Evidence for a growth zone for deep subsurface microbial clades in near-surface anoxic sediments

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Abstract

Global marine sediments harbor a large and highly diverse microbial biosphere, but the mechanism by which this biosphere is established during sediment burial is largely unknown. During burial in marine sediments, concentrations of easily-metabolized organic compounds and total microbial cell abundance decrease steadily. However, it is unknown whether some microbial clades increase with depth, despite the overall trend of abundance decrease. We show total population increases in 38 microbial families over 3 cm of sediment depth in the upper 7.5 cm of White Oak River (WOR) estuary sediments. Clades that increased with depth were more often anaerobic, uncultured, or common in deep marine sediments relative to those that decreased. Minimum turnover times (which are minimum *in situ* doubling times of growth rates) were estimated to be 2-25 years by combining sedimentation rate with either quantitative PCR (qPCR) or the product of the Fraction Read Abundance of 16S rRNA genes and total Cell counts (FRAxC). Turnover times were within an order of magnitude of each other in two adjacent cores, as well as in two laboratory enrichments of Cape Lookout Bight (CLB), NC, sediments (average difference of 28 ± 19%). qPCR and FRAxC in WOR cores and FRAxC in CLB

incubations produced similar turnover times for key deep subsurface uncultured clades *Bathyarchaeota* (8.7 ± 1.9 years) and *Thermoprofundales*/MBG-D (4.1 ± 0.7 years). We conclude that common deep subsurface microbial clades experience a narrow zone of growth in shallow sediments, offering an opportunity for natural selection of traits for long-term subsistence after resuspension events.

Significance statement: The current dogma is that the deeply-branching uncultured microbes that dominate global marine sediments do not actually increase in population size as they are buried in marine sediments – rather they exist in a sort of prolonged torpor for thousands of years. This is because no evidence has ever been found that these clades actually increase population sizes, or grow, as they are gradually buried. We discovered that they actually do increase population sizes during burial, but only in the upper few centimeters. This changes our dogma about marine sediments as a vast repository of non-growing microbes, to a vast repository of non-growing microbes with a thin and relatively rapid area of growth in the upper 10 centimeters.

Introduction

Marine sediments harbor one of the largest microbiomes on Earth, comprising ~2.9 x 10^{29} microbial cells (1). In most marine sediments, total microbial cells decrease with depth in a log-log relationship since the energy available to support the microbial community decreases as resources are depleted (1, 2). Therefore most microbes in marine sediments are nutrient-limited and in a state of near-zero growth (3, 4). Bulk sedimentary

49 microbial communities have been calculated to have turnover times on the order of tens
50 to hundreds of years, based on microbial respiration rates and energetic requirements (5,
51 6). The assembly of these deep subsurface communities over many meters of burial
52 appears to occur through selective survival (7) or environmental filtering (8), rather than
53 net growth. Only the most abundant organisms, or those best suited to the changing redox
54 regime, persist while all other clades die off (7–10). However, marine sediment microbes
55 appear to have adaptations to marine sediments, since enzyme affinity (lower K_m values)
56 to carbon substrates increases with depth and specialization shifts to highly-degraded
57 substrates with depth (11). These adaptations imply that heritable natural selection has
58 occurred in a sedimentary environment, which implies that these common subsurface
59 clades experience net growth, rather than just persistence, at some point during burial.
60 This net growth may occur in the upper few centimeters of sediments, since microbes
61 have been shown to grow and increase biomass in upper sedimentary layers (12, 13).
62 However, the methods used in these growth studies could not distinguish between
63 different clades nor whether this growth resulted in net population increases with
64 increasing depth. We therefore hypothesized that growth occurs for common subsurface
65 clades in the upper few centimeters of marine sediments, over relatively short time
66 intervals of a few tens of years. This growth would have been missed by previous studies
67 that demonstrated persistence rather than growth of subsurface clades during burial (7–
68 10) because these studies were conducted in deeper sediments, after net growth had
69 largely ceased.

