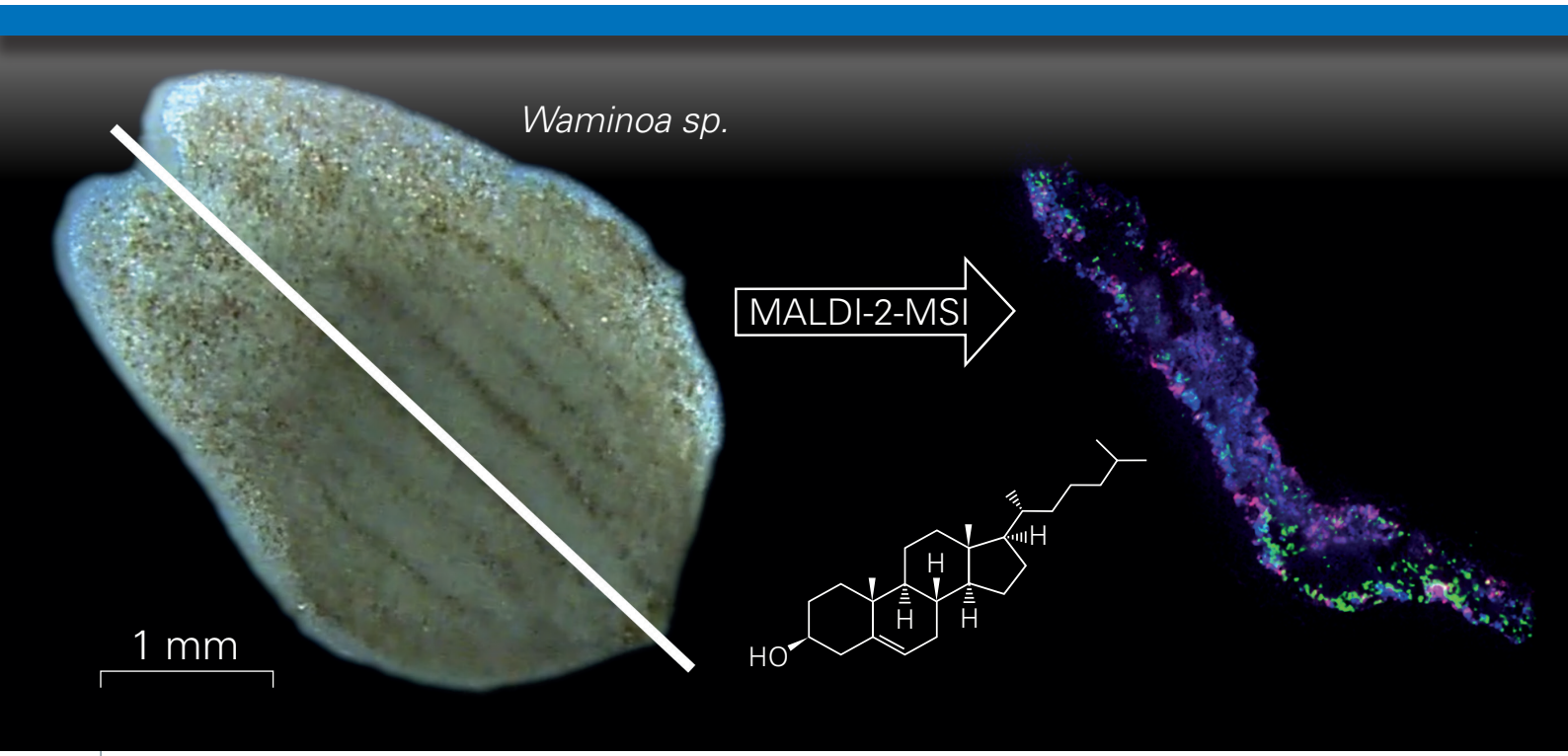

Mapping sterols in a marine flatworm-algae-system using MALDI-2 TIMS MS-imaging

Waminoa sp. acoel flatworms hosting *Symbiodiniaceae* and the related *Amphidinium* dinoflagellate algae are an interesting model system for symbiosis in marine environments.

Abstract

While the host provides a microhabitat and safety, the algae power the system by photosynthesis. In other related symbioses such as in corals, a "currency" in symbiosis is known to be the transfer of sterols between guest and host, including cholesterol and numerous phytosterols. These compounds are produced by the symbiotic dinoflagellates, but their transfer to and fate within the sterol-auxotrophic *Waminoa* worm host as well as their role in its metabolism are unknown.

