

Online $\delta^{13}\text{C}$ analysis of volatile fatty acids in sediment/porewater systems by liquid chromatography–isotope ratio mass spectrometry

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Abstract

Volatile fatty acids (VFAs) are central intermediates of anaerobic metabolism and present in aquatic environments such as marine sediments. Conceptually, the isotopic composition of volatile fatty acids is presumed to be sensitive to the specific biogeochemical processes involved in their production and consumption. However, due to generally low environmental concentrations, our knowledge on isotopic variability of VFAs is limited. We report the development and application of a new protocol for compound-specific carbon isotopic analysis of VFAs in marine porewaters and other aqueous liquids. This new protocol involves reversed-phase separation of volatile fatty acids with an aqueous mobile phase by high performance liquid chromatography (HPLC) combined with chemical oxidation of the effluents by the Finnigan™ LC IsoLink interface (Krummen et al. 2004) and subsequent online transfer of the resulting CO_2 into an isotope ratio mass spectrometer. We obtained reproducible and accurate results for pure Na-acetate dissolved in artificial seawater at concentrations as low as 2 μM , whereas minimum concentrations in marine porewaters were 10 μM . Our technique extends the previously accessible concentration range and the fully automated online operation allows for systematic analysis of large sample sets. These technical improvements make carbon isotopic analysis of selected VFAs a realistic perspective for many sedimentary environments. Our initial survey of acetate in porewaters and fluids obtained from incubations of marine sediments has revealed an unexpectedly large range of isotopic compositions from -5‰ to -85‰ and provides strong support for process-specific information encoded in the isotopic compositions of VFAs.

In recent decades, our understanding of biogeochemical processes has benefited enormously from the information

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encoded in the stable carbon isotopic composition, $\delta^{13}\text{C}$, of organic molecules that is now easily accessible for a wide range of molecules through accurate and precise online techniques (e.g., Hayes et al. 1990). For example, isotopic studies have provided valuable insight into the global methane budget. In this context, stable isotope ratios of methane have been used extensively to identify and quantify methane sources and sinks (e.g., Rosenfeld and Silverman 1959, Whiticar et al. 1986, Avery and Martens 1999, Avery et al. 1999, Whiticar 1999, Hornibrook et al. 2000, Krüger et al. 2002, Conrad 2005, Sowers 2006). The two major biochemical pathways of methanogenesis, i.e., reduction of CO_2 and acetate fermentation, are associated with distinct kinetic isotope effects and the relative importance of these two methanogenic pathways can be estimated on the basis of $\delta^{13}\text{C}$ values of methane (Whiticar et al. 1986; Whiticar 1999). Likewise, biological consumption of methane leads to strong ^{13}C -enrichment in residual methane (e.g., Barker and Fritz 1981), and the effects are detectable in environmental methane samples, e.g., in plumes of methane in the ocean (Valentine et al. 2001).

Similar to methane, the isotopic composition of acetate and other volatile fatty acids (VFAs), i.e., crucial substrates, prod-

ucts, and intermediates of anaerobic microbial metabolism, is likely to provide insight into the relative importance of sources and sinks and the associated processes. However, the investigation of stable carbon isotopic compositions of acetate is severely hampered by labor intensive analytical procedures and high detection limits (Table 1). So far, isotopic data on acetate and other VFAs are rare because these methods are not suitable for systematic analysis at typical natural concentrations (i.e., μM in sediment porewaters). Improved methods for carbon isotopic analysis of VFAs are needed to advance our understanding of substrate use and reaction networks during the degradation of organic matter. The potential of carbon isotopic compositions of VFAs as proxies for biogeochemical processes is particularly relevant for the investigation of extreme environments such as the deep marine biosphere, where cultivation-based process studies are at the limit of their applicability.

Previously reported data indicate considerable variability in the carbon isotopic composition of acetate (Table 1). The data result from three lines of investigation: analysis of samples from natural environments, incubations of soil and sediment samples, and incubation experiments with pure microbial cultures. Analyses of environmental porewaters have revealed $\delta^{13}\text{C}$ values of acetate ranging from -2.8‰ to -20.7‰ ($n = 33$) (Blair et al. 1987, Blair and Carter 1992, Gelwicks et al. 1994, Krüger et al. 2002). Similar $\delta^{13}\text{C}$ values have been reported for acetate in oil field waters ($+0.6\text{‰}$ to -19.3‰) (Franks et al. 2001) and landfill leachate (-16.9‰) (Mohammadzadeh et al. 2005). Similarly, incubation experiments with rice field soils have yielded acetate with $\delta^{13}\text{C}$ values ranging from 0‰ to -33‰ ($n = 54$) (Sugimoto and Wada 1993, Conrad et al. 2002, Fey et al. 2004). The observed isotopic variability of acetate does not correspond to variations in $\delta^{13}\text{C}$ values of total organic carbon (TOC) in the investigated soils and sediments (Table 1). Instead, data from both natural environments and incubation experiments suggest that the carbon isotopic composition of acetate is controlled by isotopic fractionations during production (i.e., organic matter fermentation, homoacetogenesis) or consumption (i.e., acetoclastic methanogenesis, sulfate reduction) and the relative importance of these processes. Support for this concept comes from the observation of carbon isotope effects in incubation experiments with pure microbial cultures (Table 1). Fermentation of glucose by *Escherichia coli* produced acetate that was enriched in ^{13}C by 12.3‰ relative to the carbon source (Blair et al. 1985). By contrast, homoacetogenesis by pure cultures of *Acetobacterium woodii* yielded acetate with $\delta^{13}\text{C}$ values ranging from -52.5‰ to -89.5‰ (Gelwicks et al. 1989, Preuss et al. 1989), and representing a ^{13}C -depletion relative to CO_2 of 39.7‰ to 58.6‰ . Acetate consumption by methanogens, on the other hand, leads to ^{13}C -enrichment of the residual acetate pool. Carbon isotopic fractionation during acetoclastic methanogenesis has been documented based on $\delta^{13}\text{C}$ values of the produced methane, which shows on average ^{13}C -depletion of 25.9‰ relative to acetate for pure cultures of *Methanosarcina barkeri* (Krzycki et al. 1987; Gelwicks et al.

1994) but less than 5‰ depletion in *Methanoseta thermophila* (Valentine et al. 2004). In contrast, only minor isotopic fractionation has been observed for consumption of acetate by the sulfate reducing bacteria *Desulfobacter hydrogenophilus* and *Desulfobacterium autotrophicum* (Londry and Des Marais 2003).

Traditionally, carbon isotopic analysis of dissolved acetate has been a demanding task because the liquid phase needed to be removed prior to sample combustion and transfer of the generated CO_2 into an isotope ratio monitoring mass spectrometer (irm-MS). Reported analytical protocols have three principal steps in common (see Table 1 for more details). (1) Acetate is separated from the liquid phase of the aqueous sample and subsequently purified. (2) The purified acetate is combusted in a gas chromatography combustion system (or alternatively pyrolyzed for separate carbon isotopic analysis of the methyl carbon in acetate). (3) The resulting CO_2 is analyzed by irm-MS, either offline from collected CO_2 using a dual-inlet mass spectrometer or online by continuous-flow mass spectrometry.