70 Microbial populations in marine sediments are phylogenetically diverse (14), so
71 clades may differ in their response to burial. One option is that the cells from each clade

72 die-off at similar rates, with no evidence of growth (Fig. 1). The second option is that
73 some clades experience growth as they are buried, allowing them to increase their
74 population size, even as whole-community cell numbers decrease (Fig. 1). In the second
75 option, the rate of increase in total population size, or turnover time, therefore represents
76 a minimum growth rate estimate. The maximum growth rate could be even higher since
77 growth and death rates may be in equilibrium at each depth layer. Marine sediments
78 contain many phylogenetically divergent non-cultured cells (PDNC) belonging to high-
79 level taxonomic groups with no cultured representatives (15). We hypothesized that 1)
80 some PDNC clades increase in cell abundance with depth in the upper 10 cm of coastal
81 sediments, 2) their turnover times are slower than the < 24 hours doubling times of most
82 microbial cultures, and 3) turnover times are specific to each clade, and are therefore
83 repeatable in different sediment cores and laboratory incubations. We measured cell
84 abundance changes for individual microbial clades with depth in the upper 10 cm (or 40
85 years of burial time) in the White Oak River (WOR) estuary. Like most marine
86 sediments, they contain diverse PDNCs, and have a steady sediment deposition rate
87 allowing a conversion between depth and time (16). As a second test, we measured the
88 growth of these organisms in 2.2 year-long incubations of sediments from Cape Lookout
89 Bight (CLB) without any nutrient additions so that time could be measured directly (17).

90 Measuring microbial growth rates in natural marine sediments is not
91 straightforward. Quantification methods, such as catalyzed reporter deposition
92 fluorescent in situ hybridization (CARD-FISH) and quantitative PCR (qPCR) are often
93 inaccurate in marine sediments, due to problems with permeabilizing cells and
94 amplification biases (18, 19). These methods are also limited because they only measure

the microbial groups targeted by the chosen probes or primers. Measuring the relative abundance of 16S rRNA gene sequence reads amplified from marine sediments encompasses a wider range of phylogenetic groups, but also does not provide accurate quantifications since DNA extraction methods and primer sequences are biased (20).

However, when considering relative population increases, these biases cancel each other out (Fig. 2). To quantify the relative population changes with depth, one can multiply the Fraction of 16S rRNA gene Read Abundance by the total Cell counts (FRAxC) at each depth or timepoint. The ratio of FRAxC at different depths or timepoints has the bias term in both the numerator and the denominator, so it cancels out, allowing the quantification of relative changes in population size (i.e., a 3-fold increase or a doubling in the example in Fig. 2), but not the exact number of cells that have been gained or lost, over the time interval (18). A second measurement for turnover time was made using qPCR assays, which can also be used to calculate relative population turnover times (18). We found that PDNC and clades that could be inferred to be anaerobic increased with population turnover times of 2-25 years, with good replication for each clade with FRAxC and qPCR, between duplicate cores from the WOR estuary, and between cores from the WOR estuary and CLB sediment incubations. Close relatives of cultures and aerobic organisms decreased with population half lives that were not replicable through the different measurements.

Methods

Sample acquisition and treatment. Four sediment cores of ~10 cm depth each were retrieved from Station H, WOR estuary (34 44.490' N, 77 07.44' W) May 16, 2016, in about 1.5 m water depth, with water salinity of 11 ppt (cores 30, 21, 32, and 34). Each core was sectioned into 1 cm intervals later that day at room temperature (roughly equal to the measured *in situ* water temperature of 25°C) at the University of North Carolina Institute for Marine Sciences. Two cores (cores 30 and 32) were sectioned for qPCR and 16S rRNA gene libraries, two cores (cores 34 and 31, which were taken adjacent to cores 30 and 32, respectively) were sectioned for sulfate concentrations and porosity. The core for cell counts was taken May 28, 2013 (core 7) from the same site (34°44.482'N, 77°7.435'W), and was sectioned in 3 cm intervals. The top 3 cm of marine sediment from CLB (34.6205°N, 76.5500°W) were collected from 10 m water depth on October 2, 2013 and combined into a 2L Erlenmeyer flask that was incubated anoxically for 802 days, as reported previously (17), without any amendment of substrates.

Geochemistry. To measure sulfate, a 15 ml plastic tube was filled completely with sediment and centrifuged at 5,000x g for 5 minutes. A syringe was used to remove the supernatant below the air interface. The porewater was filtered using a 0.2 μm syringe filter into 100 μl of 10% HCl to a final volume of 1 ml. Porewater sulfate concentrations were determined by ion chromatography (Dionex, Sunnyvale, CA). Porosity was determined by comparing dry and wet weights as described previously (21).

