Electronic Supplementary Information (ESI) for

Metallophore mapping in complex matrices by metal isotope coded profiling of organic ligands

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Content of the Supporting Material

1. Material and Methods

1.1 Reagents

All used solvents and reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany) and used without further purifications. UHPLC-grade methanol, acetonitrile, water ammonium acetate, and formic acid were purchased from Biosolve (Valkenswaard, Netherlands). All ingredients of the bacterial growth media were purchased either from Merck (Darmstadt, Germany) or Sigma-Aldrich. Ultra-pure water (0.055 µS) obtained by a MicroPure water purification system (Thermo Scientfic, Germany) was used for the preparation of aqueous solutions.

1.2 Isotopes

Stable pure isotopes of iron and molybdenum (enrichment > 98 %) were obtained from Euriso-top (Saint-Aubin Cedex, France). The molybdenum oxides (^{95}Mo and ^{98}Mo) were solved in sodium hydroxide in stoichiometric mixture. Hydrochloric acid was used to solve the iron oxides (^{54}Fe and ^{58}Fe) in stoichiometric mixture. Subsequently the prepared isotope solutions were diluted with ultra-pure water to 10^{-2} mol L^{-1} (stock solution).

1.3 Instrumentation and chromatographic conditions

All liquid chromatographic measurements were performed on an Acquity^{TM} ultra performance LC (Waters, Milford, MA, USA) coupled to a Q-ToF Micro mass spectrometer (Waters, Micromass, Manchester, UK). For the reverse phase separation, a Waters UPLC^{TM} BEH C18 Column (2.1 × 50 mm × 1.7 µm) was used with a flow rate at 600 µL min^{-1} and column temperature at 21 °C. The mobile phase consisted of solvent A with 1 mmol L^{-1} ammonium

acetate in water with 2 % of acetonitrile and solvent B which contained 1 mmol L^{-1} in ammonium acetate 90 % acetonitrile (v/v). The standard gradient started with 100 % A (0.20 min), ramped to 50 % B (4.00 min) and finally to 100 % B (4.2 min) and kept for 1.5 min. Afterwards the gradient was set back to 100 % A and held for 1.5 min.

All measurements were performed in the total ion current mode. Both the negative- and positive-ion modus were utilized for determination of metallophores using low collision energy (5.0 V) and a scan rate of 0.8 per second. Hereby, the desolvation temperatures and source temperatures were 300 °C and 120 °C, respectively. The capillary and sample cone was set at 2800 V und 25 V in negative ion mode and 3000 V and 25 V in positive ion mode. The ion energy was set at 1.8 V in both operation modes. The instrument was calibrated in the *m/z* range of 100–1500 using sodium formate. The collision was set to 18 V to achieve fragmentation for all MS^2 experiments. Upon identification of metallophore candidates, the exact mass of the pseudo-molecular ion was measured using the maXis high resolution mass spectrometer (Bruker, Germany) equipped with the Nexera XR ultra high performance liquid chromatograph (Shimadzu, Japan) in order to search for known siderophores by mass matching in data bases.

1.4 Microorganism and growth conditions

Four metallophore producing microorganisms from aquatic and terrestrial ecosystems were selected in order to establish the methodology. In all cases the bacterial growth media were spiked with the respective isotopes (= **pre-labelling**). Alternatively, for **post-labelling,** solid-phase extracts of the supernatant were spiked with the isotopically pure isotopes.

Azotocbacter vinelandii **(OP)** is a well known ubiquitous, free living nitrogen fixing soil gram-negative bacterium, which produces mainly catecholate metallophores including the tris(catechol)

protochelin (624 amu) or the bis(catechol) azotochelin (418 amu) to complex the essential metal cofactors molybdenum, iron and vanadium.[1, 2] The bacteria were grown under diazotrophic conditions according to Bellenger et al. (2008) ([Fe-EDTA] = 5.0×10^{-6} mol L^{-1} and [Mo] = 5.0×10^{-7} mol^{-1}) and constantly shaken with 120 rpm at 21°C. Cultures were harvested at the optical density (OD_{620nm}) of 0.1 (= 1.43×10^{7} cells mL^{-1}, early exponential phase) and of 1.2 (= 1.68×10^{8} cells mL^{-1}, late exponential phase) depending on the experiment.[2, 3]

***Azotobacter vinelandii* (F196)**, a transposon mutant of OP,[4] produces just the pyoverdine-like metallophore, azotobactin (1392 amu), which is a fluorescent metallophore with a catechol and hydroxamate moiety and a α-hydroxycarboyxlic acid group. It was grown under the same condition as *A. vinelandii* (OP) but at low iron concentrations ([Fe-EDTA] = 5.0×10^{-7} mol L^{-1} at 21 °C. Cultures were harvested at the optical density (OD_{620nm}) of 0.5.[5]

***Anabaena cylindrica* (CCAP 1403/2A)**, a filamentous freshwater cyanobacterium excretes anachelin (760 amu). This strain was the first identified catecholate metallophore producers in cyanobacteria. The "BG 11" growth medium[6] was slightly modified (i.e. without any nitrogen source, [Fe-EDTA] = 10^{-6} mol L^{-1}) and growth conditions were adjusted according to Beiderbeck 2000[7]. The supernatant of the culture was harvested after 3 weeks.

***Anabaena variabilis* (ATCC 29413, synonym: *Nostoc* sp. PCC 7937)** was grown under autotrophic or heterotrophic conditions. For heterotrophic and diazotropic growth at 22 °C, the "819" growth medium was modified regarding iron content and carbon source (i.e. [Fe-EDTA] = 5.0×10^{-7} mol L^{-1}, [fructose] = 10 g L^{-1}).[8] Under autotrophic and diazotropic conditions, *A.*

variabilis was growing in the modified "BG 11" growth medium (i.e. without any nitrogen source, [Fe-EDTA] = 5.0×10^{-7} mol L^{-1}).

Cladosporium cladosporioides is a ubiquitous fungus which releases the metallophore ferricrocin (717 amu) that belongs to the class of hydroxamate siderophores. The strain was cultivated in modified "MNM" growth medium ([Fe-EDTA] = 10^{-7} mol/L) and grown under continuously orbital-shaking with 120 rpm. The supernatant of the culture was harvested after 3 weeks.[9]

***Synechococcus* sp. (PCC 7002)**, a coastal marine cyanobacterium, produces three amphiphilic metallophore, synechobactins A-C, under iron-limiting growth conditions.[10] For diazotrophic growth, the modified "BG 11" growth medium (i.e. without any nitrogen source, [Fe-EDTA] = 10^{-6} mol L^{-1}) was used. This type specimen was used as reference strain for the identification of the perspective metallophores in *A. variabilis*.

1.5 Sample preparation and solid phase extraction of metallophores

Bacterial growth media were centrifuged at 6000 rpm for 5 min at room temperature. The supernatant were sterile filtered (0.2 μm) before analyses by UPHLC-MS.

If a concentration step was necessary, solid phase extraction (SPE) of metallophores was conducted: Cultures of microorganisms and fungi were harvested mostly at the late exponential phase or early stationary phase. The culture was centrifuged at 5000 rpm for 10 min at 4 °C (Hermle, Wehingen, Germany), and the supernatant (containing metal complexing organic ligands) was passed through the HLB-cartridge (200 mg sorbents, Oasis™, Waters, Milford, UK) preconditioned with 6 mL methanol and equilibrated with 8 mL water. Free metallophores and

those complexed to the applied isotopes of iron or molybdenum were eluted in one step with 6 mL of 100 % MeOH according to a well established procedure.[2, 7, 11]

1.6 Analytical process and target analysis of isotope labelled metallophore

The analytical process was performed according to the workflow (Fig. S2). Two different approaches were established:

- **pre-labelling:** The medium/matrix was spiked with the isotopically pure Mo and/or Fe *before the experimental onset*.
- **post-labelling:** The harvested medium/extract was spiked with the isotopically pure Mo and/or Fe.

Upon UHPLC-MS analyses the obtained chromatograms were analyzed by the so called "strip algorithm" provided by the software package MarkerLynx® V4.1 (Waters, UK). This program identified chromatographic peaks by specified mass difference and expected intensity ratios within a defined tolerance range. All customized parameters are summarized in Table S1.

