SUPPORTING INFORMATION

Enrichment of ANME-2 dominated anaerobic methanotrophy from cold seep sediment in an external ultrafiltration membrane bioreactor

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Methods

Mode of MBR operation

Prior to inoculation, the MBR was left under nitrogen gas for one week to check the gas leakages and then sediment was transferred anaerobically to the MBR. After inoculation, the MBR was bubbled continuously with methane gas (99.9 % CH_4, Linde gas, Schiedam, the Netherlands), stored in a gas canister and supplied to the MBR using a thermal mass flow controller. Methane supply was maintained at ~3 mL min^{-1} during the first week of MBR operation in order to provide excess methane to the reactor and to flush out the residual nitrogen used during inoculation. After that step, methane was bubbled at a constant rate of 0.5 mL min^{-1} and the reactor was operated at atmospheric pressure and a temperature of 22 (± 3) ^{o}C throughout the experimental period. Anaerobic conditions were maintained during the MBR operation, which was evidenced by the colorless redox indicator present in the medium. The artificial seawater medium was continuously recirculated in the MBR and passed from the bottom of the bioreactor to the ultrafiltration membrane and back to the bioreactor at the rate of 20 mL min^{-1}. The mineral medium was intermittently fed to MBR. During the start-up period, mineral medium was intermittently fed to the MBR to minimize the contamination and in a later period (especially after 150 days) to reduce the sulfide toxicity (Fig. 2). The key strategy for media replenishment was based on the sulfide concentration in MBR, i.e. when the sulfide concentration reached 4 mM, then effluent was extracted and new medium was fed to the reactor.

The turbidity of the effluent tank was regularly monitored and 5 mL effluent was centrifuged every 15 days at 16 g in a microcentrifuge (ThermoFisher Scientific) as well as visualized under a stereomicroscope (Olympus, Zoeterwoude, the Netherlands). However, no pellet in the centrifuged tube and no cells in the effluent were observed, showing that the ultrafiltration membrane effectively retained the biomass in the MBR.

Calculations

The volumetric sulfate consumption and the total dissolved sulfide production rates during MBR operation were calculated as described in Eqs. I and II, respectively [1]:

$$\text{Volumetric sulfate reduction rate (mM d}^{-1}) = \frac{[SO_{4\,(t)}^{2-}]-[SO_{4\,(t+\Delta t)}^{2-}]}{\Delta t} \qquad \text{(Eq. I)}$$

$$\text{Volumetric sulfide production rate (mM d}^{-1}) = \frac{S_{(t+\Delta t)}^{2-}-S_{t}^{2-}}{\Delta t} \qquad \text{(Eq. II)}$$

Where, $SO_{4\,(t)}^{2-}$ is the concentration (mM) of sulfate (SO_4^{2-}) at time (t);

$SO_{4\,(t+\Delta t)}^{2-}$ is the concentration (mM) of SO_4^{2-} at time (t+Δt);

$S_{(t)}^{2-}$ is the total dissolved sulfide concentration (mM) at time (t) and

$S_{(t+\Delta t)}^{2-}$ is the total dissolved sulfide concentration (mM) at (t+Δt).

The gradient of the slope when the sulfate/total dissolved sulfide concentration was maximum during the activity assay and MBR operation was considered for the calculation of the maximum volumetric rate.

The methane oxidation rate was estimated on the basis of the total dissolved inorganic carbon (DIC) produced during the activity assay incubation as described by Scheller et al. [2]. The amount of DIC formed at time n was indicated as $\Delta[DIC](t_n)$ and calculated from the measured ^{13}F (fractional abundance of ^{13}C) by neglecting the isotopic effects on AOM (Eq. III):

$$\Delta[DIC](t_n) \text{ in mM} = [DIC](t_0) \times \frac{^{13}F(t_n) - {}^{13}F(t_0)}{(^{13}F(CH_4) - {}^{13}F(t_n))} \qquad \text{(Eq. III)}$$

Where, [DIC] is the sum of carbonate, bicarbonate and CO_2 in mM and $[DIC](t_0)$ is the initial concentration of the DIC in the incubations (i.e. 30 mM), $^{13}F(t_n)$ is the ^{13}F fraction of DIC at time n of incubation, $^{13}F(t_0)$ is the ^{13}F fraction of DIC at time 0 of incubation and $^{13}F(CH_4)$ is the ^{13}C fraction of methane at time 0.

