A microarray for assessing transcription from pelagic marine microbial taxa

Irina N Shilova1, Julie C Robidart1, H James Tripp2, Kendra Turk-Kubo1, Boris Wawrik3, Anton F Post4, Anne W Thompson5, Bess Ward6, James T Hollibaugh7, Andy Millard8, Martin Ostrowski8, David J Scanlan8, Ryan W Paerl9, Rhona Stuart10 and Jonathan P Zehr1

1Department of Ocean Sciences, University of California Santa Cruz, Santa Cruz, CA, USA; 2DOE Joint Genome Institute, Walnut Creek, CA, USA; 3Department of Microbiology and Plant Biology, University of Oklahoma, Oklahoma, USA; 4Marine Biological Laboratory, Woods Hole, MA, USA; 5Advanced Cytometry Group, BD Biosciences, Seattle, WA, USA; 6Department of Geosciences, Princeton University, Princeton, NJ, USA; 7Department of Marine Sciences, University of Georgia, Athens, GA, USA; 8Department of Marine Microbiology, University of Warwick, Coventry, UK; 9Marine Biology Research Division, University of California San Diego, San Diego, CA, USA and 10Physical and Life Sciences, Lawrence Livermore National Laboratory, Livermore, CA, USA

Metagenomic approaches have revealed unprecedented genetic diversity within microbial communities across vast expanses of the world’s oceans. Linking this genetic diversity with key metabolic and cellular activities of microbial assemblages is a fundamental challenge. Here we report on a collaborative effort to design MicroTOOLs (Microbiological Targets for Ocean Observing Laboratories), a high-density oligonucleotide microarray that targets functional genes of diverse taxa in pelagic and coastal marine microbial communities. MicroTOOLs integrates nucleotide sequence information from disparate data types: genomes, PCR-amplicons, metagenomes, and metatranscriptomes. It targets 19,400 unique sequences over 145 different genes that are relevant to stress responses and microbial metabolism across the three domains of life and viruses. MicroTOOLs was used in a proof-of-concept experiment that compared the functional responses of microbial communities following Fe and P enrichments of surface water samples from the North Pacific Subtropical Gyre. We detected transcription of 68% of the gene targets across major taxonomic groups, and the pattern of transcription indicated relief from Fe limitation and transition to N limitation in some taxa. Prochlorococcus (eHLI), Synechococcus (sub-cluster 5.3) and Alphaproteobacteria SAR11 clade (HIMB59) showed the strongest responses to the Fe enrichment. In addition, members of uncharacterized lineages also responded. The MicroTOOLs microarray provides a robust tool for comprehensive characterization of major functional groups of microbes in the open ocean, and the design can be easily amended for specific environments and research questions.

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Introduction

Marine microbial communities are complex, composed of diverse groups of Bacteria, Archaea, Eukaryotes and viruses. Molecular techniques frequently used in marine microbial ecology have shown strain-specific differences in genetic capabilities and transcriptional responses among the most abundant representatives of microbial communities (Fuhrman et al., 2006; Giovannoni and Vergin, 2012). Clades of Prochlorococcus in the North Atlantic have more phosphorus-acquisition strategies than clades in the North Pacific, as an adaptation to chronic phosphate limitation (Coleman and Chisholm, 2010). Coastal clades of Synechococcus have higher number of regulatory systems and the use for metals than open ocean clades, the latter being adapted to relatively constant oligotrophic conditions (Palenik et al., 2006). To link ocean processes to microbial metabolism and to build better models for predicting responses to future ocean states (Azam and Malfatti, 2007), in light of this strain-level heterogeneity, new research tools are needed that assess individual and microbial community responses.

Microarray technology can complement more commonly used molecular techniques, such as PCR and
next-generation sequencing, to provide cost-effective high-throughput gene and transcript detection from several organisms in a single sample. Microarrays have the advantages of sample replication, standardization and robust interpretations of strain-level variation in functional gene transcriptional patterns and lend themselves to better comparative quantification of specific genes and transcripts, especially in rare organisms.

