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Crowd monitoring in dairy cattle—real-time VOC profiling by direct mass spectrometry

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Abstract

Volatile organic compounds (VOCs) emitted from breath, faeces or skin may reflect physiological and pathological processes *in vivo*. Our setup employs real-time proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS) to explore VOC emissions of dairy cows in stable air under field conditions. Within one herd of 596 cows, seven groups (8–117 cows per group) were assessed. Groups differed in milk yield and health status (two contained cows with paratuberculosis, a chronic intestinal infection). Each group arrived one after another in the area of air measurement in front of the milking parlour. A customised PTR-TOF-MS system with a 6 m long and heated transfer line, was used for measuring VOCs continuously for 7 h, 1.5 m above the cows. Three consecutive time periods were investigated. Twenty-seven VOCs increased while the animals were gathering in the waiting area, and decreased when the animals entered the milking parlour. Linear correlations between the number of animals present and VOC concentrations were found for $(C_4H_6)H^+$ and $(C_3H_6O)H^+$. A relatively high concentration of acetone above the cows that had recently given birth to a calf might be related to increased fat turnover due to calving and different nutrition. Changes in VOC emissions were related to the presence of animals with paratuberculosis, to different average milk yields per group and to the time of the day (morning versus noon milking time). We found that VOC monitoring of stable air may provide additional immediate information on an animal's metabolic or health status and foster novel applications in the field of breath research.

Introduction

Analysis of volatile organic compounds (VOCs) enables non-invasive, and even continuous monitoring of processes present *in vitro* or *in vivo*. VOCs can be determined from cell cultures [1], individual animals [2] or humans [3] or from crowds of humans [4] or animals [5]. VOC emissions from animals have been examined by means of individual breath gas analysis [2, 6], VOC determination in environmental chambers [7, 8], or from stable air [5, 9]. Current studies on stable air were performed to assess changes in diurnal VOC emissions of entire cattle farms [5, 10] and for the estimation of human health hazard derived from total VOC emissions of cattle farms [9]. So far, data focussing on the correlation of VOC profiles in stable air and the health status of the herd are lacking. Profiling of a broad range of trace VOCs in real time without sample preparation is possible by means of soft ionisation real-time MS e.g. proton-transfer-reaction mass spectrometry [11, 12]. In this way, fast changes in VOC concentrations with close relations to *in vivo* processes have been observed in clinical [13, 14] and environmental [15] setups.

The present pilot study aimed to investigate if continuous analysis of VOCs in room air enables monitoring of metabolic or infectious processes *in vivo*.

For stable air analysis, proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS) was applied to enable continuous VOC measurement with

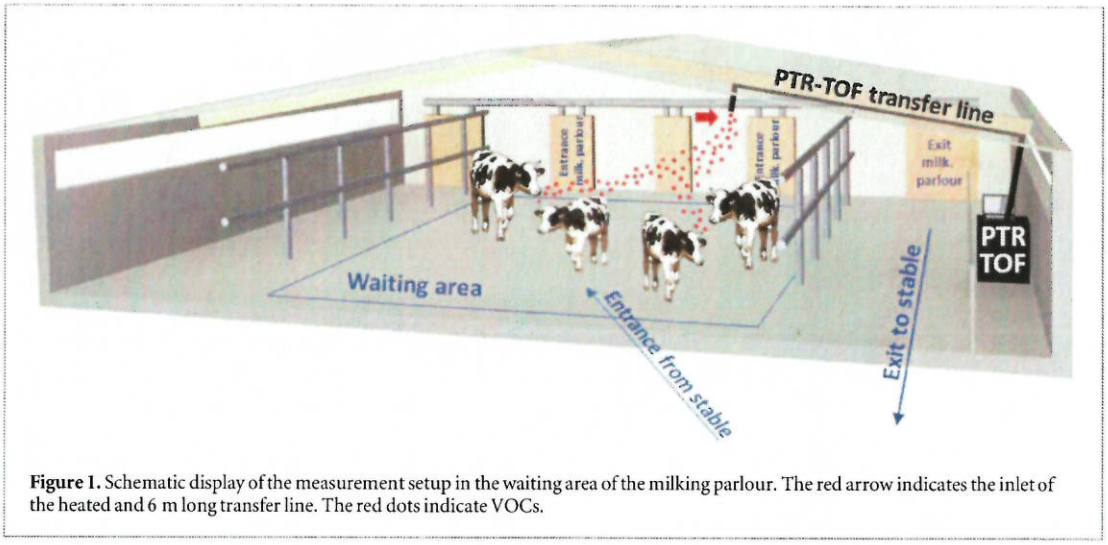

Figure 1. Schematic display of the measurement setup in the waiting area of the milking parlour. The red arrow indicates the inlet of the heated and 6 m long transfer line. The red dots indicate VOCs.

Table 1. Parameters and settings for PTR-TOF.