1.7 Biomarker extraction and multivariate comparative analysis

Growth media or solid phase extracts were divided into equally aliquots, spiked with the relevant isotopes $^{54}Fe/\ ^{95}Mo$ (= group 1) and $^{58}Fe/\ ^{98}Mo$ (= group 2) and measured by UHPLC-ESI-ToF-MS. Subsequently mass/retentions time pairs were collected automatically by e.g., MarkerLynx® or any other application that processes complex multivariate data from UHPLC/MS. These automated peak extraction routines deliver mass/retention time pairs for every biomarker detected in the chromatogram according to the selected settings listed in Table S2. Five replicates were conducted for each treatment/group ($n_{total} = 10$).

The data set obtained was analyzed using discriminant analysis such as the canonical analysis of principal coordinates (CAP) developed by Anderson and co-workers (2003).[12, 13] The data set was ln(x+1) transformed. Bray-Curtis dissimilarity was applied for distance measure. The two *a priori* defined groups can be only clearly characterized/separated by the metal complexing ligands bound to ^{54}Fe (group 1) and bound to ^{58}Fe (group 2) or to the respective molybdenum isotopes ^{95}Mo and ^{98}Mo.

1.8 Identification of metallophores

Metallophores were identified either by comparison with reference standards (i.e, co-injection) or by high resolution mass spectrometry and MS^2 experiments of the pseudo-molecular ion. Reference standards (ferricrocin, pyoverdine, schizokinen) were purchased from the EMC-Microcollection GmbH (Tübingen, Germany) or directly isolated (protochelin, azotobactin, anachelin, synechobactin A/B) from the respective bacterial reference strains according to established protocols.[5, 7, 10, 11]

1.9 Mo- and Fe-binding ability of tannic acid

The measurements with tannic acid (3×10^{-4}g mL^{-1}) were performed on an Acquity^{TM} ultra performance LC (Waters, Milford, MA, USA) coupled to a Q-ToF Micro mass spectrometer (Waters, Micromass, Manchester, UK). Reverse phase separation of the aquatic sample enriched with tannic acid was conducted on an Agilent Zorbax C_8 HPLC column (4.6 × 150 mm × 5 µm) with a flow rate at 600 µL min^{-1} and column temperature at 31 °C. The standard binary mobile phase system was used. The gradient started with 100 % A (0.20 min), ramped to 40 % B (4.00 min) and finally to 100 % B (5.2 min) and held for 1 min. Afterwards the gradient was set back to 100 % A and held for 1 min.

2. Figures

Figure S1 Chemical structures of model metallophores in this study: azotochelin (**3**), ferricrocin (**4**), azotobactin (**5**), pyoverdine (**6**), schizokinen (**7**), synechobactin B (**8**), synechobactin A (**9**). (Chemical structures of protochelin (**1**) and anachelin (**2**) are found in the main text.)

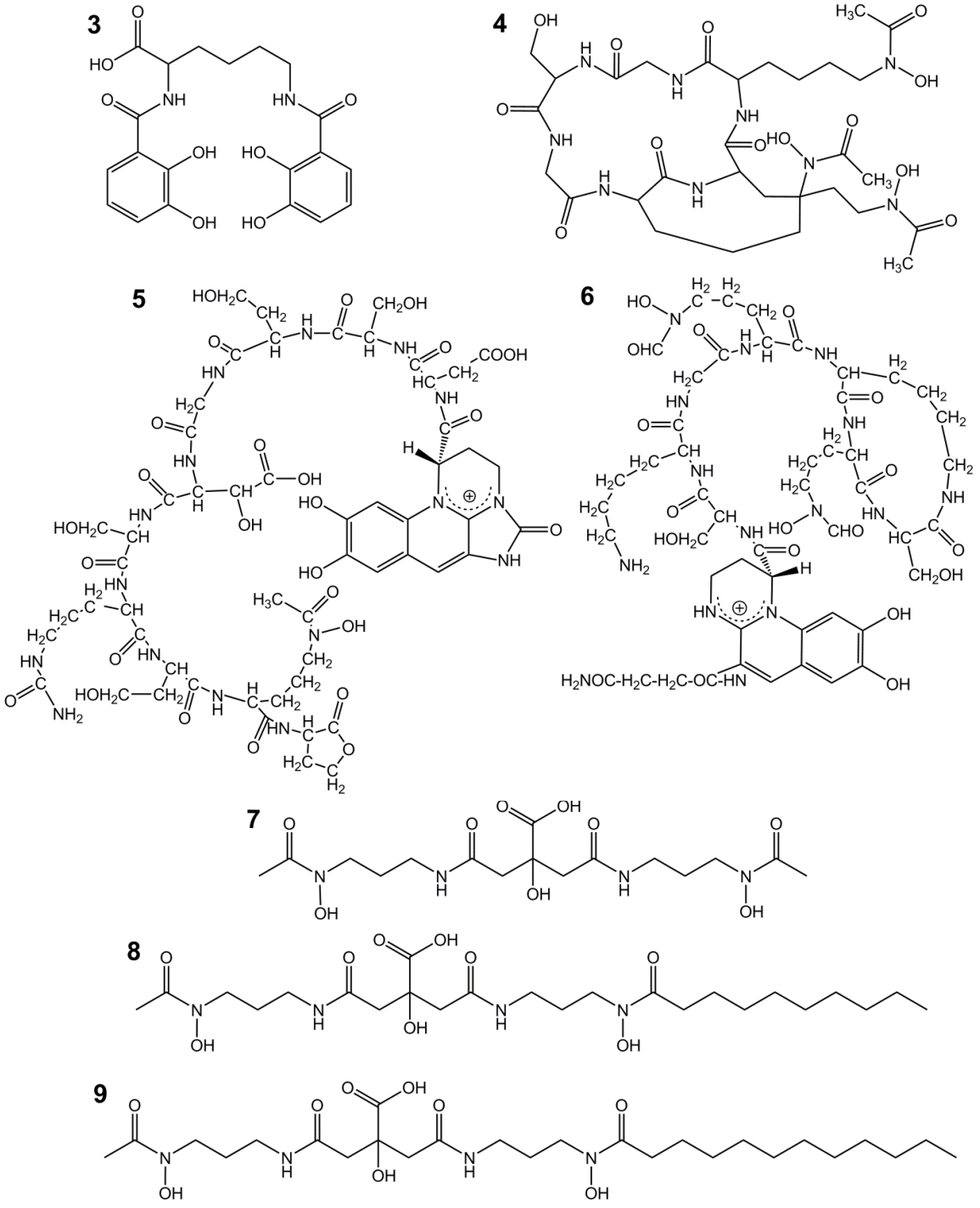


Figure S2 Comprehensive workflow of the analytical process: Growth media or extracts are spiked with isotopically pure Mo and Fe and subsequently analyzed by (**A, C**) target analysis or (B, D) multivariate comparative analysis. Metal isotopes should be applied to the medium/matrix before the experimental onset for pre-labelling (**A**, **B**) or after harvesting/extracting of the medium for post-labelling (**C**, **D**) depending on the scientific question.