Phylogenetic and functional microarrays have been developed and used for identification of microorganisms and their activity in diverse environments. The most comprehensive microbial functional microarray to date, the GeoChip 4.0, targets thousands of sequences represented by 150 genes mainly from soil microbial communities and reduction–oxidation processes (He et al., 2007, 2010; Bai et al., 2013; Zhou et al., 2013). In addition to contaminated soils, acid mine drainage sites and Antarctic soils (Yergeau et al., 2007; Mason et al., 2010; Xie et al., 2011), the GeoChip has been applied to detect microbial DNA and RNA in the marine environment (Lu et al., 2012; Wawrik et al., 2012). Aside from the PhyloChip, which targets 16S rRNA genes (Brodie et al., 2006), existing microarrays target a specific genus (Rinta-Kanto et al., 2011), a particular process (Tiquia et al., 2004; Moisander et al., 2006, 2007; Ward et al., 2007; Bulow et al., 2008; Wu et al., 2008; Bouskill et al., 2011; Abell et al., 2012) or ecosystem (Rich et al., 2008; Smith et al., 2010; Rich et al., 2011). A comprehensive microarray that targets functional genes across diverse pelagic marine microbial communities has not yet been reported.

Various strategies have been used to overcome the lack of a priori knowledge of genomic sequences in target communities, which is a major limitation in the design of an environmental microarray. The most common strategy is to search public nucleotide (nt) sequence databases (such as National Center for Biotechnology Information (NCBI)) using key words (Rhee et al., 2004; He et al., 2007; Wu et al., 2008; He et al., 2010; Smith et al., 2010). The resulting data sets, however, typically do not resemble the natural diversity of target genes, a problem that is exacerbated in undersampled environments. A second common strategy is to PCR-amplify a gene of interest and then either spot the products on a glass surface (Wu et al., 2001) or use the derived sequence data in the design of oligonucleotide probes. Although this technique results in a fingerprint for an uncharacterized community and is especially valuable for rare targets (Taroncher-Oldenburg et al., 2003; Moisander et al., 2006; Ward et al., 2007; Bulow et al., 2008), the time-consuming cloning process makes this approach suitable for studying only a limited number of genes. The large nt databases obtained with next-generation sequencing (metagenomics) present an additional opportunity to access a cross-section of the diversity of a marker gene in natural populations.

Here, we report the design and application of a high-density oligonucleotide microarray, referred to as the MicroTOOLS (Microbiological Targets for Ocean Observing Laboratories) microarray, which targets 19,400 sequences across 145 genes associated with open ocean and coastal microbial communities. The microarray is based on existing data from genomes, metagenomes, metatranscriptomes and PCR-based assays and targets functional genes responsible for biogeochemical cycling and stress responses characteristic of the oceanic photic zone. As a proof-of-concept experiment, we analyzed microbial community responses to nutrient enrichments of inorganic phosphate (P) or ferric iron (Fe) from the oligotrophic Station (Stn.) ALOHA in the North Pacific Subtropical Gyre (NPSG). The physics, chemistry and biology of this region is well characterized (Karl and Lukas, 1996; White et al., 2007), and its microbial metagenome is well represented in the nt sequence databases (DeLong et al., 2006; Frias-Lopez et al., 2008; Hewson et al., 2010). In the North Pacific, where N2 fixation is a major supply of nitrogen (Karl et al., 1997), phosphorus and iron can be the limiting nutrients for N2 fixation and primary production (Karl et al., 2001; Moore et al., 2006; Grabowski et al., 2008; Karl and Letelier, 2008; Watkins-Brandt et al., 2011). We hypothesized that if microbial taxa were Fe limited, Fe addition would result in decreased transcription of iron-stress genes and increased transcription of genes for energy, carbon and nitrogen metabolism. If microbial taxa were P limited, P addition would result in decreased transcription of P-stress genes and increased transcription of genes for energy metabolism, DNA replication and cell division. However, we expected to see heterogeneous transcriptional responses from individual taxa owing to differences in their genomic capabilities, nutrient requirements and life strategies (Tolonen et al., 2006; Dupont et al., 2008; Ilikchyan et al., 2009; Stuart et al., 2009; Tetu et al., 2009; Kamennaya and Post, 2011; Thompson et al., 2011).

Materials and methods

Design of the MicroTOOLS microarray

Target genes for oligonucleotide probe design were selected based on existing knowledge of gene markers that target microorganism interactions with their environment (for example, Lindell and Post, 2001; Webb et al., 2001; Holtzendorff et al., 2002; Chen et al., 2004; Fuller et al., 2005; Dyhrman and Haley, 2006; Zehr et al., 2007; Orchard et al., 2009; Sebastian and Ammerman, 2009; Kamennaya and Post, 2011; Mosier and Francis, 2011; Paerl et al., 2011). Several genes for hypothetical proteins that were differentially expressed in response to specific stimuli in cultured marine microorganisms were also included (Scanlan et al., 1996; Martiny et al., 2009; Shi et al., 2009; Tetu et al., 2009; Thompson et al., 2009).
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<tr>
<td>arg</td>
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<td>g20</td>
<td>Viral capsid assembly protein g20</td>
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<tr>
<td>carA</td>
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<td>5</td>
<td>gp23</td>
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<td>RdRp</td>
<td>RNA-dependent RNA pol</td>
<td>292</td>
<td></td>
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</table>

Abbreviations: DMSP, dimethylsulfoiopropionate; MicroTOOLs, Microbiological Targets for Ocean Observing Laboratories. Counts refer to gene ortholog counts on the array.

et al., 2011). A total of 145 genes provided molecular markers for metabolic and cellular processes (Table 1).

