RESEARCH ARTICLE

The dynamics of the bacterial diversity in the redox transition and anoxic zones of the Cariaco Basin assessed by parallel tag sequencing

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One sentence summary: The dominant members of the microbial community occupying the Cariaco Basin remain mostly unchanged over the nine years of observation.

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ABSTRACT

Massively parallel tag sequencing was applied to describe the bacterial diversity in the redox transition and anoxic zones of the Cariaco Basin. In total, 14 samples from the Cariaco Basin were collected over a period of eight years from two stations. A total of 244 357 unique bacterial V6 amplicons were sequenced. The total number of operational taxonomic units (OTUs) found in this study was 4692, with a range of 511–1491 OTUs per sample. Approximately 95% of the OTUs found in the redox transition zone and anoxic layers of Cariaco are represented by less than 50 amplicons suggesting that only about 5% of the bacterial OTUs are responsible for the bulk of the microbial processes in the basin redox transition and anoxic zones. The same dominant OTUs were observed across all eight years of sampling although periodic fluctuations in their proportion were apparent. No distinctive differences were observed between the bacterial communities from the redox transition and anoxic layers of the Cariaco Basin water column. The largest proportion of amplicons belongs to Gammaproteobacteria represented mostly by sulfide oxidizers, followed by Marine Group A (originally described as SAR406; Gordon and Giovannoni 1996), a group of uncultured bacteria hypothesized to be involved in metal reduction, and sulfate-reducing Deltaproteobacteria. Gammaproteobacteria, Deltaproteobacteria and Marine Group A make up 67–90% of all V6 amplicons sequenced in this study. This strongly suggests that the basin’s microbial communities are actively involved in the sulfur-related metabolism and coupling of the sulfur and carbon cycles. According to detrended canonical correspondence analysis, ecological factors such as chemosynthesis, nitrate and oxidized and reduced sulfur compounds influence the structuring and distribution of the Cariaco microbial communities.

Keywords: pyrosequencing; sulfur cycle; anoxic basin

INTRODUCTION

Initially, the development of molecular techniques such as the amplification of the 16S rRNA gene and deoxyribonucleic acid (DNA) sequencing increased considerably our knowledge of the prokaryotic world (Pace 1997). Nevertheless, only a fraction of the prokaryotic diversity has been successfully described using this approach: large groups of bacteria only rarely present
in ecosystems (sometimes called ‘rare biosphere’; Sogin et al. 2006) were missed. Parallel tag sequencing is a recently developed technique that attempts to achieve a more comprehensive inventory of microbial populations (Sogin et al. 2006). This technique allows for high-throughput sequencing of the hypervariable regions of the bacterial small subunit rDNA, generating hundreds of thousands of short DNA sequence reads that are taxonomically classified by comparing them with comprehensive reference databases of full-length 16S rRNA gene sequences of known phylotypes (e.g. Sogin et al. 2006; Huber et al. 2007).

Multiple distinctive microbial ecosystems have been studied using the parallel tag sequencing approach. Some examples include the ocean water column (Sogin et al. 2006; Kirchman, Cotrell and Lovejoy 2010; Pommier et al. 2010; Rowe et al. 2012), various vent and spring fluids/sediments (Sogin et al. 2006; Huber et al. 2007; Clingenpeel et al. 2011; Roalkvam et al. 2011; Youssef, Steidley and Elshahed 2012), soil microbial communities (Roesch et al. 2007; Youssef et al. 2009; Nemergut et al. 2010; Nacke et al. 2011; Barberan et al. 2012; Yergeau et al. 2012), coastal waters and sediments (Kim et al. 2008; Gilbert et al. 2009; Hollister et al. 2010; Campbell et al. 2011; Thompson et al. 2011; Gligorone and Murray 2012), temperature-salinity gradients in the Red Sea (Qian et al. 2011; Ngugi et al. 2012), salinity gradient in the Baltic Sea (Herlemann et al. 2011), Baltic Sea surface waters (Andersson et al. 2009), marine invertebrate microbial communities (Webster et al. 2010; Schmitt et al. 2012), estuaries (Matcher et al. 2011), acidophilic sulfur-oxidizing biofilm (Jones et al. 2012), oil fields (Kryakho et al. 2012), bioreactors (Singleton, Richardson and Aitken 2011) and gut, oral and disease-associated microbiota (Dethlefsen et al. 2008; Flores et al. 2011; Oakley et al. 2012; Takeshita et al. 2012). In many of these studies a substantial increase in the diversity of microbial communities and their structure compared to respective data obtained by the Sanger 16S rRNA gene library approach.

