Pulsed blooms and persistent oil-degrading bacterial populations in the water column during and after the Deepwater Horizon blowout

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ABSTRACT

One of the defining features of the Deepwater Horizon oil spill was the rapid formation and persistence of a hydrocarbon plume in deep water. Here we use 16S rRNA gene clone libraries and pyrosequencing of 16S rRNA gene fragments to outline the temporal dynamics of the bacterial community in the water column near the Macondo wellhead. Our timeline starts with the pre-spill (March 2010) status of the water column bacterial community, continues through the bacterial enrichments dominating the hydrocarbon plume after the blowout (DWH *Oceanospirillales*, *Cycloclasticus*, *Colwellia* in late May 2010), and leads towards post-spill bacterial communities with molecular signatures related to degradation of phytoplankton pulses (September and October 2010; July 2011) in the water column near the Macondo wellhead. We document a dramatic transition as the complex bacterial community before the oil spill was temporarily overwhelmed by a few specialized bacterial groups responding to the massive influx of hydrocarbons in May 2010. In September and October 2010, this bacterial bloom had been replaced by a diversified bacterial community which resembled its predecessor prior to the spill. Notably, the post-plume 16S rRNA gene clone libraries and pyrosequencing datasets illustrated the continued presence of oil-degrading bacteria in the water column near the Macondo wellhead which we posit to represent an inherent signature of hydrocarbon catabolic potential to the Gulf of Mexico. The pyroseqencing results detected and tracked minority bacterial populations that were not visible in the conventional 16S rRNA gene clone libraries and allowed us to identify natural reservoirs of the Deepwater Horizon *Oceanospirillales* within and outside of the Gulf of Mexico.

1. Introduction

The explosion and sinking of the Deepwater Horizon platform discharged oil and gas into the Gulf of Mexico and generated massive and long-lasting perturbations in its ecosystem (Schrope, 2011). One of the defining features of the Deepwater Horizon oil spill was the formation of a deepwater hydrocarbon-enriched plume during the multiphase ejection of gas and oil from the wellhead. The plume was positioned between approx. 1000 and 1300 m depth due to preferential entrainment of the soluble complex hydrocarbons within the deep, cold (5 °C) water, and consisted mostly of light alkanes (C_1 to C_3), BTEX, submicrometer-size oil droplets (Ryerson et al., 2012; Reddy et al., 2012); it also entrained the dispersant compound dioctyl sodium sulfosuccinate (DOSS) (Kujawinski et al., 2011). The deep plume was detected initially in early May 2010 (Diercks et al., 2010b), and its gradual spread was monitored throughout the summer of 2010 (Hazen et al., 2010, Camilli et al., 2010, Kessler et al., 2011, Joye et al., 2011a,b) by tracking local oxygen depletion and C-DOM fluorescence maxima as proxies for the presence of hydrocarbons and microbial activity (Diercks et al., 2010b; Wade et al., 2011). However, tracking the evolving composition of the bacterial community in the oil-impacted water column, including the deep hydrocarbon plume, during 2010 was an extraordinary challenge.

Initially, changes of the microbial community in the water column were inferred from Phylochip® analyses of oil degrading communities (Hazen et al., 2010), or from models of methane, ethane and propane dynamics (Valentine et al., 2010, Kessler et al., 2011). These studies did not provide exact information on

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Table 1
Samples collected on multiple research cruises near the Macondo wellhead with dates, water depths, and geographical coordinates.

Sample names with cruise-specific sampling codes in parentheses	Ship	Date	Depth (m)	Latitude (N)	Longitude (W)
Prespill-800m	R.V. *Pelican*	March 10, 2010	800	28°50.43	88°30.29
SurfaceOil-PE5	R.V. *Pelican*	May 5, 2010	0	28°44.175	88°22.335
Plumeprofile-800m (B11)	R.V. *Walton Smith*	May 31, 2010	800	28°41.686	88°26.081
Plumeprofile-1170m (B6)	R.V. *Walton Smith*	May 31, 2010	1170	28°41.686	88°26.081
Plumeprofile-1210m (B3)	R.V. *Walton Smith*	May 31, 2010	1210	28°41.686	88°26.081
Plumeprofile-1320m (B1)	R.V. *Walton Smith*	May 31, 2010	1320	28°41.686	88°26.081
Postplume I-800m (C4B8)	R.V. *Pelican*	Sept 12, 2010	800	28°41.713	88°26.073
Postplume I-1210m (C4B4)	R.V. *Pelican*	Sept 12, 2010	1210	28°41.713	88°26.073
Postplume II (GIP22)	R.V. *Cape Hatteras*	Oct 18, 2010	1052	28°40.503	87°39.250
Postplume III (E002)	R.V. *Endeavor*	July 3, 2011	1100	28°42.177	88°21.240

sampling times, water depths and geographical positions for their molecular data. Additional 16S rRNA gene clone library datasets were recently synthesized and published with precise sampling locations and times, in order to coherently survey the changing bacterial community composition over the lifetime of the deep hydrocarbon plume (Redmond and Valentine, 2012). In late May 2010, the plume-associated bacterial community was dominated by a specific cluster within the *Oceanospirillales*, subsequently termed Deep Water Horizon (DWH) *Oceanospirillales*, before changing in mid-June to a community where most clones grouped with the genera *Cycloclasticus*, obligate degraders of aromatic hydrocarbons, and *Colwellia*, known as a genus of psychrophilic marine heterotrophic generalists. By early September, the bacterial community had diversified considerably and included different *Alphaproteobacteria*, multiple lineages within the *Gammaproteobacteria*, *Flavobacteria*, and several other phylum-level lineages such as the *Actinobacteria*, *Planctomycetes*, *Chloroflexi*, and the SAR406 cluster (Redmond and Valentine, 2012).

Here we extend the timeline of microbial oil spill response with molecular analyses of samples from March 2010 to July 2011 (Table 1). By complementing clone libraries of nearly full-length 16S rRNA genes with pyrosequencing surveys of shorter 16S rRNA gene fragments, we combine the taxonomic precision of full-length 16S rRNA genes with the high-throughout resolution of bacterial community structure enabled by pyrosequencing. Specifically, we extend previous molecular analyses in three ways. (1) The pre-spill (March 10, 2010) water column bacterial community is compared to post-spill communities (September 12 and October 18, 2010; July 3, 2011) near the Macondo wellhead with 16S rRNA gene clone libraries. (2) A water column profile near the Macondo wellhead with samples above, within and below the deep hydrocarbon plume during its *Oceanospirillales*-dominated phase (May 31, 2010) is analyzed with conventional 16S rRNA gene clone libraries and by 16S rRNA gene fragment pyrosequencing. (3) The water column profile is compared to surface water samples contaminated with weathered oil from early May 2010 (May 5, 2010), and post-plume water samples (September 12 and October 18, 2010) from near the wellhead and east of the wellhead, using pyrosequencing.

