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Meta-Analysis of Quantification Methods Shows that Archaea and Bacteria Have Similar Abundances in the Subseafloor

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There is no universally accepted method to quantify bacteria and archaea in seawater and marine sediments, and different methods have produced conflicting results with the same samples. To identify best practices, we compiled data from 65 studies, plus our own measurements, in which bacteria and archaea were quantified with fluorescent *in situ* hybridization (FISH), catalyzed reporter deposition FISH (CARD-FISH), polyribonucleotide FISH, or quantitative PCR (qPCR). To estimate efficiency, we defined "yield" to be the sum of bacteria and archaea counted by these techniques divided by the total number of cells. In seawater, the yield was high (median, 71%) and was similar for FISH, CARD-FISH, and polyribonucleotide FISH. In sediments, only measurements by CARD-FISH in which archaeal cells were permeabilized with proteinase K showed high yields (median, 84%). Therefore, the majority of cells in both environments appear to be alive, since they contain intact ribosomes. In sediments, the sum of bacterial and archaeal 16S rRNA gene qPCR counts was not closely related to cell counts, even after accounting for variations in copy numbers per genome. However, qPCR measurements were precise relative to other qPCR measurements made on the same samples. qPCR is therefore a reliable relative quantification method. Inconsistent results for the relative abundance of bacteria versus archaea in deep subsurface sediments were resolved by the removal of CARD-FISH measurements in which lysozyme was used to permeabilize archaeal cells and qPCR measurements which used ARCH516 as an archaeal primer or TaqMan probe. Data from best-practice methods showed that archaea and bacteria decreased as the depth in seawater and marine sediments increased, although archaea decreased more slowly.

Marine sediments cover ~75% of Earth's surface and are estimated to contain 2.9×10^{29} microbial cells (1). The majority of these cells bear little phylogenetic resemblance to cultured microorganisms and are likely very energy limited (2). Despite the importance of this vast subsurface biome to biogeochemical cycles and our understanding of biological energy limitation, two basic questions persist: (i) how do we accurately quantify cells from a particular microbial group (e.g., bacteria versus archaea) in the methodologically challenging sediment matrix, and (ii) how many of these individuals are alive?

Attempts to answer these questions have produced highly conflicting results. In an unprecedented interlaboratory comparison beginning in 2001, sediments from Ocean Drilling Program Leg 201 in the Peru Margin were subsampled and quantified in separate laboratories. As this was the first expedition to obtain verifiably uncontaminated samples, many research groups were eager to work on the same samples. Some researchers reported an overwhelming dominance of bacteria over archaea, using catalyzed reporter deposition (CARD) fluorescent *in situ* hybridization (FISH) (3) and quantitative PCR (qPCR) (3–5). Other researchers used lipid measurements to conclude the existence of an overwhelming dominance of archaea over bacteria (6, 7). A third collection of data showed roughly equal numbers of bacteria and archaea using FISH (6), CARD-FISH (8), and metagenomic sequencing and qPCR (9). The explanation for these inconsistent results must be either that some methods were less accurate or that some methods quantified dead as well as live biomass. It now appears that detrital cell matter contributed to an overestimation of the archaea by lipid analyses (10, 11), so it is unlikely that archaea completely dominate the Peru Margin sediments. Methodological inaccuracies have been suggested to explain the qPCR discrepancies, since some TaqMan probes or PCR primers are biased against common subsurface archaea (12). However, no study has addressed the discrepancies between the results of FISH and CARD-FISH with the Peru Margin sediments or evaluated the relative accuracies of FISH, CARD-FISH, and qPCR with all marine sediments and seawater.

In FISH and CARD-FISH, an oligonucleotide probe with a taxon-specific sequence binds directly to rRNA (13). For FISH, this probe is attached to a fluorophore, allowing probe-positive cells to be counted under a microscope. For CARD-FISH, the probe is bound to a large horseradish peroxidase (HRP) enzyme, which catalyzes the deposition of many fluorescent tyramides, enhancing the fluorescence intensity. HRP is far too large (~40 kDa) to diffuse freely into cells (14), so cells must first be permeabilized by partially degrading their cell walls (15). In another variation, called polyribonucleotide FISH, nearly full-length ribosomal gene amplicons are transcribed to RNA with fluorescently tagged ribonucleotides (16). The resulting signals are very bright; however, the probe's great length makes it difficult to trust the specificity of binding to the target population (17). In principle, each of these FISH methods quantifies only viable cells by imaging intact cells

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instead of single molecules, such as DNA or lipids that may be derived from detrital cell debris, and by targeting rRNA, which is presumed to be degraded rapidly after cell death (18). Measurements are biased against any microbial population whose rRNA sequence does not completely match the probe or, in the case of CARD-FISH, whose cell wall is resistant to the permeabilization procedures.

In qPCR, total DNA must be extracted cleanly from marine sediments to minimize the amount of coextracted PCR inhibitors present. Primers for the 16S rRNA genes of specific microbial taxa are then used for PCR, while a fluorescence reader tracks the number of amplicons with either a SYBR green double-stranded DNA fluorophore or an oligonucleotide probe containing a TaqMan fluorescent molecule. If the DNA extraction and amplification are efficient, the samples are free of detrital DNA, and the primers and TaqMan probe match the expected target sequences, then qPCR measurements should be equal to total cell counts multiplied by the number of copies of 16S rRNA genes in each genome. Whether global qPCR counts are offset from cell counts by the average genomic 16S rRNA gene copy number has not been tested in marine sediments.

The high sensitivity of CARD-FISH to cells with low activity and low ribosome content makes it especially noteworthy that few CARD-FISH studies of deep subsurface sediments (>1 m below the seafloor [mbsf]) have found archaea in appreciable quantities (3, 19–22). If accurate, these results provide strong evidence that the amount of viable archaea in deep marine sediments is quantitatively negligible. If this is true, then the results of DNA and lipid analyses suggesting that archaea are abundant most likely reflect findings based on detrital archaeal cell remains.

However, to our knowledge, each study that argued for negligible populations of archaea in deep marine sediments (sediment depth, >1 m) on the basis of CARD-FISH used the enzyme lysozyme to permeabilize archaeal cell walls (3, 4, 19, 21, 22). Lysozyme hydrolyzes the β-1,4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan of Gram-negative bacterial cell walls (23). Cell walls of cultured archaea do not contain peptidoglycan. Instead, some archaea have a pseudopeptidoglycan cell wall with a β-1,3 glycosidic bond that is not hydrolyzed by lysozyme (24). Most archaeal cell walls are surrounded by a thick S layer of proteins. So, an effective method to permeabilize archaeal cell walls necessarily involves partial digestion of this protein layer. Proteinase K degrades a broad spectrum of proteins (25), making it a suitable candidate to permeabilize archaeal cell walls. Other permeabilization procedures include those involving Triton X-100, sodium dodecyl sulfate (SDS) plus lysozyme, and lysozyme plus achromopeptidase. Triton X-100 and SDS are detergents that interact nonspecifically to disrupt proteins. Achromopeptidase hydrolyzes bonds between *N*-acetylmuramic acid and the D-amino acids linking adjacent peptidoglycan strands. Achromopeptidase was originally added to environmental CARD-FISH preparations to target Gram-positive bacteria (26); archaeal pseudopeptidoglycan's interstrand L-amino acid peptide linkages are not its preferred substrate. Therefore, proteinase K and, possibly, the detergents appear to be capable of permeabilizing the cell walls of cultured archaea, whereas lysozyme and achromopeptidase do not. In direct comparisons of the permeabilization methods in the same samples, proteinase K produces better archaeal CARD-FISH signals than lysozyme in seawater (27) and in pure cultures of archaea (28). However, given that the cell walls of subsurface archaea are largely uncharacterized, we cannot assume *a priori* that they cannot be permeabilized by lysozyme and achromopeptidase.

