Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1071 (2005) 99–109
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Characterization of non-discriminating tetramethylammonium hydroxide-induced thermochemolysis–capillary gas chromatography–mass spectrometry as a method for profiling fatty acids in bacterial biomasses

Juergen Poerschmann[a,*], Ziba Parsi[b], Tadeusz Górecki[b], Juergen Augustin[c]

[a] *Center for Environmental Research, Permoserstrasse 15, 04318 Leipzig, Germany*
[b] *Department of Chemistry, University of Waterloo, Waterloo, Ont., N2L 3G1 Canada*
[c] *Leibniz-Centre for Agricultural Landscape and Land Use Research, Department of Primary Production and Microbial Ecology, D-15374 Muncheberg, Germany*

Available online 27 October 2004

Abstract

Thermochemolysis using tetramethylammonium hydroxide (TMAH) as a method for profiling fatty acids (FAs) as methyl esters (FAMEs) was studied with respect to discrimination of bacterial fatty acid patterns in Gram-negative *Pseudomonas putida* in comparison with patterns obtained by classical preparation schemes (pressurized solvent extraction, alkaline saponification). A new, non-discriminating pyrolysis approach was used in these experiments. In this method, pyrolysis is carried out inside a deactivated stainless steel capillary constituting a part of the column train. This approach made it possible to distinguish analyte discrimination brought about by (i) the pyrolyzer set-up in conventional pyrolysis and (ii) chemical thermochemolysis reaction itself. Our results showed no significant discrimination for saturated, monounsaturated and cyclopropane FAs when using thermochemolysis temperatures up to 550 °C with the non-discriminating, capacitive-discharged based approach. Likewise, the *cis*/*trans* ratio of monounsaturated FAs was preserved. This is in sharp contrast to conventional pyrolysis systems (both Curie-point or flash pyrolyzers) using much longer thermochemolysis times. When using these systems, artificial monounsaturated FAMEs were formed during heat treatment resulting in biased species identification/classification. Hydroxy-FAs, chiefly bound covalently in lipopolysaccharides, could be almost quantitatively liberated by TMAH using non-discriminating pyrolysis (beyond 90%), whereas the recoveries were lower with conventional approaches. The new method makes it possible to profile fatty acids in very small amounts of both solid and liquid samples without significant sample preparation. This was exemplified by the results of TMAH-thermochemolysis of a fermentation broth taken from an in situ bioremediation reactor to monitor enhanced natural attenuation processes, and tomato roots which were subjected to cellulolytic bacteria attacks (*Streptomyces*), resulting in adsorption of these carboxymethyl-cellulose decomposing bacteria onto the root.

Keywords: Bacteria; Fatty acids; Non-discriminating pyrolysis; Thermochemolysis; Tetramethylammonium hydroxide

1. Introduction

Fatty acids (FAs) profiling is very useful in the framework of bioremediation work [1–3], medical microbiology and other areas. Brand new applications including bioterrorism monitoring are being developed. Chemotaxonomic protocols operating on the molecular level do not necessarily require isolation and cultivation of the microorganisms. This is a significant advantage when investigating microorganisms that elude isolation/cultivation. As estimated by White [4], viable counts of bacteria in environmental samples determined on the basis of classical methods account for only a small fraction (0.1–10%) of the active microbial community (cf. also [5] and refs. cited therein). Therefore, most species in microbial matrices may never have been char-

* Corresponding author. Tel.: +49 341 235 2902; fax: +49 341 235 2492.
E-mail address: juergen.poerschmann@ufz.de (J. Poerschmann).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.10.010

acterized. Beyond this, cultivation is said to result in biased results due to selectivity of the culturing techniques [6].

Established preparation schemes for FA profiling include (i) organic solvent extraction followed by isolation of phospholipids (e.g. by solid-phase extraction or selective extraction of lipid classes using different pressurized liquid extraction regimes) or (ii) saponification of the bacterial biomass [7,8]. It was conclusively demonstrated that phospholipid FA protocols satisfy the requirements for cellular components to serve for microbial community structure analysis, as they fulfil the following requirements: (i) to show phylogenetically restricted distribution, (ii) to show quick turnover rates in detrital pools after excretion by cell death (brought about by exogenous and endogenous phospholipases to result in the formation of diacylglycerines in the case of phospholipids), (iii) to occur at relatively constant proportions in all cells, (iv) the proportion to not vary throughout the growth cycle (refs. [8,9] and refs. cited therein). Thus, phospholipid FAs provide an in situ indication of starvation, growth rate, exposure to toxicity, deficiencies of specific nutrients, aerobic/anaerobic metabolic balance, etc. [8]. Alternatively or in combination with lipid analysis, nucleic acid technologies using rDNA (or rRNA including 16S and 23S, which possess better turnover times, but are characterized by larger changes with growth state) might be used [8,10]. The "whole-cell" saponification method does not allow differentiating between fatty acids located in carbon- and energy-storage lipids on the one side (e.g. acylglycerines [11]) and membrane phospholipids in intact cellular membranes on the other side, but allows drawing conclusions about species identification and classification. Both strategies come closer together if phospholipids account for the overwhelming majority of the total lipids.

An alternative technique to whole-cell saponification is tetramethylammonium hydroxide (TMAH)-induced thermochemolysis, which produces fatty acid methyl esters (FAMEs) [12]. The method is also called sometimes "thermally assisted hydrolysis and methylation" (THM). The thermochemolysis technique is said to suffer from biased results [13,14], although – to the best of our knowledge – comprehensive methodological studies with fatty acids (including quantification) have not been carried out up to now. Biased results can be caused either by discrimination of the long-chain FAMEs due to the set-up of the conventional pyrolysis units (see refs. cited in [15]) or by discrimination due to chemical reactions (saponification, methylation) brought about by the strong alkaline TMAH reagent at the applied thermochemolysis temperature. To overcome the limitations related to the former issue, a non-discriminated pyrolysis approach was introduced recently [15,16]. The basic idea of this approach is to perform pyrolysis in a deactivated stainless steel capillary connected in-line with the GC column. The pyrolysis capillary can be heated very rapidly (∼50,000 °C/s) by capacitive discharge. The pyrolysis time in this approach (∼15 ms) is much shorter in comparison with commercial Curie-point and flash pyrolyzers (5 s as a rule). Elimination of dead volumes and cold spots in the system, combined with forced flow of the carrier gas through the pyrolyzed sample and the high heating rate, make it possible to detect high molecular weight pyrolysis products possessing high diagnostic value (in contrast to "common" pyrolysis products like phenol, which may have numerous sources/precursors). Other approaches to THM have also been tried. For example, Blokker et al. [17] introduced so-called "at-line GC–MS analysis", in which a biological sample (aqueous suspension or freeze-dried biomass) is injected into a micro-vial inside a deactivated, direct thermal desorption liner, following which the thermochemolysis reagent is added. The THM is performed by placing the liner in a programmed temperature vaporizer (PTV) injector and rapidly heating it to a high temperature. While this approach yielded good results, we believe that the method proposed in this paper performs even better due to a combination of the forced auxiliary gas flow through the pyrolysis tube and the extremely high heating rate (∼50,000 °C/s compared to the maximum heating rate of the PTV injector of about 15 °C/s), which results in very short thermochemolysis time (few milliseconds). This short time prevents many secondary reactions from occurring, leading to what we believe is non-discriminating thermochemolysis.