2. Materials and methods

2.1. Sampling

Surface and water column samples were obtained during six research cruises (Table 1). The pre-spill sample (March 10, 2010) was obtained on R.V. *Pelican* by CTD cast at 800 m depth, ca. 10 nautical miles northwest of the Macondo wellhead (28°50.43N, 88°30.29W). The water column did not show any of oxygen or CDOM anomalies (Fig. S1). From May 5 to 9, Oil spill surface water samples were collected via bucket sampling from the R/V *Pelican*, and kept at ca. 4 °C during and after immediate transport to Chapel Hill. Surface water sampled ca. 0.5 nautical miles from the wellhead (28°44.175N, 88°22.335W, May 5, 2010) showed the strongest admixture of reddish-brown weathered oil sludge, and was used for DNA sequencing. These surface seawater samples are to the best of our knowledge the first samples collected on the earliest Rapid Response cruise to the Deepwater Horizon response zone (May 5 to 9, 2010; Diercks et al., 2010a). CTD surveys during the second cruise leg (May 10 to 16, 2010) provided the first evidence of the southwest-trending hydrocarbon plume in the deep water column (Diercks et al., 2010b). About three weeks later, a water column profile with four depths bracketing the deepwater plume was obtained by CTD approx. 4.7 nautical miles southwest of the wellhead (R/V *Walton Smith*, May 31, 2010; 28°41.686N, 88°26.081W). Water samples of approx. 500 ml were collected at 800, 1170, 1210, and 1320 m depth. Immediately after shipboard recovery, they were filtered through 47 mm diameter and 0.22 μm poresize Anodisc filters; the filters were placed on dry ice until DNA extraction in Chapel Hill. The 1170 m and the 1210 m samples of this profile represent the deepwater hydrocarbon plume, as indicated by localized oxygen depletion and increased water column fluorescence measured during the CTD cast (Fig. S2). On September 12, almost two months after the Macondo wellhead had been capped on July 15, 2010, water column filter samples were collected again at the same location (R/V *Pelican*; 28°41.713N, 88°26.073W) to evaluate the water column bacterial community at 800 and 1210 m depth (Postplume I). CTD profiles no longer detected the *in-situ* indicators (localized oxygen depletion coinciding with fluorescence maximum) of the deep hydrocarbon plume (Fig. S3), consistent with the deepwater circulation of the Gulf of Mexico that moved the deep hydrocarbon plume in a southwesterly direction already at the onset of the spill (Diercks et al., 2010b). A negative control sample (Postplume II) was obtained 37 nautical miles east of the wellhead (28°40.503N, 87°39.250W) at a depth of 1052 m (R/V *Cape Hatteras*, October 18, 2010). Due to the predominantly west and southwest deepwater current pattern in this area, this sample was unlikely to have been in contact with the Macondo wellhead and any residual hydrocarbon leakage at this location. In July 2011, the water column near the Macondo wellhead was sampled again (July 3; R/V *Endeavor*; 28°42.177N, 88°21.240W), to initiate a multiannual survey of water column microbial community structure (Postplume III). In the home laboratory, DNA extraction from filters (Teske et al., 2011), 16S rRNA gene amplification with previously described 16S rRNA gene primers (Teske et al., 2002), and clone library construction were performed using standard methods, detailed in the supplementary information.

2.2. Phylogenetic analysis

Near-complete 16S rRNA gene sequences were analyzed using Sequencher (Gene Codes, Ann Arbor, MI) and compared to other

sequences via the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/) (Altschul et al., 1990). After construction of a general 16S rRNA alignment using the ARB phylogeny software package (Ludwig et al., 2004) and the SILVA v95 database (Pruesse et al., 2007), separate alignments for the *Gamma-* and *Alphaproteobacteria* were prepared with sequences for related *Gammaproteobacteria* and *Alphaproteobacteria*. Sequences of well-characterized pure cultures and described species were used for phylogenies whenever possible; otherwise, molecular phylotypes with an informative literature history were selected to anchor major phylogenetic branches of uncultured bacteria. Phylogenetic trees were constructed in ARB based on Jukes–Cantor distances. For bootstrap analysis, the ARB alignments were exported in PAUP-Nexus format, and the tree topologies in Figs. 2 and 3 were checked with 1000 neighbor-joining bootstrap runs based on Jukes-Cantor distances in PAUP4.0 (Swofford et al. 2000). Only bootstrap values above 70% are annotated in the phylogenetic trees. Sequences were deposited at NCBI Genbank with accession numbers JN015198 to JN015212 and JX878917 to JX879086 (Table S1).

2.3. Pyrosequencing of partial 16S rRNA gene sequences

Highly variable portions of 16S rRNA genes (*E. coli* positions 28 to 337) were amplified with five barcoded bacterial 16S-targeted primer pairs (Table S2) to generate ca. 300 bp-long PCR products. The PCR products were purified using MiniElute PCR Purification kit (QIAGEN) and stored in $1\times$ TE buffer for pyrosequencing analysis using the Roche 454 GS LFX Titanium Sequencer in the Microbiome Core Facility at the University of North Carolina at Chapel Hill (www.med.unc.edu/microbiome). Raw data were trimmed and filtered using LUCY to remove poor quality reads (minimum PHRED score of 27.5) and those of less than 200 nt (Kunin et al., 2010). The 8 nt barcode was used to de-multiplex and assign reads to samples using QIIME (Caporaso et al., 2010). The reads were binned into operational taxonomic units (OTUs) at 97% sequence identity with UCLUST (Edgar, 2010) followed by selection of a representative sequence based on the most abundant unique read within each cluster. After initial phylum- and family-level identification using BLAST, the 300-bp fragments were imported and aligned into ARB, using the previously prepared full-length 16S rRNA gene alignments of the water column sequences, and related published sequences, as templates. In addition, the gamma- and alphaproteobacterial alignments were manually edited, and $>90\%$ of all pyrosequencing fragments could be assigned to genus- or family-level phylogenetic branches defined by 16S rRNA gene clone library sequences (Table S3). Sequence data were submitted to the European Nucleotide Archive Sequence Read Archive under the study accession number ERP002443.

