 28%.

To examine functional differences across the sites, we homology mapped 237, 870 of the predicted membrane proteins to known annotation using Clusters of Orthologous Groups (COG) (Tatusov et al. 2000). We filtered this set to the 151 membrane families involved in transport processes (transporters, channels, permeases) as these families should be particularly sensitive to environmental perturbations and additionally, to strengthen the signal in our further analysis and prevent overfitting the data (Supplementary Table 4).

### **Standard Methods**

For the 29 sites, we computed the fraction of peptides belonging to each of the 151 families and created a Membrane Protein Families Matrix (the rows are the 29 sites, and the columns are the families) and similarly, an Environmental Features Matrix (rows are the 29 sites and the columns are the 15 environmental features) (Figure 1a). Using these matrices, there are numerous straightforward correlations we can perform to investigate the relationship between and within the features and families across the sites (Figure 1b). For example, one can compute the pair-wise correlation across sites between different families, environmental features, or even between families and environmental features. In addition, one can transpose the above, and correlate the sites on the basis of either environmental features or membrane protein families (resulting in a site-site correlation or similarity matrix, see Supplementary Figure 5). For simplicity, we refer to these site-site correlations (SS) as SS-Env or SS-Fam, for the environmental and membrane protein-based site-site correlations, respectively.

In particular, when calculating SS-Env we observed significant variation between the sites as shown in Figure 2a, where site pairs are color coded according to their similarity. Additionally, clustering the sites based on the similarity of the environmental features (see methods) revealed a distinct latitudinal influence in the data, separating the sites into three groups (Figure 2a-b): the North Atlantic, the Mid-Atlantic, and the Pacific. Such a finding is perhaps expected as the sites are not physically isolated from each other, and they were sampled from the North Atlantic through the Pacific over the course of 12 months. Thus, adjacent samples were likely subjected to similar seasonal (temporal) effects, such as phytoplankton blooms, nutrient-carrying currents, and temperature, and similar spatial effects, such as nutrient gradients. In addition, specific environmental features appeared to have distinct patterns among the clusters. For example, phosphate concentrations were generally lower in the mid-Atlantic than the other two regions while acidity was high, and pollution/shipping/climate change were all relatively low in the Pacific.

The SS-Fam matrix also showed variation across the sites (Figure 3a, color bar reference Figure 2a). Thus, even across these qualitatively similar ocean habitats, we are able to see differences in the abundance and types of membrane protein families in the genomes present. Interestingly, upon visual comparison with the site-site correlations of the environmental features matrix we observe some concordance between regions of high and low correlations (comparison of Figure 2a and 3a, sites are ordered similarly). This suggests there is a relationship between sites such that sites with similar environmental

features have similar membrane protein content and vice versa.

### ***Environmental versus Phylogenetic Variation***

A factor that could explain the observed variation across the sites is differences in species composition. The environmental differences would affect the types of species preferentially inhabiting these sites, and in turn this could explain the observed genomic variation. Thus, for comparison, we calculated the GOS SS-16S (20% 16S divergence groups) (Biers et al. 2009) to determine phylogenetic similarity of the sites (Figure 3b). However, we were unable to find a significant relationship between the phylogenetic-based and environmental-based site-site similarity (for methods see Supplementary Figure 6). The average correlation between SS-16S and SS-Env was 0.2 (Figure 3d); whereas, the average correlation between SS-Env and SS-Fam was 0.5 (Figure 3d). This suggests that the observed membrane protein variation is more a function of the measured environmental features, than phylogenetic diversity. It is important to note, however, we only had enough statistical power to look at the 20% divergence level of the 16S profiles and we cannot rule out the possibility that a lower divergence level could result in a greater concordance between environmental site similarity and 16S profile similarity.

### ***Variation in Membrane Protein Families corresponds to environmentally distinct regions***

Above, we show that the variation in membrane proteins is reflected in the variation in the environmental features; however which families and features are contributing to the association remains unanswered. There are a host of multivariate statistical techniques for understanding these types of complex (many-to-many) relationships between datasets. Thus, we began our analysis by employing a variety of standard and published techniques: 1) Principal component analysis (PCA) 2) Discriminative Partition Matching (DPM) (Gianoulis et al. 2009) and 3) regularized Canonical Correlation Analysis (CCA)(Gonzalez et al. 2008) . Further, we developed a technique which we call *Protein Families and Environmental Features Network (PEN)* to address limitations in the quantification of associations and visualization of the results of CCA.

### ***Principal Components Analysis and Discriminative Partition Matching***

As demonstrated above, hierarchical clustering of the sites based on their environmental features revealed three distinct geographical regions (Figure 2a-b). A similar pattern emerged after using a data reduction technique, principal component analysis, of the sites and the proportion of membrane proteins at each site. In brief, each principal component is a weighted linear combination of features. These weights or scores can be used as new axis allowing the projection of the sites into a new lower dimensional space. We observed that sites deemed more similar in the environmental clustering; also had a greater tendency to be closer together based on their membrane proteins. For example, in Figure 4a, the first component scores show that the occurrence of membrane proteins in the North Atlantic environmental cluster can be distinguished from the Mid-Atlantic and Pacific environmental cluster. As the clustering of the environmental features is done separately from finding variation in the membrane protein families, we independently show that the grouping of the sites based on environmental features is partially reflected in the membrane protein content.

As the PCA showed the Mid-Atlantic and Pacific to be similar, we grouped these

sites into 'Mid-Atlantic/Pacific' cluster and used DPM to determine which specific families were discriminating between them and the North-Atlantic cluster. Briefly, DPM assesses whether the distribution of a specific protein family is significantly different "discriminates" between the two partitions. Thirty families showed significant discrimination ( $q$ -value $<0.05$ ) between the two site sets, and interestingly most were enriched in the North Atlantic (28/30) (Figure 4b and 4c) (Supplementary Table 5).

In the North Atlantic environmental cluster, there is enrichment in several proteins involved in inorganic ion transport. One such protein is a magnesium transporter, which is likely related to the higher chlorophyll content ( $p$ -value  $<0.01$ ) and thus bacterial abundance (Bird and Kalff 1984) in these regions. The North Atlantic sites also have higher pollution rates ( $p$ -value  $<0.01$ ) and possible nutrient availability due to coastal proximity; such features are also indicative of regions with increased cell growth and proliferation (Kirchman 2008). Magnesium is not only contained in the center of the chlorin ring, it is a central player in the stabilization of DNA and RNA, thus one can presume in dividing cells larger quantities of the ion would be required. Most interestingly, however, there is an increase in many families involved in efflux/secretion/antimicrobial processes. The enrichment in these proteins may reflect the microbes' need to expel antimicrobials, by-products of metabolism, or environmental toxins (Neyfakh 1997). In addition, there are a set of proteins related to protection from osmotic shock (glycine/betaine,  $K^+$ , mechanosensitive channel), which may be acting to buffer against shifts in ocean solute concentrations (Poolman et al. 2002), again alluding to the increased pollutants, and possibly nutrient fluxes from land and rivers.

