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Mol Biosyst. Author manuscript; available in PMC 2010 April 4.

Published in final edited form as:
Mol Biosyst. 2008 June ; 4(6): 562–570. doi:10.1039/b720018h.

Visualizing the spatial distribution of secondary metabolites produced by marine cyanobacteria and sponges via MALDI-TOF imaging

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Summary

Marine cyanobacteria and sponges are prolific sources of natural products with therapeutic applications. In this paper we introduce a mass spectrometry based approach to characterize the spatial distribution of these natural products from intact organisms of differing complexities. The natural product MALDI-TOF-imaging (npMALDI-I) approach readily identified a number of metabolites from the cyanobacteria Lyngbya majuscula 3L and JHB, Oscillatoria nigro-viridis, Lyngbya bouillonii, and a *Phormidium* species, even when they were present as mixtures. For example, jamaicamide B, a well established natural product from the cyanobacterium *Lyngbya majuscula* JHB, was readily detected as were the ions that correspond to the natural products curacin A and curazole from *Lyngbya majuscula* 3L. In addition to these known natural products, a large number of unknown ions co-localized with the different cyanobacteria, providing an indication that this method can be used for dereplication and drug discovery strategies. Finally, npMALDI-I was used to observe the secondary metabolites found within the sponge *Dysidea herbacea*. From these sponge data, more than 40 ions were shown to be co-localized, many of which were halogenated. The npMALDI-I data on the sponge indicates that, based on the differential distribution of secondary metabolites, sponges have differential chemical micro-environments within their tissues. Our data demonstrate that npMALDI-I can be used to provide spatial distribution of natural products, from single strands of cyanobacteria to the very complex marine assemblage of a sponge.

Introduction

Today, nearly 50% of all anti-cancer agents and 75% of all antimicrobial agents in use are, or have origins from, natural products.1 Even so, the pharmaceutical industry has gone through a series of ebbs and flows with respect to their reliance on naturally derived or inspired compounds, in more recent times almost entirely depending on combinatorial chemistry for leads because of its cost effectiveness. However, in the last decade the interest in natural products has gained renewed interest.[2] The lack of novel chemistry coming from combinatorial

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approaches, the growing body of knowledge and availability of genome sequencing, and the increase (at least in public awareness) in diseases such as cancer or drug resistant pathogenic microorganisms, suggests that secondary metabolites could be the source of many significant breakthroughs in therapeutics, and underscores the importance of developing new approaches to characterize and observe secondary metabolites.

Historically, most natural product research has been directed towards terrestrial organisms, mainly plant, fungi and microbes. Among these important discoveries are the plant related anti-tumor agents taxol and colchicine, and numerous antibiotics including amphotericin B, tetracycline, erythromycin and vancomycin.[3,4] Alternatively, the marine environment remains a largely unexplored resource for novel bioactive natural products, despite the fact that our planet is composed of 70% water and that life has evolved for a much longer time in the marine environment.[5] Nevertheless, there are a few marine natural products that have been approved by the FDA, including cytarabine in 1969 to the conotoxins and bryostatins of today, and a number of others that have entered clinical trials and hold promise for a variety of diseases.[6,7,8,9] It is clear, however, that we still stand to uncover a wealth of new therapeutic agents from the marine environment, especially if we can streamline the bio-discovery process and combine and maximize the efforts of marine biologists and chemists with the power of genomics and bioinformatics.

One tool that has bridged marine biologists with chemists is MALDI-TOF mass spectrometry. MALDI-TOF is a method that can be used to analyze cyanobacterial extracts with minimal work-up.[10] This approach involves extraction of the cyanobacteria, mixing the extract with matrix and subsequent analysis for peptides and other structural classes by MALDI-TOF mass spectrometry. This approach is an excellent complement to standard LC-MS approaches, and is easier to implement.[11] Using MALDI-TOF, several new cyanopeptides were observed and characterized. Recently, MALDI-TOF was used to directly analyze cyanobacteria for the presence of cyanopeptides and toxins from 850 individual colonies.[12] In this study, a small colony of the cyanobacterium was placed on a MALDI-TOF-plate and covered with a small amount of 2,5-dihydroxybenzoic acid matrix before they were analyzed by MALDI-TOF mass spectrometry. A total of 90 individual peptides were identified from these 850 individual cyanobacteria colonies, including 18 that appear to be unique from their masses. Finally, MALDI-TOF mass spectrometry has also been used to observe sponge derived metabolites from sponge bacterial isolates or even a homogenized sponge tissue itself. [13-15]

