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Metatranscriptomic Analyses of Plankton Communities Inhabiting Surface and Subpycnocline Waters of the Chesapeake Bay during Oxic-Anoxic-Oxic Transitions

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We used metatranscriptomics to study the gene transcription patterns of microbial plankton (0.2 to 64 μm) at a mesohaline station in the Chesapeake Bay under transitions from oxic to anoxic waters in spring and from anoxic to oxic waters in autumn. Samples were collected from surface (i.e., above pycnocline) waters (3 m) and from waters beneath the pycnocline (16 to 22 m) in both 2010 and 2011. Metatranscriptome profiles based on function and potential phylogeny were different between 2010 and 2011 and strongly variable in 2011. This difference in variability corresponded with a highly variable ratio of eukaryotic to bacterial sequences (0.3 to 5.5), reflecting transient algal blooms in 2011 that were absent in 2010. The similarity between metatranscriptomes changed at a lower rate during the transition from oxic to anoxic waters than after the return to oxic conditions. Transcripts related to photosynthesis and low-affinity cytochrome oxidases were significantly higher in shallow than in deep waters, while in deep water genes involved in anaerobic metabolism, particularly sulfate reduction, succinyl coenzyme A (succinyl-CoA)-to-propionyl-CoA conversion, and menaquinone synthesis, were enriched relative to in shallow waters. Expected transitions in metabolism between oxic and anoxic deep waters were reflected in elevated levels of anaerobic respiratory reductases and utilization of propenediol and acetoin. The percentage of archaeal transcripts increased in both years in late summer (from 0.1 to 4.4% of all transcripts in 2010 and from 0.1 to 6.2% in 2011). Denitrification-related genes were expressed in a predicted pattern during the oxic-anoxic transition. Overall, our data suggest that Chesapeake Bay microbial assemblages express gene suites differently in shallow and deep waters and that differences in deep waters reflect variable redox states.

Estuaries represent the interface of marine and freshwater habitats and are host to unique and mixed communities of microorganisms that consume not only autochthonous organic materials but also terrestrially derived allochthonous nutrients that are washed in from watersheds (1–5). Most inorganic and organic nutrients that enter estuarine waters from terrestrial habitats are processed within estuaries by bacterioplankton (6–8). These nutrients are subject to chemical alterations due to varying salinity and redox states or settle into estuarine sediments where they become buried and regenerated by sediment microorganisms over longer time scales (9–13). Because of this, estuaries represent hot spots of microbial activity. In many estuaries, anthropogenic eutrophication leads to enhanced phytoplankton concentrations, leading to high concentrations of organic carbon, which stimulate heterotrophic bacterial respiration and cause net loss of dissolved oxygen (14, 15) and, in many systems, hypoxia or anoxia (14, 16). The consequences of hypoxia and anoxia are dire for aerobic organisms inhabiting these environments, especially sessile invertebrates or metazoa that are unable to migrate into oxic waters when stressed by hypoxic conditions (14). Regions within estuaries that experience suboxic conditions, referred to as oxygen-minimum zones (OMZs) or dead zones, are increasing in frequency and volume worldwide due primarily to anthropogenic inputs of limiting nutrients and possibly sea temperature rise (17–19).

The expansive Chesapeake Bay watershed is heavily populated (17.5 million people) and hosts extensive agricultural land. Despite efforts to reduce nutrient loading in the Chesapeake Bay, fertilizer use and urban sewage inputs into the Chesapeake Bay have resulted in enhanced productivity in the bay (20). The estuary is stratified in warmer months by salinity and temperature, resulting in isolation of deep bottom waters from productive and oxygenated surface waters (21). As a consequence, deeper parts of the estuary become anoxic every summer because of settled organic matter and dying phytoplankton from the euphotic zone that fuel biochemical oxygen demand and abundant quantities of S^{2-} that chemically consumes available O_2. The extent to which whole-community bacterioplankton metabolism is affected by availability of oxygen has not been previously investigated in the Chesapeake Bay.

Bacteria use energetically favorable biosynthetic reactions that enable their growth under variable oxygen regimes (22). When oxygen, the most energetically favorable electron acceptor, becomes limiting, bacteria are able to use a succession of electron acceptors, including NO_3^-, metal oxides, and SO_4^{2-}. However, there is a taxonomic spectrum of bacteria that carry out respiration using various terminal electron acceptors, and not all bacteria are able to use all alterative electron acceptors. For example, some bacteria are strictly aerobic, others are strictly anaerobic, and

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many have flexible metabolisms that enable them to exist under oxic or anoxic conditions with their metabolism most well selected for one or the other (facultative aerobic and facultative anaerobic metabolisms). The succession of bacterial taxonomic compositions and their metabolic activities along oxic-anoxic gradients have been investigated in sediments and water columns of permanently stratified ecosystems such as the Black Sea (23), Cariaco Basin (24), and the Arabian Sea (25). These investigations have detected novel pathways of autotrophy (e.g., anammox) (26), enhanced bacterial abundance and production at oxic/anoxic interfaces (27, 28), and shifts in the composition of communities between oxic and anoxic waters (3). Oceanic environments with permanently stratified waters with oxygen-minimum zones (OMZs), such as in the East Tropical South Pacific, play a crucial role in global biogeochemical cycling, removing N from pelagic waters through denitrification and anammox to N_2O and N_2 and enhancing S^{2-} concentrations through SO_4^{2-} reduction (29). Bacterial communities in these waters change from oxic to anoxic habitats, leading to a decrease in the overall diversity of both 16S rRNA and functional genes (30), suggesting that factors driving selection vary between oxic, suboxic, and anoxic conditions.

Metagenomic and metatranscriptomic investigations in these waters suggest that transcription is dominated by organisms carrying out the locally dominant biogeochemical processes. For example, *Crenarchaea* involved in ammonium oxidation dominated shallower portions of an oceanic water column, while transcripts of anammox bacteria dominated OMZ waters (31). The sensitivity of OMZ bacteria to habitat alteration was highlighted by Stewart et al. (31), who found that incubation of minimally O_2-enriched OMZ waters led to a profound increase in transcriptions indicative of cell stress, possibly as a consequence of bottle effects. These data suggest that bacteria inhabiting OMZs may be highly adapted to their local conditions and that oceanic OMZs may not be appropriate models for describing physiological or taxonomic shifts that occur in waters that become anoxic during seasonal cycles of productivity. Few studies have documented shifts in the composition and physiological activities of bacterioplankton during the transition from oxic to anoxic conditions in seasonally anoxic waters. One study reported that large-scale changes in microbial composition occur only after depletion of nitrogenous species of electron acceptors and that typical surface water taxa (including *Synechococcus*, SAR86 *Gammaproteobacteria*, and *Pelagibacter ubique*) may persist under hypoxic conditions but that under completely anoxic conditions, taxa (e.g., *Firmicutes*, *Thioalcalovibrio*, and SAR406 bacteria) unique to those conditions dominate communities (32).

Investigating the physiology and functional activities of bacterioplankton *in situ* and without *a priori* knowledge of gene sequence or metabolism has been facilitated by the advent of community gene transcription (i.e., metatranscriptomics), where randomly sequenced mRNAs from entire communities permit identification of active processes (33). Community gene transcription has been used to study activities of microbial plankton in the open ocean (34–38) and coastal waters (33, 39–42), during phytoplankton blooms (43), and in response to additions of organic and inorganic nutrients (44–47). Metatranscriptomics has also been used to study the physiology of eukaryotic phytoplankton under ambient conditions and in response to amendments (48, 49). Comparative metatranscriptomics has revealed broad-scale patterns of gene expression, e.g., conservation of expressed genes compared to nonexpressed genes, and has generally found elevated expression of genes involved in energy metabolism relative to those involved in cell growth (50).

This study applied metatranscriptomics to examine bacterial communities in deep, subpycnocline waters of the Chesapeake Bay over a seasonal cycle (March to October). During this period, subpycnocline bacterioplankton experienced entirely oxic conditions early in the sampling period, anoxic conditions during summer, and then a return to completely oxic conditions in the fall. The goal of this study was to understand how oxygen availability impacts the composition of active community constituents. It also compares transcription profiles from above-pycnocline waters, subpycnocline oxic waters, and subpycnocline anoxic waters during the time series to describe seasonality and to understand whether the impacts of oxygen concentration reflected depth-dependent processes.

MATERIALS AND METHODS

Sampling location. Samples were collected at station CB4.3 (38.22°N, 76.24°W), located 40 km down estuary of the Choptank River mouth in the main arm of the Chesapeake Bay, from a small boat or from the R/V *Hugh R. Sharp*, between late April 2010 and October 2010 and again between May 2011 and September 2011. Conductivity-temperature-depth (CTD) (Seabird SBE 25 on a small boat and SBE 911plus on R/V *Hugh R. Sharp*; Sea-Bird Electronics) profiles of salinity, temperature, and O_2 were used to determine the pycnocline depth. Seawater samples for metatranscriptomics were collected from below the pycnocline (at all sampling times) and above the pycnocline (at certain sampling times), and seawater for chemical and physical measurements was collected both above and below the pycnocline at all sampling times. To protect sample waters from O_2 exposure, a diaphragm pump (Tornado pump; Proactive Environmental Products) with a hand-deployed hose to the CTD frame was primed in surface water to remove air bubbles before being deployed at depth to collect oxic and hypoxic sample waters. Samples for bacterioplankton RNA were pumped into a 20-liter triple-rinsed carboy, prefiltered through a 64-μm Nitex mesh, and then filtered onto a 142-mm-diameter, 0.22-μm Durapore (polyvinylidene difluoride [PVDF]) filter. Duplicate samples (total volume, 0.75 to 2 liters) were processed within 30 min of collection. Filters were placed into sterile 50-ml centrifuge tubes, flash frozen on dry ice or in liquid nitrogen, and transferred to a −80°C freezer until processed.

