Detection of microbial biomass in subseafloor sediment by pyrolysis-
GC/MS

Rong Zhu^{a*}, Gerard J. M. Versteegh^b, Kai-Uwe Hinrichs^a

^a *Organic Geochemistry Group, MARUM Center for Marine Environmental Sciences & Department of Geosciences, University of Bremen, Leobener Str., 28359 Bremen, Germany*

^b *Heisenberg Group Marine Kerogen, MARUM Center for Marine Environmental Sciences & Department of Geosciences, University of Bremen, Leobener Str., 28359 Bremen, Germany*

* Corresponding author. Present address: ETH Zürich, Department of Environmental Systems Sciences, Institute of Biogeochemistry and Pollutant Dynamics, Universitätstrasse 16, CH-8092 Zürich, Switzerland. Tel: (+41) 044-633-60-46; Fax: (+41) 44-633-11-22; *E-mail address*: rongzhu1018@gmail.com (R. Zhu).

14 ABSTRACT

15 The conventional approaches for qualitative and quantitative assessment of microbial
16 biomass in sediments, e.g. via quantitative polymerase chain reaction or intact polar lipids
17 analysis, are rather tedious and time-consuming. Here we present a new, optimized and
18 simple pyrolysis-gas chromatography/mass spectrometry protocol for rapid screening of
19 sediment samples for the presence of microbial signals and quantification of bulk population.
20 Analysis of microbial cultures of different bacterial and archaeal lineages as well as reference
21 substances and model compounds provided molecular fingerprints for tracking microbial
22 biomass. The diagnostic fingerprints consist of benzyl nitrile (derived from DNA and
23 protein), 2-furanmethanol (from DNA and peptidoglycan), indole (from peptidoglycan and
24 protein), phenol (from DNA, peptidoglycan and protein) and pyrrole (from peptidoglycan).
25 Detection of microbial signals through the molecular fingerprints requires a cell density of at
26 least 10^6 cells/g, whereas at least 10^7 cells/g is necessary for quantification. The pyrolysis
27 method was applied to marine sediments from different depths (until ~ 400 m below the
28 seafloor at the Canterbury Basin) and with different organic carbon contents (0.11-6.99%).

29

30 **Keywords**

31 Py-GC/MS, diagnostic fingerprints, microbial signals, marine sediments

32

33 **1. Introduction**

34 The conventional approaches for qualitative and quantitative assessment of microbial
35 biomass in sediments, e.g. via cell counting, quantitative polymerase chain reaction, or intact
36 polar lipids analysis, are rather tedious and time-consuming. Pyrolysis-gas
37 chromatography/mass spectrometry (py-GC/MS) may provide an alternative route since it

38 can characterize non-volatile organic macromolecules in complex matrices. Potential
39 advantages of a pyrolysis route for determination of biomass in environmental samples
40 include minimal sample preparation and sample size (submilligram), rapid analysis and
41 relatively low cost [1-3].

42 During the past decades, py-GC/MS has been successfully employed to investigate a
43 wide range of biological substances, such as DNA [4,5], proteins [6-8], chitin [9-11],
44 sporopollenin, algaenan and dinosporin [12]. Additionally, different strains or clades of
45 microorganisms can be identified and differentiated through ratios of diagnostic fragments
46 and multivariate statistics (e.g. principal component analysis, linear discriminant
47 analysis)[3,13-15]. For instance, the pyrolysis product 2-picolinamide has been identified in
48 Gram-positive (G₊) bacteria with a concentration approximately ten times higher than in
49 Gram-negative (G₋) bacteria and has been employed to assign bacterial Gram-type [16].

50 Tetramethylammonium hydroxide (TMAH) has been widely applied for online
51 methylation, enabling assessment of the highly polar structural fragments of macromolecules
52 [17-21]. As a strong base, TMAH is not solely involved in pyrolytic reactions but also
53 participates in high temperature saponification or transesterification [22]. However, the
54 methyl derivatives are formed not only through hydrolysis with TMAH but also through
55 methanolysis, with the latter reaction resulting from methanol serving as solvent of TMAH
56 [23,24]. The advantage of methylation of the fragments and the easy analyses on apolar GC
57 columns, comes with a lytic process (thermally assisted hydrolysis) which is very much
58 different from pyrolysis (thermolysis) process. Since we are primarily interested in detecting
59 low amounts of polar pyrolysis products, we choose to circumvent to use TMAH for
60 methylation and analyze the polar fragments as they have been produced by using a polar
61 column instead. The use of a polar GC column enables separation and quantification of the
62 released polar compounds (e.g. alcohols) without derivatization, which provides a direct way

for assessing the released polar compounds and enables further investigation of pyrolytic mechanisms.