One interesting protein observed to increase in the North Atlantic is the ATP/ADP translocase. This protein is found in mitochondria, as well as obligate intracellular parasites of Chlamydiae and Rickettsiae where they function to exchange host ATP for ADP, thereby sequestering host nutrients (Winkler and Neuhaus 1999). It is uncertain in what capacity they function. One intriguing possibility is that they originate from marine parasites. Although a recent survey showed that a number of putative ATP/ADP translocases should have been annotated as a more general nucleotide/ $H^+$  transporters (Tjaden et al. 1999), thus simple misannotation of the class cannot be ruled out.

### ***Canonical Correlation Analysis***

Although the previous analysis is useful in finding discriminating families between environmentally distinct groups, it does not capitalize on the natural gradients in the environmental features we were able to extract. Consequently, we performed regularized CCA, which maximizes the correlation between linear combinations of the two sets of variables, to reveal a finer grained picture of the relationship between environmental features and membrane protein families (Wichern and Johnson 2003). As our analysis of site-site correlations revealed concordance of the environmental features and membrane protein families in assessing the similarity of the sites, this analysis is justified and may be able to reveal more specific correlated features.

Similar to PCA, CCA gives us a number of principal directions that describe the greatest degree of co-variation between features (Borga et al. 1992). Interpretation of CCA is commonly performed by plotting the first two of these structural correlates, schematized in Figure 1c (left) (Gonzalez et al. 2008). Those families and environmental features that are close in this structural correlation space are referred to as co-varying.

From the structural correlation plot (Figure 5a), we observed all 15 environmental features and 107 out of 151 membrane protein families varying across the 29 sites. These

points are outside the 0.3 circle and can thus be considered varying with respect to the other set of features (Borga et al. 1992; Guo et al. 2006; Wichern and Johnson 2003) (44/151 families were invariant, points inside 0.3 circle) (Supplementary Tables 6-7). No single COG functional category was over-represented in either the variant or invariant set (P-value >0.05). However, notably, 34 out of the 41 ABC transporters in the dataset were shown to co-vary with the environmental features.

Between the environmental features, we observe many intuitive relationships. As an example, ocean-based pollution and shipping lanes are highly correlated as expected due to the overlap in measurement (same direction on plot)(Halpern et al. 2008). In addition, shipping itself is a contributor to ocean pollution given emissions from fuel burning and ballast water (which can bring invasive species)(Satir 2008). Predictably, dissolved oxygen shows a negative relationship with water temperature (as oxygen more readily dissolves in colder waters, opposite direction on plot) and also, as it is a by-product of primary production, a positive relationship with chlorophyll. In addition, the positive relationship between nitrate, phosphate, and silicate reflect similarities in the gradients of nutrients across the sites.

## **PEN**

Solely using the structural correlations plot to analyze the results is problematic for several reasons. First, it is difficult to draw conclusions on the strength and directionality of a relationship between variables, especially negative relationships as they are not close in space, although such relationships can be identified by looking at the tabular form of the data. Second, the relative weight of the features' relationships can be difficult to visualize and compare. Third, there is no real means of quantifying co-variation between specific sets of features, nor do standard visualization methods allow for comparisons in more than three dimensions. To better quantify and visualize the results of CCA, we developed a new approach we call Protein Families and Environmental Features Network (PEN).

In brief, PEN creates a network from the CCA results, where each environmental feature and membrane protein is a node and the edges are weighted by taking the dot product between the structural correlations in the first and second dimensions (the procedure easily generalizes for the case of more than 2 dimensions).

We then use a simplified version of connected components analysis and prune all the edges with absolute value weights below 0.5 (see methods). This simple metric provides an intuitive means of visualizing environmental/membrane protein clusters as it gives greater weight to features closer to the correlation circle (outer circle in Figure 1c), as well as to features that have a small angle between them relative to the x-axis. Such features represent strongly co-varying pairs or sets of features. We can use the topology of the network to identify these sets of tightly (negatively, red edges and positively, green edges) correlated environmental features and membrane proteins families which we term bimodules. (Figure 1c).

In the pruned network derived from the structural correlates, we observe two distinct bimodules (Figure 5b), comprising families and environmental features that have both negative and positive relationships (see Supplementary Table 9 for edge weights) . The first bimodule contains temperature, salinity and chlorophyll with many shared connections between membrane families, and the second contains phosphate, nitrate, and silicate (which are themselves inversely related to acidity, shipping and pollution). UV, dissolved oxygen, apparent oxygen utilization, sample depth, and water depth, although

showing variation across the sites (outside 0.3 circle in Figure 5a), are not related to any specific membrane protein family and are thus not included in the graph. It is unlikely that these features are not affecting microbial diversity; it maybe the case that limiting our genomic data to membrane proteins is not allowing us to highlight these influences.

From the network, we see both intuitive and non-intuitive relationships between the features and membrane protein families. For example, chlorophyll concentration and a magnesium ABC transporter (COG0598) are positively related likely due to the relationship between chlorophyll and bacterial abundance (and thus proliferation)(Bird and Kalff 1984) and to the fact that chlorophyll molecules contain a magnesium ion at the center of the ring structure. This was inferred from the DPM analysis as these transporters were enriched in the North Atlantic (area of high chlorophyll), but here we are able to explicitly see the relationship between the two variables.

A less intuitive relationship, but nonetheless interesting, is a negative relationship between an ABC transporter involved in polyamine (putrescine/spermidine) transport (COG1176) and ocean-based pollution/shipping (Figure 5d). Polyamines are nitrogen rich compounds found in all living matter and they play an important role in the stabilization of DNA structure (Flink and Pettijohn 1975). Although their exact role is unknown, during cell growth in response to proliferative stimuli, both their uptake and biosynthesis is increased (Igarashi and Kashiwagi 2000). Possible sources of polyamines in ocean water are from the degradation of organic matter, amino acids and proteins, where they are quickly taken up by bacteria (Lee and Jorgensen 1995). The negative relationship we observe might reflect the increased amount of polyamines in the environment in polluted, nutrient rich waters, where fewer transporters would be needed for uptake. In these nutrient rich areas, cell growth and death rates may be higher, leading to increased concentrations of polyamines.