Inorganic nutrient analysis. Seawater samples for nutrient analysis were pumped to a PVC manifold on deck. The flow-controlled manifold distributed waters gently through short tubes into the bottoms of sampling syringes that were allowed to overflow for 1+ min before they were sealed. Samples were filtered through 0.45-μm-pore-size, 25-mm-diameter syringe filters and subsampled for NO_2^-, NO_3^-, NH_4^+, soluble reactive phosphate (SRP), hydrogen sulfide (H_2S) ($[S_2^-] + [HS^-] + [H_2S]$), and total soluble Fe and Mn analysis in 7-ml polyethylene vials with screw caps. Concentrations of NH_4^+, SRP, H_2S, and total Fe and Mn were analyzed colorimetrically using phenylhypochlorite, ammonium phosphomolybdate, methylene blue, chromogen ferrozine, and ammonia formaldoxime colorimetric methods, respectively (51–53). Water column and benthic sediment NO_2^- and NO_3^- analyses were done by segmented flow analysis after cadmium reduction (54) and carried out by the University of Maryland Horn Point Laboratory Analytical Services.

RNA extraction and processing. RNA samples were retrieved from −80°C storage and extracted using previously described methods (55). For 2011 samples (but not 2010 samples), filters were spiked with 25 ng of internal standard (linearized pGEM-Z transcript [56]) to account for extraction efficiency variation between samples. Briefly, tubes containing filters were amended with 9 ml ZR lysis buffer (Zymo) and 100 μl sterile glass beads, bead beaten (Biospec instruments) for 2 min, and placed on ice, and filters were manually masticated with 10-ml pipettes. Tubes were

centrifuged at 3,000 × *g* for 5 min to remove filter debris before the supernatant was transferred to a clean 15-ml tube. All ~ 8.5 ml of supernatant was run through Zymo Spin Columns II (with repeated centrifugation at 10,000 × *g* for 30 s and discarding of flowthrough). The filter columns were washed with Zymo wash buffer and then RNA eluted in two rinses of 25 μl, 45°C nuclease-free water. Total RNA extracts were then subjected to treatment with the DNA-free RNA kit (Zymo Research), following the manufacturer's recommendations. rRNAs were depleted using a previously validated approach (35, 36) employing enzymatic terminator exonuclease degradation (mRNA-ONLY; Epicentre) and bead hybridization capture (MicrobExpress; Ambion). Finally, RNA, which was rRNA depleted, was subject to amplification using the MessageAmpII-Bacteria (Ambion) kit, which employs *in vitro* transcription to linearly amplify RNAs from nanogram quantities to microgram quantities. We acknowledge that this rRNA depletion and amplification approach may bias gene expression (57). However, our approach was applied consistently between samples and does not affect our overall conclusions that compare metatranscriptomes in our study.

Our treatment of amplified RNA (aRNA) differed from 2010 to 2011 due to improvements in DNA sequencing and method development (Illumina RNAseq). In 2010, aRNA was first converted to double-stranded cDNA using the Superscript III and RNase-dependent DNA polymerase I approach outlined by Hewson et al. (35). A total of 1 μg double-stranded cDNA from 2010 samples was submitted for 100-bp paired-end Illumina Sequencing (HiSeq 200) at the Cornell University Core Laboratory Center. In 2011, aRNA (~20 μg) was submitted to the Columbia Genome Center laboratory for Illumina RNASeq, employing 100-bp paired-end sequencing.

Bioinformatic processing of metatranscriptomic sequences. Initial quality assurance/quality control (QA/QC) was carried out by the Cornell Genomics Core Laboratory Center (2010) and the Columbia Genome Sequencing Center (2011), which involved trimming low-quality reads [N(ambiguous bases) ≤ 2] and short reads (<100 bp). In addition, reads were screened and trimmed of terminal adenosine homopolymer runs (>10 bp) using MOTHUR (58). Additional QA/QC was performed using the CLC Genomics Workbench 4.0, which included trimming sequencing adapters and bar codes from sequence reads and identification of replicate reads. Because paired-end reads of 100 bp each do not offer confidence in annotation, we first assembled sequence reads into contiguous sequence (contigs) using the assembly algorithm on the CLC Genomics Workbench 4.0. Assembly was performed where length fraction (overlap) was set to 0.8 and similarity set to 0.95 and with a minimum contig length of 250 bp. We chose to annotate our metatranscriptomes to allow ontology and binning of shared function by BLASTx comparison against the SEED database using an E value cutoff of 0.001 and potential phylogeny by BLASTn comparison against RefSeq. Gene ontologies were compiled with the assistance of MG-RAST (59). However, annotations were manually curated after BLAST comparison. The best match to each contig at E values of <0.001 to either phylogenetic or functional ontologies were used as a proxy for each contig, although we recognize that multiple open reading frames (ORFs) on the same contig may have variable function. Most contigs in our analysis were short (mean size, ~346 bp); hence, we believe that this annotation approach does not strongly underestimate functional variability. The number of sequencing reads that recruited to each contig was then matched to each annotation. The expression value of total reads was calculated for each individual gene or strain and then summed ontologically for phylogenetic and functional binning.

Statistical analyses of metatranscriptomes. We generated mean functional (subsystem-level) and phylogenetic (genus-level) metatranscriptomic profiles from each sampling time and depth by averaging expression values in duplicate libraries for each genus and subsystem. Similarity between metatranscriptomes was calculated based on shared pathways from subsystem-level ontology, which provided the lowest possible functional binning based after individual gene expression, and based on genus of bacteria. The Bray-Curtis index was calculated using the XLStat (AddinSoft SARL) plug-in in Microsoft Excel and was used to compare the functional and phylogenetic profiles. The Bray-Curtis similarity matrix was used in multiple-dimensional scaling (MDS) of community potential phylogeny and function. To compare the taxonomic and phylogenetic clustering patterns, we performed a paired Mantel test on Bray-Curtis similarity indices.

We compared the expression value for each subsystem and genus between the above- and subpycnocline (from here on referred to as "surface" and "deep," respectively) waters and separately between deep oxic waters and deep anoxic waters, using paired Student's *t* tests, where we used a critical *P* value of <0.001 to correct for multiple comparisons and reduce type II error. Similarly, we compared deep oxic waters and deep anoxic waters separately to surface waters and by the same mechanism. In each case, we collated significantly ($P < 0.001$) different pathways and taxa, and we report only comparisons for which individual pathways comprised >0.01% of transcripts and for which the ratio of comparison was >0.5× (i.e., more than twice the value between comparisons).

Analysis of archaeal sequences. We analyzed the gene expression pattern of *Thaumarchaeota* in the Chesapeake Bay by first extracting contigs that had best match to *Nitrosopumilus maritimus* by RefSeq from the assembly and then identifying open reading frames using the algorithm GetORF (Wellcome Sanger). The ORFs were annotated by comparison against the nonredundant (nr) protein database at NCBI by BLASTx with an E value of <0.001. The pattern of gene expression across these loci was examined by recruiting reads by BLASTn from each metatranscriptome and corrected for total library size.

Observations of denitrification-related genes. In order to focus specifically on genes involved in the denitrification pathway, an in-house database was created from annotations downloaded from the Uniprot database (http://www.uniprot.org/) and manually curated to ensure representative gene components from a wide phylogeny. Because the extent of knowledge of different genes, and consequently accessions to Uniprot, is variable between components, database entries were limited to 500 per gene. This database was then compared by BLASTx against contigs created for each metatranscriptome, where significant hits ($E < 0.001$) were annotated and matched with recruiting sequence reads. The total number of recruits per gene was tallied and expressed as a percentage of total annotated reads per library, which was further divided by the number of query database entries to account for differences between genes.

Nucleotide sequence accession numbers. Nucleic acid sequences associated with this study have been deposited at NCBI under BioProject PRNJ222777.

RESULTS

Sequencing of 34 metatranscriptomic libraries from 2010 and 2011 resulted in a total of 876 million reads, representing 93.2 Gbp of sequence information (see Table S1 in the supplemental material). Individual libraries contained on average 27.6 million sequences. On average, 54.1% (±4.8%) of total reads sequenced assembled into contiguous sequences with an average length of 346 (±36) bp. Reads matching rRNAs (small subunit plus large subunit) represented 20.1% (±16.7%) of total sequence reads overall but represented from 0.9 to 56.1% of each library. Comparison of libraries against the SEED database and RefSeq database revealed that functional and phylogenetic annotated reads comprised on average 18.7% (± 3.7%) of assembled reads. pGEM-3Z sequences that were added to 2011 metatranscriptomes comprised a similar percentage of transcripts for all libraries (0.431% ± 0.045%). Based on the added quantity of pGEM-3Z standard (1.61×10^{10} copies), our sequencing depth represents 0.00076% ± 0.00007% of transcripts present in the samples.

