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Application of PTR-MS to an incubation experiment of the marine diatom *Thalassiosira pseudonana*

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Emission of trace gases from the marine diatom *Thalassiosira pseudonana* (CCMP1335) was continuously monitored with a proton transfer reaction-mass spectrometry (PTR-MS) in an axenic batch culture system under a 13:11-h light:dark cycle. Substantial increases in the signals at *m*/*z* 49, 63, and 69, attributable to methanethiol, dimethyl sulfide (DMS), and isoprene, respectively, were observed in response to increases in cell density. Signals at *m*/*z* 69 showed diurnal variations throughout the experiment whereas those at *m*/*z* 49 were more pronounced at the beginning of the incubation. Interestingly, the signals at *m*/*z* 49 and 69 changed immediately following the light-dark and dark-light transitions, suggesting that light plays a crucial role in the production of methanethiol and isoprene. However, in the latter half of the experiment, methanethiol showed negligible diurnal variations regardless of light conditions, suggesting the production of methanethiol from enzymatic cleavage of DMS. The trend in signals at *m*/*z* 63 was similar to that of the abundance of senescent cells plus cell debris rather than vegetative cells. The results suggest that aging or death of phytoplankton cells could also substantially control DMS production in natural waters along with the other microbial processes related to bacteria and zooplankton.

Keywords: dimethyl sulfide, isoprene, methanethiol, PTR-MS, *Thalassiosira pseudonana*, axenic culture

INTRODUCTION

While trace gases occupy less than 1% by volume of the atmosphere, they play important roles in atmospheric chemistry and the surrounding environment. The ocean surface is broadly known to act as one of the major sources for atmospheric trace gases including hydrocarbons and sulfur compounds through a variety of biogeochemical and photo-induced processes (e.g., Bonsang *et al*., 1992; McKay *et al*., 1996; Kettle *et al*., 1999). The concentrations of many trace gases in the ocean surface can be related to phytoplankton abundance, suggesting that phytoplankton activities contribute significantly to the trace gas production processes either directly or indirectly (Nguyen *et al*., 1988; Wingenter *et al*., 2004; Gist and Lewis, 2006). The production of trace gases by phytoplankton has also been confirmed through laboratory experiments using algal cultures (e.g., Stefels and van Boekel, 1993; McKay *et al*., 1996; Shaw *et al*., 2003). For example, many marine phytoplankton species produce trace gases such as isoprene, monoterpenes, and halogenated compounds with different emission rates depending on the algae species (Colomb *et al*., 2008; Yassaa *et al*., 2008). Shaw *et al*. (2003) pointed out that the production rate of hydrocarbons depends on the growth phase of the phytoplankton. Studies of dimethyl sulfide (DMS), one of the sulfur compounds, have included examination of the enzymatic cleavage of dimethylsulphoniopropionate (DMSP) to DMS, and DMS oxidation to dimethylsulphoxide (DMSO) as they relate to physicobiochemical factors such as light intensity, nutrient concentration, viral lysis, and microzooplankton grazing (Vetter and Sharp, 1993; Sunda *et al*., 2002; Evans *et al*., 2007; Harada *et al*., 2009). However, Shaw *et al*. (2010) suggested that few laboratory studies of isoprene production have been previously reported, and more are necessary to explain production mechanisms, confirm the dependence of emission rates on phytoplankton speciation, and determine the conditions that promote and inhibit production. Furthermore, in previous studies, discrete sampling (several times in a day) was commonly used for trace gas measurements. As biological processes often respond rapidly to changes in environmental conditions, high-frequency measurements are required to

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better understand the production mechanisms of biological trace gases.

Proton transfer reaction-mass spectrometry (PTR-MS) can potentially overcome this difficulty and allow high sensitivity measurements of atmospheric trace gases (at pptv levels) with a rapid response time (Lindinger *et al*., 1998; de Gouw and Warneke, 2007). In addition, this analytical technique has the advantage of eliminating sample pre-treatment steps such as dehydration or pre-concentration, resulting in a dataset with continuous and frequent observations.