We hypothesized that the low CARD-FISH counts of archaeal cells in deep subsurface sediments are an artifact of inefficient permeabilization. We also hypothesized that FISH, CARD-FISH, qPCR, or all three methods provide accurate measurements of viable cells in marine sediments and seawater. To test these hypotheses, we compiled data from 65 published studies and our own data from marine environments. Unfortunately, the accuracy of these methods for counting endogenous cell populations cannot be confidently tested on a known quantity of cells spiked into natural samples because these cells may not match the diversity of physiological states, cell envelope structures, mineral associations, and/or biofilm presence that may occur in the natural community. Therefore, we define "yield" to be the sum of bacterial plus archaeal cell counts obtained by FISH or CARD-FISH divided by the total cell counts obtained using a nonspecific stain for DNA, such as acridine orange (AO), 4′,6-diamidino-2-phenylindole (DAPI), or SYBR green. Accurate quantification methods should be associated with yields close to 1, unless the samples contain large quantities of intact but non-ribosome-containing (nonviable) cells. We tested whether the latter was likely to be true by determining whether yield was environment dependent. We then compared the precision of cell count-based methodologies to that of qPCR-based methodologies to suggest which ones might be more useful in future efforts for absolute and relative quantifications of bacteria and archaea in marine sediments. We did not include RNA slot blot, reverse transcription-qPCR (RT-qPCR), or most probable number (MPN) quantifications in this study because the first two measure total RNA, which is difficult to constrain relative to cell counts, and the third depends on organisms being amenable to common culture methods, which is not often the case in environmental samples.

MATERIALS AND METHODS

Data were compiled from published studies that included archaeal and bacterial domain-level quantifications in marine water and sediments made with qPCR, FISH, or CARD-FISH (all references and data can be found in the text in the supplemental material and Tables S1 to S3 in the supplemental material). No studies meeting these criteria were intentionally excluded from our meta-analysis. For seawater, we included 19 studies with 1,021 data points, and for sediments, we included 50 studies (including this study) with 773 data points; some studies had both seawater and sediment data. A few studies reported only relative quantities of bacteria and archaea, and so data from those studies were not used in determinations of absolute quantities or yields. Most studies also had total cell counts made with a general DNA stain, such as AO, DAPI, or SYBR green. In some cases, cell counts were not available from precisely the same sediment depth as the qPCR, FISH, or CARD-FISH counts. For these, cell counts from nearby depths of <0.25 m away at depths under 1 mbsf (5 data points), <1 m away at depths under 10 mbsf (34 data points), and <10 m away at deeper depths (147 data points) were used. Few studies included qPCR measurements of total archaea and bacteria in marine water, so qPCR was evaluated only with sediments. Where possible, data were obtained via direct contact with the authors or digitally cutting and pasting numbers from published tables; otherwise, data were extracted by manually measuring data points from printed figures or by using the freeware WebPlotDigitizer (version 2.5, 2012; http://arohatgi.info/WebPlotDigitizer). Studies where primary data were available from the authors were used to determine that data manually extracted from figures varied from primary data by <4%. For data newly reported here,

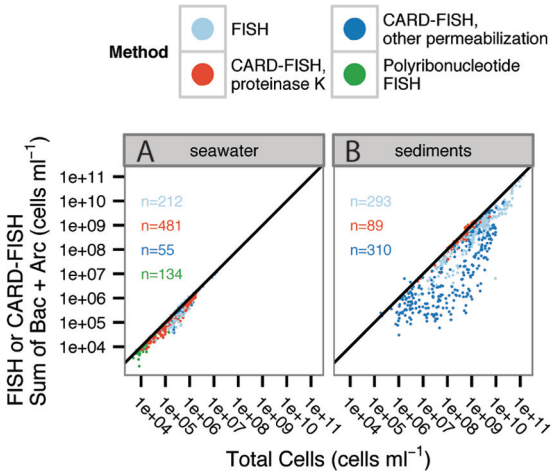

FIG 1 Yields for seawater (A) and sediments (B) obtained by FISH, CARD-FISH with proteinase K permeabilization for archaea, CARD-FISH with other permeabilization methods for archaea, and polyribonucleotide FISH. The solid line is the 1:1 line, and the numbers of data points in each method group are listed in the corresponding color in each panel. In all CARD-FISH analyses, lysozyme was used to permeabilize bacteria.

samples were obtained from Station H in the White Oak River estuary in North Carolina on 2 October 2012, a site that has been previously characterized for geochemistry and microbiology (29). CARD-FISH was performed following the method of Teira et al. (27). All statistical tests were performed using the R package, version 3.0.0 (30). Plots were generated using the ggplot2 package (31). Our database is available as supplemental Excel files (see Table S2 in the supplemental material for seawater and Table S3 in the supplemental material for sediments), and an R package to reproduce all our analyses and plots is available from the authors.

RESULTS AND DISCUSSION

Evaluating bacterial and archaeal FISH and CARD-FISH yields relative to total cell counts in seawater. In seawater, 99% of FISH and CARD-FISH measurements were within an order of magnitude of total cell counts, and the median yield was 71% (interquartile range, 59 to 82%) (Fig. 1A). No methodological variation had a large effect size on yield; however, a few methods had small but statistically significant effects ($P < 0.001$, analysis of variance [ANOVA]; see Fig. S1 and Table S4 in the supplemental material). Polyribonucleotide FISH improved the median yield by 12 percentage points over the yields obtained by FISH and CARD-FISH. FISH and CARD-FISH median yields were statistically indistinguishable from each other, but the CARD-FISH data were less scattered. Automated counting resulted in slightly higher yields (4 percentage points) and less scatter than manual counts. For archaea, methods that used mixed polyribonucleotide probes had slightly higher yields than methods that used mixed *Euryarchaeota* and *Crenarchaeota* probes, and the yields by the latter methods were slightly better than those achieved by methods that used the ARCH915 probe alone. For bacteria, methods that used mixed polyribonucleotide probes had slightly higher yields than methods that used EUB338 I or EUB338 I to III; the yields obtained by methods that used the last two probes were indistinguishable from each other. Polyribonucleotide probes span the length of the ribosome, including universally conserved regions, which could cause them to bind to nontarget taxa. We cannot determine whether the high yields obtained by methods using these probes are due to double counting of bacteria and archaea. Therefore, the best probes appear to be *Euryarchaeota* plus *Crenarchaeota* probes for archaea and either EUB338 I or EUB338 I to III for bacteria. No significant difference between AO and DAPI general DNA stains could be seen. All studies used the same fixative (formaldehyde derivatives), and all except two CARD-FISH studies permeabilized cell walls with lysozyme for bacteria and proteinase K for archaea, so the effects of these methods could not be tested robustly. Therefore, it appears that most of the cells in seawater which can be counted with general stains are alive (contain ribosomes). We found that the yields of the FISH and CARD-FISH methods in common use are accurate relative to total cell counts and can be relied upon to enumerate bacteria and archaea in environmental samples.

Evaluating bacterial and archaeal FISH and CARD-FISH yields relative to total cell counts in sediments. In contrast, FISH and CARD-FISH yields in marine sediments were highly variable, with median yields of 40% (interquartile range, 12 to 70%) (Fig. 1B). It seems reasonable to expect that cell quantifications are less precise in marine sediments than in seawater. Large volumes of seawater can be concentrated on a filter with little attending organic matter or mineral grains to either hide cells (false negatives) or adsorb fluorophore (false positives). This allows very low natural cell densities to be increased to a level high enough to count precisely. In sediments, no such concentration is possible, since filters retain sediment grains, in addition to cells. Rather, cells must be diluted from natural concentrations to avoid the formation of layers on the filter, or cells must be extracted from the sediment matrix in a method which has been used to obtain total cell counts but not in FISH and CARD-FISH (32). Researchers working in sediments must discern the difference between a true cell (small, smooth, well-defined, and highly fluorescent) and the background fluorescence of the sediment matrix (15). It is also possible that marine sediments have inherently lower yields than seawater because more of the microbial community is intact enough for cell counts yet free of ribosomes because they are dead.

