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Supporting Information for "Single cell activity reveals direct electron transfer in methanotrophic consortia"

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1 Proposed Interaction Mechanisms Between Archaea and Bacteria involved in anaerobic methane oxidation

In 1994, Hoehler and co-workers proposed the existence of a microbial consortium mediating methane oxidation coupled to sulfate reduction based on field and laboratory measurements of methane oxidation within methanogenic sediments [23]. In their hypothesis, methanogens would carry out reverse methanogenesis with concomitant production of H_2, and this H_2 would be efficiently scavenged by a sulfate-reducing bacterium, maintaining favorable thermodynamics of the redox couple. Later, using 16S rRNA FISH probes targeting putative methane-oxidizing ANME archaea, Boetius [10] observed ANME cells belonging to the Methanosarcinales in consortia with sulfate-reducing members of the Desulfobacteraceae, supporting the Hoehler proposal of a structured syntrophic relationship, where the close physical proximity observed between cells would presumably facilitate intercellular metabolic coupling. Follow up work by Nauhaus and colleagues investigated molecular hydrogen, formate, acetate and methanol as possible intermediates between the two organisms, but they were unable to find compelling evidence that these could function as intercellular electron shuttles during AOM [17]. Instead, these authors postulated that cells in direct physical contact could possibly utilize redox components positioned outside the cell as agents of electron transfer. In this scenario, direct electron transfer occurring through closely packed cells in consortia would be an alternative to the transfer of a molecular intermediate.

Other possible mechanisms, including methanethiol production and exchange [19] and, more recently zero valent sulfur transfer [20], have been proposed. In particular, the interaction mechanism hypothesized by Milucka and co-workers represents a significant departure from previously proposed interaction scenarios for sulfate-coupled AOM, where ANME archaea are proposed to independently carry out the full reaction of methane oxidation coupled to sulfate reduction, with electron transfer to sulfur atoms terminating at the S(0) oxidation state within ANME cells. This S(0) was then proposed to be disproportionated in an unprecedented reaction from HS_2^-, leading to the formation of HS^- and SO_4^{2-} in a ratio of 1:7. In this scenario, the ANME archaea were suggested to be capable of AOM independent of the associated sulfate-reducing bacteria, and the interaction occurring between organisms would be better described as commensal, rather than an obligate mutualism.

These above mentioned studies give rise to three possibilities as to the nature of ANME-SRB interactions which may be occurring during net methane oxidation:

1. An as yet unidentified molecular intermediate other than those tested is involved in syntrophic the coupling, and this unknown molecule (or mixture of molecules) obviates the thermodynamic constraints associated with a diffusible intermediate coupled with the low net energy yield of anaerobic methane oxidation.

2. SRB are dependent on ANME for the formation of HS_2^-, but thermodynamic predictions indicate ANME are not dependent on SRB for the removal of the intermediate because sulfate reduction to disulfide with methane as the electron donor is exergonic over a wide range of disulfide concentrations [20].

3. ANME-SRB consortia are syntrophic, and syntrophic coupling occurs through direct passage of electrons to the SRB which are poised at an appropriate potential.

Expected outcomes from microbial interactions within these three scenarios predict differences in the emergent spatial patterns of cellular activity which can be compared against our FISH-nanoSIMS data of individual ANME archaea and SRB cells in AOM consortia:

1. **A molecular intermediate**. In the case of a molecular intermediary of syntrophic exchange between partnering cells, consortia geometry (that is, size and cellular arrangement) will be a strong driver on both the magnitude and distribution of metabolic activity amongst partnering cells, owing to the expected rate of diffusion compared to cellular growth rates [13].

2. **Sulphate reducing ANME hypothesis**. In this case, ANME activities are not expected to be related to SRB activities, because the proposed reaction is exergonic at all reasonable HS_2^- product concentrations [20], however, bacterial activities will be related to spatial proximity to ANME cells, who are proposed to be the source of zero valent sulphur required for SRB sulfur disproportionation.

3. **Direct electron transfer**. Here, ANME-SRB activities should be positively correlated, but the magnitude and distribution of cellular activity within AOM consortia is less strongly linked to aggregate size or spatial arrangement of ANME-SRB cells as suggested by the modeling presented here.

2 Sediment composition, sample preparation, and analytical measurements

Sediment sample acquisition Sediment was obtained from a white mat covered active methane seep at Hydrate Ridge North (station HR-7; (44°40.02'N, 125°6.00'W; 600m depth) using a push coring device operated by the ROV Jason II during the AT 18-10 Hydrate Ridge Aug/Sept 2011 expedition. Push core 47 was processed shipboard immediately following recovery and the upper 9 cm of sediment stored under N_2 in mylar at 4°C until dispensing the top 9 cm of the core into a 1L overpressurized CH_4 (30 psi) large-scale microcosm incubation in anoxic filtered sea water.

^{15}N-isotope labeling experimental setup 10ml of the sediment slurry described above was aliquoted into 72ml serum vials within a Coy anaerobic chamber (2.5% H_2) and $^{15}NH_4$ was added from an anoxic 500mM solution to achieve a final concentration of 1mM. Bottles were stoppered with butyl rubber and flushed with methane, then over pressurized to 20psi methane, covered in aluminum foil, and incubated at 7°C. Geochemical analysis of the slurry was conducted by ion chromatography revealing 496 μM NH_4, 321 μM thiosulfate, and 24mM sulfate at the start of the incubation.

Microcosm sampling and embedding Aliquots of the slurry were removed from the incubations at 6, 20, and 64 days and fixed with 2% paraformaldehyde buffered with PBS. Fixation was for 1 hour on ice and followed by three PBS washes accomplished by pelleting the sediment by centrifugation followed by re-suspension (1min, 1000 x g). To separate microbial consortia from sediment matrix, fixed sediment was first diluted into PBS to achieve a final volume of 500μl and sonicated in two 15 second bursts at setting 3 (6V(rms) output power on a Branson Sonifier W-150 ultrasonic cell disruptor) on ice with a sterile remote-tapered microtip probe (Branson) inserted into the liquid.

To the resulting suspension was then added 500μl Percoll at the bottom of the tube, and this mixture was centrifuged at 4°C for 20 min. After centrifugation, the supernatant containing consortia was removed from the tube and the percoll removed from the solution by PBS buffer exchange over a 3μM TSTP filter on a 15 ml filter tower. Finally, the percolled material was concentrated by pipetting approximately 1 ml slowly from a pipet tip onto the 3μm filter in a small area (approximately 2mm diameter). After this, the material was overlaid with molten agar (2% nobel agar in 50mM Hepes pH 7.4 35g/L NaCl). The agar plug was peeled from the filter after solidifying, sliced into small pieces, and embedded in technovit 8100 resin (Heraeus Kulzer GmbH) following the manufacturer's protocol with the exception that ethanol was used rather than acetone for dehydration, and the sucrose infiltration step was omitted. Blocks were cut with a Leica microtome equipped with a glass knife, and 1 micron sections stretched on a water droplet on a polylysine coated slide with teflon wells (Tekdon Inc). The slides were then air dried depositing the sections on the polylysine slide and stored at room temperature until use.

Sulfide measurements The 6, 20 and 64 day time points were assayed for sulfide production using the method of Cline [57]. Sulfide production in the bottle was estimated to be 0.0019mM per day over the sampling period.

Average doubling time of microbial consortia in the microcosm experiments, and rational for using nitrogen isotopes for determining microbial activity At the 20 and 64-day time points, the $^{15}NH_4^+$ containing incubation was sampled to estimate biosynthetic rates within the microcosm. Microbial consortia were separated from the

sediment matrix, and embedded in Technovit for nanoSIMS analysis as described above. To estimate the doubling time of consortia in the incubation, nanoSIMS data were acquired on non-phylogenetically identified consortia at the 20 and 64 day time points and averaged to obtain a specific growth rate, with the assumption that a 50% atom percent increase in ^{15}N would represent one doubling period of the organisms. A specific growth rate of 0.0068/day was calculated, giving a doubling time of approximately 102 days.