### ***Phosphate***

The most pronounced negative relationship observed is that of phosphate concentrations and ABC-type phosphate transporters (COG0573 and COG0581) and phosphonate transporters (COG3639). These ABC transporters comprise *pstA*, *pstC*, and *phnE* of the phosphate (pho) regulon in *E. coli* that have previously been shown to be involved in the active uptake of phosphate from the environment during phosphate limitation (Karp et al. 2002). Interestingly, we also observed the converse with the phosphate/sulfate permease *PitB* in *E. coli* (COG0306; Figure 5c). The relationship between *PstA/C* and phosphate starvation conditions has been well-characterized(Martiny et al. 2006; Martiny et al. 2009); however, the positive relationship between the lower affinity *PitB* and phosphate concentration suggests a more subtle influence of environmental parameters on modulating membrane content. That is, when phosphate concentration in the environment is low, more genes are present encoding high affinity phosphate transporters (*pstA/pstC/phnE*) are present, and when phosphate concentration is high, more genes encoding a low-affinity transporter (*PitB*) are present.

Further, we observe a positive relationship between an ABC transporter predicted to be involved in Lipophospholipase L1 biosynthesis (COG3127) and phosphate levels, suggesting increased cellular activities related to phospholipids with increased phosphate concentrations. Phosphate concentrations have been shown to modulate lipid content in marine bacteria, where in organisms in low phosphate regions replace phospholipids with non-phosphorous containing lipids(Van Mooy et al. 2009).



## **Iron**

We observe a striking network of relationships between protein families involved in the active uptake of iron (COG0609: ABC Fe<sup>3+</sup> siderophore transporter, COG1178: ABC Fe<sup>3+</sup> transporter and COG4558: ABC Hemin transporter) and areas of high ocean-based pollution and shipping (Figure 5d). Iron is a critical resource essential to microorganisms for a diverse array of enzymatic reactions and cellular processes such as respiration, photosynthesis, and nitrogen fixation. As such, its depletion has been shown to limit microbial growth even in the presence of other essential nutrients, such as phosphates and nitrates. Regions with such a limitation have been termed High Nitrate/Low Chlorophyll (N/C) regions, an example of which is the Equatorial Pacific (Pacific)(Kirchman 2008). We hypothesize that the increase in gene content related to iron acquisition observed in low pollution/shipping areas may reflect a greater difficulty in attaining this nutrient. Indeed, siderophores in particular are known to be produced by bacteria under iron limited conditions to actively sequester iron from the environment (Guan et al. 2001).

The main sources of iron in the ocean are aeolian dust from land (Figure 6a), as well as terrestrial input near coastal regions, fluvial input, and upwelling from the ocean floor, all of which are lacking in these low shipping/pollution sites. Interestingly, we observed that the areas of high ocean-based pollution/shipping (North Atlantic and Mid-Atlantic) parallel areas that may have higher iron concentration. Presently, there are no means to directly measure iron concentrations; however, oceanographers have shown that models of iron-containing dust (Jickells et al. 2005) (Figure 6b) can approximate iron concentrations. We found that iron values approximated from these dust models show significant negative correlation between COG4558 (p-value < 0.01), COG0609 (p-value < 0.01), as well as the N/C ratio across the sites (Figure 6d). Such a trend is similar to our observation using shipping and pollution. In addition, searching the BRENDA database (Schomburg et al. 2002) for enzymes using iron as a cofactor revealed that an increase in these two families is negatively correlated to the amount of enzymes present that required iron. Thus, similar to phosphate, it may be that in these low pollution/shipping areas (open ocean, low aeolian dust input) microorganisms increase the production of siderophore and iron transporters to enable survival in a low iron environment.

## **Unknown Fraction**

Intriguingly, of the 1.2 million unique proteins with at least one predicted membrane spanning region, 15% had no homology to any protein currently in Genbank (e-value > 1e-10) suggesting a large and hitherto unexplored space of membrane protein diversity. To further characterize this unknown fraction, we searched for known binding motifs by running each predicted membrane protein against PROSITE (Hulo et al. 2008). Resulting in the functional characterization of 29,384 (15%) of this unknown fraction including previously unannotated ABC transporters, beta lactamases, G protein receptors, and lipocalins among others (data not shown).

## **Discussion**

We presented the Protein Families and Environmental Features Network (PEN) as a means of describing, quantifying, and exploring the relationships between and among sets of environmental features and occurrence of membrane protein families. Such graph theoretical approaches have been shown to be useful in the study of biological systems

for understanding the complexity and global topology of relationships mediating protein and many other types of interactions (Barabasi and Oltvai 2004). PEN provides a simple flexible framework for exploring these complex relationships in the context of metagenomics datasets. Although complete characterization of an environment as complex and dynamic as the ocean is highly unlikely, through careful examination of the resulting bimodules we demonstrate the usefulness of such studies even within these limitations. We are able to identify pertinent conditions affecting protein diversity and recapitulate potential explanations for the observed variation, illustrating the robustness of this type of analysis.

To date, most metagenomics studies integrating environment features have focused on the comparison of metabolic pathways or phylogenetic content among disparate habitats (DeLong et al. 2006; Dinsdale et al. 2008; Gianoulis et al. 2009; Tringe et al. 2005). Here, we focus on a specific set of membrane proteins sampled from sites with a high degree of environmental similarity (by removing outlying samples from the GOS dataset, such as estuaries and lakes), and use quantitative environmental features to differentiate factors that are affecting the genomic content. By selecting only membrane proteins, we are able to see relationships between a microorganism's (or in this case, superorganism's) external barrier, mediating the transport of molecules in and out of the cell, and features of its environment. We show that indeed there is widespread variation in most membrane protein families and these can be explicitly correlated to both nutrient availability and anthropogenic influences. In fact, the median structural correlation coefficient for the membrane proteins is 0.3 whereas for metabolic pathways it is 0.17 (Gianoulis et al. 2009) suggesting that membrane protein covariation is stronger with this set of environmental features (see Supplement).