However, despite all these difficulties, a subset of sediment studies achieved yields as high as those from seawater. By focusing on yield (as opposed to absolute amounts), we controlled for various absolute abundances of microorganisms in different environments. These studies that obtained high yields used CARD-FISH with the same permeabilization procedures that were used with seawater: lysozyme for bacteria and proteinase K for archaea (Fig. 1, red points). All 9 sediment cores analyzed with this method had median yields within or above the interquartile range of seawater yields (Fig. 2). When lysozyme or lysozyme and achromopeptidase together were used to permeabilize archaea for CARD-FISH, yields were extremely low, as none of the 17 cores with archaea permeabilized with lysozyme and only 2 out of 9 cores with archaea permeabilized with lysozyme/achromopeptidase had median yields within the interquartile range of the seawater yields (Fig. 2). CARD-FISH using detergents for archaeal permeabilization and FISH (which does not require a permeabilization step) produced variable yields relative to total cell counts (Fig. 2). The effects of the permeabilization procedure on CARD-FISH yields were significant ($P < 0.001$, ANOVA with rank-ordered yields), with the median yield obtained with proteinase K permeabilization being higher than the median yields obtained with lysozyme/achromopeptidase and detergent permeabilization, all of which were significantly higher than the median yield obtained with ly-

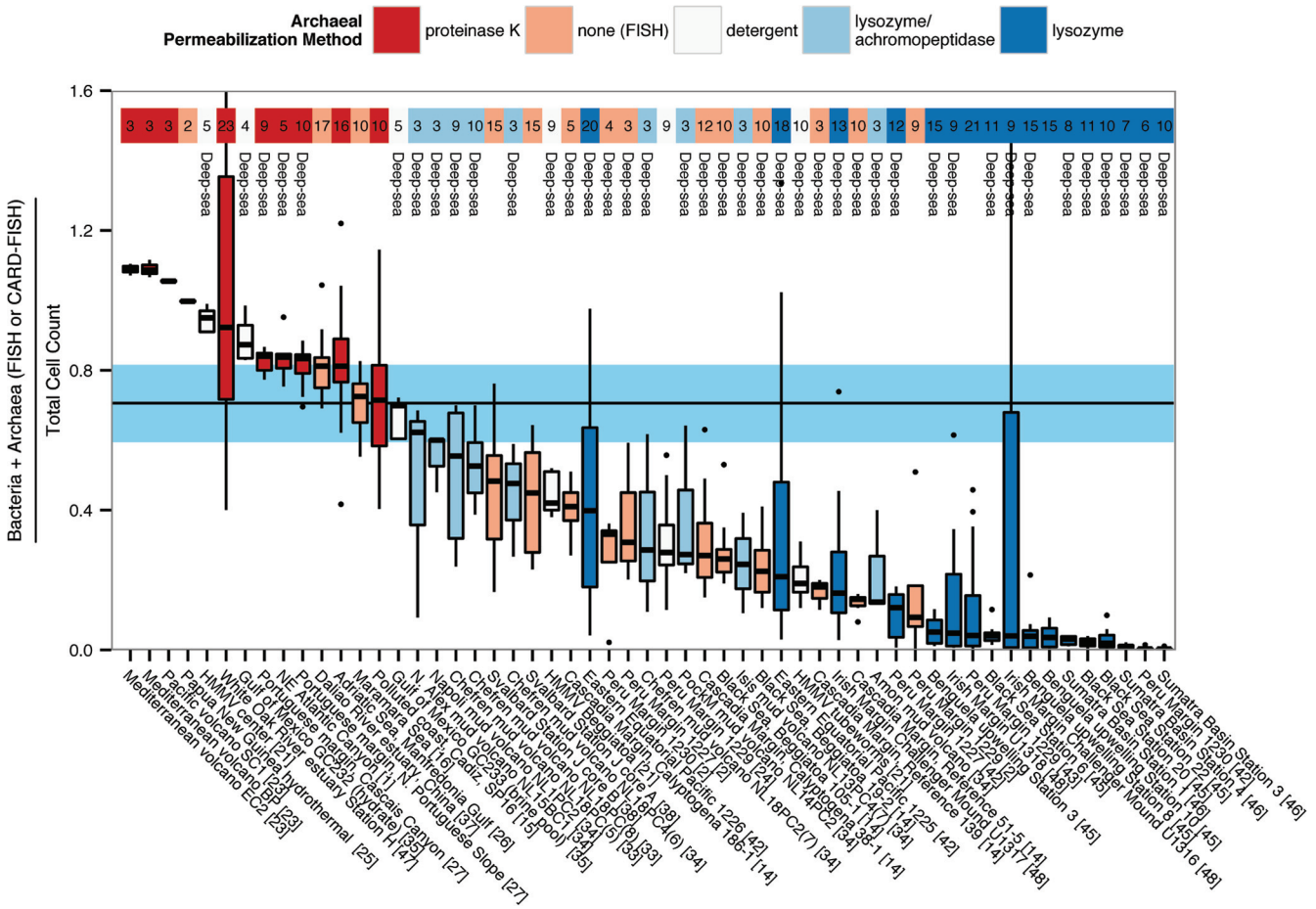

FIG 2 Sediment FISH and CARD-FISH yield box plots colored by archaeal permeabilization method and ordered by the decreasing yield of each individual study. Black line, median seawater yield; blue shading, interquartile range (i.e., the range bounded by the 25th and 75th percentiles of the yields). Samples from >250-m water depths are labeled with "deep-sea" above the data. Numbers in the color ribbon at the top indicate the number of data points in each box plot, and numbers in brackets after each core description correspond to the source citations in Table S1 in the supplemental material. Data from studies with only one data point and studies in intertidal environments (see Fig. S3 in the supplemental material) were excluded from the plot. HMMV, Haakon Mosby Mud Volcano.

sozyme permeabilization (Fig. 3A; see Table S5 in the supplemental material). The 13 lowest-yielding sediment cores (median yield, <6%) were all quantified with CARD-FISH using lysozyme to permeabilize archaea. Notably, all the Peru Margin cores where archaea were found to be quantitatively negligible (sites 1227, 1229, and 1230) had yields of <13%. As with seawater, other methodological variations had only small effects on yields, some of which were statistically significant ($P < 0.001$, ANOVA; see Fig. S2 and Table S4 in the supplemental material). Due to the overwhelming effects of archaeal permeabilization procedures on yield (Fig. 2 and 3), we considered only data from CARD-FISH methods using proteinase K for archaeal permeabilization and all FISH data in our comparisons of these other factors. SYBR green I- and (dichlorotriazinyl)aminofluorescein (DTAF)-stained cells had yields 30 percentage points greater than the yield for DAPI-stained cells, which had a yield 20 percentage points higher than that for AO-stained cells. This result agrees with reports that SYBR green I provides better cell counts due to low background staining (33). DTAF, on the other hand, may have led to a high rate of false-positive counts since DTAF stains polysaccharides rather than DNA, a property that has been used to visualize nonliving extracellular polysaccharides in seawater (34). Due to DTAF's low specificity, we do not recommend its use for cell counting. CARD-FISH improved on FISH yields by 40 percentage points, possibly due to the sensitivity of CARD-FISH to cells with low ribosome contents. No bacterial probe bias was detected (EUB338 I versus EUB338 I to III), and probe bias could not be assessed in archaea because all measurements were made with ARCH915.