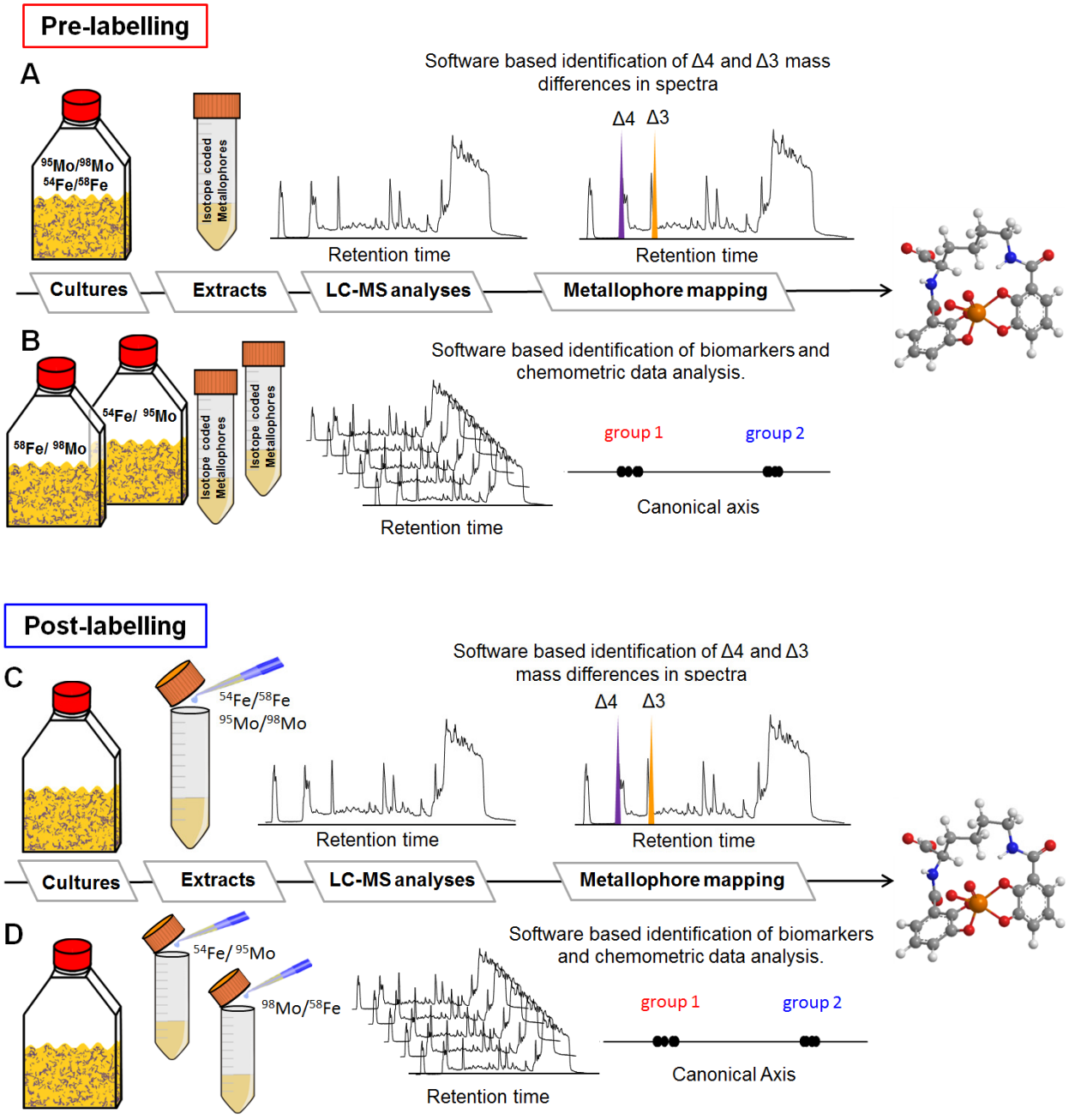


Figure S3 Metallophore mapping of *Azotobacter vinelandii* at specific growth phases.

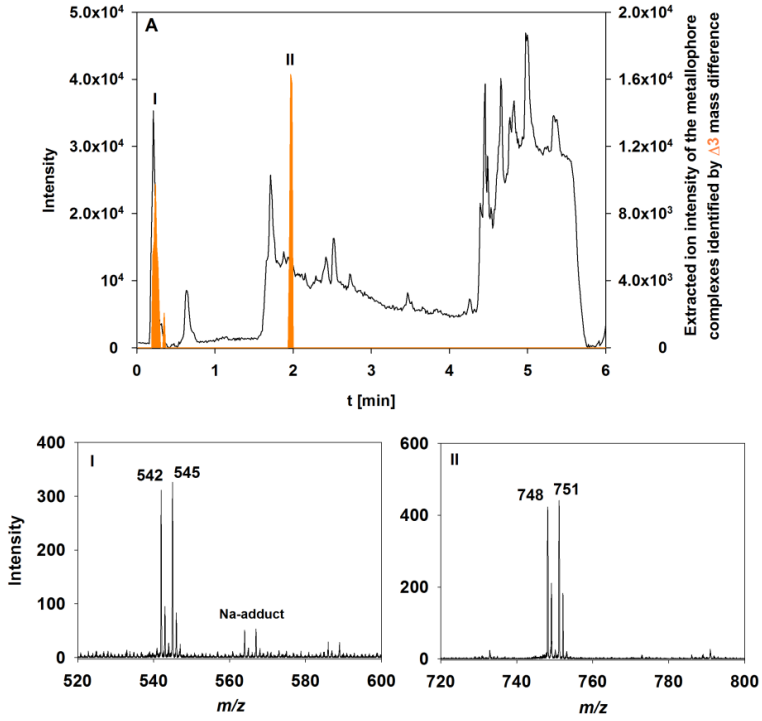
Total ion current chromatograms (TIC) of the UHPLC-MS analyses and extracted chromatograms are shown, as well as the corresponding mass spectra of the identified metallophores.

(**A**) The extracted chromatograms of the Mo-complexes of azotochelin $[MoO_2(azotochelin)+2H]^-$ (I) and protochelin $[MoO_2(H_2\text{-protochelin})+H]^-$ (II) determined in the *late* exponential phase are shown (orange line).

(**B**) Novel siderophores excreted by the bacterium could be also determined under replete iron conditions ([Fe-EDTA] = 5.0×10^{-6} mol L^{-1}) in the *late* exponential phase. The negative ion mass spectra (I and IV) show the respective candidates identified by the Δ4 mass differences with the pairs of masses of 484/488 *m/z*, 520/524 *m/z* and 688/692 *m/z* besides the known iron complexes of azotochelin $[Fe^{III}(azotochelin)+H]^-$ (II) and protochelin $[Fe^{III}(protochelin)+2H]^-$ (III) (purple line). Further minor amounts of siderophore candidates were identified by the Δ4 mass differences between 482/486 *m/z* and 702/706 *m/z* (data not shown).[1]

[1] Same putative Fe-chelators were also recently identified in *A. vinelandii* at low iron concentration by Kraepiel and co-workers (Princeton University; http://www.princeton.edu/morel/research/metal-complexing/; date 23/07/2014).