In this study we present MALDI-2 TIMS MS-imaging data to map the spatial distribution of over 30 different sterol species in thin sections of the flatworm. The novel laser post-ionization technique, which was recently implemented to the timsTOF fleX instrument, crucially increased ion yields and allowed the recording of images with very high spatial resolution. The additional dimension of Trapped ion mobility spectrometry (TIMS) helped with the assignment of the different sterol species.

Correlation with anatomical features of the worm, revealed by host-derived phospholipid signals, and the location

Keywords: MALDI-2, timsTOF flex, trapped ion mobility, imaging, sterols, laser post-ionization

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of the dinoflagellates, revealed by chlorophyll *a* signal, disclosed peculiar differences in the distribution of different sterol species (e.g., of cholesterol vs. stigmasterol) within the receiving host. These findings point to sterol species-specific roles in the metabolism of *Waminoa* sp. beyond a mere source of energy.

Introduction

Photosynthetically driven symbioses with microbes is recognized to be a widespread phenomenon with significant impacts on development, cell and marine ecosystem function and evolution. For example, symbiosis between marine animal hosts and algae allows animals to flourish in harsh environments due to symbiont transfer of photosynthetically fixed nutrients. The most widespread algal symbionts in animals are the dinoflagellate *Symbiodiniaceae*, which are well known to transfer lipids in a symbiotic system with cnidarians. The here investigated *Waminoa* sp. acoel flatworms (hereafter "*Waminoa*") host both *Symbiodiniaceae* and the related dinoflagellate *Amphidinium*. In many of these systems, the transfer of a variety of sterols between guest and host, including cholesterol, numerous phytosterols, and the gorgosterol uniquely produced by these symbiont species, plays an important role.

Mass spectrometry is arguably the most ubiquitously used technique when it comes to the analysis of sterols. In the past there have been investigations using techniques like GC-MS in combination with derivatization, ESI-MS, DESI-MS or SIMS to study sterols. Today especially Matrix assisted laser desorption ionization (MALDI) is increasingly used. However, poor ion yields still complicate or even hinder the analysis of neutral sterols like cholesterol or phytosterols especially when low concentrations are present, like when it comes to spatially resolved experiments where a mass spectrum for each pixel needs to show a good signal. To improve the ion yields for sterols in a MALDI-MS imaging experiment, MALDI-2, a recently introduced laser post-ionization technique, can be employed. It has been shown that MALDI-2 allows for the sensitive analysis of cholesterol, cholesterol esters, and vitamin D3 with high spatial resolution [1]. Compared to traditional MALDI, up to 2-3 orders of magnitude higher ion signals were obtained upon use of the laser post-ionization module.

In addition to the low sensitivity, another challenge lies in the annotation of the different known species due to the common occurrence of isomeric ion species in the sterol world which is just given by nature. A powerful extension of MALDI-2-MSI is the on-line combination with ion mobility separation (TIMS), because it introduces an additional layer of separation in analyzing highly complex samples [2]. With the ability of TIMS, ions can be separated based on their collisional cross section in a bath gas prior to MS analysis.

In this study we show a proof of concept for the MALDI-2 TIMS MSI analysis of neutral sterols from the described symbiotic system. Next to the increased sensitivity by post-ionization, the additional separation dimension allows for the confident assignment of individual analysis of ion species that are isobaric in the *m/z*-domain.