We then tested whether low yields indicate the contribution of intact dead, ribosome-free cells to total cell counts, rather than inaccurate quantifications, by determining whether yield was related to environmental factors that might predict the physiological state of the organisms. Eleven of the 13 lowest-yielding samples were from the deep sea (>250-m water depth), and all 13 included measurements from the deep subsurface (>1-m sediment depth). Here, cells are very energy limited (2), and sediments from these locations may contain a higher proportion of dead cells than sediments from other locations. However, deep-sea sediments have high CARD-FISH yields when proteinase K is used; five deep-sea sediment cores had yields that matched or surpassed seawater yields (25th percentile, 59%). Likewise, deep subsurface sediments (Peru Margin sites 1227, 1228, and 1230, which had depths up to 121 mbsf) had higher yields when FISH or CARD-FISH using detergents to permeabilize archaeal cell walls rather than

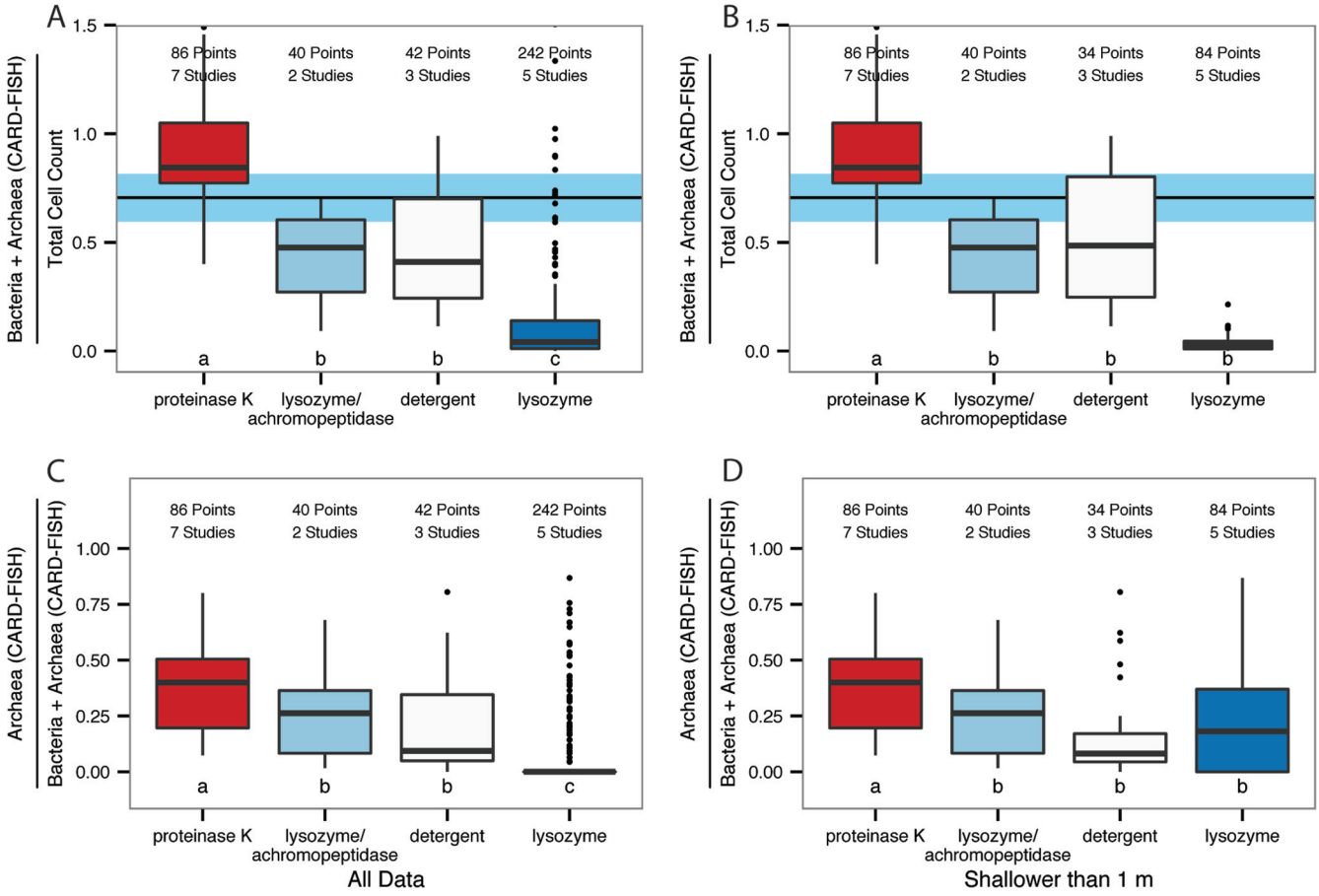

FIG 3 Evaluation of effects of archaeal permeabilization method on CARD-FISH yield (A and B) and fraction of archaea (C and D) for all data (A and C) and sediments shallower than 1 m (B and D). Letters under the box plots indicate statistically indistinguishable groups, based on ANOVA on rank-ordered data with the Tukey honestly significant difference *post hoc* analysis ($P < 0.001$), and the numbers of data points and individual studies are listed above each box plot (box plot values are listed in Table S5 in the supplemental material). Colors and shadings match those in Fig. 2.

CARD-FISH using lysozyme to permeabilize archaeal cell walls was used (Fig. 2). Another study also found archaeal cell counts to be well above the quantification limit, after permeabilization with hydrogen peroxide, at depths up to 300 mbsf in the Leg 201 samples (35), although the measurements are not included in Fig. 2 because they did not include bacterial or total cell counts. Furthermore, the lysozyme method produced significantly lower yields than the proteinase K method even when sediments from only the upper meter were compared (Fig. 3B), suggesting that low yields in the deep subsurface are an artifact of a suboptimal methodology rather than a direct reflection of the metabolic state of cells.

FISH studies, on the other hand, had a wide range of yields, even though most studies used nearly identical FISH methods. Therefore, low FISH yields appear to be associated with less active communities since environmental factors do correlate with yield; all eight deep-sea samples had FISH yields below the median yield range of seawater (Fig. 2). This sensitivity of FISH to cell activity was observed in a previous meta-analysis (36) and is consistent with this method's demonstrated low fluorescence in presumably low-activity cells (15).

We then tested whether methods with systematically low yields had low ratios of archaea to bacteria, suggesting that a systematic undercounting of archaea may have contributed to the low yield. Measurements obtained by CARD-FISH with proteinase K permeabilization had significantly higher percentages of archaea than CARD-FISH with the other permeabilization methods in the full data set (Fig. 3C) and in sediment depths restricted to less than a meter (Fig. 3D). As predicted by our hypothesis, lysozyme archaeal permeabilization reported low archaeal fractions, even when sediments from only the top meter were compared (Fig. 3D). The single seawater study that used this method (37) also had very low archaeal fractions (<5%). Notable exceptions from the correlation between low yields and low ratios of archaea to bacteria were intertidal sediments from the Wadden Sea area in Germany and the New Jersey shelf in the United States (see Fig. S3 in the supplemental material). These environments are characterized by permeable sands and large tidal ranges, which frequently expose the sediment surface to air. All 11 studies with samples from these sites had relatively high (>37%) median yields, even though they did not measure archaea at all (4 studies) or quantified archaea to be <5% of total cell counts (7 studies). No other sediment studies coupled high yields and low archaeal fractions, suggesting that for these environments and only these environments, archaea are truly a minor component and are negligible in total cell counts, as has been noted previously (15, 38). With the

