Control on rate and pathway of anaerobic organic carbon degradation in the seabed

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The degradation of organic matter in the anoxic seabed proceeds through a complex microbial network in which the terminal steps are dominated by oxidation with sulfate or conversion into methane and CO₂. The controls on pathway and rate of the degradation process in different geochemical zones remain elusive. Radiotracer techniques were used to perform measurements of sulfate reduction, methanogenesis, and acetate oxidation with unprecedented sensitivity throughout Holocene sediment columns from the Baltic Sea. We found that degradation rates transition continuously from the sulfate to the methane zone, thereby demonstrating that terminal steps do not exert feedback control on upstream hydrolytic and fermentative processes, as previously suspected. Acetate was a key intermediate for carbon mineralization in both zones. However, acetate was not directly converted into methane. Instead, an additional subterminal step converted acetate to CO₂ and reducing equivalents, such as H₂, which then fed autotrophic reduction of CO₂ to methane.

Microbial processes in most of the global seabed are directly or indirectly coupled to the degradation of organic matter (1). With increasing depth and age in the sediment, the remaining organic matter becomes increasingly recalcitrant to further degradation by microorganisms (2). Sulfate is quantitatively the most important terminal electron acceptor for the anaerobic oxidation of organic carbon in ocean margin sediments (3). When sulfate is depleted at depth, the terminal process of degradation shifts to methanogenesis with the production of methane and CO₂. Microbial sulfate reduction rates (SRR) and methanogenesis rates (MGR) can be directly determined from experiments using radioactively labeled tracers. Such measurements show that SRR drop with increasing sediment depth and age according to a reactive continuum that may be simulated by a simple power law function (3–5). This relationship reflects diagenetic alteration and increasing recalcitrance during aging of the organic matter, which limits the hydrolytic attack by extracellular enzymes and thereby the release of substrates that feed the microbial food chain (2, 6). In contrast to SRR, MGR have been difficult to determine experimentally in sediments due to extremely low rates and due to outgassing of supersaturated methane. The deep production of methane in the global seabed and the associated organic matter degradation are therefore poorly constrained.

It has been questioned whether extracellular hydrolysis of the organic matter is always limiting the rate of degradation (7, 8) or if downstream fermentative or terminal respiratory steps may also affect the overall rates (9). If the terminal electron acceptor does not affect the kinetics of organic matter degradation, then the trend of rate versus age determined in the sulfate zone should continue down into the methane zone. Thus, by extrapolation of this trend, the distribution of organic matter degradation and methane production deep within the methane zone could be estimated. To test this, we performed radiotracer-based experiments to measure (i) SRR using [35S]-labeled sulfate, (ii) MGR using [14C]-labeled dissolved inorganic carbon (MGRDIC), (iii) MGR using [2-14C]-labeled acetate (MGRAC), and (iv) acetate oxidation rates using [2-14C]-labeled acetate (AOR).

Results and Discussion

The sampling sites were located at a water depth of 88–96 m in the Bornholm Basin, SE Baltic Sea, which is characterized by seasonally hypoxic bottom water (stations BB01 to BB04; Table S1) (10). Guided by preceding seismoacoustic surveys and exploratory coring campaigns, we have carefully chosen each station according to organic matter burial history. The shallow depth of sulfate depletion (from 0.4 mbsf at BB03 to 4 mbsf at BB04) and the high rates of methane production within the top few meters (up to 140 nmol CH₄·cm⁻²·d⁻¹ at BB03) depended primarily on the thickness of the organic-rich Holocene mud layer. The Holocene mud layer was formed by stable sedimentation during the past 7,000 y and reached 4–9 m thickness, depending on the topography of the underlying organic-poor, postglacial clay from >8,500 y B.P. (Fig. S1) (10). Thus, the composition of the deposited mud was uniform across the flat basin floor, while only the sedimentation rate, 0.5–1 mm·y⁻¹, varied between stations.