By minimizing the discrimination associated with the instrumental set-up, the technique of non-discriminating pyrolysis (thermochemolysis) makes it possible to scrutinize the "chemical" discrimination. This type of discrimination may have two sources related to the dual function of the TMAH reagent, i.e. saponification and methylation. The latter is thought to consist of two steps, viz. formation of quaternary salts with the deprotonated fatty acids, followed by thermal decomposition of the salts to form methyl esters. In this contribution, discrimination attributed to TMAH-related methylation was studied using free fatty acids in commercial standard solutions as targets. Discrimination attributed to both saponification and methylation was studied by thermochemolysing bacterial biomass. Special attention was paid to study the conservation of the original *cis*/*trans* configuration and the position of the double bond in the monounsaturated FA. These two issues have been underestimated in pyrolysis/thermochemolysis work up to now, but are considered to be essential when (i) studying stress phenomena of microbial communities, which result in altered *cis*/*trans* configurations [18–20], or (ii) detecting bacterial/terrestrial origin of lipids in biological matrices. The validation criterion used in this study was the identity of the fatty acid patterns obtained by thermochemolysis with those obtained by classical alkaline saponification preparation scheme for *Pseudomonas putida*. The method of choice to locate the double bond position in monounsaturated fatty acids was thioether formation, introduced into analytical practice in the 1980s (cf. ref. [21] and refs. cited therein).

2. Materials and methods

2.1. Alkaline saponification of the biomass

Twenty-five milligram freeze-dried *P. putida* biomass was saponified with 5 mL 1 M methanolic sodium hydroxide for 1.5 h. The alkaline solution was acidified with phosphoric acid to pH ∼2 to release free fatty acids. An internal standard (1000 ppm d_2(2,2′)-palmitic acid; Promochem, Wesel, Germany) was added, then the methanolic-aqueous solution was extracted with benzene three times, and the extracts were combined The organic extract containing the acids was volume reduced using TurboVap II (Zymark; Idstein, Germany) to give a volume of 250 μL, then purified, dried and methylated with boron trifluoride-methanol (Sigma–Aldrich, Munich, Germany; for more details, see ref. [22]). Likewise, bacterial standard FAME mix (Supelco, Munich, Germany) was saponified using 1 M NaOH to obtain free acids for the methodological studies.

2.2. Pressurized liquid extraction

Twenty-five milligram *P. putida* was placed in the cartridge of a commercial pressurized liquid extration (PLE; Dionex trade name ASE for accelerated solvent extraction) system (ASE 200, Dionex, Idstein, Germany) (cf. [23]). The cartridge was filled to capacity with quartz sand (free of extractables). The cell was pressurized to 14 MPa; extraction was performed with chloroform–methanol (1:1, v/v) at 120 °C for 15 min (2 cycles). Internal standard [1000 ppm d_2(2,2′)-palmitic acid] was added to the combined chloroform–methanol mixture. The solvent mixture was washed with water, the organic phase dried with sodium sulfate, and volume reduced. Saponification of the extract was done using 1 M methanolic sodium hydroxide for 30 min. Methylation was performed using boron trifluoride-methanol.

2.3. Formation of thioether adducts

The procedure was performed according to ref. [21]. Briefly, 50 μl dimethyl disulfide (DMDS) and 10 μL iodine solution in diethyl ether (60 mg in 1 mL) were added to the FAME mixture dissolved in benzene and allowed to stand for 48 h at 55 °C. After cooling, 100 μL of sodium thiosulfate solution (5% in water) were added to remove excess iodine catalyst.

2.4. Thermochemolysis

The non-discriminating pyrolysis system used in the research [24] was a modified version of the in-column pyrolysis system first described in refs. [15,16]. A schematic diagram of the system is presented in Fig. 1. Pyrolysis was carried out in 0.53 mm i.d. deactivated stainless steel capillary tubing (Silcosteel, Restek). The sample was placed inside the tubing between two plugs of quartz wool, which in turn were kept in place by pinching the tubing slightly with wire cutters. The exact amount of the sample was determined by weighing the capillary with and without the sample. Average biomass loading was about 25 μg. Quantitation of the pyrolysis products was conducted by injecting 1000 ppm d_2-palmitic acid referred to the amount of the sample loaded into the pyrolysis capillary. For thermochemolysis, two 0.5 μL aliquots of the derivatizing reagent solution were injected into the pyrolysis tube with a syringe, one through the top and one through the bottom of the tube, thus ensuring efficient “pre-wetting” of the sample (including the internal standard). The experiments were carried out with three different reagent solutions: 25% TMAH, 2.5% TMAH and 2.5% trimethylsulfonium hydroxide (TMSH), all in methanol. The derivatizing reagent solution was added to the capillary directly before the analysis. TMAH solutions were prepared fresh every day. In the case of the 25% TMAH solution used in most of the experiments, the TMAH/sample mass ratio was about 10:1 (assuming a sample mass of 25 μg).

The upstream end of the tubing prepared in such a way was connected through a 100 μm fused silica capillary restrictor to an auxiliary carrier gas supply, while the downstream end was inserted through the injector septum into the injector of a gas chromatograph (cf. Fig. 1). For pyrolysis, a set of electrical connectors was attached to the tubing, which was then heated at a very high rate (∼50,000 °C/s) to the desired temperature by passing a pulse of electric current from a custom-made capacitive discharge power supply. Once the sample was pyrolyzed, the electrical connectors were detached and the entire pyrolysis capillary was pushed into the injector (kept at 300 °C) to thermally desorb the products that may have been sorbed onto cold spots in the tubing and/or onto the solid pyrolysis residue, if any. The capillary was pulled out of the injector after a predetermined time (typically 2 min).

To detail the discrimination of free fatty acids on thermochemolysis, 1 μL of hexane solution containing 500 ppm each of individual free fatty acids was loaded into the Silcosteel pyrolysis capillary. For a comparison between the non-discriminated pyrolysis and flash pyrolysis, a conventional flash-pyrolyzer (Pyroprobe CDS 1000, CDS, Cambridge, USA) was used. The pyrolysis time was set to 10 s in this experiment [25]. For reasons of comparison between the two systems, a similar TMAH reagent/sample ratio was used. A JHP-3f Curie-point pyrolyzer (Japan Analytical Industry) was also used in the comparison studies. Thermochemolysis experiments with the conventional pyrolyzers were run using split injection mode (1:10) to ensure efficient flushing of the interface chamber. Split mode was also applied with the capacitive discharge approach.