Most of the previous pyrosequencing-based studies of prokaryotic diversity dealt with environments other than anoxic basins. Fuchsman et al. (2011) studied free-living and aggregate-associated bacterial communities in the suboxic zone of the Black Sea, the largest brackish anoxic basin. In the Cariaco Basin, the largest truly marine anoxic basin (Richards et al. 2001), two different studies that used massively parallel sequencing have revealed greater eukaryotic species richness and ecological complexity of protist communities (Stoeck et al. 2009; Orsi et al. 2011) compared to previous clone library-based diversity surveys (Stoeck, Taylor and Epstein 2003; Orsi et al. 2011). Both studies also showed vertical stratification in eukaryotic populations. Prokaryotic communities in the Cariaco Basin redox transition zone have been previously described by the 16S rRNA gene clone library approach (Madrid et al. 2001), fluorescence in situ hybridization (FISH; Lin et al. 2006, 2008), and lipid biomarkers (Wakeham et al. 2010, 2012). In these studies, Epsilonproteobacteria have been shown to be prevalent in the redox transition and upper anoxic zones of the basin and Betaproteobacteria have been described as a dominant taxon for several cruises (Lin et al. 2008); Gammaproteobacteria and Deltaproteobacteria (specifically sulfate reducers) have been observed in much lower numbers (Lin et al. 2006, 2008). By most accounts, the depth of phylogenetic coverage of Cariaco’s prokaryotic communities to date is considered shallow compared to many other marine systems. Thus, we contend that the composition of bacteria in the Cariaco Basin remains mostly unknown.

Therefore, we decided to apply the massive parallel sequencing strategy to analyze samples from the redox transition and anoxic zones of the Cariaco Basin collected over a period of eight years. A deeper knowledge of the different bacterial groups present in the Basin’s water column could elucidate their role in the basin biogeochemistry and relate these to the various electron donors and acceptors observed at the transition zone and anoxic layer.

**METHODS AND MATERIALS**

Sampling was conducted at two geographical locations: station A (the Cariaco Basin time series station, 10.30° N, 64.40° W) situated in the eastern subbasin with a depth of 1400 m and station C (10.40° N, 65.35° W) located in the western subbasin with a depth of 1400 m. Bacterioplankton samples were collected from various depths during seven cruises (summarized in Table 1): CAR19 on 10 May 1997, CAR25 on 13 November 1997, CAR29 on 12 March 1998, CAR66 on 5 May 2001, CAR108 on 17–18 January 2005, CAR112 on 23–24 May 2005 and CAR118 on 16–17 January 2006. For the first four cruises, CAR19, CAR25, CAR29 and CAR66, samples were obtained at station A. For the last three cruises, CAR108, CAR112 and CAR118, samples were obtained at station C. Samples were retrieved with 8-liter Teflon-coated Niskin bottles under N2 atmosphere. For genomic DNA samples, 4–8 L of seawater was filtered through polyvinylidene fluoride membranes (Millipore, Durapore®, 0.22 µm pore size, 47 mm diameter), immersed in extraction buffer (20 mM Tris-HCl, pH 8.8, 60 mM EDTA, pH 8.0, 20 mM NaCl), and stored at −20°C in the field, then transferred to −80°C for a long-term storage.

**Water analyses**

Bacterial abundances, chemosynthesis, ammonia, nitrite and nitrate profiles were generated according to Taylor et al. (2001). The dissolved Fe2+, Mn2+ and intermediate oxidation state sulfur compound concentrations (S2O4−2) were determined according to Percy et al. (2008). The biogeochemical and microbiological data used in this study can be found on the CARIACO time series website (http://imars.marine.usf.edu/CAR/) and Table S1 (Supporting Information).

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**Table 1. Sampling sites and depths. Zone definition according to Lin et al. (2008).**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zone</th>
<th>Date</th>
<th>Site</th>
<th>Depth (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C19 A260</td>
<td>Redox transition</td>
<td>May 1997</td>
<td>A</td>
<td>260</td>
</tr>
<tr>
<td>C25 A330</td>
<td>Redox transition</td>
<td>November 1997</td>
<td>A</td>
<td>330</td>
</tr>
<tr>
<td>C25 A350</td>
<td>Redox transition</td>
<td>November 1997</td>
<td>A</td>
<td>350</td>
</tr>
<tr>
<td>C108 A250</td>
<td>Redox transition</td>
<td>January 2005</td>
<td>A</td>
<td>250</td>
</tr>
<tr>
<td>C108 A290</td>
<td>Anoxic</td>
<td>January 2005</td>
<td>A</td>
<td>290</td>
</tr>
<tr>
<td>C108 A320</td>
<td>Anoxic</td>
<td>January 2005</td>
<td>A</td>
<td>320</td>
</tr>
<tr>
<td>C108 C245</td>
<td>Redox transition</td>
<td>January 2005</td>
<td>C</td>
<td>245</td>
</tr>
<tr>
<td>C108 C280</td>
<td>Redox transition</td>
<td>January 2005</td>
<td>C</td>
<td>280</td>
</tr>
<tr>
<td>C108 C320</td>
<td>Anoxic</td>
<td>January 2005</td>
<td>C</td>
<td>320</td>
</tr>
<tr>
<td>C112 A260</td>
<td>Redox transition</td>
<td>May 2005</td>
<td>A</td>
<td>260</td>
</tr>
<tr>
<td>C112 A300</td>
<td>Anoxic</td>
<td>May 2005</td>
<td>A</td>
<td>300</td>
</tr>
<tr>
<td>C112 A340</td>
<td>Anoxic</td>
<td>May 2005</td>
<td>A</td>
<td>340</td>
</tr>
<tr>
<td>C112 A500</td>
<td>Anoxic</td>
<td>May 2005</td>
<td>A</td>
<td>500</td>
</tr>
<tr>
<td>C112 C290</td>
<td>Redox transition</td>
<td>May 2005</td>
<td>C</td>
<td>290</td>
</tr>
</tbody>
</table>
DNA extraction