Transfer line		Ion source		PTR drift tube				TOF-MS	
Inlet flow	Temp.	H_2O flow	Current	E/N ratio	Temp.	Pressure	Voltage	Mass resolution	Integration time
20 sccm	75 °C	6–7 ml	4–5 mA	138 Td	75 °C	2.2 mbar	606 V	>1500 m/q	200 ms

a high mass and time resolution. The following questions were addressed in detail:

- Does VOC monitoring of stable air allow to detect the appearance and disappearance of animals?

  Is there a correlation between VOC concentrations and number of cows?

  How does contamination from feaces or urine affect VOC profiles?
- Do VOC emissions provide information on the metabolic status of cows?
- Is it possible to discern groups including infected animals?

Methods

Real-time VOC measurements of stable air in the waiting area

The present study was carried out in the waiting area in front of the milking parlour prior to the milking process. In that way the influence of excretions and food emissions as well as different husbandry conditions in different stables were reduced. One by one, several groups of cows arrived and waited for their turn to be milked. As the cows moved around and thus evenly fitted into the space in the waiting area, all animals contributed equally to the measured VOCs. With this setup, similar environmental conditions for the measurement of the different groups could be established. VOCs were measured for three milking periods. The first and third milking periods took place from 2–9 AM, whereas the second took place from 2–9 PM. Within each group, individual cows entered the waiting area consecutively and went further into the milking parlour (figure 1). As there were more animals per group than places at the milking parlour, the remaining animals stayed behind within the waiting area until their turn. After being milked, the cows went back to their particular stable area.

The floor of the waiting area consisted of concrete plates with a slatted floor to enable the removal of excrement and urine. It was cleaned with water after each milking period. The waiting area was connected to the other areas of the stable via two open hallways in the side opposite to the milking plant. Some air exchange between the waiting area and other parts of the plant took place. The windows consisted of metal grids covered with slack foil. Therefore, constant permeation of fresh air could be assumed. There was no mechanical ventilation system.

A PTR-TOF-1000 (Ionicon, Austria) was used to determine VOCs above the particular groups of cows present in the waiting area. A 6 m long and heated transfer line (silcosteel, ID 1.4 mm) was attached to a wooden beam. In that way, the inlet of the transfer line could be installed with a height of ca. 3.5 m. The PTR-TOF was protected from the animals via a small wall (figure 1). The TOF-integration time was set to 200 ms, resulting in five mass spectra (0–300 amu) per second. This enabled real-time MS and recognition of fast changes in VOC emissions. Further, the PTR-TOF parameters are shown in table 1.

Table 2. Cows and housing conditions.

Group	No. of cows	Milking events per day	Milk yield	Status	Number of cows infected with MAP	Husbandry (bedding material)
1	106	2	High	First lactation	0	Pads
2	117	2	High	Lactation[a]	1	Pads
3	97	3	High	Lactation[a]	4	Pads
4	106	2	Medium	Lactation[a]	0	Pads
5	54	2	Medium	Late lactation phase	0	Pads
6	5–8	2	—	Colostral phase (1–3 days after calving) and lame animals	0	Straw
7	108	2	Mixed	Fresh cows (>3 days after calving)	0	Pads

[a] cows in second or more lactation; groups are different in energy content of feeding according to milk yield and number of milking events per day.

Table 3. Parameters and properties of selected m/z ratios. n.r. = not reported.