The GC–MS analysis parameters were the following: 30 m × 0.25 mm, 0.25 μm DB-5, DB-225 or FFAP (Agilent Technologies, Waldbronn, Germany) or SP-2330 (Supelco) columns; temperature programmed mode (initial temperature 50 °C held for 2 min, followed by linear temperature increase at 8 °C/min to the final temperature of 260 °C with

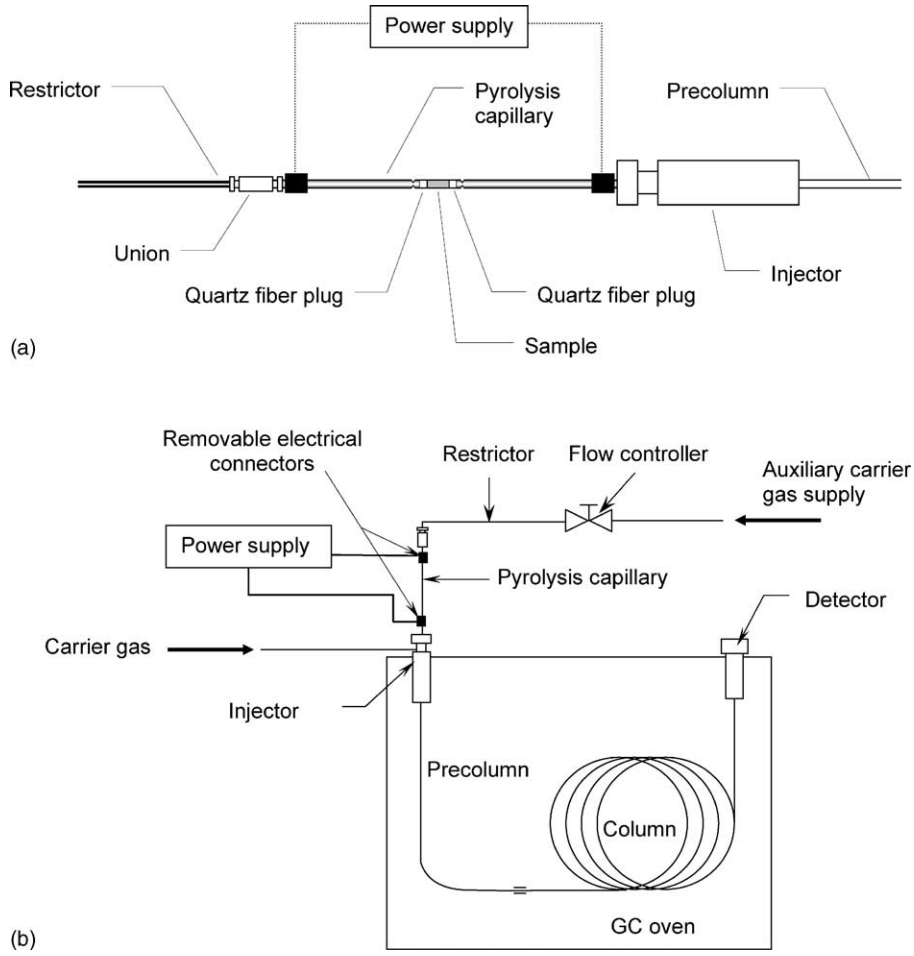

Fig. 1. Schematic diagram of the non-discriminating pyrolysis system, including a detailed view of the (a) pyrolysis tubing and its connections (rotated view) and (b) the outline of the entire system.

polar cyanopropyl phases as well as FFAP and 300 °C with DB-5); split/splitless injector (300 °C), Agilent 6890, Agilent MSD 5973B, data acquisition in Scan mode, if not otherwise stated.

3. Results

3.1. Discrimination of standard FAME mixtures using the new system

To identify the contribution of TMAH methylation towards discrimination at common thermochemolysis temperatures, free bacterial acids were thermochemolyzed with TMAH using the capacitive-discharge approach. In addition, TMSH (considered to be "milder" than TMAH [26,27]), was also studied as the thermochemolysis reagent. Fig. 2 presents – in a mirror representation – a comparison of the FAME profiles obtained by methylation of the free FA mixture using boron trifluoride-methanol (top) and by TMAH thermochemolysis (bottom). The results demonstrated that saturated fatty acids (including homomorphic and branched), as well as cyclic and monounsaturated acids did not undergo any discrimination during TMAH-induced methylation (recovery 91–105%; data not shown). Methylation reaction turned out to be complete. No free fatty acids could be detected under the conditions applied even with FFAP polar stationary phase, on which free fatty acids elute as sharp, non-tailing peaks. In slight contrast to the quantitative transformation of saturated/monounsaturated/cyclic members, the hydroxy acids revealed small discrimination (less than 10%), most likely related to residual activity in the chromatographic system. The contribution of methoxy derivatives of 2-OH-FAMEs was very small (less than 5% compared to the corresponding FAMEs with free OH-group in the alkyl chain); in the case of 3-OH-FAMEs, no methoxy derivatives were formed. Methodological studies with free standard hydroxy acids (2-OH-12:0 and 3-OH-12:0) provided strong evidence that in both cases slight defunctionalisation occurred, giving rise to breakdown products, including a 12:1 aldehyde and a cluster of 12:1 FAMEs (the latter formed by dehydration). Methylketones, supposed breakdown products of OH-functionalized fatty acids as reported in [28], could not be observed. However, it should

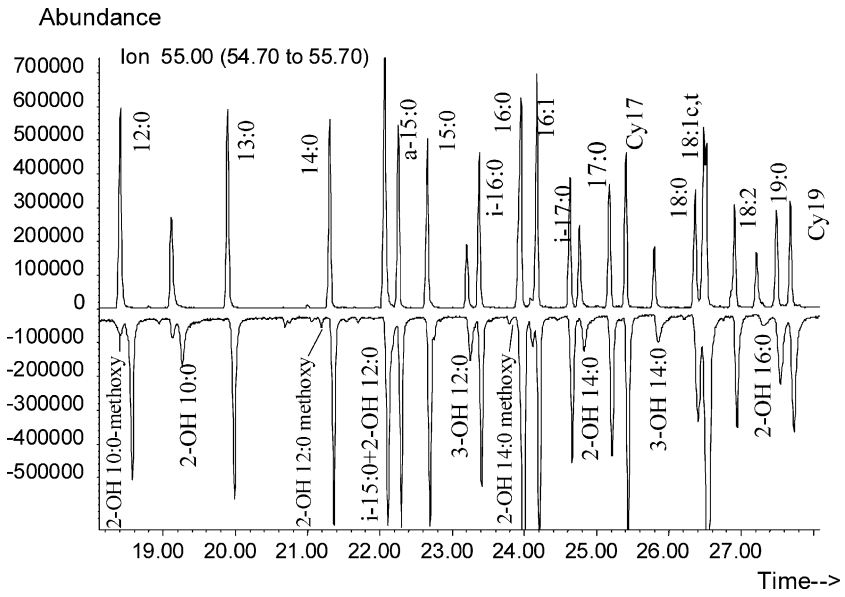

Fig. 2. Bacterial fatty acids methylated by boron trifluoride-methanol (top) and by TMAH-induced thermochemolysis at 500 °C (bottom). Extracted ion *m/z* 55; intensity of the ion chromatograms normalized to the internal standard; stationary phase DB-225.

be pointed out that the experiments reported in [28] were performed with the OH group being in ω9 and ω12 position. Additional experiments demonstrated that discrimination to a similar extent occurred also when subjecting methyl esters of 2-OH-12:0 and 3-OH-12:0 acids to thermochemolysis. Thus, the TMAH attack at 500 °C gave rise to ∼15–20% discrimination independently of the nature of the target analyte (free acid or methyl ester). The discrimination got worse when TMSH was used as the thermochemolysis reagent. Most likely, the claim that TMSH is a milder reagent than TMAH [26] refers to the protection of the GC-column, because the dimethyl sulfide formed during THM using TMSH is not harmful to the column stationary phase.