(A) Metallophore mapping in *A. vinelandii* for molybdenum binding ligands



(B) Metallophore mapping in *A. vinelandii* for iron binding ligands

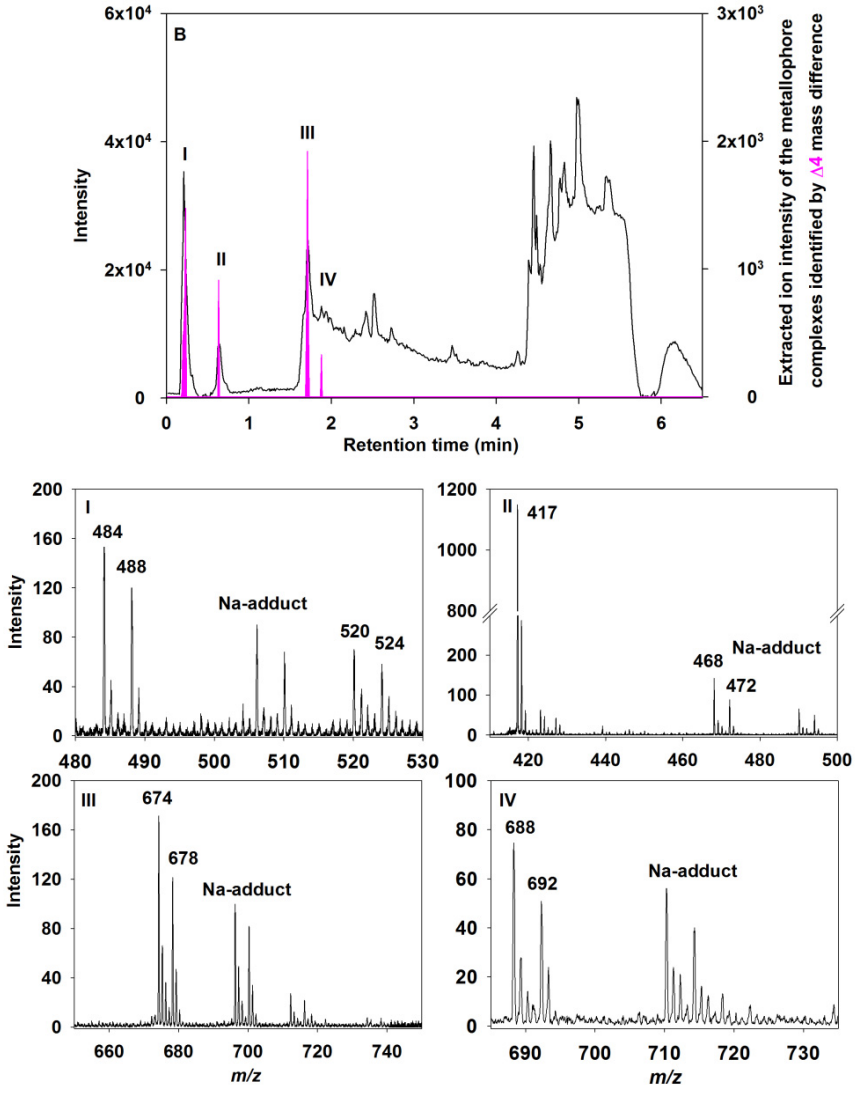
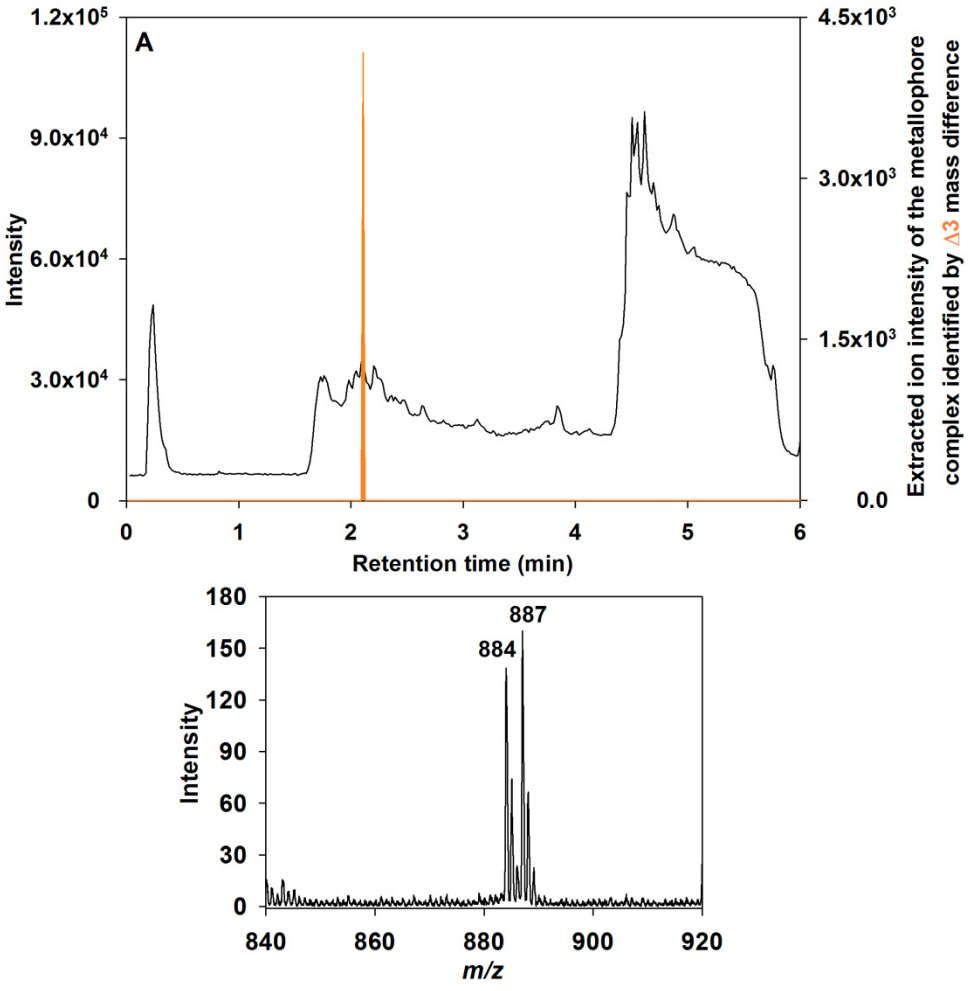


Figure S4 Metallophore mapping in *Anabaena cylindrica*. Total ion current chromatograms (**A**, **B**) of the UHPLC-MS analyses and extracted chromatograms are shown, as well as the corresponding mass spectra of the identified metallophore **2**.

(**A**) Extracted chromatogram (negative ion mode) indicates the Mo-anachelin-complex $[MoO_2(anachelin\text{-}4H)]^-$ based on the identification of the Δ3 mass difference in the isotopic signature (orange line).



(**B**) Extracted chromatogram (positive ion mode) indicates the Fe-anachelin-complex $[Fe(anachelin\text{-}3H)]^{+}$ based on the identification of the Δ4 mass difference in the isotopic signature (purple line).

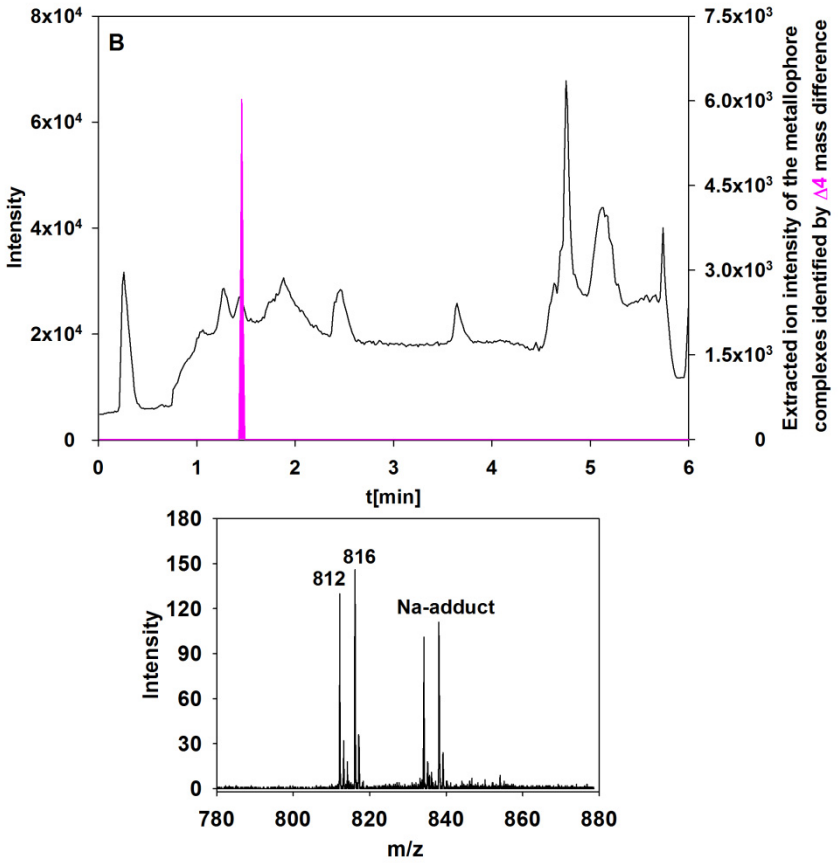
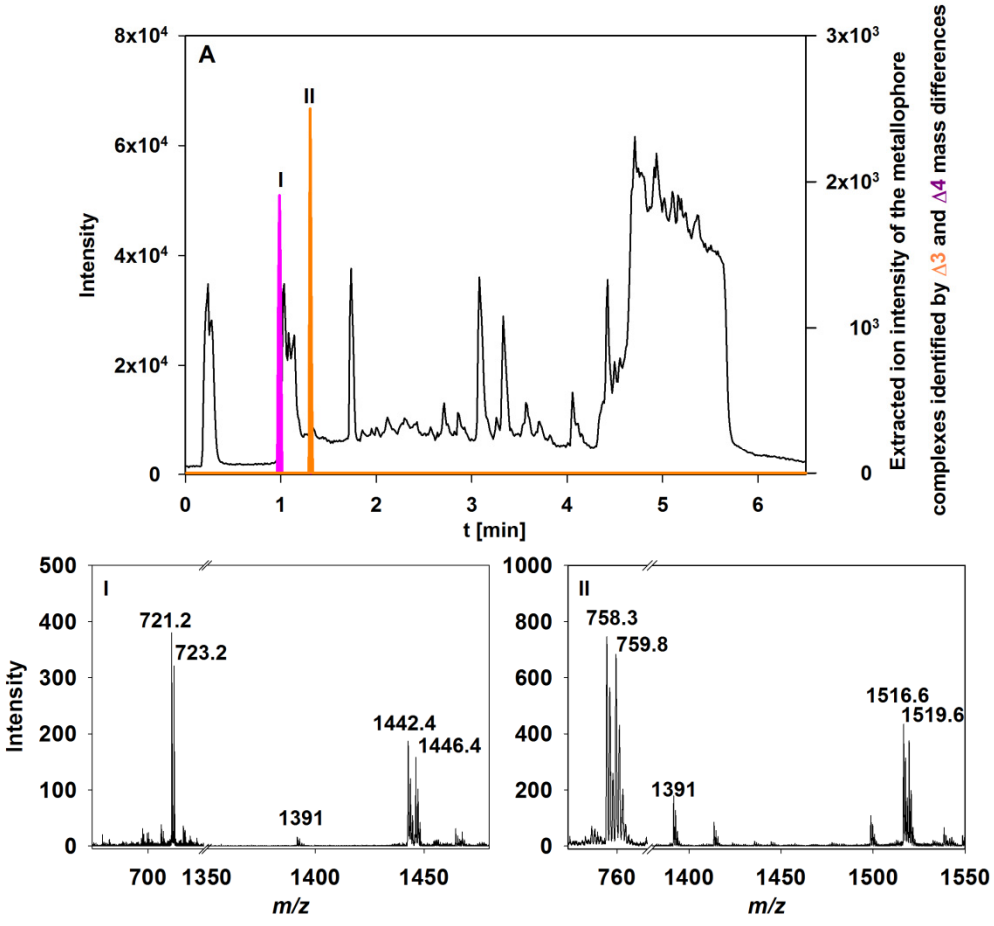


