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Authors

Hatzenpichler, Roland

Connon, Stephanie A

Goudeau, Danielle

et al.

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Visualizing *in situ* translational activity for identifying and sorting slow-growing archaeal-bacterial consortia

Roland Hatzenpichler[a,1], Stephanie A. Connon[a], Danielle Goudeau[b], Rex R. Malmstrom[b], Tanja Woyke[b], Victoria J. Orphan[a,1]

[a] Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, CA-91125, USA
[b] Department of Energy Joint Genome Institute, Walnut Creek, CA-94598, USA
[1] Address correspondence to hatzenpichler@caltech.edu and vorphan@gps.caltech.edu

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Abstract

In order to understand the biogeochemial roles of microorganisms in the environment, it is important to determine when and under which conditions they are metabolically active. Bioorthogonal non-canonical amino acid tagging (BONCAT) can reveal active cells by tracking the incorporation of synthetic amino acids into newly synthesized proteins. The phylogenetic identity of translationally active cells can be determined by combining BONCAT with rRNA-targeted fluorescence *in situ* hybridization (BONCAT-FISH). In theory, BONCAT-labeled cells could be isolated with fluorescence-activated cell-sorting (BONCAT-FACS) for subsequent genetic analyses. Here, in the first application of BONCAT-FISH and BONCAT-FACS within an environmental context, we probe the translational activity of microbial consortia catalyzing the anaerobic oxidation of methane (AOM), a dominant sink of methane in the ocean. These consortia, which typically are composed of anaerobic methane-oxidizing archaea (ANME) and sulfate-reducing bacteria, have been difficult to study due to their slow *in situ* growth rates, and fundamental questions remain about their ecology and diversity of interactions occurring between ANME and associated partners. Our activity-correlated analyses of >16,400 microbial aggregates provide the first evidence that AOM-consortia affiliated with all five major ANME-clades are concurrently active under controlled conditions. Surprisingly, sorting of individual BONCAT-labeled consortia followed by whole genome amplification and 16S rRNA gene sequencing revealed previously unrecognized interactions of ANME with members of the poorly understood phylum *Verrucomicrobia*. This finding, together with our observation that ANME-associated *Verrucomicrobia* are found in a variety of geographically distinct methane seep environments, suggests a broader range of symbiotic relationships within AOM-consortia than previously thought.

Significance statement

One of the biggest challenges in environmental microbiology is to determine the activity of uncultured cells directly in their habitat. We report on the application of bioorthogonal non-canonical amino acid tagging (BONCAT), a high-throughput approach to detecting protein synthesis in individual cells by fluorescence staining on deep-sea methane seep sediments. By combining BONCAT with fluorescence *in situ* hybridization (FISH), we visualized active archaeal-bacterial consortia catalyzing the anaerobic oxidation of methane. We further developed a novel approach that combines BONCAT with fluorescence-activated cell-sorting (FACS) to separate translationally active cells from complex samples. BONCAT-FACS enabled us to directly link the identities of anaerobic methane-oxidizing archaea with their partner bacteria for individual active consortia, uncovering previously unknown interactions between these archaea and *Verrucomicrobia*.

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Introduction

Some of the most important goals of environmental microbiology are to understand the physiology, niche differentiation, and activities of microorganisms in the context of their habitat. Studies focusing on the mere presence of a cell or gene in a sample can only provide limited information about the metabolic capabilities of an organism. Coupling the identification of an uncultured microbe with its *in situ* activity thus has been referred to as the "Holy Grail" of microbial ecology (1). While bulk techniques, such as meta-transcriptomics and meta-proteomics or stable isotope probing targeted at DNA, RNA, or proteins, have provided us with exciting new insights into microbial ecophysiology (1-3), they cannot resolve cellular activities on the micron scale. The combination of rRNA-targeted fluorescence *in situ* hybridization (FISH) with single-cell resolving stable isotope analysis offers a direct, targeted approach for detailed investigations of microbial structure-function relationships (4, 5).

Single cell methods for direct assessment of metabolic activity include three broadly applicable approaches were available for studying the *in situ* anabolic activity of individual cells: micro-autoradiography (MAR; 6), secondary ion mass spectroscopy (SIMS and nanoSIMS; 7), and Raman microspectroscopy (Raman; 8). Each of these techniques have technical challenges or limited instrument availability which have slowed their wide adoption in the field (1, 5, 9). MAR and SIMS are also destructive methods that cannot be combined with downstream analysis such as cell-sorting, sub-culturing, or genomic sequencing. Another problem is that many biomolecules are prohibitively expensive or even unavailable in their isotopically labeled form. A universally applicable approach that circumvents these limitations was recently established by combining the general labeling of active cells via heavy water (D_2O) with their subsequent identification via FISH and sorting via Raman-coupled optical tweezers (10). Complementary to detecting anabolic activity via isotope labeling, a fluorescence technique based on the visualization of bacterial reductase activity via redox sensing (Redox sensor green) has also been described (11, 12). The general applicability and exact mechanism of this proprietary staining method are, however, unknown.

An alternative approach for studying microbial ecophysiology that does not depend on isotopes is labeling active cells via chemically modifiable analogs of biomolecules (9, 13, 14). Bioorthogonal non-canonical amino acid tagging (BONCAT) is a non-destructive technique first applied in neurobiology (15, 16). Last year, BONCAT was adapted for the study of uncultured archaea and bacteria within environmental samples (9, 14). BONCAT depends on the addition of a bioorthogonal (*i.e.* non-interacting with cellular functionalities) synthetic (non-canonical) amino acid to a sample. After its uptake (the exact process is currently unknown), the amino acid is able to exploit the substrate promiscuity of specific amino acyl-tRNA synthetases, the enzymes catalyzing the esterification of amino acids with their respective cognate tRNAs, to get incorporated into *de novo* peptides (17). Protein synthesis-active cells can in the following be visualized via a highly selective click chemistry-mediated labeling reaction that conjugates a modified fluorescence dye to a chemical reporter group (an azide or alkyne) of the bioorthogonal

amino acid (Fig. 1A). While a wide range of synthetic amino acids exists, only a small number are able to exploit the natural translational machinery without the need for genetic modification of the cell (18). To date, the *L*-methionine (Met) surrogates *L*-azidohomoalanine (AHA) and *L*-homopropargylglycine (HPG) (17) have found the widest application (e.g. 16, 19, 20-24). In a proof-of-principle investigation, BONCAT was applied to environmental samples and found to be generally applicable to uncultured archaea and bacteria (9, 14). BONCAT has been demonstrated to correlate well with other, independent proxies of cellular growth, *i.e.* the incorporation of isotopically labeled compounds as detected by nanoscale SIMS (15NH4+; 9) and MAR (35S-Met; 14). In addition, a protocol for the concomitant taxonomic identification of translationally active cells via rRNA-targeted FISH (*i.e.* BONCAT-FISH), was recently developed (9; Fig. 1A,B).

In this study, we applied HPG to deep-sea methane seep sediments in which the sulfate-coupled anaerobic oxidation of methane (AOM) is occurring. AOM accounts for the removal of ~80% of the CH_4 released from ocean sediments (>400 Gt $year^{-1}$) and is a key process in the biogeochemical cycling of this highly potent greenhouse gas (25). In marine seeps AOM is predominantly catalyzed by a symbiosis of anaerobic methane-oxidizing euryarchaeotes (ANME) with sulfate-reducing bacteria (SRB), which form consortia of varying cell number (~10 to ~10^5 cells) and morphology (7, 26). Their syntrophic partnership is hypothesized to be mediated by direct electron transfer (27, 28; Scheller et al., in revision) and/or diffusible intermediates (29, 30).

Several different ANME-clades (referred to as ANME-1a, -1b, -2a, 2b, 2c, and -3) have been observed to aggregate with different representatives of SRB (particularly members of the *Desulfosarcina*, *Desulfococcus*, and *Desulfobulbus* genera), with multiple ANME-SRB consortia of different taxonomies commonly co-existing in seep sediments without apparent competitive exclusion of one another. The potential for ecological niche partitioning within these highly diverse, yet seemingly functionally redundant associations is not well understood. Previously, it was demonstrated that temperature (31), CH_4 partial pressure (31), concentrations of sulfate (32, 33) and sulfide (33), as well as the availability of nitrogen (34, 35) may influence distribution and activity of AOM-consortia. In addition, ANME-community structure can vary dramatically between geographically proximate sites as well as distinct sediment layers (36, 37). Whether different ANME-subgroups in a given sample show variable activities depending on specific physico-chemical or ecological conditions or are all metabolically active at a given time is unknown. In addition to sulfate reducers, other bacterial lineages, including members of the alpha-, beta-, gamma-, and epsilon-proteobacteria, have been observed to form physical associations with ANME (38; Trembath-Reichert et al., in prep.). The metabolic interactions underlying these relationships are, however, yet to be determined.

Our study represents the first research application of BONCAT-FISH in environmental microbiology. We tested the influence of methane on the metabolic activity (protein synthesis) of diverse AOM-consortia in three sediment samples from two geographically and geochemically distinct locations. We further developed a novel approach for isolating protein-synthesizing cells

identified using BONCAT via fluorescence-activated cell-sorting (FACS). Subsequent 16S rRNA gene-based identification of individual AOM-consortia provided detailed aggregate-specific information into specific archaeal-bacterial partnerships. This approach revealed previously unrecognized metabolically active associations between ANME and new bacterial groups outside the deltaproteobacteria.

Results and Discussion

Establishment of HPG incubation experiments with methane seep sediment. Samples were obtained from a methane seep from Hydrate Ridge, Oregon, (sample #3730), and a seep site in the Santa Monica basin, California, (samples #7135, 7136-37, and 7142). Anoxic sediment microcosms were set up in the presence or absence of HPG (5 or 50 μM) and over-pressurized (2 bars) with either N_2 or CH_4 followed by incubation in the dark at 4°C for 114 (#3730), 171 (#7135), 31 (#7136-37), and 25 (#7142) days. These long incubation times were necessary due to the slow growth of ANME-SRB consortia (3-7 months; e.g. 39, 40, 41), which is attributed to the very low free energy yield of sulfate-coupled AOM (40, 42). Subsampling for molecular and geochemical analyses as well as exchange of gaseous headspace and seawater were conducted at regular intervals. Details on incubation setup and sampling is provided in Tab. S1.

HPG amendment had no detectable effect on microbial community composition or activity. Adding a compound to an environmental sample always bears the risk of altering the structure or function of the microbial community. Three independent lines of evidence suggest that at the concentrations used in this study (≤ 50 μM), the methionine analog HPG did not affect the geochemical activity or community structure of microbes within methane seep sediment for up to 171 days of incubation. First, HPG addition had no effect on sulfide production, a reliable proxy for sulfate-dependent AOM-activity (43) in time course experiments (up to 6 months). In contrast, the removal of CH_4 led to a notable and expected decrease (Fig. S1A) in sulfide production as previously reported (43; Fig. S1A). Second, seep sediment from Santa Monica basin (sediment #7142) incubated with and without HPG over a period of 25 days showed equivalent rates of AOM, as measured by ^{13}C-DIC production following $^{13}CH_4$ amendment (Fig. S1B). Third, Illumina tag sequencing of the 16S rRNA V4 region of archaea and bacteria revealed HPG additions had no statistically significant effect on sediment #3730 and #7135 community compositions (Fig. S2-4). More specifically, control incubations of #3730 are indistinguishable from samples containing 50 μM HPG after 114 days of incubation (effect of HPG, $p=0.20$), with an average Bray Curtis similarity of the communities of 94.4% between all samples (Fig. S2-3). Sequences related to ANME-2c archaea make up a slightly higher proportion in HPG-containing samples as compared to incubations without HPG, however this observation was not statistically supported (Fig. S3 and S5). Similarly, for #7135, no differences in the composition of the microbial community in general or ANME- and SRB-related lineages in particular between samples incubated in the presence (5 or 50 μM) or absence of HPG were observed (Fig. S2, S4, and S5). After 41 days, communities in incubations with or without HPG were on average 92.1% similar (Bray Curtis; effect of HPG, $p=0.56$). This similarity decreased to 87.9-89.3% after an additional 130 days of incubation, but without a detectable effect from HPG ($p=0.25$; Fig. S4A). The absence of CH_4 in the headspace, however, did result in a statistically significant change in the microbial community ($p=0.042$; Fig. S2B).

These independent activity and community composition analyses all indicate that the addition of the bioorthogonal amino acid HPG at concentrations up to 50 μM did not result in any detectable changes in the seep microbial diversity or *in situ* activity up to 171 days.

Fluorescence detection of translationally active cells. To fluorescently label microbes undergoing active protein synthesis during the incubation, a recently established BONCAT-protocol based on the Cu(I)-catalyzed conjugation of HPG with an azide-dye (9, 14, 44) was employed. In initial tests, no difference in fluorescence intensity or signal-to-noise ratio was observed between ethanol-fixed, paraformaldehyde-fixed, or non-fixed AOM-consortia (data not shown). All further experiments were thus performed on ethanol-fixed biomass, unless stated otherwise. While several factors prohibit the absolute quantification of new proteins from fluorescence data (discussed in the SI Appendix), semi-quantitative comparisons between different cells of the same taxonomic group may offer information on the functional (translational) activity of uncultured cells in the environment. Throughout the text, we refer to DAPI-stained cell clusters that bound an ANME-specific (CARD)FISH-probe as "microbial (AOM) consortia". When cells within a DAPI-stained cluster could not be unambiguously identified, we use the term "microbial aggregate" instead.

BONCAT as a novel approach to study anabolic activity of AOM-consortia. To establish whether BONCAT can be used as a proxy for CH_4-dependent translational activity of AOM-consortia, we used sample #7136-37, representing the 9-15 cm depth horizon immediately underlying sediment horizon #7135 (6-9 cm). After pre-incubation of the sediment in the absence of CH_4 for 124 days, microcosm experiments were established with 50 μM HPG in the presence or absence of CH_4. After 31 days of incubation, microbial aggregates were extracted and analyzed by microscopy (n=1,554). Under CH_4-replete conditions, 24.9% of all microbial aggregates were BONCAT-stained, while only 2.3% exhibited detectable translational activity when CH_4 was absent from the headspace (Fig. 2). This initial test demonstrated that BONCAT can be used to study the CH_4-dependent anabolic activity of AOM-consortia.

Sensitivity of BONCAT. In all incubations, the relative abundance of translationally active DAPI-stained microbial aggregates increased with time, with 65.8% and 48.5% of aggregates in #3730 and #7135 showing a positive BONCAT signal at the end of incubation (114 and 171 days, respectively. It is interesting to note that already after 7 days of incubation, 5.6% of the microbial aggregates in sediment #7135 were labeled by BONCAT (Fig. 2). Assuming that the AOM-consortia studied here have growth rates comparable to those previously reported (2-7 months; 39, 40, 41), we estimate that BONCAT is able to detect the *in situ* activities of cells within ANME-SRB consortia after 3.3-7.7% of their doubling time. Considering the differences in the physiology of the organisms as well as the lower fluorescence signal-to-noise ratios observed in environmental systems, this estimate is consistent with our finding of a BONCAT-detection limit of <2% of generation time for slow-growing *E. coli* (9).

At this time, it is unclear why up to half of the DAPI-stained consortia were not BONCAT-stained during the AOM incubation experiments. This may have been the result of storing these deep-sea samples in the lab, variable amino acid uptake, or low levels of protein synthesis that

are below detection with BONCAT. The combined application of BONCAT and rRNA-targeted FISH demonstrated that all ANME-lineages present in our samples are able to incorporate HPG into their biomass (discussed below). This suggests that an inability to take up HPG into cells is likely not a problem in our AOM microcosms. This idea is further supported by the observation that AHA and HPG are taken up and incorporated by a range of bacterial and archaeal cultures, including methanogens and sulfate reducers (9, 44).

