Detection of mycobacteria by volatile organic compound analysis of in vitro cultures using differential ion mobility spectrometry

Roman Purkhart,¹ Gunther Becher,² Petra Reinhold³ and Heike U. Köhler³,*

Abstract

Purpose. Differential ion mobility spectrometry (DMS) is an analytical technique used to detect volatile organic compounds (VOCs) in gaseous samples at very low concentration ranges from ppb to ppt. The aim of this study was to investigate whether VOC analysis by DMS is capable of detecting Mycobacterium avium subsp. paratuberculosis (MAP).

Methodology. Headspaces of in vitro cultures of two different MAP strains at 1, 2, 3, 4 and 6 weeks after inoculation (each at two dilutions) were analysed with DMS in comparison to control samples without viable bacteria [(i) blank medium, (ii) medium inoculated with heat-inactivated MAP and (iii) sterile-filtered MAP culture broth]. Furthermore, VOC patterns in the headspace over cultures of six non-tuberculous mycobacterial species were compared to MAP-derived VOC patterns. Data analysis included peak detection, cluster analysis, identification of discriminating VOC features (Mann–Whitney U test) and different cross-validated discriminant analyses.

Results. VOC analysis resulted in up to 127 clusters and revealed highly significant differences between MAP strains and controls at all time points. In addition, few clusters allowed differentiation between MAP and other non-tuberculous mycobacteria and even between different MAP strains. Compounds have not been characterized. VOC analysis by DMS was able to identify MAP-positive samples after 1 week of in vitro growth.

Conclusions. This study provides strong evidence that VOC analysis of headspace over mycobacterial cultures in combination with appropriate data analysis has the potential to become a valuable method to identify positive samples much earlier than with current standard procedures.

INTRODUCTION

Diagnosis of infectious diseases is time consuming and expensive. Traditional methods are based on culture of bacteria from samples of sputum, tissues or other specimens on non-selective or selective media. In the case of mycobacterial infections, the time until a negative result is finally confirmed will take between 3 weeks and about 1 year depending on the mycobacterial species; some do not grow in culture medium at all (e.g. Mycobacterium leprae). Mycobacterium avium subsp. paratuberculosis (MAP) is the causative agent of paratuberculosis, a chronic enteritis of ruminants accompanied by high economic losses for the livestock industry worldwide. MAP belongs to the group of slow-growing mycobacteria with cultivation times of up to 12 months [1]. This compromises the utilization of cultural isolation of the pathogen from specimens like faecal samples, which otherwise is still the most sensitive diagnostic procedure for living animals. The acceptance of cultural isolation as a diagnostic tool would be enhanced if the time to detection could be reduced.

Automated liquid culture systems are available for different pathogens including MAP using non-specific read-out parameters for bacterial growth, like pressure changes in the headspace by gas production or gas consumption [2] or consumption of dissolved oxygen in broth medium by respiring bacteria [3]. Additional subsequent identification steps are required for species confirmation. Novel developments aim at...
shortening of the duration and improvement of specificity of the primary cultivation technique.

All organisms produce volatile organic compounds (VOCs) as part of their normal metabolism [4, 5]. Exploitation of this phenomenon for diagnostic purposes has gained growing attention during recent years. In slow-growing microorganisms, such as mycobacteria, early detection of characteristic VOC patterns has special potential [6]. Proof of principle has been provided by several studies that specific VOC profiles can be detected, and differentiation between bacterial species is possible in the headspace of bacterial cultures [7, 8]. Different analytical methods have been used, such as electronic noses (e-nose) [9, 10], MonoNose [11], ion molecule reaction mass-spectrometry (MS) [12], gas chromatography MS [13, 14], differential ion mobility spectrometry (DMS [15]), proton transfer reaction MS [16] or multi-capillary column-ion mobility spectrometry [17]. Several volatile compounds have been identified in the headspace of bacterial cultures by spectral library match and retention time [14, 18, 19]. However, technical and biological factors that influence the composition of VOCs over bacterial cultures have not been examined to a large extent so far.