Extraction of genomic DNA was done as described elsewhere (Xu and Tabita 1996; Rodriguez-Mora et al. 2013). Briefly, to the thawed and shredded membranes immersed in extraction buffer, 0.5 mL of 10% sodium dodecyl sulfate and 250 μL of proteinase K solution (10 mg/mL) were added, followed by three cycles of freeze–thaw incubation, 50°C for 10 min and –80°C for 15 min. The thawed mixture was then extracted once with water-saturated phenol (neutralized with 0.5 M Tris-HCl buffer, pH 7.4) and once with chloroform. DNA was precipitated adding 1/10 volume of sodium acetate 3 M (pH 5.2) and two volumes of chilled absolute ethanol. To facilitate DNA precipitation, the mixture was incubated at –80°C overnight, and then centrifuged at 15 000 rpm for 30 min in an Eppendorf microcentrifuge. The DNA pellet was washed with 70% ethanol and left to dry in a desiccator for 20 min. The pellet was dissolved in 200 μL of sterile HPLC-quality water (Fisher Scientific, USA) and stored at –20°C.

Massively parallel tag sequencing

V6 amplicon constructs were constructed and sequenced as described in Sogin et al. (2006). Modifications to primers and tags were included as described in Huber et al. (2007). The individual PCR reactions contained a mix of forward and reverse primers from a set of universal primers designed to target bacteria, as described in Huber et al. (2007). Sequences were trimmed and low-quality reads were removed based on estimations of pyrosequencing error rates according to Huse et al. (2007). Taxonomy assignment was done using GAST (Global Alignment for Sequence Taxonomy) as described in Sogin et al. (2006). DOTUR (Schloss and Handelsman 2005) was used to create clusters at the unique, 0.03, 0.06 and 0.1 levels and rarefaction curves were calculated from the generated frequencies of OTUs at different distances as described in Huber et al. (2007). The species richness estimator Chao1 was also calculated using DOTUR.

Phylogenetic analysis of Car166 and Car172f 16S rRNA genes

An alignment, which includes Car166 (AF285812) and Car172f (AF285614) and selected Gammaproteobacterial sequences, was generated using the ClustalW v2.0 aligner (Larkin et al. 2007). Shorter sequences of interest (<1000 nucleotides) were added to the alignment and the alignment was then manually edited. Phylogenetic trees of almost full-length sequences were calculated with the neighbor-joining and maximum likelihood algorithms using the MEGA v5 (Tamura et al. 2011).

Statistical analysis

A hierarchical cluster was calculated using Ward’s method (Ward 1963) on the programming language R v2.14.0 (R Development Core Team 2008). The R package `prclust` v1.2–2 (Suzuki and Shimodaira 2011) was used to calculate approximately unbiased (AU) p-values as well as bootstrap probabilities (BP) values via multiscale bootstrap resampling.

Because of environmental data availability, only 11 samples obtained in January and May 2005 were included in further statistical analysis. We performed a constrained ordination analysis to study the interaction between different environmental parameters and the microbial communities. The environmental factors included are O₂, H₂S, NO₃⁻, NO₂⁻, NH₄⁺, sulfur species with intermediate redox states (S₆O₅²⁻, SO₄²⁻, S₂O₇²⁻), dissolved metals (Mn²⁺, Fe²⁺) and PO₄³⁻, as well as dark inorganic carbon assimilation rates (Taylor et al. 2001; Percy et al. 2008). A detrended canonical correspondence analysis (DCCA) (ter Braak 1986) was performed using CANOCO with polynomial detrending (ter Braak and Smilauer 2002). Analysis was done with Hill’s scaling focusing on inter-sample distances. Ordination diagrams were drawn to show the relationships observed, with samples (microbial communities) and phyla represented by points and environmental variables represented by arrows. The angle and length of the arrows indicate the direction and strength of the relationship among the environmental variables and the ordination scores for the samples (ter Braak 1986).

RESULTS

OTU distribution

Table 1 lists the different samples studied and the depths at which they were taken. A total of 244 357 bacterial V6 amplicons were sequenced from the two different sampled stations. The total numbers of V6 tag sequences and OTUs (defined as those amplicons that differed by no more than 3% as per Huber et al. 2007) found for each of the samples studied are shown in Table 2. The number of unique OTUs found across all samples was 4692.

The non-parametric richness estimator (Chao1; Chao 1984; Table 2) indicated that our sampling of bacterial richness is not complete but is fairly comprehensive. In general, the number of OTUs found is between 50 and 77% of the estimate by Chao1. Similarly, even though the curves for the unique sequences sampling saturation profiles show a non-asymptotic slope (particularly for Car112-C290), when clustering the OTUs at the 3, 6 and 10% similarity we observe a nearly asymptotic slope (data not shown).

Figure 1 shows the observed rank abundance distribution. In all samples, the dominant OTUs (<5 reads) account for the majority of the total sequences analyzed (more than 90% in every sample), while the low-abundance phylotypes are represented by only ~5 to 7% of all reads (Table 3). In fact, at any location at any specific time, no more than 80 abundant (considering 50% coverage) bacterial OTUs are present. The diversity of the ‘rare biosphere’ in the Cariaco Basin is very extensive: rare OTUs (<5 reads) comprise >71% of total OTUs for each of the samples.