BONCAT-FISH as a tool for microbial *in situ* activity studies. While the quantitative isotopic analysis provided by secondary ion mass spectrometry (SIMS and nanoSIMS) is currently unparalleled by other single cell techniques, the specialized instrumentation and cost along with comparatively lower sample throughput have restricted its widespread application in the field (10-100 consortia per study; e.g. 7, 27, 34, 35). Many fundamental questions in microbial ecology regarding the activity and metabolic potential of environmental microbes *in situ*, however, can be addressed with lower precision methods.

To demonstrate the utility of BONCAT as a comparatively higher throughput method for tracking microbial biosynthetic activity, we used epifluorescence microscopy combined with FISH-BONCAT to investigate the *in situ* translational activities of 16,438 individual microbial aggregates in the presence or absence of CH_4. In total, 12,652 aggregates from four #3730 microcosms incubated for 30 and 114 days were analyzed. In addition, the activities of 2,232 aggregates from six incubations of sediment #7135 after 7, 41, and 171 days of incubation were determined. To assess whether the activity of individual consortia is influenced by the phylogenetic affiliation of their partners we combined BONCAT with rRNA-targeted FISH (Fig. 1B). Following our recently established protocol (9, 44), we used 11 different 16S and 23S rRNA-targeted oligonucleotide probes labeled with either a fluorescence dye or horseradish peroxidase (for catalyzed reporter deposition FISH, CARD-FISH) to target the different ANME-populations and their associated bacteria present in our samples. These were applied in 11 different combinations (see SI Appendix and Tab. S2 and S3 for details) to study the activity of AOM-consortia in the presence or absence of CH_4. In total, we visualized the translational activities of 1,346 taxonomically identified AOM-consortia (representative images in Fig. 3; results in Fig. 2, Tab. 1, Tab. S2).

BONCAT-FISH reveals the co-occurrence of diverse and active AOM consortia. Domain-specific FISH-probes (Arch915 and EUB338mix) hybridized 37.6% (114 days) and 51.8% (41 days) of microbial aggregates in CH_4-containing incubations of sediment #3730 and #7135, respectively. In contrast, the combined application of 5 fluorescently labeled oligonucleotide probes specific for the main ANME-subpopulations present in our samples (ANME-1, -2a, -2b, and -2c) yielded positive results for only 5.4% (#3730, 114 days) and 5.8% (#7135, 41 days) of microbial aggregates in CH_4-containing samples. Two other groups of ANME archaea, nitrate-dependent ANME-2d (45) as well as ANME-3 were absent from our methane seep 16S rRNA iTAG datasets and thus were not probed by FISH. As compared to FISH, CARD-FISH yielded slightly lower detection rates for probe Arch915, specific for domain *Archaea* (55.7% in FISH vs. 53.2% for CARD-FISH for #3730, 114 days of incubation). Similar

results were obtained for the combined application of ANME-specific probes, which together hybridized 4.2% of microbial aggregates (5.4% with FISH; Fig. 2 and Tab. 1). Comparable hybridization efficiencies were found for samples that had been incubated in the absence of HPG (data not shown). Independent CARD-FISH experiments (see Tab. 1 and SI Appendix), corroborated these findings.

The stark discrepancy between domain- and subpopulation-specific probes might be explained by a combination of factors. First, several available probes specific for different ANME-clades are predicted to have low accessibilities to their rRNA-target sites (46) and thus may exhibit low fluorescence signal-to-noise ratios. In addition, while most published ANME-probes are predicted to comprehensively and specifically detect the diversity of sequences in rRNA-databases, we do not know how well these probes cover the full diversity of ANME in the environment. For one subpopulation, ANME-2b, which was represented by 18-20% of all ANME-related tag-sequences in our sediments (Fig. S5), we tried to overcome this problem by designing a new probe, ANME-2b-729. When tested *in silico* this probe binds to 93% of all ANME-2b 16S rRNA sequences and has at least two mismatches to all non-ANME-2b-related rRNA-sequences in public and in-house databases. We successfully applied this new probe to our sediment samples (Fig. 3E-F) and the results are described in Tab. 1 and Tab. S2. Alternatively, it is possible that a fraction of DAPI-stained aggregates contained archaea unaffiliated to ANME. Similar to most other marine sediments (37), seep sediment #7135 from Santa Monica Basin hosts a variety of archaea unrelated to ANME (9-17% of all iTAG sequences in our dataset). The archaeal population in Hydrate Ridge sediment #3730, in contrast, is dominated by ANME (93.2-97.7% of all archaeal i-tags). This makes it unlikely that archaea unrelated to ANME constituted a substantial number of microbial consortia in #3730. Furthermore, to our knowledge, no other sediment-dwelling archaea have previously been reported to occur in multicellular associations with bacteria. Additional discussions on FISH using ANME-specific probes can be found in the SI Appendix.

Of all consortia detectable with an ANME-specific FISH probe, 91.4% (sample #3730) and 81.8% (sample #7135), were also BONCAT-positive. In contrast, only 81.4% (#3730) and 58.3% (#7135) of all positively hybridized microbial aggregates (using either domain- or ANME-specific FISH probes) could be detected via BONCAT (Fig. 2 and Tab. S2). This lends support to the view that rRNA-targeted FISH is not always a reliable proxy for cellular activity. Notably, there was no statistically supported relationship between BONCAT- and FISH-signal intensities of individual cells within AOM-consortia (n=5 consortia, each composed of 50-200 cells; not shown).

Multiple co-occurring ANME subgroups were active under identical incubation conditions. Methane seeps commonly harbor a wide range of taxonomically distinct ANME-clades (e.g. 26, 37, 47, 48). The activity patterns and niche differentiation of these apparently functionally redundant groups are, however, not understood. So far, temperature (31), CH_4 partial pressure (31), concentration of sulfate (32, 33) and sulfide (33), and nitrogen availability (34, 35) have directly or indirectly been shown to drive the abundance and activity of different

ANME-lineages. Using BONCAT, we were able to demonstrate that representatives of all major ANME-subpopulations (ANME-1 and ANME-2a, -2b, and -2c) co-occurring in a sediment incubation were biosynthetically active under controlled AOM incubation conditions in the lab (Tab. 1). This result raises questions about the ecology underlying this apparent functional redundancy and factors influencing niche specialization. A promising approach for future studies of the functional capacities and niche adaptations of different ANME-lineages will be to employ isotopically labeled substrates in combination with BONCAT-based activity-screening in targeted physiological experiments (9).

Activity of ANME-SRB consortia in the absence of CH_4. To date, members of ANME archaea have only been shown to conserve energy through methane oxidation. Some studies, however, have suggested that select ANME-populations might be capable of methanogenesis (33, 49-53). Anabolic activity of individual ANME-SRB consortia in the absence of CH_4 has, however, yet not been directly demonstrated. In our BONCAT-FISH studies, we found that after 30 days of incubation without the addition of CH_4, 7.9% of DAPI-stained microbial aggregates from sediment #3730 were BONCAT-stained, and after 114 days, this proportion nearly doubled to 14.6% (~50-95% of doubling time; 39, 40, 41). A similar trend was observed for sample #7135 (14.5%, 30.4%, and 24.2% BONCAT-positive aggregates after 7, 41, and 171 days). This observation was in stark contrast to our #7136-37 in incubations in the absenece of methane, in which only a very small percentage (2.3%) of microbial aggregates were fluorescently labeled. While #3730 and #7135 consortia were clearly BONCAT-stained, their fluorescence intensity was roughly 10-fold lower (n=20) than consortia incubated in the presence of CH_4. Consistent with samples incubated in the presence of CH_4, the relative proportion of BONCAT-stained aggregates that could be attributed to a specific ANME-population via (CARD)FISH was also very low in the absence of CH_4 (0.3-9.8%, Fig. 2).

An explanation for activity of AOM-consortia in the absence of CH_4 headspace could be that some consortia may have the potential to consume previously accumulated energy storage compounds. This is supported by the observation that some representatives of ANME-2b but not ANME-2c cells feature polyphosphate granules (McGlynn *et al*., in prep.). Similarly, some ANME-associated SRB might harbor carbon storage compounds (54). Our results suggest that more AOM-consortia than previously thought are able to build up substantial amounts of energy storage during times of substrate repletion. This is surprising, given the very low energy yield of the AOM-reaction (ΔG^0 = -16 kJ mol^{-1} at standard conditions) (55). Our finding, however, is consistent with the observation that some AOM-consortia are able to invest substantial amounts of metabolic energy in N_2-fixation (34, 56), one of the most energy-intensive reactions known (16 ATP per N_2).

The observation of an increase in active consortia in sediment #3730 over long periods of time (from 7.9% after 30 days to 14.7% after 114 days; Fig. 2) is, however, inconsistent with the predicted progressive depletion of storage compounds. Another possible explanation for results from #3730 could be attributed to an internal CH_4-cycle, in which methanogens produced CH_4, which was then re-oxidized by AOM-consortia. Alternatively, members of ANME-SRB

consortia might themselves engage in the production of CH_4 rather than in sulfate-coupled AOM, as previously suggested (49-53). Recent experiments on the methanogenic potential of ANME-1 and ANME-2 enrichment cultures, however, have not supported this idea (57). A minor amount of CH_4 was detected in our N_2-containing #3730 and #7136-37 microcosms, however these levels were similar to concentrations measured as a trace contaminant in the N_2-tank (approximately 1-10 ppm CH_4). The reported CH_4 partial pressures capable of supporting AOM by methane seep consortia is 1 bar (*i.e.* 1.4 mM in solution) (31, 43), 5 orders of magnitude higher than concentrations in our incubations. Another intriguing possibility to explain the activity of BONCAT-stained ANME-SRB consortia in the absence of CH_4 is that we yet might not recognize the full extent of physiological capabilities of ANME-SRB consortia and that they might be involved in energy conservation pathways unrelated to the oxidation or generation of CH_4. A first step towards formulating hypotheses about potential energy-yielding substrates is to identify specific archaeal-bacterial partnerships that might differ in their substrate range for targeted genomics.

Identification of microbial partners within activity-sorted consortia. With few notable exceptions (10, 11, 38, 58-60), single cell genome sequencing efforts so far have been "target-blind"; they did not select for a specific taxon of interest or focus on metabolically active cells that could be considered key species for ecosystem functioning. The power of these techniques to investigate microbial partnerships involved in AOM was recently demonstrated by the separation of FISH-identified ANME-consortia from methane seep sediments via immuno-magnetic capture (magneto-FISH; 38, 61) and fluorescent-activated cell-sorting (FISH-FACS; 59, 62). Using sorting approaches, new associations between ANME and bacteria not known to be capable of sulfate reduction, including members of the alpha-, beta-, gamma- and epsilon-proteobacteria as well as *Planctomyces*, have been revealed (38). In addition, the potential of targeted sorting approaches for uncovering rare populations has been demonstrated by the successful enrichment of an ANME-2d population from ~2 to ~94% (59).

To demonstrate the potential of BONCAT to separate the functionally active fraction of a microbial community from complex samples, we sorted individual, translationally active consortia from sediments #3730 and #7142 by fluorescence-activated cell-sorting (FACS). We refer to this activity-based cell-sorting approach as BONCAT-FACS (Fig. 1C). To test the specificity of the chosen gates, 200 events per gate were sorted into individual tubes and analyzed using fluorescence microscopy. For the BONCAT++ gate, which was used for all downstream analyses, 204 DAPI-stained microbial aggregates were identified, all of which were BONCAT-stained (Fig. 4). The slightly higher number of consortia (204 vs. the expected 200) may be due to co-sorting of physically attached aggregates or the partial disaggregation of some aggregates after sorting.

Consortia sorted into individual wells of micro-titer plates were lysed and their genomes amplified via multiple displacement amplification (MDA) following established protocols (63). For ethanol-fixed biomass from sediment #3730, 76 out of 168 sorted events resulted in a MDA product. This efficiency (45%) is comparable with that of a recent study that reported a success

rate of 50% when DNA from cells stored in 70% EtOH was used as PCR template (64). By avoiding any cell fixation prior to sorting of individual consortia (Fig. 4), the efficiency of MDA reactions improved to 93% (78 out of 84 sorted events) for the same sample. Together, these results suggest that BONCAT and click chemistry-mediated dye-labeling have no detrimental effect on DNA-quality. Fixation of biomass was avoided for sediment #7142.

Diluted DNA from MDA reactions was used as the template in PCR amplifications targeting the V4 region of archaeal and bacterial 16S rRNA genes (65, 66). Amplicons from PCR reactions that yielded a single band during gel electrophoresis examination were barcoded, sequenced, and phylogenetically analyzed (Fig. 5A and Fig. S6). The majority of reactions contained both ANME- and SRB-related sequences in high numbers. In several reactions, however, only sequences from either the ANME- or the SRB-partner were obtained (sequences from the other cell type were <0.1%). Given the observed specificity for active microbial aggregates of our sorting approach (Fig. 4B), preferential amplification of a single sequence type for this subset may be indicative of bias during cell lysis and/or the MDA reaction, a problem well discussed in the literature (64, 67, 68). Reactions that only recovered SRB sequences (n=5) were not considered further in our analysis. For each of the remaining 45 consortia, a single ANME operational taxonomic unit (OTU; defined here as a unique sequence) represented 93-100% of all ANME-associated sequences. Similarly, in each individual consortium from sediment #3730 a single OTU always contributed 94-100% of all SRB-related sequences. Despite the relatively low number of consortia analyzed (n=34), representatives of the all ANME-clades found in #3730 samples (according to 16S rRNA iTAG data) were retrieved from a single incubation, corroborating the findings of phylogenetic diversity from our BONCAT-FISH studies (findings are summarized in Tab. 1). Consistent with previous reports (26, 35, 38, 47, 69), ANME-1 and ANME-2 were predominantly associated with putative sulfate reducers within the delta-proteobacterial lineages *Desulfobacterium*, *Desulfobulbus*, *Desulfococcus*, and *Desulfosarcina* (Fig. 5A and FIG. S6). Analysis of single consortia further revealed that individual ANME-subpopulations (single OTU) are often associated with different bacterial partners, and *vice versa*. For example, the seven observed #3730 ANME-2b consortia (identical in their V4 region) have partners within three different bacterial families. Similarly, three consortia (A03Uf, C08Uf, and H01Uf) from sediment #7142 each harbored two SRB OTUs (94.9-98.8% identical in sequence).

In addition to sequences from SRB, 5 consortia from sediment #3730 also yielded low proportions of sequences from bacteria related to *Pseudomonas* or other gamma-proteobacteria (Fig. 5A and FIG. S6). These sequences were taxonomically different than commonly observed lab-contaminants and were distinct from sequences detected in over-amplified negative controls or control mock communities. This result, together with (i) the finding of highly similar gamma-proteobacterial sequences in a magneto-FISH study of AOM-consortia (38), (ii) our demonstration that translationally active gamma-proteobacteria and archaea form consortia in sediment #3730 (Tab. S2), and (iii) the observation that individual ANME-OTUs in different consortia were associated with diverse bacterial groups, lend strong support to the idea that

AOM-partnerships are dynamic, both in terms of partner affiliation and possibly their ecophysiology (38).

Discovery of a new inter-domain partnership. rRNA gene sequences obtained from an ANME-1a consortium (#E06) from #3730 did not contain sequences related to known sulfate reducers, but instead recovered sequences associated with *Verrucomicrobia*. Sequences retrieved from a second consortium (#D05) also contained the same ANME-1a OTU, in addition to both *Desulfobulbaceae* sequences and another *Verrucomicrobia*-affiliated OTU (sequence 80% identical to the E06 OTU). Both *Verrucomicrobia*-affiliated sequences had ≤81% identity to rRNA genes from previously described species (Fig. 5A and FIG. S6).