In this study, we used PTR-MS to monitor the trace gases released from an axenic culture of the marine centric diatom *Thalassiosira pseudonana* (CCMP1335). This species has been used often in laboratory culture experiments (see the review by Sarthou *et al*., 2005) and was chosen as the first eukaryotic marine phytoplankton for whole genome sequencing (Armbrust *et al*., 2004). The genus *Thalassiosira* is found throughout the world's oceans (Hasle and Syvertsen, 1996). We evaluated variations in the release of the trace gases in response to changes in cell abundance and light conditions.

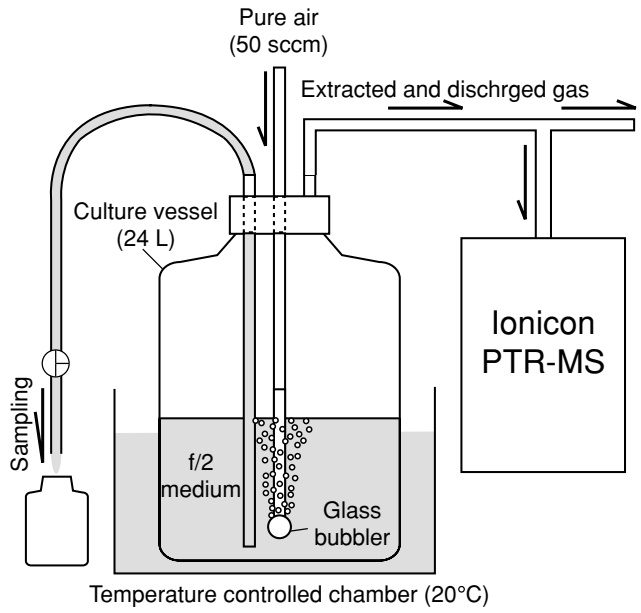

Fig. 1. Schematic diagram of the culture experiment and measurement system.

Culture Conditions

An axenic culture of the marine centric diatom *Thalassiosira pseudonana* (Hustedt) Hasle et Heimdal (CCMP 1335) isolated from Moriches Bay (Long Island, New York, USA) in 1958 was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbor Maine, USA). Figure 1 shows a schematic of our experiment system. Ten liters of f/2 medium (Guillard, 1975) was prepared and placed in a 24-L polycarbonate carboy, which was first cleaned with a neutral detergent and 1 M hydrochloric acid and then carefully rinsed with Milli-Q water and f/2 medium before the experiment. To prevent contamination from ambient air, the carboy was sealed with an autoclaved screw cap, which had three fittings to permit bubbling of the medium, transport of the extracted gas directed to PTR-MS, and sample collection for biological analysis, which was only opened upon samplings. The medium was degassed by purging with 500 standard cubic centimeters per minute (sccm) of pure air for a day by using an autoclaved glass bubbler and Tygon tubing, and then *T. pseudonana* was added to the medium. The culture was grown and incubated at 20°C under a 13:11-h light:dark cycle at 145 μmol photons m^{-2} s^{-1} from fluorescent lamps, with constant bubbling of purified air at 50 sccm.

All samples were collected via autoclaved Tygon tubing after first gently shaking the medium. Duplicate water samples (2 mL) for measurement of cell density were collected daily, preserved with paraformaldehyde (0.2% final concentration) and stored in a deep-freezer at –80°C. A 2-mL and a 10-mL water sample for nutrient concentration measurements were collected every other day and stored in a freezer at –20°C. The cell density of vegetative cells and senescent cells plus cell debris was analyzed by a flow cytometer (XL ADC system, Beckman Coulter, Brea, California, USA). Details of the flow cytometry analytical procedure are described by Suzuki *et al*. (2005). Vegetative cells were distinguished from senescent cells plus cell debris by the intensity of *in vivo* chlorophyll fluorescence. Concentrations of nutrients were measured by using an auto-nutrient analyzer (QuAAtro, BRAN+LUEBBE, Hamburg, Germany).