The most prominent phylum of bacteria present in the Cariaco Basin is Proteobacteria (28–80% of all the sequences, Fig. 2) followed by SAR406 (Marine group A; 10–42%). In fact, combined they comprise 70–91% of all retrieved sequences. Other significantly represented phyla include Actinobacteria, Planctomycetes and Chloroflexi. Less than 1% of all sequences are cataloged as unclassified bacteria, non-assignable to any of the previously described bacterial phyla. Within Proteobacteria, the class showing the highest proportion of the sequences is Gammaproteobacteria. The OTUs of this proteobacterial class comprise 32–91% of the total sequences across all samples. Deltaproteobacteria comprise 5–62% of all proteobacterial sequences obtained. Gamma and Deltaproteobacteria combined account for 71–97% of all proteobacterial sequences, while Alpha, Epsilon and Betaproteobacteria are represented on average by 4, 1 and 0.3% of all proteobacterial sequences retrieved, respectively (Fig. 3). In fact, Alphaproteobacteria are present in large numbers only in two samples (C108 A320 and C108 C245).
Table 2. Number of sequences, OTUs and diversity estimates for bacteria across all samples. Sample names in bold indicate those considered to be collected in the redox transition zone.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total number of V6 tag sequences</th>
<th>Number of total unique V6 tag sequences</th>
<th>Number of total OTUs at 97% identity</th>
<th>Chao1 estimator of richness at 97% identity (95 CI)</th>
<th>Number of OTUs at the 97% identity unique in the sample (% in the sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Car19-A260</td>
<td>12 515</td>
<td>1289</td>
<td>511</td>
<td>825 (715–957)</td>
<td>96 (19)</td>
</tr>
<tr>
<td>Car25-A330</td>
<td>11 573</td>
<td>1394</td>
<td>544</td>
<td>1035 (891–1226)</td>
<td>102 (19)</td>
</tr>
<tr>
<td>Car25-A350</td>
<td>13 592</td>
<td>1566</td>
<td>594</td>
<td>1004 (888–1163)</td>
<td>147 (25)</td>
</tr>
<tr>
<td>Car108-A250</td>
<td>16 981</td>
<td>1780</td>
<td>743</td>
<td>1475 (1298–1709)</td>
<td>136 (18)</td>
</tr>
<tr>
<td>Car108-A290</td>
<td>15 791</td>
<td>2335</td>
<td>1045</td>
<td>1641 (1493–1831)</td>
<td>186 (18)</td>
</tr>
<tr>
<td>Car108-A320</td>
<td>18 131</td>
<td>2404</td>
<td>1120</td>
<td>1977 (1792–2210)</td>
<td>450 (40)</td>
</tr>
<tr>
<td>Car108-C245</td>
<td>12 551</td>
<td>1911</td>
<td>855</td>
<td>1518 (1347–1743)</td>
<td>169 (20)</td>
</tr>
<tr>
<td>Car108-C280</td>
<td>16 519</td>
<td>2132</td>
<td>923</td>
<td>1520 (1378–1703)</td>
<td>154 (17)</td>
</tr>
<tr>
<td>Car108-C320</td>
<td>15 465</td>
<td>1794</td>
<td>717</td>
<td>1451 (1258–1714)</td>
<td>84 (12)</td>
</tr>
<tr>
<td>Car112-A260</td>
<td>16 699</td>
<td>2284</td>
<td>1100</td>
<td>1680 (1542–1857)</td>
<td>192 (17)</td>
</tr>
<tr>
<td>Car112-A300</td>
<td>15 497</td>
<td>1718</td>
<td>706</td>
<td>1255 (1109–1450)</td>
<td>114 (16)</td>
</tr>
<tr>
<td>Car112-A340</td>
<td>15 563</td>
<td>2458</td>
<td>1157</td>
<td>1689 (1563–1847)</td>
<td>337 (29)</td>
</tr>
<tr>
<td>Car112-A500</td>
<td>23 510</td>
<td>2560</td>
<td>1044</td>
<td>1369 (1237–1541)</td>
<td>170 (16)</td>
</tr>
<tr>
<td>Car112-C290</td>
<td>39 970</td>
<td>3702</td>
<td>1491</td>
<td>1940 (1720–2227)</td>
<td>368 (25)</td>
</tr>
</tbody>
</table>

Figure 1. Rank abundance distribution of unique bacterial OTUs in the redox transition and anoxic zones of the Cariaco Basin.

Within Gammaproteobacteria, a single OTU related to sulfide-oxidizing symbionts [herein called sulfide-oxidizing symbiont relative (SOSR) GS03–18], comprised 21–81% of all Gammaproteobacteria sequences (Fig. 4) and 2–44% of total OTUs found across all samples. It accounted for as much as 44% of the total sequences in the C19 260 sample. The two next most abundant Gammaproteobacteria were classified as a member of the SUP05 clade (clade SUP05 species G03–103) and Pseudomonas sp. G03–4. They account for 1–17% and 0.3–13% of total proteobacterial sequences, respectively. The sample C112 A340 is rather an exception in which Pseudomonas sp. G03–4 comprises 46% of all proteobacterial sequences.