3. Results and discussion

3.1. Bacterial community timeline

The timeline of bacterial community composition in the aftermath of the Deepwater Horizon blowout reveals a complex pattern of microbial community succession within the oil and gas-impacted water column of the Gulf of Mexico. The baseline for bacterial community composition in the Gulf of Mexico water column on the eve of the Deepwater Horizon blowout is accessible thanks to a serendipitous water sample, collected on March 10, 2010 at 800 m depth at the Mississippi Canyon 118 Microbial Observatory, ca. 9 nautical miles northwest of the Macondo wellhead (Table 1). The 16S rRNA clone library results indicated a water column bacterial community where SAR11 and other *Alphaproteobacteria*, the SAR 406 lineage, the deltaproteobacterial SAR324 lineage, and a complex gammaproteobacterial assemblage of cultured and uncultured lineages, often within the families *Oceanospirillales* and *Alteromonadales*, constituted the dominant proportion (76%) of all clones. Other phyla, such as *Chloroflexi*, *Bacteroidetes*, *Acidobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Gemmatimonadetes* and *Cyanobacteria* were also present (Fig. 1). This bacterial community matches, in phylum-level composition and in relative abundance of the major community members, the open-ocean Atlantic and Pacific bacterial communities from the same depth (800 m), as determined by single-cell genome amplification and sequencing (Swan et al., 2011) (Fig. S4). Thus, the pre-spill deepwater column near the Macondo wellhead shared the microbial community of the ultimate source reservoir of the Gulf of Mexico, the Atlantic Ocean.

In the course of the oil spill, this complex bacterial community was temporarily overprinted by blooms of opportunistic bacteria that responded to the massive influx of hydrocarbons. The pre-plume bacterial 16S rRNA gene clone library contrasted sharply with the bacterial community composition of the oil-contaminated surface water sample (May 5, 2010) and the hydrocarbon-enriched deepwater plume samples (May 31, 2010). The 16S rRNA gene and pyrosequencing analyses of oil slick-contaminated surface water samples collected shortly after the beginning of the discharge (May 5–9 2010; R.V. *Pelican*) demonstrated rapid colonization of the surficial oil slick-seawater mixture by PAH-degrading bacteria of the genus *Cycloclasticus*, by oil-degrading members of the genera *Pseudoalteromonas*, *Alteromonas* and *Colwellia*, and by other heterotrophic bacterial groups (Fig. 1). This microbial community formed extensive flocs of microbial exopolymeric substances (EPS), observed in the field as microbial flocs developed ubiquitously in the oil-contaminated surface waters in early May 2010 (Passow et al., 2012), and in the laboratory in roller table bottle incubations using fresh oil slick samples and Gulf of Mexico surface water (Ziervogel et al., 2012). Sinking flux of these oil slick-derived microbial EPS flocs exported the associated microbial communities into the deep Gulf of Mexico (Passow et al., 2012).

The clone libraries and pyrosequencing datasets from deep hydrocarbon plume samples (1170 and 1210 m depth) collected on May 31, 2010, were strongly dominated by members of the DWH *Oceanospirillales* cluster; *Cycloclasticus* and *Colwellia* were detected as the most substantial minority population in the pyrosequencing datasets (Fig. 1; Table S3). The pyrosequencing datasets detected many bacterial groups in the plume layer that were not visible in the clone libraries, such as Deltaproteobacteria and the SAR406 lineage. The *Oceanospirillales*-dominated enrichment within the plume layer contrasted with the bacterial communities above and below the deep hydrocarbon plume (800 and 1320 m) that resembled the pre- and post-plume clone libraries by the presence – in variable proportions – of SAR11 and other *Alphaproteobacteria*, *Gammaproteobacteria*, and SAR406; these samples above and below the plume also showed unusually high clone library representation of *Actinobacteria* (14% and 11%), *Planctomycetes* (8% in both depths), and uncultured *Deltaproteobacteria* (5% and 13%). Similar bacterial groups were recovered by pyrosequencing (Fig. 1).

Based on sampling time and location, these water column samples are congruent with previous sampling surveys and bacterial community analyses of the well-documented deep hydrocarbon plume near the Macondo wellhead. Hazen et al. (2010) reported that uncultured members of the gammaproteobacterial order *Oceanospirillales* dominated 16S rRNA gene clone libraries in the deepwater plume between 1100 and 1220 m depth at the end of May 2010 (May 25 to June 2). Subsequent single-cell genome sequencing of two *Oceanospirillales* single cells revealed that they possessed genes involved in the degradation of *n*-alkanes