PTR-MS Measurements

We used a commercially available PTR-MS instrument (PTRMS-FDT-hs, IONICON Analytik GmbH, Innsbruck, Austria) as described by Inomata *et al*. (2008). Briefly, the PTR-MS instrument consists of an ion discharge source to produce H_3O^+ ions, a drift tube, in which the proton-transfer reactions between H_3O^+ and trace gases take place, and a quadrupole mass spectrometer (QMS) for detection of reagent and product ions. Trace gases were extracted from the culture medium by the 50 sccm constantly bubbling air controlled by a mass flow controller (FC-795C, Advanced Energy Japan, Tokyo, Japan), collected in the headspace of the culture vessel where they were diluted by mixing with other gases, and then discharged. A portion of the gases (approximately 22 sccm)

discharged from the carboy was continuously directed to the PTR-MS at ambient pressure without pre-treatment such as dehydration or pre-concentration. The drift voltage was set to 400 V, and the field strength, E/N, of the drift tube at the voltage setting, where E is the electric field strength (V cm^{-1}) and N is the buffer gas number density (molecule cm^{-3}), was 108 Td (Td = 10^{-17} cm^2 V $molecule^{-1}$). The field strength was set as low as possible to minimize fragmentations of detected VOCs. The analysis was performed with the instrument in scan mode (from m/z = 21 to m/z = 120, with 0.5-s integration per mass), and the mass signals obtained at 50-s intervals were used in this study. The water vapor concentrations in the sample air, $[H_2O]_{sample}$, were determined by the best-fit curve for the plot of $[H_2O]_{sample}$ versus the relative intensity of $H_3O^+ \cdot H_2O$ to H_3O^+ (the ratio of m/z 37 to m/z 19), as shown by Inomata *et al*. (2008). The humidity dependence of PTR-MS detection sensitivities were taken from Kameyama *et al*. (2009) and Kameyama *et al*. (2010). Similarly, the humidity dependence for methanethiol indicated by the signals at m/z 49 (described in detail later) was estimated in the laboratory by the same procedure shown by Inomata *et al*. (2008), using the following equation:

$$RS = (0.000292 \pm 0.000012) \times \{[H_2O]_{sample}\}^2 + 1,$$
$$R^2 = 0.97 \ ([H_2O]_{sample} = 0\text{–}15 \text{ mmol mol}^{-1});$$

where RS is the detection sensitivity relative to that under dry conditions.

Temporal Variations in Cell Density, Nutrients and Organic Species

The growth curve of vegetative *T. pseudonana* cells was divided into two phases: an exponential growth phase up to day 5 (i.e., 5 days from the beginning of the experiment) and a stationary phase after day 5 (Fig. 2a). The abundance of senescent cells plus cell debris increased until day 11. Silicate concentrations rapidly declined during the exponential growth phase and thereafter remained almost constant at about 10 μmol L^{-1} (Fig. 2b), indicating a balance between nutrient utilization by the strain and the dissolution of biogenic silica. Nitrate plus nitrite and phosphate concentrations gradually decreased with time (Figs. 2c and d).

The signals of trace gases in the discharged air were measured starting about 30 h after inoculation. This was to allow replacement of headspace gas in the carboy with the purging air because the headspace might have been contaminated by ambient air during inoculation. After 30 h, approximately 90% of the headspace air had been replaced by the purified purging air. Increases and decreases in measured signals of the trace gases reflect increases and decreases in their production rates, respectively.