Figure S5 Metallophore mapping of the pyoverdines, 5 and 6, in (A) *Azotobacter vinelandii* (F196) and (B) *Pseudomonas fluorescence*. Total ion current chromatograms (TIC) of the UHPLC-MS analyses and extracted chromatograms are shown, as well as the corresponding negative ion mass spectra of the identified metallophores.

(A) Metallophore mapping in *Azotobacter vinelandii* (F196)

Extracted chromatograms showing siderophores (purple line) and molybdophores (orange line) are based on the simultaneous identification of Δ4 (1442.4/1446.4 *m/z*, Fe-azotobactin[5]) and Δ3 (1516.6/1519.6 *m/z*, Mo-azotobactin) mass differences in the isotopic signature. The doubly charged Fe-complex and Mo-complex can be identified by Δ2 (721.2/723.2 *m/z*) and Δ1.5 (758.3/759.8 *m/z*).



(B) Metallophore mapping in *Pseudomonas fluorescence*

Extracted chromatogram indicates the iron complex of pyoverdine **6**. The pairs of *m/z* values 1210.4/1214.4 and 605.2/607.2 correspond to the singly and doubly charged Fe-complex.

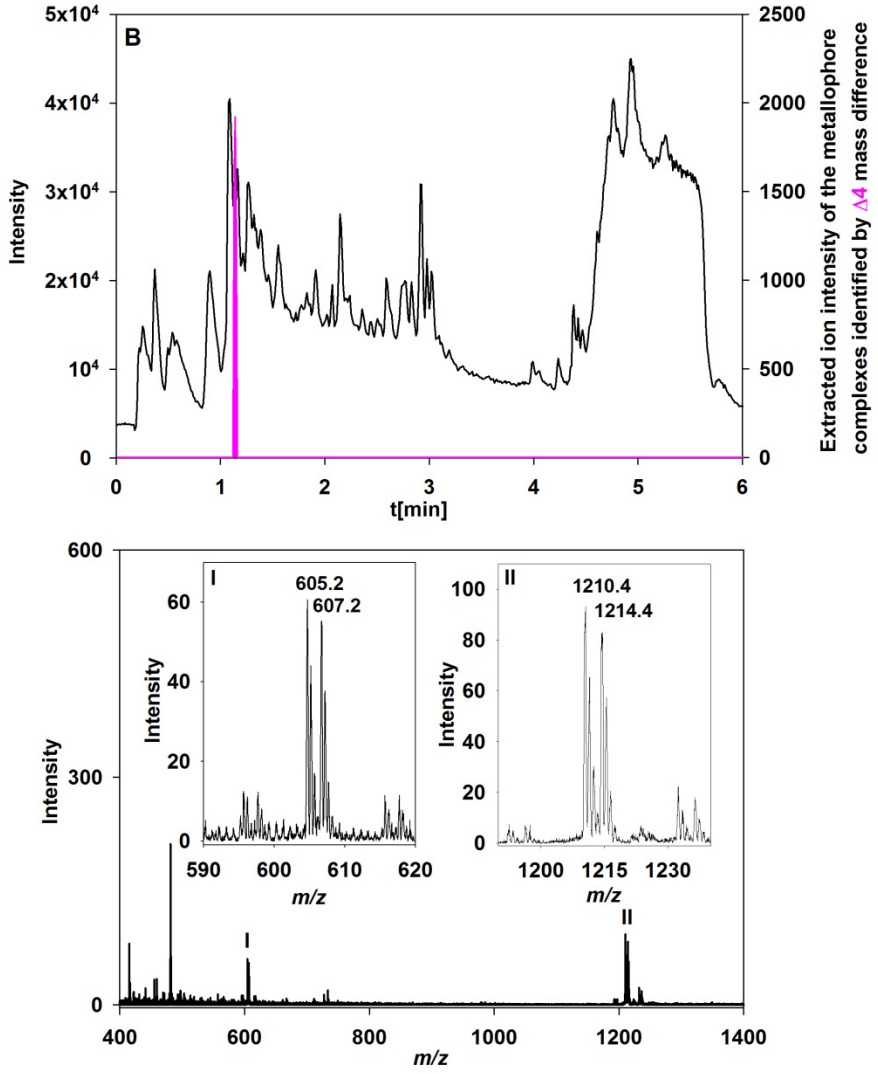


Figure S6 Metallophore mapping in *Cladosporium cladosporioides.* Total ion current chromatograms of the UHPLC-MS analyses and extracted chromatograms are shown, as well as the corresponding negative ion mass spectrum of the identified metallophore **4**. Extracted chromatogram indicates the iron complex $[Fe(ferricrocin\text{-}4H)]^-$ (purple line) based on the identification of the Δ4 (767/771 *m/z*) mass differences in the isotopic signature.

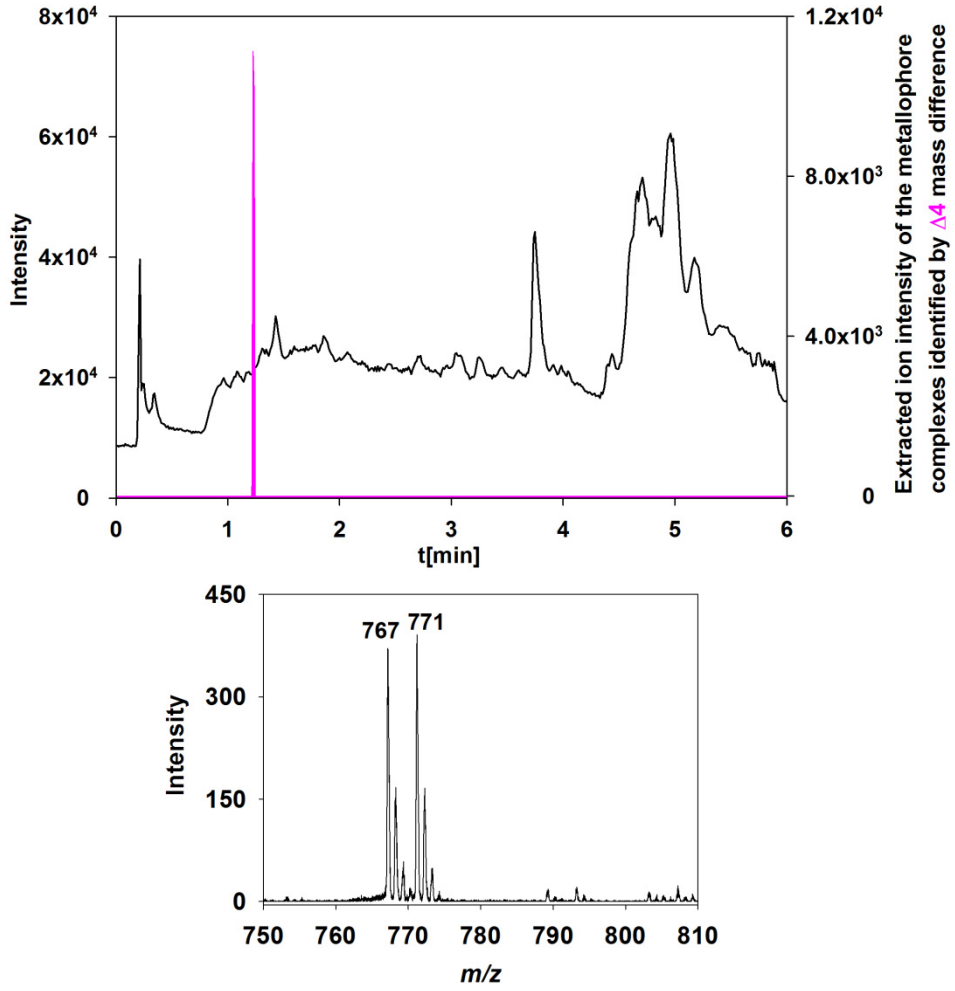
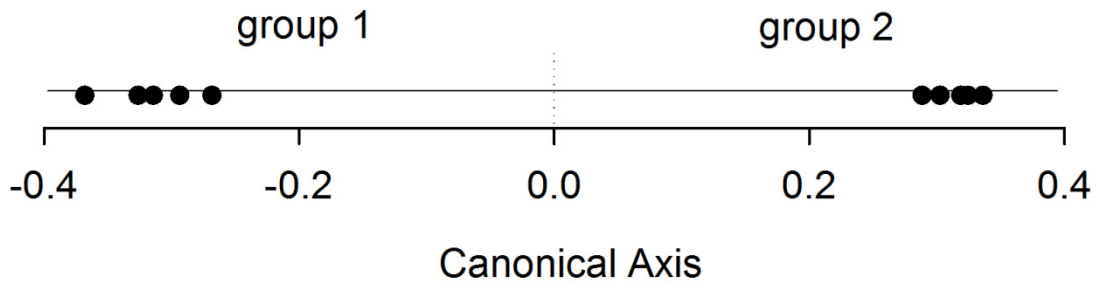