In this communication we expand upon the approaches described above which were primarily developed by the von Dohren lab, and demonstrate that MALDI-TOF-imaging can be applied to the detection of natural products even from very complex marine systems such as sponges. In this paper we show that MALDI-TOF imaging is a valuable tool in the de-replication of cyanobacteria for low intensity signals, allowing the detection of a broad range of known and unknown metabolites in both homogenous and heterogeneous assemblages. This approach provides not only the mass information on the ions analyzed but also the spatial localization of these metabolites. Such an imaging approach has been used to identify biomarkers of disease as well as the accumulation of drugs in whole animals but has not yet been applied to the analysis of natural products,. [16-18] The visualization and spatial information this technique provides could allow the identification and enrichment of specific organisms, allowing the isolation and characterization of genomes via single cell genomic sequencing. It could also become an important tool in the study of host-microbe interactions, such as those involving complex assemblages of species (e.g. sponges, tunicates). Finally the new MALDI-TOF-imaging approach outlined in this paper could aid in the discovery of new therapeutic agents as numerous uncharacterized ions are detected in these experiments.

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Results and discussion

MALDI-TOF-imaging of natural products from single cyanobacterial filaments

We first needed to establish the reasonableness of our goal to determine the presence and localization of natural products from samples of marine origin with MALDI-TOF-imaging. Because the lower *m/z* region is typically obscured from ions deriving from matrix, it is usually not recommended that MALDI-TOF mass spectrometry be used when the ions under examination are in the low *m/z* region of the spectrum. Unfortunately, most natural products are observed in this region. The observations of matrix clusters within the lower *m/z* window is particularly problematic when small amounts of material are analyzed, as would be expected from single cyanobacterial filaments.[19,20] To demonstrate that secondary metabolites can be observed from intact marine organisms, *Lyngbya majuscula* JHB was analyzed via MALDI-TOF-imaging, referred to here as Natural Product MALDI-TOF-imaging or npMALDI-I throughout the rest of the manuscript. *Lyngbya majuscula JHB* is known to produce several bioactive natural products with masses below *m/z* 600. For example, jamaicamides A-C are sodium channel blockers (Fig. 1A).[33] Therefore, this strain serves as an excellent example to demonstrate that npMALDI-I can be utilized to show the location of secondary metabolites even when the molecular species is less than *m/z* 600,(the $M+H^+$ of jamaicamide B has a mass of 489 Da).

To image natural products from intact cyanobacteria, a small colony of *Lyngbya majuscula JHB* was grown for ~20 days. Single filaments were removed, washed in distilled water to remove most of the salty growth media and placed on top of the MALDI plate. The plate was dried and a matrix (composed of a-cyano-4-hydroxycinnamic acid, 2,5 dihydroxybenzoic acid) was airbrushed on the plate until a uniform crystalline layer appeared. This matrix composition was deemed to be optimal for minimizing the crystal size and therefore increased our spatial resolution, while retaining enough ionization so that a mass spectrum can be obtained directly from a single filament (we estimate that we are only analyzing 25-40 individual cyanobacterial cells at one time as the cells are 20-50 μm in width and 2-4 μm in length).

To demonstrate the npMALDI-I approach, a 0.6 *1.5 mm area that contained a section of a single *Lyngbya majuscula* JHB filament was imaged with a 100*100 μm raster area (Fig. 1B and 1C). At each of those raster points, a single MALDI spectrum from *m/z* 350 to 1000 was obtained. Following the acquisition of all the spectra, an average spectrum was generated from all the individual spectra. The individual masses can then be displayed on a pre-imported image of the cyanobacterial filament by designating a specific color to an *m/z* window (Fig. 1C). The higher the relative intensity of a specific species with respect to other ions at any one raster point, the darker the color. Manual scanning at *m/z* 0.5-3.0 windows revealed that several masses co-localized with the cyanobacterial filament, while others were localized throughout the entire sample. Once a particular isotope peak is shown to co-localize with the filament, the *m/z* window is expanded to include the neighboring peaks as long as they show the same co-localization pattern. Keeping in mind the resolution of the instrument-R, by the FWHM method, is determined to be about 5000 for each individual raster point and closer to 4000 for the average spectrum, it is difficult to distinguish between different metabolites with similar masses (within 0.05-0.1 Da), however the isotopic signatures combined with the localization pattern is usually enough to suggest if there is more than one compound present in a particular *m/z* window. For example, the ions *m/z* 511 and 612 are localized with the filament while other ions such as *m/z* 441, originating from the matrix, are localized throughout the sample (Fig. 1C). The masses and the isotopic distributions at *m/z* 511 and 612 are in agreement with the natural products jamaicamide B and yanucamide, and demonstrate that even low molecular weight natural products can be observed by npMALDI-I of intact marine cyanobacterial filaments (Fig 1D).[21] In addition to ions that are in agreement with jamaicamide B and yanucamide B, other ions at *m/z* 589 (jamaicamide A), 526, 629 were also observed and co-

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localized with the cyanobacterium (Fig 1E.). It is not yet known what molecular entities are represented by the ions at *m/z* 526 and 629.