Clustering by potential phylogeny was significantly related to clustering by functional annotation ($P < 0.00001$ [Mantel test]; df = 32). The mean similarities between duplicate metatranscrip-

tomes based on potential phylogeny (mean Bray-Curtis index = 0.319 ± 0.048) and function (mean Bray-Curtis index = 0.427 ± 0.058) were greater than the mean similarities between metatranscriptomes from other sampling dates and depths (0.205 ± 0.011 for potential phylogeny and 0.282 ± 0.014 for function). Based on this information, we examined the clustering pattern of metatranscriptomes based on mean representation of each genus (for potential phylogeny) or SEED subsystem level 3 (for function) between replicate metatranscriptomes (Fig. 1A and B). Samples generally clustered by year, with clear differences between 2010 and 2011 samples. Progression of phylogenetic and functional profiles generally followed oxygen status in deep oxic and anoxic waters, with greater similarity between metatranscriptomes from similar oxygen conditions. In 2010, similarity between metatranscriptomes was negatively correlated with monthly change in oxygen concentration ($r^2 = -0.87$ for potential phylogeny [$P < 0.05$] and $r^2 = -0.77$ for function [not significant]). The metatranscriptomes in surface waters showed no clustering pattern with deep oxic or anoxic water metatranscriptomes. Samples from 30 August 2011 (3 m and 18 m) and 21 September 2011 (17 m) were the most dissimilar to all other metatranscriptomes.

The domain-level affiliation of sequence reads demonstrated different patterns in 2010 and 2011 (Fig. 2). In 2010, bacterial transcripts comprised the bulk of reads throughout the sampling period, while eukaryotic transcripts exceeded annotated bacterial transcripts in 2011 early and late in the sampling period. Archaeal transcripts comprised a very small percentage (<0.05% of transcripts) early in the season in both 2010 and 2011 but increased to >1% of transcripts in late August and September. Viral transcripts always comprised <1% of total sequence reads. The pattern in surface waters was similar to the pattern in deep oxic and anoxic waters. Potential phylum-level annotation of surface water and of deep oxic and anoxic water transcripts from 2010 and 2011 revealed differences between depths and between years, particularly more proteobacterial transcripts in 2010 than in 2011, when putative *Chordate* and *Ascomycete* transcripts became a larger proportion of metatranscriptomes than in 2010 (Fig. 3A). Functional annotation of transcripts revealed that the majority of transcripts from 2010 and 2011 were dominated by carbohydrate metabolism, amino acid metabolism, RNA metabolism, and protein metabolism. However, the distribution of transcripts among individual subsystems varied between 2010 and 2011 (Fig. 3B). Transcripts associated with the oxidative C_2 cycle and ribulose monophosphate pathways were more highly transcribed in 2011 than in 2010, while genes involved in branched-chain amino acid degradation and respiratory complex I were highly expressed in 2010 but nearly absent in 2011.

Comparison of transcripts between surface waters and deep oxic and anoxic waters and between deep oxic waters and deep anoxic waters reflected expected patterns of transcription related to photosynthesis and heterotrophic respiration (Fig. 4; see Fig. S1 in the supplemental material). Gene transcripts involved in cytochrome b_6 formation, photosystem I, and accessory pigments were always more highly expressed in surface waters than in deep oxic and anoxic waters. In contrast, genes involved in oxidation of organic acids, electron transport (notably menaquinone biosynthesis), fermentation, and methanogenesis were more expressed in deep oxic and anoxic waters than in surface waters. Despite measured differences in oxygen concentration in deep oxic and anoxic waters between samples, waters with higher-oxygen conditions in deeper waters (i.e., deep oxic waters) had significantly more genes transcribed that were related to anaerobic degradation of organic material than surface waters, while deep anoxic waters had significantly greater expression of genes involved in sulfate reduction, anaerobic respiratory reductases, high-oxygen-affinity cytochromes, and formation of cell walls of anaerobic bacteria than deep oxic waters (Fig. 4). Comparison of deep metatranscriptomes revealed sulfate reduction and genes involved in Fe-S cluster assembly as more transcribed in deep anoxic waters than in deep oxic waters, while genes involved in biofilm formation and chlorophyll synthesis were more expressed in deep oxic waters than in deep anoxic waters. Structural, secretory, and replication-associated transcripts were also more highly expressed in deep anoxic waters than in deep oxic and surface waters.

Archaeal gene transcription was examined by recruiting reads to genes of *Nitrosopumilus marinus* SCM1 by BLASTn analysis (Fig. 5). The patterns of *Nitrosopumilus*-like gene expression were different in 2010 and 2011. Ammonium monooxygenase (*amo*) gene transcripts were among the most highly expressed archaeal transcripts in late 2010, while transcripts of the thrombospondin type 3 repeat gene (putatively involved in cell adhesion) were present in 2010 but not 2011. Similarly, ammonium transporter genes were detected only in 2011 and the nitrogen regulatory protein P-11 gene only in 2010.

The relative abundance of genes involved in the denitrification pathway varied between sampling dates and depths in 2010, with greatest expression of dissimilatory nitrate reductase (*nar*) and dissimilarity copper-containing nitrite reductase (*nirK*). Total expression of genes involved in the denitrification pathway generally increased over the course of the sampling period, but the relative expression of different components did not vary between sampling times (Fig. 6).

DISCUSSION

Our data suggest that bacterioplankton community gene transcription in surface waters and deep oxic and anoxic waters of the Chesapeake Bay varies in potential phylogeny and function according to sampling date and oxygen concentration in the water masses that were sampled. These data also suggest that functional variability is related to potential phylogenetic variability, where the balance between eukaryotic and prokaryotic transcripts causes the greatest variability in gene transcription pattern. Comparison between surface waters and deep oxic and anoxic waters, and between deep oxic waters and deep anoxic waters suggests that oxygen concentration is a key driver of microbial community gene expression, where genes involved in predicted anaerobic activity are more well represented in deep oxic and anoxic waters than genes involved in aerobic metabolism. Furthermore, our data suggest that *Archaea*, which comprise a small proportion of transcripts for most of the summer, increase in relative representation upon pycnocline degradation. These observations support the concept that active constituents of microbial communities undergo dramatic shifts in their gene expression during transitions between oxic and anoxic conditions.

Microbial communities sampled as part of this study (<64 μm) represent free-living bacterioplankton, single-celled eukaryotes, and possibly particle-associated bacteria but exclude larger metazoa, which were removed by prefiltration. In both 2010 and 2011, the ratio of eukaryotic to prokaryotic transcripts increased after the breakdown of stratification (in October 2010 and due to Hurricane Irene in late August 2011), which increased nu-