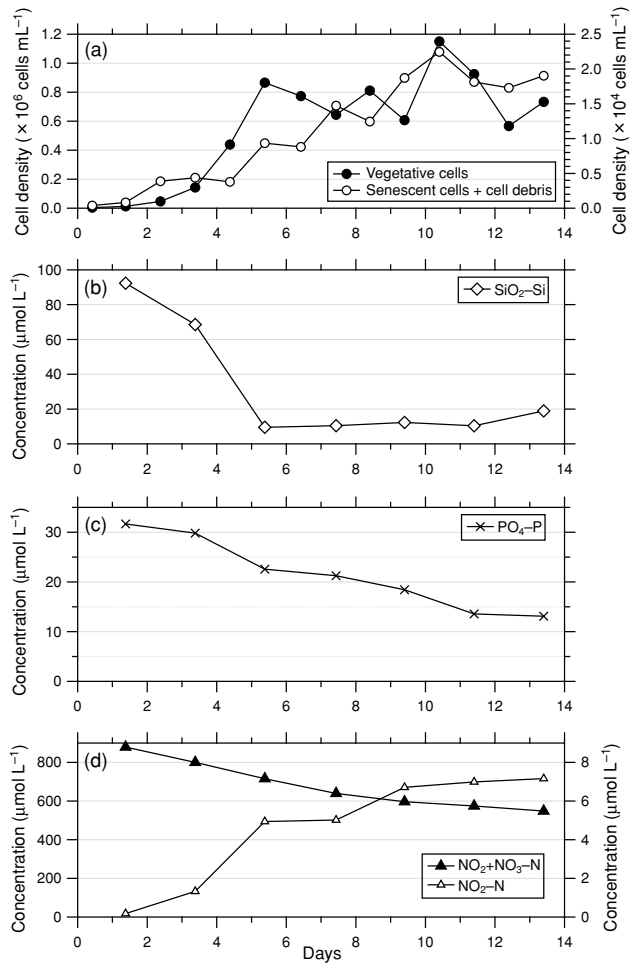

Fig. 2. Time course of (a) cell density of vegetative cells (left axis) and senescent cells plus cell debris (right axis), and concentrations of (b) silicate, (c) phosphate, and (d) nitrite plus nitrate (left axis) and nitrite (right axis) during the culture period.

Figure 3 shows examples of mass spectra for background and measurements. The mass spectra for the sample measurements shown here were at 0 o'clock on days 4, 8, 12. The background signals were obtained for the discharged gas before the inoculation of the diatom strain. In the background mass spectrum, signal intensities more than 100 ncps (normalized counts per second, the count rates of the product ions expressed as ion count rates normalized to an H_3O^+ intensity of 10^6 cps) were detected at m/z 45, 59, 73, except for the signals influenced by primary ions (m/z = 30, 32, 37, 39, 55, 57). These signals are attributable to acetaldehyde, sum of acetone and propanal, and butanal, respectively, and are likely due to some organic contamination in the culture medium. During the experimental period, we found substantial in-