SRR decreased in a log-log linear relationship with sediment age at all stations in accordance with an expected control by organic matter age on the overall metabolic rate (Fig. L4) (2). This trend continued down toward the sulfate–methane transition (SMT), where sulfate dropped below 1 mM with a concomitant rise of methane. Below the SMT, SRR dropped sharply away from the log-log linear depth trend. In the sulfate zone, above the SMT, ...
were detectable but very low, <10^{-3} \text{ nmol CH}_4 \text{ cm}^{-3} \text{ d}^{-1}, or two to three orders of magnitude lower than SRR (Fig. 1B). The MGR_{DOC} rose sharply and peaked in the SMT with rates similar to the SRR in that zone (up to ~4 \text{ nmol CH}_4 \text{ cm}^{-3} \text{ d}^{-1} at station BB03). Thus, the highest rates of MGR_{DOC} occurred in the SMT because the organic matter here had the youngest age and the highest reactivity. In the methane zone below, MGR_{DOC} continued to drop along the same log–log linear depth trend of organic carbon mineralization as was found for SRR in the sulfate zone. By contrast, MGR_{AC} were extremely low throughout the sediment at all investigated stations (<10^{-3} \text{ nmol CH}_4 \text{ cm}^{-3} \text{ d}^{-1}) (Figs. S7–S9).

We calculated total anaerobic carbon oxidation rates (COR) from the sum of SRR and MGR based on their stoichiometry during organic carbon mineralization (Fig. S2) (11). The resulting vertical depth trend of COR (Fig. 1C) revealed that rates of organic carbon mineralization decreased smoothly and continuously with organic matter age throughout the sediment, irrespective of the prevailing redox zonation and the associated change in the terminal pathway of the microbial food chain. This trend has previously been assumed but not experimentally demonstrated. The data thereby provide direct support for the validity of commonly applied organic matter decay models, such as the time-continuous and reactive-continuous approaches (2, 6), without the necessity to correct organic matter degradation rate with pathway-specific inhibition factors (1). The depth trend, based on activity measurements from all four stations in the Bornholm Basin, corresponded to the following power law dependence on sediment age (in years B.P.):

\[ \text{COR} = 1,640 \times \text{age}^{1.21} \text{ (nmol C cm}^{-3} \text{ d}^{-1}) \]  

In a uniform and continuously deposited sediment, such a relationship can be extrapolated to estimate organic carbon mineralization also in deeper sediment layers where process rates are difficult to quantify by radiotracer methods due to exceedingly low microbial activity and due to supersaturation of methane. Total depth-integrated MGR contributed to the total MGR, MGR_{DOC} + MGR_{AC}, that is, variations of the Wood–Ljungdahl pathway—might therefore be widespread among anaerobic prokaryotes, including sulfate reducers and methanogens (17, 21, 22).

Consistent with our findings, incubation studies and investigations of stable carbon isotopic compositions of CH$_4$ in marine
sediments suggested a predominance of methanogenesis via CO$_2$ reduction in marine sediments (4, 13, 23, 24), thus indicating a global relevance of syntrophic methanogenesis from acetate. However, minor contributions of acetoclastic methanogenesis (<20% of total methane production) have also been detected in marine sediments (4, 24, 25), thereby leading to the question of what controls the relative contribution of the two acetate degradation pathways in methanogenic environments. There is no obvious thermodynamic rationale for a syntrophic coupling between acetate oxidation and hydrogenotrophic methanogenesis, as there is no difference in the overall energy yield of the sum of these processes compared with that of direct acetoclastic methanogenesis. In Bornholm Basin sediments, methanogenesis from acetate was exergonic beneath the SMT (Fig. 2) but was close to the proposed energetic limit of methanogenic archaea (26), $-10$ kJ·mol$^{-1}$. If H$_2$ is the exchanged reducing equivalent, then the syntrophic relationship would require a narrow concentration range of 1–4 nM to allow both reactions to be exergonic so that the low overall energy yield can be shared between the partner organisms. Such low H$_2$ concentrations have, indeed, been reported below the SMT (e.g., <10 nM in the Cape Lookout Bight) (26). Consistent with our findings, that site was also characterized by a conspicuously high contribution of methanogenesis via CO$_2$ reduction (70–80% of total methane production) (25) and by similar energy yields for methane production from acetate (27).

Our results highlight the importance of a syntrophic relationship between acetate oxidizers and methanogens to enable acetate degradation under sulfate-limiting conditions. Intriguingly, at stations BB04 and BB02, where the methane zone is still in early development, very high acetate concentrations (up to >1 mmol·L$^{-1}$; Figs. S5 and S6) indicate a non-steady state situation where syntrophic degradation is not yet able to balance, at the energetic limit, the continuous acetate production by fermenters.