To complement our sequence-based observations, we used FISH to screen 27 samples from 9 methane seep sediment cores obtained from a variety of geographic locations as well as two carbonates retrieved from a methane vent site for the association of *Verrucomicrobia* with AOM-consortia. We used two 16S rRNA-targeted probes specific for the phylum *Verrucomicrobia* (EUB338-III and Ver47) (70-72), in combination with the general archaeal probe Arch915 to screen archaeal aggregates from these samples. In four sediments (#3730 incubated for 30 and 114 days in the presence of HPG and CH_4; sediments #5119, #5202, and #7142) as well as both carbonate samples (#2450 and #3439), verrucomicrobial cells were associated with 10-20% of AOM-consortia (Tab. S3; Fig. S7). While the vast majority (~98%) of these aggregates contained only low numbers of *Verrucomicrobia* (<20 per aggregate), a small number (n=14) of aggregates exhibited very high ratios of verrucomicrobial to archaeal cells (Fig. 5; Fig. S7; Tab. S3).

Despite the near-ubiquitous distribution of *Verrucomicrobia* in marine sediments (73), including all 23 methane seeps recently surveyed via 16S i-tag sequencing (37), archaeal-verrucomicrobial associations have not been described previously. Because of the loss of structural information during DNA-extraction and the typically low abundance of consortia consisting of ANME and non-traditional partner bacteria (38; Trembath-Reichert, in prep.; and this study), metagenomic sequencing has yet failed to provide metabolic predictions on the whole diversity of these microbial associations. This reinforces the imperative to combine genomic sequencing with spatially resolved approaches, such as microscopy and cell-sorting (e.g. 10, 67, 74), to gain access to the genetic potential of these important associations.

At this time, we can only speculate about the biological necessities driving the physical association of *Verrucomicrobia* with ANME. The low relative abundance of *Verrucomicrobia* associated with AOM-consortia, however, suggests that these cells might be heterotrophs, consuming organic exudates or exo-polymers of the archaeal cells. A similar cross-feeding relationship has previously been hypothesized for anammox bacteria co-occurring with ANME-2d archaea (45). Genomic sequencing of individual ANME-*Verrucomicrobia* aggregates sorted via BONCAT-FACS combined with additional microcosm experiments may assist with expanding our understanding of the nature of these ANME-verrucomicrobial interactions.

Conclusion and Outlook

The possibility to detect anabolic activity of taxonomically identified cells using fluorescence-staining offers a valuable compliment to existing fluorescence microscopy methods for microbial ecology. BONCAT-FISH allows the activity-screening of thousands of cells within a few hours, rather than a few dozen per day as achieved by isotope-labeling techniques. Both bioorthogonal (this study) as well as non-destructive isotope-labeling (10, 58) approaches further allow the sorting of individual cells and aggregates from complex samples, although sorting throughput for BONCAT-FACS is much higher. Sorted cells can be subjected to whole genome amplification and sequencing, thus allowing direct access to the genetic potential of cells functionally important under defined conditions. In contrast to isotope labeling approaches, which test whether cells are able to assimilate a specific substrate of interest (1, 5, 6, 75), BONCAT is an un-targeted marker of translational activity. Hence, BONCAT cannot directly provide information about specific substrate metabolism as is possible with SIP. However, the possibility to combine BONCAT-incubations with the addition of any other compound allows for *in situ* metabolic screening and comparative analysis of organisms stimulated by the compound (9). Thus, we anticipate that BONCAT-FISH will be particularly attractive if non-assimilatory pathways or substrates that are not (or only very expensively) available as stable isotope-labeled derivatives are to be studied. Growth-promoting substrates could in the following be used for the targeted cultivation of translationally responsive cells. In contrast to isotope labeling approaches, which require specialized instrumentation, BONCAT-FISH and BONCAT-FACS use standard-configuration microscopes and flow-cytometers that are more readily available to molecular biological labs.

Here, we established that HPG at concentrations up to 50 μM can be applied to marine methane seep sediments without detectable effects on the structure and function of the microbial community. We demonstrate that the subsequent detection of HPG-containing *de novo* synthesized proteins via click chemistry is a powerful approach to visualizing and identifying translationally active microbes *in situ*, separating them from complex samples via activity-based cell-sorting, and studying microbial interactions. We demonstrate that representatives of all major subgroups of ANME are functionally active in Hydrate Ridge seep sediments. In addition, we show that in two geographically distinct sediments some consortia are active in the absence of CH_4. It remains to be tested whether these findings are entirely the result of the use of cellular storage materials or a physiological flexibility of these archaea that is yet unaccounted for. Furthermore, we provide the first evidence for the existence of previously unrecognized interactions of archaea and *Verrucomicrobia* in marine sediments. We anticipate that genomic characterizations of these as well as other diverse ANME-SRB consortia will soon provide hypotheses about potential growth-supporting substrates, and that BONCAT-FISH and BONCAT-FACS will play important roles in experimentally testing the ecophysiological properties of these globally relevant microbial partnerships.

Materials and Methods

Incubation of methane seep sediment. Samples were obtained from three sediment cores taken off the coasts of California (Santa Monica Basin) and Oregon (Hydrate Ridge) and incubated in the presence of absence of *L*-homopropargylglycine (HPG) and CH_4 for up to 6 months. Samples for geochemical characterization, whole-community composition, and microscopic analyses were taken in regular intervals. Details on these experimental procedures are described in the *SI Appendix*.

Bioorthogonal non-canonical amino acid tagging (BONCAT). BONCAT was performed following our recently established protocol without modifications (44). Succinctly, fixed biomass (whole sediment or extracted consortia) was immobilized on Teflon-coated glass-slides and dried at 46°C. An increasing ethanol series (50, 80, and 96% ethanol in ddH_2O) was performed and the slides air-dried. Solutions were prepared as recently described (44) and the "click cocktail" was always freshly mixed. This solution contained 5 mM sodium ascorbate (Sigma-Aldrich), 5 mM amino-guanidine hydrochloride (Sigma-Aldrich), 500 μM Tris[(1-hydroxypropyl-1H-1,2,3-triazol-4-yl)methyl]amine (THPTA; Click Chemistry Tools), 100 μM $CuSO_4$ (Sigma-Aldrich) and 2 μM of azide-modified dye carboxyrhodamine 110 (CR-110; Click Chemistry Tools) or azide-modified 5(6)-carboxytetramethylrhodamine (TAMRA; Click Chemistry Tools) [used only in combination with CARD-FISH] in 0.2 μm-filtered 1x PBS, pH 7.4. 20 μL of this solution were applied atop of biomass and the glass slides incubated for 60 min at RT in a humid chamber. Afterwards, slides were washed repeatedly in 1x PBS and an increasing ethanol series (50, 80, and 96%), before being air-dried. For details on this protocol see Hatzenpichler and Orphan (2015) and Hatzenpichler *et al*. (2014). Non-fixed samples were processed following the same protocol with the difference that all ethanol-washing steps were omitted.

Fluorescence *in situ* hybridization (FISH) and Catalyzed Reporter Deposition (CARD) FISH. Following BONCAT, ethanol-washed samples were hybridized with oligonucleotide-probes. For FISH-experiments, double- (76) or mono-labeled probes with either Cy3 or Cy5 fluorescence dyes were used. FISH hybridizations were performed overnight (14-18 hours) according to standard protocols (9, 77). The quality of all probe solutions (except ANME-2-932) was checked using other seep samples before they were applied to #3730 and #7135-37 sediments. All tested probe solutions yielded a large number of positive hybridizations to AOM-consortia in these control samples. Each probe or probe-set was hybridized in a technical duplicate or triplicate. No differences in fluorescence intensity or relative proportion of (CARD)FISH-positive consortia could be observed between replicate hybridizations.

CARD-FISH was performed as recently described (78). Three different cell wall digestion protocols were tested, but only one, a modified version of a recently published protocol (35), was found to be successful. Permeabilization was performed at RT as follows: incubation in 0.01 M HCl for 15 min; 2 washing steps in water, 1 min each; incubation in 0.5% sodium-dodecyl sulfate for 5 min; 3 washing steps in water, 1 min each; incubation in 50% EtOH for 1 min. After air-drying, samples were hybridized for 14-18 with horseradish peroxidase-conjugated

oligonucleotides (purchased from Biomers), before signal amplification using self-synthesized fluorescein-labeled tyramide was carried out.

Microscopy and image analysis. Samples were mounted with 1 mg mL^{-1} 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) in Citifluor AF-1 anti-fading solution (Electron Microscopy Sciences) and analyzed using either an Olympus BX51 epifluorescence microscope or a Zeiss LSM-510-Meta confocal laser-scanning microscope. Images were analyzed using the imageJ software (NIH). In total, 12,652, 2,232, and 1,554 consortia were analyzed for sediment #3730, #7135, and #7136-37, respectively. The lower number of consortia analyzed for the latter samples is explained by the much lower concentration of AOM-consortia in that sediment core. Each DAPI-stained microbial aggregate was manually inspected for BONCAT- and FISH-signals, and representative images were taken for each probe-set. Consortia were always identified via DAPI, before switching to the other fluorescent channels to not preferentially select for BONCAT-positive or FISH-positive consortia. A detailed list of BONCAT-FISH counts can be found in the Tab. S2 and Tab. S3. In every BONCAT-positive aggregate either (i) (nearly) all cells within an aggregate were stained, or, in rare cases, (ii) (nearly) all cells of one (but not the other) cell-type (archaea or bacteria) were stained. Given the complex three-dimensional structure of the aggregates, which sometimes grow many thousands of cells large (see Fig. 2A), we cannot exclude that in rare cases individual cells (<5%) of a specific cell-type were not stained (hence, the term "nearly all").

After shipping and storage at 4°C for three days, activity-sorted consortia were re-suspended in 1 mL 1x PBS, before being harvested by centrifugation (5 min at 16,100 g, RT), re-suspended in 1:1 1x PBS:EtOH, and immobilized on a glass slide. The slide was washed for 1 min in 50% EtOH (in ddH_2O), air-dried, DAPI-embedded, and microscopically analyzed. Consortia were counted following the same procedure as outlined above and representative images were taken.

Activity-based cell-sorting. Initial tests were performed using pure cultures and mixtures of *Escherichia coli* K-12, *Methyloprofundus sedimenti* WF-1 (79), and *Desulfovibrio alaskensis* G20 that had been incubated in the absence or presence (250 μM) of HPG for ~1 generation and stained using an azide-modified version of dye CR110 as recently described (9, 44). After successful tests, BONCAT-treated sediment-extracted consortia from samples #3730 and #7142 were analyzed. A BD Influx cell sorter was sterilized, and sheath fluid was prepared using 1x PBS as recently described (63). After passing through a 70 μm nylon mesh filter, samples were sorted using a 200 μm nozzle at 0.21 bar. The sort mode was "1.0 drop single". 2x 84 single events were deposited into wells E4-L15 of two 384-well plates, with Rows D and L serving as negative control wells. BONCAT-dye CR110 was excited using a 488 nm laser and fluorescence was captured with a 531 nm / 30 nm filter. Gates were defined using a forward scatter (FSC) vs. 531 nm emission plot, and events with a BONCAT signal brighter than >90% of aggregates in the negative control were captured (see Fig. 4). For quantification of sorted consortia, 200 events identified within each gate were sorted into 1.5 mL tubes that contained 10 μL 1x PBS. Tubes were stored at 4°C until further processing.

582 **Sequence access.** Sequences have been deposited in the National Center for Biotechnology
583 Information database under accession numbers KT945170-KT945234 and KU564217-
584 KU564240 (16S rRNA iTAG sequences from activity-sorted consortia in #3730 and #7142,
585 respectively) and SRP066109 (whole community 16S rRNA iTAG sequences).
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606 ## Figure legends
607
608 **Fig. 1.** Concept for the visualization, identification, and sorting of translationally active cells.
609 *(A)* The bioorthogonal amino acid *L*-homopropargylglycine (HPG) is added to an environmental
610 sample, which is then incubated under *in situ* conditions. After HPG has entered the cell, the
611 exact process of which is currently unknown, it competes with methionine (Met) for
612 incorporation into newly made peptides. HPG-containing proteins are then fluorescently labeled
613 via a Cu(I)-catalyzed azide-alkyne click reaction, thus marking cells that have undergone protein
614 synthesis during time of incubation. *(B)* Next, rRNA-targeted fluorescence *in situ* hybridization
615 (FISH) is performed to taxonomically identify anabolically active cells. In this example, the
616 yellow cell is detected via both BONCAT and FISH, while the other two were either not
617 translationally active (red cell) or are taxonomically unidentified but active (green cell). *(C)*
618 Individual labeled cells or consortia can be separated using fluorescence-activated cell-sorting
619 (FACS). Cells are lysed and their genomes amplified using multiple displacement amplification
620 (MDA). The resulting amplified genomes are taxonomically screened via amplification and
621 sequencing of the V4 region of the 16S rRNA gene. This information may then guide the
622 selection of cells or consortia for genomic sequencing.
623
624 **Fig. 2.** Temporal dynamics of translational activities of microbial consortia as revealed by
625 BONCAT-FISH. Three geographically distinct sediment samples, #3730, #7135, and #7136-37
626 were incubated in the presence of CH_4 or N_2 for 114, 171, and 31 days, respectively. 2.3-30.4%
627 of aggregates were translationally active in absence of CH_4, suggesting either the use of storage
628 compounds, the presence of an endogenous methane source, or a potential physiological
629 flexibility of these microbial partnerships. Green bars show the relative abundance of aggregates
630 detectable via BONCAT. Red bars indicate the detection rate of DAPI-stained aggregates by
631 FISH and CARD-FISH. Yellow bars give the relative abundance of aggregates that were both
632 BONCAT-positive as well as FISH- or CARD-FISH stained. n, number of microbial aggregates
633 analyzed per experiment. For a list of probes and detailed results, see Tab. S2.
634
635 **Fig. 3.** Single cell-resolved visualization of the translational activity of morphologically and
636 taxonomically distinct methane-oxidizing consortia in sediment #3730. Protein synthesis-active
637 cells were identified via BONCAT (green). 16S rRNA-targeted oligonucleotide FISH-probes,
638 specific for the domain *Archaea (A,B)*, most delta-proteobacteria *(C,F)*, and ANME-subgroup 2b
639 *(D,E)*, were used to taxonomically identify the two microbial partners (red). DAPI-staining of
640 DNA is shown in blue. An overlay of the three fluorescence channels and a 10 µm scale bar are
641 shown in the lower right image of each panel. BONCAT-negative cells were either
642 translationally inactive or have not taken up or incorporated HPG into new proteins.
643
644 **Fig. 4.** Flow cytometric analysis of unfixed microbial cells and consortia extracted from
645 sediment #3730 after incubation with *(A)* or without *(B)* of HPG. *(C)* Microbial consortia

detected by fluorescence microscopy after sorting of 200 events per gate. *(A)*. Only 'BONCAT' and 'BONCAT++' gates were used for activity-based sorting. Microscopic images of representative tube-sorted consortia are on the right. Note that the relative fluorescence intensity differs between gates (for gate 'P1' events, exposure time had to be increased 10-fold as compared to gate 'BONCAT' to yield visually detectable BONCAT-fluorescence). FSC, forward scatter; RFI 488nm, relative fluorescence intensity at an excitation of 488 nm; 'BONCAT++', 'BONCAT', 'P1', 'P2', and 'P3', gates using for counting and sorting; Gate 'P3' did only contain individual cells as well as sediment and Percoll particles. Percent-values indicate the relative abundance of events within each gate.