Among Deltaproteobacteria, the most abundant OTU belonged to the SAR324 group bacterium D03–3 (Fig. 5). It accounted for 2–40% of all Deltaproteobacteria tags sampled. All SAR324 sequences combined comprise 3–60% of all Deltaproteobacterial OTUs. Only one sample, C112 A340, had a very low proportion (3%) of the SAR324 sequences. In the Cariaco Basin, the evenness of Deltaproteobacteria is higher than that of Gammaproteobacteria. At least 11 Deltaproteobacterial OTUs were ubiquitously found across all samples.

Thus, the bacterial communities in the Cariaco Basin do not appear to be very diverse. Sequences unique to a specific library never exceeded 40% of total OTUs and comprised on average ~18% of total OTUs. Those are usually represented by a few sequence tags in each library. On the other hand, a common group of 64 OTUs (usually numerically dominant) were found in every sample.

A slight shift in community composition is apparent when comparing the OTU distribution in the samples from 1997 and the samples from 2005 (Fig. 2). Microbial communities in samples collected in 1997 are more similar to each other than to any sample from 2005 (Fig. 6), with stronger, more marked dominance by Proteobacteria OTUs in 1997 samples (Fig. 2). It appears that after 1997, Marine Group A representatives dramatically increased their proportion in the Cariaco community at the expense of Proteobacteria. Within Proteobacteria, a relative increase in Deltaproteobacterial numbers occurred at the same time. There is no clear differentiating pattern detected in
Table 3. Distribution of tags across samples. ≤5 tags, OTUs represented by five amplicons or less; 5< tags >50, OTUs containing between 5 and 50 amplicons; ≥50 tags, OTUs represented by 50 amplicons or more. Sample names in bold indicate those considered to be collected in the redox transition zone.

<table>
<thead>
<tr>
<th>Sample</th>
<th>≤5 tags</th>
<th>% Respective to number of total OTUs</th>
<th>% Respective to number of total tags</th>
<th>5&lt; tags &gt;50</th>
<th>% Respective to number of total OTUs</th>
<th>% Respective to number of total tags</th>
<th>≥50 tags</th>
<th>% Respective to number of total OTUs</th>
<th>% Respective to number of total tags</th>
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<tbody>
<tr>
<td>Car19-A260</td>
<td>77.25</td>
<td>4.66</td>
<td></td>
<td>17.75</td>
<td>6.98</td>
<td></td>
<td>5.00</td>
<td>88.36</td>
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</tr>
<tr>
<td>Car25-A330</td>
<td>76.24</td>
<td>5.54</td>
<td></td>
<td>18.78</td>
<td>9.63</td>
<td></td>
<td>4.98</td>
<td>84.83</td>
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</tr>
<tr>
<td>Car25-A350</td>
<td>74.95</td>
<td>4.94</td>
<td></td>
<td>19.79</td>
<td>8.49</td>
<td></td>
<td>5.26</td>
<td>86.57</td>
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<tr>
<td>Car108-A250</td>
<td>77.02</td>
<td>7.09</td>
<td></td>
<td>18.35</td>
<td>12.15</td>
<td></td>
<td>4.63</td>
<td>80.75</td>
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<td>5.72</td>
<td></td>
<td>22.60</td>
<td>7.48</td>
<td></td>
<td>4.69</td>
<td>86.80</td>
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<td>71.60</td>
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<td>13.67</td>
<td></td>
<td>4.83</td>
<td>79.36</td>
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<td>73.30</td>
<td>4.89</td>
<td></td>
<td>21.66</td>
<td>8.65</td>
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<td>5.04</td>
<td>86.46</td>
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<td>4.03</td>
<td>81.42</td>
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<td>Car112-A260</td>
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<td>7.00</td>
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<td>24.87</td>
<td>11.28</td>
<td></td>
<td>3.19</td>
<td>81.72</td>
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<td>Car112-A300</td>
<td>74.43</td>
<td>9.17</td>
<td></td>
<td>20.52</td>
<td>19.92</td>
<td></td>
<td>5.04</td>
<td>70.91</td>
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<tr>
<td>Car112-A340</td>
<td>73.46</td>
<td>9.47</td>
<td></td>
<td>22.75</td>
<td>17.73</td>
<td></td>
<td>3.79</td>
<td>72.79</td>
<td></td>
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<tr>
<td>Car112-A500</td>
<td>70.44</td>
<td>5.19</td>
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<td>24.08</td>
<td>11.24</td>
<td></td>
<td>5.49</td>
<td>83.57</td>
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</tr>
<tr>
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<td>68.59</td>
<td>4.04</td>
<td></td>
<td>25.72</td>
<td>9.41</td>
<td></td>
<td>5.69</td>
<td>86.55</td>
<td></td>
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Figure 2. Relative abundance of OTUs at the phylum level in the studied samples. Sample names in bold indicate those collected within the redox transition zone at the time of sampling.

bacterial population distribution between the redox transition zone and the anoxic layer.