Methods

Full experimental details including all mass spectra and detailed supporting

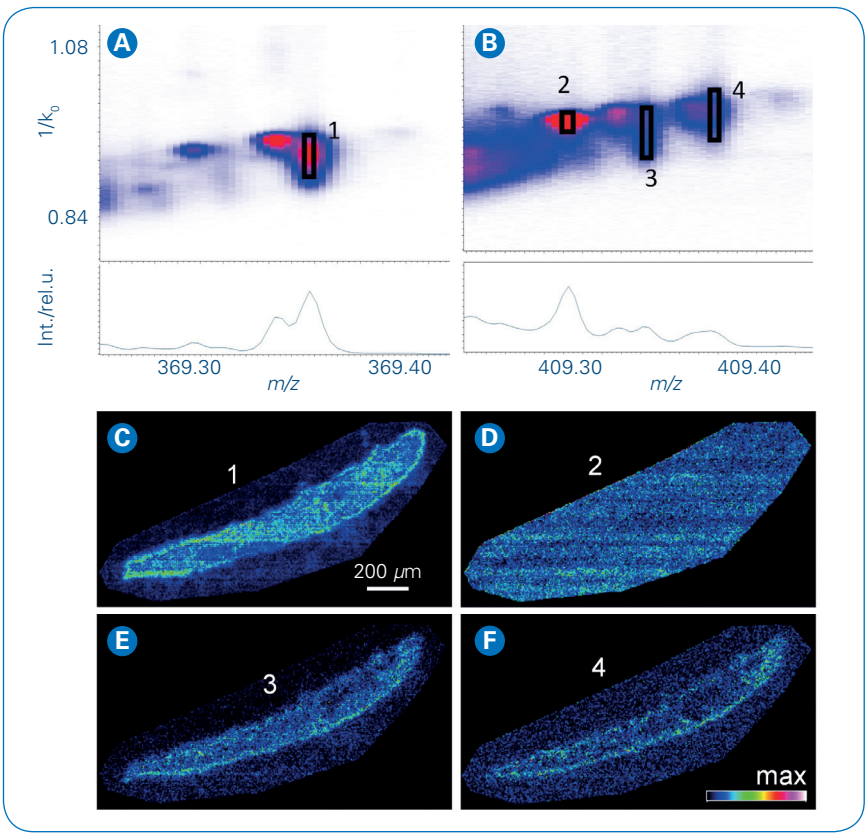

Figure 1. Mobilograms and mass spectra of cholesterol (Ⓐ, feature 1), sterol $C_{29}H_{46}O_2$ (Ⓑ, 3), gorgosterol (Ⓑ, 4). Also shown is an exemplary feature assigned to matrix-derived chemical noise (Ⓑ, 2); Ⓒ,Ⓓ, Ⓔ,Ⓕ show the respective ion images; other matrix- as well as non-sterol derived endogenous analyte signals showed similar compact features. Data were summed across a full section of Waminoa *but also include areas of matrix-coated substrate next to the tissue.*