The predominance of acetate oxidizing prokaryotes over acetoclastic methanogens might be due to substrate kinetics and/or other competitive factors, such as the ability to engage in alternative forms of energy metabolism like acetogenesis and fermentation (17). Methanogens have a very narrow substrate

![Diagram of acetate turnover](image)

**Fig. 3.** Pathways of acetate turnover. The experiments with radiotracers (red) show how the key intermediate, acetate, may be degraded in the sulfate zone and the methane zone: (A) When sulfate was above a threshold concentration, acetate was oxidized to CO$_2$, coupled with sulfate reduction to sulfide. (B) Depletion of sulfate did not lead to direct methane production from acetate. (C) Instead, acetate was completely oxidized to CO$_2$. (D) Reducing equivalents, symbolized by electrons [e$^-$], from acetate were used for methane production from CO$_2$.
Our results have important implications for the interpretation of organic matter degradation in the methane zone of marine sediments: (i) The terminal process of anaerobic organic matter mineralization does not control the overall rate of degradation, (ii) fermentation produces acetate as the main potential substrate for the terminal degradation step in both the sulfate and the methane zones, and (iii) methanogenesis from acetate proceeds through a syntrophic pathway via CO₂ rather than by a simple conversion of the methyl-carbon in acetate to CH₄. The reasons for this indirect pathway and the key parameters controlling it remain to be identified. In fact, it also remains to be shown whether the oxidation of acetate, as the single most important substrate feeding sulfate reduction, may also partly take place in syntrophy. Similar to methanogenesis, a syntrophic acetate oxidation coupled to sulfate reduction would be exergonic for both partners under the low H₂ concentrations available.

Materials and Methods
Site Description and Sediment Coring. Cruises were undertaken with the R/V Aurora to the Bornholm Basin (Stations BB01, BB02, BB03, and BB04; Table S1). After initial exploratory sampling campaigns in 2014 and 2015, stations were revisited in 2016 with a keen focus on the transition in mineralization processes across the SMT. If not indicated otherwise, each site was sampled with a gravity corer of up to 9 m in length and complementary Rumohr cores (31). All cores at each station were sampled with the vessel positioned within 5 m diameter. The Rumohr corer allows nearly undisturbed sampling of the top meter of sediment below the seafloor. The extent of the unavoidable sediment loss from the top of the gravity cores (ca. 10–20 cm) was determined by aligning the sulfate concentration profiles of parallel gravity and Rumohr cores. All cores were processed on board the R/V Aurora, with high priority given to samples for methane and microbial activity analysis to ensure that samples could be taken within minutes after core retrieval. Gravity cores were cut into 1- or 2-m subsections, capped, and processed immediately depending on the type of analysis, as described below. In Rumohr cores, sediment material was sampled by stepwise extrusion from the core liner with a piston, while sediment material in gravity-core subsections was sampled, starting from the bottom, by drilling holes (for methane samples) or by cutting windows in the core liner (for microbial activity samples). The outermost part of the sediment that had been in contact with the core liner was avoided during sampling.

Methane Analysis. A 2.5-mL sediment sample was taken with a cutoff plastic syringe, immediately transferred to 20-mL preweighed glass vials containing 4 mL of saturated NaCl solution, and crimp-capped with butyl rubber stoppers. Samples were then shaken thoroughly and stored upside down at −20 °C until analysis. Before analysis, the samples were weighed, thawed, and shaken again to equilibrate methane between slurry and headspace of the glass vial. We injected 100–500 μL of the headspace with a gas chromatograph equipped with a 0.9 m packed silica gel column (3.1 mm inner diameter) and a flame ionization detector (GC-FID; SRI 310C; SRI Instruments).

Pore-Water Analyses. Rhizon samplers (32) for pore-water analysis were flushed with at least 50 mL Milli-Q water, dried, and stored in gas-tight foil bags until use. In Rumohr and gravity cores, small holes were drilled into the core liner of undisturbed sections to collect pore water with the Rhizon samplers by creating a vacuum with a 20-mL syringe. Each subcore was injected with 5 mL carrier-free (14)C-sulfate solution containing up to 140 kBq of radioactivity, depending on the expected microbial activity.