Figure S7 Chemometric data analysis of (**A**) *Azotobacter vinelandii*, (**B**) *Anabaena variabilis,* (**C**) *Anabaena cylindrica*, and (**D**) *Cladosporium cladosporioides.*

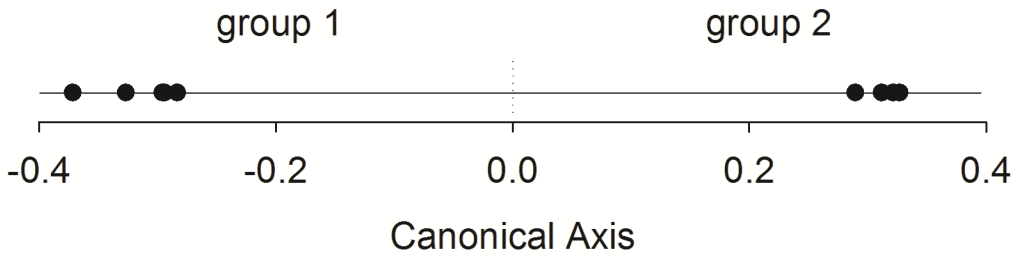
(A) *Azotobacter vinelandii*: simultaneous determination of molybdophores and siderophores

Retention time [min]	*m/z*	Pearson's correlation	*m/z*	Pearson's correlation	Δ
	group 1: ^{54}Fe /^{95}Mo		group 2: ^{58}Fe /^{98}Mo		
1.59	338.51	-0.996	336.51	0.989	2.00
1.59	678.04	-0.997	674.04	0.996	3.99
1.88	375.00	-0.996	373.50	0.997	1.50
1.88	751.04	-0.996	748.03	0.997	3.01
2.79	593.18	-0.991	-	-	-
2.88	-	-	187.09	0.978	-
3.49	-	-	213.10	0.985	-
3.52	765.03	-0.995	-	-	-



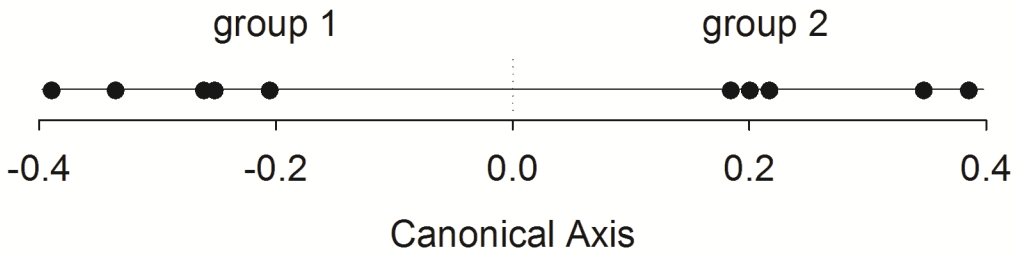
(B) *Anabaena variabilis*: determination of siderophores

Retention time [min]	*m/z*	Pearson's correlation	*m/z*	Pearson's correlation	Δ
	group 1: ^{54}Fe		group 2: ^{58}Fe		
2.36	631.22	0.9014	-	-	-
2.38	-	-	626.19	0.9865	-
2.76	658.20	-0.985	654.19	0.9858	4.01
2.86	582.34	-0.810	586.31	0.8233	4.04
3.41	614.17	-0.9981	610.14	0.9852	4.03
3.75	656.18	-0.8625	652.20	0.9852	3.98
4.05	643.23	-0.9874	639.21	0.9758	4.02
4.06	642.19	-0.9857	638.18	0.9717	4.01

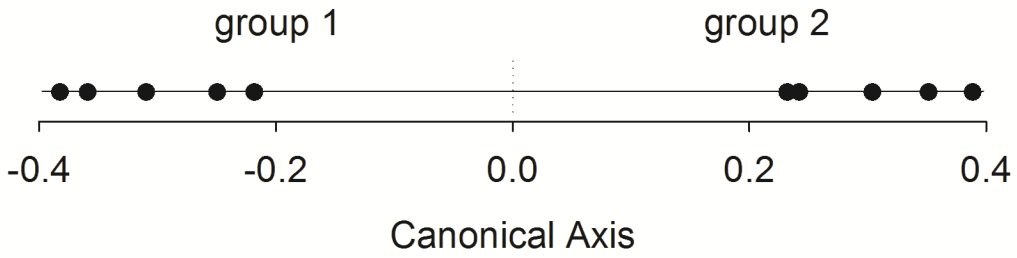


(C) *Anabaena cylindrica*: determination of molybdophores and siderophores

Retention time [min]	*m/z*	Pearson's correlation	*m/z*	Pearson's correlation	Δ
	group 1: ^{95}Mo		group 2: ^{98}Mo		
1.95	884.07	0.9453	887.08	-0.955	3.01
1.96	885.06	0.9411	888.06	-0.9519	3.00
2.43	-	-	789.25	-0.959	-
2.49	-	-	827.21	-0.9037	-



Retention time [min]	*m/z*	Pearson's correlation	*m/z*	Pearson's correlation	Δ
	group 1: ^{54}Fe		group 2: ^{58}Fe		
1.47	812.22	-0.9796	816.17	0.9782	4.03
1.48	834.24	-0.979	838.16	0.9779	4.08
1.52	812.30	-0.973	-	-	-
2.46	-	-	169.64	0.9777	-



(D) *Cladosporium cladosporioides*: determination of siderophores

Retention time [min]	*m/z*	Pearson's correlation	*m/z*	Pearson's correlation	Δ
	group 1: ^{54}Fe		group 2: ^{58}Fe		
1.33	-	-	772.24	0.958	-
1.34	768.29	-0.9853	771.30	0.9583	3.01
1.34	767.42	-0.9539	-	-	-
1.34	767.17	-0.962	-	-	-
1.37	-	-	755.36	0.955	-
1.38	751.24	-0.9069	-	-	-
4.80	-	-	371.15	0.8507	-