To obtain gene probes that adequately represent environmental nt diversity, we searched all marine metagenomic and metatranscriptomic databases along with sequences from clone libraries for known genes. For metagenomic and metatranscriptomic searches, a seed amino-acid sequence data set was built for selected taxa (Supplementary Material) for each targeted gene. This seed data set was used for a TBLASTN query against ‘All Sanger reads’ and ‘All 454 reads’ in the Community Cyberinfrastructure for Advanced Microbial Ecology Research and Analysis (CAMERA, http://camera.calit2.net/, Sun et al., 2011) with loose criteria: an E value cutoff of 1e-03 and up to 1000 hits per query. All TBLASTN hits with lengths > 150 nt were used as queries in reciprocal BLASTX in CAMERA to confirm gene annotation. For this analysis, an E value cutoff of 1e-10 and 60% identity over a minimum 40 amino acid alignment were used. Subsequent BLASTN searches in CAMERA were used to retrieve corresponding taxonomic information. Sequences with > 85% identity over 100 nt alignment length to targeted marine microorganisms were selected. Taxonomic affiliation means that the target sequence had the highest similarity to a specific organism by BLASTN search against the ‘nr’ database. Thus, the accuracy of affiliation to an organism depended on gene conservation and their representation in sequence databases. NimbleGen technology allows 5% nt mismatch in the whole probe region, thus sequences within a range of 95–100% nt identity to the target gene were detected. We use the phrase ‘organism-like genotype’ to refer to target gene affiliation if nt identity of the target gene to this organism was < 95%. After reciprocal BLAST, sequences originating from metagenomic libraries that contained non-transcribed regions were trimmed at 5’- and 3’-ends.
of the open reading frame region. Custom Java applications and R scripts were developed to filter all BLAST results and to trim the ends of sequences and are available upon request. Additionally, target sequences were added that derived from the clone libraries of genes (Supplementary Material) and from genomes of marine microorganisms contained in NCBI Genbank. Combined sequences were clustered using CD-HIT-EST (Li and Godzik, 2006; Huang et al., 2010) at 95% nt similarity. The longest representative sequence from each cluster was selected as the target sequence for oligonucleotide probe design. Probe design was performed at Roche NimbleGen (Madison, WI, USA), and six probes of 60-nt length were designed for each target. Random oligonucleotide probes were included in addition to standard control and alignment NimbleGen probes. All oligonucleotide probes were tested in silico for possible cross-hybridization (Supplementary Material). Before the MicroTOOLs microarray design, we tested the specificity of oligonucleotide microarray technology in detecting transcripts from a mixed community and from environmental samples. The results showed cross-hybridization for highly conserved genes (such as the photosystem II psbA gene across cyanobacteria) when nt sequences were >5% dissimilar (Supplementary Material, Supplementary Figure S1). The final design of the MicroTOOLs microarray comprised of ca. 116 000 experimental and 19 000 control probes with one replication synthesized on a 12-plex 12 × 135 K NimbleGen array. The platform is available at NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession number GPL16706.