Given the prior successes of pyrolysis on investigation of standard biological components and pure microbial cultures [e.g., 3,6,10,15], we aim to explore the application of py-GC/MS as an assay for direct detection and quantification of microbial biomass in complex marine sediments. Minimized sample preparation steps, application of a polar GC column and without TMAH derivatization enable this protocol rapid screening of microbial signals in environmental samples.

2. Material and methods

Exploration and validation of the py-GC/MS protocol for rapid assessment of microbial biomass in marine subseafloor sediments generally consisted of the following tasks. (1) We firstly optimized pyrolysis and GC programs and analyzed a variety of materials, including standard compounds, biopolymers and microbial cultures, to select diagnostic pyrolysis fragments with maximum recoveries as microbial fingerprints. (2) Microbial cultures were diluted in a concentration series with de-activated silica gel and analyzed using py-GC/MS to determine the detection limit of the microbial signals and validate the py-GC/MS approach. (3) We analyzed marine sediment samples from different depths and with different total organic carbon (TOC) contents to test the method for detecting microbial signals in the form of the microbial fingerprints.

2.1. Material

To obtain pyrolysis fingerprints for detection of microbial biomass, materials selected for analysis were (i) amino sugars (e.g. *N*-Acetyl-D-glucosamine, GlcNAc); (ii) biopolymers (e.g. chitin from crab shells, peptidoglycan from *Staphylococcus aureus*, peptidoglycan from

87 *Micrococcus luteus*, pBR322 DNA isolated from *Escherichia coli*, Bovine Serum Albumin)
88 and (iii) microbial cultures (three bacteria and seven archaea were selected to investigate the
89 compositional differences of pyrolysis fingerprints among diverse lineages of microbial
90 biomass. These bacteria belong to three bacterial orders in three bacterial phyla *Firmicutes*,
91 *Proteobacteria* and *Thermotogae* and include different Gram types; the selected archaeal
92 cultures are representatives of seven archaeal orders spanning the archaeal phyla
93 *Euryarchaeota* and *Crenarchaeota*; Table 1). The following low-molecular-weight
94 compounds were used as proxies for the quantification of microbial biomass: benzyl nitrile,
95 2-furanmethanol, indole, phenol and pyrrole (see Fig. 1 for the structures). Naphthalene-D8
96 was employed as an internal standard and added before analysis. All the standards, biological
97 material and the other chemicals were obtained from Sigma-Aldrich (Munich, Germany) and
98 MBI Fermentas GmbH (St. Leon-Rot, Germany). The microbial cultures were provided by
99 Prof. Michael Thomm (University of Regensburg, Germany), Prof. Christopher H. House
100 (Pennsylvania State University, USA), or from our lab collections. Sediment samples from
101 three locations, R/V Sonne cruise 147 Station 2MC, ODP Leg 201 Site 1229 and IODP
102 Expedition 317 Site U1352, were used during method development and validation (Fig. 2;
103 Table 2).

104

105 2.2. Sample preparation

106 Each sample was lyophilized over night, milled and homogenized using a CryoMill
107 (Retsch, Haan, Germany; milling conditions: frequency of rod vibration, 20 s⁻¹; milling
108 duration, 2 min; under liquid nitrogen) and loaded into a quartz tube (CDS Analytical Inc.,
109 Kamp Lintfort, Germany) prior to analysis.

The detection limit of the microbial signals was determined by diluting freeze-dried cultures of *Thermotoga maritima* and *Methanothermobacter thermoautotrophicus* with de-activated silica gel in a concentration series of 10^4 - 10^{12} cells/g using one order of magnitude intervals; the respective py-GC/MS analysis were performed from low to high cell concentrations with a blank between each analysis. Silica gel was pre-combusted at 450°C overnight for de-activation in order to impede any adsorption of pyrolysis fragments, and analyzed by py-GC/MS for blank check.