Our results comparing membrane protein content to environmental features and species diversity add to the growing body of evidence suggesting that genome plasticity may be largely driven by environmental factors and less a result of species specificity. Given the large amount of horizontal gene transfer, observed intraribotype diversity, and the growing appreciation of the impact and prevalence of ocean viruses in surface waters (Williamson et al. 2008) (Sharon et al. 2009), it might be expected that phylogenetic composition could play less of a role in determining membrane protein functions present in an organism. There are a number of instances in the literature suggesting genome content differences even within species ('ecotypes'), reflect the environment conditions in which they were extracted (Thompson et al. 2005; Martiny et al. 2006; Van Mooy et al. 2009). As an example, two ecotypes of the ocean dominating *Prochlorococcus*, high-light (HL) and low-light (LL), are adapted to inhabit different levels of the water column, reflecting genomic adaptation to environmental characteristics (West and Scanlan 1999). In addition, in the GOS analysis of whole genomic content (Rusch et al. 2007), it was observed that there was a clear distinction between sites, and this was still evident upon limiting to or removing reads from dominant species, suggesting more global niche differences.

An advantage to our analysis is that it reveals not only the environmentally-influenced fraction of the membrane proteins but also provides a window into those membrane proteins that appear insensitive to this set of environmental features, suggesting an importance to their function. For example, in our CCA analysis, we find 44 out of the 151 families to be invariant across the sites, including the ubiquitous chloride channel and type III secretion proteins involved in virulence, as noted previously to be abundant in marine bacteria (Persson et al. 2009). Within these invariant proteins there is

a suggestion of functional importance, whether for essential cellular processes or processes intrinsic to their ocean habitats.

Across the variant set, we observed a significant proportion of ABC-type transporters (34/41) co-varying with the environment, illustrating a possible case of streamlining for optimization and energy conservation. Responsible for the high affinity transport of a wide array of substrates, and in some cases having broad specificity, these proteins provide an efficient means of transport in oligotrophic surface waters. As noted, these proteins had a strong tendency to be inversely correlated with the prevalence of their substrate, as in the case of phosphate, showing possible adaptation to phosphate rich/poor conditions. Recently, Martiny et al. (Martiny et al. 2009), showed that the proteins surrounding the PhoB gene (the phosphate response regulator) in *Prochlorococcus* are enriched in GOS samples found in waters with low phosphate content. They limited sites selection to those sites with a high *Prochlorococcus* hit counts (2.5 hits per 1000 bp), thus focusing on nutrient adaptation in this species (only 11 sites overlapped between studies). We observed the same trend in our results, however we did not address any particular species, instead treating the environmental sites as a 'superorganism'.

Through the GPS coordinates provided by the GOS project, we were able to tap into a wealth of available geospatial data from those measuring natural fluxes to those assessing human impact. It is important to note that due to the nature of collection only 5 out of the 15 environmental features used in this study were collected at the same time as the metagenomics sampling was performed. The remainder of the environmental features were derived from historical information resulting in sometimes large differences in time and space resolution between the environmental feature data and the metagenomics survey (Supplementary Figure 1). However, the characteristics of microbial communities are affected not just by the features at the time of sample collection but the history and flux of the features. We have only begun to skim the surface of the question of how much environmental history these communities carry, how much of a microbial footprint the environment reflects, and how much of our own footprint is reflected in both of them. The true test of these questions can only come through detailed examination of both microbial and environmental dynamics.

In addition, the analysis presented here is of the linear interactions between the environment and membrane proteins. Capturing the nonlinear interactions will require some modifications to existing techniques (e.g. kernel CCA) making it a particularly promising avenue for future research. We chose to first explore the linear interactions for the ease of their interpretability. We hope this work serves as a motivation for collecting additional oceanographic and metagenomics datasets and exploring higher order relationships.

We have used metagenomic data to quantitatively investigate the relationship between gene content and abundance in differing habitats. The questions we have addressed here are certainly not new, however, metagenomics studies are beginning to reveal these relationships on much larger scales. Thus, the strength of metagenomics studies is not only in their ability to study uncultivable organisms, but also in their ability to integrate layers of data in the study of whole community dynamics, and to untangle the intricate web of dependencies within habitats.

## **Methods**

### Preprocessing GOS data

Sequences and Metadata (salinity, chlorophyll, sample depth, water depth, temperature) from the GOS Expedition (Rusch et al. 2007) were downloaded from CAMERA (Seshadri et al. 2007). Sites were initially selected as in (Gianoulis et al. 2009). All sites used a filter size of 0.1-0.8  $\mu\text{m}$ . Peptides were mapped to sites as in (Gianoulis et al. 2009). Briefly, each peptide was mapped to its open reading frame (ORF) and back to its read (which mapped to a site) through the scaffolds. If a peptide originated from two reads from different sites combined in a scaffold, they were placed in both sites. Cluster annotation in CAMERA was used to remove clusters of peptides that were labeled as spurious and that contained fewer than four sequences.

#### Environmental Data Integration

UV, shipping, pollution, climate change, and ocean acidification impact values for each of the sites were extracted using ArcGIS from maps from the National Center for Ecological Analysis and Synthesis (NCEAS) (Halpern et al. 2008). Each value represents the impact of the particular factor at the site based on the type of ecosystems present. The resolution of the data is 1 km square, and thus the value for the km square in which the site was contained was used. The other factors analyzed were not used due to the sparsity of the data at the GOS sites.

Phosphate, silicate, nitrate, dissolved oxygen, and apparent oxygen utilization annual values of the objectively analyzed mean for each site at surface levels were extracted from maps provided by the World Ocean Atlas 2005 (Antonov et al. 2006; Garcia et al. 2006; Locarnini et al. 2006). These environmental features are based on historical data regardless of year of observation, from various sources, with a resolution of 1 degree latitude/longitude.

#### Site Selection

Sites were filtered for three main reasons: insufficient sample coverage, missing or nonrepresentative metadata, and metagenome composition outliers (see Supplementary Table 3 for a site-by-site breakdown; Supplementary Figures 2-4). We selected the 29 sites based on availability of the environmental data as well as to measure subtle differences in genomic content across habitats. For example, Lake Gatan, a freshwater lake, and Punta Cormorant, a hypersaline lagoon, were removed as they were extreme environmental outliers with very different membrane protein (Supplementary Figure 2) and genomic content (Rusch et al. 2007) with no representative metadata. While these features of the outlier sites in themselves are interesting, we wanted continuous differences in terms of environmental data and sequence data for further analysis.