Observing natural products from intact cyanobacteria

Following the detection of ions with the same isotopic profile that correspond to the expected mass of jamaicamide A and B, and yanucamide B in the *Lyngbya majuscula* JHB, it was important to demonstrate that npMALDI-I would work on other marine cyanobacteria, to establish that this may in fact be a general method for observing natural products from these organisms. To demonstrate the generality of this approach, the marine cyanobacteria, Lyngbya majuscula 3L, Oscillatoria nigro-viridis, Lynbya bouillonii, and *Phormidium* species were investigated in a similar fashion as *Lyngbya majuscula JHB* described in the previous section. Imaging showed that some of the ion intensities co-localized with the cyanobacteria and a selection is shown in Figure 2. Some of the masses that co-localized to the *Lyngbya majuscula* 3L filament were observed at *m/z* 372, 374, 592 and 610. The *m/z* 372 and *m/z* ions are in agreement with the natural products curazole and curacin (Fig. 2).[34] Ions *m/z* 592 and 610 are unknown but have to come from the filament as they are co-localized. Among the ions that were observed to co-localize with the image of the cyanobacteria *Oscillatoria nigro-viridis* were *m/z* 593, 871 and 885. Of these masses, *m/z* 871 and 885 are in agreement with the unpublished natural products viridamides A and B (Fig. 1E), demonstrating that this approach could be used to discover new natural products (Gerwick, unpublished results). The *m/z* 593 ion represents an unknown that is associated with this cyanobacteria and similar ions of this mass were detected in a previous MALDI-TOF study of cyanobacteria.[10]

Phormidium sp. and *Lynbya bouillonii*, just like the previous cyanobacteria, showed a large number of associated unidentified ions. Because there are a large number of ions observed (e.g. 24 unique ions were observed for *Phormidium*), only some representative co-localizations are shown in Figure 2 F and G, demonstrating that npMALDI-I of intact cyanobacterial species can be used to detect metabolites in a spatial fashion. In some cases additional information can be gleaned from just analyzing the mass spectrum alone. For example, the *m/z* 364 ion is halogenated, judged from the isotopic pattern that this ion displays (Fig. 1H). Since none of these metabolites could be correlated to specific natural products, it underscores the untapped therapeutic potential that the ocean represents.

One of the main challenges with the npMALDI-I approach is the spatial resolution of the instrument and sample preparation. Currently our technique is limited to about 100-200 μM resolution, hinging on the size of the matrix crystals applied to the specimens. While the size of the crystals is one of the factors in determining resolution, the N_2-laser used for these experiments is also a limitation. A typical N_2 laser has a diameter of 50-100 μM, although in the near future it should be possible to get 10 μm resolution.[25] Many laboratories are still developing new approaches to matrix application and, undoubtedly, future implementation of these techniques will greatly improve the resolution in our experiments.[22-24]

Even though npMALDI-I has this inherent spatial resolution, npMALDI-I can indeed identify ions for which the masses are in agreement with unique natural products even at low *m/z* regions (we have shown ions as low as *m/z* 372 can be observed by this approach). We currently do not see the low *m/z* as a major limitation for npMALDI-I as we can confidently say that the ions detected are associated with the cyanobacteria via co-localization. Therefore, the npMALDI-I approach is applicable in dereplication or taxonomic strategies.

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Using MALDI-TOF imaging for the dereplication of individual marine cyanobacteria from mixed assemblages

Thus far, we have only demonstrated that the npMALDI-I approach works on single cyanobacteria but if the analysis from heterogeneous mixtures or the ability to dereplicate individual cyanobacteria is desired, it is important to show that the spatial information is not lost when multiple organisms are present in one image. Therefore, a mixture of *Lyngbya majuscula* 3L *and* JHB, *Oscillatoria nigro-viridis, Lyngbya bouillonii*, were laid down on the MALDI-plate and imaged using MALDI-TOF in an identical fashion described for the individual cyanobacteria. The ions at *m/z* 511 (*Lyngbya majuscula JHB*), 592 (*Lyngbya majuscula 3L*), 611 (*Lyngbya bouillonii*), and 885 (*Oscillatoria nigro-viridis*) were displayed with a different color. npMALDI-I could readily distinguish these known ion masses known to be associated with each cyanobacteria. This proof-of-principle experiment demonstrates that we can observe natural products in a spatial fashion within mixed assemblages.