Tentative VOCs	Chemical formula	Protonated mass [m/z]	Measured concentration range [nmol l^{-1}]	Potential main origin
Methanol	$(CH_4O)H^+$	33.034	52.24–303.89	Eructation [16]
Acetonitrile	$(C_2H_3N)H^+$	42.034	1.26–7.37	Eructation [16]
Hydrazoic acid	$(HN_3)H^+$	44.024	3.71–12.40	n.r
Oxirane	$(C_2H_4O)H^+$	45.034	12.47–64.76	Eructation [16]
Formic acid	$(CH_2O_2)H^+$	47.013	15.60–36.84	Eructation [16]
Ethanol	$(C_2H_6O)H^+$	47.049	4.04–63.65	Eructation [16]
Methanthiole	$(CH_4S)H^+$	49.011	1.15–24.74	Faeces, urine [17, 18]
1,3-Butadiene	$(C_4H_6)H^+$	55.054	6.59–16.93	Eructation [16]
Butene	$(C_4H_8)H^+$	57.070	3.02–27.94	Eructation [16]
Acetone	$(C_3H_6O)H^+$	59.049	52.32–981.38	Breath, eructation [6, 16]
Trimethylamine	$(C_3H_9N)H^+$	60.081	19.81–568.382	Eructation [19] urine, faeces [10]
Acetic acid	$(C_2H_4O_2)H^+$	61.028	27.97–368.79	Eructation [16, 20], faeces [21–23], urine [22, 24]
Isopropanol	$(C_3H_8O)H^+$	61.065	5.12–48.82	Breath [6]
Nitromethane	$(CH_3NO_2)H^+$	62.024	2.88–10.19	Eructation [16]
Dimethyl sulphide	$(C_2H_6S)H^+$	63.026	18.88–252.05	Eructation [16, 20], faeces [23, 25]
1,1-Difluorethene	$(C_2H_2F_2)H^+$	65.020	1.39–11.69	Eructation [16]
Isoprene	$(C_5H_8)H^+$	69.070	2.13–7.36	Breath, eructation [16]
2-Butenal	$(C_4H_6O)H^+$	71.049	1.12–4.45	n.r
2-Butanone	$(C_4H_8O)H^+$	73.065	4.21–66.03	Breath, eructation [6, 16, 20]
Propanamid	$(C_3H_7NO)H^+$	74.060	2.46–9.13	Eructation [16]
Propionic acid	$(C_3H_6O_2)H^+$	75.044	5.72–65.80	Breath, eructation [6, 16, 20], faeces [21–24]
Butanoic acid	$(C_4H_8O_2)H^+$	89.060	5.00–64.89	Eructation [16, 20], faeces [21–24]
Phenol	$(C_6H_6O)H^+$	95.049	4.79–65.50	Breath, eructation [6, 16, 20], faeces[6], urine [21, 22, 24]
Isovaleric acid	$(C_5H_{10}O_2)H^+$	103.075	1.72–14.94	Breath [6, 20], faeces, urine [21, 22, 24]
Methylphenol	$(C_7H_8O)H^+$	109.065	13.73–136.02	Eructation [20], faeces [23], urine [21, 22, 24]
Aminophenol	$(C_6H_7NO)H^+$	110.060	1.73–12.81	n.r
Nicotinamide	$(C_6H_6N_2O)H^+$	123.055	1.55–6.04	n.r

Animal housing conditions and welfare

This study was carried out in strict accordance with European and national laws for the care and use of animals. The protocol was approved by the Animal Health and Welfare Unit of the *Thüringer Landesamt für Verbraucherschutz* (Permit Number: 04-102/16). The experiments were done under the supervision of the authorised institutional Animal Protection Officer. No animal was harmed or disturbed in its daily routine. The herd of observation was kept in a half open stable, and was grouped for milking by milk yield (table 2). Two groups contained animals infected with *Mycobacterium avium* ssp. *paratuberculosis* (MAP), the causative agent of paratuberculosis, a chronic intestinal infection (table 2). Food and water were provided ad libitum whereas the energy content of the food for each group of cows was adjusted according to the milk yield.

VOC selection and statistical analysis

Only m/z ratios with significant changes in concentration during the measurements were selected for further analysis. Unequivocal identification of VOCs with identical m/z ratios is not possible due to the