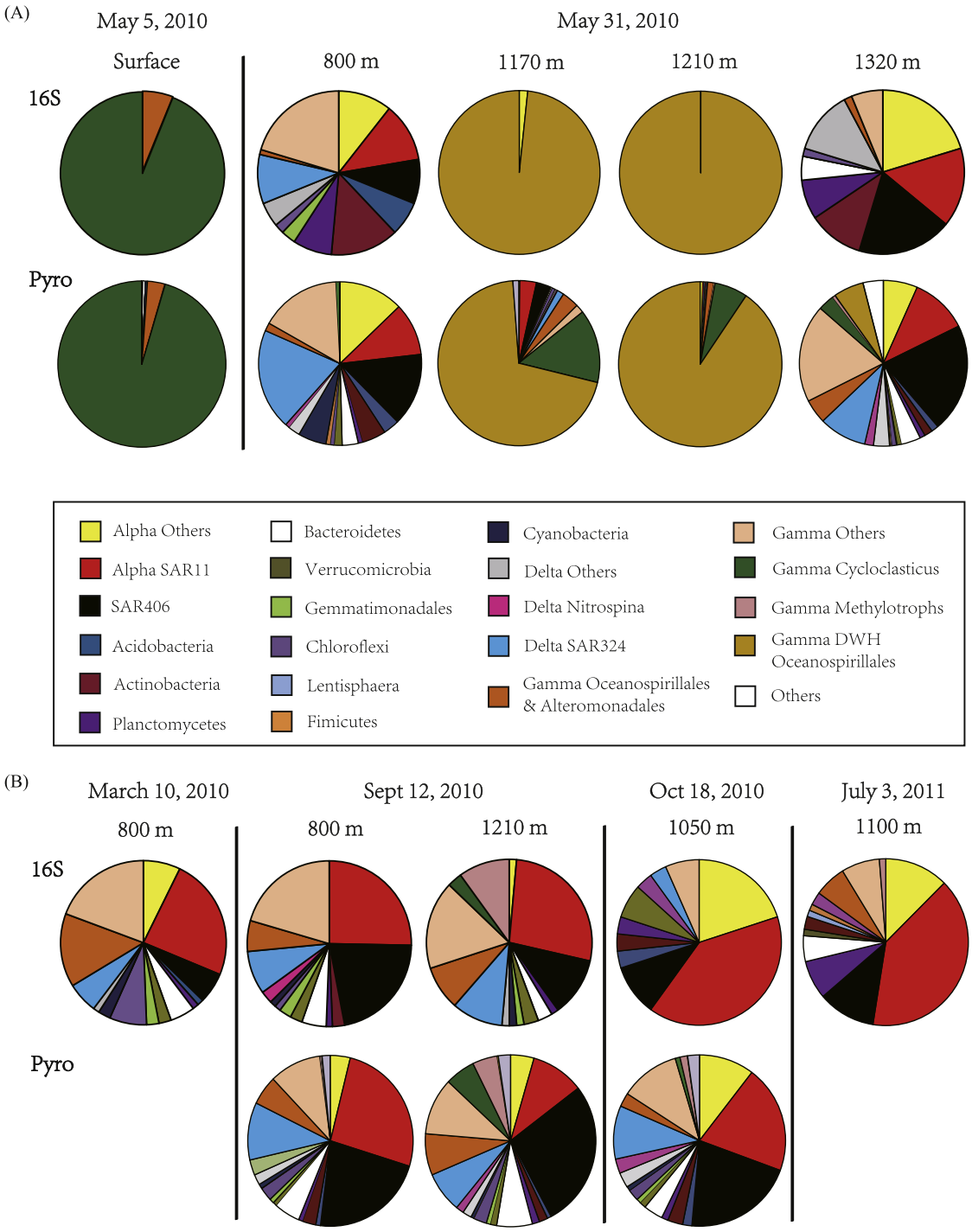

Fig. 1. Pie charts of phylum- and subphylum composition of bacterial 16S rRNA gene clone libraries and bacterial 300-bp pyrosequencing fragments from the Gulf of Mexico water column near the Macondo wellhead. Within the *Gammaproteobacteria*, the genus *Cycloclasticus*, the methylotroph-affiliated lineages, the DWH *Oceanospirillales*, and other *Oceanospirillales* and *Alteromonadales* are highlighted at family- or genus-level resolution. (A) Surface water sample collected on May 5, 2010; hydrocarbon plume water column samples near Macondo wellhead from 800, 1170, 1210, and 1320 m depth collected on May 31, 2010. (B) Pre-plume March 2010 sample from 800 m depth near MC118; water column samples from 800 and 1210 m depth near Macondo wellhead, collected September 12, 2010; water column samples from October 18, 2010, and July 3, 2011. The upper pie charts in (A) and (B) show 16S rRNA gene clone library composition, the lower pie charts show the corresponding pyrosequencing results.

and cycloalkanes (Mason et al., 2012). This genomic potential of the DWH *Oceanospirillales* is also consistent with the physiological capabilities of their close cultured relatives, *Thalassolituus oleivorans* (Yakimov et al., 2004) and *Oleispira antarctica* (Yakimov et al., 2003), which oxidize long-chain *n*-alkanes aerobically (Fig. 2). Alkane oxidation remains to be checked in the cultured marine relatives *Bermanella marisrubri* (Pinhassi et al. 2009), *Spongiispira norvegica* (Kaesler et al. 2008), and *Oceanoserpentilla haliotis* (Schlösser et al. 2008). An integrated physiological and genomic characterization of the DWH *Oceanospirillales* in the context of its cultured relatives should allow for a more finely resolved taxonomic designation of this group, beyond order level.

Previous analyses show that the hydrocarbon plume had a strong enrichment effect on many heterotrophic genera of marine *Gammaproteobacteria*, whose 16S rRNA gene frequency had increased by 100 to 300% within the plume (Hazen et al., 2010);