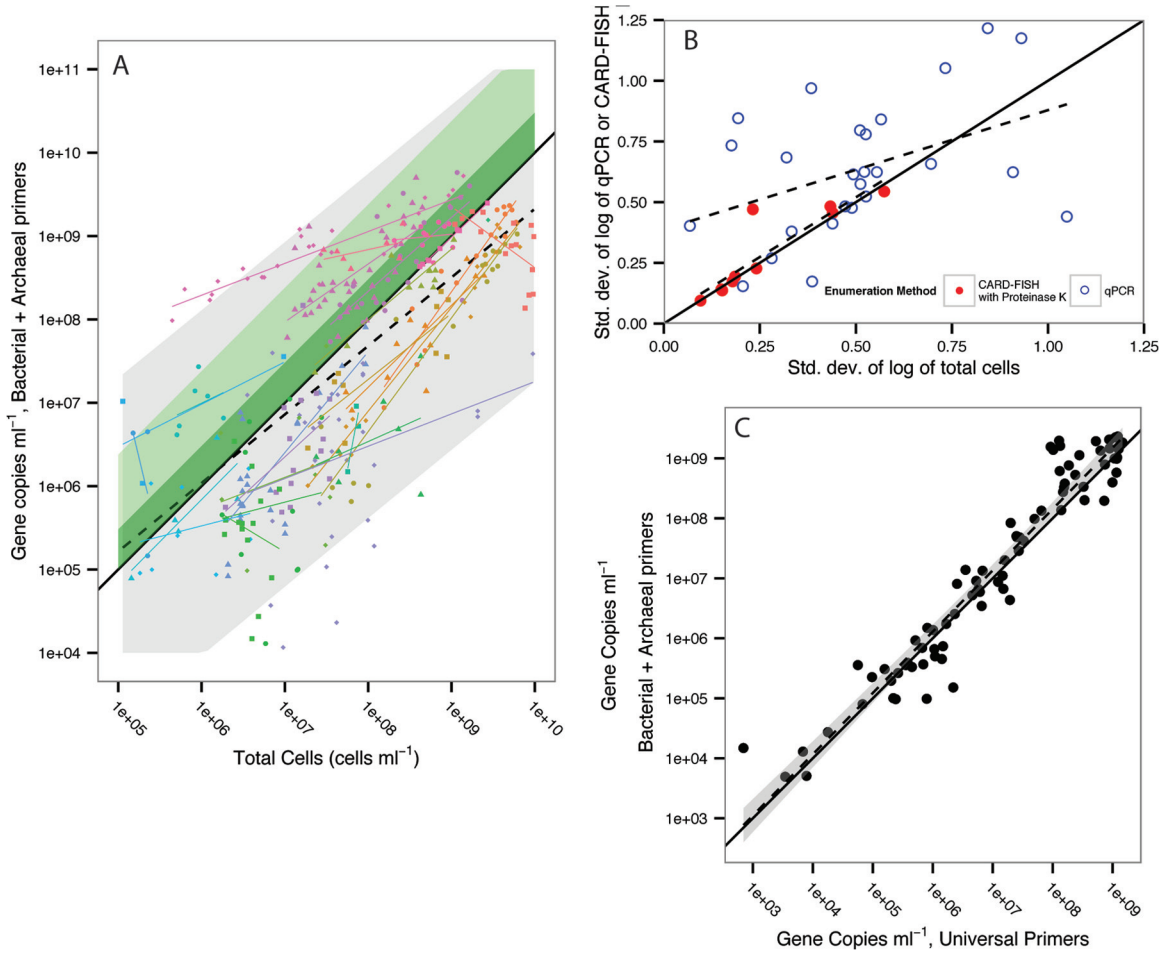

FIG 4 Evaluation of qPCR counts relative to cell counts. (A) Sum of separately measured bacterial and archaeal 16S rRNA gene copy numbers versus cell counts colored by core. Gray-shaded region, 95% prediction interval for the data in aggregate (i.e., 95% of future measurements are predicted to be within this interval); green-shaded areas, known variation in the range of 16S rRNA gene copy numbers per genome (dark green = 3.04 per genome; light green = 24 per genome). Each sediment core is coded by color and has its own fit line; data source citations are in Fig. S4 and Table S1 in the supplemental material. (B) Standard deviation (Std. dev.) of qPCR counts (open blue circles) or yields by CARD-FISH with proteinase K archaeal permeabilization (closed red circles) versus standard deviation for cell counts for each core. (C) Sum of separately measured 16S rRNA gene copy numbers of archaea and bacteria versus a single measurement obtained with universal primers, with the 95% confidence interval shown in gray. In all panels, solid black lines are the 1:1 line and dashed black lines are linear regressions of the data.

exception of these sandy intertidal zones, proper archaeal permeabilization procedures appear to be necessary to achieve both high fractions of archaea and high yields.

Evaluating archaeal and bacterial qPCR measurements relative to total cell counts. High CARD-FISH yields relative to total cell counts do not necessarily mean that CARD-FISH is the most accurate method because CARD-FISH and total cell counts are both based on the visual counting of cells in microscopic images and may therefore suffer from similar biases. Therefore, we compared the measurements obtained by cell counting methods to the measurements obtained by qPCR, which is the only other commonly used quantification method.

In the 10 studies (352 samples) that included cell counts in addition to archaeal and bacterial qPCR measurements, 16S rRNA gene copy numbers were not closely related to total cell counts (Fig. 4A; see Fig. S4 in the supplemental material). The correlation was positive and significant (log-log, $P \ll 0.001$, $R^2 = 0.41$), but the large 95% prediction interval showed that qPCR measurements are poorly predicted by total cell counts. For a given cell count, the narrowest interval that contained 95% of qPCR copy numbers spanned 4 orders of magnitude (Fig. 4A, gray band). This variability is too large to be explained by different 16S rRNA gene copy numbers per cell (mean = 3.04, maximum = 24, excluding the results for *Streptococcus agalactiae* at 85 gene copies per cell, based on the findings obtained with 5,669 published genomes [39]). In fact, many of the data points lie under the 1:1 line, which indicates either highly variable undercounting by qPCR or highly variable overcounting of total cells. To test which of the two methods drove the observed variability, we compared the standard deviations of all log-transformed qPCR measurements within a sediment core with the standard deviations of all log-transformed cell counts within the same core. For the majority of sediment cores (15/25), qPCR measurements were more variable than cell counts (Fig. 4B), showing that qPCR and not cell counts drove the high variability seen in Fig. 4A. Overall, the variance in qPCR counts was weakly correlated ($P = 0.03$, $R^2 = 0.19$, $n = 26$)

to the variance in total cell counts from the same core. As a comparison, the variance in CARD-FISH counts using proteinase K to permeabilize archaea was very closely related ($P < 0.001$, $R^2 = 0.80$, $n = 10$) to the variance in total cell counts with a fit line that was indistinguishable from a 1:1 line (slope = 0.98 ± 0.18, intercept = 0.03 ± 0.05; Fig. 4B).

This high variability of qPCR data is well within that expected from the variability of DNA extraction yields. Two commonly used kits, MoBio UltraClean and FastDNA Spin kits, had yields of DNA retrieved from a known quantity of cells added to sediments of 15% ± 16% and 28% ± 11%, respectively (40). This means that qPCR is not a reliable method to measure absolute quantities of cells in marine sediments, most likely due to variable extraction yields. However, for a given extraction, qPCR of 16S rRNA is quantitative relative to that of other genes, since the sum of bacterial and archaeal 16S rRNA gene copy numbers closely matches the 16S rRNA gene copy numbers obtained using a primer set universal for all prokaryotes ($P < 0.001$, $R^2 = 0.74$, $n = 80$; Fig. 4c). This suggests that, at least for these primers, measurements of extracted DNA are precise, if not accurate. Previous work agrees that although absolute quantities of qPCR measurements vary by extraction (35), relative values are repeatable across different primer sets (35) and cores (5, 29).