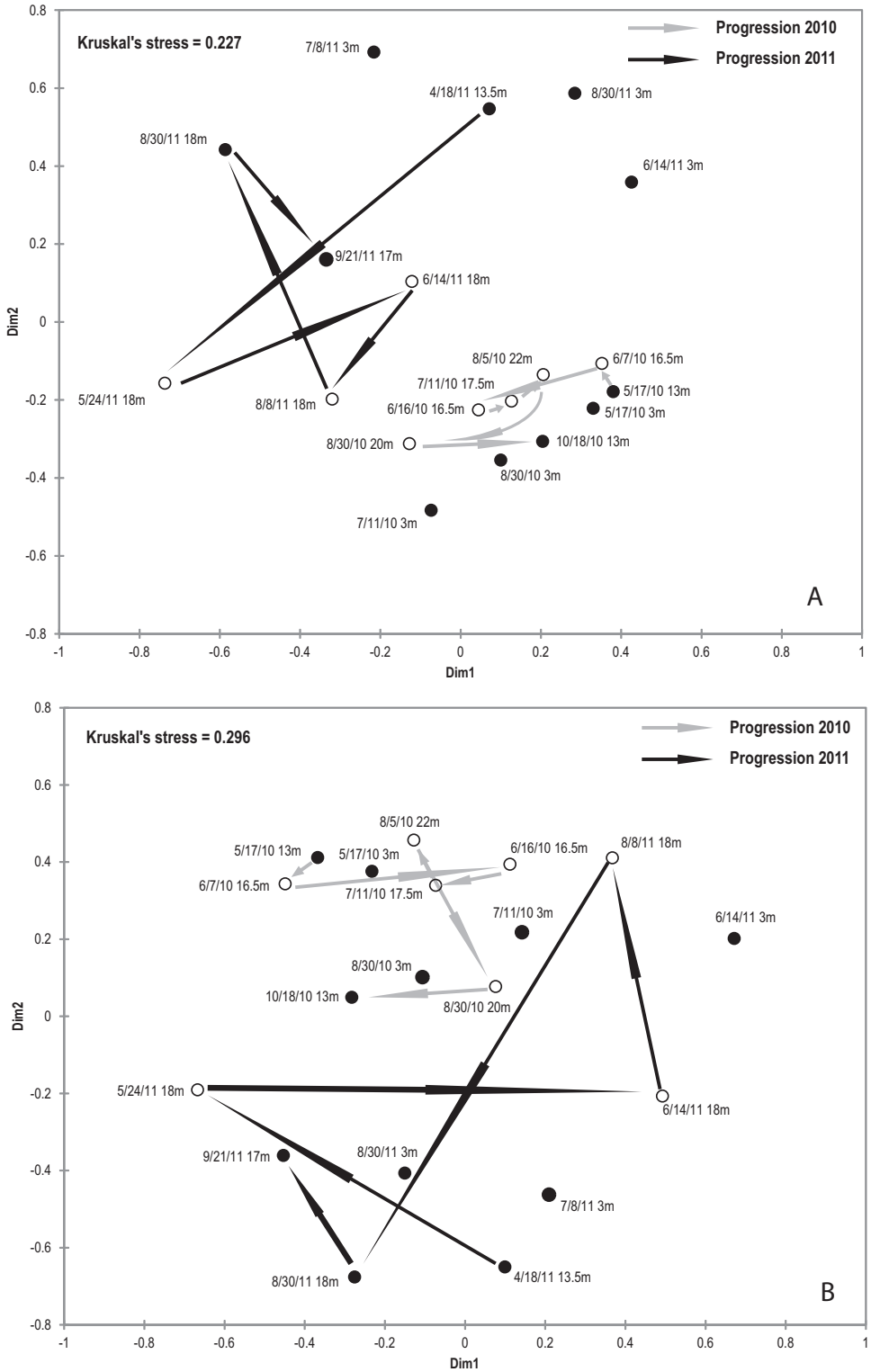

FIG 1 Multiple-dimension scaling (MSD) of functional (A) and phylogenetic (B) profiles of metatranscriptomes collected in the Chesapeake Bay in 2010 and 2011. Clustering is based on the Bray-Curtis similarity index, which was calculated based on subsystem- and genus-level annotations of function and potential phylogeny (respectively). Each data point is annotated with its collection date and depth. Closed circles indicate oxic waters (surface waters and deep oxic waters), while open circles indicate anoxic waters (deep anoxic waters). The progression of metatranscriptomic profiles between dates in deep oxic and anoxic waters is indicated by arrows.

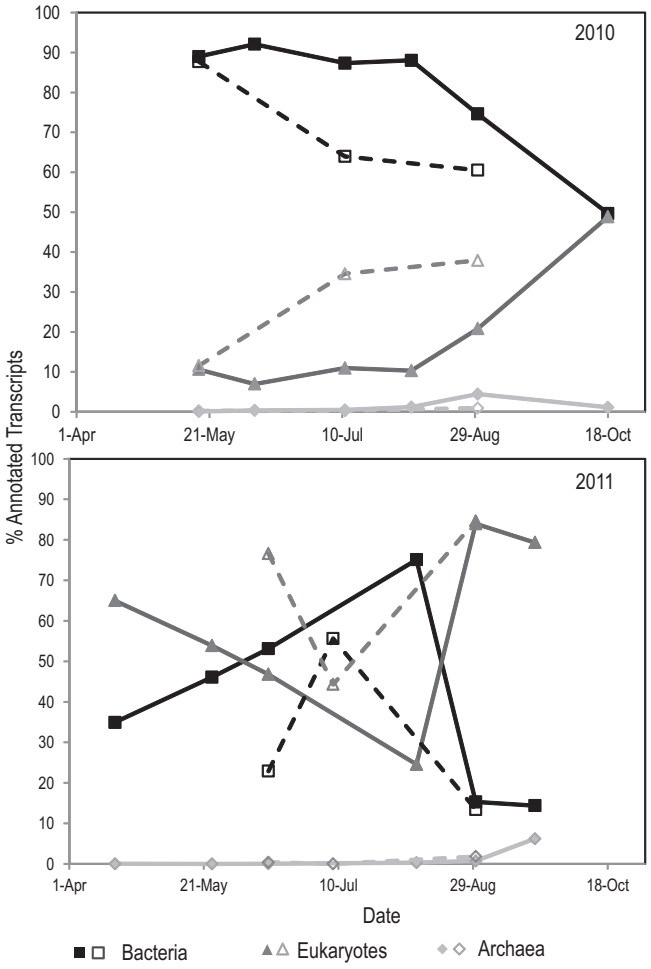

FIG 2 Domain-level affiliations of metatranscriptomic reads in 2010 (top) and 2011 (bottom). Open symbols with dashed lines indicate surface water communities, and solid symbols with solid lines indicate deep oxic and anoxic water communities.

trient concentrations in surface waters. In both 2010 and 2011, the abundance of *Archaea*, which comprised a small fraction of microbial transcripts for most of the summer, increased late in August, which may be a result of the increased availability of reduced nitrogen compounds for chemoautotrophic activities (i.e., nitrification) due to annual or episodic wind events (60).

The overall pattern of gene transcription was different between 2010 and 2011 in both potential phylogenetic and functional compositions, which was possibly due to interannual differences in the composition of microbial assemblages and the geochemical conditions in both years. However, we acknowledge that the different methods used to prepare metatranscriptomes in 2010 and 2011 may have led to heterogeneous metatranscriptomes between these years. The annual average of Susquehanna River flow at the head of the Chesapeake Bay, providing >50% of freshwater flow and having a critical role in determining the duration and volume of hypoxia, was much larger in 2011 (1,861.1 $m^3 s^{-1}$) than in 2010 (963.6 $m^3 s^{-1}$) and larger than the long-term average (1,173.1 $m^3 s^{-1}$) (U.S. Geological Survey at the Conowingo Dam; http://waterdata.usgs.gov/md/nwis). Annual repeating patterns of microbial assemblage composition have been reported in coastal waters of southern California and elsewhere (61–63). However, within the Chesapeake Bay, variability in physicochemical structure and inputs results in patchy distributions of microorganisms over small temporal and spatial scales (64–67). Within the sampling periods, functional and potential phylogenetic profiles changed over the season, with the most dramatic changes in 2011 and less change in 2010, and in both years between profiles of deep anoxic waters and reoxygenated deep oxic waters. A previous study (32) found that shifts in microbial composition tracked the transition from deep oxic waters to deep anoxic waters. However, many community constituents remained the same during this transition. Our results suggest that ecosystem-wide shifts in oxygen status can result in variable microbial function. However, the slow transition from deep oxic water to deep anoxic water after onset of stratification did not cause a dramatic shift in overall gene transcription. In addition, our data suggest that the functional profiles of the permanently oxic and sunlit surface waters are different from those of deep oxic and anoxic waters regardless of establishment of a pycnocline.

Our data support the idea that microbial phylogenetic diversity corresponds with functional diversity, since variation in the functional profile of microbial communities was significantly related to the potential taxonomic composition of the transcript pool (Mantel test, $P < 0.0001$, df = 32). Previous studies have found that function was not significantly related to strain-level taxonomic composition in the open ocean and freshwater habitats, which suggested that there may be some degree of functional redundancy in bacterioplankton communities (35, 68). However, the potential taxonomic changes in the transcript pool over time observed in the Chesapeake Bay were more extensive and corresponded with significant variation in functional activities. This does not discount the possibility of functional redundancy at the strain level. However, it is clear from these data that different genera utilize different functional genes in mixed communities and that their variation is linked.

Previous examinations of the compositions of functional gene transcript pools have similarly identified vertical stratifications of both potential phylogeny and function (38). However, in previous studies of the surface open ocean, core metabolic functions, which made up the greatest fraction of community gene transcripts (34–36), were relatively invariant spatially and from day to night when sampled in the North Atlantic and Pacific Oceans, while most variation was driven by rarer transcripts (35). In this study, the most abundant dominant transcripts were those involved in metabolism (i.e., F_oF_1-type ATP synthase, oxidative C_2 cycle, respiratory complexes, photosystems I and II, ribulose monophosphate pathway, and methanogenesis), replication (YgfZ, purine conversions, chaperones, translation-elongation factors, and RNA polymerase), and the synthesis of biotin and methanoproteins. Dominant taxonomic families in metatranscriptomes were also different from those in the open ocean. In the open ocean, metatranscriptomes were dominated by transcripts of cyanobacteria (34–37), while in this study, they were primarily from *Proteobacteria*, unicellular eukaryotes, and interestingly, *Thaumarchaeotes*. This difference may reflect dominance of heterotrophic metabolic processes in the bay, where respiration by heterotrophic organisms outweighs photosynthetic activities by unicellular prokaryotes and eukaryotes on a community basis, in line with geochemical measurements (69, 70).

While the vast majority of gene transcripts were shared between surface waters and deep oxic and anoxic waters, compari-