DNA extraction, qPCR, sequencing, and data analysis. Each 1 cm depth interval of cores 30 and 32 was placed into a Whirlpak bag and frozen immediately on dry ice for later DNA extraction after storage at -80°C. DNA was extracted from frozen sediments (MoBio RNA Powersoil kit with DNA accessory) and enumerated on a

NanoDrop 3000. At the timepoints from the CLB incubations, DNA was extracted from frozen samples using the FastDNA kit for Soil (MP Bio, Santa Ana, CA). For both the WOR cores and the CLB incubations, the V4 region of each DNA extract was amplified using the universal primers 515f and 806r (22), prepared via Nexterra kit and sequenced at the Center for Environmental Biotechnology at the University of Tennessee (Knoxville, TN) on an Illumina MiSeq. 16S rRNA gene reads were processed, chimera-checked, and classified via Silva taxonomy (v126; (23)) in mothur (24). All calculations were performed on clades at the fifth taxonomic level, which is roughly the family level, only from clades with >150 total reads in the Miseq run, which left 327 clades from core 30 and 288 clades from core 32. 16S rRNA gene reads were deposited at the Short Read Archive (SRA) at NCBI's Genbank with BioProject ID PRJNA614649 for WOR sediments and PRJNA321388 for CLB incubations.

Quantitative PCR (qPCR) was used to determine the 16S rRNA gene copy numbers of bacteria, archaea, *Bathyarchaeota*, and Marine Benthic Group – D in the *Thermoplasmata* (with the latter for core 32 only) using the Quantifast SYBRGreen kit (Qiagen) on a BioRad Opticon2 thermocycler. Since absolute copy numbers cannot be accurately measured for DNA in marine sediments (18, 25), only the relative changes in copy number were measured. Our primers were MCG528F and MCG732R for *Bathyarchaeota* (26), MBG-D322F and MBG-D569R for Marine Benthic Group – D (27), ARCH915F (28) and ARC1059R (29) for archaea, and BAC340F and BAC515R for bacteria (30).

Population turnover time calculations. Population turnover times for a family-level classification were calculated from relative 16S rRNA gene abundances and total cell counts as described previously (17), with the following relationship:

$$t_{d,i} = \frac{t\ln(2)}{\ln\left(\frac{FRA_{i,t}C_{tot,t}}{FRA_{i,0}C_{tot,0}}\right)} \quad \text{Equation 1}$$

where $t_{d,i}$ is the doubling time of the *i*th family clade, t is the elapsed time, $FRA_{i,t}$ is the fraction read abundance of the *i*th clade at time t, and $C_{tot,t}$ is the total number of cells at time t. The product of the fraction 16S rRNA gene abundance of a particular clade and the total cell abundance will be referred to as the FRAxC of a particular clade. To calculate the population turnover for a specific group of target organisms using qPCR, we used the following relationship:

$$t_{d,i} = \frac{t\ln(2)}{\ln\left(\frac{copies_{i,t}}{copies_{i,0}}\right)} \quad \text{Equation 2}$$

where *copies* is the number of copies of target 16S rRNA gene quantified via qPCR. Equations 1 and 2 are equivalent to ln(2) divided by the slope time vs. the natural log of FRA_iC_{tot} or the natural log of $copies_i$. For incubations from CLB, time from the start of the experiment was known. For samples taken from the WOR estuary, time at each depth was determined based on an age model (31), using the porosity measurements for core 31 and a sedimentation rate of 0.26 cm/year (32).

Cell counts. Sediments were fixed in 3% paraformaldehyde for a few hours, washed twice with PBS, and stored at -20°C in PBS:ethanol. Sediments were sonicated at 20% power for 40 seconds, diluted to 1:40 in PBS, filtered onto a 0.2 μm polycarbonate filter (Fisher Scientific, Waltham, MA), stained with SYBR Gold (Invitrogen, Carlsbad, CA),

and counted at 100x magnification on an epifluorescent microscope. To interpolate cell counts into 1 cm intervals, an exponential curve fit was applied to the samples within the 0-10 cm interval with the following fit: depth = 137.77 exp(-1x10^{-9} x cells/ml), R^2=0.94. Two more cell count curves were used to test the sensitivity of turnover times to cell count profile variations. In one, cell counts from a 2012 WOR core were previously published (17), and resulted in the following curve fit over 0-10 cm: depth = 14.13 exp(-4x10^{-10} x cells/ml), R^2=0.93. In the second, cell counts were taken using WebPlotDigitizer from previously published estuarine sediments from Ashleworth Quay (33). For the CLB incubations, SYBR Gold direct counts of paraformaldehyde-fixed cells were performed as described previously (17).