#### Prediction of Membrane Proteins/Mapping to COG

Each non-redundant sequence was run through PRODIV-TMHMM (Eddy 1998) to predict membrane spanning regions and subsequently mapped to a Clusters of Orthologous Group (COG) using blastp (e-value threshold  $1\text{e-}10$ ) (Altschul et al. 1990). Only for 0.2% of the sequences the top two COG hits were inconsistent, thus the top hit for each sequence was used. If greater than 80% of the sequences in a COG were annotated as a membrane protein by PRODIV-TMHMM, the COG was labeled as a membrane COG (high confidence membrane proteins). This threshold was chosen arbitrarily given the number of partial protein sequences on GOS and error rate of PRODOV-TMHMM, and upon manual inspection of the COG descriptions. Membrane proteins that were not transporters, permeases, and channels (for example, oxidative

phosphorylation proteins) were manually removed to focus on transport and efflux processes. In addition, COGs that mapped to less than 1% of all sequences in the resulting sequence dataset were removed during the further analysis (peptides mapping to viral sequences, 0.01% (Williamson et al. 2008) were included due to the insignificant number and prevalence of horizontal gene transfer).

#### 16S Gene Data

16S data was taken from (Biers et al. 2009) at the 20% divergence level. Each site had an 18 element vector of counts for each 'phylum' (as referred to in (Biers et al. 2009)).

#### Pair-wise Correlations/Clustering

Matrices (rows are sites, columns are either percentage of membrane protein families, 16S diversity, or environmental features) were standardized prior to performing pair-wise correlations (Pearson) of the sites (rows) and hierarchical clustering.

#### PEN

The membrane protein families and environmental features network was constructed using the structural correlations from regularized CCA. The dot product of the structural correlations in the first and second dimension between and within the membrane protein families and environmental features were calculated. The distance (dot product) threshold was set to  $>|0.5|$  and between every satisfying pair (nodes) an edge was placed.

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Figure 1 (a) Environmental and Membrane Family Matrix construction. (b) Types of correlations that can be performed: (1) between either environmental features (Env-Env) or membrane protein families (Fam-Fam), (2) between an environmental feature and a membrane protein family (Env-Fam), or (3) between two sites (Site-Site) defined either through their membrane protein families (SS-FAM) or their environmental features (SS-Env; see figures 2 for larger resulting heatmap and labels). (c) Quantification of relationships between environmental features and membrane protein families by construction of Env-Fam network from structural correlation coefficient plot.

Figure 2 (a) Clustering of site-site correlations where each site is defined by a vector of 15 environmental features (Site-Site Env heatmap). (b) Sites color coded by environmental clustering, shows strong concordance with geographic location, North-Atlantic (blue), Mid-Atlantic (red), Pacific (orange)

Figure 3 Site-site correlations where each site is defined by (a) 151 membrane protein families (Site-Site-Fam, (SS-FAM)) and (b) 16S genes at the 20% divergence level (SS-16S) (sites ordered as in Figure 2a). For each row of SS-FAM, we sort the correlation coefficients and convert them to rank-order. We then repeat this procedure for SS-16S and SS-Env (Figure 2a). We then compare the ranks of SS-FAM and SS-Env, as well as, SS-16S and SS-Env. If the rank vectors are similar to one another, this implies that differences in one set of features are reflected in differences in a second set of features. For the FAM/Env, this is indeed the case; however, the low rank correlation between 16S/Env implies that 16S is not reflective of changes in environment as seen by the boxplot in (d).

Figure 4 (a) Boxplot of PCA first component scores on Membrane Protein Family matrix. Separating sites by environmental clusters from Figure 2a shows the North Atlantic scores are distinguishable from the Mid-Atlantic/Pacific. Discriminate Partition Matching. Membrane protein families enriched in the (b) North Atlantic and (c) Mid-Atlantic/Pacific

Figure 5 (a) Plot of first and second dimension of CCA with labeled environmental features (blue) and membrane protein families (gray). Within inner circle (0.3 circumference) features are invariant across the sites. (b) PEN construction from CCA structural correlations in the first and second dimension using a distance cutoff  $> |0.05|$  between all nodes (environmental features and membrane protein families) Red edges represent negative associations and green edges represent positive associations (c)

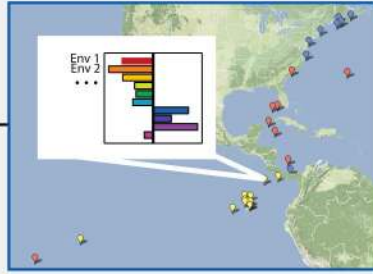
Phosphate sub-network. (d) Iron/Polyamine sub-network

Figure 6 (a) Image of dust storm off Sahara desert (NASA) (b) Model of dust concentrations (color-coded) across GOS sites, adapted from Jickells et al. (Jickells et al. 2005). (c) Pollution levels (\* impact value, see Halpern et al., 2008), dust concentrations, % of ABC-type hemin transport system proteins (# of COG4558 proteins/# total proteins at site), and nitrate/chlorophyll ratio values across the 29 GOS sites. Black line shows separation of sites into two sets, one with high pollution and dust and low N/C and iron transporters and vice versa.