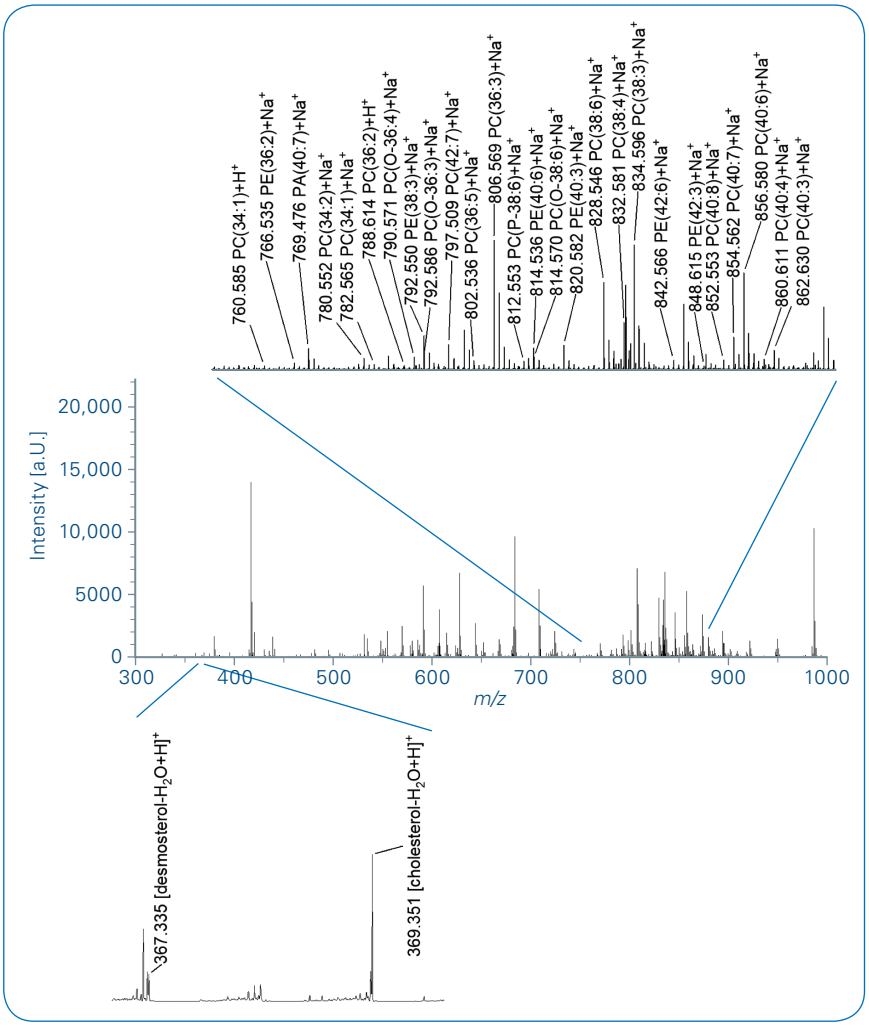

Figure 2. Full mass spectrum of a full MALDI-2 MSI experiment on a section of Waminoa *sp. (middle) and cut-outs representing the phospholipid region (top) and a 2.5 Da wide window including two sterol ion species (bottom). In the top cut-out PC and PE species were tentatively assigned based on accurate mass.*

information have been described in detail recently [3]. Only the most relevant experimental aspects are summarized here.

Symbiotic systems of *Waminoa* sp. flatworms hosting intracellular symbiotic *Amphidinium* and *Symbiodiniaceae* dinoflagellates were cultured in artificial seawater aquaria (25°C with 12:12 L:D illumination). They were embedded in a mixture of 5% 2-hydroxyethylcellulose / 10% gelatin prior to cryosectioning to 14 µm thickness. Cryosections were prepared on ITO-slides. Matrix was applied using a home build apparatus for sublimation. 2,5-dihydroxyacetophenone (DHAP) matrix was sublimated for 6 minutes at 130°C and 3 mbar. The 6 min deposition resulted in a matrix coverage of 236 ± 34 µg/cm^2.

Standards were prepared in chloroform to concentrations of 2.5 mM. For dried droplet preparations on ground steel plates a 15 mg/mL 2,5-DHAP matrix in ACN:MeOH:H_2O (8:1:1; v/v/v) was used in a sample:matrix ratio of 1:1 (v/v).

MALDI-2-MS images were acquired on a timsTOF fleX instrument (Bruker) that has been modified to allow for MALDI-2 at 266 nm and 1 kHz repetition rate (10 µs delay). Due to the highly modified setup it was possible to use a pixel size of 5 µm without oversampling. The mass range was adjusted to *m/z* 300-1000 (without TIMS separation) or *m/z* 250-655 (with TIMS separation). 1/k_0 values were measured between 0.6 and 1.6 with a ramp time of 659 ms. Data analysis was performed using SCiLS Lab (2020b) and TDF viewer software (+FlexImaging for visualization).

Results

Characteristics of Sterol Signals Detected with MALDI-2 TIMS MSI

As detected with regular MALDI without further derivatisation, and also with SIMS, with MALDI-2 sterols are, in the positive ion mode, predominantly detected as protonated species with the loss of water [sterol-H_2O+H]$^+$. In addition most sterol species also produced radical ions of the form [sterol-H_2O]$^{\bullet+}$. Sterol signals are expected in the *m/z*-region between about *m/z* 250-500, according to what has been detected in marine systems in the past. MALDI-2-MSI spectra generated directly from tissue in this *m/z*-region are generally highly complex due to matrix-derived signals mixed with signals generated from tissue-derived metabolites, as well as possible fragments from larger analyte molecules. Therefore, TIMS is included as an additional orthogonal separation technique into our MALDI-2-MSI workflow to aid with tentative assignment of molecular IDs. The analysis of sterol standards (e.g., cholesterol and stigmasterol) revealed that no sizeable in-source fragmentation other than the loss of water is generated with MALDI-2. Using TIMS, these sterol ion species produce characteristic features in a mobilogram. While their peak shape in the *m/z*-dimension is normal, the peak shape in the mobility domain is peculiar. Compared to phospholipid or matrix-derived ions, peak shapes are considerably broadened for all tested

sterols. Beneficially, it leads to a characteristic feature shape that appears exclusive to sterols in the mobilogram as demonstrated in Figure 1. While cholesterol (A, feature 1), $C_{29}H_{46}O_2$ (B, feature 3) and gorgosterol (B, feature 4) display a feature "drawn-out" in the mobility dimension, the feature tentatively assigned to chemical noise in (B, feature 2), is much more compact.

MS Imaging of Sterol Species Detected from the Symbiotic System

Because of the small size of *Waminoa*, with typical maximum dimensions of 4 mm length, 2 mm width, and 200 μm thickness, sections were measured with the smallest available pixel size of 5 μm. This enables to resolve the algae and their direct surroundings as the dinoflagellate cells have diameters of approx. 9-13 μm. Apart from the variety of sterols, numerous signals are identified in the MSI data that correlate well with the structure of the worms and can be used to outline the morphology of the flatworm. Based on accurate mass, a number of these signals can tentatively be assigned as phospholipids. In Figure 2 an example spectrum of a MALDI-2 analysis of a worm section is shown.