Results and Discussion

Sulfate concentrations and porosity were constant with depth in the upper 5 cm of core 34 and the upper 3 cm of core 31 (Fig. S1) due to bioirrigation and bioturbation. Below this, sulfate concentrations decreased due to microbial sulfate reduction as has been observed in other cores at this site (21, 34). Total cell abundance decreased with depth (Fig. S2). The FRAxC and qPCR of total archaea, *Bathyarchaeota* (previously called MCG or MG 1.3 (35)), and *Thermoprofundales* (previously called MBG-D (36)) increased slightly below the depth of bioirrigation in both WOR cores (Fig. S3). The FRAxC and qPCR of total bacteria decreased with depth in both cores. qPCR values were lower than FRAxC values, demonstrating that these methods are subject to amplification biases and are best used to measure relative changes rather than absolute values (18). The turnover times measured with FRAxC and qPCR methods were within a year of each

other for *Bathyarchaeota* and MBG-D (Table 1), suggesting that FRAxC is as good a measure of relative abundance as qPCR. FRAxC, however, opens the possibility of measuring turnover times for any clade represented in the 16S rRNA gene libraries, without developing primer sets for each one.

Using FRAxC in the WOR cores, the doubling time (defined as a positive value for turnover time) or half-life (defined as negative a value for turnover time) was calculated for each family-level clade across the three centimeters below the bioirrigated upper layer (5.5-7.5 cm for core 30 and 3.5-5.5 cm for core 32, Table S1). These three depths produced the highest number of clades that increased with depth. A similar increase in the relative contribution from anaerobic organisms was observed immediately below the bioirrigation zone in the marine sediments of Aarhus Bay, Denmark (8). Of the 315 clades, 278 were present in both cores, and 60% of these had $R^2 > 0.4$ to the curve fit to equation 1, meaning that a change in FRAxC over depth could be observed. The lenient cut-off of $R^2 > 0.4$ was chosen in order to perform downstream analyses on the largest possible selection of clades. Of these, 80% had doubling times or half-lives between 2 and 25 years. This means that the sampling interval of ~8 years was sufficient to resolve clades that underwent at least a third of one doubling or halving in that interval. Clades that increased faster or more slowly with depth were outside the detection limit. Read abundance between different clades did not correlate with turnover time (Fig. S4). In other words, a clade's relative abundance in the 16S rRNA gene libraries did not predict whether it would increase or decrease with depth.

To determine how sensitive turnover times were to differences in the cell abundance curves used to calculate FRAxC, comparisons were made with cell counts

228 from a 2012 WOR core (17) and estuarine sediments from Ashleworth Quay (33). For
229 core 30, 92% and 100% of the clades with turnover times of 2-25 years still fell within
230 that range using the 2012 and Ashleworth Quay cell counts. For core 32, these numbers
231 were 98% and 100%. This suggests that this method is fairly robust to variations in cell
232 abundance curves.

233 The direction of either increase or decrease was replicable in WOR cores 30 and
234 32 for 131 out of 133 clades (Fig. 3a). Clades that increased with depth were common
235 marine subsurface inhabitants: *Chloroflexi*, *Deltaproteobacteria*, *Planctomycetes*,
236 *Bathyarchaeota*, *Lokiarchaeota*, MBG-D, etc (37). Those that decreased with depth were
237 common seawater inhabitants: *Betaproteobacteria*, *Alphaproteobacteria*,
238 *Gammaproteobacteria*, *Thaumarchaeota* (MG-I), *Bacteroidetes*, *Acidobacteria* (38).
239 Clades from uncultured phyla were more likely to increase rather than decrease with
240 depth (9/11 clades). Clades from cultured orders, families, genera, or species were more
241 likely to decrease rather than increase with depth (57/60 clades). This suggests that easily
242 cultured organisms were dying off and difficult-to-culture clades, or PDNC, were
243 growing. Aerobes and nitrate reducers were more likely to decrease with depth (23/23
244 clades where all cultures are obligate aerobes or nitrate reducers, Fig. 3a, inset). Doubling
245 times for clades that increased were well-correlated for individual clades between the two
246 WOR cores (slope = 0.87, $R^2 = 0.64$), suggesting that they may have been growing at
247 rates determined by their particular physiological traits. The mean deviation from the
248 mean between the duplicate cores was 28 ± 19%. This means that turnover times were
249 accurate at least within an order of magnitude. For instance, a clade with a turnover time
250 of 10 is very likely to have an actual turnover time within at least a range of 5-15 years.

In contrast, half-lives for clades that decreased were poorly correlated for individual clades between cores (slope = 0.27, $R^2 = 0.11$). Clades that decreased with depth may have been dying or decaying because they could not meet their energetic requirements in this anoxic environment, and, unlike the turnover times of the growing organisms, their rates of decay/death were not specific to their physiologies. These results support the conclusion that FRAxC doubling times represent real population changes during burial and not random variations.