3.2. Discrimination of the fatty acid pattern during thermochemolysis of the biomass.

Table 1 illustrates the FA patterns obtained by thermochemolysis (TMAH 25% and TMAH 2.5%, 500 °C) along with patterns obtained by (i) alkaline saponification and (ii) PLE followed by saponification (fatty acid methyl ester listed in shorthand designation in Table 1; e.g. 12:0 for lauric acid methyl ester). Minor analytes including cyclo 19, branched

Table 1
Fatty acid pattern of *Pseudomonas putida* using alkaline saponification and thermochemolysis at 500 °C (in ppm) ($n = 3$, R.S.D. between 10 and 22% for thermochemolysis, between 5 and 11% for PLE/saponification)

FAME[a]	Alkaline saponification	PLE/saponification	Conventional 25% TMAH	In-column 25% TMAH	In-column 2.5% TMAH
n12:0	1330	1310	1180[b]	1220	890
n14:0	165	145	170	150	104
3OH-10:0[c]	1585	55	1020	1250	260
2OH-12:0[c]	435	n.n.	330	380	48
n16:0	8230	7190	8720	8380	3030
Σ16:1	3420	3270	2975	3425	1140
Cy17	2195	2030	1910	2040	1080
n18:0	210	190	230	210	110
Σ18:1	1005	955	760	955	470
ΣFAME	18740	15415	17670	18140	7220
ΣSaturated	10015	8930	10450	10120	4190
Σn:1	4510	4340	3770	4410	1610
ΣSaturated/Σ FAME	0.535	0.58	0.59	0.56	0.58
Σn:1/Σ FAME	0.24	0.28	0.21	0.25	0.22
3OH/Σ FAME.	0.08	0.003	0.06	0.07	0.04
ΣCy17/Σ FAME	0.12	0.13	0.11	0.11	0.15

[a] Shorthand designation.
[b] External calibration using palmitic and myristic acid ME with conventional pyrolysis, due to difficulties in calibrating with internal standards when using hot pyrolyzer interfaces.
[c] Free hydroxy group in the alkyl chain when using capacitive discharge; methoxy derivative when using the Curie-point or flash pyrolyzers.

15:0 and 3-OH-12:0 were not considered explicitly in Table 1. To exclude sample heterogeneity, the freeze–dried biomass was homogenized and five subsamples were taken, all of them subjected to PLE (cf. Section 2). The FA patterns obtained for the subsamples were very similar to each other, with the R.S.D. values typically around 10%.

3.2.1. Saturated, monounsaturated and cyclic fatty acids

Thermochemolysis with the new system using 25% TMAH methanolic solution gave very similar results to those of alkaline saponification and PLE/alkaline saponification. It should be pointed out that as our experience with biomasses indicates, the PLE data might have been too low by ∼2–5%, because only two extraction cycles were performed. When 2.5% TMAH was used (of about 1:1; cf. Table 1), generally lower recoveries were obtained for all analytes. The lower ratio was used to evaluate the effect of the TMAH amount on the completeness of thermochemolysis, as well as to facilitate comparisons with literature data. For example, similarly low TMAH/bacterial biomass ratios were used in ref. [29], and a 0.2 M solution of TMSH (roughly 2%) was applied in ref. [26]. The lower FAME recoveries observed for the lower TMAH concentration point to insufficient "thermal saponification" of bound fatty acids caused by the low TMAH/biomass ratio. Free fatty acids could not be observed. Our experiments indicated that conventional approach with 25% TMAH (10 s pyrolysis time) – both with Curie-point (thermochemolysis temperature 530 °C) and flash pyrolyzers (500 °C) – revealed slight, but significant (10–20%) discrimination of monounsaturated fatty acids. Since the degree of saturation is one of the decisive parameters for microbiological considerations (cf. [20]), fatty acid profiles obtained by TMAH-induced thermochemolysis with conventional setups should be considered with some caution. This is further evidenced by the formation of additional isomeric monounsaturated FAMEs. As detailed below, the *P. putida* under study possessed only two significant pairs of monounsaturated FAMEs: 18:1ω7c/18:1ω7t and 16:1ω7c/16:1ω7t. However, we could detect two additional monounsaturated 18:1 isomers among the products of thermochemolysis performed with flash and Curie-point pyrolyzers when using the SP-2560 polar cyanopropyl stationary phase characterized by high selectivity towards unsaturated FAMEs. This peak cluster of 18:1 isomers could not be resolved using non-polar stationary phases. Thus, this unwanted isomerization, negatively affecting the structural information and microbial community structure analysis, might have been overlooked, because most of the thermochemolysis work reported on in the literature up to now has been performed with non-polar phases, considering monounsaturated FAMEs of the same carbon number as a total measure. Similar phenomena were observed at different thermochemolysis times (5, 15 s) or temperatures. It should be emphasized that no such isomerization was observed when using the capacitive discharge approach, i.e. the pattern of monounsaturated isomers (in this case the ratio between n:1ω7c/n:1ω7t) mirrored precisely the pattern

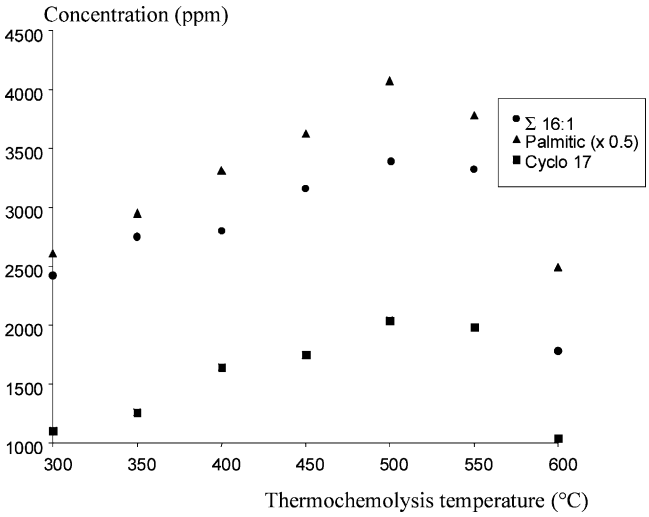

Fig. 3. Selected FAME concentration profiles as a function of thermochemolysis temperature (data for FAME 16:0 multiplied by a factor of 0.5 for reasons of better presentation).

obtained when using solvent extraction. This is a significant advantage over traditional flash and Curie-point pyrolyzers. The reasons for this difference are unclear; one possible explanation is the lower amount of energy supplied by the capacitive discharge heating. However, this hypothesis should be addressed elsewhere.

Fig. 3 depicts the concentrations of selected analytes (16:0, cyclo 17, Σ16:1) after TMAH thermochemolysis as a function of pyrolysis temperature for the non-discriminated pyrolysis system. The temperature profiles were very similar to each other; the remaining analytes listed in Table 1 shared similar dependencies. Optimum temperature turned out to be in the range of 450–550 °C. Although the strongly alkaline TMAH reagent allows thermochemolysis to be performed at sub-pyrolysis temperatures [30], the temperature of 300 °C was definitely too low for the capacitive-discharge approach with regard to thermochemolysis efficiency. Incomplete saponification most likely accounted for this finding (as no free acids were observed), whereas unwanted degradation side reactions were probably responsible for losses at temperatures above 550 °C.

3.2.2. Hydroxy acids

High concentrations of 3OH-10:0 and 2OH-12:0 obtained on saponification of the biomass sample, along with low values obtained for PLE followed by alkaline saponification (see Table 1), indicate that these acids were covalently bound in lipopolysaccharides [31,32], thus eluding solvent extraction. These bound FA are of utmost interest when assigning taxonomic groups [33]. For example, 3-OH-14:0 FA points to waterborne Gram-negative pathogens; water biofilms including *Pseudomonas* have 3-OH-10:0 and 3-OH-12:0 FA [7]. As shown in Table 1, TMAH-induced thermochemolysis could release hydroxy acids from this "network" almost completely (cf. 3OH/Σ FAME ratio: 0.08 versus 0.07 in Table 1).