Ion mobility spectrometry is a fast and mobile analytical technique for detection of VOCs in gaseous samples. The molecules are ionized and separated based on their ion mobility. For selected classes of compounds, detection limits of 5 to 10 ppb, were reported [20]. DMS uses the dependence of ion mobility on electric field strength and detects both positive and negative ions simultaneously. A DMS microanalyzer as a highly mobile device combines DMS technology with a thermal adsorption/desorption inlet and a capillary gas chromatographic column for very high sensitivity (down to ppb or ppt). It is suitable for use as a point-of-care device with no need for special laboratory equipment and additional consumables such as high-purity carrier gases. It allows measurement of samples online without storage in collection bags or traps, thus reducing the impact of confounding factors.

The overall aim here was to investigate whether VOC analysis by DMS is capable of detecting the growth of MAP in vitro and to identify inherent factors that influence VOC composition. In the first part (study I), the appearance and temporal changes of growth-related VOC patterns in the headspace over MAP cultures were analysed, and their dependence on inoculum (bacterial counts) and bacterial strain was determined. Additionally, the impact of sample handling and culture time on VOC patterns was elucidated. In the second part (study II), VOC patterns in the headspaces of MAP cultures were compared to those assessed in the headspaces of six other non-tuberculous mycobacterial species.

**METHODS**

**Study I: growth-dependent VOC patterns of MAP**

Two strains of MAP [strain DSM 44133 (German Collection of Microorganisms and Cell Cultures, DSMZ) and strain JII-1961 (field isolate from cattle)] were cultured on Herrold’s egg yolk medium (HEYM) containing mycobactin J and amphotericin B, nalidixic acid and vancomycin (Becton Dickinson). Colony material was suspended in 4 ml Middlebrook 7H9 broth containing mycobactin J and oleic acid–albumin–dextrose–catalase. Optical density at 580 nm was adjusted to 0.3. After incubation for 1 week under constant shaking, the cultures were split into three aliquots, of which two were used for the preparation of controls without viable bacteria. One aliquot was sterile filtered through a 0.2 µm filter, and the filtrate was collected. Another aliquot was heat inactivated at 100 °C for 60 min. The third aliquot was left untreated. HEYM tubes were inoculated with 200 µl of the original or a 10⁻⁴ dilution of the untreated bacterial suspensions. Control tubes received 200 µl of heat-

![Image](https://via.placeholder.com/150)

**Fig. 1.** Two representative clusters (a, b) with significant differences between control groups (between Aa/Ba (1+2) and Ca [1+2], all time points).
inactivated bacterial suspensions, filtrates or pure medium. The tubes were incubated at 37°C up to 6 weeks.

Colony counts of the original suspensions and the 10⁻⁴ dilutions were determined. These amounted to 3.23×10⁷ ml⁻¹ and 7.20×10⁵ ml⁻¹ for DSM 44133 and to 2.21×10⁷ ml⁻¹ and 5.10×10⁵ ml⁻¹ for JII-1961, respectively.

This part of the study had a randomized, negatively controlled design. Ten tubes were inoculated for each strain and each concentration, resulting in 40 positive samples. Two tubes were inoculated for each strain with the sterile-filtered bacterial suspension (Ba), and two with the heat-inactivated bacterial suspensions (Ca). Furthermore, two tubes were inoculated with pure growth medium (Aa), resulting in 10 negative samples (controls).

Eight positive samples (two per strain and concentration) and the ten control tubes were firstly analysed 1 week after inoculation. These 18 samples were then repeatedly analysed 2, 3, 4 and 6 weeks after inoculation (tubes containing pure medium were measured twice per date). In addition, eight corresponding positive samples (two for each strain and concentration) were newly opened at each time point and measured once only.