Spatial distribution of secondary metabolites within the marine sponge, *Dysidea herbacea*

Sponges are often regarded as one of the oldest, most successful and most complex assemblages in the marine environment, with some species often comprised of a several species of cyanobacteria, red algae, and many other microorganisms. Up to 40% of a sponge's mass is widely thought to be attributed to coexisting organisms.[26,31,32] This observation, combined with the fact that sponges are a rich source of new natural products with potent bioactivities, leads us to the question as to which organisms are responsible for the observed bioactive compounds. Our goal was to determine if npMALDI-I could be accomplished on a sponge and if the resulting data could provide us with insight regarding not only the presence of natural products, but their localization as well. We choose to study the sponge, *Dysidea herbacea,* because it is known to be rich in cyanobacteria and numerous natural products have been isolated from this sponge.[27–30] To accomplish the imaging on the sponge, a 14 μM thick section was prepared using a cryo-microtome and mounted onto a MALDI plate, the sample was then dessicated and covered with a uniform crystalline matrix and subjected to npMALDI-I. Using this approach, at least 40 ions localized specifically to the sponge, many of which are halogenated judging from the isotopic profile (Figure 4A). In addition, differential localization of ions throughout is observed. The relative variation in the MALDI-TOF signal from region to region can be directly observed from the individual spectra. Three mass spectral traces from the indicated raster points as shown in Fig. 4B indicate that they have dramatically different ion concentration profiles, these abundances are then reflected by the use of colors on those relative (but not absolute) ion intensities to provide a spatial localization. Some representative ions we have observed with differential localization are shown in Fig. 4C (and supporting information Fig. S1). The data in Figure 4C clearly provides evidence that there is a differential distribution of secondary metabolites within a cross section of the sponge. Some ions are localized on the outer edges, tentatively the pinacoderm and edges of the ostia, while others have a more complete and uniform distribution, and others appear to have distinct, internal localization concentrated in what appears to be possibly the mesohyl. The idea that secondary metabolites have different spatial distributions within the sponge tissue is not surprising given that it has been shown that co-existing microorganism tend to populate specific regions of sponge tissue and that it is becoming increasingly clear that these same microorganisms are at least responsible for the biosynthesis of some of the secondary metabolites.[5,29,31,32] A detailed report on npMALDI-I, combined with microscopy studies, single cell genomics and LC-MS analysis of the *Dysidea* sponge is forthcoming (Gerwick, Dorrestein unpublished).

Conclusions

In this paper we have introduced npMALDI-I to observe, in a spatial fashion, natural products from intact marine organisms such as cyanobacteria and heterogenous assemblages such as the

Dysidea herbacea sponge. We have demonstrated that it can be used to observe these metabolites from intact cyanobacterial filaments and sponges with high spatial resolution even though the ions are in the low *m/z* window. In addition, npMALDI-I has advantages over traditional MALDI-TOF screening because we can say with confidence that even the very low intensity ions are originating from the sample because of the specific co-localization with the target tissue. The data on the sponge *Dysidea herbacea* indicates that, based on the differential distribution of secondary metabolites, sponges have differential (chemical) micro-environments within their tissues. Our laboratories are planning to use npMALDI-I to localize previously described specific natural products with potent therapeutic properties from heterogeneous marine assemblages, a step towards allowing us to collect the genetic material and pinpoint the specific gene clusters responsible for their synthesis. Finally, the approach outlined in this paper should be readily adapted to secondary metabolomic studies, the chemical communication of symbionts or therapeutic discovery programs.

Materials and Methods

Cyanobacteria Cultures

3L Lyngbya was collected at Las Palmas beach near the CARMABI Research Station in Curacao, Netherlands. JHB was collected in Hector's Bay, Jamaica. *Oscillatoria nigroviridis* was isolated as a contaminant of the 3L *Lyngbya majuscula* strain *Lyngbya bouilonii* was collected on Pigeon Island in Papau New Guinea. *Phormidium sp*. was collected in Indonesia. All cultures were subsequently isolated to a monoclonal culture using standard microbiological isolation techniques (Rossi et al 1997, Edwards *et al* 2004). Approximately 3 g of each strain were inoculated into 2-L Fernbach flasks containing 1 L of SWBG11 medium. These static cultures were grown at 28°C under uniform illumination (4.67 μmol photon s^-1 m^-2) with a 16hr/8hr light/dark cycle for 30 days. About 10-20 individual filaments were transferred to 50ml polystyrene tissue culture flasks.

Filament Sample Preparation

Using blunt-tip tweezers, a single filament was removed from the small colonies in the 50ml flasks and transferred to a petri dish containing distilled water to remove excess salt water attached to the cyanobacterial filaments. Again, using blunt-tip tweezers, the filament was removed and carefully laid out onto a Bruker MSP 96 anchor plate, making sure that the filament laid flat against the plate. Any excess liquid on the surface was absorbed using the corner of a Kimwipe ®. If multiple filaments were being examined, particular attention was given to the orientation and location of the filaments on the plate. The plate was then placed in a desiccator at 38°C for 5-10 minutes or until visibly dry. Prior to matrix application, a photograph (Nikon Coolpix, 1-3 mp image) of the plate was taken to use as teach reference for the Bruker MALDI MS.