We did not systematically examine primer bias across all primers because bias depends on how well a primer or TaqMan probe matches the *in situ* microbial community, which varies greatly across ecosystems. Also, unlike with FISH and CARD-FISH, researchers utilized a wide range of qPCR primers and probes, making spurious correlations likely for each small data set. However, ARCH516, which was developed to target hydrothermal vent archaea (41), has previously been suggested to be biased against the type of archaea found in organic-rich marine sediments (12). Therefore, we tested whether this bias was borne out in the whole data set. We found that using ARCH516 as a qPCR primer or probe was associated with lower percentages of archaea ($P < 0.001$, $n = 430$; see Fig. S5A and Table S6 in the supplemental material). This bias was depth dependent; we found that the use of ARCH516 systematically resulted in low percentages of archaea at every depth except for the top centimeter, in which all qPCR primers/probes resulted in low archaeal fractions (see Fig. S5B and Table S6 in the supplemental material). An exception that supports the conclusion that the use of ARCH516 is inadequate for archaea in organic-rich sediments is that in the few studies of oligotrophic sediments available, the use of ARCH516 resulted in more archaea than bacteria (42, 43). This reflects the fact that the ARCH516 sequence is a good match to archaeal sequences retrieved from oligotrophic deep-sea sediments (84 out of 85 archaeal sequences in the Silva 111NR database from the South Pacific Gyre contained perfect matches to the ARCH516 sequence).

Using best-practice methods to examine changes in abundance of archaea and bacteria with depth in marine environments. We conclude that the best practice to determine the absolute densities of viable bacteria and archaea in marine sediments is CARD-FISH using lysozyme and proteinase K to permeabilize bacteria and archaea, respectively. For seawater, FISH measurements are also acceptable. The best practice for qPCR is to report values relative to those for comeasured genes. For anoxic marine sediments, ARCH516 should be avoided, although other environments will have different primer biases. These best-practice methods provide initial answers to the two basic questions about bacteria and archaea in marine environments: what is their abundance, and what fraction is viable?

With CARD-FISH, bacterial cell densities decreased with depth in sediments and also below the euphotic zone in seawater (Fig. 5A and D). In sediments, we used qPCR measurements to estimate total bacteria by multiplying the best-practice qPCR fraction for bacteria by the total cell count for that sample and the median yield for best-practice method CARD-FISH (0.84). With this method, bacterial cell densities decreased with depth at a slope nearly identical to that for the CARD-FISH data. However, qPCR estimations were systematically higher than CARD-FISH measurements, suggesting that bacteria are overrepresented in qPCR measurements due to more efficient DNA extraction, better matches to primers, or higher 16S rRNA gene copy numbers than those for archaea. Unexpectedly, the trend of decreasing bacteria with depth in marine sediments ended at ~10 mbsf; below this depth, bacterial cell density was not correlated with sediment depth. This may indicate the presence of a more stable deep subsurface population that is better equipped for subsurface living.

Archaeal qPCR-estimated cell densities also decreased with depth in marine sediments and below the euphotic zone in seawater (Fig. 5B and E), but this decrease was more gradual than that of bacteria, causing the ratio of archaea to bacteria to increase significantly with depth in the upper 10 m of marine sediments and below the euphotic zone in seawater (Fig. 5C and F). The trend of increasing ratios of archaea to bacteria with depth was true within individual sediment cores as well (13/16 cores; the results for 6 were significant to a P value of <0.05). No core had a statistically significant decrease in the ratio of archaea to bacteria with depth. Increasing ratios of archaea to bacteria with depth have been well documented in seawater (44, 45), and we show that is upheld in both seawater and sediments when all published data are considered together. Archaeal CARD-FISH counts increased slightly with depth (Fig. 5E); although the data coverage was low, the slope was barely significant, and the trend explains only 4% of the variation. Low data coverage also makes it difficult to know if a more persistent population of archaea is found below 10 mbsf, similar to the findings for bacteria.

To answer the second question about cell viability in the marine subsurface requires the assumption that cells or spores containing ribosomes, even at a very low concentration, are alive, although cells may not be very active relative to human timescales. The extremely slow biomass turnover in the deep subsurface makes it difficult to test this hypothesis (46), but the high chemical lability of RNA and its rapid degradation (47) make RNA a good candidate for an indicator of living biomass. In both seawater and sediments, the majority of cells producing DAPI, AO, or SYBR green signals also had positive CARD-FISH signals, showing that they contain ribosomes. If ribosome-free yet intact cells are present, they must therefore represent a minority of archaea and/or bacteria. This agrees with metatranscriptomic data (48) from Peru Margin sediments, showing that at all sediment depths assayed (down to 159 m) both bacteria and archaea contain mRNA.

Conclusions. The near absence of viable archaea in published CARD-FISH quantifications of the deep marine subsurface is likely to be an artifact of improper archaeal permeabilization procedures and does not indicate archaeal inactivity or absence. qPCR measurements for deep subsurface sediments obtained using ARCH516 as a primer or probe also systematically underestimate the fraction of archaea. These conclusions are supported by