**Study II: differentiation of MAP from other mycobacterial species**

One strain each of *M. fortuitum* (DSM 46621), *M. phlei* (DSM 43239), *M. smegmatis* (DSM 43756), *M. terrae* (DSM 43227), *M. avium* subsp. *avium* (DSM 44156), all purchased from the DSMZ, one field isolate of *M. avium* subsp. *hominaissuis* (03A2893) from cattle and two strains of MAP [strain DSM 44133 (DSMZ) and strain JII-1961 (field isolate from cattle)] were included. MAP strains were maintained on HEYM as described above. The other mycobacteria were maintained on Stonebrink medium without glycerol, containing pyruvate and polymyxin B, amphotericin B, carbencillin and trimethoprim (Bioservice Waldenburg). Colony material was suspended in 4 ml Middlebrook 7H9 broth. The suspensions were vortexed in the presence of glass beads, the remaining clumps were allowed to settle and the supernatants were transferred to new tubes. The optical density of the suspensions at 580 nm was adjusted to 0.3 (0.245–0.352, minimum–maximum). Two HEYM tubes per strain were inoculated with 200 µl of the bacterial suspensions and incubated at 37°C. After 4 weeks of incubation, tubes were measured in duplicate and after another 4 weeks once.

**DMS methodology**

In these studies, the DMS microanalyser (serial no. 0149; Sionex) was used, consisting of a differential mobility spectrometer coupled to a thermal adsorption/desorption inlet and a capillary gas chromatographic column for increased sensitivity.

The gaseous sample was aspirated into the carbon adsorption trap by a pump with a flow rate of 1.5 ml s⁻¹. After an aspiration time of 20 s, the inlet closed and the sample was thermally desorbed into the capillary gas chromatographic column and into the ionization chamber using air as carrier gas. The adsorbent trap and the chromatographic column were programmed to heat in a specific temperature/time profile to 300°C (trap) and accordingly 120°C (column). A radioactive ⁶³Ni source (93 MBq) was used for ionization of the sample at the inlet of the detector. To identify different ions, an oscillating asymmetric radiofrequency electric field (1000 V) and a direct current (DC) compensation electric field were applied across two parallel plates. The tunable DC field was superimposed on the oscillating asymmetrical field and kept the ions of interest centre between the plates and thus detectable by electrometry (other ions coming in contact with the plates were neutralized). The DC field changed stepwise between −20 and +5 V once per second (100 steps). Consequently, one positive and one negative spectrum between −20 and +5 V were stored per second. Measurement time was set to 270 s, resulting in 540 (270 positive and 270 negative) spectra stored per measurement (eg see Fig. S1, available in the online Supplementary Material).

**Fig. 2.** Changes in the signal intensity of two representative VOC clusters (a, b) from pure growth medium (Aa) over time.
Analysis of sample tubes

Sample tubes (with approximately 15 ml headspace) removed from the incubator were placed directly on a heating block warmed to 37 °C. The tubes were opened, and the caps were immediately replaced by a two-holed stopper prepared with two polytetrafluoroethylene tubes of 300 mm length, which were passed through the holes and tightly sealed. DMS was connected to one hole, and a multi-stage air filter (combination of carbon filter and fine dust particle filter) was connected to the other hole. The DMS drew ambient air through the filter and then the filtered air through the sample tube and finally onto its pre-concentration trap (see Fig. S2).

Data analysis

The entire data set of study I consisted of 132 measurements (28 samples at 4 dates, 20 samples at week 1), and the data set of study II consisted of 48 measurements (12 MAP samples, 36 other samples). Ionization of molecules by the radioactive $^{63}$Ni source mainly resulted in positive ions (proton transfer). Consequently, the positive spectra of each measurement contained most of the information, and only positive spectra were used for further analysis.

Spectra had been background adjusted and filtered using a moving average filter to reduce data noise. Peaks were calculated as local maxima beyond a certain threshold (0.005 arbitrary units). Because peak positions of the same VOC can vary slightly between measurements, a cluster analysis of all peak positions was performed to find groups (clusters) of corresponding peaks (cluster centre position, see Table S1), assuming that these peaks are representing the same VOC [21].