There have been numerous estimates of the growth rate of consortia involved in AOM [16, 61, 62, 24, 63, 64]. Variability in these values may stem from differences in the incubation set up (for example temperature or methane partial pressure), as well as geographic, and microbial composition related differences between samples and set ups. In general though, the relative agreement between methods based on counting biomass and isotope incorporation amidst significant sample diversity seems to indicate that all these techniques function as decent indicators of cell growth.

Fluorescence in situ hybridization The phylogenetic identity of microorganisms within consortia within the sample was determined using conventional FISH with the probes described in the table below. FISH hybridization was conducted using standard protocols [60] with percoll separated aggregates immobilized on 3 micron TSTP filters. Visualization via epi-fluorescence was accomplished by mounting FISHed material with a mixture of DAPI-citifluor (5μg DAPI/ml) and imaging with a 60x objective (Olympus).

Hybridization with the ANME-2_538 and DSS member seep1a_1441 probes showed that approximately 11% of the aggregates in the incubation were ANME-2_538:seep1a_1441 pairs. A similar amount (12%) of ANME-2_538 targeted consortia were found to pair with a non seep1a_1441 targeted bacterial partner. 3% of the incubation was found to be seep1a_1441 paired with a non ANME-2_538 targeted archaea or with unidentified cells (non archaeal). FISH with the ANME-2c-760 probe gave a similar results: 12% of ANME-2c consortia were found with DSS-658 hybridized cells (likely seep1a given the above mentioned results), and 9% were with an unidentified partner. 4% of the incubation was ANME-2c paired with a non-EUB probe identified partner. It was concluded that approximately half of the ANME-2c-bacterial consortia exists partnerships with the specific seep1a-DSS group (those hybridized by the ANME-2_538 and ANME-2c-760 probes) and another half with a non-identified partner.

Use of the newly designed ANME-2b_729 probe showed that approximately 14% of the consortia in the incubation were ANME-2b paired with a delta_495 targeted bacterial partner. 3% of the incubation was ANME-2b paired with a non delta_495 targeted partner.

From the above, it was concluded that the ANME-2c and ANME-2b - deltaproteobacterial pairs are prominent aggregate types in the incubation. These were selected for nanoSIMS analysis. Prior to nanoSIMS, FISH with the seep1a-1441, arc915, and delta-495a probes was used to identify seep1a-archaeal pairs (very likely seep1a-ANME-2c pairs given the above results) and archaea paired with deltaproteobacteria (very likely a mixture of ANME-2b and ANME-2c deltaproteobacteria pairs given the above results). Thus, two major groups of organisms were identified for nanoSIMS analysis: the specific seep1a-ANME-2c pairs, and a mixture of ANME-2b/2c paired with an unknown deltaproteobacterial partner targeted with delta_495.

Fish probes used in this study

1. Arc915 [66]
2. Anme2:538 [67]
3. Anme2c:760 [15]
4. Anme2b:729 (This study)
5. Eub_mix [68]
6. d495a and competitor [69] [70]
7. seep1a_1441 [25]

Preparation of FISH hybridized samples for nanoSIMS analysis The 20-day time point sample - where measured sulfide in the incubation was approximately 2.3mM was the subject of detailed analysis by FISH-nanoSIMS. Mapping of FISH stained consortia for nanoSIMS analysis was carried out on thin sectioned aggregates in Technnovit 8100 resin (1 μm thickness) which were mounted onto teflon coated microscope slides. Identification of consortia was made using the arc-915(fitc), anme-1-350(cy3), seep1a-1441(cy3), and delta495 (cy5) probes mixed in a 40% formamide hybridization buffer. After FISH, the coverslip was removed, and the slide was washed gently in DI water. After air drying, the slide was carefully broken into the dimensions of the nanoSIMS holder (filing was necessary for precise fitting) and the sample was gold coated with 30nM gold by sputter coating for conductivity.

nanoSIMS operation Prior to analysis, the areas for analysis were pre-sputtered with a 90pA beam until approximately 15,000cps $^{12}C^{14}N$ were reached at the analytical settings: approximately 0.3pA primary Cs^+ ion beam at (D1=4, ES=2) and a 25 micron raster. At least two image frames were collected for each consortia analyzed. Images were acquired at either 256x256 or 512x512 pixel resolution, depending on the size of the image captured.

^{15}N accumulation by cells was determined from measurement of the $^{12}C^{14}N^-$ and $^{12}C^{15}N^-$ ions.

Transmission Electron Microscopy A modified protocol from Ghineas and Simionescu was followed for heme staining [58, 59, 35]. Fixation was accomplished on ice by mixing one volume sediment slurry with 1/2 volume each of 1) 5 % gluteraldehyde in 25mM Hepes pH 7.4, 17.5 g NaCl and 2) 8% paraformaldehyde in 37.5mM Hepes pH 7.4, 26.25 g/L NaCl to achieve final aldehyde concentrations of 2% paraformaldehyde and 1.25% gluteraldehyde. After fixation, washing was completed by 5x 1ml washes with resuspension and centrifugation (1 minute, 1000 x g) in 50mM Hepes pH 7.4, 35g/L NaCl. The sediment was then percolled to separate cellular aggregates from inorganic particles and embedded in molten nobel agar (see above section "Microcosm sampling and embedding" for more information). A solution of 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO (DAB) at a concentration of 0.0543g DAB/ml was made in 1M HCl by sonication at setting 3 until the powder was dissolved. After sonication, the dissolved DAB solution was added into 50mM Tris HCl pH 8 to achieve a final concentration of 0.0015g DAB/ml buffer. The solution was briefly sonicated again and immediately filtered through a 0.22μm syringe filter. H_2O_2 was added from a 30% aqueous stock to the DAB solution to achieve a final concentration of 0.02%. This H_2O_2/DAB solution was added to agar embedded sediment and incubated for 2.5 hours at room temp on a rocker. A DAB solution without H_2O_2 was added to a separate set of samples for comparison. The DAB solution was removed with 5x 1ml washes with 100mM Hepes pH 7.8.

Next, a 1% OsO_4 solution was made by dilution of a 4% aqueous stock into 100mM Hepes pH 7.8. 1/2 ml of the 1% OsO_4 solution was added to each of the tubes containing the agar embedded sediment samples and this was incubated 90 min on ice. The samples were then washed with 5x 1ml changes of 100mM Hepes pH 8 solution and the samples embedded into LR white by a graded ethanol series (15 minutes each of 25%, 50%, 75%, 100% x 3 times, follwed by 50% LR White Resin, 50% ethanol on a rocker for 30min. The samples were then moved to 100% LR White Resin for 1 hour on a rocker followed by a LR white replacement and placement at 56°C for 2 days for polymermization. The blocks were then sectioned at 200nm and floating sections were mounted on copper grids which had been briefly flamed and rinsed in water. Thin sections were examined and imaged by a FEI Tecnai Spirit TEM operated at 120 kV. Conventional transmission electron microscopy 2K by 2K images were acquired using TVIPS F224 CCD camera.

3 Analysis of NanoSIMS data

Introduction Previous SIMS measurements have not indicated that growth in these consortia was limited to syntrophic partner interfaces [16], however that work was not conducted at sufficient resolution to quantitatively determine activity relationships as was done here. In this work, our goal was to specifically analyze the activity of individual cells as it relates to their surroundings - below we describe how this analysis was performed.

This section covers our workflow for taking a FISH image and a corresponding nanoSIMS isotope map and producing a finalized dataset consisting of cell locations, phylogenetic identities and isotope ratios. This data is then used as a starting point for all downstream spatial analyses. Briefly, this process involves a transformation of the FISH image onto the nanoSIMS image; manual drawing of regions of interest (ROIs) around individual cells on the nanoSIMS image; phylogenetic classification of those cells based on the FISH image; extraction of nanoSIMS isotope information for each ROI; and finally the inverse-transformation of the ROI centroids. Each process is explained in detail below, and **Extended Figure 1** illustrates the process.