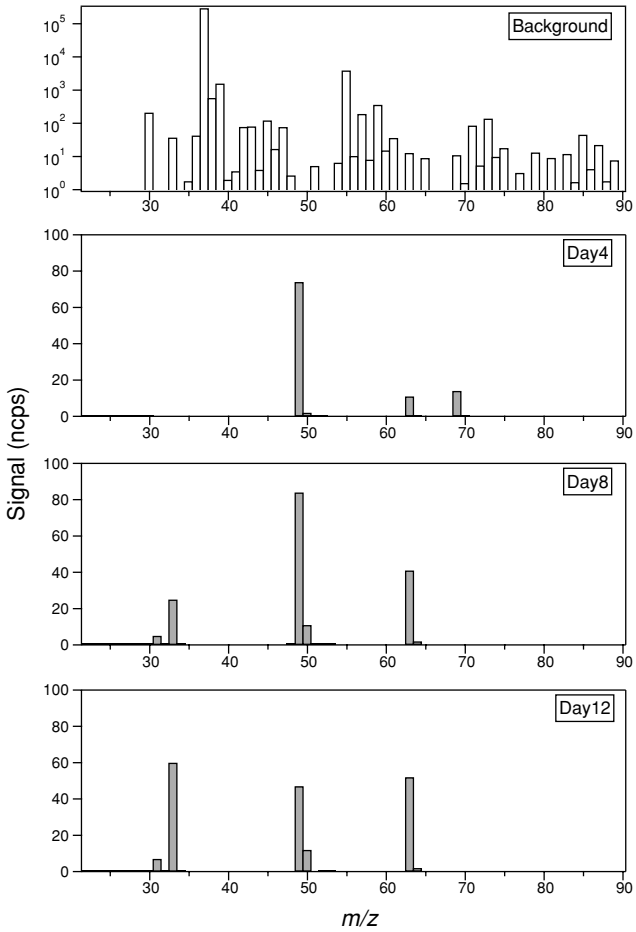

Fig. 3. Mass spectra for background and sample measurements. The mass spectra for the sample measurements were at 0 o'clock on days 4, 8, and 12. The background signals were subtracted for the sample mass spectra.

creases in the signals at *m/z* 33, 49, 63, and 69 along with incubation time. The signals at *m/z* 33 are likely originated from methanol (de Gouw and Warneke, 2007; Warneke *et al.*, 2007; Inomata *et al.*, 2010), and increased in the latter period (after day 8) following the increase in phytoplankton abundance. While methanol emissions from primary biogenic and oceanic sources exhibit the greatest uncertainty (Heikes *et al.*, 2002), Warneke *et al.* (1999) showed evidence that oxidized volatile organic carbons including methanol are produced from the decay of dead plant material through abiological processes. Therefore, the increase in methanol emissions during the latter period may result from the increase in degradation of organic matter due to the increase in leakage of organic matter from the senescent cells.

The signals at *m/z* 49, 63, and 69 are attributed to methanethiol, DMS, and isoprene, respectively, on the basis of literature evidence (Lindinger *et al.*, 1998; Williams *et al.*, 2001; Hayward *et al.*, 2002; Boscaini *et al.*, 2003; Aprea *et al.*, 2007; Feilberg *et al.*, 2010), even though the signals at *m/z* 69 have minor contributions from other compounds (e.g., 2-methyl-3-buten-2-ol) cannot completely be ruled out (de Gouw and Warneke, 2007). Oxidation products including DMSO (detected at *m/z* 79, oxidized from DMS) and methyl vinyl ketone and methacrolein (detected at *m/z* 71, oxidized from isoprene), were not detected, suggesting that oxidation effects on these species in the headspace of the culture vessel are negligible.

In order to further examine production processes, we compared these signals to biological properties. Figure 4 shows time series of the signals of isoprene, methanethiol, and DMS over the course of the experiment. The maximum concentrations were seen on day 5–7 (the beginning of stationary phase) for methanethiol, day 9–12 for DMS, and day 3 (during the exponential growth phase) for isoprene. We found clear diurnal variations in the signals at *m/z* 49 and 69, and will discuss the feature in the next section.

Diurnal Variations

To better describe and explain diurnal variations in the production of the compounds, we used the "apparent production rate", defined as the quantity discharged from the culture vessel per unit time (nmol h^{-1}). Detected signals (ncps) were converted to concentrations (ppbv) based on detection sensitivity (5.3, 6.0, and 5.1 ncps $ppbv^{-1}$ for methanethiol, DMS, and isoprene, respectively) determined in the laboratory experiments. The apparent production rate was calculated by multiplying the concentration (ppbv) by the flow rate of the purging gas (50 sccm). As this rate was influenced by both the actual production rate and dilution in the headspace of the culture vessel, we could not directly obtain the actual rate of production by the organisms in culture. However, the apparent production rate permits detection of variations in the actual production rate. The apparent production rate increases if the actual production rate exceeds the apparent production rate at any given time, and *vice versa*.