Fig. 5. Identification of microbial partners within AOM-consortia after activity-based sorting. Taxonomic affiliation of the archaeal (left) and bacterial (right) partners within 45 individually sorted, translationally active consortia from sediments #3730 (black) after 114 days and #7142 (red) after 25 days of incubation with HPG. The potential to discover yet unrecognized microbial interactions with this approach is evidenced by our finding of two consortia containing *Verrucomicrobia*-derived sequences. Using FISH, we independently confirmed the presence and activity of microbial consortia composed of *Archaea* and *Verrucomicrobia* in several sediment and carbonate samples. Trees represent maximum likelihood reconstructions onto which bootstrap values are projected. Green and white colored boxes show support ≥90% and ≥70%, respectively. Values <70% are not shown. Left and right boxes indicate max. parsimony (100x) and neighbor joining (1,000x) values, respectively. Tag sequences were added after tree construction without changing overall tree topology. Solid and dashed lines indicate individual archael-bacterial partnerships. Scale bars equal 10% estimated sequence divergence. Detailed trees and additional FISH-images are available in the SOI (Fig. S6 and S7).

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Tab. 1. Comparison of 16S rRNA gene-based affiliations of ANME-related archaea in sediment #3730 after 114 days of incubation in the presence of CH_4 reveals that members of all ANME-subclade are active under controlled conditions.

Number of aggregates	FISH	CARDFISH	FACS
DAPI	2,946	1,130	
BONCAT+	1,922	678	34
CARD(FISH)+	167	48	
BONCAT+ and (CARD)FISH+	163	42	
% ANME 1	1	79	29
% ANME 2a	34	0	3
% ANME 2b	27	14	21
% ANME 2c	38	7	47

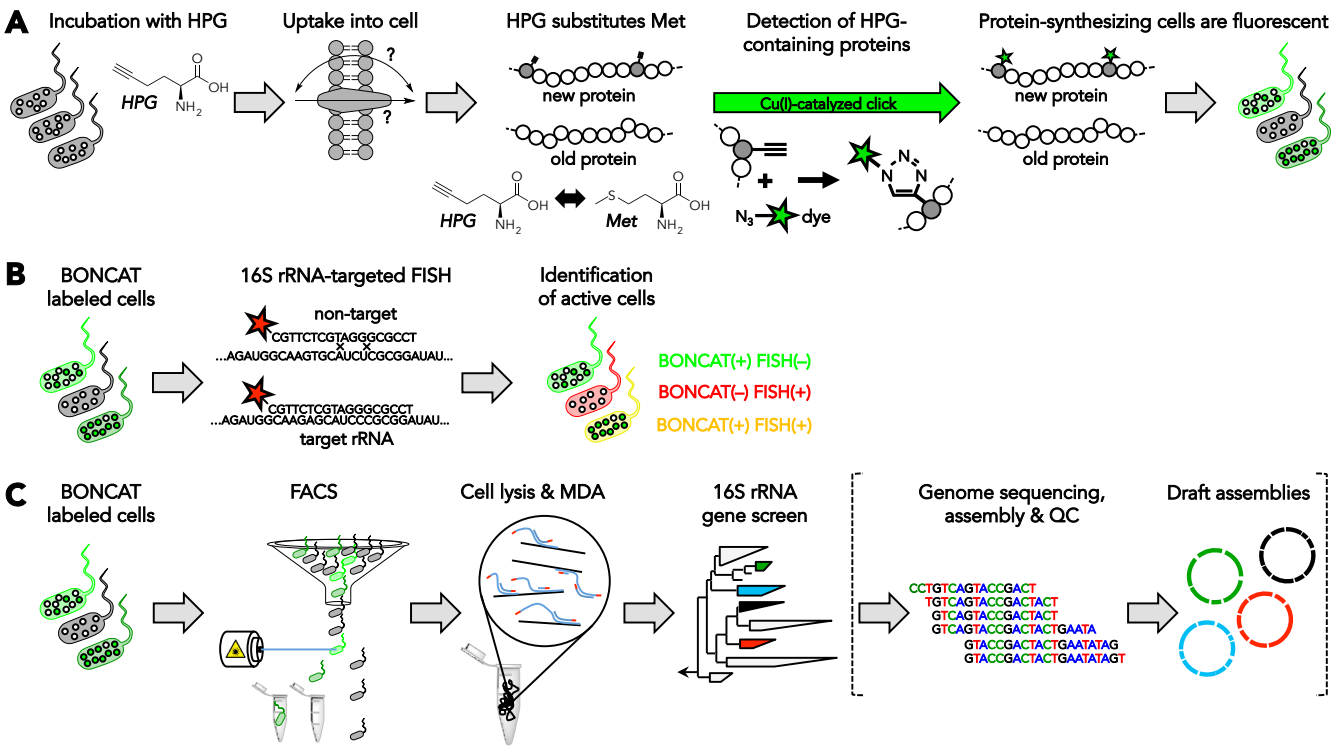

A
Incubation with HPG
HPG
Uptake into cell
HPG substitutes Met
new protein
old protein
HPG
Met
Detection of HPG-containing proteins
Cu(I)-catalyzed click
N3
dye
Protein-synthesizing cells are fluorescent
new protein
old protein
B
BONCAT labeled cells
16S rRNA-targeted FISH
non-target
CGTTCTCGTAGGGCGCCT
...AGAUGGCAAGTGCAUCUCGCGGAUAU...
CGTTCTCGTAGGGCGCCT
...AGAUGGCAAGAGCAUCCCGCGGAUAU...
target rRNA
Identification of active cells
BONCAT(+) FISH(–)
BONCAT(–) FISH(+)
BONCAT(+) FISH(+)
C
BONCAT labeled cells
FACS
Cell lysis & MDA
16S rRNA gene screen
Genome sequencing, assembly & QC
CCTGTCAGTACCGACT
TGTCAGTACCGACTACT
GTCAGTACCGACTACT
GTCAGTACCGACTACTGAATA
GTACCGACTACTGAATATAG
GTACCGACTACTGAATATAGT
Draft assemblies

Figure 1

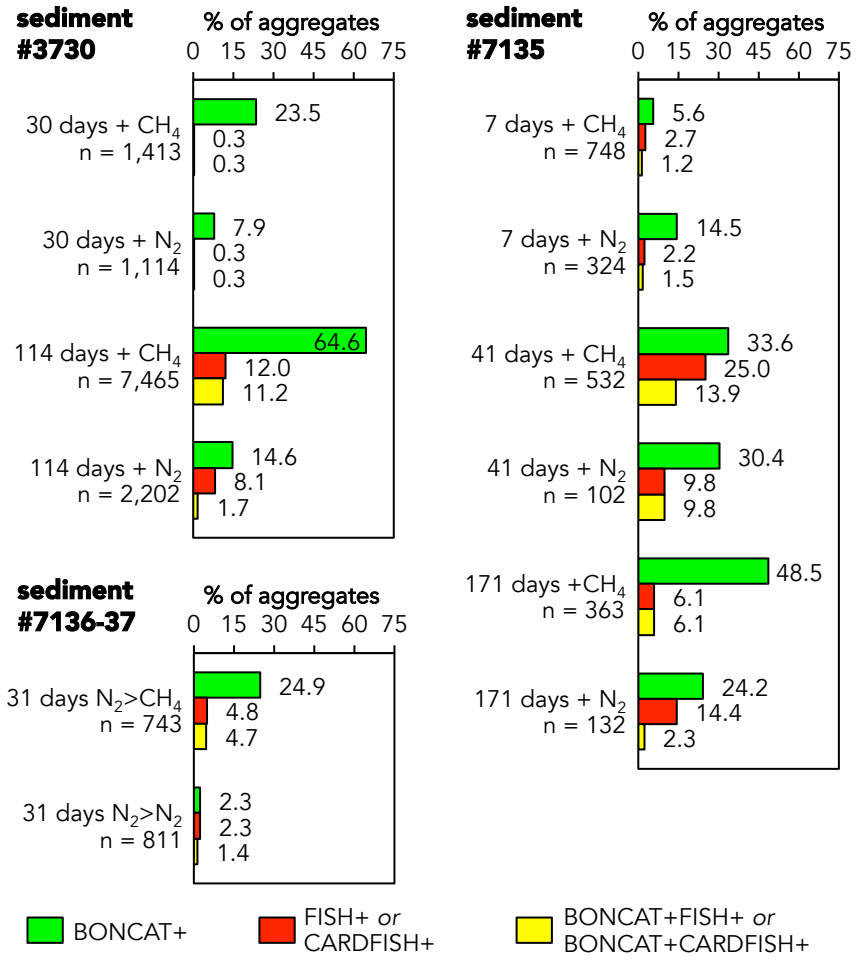

sediment #3730
% of aggregates
0 15 30 45 60 75
30 days + CH4 n = 1,413
23.5
0.3
0.3
30 days + N2 n = 1,114
7.9
0.3
0.3
114 days + CH4 n = 7,465
64.6
12.0
11.2
114 days + N2 n = 2,202
14.6
8.1
1.7
sediment #7135
% of aggregates
0 15 30 45 60 75
7 days + CH4 n = 748
5.6
2.7
1.2
7 days + N2 n = 324
14.5
2.2
1.5
41 days + CH4 n = 532
33.6
25.0
13.9
41 days + N2 n = 102
30.4
9.8
9.8
171 days +CH4 n = 363
48.5
6.1
6.1
171 days + N2 n = 132
24.2
14.4
2.3
sediment #7136-37
% of aggregates
0 15 30 45 60 75
31 days N2>CH4 n = 743
24.9
4.8
4.7
31 days N2>N2 n = 811
2.3
2.3
1.4
BONCAT+
FISH+ or CARDFISH+
BONCAT+FISH+ or BONCAT+CARDFISH+

Figure 2

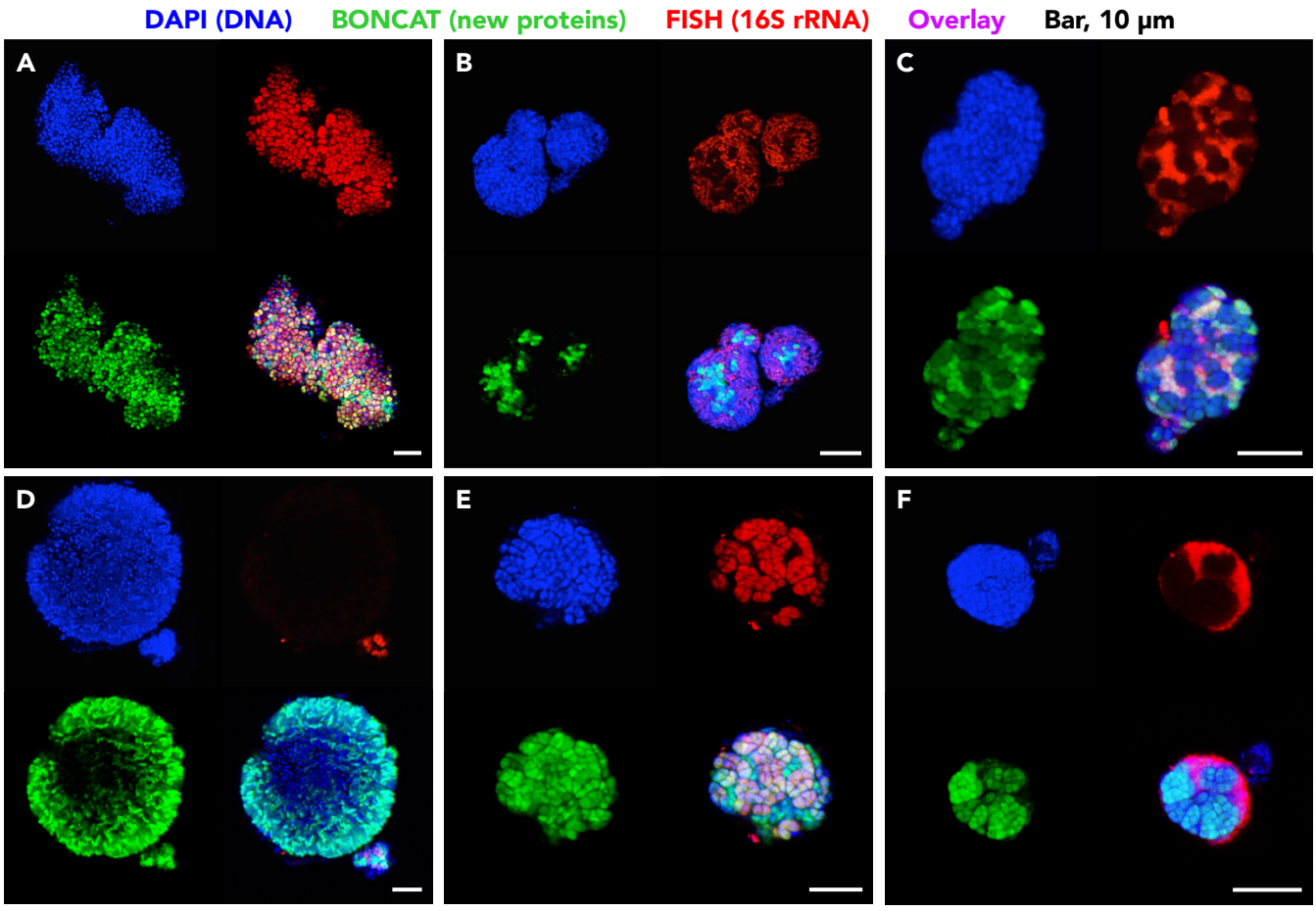

DAPI (DNA)
BONCAT (new proteins)
FISH (16S rRNA)
Overlay
Bar, 10 µm
A
B
C
D
E
F

Figure 3

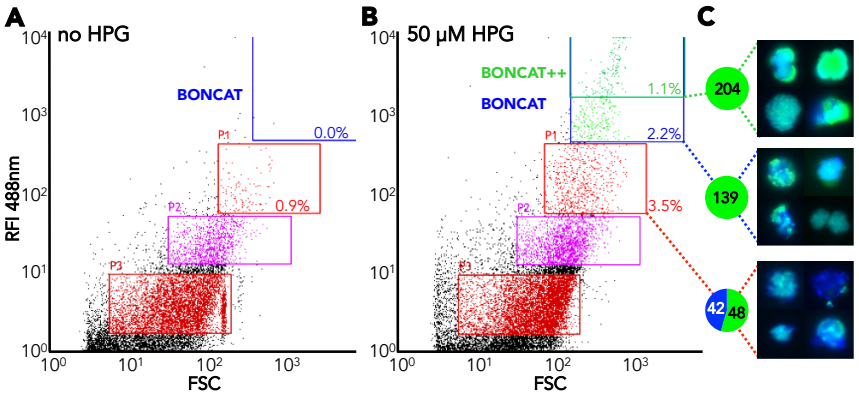
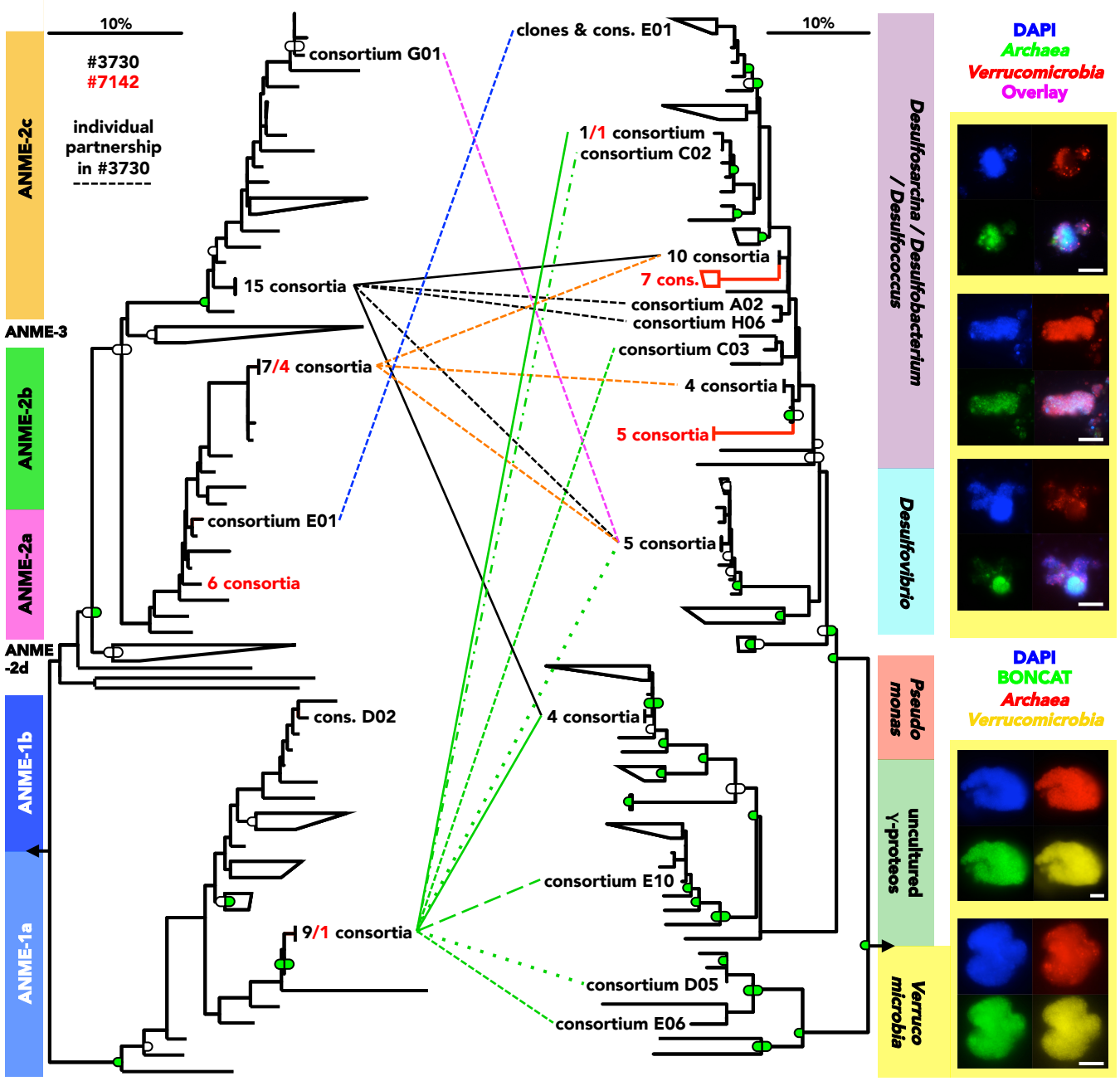