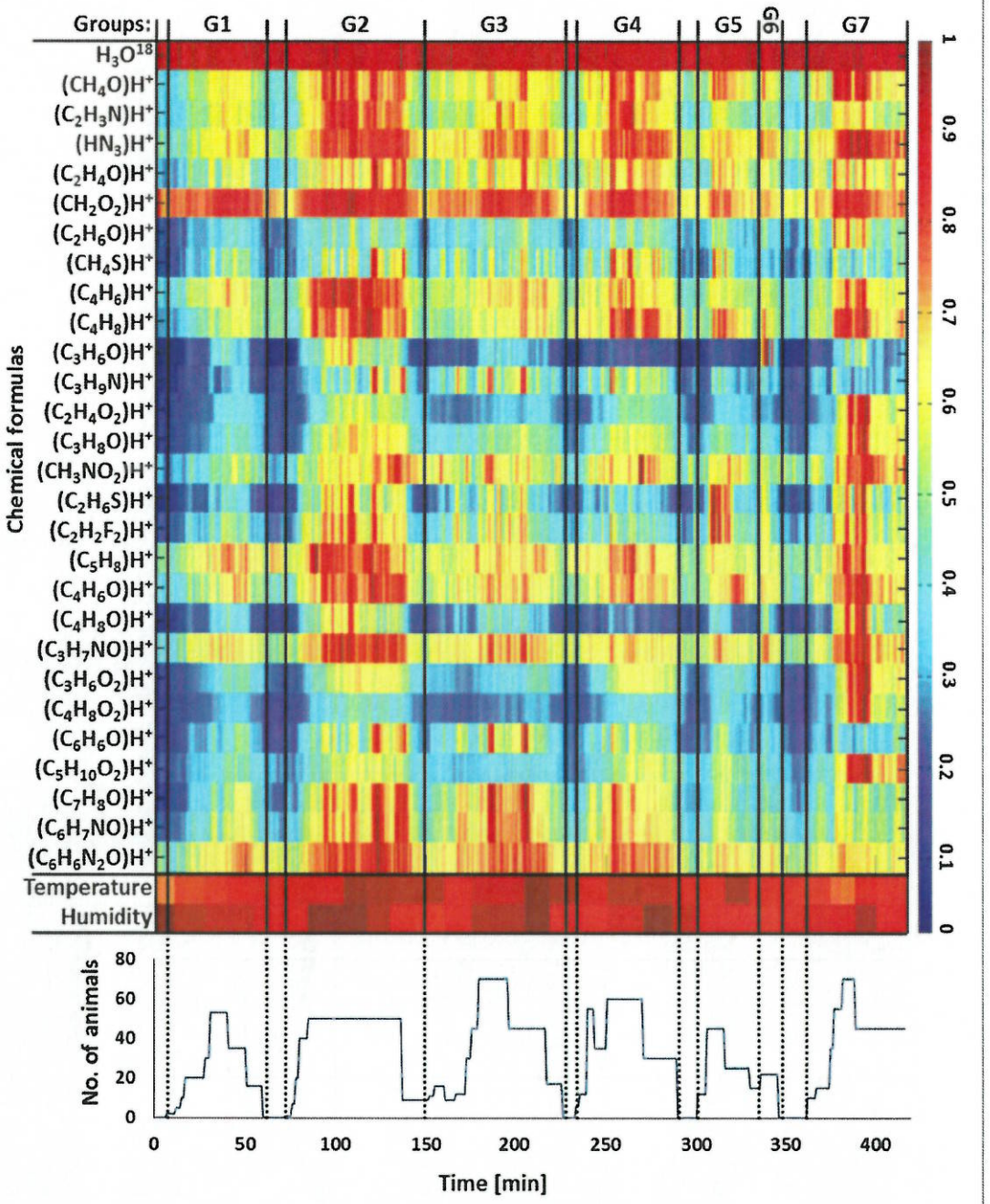

Figure 2. Heatmap of VOC concentration changes during the third milking period. For every m/z ratio and its corresponding chemical formula, the concentration was normalised onto its maximum. Corresponding number of cows in the waiting area are shown in the graph below. G1–G7: groups of cows (see table 2).

functional principle of PTR-TOF-MS. Thus, m/z ratios were tentatively assigned to VOCs according to knowledge obtained from the scientific literature (table 3). The data from this study was non-parametric. The Kruskal–Wallis test with a two-tailed comparison to a control group was applied to evaluate significant differences. R, the language and environment for statistical computing for further statistical investigations, was used for statistical computing. *P*-values below 0.05 were defined as statistically significant.

Results

VOC profiles of the different groups of cows

The arrival, presence, and departure of cows in the waiting area were recognisable by VOC concentration changes in the stable air. Twenty-seven VOCs increased when the animals stayed in the waiting area, and decreased when the animals left the area to enter the milking parlour (figure 2). The magnitude of the increase differed for each VOC. The increase in VOC concentrations was slightly shifted in time by a few

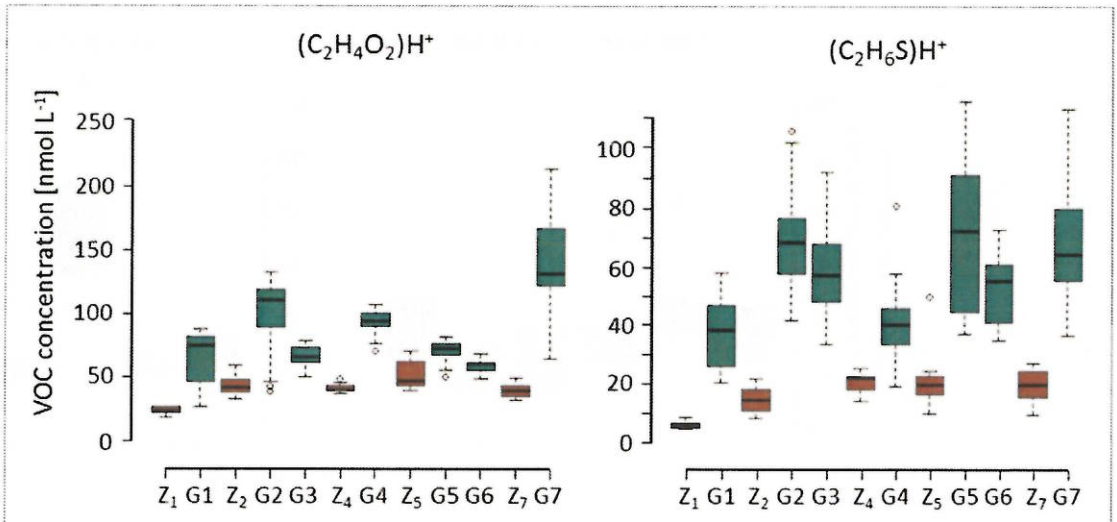

Figure 3. Acetic acid ($(C_2H_4O_2)H^+$) and dimethyl sulphide ($(C_2H_6S)H^+$) concentrations in stable air in the presence (green) and absence (brown) of animals. Data from the three milking periods are shown. 'Z_i' indicates phases without the presence of animals. G1–G7: groups of cows. Data is presented as medians with percentiles of 25% and 75%.