Thus, the discrimination with the capacitive-discharge approach was very similar to that observed with methylation of free hydroxy fatty acids (cf. above). This finding provides evidence that the saponification reaction, i.e. the space release of the covalently bound hydroxy acids from the polymeric network, is quantitative. Shifting to lower TMAH-concentrations (to make methylation reaction milder, thus expecting less discrimination) turned out to be counterproductive in terms of recoveries (see Table 1). Following this line, findings reported in [29], according to which TMAH-thermochemolyzed *E. coli* cells did not reveal the characteristic 3-OH-14:0 FAME, might be interpreted in terms of insufficient TMAH reagent/sample ratio. Another crucial point is to flush the thermochemolyzate rapidly out of the pyrolysis chamber, so as not to allow the susceptible analytes to react or to sorb onto active sites. Data in Table 1 provide evidence that utilization of a conventional pyrolysis device resulted in slightly lower recoveries for hydroxy surrogates. In this case, they occurred in the form of methoxy derivatives, which was also observed for off-line thermochemolysis [34].

As with hydroxy FA located in lipopolysaccharides, our method is also capable of detecting monomeric hydroxy-FA occurring in poly-β-hydroxyalkanoic acids in both freeze-dried biomass and fermentation broth. Bacteria which undergo unbalanced growth in the absence of essential nutrients (phosphate, nitrate) can form these polymers. Therefore, detecting monomeric 3-hydroxy acids including 3-hydroxy butyric acid is of special importance in monitoring and controlling the fermentation processes [35]. In the *P. putida* under study, no such polymers were formed.

3.2.3. Preservation of the double bond location and configuration

The *P. putida* FA pattern is characterized by high amounts of 16:1 and 18:1 monounsaturated FA, and a strong even-over-odd discrimination (cf. Table 1). Unambiguous identification of monounsaturated FAMEs by GC–MS with regard to the location and configuration of the double bonds is very difficult due to their migration during electron-impact fragmentation process [36–38]. Beyond this, baseline separation of the pairs of monounsaturated FAMEs gets more difficult when the abundances of the monounsaturated FAMEs possessing similar equivalent chain length (ECL) values are very different. Formation of thioethers of monounsaturated FAMEs using dimethyl disulphide (DMDS) in the extracts obtained by PLE and alkaline saponification allows the detection of the position of the double bond in monounsaturated FAMEs by using extracted ions (cf. [21] and refs. cited therein). Fig. 4 presents the extracted ion chromatograms obtained in the experiment. The trace of the ion *m*/*z* 145 (fragmentation patterns of thioether adducts, cf. [21]) allowed the detection of the n:1ω7 FAMEs, the *threo* and the *erythro* forms possessing different retention indices. Extracted ion traces for *m*/*e* 159 and *m*/*e* 173 were empty (cf. Fig. 4), indicating the absence of n:1ω8 and n:1ω9 members, respectively, in the biomass under study. Fig. 4 indicates that the elution order of the *erythro* and *threo* adducts was identical on polar DB-225 and non-polar HP-5ms stationary phases. Attention should be paid to the elution sequence—the erythro form, which translates into trans, elutes last on both polar and non-polar phases, whereas the native *trans*-FAME elutes last only on polar phases. The *threo* and *erythro* isomers cannot be differentiated on the basis of their mass spectra. Cyclic FAMEs were observed not to react with DMDS. This finding, which is in some contrast to the results published in ref. [39], can be regarded a useful indication in distinguishing monounsaturated and cyclic FAMEs, both types having almost identical retention indices and fragmentation patterns.

To scrutinize both the potential migration of the double bonds and the potential *cis*/*trans* isomerization as a result of TMAH thermochemolysis – which, to the best of our knowledge, has not been addressed up to now – a combination of TMAH-thermochemolysis with formation of thioethers is called for. Unfortunately, the DMDS procedure cannot be

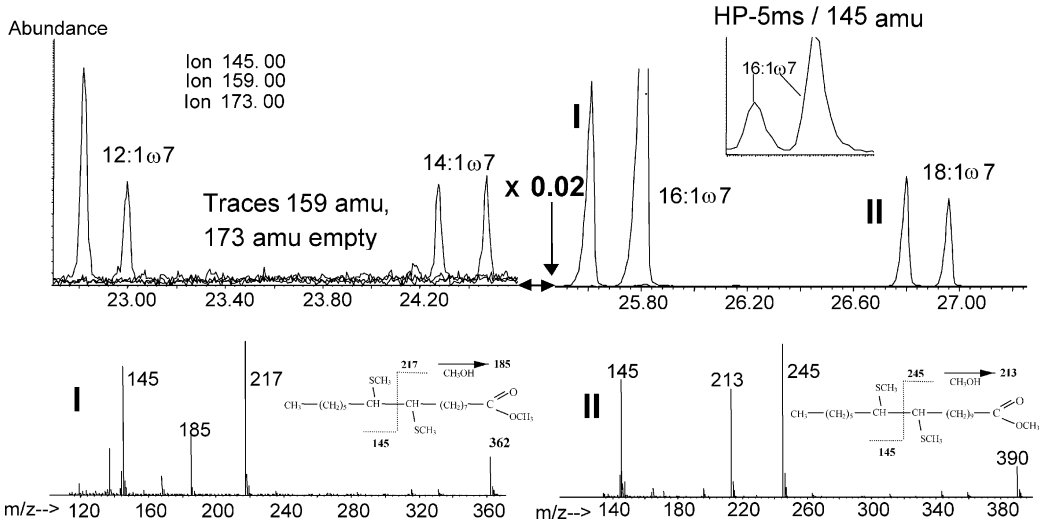

Fig. 4. Identification of monounsaturated FAMEs using thioether adducts (see text). Stationary phase: DB-225.

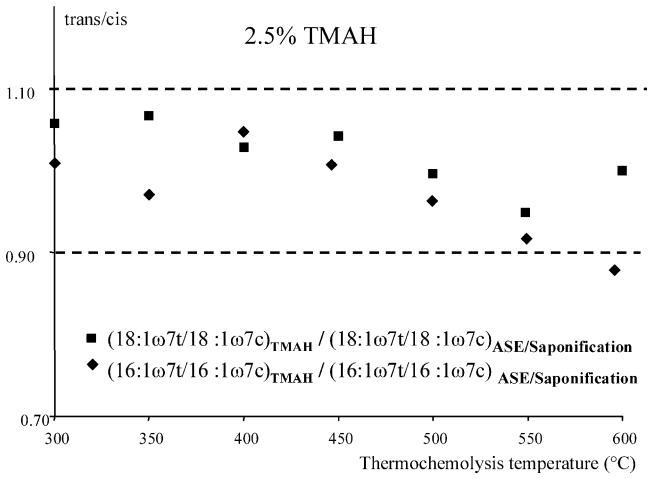

Fig. 5. The dependence of the *cis*/*trans* isomerization of monounsaturated 16:1ω7 and 18:1ω7 geometrical isomers on TMAH-thermochemolysis temperature (2.5% TMAH) relative to the results obtained by PLE/mild alkaline saponification.

easily transferred to an on-line method combined with thermochemolysis. Preliminary results, which will be outlined in more detail elsewhere, indicate that injection of DMDS into the hot splitless injector just before thermochemolyzing the sample does not allow the thioether products of monounsaturated FAMEs to form in a reproducible way during the residence time in the injector. Therefore, the thermochemolyzate was trapped via a ~3 m × 0. 25 mm i.d. piece of uncoated fused silica capillary in a 2 mL vial filled with about 1 mL *n*-hexane. The *n*-hexane solvent containing the trapped FAMEs was then subjected to off-line DMDS procedure as described above. The results clearly showed no migration of the double bonds (explicit data not shown here). The ratios of the *threo* and the *erythro* forms were identical to those depicted in Fig. 4. This is a conclusive proof that there is no migration of double bonds in monounsaturated FAMEs on thermochemolysis using the capacitive-discharge approach.