**RNA extraction and processing for hybridization to the microarray**

RNA was extracted using the Ambion RiboPure kit (Life Technologies, Grand Island, NY, USA) with modifications that included mechanical lysis using glass beads (Supplementary Material). Extracted RNA was treated with DNase to remove genomic DNA. RNA quantity and quality were determined with a NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA) and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using the RNA 6000 Nano kit (Agilent Technologies). Only samples with RNA Integrity Number > 7.0 and ratios of A260/A230 and A260/A280 ≥ 1.8 were processed further. RNA yield from the incubations of the surface community at Stn. ALOHA ranged from 280 to 1130 ng l⁻¹, an amount insufficient for the NimbleGen microarray. cDNA was synthesized from 400 ng RNA from each sample, which was then amplified using the TransPlex Whole Transcriptome Amplification kit (WTA-1, Sigma-Aldrich, St Louis, MO, USA) and antibody-inactivated hot-start Taq DNA Polymerase (Sigma-Aldrich). The amplified cDNA was purified with the GenElute PCR cleanup kit (Sigma-Aldrich), and the quality and quantity of double-stranded (ds) cDNA was determined with NanoDrop 1000 and a 2100 Bioanalyzer using the Agilent DNA 7500 kit (Agilent Technologies). Four hundred nanograms of total RNA yielded on average 12 μg of ds-cDNA. The amplification efficiency was determined with a TaqMan quantitative PCR (qPCR) assay targeting a spike-in transcript ERCC-00116 (Invitrogen, Life Technologies). One microliter of 1:100 dilution (corresponding to 4.7 attomoles of ERCC-0016) of the ERCC (External RNA Control Consortium, Lemire et al., 2011) RNA spike-in mix 1 (Ambion) was added to RNA samples before amplification. Amplification of one replicate for Fe treatment was seven times less than the average amplification (150-fold), and the sample was excluded from microarray hybridization. The labeling and hybridization of cDNA samples (1.0 μg of ds-cDNA) to the microarray was done at the Sandler Center Functional Genomics Core Facility (University of California, San Francisco, CA, USA) according to the protocol in NimbleGen Arrays User’s Guide: ‘Gene Expression Arrays, version 6.0’.

**DNA extraction and estimation of cell abundances for diazotrophs and Synechococcus spp.**

DNA was extracted from the organic phase of the nucleic acid extract after RNA separation using the RiboPure kit (Ambion) according to the
manufacturer’s instructions (Supplementary Material). DNA yield ranged from 9.2 to 26.6 μg l\(^{-1}\). Diazotroph abundances were determined with Taqman qPCR assays targeting \(nifH\) as described in Moisander et al. (2010). *Synechococcus* spp. cell abundances were estimated using the qPCR assay targeting \(narB\) (group G) as described in Paerl et al. (2012). The Tukey’s test was used to compare the qPCR data.

**Microarray data analysis**

All data analyses were performed with R (www.R-project.org) and the Bioconductor project (Gentleman et al., 2004), specifically using the Linear Models for Microarray (LIMMA; Smyth, 2005), affy (Gautier et al., 2004) and samr (Tusher et al., 2001) packages; plots were made using gplots package. Transcription values were obtained using the robust multi-array average algorithm (Irizarry et al., 2003) and using Li–Wong across-chip normalization (Li and Wong, 2001) (Supplementary Figure S2). The signal-to-noise ratio (SNR) of each chip was calculated as: \(\text{SNR} = (S - \text{BG})/\text{BG}\); where \(S\) is the hybridization signal for the gene and \(\text{BG}\) is the chip background signal determined as average of the lowest 5% of all signals. Transcription was considered detected if SNR of a transcript was \(\geq 5\). The detection range was estimated based on the ERCC hybridization data (Supplementary Figure S3). The detection limit as percentage of total mRNA was calculated for 1000 nt long mRNA and considering that rRNA constitutes 95% of total RNA. The relative cell sensitivity limit was estimated based on the assumption of 1380 mRNA per cell (Neidhardt and Umbarger, 1996). For each group (Eukaryota, Bacteria non-picocyanobacteria, Prochlorococcus, *Synechococcus*, Viruses, Archaea), gene transcription was scaled to the median of the group in each sample. To identify differentially transcribed genes, the nonparametric method, Significance Analysis of Microarray (Tusher et al., 2001) was used with the following parameter settings: \(d = 0.3\), 100 iterations, false discovery rate (FDR) = 0.05 (Supplementary Figure S4). In addition, LIMMA (Smyth, 2005) was used with the following parameters: fold change in log, scale = 1; FDR = 0.05; \(P < 0.1\) (Benjamini–Hochberg adjusted, Benjamini and Hochberg, 1995). The separation of samples by treatment was supported by a Wilcoxon’s test (Bauer, 1972) performed with at least a 100 random resamplings of 1000 gene probes (Supplementary Figure S4). Transcription data was centered and scaled across genes, and a distance matrix was calculated by Pearson’s correlation coefficient. The distance matrix was then used in hierarchical clustering by a complete agglomeration method. Raw and normalized microarray data were prepared in accordance to the MIAME standards (Brazma et al., 2001) and submitted to NCBI GEO under accession number GSE44448.

**FRRF measurements**

Chlorophyll \(a\) variable fluorescence (Fv) and maximal fluorescence (Fm) were measured using FRRF as described in Kolber et al. (1998). FRRF measurements were taken for each sample in the beginning and after 48 h of incubation in six replications and using blue light (470 nm) for excitation. The Tukey’s test was used to compare the FRRF data.