ROI isotope data generation The initial processing of all FISH and nanoSIMS data was done in the Matlab program Look@nanoSIMS (LANS), which is designed to read nanoSIMS .im data files, and has a range of analysis tools [48]. In this study, the LANS interface was used for pre-processing the data, and ROI drawing, while the rest of the analysis was left to custom Matlab scripts. Below are the steps conducted in LANS.

First, a nanoSIMS .im file is loaded into LANS, and the planes of data are aligned and combined following the program manual [48]. Next, the "Align External Template Image" option is used to warp the FISH image of the aggregate onto the nanoSIMS image. This process is essential for correct identification of cells on the nanoSIMS image because in the process of acquiring data it is common for the isotope map to appear slightly warped when compared to the corresponding light microscopy image. Warping was accomplished by selecting well-resolved fiducial marker points in side-by-side FISH and nanoSIMS images, and then constructing a transform function from these points. **Extended Figure 1a** and **1b** shows example markers on the FISH and the nanoSIMS image respectively. **Extended Figure 1c** illustrates the resulting warp of the FISH image onto the nanoSIMS image.

To illustrate the necessity for using a transform function instead of manually overlaying the FISH and the nanoSIMS images, **Extended Figure 1d** shows a manual overlay of the original FISH image in yellow, and the transformed FISH image in blue. The effect is slight, but it is clear that the overlay is quite accurate at the top of the image, and off by approximately one cell length at the bottom of the aggregate. This precludes the use

of simple image manipulations such as resizing or rotating to attain an accurate overlay. Also, such manual transformations cannot be accurately inverted, which is an integral part of this analysis (as described later in this section).

With the FISH image transformed onto the nanoSIMS image, regions of interest (ROIs) corresponding to single cells are selected. Each cell in an aggregate is outlined by hand, and this process is exclusive, i.e. no pixel can then be assigned to two different ROIs.

After ROIs were defined the following data was exported for each aggregate. First, a ".mat" file was saved that contained the counts for each isotope of interest (in our case, $^{14}N^{12}C$, $^{15}N^{12}C$, ^{12}C, ^{13}C, ^{31}P, ^{32}S, and ^{34}S). Next, the transformed FISH image, as well as the points that were used to define the transform function were saved. Finally, a file was saved that contains the index number and spatial information for each ROI.

Each ROI needs to be phylogenetically classified based on the information from the FISH image. This is completed in a semi-automated fashion by a custom Matlab script. This script takes a warped FISH image and the ROI data, and for each ROI the average intensity of each channel in the FISH image is calculated, and the phylogenetic identity is assigned automatically based on which channel is most intense over the entirety of the ROI. Occasionally the background fluorescence is such that this automated selection fails for some of the ROIs, and these were then re-assigned phylogenies by hand based on manual inspection of the FISH image.

Extraction of nanoSIMS Data After ROIs have been classified, each ROI is used as a mask to extract the isotope counts contained within it. For each ROI the raw counts for each isotope is stored for each pixel, as well as the average counts across the whole ROI. This data is combined with the ROI classification data and exported.

Inverse Transformation of ROI Spatial Data Much of the subsequent analysis for this study involves the spatial distance between cell centroids. This is a trivial calculation, however the ROIs and their centroids must again be transformed, this time using the inverse of the original transform applied to the FISH image. This is because, as noted above, the nanoSIMS introduces slight, non-linear spatial warping. As a result, for the accurate measurement of distance between centroids in microns, the x,y coordinates of centroids were transformed back into the "FISH space" with the inverse of the original transform **Extended Figure 1f**. Reliable distance calculations can be made in units of microns using our pixel to micron conversion for microscope images.

On the relationship between two-dimensional slices and whole aggregate behavior It has not escaped our attention that the data presented in this paper represents two-dimensional slices of aggregates that are three-dimensional and spheroidal in shape. A potential concern within our empirical observations is that the inferences made from two-dimensional slices could be missing the effects of the layers above and below the slice. However, if strong gradients, consistent with previous diffusive models, existed in the three-dimensional aggregate these should be apparent in the two-dimensional slice for the correlations between various distances and activity. If the three-dimensional structure represented a shell of the two types with strong gradients then any two-dimensional slice would be the same given symmetry. If the geometry were completely random then the surrounding cells should on average match what surrounds a cell in two-dimensions. The basic assumption for any geometry in between these two extremes is that the two-dimensional slice gives representative statistics for the three-dimensional spatial arrangement.

4 Metrics for Degree of Mixing

Introduction ANME-SRB consortia display spatial arrangements that vary substantially even within narrow sediment horizons. The canonical aggregate is the so-called "shell-type", in which an archaeal core is surrounded by a layer of bacteria. Aggregates are also found in the "mixed-type" however, in which the archaea and bacteria are more evenly distributed throughout, with the perfectly mixed end-member resembling a checkerboard of the two species. Consortia fall somewhere along a continuous spectrum of mixing between the perfect shell and the perfect checkerboard morphologies.

It has been proposed in the literature that syntrophic communities optimize their activity by achieving the highest degree of mixing between the partners (references and discussion in the main text). In order to test whether we could detect such a pattern in our unique dataset, we set out to develop a metric that captures the degree of mixing of an aggregate in a single analytical value. An extensive literature search did not reveal anything specifically designed for microbial communities that fit this description. The closest measure we could find is available in the Daime software package [49], which has a spatial arrangement analysis function designed for FISH images. Unfortunately this function outputs a plot that describes how clustering varies with distance. While this is a very useful tool in some instances, it does not satisfy our requirement for a single metric value for the degree of spatial mixing within each consortium.

After our literature search failed to yield an acceptable metric, we designed our own based on statistics originally developed for measuring spatial autocorrelation. For this we assigned an identity value of 1 or 0 to each ROI, then examined how spatially autocorrelated the identity values were over the entire aggregate. Two metrics were developed which approached the problem in slightly different ways, but behaved similarly on both computer-simulated mock aggregates and observed aggregate data (see **Extended Figure 4**). One of the metrics, Moran's I [50], is usually applied to continuous data, while the second, join counting [51], is used for categorical data. Both metrics compare the value of a measurement at a specific location with other nearby measurements. In our case, the measurement is the phylogenetic affiliation of the ROIs in question, and for Moran's I the value of 1 for archaea and 0 for bacteria was arbitrarily chosen.

The formulations of the metrics are shown below. In both cases a weight function must be applied which describes how the neighboring ROIs are weighted in the calculation of the spatial autocorrelation. This weight function can either be a continuous function of distance (decreasing weight with increasing distance), or a function which gives equal

weight to measurements occurring in the predefined neighborhood of the measurement in question, a zero weight to all measurements outside that neighborhood. For the results presented in the main text the common weight function of inverse square of the distance between the measurements was used, although other powers of inverse distance were tested, and they had no effect on the general trends presented in the text (data not shown).