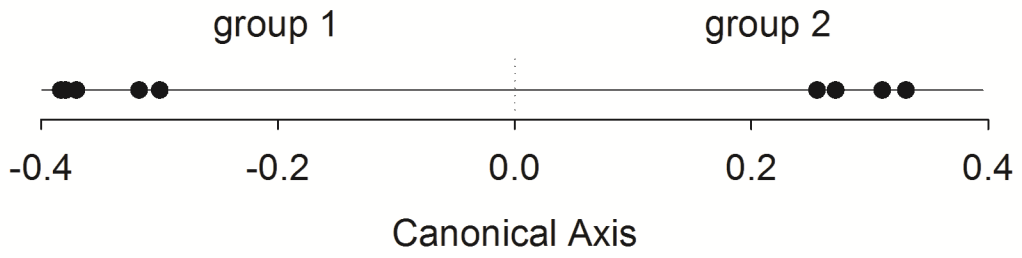
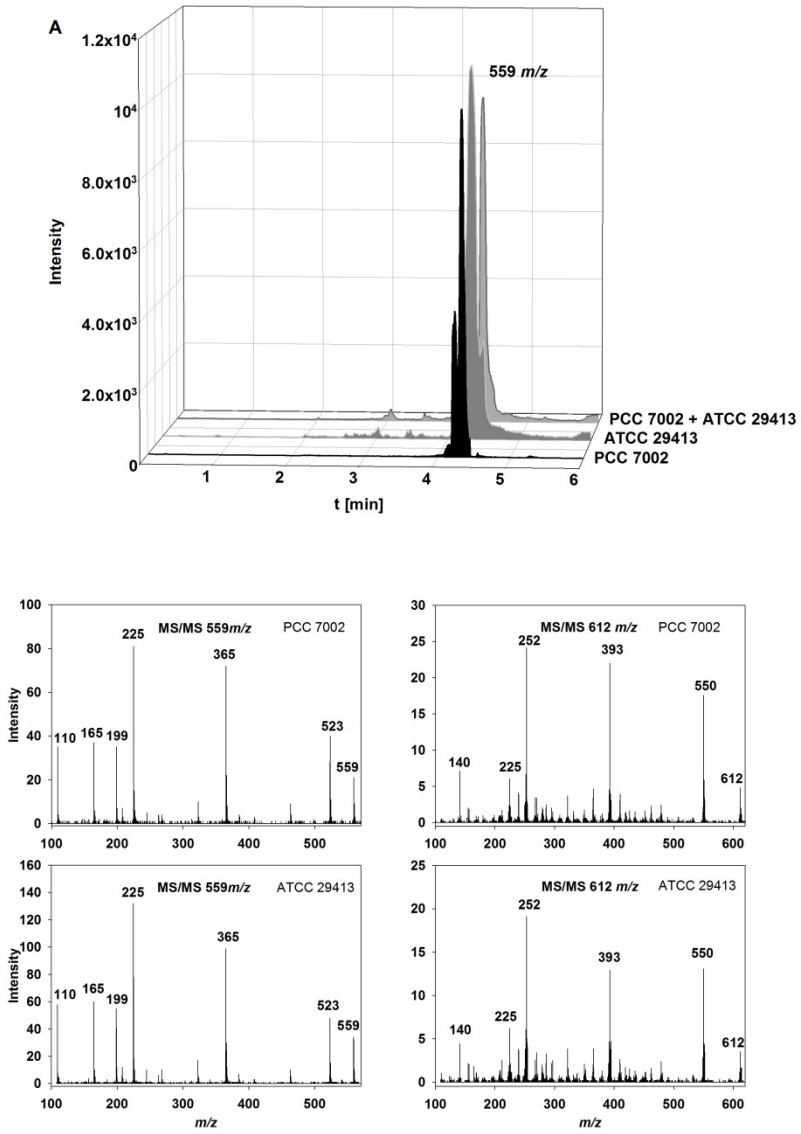
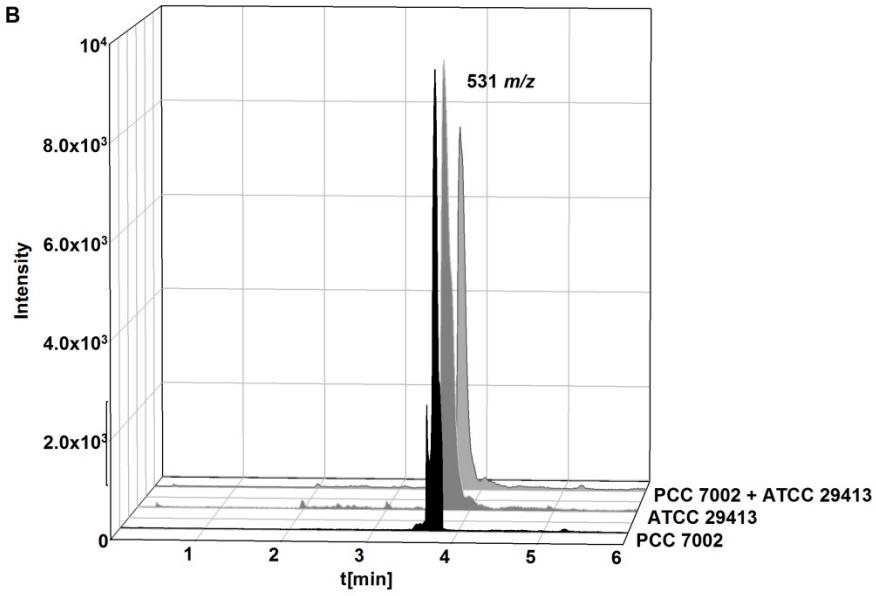


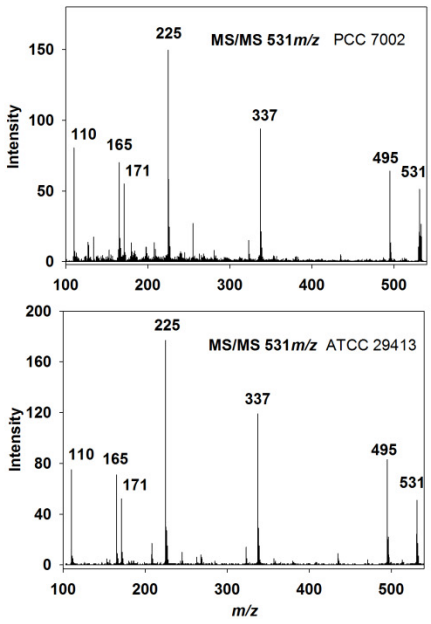
Figure S8: Identification of synechobactin A and B in *A. variabilis* by co-injection and MS² experiments. Equal amounts of the compound extracted from the growth media of *A. variabilis* (ATCC 29413) and of the reference strain *Synechococcus* sp. (PCC 7002) were co-injected (A, B). Extracted ion trace chromatograms of the molecular ions of the ligands are shown.

(**A**) Identification of synechobactin A at 4.32 min by co-injection and comparison of the negative ion MS² spectra of the ligand (559 *m/z*, $[M-H]^-$) as well as its ^{56}Fe-complex (612 *m/z,* $[^{56}Fe^{III}(synechobactin\text{-}4H)]^-$) with the respective reference spectra obtained from extracts of *Synechococcus* sp.



(**B**) Identification of synechobactin B at 3.70 min by co-injection and comparison of the negative ion MS² spectra of the ligand (531 *m/z,* $[M-H]^-$) with the respective reference spectra obtained from extracts of *Synechococcus* sp. (PCC 7002). The concentration of the corresponding Fe-complex was below the limit of detection for the MS^2 experiments.





(C) Positive ion ESI MS^2 spectrum of deferriated synechobactin B. Fragmentation was annotated accordingly to previous studies[14] with schizokinen **7**.