The previously reported methods for carbon isotopic analysis of acetate vary in their degree of automation and in their requirements for sample volume and acetate concentration (Table 1). For all protocols, it is important to note that the initial separation steps require special caution, because incomplete recovery of acetate from the aqueous sample may cause isotopic fractionation. This is of particular concern for extraction of acetate by solid-phase microextraction (SPME) (Dias and Freeman 1997, Franks et al. 2001). However, with careful calibration, the SPME method is suitable for the analysis of small samples (3 to 5 mL) if acetate concentrations are sufficiently high ($> 700 \mu\text{M}$). The methods established by Gelwicks and Hayes (1990) and Blair and Carter (1992) allow carbon isotopic analysis of acetate at concentrations down to $20 \mu\text{M}$ and $30 \mu\text{M}$, respectively, but require large sample volumes ($> 40 \text{mL}$). The demanding analytical protocols impede generation of large data sets and the required sample size is in conflict with the small sample volumes that are usually available for porewater analysis. Therefore, none of these methods is suitable for systematic carbon isotopic analysis of acetate in natural sediment/porewater systems.

Here we report a new analytical protocol for sensitive, accurate, and precise carbon isotopic analysis of dissolved acetate and other VFAs by isotope ratio monitoring liquid chromatography/mass spectrometry (irm-LC/MS). This technique is based on the FinniganTM LC IsoLink interface that couples high-performance liquid chromatography (HPLC) directly to a commonly used irm-MS system (Krummen et al. 2004). In contrast to previously available methods, the liquid phase is not removed from the sample prior to oxidation. Instead, the target compounds are separated by liquid chromatography and chemically oxidized while still in the aqueous mobile phase. The generated CO_2 is separated online from the solvent stream through a gas-exchange membrane into a stream of He, subsequently dried by Nafion tubes, and finally introduced into the irm-MS. Our protocol allows fully automated online

Table 1. Compilation of $\delta^{13}\text{C}$ -acetate values reported for (a) samples from natural environments; (b) incubation experiments with soil and sediment samples; (c) incubation experiments with acetate producing microbial cultures; (d) incubation experiments with acetate consuming microbial cultures

| (a) Natural environments | | | | | | |
|---|---|--|-----------------------------|--------------------|--------|--|
| Environment | $\delta^{13}\text{C}$ -TOC (‰ PDB) | $\delta^{13}\text{C}$ -acetate (n) ^a (‰ PDB) | Acetate concentration | Sample size | Method | Reference |
| Anoxic coastal sediment (Cape Lookout Bight, North Carolina, USA) | -19.0±0.3 | -11.6±0.4 to -16.2±0.4 (5) | ≥ 80 μM | 100 mL | A | Blair et al., 1987 |
| Anoxic coastal sediment (Cape Lookout Bight, North Carolina, USA) | -19.08±0.26 | -2.8±1.6 to -17.6 (7) | > 30 μM | 40 mL | A | Blair and Carter, 1992 |
| Freshwater lake sediment (Wintergreen Lake, Michigan, USA) | | -18.1±0.1 (1) | > 20 μM | | B | Gelwicks et al., 1994 |
| Rice field soil (Vercelli, North Italy) | -26.5 to -27.3 | -16.2±0.3 to -20.7±2.3 (20) | 5-10 μM | 1 mL | C | Krüger et al., 2002 |
| Oil field waters (San Joaquin Basin, California, USA) | -23.6 to -29.4 ^b | +0.57 to -19.25 (17) | > 700 μM | 3-5 mL | D | Franks et al., 2001 Dias and Freeman, 1997 |
| Landfill leachate | | -16.9±0.1 (3) | 958-1042 μM | 18 ml | E | Mohammadzadeh et al., 2005 |
| Murchison meteorite | | +22.7±0.2 (1) -7.7±0.2 (1) | 1730 μmol/kg 770 μmol/kg | 23.5 g ca. 10 g | A D | Yuen et al., 1984 Huang et al., 2005 |
| (b) Incubation experiments | | | | | | |
| Environment | $\delta^{13}\text{C}$ -TOC (‰ PDB) | $\delta^{13}\text{C}$ -acetate (n) ^a (‰ PDB) | Acetate concentration | Sample size | Method | Reference |
| Rice field soil (Konosu, Japan) | -26.5 | -22.2 to -32.9 (7) | ca. 1.9–4.2 mM | 100 mL | A | Sugimoto and Wada, 1993 |
| Rice field soil (Vercelli, North Italy) | -26.70±0.39 | 0 to -33 (13) | 0.2 to 16 mM | 1-2 μL | C | Conrad et al., 2002 |
| Rice field soil (Vercelli, North Italy) | -26.0±0.3 | -7.5 to -27.5 (34) | < 0.1 to 1.5 mM | 1 mL | C | Fey et al., 2004 |
| (c) Acetate production in pure microbial cultures | | | | | | |
| Organism | $\delta^{13}\text{C}$ -substrate (n) ^a (‰ PDB) | $\delta^{13}\text{C}$ -acetate (n) ^a (‰ PDB) | Acetate concentration | Sample size | Method | Reference |
| <i>Escherichia coli</i> | Glucose: -9.0±0.1 | +3.3±0.9 (1) | | 10 mL | A | Blair et al., 1985 |
| <i>Acetobacterium woodii</i> | CO ₂ : 34.2 to -13.93 | -52.51 to -70.28 (13) | | 200 mL | B | Gelwicks et al., 1989 |
| <i>Acetobacterium woodii</i> | CO ₂ : -47.6/-49.8 | -87.2/-89.5 (2) | | | E | Preuß et al., 1989 |
| (d) Acetate consumption in pure microbial cultures | | | | | | |
| Organism | $\delta^{13}\text{C}$ -acetate (n) ^a (‰ PDB) | $\delta^{13}\text{C}$ -product (n) ^a (‰ PDB) | Acetate concentration | Sample size | Method | Reference |
| <i>Methanosarcina barkeri</i> | Total: -17.7 (2) CH ₃ -: -22.4/-22.1 | CH ₄ : -43.6/-43.3 | 200 mM | | A | Krzycki et al., 1987 |
| <i>Methanosarcina barkeri</i> | Total: -20.42 to -35.76 (10) CH ₃ -: -22.1 to -37.4 (10) | CH ₄ : -51.80 to -57.02 | ca. 18-487 μM | 83 mL | B | Gelwicks et al., 1994 |

Continued

TABLE 1. Continued

| | | | | | |
|---------------------------------------|--|-----------------------------|----------|---|-----------------------------|
| <i>Methanosaeta thermophila</i> | Total: ca. -30 to -20 CH ₃ :- -28.3 to -31.0 | CH ₄ :-28 to -35 | 31-34 mM | F | Valentine et al., 2004 |
| <i>Desulfobacter hydrogenophilus</i> | | | | | |
| <i>Desulfobacterium autotrophicum</i> | Total: -34.2 | Biomass: -35.0 | | G | Londry and Des Marais, 2003 |
| <i>Desulfotomaculum acetoxidans</i> | Total: -34.2 | Biomass: -25.4 | | G | Londry and Des Marais, 2003 |