Fig. 5 illustrates the dependence of the *cis*/*trans* isomerization on thermochemolysis temperature using 2.5% TMAH. The values of the *trans*/*cis* ratio plotted in the figure are relative to those obtained by PLE/mild alkaline saponification. Findings for 25% TMAH were similar (data not shown), except for higher R.S.D. values. It is evident from the results that the thermochemolysis temperature had little effect on the *cis*/*trans* ratio.

Discrimination of cyclic FAMEs (cyclo 17 and cyclo 19; the latter not shown in Table 1 because of significantly lower concentration in the 300 ppm range) was not observed, indicating that TMAH had no impact on these cyclic structures. Keeping in mind the non-biased results for the monounsaturated and cyclic structures, TMAH-induced thermochemolysis can be regarded a useful method in detecting the formation of cyclopropane acids from monounsaturated ones, e.g. in carbon source limited fermentation regimes.

To draw conclusions on both species identification and microbial community analysis based on fatty acid profiling in environmental and microbiological practice, bioremediation work, etc., a deeper insight into the diagnostic fatty acids is necessary (see above). In the framework of studies on bioremediation of contaminated sites and the behaviour of microbial communities under toxic stress performed at the Center for Environmental Research and the University of Waterloo, the determination of the *cis*/*trans* ratio of monounsaturated FAMEs has been considered essential [18,20,40]. The changes in the *cis*/*trans* ratio can be regarded as the bacterial response to the interaction of lipophilic chemicals with the cell membranes, modifying membrane fluidity. In the ideal case scenario, the ratio of monounsaturated to saturated FAMEs obtained by PLE/mild alkaline saponification on the one hand, and thermochemolysis on the other hand, should be identical. Fig. 6a and b presents the concentration ratios of monounsaturated to homomorphic FAMEs (normalized to concentration ratios obtained by PLE/mild saponification of the biomass). The dashed lines indicate the arbitrarily chosen 10% interval (R.S.D. of the method amounts to at least 10%). As can be concluded from Fig. 6a, there was no significant discrimination of monounsaturated FAMEs to

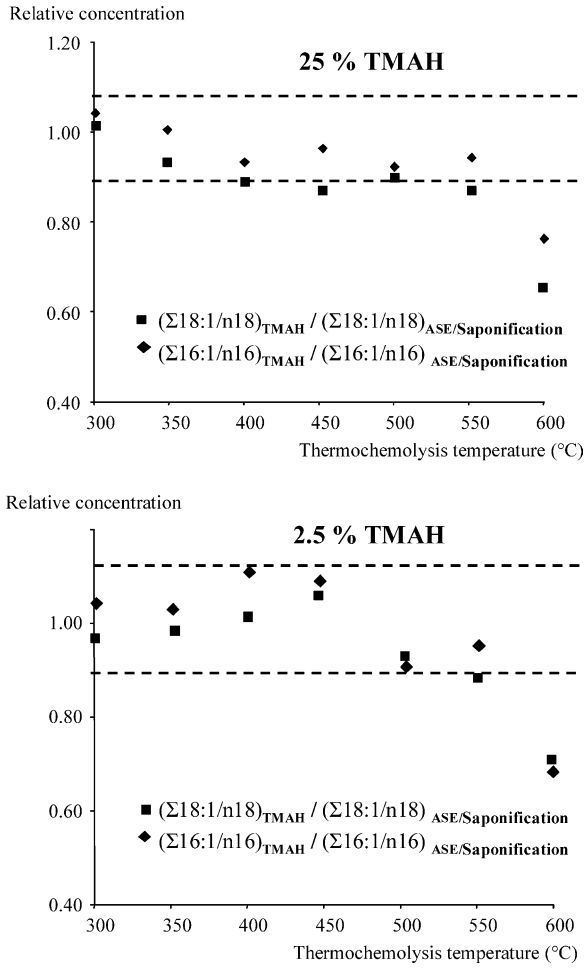

Fig. 6. Relative concentrations of monounsaturated 16:1ω7 and 18:1ω7 FAMEs obtained by TMAH thermochemolysis in comparison with PLE/mild alkaline saponification (a: 25% TMAH; b: 2.5% TMAH).

the corresponding homomorphic FAMEs as long as the thermochemolysis temperature (using 25% TMAH) did not exceed 550 °C. The situation got somewhat better when turning to a lower reagent concentration (2.5% TMAH, see Fig. 6b). However, this slight improvement occurred at the expense of lower recoveries (cf. Table 1).

With both approaches (capacitive discharge on the one hand, and conventional "flash" and Curie-point pyrolyzers on the other hand), no unwanted α-methylated and α,α-dimethylated FAMEs, as observed with oils/paints [13], were detected. Obviously, this phenomenon is likely not to occur with bacterial lipids, where both polyunsaturated fatty acids and diacids are scarce.

3.3. Conclusions about the significance of TMAH-thermochemolysis towards microbial community structure analysis

As detailed in the Section 1, the "whole cell" saponification method generally does not meet the criteria for microbial community structure analysis. The situation changes, however, when phospholipids account for a vast majority of the total lipids (more than 95%, as was the case with the sample used in this study). TMAH-induced thermochemolysis as a "whole cell" technique suffers similar shortcomings at the present stage as whole cell saponification (cf. above). However, our most recent results indicate that upon thermochemolyzing the biomass sample at 500 °C—phosphoric acid (in the form of the trimethyl ester) as well as glycerine (as trimethoxy derivative) might be useful as markers to determine the acylglycerines/phospholipids ratio. Such results indicate that phospholipids accounted almost exclusively for the total lipids in the *P. putida* sample under study. This finding was in-line with the conclusions based on the abundant presence of the carbon source, electron acceptor and essential nutrients during fermentation [41].

In general, the role of fatty acids in classification can be supplemented by patterns of other lipid classes, including hopanoids for bacteria or sterols for algae. The method of non-discriminating TMAH-induced thermochemolysis is a potential candidate for simultaneous detection of different lipid classes of interest in biomasses, without the need for cultivation, complicated sample preparation or large sample amounts.