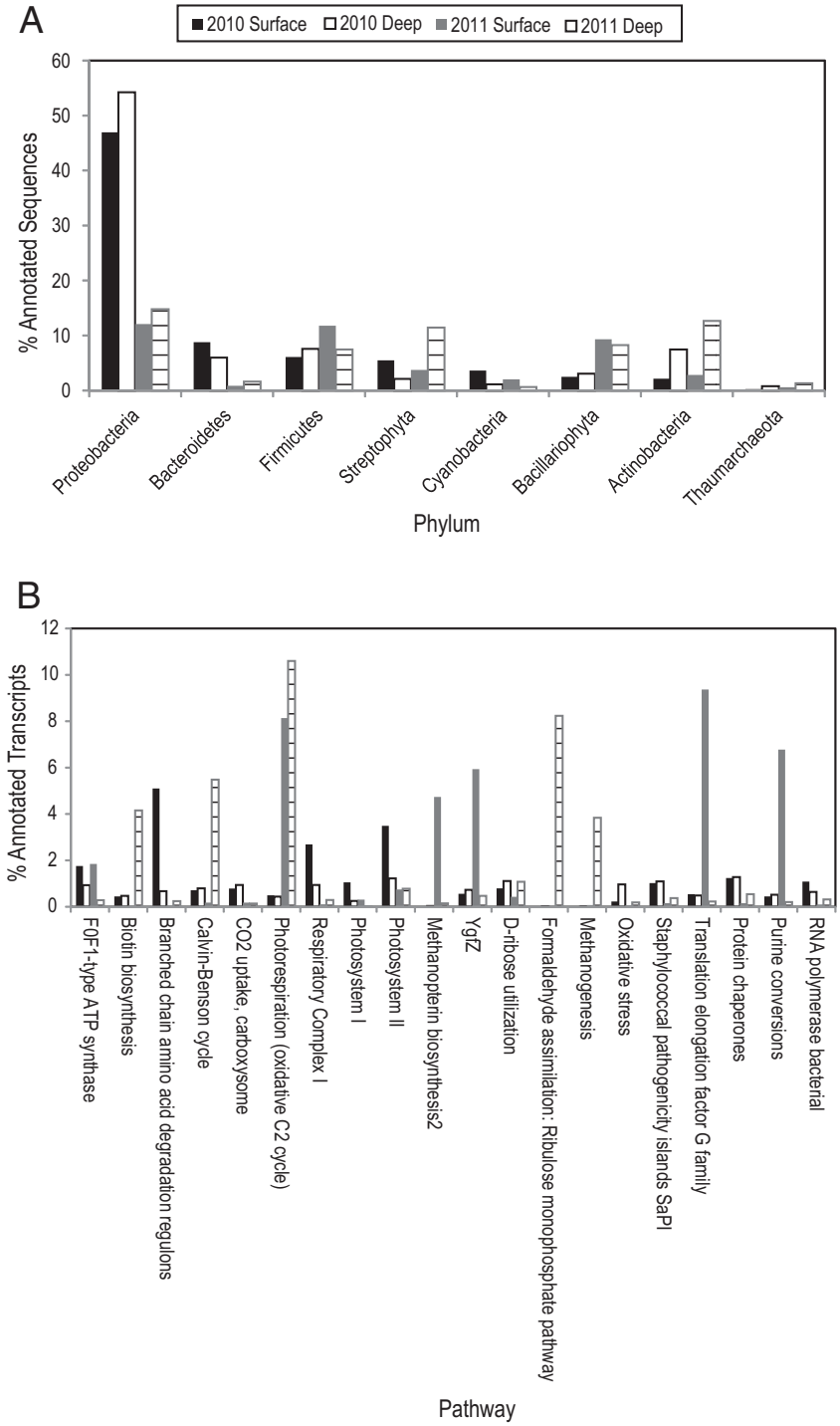

FIG 3 Dominant phylogenetic transcripts (A) and functional transcripts (B) in 2010 and 2011. Data are given as the percentage of total annotated transcripts, which represented a variable fraction of total library size (see Table S1 in the supplemental material).

son of these two compartments revealed a greater expression of genes involved in photoautotrophic metabolism in surface waters but greater expression of genes involved in respiratory activities, DNA recycling, and regulation in deep oxic and anoxic waters. Among transcripts that varied in greatest magnitude, the photoautotroph-related transcripts of phycobilisome formation and nucleoside diphosphate epimerases comprised >0.05% of transcripts and demonstrated more than an order of magnitude greater expression in surface waters than in deep oxic and anoxic waters, while two-component regulatory systems, deoxyribose and deoxynucleoside catabolism, C storage metabolism, thiol peroxidase, and glycosyltransferases comprised >0.05% of deep oxic and anoxic water transcripts and were at >5 times higher levels than in surface waters. When we compared gene expression in

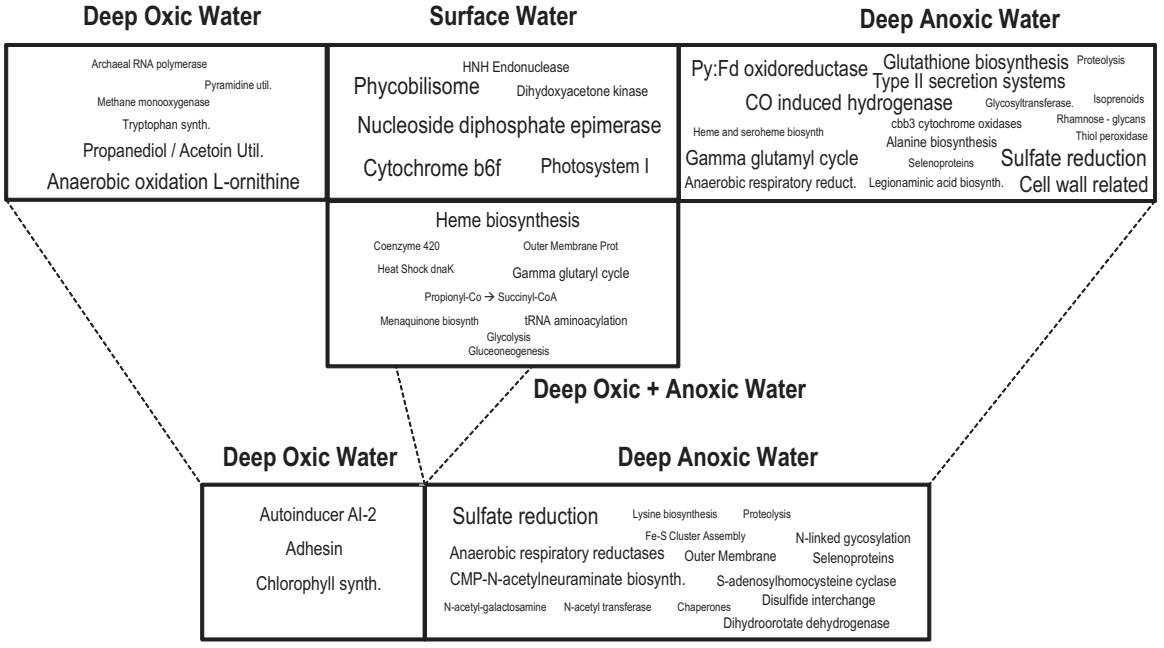

FIG 4 Graphical representation of significant changes in metatranscriptomes between surface waters and deep oxic and anoxic waters (top) and between deep oxic waters and deep anoxic waters (bottom). Significant changes were determined by comparing the expression levels in each subsystem between all samples from both 2010 and 2011 in each water state by Student *t* tests corrected for type II error with a critical *P* value of 0.0001. Only significantly changing subsystems are illustrated. Word size indicates average relative proportion of total transcripts in the sample types with maximum values. The data used to generate this representation are given in Fig. S1 in the supplemental material.

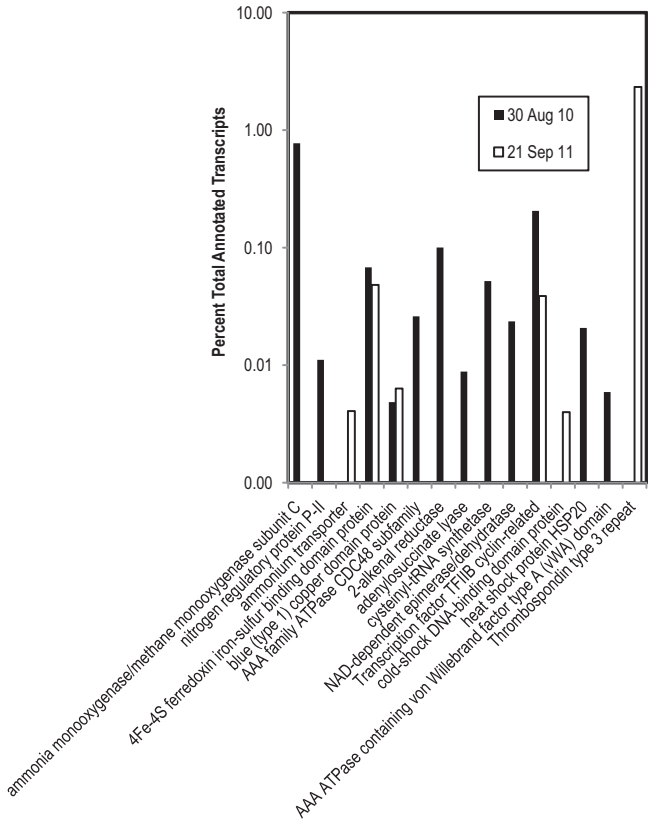

FIG 5 Reads recruiting to the *Nitrosopumilus* SCM1 gene during maximum archaeal abundance (August 2010 and August 2011). Gene frequency was calculated by recruiting reads onto the *Nitrosopumilus* SCM1 genome and then expressing total recruits as a percentage of total annotated reads.

surface waters with that in deep anoxic waters and deep oxic waters separately, we found that genes that were enhanced in expression in deep oxic and anoxic waters were primarily those involved in anaerobic metabolism, even when deep waters were oxic. For example, in deep oxic waters, we found that genes involved in anaerobic oxidation of L-ornithine, propanediol, and acetoin utilization (fermentation under anaerobic conditions) and methane (also formed under anaerobic conditions) oxidation were at higher levels than in surface waters, while in deep anoxic waters, genes involved in sulfate reduction, anaerobic respiratory reductases, glutathione biosynthesis, and high-affinity (cbb_3) cytochrome oxidases were at higher levels than in surface waters. The greater expression of these genes reflects the absence of photosynthetic processes in deep oxic and anoxic waters but also the presence of anaerobic or low-O_2-adapted energy-generating processes.