^an denotes the number of data points reported

^bcarbon isotopic value of the accompanying kerogen and oil

Method A: (1) extraction of VFA fraction by distillation of the acidified water sample and subsequent drying of the basified distillate; (2) separation of acetate from the distillate by preparative liquid chromatography and additional purification by distillation; (3) combustion of acetate or pyrolysis of acetate and combustion of the resulting CH₄ for carbon isotopic analysis of total carbon and methyl-carbon, respectively, using a gas chromatography combustion system; (4) online or offline analysis of the resulting CO₂ by irm-MS. **Method B:** (1) drying of the basified sample; (2) extraction of acetic acid after addition of oxalic acid to the residue and heating (100°C); (3) purification of acetate followed by combustion or pyrolysis and combustion of the resulting CH₄ using a gas chromatography combustion system; (4) online or offline analysis of the resulting CO₂ by irm-MS. **Method C:** (1) drying of the basified sample; (2) addition of formic acid and n-propanol to the residue; (3) separation of acetate followed by combustion using a gas chromatography combustion system; (4) online analysis of the resulting CO₂ by irm-MS. **Method D:** (1) extraction of VFAs from the water sample by solid-phase microextraction (SPME); (2) thermal desorption and separation of acetate followed by combustion or pyrolysis and combustion of the resulting CH₄ using a gas chromatography combustion system; (3) online analysis of the resulting CO₂ by irm-MS. **Method E:** (1) separation of acetate from the water sample by preparative HPLC; (2) chemical oxidation of acetate to CO₂ in the liquid phase; (3) online analysis of purged CO₂ by irm-MS. **Method F:** (1) separation of acetate from the culture medium by diethylether extraction and distillation of the extract; (2) combustion and precipitation of the resulting CO₂ as BaCO₃; (3) carbon isotopic analysis of the precipitate by elemental analyzer. **Method G:** (1) separation of acetate from the culture medium by preparative HPLC followed by drying of the basified acetate fraction; (2) combustion of acetate or pyrolysis of acetate and combustion of the resulting CH₄; (3) online analysis of the resulting CO₂ by irm-MS.

analysis of aqueous samples with minimal or no prior treatment and thus minimizes the required sample volume (0.3 to 3 mL, for triplicate analysis), the risks of isotopic fractionation during sample preparation, and labor.

To assess our method, we have systematically analyzed samples from a variety of natural environments and from laboratory-based incubations of natural sediments. The generated database indicates wide variations of the isotopic composition of acetate and other VFAs that appear to be linked systematically to biogeochemical processes that produce and consume them.

Materials and procedures

Instrumentation—Our method for carbon isotopic analysis of VFAs by irm-LC/MS utilizes the Finnigan™ LC IsoLink interface (Krummen et al. 2004), which couples a ThermoFinnigan Surveyor HPLC to a continuous-flow ThermoFinnigan Delta Plus XP irm-MS. Because all carbon-bearing compounds are detected, organic solvents, organic buffers, or other carbon-bearing compounds (incl. inorganic carbon) must be absent. We took care to avoid contamination of the HPLC pumps by organic solvents and bleeding of the HPLC column. Such factors increase the analytical background and limit the precision attainable for small samples. Columns that were shipped in methanol/water mixtures were only used after thorough equilibration with MilliQ water on a separate HPLC system.

HPLC—For liquid chromatography, we used a Reprosil-Pur C18-AQ column (250 mm length; 4.0 mm i.d.; 5 μm particle

size; Wicom) equipped with a guard column (CC8/4 Nucleosil 100-5 C8 ec, Macherey-Nagel) and a ThermoFinnigan Surveyor HPLC system, consisting of a temperature-controlled autosampler and column holder, a pulsation-free MS pump, and a photodiode array (PDA) detector. During analytical sequences samples were stored at 10°C and injected via needle port, 50-μL sample loop, and 6-port valve (with a syringe and loop loading speed of 2.0 μL s⁻¹ operated in no-waste-mode). After each injection, the autosampler syringe was flushed with 500 μL of MilliQ water. As mobile phase, we used an aqueous phosphate buffer (pH 2.5) made up of 13.8 g NaH₂PO₄·H₂O (p.a., Merck) and 1 mL H₃PO₄ (85 wt-%, ACS reagent, Aldrich) in 500 mL of MilliQ water. The buffer was freshly prepared every day and stored in an amber coated 1 L glass bottles (Schott) while in use. To obtain a CO₂-free mobile phase, the buffer solution was degassed under vacuum in an ultrasonic bath (10 min at 40°C) and purged with a constant flow of He during operation. In this study, the mobile phase was pumped isocratically at a steady flow rate in the range of 300 to 500 μL min⁻¹, and the column was kept at a constant temperature between 10°C and 35°C for analysis times of 70 to 90 min, depending on sample type. For pore-water samples from the natural environment, we used a combination of low flow rates and low temperatures to optimize separation of lactate and acetate. When chromatographic separation of acetate and lactate was unproblematic in certain sample sets, e.g., in incubation experiments, flow rates and

Table 2. $\delta^{13}\text{C}$ values of pure VFAs in artificial seawater measured by irm-LC/MS

| Compound | Concentration range (μmol) | Injected carbon (ng) | irm-LC/MS | | | | irm-EA/MS | | $\Delta\delta^{13}\text{C}$ ‰ |
|------------|---|----------------------|-----------------|---|---|-------------------------|---|------|-------------------------------|
| | | | Retention index | Sensitivity ($\text{Vs}\cdot\text{ng}^{-1}\cdot\text{C}$) | $\delta^{13}\text{C} \pm 1$ SSD (n), ‰ VPDB | 95% Confidence interval | $\delta^{13}\text{C} \pm 1$ SSD (n), ‰ VPDB | | |
| Acetate | 10 to 1670 | 12.5 to 2000 | 1 | 0.23 ± 0.02 | -32.1 ± 0.7 (24) | 0.2 | -32.1 ± 0.0 (3) | 0.0 | |
| Butyrate | 21 to 833 | 50 to 2000 | 5.89 to 6.09 | 0.48 ± 0.04 | -27.3 ± 0.4 (15) | 0.2 | -27.1 ± 0.8 (5) | -0.2 | |
| Formate* | 418 to 2500 | 250 to 1500 | 0.72 | 0.21 ± 0.01 | -22.8 ± 1.5 (33) | 0.5 | -20.8 ± 0.3 (3) | -2.0 | |
| Lactate | 28 to 1110 | 50 to 2000 | 0.77 to 0.80 | 0.22 ± 0.02 | -24.8 ± 0.4 (6) | 0.3 | -24.5 ± 0.2 (3) | -0.3 | |
| Malic acid | 21 to 833 | 50 to 2000 | 0.81 to 0.83 | 0.24 ± 0.05 | -25.4 ± 0.6 (6) | 0.5 | -25.4 ± 0.1 (2) | 0.0 | |
| Malonate | 28 to 1110 | 50 to 2000 | 0.82 to 0.83 | 0.24 ± 0.02 | -24.4 ± 0.6 (10) | 0.5 | | | |
| Oxalate | 42 to 1670 | 50 to 2000 | 0.66 | | -24.5 ± 0.5 (8) | 0.3 | -23.9 ± 0.4 (3) | -0.6 | |
| Propionate | 28 to 1110 | 50 to 2000 | 2.06 to 2.13 | 0.20 ± 0.02 | -20.6 ± 0.5 (19) | 0.3 | -20.5 ± 0.1 (3) | -0.1 | |
| Pyruvate | 28 to 1110 | 50 to 2000 | 0.77 to 0.80 | 0.19 ± 0.01 | -24.0 ± 0.4 (16) | 0.2 | -24.0 ± 0.0 (3) | 0.0 | |
| Succinate | 21 to 833 | 50 to 2000 | 1.42 to 1.44 | 0.23 ± 0.03 | -31.2 ± 0.7 (19) | 0.3 | -31.7 ± 0.3 (4) | 0.5 | |

SSD denotes sample standard deviation (1 sigma), n denotes the number of replicate analysis. Retention index: $\text{RI} = R_i(\text{VFA})/R_i(\text{Acetate})$ where R_i refers to the respective retention time.