Diffusive Fluxes and Stoichiometric Ratios During Organic Matter Mineralization. Diffusive fluxes of methane were calculated from the concentration gradients using Fick’s First Law:

\[ J = -D \cdot \nabla C - \phi \cdot D_x \cdot \nabla C \cdot dz \]

where J is the diffusive flux, C is the concentration, z is depth (cm), D is the whole-sediment diffusion coefficient, and \( \phi \) is porosity. \( D_x \) was calculated from porosity, \( \phi \), and molecular diffusion coefficients of individual species (36), \( D_z \) at 8 °C in situ temperature and a salinity of 15 (37). \( D_{SO_4} \) was 0.566 cm²/d, \( D_{DIC} \) was 0.609 cm²/d, as described before (36, D = 0/(1 + (31 - \( \eta \)j)) (37).

The concentration profiles of pore-water DIC and SO₄²⁻ (Fig. S2) were used to calculate the stoichiometry of organic carbon oxidation to sulfate reduction (COR:SROR or fᵥ₂SO₄ in the sediment (11):

\[ r_C - SO_4 = -(dDIC/dSO_4) \cdot (DIC/DIC) - (SO_4/DO_2) \]

where \( dDIC/dSO_4 \) is the slope of the linear regression. Because \( r_C - SO_4 \) is determined by the oxidation state of the organic matter (ox), we can use \( r_C - SO_4 \) to calculate the stoichiometry of organic carbon oxidation to methanogenesis (COR:MRG or fᵥ₂CO in (11):

\[ r_C - SO_4 = (B/4 - ox) = r_C - CH_4 \]

Process Rate Measurements Using Radiotracers. As processing under strictly temperature-controlled conditions was not possible on board, the temperature of the cores was monitored continuously. Over the course of the entire sampling procedure (1–2 h), the temperature in the cores increased by only 1–3 °C. SRR were determined in subcores collected in butyl rubber-stoppered 5-mL sterile cutoff polycarbonate syringes, as described before (33). Each syringe subcore was injected with 5 μL carrier-free (35)S-sulfate solution containing up to 140 kBq of radioactivity, depending on the expected microbial activity. MGRas or MGRas, as well as AOR, were determined in subcores collected with butyl rubber-stoppered glass tubes equipped with acrylic plungers similar to the cut syringes. Each subcore was injected with 5 μL (14)C-DIC solution, containing up to 170 kBq of (14)C radioactivity, or 5 μL (14)C-acetate solution, containing up to 10 kBq (14)C radioactivity. The addition of (14)C-acetate led to an increase of the acetate concentration by only ~20%. All samples for measurement of microbial activity were incubated in the in situ temperature of 8 °C (281 K) within gas-tight plastic bags containing an Oxoid AnaeroGenTM pad (Oxoid A5). This oxygen scrubber effectively removes O₂ but does not generate H₂. The incubation times were chosen according to the expected microbial activity and differed from 12 h to 30 h. For example, in samples from the shallow Rumohr cores, we expected a fast turnover of the typically small acetate pool, and, therefore, incubations were kept relatively short (~12 h).

SRR incubations in cut syringes were terminated by freezing the intact samples at −20 °C, without opening the bags. After thawing, the total reduced inorganic sulfur (TRIS = H₂S, S, FeS, and FeS₂) was separated from the (35)S-sulfate and (35)TRIS was counted in 15 mL scintillation mixture (Gold Star, Meridian Biotechnologies) on a TriCarb 2900TR liquid scintillation analyzer (Packard Instrument Company).

To terminate (14)C-DIC and (14)C-acetate incubations, the entire glass syringe was transferred into glass vials, containing 5 mL NaOH (2.5%), without extracting the mud, and immediately crimp-capped with butyl rubber stoppers. The glass vials were then shaken thoroughly to wash the sample out of the glass syringe and stored upside down at −20 °C until analysis. (14)CH₄ in these vials was determined by flushing the headspace with CO₂-free air at 25 mL·min⁻¹ for 15 min and oxidation of the (14)CH₄ in the

spectrum (28), and the direct conversion of acetate to methane has so far only been ascribed to two microbial groups, Methanosarcina and Methanosaeta, of which only the latter is reported to grow at in situ acetate concentrations (<0.2 mM) (29, 30). Thus, the conversion of high levels of [2,3-14C]-acetate to [1-13C]-CO₂ in batch incubations of manure and wastewater sludge was only detected in the absence of Methanosaeta (19).