Phylogenetic assignment of Car166 and Car172f sequences

The two most abundant Gammaproteobacterial sequences, SORS GS03–18 and clade SUP05 species GS03–103, found in the V6 tag libraries were 100% identical to the Car166 and Car172f sequences from the GenBank database, respectively. The Car166 and Car172f sequences were retrieved from a 16S rDNA library generated in an earlier study (Madrid 2000) for a sample (C25 A310) from the same C25 sample sets that was used in this study (i.e. C25 330 and C25 350). In this library, the Car166 and Car172f were also the two most dominant Gammaproteobacteria. Phylogenetic analyses (Fig. 7) unambiguously placed both sequences within sulfide-oxidizing bacterial clades.

Ecological gradients and bacterial community structure

A DCCA was performed to assess the interactions between several ecological factors and the microbial communities studied. At the phylum level, the first and second axis of the ordination analysis explain 57.8 and 13.2% of the species-environment variance, with a cumulative 73.1% of the total variation in the data set explained by the ordination analysis (Fig. 8). Such a high percentage for the first axis implies that it represents a strong gradient. In general, the most dominant ecological variable (among the variables included in the analysis) shaping the microbial communities distribution at the phylum level at the redox transition and anoxic zones is the nitrate ion concentration, followed...
Figure 3. Relative abundance of OTUs belonging to phylum Proteobacteria in the studied samples. Sample names in bold indicate those collected within the redox transition zone at the time of sampling.

Figure 4. Relative abundance of Gammaproteobacterial OTUs in the studied samples. Sample names in bold indicate those collected within the redox transition zone at the time of sampling.

Figure 5. Relative abundance of Deltaproteobacterial and Nitrospina OTUs in the studied samples. Sample names in bold indicate those collected within the redox transition zone at the time of sampling.
by chemolithotrophic processes. Among the most prevalent metabolic processes are those associated with sulfur redox cycling, including 

**DISCUSSION**

Marine anoxic basins are environments dominated by coupling of photosynthesis and nitrogen fixation, which sometimes exceeds the levels of photosynthetic CO₂ fixation observed in the photic zone of the Basin (Taylor *et al.* 2001). The Cariaco Basin is the largest typical marine anoxic basin with a redox transition zone sufficiently deep enough to be out of the basin’s photic zone. This distinguishes it from the Black Sea, the largest anoxic basin on Earth, whose redox transition zone is sufficiently shallow to be in the photic zone.

Approximately 95% of the OTUs found in the redox transition zone and anoxic layers of Cariaco are represented by less than 50 amplicons (Table 2). Even considering undersampling for some Cariaco communities, this means that likely only about 5% of the bacterial OTUs are responsible for the bulk of the microbial processes in the basin’s redox transition and anoxic zones. This large pool of low abundance OTUs also found in the Cariaco Basin is known as the ‘rare biosphere’ (Sogin *et al.* 2006) or ‘biological detritus’ (Falkowski *et al.* 2008). Some researchers suggest that these rare species may be living but metabolically inactive cells (Falkowski *et al.* 2008), or even dead cells that can persist in the environment (Stoeck and Epstein 2009). However, another study (Campbell *et al.* 2011) has demonstrated that ‘one half of all bacterial taxa actively cycled between abundant and rare’ in the oxic water column of the Delaware Bay over a period of three years. Clearly, ‘rare biosphere’ could represent a source for potential new metabolisms in response to environmental change, either seasonal or in the longer term (Andersson *et al.* 2009) or form a reservoir of functional redundancy and ensure that the microbial community always contains members that can take over and fill the ecological role of others, if needed. The latter scenario is likely valid for microbial communities in small confined environments such as human gut microflora (David *et al.* 2014), sponge symbionts (Fan *et al.* 2012) or bacterial communities colonizing *Ulva* surfaces based on the ‘first come first serve’ principle (Burke *et al.* 2011).

Compared to the oxic water column bacterial communities, the abundant component of the bacterial community in the Cariaco Basin is very stable although periodic fluctuations in the proportion of the most abundant OTUs did take place. A noticeable shift seems to have taken place in the microbial community composition of the redox transition zone and anoxic
Figure 7. A consensus neighbor-joining phylogenetic tree of the 16S rRNA gene from two Cariaco Gammaproteobacteria (shown in bold) and selected bacteria from the GenBank database generated by the Jukes-Cantor method. Filled square indicates bootstrap values > 75% for neighbor-joining analysis; filled circle indicates values between 50 and 75% for neighbor-joining analysis. Bootstrap values were calculated based on 1000 replicates.
layers of the Cariaco Basin: an increase in the proportion of taxa other than Proteobacteria was observed in samples obtained in 2005 compared to those from 1997. Nevertheless, the same dominant OTUs were still being observed across all eight years of sampling. This stability seems to be an attribute of the anoxic basins: Fuchsman et al. (2011) made similar observations for the Black Sea, where a continuity in the microbial community composition was shown between 1988 and 2005, with only some differences observed.