*Note that no baseline separation was achieved for the formate peak using the described HPLC method.

column temperatures were increased to reduce retention times of less polar VFAs, such as propionate and butyrate. We verified the accuracy of the carbon isotopic analysis for individual flow rates and used a fixed flow rate for each sample type and for the corresponding calibration because flow rates can influence the sensitivity and accuracy of the irm-MS analysis. The PDA detector is not obligatory for the quantification of analytes since both carbon isotopic analysis and quantitative analysis can be achieved by irm-MS. However, PDA analysis offers the additional option to monitor, identify, and quantify eluting sample constituents before they are oxidized to CO_2 in the interface. The pump head of the MS pump was rinsed with water at least once a day to avoid crystallization of buffer. In addition, all lines of the HPLC system were thoroughly purged with water once a week to prevent contamination from microbial growth.

LC IsoLink Interface—The outlet of the PDA detector was coupled to the Finnigan™ LC IsoLink interface, where eluents and mobile phase are mixed with an oxidation reagent and acid (Krummen et al. 2004). To avoid premature degradation of the final oxidation mixture, oxidation reagent and acid were pumped separately by two-head pumps and combined in a T-piece just before entering the HPLC eluent flow. This mixture flowed through a heated capillary oxidation reactor where organic compounds are quantitatively converted to CO_2 at 99.9°C . After passing the oxidation reactor, the liquid phase was cooled and individual CO_2 peaks were separated from the liquid phase by a membrane exchanger and transferred into a counter flow of helium (1 mL min^{-1}). As reagents, we used sodium peroxodisulfate as oxidant and ortho-phosphoric acid. In contrast to previous reports (Krummen et al. 2004), no AgNO_3 was added as catalyst because the oxidation potential of $\text{Na}_2\text{S}_2\text{O}_8$ is high enough for complete oxidation of the inves-

tigated substances, and chloride-containing samples would cause precipitation of AgCl in connecting tubes and membranes, resulting in damage of the system. The oxidation reagent was prepared from 60 g $\text{Na}_2\text{S}_2\text{O}_8$ (p.a., Merck) in 200 mL MilliQ water, and acid that was mixed from 30 mL H_3PO_4 (85 wt-%, ACS reagent, Aldrich) with 200 mL MilliQ water. Both reagents were freshly prepared for prompt consumption and stored in amber-coated 500-mL glass bottles (Schott) while in use. To eliminate CO_2 , both solutions were degassed under vacuum in an ultrasonic bath (10 min at 40°C) and purged with a constant flow of He during operation. In this study, the oxidation reagent and acid were added to the mobile phase with flow rates of approximately 30 to $70\ \mu\text{L min}^{-1}$. We adjusted optimal flow rates at the beginning of each analytical sequence by monitoring O_2 entering the mass spectrometer. Ideally, the oxidation reaction produced enough O_2 to yield a voltage of 9 to 12V when monitored as $m/z\ 32$ on cup nr 2 (resistor of $3\cdot 10^8\ \Omega$). Due to mechanical constraints, the combined flow of mobile phase, oxidation reagent, and acid must be kept below $700\ \mu\text{L min}^{-1}$. The pump heads of the oxidant and acid pumps were rinsed with water at least twice a day to avoid crystallization of reagents.

irm-MS—The outlet of the LC IsoLink interface was coupled to a ThermoFinnigan Delta Plus XP irm-MS via a routine open split. The ion source was held at a pressure of $2\cdot 10^{-6}$ Torr, and ions were generated by electron impact at 150 eV. Primary standardization of CO_2 isotope analyses on the Delta Plus XP is based on multiple (three to seven) injections of reference CO_2 ($\delta^{13}\text{C} = -28.0\text{‰} \pm 0.1\text{‰}$, $4.5 \pm 0.5\text{ V}$ at $m/z\ 44$) from a tank before and after the analysis of each sample. Isotope ratios are reported in $\delta^{13}\text{C}$ notation (permil, ‰) relative to the Vienna peedee belemnite standard (VPDB) (Slater et al. 2001), with $\delta^{13}\text{C} = [(R_{\text{sample}} - R_{\text{VPDB}})/R_{\text{VPDB}}] \cdot 10^3$, where $R = {}^{13}\text{C}/{}^{12}\text{C}$ and

$$R_{\text{VPDB}} = 0.0112372 \pm 2.9 \cdot 10^{-6}$$

Instrument control and data acquisition—The HPLC system, LC IsoLink pumps, irm-MS, and data acquisition system were controlled using software Isodat 2.0 SP 2.38 supported by Xcalibur 1.4 (both Thermo).

Standard solutions of VFAs—We tested the applicability of our method for qualitative, quantitative, and carbon isotopic analysis with aqueous standard solutions prepared from sodium salts of VFAs (Aldrich) in sulfate-free artificial seawater (26.4 g L⁻¹ NaCl, 11.3 g L⁻¹ MgCl₂·6H₂O, 0.682 g L⁻¹ KCl, and 0.099 g L⁻¹ KBr; degassed under vacuum in an ultrasonic bath for 10 min at 40°C). Standard solutions were acidified to pH 1.5 using H₃PO₄ (85 wt-%, ACS reagent) to eliminate dissolved inorganic carbon (DIC). For qualitative analysis, we identified peaks by retention time and calculated retention indices by dividing retention times of individual VFAs through that of acetate (Table 2). For quantitative analysis, we calibrated the relationship between peak area and VFA concentration in the standard solution. To assess the precision and accuracy of our carbon isotopic analysis, we performed multiple analyses of dissolved VFA standards and compared the $\delta^{13}\text{C}$ values obtained by irm-LC/MS to those of the corresponding salts determined via irm-elemental analyzer-MS (irm-EA/MS). For each VFA, we assessed the accuracy and precision of our method for a concentration range that corresponds to a total of 50 to 2000 ng carbon delivered to the irm-LC/MS. For acetate, lower concentrations were tested as well (12.5 ng C injection). For formate, only higher concentrations (> 250 ng C injection) were analyzed successfully and included in the method assessment. The investigated VFAs, their $\delta^{13}\text{C}$ values, retention times, and the tested concentration ranges are listed in Table 2.