Pore-Water Analyses. Rhizon samplers (32) for pore-water analysis were flushed with at least 50 mL Milli-Q water, dried, and stored in gas-tight foil bags until use. In Rumohr and gravity cores, small holes were drilled into the core liner of undisturbed sections to collect pore water with the Rhizon samplers by creating a vacuum with a 20-mL syringe. The first 1 mL of the extracted pore water was discarded, and then up to ~10 mL was collected before the samples were aliquoted for analysis of individual pore-water parameters. For sulfate analysis, the extracted pore water was acidified and stripped of hydrogen sulfide (H₂S) by bubbling with a stream of moist CO₂. Sulfate samples were stored at 4 °C until analysis as described before (33). DIC samples were stored in 2-mL glass vials without headspace at 4 °C. Samples were transferred to sealed Exetainers, acidified with 85% (v/v) phosphoric acid, and after 24 h of equilibration time, the produced CO₂ was measured from the headspace of the Exetainer by a DeltaV isotope ratio mass spectrometer. Samples for acetate analysis were stored in combusted glass vials at −80 °C until analysis by 2D ion chromatography–mass spectrometry (29, 34). Pore water for ammonium (NH₄⁺) analysis was stored in 2 mL Eppendorf vials at −20 °C, and NH₄⁺ was measured photometrically according to the Danish Standard DS 224 protocol adapted for seawater samples (35). The detailed pore-water geochemistry and the measurements of microbial activity were derived from different cores. To ensure comparable geochemistry between replicate cores, additional pore-water samples for sulfate and acetate analysis were extracted from selected sediment samples before radiotracer incubation. For this, sediment aliquots (up to 1 g) were centrifuged at 14,000 × g with an Eppendorf Minispin benchtop centrifuge, and the supernatant was stored and processed as described above.
evolving gas stream to \(^{14}C\)O\(_2\) in a quartz glass tube containing CuO pellets, heated to 900 °C. The efficiency of methane oxidation was tested by adding known amounts of \(^1\)H\(_2\) to a 0.5-mL subsample of sediment slurry and following its conversion to CO\(_2\) in the exhaust gas by GC-FID (see Methane Analysis). Conversion efficiencies were always >99%. \(^{14}C\)O\(_2\) from the oven exhaust gas was trapped in 5 mL Carbosorb (Perkin-Elmer). In contrast to our earlier application of \(^{14}C\) for determination of methanogenesis, the gas stream was subjected to a wash step in 1 M NaOH before combustion to prevent trace amounts of labeled DIC to penetrate into the oven. In addition, the entire gas line was made of glass, which does not absorb CO\(_2\). These precautions lowered the background by at least two orders of magnitude and thus enhanced the sensitivity of the method by a similar factor. This improvement of the method was a prerequisite for the precise measurement of extremely low rates of methanogenesis in the deep part of the sediment.

To determine the \(^{14}C\)-DIC remaining in the glass vial, after extraction of CH\(_4\) at the end of \(1.08/2^{1/2}\) produced was flushed out of the vial headspace with N\(_2\) and AOR were then calculated as

\[
\text{AOR} = \frac{\text{A} \times \text{TRIC}}{\text{A} \times \text{ACh} + \text{A DIC}}\]

where [DIC] is the DIC concentration, \(A_{\text{TRIC}}\) is the radioactivity of TRIS at the end of the incubation, \(A_{\text{DIC}}\) is the radioactivity of DIC at the end of the incubation, and \(t\) is the length of the incubation. Similarly, the MGR\(_{\text{DIC}}\) were calculated as described before (25, 40):

\[
\text{MGR}_{\text{DIC}} = \frac{[\text{DIC}] \times \text{AC} \times 1.08 \times \exp(t/\text{ACh} + \text{A DIC})}{[\text{DIC}] \times \text{AC} \times 1.08 \times \exp(t/\text{ACh} + \text{A DIC})}\]