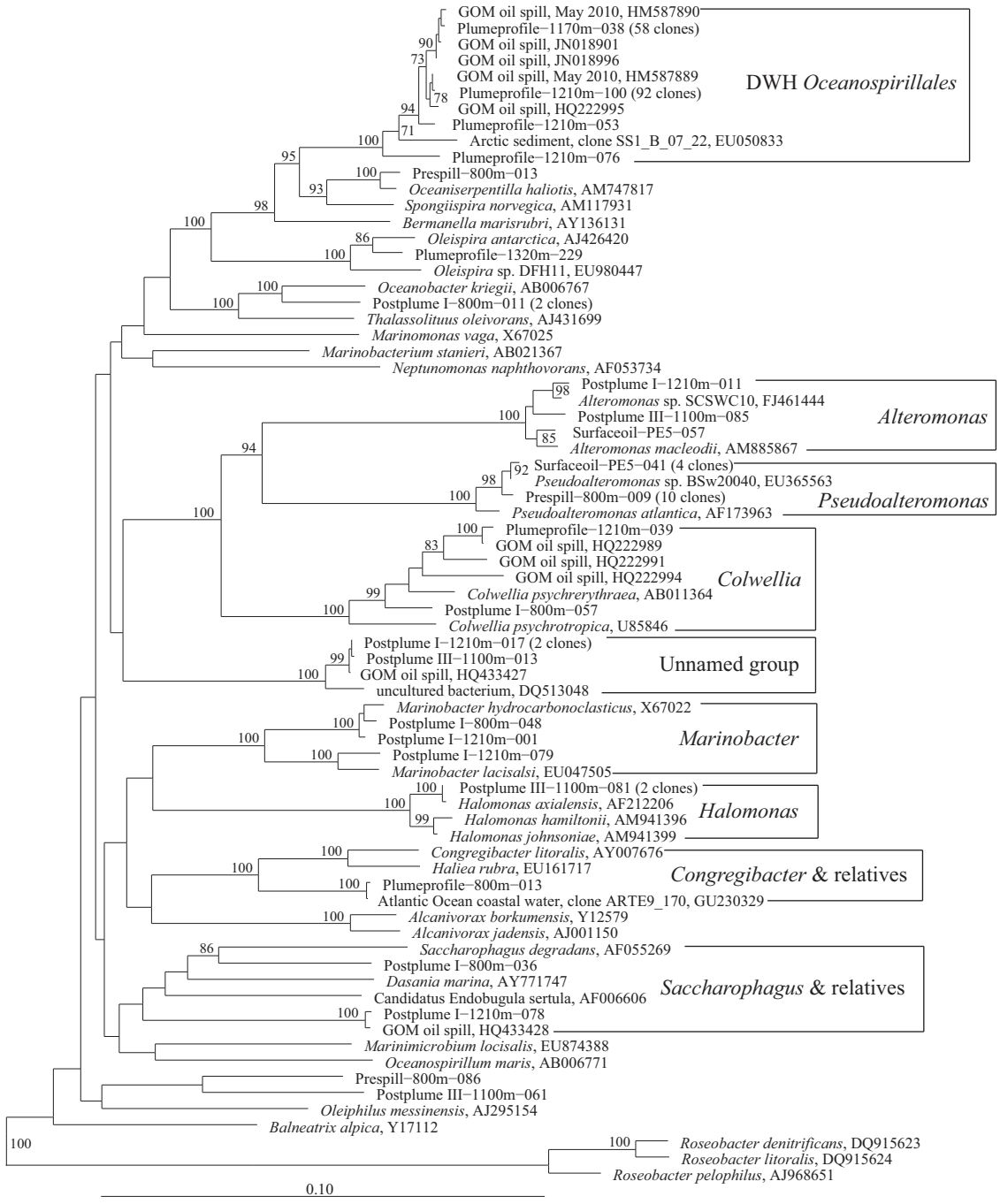

Fig. 2. Phylogeny of *Gammaproteobacteria* (*Oceanospirillales* and *Alteromonadales* including DWH *Oceanospirillales*) in the Gulf of Mexico water column near the Macondo wellhead, based on near-full length 16S rRNA genes. Clones from the pre-spill water column sample (March 10, 2010) are labeled "Prespill"; clones from surface oil slicks (May 5, 2010) are labeled "Surfaceoil"; clones from plume water column samples (May 31, 2010) are labeled "Plumeprofile". Clones from September 12 and October 18, 2010, and from July 3, 2011, are labeled Postplume I, II and III, respectively. The clone designations are followed by sampling depth in meters, and a 3-digit clone ID (Table S1). The scale bar corresponds to 10% sequence distance.

subsequent microarray-based phylochip analysis of DNA from hydrocarbon plume samples showed increased normalized signal intensity for functional genes involved in hydrocarbon degradation, especially alkane-1 monooxygenase among the alkane and cycloalkane-degrading genes, and a wide spectrum of dehydrogenases, dioxygenases and decarboxylases involved in aromatic carboxylic acid degradation (Hazen et al., 2010; Lu et al., 2012). Most likely, source populations for these genes include cultured heterotrophs and hydrocarbon-degrading bacteria that were found in our 16S rRNA gene surveys either in plume or post-plume samples, such as *Marinobacter*, *Alteromonas*, *Oleispira*, *Oceanobacter*, *Cycloclasticus*, and uncultured sister lineages of the genera *Saccharophagus*, *Congregibacter* and *Fangia*.

The detection of *Cycloclasticus* and *Colwellia* spp. in our pyrosequencing surveys of the plume samples (May 31, 2010) is consistent with the previously published clone library detection of these genera in plume samples from May 26 to June 5 (Redmond and Valentine, 2012), and shows that these two oil-degrading genera co-occurred with DWH *Oceanospirillales* in the deep plume (Fig. 1). In plume samples collected two weeks later (June 13 to 16, 2010), 16S rRNA gene phylotypes of the genera *Cycloclasticus* and *Colwellia* predominated (Redmond and Valentine, 2012); these genera were

discussed as bacterial catalysts of the dominant oxygen-consuming process, ethane and propane oxidation, in the deep-water plume (Valentine et al., 2010). This interpretation contrasts with the known substrate spectrum of *Cycloclasticus*, a genus described originally as aerobic degraders of polycyclic aromatic hydrocarbons (Dyksterhouse et al., 1995). *Cycloclasticus* remains recognized as an obligate degrader of these compounds (Yakimov et al., 2007); several *Cycloclasticus* strains were previously isolated from Gulf of Mexico sediments by enrichment with PAH substrates (Geiselbrecht et al., 1998). Thus, a likely role for *Cycloclasticus* is the degradation of BTEX compounds in the plume. The moderately psychrophilic genus *Colwellia*, consistently present in plume- and post-plume samples (Fig. 2), was selectively enriched on crude oil at 4°C (Redmond and Valentine, 2012) and was capable of oil degradation at in-situ temperatures of 5 °C (Bælum et al., 2012), consistent with the in-situ temperature of the deep Gulf of Mexico water column. Viewed in context, the bacterial community in the deep plume apparently changed within two weeks from being dominated by DWH *Oceanospirillales* in late May to becoming dominated by *Colwellia* and *Cycloclasticus* in mid-June (Valentine et al., 2010; Redmond and Valentine, 2012).

By mid-September 2010, oxygen depletion signals, CDOM fluorescence and DOSS concentrations showed that the slowly decaying deep hydrocarbon plume drifted in a generally west-southwesterly direction away from the Macondo wellhead area (Kessler et al., 2011; Kujawinski et al., 2011); this is consistent with our CTD profile of the water column near the Macondo wellhead, recorded on Sept 12 2010, that lacks hydrocarbon plume signatures (Fig. S3). The post-plume 16S rRNA gene clone libraries and pyrosequencing surveys of September and October 2010, and the 16S rRNA gene clone library of July 2011 shared dominant bacterial groups with the clone library of March 2010, indicating a partial recovery towards the pre-spill bacterial community. The SAR11 *Alphaproteobacteria*, the SAR406 lineage, the deltaproteobacterial lineage SAR324, and a complex assemblage of *Gammaproteobacteria* dominated the clone libraries and accounted together for 81 to 88% of all post-plume clones. The *Planctomycetes*, *Bacteroidetes*, *Verrucomicrobia*, *Actinobacteria*, *Chloroflexi* and *Gemmatimonadetes* accounted for smaller proportions or remained undetected in some samples (Fig. 1).

Not only dominant phylum-level lineages, but also specific pelagic alpha- and gammaproteobacterial lineages, reappeared in post-spill clone libraries: the SAR11 subclusters (Field et al., 1997; Fig. S5); the Arctic96BD-19 group of sulfur-oxidizing heterotrophs (Marshall and Morris, 2013) that is prevalent in stratified, oxygen-depleted conditions (Walsh et al., 2009); the uncultured AGG47 cluster associated with marine snow (DeLong et al., 1993); the uncultured North Sea ZD0417 cluster (Stevens and Ulloa, 2008), and the uncultured SAR156 lineage (Mullins et al., 1995) (Fig. 3). The widely distributed SUP05 lineage, a presumable sulfur oxidizer typical of oxygen-depleted water columns (Walsh et al., 2009; Canfield et al., 2010), was found during and after the plume stage.