Clusters were tested statistically for differences in peak heights between certain groups by Mann–Whitney U test,

Fig. 3. Differences in the peak intensity of three representative VOC clusters (a–c) between controls and MAP-positive samples (dilution $10^0$).
because most of the data were not normally distributed. Since testing for significant differences does not mean that these differences are really group induced, we further used a prediction model based on cross-validation and discriminant analysis to estimate the classification results on new, unknown samples (the data were analysed without one certain sample, and this sample was then treated as unknown and classified; repeated for each sample). Spearman rank correlation was applied to assess time-dependent effects.

RESULTS

Study I: growth-dependent VOC patterns of MAP

The analysis of DMS data resulted in 160 different clusters. In the subsequent sections, maximal peak heights in these clusters were tested for significant differences.

No differences between reopened and newly opened samples

The first question to be answered was whether differences existed between the VOC patterns of newly opened and reopened samples. The reopened samples did not show any significant difference compared to newly opened samples, indicating that multiple opening did not affect future measurements. This observation applied to all groups and all time points (data not shown). Therefore, these two groups were merged for further analyses.

Heat-inactivated bacterial suspensions differed from bacteria-free medium

The three control groups were tested for general differences between each other (all time points together).

Tubes inoculated with sterile-filtered bacterial suspension (Ba) showed almost no differences compared to tubes inoculated with pure growth medium (Aa). Only 1 of 160 clusters showed significant differences (cluster 6, \( P = 0.014 \), Mann–Whitney U test).

In contrast, tubes inoculated with heat-inactivated bacterial suspensions (Ca) showed clear differences compared to tubes inoculated with pure growth medium. In total, 24 of 160 clusters differed significantly, 6 clusters even with \( P < 0.001 \) (clusters 6, 10, 12, 42, 54 and 70). Deviations occurred in both directions (increase or decrease in peak height); representative examples are given in Fig. 1.

VOC emission of pure growth medium over time

To assess the stability of VOC emission from pure growth medium over time, Spearman correlation was calculated for all 160 clusters. Six clusters showed a significant correlation

Table 1. Results of a cross-validated discriminant analysis (first 20 clusters) between control groups and positive samples (dilution \( 10^0 \))

<table>
<thead>
<tr>
<th>Actual group</th>
<th>Predicted group membership</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-validated Count</td>
<td>Aa/Ba/Ca (1+2)</td>
<td>MAP-1W</td>
</tr>
<tr>
<td>Aa/Ba/Ca (1+2)</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>MAP-1W</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>MAP-2W</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAP-3W</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAP-4W</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>MAP-6W</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>%</td>
<td>Aa/Ba/Ca (1+2)</td>
<td>96.7</td>
</tr>
<tr>
<td>MAP-1W</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>MAP-2W</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MAP-3W</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MAP-4W</td>
<td>0.0</td>
<td>12.5</td>
</tr>
<tr>
<td>MAP-6W</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*94.8 % of cross-validated grouped cases correctly classified.

Table 2. Results of a cross-validated discriminant analysis (first 20 clusters) between control groups and positive samples (dilution \( 10^0 \), week 1)

<table>
<thead>
<tr>
<th>Actual group</th>
<th>Predicted group membership</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-validated Count</td>
<td>Aa/Ba/Ca (1+2)</td>
<td>MAP-1W</td>
</tr>
<tr>
<td>Aa/Ba/Ca (1+2)</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>MAP-1W</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>%</td>
<td>Aa/Ba/Ca (1+2)</td>
<td>100.0</td>
</tr>
<tr>
<td>MAP-1W</td>
<td>0.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

*100.0 % of cross-validated grouped cases correctly classified.
with time points [four with increasing (clusters 26, 64, 87 and 145) and two with decreasing (clusters 29 and 40) peak height, \(P<0.001\), examples shown in Fig. 2], while most features stayed constant. Five of these clusters also showed significant correlations in the other control groups.