## a. Matrix Construction

Environmental Feature (Env) Matrix

	Env 1	Env 2	...	...	Env 15
Site 1	30	.2			
Site 2	29	.6			
...					
Site 29					

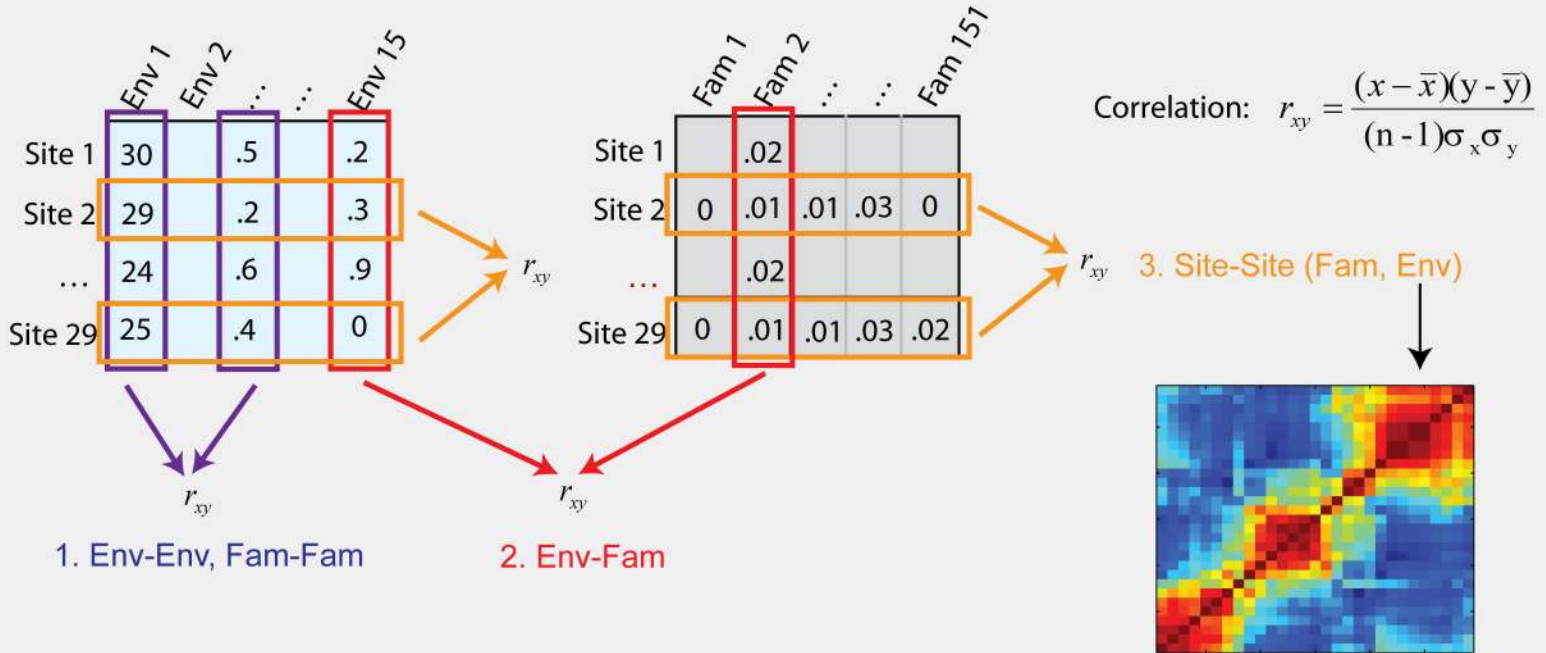


Membrane Protein Family (Fam) Matrix

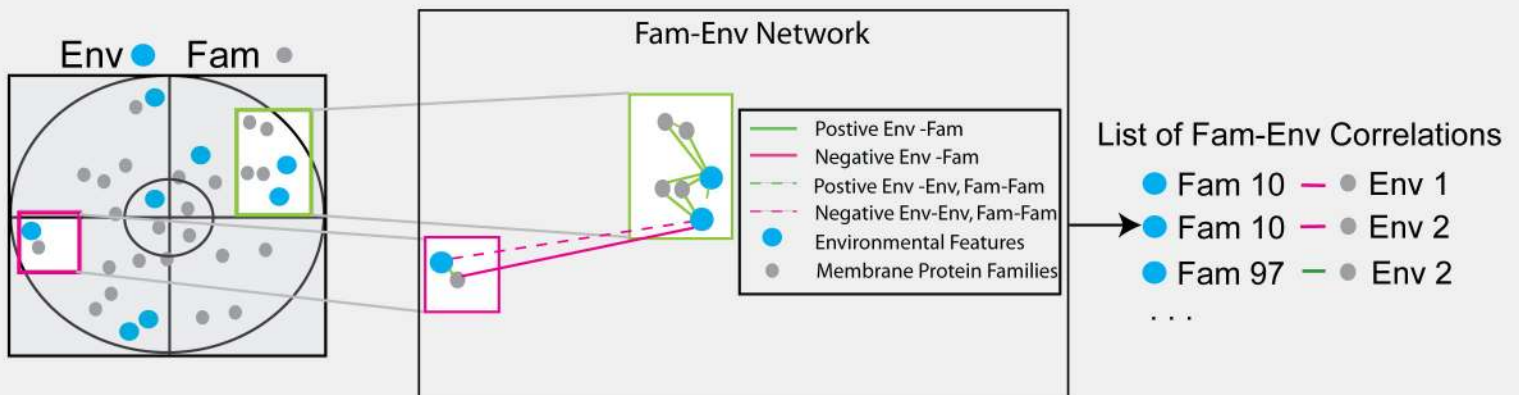
	Fam 1	Fam 2	...	...	Fam 151
Site 1	.01	.02			
Site 2	0	.01			
...					
Site 29					

# counts Fam 2  
#total protein counts at site 2

## b. Pairwise Correlations

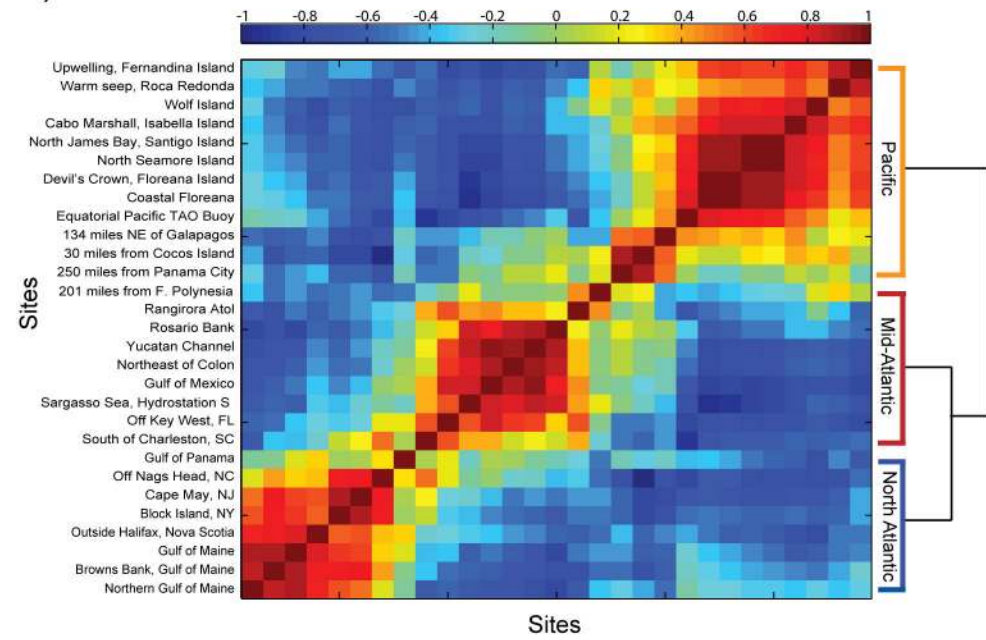


## c. Membrane Protein Families and Environmental Features Network (PEN) Construction



a)