3.4. Rapid FAME profiling directly from complex biological samples

Data reported here provide strong evidence that the new pyrolysis approach opens up the possibility of microgram-scale, rapid bacterial identification without the need for time-consuming and error-prone culturing and sample preparation schemes. Fig. 7 shows fatty acid patterns of a microorganism consortium isolated from an in situ pilot plant reactor obtained by alkaline saponification (top) and thermochemolysis (bottom). Indigenous bacteria in native aquifer matrices

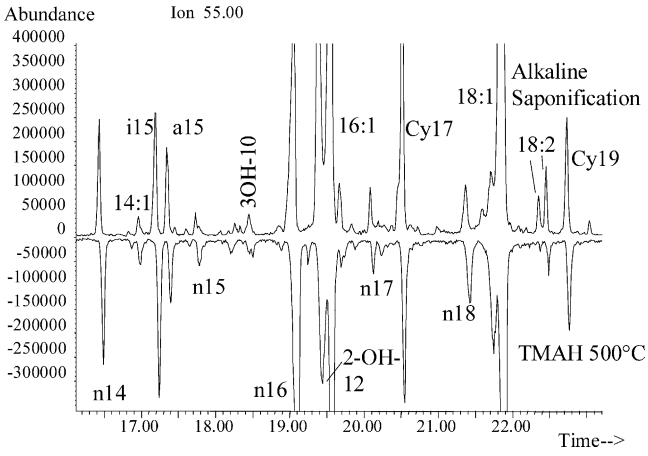

Fig. 7. Fatty acid pattern of a biofilm sample (see text) obtained on alkaline saponification (top) and thermochemolysis (25% TMAH, 500 °C; bottom). Stationary phase: SP 2330. Data acquisition: scan mode; extracted ion *m/z* 55 for both alkaline saponification and TMAH thermochemolysis. Peak labelling: shorthand designation (cf. text and Table 1).

(depth about 20 m) were cultivated by adding hydrogen peroxide as oxygen supplier and nitrate as electron acceptor in the lower part of the reactor [42]. Remediation of a groundwater plume contaminated with chlorinated aromatics took place in the biofilm attached to the aquifer material. This enhanced natural attenuation remediation approach has been aimed at complete removal of chlorobenzene using oxygen concentration as low as possible [43]. An aliquot of 1 μL of the liquid biofilm sample was loaded directly into the Silcosteel tube for thermochemolysis. The results were compared to those of alkaline saponification/methylation. As depicted in Fig. 7, there was a high degree of similarity between the two patterns obtained by completely different methods. Cyclic surrogates (Cy-17 and Cy-19, originating from palmitoleic and *cis*-vaccenic acids, respectively, when microorganisms move from the logarithmic to the stationary phase of growth; cf. above) and monounsaturated surrogates of the palmitoleic family (n:1ω7) point to bacterial origin (chiefly Gram-negative bacteria). *Pseudomonad* strains are expected to account for that, because (i) they have been known to degrade aromatic pollutants (cf. [44] and refs. cited therein) and (ii) their fatty acid patterns (cf. [45]) are similar to that depicted in Fig. 7. Significant concentrations of 18:2 acids (cf. Fig. 7) point to sources other than bacteria, for example fungi or plants. The absence of 18:3 acids (which usually accompany 18:2 acids in plants) seems to indicate that the 18:2 acids originated from fungi, but this finding would have to be confirmed by further studies, outside the scope of this paper. Both FAME patterns in Fig. 7 are characterized by significant concentrations of 3-OH-10:0 and 2-OH-12:0.

Another example is given in Fig. 8, which shows the FA pattern obtained for tomato roots attacked by carboxymethylcellulose degrading soil bacteria [46]. The bacteria, capable of degrading both colloidal and highly crystalline cellulose derivatives, underwent colonization onto the root. To perform the analysis, about 100 μg of the freeze-dried sample was

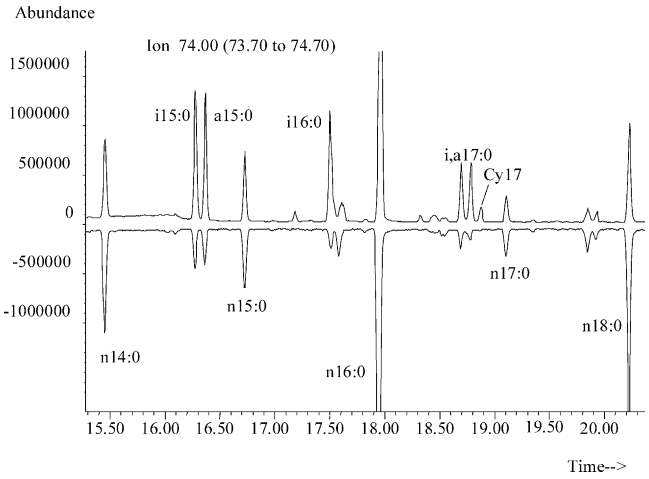

Fig. 8. Fatty acid pattern of a tomato root treated by carboxymethyl-cellulose decomposing bacteria (top) and in non-treated form (bottom). Sample: freeze-dried, sample mass: each 100 μg, TMAH 25%, DB-5ms, peak abundances normalized to the internal standard.

loaded into the pyrolysis capillary and treated by 25% TMAH solution. Thermochemolysis was carried out at 500 °C using a 1:5 split ratio rather than the 1:10 split ratio as described in the Experimental part because of the lower lipid concentration in the tomato root matrix. To simplify the comparison, the chromatograms were normalized to the internal standard (deuterated palmitic acid).

The solid–water interface adsorption of microorganisms onto the tomato root could be clearly traced by TMAH-induced thermochemolysis. Fig. 8 (top) shows the FA profile for the treated tomato root, indicating high concentrations of iso- and anteiso-branched C-15 and C-17 surrogates, as well as the cyclic C-17 member, all of them indicating the *Streptomyces* strains. In contrast to that, the non-treated tomato root (Fig. 8 bottom) shows a pattern common for higher plants.

These examples give strong evidence, that – in contrast to the established monitoring methods including polymerase chain reaction, which are labour intensive, very selective and not quantitative (cf. above) – this chemotaxonomic approach allows both absolute quantitative considerations as well as rapid screening, thus in turn allowing to draw conclusions about the course of bioremediation.

Fatty acid profiling using the traditional techniques has significant disadvantages, including lengthy steps in sample preparation, high amount of biomass needed to get adequate masses of the fatty acids, etc. Using our approach, only small amounts of the bacterial biomass (about 25 μg or less, e.g. when applying the selected-ion monitoring acquisition mode) are required, thus eliminating the need for biomass culturing and the complicated and time-demanding sample preparation.

The approach described in this paper, focused on speeding up and simplifying sample preparation, can also form the basis for the development of a real-time, on-site and field portable device for the identification of microorganisms, e.g. in early warning systems to detect the onset of biological weapons release. To meet the aim of real-time measurements with duty cycles of a few minutes, the application of short capillary columns (5 m or less) should be preferred, as e.g. in ref. [47].

The time-demanding MIDI system (Sherlock Microbial Identification System, MIDI Inc., Newark, DE, USA; see refs. in [48]), which requires isolation and culturing of the species prior to the analysis (thus not representing the species which elude cultivation, cf. Section 1), does not meet the needs for real-time or near-real-time analysis. In this context, a field-portable membrane inlet thermochemolysis–MS technique for whole cell characterization proved to be better, but more demanding [12]. A significant shortcoming of this GC-less approach is brought about by the lack of information regarding isomeric analytes (e.g. oleic and *cis*-vaccenic acids, which usually represent different origins). A sensible alternative might be to utilize a combination of a Silcosteel capillary filled with a temperature-stable sorbent serving as the aerosol collector, followed by pyrolysis/thermochemolysis of the collected cells (aerosols) and GC–MS analysis using a field-portable system. The MIDI systems, which rely on the "whole-cell" saponification approach, should be compatible with non-discriminated pyrolysis based on capacitive discharge, whereas conventional pyrolysis units based on either flash or Curie-point heating are not expected to meet this criterion (due to discrimination of unsaturated FAMEs and conversion of hydroxyl-FAMEs to the methoxylated form, which cannot be identified by the database). Overall, the new approach proposed in the paper integrates well into the development of chemical characterization techniques to species identification and classification without prior isolation and cultivation, owing to the very small amount of the sample required (microgram method).