The apparent production rates were averaged for each one-hour period to evaluate the diurnal variations (Fig. 5). Diurnal variations in isoprene and methanethiol production rates was observed, although no clear variations in DMS production were evident over the course of the experiment. In this study, because the medium was continuously purged and the extracted gas was diluted in the headspace, the monitored signals might show a somewhat weakened response to changes in the production rates. Thus, the enhanced production rates could be detected in the daytime when the productivity became relatively high.

Isoprene production rate showed clear diurnal varia-

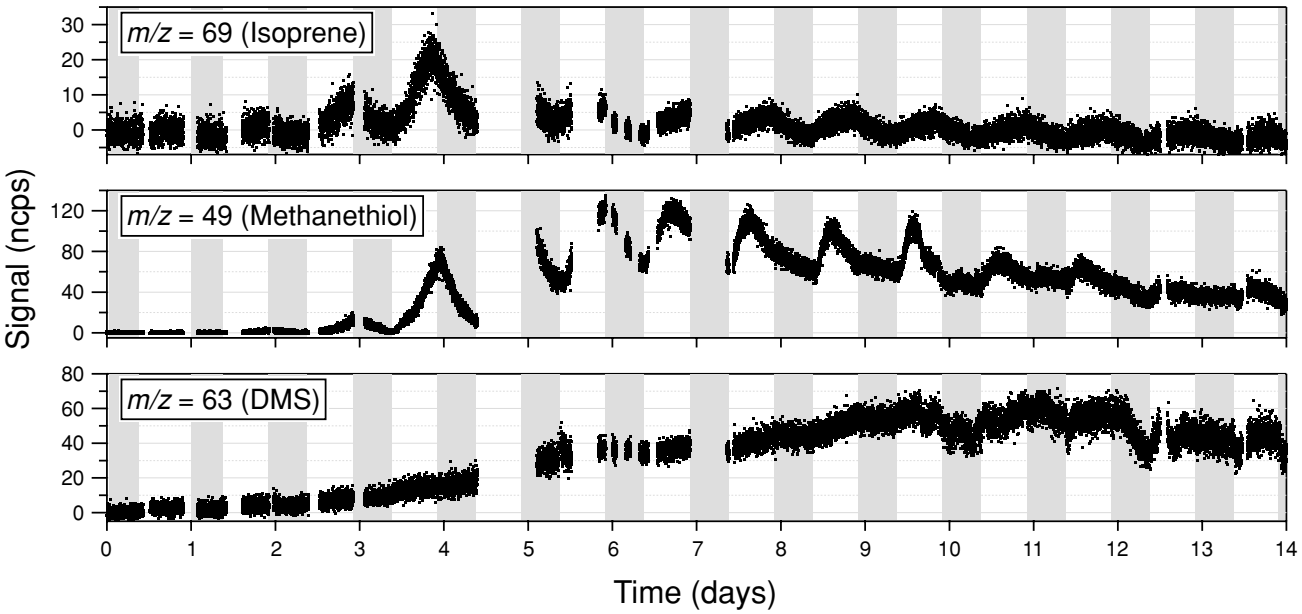

Fig. 4. Time series of PTR-MS signals at m/z 69, 49, and 63. Shaded areas indicate dark periods. Time-zero corresponds approximately to 30 h after inoculation to allow time for purging of possible gas contaminants.

tions, indicating that rapid enhancement of the photosynthetic activity of the diatom with light availability resulted in the increased isoprene production in the daytime. Sinha *et al.* (2007) reported a similar correlation between isoprene emission and light intensity during a mesocosm experiment. The apparent production rate decreased to nearly background level after dark, suggesting that isoprene was not produced under dark conditions. It is possible that other metabolic processes in addition to photosynthetic activity, such as cell division and mechanisms for protection from light stress, might be responsible for isoprene production in the daytime.