2.3. Instrumentation

Py-GC/MS was performed using a CDS Pyroprobe 5000 series system (CDS Analytical Inc.) coupled to an Agilent 7890 GC/5975C VL MSD in electron ionization mode (Agilent Technologies Deutschland GmbH, Böblingen, Germany) equipped with an Optima Wax column (25 m × 0.25 mm, 0.25 µm film thickness; Macherey-Nagel GmbH & Co. KG, Düren, Germany). The pyrolysis program was initiated at 200 °C and held for 1 s, then increased to 500 °C at 20 °C/min and held for 1 min. The GC temperature program was initiated at 50 °C and held for 0.5 min, increased to 200 °C at 4 °C/min and held for 5 min, then raised to 250 °C at 4 °C/min and held for 5 min, and finally decreased to 50 °C at 100 °C/min and held for 1 min. The MS was programmed in full scan mode (40-500 m/z) for characterization of the pyrolysis fragments and in selective ion monitoring mode for quantification. The ions selected for monitoring pyrolysis fingerprints were 67 (for pyrrole), 66, 94 (for phenol), 98 (2-furanmethanol), 90 and 117 (for benzyl nitrile and indole). The other pyrolysis fragments and the corresponding ions monitored are listed in Table 3.

3. Results

3.1. Pyrolysis fragments for tracking microbial biomass

A number of characteristic pyrolysis fragments have been identified from different biological sources in previous work [6,10,11,15,25,26,27,29,30,31,32] as well as in this study (see details in Table 3). The pyrolysis fragments were usually generated from multiple biopolymers, except for acetamidoacetaldehyde (only detected from GlcNAc), 2-picolinamide (only from peptidoglycan; Fig. 1), methyl-phenol, 3-methyl-butanenitrile and 4-methyl-pentanenitrile (from protein only). Most pyrolysis fragments were also detectable in the microbial cultures, with their relative abundances differing significantly (e.g. Fig. 3a, b, c). Levoglucosenone and acetamidoacetaldehyde could not be detected in the pyrolysis products of any of the microbes, whilst 2-picolinamide was detectable in all cultures except in the two archaeal strains *Pyrobaculum oguniense* and *Sulfolobus acidocaldarius*.

Clear differences in pyrolysate relative compositions were observed among microorganisms. However, multivariate analysis revealed no significant correlation between these differences and lineages of microbial biomass (data not shown). Five pyrolysis fragments, i.e. benzyl nitrile (from DNA and protein), 2-furanmethanol (from DNA and peptidoglycan), indole (from peptidoglycan and protein), phenol (from DNA, peptidoglycan and protein) and pyrrole (from peptidoglycan; Table 3), occurred in all pyrolysates and were selected as indicators for microbial biomass in sediments. Pyrolysis of these compounds exhibited highly linear relationships in a concentration range of 1-200 ng/μl for each standard and respective peak area (relative to the internal standard naphthalene-D8; Fig. 4).

3.2. Detection of microbial biomass in marine sediment

Analysis of microbial biomass *Thermotoga maritima* and *Methanothermobacter thermoautotrophicus* in a dilution series demonstrated that the detection limit using the

selected pyrolysis fragments was equivalent to a cell density of 10^6 cells/g (e.g. *Thermotoga*
maritima; Fig. 5). Concentrations of the selected pyrolysis fingerprints were nonlinearly
correlated with microbial biomass for cell densities between 10^6 - 10^9 cells/g, whereas they
increased linearly with cell densities above 10^9 cells/g. Relationships between the responses
of the combined pyrolysis fingerprints and cell density exhibited a similar trend as the
individual compounds, but the former started to increase at a cell density of 10^7 cells/g.
Pyrolysis of marine sediments from different depths and different TOC contents (Table 2)
showed that the selected pyrolysis products were detectable until a sample depth of ~ 400 m
below the seafloor (mbsf), with strongly different distributions of pyrolysis fingerprints
among the samples (e.g. Fig. 3d, e).

4. Discussion

The pyrolysis products generated from standards and biopolymers are generally
identical to those reported previously in studies of isolated chitin, protein, bacterial cell walls,
and whole microorganisms (Table 3). We note that most of these products are also produced
by pyrolysis of microbial biomass. The absence of levoglucosenone and
acetamidoacetaldehyde in microbial biomass is ascribed to the low proportions of their
precursors in combination with the limited sample sizes employed for pyrolysis.
Peptidoglycan, for example, only accounts for $< 2\%$ of the dry cell weight for *Escherichia*
coli [33]. Although the proportion of peptidoglycan can be up to eight-fold higher in Gram-
positive bacteria [34,35], levoglucosenone is still barely detectable due to the intense adjacent
peaks. In contrast, 2-picolinamide is more abundant than levoglucosenone in the pyrolysis
products of peptidoglycan, and consequently is detectable in the majority of investigated
microbial biomass samples. Since Archaea lack peptidoglycan [34,35], the presence of 2-
picolinamide both in bacteria and in most archaea suggests that 2-picolinamide also

originates from rearrangement of peptide-derived amino acid-residues, since the peptides are significant components in bacterial peptidoglycan and most archaeal cell walls [36].