In the following equations, the weight function w_{ij} is always equal to $1/r_{ij}^2$, where r_{ij} is the distance in microns between ROIs i and j. The functions of the form f_{aa} are piecewise functions, which return 1 if the subscript condition is met. For example, if ROIs i and j are an archaea and bacteria, $f_{aa} = f_{bb} = 0$ and $f_{ab} = 1$. The functions J_{aa}, J_{bb} and J_{ab} simply add up all the weights associated with the specific joins, archaea-archaea, bacteria-bacteria, and archaea-bacteria, respectively. Since we need a single value to capture the spatial mixing of partners within an aggregate we combined J_{aa}, J_{bb} and J_{ab} for the calculation of "J". As shown below these sums are normalized to the number of joins of that type (n_a choose 2, etc), and ratio of the average within-species join to the average between-species join is calculated. If this ratio is large, it means that on average the joins within a species are closer, and overall the consortia consists of segregated populations of bacteria and archaea.

$$I = \frac{n}{\sum_{ij} w_{ij}} \frac{\sum_{ij} w_{ij}(x_i - \overline{x})(x_j - \overline{x})}{\sum_i (x_i - \overline{x})^2} \tag{S1}$$

$$J_{aa} = \sum_{ij} w_{ij} f_{aa}(x_i, x_j) \tag{S2}$$

$$J_{bb} = \sum_{ij} w_{ij} f_{bb}(x_i, x_j) \tag{S3}$$

$$J_{ab} = \sum_{ij} w_{ij} f_{ab}(x_i, x_j) \tag{S4}$$

$$\tag{S5}$$

$$J = \frac{\frac{J_{aa}}{\binom{n_a}{2}} + \frac{J_{bb}}{\binom{n_b}{2}}}{2\frac{J_{ab}}{n_a n_b}} \tag{S6}$$

Testing spatial mixing metrics on simulated aggregates The spatial mixing metrics shown above have different ranges of values. Moran's I and the J value approach -1 and 0, respectively, for cases of perfect mixing. In the other extreme, Moran's I and J approach 1 and ∞, respectively, for cases of perfect segregation. For random distributions of cells, Moran's I and J equal 0 and 1, respectively. To make sure the spatial

mixing metrics we developed performed as predicted, we constructed a number of mock aggregates comprised of 8x8 grids where each point was assigned a 1 for archaea or 0 for bacteria. The mock aggregates were made to span the full spectrum from full segregation to perfect mixing. Examples of mock aggregates and their corresponding mixing metrics are shown in **Extended Figure 4a**. The metrics behaved as expected.

Permutation tests for significance of mixing in the ANME-SRB consortia
Methods exist for Moran's I for calculating the statistical significance of spatial autocorrelation, however, since this metric has an expectation of normally distributed continuous data, we were not able to use these significance calculations to determine which aggregates were significantly mixed or segregated beyond what would be expected from random variations.

To address this question we turned to non-parametric statistics and performed 300 permutation tests per AS and AD consortia. In these permutation tests the phylogenetic identifications of the ROIs were randomly redistributed to the (x,y) indices of the ROIs, and Moran's I and J were calculated for all permuted aggregates. If the calculated value of a metric for an aggregate was greater or less than the permuted values more than 95% of the time it was considered to be significantly more or less segregated than random, respectively. **Extended Figure 4b** and **4c** display the results of this test for the AS and AD datasets. The small black dots show the values for the various permutation tests, and the larger colored dots show the actual value for that aggregate. If an aggregate was more segregated than random ($p < 0.05$) it is colored green, if it was more mixed than random ($p < 0.05$) it is colored purple, and if it was not significantly different than the random permutations it is colored red. It is worth noting that the spatial arrangements of the majority of consortia was more segregated than random, whereas there was only a single aggregate in our dataset which was more mixed than random.

5 Spatial Effects on Activity of Single Cells

5.1 Introduction

Our data on the location, relative activity, and identity of syntrophic partners allowed us to address questions pertaining to the controls on single cell anabolic activities. The spatial analyses performed are described in the following sections. To compare patterns of activity across the entire dataset, the z-score of the activities within populations within aggregates are often used instead of the raw activity values. This normalization allowed us to compare the controls on ROI activities between aggregates, and between types, without the confounding effects of overall aggregate activity differences.

5.2 Relationship Between Cell Activity and Distance to Surface

One simple question that we were able to address with our dataset was: how do archaea or bacterial activities vary as a function of distance to the exterior surface of the aggregate? To answer this question each cell that was on the surface of the aggregate was marked by hand, these were assigned 0 microns as their distance to the surface. The rest of the cells were assigned distances to the surface by finding the shortest distance from their centroid to a centroid of a surficial cell. **Extended Figure 8** shows archaea and bacteria ROI z-score activities as a function of distance to the surface of the aggregate for both AS and AD consortia. It is apparent from our data is that there is no significant concentration of above average or below average activity cells of either type near or far from the surface of the aggregate. Note: as with all analyses conducted in this study, we only have a two dimensional slice through a three dimensional body, so all measurements of distance are best approximations.

5.3 Relationship Between Cell Activity and Distance to Partner

In previous modeling studies it was found that the proximity to syntrophic partner was a strong determinant of cellular activity within AOM consortia [13]. We were quite interested to see to what extent this spatial effect was present in our empirical dataset. We plotted ROI activity z-scores vs. the distance to nearest partner for archaeal and bacterial ROIs for AS **Figure 2** and AD **Extended Figure 6**. We also plotted the activity z-scores vs. the average distance to three nearest partners, with similar results (data not shown). Unlike the models previously developed based on diffusible substrates, there appears to be no significant trends in activity with distance to syntrophic partner for either cell type, in either the AS or AD datasets.

5.4 Interfaces in cellular neighborhoods

It is often informative to examine patterns in spatially indexed data by constructing neighborhoods and asking questions about how the values of observations are dependent on the characteristics of their neighborhoods. To this end we applied numerous neighborhood construction algorithms including Delaunay Triangulation; Spheres of Influence; Gabriel Neighbors; Relative Neighbors; and 1, 2, 3 and 4 nearest neighbors. All neighborhoods were constructed by importing x,y coordinates of the ROI centroids into the R statistical package [54]. Neighborhoods were made with the spdep package [52][53]. The choice of neighborhood method is largely arbitrary, and seemed to have little effect on the outcomes of the analyses presented in this study. For the presented data we used the Spheres of Influence neighborhoods, as they seems the most reasonable and free from artifacts by visual inspection (see **Extended Figure 9a**).

As described in the main text, we would predict that there was an enhancement in ^{15}N enrichment for those cellular ROIs which had syntrophic partners in their neighborhoods. To test this effect across the entire dataset, we split each partner into two groups, those at syntrophic interfaces (with a syntrophic partner in their neighborhood) and those without (see **Extended Figure 7c and d** for depiction of a neighborhood and the resulting interfacial cells). For each archaea or bacteria in each aggregate we then conducted a 2-sample t-test to determine whether there was a significant difference between the mean activities of the interfacial or non-interfacial cells. The results of these test are displayed in supplementary tables 2-5. Significance was determined at a $P<0.05$ level, with a Bonferroni correction applied for the multiple comparisons within aggregate types (41 AS and 21 AD). Consortia with significant differences between the interfacial and non-interfacial cells are bolded and underlined. Two observations from this analysis are clear: 1) in very few consortia is there a significant difference in the activity of cells with and without partners in their immediate neighborhood, and 2) the ratio of activities between the interface and non-interfacial cells is nearly 1 in all cases, even those with significant differences in partner activities. Both of these observations contradict the classic assumptions of steep gradients of cellular activity caused by diffusible intermediates.