Concentrating samples—If VFA concentrations are below the limits indicated in Table 2, samples need to be concentrated prior to analysis. Our approach follows previously reported techniques (Blair et al. 1987, Gelwicks and Hayes 1990). To prevent evaporative loss of VFAs, the sample solution was basified to pH 12 by addition of 2 N KOH and dried by lyophilization. The resulting residue was dissolved in at least 300 μL of MilliQ water and acidified to pH 1.5 using H₃PO₄ (85 wt-%, ACS reagent). We have analyzed 2 to 20 μM solutions of acetate and propionate in artificial seawater after concentrating them 12.5-fold, i.e., by the maximum factor possible at the given salinity of the sample matrix. Since contaminants are amplified by the concentration procedure as well, purity of reagents and vials is critical. During lyophilization, sample solutions were kept in pre-combusted glass vials capped with Teflon-coated septa in open-hole screw-caps. Sterile needles were inserted into the septa to allow for the exchange of the gas phase. We obtained best results when the 2 N KOH solution was prepared just before use from MilliQ water and KOH pellets (85 wt-%, p.a., Roth) that had been heated to 450°C for 30 min to remove organic contaminants.

Sample collection, processing, and analysis—We applied our method to two types of “real-world” samples, i.e., natural pore-

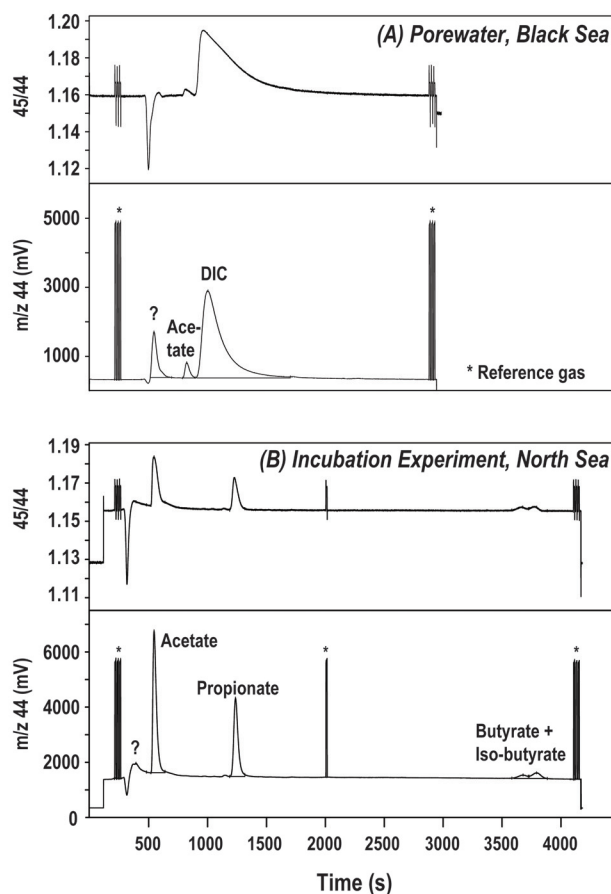


Fig. 1. Application of irm-LC/MS for analysis of VFAs in marine sediment/porewater systems. (A) The chromatogram of a natural porewater sample obtained from the Black Sea (41°57.26 N 41°16.73 E, 888 m water depth, 5.53 m sediment depth) shows elution and successful separation of an unidentified compound or group of compounds, acetate (eluting as acetic acid, $\delta^{13}\text{C} = -29.4\text{‰}$, 71 μM), and DIC. Analytical conditions: no sample acidification prior to analysis, HPLC flow 300 $\mu\text{L min}^{-1}$, oxidation reagent and acid flow 30 $\mu\text{L min}^{-1}$ each, column temperature 15°C. (B) Incubation of surface sediment from a tidal flat of the North Sea (1:1 slurry incubated under N₂ atmosphere for 14 days at 50.5°C) yields an unidentified compound, acetate (eluting as acetic acid, $\delta^{13}\text{C} = -11.0\text{‰}$, 809 μM), propionate (eluting as propionic acid, $\delta^{13}\text{C} = -22.4\text{‰}$, 383 μM), and butyrate and iso-butyrate (eluting as butyric and iso-butyric acid, $\delta^{13}\text{C}$ of the mixture of two isomers = -26.4‰ , 82 μM). Analytical conditions: acidification of sample to remove DIC prior to analysis, HPLC flow 500 $\mu\text{L min}^{-1}$, oxidation reagent flow 70 $\mu\text{L min}^{-1}$, acid flow 50 $\mu\text{L min}^{-1}$, column temperature 35°C. Both analyses were conducted using a Reprosil-Pur C18-AQ column (250 mm length; 4.0 mm i.d.; 5 μm particle size; Wicom) equipped with a guard column (CC8/4 Nucleosil 100-5 C8 ec) and an aqueous phosphate buffer (pH 2.5) as mobile phase.

water samples and aqueous liquids obtained from anaerobic incubation experiments with marine sediments. Sediment and porewater samples resulted from Ocean Drilling Program (ODP) Leg 201 (Peru Margin, Pacific Ocean, 27 January–29 March 2002), Integrated Ocean Drilling Program (IODP) Expedition 301 (Juan de Fuca, Pacific Ocean, 27 June–20 August 2004), IODP Expedition 302 (Lomonosov Ridge, Arctic Ocean, 7

August–13 September 2004), RV *Somme* Cruise So 174 (Gulf of Mexico, 1 October–12 November 2003), and from field trips to tidal flats of the coastal North Sea. In the experiments, we incubated 1:1 slurries of sediments and sulfate-free artificial seawater ($26.4 \text{ g L}^{-1} \text{ NaCl}$, $11.3 \text{ g L}^{-1} \text{ MgCl}_2 \cdot 6\text{H}_2\text{O}$, $0.682 \text{ g L}^{-1} \text{ KCl}$, and $0.099 \text{ g L}^{-1} \text{ KBr}$) at temperatures ranging from 30°C to 80°C . Incubations were stopped by freezing the incubation vials. Liquid samples were collected from defrosted samples by centrifugation (10 min at 2000 rpm) and, if necessary, further purified by filtration (Nylon syringe filters, $0.2 \mu\text{m}$, Roth). Porewater samples were obtained by standard procedures involving whole core squeezing (e.g., D'Hondt et al. 2003, Shipboard Scientific Party 2004) or sectioning of the core and pressure filtration (Grasshoff et al. 1999) using regenerated cellulose filters (membrane filter RC58, $0.2 \mu\text{m}$, Schleicher & Schüll). ODP and IODP samples were processed at room temperature. All other porewater samples were obtained in cooled shipboard laboratories (4°C) to minimize metabolic activity during sample collection. Subsequently, samples were stored in pre-combusted glass vials at -25°C . Note that the concentration and isotopic composition of VFAs may be altered in the course of sample retrieval. We did not validate the sample collection methods for this application. For analysis, 1 mL aliquots of the samples were transferred to pre-combusted 2 mL glass vials (closed with Teflon-coated silica septa and open-hole screw caps, Rotilabo) and kept at 10°C in the autosampler in between duplicate runs. All samples from incubation experiments were acidified to pH 1.5 using H_3PO_4 (85 wt-%, ACS reagent, Aldrich) and allowed to equilibrate for at least 12 h to remove DIC. Porewater samples were only acidified if bicarbonate interfered with peaks of VFAs.

Assessment

Compound specific analysis—We successfully separated VFAs dissolved in marine sediment/porewater systems for compound specific carbon isotopic analysis (Fig. 1). In samples resulting from porewater squeezing and incubation of marine sediments, acetate, lactate, propionate, and butyrate were the most prominent VFAs. For butyrate and iso-butyrate, peak separation was not sufficient for isotopic distinction of individual isomers. Therefore, $\delta^{13}\text{C}$ values of the combined C_4 -components are reported. Many chromatograms of natural samples showed an early eluting, unidentified substance (Fig. 1). This peak obstructs the analysis of coeluting formate. However, irm-LC/MS analysis of pure formate in artificial seawater yields precise and accurate $\delta^{13}\text{C}$ values (Table 2). The same holds for malate, malonate, oxalate, pyruvate, and succinate, i.e., compounds that were not detected in incubations and natural samples.