**Results and discussion**

**Microarray design**

The MicroTOOLs microarray targets marker genes for three domains of life in marine microbial communities along with known viruses. Using our approach, a total of 19 400 target sequences representing 145 genes (Table 1) were obtained from genomes (~8%) and from metagenomes/metatranscriptomes (~92%). The design was biased toward the picocyanobacteria *Prochlorococcus* and *Synechococcus* spp. and the Alphaproteobacteria clade SAR11 sequences (Figure 1, Supplementary Table S1) owing to their high abundances in surface oceanic waters, resulting in high abundances of their nt sequences in metagenomes and metatranscriptomes (for example, DeLong et al., 2006; Hewson et al., 2010) and the fact that gene function and diversity of these microorganisms have been relatively well studied (Scanlan and West, 2002; Scanlan et al., 2009; Sun et al., 2011; Brown et al., 2012). Genes from less abundant prokaryotic microorganisms, such as from marine \(N_2\)-fixing cyanobacteria, were also included. Marine eukaryotic phytoplankton were primarily represented by the genes encoding the large subunit of RuBisCO (\(rbcL\)) and nitrate reductase (\(NR\)) (Figure 1). Additional eukaryotic genes were selected based on available genomes and/or expressed sequence tags for diatoms (Armbrust et al., 2004; Bowler et al., 2008).

Genes for marine DNA and RNA viruses included genes for DNA polymerase, major capsid protein and RNA-dependent RNA polymerase. Probes for Archaea targeted genes encoding ammonia monooxygenase, RuBisCO and urease (Supplementary Table S1).

As a proof of concept, the transcriptional responses of the surface microbial community at Stn. ALOHA to phosphate (P) or iron (Fe) amendments were analyzed using the MicroTOOLs microarray. During the time of sampling, surface waters at Stn. ALOHA had 54–79 nmol l\(^{-1}\) of P, 4–33 nmol l\(^{-1}\) of nitrate plus nitrite, 0.79–1.07 μmol l\(^{-1}\) of silicon and 67–78 ng l\(^{-1}\) of chlorophyll \(a\) (data from 5–10 m depths). Fe concentrations were not measured during the time of study, but average near-surface Fe concentration at Stn. ALOHA is 0.44 nmol l\(^{-1}\) (Boyle et al., 2005). The long-term mean P concentrations at Stn. ALOHA are 58 ± 3 nmol l\(^{-1}\) (Björkman et al., 2012), and thus, out of P and Fe, Fe was believed to be the limiting nutrient at the time of incubation, at least for the diazotrophic community.

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Microbial community transcription at Stn. ALOHA detected with the MicroTOOLS microarray
Hybridization signals above background were detected for a total of 15,507 genes (68% of the microarray set), with an average detection of 40% of target orthologs for each gene (Supplementary Table S3). The range of detection for the microarray was from 700 to 11E+06 transcript copies based on the spike-in ERCC data (Supplementary Figure S3). Calculated for a 1000-nt long mRNA, the 700 transcript copies (absolute sensitivity) corresponded to 1.8E-06% of the total community mRNA and to 0.0025% as the lowest relative abundance of cells within the community that can be detected. Average transcription from pelagophytes (Aureococcus) and prymnesiophytes (Chrysocromulina, Phaeocystis, Helicosphaera) was up to sixfold higher than the median transcription in all eukaryotes across samples (Figure 2a, Supplementary Table S2). Average transcription among prokaryotes (Prochlorococcus, Synechococcus, Proteobacteria,) was distributed around the median transcription of the corresponding transcriptome in all samples (Figure 2a, insert). The exceptions were genes related to an uncultured Prochlorococcus species represented by a fosmid clone HOTOM, which had 16-fold higher transcription than the median (Figure 2a). The most highly transcribed genes across all treatments were psaA (photosystem I), psbA (photosystem II), amt (ammonium transport), ursA (urea transporter) and rbcL (Figure 2b). Among relatively low abundance taxa, diazotrophic cyanobacteria, Trichodesmium erythraeum IMS101, Candidatus Atelocyanobacterium thalassa (Ca. A. thalassa or unicellular cyanobacterial group A, UCYN-A) and an uncultured heterocystous cyanobacterium (NCBI 112280460, Moisander et al., 2007) had high nifH (Fe-nitrogenase reductase) transcription (Supplementary Table S2). The maximum nifH transcription in these cyanobacteria occurs during the early morning hours (Church et al., 2005), when samples from incubations were collected. Another N2-fixing cyanobacterium Crocosphaera watsonii (Crocosphaera) had high transcription of pstS (high-affinity phosphate binding) and ftsZ (cell division) (Supplementary Table S2). Ca. A. thalassa and Crocosphaera cell abundances (Table 2) in the incubations ranged from 0.0002% to 0.04% of the total prokaryotic community (8.0E+08 cells l-1, Björkman et al., 2012), overlapping the estimated relative cell detection limit for the microarray (0.0025%).