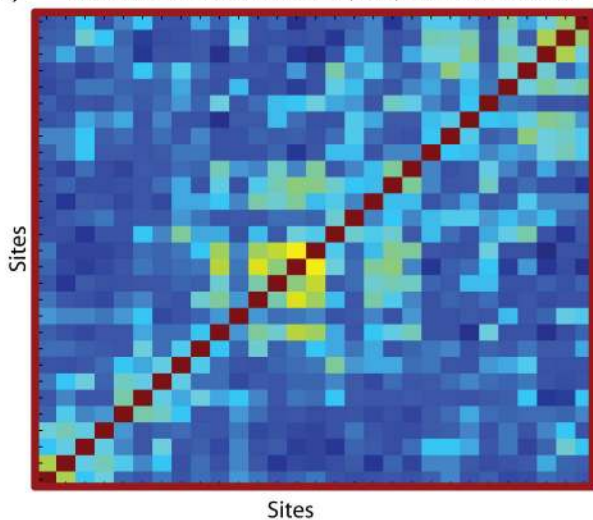
### Environmental Features (Env) Site Correlations and Clustering



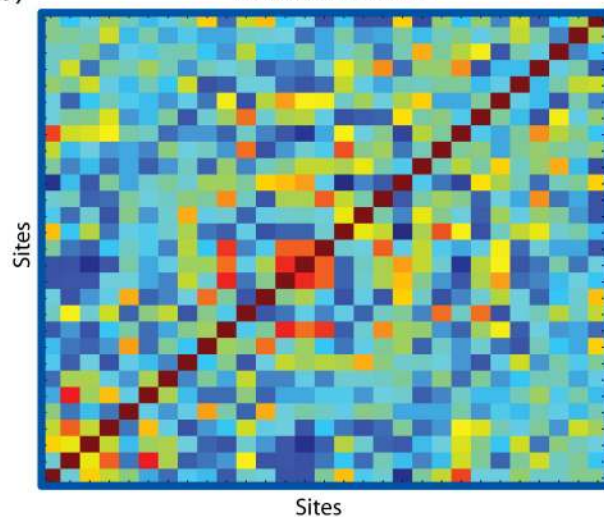
b)



a) Membrane Protein Families (Fam) Site Correlations



b) 16S Site Correlations



c) Environmental Features Site Correlations

	Site 1	Site 2	Site 3	Site 4	...
Site 1	1	.8	.5	.7	
Site 2	.8	1	.3	.4	
Site 3	.5	.3	1	.7	
...	.7	.4	.7	1	

Membrane Protein Families Site Correlations

	Site 1	Site 2	Site 3	Site 4	...
Site 1	1	.2	.6	.3	
Site 2	.2	1	.9	.8	
Site 3	.6	.9	1	.6	
...	.3	.8	.6	1	

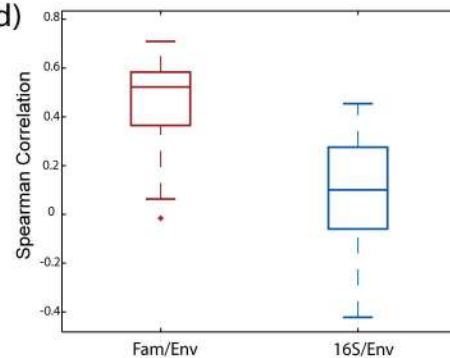
Spearman rank correlation:

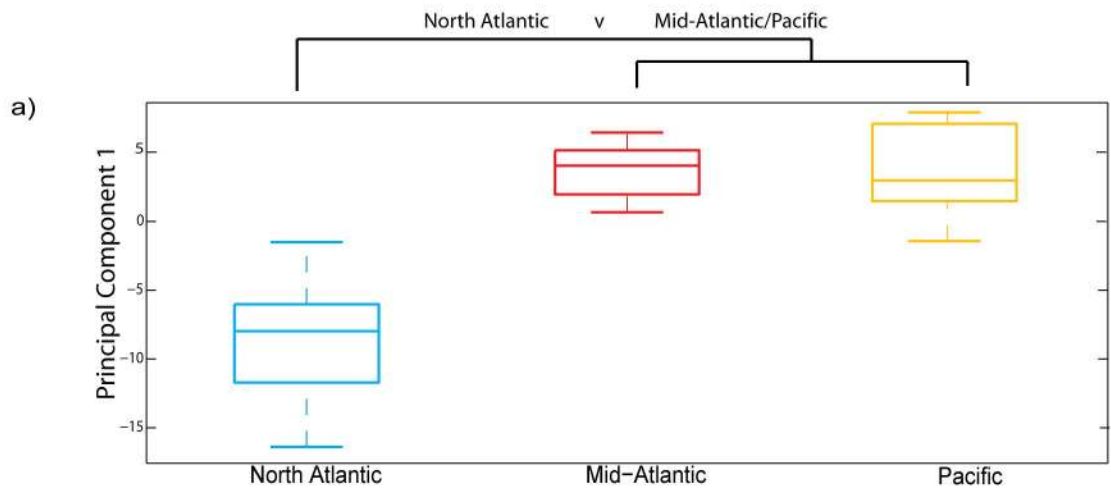
1	2	4	3	...		
---	---	---	---	-----	--	--

1	4	2	3	...		
---	---	---	---	-----	--	--

$$r_{sp} = \frac{\sum (x - \bar{x})(y - \bar{y})}{(n-1)\sigma_x\sigma_y}$$

d)





Membrane Protein families discriminating between Site Groups

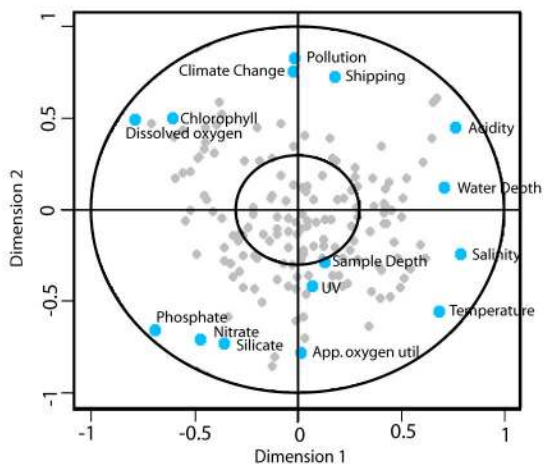
b)