Accuracy and precision—In general, standard deviations for repeated carbon isotopic analysis were $< 0.7\text{‰}$, and mean $\delta^{13}\text{C}$ values of dissolved VFAs deviated by $< 0.6\text{‰}$ from those determined by irm-EA/MS (Table 2). Only analysis of formate yielded a relatively low precision (1.5‰) and accuracy (2‰). This analytical uncertainty is likely due to the short retention time of formate using the described method, which impaired baseline

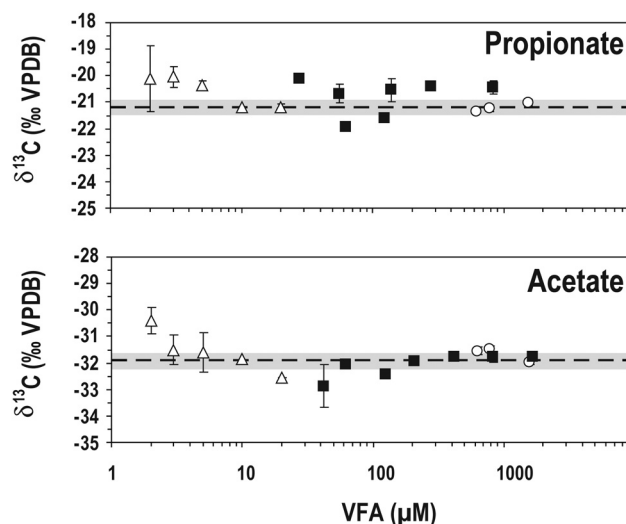


Fig. 2. Accessible concentration range: irm-LC/MS analysis of 2 to 1667 μM aqueous solutions prepared from pure Na-acetate and Na-propionate salts yields mean $\delta^{13}\text{C}$ values (dashed lines) of $-31.7\text{‰} \pm 0.6\text{‰}$ ($n = 28$) for acetate and $-21.0\text{‰} \pm 0.6\text{‰}$ ($n = 27$) for propionate. The reproducibility of the overall analyses is $\pm 0.2\text{‰}$ for both acetate and propionate (95% confidence interval, indicated by the shaded areas). Within analytical error, irm-LC/MS results agree with the $\delta^{13}\text{C}$ values obtained from the pure salts by irm-EA/MS, i.e., $-32.1\text{‰} \pm 0.0\text{‰}$ ($n = 3$) for Na-acetate and $-20.5\text{‰} \pm 0.1\text{‰}$ ($n = 3$) for Na-propionate. Open triangles represent 2 to 20 μM VFA solutions, analyzed after 12.5-fold pre-concentration. Solid squares symbolize 42–1667 μM standards. Open circles designate 625 to 1563 μM solutions obtained from 12.5-fold pre-concentration of 50 to 250 μM standards and document negligibility of isotopic fractionation. The analyzed concentration range is equivalent to 30 to 2000 ng acetate-carbon and to 45 to 2813 ng propionate-carbon delivered to the ion source.

separation essential for precise carbon isotope analysis.

For the concentration ranges reported in Table 2, we observed a linear correlation between signal area and injected amount of carbon as reported previously for benzoic acid (Krummen et al. 2004), angiotensin III, and leucine (Godin et al. 2005). Therefore, irm-LC/MS can be used to combine stable carbon isotopic and quantitative analysis of VFAs. Repeated analysis of aqueous standard solutions shows that the signal response remains stable over time and relative analytical error of quantitative analysis is $< 5\%$. For verification of qualitative, quantitative, and carbon isotopic analysis, we recommend one calibration per week based on a set of VFA standards that cover the concentration range of interest, followed by two daily quality checks via single analysis of a standard resembling the qualitative and quantitative composition of the analyzed samples.

Sample size requirements—The sensitivity of the irm-LC/MS method averages around $0.25 \text{ Vs ng}^{-1} \text{ C}$ injected on the HPLC column (Table 2) and is comparable to previously reported values (Krummen et al. 2004; Godin et al. 2005). During evaluation of our method, peak areas showed a constant signal response to the injected amount of carbon for a given flow rate. However, peak areas decreased with increasing flow rate as has been reported for irm-LC/MS analysis for amino acids (Godin et