A key aim of this research was to track changes in gene expression in subpycnocline waters as they pass from oxic to anoxic and return to oxic states. Comparison of genes that changed repeatably and statistically significantly between deep oxic waters and deep anoxic waters revealed genes typical of anaerobic metabolism in deep anoxic waters. However, there were few genes that were significantly more expressed in deep oxic waters than in deep anoxic waters. Genes in deep oxic waters were those involved in biofilm initiation and development and photosynthesis (chlorophyll synthesis), while those in deep anoxic waters were primarily genes involved in respiration and anaerobic metabolism. The greatest difference between deep oxic waters and deep anoxic waters was for genes involved in sulfate reduction, anaerobic respiratory reductases, formation of cell wall material, protein degradation, and metallo- and selenoprotein formation. This is

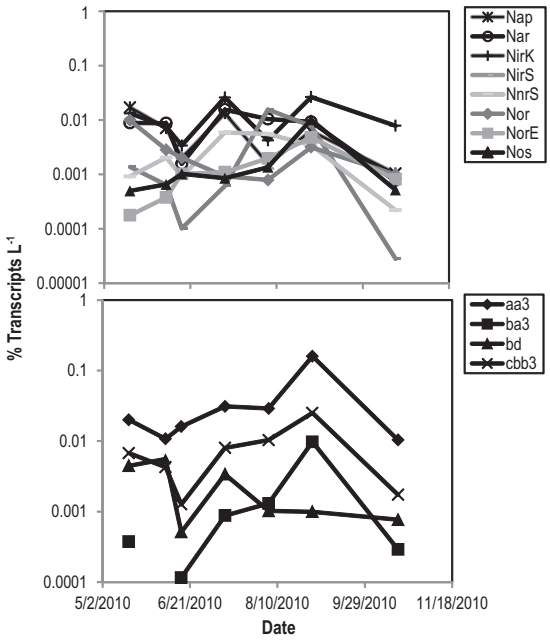

FIG 6 Expression of genes involved in the denitrification pathway, including nitrogen transformation-associated genes (top) and high- and low-affinity cytochrome oxidases (bottom). Expression values are adjusted for sample volume (variable throughout the study [see Table S1 in the supplemental material]) and expressed as a percentage of total annotated transcripts for each library and volume of sample filtered.

consistent with the observation of greater levels of high-affinity cytochrome oxidase genes in deep anoxic water than in surface waters, where low-affinity oxidase genes were in greater proportion. Other genes in deep anoxic waters that were more highly expressed than in deep oxic waters were involved in protein synthesis and degradation (proteolysis, chaperones, protein folding, and N-glycosylation) and cell wall synthesis (*N*-acetyltransferase, the glycan-chain associated CMP-*N*-acetylneuraminate, outer membrane proteins, and the gene for YbbK of undefined function within cell wall synthesis), along with genes involved in amino acid and carbohydrate metabolism (lysine synthesis and *N*-acetylgalactosamine utilization). Overall, the differential expression of these genes reflects greater anaerobic metabolism and respiratory activities in deep anoxic waters than in deep oxic waters and surface waters.

Our data provide evidence that *Archaea* have a repeating pattern of seasonal expression, with low representation during the oxic-to-anoxic transition but greater expression during the anoxic-to-oxic transition in late summer and after pycnocline breakdown. In 2010 and 2011, the relative contribution of archaeal gene transcripts increased over an order of magnitude from levels in early summer. Almost all archaeal transcripts recruited to the thaumarchaeote *Nitrosopumilus marinus* SCM1, which has been previously demonstrated as an important nitrifier in coastal waters. However, the functional compositions of the thaumarchaeote transcript pool were different in 2010 and 2011. The ammonium monooxygenase subunit C (*amoC*) gene, which is a commonly used marker gene for nitrification, was observed only in 2010 and was absent in 2011, while thrombospondin type 3 repeats were highly expressed in 2011 but absent in 2010. The function of thrombospondin in *Archaea* and *Bacteria* is in Ca^{2+} ion binding and cell adhesion. Thrombospondin protein-encoding regions also occur in isolates of *Archaea* that do not carry out nitrification, and hence there is no reason to expect that its expression in bacterioplankton is related to biogeochemical function. Archaeal genes that were shared in 2010 and 2011 include those for cyclin-related transcription factors, blue copper domain proteins, and Fe-S binding domain proteins; the last two encode electron transfer reactions and may be involved in nitrification activities. Other transcribed archaeal genes include those involved in metabolic activities that support nitrification, including ATPase, nitrogen regulatory protein P-II (which binds ATP to ammonium monooxygenase), and ammonium transport (all present in 2010 but absent in 2011). There are also genes involved in cell adhesion or nitric oxic reduction (von Wildenbrand factor), oxidoreduction (2-alkenyl reductase), purine biosynthesis, and heat shock proteins. Overall, these data suggest that while archaeal transcripts increased in relative expression in both 2010 and 2011, the stimulus for this increase may not be enhanced ammonium availability, since in 2011, metabolic genes involved in nitrification were not expressed highly.

Denitrification is a key process in nitrogen cycling under hypoxic or anaerobic conditions. Nitrate is the next most energetically favorable electron acceptor in respiration after oxygen, and hence denitrification is a sensitive indicator of low-oxygen conditions. Denitrification-related genes are widespread in bacterioplankton. However, there have been few studies of transcription of the entire suite of genes involved in the process of denitrification. In this survey, we examined transcripts of high-affinity (aa_3 and perhaps ba_3) and low-affinity (cbb_3 and *bd*) cytochrome oxidases as a proxy for oxygen levels as well as transcripts for nitrate reduction (*nar* and *nap*), nitrite reduction (*nirS* and *nirK*), nitrous oxide reduction (*nos*), nitric oxide reduction (*nor*), and accessory proteins required for optimal activity (*nnrS* and *norE*). The pattern of change in denitrification-related gene expression over the course of the season in 2010 reflected overall oxygen availability, with increasing high-affinity cytochrome oxidase gene expression with progression of anoxia and then a decrease with the reestablishment of oxic conditions, an increase in nitrate reductases early in summer and then a decline later in the period during anoxia, and then an increase in nitric and nitrous oxide reductases. These gene expression patterns reflect expected oxic-to-anoxic and anoxic-to-oxic transitions during the season.

Our results suggest that estuarine microbial communities undergo potential taxonomic and functional expression shifts between spring and late summer, where surface water and deep oxic and anoxic water communities comprise distinct transcript pools in which all communities are responsive to salinity variation and subpycnocline communities vary with oxygen concentrations between oxic, anoxic, and return-to-oxic states. These data also demonstrate that the transcript pools in microbial communities have variable representation of eukaryotic and prokaryotic constituents, likely depending on algal bloom phenomena, but that archaea have a repeatable pattern of abundance increase late in summer which is not intimately linked with nitrification activities. Finally, our data suggest that the removal of NO_3^- to N_2 is carried out by microorganisms that follow patterns of oxygen concentration in subpycnocline waters. Overall, these data illustrate the profound changes in microbial community activities that occur during the stark physicochemical condition changes that occur in summer in the stratified Chesapeake Bay and that the shift in oxic status of waters and expected change in metabolism from aerobic to anaerobic is reflected in gene transcriptional activities.