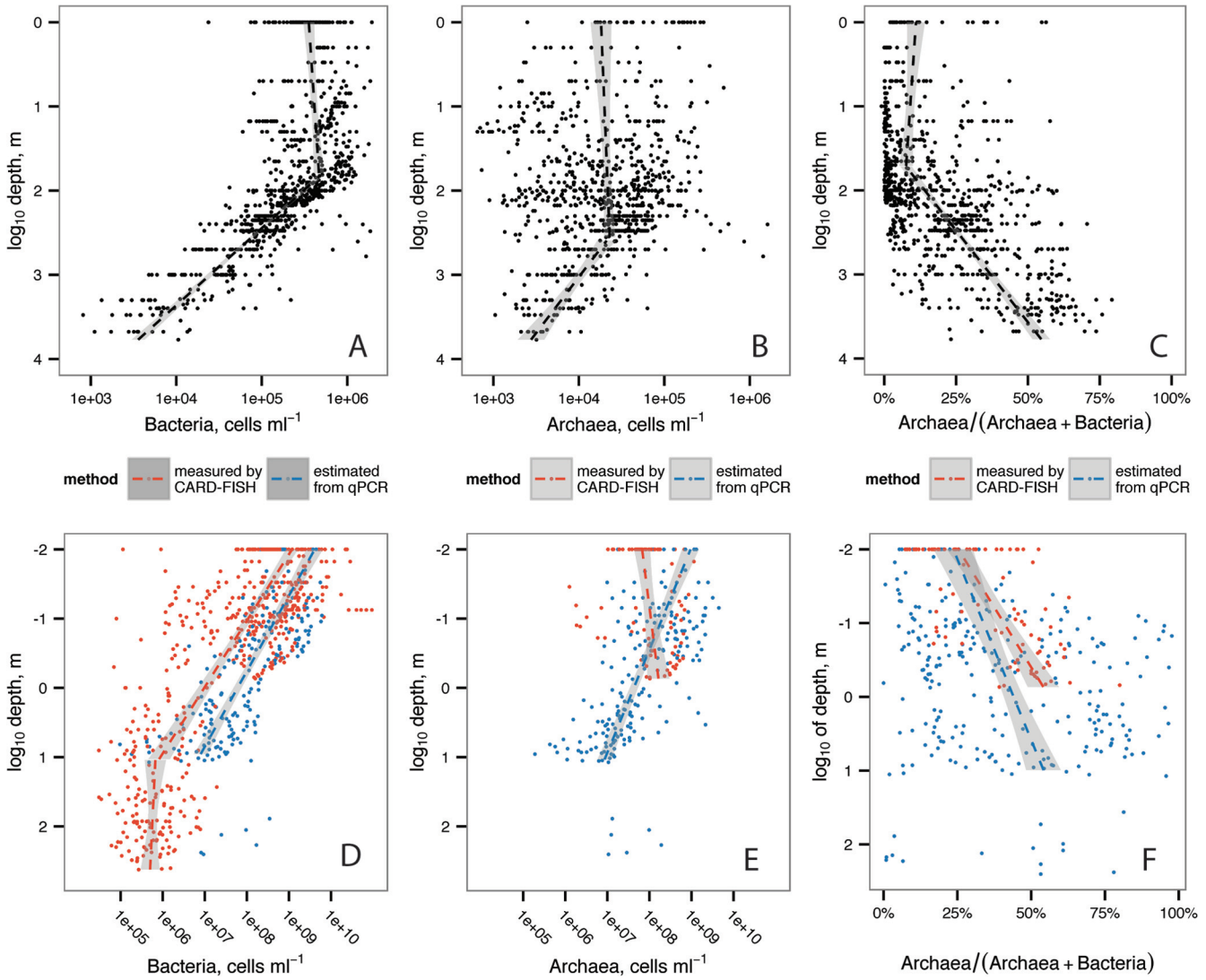

FIG 5 Results of best-practice quantification methods. (A to C) CARD-FISH and FISH quantifications of bacteria (A), archaea (B), and the fraction of archaea relative to the sum of bacteria and archaea (C) in seawater; (C to F) cell densities measured with CARD-FISH with proteinase K for archaeal permeabilization (red) or estimated from relative qPCR measurements and total cell counts (blue) for bacteria (D), archaea (E), and the fraction of archaea relative to the sum of bacteria and archaea (F) in marine sediments. Dotted lines, linear regressions of data of the same color; gray shading, 95% confidence interval. Fit parameters, breakpoints, and statistical parameters are provided in Table S7 in the supplemental material.

the fact that lysozyme cannot hydrolyze the compounds in archaeal cell walls (24) and the fact that the ARCH516 sequence is a poor match to the 16S rRNA gene sequences of most archaea in anoxic sediments (12). Despite the inherent difficulties of quantifying cells in sediment, it appears to be possible to reach the accuracy of CARD-FISH yields of seawater if cells are permeabilized using lysozyme and proteinase K for bacteria and archaea, respectively. Although absolute qPCR quantifications are highly variable, likely due to the variability introduced during the DNA extraction step, this method provides accurate values relative to other qPCR measurements, as long as appropriate primers and probes are employed. We propose that gene copy numbers per volume of sample measured by qPCR should be reported only relative to other qPCR-measured values for samples with similar extraction biases (i.e., the same sample or samples extracted alongside each other with the same methods). We predict that CARD-FISH measurements made below 1 mbsf using best practices will show that the abundance of bacteria and archaea are similar for the following reasons: archaeal abundance in the deep subsurface (up to 120 mbsf) is supported by qPCR and FISH data, qPCR and CARD-FISH data are in good agreement where bacteria and archaea co-occur, and qPCR and CARD-FISH show that the fraction of archaea increases with depth. These predictions are in line with the conclusions of previous qPCR-based studies (22, 49, 50). Therefore, we propose a solution to the Peru Margin conflict: data from best-practice qPCR and CARD-FISH agree with data from lipid (11) and metagenome (9) analyses that both archaea and bacteria are abundant, alive, and important in shaping the ecology and biogeochemistry of the vast microbial subsurface biome.

When all data collected with best-practice methods are considered, both bacteria and archaea decrease with depth below the

euphotic zone in seawater and in the upper 10 m of sediment, although the archaea increase relative to bacteria. In the presence of dwindling carbon and energy substrates with depth, something in the nature of archaea may make them more resistant to decay than bacteria in this energy-starved environment, as has been suggested previously (51). There is some evidence that uncultured archaea degrade detrital proteins in marine sediments (52), a metabolism shown to confer resistance to *Escherichia coli* populations undergoing energy starvation (53). Another option is that archaea may have lower total mRNA concentrations per cell (48), suggesting that archaea tolerate starvation simply by functioning at a lower metabolic rate. A third option is that subpopulations of archaea are able to find metabolically productive niches, even though the larger population is decaying. Interestingly, the major control of bacterial population size appears to shift at a sediment depth of about 10 m, suggesting that a subset of bacteria may be adapted to life in the deep subsurface. However, more data collected with best practices are required to determine whether that is also true for archaea or for sediments in environments with extremely small amounts of organic matter, such as the midocean gyres.

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REFERENCES

1. **Kallmeyer J, Pockalny R, Adhikari RR, Smith DC, D'Hondt S.** 2012. Global distribution of microbial abundance and biomass in subseafloor sediment. Proc. Natl. Acad. Sci. U. S. A. **109:**16213–16216.
2. **Hoehler TM, Jørgensen BB.** 2013. Microbial life under extreme energy limitation. Nat. Rev. Microbiol. **11:**83–94.
3. **Schippers A, Neretin LN, Kallmeyer J, Ferdelman TG, Cragg BA, Parkes RJ, Jørgensen BB.** 2005. Prokaryotic cells of the deep sub-seafloor biosphere identified as living bacteria. Nature **61:**861–864.
4. **Schippers A, Neretin LN.** 2006. Quantification of microbial communities in near-surface and deeply buried marine sediments on the Peru continental margin using real-time PCR. Environ. Microbiol. **8:**1251–1260.
5. **Inagaki F, Nunoura T, Nakagawa S, Teske A, Lever M, Lauer A, Suzuki M, Takai K, Delwiche M, Colwell FS, Nealson KH, Horikoshi K, D'Hondt S, Jørgensen BB.** 2006. Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. Proc. Natl. Acad. Sci. U. S. A. **103:**2815–2820.
6. **Biddle JF, Lipp JS, Lever MA, Lloyd KG, Sørensen KB, Anderson R, Fredricks HF, Elvert M, Kelly TJ, Schrag DP, Sogin ML, Brenchley JE, Teske A, House CH, Hinrichs K-U.** 2006. Heterotrophic archaea dominate sedimentary subsurface ecosystems off Peru. Proc. Natl. Acad. Sci. U. S. A. **103:**3846–3851.
7. **Lipp JS, Morono Y, Inagaki F, Hinrichs K.** 2008. Significant contribution of Archaea to extant biomass in marine subsurface sediments. Nature **454:**991–994.
8. **Mauclaire L, Zepp K, Meister P, McKenzie J.** 2004. Direct in situ detection of cells in deep-sea sediment cores from the Peru Margin (ODP Leg 201, Site 1229). Geobiology **2:**217–223.
9. **Biddle JF, Fitz-Gibbon ST, Schuster SC, Brenchley JE, House CH.** 2008. Metagenomic signatures of the Peru Margin subseafloor biosphere show a genetically distinct environment. Proc. Natl. Acad. Sci. U. S. A. **105:**10583–10588.
10. **Schouten S, Middelburg JJ, Hopmans EC, Sinninghe Damsté JS.** 2010. Fossilization and degradation of intact polar lipids in deep subsurface sediments: a theoretical approach. Geochim. Cosmochim. Acta **74:**3806–3814.
11. **Xie S, Lipp JS, Wegener G, Ferdelman TG, Hinrichs K.** 2013. Turnover of microbial lipids in the deep biosphere and growth of benthic archaeal populations. Proc. Natl. Acad. Sci. U. S. A. **110:**6010–6014.
12. **Teske A, Sørensen KB.** 2008. Uncultured archaea in deep marine subsurface sediments: have we caught them all? ISME J. **2:**3–18.
13. **Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
14. **Braithwaite A.** 1976. Unit cell dimensions of crystalline horseradish peroxidase. J. Mol. Biol. **106:**229–230.
15. **Pernthaler A, Pernthaler J, Amann R.** 2002. Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl. Environ. Microbiol. **68:**3094–3101.
16. **DeLong EF, Taylor LT, Marsh TL, Preston CM.** 1999. Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent in situ hybridization. Appl. Environ. Microbiol. **65:**5554–5563.
17. **Pernthaler A, Preston CM, Pernthaler J, DeLong EF, Amann R.** 2002. Comparison of fluorescently labeled oligonucleotide and polynucleotide probes for the detection of pelagic marine bacteria and archaea. Appl. Environ. Microbiol. **68:**661–667.
18. **Davis BD, Luger SM, Tai PC.** 1986. Role of ribosome degradation in the death of starved Escherichia coli cells. J. Bacteriol. **166:**439–445.
19. **Siegert M, Krüger M, Teichert B, Wiedicke M, Schippers A.** 2011. Anaerobic oxidation of methane at a marine methane seep in a forearc sediment basin off Sumatra, Indian Ocean. Front. Microbiol. **2:**249. doi:10.3389/fmicb.2011.00249.
20. **Breuker A, Köweker G, Blazejak A, Schippers A.** 2011. The deep biosphere in terrestrial sediments in the Chesapeake Bay area, Virginia, USA. Front. Microbiol. **2:**156. doi:10.3389/fmicb.2011.00156.
21. **Schippers A, Kock D, Höft C, Köweker G, Siegert M.** 2012. Quantification of microbial communities in subsurface marine sediments of the Black Sea and off Namibia. Front. Microbiol. **3:**16. doi:10.3389/fmicb.2012.00016v.
22. **Webster G, Blazejak A, Cragg BA, Schippers A, Sass H, Rinna J, Tang X, Mathes F, Ferdelman TG, Fry JC, Weightman AJ, Parkes RJ.** 2009. Subsurface microbiology and biogeochemistry of a deep, cold-water carbonate mound from the Porcupine Seabight (IODP Expedition 307). Environ. Microbiol. **11:**239–257.
23. **Visweswaran GRR, Dijkstra BW, Kok J.** 2011. Murein and pseudomurein cell wall binding domains of bacteria and archaea—a comparative view. Appl. Microbiol. Biotechnol. **92:**921–928.
24. **Albers S-V, Meyer BH.** 2011. The archaeal cell envelope. Nat. Rev. Microbiol. **9:**414–426.
25. **Ebeling W, Hennrich N, Klockow M, Metz H, Orth HD, Lang H.** 1974. Proteinase K from *Tritirachium album* Limber. Eur. J. Biochem. **47:**91–97.
26. **Sekar R, Pernthaler A, Pernthaler J, Posch T, Amann R, Warnecke F.** 2003. An improved protocol for quantification of freshwater actinobacteria by fluorescence in situ hybridization. Appl. Environ. Microbiol. **69:**2928–2935.
27. **Teira E, Reinthaler T, Pernthaler A, Pernthaler J, Herndl GJ.** 2004. Combining catalyzed reporter deposition-fluorescence in situ hybridization and microautoradiography to detect substrate utilization by bacteria and archaea in the deep ocean. Appl. Environ. Microbiol. **70:**4411–4414.
28. **Molari M, Manini E.** 2012. Reliability of CARD-FISH procedure for enumeration of Archaea in deep-sea surficial sediments. Curr. Microbiol. **64:**242–250.
29. **Lloyd KG, Alperin MJ, Teske A.** 2011. Environmental evidence for net methane production and oxidation in putative anaerobic methanotrophic (ANME) archaea. Environ. Microbiol. **13:**2548–2564.
30. **R Core Team.** 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
31. **Wickham H.** 2009. ggplot2: elegant graphics for data analysis. Springer, New York, NY.
32. **Kallmeyer J, Smith DC, Spivack AJ, Hondt SD.** 2008. New cell extraction procedure applied to deep subsurface sediments. Limnol. Oceanogr. Methods **6:**236–245.
33. **Noble RT, Fuhrman JA.** 1998. Use of SYBR green I for rapid epifluores-