A
no HPG
BONCAT
P1
P2
P3
0.0%
0.9%
RFI 488nm
FSC
B
50 µM HPG
BONCAT++
BONCAT
1.1%
2.2%
3.5%
C
204
139
42
48

Figure 4

Figure 5


10%
#3730
#7142
individual partnership in #3730
ANME-2c
ANME-3
ANME-2b
ANME-2a
ANME -2d
ANME-1b
ANME-1a
consortium G01
15 consortia
7/4 consortia
consortium E01
6 consortia
cons. D02
9/1 consortia
clones & cons. E01
1/1 consortium
consortium C02
10 consortia
7 cons.
consortium A02
consortium H06
consortium C03
4 consortia
5 consortia
5 consortia
4 consortia
consortium E10
consortium D05
consortium E06
10%
Desulfosarcina / Desulfobacterium / Desulfococcus
Desulfovibrio
Pseudomonas
uncultured γ-proteos
Verrucomicrobia
DAPI
Archaea
Verrucomicrobia
Overlay
DAPI
BONCAT
Archaea
Verrucomicrobia

SI Appendix

Visualizing *in situ* translational activity for identifying and sorting slow-growing archaeal-bacterial consortia

Roland Hatzenpichler[a,1], Stephanie A. Connon[a], Danielle Goudeau[b], Rex R. Malmstrom[b], Tanja Woyke[b], Victoria J. Orphan[a,1]

[a] Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, CA-91125, USA

[b] Department of Energy Joint Genome Institute, Walnut Creek, CA-94598, USA

[1] Address correspondence to hatzenpichler@caltech.edu and vorphan@gps.caltech.edu

SI Appendix Results and Discussion

Factors limiting the absolute quantification of newly synthesized proteins. BONCAT has the theoretical potential to detect all *de novo* synthesized proteins that contain at least one Met, *i.e.* >99% of proteins in an average archaeal and bacterial proteome. However, in practice several factors reduce this sensitivity and prohibit the absolute quantification of the amount of new protein, and, in consequence, of cell doubling times, from fluorescence data. (i) the process(es) by which HPG enters the cell is currently unknown and might depend on the physiological state of the cell or differ between different taxonomic groups; (ii) the rate at which HPG substitutes Met during protein synthesis has so far only been studied for *E. coli* (1) and might deviate in other organisms; (iii) due to varying contents of Met and contrasting copy numbers of proteins, distinct peptides contribute differently to overall fluorescence; and (iv) the extent by which protein-recycling and post-translational modification affect the stability of HPG (in particular its alkyne group) is currently unknown.

CARD-FISH vs. FISH. Our CARD-FISH experiments revealed marked discrepancies in the efficiencies to permeabilize and fluorescently detect different ANME-subgroups. Most importantly, while ANME-1 constituted only 0.6% of all taxonomically identified consortia in our FISH experiments on sample #3730 (114 days sample; n=167 consortia), the same sub-group represented 81.3% of detected ANME-consortia in our CARD-FISH dataset (n=48) (Tab. 1). This result is mainly explained by the inability of the employed CARD-FISH protocol to detect ANME-2a and -2c consortia in our samples. This finding is in contrast to two recent studies that employed near-identical permeabilization protocols for the successful visualization of these ANME-groups in other methane seep sediments (2, 3).

Comparing ANME-community structure in sediment #3730 to previous studies. The very low proportion of aggregated ANME-1 in our Hydrate Ridge sediment (#3730) is consistent with a previous study on this methane seep, that found ANME-1 occurred mostly as planktonic cells rather than in multicellular associations (4, 5). Because of our focus on syntrophic consortia in this study, we initially separated microbial aggregates >3 μm from sediment particles. Planktonic ANME-1 cells thus might have partly evaded FISH-detection. It should however be noted, that our filtered samples contained large numbers of individual cells and that in no #3730 sample individual cells were found to bind the ANME-1-specific probe.

ANME-2a and -2c probes used in this study have been successfully employed in previous mono-FISH studies of geographically distinct Hydrate Ridge sediment samples with hybridization rates of 20-80% of all DAPI-stained AOM-consortia (5, 6). Because of the high spatial variance in ANME-community structure (5, 7) it is, however, possible that our specific sediment samples hosted a unique combination of ANME-clades. Alternatively, ANME-community structure might have diverged from its original composition during the nearly four years of incubation in the lab before the experiments described herein where conducted.

Considerations on the environmental application of BONCAT. In contrast to the well-established stable isotope probing approach, the universal applicability of BONCAT is currently untested and several questions demand rigorous investigation in future studies: (i) the

mechanism(s) by which bioorthogonal amino acids are taken up by cells is currently unknown. If active transporters are required for their uptake, their absence would prohibit the application of BONCAT to that particular cell. To that effect, the recent report of up to 100% BONCAT-labeling efficiency of planktonic microbes in surface seawater is encouraging (8). (ii) HPG and AHA, the bioorthogonal amino acids that have been used in environmental studies so far (8, and this study, 9), have to compete with intracellular Met for incorporation into newly being made proteins. The preference of the translational machinery for Met over its synthetic surrogates (1) might therefore restrict the use of BONCAT in habitats featuring high concentrations of free Met. (iii) Lastly, substituting proteins with synthetic amino acids bears a high risk of interfering with the cellular machinery. We recently demonstrated that the addition of up to 1 mM of HPG or AHA had no detectable effect on the growth of several different, physiologically distinct archaeal and bacterial pure cultures for at least one cell generation. At longer incubation times, however, inhibition of growth could be observed at these high concentrations (9, 10, and Hatzenpichler, unpubl.). For environmental applications we thus recommend that low concentrations of bioorthogonal amino acid should be employed, incubation times kept to a minimum, and complementary experiments testing for potential community shifts be performed (for details see 10).

SI Appendix Material and Methods

Environmental sampling and storage. Sediment sample #3730 was obtained from Hydrate Ridge South methane seep field (Atlantis cruise AT-15-68, Alvin Dive 4635; push-core 16; 44°34.09 N, 125°9.14 W; 775 m water depth; sediment horizon 0-6 cm; 4°C *in situ* temperature) on August 7th 2010. Sediment was stored under argon headspace in a Mylar bag for five weeks, before being transferred to a 1 L glass bottle with a 1.38 bar 100% CH_4 headspace, which was stored at 4°C. Seawater and headspace were exchanged in regular intervals to prevent the accumulation of inhibitory compounds.

Sediment samples #7135 and #7136-37/37 were collected from Santa Monica basin on May 9th 2013 (R/V Western Flyer MBARI Cruise 2013; dive 463; push-core 43; 33°47.34 N, 118°40.10 W; 860 m water depth; horizons 6-9 cm (#7135) and 9-15/15-22 cm (#7136-37/37, pooled) below a pink and white microbial mat; 4°C) . Sediment slurry were stored in a glass bottle for ~1 year under argon at 4°C, before the experiments described below were carried out.

Sediment sample #7142 was collected from Santa Monica basin on May 7th 2013 (R/V Western Flyer MBARI Cruise 2013; dive 459; push-core 74; 35°47.34 N, 118°40.09 W; 863 m depth; sediment horizon 4-6 cm; 4°C *in situ* temperature). The sediment was sealed under argon and stored at 4°C. After 40 days of storage, the sediment was suspended in anaerobic natural bottom seawater from the site in an anaerobic chamber (3% H_2 in N_2) and aliquots were over-pressured with 1.5 bar CH_4. The sediment was kept for 12 months under 1.5 bar 100% CH_4 in natural bottom seawater that was exchanged every three months.

Sediment sample #5119 was collected from the Hydrate Ridge methane field during Atlantis Cruise AT-18-10 on September 1st 2011. (44°40.02 N, 125°6.00 W; dive J2-593 E4A; push-core 36 through a yellow microbial mat; water depth 600 m; sediment horizon 9-12 cm). Sediment #5202 was collected during the same cruise on September 3rd 2011, dive J2-593 E6B (44°40.02 N, 125°7.51 W; push-core 18 through a pink-white microbial mat; water depth 601 m; horizon 3-6 cm;).

Carbonate #3439 was collected from atop an active seep at the Hydrate Ridge methane field during cruise AT-15-68 on August 1st, 2010 (44°34.09 N, 125°9.14 W; dive AD4629; water depth 775 m). Carbonate sample #2450 was retrieved from sediment sample #2450 collected at Eel River Basin on July 27th 2005 (40°48.68 124°36.73 W; dive T-864; push-core 49; horizon 0-2 cm; water depth 516 m).

Information on the geochemical characteristics of the sampling sites may be requested from the corresponding authors.

Setup of incubations. All samples were kept in an ice bath at all times during handling. Artificial sea-water (ASW) consisted of 10.9 g $MgCl_2$ $6H_2O$, 0.2 g $NaHCO_3$, 0.76 g KCl, 25.9 g NaCl, 1.47 g $CaCl_2$ $2H_2O$, 3.98 g Na_2SO_4, and 26.73 mg NH_4Cl L^{-1} ddH_2O at pH 7.4. 1 mL vitamin solution (see medium 141, www.dsmz.de) and 1 mL trace element solution SL-10 (see www.dsmz.de) were also added. Before use, ASW was filtered through a 0.2 µm filter and N_2-bubbled for 10 minutes. ASW was kept on ice during handling.

~50 ml of wet sediment #3730 were re-suspended in ASW, yielding a total volume of ~130 mL. 20 mL aliquots of homogenized slurry were transferred into 160 mL serum bottles and 30 mL of ASW were added. Bottles were sealed with rubber stoppers, headspaces flushed with either 100% CH_4 or 100% N_2 for 5 min before being pressurized with 2 bar 100% CH_4 or 100% N_2. Sediment was allowed to equilibrate over-night (~18 h) at 4°C in the dark. 0.2 μm-filtered *L*-homopropargylglycine (HPG; Click Chemistry Tools) in ddH_2O was added to reach a final concentration of 50 μM. Control incubations without HPG were supplemented with sterile ddH_2O to reach equal volumes. All bottles were then flushed for 5 min, pressurized with 2 bar CH_4 or N_2, and incubated in the dark at 4°. In total, 6 incubations were performed: 2x without HPG plus CH_4; 2x 50 μM HPG plus CH_4; and 2x 50 μM HPG plus N_2.

~100 mL of wet sediment #7135 were re-suspended in ASW, yielding a total volume of ~300 mL, and incubated for 15 days under 2 bar 100% CH_4. After this pre-incubation, 100 mL of ASW were added and the slurry homogenized. Under constant N_2-flushing, 35 mL aliquots were transferred into 160 mL serum bottles, bottles sealed with rubber stoppers, and headspaces flushed for 5 min with either 100% CH_4 or 100% N_2, depending on incubation setup. HPG was added to reach a final concentration of either 5 or 50 μM. In addition, controls without HPG were supplemented with sterile ddH_2O to reach equal incubation volumes. Then, all bottles were flushed for 5 min, over-pressurized with 2 bar CH_4 or N_2, and incubated in the dark at 4°C. In total, 12 incubations were performed: 4x without HPG plus CH_4; 4x 50 μM HPG plus CH_4; 2x 50 μM HPG plus N_2; and 2x 5 μM HPG plus CH_4.

20 mL of wet sediment #7136-37 were re-suspended in ASW, yielding a total volume of ~50 mL. The slurry was bubbled with N_2 for 10 min (this was repeated after 5 days), before the bottle was incubated for 124 days under 2 bar N_2 (detectable but non-quantifiable amount of CH_4, 1-10 ppm). After this pre-starving, 10 mL aliquots were transferred into 75 mL bottles. HPG was added to reach a final concentration of 50 μM and 2 bar 100% CH_4 or 100% N_2 (2 bar) were added to the headspace of 2 aliquots each. In addition, a control mesocosm was incubated without HPG under 100% CH_4 (2 bar). All bottles were incubated at 4°C in the dark for 31 days.

1 mL aliquots of wet sediment #7142 in 5 mL ASW containing 25 mM HEPES buffer (pH 7.5), 5 mM sulfide, and 5 mM dissolved inorganic carbon were re-suspended in serum vials, which were then sealed with rubber stoppers (12.9 mL final volume). The headspace was flushed with $^{12}CH_4$ before 1.0 mL $^{13}CH_4$ (99% ^{13}C, containing 0.05 vol% $^{13}CO_2$ as impurity; Cambridge Isotope Laboratories) was added. After ~5 days of pre-incubation, HPG was added to two of the four bottles to reach a concentration of 50 μM. In addition, one incubation was performed at 250 μM HPG for 25 days and later used for activity-based cell-sorting.

Sampling for molecular and geochemical analyses. Sampling of sediment microcosms was undertaken at incubation start as well as after 30, 73, and 114 days and 7, 14, 41, 56, and 171 days for sediment #3730 and #7135, respectively. Samples for molecular, cellular, and geochemical analyses were removed using sterile syringes while the incubation bottles were kept in an ice bath.