where [DIC] is the DIC concentration, \(A_{\text{AC}}\) is the radioactivity of CH\(_4\) at the end of the incubation, and \(A_{\text{DIC}}\) is the radioactivity of DIC at the end of the incubation. In contrast to incubations with \(^{14}C\)-DIC, a significant fraction of the \(^{2}C\)-acetate tracer was consumed during the incubation of samples close to the sediment surface, where activities were high and acetate concentrations were low. To account for this consumption, we assumed a first-order decrease in the specific radioactivity of the acetate pool (25, 40). The acetate turnover rate (ATR) was therefore calculated as

\[
\text{ATR} = \frac{[\text{Ac}] \times 1.08 \times \exp(-t/\text{ACh} + \text{A DIC})}{[\text{Ac}] \times 1.08 \times \exp(-t/\text{ACh} + \text{A DIC})}\]

where [Ac] is the acetate concentration and \(A_{\text{Ac}}\) is the radioactivity of acetate at the end of the incubation. MGR\(_{\text{Ac}}\) and AOR were then calculated as

\[
\text{MGR}_{\text{Ac}} = \text{ATR} \times \text{ACh}/(\text{ACh} + \text{A Ac})\]

\[
\text{AOR} = \text{ATR} \times \text{ACh}/(\text{ACh} + \text{A Ac})\]

COR were calculated from SRR and MGR based on the estimated stoichiometry of organic matter mineralization during sulfate reduction and methanogenesis (\(r_{\text{COR}}\)). 

\[
\text{COR} = \frac{\text{SRR} \times (C_{\text{DIC}}/C_{\text{DIC}}) + ([\text{MGR}_{\text{Ac}} + \text{MGR}_{\text{DIC}}] \times c_{\text{CH}_4})}{\text{AOR} \times (C_{\text{DIC}}/C_{\text{DIC}}) + ([\text{MGR}_{\text{Ac}} + \text{MGR}_{\text{DIC}}] \times c_{\text{CH}_4})}\]

The power law correlation lines describing the age or depth trend of COR were generally calculated under omission of elevated rates within the SMT, fed by upwards diffusing methane, and those from 0 to 10 cmbsf (0–100 y sediment age) where sulfate might not be the main electron acceptor. The linear regression after log–log transformation was corrected for logarithmic skewing as described before (5).

Calculation of Gibbs Free Energy. The standard Gibbs energy (\(\Delta G_{\text{mic}}\)) of sulfate reduction from acetate (\(\text{SO}_{4}^{2-} + \text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{H}_2\text{CO}_3 + \text{H}_2\text{O}\)) and methanogenesis from acetate (direct: \(\text{CH}_3\text{COO}^- + \text{H}_2\text{O} \rightarrow \text{CH}_4 + \text{HCO}_3^-\)) or syntrophic: \(\text{CH}_3\text{COO}^- + 4\text{H}_2\text{O} \rightarrow 4\text{H}_2 + 2\text{CO}_2 + 8\text{H}_{\text{ac}}^+\), coupled to \(4\text{H}_2 + \text{HCO}_3^- \rightarrow \text{CH}_4 + 3\text{H}_2\text{O}\)) was calculated using the SUPERT92 software package (41) and reported thermodynamic data (42–44) for a mean pressure of 1.1 MPa and 8 °C in situ temperature. The energy of reactions at nonstandard conditions \(\Delta G_{\text{insitu}}\) was calculated according to

\[
\Delta G_{\text{insitu}} = \Delta G_{\text{mic}} + R \times T \times \ln (Q)\]

where \(R\) is the ideal gas constant, \(T\) is the in situ temperature, and \(Q\) denotes the activity quotient of the reactants and reaction products. Activities in the saline pore fluids were estimated by multiplying the measured concentration of the species with activity coefficients (0.245 for \(\text{SO}_4^{2-}\), 0.725 for \(\text{HCO}_3^-\), 0.699 for \(\text{H}^+\), 0.725 for \(\text{CH}_3\text{COO}^-\), 1.08 for \(\text{CH}_4\), and 1 for \(\text{H}_2\text{O}\)) calculated from an extended version of the Debye–Hückel equation (45) for anionic strength of \(I = 0.28\) and at 8 °C using the Geochemistry Workbench Software (www.gwb.com).

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