No apparent differences were observed between the bacterial communities from the redox transition and anoxic layers of Cariaco Basin water column. The most prominent taxa of bacteria were found at all sites and redox conditions with only slight proportion differences (Figs 2–5). However, these findings are in contrast with the results obtained by previous studies in the Cariaco Basin that used FISH and lipid markers profiles (Lin et al. 2006, 2008; Wakeham et al. 2010, 2012) where a vertical zonation of bacterial groups coincident with the different redox conditions has been observed. Two separate studies also showed a vertical stratification of protistan communities in the Cariaco Basin based on pyrosequencing (Stoeck et al. 2009; Orsi et al. 2011) and extensive clone libraries of nearly full-length 18S rDNA (Orsi et al. 2011). However, protists observed in the Cariaco Basin are for the most part motile and can actively
position themselves within the redox gradient at least to some degree. Additionally, it has been hypothesized that the anaerobic protistan communities present in the Cariaco Basin may have developed habitat specialization due to a lack of plasticity for hydrogen sulfide detoxification/oxygen tolerance (Stoeck et al. 2009; Orsi et al. 2011). Another possible explanation for a lack of major differences in microbial communities of the redox transition and anoxic zones is that the short V6 tag amplicons could easily come from living bacteria and also from bacteria in various stages of decomposition. Then, it would appear plausible that a particle flux ‘smears’ the bacterial community signature from the redox transition zone into the anoxic water column.

A previous PCR-DGGE characterization of the Cariaco Basin bacteria community (Rodriguez-Mora et al. 2013) revealed similar most prominent taxa. Even though the numerical proportions differ, representatives of Chloroflexi, Proteobacteria (Alpha, Gamma, Delta and Epsilonproteobacteria), Firmicutes, Actinobacteria, Planctomycetes, Marine Group A, Bacteroidetes and the candidate divisions OP11 and OD1 were found by both pyrosequencing and PCR-DGGE techniques. The 16S rDNA library data available for the C25 330 sample (Madrid 2000) qualitatively correspond with pyrosequencing data (Table S2, Supporting Information). Although, there is no exact quantitative match between the two libraries (probably due to use of different primer sets), a general congruency is present. Consistent microbial community profiles by both methods would argue forcefully against the notion that the V6 tag signal is distorted by too much degraded DNA and cell material. The majority of 16S rRNA sequences from the 16S rRNA Cariaco libraries (Madrid et al. 2001) were represented in the V6 pyrosequencing tags. Orsi et al. (2011) have compared an extensive protistan 18S rRNA gene clone library with a pyrosequencing library of the V9 18S rRNA gene hypervariable region constructed with a set of samples collected in parallel with those used in this study and demonstrated that, similar to our results, both libraries showed the same dominant taxa.

Gammaproteobacteria, Deltaproteobacteria and Marine Group A make up 67–90% of all V6 tags sequenced in this study. These data somewhat contrast with the FISH results previously obtained by Lin et al. (2006, 2008), who found that Gammaproteobacteria represented only about 3.5% and Epsilonproteobacteria accounted for as much as 27% of total DAPI cell counts in the Cariaco Basin in 2004 and 2005. The most prominent class of Proteobacteria (up to 40% of all DAPI) found by Lin et al. (2008) in May 2005 samples was Betaproteobacteria. Very few betaproteobacterial V6 tags were detected in the same samples in this study, which can likely be explained by the lack of specificity of the BET42a probe used by Lin et al. (2008). According to BLAST analyses, the probe has a 100% match with multiple Gammaproteobacteria including members of order Thiotrichales found in the Cariaco Basin. Moreover, the probe for Epsilonproteobacteria used in the study of Lin et al. (2008) amplifies 16S rRNA genes from several candidate division ampli cons presently deposited in the Cariaco Basin. Interestingly, Fuchsmann et al. (2011) have also found discrepancies between their V6 tags numbers and FISH numbers for the Black Sea samples. In that study, Gammaproteobacteria comprised 25% of V6 tag sequences, while only 6% of bacterial cells were found by Lin et al. (2006) to belong to Gammaproteobacteria using FISH. Additionally, Bacteroidetes and Epsilonproteobacteria were found to represent 5 and 6% of DAPI stained cells by FISH, but only 0.4 and 1.4% of V6 tags correspond to these groups, respectively (Fuchsmann et al. 2011). Unexpectedly, low V6 tag abundances of certain bacterial groups could also result from the overabundance of other V6 tag fragments contributed by inactive bacteria and chemocline detritus; a large background will decrease the relative V6 tag representation of key microbial community members.

A large proportion of gamma- and Deltaproteobacteria V6 tags could be assigned to bacteria involved in sulfur metabolism. The two most abundant Gammaproteobacteria are closely related to sulfur-oxidizing symbionts of various invertebrates and the ubiquitous sulfide-oxidizing bacterium SUP05 as well as free-living sulfur-oxidizing bacteria (Fig. 8). A sizable proportion of Gammaproteobacteria were heterotrophs (i.e. Pseudomonas, Acinetobacter and Pseudoalteromonas spp.) more typical for eutrophic environments such as estuaries than for the open ocean water of the Cariaco Basin. Perhaps, high levels of chemolithoautotrophy and primary production (Taylor et al. 2001) create an

Figure 10. Relative abundance of bacteria with inferred types of metabolism in the redox transition and anoxic zones of the Cariaco Basin. Sample names in bold indicate those located within the redox transition zone at the time of sampling.
environment, which, at least locally, is rich in organic carbon. The most abundant group of Deltaproteobacteria in the Cariaco Basin, SAR324, is also involved in chemolithotrophic oxidation of sulfur species (Swan et al. 2011). Sulfate reducers in the Cariaco Basin are represented by several species of incomplete oxidizers from families Desulfobacteriaceae and Desulfobulbaceae (Desulfurhopalus spp.). A potential elemental sulfur and thiosulfate disproportionator, Desulfocapsa sp. D03–222, also was among the most abundant Deltaproteobacteria. To complete the list of bacteria involved in sulfur metabolism in the Cariaco Basin, the presence of sulfur-metabolizing Epsilonproteobacteria should be mentioned.