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REFERENCES

1. **Cissoko M, Desnues A, Bouvy M, Sime-Ngando T, Verling E, Bettarel Y.** 2008. Effects of freshwater and seawater mixing on virio- and bacterioplankton in a tropical estuary. Freshw. Biol. **53:**1154–1162. http://dx.doi.org/10.1111/j.1365-2427.2007.01930.x.
2. **Crump BC, Armbrust EV, Baross JA.** 1999. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary and the adjacent coastal ocean. Appl. Environ. Microbiol. **65:**3192–3204.
3. **Crump BC, Hopkinson CS, Sogin ML, Hobbie JE.** 2004. Microbial biogeography along an estuarine salinity gradient: combined influences of bacterial growth and residence time. Appl. Environ. Microbiol. **70:**1494–1505. http://dx.doi.org/10.1128/AEM.70.3.1494-1505.2004.
4. **Hewson I, Fuhrman JA.** 2004. Richness and diversity of bacterioplankton species along an estuarine gradient in Moreton Bay, Australia. Appl. Environ. Microbiol. **70:**3425–3433. http://dx.doi.org/10.1128/AEM.70.6.3425-3433.2004.
5. **Stepanauskas R, Moran MA, Bergamaschi BA, Hollibaugh JT.** 2003. Covariance of bacterioplankton composition and environmental variables in a temperate delta system. Aquat. Microb. Ecol. **31:**85–98. http://dx.doi.org/10.3354/ame031085.
6. **Hoch MP, Kirchman DL.** 1993. Seasonal and inter-annual variability in bacterial production and biomass in a temperate estuary. Mar. Ecol. Progr. Ser. **98:**283–295. http://dx.doi.org/10.3354/meps098283.
7. **Jonas RB.** 1997. Bacteria, dissolved organics and oxygens consumption in salinity stratified Chesapeake Bay, an anoxia paradigm. Am. Zool. **37:**612–620.
8. **Kirchman D, Dittel A, Findlay E, Fisher D.** 2004. Changes in bacterial activity and community structure in resonse to dissolved organic matter in the Hudson River, New York. Aquat. Microb. Ecol. **35:**243–257. http://dx.doi.org/10.3354/ame035243.
9. **Abril G, Nogueira M, Etcheber H, Cabecadas G, Lemaire E, Brogueira MJ.** 2002. Behaviour of organic carbon in nine contrasting European estuaries. Estuar. Coast. Shelf Sci. **54:**241–262. http://dx.doi.org/10.1006/ecss.2001.0844.
10. **Cottrell MT, Kirchman DL.** 2004. Single-cell analysis of bacterial growth, cell size, and community structure in the Delaware River estuary. Aquat. Microb. Ecol. **34:**139–149. http://dx.doi.org/10.3354/ame034139.
11. **Dong LF, Thornton DCO, Nedwell DB, Underwood GJC.** 2000. Denitrification in sediments of the River Colne estuary, England. Mar. Ecol. Progr. Ser. **203:**109–122. http://dx.doi.org/10.3354/meps203109.
12. **Findlay S, Pace ML, Lints D, Howe K.** 1992. Bacterial metabolism of organic-carbon in the tidal freshwater Hudson estuary. Mar Ecol. Progr. Ser. **89:**147–153. http://dx.doi.org/10.3354/meps089147.
13. **Guldberg LB, Finster K, Jorgensen NOG, Middelboe M, Lomstein BA.** 2002. Utilization of marine sedimentary dissolved organic nitrogen by native anaerobic bacteria. Limnol. Oceanogr. **47:**1712–1722. http://dx.doi.org/10.4319/lo.2002.47.6.1712.
14. **Cooper SR, Brush GS.** 1993. A 2,500-year history of anoxia and eutrophication in Chesapeake Bay. Estuaries **16:**617–626. http://dx.doi.org/10.2307/1352799.
15. **Cowan JLW, Boynton WR.** 1996. Sediment-water oxygen and nutrient exchanges along the longitudinal axis of Chesapeake Bay: seasonal patterns, controlling factors and ecological significance. Estuaries **19:**562–580. http://dx.doi.org/10.2307/1352518.
16. **Howarth RW.** 2008. Coastal nitrogen pollution: a review of sources and trends globally and regionally. Harmful Algae **8:**14–20. http://dx.doi.org/10.1016/j.hal.2008.08.015.
17. **Breitburg DL.** 1990. Nearshore hypoxia in the Chesapeake Bay: patterns and relationships among physical factors. Estuar. Coast. Shelf Sci. **30:**593–609. http://dx.doi.org/10.1016/0272-7714(90)90095-9.
18. **Diaz RJ, Rosenberg R.** 2008. Spreading dead zones and consequences for marine ecosystems. Science **321:**926–929. http://dx.doi.org/10.1126/science.1156401.
19. **Najjar RG, Pyke CR, Adams MB, Breitburg D, Hershner C, Kemp M, Howarth R, Mulholland MR, Paolisso M, Secor D, Sellner K, Wardrop D, Wood R.** 2010. Potential climate-change impacts on the Chesapeake Bay. Estuar. Coast. Shelf Sci. **86:**1–20. http://dx.doi.org/10.1016/j.ecss.2009.09.026.
20. **Bricker SB, Clement CG, Pirhalla DE, Orlando SP, Farrow DRG.** 1999. National estuarine eutrophication assessment: effects of nutrient enrichment in the nation's estuaries. National Ocean Service, Special Projects Office, and the National Centers for Coastal Ocean Science, Silver Spring, MD.
21. **Murphy RR, Kemp WM, Ball WP.** 2011. Long-term trends in Chesapeake Bay seasonal hypoxia, stratification, and nutrient loading. Estuaries Coasts **34:**1293–1309. http://dx.doi.org/10.1007/s12237-011-9413-7.
22. **Morris RL, Schmidt TM.** 2013. Shallow breathing: bacterial life at low O_2. Nat. Rev. Microbiol. **11:**205–212. http://dx.doi.org/10.1038/nrmicro2970.
23. **Jetten MSM, Sliekers O, Kuypers M, Dalsgaard T, van Niftrik L, Cirpus I, van de Pas-Schoonen K, Lavik G, Thamdrup B, Le Paslier D, Op den Camp HJM, Hulth S, Nielsen LP, Abma W, Third K, Engstrom P, Kuenen JG, Jorgensen BB, Canfield DE, Damste JSS, Revsbech NP, Fuerst J, Weissenbach J, Wagner M, Schmidt I, Schmid M, Strous M.** 2003. Anaerobic ammonium oxidation by marine and freshwater planctomycete-like bacteria. Appl. Microbiol. Biotechnol. **63:**107–114. http://dx.doi.org/10.1007/s00253-003-1422-4.
24. **Lin XJ, Wakeham SG, Putnam IF, Astor YM, Scranton MI, Chistoserdov AY, Taylor GT.** 2006. Comparison of vertical distributions of prokaryotic assemblages in the anoxic Cariaco Basin and Black Sea by use of fluorescence in situ hybridization. Appl. Environ. Microbiol. **72:**2679–2690. http://dx.doi.org/10.1128/AEM.72.4.2679-2690.2006.
25. **Jayakumar DA, Francis CA, Naqvi SWA, Ward BB.** 2004. Diversity of nitrite reductase genes (*nirS*) in the denitrifying water column of the coastal Arabian Sea. Aquat. Microb. Ecol. **34:**69–78. http://dx.doi.org/10.3354/ame034069.
26. **Dalsgaard T, Canfield DE, Petersen J, Thamdrup B, Acuna-Gonzalez J.** 2003. N_2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. Nature **422:**606–608. http://dx.doi.org/10.1038/nature01526.
27. **Casamayor EO, Garcia-Cantizano J, Pedros-Alio C.** 2008. Carbon dioxide fixation in the dark by photosynthetic bacteria in sulfide-rich stratified lakes with oxic-anoxic interfaces. Limnol. Oceanogr. **53:**1193–1203. http://dx.doi.org/10.4319/lo.2008.53.4.1193.
28. **Drake LA, Choi KH, Haskell AGE, Dobbs FC.** 1998. Vertical profiles of virus-like particles and bacteria in the water column and sediments of Chesapeake Bay, USA. Aquat. Microb. Ecol. **16:**17–25. http://dx.doi.org/10.3354/ame016017.
29. **Ulloa O, Canfield DE, DeLong EF, Letelier RM, Stewart FJ.** 2012. Microbial oceanography of anoxic oxygen minimum zones. Proc. Natl. Acad. Sci. U. S. A. **109:**15996–16003. http://dx.doi.org/10.1073/pnas.1205009109.
30. **Bryant JA, Stewart FJ, Eppley JM, DeLong EF.** 2012. Microbial community phylogenetic and trait diversity declines with depth in a marine oxygen minimum zone. Ecology **93:**1659–1673. http://dx.doi.org/10.1890/11-1204.1.
31. **Stewart FJ, Dalsgaard T, Young CR, Thamdrup B, Revsbech NP, Ulloa O, Canfield DE, DeLong EF.** 2012. Experimental incubations elicit profound changes in community transcription in OMZ bacterioplankton. PLoS One **7:**e37118. http://dx.doi.org/10.1371/journal.pone.0037118.
32. **Crump BC, Peranteau C, Beckingham B, Cornwell JC.** 2007. Respiratory succession and community succession of bacterioplankton in seasonally anoxic estuarine waters. Appl. Environ. Microbiol. **73:**6802–6810. http://dx.doi.org/10.1128/AEM.00648-07.
33. **Helbling DE, Ackermann M, Fenner K, Kohler HP, Johnson DR.** 2012. The activity level of a microbial community function can be predicted from its metatranscriptome. ISME J. **6:**902–904. http://dx.doi.org/10.1038/ismej.2011.158.
34. **Frias-Lopez J, Shi Y, Tyson GW, Coleman ML, Schuster SC, Chisholm SW, DeLong EF.** 2008. Microbial community gene expression in ocean surface waters. Proc. Natl. Acad. Sci. U. S. A. **105:**3805–3810. http://dx.doi.org/10.1073/pnas.0708897105.
35. **Hewson I, Poretsky RS, Tripp HJ, Montoya JP, Zehr JP.** 2010. Spatial patterns and light-driven variation of microbial population gene expression in surface waters of the oligotrophic open ocean. Environ. Microbiol. **12:**1940–1956. http://dx.doi.org/10.1111/j.1462-2920.2010.02198.x.
36. **Poretsky R, Hewson I, Sun S, Allen AE, Moran MA, Zehr JP.** 2009. Comparative day/night metatranscriptomic analysis of microbial com-