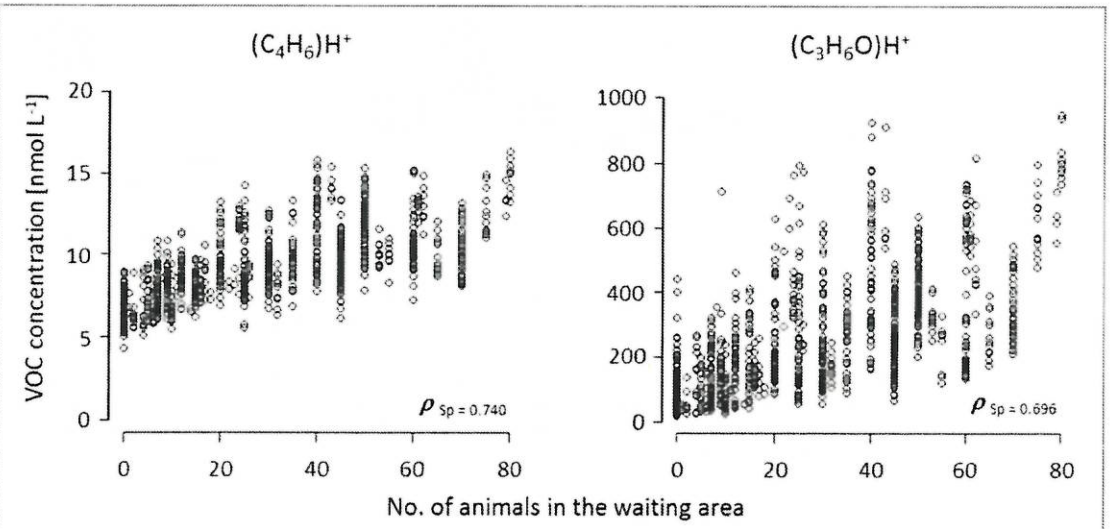

Figure 4. Correlation of 1,3-Butadiene ($(C_4H_6)H^+$) and acetone ($(C_3H_6O)H^+$) concentration with number of animals. Data from all three milking periods are shown in the plots. Spearman correlation coefficient is given as ρ_{Sp}.

minutes compared to the increase in the number of cows in the waiting area. The waiting area got dirtier due to excretion from cows but faeces-related VOCs (table 3) did not disturb the analysis during the measurement period (see figure 3). Concentrations of marker substances returned to baseline levels when the animals had left the area. This proves that the environment (e.g. liquid manure) had no significant effect on peak concentrations of the selected marker substances. The temperature and relative humidity showed only minor changes over the measurement period (see figure 2; third milking period $T = 16.90\,°C \pm 1.15\,°C$ SD; rel. humidity 71.30% ± 3.95%, SD).

Tentatively assigned VOCs may originate from different sources: breath, eructation, faeces, urine or skin (table 3). Some VOCs may originate from multiple sources.

Correlation of VOC concentration and number of cows

A linear correlation between the number of animals and VOC concentration could be found for two VOCs (figure 4). Spearman correlation revealed a coefficient of 0.740 for 1,3-Butadiene ($(C_4H_6)H^+$) and 0.696 for acetone ($(C_3H_6O)H^+$). Other VOCs showed smaller correlation coefficients.

Differences between groups

For further analysis, the section of maximum VOC concentrations of each animal group was selected for analysis and normalised to the mean number of cows present in the measurement area at these time points.