Aggregate	Total ROIs	Total Interface ROIs	Total Non-interface ROIs	Interface mean activity	Non-Interface mean activity	Interface:Non-interface activitiy ratio	P-value	Pass with Bonferroni Correction
1	218	179	39	0.049	0.046	1.063	0.0443	0
2	11	9	2	0.098	0.090	1.082	0.1353	0
3	17	5	12	0.058	0.057	1.021	0.8509	0
4	91	56	35	0.082	0.081	1.020	0.3328	0
5	93	82	11	0.071	0.069	1.026	0.7219	0
6	68	58	10	0.064	0.054	1.183	0.2266	0
7	61	45	16	0.043	0.038	1.145	0.1402	0
8	6	6	0	0.008	NaN	NaN	NaN	NaN
9	50	28	22	0.073	0.070	1.042	0.0720	0
10	42	29	13	0.072	0.061	1.188	0.0658	0
11	30	26	4	0.078	0.079	0.986	0.7812	0
12	11	11	0	0.051	NaN	NaN	NaN	NaN
13	14	14	0	0.091	NaN	NaN	NaN	NaN
14	12	11	1	0.088	0.078	1.131	NaN	NaN
15	21	21	0	0.087	NaN	NaN	NaN	NaN
16	72	72	0	0.057	NaN	NaN	NaN	NaN
17	37	34	3	0.107	0.101	1.056	0.3044	0
18	13	4	9	0.069	0.057	1.206	0.4364	0
19	14	12	2	0.082	0.097	0.845	0.0725	0
20	18	17	1	0.099	0.105	0.942	NaN	NaN
21	10	5	5	0.085	0.081	1.047	0.6286	0
22	38	35	3	0.081	0.078	1.040	0.3087	0
23	70	63	7	0.113	0.108	1.045	0.2809	0
24	5	4	1	0.084	0.089	0.941	NaN	NaN
25	34	32	2	0.048	0.033	1.441	0.5533	0
26	69	67	2	0.020	0.015	1.381	0.6040	0
27	154	153	1	0.079	0.082	0.966	NaN	NaN
28	13	11	2	0.040	0.036	1.099	0.6055	0
29	31	21	10	0.090	0.075	1.200	0.2236	0
30	120	114	6	0.037	0.036	1.015	0.8316	0
31	18	18	0	0.106	NaN	NaN	NaN	NaN
32	43	36	7	0.095	0.095	1.003	0.9038	0
33	37	36	1	0.068	0.052	1.302	NaN	NaN
34	90	89	1	0.114	0.120	0.950	NaN	NaN
35	44	27	17	0.079	0.079	1.000	0.9868	0
36	**58**	**43**	**15**	**0.112**	**0.103**	**1.095**	**0.0006**	**1**
37	38	35	3	0.053	0.055	0.968	0.5090	0
38	30	24	6	0.045	0.052	0.868	0.4419	0
39	103	90	13	0.058	0.055	1.054	0.5913	0
40	44	39	5	0.084	0.086	0.983	0.3668	0
41	19	14	5	0.101	0.098	1.032	0.3972	0

Table S1: AS Consortia: Archaea Interface vs. Non-Interface Activity Comparison

Aggregate	Total ROIs	Total Interface ROIs	Total Non-interface ROIs	Interface mean activity	Non-Interface mean activity	Interface:Non-interface activitiy ratio	P-value	Pass with Bonferroni Correction
1	**203**	**168**	**35**	**0.064**	**0.053**	**1.209**	**0.0001**	**1**
2	36	21	15	0.113	0.122	0.926	0.0297	0
3	11	4	7	0.148	0.137	1.079	0.2532	0
4	71	54	17	0.115	0.114	1.009	0.8041	0
5	88	77	11	0.070	0.055	1.278	0.0053	0
6	60	52	8	0.071	0.069	1.028	0.7875	0
7	76	46	30	0.085	0.086	0.993	0.8771	0
8	21	10	11	0.013	0.012	1.047	0.7600	0
9	**24**	**22**	**2**	**0.093**	**0.084**	**1.102**	**0.0002**	**1**
10	55	36	19	0.102	0.106	0.961	0.3770	0
11	29	27	2	0.068	0.054	1.255	0.7180	0
12	13	11	2	0.058	0.044	1.297	0.1469	0
13	21	14	7	0.107	0.114	0.943	0.0233	0
14	22	14	8	0.097	0.075	1.293	0.1447	0
15	45	25	20	0.091	0.087	1.040	0.2170	0
16	106	100	6	0.044	0.041	1.080	0.3318	0
17	25	24	1	0.115	0.062	1.860	NaN	NaN
18	11	5	6	0.134	0.135	0.990	0.8599	0
19	12	12	0	0.081	NaN	NaN	NaN	NaN
20	15	15	0	0.093	NaN	NaN	NaN	NaN
21	11	7	4	0.147	0.168	0.871	0.1118	0
22	34	32	2	0.097	0.084	1.152	0.0283	0
23	65	60	5	0.116	0.093	1.245	0.1118	0
24	10	6	4	0.064	0.063	1.017	0.9069	0
25	39	30	9	0.064	0.063	1.002	0.9841	0
26	78	75	3	0.033	0.027	1.247	0.4740	0
27	212	194	18	0.082	0.052	1.578	0.0025	0
28	33	14	19	0.060	0.064	0.933	0.6379	0
29	11	11	0	0.130	NaN	NaN	NaN	NaN
30	101	98	3	0.033	0.036	0.907	0.4406	0
31	11	11	0	0.131	NaN	NaN	NaN	NaN
32	42	36	6	0.086	0.070	1.225	0.0112	0
33	**54**	**40**	**14**	**0.060**	**0.053**	**1.136**	**0.0008**	**1**
34	100	97	3	0.107	0.112	0.957	0.0895	0
35	25	20	5	0.086	0.091	0.950	0.1769	0
36	61	50	11	0.122	0.105	1.167	0.1025	0
37	50	39	11	0.084	0.084	1.000	0.9897	0
38	49	35	14	0.076	0.063	1.204	0.0987	0
39	99	86	13	0.065	0.059	1.098	0.4257	0
40	28	28	0	0.080	NaN	NaN	NaN	NaN
41	6	6	0	0.130	NaN	NaN	NaN	NaN

Table S2: AS Consortia: Bacteria (Seep SRB 1a) Interface vs. Non-Interface Activity Comparison

Aggregate	Total ROIs	Total Interface ROIs	Total Non-interface ROIs	Interface mean activity	Non-Interface mean activity	Interface:Non-interface activitiy ratio	P-value	Pass with Bonferroni Correction
1	66	25	41	0.041	0.042	0.974	0.7713	0
2	41	34	7	0.052	0.037	1.411	0.1061	0
3	45	15	30	0.060	0.078	0.776	0.0144	0
4	24	20	4	0.050	0.033	1.540	0.0467	0
5	56	50	6	0.070	0.062	1.130	0.3636	0
6	27	19	8	0.067	0.069	0.970	0.5758	0
7	**93**	**74**	**19**	**0.051**	**0.029**	**1.749**	**0.0002**	**1**
8	4	2	2	0.092	0.085	1.080	0.646	0
9	28	28	0	0.026	NaN	NaN	NaN	NaN
10	4	4	0	0.117	NaN	NaN	NaN	NaN
11	7	7	0	0.073	NaN	NaN	NaN	NaN
12	21	16	5	0.007	0.005	1.326	0.0718	0
13	35	31	4	0.040	0.052	0.756	0.3512	0
14	21	19	2	0.027	0.037	0.742	0.1254	0
15	19	19	0	0.031	NaN	NaN	NaN	NaN
16	27	8	19	0.027	0.043	0.629	0.0726	0
17	10	7	3	0.120	0.122	0.984	0.6861	0
18	12	7	5	0.004	0.004	1.034	0.7902	0
19	15	14	1	0.073	0.056	1.301	NaN	NaN
20	94	28	66	0.055	0.055	1.001	0.9877	0
21	9	4	5	0.121	0.101	1.203	0.7292	0

Table S3: AD Consortia: Archaea Interface vs. Non-Interface Activity Comparison

Aggregate	Total ROIs	Total Interface ROIs	Total Non-interface ROIs	Interface mean activity	Non-Interface mean activity	Interface:Non-interface activitiy ratio	P-value	Pass with Bonferroni Correction
1	65	23	42	0.048	0.049	0.987	0.8216	0
2	40	39	1	0.056	0.084	0.672	NaN	NaN
3	28	12	16	0.047	0.048	0.965	0.4372	0
4	11	11	0	0.062	NaN	NaN	NaN	NaN
5	47	44	3	0.052	0.034	1.510	0.2217	0
6	54	17	37	0.061	0.062	0.989	0.6446	0
7	70	59	11	0.086	0.077	1.116	0.1846	0
8	7	4	3	0.062	0.073	0.841	0.532	0
9	53	45	8	0.036	0.034	1.054	0.5196	0
10	20	8	12	0.097	0.101	0.964	0.7594	0
11	16	6	10	0.075	0.074	1.009	0.7576	0
12	15	15	0	0.006	NaN	NaN	NaN	NaN
13	41	30	11	0.063	0.065	0.967	0.771	0
14	36	28	8	0.011	0.015	0.760	0.3623	0
15	26	26	0	0.039	NaN	NaN	NaN	NaN
16	35	8	27	0.051	0.053	0.971	0.2181	0
17	5	4	1	0.119	0.118	1.007	NaN	NaN
18	48	9	39	0.004	0.004	0.956	0.2593	0
19	58	28	30	0.073	0.069	1.069	0.0237	0
20	58	21	37	0.054	0.044	1.243	0.0246	0
21	32	6	26	0.056	0.060	0.932	0.5416	0

Table S4: AD Consortia: Bacteria (deltaproteobacteria) Interface vs. Non-Interface Activity Comparison

6 Genomic Evidence for Direct Electron Transfer in ANME-2 Archaea

6.1 Distribution of putative multiheme cytochromes in microbial genomes

Organisms which conduct direct electron transfer as part of their energy metabolism often contain an abundance of multiheme cytochrome c (MHC) proteins encoded in their genomes. Heme-binding motifs in cytochrome c proteins are most often CxxCH; the two cysteines forming covalent bonds to the porphyrin ring and the histidine acts as an axial ligand for the iron atom. The presence of a CxxCH motif is often taken as very strong evidence for heme binding, although ultimately biochemical characterization of a protein is necessary to make a definitive conclusion.