MALDI Matrix Deposition

After the desiccation and image capture, matrix composed of 35mg/ml a-cyano-hydroxycinnamic acid, 15 mg/ml DHB, 78% ACN and 0.1% TFA was coated onto the MALDI MSP 96 plate using an airbrush (www.paascheairbrush.com) and repeated side to side strokes until an even, thin crystalline layer occluded the background of the plate The Bruker MSP 96 anchor plate containing the sample and matrix was placed in an empty Petri dish until analysis.

MALDI MS and Imaging

The Bruker MSP 96 anchor plate containing the sample was inserted into a Microflex Bruker Daltonics mass spectrometer outfitted with Compass 1.2 software suite (Consists of FlexImaging 2.0, FlexControl 3.0, and FlexAnalysis 3.0). The sample was run in positive mode,

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with 100μm raster intervals in XY and roughly 35-62% laser power. Briefly, a photomicrograph of the sample to be imaged by mass spectrometry was loaded onto the *Fleximaging* command window. Three teach points were selected in order to align the background image with the sample target plate. After the target plate calibration was complete, the *AutoXecute* command was used to analyzed the samples. The settings under the FlexControl panel where as follows: For the **Autoexecute**. **Method**: Our own. Consisting of the following settings: *General*: Flex-Control Method-RP_pepMix.par. *Laser*: Fuzzy Control-On, Weight - 1.00; Laser Power-varied between 35-62%; Matrix Blaster- 0. *Evaluation*: Peak Selection- Masses from *m/z* 350-3000, mass control list- Off. Peak Exclusion-Off. Peak Evaluation- Processing Method-Default, Smoothing-Off, Baseline Subtraction-On, Peak-Resolution higher than 100. *Accumulation*: Parent Mode: On, Sum up to 20 satisfactory shots in 20 shots, Dynamic Termination- Off. *Movement*: Random Walk- 2 shots at raster spot. Quit sample after- 2 subsequent failed attempts. *Processing*: Flexanalysis Method- none, Biotools MS method-none. **Sample Carrier**: nothing **Spectrometer**: On, Ion Source 1- 19.00mV, Ion Source 2-16.40mV, Lens- 9.45mV, Reflector 20.00, Pulsed Ion Extraction- 190ns, Polarity-Positive. Matrix Suppression: Deflection, Suppress up to: *m/z* 350. **Detection**: Mass Range-350-1000, Detector Gain- Reflector 3.7X. Sample Rate- 2.00 GS/s, Mode- low range, Electronic Gain-Enhanced, 100mV. Real time Smooth- Off. Spectrometer, Size: 81040, Delay 42968. **Processing Method**: Factory method RP_2465. **Setup**: Mass Range- Low. Laser Frequency-20Hz, Autoteaching-off. Instrument Specific Settings: Digitizer- Trigger Level- 2000mV, Digital Off Linear- 127 cnt, Digital Off Reflector- 127cnt. Detector Gain Voltage Offset, Linear- 1300V, Reflector- 1400V. Laser Attenuator, Offset −12%, Range- 30%, Electronic Gain Button Definitions, Regular: 100mv (offset lin) 100mV (offset ref) 200mV/full scale. Enh: 51mV (offset lin), 51mV (offset ref) 100mV/full scale. Highest: 25mV (offsetlin) 25mV (offset ref) 50 mV/full scale. **Calibration**: Calibration was accomplished using a BSA digest as external standard. Zoom Range +−1.0% Peak Assignment Tolerance-User Defined-500ppm.

After data acquisition, the data was analyzed using the FlexImaging software. The resulting mass spectrum was filtered manually in 0.5-3.0 Da increments with individual colors assigned to the specific masses associated with the filaments.

Dysidea herbacea Preparation

Sample Collection and Storage Dysidea herbacea (Collection code 02158) was collected in Papua New Guinea in 2002 by Phil Crews lab (UCSC). It was stored and frozen in EtOH/Sea water (1:1)

Cryosectioning of Sponge Tissue—The sample was thawed and precut then embedded in 1X Dulbecco's PBS and placed in the cryostat at −20C. Once the tissue and embedding block had frozen completely, 14-20 μm thick coronal sections were cut and mounted on to a semi-thawed MALDI MSP 96 plate. The plate was desiccated at 38°C for 10 minutes. Prior to matrix application, a photograph (Nikon Coolpix, 3mp image) of the plate is taken as teach reference for the Bruker MALDI MS.