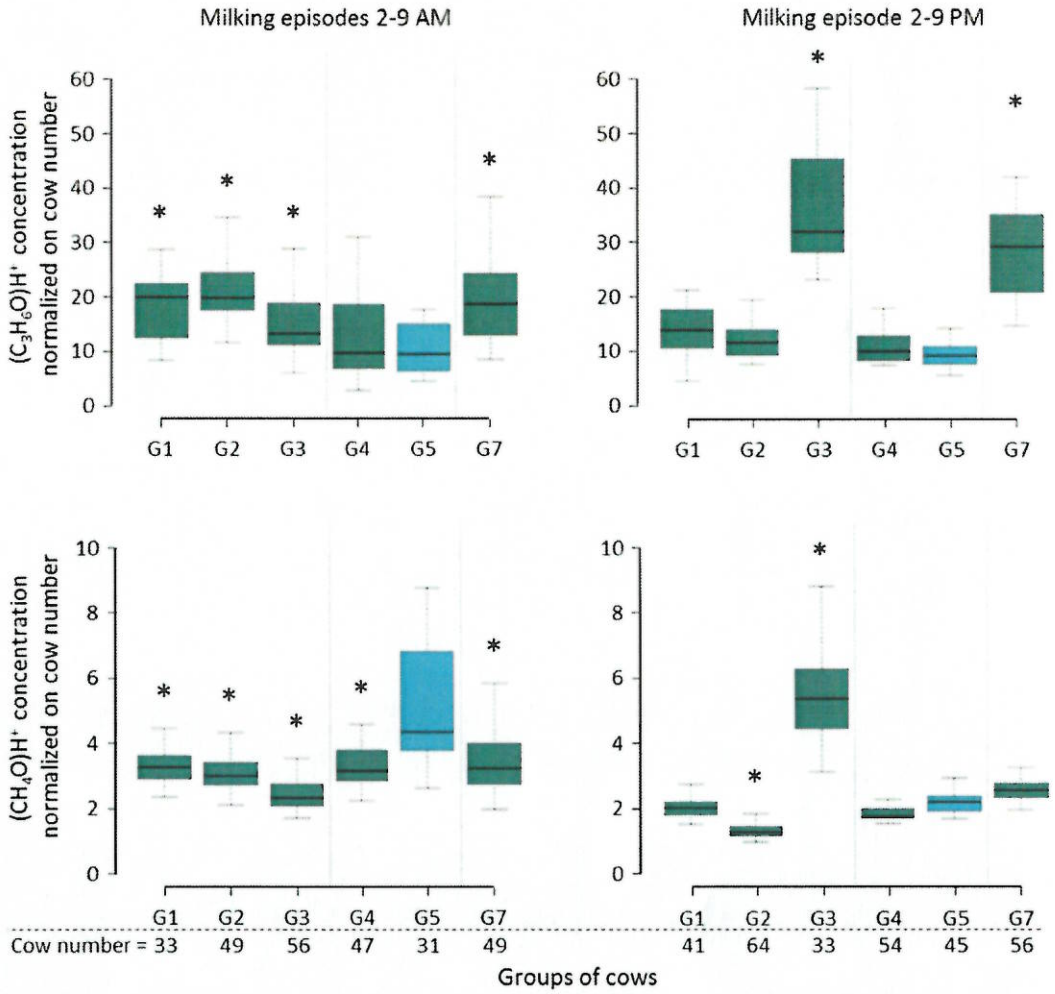

Figure 5. Emissions of acetone ($(C_3H_6O)H^+$) and methanol ($(CH_4O)H^+$) in different groups. Significant differences in cows in the late lactation phase (G5, blue coloured box) are marked with asterisks. Grey dashed lines separate different milk yields of groups (table 2). G1–G7: groups of cows. The number of cows indicates the mean number of cows present in the waiting area during the respective section of maximum VOC emission.

Data of the consecutive days, i.e. milking periods 1 and 3, were pooled, as VOC concentrations differed only slightly (see figure S2 in the supporting information, available online at stacks.iop.org/JBR/13/046006/mmedia), and the conditions in terms of activity and food intake were similar.

Metabolic status

The high concentration of acetone ($(C_3H_6O)H^+$) measured for group 6 was remarkable because of the comparably low number of cows (n = 5–8, figure 2). Even the short presence (<10 min) of very few cows sufficed to measure a high concentration of acetone ($(C_3H_6O)H^+$). As this group was very special regarding the number of animals, husbandry, and status it was excluded from further comparisons.

Changes in VOC emissions differed significantly between the groups (figures 5 and 6). VOC emissions above the cows in the late lactation phases were different from those of other cows. Acetone emissions were the lowest in cows with a medium milk yield (G4, G5) and highest in those with a high (G1, G2, and G3) or mixed (G7) milk yield. With respect to the different milking phases (morning and afternoon/evening) the distribution of acetone emissions was similar, but differences between the groups of cows varied. Methanol ($(CH_4O)H^+$) emissions depended more on the time of milking than on milk yield.

Presence of paratuberculosis (MAP infection)

Concentrations of 2-butanone ($(C_4H_8O)H^+$) and methylacetate ($(C_3H_6O_2)H^+$) have been reported in the context of MAP infections [26–28]. Figure 6 shows emission profiles of these substances above the groups. Partially infected groups 2 and 3 (with 1 and 4 infected animals, respectively) were compared to groups without infected animals.