As a separate check on the feasibility of these turnover times, we used the depth-integrated sulfate reduction rate (0.93 µM cm^{-3} d^{-1}, Fig. S1) to estimate a turnover time for sulfate reducers of 12 years, assuming that 10% of total cells were sulfate reducers with growth yields of 2 g cell C/mol (3), and cell carbon contents of 23 fg/cell (39). This falls well within our results from FRAxC of 2-25 years doubling times, including one clade with cultured sulfate reducers, *Desulfarculaceae*, that had doubling times of 15 and 18 years in the two cores. Previous work in arctic and temperate marine sediments also supports generation times of over a year in marine sediment incubations where total cell abundance of sulfate reducing bacteria held constant despite increasing metabolic rates over a year-long incubation (40).

Should the population turnover times estimated here approximate the growth rate of these clades, then these microbes would be considered dormant relative to pure cultures (4). However, if the populations are in steady state with some resource that gradually increases with depth (such as a lessening influence of bioturbation, for example), then cell replication is roughly balanced by cell death, so an increase in total population size does not necessarily reflect the growth rate, which may be much faster.

To estimate how much the growth rate is likely to differ from doubling times, imagine a clade which increased in relative population from 4% to 50% of the population during a drop in total community size of 1,000,000 cells to 100,000 cells over 2 years. This clade would have a doubling time of 6.21 years. During those two years, the net gain was therefore 10,000 cells. If no cells died, then 10,000 divisions occurred and the doubling time is a growth rate. Death rates can vary greatly among different microbial groups (41), but if we estimate the death rate for a clade to be equal to the total community death ratio of 10:1 for this example, then 100,000 divisions would have occurred and the true growth rate would have been 1.16 years. So, it is possible that the actual growth rates of the clades in our study were faster than 2-25 years, but they may not have been orders of magnitude faster if the decay rates are slow for cells adapted to low energy environments.

Two alternative possibilities could also explain an increase with depth in sediment cores, without indicating population growth. First, this increase could represent a mixing curve between a deep source of microbial cells diffusing up toward the surface sediments. Second, the increase could be the result of depositional changes, with greater loading of these subsurface clades in previous depositional events. To rule out these alternative possibilities, we tracked community succession in marine sediment from Cape Lookout Bight, NC, in a laboratory mesocosm under methanogenic conditions over 2.2 years (Fig. 3b). Doubling times or half-lives were calculated with FRAxC for each family level clade using timepoints between 40 and 802 days (40, 47, 54, 61, 75, 80, 86, 94, 107, 114, 122, and 802 days, Table S2). All 601 taxa were present in both incubations. R-squared was not a good method of quality control for the CLB incubation data because the data were not evenly distributed across the time interval. Instead, an abundance cutoff was imposed

297 such that each clade must have had at least one timepoint with a value of at least 14 for
298 ln(FRAxC), which left 195 taxa remaining. Of these, 96%, or 188, had doubling times or
299 half-lives between 0.1 and 7 years (Fig. 3b, Table S2). As with the WOR cores, the
300 sampling interval of 0.1 to 2.2 years was sufficient to resolve clades that underwent a
301 least a third of one doubling or halving in that interval, with slower or faster clades
302 outside the detection limit. Also, similarly to the WOR cores, the direction of increase or
303 decrease was replicable in duplicate incubations (182/188 clades), clades that increased
304 were more similar between replicate incubations (slope = 1.05, R^2 = 0.96), and clades
305 that decreased were less similar between replicate cores (slope = 0.83, R^2 = 0.53).

306 The clades that increased over time in the CLB incubations were largely methane-
307 cycling archaea (*Methanosarcinales*, *Methanomicrobiales*, and ANME-1), which was
308 expected since the onset of methanogenesis occurred during the sampling interval. The
309 turnover times of methanogen-like archaea of 0.3 to 0.8 years, were consistent with
310 previously measured doubling times of one of these clades, ANME-1, grown in an
311 enrichment, at 0.6 years (42). Of the five clades that increased in both the WOR
312 sediments and the CLB incubations (*Bathyarchaeota*, C3, MBG-D, MG-III, and 20a-9),
313 four increased faster in the CLB incubations, but most had multi-year turnover times in
314 both types of experiments and measurement methods (Table 1). Two clades
315 (*Bathyarchaeota* and MBG-D) had both types of measurements (qPCR and FRAxC) in
316 both types of experiments (WOR cores and CLB incubations). The mean of all these
317 measurements resulted in turnover times of 8.7 ± 1.9 years for *Bathyarchaeota* and 4.1 ±
318 0.7 years for MBG-d; the relatively small standard deviation suggests that these turnover
319 times are accurate within a few years. Many of the clades with slower turnover times in