The genomes of known metal oxide and anode reducing organisms often encode MHCs with 10 or more CxxCH domains, which function as molecular wires for the export of electrons across the insulating outer cell membranes [33]. To perform this function the protein must position its heme groups within around 14 angstroms of one another to allow for rapid electron tunneling between the iron atoms [65]. While there are other proposed mechanisms for direct electron transfer, such as conductive pili in Geobacter species, these are not straightforward to predict from genome sequences, so we have not included this mechanism in our exploration of available genomes.

To determine if there were any MHCs present in the two publically available ANME genomes we downloaded all predicted proteins from the IMG website (taxon IDs 2515154041 and 2565956544 for ANME-2d and ANME-2a, respectively), as well as proteins from the ANME-1 metagenome [29]. We have also included an ANME-2b metagenomic bin from methane seep sediments from Hydrate Ridge (GenBank accession KR811028). CxxCH domains were counted for each protein. For comparison, all sequenced archaeal genomes on the NCBI's ftp site were retrieved as well (ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/archaea/. 256 genomes retrieved in September 2014 and their largest putative multiheme cytochromes were tabulated using a custom python script. A description of the CxxCH motif identifying script can be found on github along with the python code at: `https://github.com/gchadwick/cxxch_counter/`.

Below is a list of genomes and accession of the archaea and bacteria that appear in figure 3b: ANME_2a: 2565956544, *Ferroglobus placidus*: 646564534, ANME_2d: 2515154041, *Geobacter sulfurreducens* PCA:637000120, *Geobacter metallireducens* GS-15: 637000119, ANME_2b: KR811028, *Pyrolobus fumarii* 1A: 2505679005, *Shewanella putrefaciens* CN-32: 2524023073, *Shewanella oneidensis* MR-1: 637000258, *Archaeoglobus veneficus* SNP6: 2504136002, *Methanolobus tindarius* DSM 2278: 2515075008, ANME-1 Meyerdierks: FP565147, *Ignicoccus hospitalis* KIN4/I: 640753029, *Methanohalophilus mahii* SLP: 646564550, *Methanococ-*

coides burtonii: 637000161, *Pyrobaculum calidifontis* JCM 11548: 640069326, *Hyperthermus butylicus*: 640069314, *Pyrobaculum oguniense* TE7: 2512047039, *Pyrobaculum sp.* 1860: 2511231117, *Methanohalobium evestigatum* Z-7303: 648028039, *Thermoplasmatales archaeon* SCGC AB-540-F20: 2517572172, *Methanomethylovorans hollandica*: 2509601008, *Methanosalsum zhilinae* WeN5: 2502790017, *Methanolobus psychrophilus* R15: 2519103099, *Methanosarcina acetivorans* C2A: 638154508, *Methanosarcina mazei* Go1: 638154509, *Methanofollis liminatans* GKZPZ: 2506783068, *Archaeoglobus fulgidus* 7324: 2528311132, *Methanobrevibacter smithii* PS: 640427121, *Picrophilus torridus*: 638154512, *Natronobacterium gregoryi* SP2: 2529293212, *Archaeoglobus sulfaticallidus* PM70-1: 2522125074, *Pyrococcus abyssi* GE5: 638154514, *Pyrococcus sp.* ST04: 2521172719, *Halorhabdus tiamatea* SARL4B: 2562617191, *Archaeoglobus profundus* Av18: 646311906.

6.2 ANME-2 multiheme cytochrome proteins with putative s-layer domains

The presence of MHC proteins encoded in ANME genomes supports the idea that an important part of their physiology may involve direct electronic contact with the extracellular environment. As noted in the main text, ANME-2a, ANME-2b and ANME-2d all encoded MHC proteins with predicted s-layer domains, which the authors find highly suggestive of electron transport to (or from) the extracellular environment. The s-layer domains were predicted by subjecting the putative MHC sequences to conserved domain searches against the NCBI's Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [55]. Each s-layer domain contains a heme-binding motif in the middle of the domain (see **Figure 3**). In addition, the CDD search identified a short putative PGF-pre-PGF which could suggest interactions with extracellular protein modification systems such as archaeosortase.

7 Modeling Overview

Within the AOM literature there are a variety of proposed mechanisms (reviewed in section 1) describing the coexistence of archaea and bacteria within spatially structured consortia. Hypothesized mechanisms range from obligate syntrophy [10, 11, 13, 12] to commensalism [20]. At present the proposed mechanisms can be divided into two categories, the diffusible exchange of chemical intermediates, and direct electron transfer, where the range of specific interactions and dependencies between the two partners is diverse [10, 11, 13, 12, 42, 43]. We developed two simplified classes of modeling frameworks to address the essential spatial relationships arising from these two classes of interaction where our goal is to broadly compare modeling results with our empirical observations in order to infer the types of mechanistic processes that could be at play. The first modeling framework is based on the diffusive exchange of chemicals and the second is reliant on direct electron exchange. In both cases we have idealized the physics and physiology in order to focus on the general features of different types of models and to avoid complications related to unknown physical mechanisms or biological processes. In general, our modeling efforts illustrate that fast transport relative to growth rate is the dominant feature in achieving spatial activity relationships that match our empirical findings.

7.1 Diffusive chemical exchange

The most well-studied set of AOM models is the scenario where archaea and bacteria each perform half of a metabolic reaction with the diffusive exchange of a chemical intermediate between the two types, where the intermediate may also be present in the environment. Previous work [13, 12] shows that this co-metabolism will only work if the intermediate can be kept in a regime where both partner reactions are thermodynamically favorable and this will depend on the chemical concentrations within the environment and rates of metabolic activity for each cell type. The recent work of Milucka et al. [20] would be a subset of this scenario where the archaea are either independent from bacteria or are only weakly benefitted by the bacterial activity. In general, for single-cell organisms living in close proximity there are a variety of more complicated interactions arising from a potential array of metabolites that could serve as interspecies toxins or nutrients. However, our main interest is in the spatial patterns in cellular activity that are likely to arise from interspecies interaction via a diffusible intermediate.

To capture the most likely set of interactions we employ a model where a diffusive intermediate is consumed by bacteria and produced by archaea, where each cell type's ability to produce or consume this intermediate will shut down above (archaea) or below (bacteria) a given threshold concentration, and where the environment contains some background concentration of the intermediate. Mathematically this model can be written,

for the simplest cases, as

$$\frac{\partial C}{\partial t} = D_c \nabla^2 C + p\left(\mathbf{x}\right) \mu_A\left(C\right) - c\left(\mathbf{x}\right) \mu_B\left(C\right) \tag{S7}$$

where C is the intermediate concentration, $p\left(\mathbf{x}\right)$ and $c\left(\mathbf{x}\right)$ are, respectively, production and consumption proportionalities based on the growth rates of archaea, μ_A, and bacteria, μ_B. The production and consumption rates depend on whether point in space is occupied by archaea or bacteria:

$$p\left(\mathbf{x}\right) = \begin{cases} p & : \text{point occupied by archaea} \\ 0 & : \text{point occupied by bacteria} \end{cases}$$

$$c\left(\mathbf{x}\right) = \begin{cases} c & : \text{point occupied by bacteria} \\ 0 & : \text{point occupied byarchaea} \end{cases}$$

We chose growth rate functions to be steep responses curves that act as "switches" in agreement with the previous efforts [13, 12]:

$$\mu_A\left(C\right) = \mu_{Amax}\left(1 - \frac{1}{1 + e^{-\gamma\left(C - C_{amax}\right)}}\right) \tag{S8}$$

$$\mu_B\left(C\right) = \mu_{Bmax}\frac{1}{1 + e^{-\gamma\left(C - C_{bmin}\right)}} \tag{S9}$$

where γ is a parameter that adjusts how steep the growth response is (typically this is picked to be fairly large) and C_{amax} and C_{bmin} are the archaea and bacteria maximum and minimum concentrations.