MALDI Imaging of Dysidea herbacea—The Data acquisition, Flex control settings and data processing was performed as described for the analysis of single filaments with a few notable exceptions. The sample was run in positive mode, with 100 μm raster intervals in XY and 55-65% laser power. **Autoexecute**. **Method**: *Movement*: Random Walk- 3 shots at raster spot. Sum up to 30 satisfactory shots in 30 shots.

Supplementary Material

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Acknowledgments

We thank P. Crews and K. Tenney (UC Santa Cruz) for samples of the sponge *Dysidea herbaceae*, We thank T. Matainaho and the captains/crews of the Golden Dawn and Teleta Dive Boats for their assistance with cyanobacterial collections, and the governments of Papua New Guinea, Curaçao, and Indonesia for permission to make these collections. We thank Mark Ellisman, Tom Deerinck, John Crum and Andrea Thor at The National Center for Microscopy and Imaging Resource for their support and expertise in cryo-sectioning. This work was supported by grants NIH CA52955, CA100851, American Cancer Society for maintenance of the instrumentation (IRG-70-002-29) and funding to the Skaggs school of pharmacy and pharmaceutical sciences.

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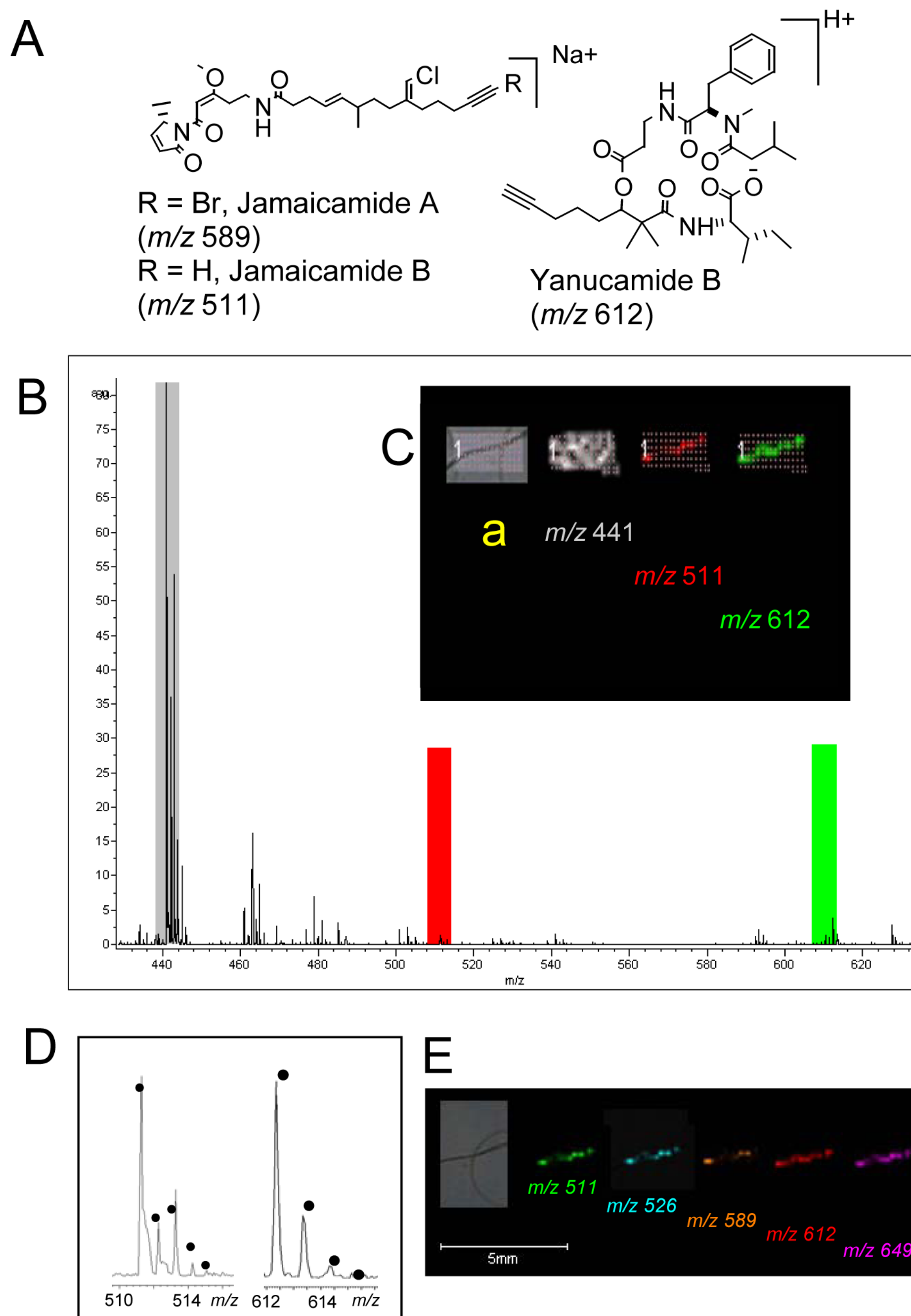

Fig. 1.