Acknowledgements

We wish to thank Dr. C. Haertig (UFZ, Department of Environmental Microbiology) for culturing *P. putida* biomass and helpful discussions. We are very grateful to Dr. K. Vogt (UFZ, also Department of Environmental Microbiology) for providing the field sample from the in situ reactor. Thanks are also due to Marion Hoyer, Evelyn Becker and Ursula Bachmann for excellent technical assistance. Dr. K. Mueller (Institute for Tropospheric Research, Leipzig) is gratefully acknowledged for providing access to the Curie-point pyrolyzer. Natural Sciences and Engineering Research Council of Canada provided financial assistance for the project. Finally, thanks to Restek for supplying the Silcosteel tubing free of charge.

References

[1] S.J. Macnaughton, T.L. Jenkins, S. Alugupalli, D.C. White, Am. Ind. Hyg. Assoc. J. 58 (1997) 270.

[2] M. Steele, W.B. McNab, S. Read, C. Poppe, L. Harris, A.M. Lammerding, J. Odumeru, Appl. Environ. Microbiol. 63 (1997) 757.
[3] F. Xiang, G.A. Anderson, T.D. Veenstra, M.S. Lipton, R.D. Smith, Anal. Chem. 72 (2000) 2475.
[4] D.C. White, FEMS Microbiol. Rev. 20 (1997) 371.
[5] S.N. Dedysh, N.S. Panikov, J.M. Tiedje, Appl. Environ. Microbiol. 64 (1998) 922.
[6] P. Farrimond, P.A. Fox, H.E. Innes, I.P. Miskin, I.M. Head, Ancient Biomol. 2 (1998) 147.
[7] D.C. White, D. Hedrick, A. Peacock, J. Stephen, S. Macnaughton, J.J. Bruggemann, Microbiol. Methods 41 (2000) 235.
[8] D.C. White, H.C. Pinkart, D.B. Ringelberg, in: C.J. Hurst (Ed.), Manual of Environmental Microbiology, ASM Press, Washington, DC, 1997, pp. 91–101.
[9] J.B. Guckert, J. Microbiol. Methods 25 (1996) 101.
[10] A.M. Ibekwe, A.C. Kennedy, P.S. Frohne, S.K. Papiernik, C.-H. Yang, D.E. Crowley, FEMS Microbiol. Ecol. 39 (2002) 183.
[11] H.M. Alvarez, A. Steinbuechel, Appl. Microbiol. Biotechnol. 60 (2002) 367.
[12] F. Basile, M.B. Beverly, K.J. Voorhees, Trends Anal. Chem. 17 (1998) 95.
[13] J.V.D. Berg, J.J. Boon, J. Anal. Appl. Pyrol. 61 (2001) 45.
[14] W.J. Jun-Kai Ding, T.-Z. Zou, M. Song, X.-G. Yu, C.-C. Fan, J. Anal. Appl. Pyrol. 42 (1997) 1.
[15] T. Górecki, J. Poerschmann, Anal. Chem. 73 (2001) 2012.
[16] T. Górecki, J. Poerschmann, System for in-column pyrolysis, German Patent DE 10111854.6 (2001).
[17] P. Blokker, R. Poel, L. Akoto, U.A.Th. Brinkman, R.J.J. Veuls, J. Chromatogr. A 959 (2002) 191.
[18] H.J. Heipieper, P.D. Waard, P.V.D. Meer, J.A. Killian, S. Isken, J.A.M.D. Bont, G. Eggink, Appl. Microbiol. Biotechnol. 57 (2001) 541.
[19] H. Keweloh, H.J. Heipieper, Lipids 31 (1996) 129.
[20] N. Loffhagen, C. Hartig, W. Babel, Ecotoxicol. Environ. Saf. 50 (2001) 65.
[21] C. Pepe, Eur. Mass Spectrom. 1 (1995) 209.
[22] J. Poerschmann, T. Welsch, R. Herzschuh, W. Engewald, K. Müller, J. Chromatogr. 241 (1982) 73.
[23] B.E. Richter, J.L. Ezzel, D.E. Knowles, F. Hoefler, Chemosphere 34 (1997) 975.
[24] Z. Parsi, T. Górecki, J. Poerschmann, J. Anal. Appl. Pyrol., submitted for publication.
[25] J. Poerschmann, F.-D. Kopinke, M. Remmler, K. Mackenzie, W. Geyer, S. Mothes, J. Chromatogr. A 750 (1996) 287.
[26] Y. Ishida, S. Wakamatsu, H. Yokoi, H. Ohtani, S. Tsuge, J. Anal. Appl. Pyrol. 49 (1999) 267.
[27] D. Drechsel, K. Dettmer, W. Engewald, Chromatogr. Suppl. 57 (2003) S-283.
[28] W.A. Hartgers, J.S. Sinninghe, J. Damste, J. Anal. Appl. Pyrol. 34 (1995) 191.
[29] J.P. Dworzanski, L. Berwald, H.L.C. Meuzelaar, Appl. Environ. Microbiol. 56 (1990) 1717.
[30] J.M. Challinor, J. Anal. Appl. Pyrol. 61 (2001) 3.
[31] J.P. Bowman, J.H. Skerratt, P.D. Nichols, L.I. Sly, Microbiol. Ecol. 85 (1991) 15.
[32] A. Monilaro, R. Lanzetta, A. Evidente, M. Parrilli, O. Holst, FEMS Microbiol. Lett. 181 (1999) 49.
[33] L. Zelles, Chemosphere 39 (1999) 665.
[34] L. Grasset, A.J. Ambles, Anal. Appl. Pyrol. 47 (1998) 1.
[35] K.D. Wendlandt, M. Jechorek, J. Helm, U. Stottmeister, J. Biotechnol. 86 (2001) 127.
[36] N.R. Filho, F.M. Lancas, J. High Resolut. Chromatogr. 18 (1995) 167.
[37] K. Stransky, T. Jursik, A. Vitek, J. High Resolut. Chromatogr. 20 (1997) 143.
[38] R.H. Thompson, J. Chromatog. Sci. 34 (1996) 495.
[39] G. Dobson, W.W. Christie, Trends Anal. Chem. 15 (1996) 130.
[40] A.G. Werker, J. Becker, C. Huitema, Water Res. 37 (2003) 2162.
[41] C. Haertig, UFZ, personal communication, 2002.
[42] A. Alfreider, C. Vogt, W. Babel, Syst. Appl. Microbiol. 25 (2002) 232.
[43] C. Vogt, A. Alfreider, H. Lorbeer, L. Wünsche, W. Babel, Grundwasser 3 (2002) 156.
[44] H. Shim, S.T. Yang, J. Chem. Tech. Biotechnol. 77 (2002) 1308.
[45] J. Fang, M.J. Barcelona, P. Alvarez, Org. Geochem. 31 (2000) 881.
[46] S. Wirth, A. Ulrich, System. Appl. Microbiol. 25 (2002) 584.
[47] G. Matz, G.G. Kibelka, J. Dahl, F. Lennemann, J. Chromatogr. A 830 (1999) 365.
[48] F. Basile, M.B. Beverly, C. Abbas-Hawks, C.D. Mowry, K.J. Voorhees, Anal. Chem. 70 (1998) 1555.