As described, mass spectra in the investigated *m/z* range are highly complex. Sometimes sterols are not clearly identified, therefore in this application note only clear features with high S/N ratio and given distributions (clarity and contrast) in tissue are considered. Overall, 32 tentatively assigned sterols met these conditions and were therefore identified with their grading and chemical sum formula.

Figure 3 d-f shows the distributions of cholesterol ($C_{27}H_{46}O$), stigmasterol ($C_{29}H_{48}O$), and saringosterol ($C_{29}H_{48}O_2$) in three sections of three different flatworms recorded using

Table 1: List of sterols detected by MALDI-2 TIMS MSI in the Waminoa*/dinoflagellate system based on the individual image distribution quality and feature clarity; All tentative structural assignments based on accurate mass (5 ppm) and the cited literature; data were extracted from a single MALDI-2 TIMS MSI measurement of a section of a* Waminoa *sp.*

Chemical Composition							
C	H	O	$[M-H_2O+H]^+$ (calc)	$[M-H_2O+H]^+$ (exp.)	Mass error/ppm	$1/k_0$	Tentative Sterol ID
26	42	1	353.321	353.320	3.8	0.91	24-nor-cholest-5,22E-dien-3β-ol
27	42	1	365.321	365.321	1.0	0.9	glaucasterol, cholest-5-en-23-yn-3β-ol,
27	44	1	367.336	367.337	0.9	0.92	desmosterol
27	46	1	369.352	369.353	2.9	0.93	cholesterol
28	42	1	377.321	377.319	4.8	0.92	dehydroergosterol
28	44	1	379.336	379.336	1.0	0.93	ergosterol
28	46	1	381.352	381.352	1.4	0.94	brassicasterol
28	48	1	383.368	383.367	1.2	0.95	campesterol
29	44	1	391.336	391.335	4.5	0.93	conicasterol B
29	46	1	393.352	393.352	0.4	0.94	calysterol
29	48	1	395.368	395.369	3.4	0.95	stigmasterol
29	50	1	397.383	397.382	3.8	0.97	β-sitosterol
29	52	1	399.399	399.398	3.7	0.99	stigmastanol
30	46	1	405.352	405.351	2.0	0.96	theonellasterol B
30	48	1	407.368	407.367	2.4	0.97	nervisterol
30	50	1	409.383	409.382	3.7	0.98	gorgosterol
32	54	1	437.415	437.413	4.6	1.02	24-(1-ethyl-2-methyl-2-propenyl)-27-norcholest-7-en-3-ol
21	28	2	295.206	295.206	0.1	0.79	18-hydroxypregna-1,4,20-trien-3-one
23	38	2	329.284	329.286	3.5	0.87	
24	42	2	345.316	345.315	3.0	0.89	
27	42	2	381.316	381.317	2.5	0.91	chabrosterol
27	44	2	383.331	383.332	1.6	0.92	24-ketocholesterol
27	46	2	385.347	385.348	1.2	0.94	24-ketocholestanol
28	46	2	397.347	397.348	1.9	0.95	22,23-epoxycampesterol
28	48	2	399.363	399.362	0.7	0.96	22,23-epoxy-5β-campestan-3β-ol
29	46	2	409.347	409.347	0.6	0.95	22S,23S-epoxy-5α-stigmast-8(9),14(15)-dien-3β-ol
29	48	2	411.363	411.364	2.2	0.96	saringosterol
29	50	2	413.378	413.378	0.3	0.98	22S-hydroxysitosterol
30	50	2	425.378	425.379	2.0	0.98	
21	32	3	315.232	315.233	2.9	0.82	3β,6α-dihydroxy-5α-pregn-9(11)-en-20-one
23	38	3	345.279	345.280	1.9	0.88	
29	50	3	429.373	429.375	3.4	0.99	nebrosteroid M