320 the WOR cores were present in the CLB incubations, but did not have turnover times that
321 met our quality cut-off. We may have been able to resolve their growth rates if we had
322 waited ~8 years. The lab incubation experiment therefore also supports a multi-year
323 population turnover time for anoxic uncultured marine subsurface organisms, in an
324 experimental system that rules out diffusional mixing and depositional changes as drivers
325 for the population changes.

326 These results suggest that the log-log decay rate with depth for the total microbial
327 community does not apply to some clades within the marine sediment biosphere. Some
328 clades experience periods of net growth, resulting in population increases during burial
329 (Option 2 in Fig. 1). This has important implications for microbial adaptations to the
330 marine subseafloor biosphere. Many of these clades have been shown to be dormant or
331 non-growing for most of the sediment column (3, 9). In other environments, such as
332 agricultural soils, periods of dormancy are interspersed with periods of growth (4). Our
333 results suggest that the period of growth for marine sediment microbes may occur in the
334 upper few cm, over a time period that is much shorter than the full marine sediment
335 column, which can be millions of years old.

336 This suggests a possible mechanism for adaptation to long-term dormancy in
337 marine sediments, building on the Growth Advantage in Stationary Phase (GASP), where
338 *E. coli* cells that have been starved for years outcompete fresh cells under low substrate
339 conditions (43). Our results suggest that these GASP-like adaptations to long-term
340 starvation could also be selected for after resuspension events, allowing GASP to be
341 extrapolated over much longer timescales in marine sediment, as was predicted
342 previously (44). If deep sedimentary cells with subsistence-promoting or GASP-like

343 mutations are resuspended either by a storm or sediment slumping, these organisms
344 would have a growth advantage over others as they were buried a second time through
345 the relatively brief and shallow growth zone. This means that although evolutionary
346 changes do not occur during the process of burial and near-zero cell growth over
347 thousands of years (7), the ability to subsist over thousands of years in marine sediment is
348 a trait that could have been selected for within the growth zone in shallow sediments.
349 Microbes might even be ejected from deeper sediments depths by mud volcanoes into
350 overlying seawater, where they can be carried by currents and re-deposited at the seafloor
351 (45). Our results suggest that such organisms may revive and begin growing shortly after
352 deposition.

353 If these turnover times are similar to growth rates, then these organisms grow
354 much more slowly than most laboratory cultures, which double in less than a day. This
355 agrees with previous work showing that microbes grow much more slowly in the
356 environment than in laboratory culture. *Staphylococcus aureus* grows 48-fold faster in
357 culture than it does in an infected lung (46) and *Leucothrix mucor* grows 7-fold faster in
358 culture than it does in seawater (47). The fact that so many of the clades that grew in our
359 study belong to uncultured clades suggests that slow growth rates may be a reason why
360 so many of them remain uncultured. Recent successes with culturing slow-growing
361 strains from previously-uncultured phyla *Lokiarchaeota* and *Atribacteria* attest to the
362 necessity of extremely long wait times for growth; 2000 days in one case (48, 49).

363 Even if it is possible to speed up such organisms sufficiently to culture them, their
364 traits expressed under such conditions are likely to greatly diverge from those expressed
365 in their natural environment. Therefore, even when cultures are available, it is crucial to

366 study these organisms in their natural environmental settings. Using label incorporation
367 (46, 50, 51), or tracking slow growth in a mixed population under different conditions,
368 will allow for growth experiments on the environmentally abundant, evolutionarily
369 distinct, uncultured microbes from resource-limited environments. Such experiments are
370 necessary to assess modifications to cell cycle regulation, proteins, or plasma membranes
371 that enable such extraordinarily slow growth, as well as determine how this narrow
372 growth zone in surficial sediments may select for the traits that enable long-term
373 subsistence of the deep subsurface biosphere.

374

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384

385 **Table 1.** Doubling times for all clades that increased in both cores of White Oak River
386 (WOR) estuary sediments and replicate incubations from the Cape Lookout Bight (CLB)
387 marine sediments. Data are expressed in years. FRAxC denotes values calculated by the

388 increase of the product of 16S rRNA gene read percentages and total cells over depth
389 (WOR) or time (CLB). qPCR denotes values calculated by the increase of qPCR of 16S
390 rRNA genes over depth. Hyphen indicates no data because qPCR was not attempted for
391 that clade or sample.