At each sampling-point, 0.25 mL of sediment slurry were transferred into a sterile 1.5 mL tube and centrifuged at 16,100 g for 10 sec at room temperature (RT). The supernatant (SN) was removed, mixed in a 1:1 ratio with 0.5 M Zn-Acetate solution and stored for later sulfide analysis. The pellet was flash-frozen using liquid N_2 and stored at -20°C for DNA extraction. 0.25 mL of sediment slurry were removed, centrifuged as described above, the SN wasted, and the pellet re-suspended in a 1:1 mix of 1x PBS and absolute ethanol (EtOH). Another 0.25 mL were processed in the same way, but re-suspended in 3% paraformaldehyde (PFA; Electron Microscopy Sciences) in 1x PBS and incubated for 1 h at RT for chemical fixation of cells. Afterwards, biomass was harvested by centrifugation, the SN wasted, and the pellet washed with 1.5 mL of 1x PBS. Finally, sediment was centrifuged, the SN wasted, and the biomass re-suspended in a 1:1 mix of 1x PBS and EtOH. All EtOH- or PFA-fixed samples were kept at -20°C until further processing. After sampling (30, 73, and 114 days and 7, 14, 41, 56, and 171 days for #3730 and #7135, respectively), the headspace of bottles was flushed for 3 min with either CH_4 or N_2 before the sediment was again incubated at 4°C with 2 bar of either 100% CH_4 or 100% N_2, depending on incubation setup. In addition, after 73 days (#3730) and 41 and 130 days (#7135) ~90% of artificial seawater overlying sediment was exchanged. The slurry volume and sediment-to-water ratio of all incubations was identical at all times for each sediment type (#3730 or #7135, respectively). When appropriate, newly added seawater was then supplemented with 5 or 50 μM (final) HPG.

Geochemical analyses. Sulfide (H_2S plus HS^-) concentrations were determined via the cline assay (11). Samples were analyzed for statistically relevant differences via student's t-test. Differences were considered to be significant at $p \leq 0.05$. Methane oxidation rates for sediment #7142 were determined as described by Scheller *et al*. (12) by measuring the formation of ^{13}C-dissolved inorganic carbon ($DI^{13}C$) from $^{13}CH_4$ over time. Succinctly, 0.25 mL of ASW overlying settled sediment was removed and centrifuged (16,000 g for 5 min). The SN was transferred into 0.6 mL tubes, flash frozen in N_2, and stored at -20 °C until further processing. 150 μL of thawed SN was then added to He-flushed vials containing 100 μL H_3PO_4 (85%). The resulting CO_2 was analyzed for isotopic enrichment on a GC-IR-MS GasBench II (Thermo Scientific).

Extraction of microbial aggregates. To separate microbial aggregations and individual cells from sediment particles, 50 μL of sediment slurry were re-suspended in 450 μL of 1x PBS in a 2 mL tube. This solution was chilled in an ice-bath for 15 min before being sonicated 3x for 10 sec at 3-6 W output using a Branson Sonifier 150 (Branson Ultrasonics Corporation). Between pulsing intervals the sample was allowed to cool for 10-30 sec. After sonication, the sample was applied atop of 500 μL Percoll (Sigma-Aldrich) and centrifuged at 16,100 g for 20 min at 4°C. To remove Percoll particles and the majority of individual planktonic cells, the entire SN was re-suspended in 15 mL 1x PBS and filtered through a 3 μm TSTP white polycarbonate filter (EMD Millipore) using a filter tower at ~0.3 bar under-pressure. Each filter was washed with a total volume of 50 mL 1x PBS without letting the filter run dry. Then, particles and biomass that had been retained by the filter were transferred into a 2 mL tube using 1x PBS by repeatedly and

vigorously pipetting up-and-down using a 1 mL pipette. DAPI-staining confirmed that this protocol leads to the near-complete transfer of microbial aggregates from the filter into solution (99-100% of DAPI-stained consortia), without selecting for or against a particular type of consortium morphology or size (not shown). After transfer into 1x PBS, biomass was harvested via centrifugation (16,100 g, RT), re-suspended in either 1x PBS (for 'nonfixed' BONCAT analyses) or a 1:1 ratio of 1x PBS and EtOH (fixed biomass), and stored at either 4°C (nonfixed) or -20°C (fixed).

List of oligonucleotide probes for FISH and CARD-FISH. In FISH-experiments combination of mono- and dual-labeled (indicated with ** in the list below) probes were used in different combinations (see Tab. S2): Arch915, specific for most members of the domain *Archaea* (13), used at 35% formamide (FA); EUB338, -II, and -III (*a.k.a.* EUB338mix), which together cover most of the known bacterial diversity (14, 15), used at 35% FA; EUB338-III, specific for most members of the *Verrucomicrobia* (15), used at 35% FA, in combination with EUB338-I and –II as competitor probes; Delta495a** together with its competitor probe, specific for most delta-proteobacteria (16), used at 35% FA; Gam42a, together with its competitor, specific for most gamma-proteobacteria (17), used at 35% FA; Ver47**, specific for *Verrucomicrobia* (18) together with its helper probe H64 (19) at 15% FA; as well as ANME-1-350 (4) (40% FA), ANME-2-932 (a.k.a. EelMS-932; 4) (40% FA), ANME-2a-647 (50% FA) (5), and ANME-2c-760** (60% FA) (5), specific for different subpopulations of anaerobic methane-oxidizing euryarchaeotes. In addition, a new probe, ANME-2b-729, was designed, which detects >93% of all ANME-2b-affiliated 16S rRNA sequences in online and lab-internal databases. The new probe has at least 2 mismatches to all other archaeal or bacterial 16S rRNA sequences (tested using probeCheck, ref. 20). After careful evaluation, ANME-2b-729 was used at 20% formamide concentration. Note that this probe has a one-nucleotide overlap with probe ANME-2-712 (5) and should thus not be used in conjunction with this probe. Hybridizations without probe addition or probe NONEUB388 (21) were used as negative controls.

With the exception of probe ANME-2a-647, which was used at 40% FA (rather than 50%), all probes employed in CARD-FISH were used at the same FA concentrations as in FISH-experiments. For CARD-FISH, hybridizations with probe NONEUB388 were used as negative controls.

Multiple displacement amplification. Individual sorted consortia were lysed and subjected to whole genome amplification (WGA) as previously described (22) with the following modifications: WGA was performed with a REPLI-g Single Cell Kit (Qiagen) with a scaled-down reaction volume of 2 µl and DNA-dye SYTO-13 added at 1x for real-time tracking. The cell lysis procedure followed a recently described protocol (22), which was modified by lysozyme treatment. This step included a 15 min RT incubation with 300 nl of 50 U/µl ReadyLyse lysozyme (Epicentre R1810M), which was followed by the addition of 50 nl concentrated DLB buffer (22). Lysis and stop reagents were UV-treated as described (22), while the Master Mix was used as obtained from the manufacturer (Qiagen). The amplification reaction was incubated for 6 hours at 30°C.

16S rRNA gene tag sequencing. Sediment DNA was extracted using the Power Soil DNA Isolation Kit according to the manufacturer's protocol (MoBio, Carlsbad, CA) and diluted DNA from genome-amplified sorted consortia was used directly. The V4 region of the 16S rRNA gene was amplified from each extract using archaeal and bacterial primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (23, 24). Sediment samples were amplified in duplicate. The non-barcoded primers were used with Q5 Hot Start High-Fidelity 2x Master Mix (New England Biolabs, Ipswich, MA) according to the manufacturer's directions using annealing conditions of 54°C for 30 cycles and 58°C for 32 cycles for sediments and MDAs, respectively. Duplicates of sediment sample amplifications were then pooled. The barcoded 806R primer (CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT) was paired with 515F in a reconditioning reaction (same conditions as above except for 5 cycles of PCR) to barcode the PCR products. Samples were mixed together in equimolar amounts and purified in bulk through a Qiagen PCR purification kit before submission to Laragen (Culver City, CA) for analysis on an Illumnia MiSeq platform. The resulting paired-end sequence data, 2x 250 base pairs (bp), was de-multiplexed and sequences with >1 bp mismatch on the 12 bp barcode were removed. The resulting sequences were passed through Illumina's MiSeq Recorder software to assign quality scores to each base call and remove adapter, barcode and primer sequence.

Analysis of 16S rRNA gene tag sequences. Sequence data was processed in QIIME version 1.8.0 (25) following a recently published protocol (26). Raw sequence pairs were joined and quality-trimmed using the default parameters in QIIME. Sequences were clustered into *de novo* operational taxonomic units (OTUs) with 99% similarity using UCLUST open reference clustering protocol (27). Then, the most abundant sequence was chosen as representative for each *de novo* OTU (28). Taxonomic identification for each representative sequence was assigned using the Silva-115 database (29, 30) clustered at 99% similarity. This SILVA database had been appended with 1,197 in-house high-quality, methane-seep derived bacterial and archaeal clones. Any sequences with pintail values >75 were removed. The modified SILVA database is available upon request from the corresponding authors. OTUs were then filtered to remove singletons from the combined MDA dataset. A threshold filter was used to remove any OTU that occurred below 0.01% of the entire combined sediment samples dataset. Known contaminants in PCR reagents as determined by the analysis of negative and positive controls run with each MiSeq set were also removed (31). For the sediment samples, the sequence data was rarified by random subsampling to equal the sample with the least amount of sequence data, resulting in 12,115 and 3,707 sequences per sample for sediments #3730 and #7135, respectively. Tables of both absolute and relative abundance were generated at the family level for each sample. For statistical and similarity percentage analyses (Non-metric Multi Dimensional Scaling (NMDS), Analysis of Similarity (ANOSIM), and Similarity Percentage (SIMPER)), family level abundance tables were square-root transformed prior to generation of Bray Curtis similarity matrices and analyzed using Primer-E software (http://www.primer-e.com). Differences were considered to be significant at $p \leq 0.05$.

Phylogenetic analysis. 16S rRNA gene tag sequences from each consortium as well as closely related sequences from online databases (identified via the BLAST-algorithm of NCBI) were imported into and analyzed via the ARB software package (32). Sequences were automatically aligned to reference sequences of all ANME-subpopulations as well as relevant bacterial clades contained within the SILVA-115 database that had been amended with 1,197 in-house seep derived clones. Sequences from cultured representatives of archaeal phyla *Thaum-* and *Euryarchaeota* were used as outgroup for reconstruction of the archaeal tree. Members of the *Planctomycetes* where chosen as outgroup for the bacterial tree. Both alignments were manually curated and termini-filters were created. During the phylogenetic reconstruction of archaeal (all >1,100 nt in length) and bacterial (all >1,000 nt in length) sequences, 958 and 1,255 positions were considered, respectively. Phylogenies were modeled using RaxML and short tag-sequences were individually added to the tree using the parsimony interactive tool in ARB without changing the overall topology of the tree. In addition, maximum parsimony (100x replications) and Neighbor Joining (1,000x replications) trees were calculated and bootstrap values projected onto the RaxML tree.

SI Appendix Figure and Table Legends

Fig. S1. Sediment sulfide production rates and methane oxidation rates are not affected by the presence of HPG. *(A)* Sulfide [H_2S + HS^-] levels cannot be directly compared between different time-points, because seawater and headspace of incubations were refreshed in regular intervals. In contrast to HPG, CH_4 has a statistically significant effect on sulfide production ($p=0.0183$ and $p=0.0063$ for #3730 and #7135 after 114 and 56 days of incubation, respectively). Sulfide levels in sediment #7135 samples #09 and #10 (both without CH_4) were below detection limit (bd) after 171 days of incubation. *(B)* Sediment methane oxidation rates are not affected by the presence of HPG over a course of up to 25 days. Four separate aliquots of sediment #7142 were incubated in the absence of HPG for ~5 days, after which 50 μM HPG (final concentration) were added to two incubations. Note that one of the HPG-containing incubations exhibited low rates of AOM from the start of the experiment on. Because of this, the experiment was stopped after ~10 days of incubation. Compilations of representative AOM-consortia from the end-points of two incubations are shown on the right. Green fluorescence indicates that cells have been translationally active during time of incubation. DAPI-stained of DNA is in blue. Methane oxidation rates were measured as ^{13}C-dissolved inorganic carbon [DIC] formed from $^{13}CH_4$. Sampling on day 5 was performed immediately after addition of HPG.

Fig. S2. Bray Curtis similarity indexes of the microbial communities of sediments #3730 *(A)* and #7135 *(B)* after incubation in the absence or presence of HPG for 114 and 171 days, respectively. For statistical analyses see Fig. S3 and S4.

Fig. S3. Non-metric Multidimensional Scaling (NMDS) ordinations of 16S rRNA gene tag sequences demonstrated that neither HPG *(A)* nor CH_4 *(B)* have a statistically relevant effect on the microbial community of sediment #3730 after 114 days of incubation. Stress values of NMDS-ordinations and p-values of concomitant Anosim analyses for whole communities and ANME-SRB-related lineages specifically are shown next to the plots. n, number of sequences per sample. The more similar microbial communities from two samples are, the closer they lie together. Differences between samples were considered to be significant at $p \leq 0.05$.

Fig. S4. Non-metric Multidimensional Scaling (NMDS) ordinations of 16S rRNA gene tag sequences demonstrated that HPG *(A)* does not have a statistically relevant effect on the microbial community of sediment #7135 after 171 days of incubation. *(B)* The absence of CH_4, on the other hand, has a clear effect on the community composition. Stress values of NMDS-ordinations and p-values of concomitant Anosim analyses for whole communities and ANME-SRB-related lineages specifically are shown next to the plots. Dotted lines connect the individual sampling points for each incubation bottle. n, number of sequences per sample. The more similar microbial communities from two samples are, the closer they lie together. Differences between samples were considered to be significant at $p \leq 0.05$.

Fig. S5. Relative abundances of ANME-related archaea and sulfate-reducing bacteria in sediment #3730 *(A)* or #7135 *(B)* over time. Abundance-based color-coding indicates relative abundance of taxa within a sample. Sequences summarized as "other archaea", which are slightly enriched in #7135 samples incubated in the absence of CH_4, were related to rRNA genes from Marine Benthic Group D / Deep Sea Hydrothermal Vent Euryarchaeotal Group 1 as well as the Marine Hydrothermal Vent Group and Miscellaneous Euryarchaeotal Group. The physiology of these uncultured, yet environmentally widely distributed clades is currently unknown. However, recent genomic data suggest an implication of members of Marine Benthic Group D in the degradation of detrital proteins in marine sediments (33).

Fig. S6. Extended versions of the phylogenetic trees shown in Fig. 5. Green and white colored boxes show support ≥90% and ≥70%, respectively. Values <70% are not shown. Left and right boxes indicate max. parsimony (100x) and neighbor joining (1,000x) values, respectively. Scale bars equal 10% estimated sequence divergence. Numbers in boxes give the number of sequences within a group. 16S rRNA gene tag sequences were added after tree construction without changing overall tree topology. Doted lines indicate individual partnerships. The scales bars represent 10% estimated sequence divergence.

Fig. S7. Representative images of our FISH-based screening of methane seep sediment and carbonate samples for associations of *Archaea* and members of the *Verrucomicrobia*. Arch915, probe specific for archaea; EUB338-III, a probe specific for most *Verrucomicrobia*; comp, unlabeled competitor probes EUB338-I and II; Ver47, a *Verrucomicrobia*-specific probe, used together with an unlabeled helper probe (helper). For references of FISH-probes and detailed aggregate-counts for specific samples refer to *SI Appendix* and Tab. S3.

Tab. S1. Incubation setup and sampling details. -, not determined; d, days of incubation; Y, yes. 5/50, 5/50 μM of HPG.

Tab. S2. Details on BONCAT-FISH experiments summarized in Fig. 2 and 4. For probe specificities, see Materials and Methods section. comp, competitor probe; nd, not determined; d, days of incubation.

Tab. S3. Details on *Verrucomicrobia*-FISH experiments. For probe specificities, see Materials and Methods section. comp, competitor probe; help, helper probe; cons, consortium.