### Differences in VOC emission of MAP cultures in comparison to control samples

Positive samples (both strains together, representing MAP) showed a high proportion of significant differences compared to all groups of controls. With increasing incubation time, the number of clusters with significant differences between positive samples and control samples increased. The maximum was reached at week 4 with 44 of 160 clusters showing significant differences and 16 clusters with \(P<0.001\) (clusters 10, 13, 15, 17, 26, 27, 30, 32, 34, 38, 39, 44, 63, 116, 139 and 146). Twenty-eight clusters were already showing significant differences after week 1, among these nine clusters with \(P<0.001\) (clusters 10, 11, 33, 34, 44, 82, 83, 84 and 85). In positive tubes, clusters with both increasing and decreasing peak intensity could be found (Fig. 3).

A cross-validated (leave-one-out) discriminant analysis (only the first 20 clusters of importance) between positive samples at different dates and control samples showed a maximum correct classification rate of 94.8 % (Table 1). This demonstrates that not only do VOC patterns for differentiating MAP from control samples exist, but even specific markers for different propagation stages. Only the first 20 clusters (clusters with most peaks along all measurements based on the used cluster analysis) were included, because classification algorithms tend to ‘overfit’ if too many features are used in comparison to group size. This did not affect the value of the results because the used clusters were not selected by their group separation capabilities, and a cross-validation was applied.

A cross-validated (leave-one-out) discriminant analysis (first 20 clusters) was also calculated between the week 1 positive samples and the controls. This showed a maximum correct classification rate of 100 % (specificity of 100 % and sensitivity of 100 %). Consequently, a positive sample could be identified after just 1 week when inoculated with pure bacterial suspension (Table 2).

### Differences between the two MAP strains (DSM 44133 and JII-1961)

Although most of the clusters that showed specific differences for the positive samples had similar courses for the two different MAP strains, we wanted to test whether significant differences between the two strains could be identified.

The differences between the two MAP strains were calculated by combining results for weeks 3 and 4 and for both concentrations (these two weeks showed the fewest differences in potential biomarker clusters). Of 160 clusters, 16 showed significant differences, 2 with \(P<0.001\) (clusters 48 and 73; Fig. 4). In contrast, almost no significant differences between the two strains were noted after week 1 (only one cluster with \(P=0.015\)).

### Differences caused by MAP concentrations

The differences between the two concentrations of MAP were also calculated for weeks 3 and 4 and for both strains in a combined way (these two weeks showed the fewest differences between each other). Of 160 clusters, 21 showed significant differences between the two concentrations, seven clusters with \(P<0.001\) (clusters 17, 26, 27, 36, 38, 83 and 84). Some clusters showed a fairly similar course over the whole time (Fig. 5a); interestingly, others showed a delayed course for samples with lower bacterial count (Fig. 5b, c).

### Principal component analysis (PCA)

A PCA was calculated for the original concentration of both strains over all weeks, and the controls to identify possible

---

![Fig. 4. Two representative clusters (a, b) with significant differences between the two strains (weeks 3 and 4 combined).](image-url)
causes for variance and to investigate whether the groups would cluster together even without inputting any pre-information to the algorithm.

Controls and MAP-positive samples could be separated efficiently just by the first two principal components (components with great influence on data variance, Fig. 6). Interestingly, despite clear discrimination, positive samples of 1 and 6 weeks of incubation were located next to the controls.

A cross-validated (leave-one-out) discriminant analysis for the first two principal components between positive samples at different dates and control samples showed a maximum correct classification rate of 84.4% (Table 3).

**Study II: differentiation of MAP from other mycobacterial species**

Separate analysis of these additional data resulted in 102 clusters. Twenty-three of these clusters showed highly significant differences between MAP and other mycobacterial cultures combined ($P<0.001$, Mann–Whitney $U$ test).

A PCA was performed for all mycobacteria. The PCA showed that most of the variance in measurements was caused by the differences between different species of mycobacteria. Most of the species (including MAP) could already be separated (except for some outliers) by the first two principal components (Fig. 7).

Therefore, the VOC patterns of MAP showed highly significant differences compared to the VOC patterns of other mycobacteria. A cross-validated (leave-one-out) discriminant analysis (the first 20 clusters) between MAP samples and samples of all other mycobacteria showed a maximum correct classification rate of 85.4% (96.4% sensitivity; 83.3% specificity; Table 4).