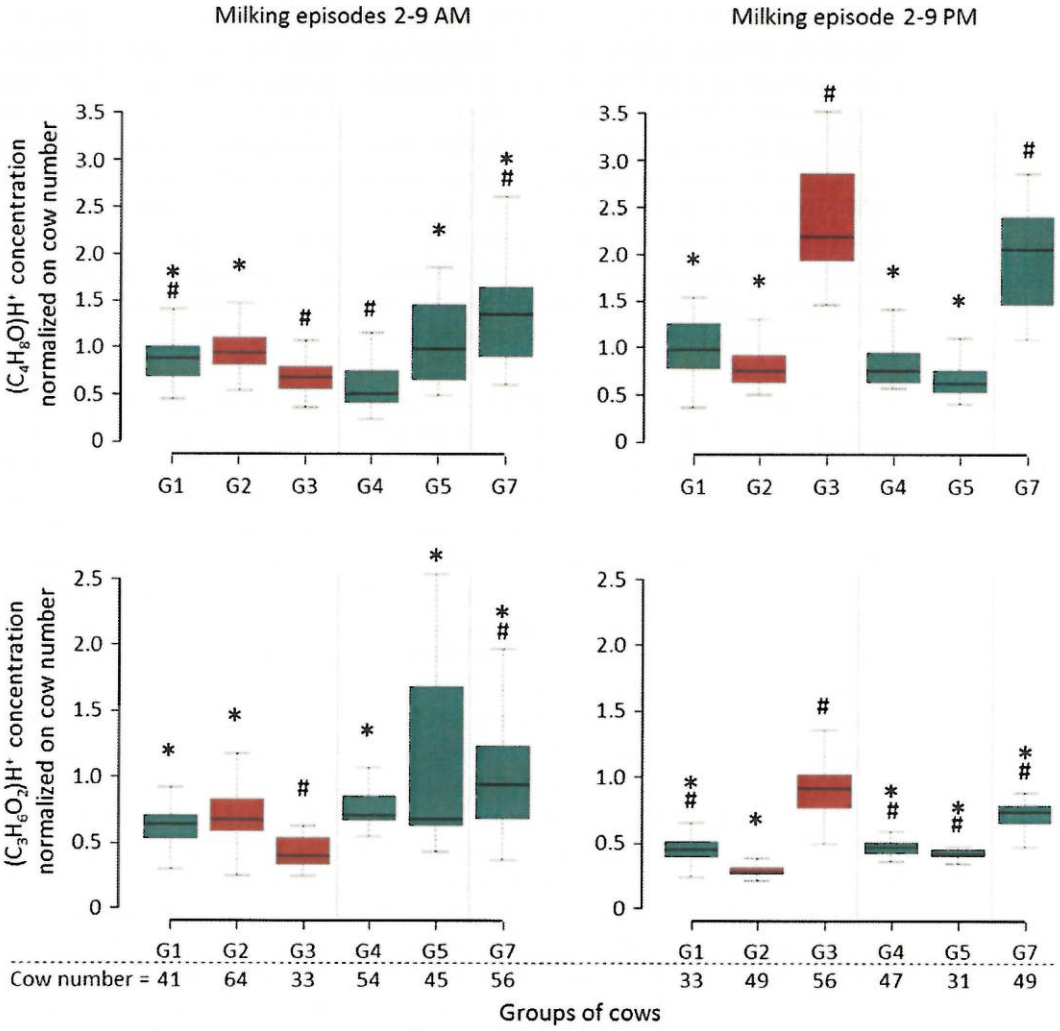

Figure 6. Comparison of normalised 2-butanone ($(C_4H_8O)H^+$) and methylacetate ($(C_3H_6O_2)H^+$) emissions in stable air above groups of cows with and without MAP-infected animals. VOC concentration was normalised to number of animals. G1–G7: groups of cows. Groups with MAP-infected animals (G2, G3) are coloured in red. Significant differences in comparison to G2 and G3 are shown with rhombus and asterisks, respectively. Grey dashed lines separate the different milk yields of groups (table 1). Number of cows indicates the mean number of cows present in the waiting area during the respective section of maximum VOC emission.

Both substances expressed differences between groups containing MAP-infected animals and non-infected animals. Especially group 3 stood out and emitted less VOCs in the morning and more VOCs in the afternoon. Group 4 was defined as a control group as it consisted of a comparable numbers of cows, with similar nutrition and without any special notifications on their current status. Compared to group 4, group 3 always had higher VOC emissions in the afternoon and mostly lower emissions in the morning (see table S1).

Discussion

Continuous VOC analysis from stable air sampled in the presence of varying numbers of cows in the waiting area in front of the milking parlour was possible by means of PTR-TOF-MS. Depending on the presence or absence of animals, changes in the concentration of 27 VOCs in the nmol l^{-1} range could be observed. The return of VOC concentrations to baseline levels occurred within minutes after the cows had left the area. VOC profiles were different when cows with different metabolic or health related status were present.

Previous studies which assessed VOCs in stable air focused on environmental issues [5, 7]. In the present study we investigated the possibility of monitoring metabolic and health related processes via VOC profiles in stable air. One of the main features of this study was the determination of VOC profiles by means of real-time MS in a field setup under realistic conditions. In contrast to studies done in the laboratory or environmental chambers, not all parameters (such as air exchange, ventilation, proximity to the inlet, or movement of single individuals) can be fully controlled. Nevertheless, we were able to acquire a

continuous and more differentiated VOC profile due to the utilisation of a real-time TOF-MS providing a high mass resolution. Additionally, the identical area of measurement allowed VOC assessment of different groups of cows under comparable conditions.