	WOR marine sediment				**CLB marine sediment**	
	Core 30		Core 32		Incubation 2	Incubation 3
Clade	FRAxC	qPCR	FRAxC	qPCR	FRAxC	FRAxC
Bathyarchaeota, or MCG	10.1	10.3	8.9	10.0	6.4	6.3
Group C3, MCG-related	16.2	-	14.9	-	2.1	1.8
Thermoprofundales (MBG-D)	4.6	-	3.4	4.8	3.4	4.4
Thermoprofundales (MG-III)	3.7	-	2.8	-	1.4	1.5
Thermoprofundales (20a-9)	7.1	-	3.7	-	0.8	0.7

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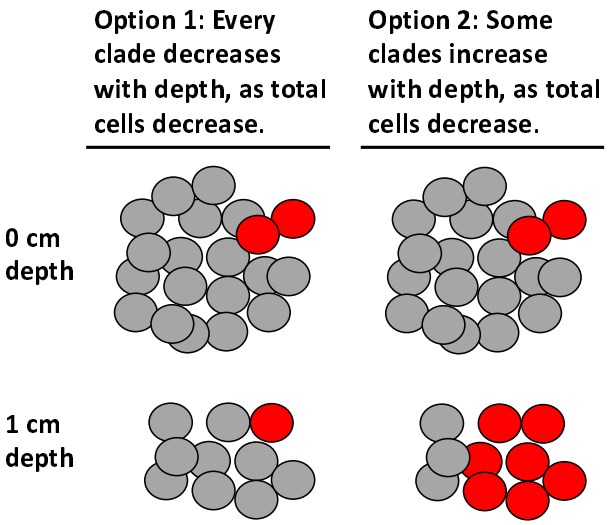

Figure 1. Options for how an individual clade of microbes (red cells) either decrease (option 1) or increase (option 2) with total population decrease with 1 cm sediment depth.

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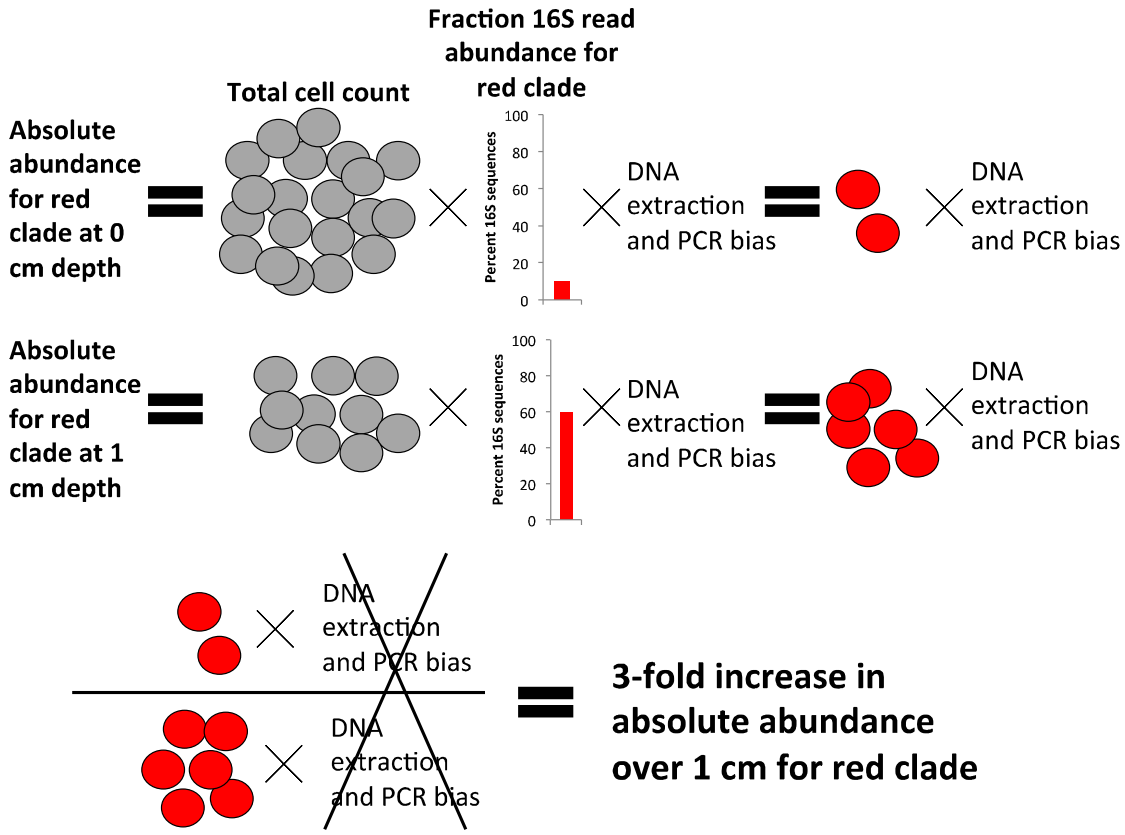