The production rate of methanethiol decreased almost to background level under dark conditions before day 4, resulting in clear diurnal variations. After day 6, the maximum production rate occurred progressively earlier in the light period, the production rates of methanethiol decreased, and differences between daytime and nighttime levels became smaller. These observations suggest that production rate of methanethiol through the production process, which contributed to the diurnal variations during the early period, became lower during the latter period. The production rate remained at 1–2 nmol h^{-1} even in the dark after day 6. This indicates that methanethiol was produced even in the nighttime during the latter part of the incubation period. In addition, our results suggest that biological responses to light availability contributed substantially to the production of methanethiol during the early part of the incubation; the productivity became weaker with the accumulation of senescent and dead cells, and other light-independent processes might have contributed to the production of methanethiol during the later incubation period.

Table 1. Change in apparent production rate of DMS under light and dark conditions. The changes were calculated as difference in production rates just before the dark-light and light-dark transitions.

Day	Change in production rate	
	Light period	Dark period
0	0.035	0.009
1	0.029	0.016
2	0.075	0.045
3	0.116	0.066
4		
5	0.132	–0.050
6	0.052	–0.051
7	0.198	–0.007
8	0.228	–0.002
9	–0.036	–0.098
10	0.285	–0.107
11	0.031	–0.519
12	0.277	–0.078
13	0.002	

Recently, Stefels *et al.* (2007) proposed two major production processes for methanethiol: demethiolation of methylmercaptopropionate (MMPA) and degradation of