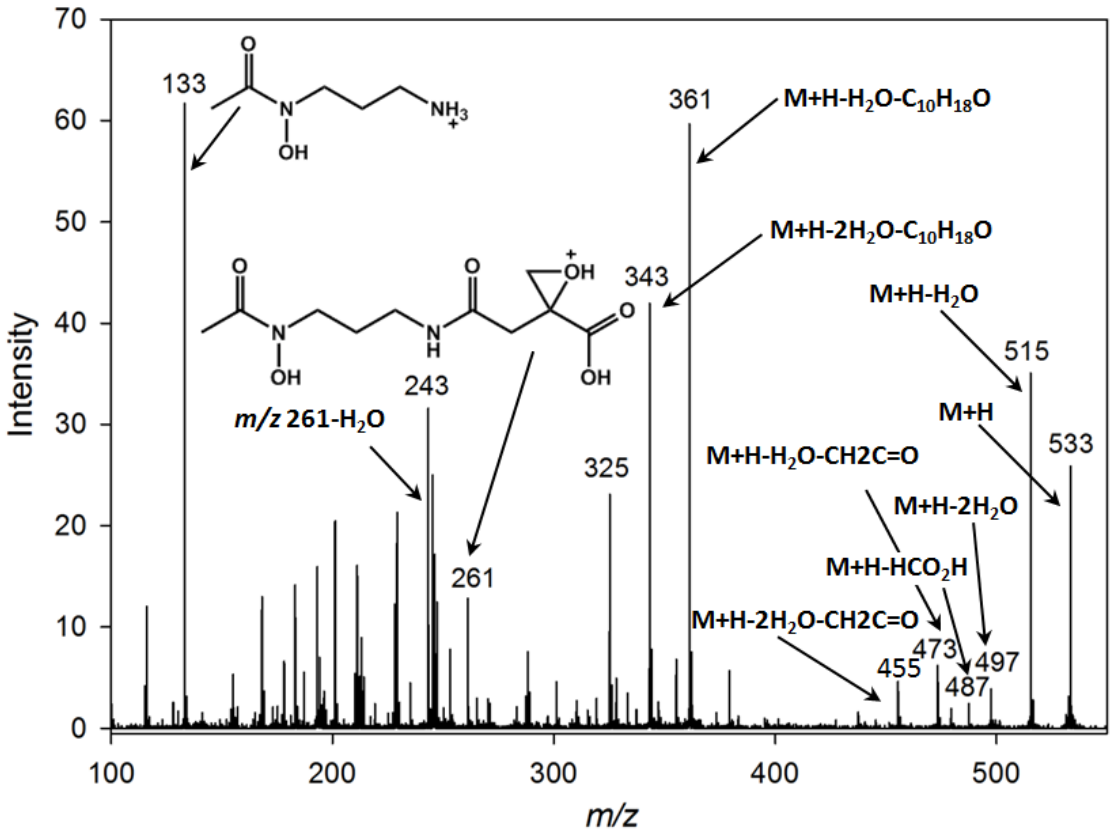
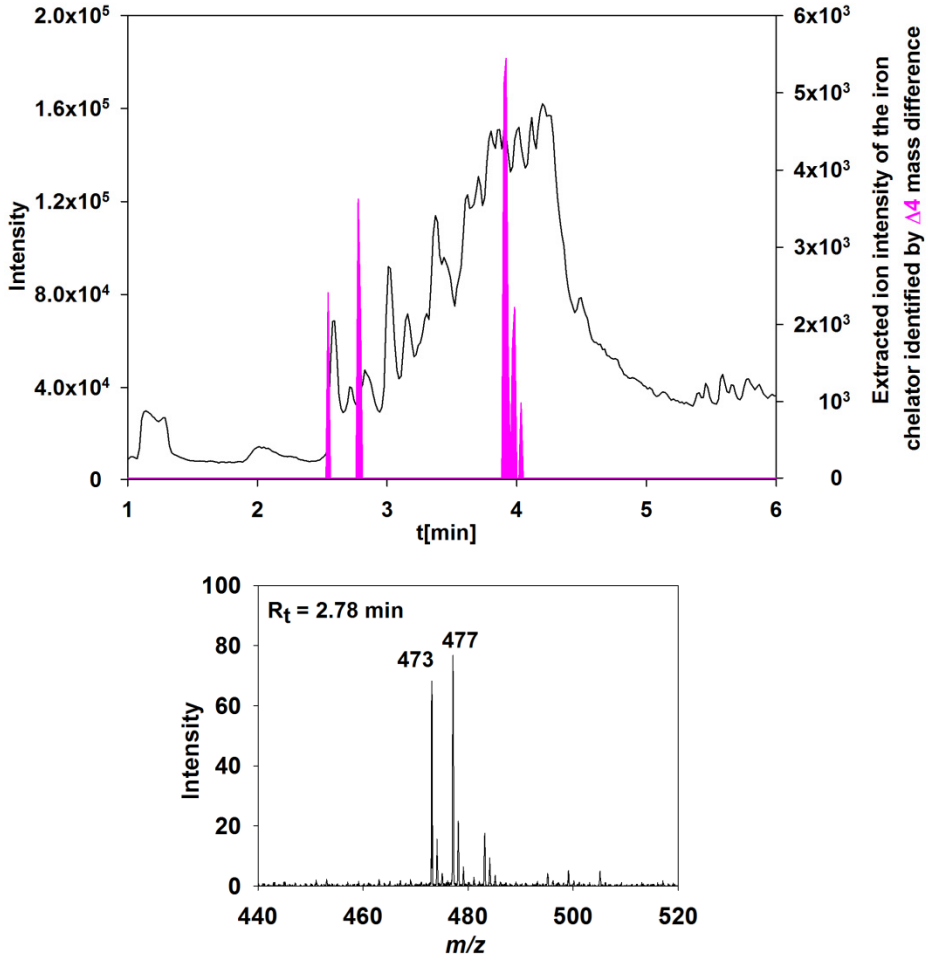


Figure S9 Extracted chromatogram of Fe-isotope coded tannic acid. Total ion current chromatograms of the UHPLC-MS analyses and extracted chromatograms are shown, as well as the representative positive ion mass spectra of the identified metal binding ligand 473/477 *m/z* (purple line).



Tables

Table S1: Selected parameters for the automated collection isotope labelled metallophores from chromatographic data through the "Strip Function" in MarkerLynx® V4.1.

Parameter	Iron isotopes	Molybdenum isotopes
Mass Difference [amu]	4	3
Ratio	1	1
Mass Tolerance [amu]	0.03	0.03
Ratio Tolerance [%]	40	40
Time Window	0.00	0.00
Threshold [%]	4.00	4.00

Table S2: Selected parameters for the collection of the mass retention time pairs through MarkerLynx® V4.1.

Parameter	Value
Function	1
Initial Retention Time	depended on the gradient
Final Retention Time	depended on the gradient
Low Mass [*m/z*]	100.00
High Mass [*m/z*]	1500.00
Mass Tolerance	0.05
Masses per retention time	30
Minimum intensity (% of BPI)	1.00
Mass window	0.30
Retention time window	0.50
Peak-to-Peak baseline Noise	0.00

Table S3: High resolution mass spectrometry (positive-ion mode) of potential metallophore complexes released by *Anabaena variabilis* (ATCC 29413). The measured mass was compared with the calculated molecular mass. The elemental composition of the molecular ion of the $^{54}Fe^{III}$-metallophore complexes is given. Numbering according the chromatogram (Fig. 5, main text).

Potential Metallophore complexes	Measured mass of the $^{54}Fe^{III}$-metallophore complexes [amu]	Calculated mass of the $^{54}Fe^{III}$-metallophore complexes [amu]	Error [ppm]	Molecular ion of the $^{54}Fe^{III}$-metallophore complexes
I	472.1093	472.1096	0.6	$[C_{16}H_{26}N_4O_9\,^{54}Fe^{III}]^+$
II	656.2925	656.2923	0.3	$[C_{28}H_{50}N_4O_{10}\,^{54}Fe^{III}]^+$
III	584.2350	584.2348	0.3	$[C_{24}H_{42}N_4O_9\,^{54}Fe^{III}]^+$
IV	612.2653	612.2661	1.3	$[C_{26}H_{46}N_4O_9\,^{54}Fe^{III}]^+$
V	654.2758	654.2767	1.4	$[C_{28}H_{48}N_4O_{10}\,^{54}Fe^{III}]^+$
VI	640.2971	640.2974	0.5	$[C_{28}H_{50}N_4O_9\,^{54}Fe^{III}]^+$

Table S4: High resolution mass spectrometry (positive-ion mode) of identified metallophores released by *Anabaena variabilis* (ATCC 29413).

Metallophores	Measured mass $[M+H]^+$ [amu]	Calculated mass $[M+H]^+$ [amu]	Error [ppm]	Pseudo molecular ion
Synechobactin A	561.3497	561.3500	0.5	$[C_{26}H_{49}N_4O_9]^+$
Synechobactin B	533.3186	533.3187	0.2	$[C_{24}H_{45}N_4O_9]^+$
Schizokinen	421.1932	421.1937	0.7	$[C_{16}H_{29}N_4O_9]^+$

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