Figure 2. Example to explain how Fraction Read Abundance times Cell abundance (FRAxC) works. The total cell count (grey) decreases with depth. The absolute abundance of an imaginary clade of organisms (red) is equal to the total cell abundance multiplied by the fraction of 16S rRNA gene reads of the red clade and a bias term that cannot be measured accurately and is different for each clade of microbes. Assuming that this bias term is similar over adjacent sediment depths for the same clade of microbes, the relative increase in the red clade with depth can be measured absolutely, since the bias term cancels out.

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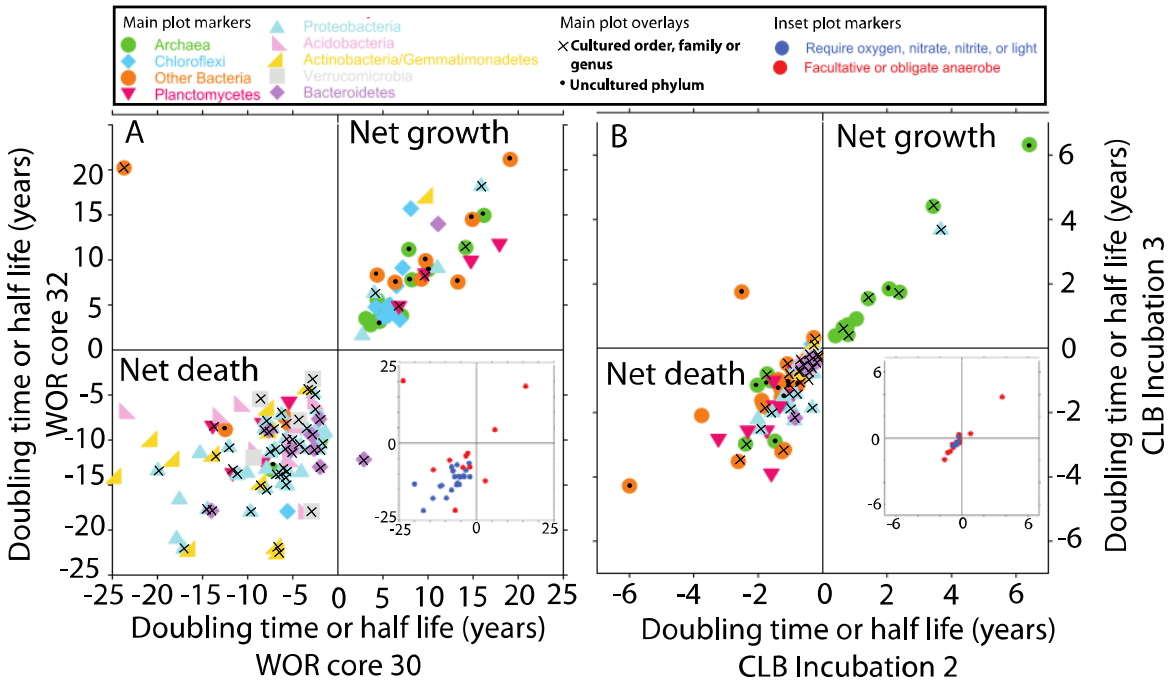

Figure 3. Multi-year doubling times for uncultured microbes in marine sediments, measured in A) down-core trends in White Oak River estuary or B) incubation time course experiments from Cape Lookout Bight. In each panel, replicates are plotted against each other to show good correlation between clades that increase (upper right quadrants) or decrease (lower left quadrants) with depth. In A) clades that grow tend to be common subsurface clades (in dark colors) and uncultured phyla (black dots); clades that decay tend to be common seawater clades (in pastels) and more closely related to cultures (x's). Insets show the subset of clades belonging to cultured families where all cultured relatives are oxygen- or nitrate-reducing (blue), or contain at least some non-nitrogen-cycling facultative anaerobes (red). Note different timescales for WOR and CLB data.

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