For each incubation, the methane oxidation rate per volume of bioreactor suspension was estimated by linear regression with 95% confidence intervals and the estimated $\Delta[DIC](t_n)$ was plotted as derived from the regression equation. The total amount of DIC was estimated according to Eq. IV:

$$\text{Amount of DIC per vial } (\mu\text{mol}) = \Delta[DIC](t_n) \times 60 \text{ ml} \qquad \text{(Eq. IV)}$$

The AOM rate on the basis of DIC production (μmol d^{-1}) was obtained from the slope gradient of the plot of $\Delta[DIC](t_n)$ versus time and then estimated on the basis of gram dry weight of biomass in each vial.

Fluorescence in situ hybridization (FISH) analysis

Sediment samples obtained from inoculated to BTF were analyzed by FISH . About 0.5 mg of the sediment was obtained diluted with nuclease free water and FISH was performed by following the standard protocol described previously [3]. Prior to the fixation, the obtained sediment were washed three times with phosphate buffer saline solution (PBS, 1X) to avoid potential artifacts in the samples.

The biomass was hybridized with the Arc-FAM probe [4], a mixture of Cy3-labeled ANME probes: ANME-1 350 [5], ANME-2 538 [6], ANME-3 1249 [7] and Cy5-labelled *DBB* 660 [7]. Following the removal of unbound probes, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) [8]. The hybridization and microscopic visualization of fluorescent cells was performed as described previously [3].

Results

Visualization of archaeal cells in initial inoculum

On the contrary to the MBR enriched biomass, in the samples from initial inoculum, ANME cells could not be clearly visualized even after several attempts of hybridization (Fig. S1). Only few archaeal cells were hybridized during the FISH and there was some fluorescence signal was observed during microscopic visualization, when ANME-2 probe was used solely for the hybridization (Fig. S1). Nevertheless, diverse cells were observed under microscope after staining by DAPI.

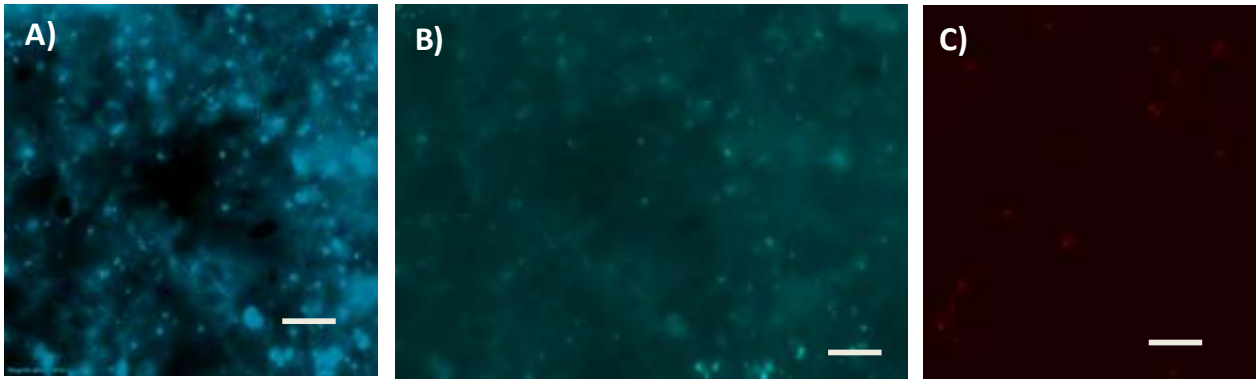

Fig. S1: Photomicrographs of microbial cells in the sediment of Ginsburg mud volcano (initial inoculum): Photomicrographs of DAPI stained cells are in blue color (A and B), archaeal cells hybridized by Arc-FAM probe are in red (C)

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