In addition, gene transcripts were detected for members of the Phycodnavirus family, which infects a number of eukaryotic phytoplankton, including Micromonas (Mayer and Taylor, 1979) and Aureococcus (Milligan and Cosper, 1994) and also for cyanophages from the Myoviridae family (DNA polymerase and viral capsid genes). Overall, the genes with detected transcription reflected the composition and activity of the microbial community at Stn. ALOHA as previously described (DeLong et al., 2006; Frias-Lopez et al., 2008; Church et al., 2009; Hewson et al., 2010). Moreover, the high activity of the pelagophytes and prymnesiophytes was consistent with the detection of a eukaryotic phytoplankton bloom at that time (Björkman et al., 2012).

Transcription by members of unknown lineages
A wide diversity of genotypes of Prochlorococcus, Synechococcus and Alphaproteobacteria were detected at the study site. Prochlorococcus probes that yielded detectable signals had a wide range of nt similarity to sequenced genomes (Figures 3a and d), indicating a broad representation of the known natural genetic diversity in Prochlorococcus, as well as transcriptional activity in genotypes with no currently sequenced genome. Gene probes with detectable transcription had a median of 91% nt identity to Prochlorococcus genome sequences, such as strain CCMP1986 (Figure 3a).

Detected Synechococcus-like transcripts had a median of 88.5% similarity at the nt level to their orthologs in known genomes (Figures 3b and d). Especially high transcriptional activity (normalized transcription >2) was detected for genes similar to Synechococcus sp. RCC307, a strain from Synechococcus sub-cluster 5.3A. The low percentage of similarity to known genomes indicates the existence of an uncharacterized lineage, potentially within the sub-cluster 5.3, at Stn. ALOHA. The presence of this clade has recently been reported in the open ocean and in the Mediterranean Sea, with higher abundances in warm, low-nutrient waters (Mella-Flores et al., 2011; Post et al., 2011; Ahlgren and Rocap, 2012; Huang et al., 2012).

With a median of 80.0% similarity, probe sequences targeting Alphaproteobacteria also displayed a degree of degeneracy relative to known genome sequences (Figure 3c), suggesting that new, active strains remain uncharacterized. This is consistent with another recent study, where a new group of the SAR11 clade was proposed based on 16S rRNA gene phylogeny (Allen et al., 2012). Such uncharacterized genotypes with detectable transcription are candidates for further targeted genomic studies.

Response to nutrient amendments
Maximum chlorophyll a fluorescence (Fm) was not significantly different between each of the amendments and the control and between P and Fe amendments (P>0.2, Table 2). The lower ratio of variable to maximum fluorescence (Fv/Fm) in the Fe treatment (P<0.05) may be a result of either a shift in phytoplankton community composition or lower photosynthetic efficiency in Fe-enriched samples due to nutrient limitation (Vogel et al., 2003; Sylvan et al., 2007, 2011).
Cyanobacterial abundances increased in response to Fe amendments. Diazotrophic cyanobacteria *Crocosphaera* and *Ca. A. thalassa* and the non-diazotrophic cyanobacteria *Synechococcus* were 8, 19 and 23 times, respectively, more abundant in Fe-amended versus P-amended samples. This high increase in cell abundances in response to Fe and low response to P is consistent with previously reported variability in responses of diazotrophs to P and Fe availability in the NPSG (Zehr et al., 2007; Grabowski et al., 2008; Watkins-Brandt et al., 2011).
Differential transcriptional responses to P and Fe amendments

After 48 h of incubation, Fe and P amendments yielded significant differences in transcript levels across the microbial community despite the high variability among biological replicates. When transcription of all genes was compared, biological replicates had a weak-positive-to-weak-negative correlation (Supplementary Table S4). Strong correlations between replicates were obtained at the level of specific phylogroups (eukaryotic, Prochlorococcus and Synechococcus) and metabolic functions (energy and N metabolism) (Figure 4). Factors that may have caused discrepancies in transcription profiles between replicates include differences in biological processes within individual bottles (such as protist grazing, viral lysis) and biases of sample collection and processing.