We solved these equations for the steady-state aggregates of various spatial arrangements representative of the observed mixing geometries for the two cell types. For these solutions we enforced a fixed boundary concentration of the intermediate set between the two threshold concentrations where both cell types are equally suited for growth.

A key feature of this system is the relationship between maximum growth rate and diffusivity which can be seen more easily by non-dimensionalizing the system (shown here in one dimension for simplicity):

$$\frac{\partial C^*}{\partial t^*} = \frac{\partial^2 C^*}{\partial x^{*2}} + \zeta f_A\left(C^*\right) - f_B\left(C^*\right) \tag{S10}$$

where

$$\zeta = \frac{p\mu_{Amax}}{c\mu_{Bmax}} \tag{S11}$$

the intermediate concentration has been rescaled as $C^* = C/C_{amax}$, the spatial dimensions have been rescaled as $x^* = x_{fac}x$ with

$$x_{fac} = \left(\frac{c\mu_{Bmax}}{C_{amax}D}\right)^{1/2} \tag{S12}$$

and the temporal dimension has been rescaled $t^* = t_{fac}x$ with

$$t_{fac} = \frac{c\mu_{Bmax}}{C_{amax}}. \tag{S13}$$

From x_{fac} it can be seen that all of the spatial scales will depend on the ratio of the maximum consumption activity, $c\mu_{Bmax}$, to the diffusivity of the intermediate times the typical concentration. Given this dependence on the relative activity and diffusivity our modeling efforts focused on comparing both the effect of aggregate geometry and also the relative diffusive regime of the system which we define as variations in x_{fac}. For the constants involved in the equations above we have the following: from this study the cellular radius is roughly 0.5×10^{-6} m^3 which we used to calculate a cellular density of $a = 4.8 \times 10^5$ g m^{-3} using the conversion between volume and mass found in reference [45]. Given a yield coefficient, Y, for the intermediate we can find the effective consumption coefficient as $c = a/Y$. The yield coefficient for growth on sulfate reacting with hydrogen has been shown to be 2 g cell mol sulfate^{-1} [44] which implies that the yield coefficient for hydrogen should be $Y = 0.5$ g cell mol hydrogen^{-1} and thus $c = 9.6 \times 10^5$ mol m^{-3}. The maximum growth rates of these organisms are not well-characterized, but for the purposes of calculating an effective x_{fac} we used the observed growth rates of $\mu = 7.9 \times 10^{-8}$ s^{-1}. The diffusivity was taken to be the value for hydrogen which is the fastest value for a chemical intermediate $D = 2.4 \times 10^{-9}$ m^2 s^{-1} [13]. For hydrogen an environmental value that is equally favorable for both types of cells would be $C_{env} \approx 3 \times 10^{-7}$ mol m^{-3} [13]. Taking these values together gives $x_{fac} = 1.0 \times 10^7$ m. It is important to note that since we are simulating these dynamics in two dimensions the effective diffusivities will be higher. Again our goal here is not to compare the effects of specific parameter values but rather to explore the types of spatial patterning that arise in these systems across a range of ratios of transport to metabolic activity and as it can be seen in Extended Figure 2 we cover the appropriate range of possibilities: a transition from low relative diffusivity where geometry strongly affects activity to high relative diffusivity where geometry matter little. The underlying physiological parameters are often unknown or poorly quantified at present and we provide these values mostly as reference and to give a sense of rates and scales. We varied x_{fac} from 1.7×10^4 to 1.7×10^7 which covers a wide range of combinations of the specific chemical intermediate being used, the environmental concentrations of this intermediate, the cellular activities, and chemical diffusivities. Please note that the largest values of x_{fac} represent the slowest relative diffusivity (left end of **Extended Figure 2**) because here the numerator of x_{fac} (capturing activity rates) is large compared to the denominator (capturing diffusive transport). In our models we take $\zeta = 1$ for simplicity. To model a sharp thermodynamic cutoff we set $\gamma = 50$, and we typically take $C_{amax} = 1.1 \times C_{mid}$ and $C_{bmin} = 0.9 \times C_{mid}$ where C_{mid} is the environmental concentration chosen to be in the middle of the thermodynamic range.

For the results presented in **Extended Figure 2** we simulated a variety of aggregate geometries covering a range of cellular mixing coefficients and spatial structures for a

variety of diffusive regimes. We modeled all aggregate dynamics within a two dimensional slice. We find that the total activity of aggregates depends strongly on the overall diffusive regime. When diffusion is slow compared with the biological reaction rates then the overall aggregate activity is low compared with a relatively fast diffusive regime. The overall diffusive regime also determines how strongly aggregate activity is associated with the mixing and geometry of the two cell types. When diffusion is relatively slow there is a steep drop in activity for aggregates with increasing amounts of segregation as illustrated in **Extended Figure 2a** which gives activity as a function of the J metric for mixing. However, it is interesting to note that despite these differences in total activity, in all of the diffusive regimes archaea activity is positively correlated with bacterial activity although there is much more deviation in the fast diffusive regimes where the cells all have nearly identical activity levels (**Extended Figure 2b**). These results suggest that the correlation between archaea and bacterial activity may be achievable under a wide range of environmental and cellular interaction regimes, and this is further supported by our modeling of direct electron transfer as described below. The correlation between the activity of individual cells and their nearest partner is also strongly effected by the diffusive regime where fast diffusion minimizes the connection between partner distances and activity (**Extended Figure 2c**).

From the diffusive perspective it is scenarios of fast exchange relative to biological rates that most strongly resemble our empirical observations. However, previous work shows that for the chemical concentrations observed *in situ*, and for a variety of likely chemical intermediates, the estimated biological rates and diffusivities will produce strong spatial gradients [13, 12] inconsistent with the fast end of our diffusive regime. However, examining our analysis of x_{fac} above, the fast end of the diffusive regime considered here could be achieved if slower effective activity rates were observed ($c\mu_{max}$), or if it were possible to increase the environmental concentrations while still meeting the necessary thermodynamic constraints (e.g. increasing C_{amax}). For example, taking a lower effective density of $a = 12,000$ g m^{-3} [56] would put x_{fac} closer to the middle of our modeled range.

Finally, it is interesting to note that as relative diffusivity increases the correlation between cellular activity and the distance to the surface is enhanced (**Extended Figure 2d**). This is a subtlety owing to the importance of the environmental concentrations and dominated by the cells that are in direct contact with the environment. It should also be noted that previous efforts of three dimensional aggregates using best guesses for the environmental concentrations and physiology demonstrate very steep spatial gradients in cellular activity with strongly mixed aggregates having higher activity [13].