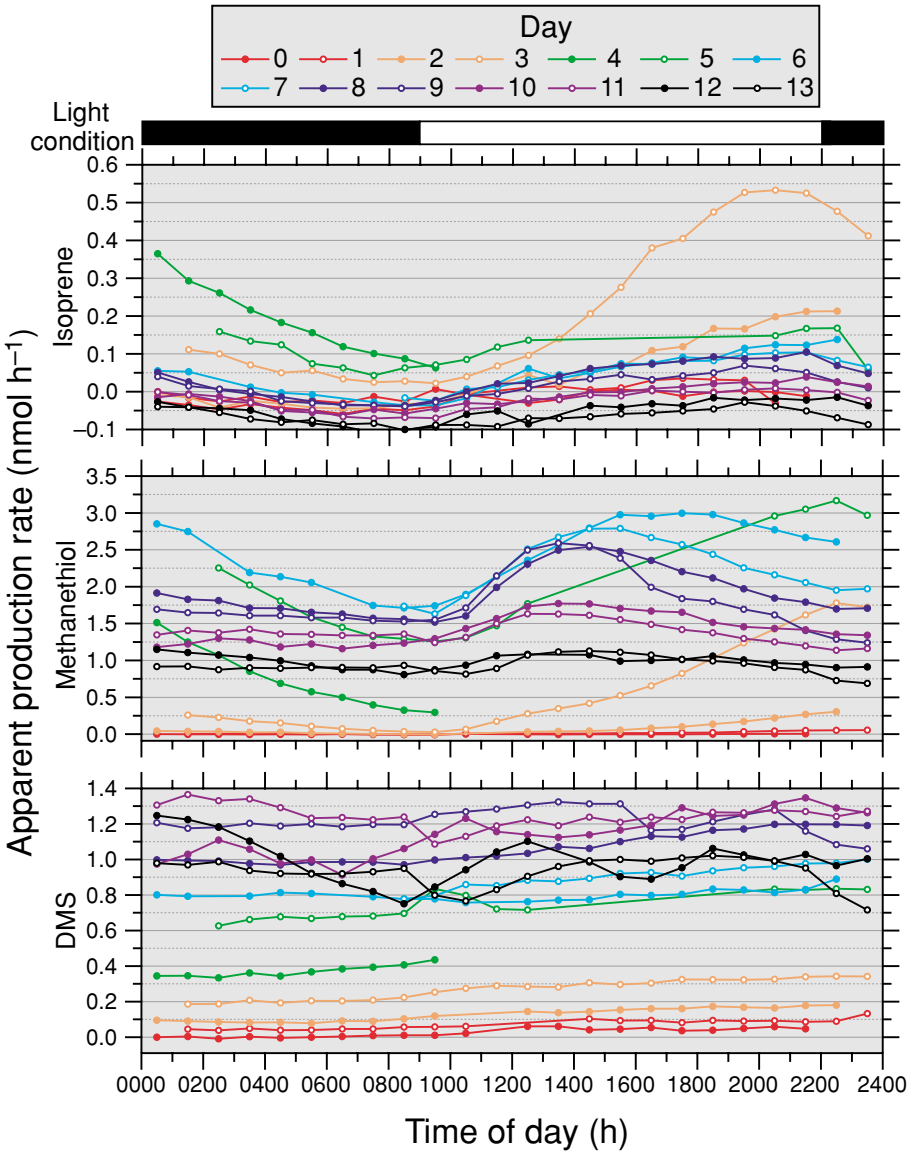

Fig. 5. Diurnal variation in apparent production rates of isoprene (m/z 69), methanethiol (m/z 49), and DMS (m/z 63). The data are averages for each hour. A color version of this figure is available online.

DMS. Both processes originate with DMSP. In the present study, the demethiolation of MMPA was possibly the dominant methanethiol production process during the early part of the incubation period because the diurnal variation in the methanethiol production rate differed from that of DMS. Degradation of DMS probably also influenced the production of methanethiol during late incubation period as nighttime DMS production was observed and the production rate of methanethiol increased with the increase in DMS productivity. Detailed studies at the biochemical and molecular level are needed to improve our understanding of the methanethiol production processes.

In great contrast to the above-mentioned two compounds, there was no clear diurnal variation in DMS production during the early incubation period. To evaluate changes in the production rate under light/dark conditions in detail, we calculated the differences between the production rates immediately before the dark-light and light-dark transitions (0800–0900 and 2100–2200, respectively) for light period and immediately before the light-dark transition and the dark-light transition of the following day for dark period as follows:

$[\text{Changes in production rate}]_{\text{light}}$ = [Average production rate 2100–2200] – [Average production rate 0800–0900],

[Changes in production rate]$_{dark}$ = [Average production rate 0800–0900] – [Average production rate 2100–2200].

The positive and negative values obtained from the equations indicate higher and lower productivity at each condition, respectively. Although the production rate increased during light periods throughout the experimental period, except for day 9, we found measurable decreases during the dark periods late in the experimental period (Table 1). Vetter and Sharp (1993) demonstrated a significant relationship between DMS production rate and light intensity from laboratory experiments using the centric diatom *Skeletonema costatum*. They found that DMS production per cell under strong illumination was significantly higher than that under weak illumination, especially for senescent cells. This implies a possible difference in DMS production rate between daytime and nighttime, particularly from senescent cells, and is consistent with our results. The trend of DMS production rates showing the continuous increase until day 10 is similar to the temporal changes in cell density of senescent cell plus cell debris. Therefore, for possible DMS production processes in this study, cell aging and/or cell death are likely crucial factors, controlling the quantity of DMSP and the subsequent DMS production.

Concluding Remarks

From the high-frequency datasets obtained by PTR-MS, we found that the production rates of the chemical compounds of interest released from *T. pseudonana* were influenced by the growth phase of the diatoms and by light availability. The headspace volume of the culture vessel used in this study increased with time as a result of our repeated sampling, therefore the actual production rates of the target gases could not be determined from our measurements because the detected signals were controlled by not only the variations in the actual production rates but also by the dilution effect in the headspace. This issue could be resolved if the headspace volume was minimized and adjustable.

We accomplished the continuous measurement of biogenic trace gases released from a diatom species, but those from other phytoplankton groups such as coccolithophores and dinoflagellates should also be evaluated in future studies. Detailed production rate measurements and understanding of the production processes will greatly contribute to the estimation of the flux of biogenic gases from the ocean to the atmosphere.

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