Our selection of the pyrolysis fingerprints, i.e. benzyl nitrile, 2-furanmethanol, indole, phenol and pyrrole, for identification and quantification of microbial biomass, is based on the detectability of the compounds under our current analysis conditions. With the methodology applied, the minimum cell density required for tracking microbial signals, i.e. 10^6 cells/g, is consistent with the low range of cellular concentrations previously reported in marine continental margin sediments [37], whereas the total responses of the selected pyrolysis products suggest that the threshold for quantification of microorganisms requires $> 10^7$ cells/g. The nonlinear relationship between the cell numbers and the yields of the target compounds among 10^6 - 10^9 cells/g is presumably due to a concentration dependent kinetic mechanism involved in formation of these compounds by py-GC/MS. It also can be attributed to limitation of the dilution technique, i.e. the calculated cell densities may not be accurate after 10^7 -fold dilution. Microbial populations in surface sediments are in the orders of 10^8 - 10^9 cells [37,38], which fall in the range for reliable quantification of microbial biomass by the current py-GC/MS approach. However, implementation of the approach for accurate determination of microbial population sizes in deep marine sediments, where cell densities are presumed to be lower, will be a challenge. Unexpectedly, the selected biomass fingerprints are detectable in deeply buried marine sediment until ~ 400 mbsf (Fig. 3e), where cell density is less than 10^5 cells/g [39], suggesting that a proportion of microbial debris or microbe-related compounds are preserved in marine sediments and the total determined microbial signals include both active cells and necromass. This evidence also appears to the samples from the Peru margin and is in agreement with prior studies on intact polar lipids and amino acids [40,41]. In case this appears to be so, combination of py-GC/MS

and cell-counts could provide a way to separate between living and total microbial organic matter content.

5. Conclusion and outlook

By analyzing a wide range of biological material using py-GC/MS, five pyrolysis products, i.e. benzyl nitrile, 2-furanmethanol, indole, phenol and pyrrole, appear suitable for rapid detection and quantification of microbial biomass in marine sediment. Within a concentration range of 10^4 - 10^9 microbial cells per sample (equivalent to cell densities of 10^7 - 10^{12} cells/g), the GC/MS response of the selected pyrolysis products reliably reflects the concentrations of the source biomass, which validates the analytical approach. The detection limit of the approach is equivalent to a cell density of at least 10^6 cells/g, but at least 10^7 cells/g are necessary for quantification of microbial biomass. Analyses of sediment samples from different depths and with different TOC contents show that microbial signals can be tracked until ~ 400 mbsf at the Canterbury Basin (TOC 0.11%), indicating assemblages of active cells and necromass in the deep sediment. Combination of the pyrolysis-based approach with other analyses on microbial biomass using microscopy, lipids, DNA and RNA may provide an efficient way to shed more light on the contributions of active cells and necromass to the total organic matter in the deep biosphere and sediments in general. Combination with additional sample preparation steps (e.g. demineralization, density separation), or using a more advanced technique (e.g. multiple pyrolyses of one sample with cryotrapping, a more sensitive MS) may reduce the detection limit and enable accurate determination of microbial biomass in deeply buried sediments.

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359

Figure captions

Fig. 1. Structures of the selected pyrolysis fragments from microbial biomass: (I) pyrrole, (II) indole, (III) phenol, (IV) benzyl nitrile, (V) 2-furanmethanol, and (VI) 2-picolinamide.

Fig. 2. Maps of the investigated area and the sampling sites: (a) R/V Sonne cruise 147 Station 2MC and ODP Leg 201 Site 1229 at the Peru Margin; (b) IODP Expedition 317 Site U1352 at the Canterbury Basin.

Fig. 3. Partial py-GC/MS chromatograms of (a) Gram-positive bacterium *Acetobacterium woodii*, (b) Gram-negative bacterium *Thermotoga maritima*, (c) archaeon *Methanosarcina barkeri*, (d) a surface sediment sample from Peru Margin (2MC 0-1, 0.005 mbsf) and (e) a subsurface sediment sample from Canterbury Basin (Site U1352B, 48X3, 402.9 mbsf). The filled peaks indicate the selected pyrolysis fragments for tracking microbial signals (1: pyrrole; 2: 2-furanmethanol; 3: benzyl nitrile; 4: phenol; 5: indole).

Fig. 4. Calibration curves of selected pyrolysis fragments between concentrations and corresponding peak area ratios (relative to internal standard naphthalene-D8). Error bars represent standard errors from triplicate measurements. The error bars of abscissa are too small to be visible when using a log scale.