munities in the North Pacific subtropical gyre. Environ. Microbiol. **11:** 1358–1375. http://dx.doi.org/10.1111/j.1462-2920.2008.01863.x.
37. **Shi YM, Tyson GW, DeLong EF.** 2009. Metatranscriptomics reveals unique microbial small RNAs in the ocean's water column. Nature **459:** 266–U154. http://dx.doi.org/10.1038/nature08055.
38. **Shi Y, Tyson GW, Eppley JM, DeLong EF.** 2011. Integrated metatranscriptomic and metagenomic analyses of stratified microbial assemblages in the open ocean. ISME J. **5:**999–1013. http://dx.doi.org/10.1038/ismej.2010.189.
39. **Feike J, Jurgens K, Hollibaugh JT, Kruger S, Jost G, Labrenz M.** 2012. Measuring unbiased metatranscriptomics in suboxic waters of the central Baltic Sea using a new *in situ* fixation system. ISME J. **6:**461–470. http://dx.doi.org/10.1038/ismej.2011.94.
40. **Hollibaugh JT, Gifford SM, Sharma S, Bano N, Moran MA.** 2011. Metatranscriptomic analysis of ammonia-oxidizing organisms in an estuarine bacterioplankton assemblage. ISME J. **5:**866–878. http://dx.doi.org/10.1038/ismej.2010.172.
41. **Ottesen EA, Marin R, III, Preson CM, Young CR, Ryan JP, Scholin CA, DeLong EF.** 2011. Metatranscriptomic analysis of autonomously collected and preserved marine bacterioplankton. ISME J. **5:**1881–1895. http://dx.doi.org/10.1038/ismej.2011.70.
42. **Poretsky RS, Bano N, Buchan A, LeCleir G, Kleikemper J, Pickering M, Pate WM, Moran MA, Hollibaugh JT.** 2005. Analysis of microbial gene transcripts in environmental samples. Appl. Environ. Microbiol. **71:**4121–4126. http://dx.doi.org/10.1128/AEM.71.7.4121-4126.2005.
43. **Rinta-Kanto JM, Sun S, Sharma S, Kiene RP, Moran MA.** 2012. Bacterial community transcription patterns during a marine phytoplankton bloom. Environ. Microbiol. **14:**228–239. http://dx.doi.org/10.1111/j.1462-2920.2011.02602.x.
44. **Mason OU, Hazen TC, Borglin S, Chain PS, Dubinsky EA, Fortney JL, Han J, Holman H-YN, Hultman J, Lamendella R, Mackelprang R, Malfatti S, Tom LM, Tringe SG, Woyke T, Zhou J, Rubin EM, Jansson JK.** 2012. Metagenome, metatranscriptome, and single-cell sequencing reveal microbial response to Deepwater Horizon oil spill. ISME J. **6:**1715–1727. http://dx.doi.org/10.1038/ismej.2012.59.
45. **McCarren J, Becker JW, Repeta DJ, Shi YM, Young CR, Malmstrom RR, Chisholm SW, DeLong EF.** 2010. Microbial community transcriptomes reveal microbes and metabolic pathways associated with dissolved organic matter turnover in the sea. Proc. Natl. Acad. Sci. U. S. A. **107:** 16420–16427. http://dx.doi.org/10.1073/pnas.1010732107.
46. **Mou X, Vila-Costa M, Sun S, Zhao W, Sharma S, Moran MA.** 2011. Metatranscriptomic signature of exogenous polyamine utilization by coastal bacterioplankton. Environ. Microbiol. Rep. **3:**798–806. http://dx.doi.org/10.1111/j.1758-2229.2011.00289.x.
47. **Vila-Costa M, Rinta-Kanto JM, Sun SL, Sharma S, Poretsky R, Moran MA.** 2010. Transcriptomic analysis of a marine bacterial community enriched with dimethylsulfoniopropionate. ISME J. **4:**1410–1420. http://dx.doi.org/10.1038/ismej.2010.62.
48. **Cuvelier ML, Allen AE, Monier A, McCrow JP, Messie M, Tringe SG, Woyke T, Welsh RM, Ishoey T, Lee JH, Binder BJ, DuPont CL, Latasa M, Guigand C, Buck KR, Hilton J, Thiagarajan M, Caler E, Read B, Lasken RS, Chavez FP, Worden AZ.** 2010. Targeted metagenomics and ecology of globally important uncultured eukaryotic phytoplankton. Proc. Natl. Acad. Sci. U. S. A. **107:**14679–14684. http://dx.doi.org/10.1073/pnas.1001665107.
49. **Marchetti A, Schruth DM, Durkin CA, Parker MS, Kodner RB, Berthiaume CT, Morales R, Allen AE, Armbrust EV.** 2012. Comparative metatranscriptomics identifies molecular bases for the physiological responses of phytoplankton to varying iron availability. Proc. Natl. Acad. Sci. U. S. A. **109:**E317–E325. http://dx.doi.org/10.1073/pnas.1118408109.
50. **Stewart FJ, Sharma AK, Bryant JA, Eppley JM, DeLong EF.** 2011. Community transcriptomics reveals universal patterns of protein sequence conservation in microbial communities. Genome Biol. **12:**R26. http://dx.doi.org/10.1186/gb-2011-12-3-r26.
51. **Brewer PG, Spencer DW.** 1971. Colorimetric determination of manganese in anoxic waters. Limnol. Oceanogr. **16:**107–110. http://dx.doi.org/10.4319/lo.1971.16.1.0107.
52. **Gibbs MM.** 1979. A simple method for the rapid determination of iron in natural waters. Water Res. **13:**295–297. http://dx.doi.org/10.1016/0043-1354(79)90209-4.
53. **Parsons TR, Maita Y, Lalli CM.** 1985. A manual of chemical and biological methods for seawater analysis. Pergamon Press, Oxford, United Kingdom.
54. **Lane L, Rhoades S, Thomas C, Van Heukelem L.** 2000. Standard operating procedures 2000. Horn Point technical report. University of Maryland, Cambridge, MD.
55. **Hewson I, Poretsky R, Beinart R, White AE, Shi T, Bench SR, Moisander PH, Paerl RW, Tripp HJ, Montoya JP, Moran MA, Zehr JP.** 2009. *In situ* transcriptomic analysis of the globally important keystone N_2-fixing taxon *Crocosphaera watsonii*. ISME J. **3:**618–631. http://dx.doi.org/10.1038/ismej.2009.8.
56. **Gifford SM, Sharma S, Rinta-Kanto JM, Moran MA.** 2011. Quantitative analysis of a deeply sequenced marine microbial metatranscriptome. ISME J. **5:**461–472. http://dx.doi.org/10.1038/ismej.2010.141.
57. **He S, Wurtzel O, Singh K, Froula JL, Yilmaz S, Tringe SG, Wang Z, Chen F, Lindquist EA, Sorek R, Hugenholtz P.** 2010. Validation of two ribosomal RNA removal methods for microbial metatranscriptomics. Nat. Methods **7:**807–858. http://dx.doi.org/10.1038/nmeth.1507.
58. **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF.** 2009. Introducing mothur: open source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. **75:**7537–7541. http://dx.doi.org/10.1128/AEM.01541-09.
59. **Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Rodriguez A, Stevens R, Wilke A, Wilkening J, Edwards RA.** 2008. The metagenomics RAST server—a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics **9:**386. http://dx.doi.org/10.1186/1471-2105-9-386.
60. **McCarthy JJ, Kaplan W, Nevins JL.** 1984. Chesapeake Bay nutrient and plankton dynamics. 2. Sources and sinks of nitrite. Limnol. Oceanogr. **29:**84–98. http://dx.doi.org/10.4319/lo.1984.29.1.0084.
61. **Fuhrman JA, Hewson I, Schwalbach MS, Steele JA, Brown MV, Naeem S.** 2006. Annually reoccurring bacterial communities are predictable from ocean conditions. Proc. Natl. Acad. Sci. U. S. A. **103:**13104–13109. http://dx.doi.org/10.1073/pnas.0602399103.
62. **Kent AD, Jones SE, Yannarell AC, Graham JM, Lauster GH, Kratz TK, Triplett EW.** 2004. Annual patterns in bacterioplankton community variability in a humic lake. Microb. Ecol. **48:**550–560. http://dx.doi.org/10.1007/s00248-004-0244-y.
63. **Riemann L, Middelboe M.** 2002. Stability of bacterial and viral community compositions in Danish coastal waters as depicted by DNA fingerprinting techniques. Aquat. Microb. Ecol. **27:**219–232. http://dx.doi.org/10.3354/ame027219.
64. **Chen F, Wang K, Huang S, Cai H, Zhao M, Jiao N, Wommack KE.** 2009. Diverse and dynamic populations of cyanobacterial podoviruses in the Chesapeake Bay unveiled through DNA polymerase gene sequences. Environ. Microbiol. **11:**2884–2892. http://dx.doi.org/10.1111/j.1462-2920.2009.02033.x.
65. **Heidelberg JF, Heidelberg KB, Colwell RR.** 2002. Seasonality of Chesapeake Bay bacterioplankton species. Appl. Environ. Microbiol. **68:**5488–5497. http://dx.doi.org/10.1128/AEM.68.11.5488-5497.2002.
66. **Winget DM, Wommack KE.** 2009. Diel and daily fluctuations in virioplankton production in coastal ecosystems. Environ. Microbiol. **11:**2904–2914. http://dx.doi.org/10.1111/j.1462-2920.2009.02038.x.
67. **Yeager CLJ, Harding LW, Jr, Mallonee ME.** 2005. Phytoplankton production, biomass and community structure following a summer nutrient pulse in Chesapeake Bay. Aquat. Ecol. **39:**135–149. http://dx.doi.org/10.1007/s10452-004-4767-6.
68. **Steffen MM, Li Z, Effler TC, Hauser LJ, Boyer GL, Wilhelm SW.** 2012. Comparative metagenomics of toxic freshwater cyanobacteria bloom communities on two continents. PLoS One **7:**e44002. http://dx.doi.org/10.1371/journal.pone.0044002.
69. **Lee DY, Keller DP, Crump BC, Hood RR.** 2012. Community metabolism and energy transfer in the Chesapeake Bay estuarine turbidity maximum. Mar. Ecol. Prog. Ser. **449:**65–82. http://dx.doi.org/10.3354/meps09543.
70. **Shiah F-K, Ducklow H.** 1994. Temperature regulation of heterotrophic bacterioplankton abundance, production, and specific growth rate in Chesapeake Bay. Limnol. Oceanogr. **39:**1243–1258. http://dx.doi.org/10.4319/lo.1994.39.6.1243.