Marine Group A is the second most abundant bacterial taxon found in both the redox transition zone and anoxic layers of Cariaco. The taxonomy of this group is a matter of debate. Gordon and Giovannoni (1996) first reported SAR406 to be a deeply diverging lineage most closely associated with green sulfur bacteria and Fibrobacter spp. The National Center for Biotechnology Information (NCBI) Taxonomy Browser (www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) places SAR406 within the Fibrobacteres/Acidobacteria group, although it does not assign a rank to it. The GAST taxonomic assignment (Sogin et al. 2006) utilizes the SILVA database taxonomy (Pruesse et al. 2007), which classifies SAR406 as Marine Group A, and places it within the order Deferribacteriales, based on its proximity to Caldithrix sp. (Euzéby 1997). However, Miroshnichenko et al. (2003) when first describing the genus Caldithrix stated that its affiliation within the Bacteria domain was still to be determined since neither physiological nor morphological characteristics support placing of this genus within any recognized phyla. More recently, Caldithrix abyssi, the only described species of the genus, was shown to form a separate clade from the Deferribacteres and Synergistetes phyla (Jumas-Bilak, Roudi & Proksa 2009). In the same study, Marine Group A is, in fact, shown to be closely associated with Caldithrix, with both groups forming separate clades from Deferribacteres. Even more, Fuchsman et al. (2011) performed a neighbor-joining phylogenetic analysis of non-proteobacterial groups, using sequences that included, among others, an uncultured bacterial clone from the Cariaco Basin (Car53c; Madrid et al. 2001, also found in the Cariaco Basin in this study), and they also found that, indeed, Marine Group A forms a separate clade from the Deferribacteres.

According to Fuchsman et al. (2011), the Black Sea’s suboxic zone harbors Gammaproteobacteria, Marine Group A and Deltaproteobacteria among the most prominent taxa, which involved in the sulfur biogeochemistry. Taylor et al. (2001) suggested that, given the Mn$^{2+}$ and Fe$^{3+}$ profiles found in Cariaco and their coincidence with the chemooautotrophic production peak, metal-reducing sulfide-oxidizing populations may be important in dark inorganic carbon assimilation. Madrid (2000) was able to show autotrophic thiosulfate oxidation with manganese oxide as the electron acceptor in cultures isolated from Cariaco’s redox transition zone, while abiotic reduction of manganese oxide by thiosulfate did not occur under experimental conditions.

At this point, it is difficult to ascertain the role of the nitrogen cycle in the Cariaco biogeochemistry. Nitrite accumulates in the Cariaco redox transition zone (Taylor et al. 2001; Wakeham et al. 2012) and as a result 16S rRNA genes of bacteria-utilizing nitrite were identified among the V6 tags. These include nitrite-oxidizing Deltaproteobacteria from genus Nitrospina (Fig. 5) and anammox planctomycetes (Fig. 2). Wakeham et al. (2012) also have detected lipid biomarkers of anammox planctomycetes in the Cariaco Basin.

Based on the type of metabolism of cultured bacteria closely related to the most prominent OTUs found by massive parallel tag sequencing, we inferred metabolic properties of the microbial communities present in the redox transition zone and anoxic layers of the Cariaco Basin (Fig. 10). Considering this and previously described chemical profiles for the Cariaco Basin (Taylor et al. 2001; Li et al. 2012), it is safe to conclude that the Basin’s microbial communities are actively involved in sulfur-related metabolism and coupling of the sulfur and carbon cycles. Our results support the existence of an internal sulfur cycle in the redox transition/upper anoxic zones of the Cariaco Basin, as postulated by Li et al. (2012): Deltaproteobacteria provide the source of sulfide, which is then oxidized at the redox transition zone by Gammaproteobacteria and representatives of Marine Group A. The latter two groups may use intruding oxygen, nitrates and oxidized manganese and iron species as potential electron acceptors.

According to the DCCA, microbial communities in the samples from the redox transition zone are shaped by different environmental parameters than those from the anoxic zone (Fig. 9). Nitrate and dissolved metals seem to directly correlate with the redox transition microbial community composition, while sulfide and intermediate sulfur species show an inverse correlation. On the other hand, chemooautotrophy seems to have the strongest correlation with the anoxic zone microbial community with an exception of the redox transition zone microbial community from the C112 C290 sample collected in May 2005, which seems to have a strongest correlation with chemooautotrophy and sulfur species. Further metagenomic analyses are required to ascertain a specific role of bacteria in individual biogeochemical reactions identified in the Cariaco Basin.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSEC online.

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**Conflict of interest.** None declared.

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