3.2. Pyrosequencing results for surface oil slick and the plume-impacted water column

The pyrosequencing results for the weathered oil mixture at the surface from May 5, 2010, and the water column samples of May 31, 2010 were broadly consistent with the 16S rRNA gene clone libraries for the same samples (Fig. 1), but in addition revealed bacterial populations that had remained undetected in the clone libraries (Table S3). In the surface sample, pyrosequencing representation for *Cycloclasticus* (> 93%), *Alteromonas* (1.45%) and *Pseudoalteromonas* (1.2%) resembled the clone library results, whereas *Colwellia* and *Halomonas* were detected in smaller proportions (Table S1). In contrast, the alkane-degrading DWH *Oceanospirillales* accounted for near 90 and 70% of the pyrosequencing reads in the two deep plume samples of late May 2010 (Table S3).

The DWH *Oceanospirillales* pyrosequencing reads were congruent with full-length 16S rRNA gene clones of DWH *Oceanospirillales* from the Gulf of Mexico (Redmond and Valentine, 2012) and from the Atlantic Ocean offshore North Carolina (D'Ambrosio, 2011), and formed at least three distinct phylogenetic clusters (Fig. 4). The pyrosequencing survey also validated a diverse community of hydrocarbon-degrading bacteria in the plume profile that went largely undetected in the clone libraries (Table S3). The PAH-degrading genus *Cycloclasticus* remained variably detectable throughout the water column. Psychrophilic heterotrophs of the genus *Colwellia* (the only group detected in the plume clone libraries besides the DWH *Oceanospririllales*) accounted for approx. 1 to 3% of the pyrosequencing reads within the plume. The alkane-degrading genera *Oleiphilus* and *Oleispira* were found in low abundances below and within the plume. The pyrosequencing representation of the uncultured gammaproteobacterial groups (AGG47, Arctic96BD19, SUP05, ZD0417, SAR156) above and below the plume was strongly reduced within the plume (Table S3). A similar trend was observed for *Alphaproteobacteria*. While SAR11 bacteria accounted for a tenth of the pyrosequencing fragments above and below the plume, their representation decreased within the plume (Fig. 1). In general, pyrosequencing analysis indicated a functionally and phylogenetically diversified alpha- and gammaproteobacterial community in the hydrocarbon plume; pre-spill populations of uncultured bacteria and oil-degrading bacteria remained detectable against the dominant plume populations of DWH *Oceanospirillales*. This result is compatible with a complex functional gene repertoire of plume microbial communities sampled at the same time (Lu et al., 2012).

3.3. Pyrosequencing results for the post-plume water column

The pyrosequencing results for the post-plume water column samples of September 12, 2010, and October 18, 2010, were broadly consistent with the corresponding 16S rRNA gene clone libraries (Fig. 1), but revealed additional bacterial populations that had not been observed in the clone libraries (Table S3). The DWH *Oceanospirillales* that had disappeared from the clone libraries remained detectable at low levels in the pyrosequencing dataset (up to 0.2% at 1200 m, Sept. 12 sample). Interestingly, the post-plume pyrosequencing datasets showed that oil-degrading bacteria persisted in the water column near the Macondo wellhead, although the deep hydrocarbon plume had been drifting in a southwesterly direction, and was no longer detectable in the wellhead region as indicated by CTD profiling in September 2010 (Fig. S3). Bacterial alkane degraders (*Alcanivorax*, *Oleiphilus*, *Marinobacter*) remained detectable in low proportions (< 1%), and the PAH oxidizer *Cycloclasticus* and relatives of gammaproteobacterial methylotrophs accounted for near 5% of pyrosequencing reads in the 1210 m sample (Table S3). These results suggest local sources that re-inject reservoir populations of these bacteria into the water column, either from small-scale accidental leakage, natural hydrocarbon seepage (Joye et al. 2011b), or resuspension and import of oil-impacted particles (Passow et al. 2012).

Most pyrosequencing fragments from the post-plume water column do not represent specialized oil degraders; these pyrosequencing results resemble (and extend) the diversified 16S rRNA gene clone library results for the same samples. Within the *Gammaproteobacteria*, the cultured genera *Oceanobacterium*, *Oceanobacter*, *Oceanospirillum*, *Alteromonas*, *Pseudoalteromonas*, *Halomonas*, *Idiomarina*, *Marinimicrobium*, *Congregibacter*, were complemented by uncultured water column lineages (two different AGG47 clusters; Delong et al., 1993; Arctic96BD19 and SUP05, Walsh et al., 2009;