MALDI-TOF-imaging of the intact marine cyanobacterium *Lyngbya majuscula JHB* filament. A) The molecular structures of jamaicamide A, B and yanucamide B. B) The average mass spectrum of a 0.6 *1.5 mm area of the MALDI imaging experiment. The colors indicate the regions visualized in C. C) The differential localization of the indicated masses with respect to the cyanobacterial filament. A shows the raster points in this experiment. D) Comparison of the theoretical isotopic distribution of jamaicamide B and yanucamide B indicated by the black dots with the observed average spectrum in this experiment. E) The spatial distribution for several molecular ions co-localized with *Lyngbya majuscula JHB*.

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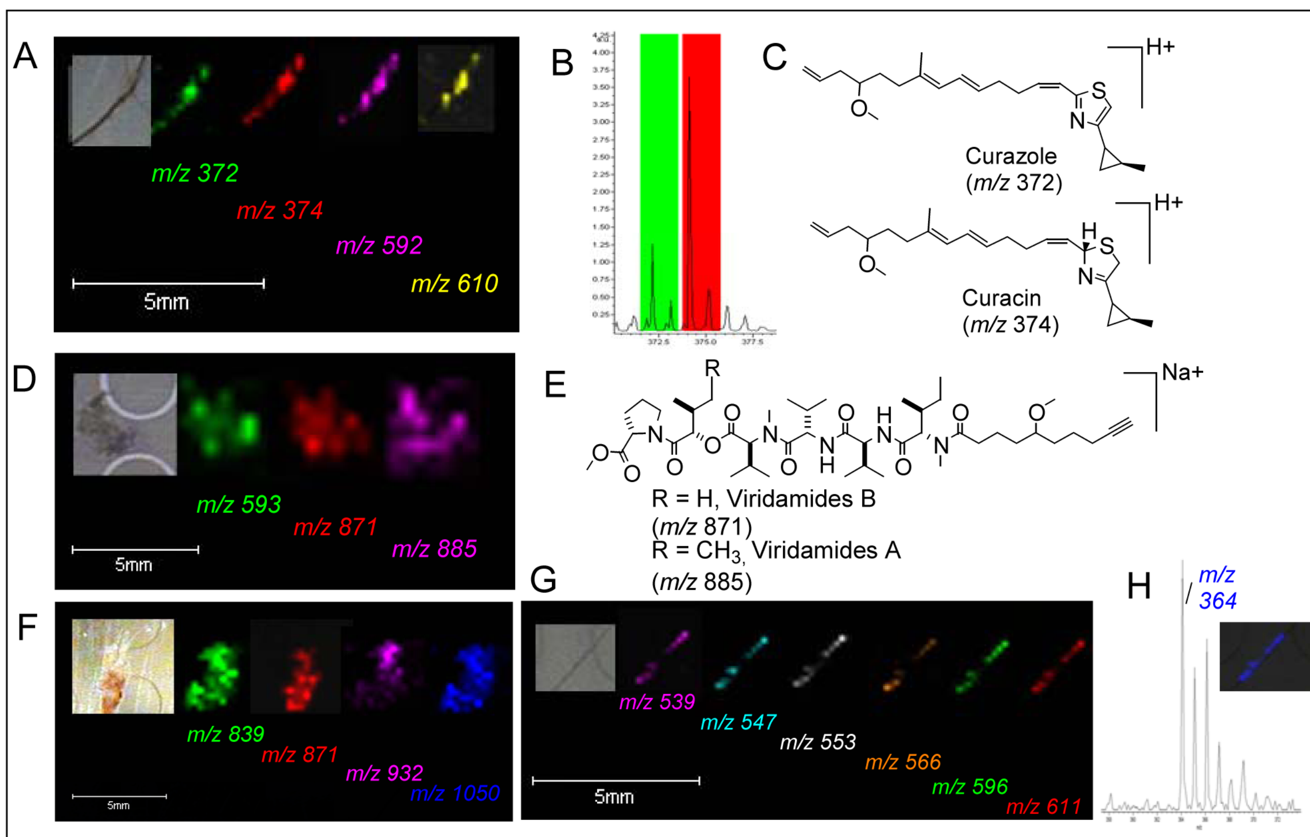

Fig. 2.