Efflux	COG3696	Putative silver efflux pump
	COG0534	Na <sup>+</sup> -driven multidrug efflux pump
	COG0841	Cation/multidrug efflux pump
	COG1566	Multidrug resistance efflux pump
	COG3267	Type II secretory pathway, component ExeA (predicted ATPase)
	COG2095	Multiple antibiotic transporter
Osmotic balance	COG4168	ABC-type antimicrobial peptide transport system, permease component
	COG1292	Choline-glycine betaine transporter
	COG3158	K <sup>+</sup> transporter
Magnesium	COG3264	Small-conductance mechanosensitive channel
	COG0598	Mg <sup>2+</sup> and Co <sup>2+</sup> transporters
Sulfur	COG4536	Putative Mg <sup>2+</sup> and Co <sup>2+</sup> transporter CorB
	COG2981	Uncharacterized protein involved in cysteine biosynthesis
Sugars	COG0659	Sulfate permease and related transporters (MFS superfamily)
	COG0531	Amino acid transporters
	COG0738	Fucose permease
	COG1593	TRAP-type C4-dicarboxylate transport system, large permease component
	COG3090	TRAP-type C4-dicarboxylate transport system, small permease component
	COG4213	ABC-type xylose transport system, periplasmic component
	COG4214	ABC-type xylose transport system, permease component
	COG2031	Short chain fatty acids transporter
	COG3202	ATP/ADP translocase
	COG5265	ABC-type transport system involved in Fe-S cluster assembly, permease and ATPase components
COG0370	Fe <sup>2+</sup> transport system protein B	
COG4623	Predicted soluble lytic transglycosylase fused to an ABC-type amino acid-binding protein	
COG1757	Na <sup>+</sup> /H <sup>+</sup> antiporter	
COG3127	Predicted ABC-type transport system involved in lysophospholipase L1 biosynthesis, permease component	
COG2972	Predicted signal transduction protein with a C-terminal ATPase domain	

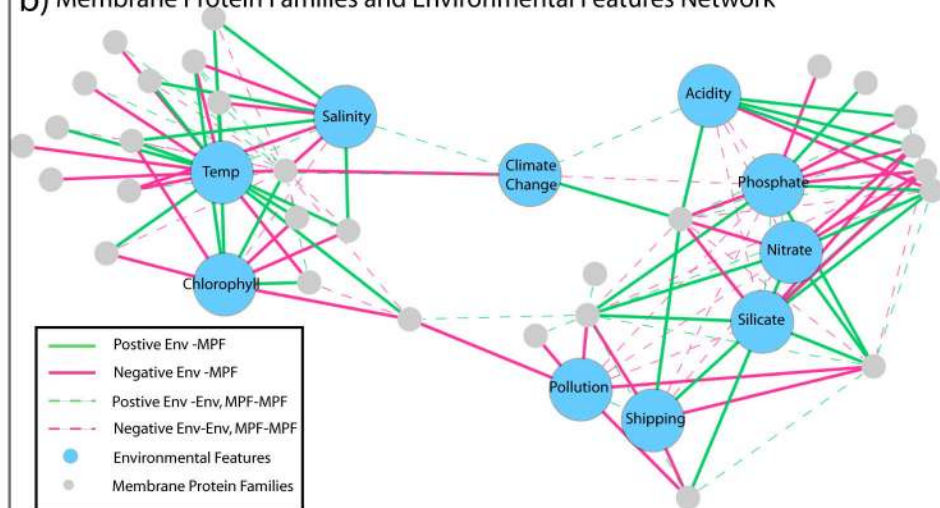
c)

COG1792	Cell shape-determining protein
COG2076	Membrane transporters of cations and cationic drugs

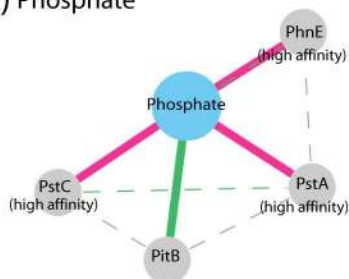
### a) CCA Structural Correlations



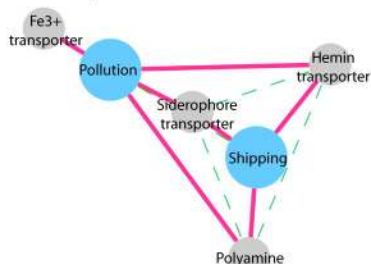
### b) Membrane Protein Families and Environmental Features Network



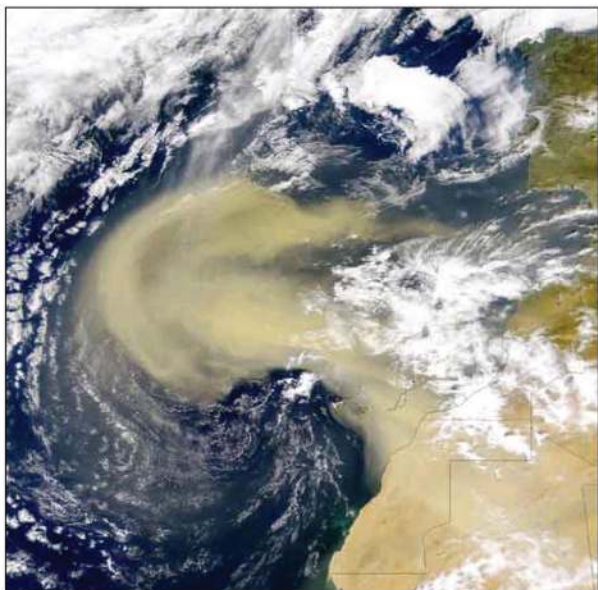
### c) Phosphate



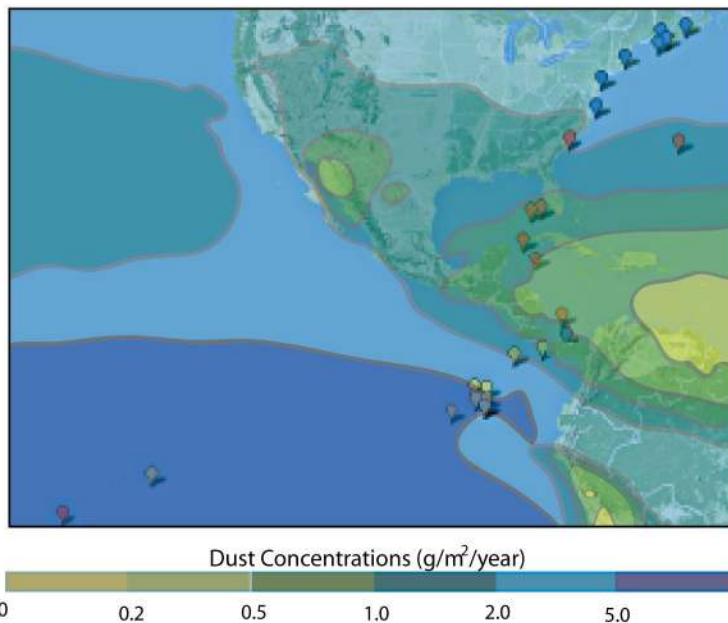
### d) Iron/Polyamines



a)



b)



c)