---

**Fig. 5.** Development of the peak intensity of three representative clusters (a–c) over time depending on the concentration of MAP.
DISCUSSION

In the present study, we verified that detection of MAP growth and discrimination from other mycobacterial species is reliable using DMS analysis of VOC patterns in the headspace of mycobacterial cultures on solid media. Methodological factors that influence VOC composition were identified.

Multiple opening of tubes causing ventilation and air exchange did not influence the VOC patterns significantly. This indicates that VOCs are produced continuously during bacterial cultivation. Consequently, repeated analysis of markers at different time points using the same cultures is possible.

Several inherent factors influence the VOC composition in the headspace above cultures and have to be considered during data analysis. Differences between bacteria-free controls (Aa and Ba) and tubes that were inoculated with heat-inactivated bacteria indicate that even dead bacteria emit significant numbers of VOCs into the headspace. Hence, large concentrations proportions of dead bacteria can modify VOC patterns, e.g. overaged cultures that are characterized by a balance between bacterial replication and death. Even after 6 weeks of incubation, the MAP cultures in the present study did not reach this stage. Furthermore, all control samples showed a significant shift in the emission of VOCs within the 6 weeks of incubation. Data are lacking in the literature; however, we hypothesize that this is due to degradation of medium components over time, affecting especially the biological components of HEYM, e.g. egg yolk. Consequently, samples with unknown growth characteristics must always be compared to similarly aged control tubes. During data analysis, peaks of unique clusters were found only in control samples, meaning that the growth medium itself emits more VOCs than the mycobacterial cultures. The growth of bacteria even reduced the number of emitted VOCs. Consequently, the data analysis of study II resulted in fewer clusters.

The composition and concentration of growth-dependent VOCs in the headspace over MAP cultures resulting in characteristic patterns changed over time. Up to the third week of cultivation, the rates of correct classification of bacterial growth were high (about 95%) but declined at later time points. It seems that volatile biomarkers of bacterial growth were at their maximum after about 3 to 4 weeks depending on the inoculum. Although nothing is known about the growth phases of MAP on solid media, it can be assumed that reduction or slowing of bacterial growth occurred later (comparable to the stationary phase of liquid batch cultures). To date, we can only speculate about the

---

Table 3. Results of the cross-validated discriminant analysis for the first two PCA factors between positive samples and controls (dilution 10^0)*

<table>
<thead>
<tr>
<th>Actual group</th>
<th>Predicted group membership</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aa/Ba/Ca (1+2)</td>
<td>MAP-1W</td>
</tr>
<tr>
<td></td>
<td>MAP-2W</td>
<td>MAP-3W</td>
</tr>
<tr>
<td></td>
<td>MAP-4W</td>
<td>MAP-6W</td>
</tr>
<tr>
<td>Cross-validated Count</td>
<td>51</td>
<td>6</td>
</tr>
<tr>
<td>Aa/Ba/Ca (1+2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAP-1W</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAP-2W</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAP-3W</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAP-4W</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAP-6W</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>%</td>
<td>85.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Aa/Ba/Ca (1+2)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MAP-1W</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>MAP-2W</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MAP-3W</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MAP-4W</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MAP-6W</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*84.4 % of cross-validated grouped cases correctly classified.
metabolic sources of VOCs of MAP and of mycobacteria in general. Different metabolic pathways are activated during the various growth phases of mycobacteria, which have been shown at the gene expression level [22] and with regard to antigen production [23]. This may also be mirrored in the VOC emissions. Differences in the short-chain VOC concentration over time have been reported by Crespo et al. [24] for cultures of M. kansasii, M. avium and M. smegmatis. Nonetheless, the discrimination of bacterial growth of MAP against controls was possible at all time points of the present study (see PCA; Fig. 6).