The Significance Analysis of Microarray analysis (see Materials and methods section) identified 3742 genes as significantly differentially transcribed between treatments (FDR = 0.05) from a total of 15 507 genes with detected transcription (Supplementary Figure S5, Supplementary Table S5). Overall, the addition of Fe resulted in the increased transcription of 1699 genes encoding for N metabolism, photosynthesis, oxidative phosphorylation and ABC-type transporters (Figure 4f). The category of genes not assigned to a KEGG pathway (NA) and upregulated in the Fe amendment included genes for ammonium transport, organic P assimilation, DNA replication and cell division. The 2043 genes upregulated in the P amendment were enriched in genes for carbon fixation (Figure 4g).

It is important to note that highly transcribed genes in all samples (for example, eukaryotic nitrate reductase NR NCBI GI: GU203403 (Supplementary Table S2) oversaturated the hybridization signal, and the difference in transcription could not be estimated.

Relief from Fe limitation in oligotrophic taxa

Transcriptional patterns showed that Fe amendment resulted in relief from Fe limitation in taxa common to oligotrophic waters.
Transcription of genes for energy metabolism increased in picocyanobacteria upon Fe amendment (Figures 5a and b). Fe additions resulted in a twofold increased transcription of \textit{psaA} (photosystem I) in both \textit{Prochlorococcus} and \textit{Synechococcus} spp, with an up to fivefold increase in transcript level for the \textit{psaA} gene in the \textit{Synechococcus} RCC307-like genotype. Transcription of the \textit{coxA} gene (cytochrome c oxidase) in \textit{Prochlorococcus} eHLI and eHLII genotypes was up to fivefold higher in the Fe-amended treatment. The role of Fe as a cofactor in electron transport is consistent with increases in transcripts for energy-metabolism genes in \textit{Prochlorococcus} and \textit{Synechococcus} in Fe amendments, a pattern also observed in cultures (for example, Singh \textit{et al.}, 2003; Thompson \textit{et al.}, 2011).

This increased transcription of energy-metabolism genes was accompanied by the increased transcription of genes for cellular activity (DNA replication \textit{recA}, cell division \textit{ftsZ}) in \textit{Prochlorococcus} eHLI and eHLII genotypes was up to fivefold higher in the Fe-amended treatment. The role of Fe as a cofactor in electron transport is consistent with increases in transcripts for energy-metabolism genes in \textit{Prochlorococcus} and \textit{Synechococcus} in Fe amendments, a pattern also observed in cultures (for example, Singh \textit{et al.}, 2003; Thompson \textit{et al.}, 2011).

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The downregulation of \textit{idiA} in oligotrophic taxa such as \textit{Prochlorococcus} AS9601 (eHLII) and Alphaproteobacteria SAR11 clade strain HIMB59 (Supplementary Figure S6) in Fe amendments was consistent with the repression of the gene in Fe-replete cultures (Begg and Neilands, 1987; Webb \textit{et al.}, 2001; Smith \textit{et al.}, 2010; Thompson \textit{et al.}, 2011). In contrast, \textit{idiA} transcription by more eutrophic \textit{Synechococcus} RCC307-like (sub-cluster 5.3A, Mella-Flores \textit{et al.}, 2011) and \textit{Pelagibacter} HTCC7211-like genotypes was higher in the Fe-amendment. The differences between coastal and open ocean species have been reported before in cultures and include differences in Fe requirements and sensing (for example, Sunda \textit{et al.}, 1991; Palenik \textit{et al.}, 2006), uptake of siderophore- or porphyrin-bound Fe (Hutchins \textit{et al.}, 1999) and post-translational regulation by antisense RNA (Hernández \textit{et al.}, 2006).

This is the first report that validates differential Fe responses across a multitude of taxa in a mixed community. The relatively high (0.44 nmol l$^{-1}$) average Fe concentrations in the surface waters at Stn. ALOHA are not usually considered limiting for microbial communities (Boyle \textit{et al.}, 2005). The response to Fe amendments reported here indicates that either Fe concentrations at the time of study were lower than average or that much of this Fe was not bioavailable, at least for some taxa.
Increased N metabolism and N limitation in Fe amendments

The availability of Fe led to an increased transcription of N transport and metabolism genes (amt, urtA, nrtP, glnA, nirA, sigAII) in both Synechococcus and Prochlorococcus and possibly led to N limitation in Prochlorococcus eHL (Figure 5c, Supplementary Table S6). The observed fourfold downregulation of the rbcL transcripts in Prochlorococcus eHLI and eHLII in the Fe amendment (Figure 5d) might have resulted from N limitation (Tolonen et al., 2006). In contrast, Synechococcus spp. had twofold higher rbcL transcription in Fe amendments than in P amendments (Supplementary Table S6). We speculate that the differences in rbcL transcription were due to the ability of the majority of Synechococcus spp. to assimilate nitrate, while only few uncultured Prochlorococcus spp. have that capability (Moore et al., 2002; Martiny et al., 2009). Transcription of nitrite/nitrate-utilization genes (nrtP, nirA and narB) was detected in Synechococcus spp. and was upregulated in Synechococcus WH8109 (clade II) in Fe amendments (Supplementary Tables S2 and S6). Transcription of the narB gene was also detected for a few uncultured Prochlorococcus spp., but the precise affiliation of these genes is unknown.