In order to compare these results to the recent work of Milucka et al. [20] we constructed a modified version of our diffusive model where there is no feedback on the activity of the archaea. The results for the activity of bacteria in this scenario are similar to the full interdependent case in that bacteria will greatly depend on the distance to the partner except in cases of very fast relative diffusion. The thermodynamic range (range

of intermediate concentrations at which bacteria and archaea both have a thermodynamically favorable reaction) is an important parameter of this system which we have not yet discussed. We have set this range to be fairly narrow which is supported by previous considerations of various diffusible chemical intermediates [13, 12]. However the recent work of Milucka et al. proposes a mechanism of interaction with an intermediate that is thermodynamically favorable for the entire range of feasible chemical concentrations [20]. In the context of the intermediate then, the archaea should have saturated growth from an energetic perspective and this could eliminate the appearance of spatial gradients, but this condition would also imply that cells are either 1.) growing at their maximum rate and thus minor concentration gradients in the intermediate do not matter because the cells do not have extra capacity for growth or 2.) are limited by some other resource in which case we should see some gradient in this resource along with activity gradients. For scenario 1, we do not think that the observed growth rates in our system represent maximal rates for these cells.

It should be noted that individual cellular growth rates often vary in a population even when all of the cells are experiencing very similar nutrient conditions (e.g. [72, 71, 45]) and it is important to consider whether cell-level metabolic differences could disrupt the dependence of aggregate activity on geometry in systems with low relative diffusivity. We tested this by assigning a random activity multiplier to the growth rates μ for each individual cell. We observed that in the situations where the relative diffusivity implies strong geometric dependence (e.g. strong decrease in activity with increasing J) that these randomizations are not enough to disrupt these dependencies. We test this using several ranges from which the random multiplier could be chosen: $0.24 - 1$; $0.49 - 1$; $0.74 - 1$; and $0.99 - 1$. There was a slight decrease in geometric dependencies for larger ranges of the multiplier but these scenarios still demonstrated a strong dependence between J and total activity. The fact that this randomization does not eliminate the strong dependence between geometry and activity also addresses concerns about possible contributions from the layers above and below the slice by considering these as a random increase or decrease to the activities controlled by the cells modeled in the plane.

7.2 Conceptual Model for Interspecies Direct Electron Transfer

Here again our goal is to understand how a class of mechanisms contributes to the overall interaction and spatial activity patterns of archaea and bacteria. Our model treats the consortia of archaea and bacteria as being embedded within a continuously conductive material where electrons are able to flow and be produced or consumed by archaea or bacteria respectively. The dynamics of individual electron movement at the spatial scales of single proteins or cell lengths is admittedly complex and different mechanistic descriptions may be the most appropriate depending on the specific scenario. For example previous efforts have shown that the direct electron transfer between two cells can vary from nanowire like structures to extracellular conductive materials (e.g. [42, 43]). It is

unclear how these various mechanisms may change the mechanistic description of the flow of electrons for different intercellular spatial scales, or different types of electron exchange. For example, even the specific structure of a nano-wire may dramatically effect the appropriate mathematical description for electron flow. Detailed work at the intercellular and aggregate scales is needed to fully understand electron flow dynamics and is beyond the scope of this study. Our goal in this first attempt to infer mechanism from the spatial pattern of AOM cellular activity is to capture the key differences between direct electron transfer and diffusive processes. In our treatment the essential difference is that electron flow responds to the global electric potential rather than solely to the local gradient as would be the case for diffusion.

We model the dynamics of charge density, ρ, via the flow, consumption, and production of electrons as

$$\frac{\partial \rho}{\partial t} = -\sigma \nabla \cdot \mathbf{E}(\mathbf{r}) + p(\mathbf{r}) f_A(\nabla \cdot \mathbf{E}(\mathbf{r})) - c(\mathbf{r}) f_B(\nabla \cdot \mathbf{E}(\mathbf{r})) \tag{S14}$$

where the consumption and production terms are again given by

$$p(\mathbf{r}) = \begin{cases} p & : \text{point occupied by archaea} \\ 0 & : \text{point occupied by bacteria} \end{cases}$$

$$c(\mathbf{r}) = \begin{cases} c & : \text{point occupied by bacteria} \\ 0 & : \text{point occupied by archaea.} \end{cases}$$

σ is the conductivity, and $\mathbf{E}$ is the electric field given by

$$\mathbf{E}(\mathbf{r}) = \frac{1}{4\pi\epsilon_0} \int \rho(\mathbf{r}') \frac{\mathbf{r} - \mathbf{r}'}{|\mathbf{r} - \mathbf{r}'|^3} d\mathbf{r}', \tag{S15}$$

where ϵ_0 is the permativity in free space. The functions $f_A(\mathbf{E})$ and $f_B(\mathbf{E})$ represent the response of metabolic activity to the electric potential at a given point in space and could take on a variety of forms based on the mechanisms of electron exchange (e.g. [46, 47]). p and c here should each be the maximum charge density production or consumption rate that a cell is capable of. For f_A and f_B we chose Monod kinetics and modeled our system in arbitrary units for a system that is representative of the above equations in a regime where we observe that the spatial dependencies shift from a strong dependency on cell-type mixing to a weak dependency. We simulated the dynamics of in a two dimensional slice (**Extended Figure 10**) along various conductive regimes defined by the ratio of p to σ.

Here we find that the total activities of aggregates are much more constant across the different conductive regimes and that the connection between mixing geometry and total activity is also diminished especially in the relatively high conductivity regime (**Extended Figure 10a**). The correlation between archaeal and bacterial activity is more tightly coupled across all conductive regimes (**Extended Figure 10b**). Finally, and in most

contrast to the diffusive scenarios, the dependence of cellular activity on distances to either the surface or the nearest partner are greatly diminished especially for cases with high relative conductivity (**Extended Figure 10c-d**). The simulations for the high conductivity regime agree with all of our empirical findings for whole aggregate and single cell activity patterning. It should be noted that we have not assigned values to the constants in this system and are using a representative model, and thus, it is possible that the top end of the conductivity range is well below realistic scenarios. Our aim is to show the types of spatial patterns that arise from different mechanisms and to emphasize that fast metabolic exchange between the two cell types relative to cellular metabolic activities is the key for matching our empirical results. However, we can roughly quantify the conductive regime by estimating the rate of change in charge density between two cells and comparing this with expected rates of charge density production. Considering $\frac{\sigma q}{4\pi\epsilon_0 r^3}$, where r is the separation between cells and q is the number of free electrons at one of the cells, we take a separation of 1 micron, a charge difference of only a single electron, and a conductivity range from a previous study of $\sigma \approx 10^{-9}$ to 10^{-1} A V^{-1} m^{-1} [74], to find an expected range of $\partial\rho/\partial t \approx 1.43$ to 1.43×10^8 C s^{-1} m^{-3}. To give a sense of how large this rate is we use the methane oxidation rate to estimate the charge production rate as $\frac{8\mu a N_A e}{Y_{CH4}}$ where 8 is the number of electrons for each methane oxidized, e is the electron charge, N_A is Avogadros number, and Y_{CH4} is the growth yield for methane. Taking $Y_{CH4} = 0.6$ g cell (mol CH4 oxidized)$^{-1}$ [44], and using a and μ from above, gives a rate of 4.8×10^4 C s^{-1} m^{-3} which is below most of the range described above, which is already likely to be underestimate given that we used a potential difference of only one electron. Using the methane oxidation rates from [44] of 5.8×10^{-8} mol CH_4 s^{-1} (g cell)$^{-1}$ would give a similar rate of 2.1×10^4 C s^{-1} m^{-3}.

It may seem counterintuitive in the low conductivity regime that activity increases for more spatial organization, and separation, of the two cell types. This result occurs because the flow of electrons depends on the global electric field. When the two cell types are highly mixed the overall electric field is more random and less organized compared to when the two cell types are segregated into distinct regions. As an analogy, this result is similar to effects that the degree of organization of individual dipoles in a magnet has on the overall strength of the magnetic field.

Finally, it should be noted that the dynamics described in several earlier papers for direct electron exchange would behave diffusively [28, 73, 27], although the diffusivity should be far higher than for a chemical intermediate and this mechanism could also match our results [27]. For example, it has been estimated that the effective diffusivity in a conductive filament network would be $D_E = 3.5 \times 10^{-7}$ m^2 s^{-1} [28] which is two orders of magnitude larger than the diffusivity for hydrogen and would occupy the upper end of our diffusive spectrum given the same biological rates.

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