References to *SI Appendix*

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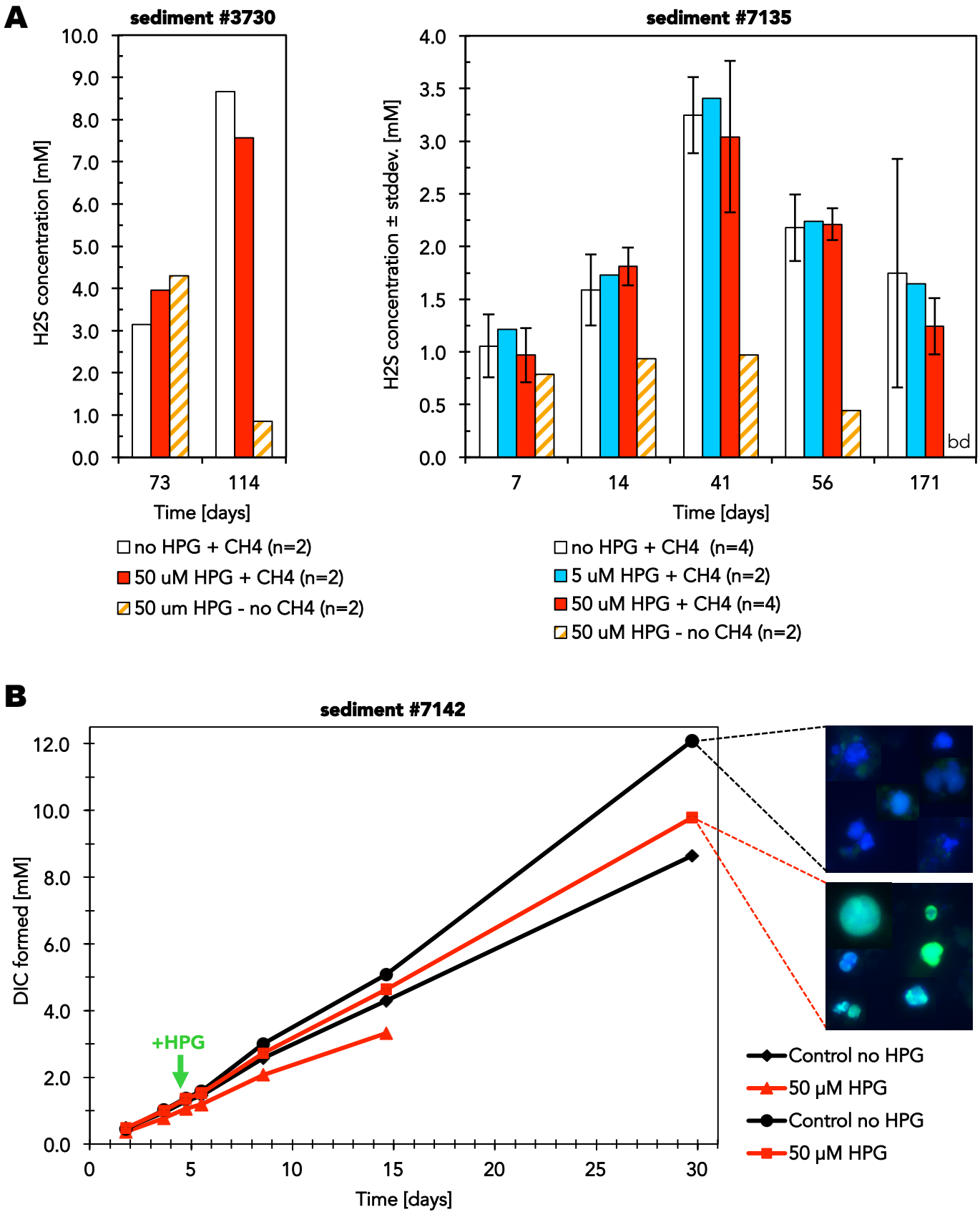
A
sediment #3730
H2S concentration [mM]
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
73
114
Time [days]
no HPG + CH4 (n=2)
50 uM HPG + CH4 (n=2)
50 um HPG - no CH4 (n=2)
sediment #7135
H2S concentration ± stddev. [mM]
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
7
14
41
56
171
bd
Time [days]
no HPG + CH4 (n=4)
5 uM HPG + CH4 (n=2)
50 uM HPG + CH4 (n=4)
50 uM HPG - no CH4 (n=2)
B
sediment #7142
DIC formed [mM]
12.0
10.0
8.0
6.0
4.0
2.0
0.0
+HPG
0
5
10
15
20
25
30
Time [days]
Control no HPG
50 µM HPG
Control no HPG
50 µM HPG

Figure S1

A

3730			HPG	NO	NO	NO	50	50	50	50
			CH4	Y	Y	Y	Y	Y	NO	NO
			Time	0	114	114	114	114	114	114
HPG	CH4	Time	Sample	#0	#1	#2	#3	#4	#5	#6
NO	Y	0	#0							
NO	Y	114	#1	91.7						
NO	Y	114	#2	92.0	94.5					
50	Y	114	#3	90.8	94.3	94.2				
50	Y	114	#4	91.0	94.4	93.1	94.8			
50	NO	114	#5	88.4	92.6	91.8	93.2	92.2		
50	NO	114	#6	91.1	93.6	92.8	93.6	92.7	92.8	

3730: 12,115 sequences

7135: 3,707 sequences

Bray Curtis similarity [%]

85.7 — 94.8

HPG
- NO no HPG addition
- 5 5 μM HPG
- 50 50 μM HPG

CH4
- NO 30 psi N2
- Y 30 psi CH4

Time
- 0 start
- 114 114 days
- 41 41 days
- 171 171 days

B

7135			HPG	NO	NO	NO	NO	NO	NO	NO	NO	50	50	50	50	50	50	50	50	50	50	50	5	5	5	5
			CH4	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NO	NO	NO	NO	Y	Y	Y	Y
			Time	41	41	41	41	171	171	171	171	41	41	41	41	171	171	171	171	41	41	171	171	41	41	171	171
HPG	CH4	Time	Sample	#01	#02	#03	#04	#01	#02	#03	#04	#05	#06	#07	#08	#05	#06	#07	#08	#09	#10	#09	#10	#11	#12	#11	#12
NO	Y	41	#01																								
NO	Y	41	#02	92.7																							
NO	Y	41	#03	92.9	92.3																						
NO	Y	41	#04	91.2	91.9	91.8																					
NO	Y	171	#01	87.9	88.2	87.7	88.5																				
NO	Y	171	#02	87.3	87.8	89.0	87.6	86.6																			
NO	Y	171	#03	87.6	88.2	87.7	88.1	91.2	88.9																		
NO	Y	171	#04	87.1	88.2	88.1	87.3	89.9	87.3	90.1																	
50	Y	41	#05	91.8	91.4	92.3	90.7	87.8	88.3	87.2	87.1																
50	Y	41	#06	92.1	92.2	93.7	90.9	87.6	89.2	87.7	87.9	90.7															
50	Y	41	#07	93.4	92.4	92.6	92.3	87.9	87.0	87.6	87.5	91.3	92.8														
50	Y	41	#08	90.9	92.7	92.5	91.7	88.8	88.8	88.5	88.7	91.4	91.5	91.6													
50	Y	171	#05	89.3	88.9	89.2	88.6	89.9	88.0	89.6	88.4	88.5	88.7	89.4	88.4												
50	Y	171	#06	88.8	89.0	89.3	88.5	90.3	87.3	90.8	90.0	87.5	88.8	88.5	88.9	90.7											
50	Y	171	#07	90.0	91.0	90.4	89.4	90.3	87.5	89.7	89.8	89.5	89.5	89.2	89.9	89.7	89.8										
50	Y	171	#08	89.9	90.6	90.5	89.6	89.2	89.8	89.1	88.8	90.9	90.3	90.5	90.4	90.9	89.1	90.1									
50	NO	41	#09	89.9	89.3	90.2	90.4	86.7	85.7	88.6	86.7	89.0	87.9	88.7	90.1	87.4	88.3	89.9	87.4								
50	NO	41	#10	91.3	91.8	92.1	89.8	86.8	89.1	86.4	86.2	90.8	91.6	91.0	91.3	88.1	87.8	89.4	90.7	89.2							
50	NO	171	#09	89.3	89.9	88.8	88.1	89.4	87.7	88.9	88.4	88.1	88.2	88.6	88.3	90.5	89.0	90.5	90.1	88.4	88.5						
50	NO	171	#10	89.2	88.9	89.3	88.2	89.4	86.8	88.8	88.9	88.0	87.7	88.7	89.0	89.0	89.5	91.2	88.5	89.4	88.3	90.4					
5	Y	41	#11	91.0	92.1	91.9	90.8	88.1	88.5	88.2	86.5	90.2	90.6	90.8	91.3	87.5	88.7	89.5	89.1	89.8	90.8	88.5	88.4				
5	Y	41	#12	92.6	92.8	93.2	92.2	88.0	88.0	88.3	87.0	92.2	91.2	92.2	91.8	89.6	88.8	90.6	89.7	91.1	91.4	88.7	89.0	91.8			
5	Y	171	#11	88.5	88.5	90.4	89.9	86.5	87.5	86.6	86.0	89.4	89.2	88.7	88.8	87.9	86.7	90.1	89.8	88.7	88.4	86.8	87.1	88.3	89.3		
5	Y	171	#12	89.4	90.1	89.6	89.6	90.9	87.9	91.2	90.7	89.1	89.3	89.3	90.2	89.0	91.1	90.1	90.2	89.1	89.6	89.6	89.5	89.3	89.2	87.2	

Figure S2

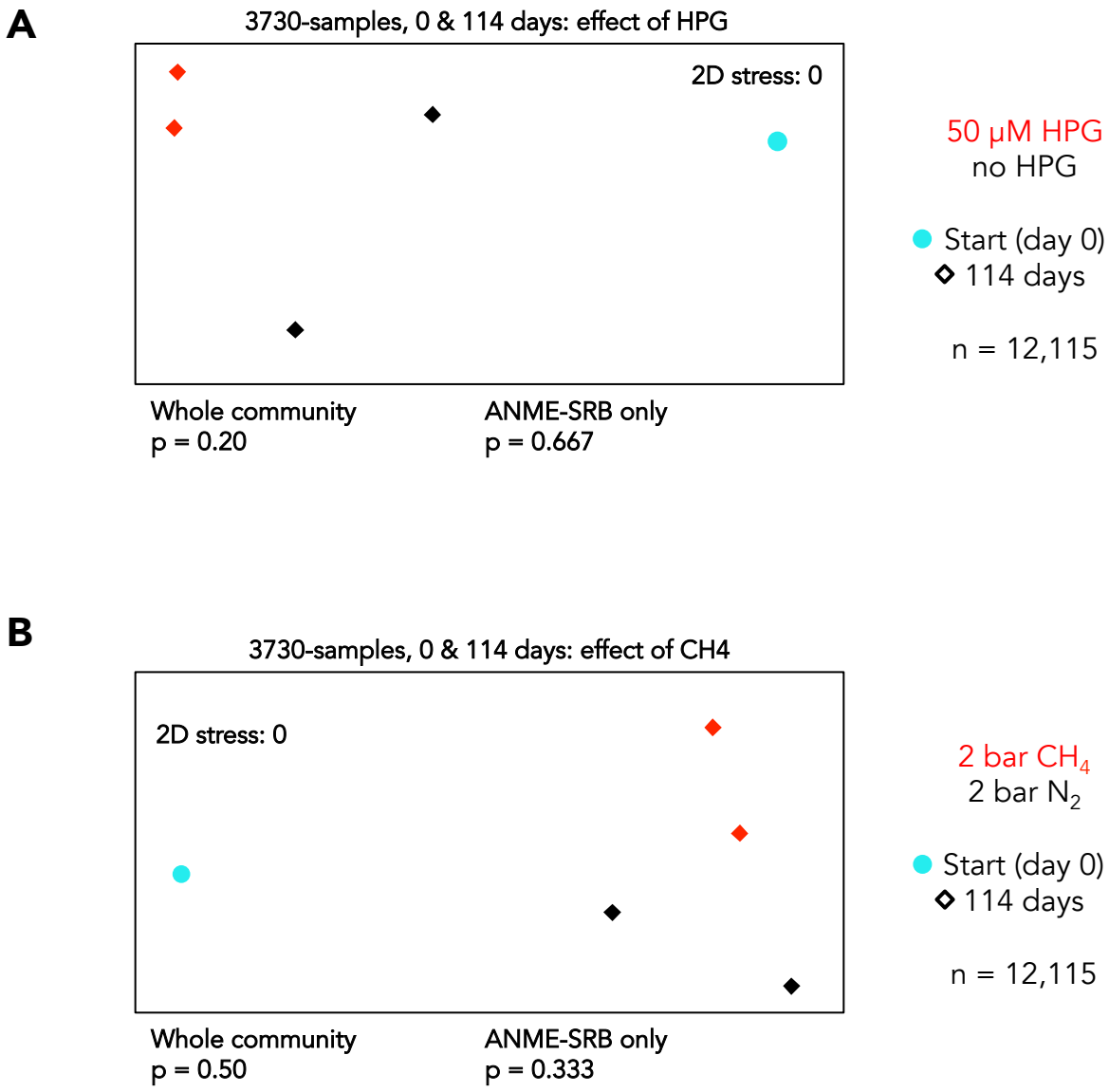
A
3730-samples, 0 & 114 days: effect of HPG
2D stress: 0
50 µM HPG
no HPG
Start (day 0)
114 days
n = 12,115
Whole community
p = 0.20
ANME-SRB only
p = 0.667
B
3730-samples, 0 & 114 days: effect of CH4
2D stress: 0
2 bar CH4
2 bar N2
Start (day 0)
114 days
n = 12,115
Whole community
p = 0.50
ANME-SRB only
p = 0.333

Figure S3

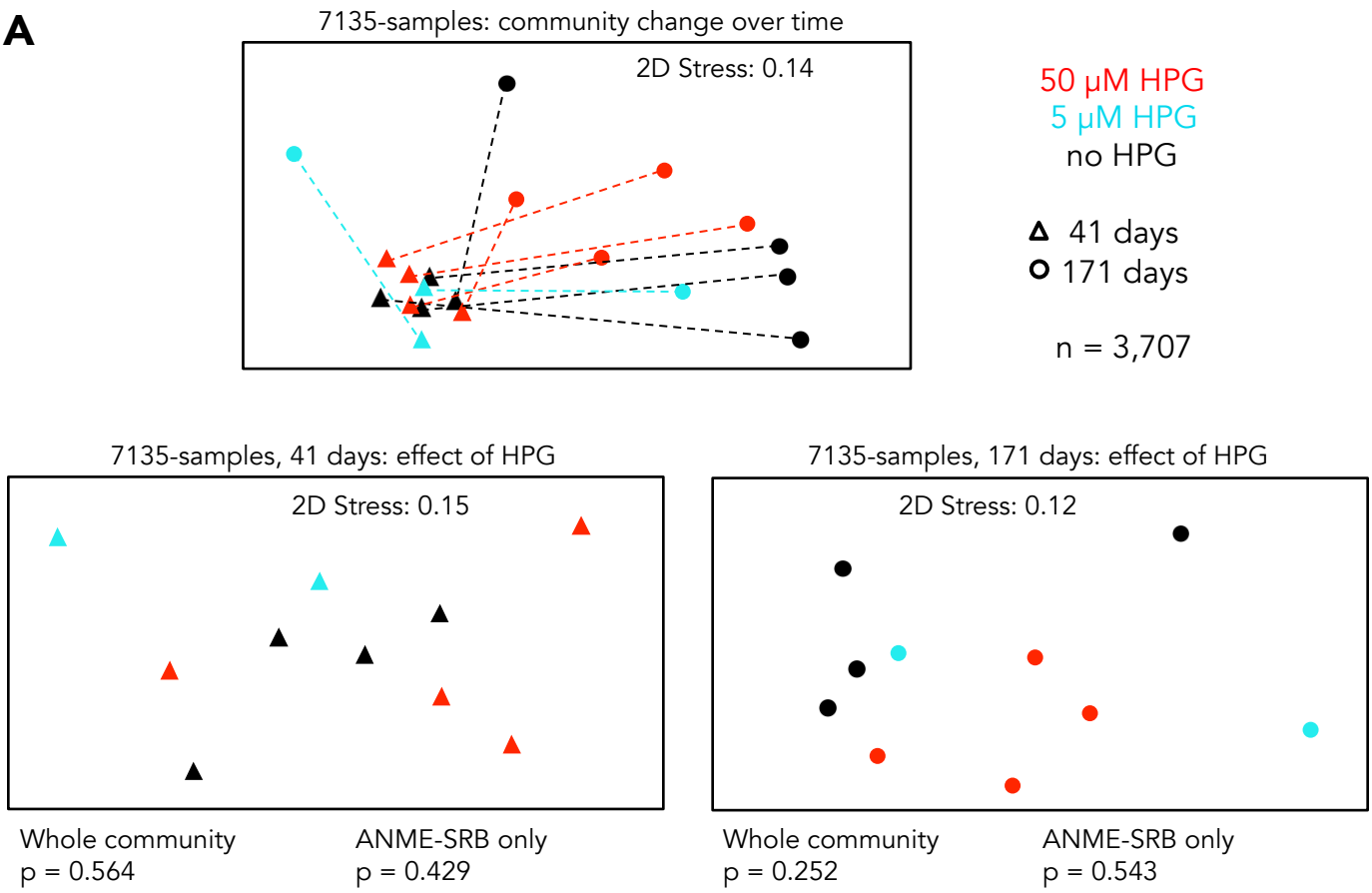
A
7135-samples: community change over time
2D Stress: 0.14
50 µM HPG
5 µM HPG
no HPG
41 days
171 days
n = 3,707
7135-samples, 41 days: effect of HPG
2D Stress: 0.15
Whole community
p = 0.564
ANME-SRB only
p = 0.429
7135-samples, 171 days: effect of HPG
2D Stress: 0.12
Whole community
p = 0.252
ANME-SRB only
p = 0.543
B
7135-samples with 50 µM HPG: effect of CH4
2D Stress: 0.11
2 bar CH4
2 bar N2
41 days
171 days
n = 3,707
Whole community
p = 0.042
ANME-SRB only
p = 0.20