The spatial distribution of selected ions observed to co-localize with *Lyngbya majuscula* 3L (A), *Oscillatoria nigro-viridis* (D), and a *Phormidium* species (F), *Lyngbya bouillonii* (G). The average mass spectral trace showing curacin and curazole and the respective colors indicated is shown in figure B. The structures of curacin, curazole (C) and viridamides (E) are also shown. H) The isotopic distribution for the 364 *m/z* molecular ion.

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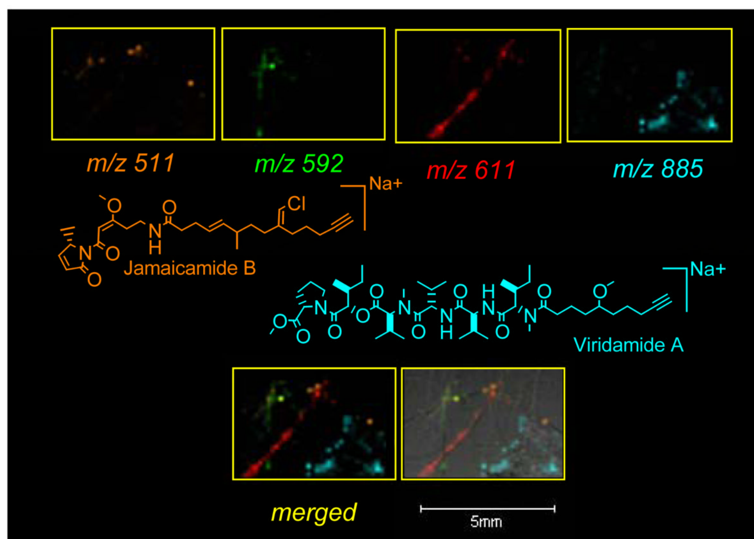

Fig. 3.
npMALDI-I of a complex mixture of cyanobacteria: a single npMALDI-I run on a mixture of *Lyngbya majuscula* JHB (orange),and 3L (green), *Lynbya bouillonii* (Red) *Oscillatoria nigro-viridis* (Blue). The top panels represent detection of two known masses-Jamaicamide B (orange-structure shown) and viridamide A (blue- structure shown) as well as two unknown masses (red and green), each specifically and differentially locates to a particular organism. The bottom panels emphasizes the scale and spatial resolution, as well as the ability to visualize various different secondary metabolites from multiple organisms.

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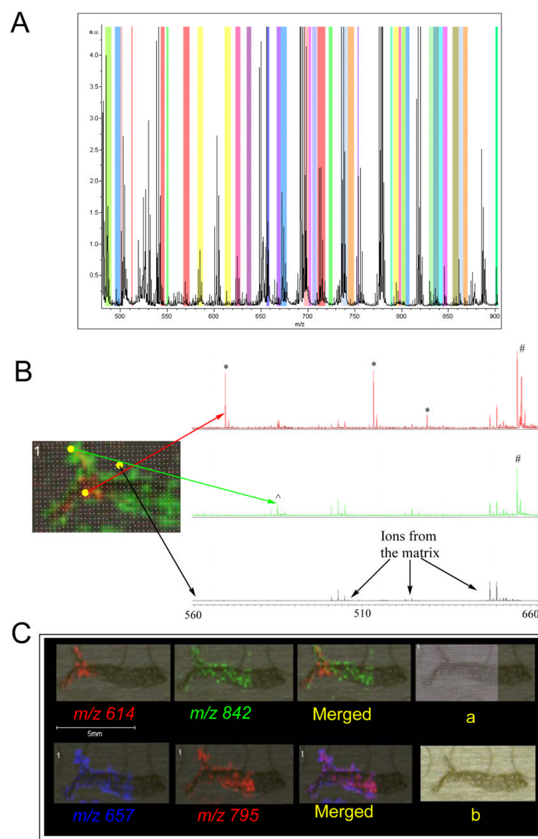

Fig. 4.

npMALDI-I on the sponge *Dysidea herbacea*. A) The average mass spectrum. The colors indicate all the ions that specifically localized to the sponge section, the colors themselves have no meaning other than that they are a means to show the differential localization. B) An image of the *Dysidea* section with the laser raster points and selected masses shown. In this image we show the relative ion intensities in the region from 560 to 660 m/z for three different areas of the sampling area. This image shows the different ion localizations and ion clusters associated with the matrix. Ions indicated with a # are co-localized throughout the sponge, ions that are localized near the edges of the sponge are indicated with a ^ and ions found on the inner section of the sponge are shown with a *. C) Some representative differential localizations and ion masses associated with the sponge, suggesting a differential chemical microenvironments. “a” shows the raster on the image. “b” shows the photomicrograph of the sponge-section itself.