Fig. 5. Yields of the selected pyrolysis fragments upon pyrolysis of different numbers of *Thermotoga maritima* cells.

TABLES

Table 1.

Microbial cultures analyzed.

Domain	Phylum	Order	Species*	Strain
Bacteria	<i>Firmicutes</i>	<i>Clostridiales</i>	<i>Acetobacterium woodii</i> (G ₊)	WB1
	<i>Proteobacteria</i>	<i>Desulfovibrionales</i>	<i>Desulfovibrio gigas</i> (G ₋)	LeGall
	<i>Thermotogae</i>	<i>Thermotogales</i>	<i>Thermotoga maritima</i> (G ₋)	MSB8
Archaea	<i>Euryarchaeota</i>	<i>Archaeoglobales</i>	<i>Archaeoglobus fulgidus</i>	VC-16 ^T
		<i>Methanobacteriales</i>	<i>Methanothermobacter thermoautotrophicus</i>	Delta H ^T
		<i>Methanococcales</i>	<i>Methanothermococcus thermolithotrophicus</i>	SN-1 ^T
		<i>Thermococcales</i>	<i>Pyrococcus furiosus</i>	Vc 1 ^T
		<i>Methanosarcinales</i>	<i>Methanosarcina barkeri</i>	MS ^T
	<i>Crenarchaeota</i>	<i>Thermoproteales</i>	<i>Pyrobaculum oguniense</i>	TE7
		<i>Sulfolobales</i>	<i>Sulfolobus acidocaldarius</i>	98-3 ^T

* Bacterial Gram-types in parentheses; G₊, Gram-positive; G₋, Gram-negative.

Table 2.

Marine sediment samples analyzed.

Location	Site	Depth (mbsf*)	TOC (%)
Peru Margin	R/V Sonne cruise 147		
	Station 2MC	0.005	3.96
	ODP Leg 201	1.4	6.99
	Site 1229	102.2	2.28
Canterbury Basin	IODP Expedition 317 Site U1352	1.1	0.48
		11.6	0.13
		40.1	0.11
		137.3	0.18
		231.4	0.18
		403.0	0.20
		633.4	0.26
		816.3	0.15
		1113.4	0.15
		1164.2	0.30

* m below the seafloor.

Table 3.

Selected pyrolysis fragments identified from the standards and microbial biomass in this study.

Formula	Pyrolysis fragment	Ions (m/z)	Major source ^a				
			Protein	Peptidoglycan	DNA	Chitin	GlcNAc ^b
C ₈ H ₈	styrene	78, 104	25, 26, 30	*			
C ₆ H ₆ O	phenol ^c	66, 94	6, 15, 25, 29, 30, *	*	*		
C ₇ H ₈ O	methyl-phenol	77, 90, 107	6, 15, 25, 29, 30				
C ₂ H ₄ O ₂	acetic acid	43, 60	*	*	*	10, *	
C ₃ H ₆ O ₂	1-hydroxy-2-propanone	43, 74	*	*		10	
C ₅ H ₆ O ₂	2-furanmethanol ^c	98		15, *	15, *		
C ₆ H ₆ O ₃	levoglucosenone	53, 98		*		10, *	
C ₅ H ₉ N	3-methyl-butanenitrile	43, 68	25				
C ₆ H ₁₁ N	4-methyl-pentanenitrile	43, 55, 82	25				
C ₈ H ₇ N	benzyl nitrile ^c	90, 117	15, 25, 26, *		25		
C ₅ H ₅ N	pyridine	52, 79	15, 25, 32		15	10, 11, *	31
C ₆ H ₇ N	methyl-pyridine	66, 78, 93	25				31
C ₄ H ₅ N	pyrrole ^c	67	25, 30, *			11	
C ₅ H ₇ N	methyl-pyrrole	80, 81	25, 32			11	
C ₈ H ₇ N	indole ^c	90, 117	6, 25, 26, 29, 30, 32	*			
C ₂ H ₅ NO	acetamide	44, 59	*	27, 32, *	*	10, 11, *	

$C_4H_7NO_2$	acetamidoacetaldehyde	43, 72, 73	28, *
$C_6H_6N_2O$	2-picolinamide	79, 122	16, *

^a Numbers refer to studies reported sources of the corresponding pyrolysis fragments. *, this study.

^b GlcNAc refers to N-Acetylglucosamine.

^c Compounds selected for identification and quantification of microbial biomass.

Figures

I

II

III

IV

V

VI

Fig 1

sum of five compounds

pyrrole

2-furanmethanol

benzyl nitrile

phenol

indole

Fig 5