Figure S4

A

3730	HPG	NO	NO	NO	50	50	50	50
	CH4	Y	Y	Y	Y	Y	NO	NO
	TIME	0	114	114	114	114	114	114
	Sample	#0	#1	#2	#3	#4	#5	#6
ANME-1;Other		0.7	0.3	0.4	0.4	0.4	0.2	0.3
ANME-1;ANME-1a		20.3	14.6	15.0	12.8	15.7	9.8	12.4
ANME-1;ANME-1b		1.3	1.2	1.5	1.5	0.9	1.0	0.9
Methanosarcinales;ANME-2a-2b		9.7	6.0	7.9	6.1	5.2	4.5	6.5
Methanosarcinales;ANME-2c		6.1	5.8	5.0	6.9	7.0	4.2	5.6
Methanosarcinales;Methanosaetaceae		0.0	0.0	0.0	0.1	0.0	0.0	0.0
Desulfarculales;Desulfarculaceae		0.4	0.9	0.6	0.9	0.8	1.0	0.8
Desulfobacterales;Desulfobacteraceae		11.9	12.5	10.7	10.8	11.6	14.4	16.8
Desulfobacterales;Desulfobulbaceae		3.3	4.4	5.0	4.5	4.3	5.1	4.8
Desulfuromonadales;Desulfuromonadaceae		0.2	0.2	0.5	0.3	0.3	0.5	0.2
Other archaea		0.9	1.1	1.0	1.0	1.2	1.4	0.7
Other bacteria		45.1	52.9	52.3	54.7	52.6	58.0	50.9

3730: 12,115 sequences

7135: 3,707 sequences

Relative abundance of taxon [%]

low — high

HPG

NO no HPG addition

5 5 µM HPG

50 50 µM HPG

CH4

NO 30 psi N2

Y 30 psi CH4

Time

0 start

114 114 days

41 41 days

171 171 days

B

7135	HPG	NO	NO	NO	NO	NO	NO	NO	NO	50	50	50	50	50	50	50	50	50	50	50	50	5	5	5	5
	CH4	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NO	NO	NO	NO	Y	Y	Y	Y
	Time	41	41	41	41	171	171	171	171	41	41	41	41	171	171	171	171	41	41	171	171	41	41	171	171
	Sample	#01	#02	#03	#04	#01	#02	#03	#04	#05	#06	#07	#08	#05	#06	#07	#08	#09	#10	#09	#10	#11	#12	#11	#12
ANME-1;_ANME-1a		2.3	1.7	1.9	4.2	3.2	1.3	2.1	5.4	3.3	2.8	3.4	1.8	3.3	2.1	1.5	3.6	0.7	1.6	1.5	1.2	1.2	1.9	2.6	2.4
ANME-1;_ANME-1b		0.3	0.2	0.3	0.2	0.1	0.0	0.1	0.1	0.3	0.1	0.2	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.2
Methanomicrobia;D-C06;__f		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0	1.1	0.0	0.0	0.1
Methanosarcinales;ANME-2a-2b		0.7	0.7	0.9	0.7	1.3	0.5	1.5	1.0	0.4	0.8	0.5	0.9	0.6	1.4	0.8	0.5	0.3	0.2	0.3	0.4	0.7	0.5	0.7	1.1
Methanosarcinales;ANME-2c		1.1	1.8	1.4	1.6	4.9	7.8	7.5	3.4	1.5	2.0	1.2	1.7	3.0	4.8	2.4	3.3	0.4	0.6	2.1	1.3	1.1	0.8	1.8	3.5
Desulfarculales;Desulfarculaceae		1.1	0.9	1.0	0.9	0.8	1.0	0.9	0.9	1.0	1.1	0.8	0.9	0.9	1.0	1.2	1.2	1.5	1.0	0.9	1.2	0.9	1.1	2.0	0.9
Desulfobacterales;Desulfobacteraceae		3.3	3.5	3.4	3.4	4.4	6.0	5.9	5.8	3.8	3.2	3.3	3.4	4.7	4.3	5.6	4.3	4.9	4.4	4.5	3.5	3.6	3.6	6.8	5.2
Desulfobacterales;Desulfobulbaceae		3.2	2.8	2.8	2.8	2.8	2.6	2.3	2.3	2.5	3.0	3.3	2.6	2.2	3.0	2.9	2.9	2.4	3.3	2.1	2.0	2.6	2.6	3.2	2.9
Other archaea		13.6	14.1	12.8	12.8	14.2	9.1	11.9	12.6	13.2	11.3	12.8	13.7	12.1	12.3	13.3	12.2	12.8	11.7	17.3	17.1	12.1	12.0	9.6	12.4
Other bacteria		74.3	74.2	75.2	73.3	68.2	71.5	67.5	68.2	73.9	75.5	74.2	74.6	72.8	70.8	72.0	71.7	76.6	76.7	71.1	72.8	76.2	77.3	73.1	71.3

Figure S5

Figure S6


10%
#3730
#7142
Uf, unfixed biomass
ANME-2c
ANME-3
ANME-2b
ANME-2a
ANME-2d
ANME-1b
ANME-1a
#3730 methane seep clone
AM888264, marine sediment clone
#3730: G01
GU553521, methane seep clone
DQ058815, marine sediment clone
methane seep clones (including #3730)
AF354131, methane seep clone
FN820374, marine mud volcano clone
FN820372, marine mud volcano clone
HQ588667, marine mud volcano clone
gas hydrate and mud volcano clones
AM888269, marine sediment clone
AM888268, marine sediment clone
AM888270, marine sediment clone
#3730: A02, A07, A11, A12, B04, B07, B09, B10, B12, C09, C10, D03, E07, F06, H06
AM888265, marine sediment clone
ANME-3
#3730: A10, C04, C08, F03, D10Uf, E01Uf, F07Uf; #7142: C02Uf, C08Uf, C10Uf, D12Uf
#3730 methane seep clone
#3730 methane seep clone
FJ712376, marine mud volcano clone
AM888262, marine sediment clone
AY454546, tropical estuary sediment clone
DQ837287, coastal aquifer clone
EF644784, hydrothermal vent clone
DQ837289, coastal aquifer clone
#3730: E01
FM179915, methane seep clone
FJ555686, marine sediment membrane bioreactor clone
AF354130, marine sediment clone
#7142: A03Uf, G01Uf, G09Uf, H01Uf, H03Uf, H05Uf
DQ058812, methane seep clone
AY592057, marine mud volcano clone
AJ578088, methane seep clone
ANME-2d, AAA, GoM clade
AP011532, Methanocella paludicola
AF027738, Halorubrum trapanicum
CP000559, Methanocorpusculum labreanum Z
AM229232, pure gas hydrate clone
#3730: D02
DQ521783, hypersaline marine sediment clone
FM179858, methane seep clone
#3730 methane seep clone
AJ578120, methane seep clone
AF412943, methane seep clone
marine sediment and freshwater clones
HQ700682, marine sediment clone
clones: hot vent; mud volcano; seeps, including #3730
seawater and marine sediment clones
#3730: A01, C02, C03, C12, D05, E06, E12, B06Uf, D02Uf; #7142: A12Uf
#3730 methane seep clone
#3730 methane seep clone
AJ578145, methane seep clone
DQ521778, marine hypersaline sediment clone
GU553634, marine sediment clone
AF419649, hydrothermal sediment clone
AF419627, hydrothermal sediment clone
AM746092, gas seep clone
10%
#3730 methane seep clones & #3730: E01
#3730 methane seep clone
#3730 methane seep clone
AJ535235, Hydrate Ridge methane seep clone
#3730 methane seep clone
Desulfosarcina, Desulfobacterium, Desulfococcus sp.
#3730: C12; #7142: A12Uf
#3730: C02
#3730 methane seep clone
#3730 methane seep clone
#3730 methane seep clone
#3730 methane seep clones
#3730 methane seep clones
#3730: A11, A12, B07, B09, B12, C08, C09, C10, D03, H06
#7142: A03Uf, C08Uf, G01Uf, G09Uf, H01Uf, H05Uf
#7142: A03Uf
CP000859, Desulfococcus oleovorans
#3730: A02, H06
#3730 methane seep clone
#3730: C03
#3730 methane seep clone
#3730: A10, C04, D10Uf, E01Uf
#3730 methane seep clone
#3730 methane seep clone
#7142: C02Uf, C08Uf, C10Uf, D12Uf, H01Uf
AJ237601, Desulfobacterium anilini
AJ441316, Desulfobacula toluolica
3#730 methane seep clones
AF354150, methane seep clone
AF354152, methane seep clone
#3730: A07, D05, E10, F03, G01
AF354156, methane seep clone
AM746088, cold seep sediment clone
DQ521792, hypersaline methane seep clone
Desulfocapsa, Desulfotalea, Desulfovibrio sp.
Syntrophus sp.
Pseudomonas sp.
EU391388, Pseudomonas brassicacearum
#3730: B04, C09, C10, C12
KF782794, Pseudomonas aeruginosa
#3730 methane seep clone
Oceanospirillum sp.
#3730 methane seep clones
#3730 methane seep clones
#3730 methane seep clone
K616794, methane seep carbonate clone
#3730: E10
#3730 methane seep clone
FN554084, uncultured sediment bacterium
AB188770, Maorithyas hadalis symbiont
#3730 methane seep clone
AM490658, sulphidic cave spring microbial mat clone
FM200890, fouling biofilm; membrane bioreactor clone
#3730: D05
DQ337041, subsurface water clone
#3730: E06
GQ262724, Verrucomicrobium sp., marine sediment
X90515, Verrucomicrobium spinosum
Desulfosarcina / Desulfobacterium / Desulfococcus related seep clones
Desulfovibrio et al. related clones
Pseudomonas
uncultured γ-proteos
Verrucomicrobia

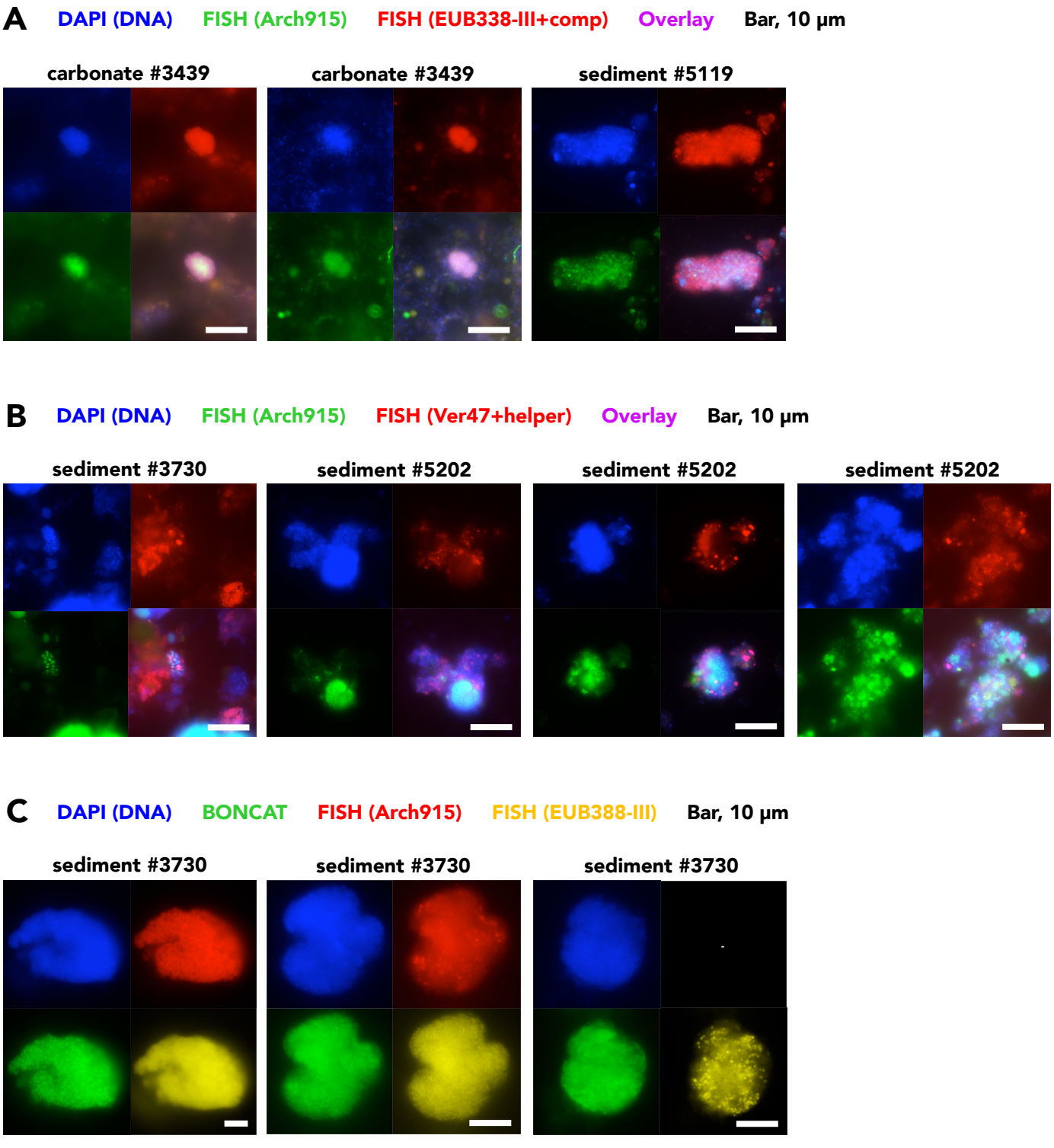
A
DAPI (DNA)
FISH (Arch915)
FISH (EUB338-III+comp)
Overlay
Bar, 10 µm
carbonate #3439
carbonate #3439
sediment #5119
B
DAPI (DNA)
FISH (Arch915)
FISH (Ver47+helper)
Overlay
Bar, 10 µm
sediment #3730
sediment #5202
sediment #5202
sediment #5202
C
DAPI (DNA)
BONCAT
FISH (Arch915)
FISH (EUB388-III)
Bar, 10 µm
sediment #3730
sediment #3730
sediment #3730

Figure S7