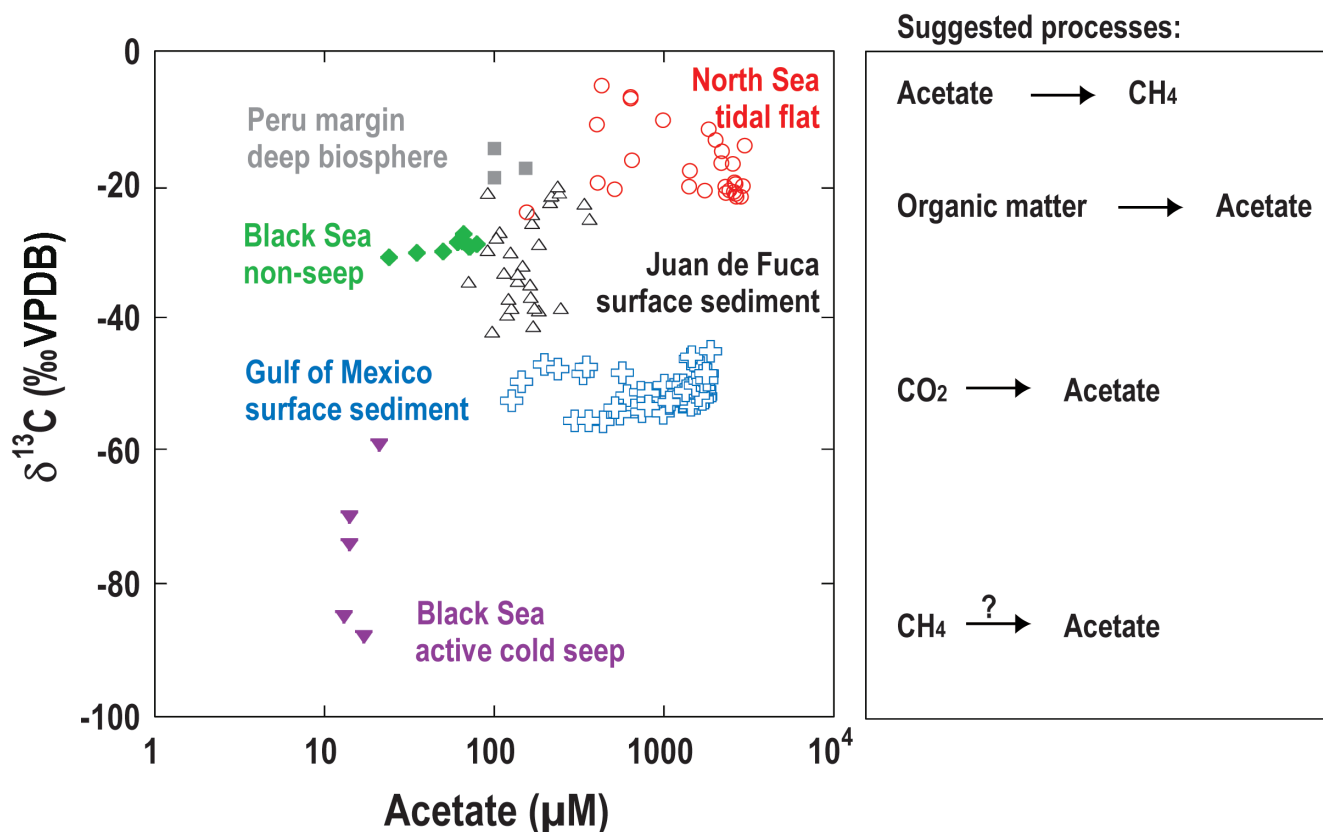


Fig. 3. Carbon isotopic variability of acetate observed in marine porewaters (closed symbols) and in incubation experiments with marine sediments (open symbols). Also shown are the biogeochemical processes related to acetate production and consumption. See text for more details.

al. 2005). Using a high flow rate of $500 \mu\text{L min}^{-1}$, we obtained precise and accurate $\delta^{13}\text{C}$ values for injections of individual VFAs equivalent to 50 to 2000 ng carbon (Fig. 2, Table 2). With a $50\text{-}\mu\text{L}$ sample loop, these amounts correspond to VFAs concentrations of 42 to 1670 μM (C_2), 28 to 1110 μM (C_3), and 21 to 833 μM (C_4). In general, samples that under typical analytical conditions would introduce > 2000 ng of carbon to the irm-LC/MS need to be diluted, whereas samples yielding less than 50 ng carbon need to be evaluated carefully. Decreasing the flow rate to $300 \mu\text{L min}^{-1}$ (as typically applied for porewater analysis) allowed us to obtain accurate $\delta^{13}\text{C}$ values for 10 μM solutions of acetate. In this study, peak areas < 2.8 Vs (corresponding to < 12.5 ng C delivered by acetate) did not yield accurate $\delta^{13}\text{C}$ values. The detection limit for quantitative analysis was found to be a peak area of 1.3 Vs resulting from 6.25 ng of C, i.e., introduced by injection of 5 μM acetate solution via a $50\text{-}\mu\text{L}$ sample loop.

Concentrating samples—Samples with VFA concentrations too low for irm-LC/MS measurement can be assessed if samples are concentrated by lyophilization prior to analysis. We have successfully tested this step (Fig. 2) and obtained precise $\delta^{13}\text{C}$ values for acetate ($-31.6\text{‰} \pm 0.9\text{‰}$, $n = 9$) and propionate ($-20.6\text{‰} \pm 0.8\text{‰}$, $n = 10$) after 12.5-fold concentration

of initially 2 to 20 μM aqueous solutions. The achieved precision is comparable to the precision of irm-LC/MS analysis without prior sample treatment (Table 2). Within analytical error, the $\delta^{13}\text{C}$ values obtained by irm-LC/MS after lyophilization agreed with the irm-EA/MS analysis conducted on the sodium salts of acetate ($-32.1\text{‰} \pm 0.0\text{‰}$) and propionate ($-20.5\text{‰} \pm 0.1\text{‰}$), indicating the preparative procedure does not result in isotopic fractionation (Fig. 2). However, the quantitative recovery of dissolved compounds was incomplete and variable. Therefore lyophilization cannot be used for quantitative analysis.

Application to natural samples/real-world problems—Our systematic analysis of porewaters and fluids from incubations of marine sediments (Fig. 3 and 4) demonstrates a wide range of $\delta^{13}\text{C}$ values for acetate, lactate, propionate, and butyrate and stresses both the need for and potential of further investigations of the carbon isotopic variability of VFAs. Using the outlined protocol, approximately 40 samples were analyzed per week with minimal manual sample handling. Samples were solely acidified to remove inorganic carbon prior to analysis if a co-elution of VFAs and DIC was observed.

Though we demonstrated the extension of the accessible concentration range to 2 μM levels of VFAs by concentrating

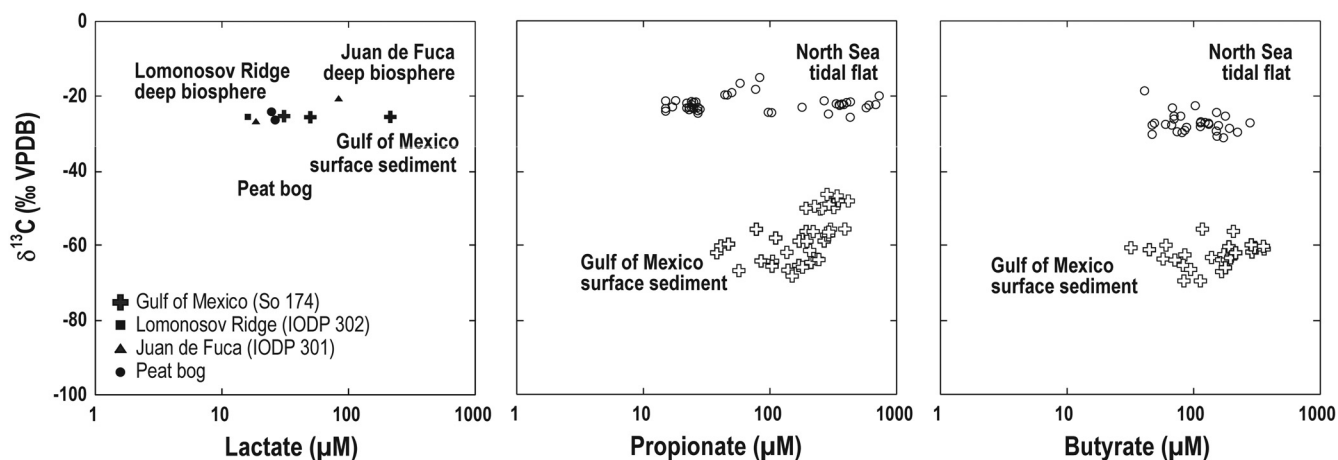


Fig. 4. Carbon isotopic variability of lactate, propionate, and butyrate observed in marine porewaters (closed symbols) and in incubation experiments with marine sediments (open symbols).

samples prior to irm-LC/MS analysis (Fig. 2), the application of this method to natural porewater samples with very low VFA concentrations is presently limited by the elution of an unidentified peak at the beginning of the chromatogram. At high concentrations, this peak tends to tail, which hampers analysis of lactate and acetate in the concentrated samples. Other stationary phases are probably required to optimize chromatographic separation.

Discussion

Irm-LC/MS is a highly promising technique to study the carbon isotopic composition of VFAs in aqueous samples from the natural environment. The method is precise, accurate, and provides a sensitivity that allows direct analysis without manipulation of VFAs in the samples. The protocol requires only minor sample treatment and minimizes the risk of isotopic artifacts resulting from sample preparations. While previously available methods aimed to selectively separate acetate off-line prior to its carbon isotopic analysis, the separation by HPLC coupled online to an irm-MS allows screening of other VFAs in the sample as well. However, intramolecular carbon isotopic analysis of VFAs, i.e., distinction between carboxyl- and alkyl-C (Gelwicks and Hayes 1990, Dias and Freeman 1997), cannot be performed because HPLC is not compatible with online pyrolysis. Our protocol enables high sample throughput and is suitable for routine analysis. Only small sample volumes (0.3-3 mL) are required and, thus, carbon isotopic analysis of VFAs can be integrated into routine porewater analysis programs (e.g., within the Integrated Ocean Drilling Program).