While not measured directly in the incubations, nitrate/nitrite concentrations at Stn. ALOHA during incubation were relatively high. It is possible that Synechococcus spp. were able to obtain sufficient nitrogen, and thus carbon fixation in Synechococcus was not downregulated.

The eukaryotic NR gene was upregulated in Fe amendments (Supplementary Table S6) possibly resulting from increased energy production, by sufficient Fe requirements for the nitrate reductase enzyme and/or by subsequent N limitation. Although transcription of NR can be induced by the presence of nitrate alone in eukaryotes (Song and Ward, 2004; Poulsen and Kröger, 2005), the lower Fv/Fm ratio was possibly due to insufficient N (Tolonen et al., 2006) for the dominant phytoplankton in chlorophyll a measurements, which at that time were eukaryotic phytoplankton. Similar to Prochlorococcus, eukaryotic phytoplankton downregulated rbcL genes in the Fe treatment, especially two Chrysophytes, Epipyx pulchra and Ochromonas aestuarii, and two Prymnesiophytes, Chrysochromulina alifera and Chrysochromulina flava (Figure 5d). The downregulation of rbcL could be due to a reduced cellular N:C ratio in these organisms. Alternatively, if photosynthetic eukaryotic...
Phytoplankton were utilizing nitrate, they would have had to divert ATP and reductant away from carbon fixation, which would also result in decreased carbon fixation (Laws, 1991) by down-regulating \( rbcL \) transcription.

P limitation or increased P metabolism followed Fe addition

Individual taxa in the Fe treatment showed upregulated transcription of P stress-response genes, despite the generally high availability of phosphate. Transcription of \( pstS \) (the high-affinity phosphate-binding protein) in Prochlorococcus MIT9301-like genotypes (eHLII) was twofold higher in the Fe amendments in comparison to the P amendments (Supplementary Figure S6). The set of iron upregulated genes was enriched in \( pstS \) transcripts from Proteobacteria (especially, Alphaproteobacterium HIMB5) and cyanobacteria (Synechococcus WH8102) (Supplementary Table S6). The phosphonate utilization genes \( phnJ \) and \( phnA \) in Alphaproteobacteria and the alkaline phosphatase gene \( phoD \) in Gammaproteobacteria were upregulated in the Fe addition (Supplementary Figure S6, Supplementary Table S6). The 2-\( \mu \)m size fraction of phytoplankton was not P-depleted at the time of incubation (Björkman et al., 2012), but it is possible that P limitation was induced by the fast removal of P in the Fe amendment by some members of the community, such as nitrate utilizers. Alternatively, increased energy generation in microorganisms in the Fe treatment might have provided energy for phosphate acquisition and membrane translocation systems for proteins involved in alternative P source assimilation (Cembella et al., 1982; Jansson, 1988; Tetu et al., 2009). Taxa that upregulated P transport and metabolism genes were largely different from taxa that demonstrated N limitation, but manifestations of both N and P limitations occurred after Fe was supplied, suggesting Fe as a primary limiting nutrient at that time.

Figure 5 Normalized transcription for top differentially transcribed genes for (a) photosynthesis, (b) oxidative phosphorylation, (c) nitrogen metabolism and stress and (d) carbon fixation. Transcription was normalized to the mean transcription across samples.
Conclusions

This study reports the design of a high-density oligonucleotide microarray (MicroTOOLs) that targets marine microbial communities and was enabled by currently available environmental sequence data. We detected differential microbial community responses to nutrient amendments in the NPSG, ultimately demonstrating strain-specific community responses to relief of Fe stress that was followed by N or P limitation in some taxa. Such data provide a mechanistic understanding of changes in microbial communities in response to nutrient fluxes or other environmental factors.

Future technological improvements, such as automated probe design including probes for other marker genes and exploratory probes (Chung et al., 2005; Dugat-Bony et al., 2011), would improve the performance of the microarray. In addition to the utility of the MicroTOOLs array for incubation experiments, this microarray could be applied as a tool for pelagic marine microbiological studies for standardized information across study types and ocean basins. This would result in a high-resolution map of microbial genes and their transcriptional activities in the environment and provide the baseline for assessing the impacts of future perturbations of the global ocean.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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References


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