Lower levels of VOCs were found in the headspaces of tubes with lower bacterial numbers in the inoculum [24]. Similar VOC patterns were detectable with a delay of about 1 week of incubation compared to tubes with higher bacterial concentrations. In general, the growth kinetics and metabolism of bacteria depend on the bacterial concentrations. The generation time of MAP was observed to be dependent upon the number of organisms in the inoculum [24]. Furthermore, chemical signal molecules involved in quorum sensing of bacteria increase in concentration as a function of cell density [25].

Table 4. Results of a cross-validated discriminant analysis (first 20 clusters) between MAP and other mycobacteria*

<table>
<thead>
<tr>
<th>Actual group</th>
<th>Predicted group membership</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAP</td>
<td>Other</td>
</tr>
<tr>
<td>Cross-validated Count</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>OTHER</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>% MAP</td>
<td>91.7</td>
<td>8.3</td>
</tr>
<tr>
<td>OTHER</td>
<td>16.7</td>
<td>83.3</td>
</tr>
</tbody>
</table>

*85.4 % of cross-validated grouped cases correctly classified.

The two MAP strains under investigation represent different genotypes of MAP [26]. Furthermore, DSM 44133 is a laboratory-adapted strain with a long history of in vitro propagation, while JII-1961 is a field isolate from a bovine intestinal lymph node which underwent only a few in vitro passages before it was included in this study. Differentiation between the two strains was poor, although significant differences in a few clusters were detectable (clusters 48 and 73 – see Fig. 4). These findings emphasize that MAP strains share common features that can be used for (sub)species identification.

PCA demonstrated that discrimination of MAP-positive cultures (high inoculum concentration) from control samples is possible even using only the first two principal components. The dots of the MAP-positive samples are close, reflecting the similarity of VOC patterns at each measurement point. Discrimination becomes complicated in very short (1 week) and mature MAP cultures, since dots of positive samples and controls are more closely located, indicating the possibility of false-positive results. This mis-discrimination disappeared after 2 weeks of MAP cultivation. Using more components for discrimination can reduce false-positive results. Interestingly, false-negative discrimination did not occur in the study, even when using just the first two components.

Growing MAP were clearly differentiated from other mycobacterial species, including other closely related members of the M. avium complex, M. avium subsp. avium and M. avium subsp. hominisuis. This may be due to differences in cell wall composition, since the mycobacterial cell wall is a complex of carbohydrates and lipids that are biologically active [27, 28]. It has been shown recently that analysis of mycolic acids of the mycobacterial cell wall by different analytical methods (MS, nuclear magnetic resonance and Raman spectroscopy) allowed differentiation of tuberculous and non-tuberculous mycobacterial strains [29].

As a future prospect, DMS-based analysis of mycobacterial species-specific VOC patterns has the potential for direct classification of cultures without the necessity of additional tests to confirm species identity (e.g. PCR). This is an important advantage in comparison to the non-specific detection methods used in commercial culture systems to date. The consequences would be a shorter time until detection and a reduced workload.

In conclusion, this study provides strong evidence that detection of mycobacterial growth is possible by analysis of VOCs in the headspace of culture vessels by DMS. Furthermore, it was shown that a reliable identification of MAP in pure culture is possible already after 1 week of incubation. When this is applicable also to field samples, which has yet to be proved, it would be much faster than the standard method of visual evaluation of culture slopes and non-specific detection of bacterial growth in automated liquid culture systems. Despite sharing of the majority of VOC clusters by all included mycobacterial species, differentiation...
of MAP from other non-tuberculous mycobacteria was possible based on a few specific VOC clusters. The applicability of the technique for members of the Mycobacterium tuberculosis complex and for clinical diagnostic samples has to be evaluated in future studies.

Funding information
The authors received no specific grant from any funding agency.

Acknowledgements
The authors are very grateful to Sandy Werner for skilful technical assistance during measurements with DMS. We further thank Heike Friedrich for assistance in formatting the manuscript. Last but not least, the authors are thankful to Professor (em.) Dr Terence Risby (The Johns Hopkins University, USA) for the English editing.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References