Our preliminary analysis of a wide range of sedimentary aqueous fluids has greatly extended the database on marine VFA carbon isotopic compositions and reveals a hitherto unknown variability in different geochemical environments (Fig. 3 and 4). For acetate, we observe $\delta^{13}\text{C}$ values in the range from $\sim -5\text{‰}$ to -85‰ (Fig. 3), i.e., a similarly large isotopic range as known from methane. Within this data population,

acetate is both significantly enriched and depleted in ^{13}C relative to sedimentary organic carbon. This large carbon isotopic range and its apparent relation to patterns of carbon flow are highly relevant for future examinations and interpretations of biogeochemical processes in anaerobic aquatic environments. For example, highest $\delta^{13}\text{C}$ values of up to -5‰ were found during incubation of North Sea sediment, which exhibited strong evidence for acetate consumption by acetoclastic methanogenesis (Fig. 3). In contrast, low $\delta^{13}\text{C}$ values of around -50‰ were found in incubation experiments of surface sediments from the Gulf of Mexico (Tille 2005). Supporting data (not shown) strongly suggest that in this system homoacetogenesis strongly contributes to acetate production. Notably, acetate is depleted in ^{13}C relative to TOC and DIC by about 27‰ and 21‰ , respectively, which is consistent with CO_2 -reduction rather than fermentation of sedimentary organic matter.

Carbon isotopic compositions of acetate between -15‰ and -30‰ as found in porewater samples of ODP Site 1230 (Peru Margin), surface sediments from the Black Sea, and incubations of sediments from the North Sea are presumed to reflect production of acetate by fermentation of sedimentary organic compounds combined with the consumption of acetate in these systems by processes with little or no isotopic fractionation. Acetate production in deep subsurface settings is a globally important process since this extreme environment is generally lacking small, utilizable organic molecules that can serve as carbon and energy substrates for the growth of microorganisms (Wellsbury et al. 1997). Incubation of a sediment sample from IODP Site U1301, eastern flank of the Juan de Fuca Ridge, yielded acetate with an isotopic composition between -20‰ to -40‰ and an isotopic relationship to other sedimentary carbon pools that suggest production of acetate by homoacetogenesis and organic matter fermentation with possibly somewhat greater importance of the latter process compared to the Gulf of Mexico sediments.

A special case are porewater samples from a methane seep environment in the Black Sea where turnover of ^{13}C -depleted methane carbon seems to affect the acetate pool, resulting in $\delta^{13}\text{C}$ values as low as -85% . The mechanism underlying the formation of such strongly ^{13}C -depleted acetate remains elusive, but plausible explanations involve a role of acetate as intermediate in the anaerobic oxidation of methane (cf Sørensen et al. 2001) and/or production of acetate from strongly ^{13}C -depleted organic or inorganic precursors.

Not only acetate exhibits a remarkable carbon isotopic variability; isotopic compositions of lactate, propionate, and the two combined butyrate isomers suggest that these compounds carry valuable biogeochemical information as well (Fig. 4). Whereas $\delta^{13}\text{C}$ values of porewater lactate appear largely similar to those of total organic carbon in various environments, values for both propionate and butyrate vary distinctly during incubation experiments with different sediments. Strikingly, in Gulf of Mexico sediments, propionate and butyrate co-vary with acetate but are even more depleted in ^{13}C than acetate in identical samples. We suggest that this might reflect a pathway of propionate and butyrate formation that is related to that of acetate in these samples.

These preliminary observations stress the potential of exploiting the carbon isotopic compositions of VFAs as proxies for biogeochemical processes. We foresee fruitful applications in areas of aquatic biogeochemistry where isotopic information from VFAs can provide important details on patterns of carbon flow in natural ecosystems, in particular when combined with isotopic information on other environmental carbon pools, i.e., DIC, TOC, methane, microbial biomass, etc. (e.g., Biddle et al. 2006). Complementary laboratory-based incubations will yield independent information on the potential for generation of VFAs and related pathways and, possibly, the presence of microbial functional groups involved in biogeochemical turnover of VFAs in sedimentary environments. Systematic evaluation of quantitative and isotopic patterns of VFAs in various environments and their relationship to the type of organic matter and microbial community composition will ultimately provide a more rigorous understanding of carbon cycling in the environment.

Comments and recommendations

Using the outlined method, we have successfully analyzed the carbon isotopic composition of acetate, lactate, propionate, and butyrate in natural sediment/porewater systems. Moreover, irm-LC/MS analysis of pure formate, malate, malonate, oxalate, pyruvate, and succinate in artificial seawater yield precise and accurate $\delta^{13}\text{C}$ values. While the latter five compounds were not detected in incubations experiments and natural samples, isotopic analysis of formate was obstructed by a coeluting unidentified peak. Further sample cleanup procedures and/or other chromatographic stationary phases will be tested to overcome this problem and extend the isotopic analysis to formate.

We expect that irm-LC/MS will allow carbon isotopic analy-

sis of other water-soluble biomolecules from natural environments as well. In the development of further methods, achieving baseline separation of the targeted analytes will be the most challenging analytical step since the Finnigan™ LC IsoLink strongly limits modifications of the chromatographic protocol. In particular, no organic solvents, organic buffers, or other carbon-bearing compounds (including inorganic carbon) can be used because all carbon-bearing compounds are detected. Care must be taken when operating with variable flow rates of the mobile phase. Changing flow rates of the mobile phase may require corresponding adjustment of the oxidant and acid flow of the system. In addition, the calibration for quantitative analysis should be verified. Ideally, the final reagent flow should produce enough O_2 to yield a voltage of 9 to 12 when monitored at m/z 32 on cup nr 2 (resistor: $3 \cdot 10^8 \Omega$). If flow rates of oxidant and acid are too low, analytical errors will be introduced because of incomplete oxidation of the analytes. In this study, we monitored O_2 and, if necessary, adjusted optimal flow rates manually at the beginning of each analytical sequence (i.e., once a day).

With respect to the stationary phase, column bleeding has to be considered. After installing new Reprosil-Pur C18-AQ columns (thoroughly equilibrated with MilliQ water on a separate HPLC system), we noticed an initial background signal intensity of up to 2.3 V for m/z 44, which decreased to a constant level of ~ 0.7 V within 5 to 7 days of operation. Thereafter, the analytical background was stable (background signal for m/z 44 changing $< 0.5\%$ per hour).

When interpreting $\delta^{13}\text{C}$ values of VFAs, further isotopic information on their potential precursors and products (e.g., DIC, TOC, methane) is important. Though we observe distinct DIC peaks in our chromatograms (Fig. 1), we do not recommend exploiting irm-LC/MS for $\delta^{13}\text{C}$ -DIC analysis, in particular because co-elution of organic carbon compounds cannot be excluded. For precise $\delta^{13}\text{C}$ -DIC analysis from small sample volumes (0.03–0.5 mL), we refer to the protocol by Torres et al. (2005).

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