The applied analytical setup was suitable to measure rapid VOC concentration changes in trace levels (ppbV) from stable air continuously and with a subsecond time resolution. We applied a high time resolution for VOC measurements as VOC concentration changes may happen fast due to eructation [16], excretion, or urination. Our results show that changes in the concentration of the VOC profiles from stable air occurred in the range of minutes rather than in the range of seconds. This is probably due to the time the VOCs needed to distribute within the stable air rather than from the delay due to the analytical setup with long transfer lines (<5 s [13]). Punctual sampling above a group of animals at a fixed time point only, may, however, neglect important concentration changes due to different metabolic or nutrition conditions. In order to gain even more sensitivity, TOF-integration times may, however, be increased to the range of ~60 s. To adapt the setup to more general conditions or larger stables, a longer transfer line similar to modifications reported by Trefz *et al* [13] may be feasible.

The limited correlation between some VOC concentrations and the number of animals may be due to the combined effects of time shift, distribution of cows in the waiting area (proximity to PTR-TOF transfer line), or possible dilution effects from fresh air into the waiting area. The time shift of some minutes between the increase in the number of cows within the waiting area and changes in VOC emissions can be explained by the time needed for VOC distribution within stable air. It is also possible that, due to all those factors, the correlation between VOC concentrations and the number of cows is not only linear. In general, VOC profiles are therefore related to the number of animals as well as to different physiological and analytical effects. To reduce the effects related to the number of cows, data were normalised onto numbers of animals present in the waiting area.

As VOC profiles quickly returned to base levels when the animals left the waiting area, selected VOCs are likely to originate mainly from breath, eructation, or skin rather than from faeces or urine. Although the waiting area potentially was polluted by excretions from cows over time, faeces-related VOCs did not increase relevantly over the whole measurement period. This was most probably due to the fact that excrements disappeared through the grid of the floor. These results are in agreement with the investigations of Shaw *et al* [8]. They reported that faecal emissions only minimally contributed to air VOC profiles of cows in environmental chambers. Apart from faecal or urine emissions, VOCs emitted from cows into ambient air may originate from breath or eructation [16]. That is, deviations and differences in VOC emissions between different animal groups have to be interpreted with respect to potential changes in blood borne VOCs and with respect to altered composition of rumen gas. Hence, investigation of ambient air in the presence of cows ('herd monitoring') may simultaneously provide information on systemic metabolism and on fermentation and biochemistry in the intestinal tract, particularly in the forestomaches. Substances linked to systemic metabolism were alcohols (methanol, ethanol, isopropanol), ketones (acetone), and aldehydes (butenal), substances typically linked to fermentation and ruminal microbiome were (fatty) acids (acetic, propionic and butanoic acid), and sulphur containing compounds (thioethers) [29].

The increase in acetone emission in group 6 was remarkable as a comparatively low number of animals was sufficient to cause high concentrations in ambient air. Group 6 consisted of animals 1–3 days after first calving, and very high acetone emissions may be related to changes of metabolic pathways in these animals. As acetone production is linked to glucose metabolism and lipolysis, its concentration will increase when fat metabolism (lipolysis) is intensified due to calving and increased lactation [30]. Additional effects potentially influencing metabolism in these animals were bedding on straw and being fed with different feeds [31]. The lowest acetone concentrations in cows in the late lactation phase are most probably due to the same effect. Cows with a high milk yield or those with recent calving have higher metabolic activity, and thus produce more acetone than those in the late lactation phase. Variation in differences of acetone emissions between morning and afternoon milking mirrors the dependency of metabolic activity on the time of day. Concerning methanol emissions, this effect seems to override any other potential effect of milk yield or metabolic status. Methanol can be derived from the degradation of pectin, an ubiquitous part of non-wooden plants [32]. Hence, it is not surprising that methanol excretion mainly depends on food intake and digestive status which will be different at different times of the day.

VOC profiles of groups containing MAP-infected cows showed changes in 2-butanone ($((C_4H_8O)H^+)$) and methylacetate ($((C_3H_6O_2)H^+)$) emissions. When compared to a non-infected group with similar metabolic status in terms of milk yield and lactation, ketone emissions were higher and acid emissions were lower in groups including cows with paratuberculosis. Oxygenated compounds in the breath have been linked to oxidative stress and may, thus, indicated increased oxidative (inflammatory) activity in the infected animals. (Fatty) acids in ruminant's digestion are typically linked to digestive processes in the forestomaches. Comparison of the infected animals with healthy cows having a high milk yield and high energy intake due to first calving indicated that metabolic conditions might interfere with infection-related phenomena.

In conclusion, the described setup was appropriate to monitor dynamic changes in trace VOC profiles above cows in a realistic field scenario. Herd monitoring provided promising information on metabolic and inflammatory processes in different groups of animals. Remarkably, even the presence of some infected cows in a larger group may impact the composition of emitted VOC profiles. 'Crowd VOC monitoring' may foster the field of VOC research and serve as a first, non-invasive, simple, and fast screening approach well before individual investigations *in vivo*.

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