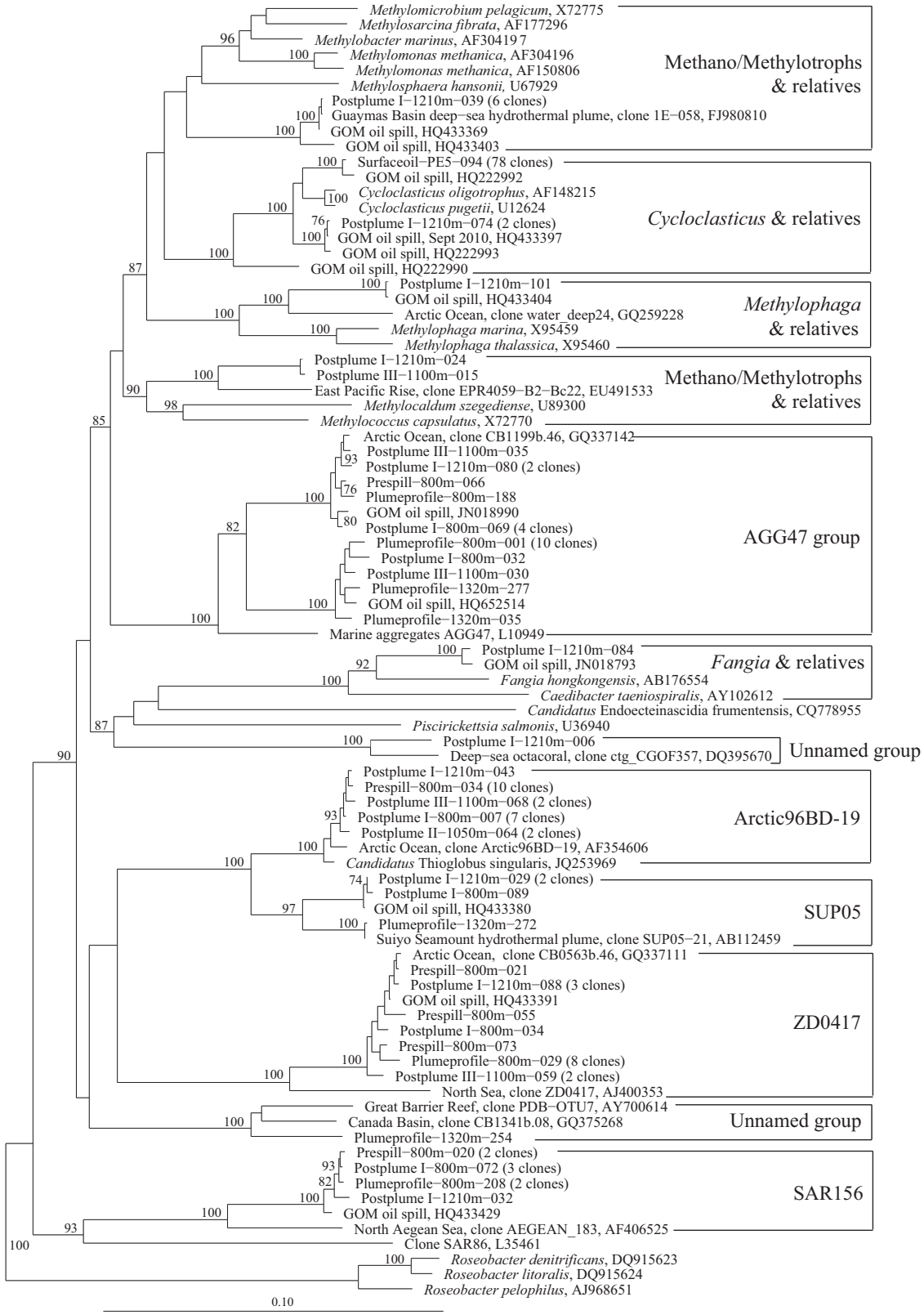

Fig. 3. Phylogeny of *Gammaproteobacteria* (Uncultured lineages, *Cycloclasticus* and methaneotrophs/methylotrophs) in the Gulf of Mexico water column near the Macondo wellhead, based on near-full length 16S rRNA genes. Clones from the pre-spill water column sample (March 10, 2010) are labeled "Prespill"; clones from surface oil slicks (May 5, 2010) are labeled "Surfaceoil"; clones from plume water column samples (May 31, 2010) are labeled "Plumeprofile". Clones from September 12 and October 18, 2010, and from July 3, 2011, are labeled Postplume I, II and III, respectively. The clone designations are followed by sampling depth in meters, and a 3-digit clone ID (Table S1). The scale bar corresponds to 10% sequence distance.

SAR156, Mullins et al., 1995; a ZD0417-related group, Stevens and Ulloa, 2008). Within the *Alphaproteobacteria*, relatives of the genera *Oceanibaculum* and *Roseobacter*, of the *Rhizobiales*, *Rhodoplanes*, *Rhodospirillales*, *Sphingomonadales*, several uncultured clusters, and the SAR11 lineage (the latter in the 10 to 25% range) were found in all post-plume samples (Table S3). The *Deltaproteobacteria* (dominated

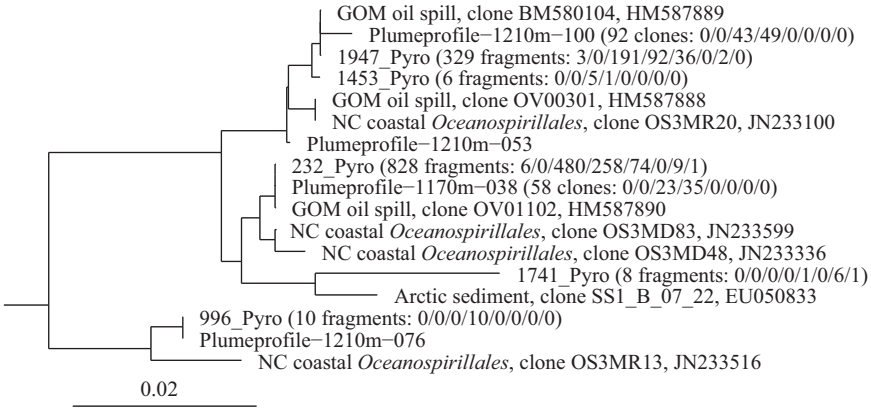

Fig. 4. Phylogeny of DWH *Oceanospirillales* based on 300 bp pyrosequencing fragments and corresponding sections of 16S rRNA gene clones, showing the phylogenetic fine structure of this cluster. The phylogeny was obtained with an alignment mask that excluded all sequence regions except *E. coli* 16S rRNA gene positions 28-337, equivalent to the pyrosequencing fragment. The tree was rooted with the gammaproteobacterial North Sea clone ZD0417 (AJ400353). The number of occurrence for each type of pyrosequencing fragment and 16S rRNA gene clone in the different samples is listed in brackets in the following order: Surface sample; Plume profile at 800 m; Plume profile at 1170 m; Plume profile at 1210 m; Plume profile at 1320 m; Postplume I at 800 m; Postplume I at1210 m; Postplume-II at 1050 m. The scale bar corresponds to 2% sequence distance.

by SAR324) and the SAR406 lineage accounted for ca. 10 to 25% of the pyrosequencing dataset, similar to their representation in the 16S rRNA clone libraries (Fig. 1). A wide range of phylum-level lineages, the *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Gemmatimonadetes*, *Planctomycetes*, and *Verrucomicrobia* accounted for approx. 0.5% to 5% of the pyrosequencing reads (Table S3), and appeared to a limited extent in the corresponding clone libraries (Fig. 1). Other phylum-level lineages (Candidate Division OD1, *Epsilonproteobacteria*, *Lentisphaerae*) were barely detected in the pyrosequencing dataset, and were not observed in the clone libraries (Table S3).