cence counts of marine viruses and bacteria. Aquat. Microb. Ecol. **14:**113–118.
34. **Schumann R, Rentsch D.** 1998. Staining particulate organic matter with DTAF—a fluorescence dye for carbohydrates and protein: a new approach and application of a 2D image analysis system. Mar. Ecol. Prog. Ser. **163:**77–88.
35. **Kubo K, Lloyd KG, Biddle J, Amann R, Teske A, Knittel K.** 2012. Archaea of the miscellaneous crenarchaeotal group are abundant, diverse and widespread in marine sediments. ISME J. **6:**1949–1965.
36. **Bouvier T, Del Giorgio PA.** 2003. Factors influencing the detection of bacterial cells using fluorescence in situ hybridization (FISH): a quantitative review of published reports. FEMS Microbiol. Ecol. **44:**3–15.
37. **Garneau M-È, Vincent WF, Terrado R, Lovejoy C.** 2009. Importance of particle-associated bacterial heterotrophy in a coastal Arctic ecosystem. J. Mar. Syst. **75:**185–197.
38. **Ishii K, Mussmann M, MacGregor BJ, Amann R.** 2004. An improved fluorescence in situ hybridization protocol for the identification of bacteria and archaea in marine sediments. FEMS Microbiol. Ecol. **50:**203–213.
39. **Markowitz VM, Korzeniewski F, Palaniappan K, Szeto E, Werner G, Padki A.** 2006. The integrated microbial genomes (IMG) system. Nucleic Acids Res. **34:**D344–D348.
40. **Mumy KL, Findlay RH.** 2004. Convenient determination of DNA extraction efficiency using an external DNA recovery standard and quantitative-competitive PCR. J. Microbiol. Methods **57:**259–268.
41. **Takai K, Horikoshi K.** 2000. Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. Appl. Environ. Microbiol. **66:**5066–5072.
42. **Breuker A, Stadler S, Schippers A.** 2013. Microbial community analysis of deeply buried marine sediments of the New Jersey shallow shelf (IODP Expedition 313). FEMS Microbiol. Ecol. **85:**578–592.
43. **Breuker A, Schippers A.** 2013. Data report: total cell counts and qPCR abundance of Archaea and Bacteria in shallow subsurface marine sediments of North Pond: gravity cores collected during site survey cruise prior to IODP Expedition 336. Proc. Integr. Ocean Drill. Progr. **336:**1–7.
44. **Karner MB, DeLong EF, Karl DM.** 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature **409:**507–510.
45. **Kirchman DL, Elifantz H, Dittel AI, Malmstrom RR, Cottrell MT.** 2007. Standing stocks and activity of Archaea and Bacteria in the western Arctic Ocean. Limnol. Oceanogr. **52:**495–507.
46. **Lomstein BA, Langerhuus AT, D'Hondt S, Jørgensen BB, Spivack AJ.** 2012. Endospore abundance, microbial growth and necromass turnover in deep sub-seafloor sediment. Nature **18:**101–104.
47. **Srivastava AK, Schlessinger D.** 1990. Mechanism and regulation of bacterial ribosomal RNA processing. Annu. Rev. Microbiol. **44:**105–129.
48. **Orsi WD, Edgcomb VP, Christman GD, Biddle JF.** 2013. Gene expression in the deep biosphere. Nature **499:**205–208.
49. **Schippers A, Köweker G, Höft C, Teichert BMA.** 2010. Quantification of microbial communities in forearc sediment basins off Sumatra. Geomicrobiol. J. **27:**170–182.
50. **Engelen B, Ziegelmüller K, Wolf L, Köpke B, Gittel A, Cypionka H, Treude T, Nakagawa S, Inagaki F, Lever MA, Steinsbu BO.** 2008. Fluids from the oceanic crust support microbial activities within the deep biosphere. Geomicrobiol. J. **25:**56–66.
51. **Valentine DL.** 2007. Adaptations to energy stress dictate the ecology and evolution of the Archaea. Nat. Rev. Microbiol. **5:**316–323.
52. **Lloyd KG, Schreiber L, Petersen DG, Kjeldsen KU, Lever MA, Steen AD, Stepanauskas R, Richter M, Kleindienst S, Lenk S, Schramm A, Jørgensen BB.** 2013. Predominant archaea in marine sediments degrade detrital proteins. Nature **496:**215–218.
53. **Zinser ER, Kolter R.** 1999. Mutations enhancing amino acid catabolism confer a growth advantage in stationary phase. J. Bacteriol. **181:**5800–5807.