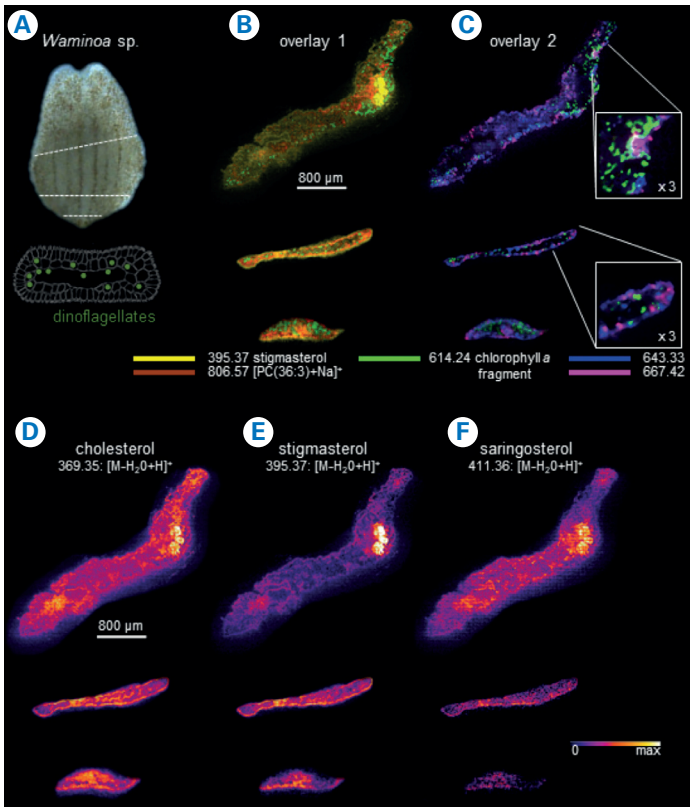

Figure 3. MALDI-2 MS imaging of Waminoa *flatworms. Data were collected from sections of three individual flatworms and represent different "depth profiles". (A) Snapshot of a* Waminoa *flatworm with approximate positions of the three sections produced for the MSI analyses, indicated by dotted lines, and schematic of a* Waminoa *cross-section with symbiotic dinoflagellates. (B) Overlay of ion images of stigmasterol $[M–H_2O+H]^+$ (yellow) with $[PC(36:3)+Na]^+$ (brown), representative of a group of phospholipids that are detected from animal tissue, and a chlorophyll a fragment,* m/z *614.24 (green), representative of the two algae species. (C) Overlay ion images of the chlorophyll a fragment and two currently unknown species,* m/z *values of 643.33 (blue) and 667.42 (pink). MALDI-2 MS images of cholesterol (D), stigmasterol (E), and saringosterol (F). MS images are presented with weak denoising, no normalization was applied. The scale bars plotted in (B) and (D) apply to all MS images in the figure.*

MALDI-2-MSI. The approximate positions of the sections and a schematic of the dinoflagellate symbionts within the animal are shown in Figure 3A. While cholesterol is distributed almost homogenously throughout the tissue, the cross section through the center of one of the animals (top) reveals a higher concentration of stigmasterol within an area corresponding to the syncytial gut, possibly containing gonads. Saringosterol is distributed similar to cholesterol in the center of the animal but shows depleted signal intensity in the outer parts.

As already described, different phospholipids can be used to outline the morphology of the worm itself. Other signal intensity distributions correlate with the dinoflagellates. Interestingly, three groups of spatial features, putatively assigned to the single-cellular algae by their shape and size, can clearly be discerned based on these signals (Figure 3C, insets). The most intense of these signals can be assigned to the photosynthetic molecule chlorophyll *a*, which appears to undergo in-source fragmentation.

Conclusion

- The combination of MALDI-2 and TIMS MS Imaging enables the sensitive analysis of 32 individual sterol species directly from tissue without prior chemical derivatization. The signal intensity boost enabled by laser post-ionization allows to perform MS imaging experiments with a pixel size as low as 5 µm, approaching a cellular resolution for algal systems. While MALDI-2 critically increases signal intensity, TIMS crucially aids with the tentative assignment of sterols. Besides the targeted analysis of sterols, collected MALDI-2-MSI data contain spatial information about a great amount of other molecular ion species. Next to different phospholipid species colocalized with regions of the *Waminoa* host, signals were detected that exclusively colocalized with the symbiotic dinoflagellates, including intact chlorophyll *a* and some of its fragments as well as yet unidentified species.
- The observed distribution of symbiont-produced sterols within host flatworm tissues reveals that sterol transfer is a conserved element of *Symbiodiniaceae* symbioses in host backgrounds. Surprisingly, the different spatial distribution of stigmasterol compared to cholesterol in an area corresponding to the syncytial gut region could indicate an unprecedented differential use of the various symbiont-produced sterols. The accumulation of these sterols could be based on several reasons, yet the mechanisms and function remain unknown.

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