3.4. Contrasting interpretations of the post-plume bacterial community

The interpretation of bacterial communities in the water column of late summer 2010 remains contested. Bacterial 16S rRNA gene clone libraries from post-plume water column samples (Sept. 7 to 17, 2010) detected diverse *Alpha-* and *Gammaproteobacteria*, *Flavobacteria*, *Chloroflexi*, and *Planctomycetales* (Kessler et al., 2011); the *Gammaproteobacteria* included *Cycloclasticus*, members of the *Oceanospirillales* (not the DWH group), and members of the *Methylophilaceae*, *Methylococcaceae* and the genus *Methylophaga*. The latter three lineages constitute a phylogenetic assemblage of C_1-oxidizing marine bacteria; this assemblage was regarded as evidence for bacterial methane oxidation as the dominant hydrocarbon-degrading process in the water column during the decay of the deep plume (Kessler et al., 2011), although re-examination of the clone libraries and comparison with substrate spectra of cultured C_1-oxidizing bacteria suggested that methylotrophy was at least as likely (Joye et al., 2011b). The phylogenetic analysis of these clones and their closest matches reported here shows that they are not representatives of cultured methylotrophic and methanotrophic genera. Instead, they form two separate sister lineages to the methylo- and methanotrophic genera *Methylobacterium*, *Methylosarcina*, *Methylobacter*, *Methylomonas*, and *Methylosphaera*, and to the separately branching, obligately methylotrophic genus *Methylophaga* (Fig. 3). If these uncultured bacteria represent methylotrophs or methanotrophs, they would constitute new genera with potentially novel physiological properties. Assuming that these uncultured lineages represent C_1-oxidizing bacteria, the sampling campaign appears to have caught the last stages of a methanotrophic bacterial bloom that pushed the methane concentrations to below typical Gulf of Mexico ambient levels at the time of sampling in September 2010 (Kessler et al., 2011). However, alternative interpretations are possible. Transcriptomics studies that explored the impact of high molecular weight dissolved organic matter on microbial community structure and activity showed a selective enrichment of marine heterotrophs within the *Gamma-* and *Alphaproteobacteria* (*Alteromonas*, *Thalassobius*) and gammaproteobacterial methylotrophs (*Methylophaga*) after a short incubation time (27 h) under DOM-amendment (McCarren et al., 2010). These strains could be enriched in consequence of a DOM-degrading heterotrophic cascade that releases naturally abundant methylated sugars from DOM, and leads to the frequently observed high abundance of methylotrophic bacteria in clone libraries from DOM-rich coastal waters (McCarren et al., 2010). In this interpretation, the combined presence of DOM-degrading methylotrophic and heterotrophic *Gammaproteobacteria* and *Alphaproteobacteria* marks the microbial degradation of a DOM pulse; this explanation is consistent with dissolved oxygen and fluorescence anomalies and the lack of detectable methane at the sampling stations that yielded this bacterial signature (Kessler et al., 2011). The methylotroph-related clones disappeared from the October 2010 clone library, but reappeared in July 2011 (Figs. 1 and 3). Methylotroph-related sequences remained detectable among the pyrosequencing reads in September and October 2010 (Table S3). Their continued occurrence near the Macondo wellhead and in other widely dispersed marine habitats (for a high-arctic example see Teske et al., 2011) may not be specifically linked to methanotrophy or methylotrophy sustained by fossil hydrocarbons; seasonal phytoplankton blooms provide an alternative explanation that requires systematic investigation.

3.5. Natural reservoirs of DWH Oceanospirillales

The rapid enrichment of specific bacterial types associated with the deep hydrocarbon plume indicates the existence of easily accessible natural reservoirs or seed populations of these bacteria in the Gulf of Mexico. Identifying their natural reservoir is of particular interest toward a more complete understanding of their ecology and adaptability to a massive and prolonged input of oil. The DWH *Oceanospirillales*, for example, lacked closely related representatives in Genbank when first reported (Hazen et al., 2010). The closest relatives in GenBank (EU050833) were a clone from Arctic marine sediments (Tian et al., 2009) and cultured sister groups within the *Gammaproteobacteria*, including the

hydrocarbon degraders *Oleispira* and *Thalassolituus*, and the genera *Bermanella*, *Spongiispira* and *Oceanoserpentilla* (Hazen et al., 2010). While our pre- and post-plume 16S rRNA gene clone libraries did not contain any full-length DWH *Oceanospirillales* clones, the DWH *Oceanospirillales* were detected by pyrosequencing in the post-plume samples (September and October 2010), indicating a low-level background population and reservoir of these bacteria in the Gulf of Mexico water column.

Unexpectedly, members of the DWH *Oceanospirillales* were found in bacterial 16S rRNA gene and rRNA transcript libraries from the Atlantic shelf break offshore North Carolina, sampled on December 4th, 2009 (D'Ambrosio, 2011), at a depth of 146 m in a distinct water mass known as the Subtropical Underwater (SUW) layer and distinguished by high salinity and warm temperature (Cléroux et al., 2009). They constituted a substantial proportion (around 20% to 25%) of all clone libraries from the SUW sample, regardless of whether these were derived from 16S rRNA genes or 16S rRNA transcripts of the particle-associated or free-living fraction (D'Ambrosio 2011). The North Carolina *Oceanospirillales* 16S rRNA genes fell into the same phylogenetic clusters as the *Oceanospirillales* 16S rRNA genes and pyrosequencing fragments from the DWH oil spill (Fig. 4). Since the North Carolina *Oceanospirillales* were sampled in December 2009, they do not originate from the DWH oil spill; yet they are members of the DHW *Oceanospirillales* cluster by phylogenetic affiliation. The conspicuous enrichment of DWH *Oceanospirillales* in the Subtropical Underwater layer might be the consequence of natural hydrocarbon seepage and hydrocarbon enrichment in this water layer in the southwest North Atlantic (Harvey et al., 1979; Requejo and Boehm, 1985). This North Atlantic population of DWH *Oceanospirillales* could be in constant exchange with the Gulf of Mexico, and might represent a parent population. More generally, the North Atlantic and the Gulf of Mexico occurrences of this microbial group could be the result of localized enrichments from a widely distributed low-abundance seed population.

4. Conclusions

Pyrosequencing and clone library analyses of PCR-amplified 16S rRNA genes and gene fragments have revealed strong microbial community stratification in the deep-plume water column, dominated by abundant populations of alkane-oxidizing DWH *Oceanospirillales* and aromatics-degrading *Cycloclasticus* spp. After the Macondo wellhead was capped and the source for the deep plume extinguished, the pre-spill pelagic microbial community re-established itself near the vicinity of the Macondo wellhead. However, even after the deep hydrocarbon plume was no longer detectable in the wellhead area in September and October 2010, small populations of oil-degrading *Gammaproteobacteria* and of the DWH *Oceanospirillales* remained detectable by pyrosequencing, indicating persistent and widely occurring seed populations in the water column that respond quickly to natural or anthropogenic hydrocarbon pulses.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dsr2.2014.01.014.

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