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Metabolomic Analysis of Marine Microalgae Using High Resolution Mass Spectrometry for Taxonomic Comparisons and Screening of Marine Biotoxins

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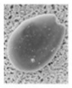
Introduction

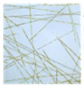
Out of the circa 5000 microalgae known, around 100 marine species of unicellular algae have been shown to produce several hundred biotoxins worldwide. Such biotoxins may be harmful to man through consumption of fish and shellfish exposed to such algae or through direct contact. Many official methods aimed at the detection of such toxins had traditionally been based on biological assays, due to the lack of reference compounds. Recently, legislation for shellfish safety in Europe introduced targeted MS screening for lipophilic toxins. Due to globalization of trade and climate change, there is a need to develop methods for untargeted screening to fully protect consumer safety, e.g. LC-HRMS. For this purpose we studied the metabolomes of marine microalgae using HRMS techniques.

Methods

We have developed an LC-HRMS method in full scan mode to characterize microalgal fingerprints or footprints. Several extraction solvents were evaluated for their recovery and artefact formation. Extracts were injected into the HPLC-QTOF-MS system using a generic gradient. The QTOF system was operated in either TOF and/or MS/MS modes using full scan and targeted modes. The *Find-by-Molecular-Feature* data mining algorithm has been used for the metabolomics workflow for compound finding and noise removal.. An in-house database was created for ca. 300 toxins and complemented with spectra of EU-regulated toxins for automatic library screening and comparison to a marine natural products database. Direct comparison of different species and PCA were used for chemotaxonomic interpretation and clarification of biological sources of natural products.

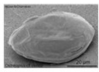
Microalgae studied

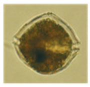

Prorocentrum lima: producer of okadaic acid and anlogues (toxic)


Pseudo-nitzschia: producer of domoic acid (toxic)

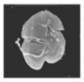

Vulcanodinium rugosum: producer of pinnatoxin (toxic)


Azadinium spinosum: producer of azaspiracid 1 and -2 (toxic)

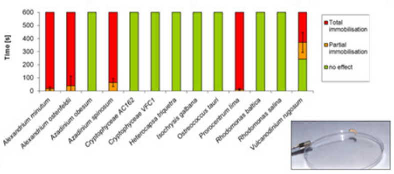

Ostreopsis ovata: producer of ovatoxin (toxic)


Alexandrium ostenfeldii: producer of spirolides and saxitoxins (toxic)


Azadinium obesum: non-toxic alga


Karenia selliformis: producer of gymnodimine (toxic to mice)

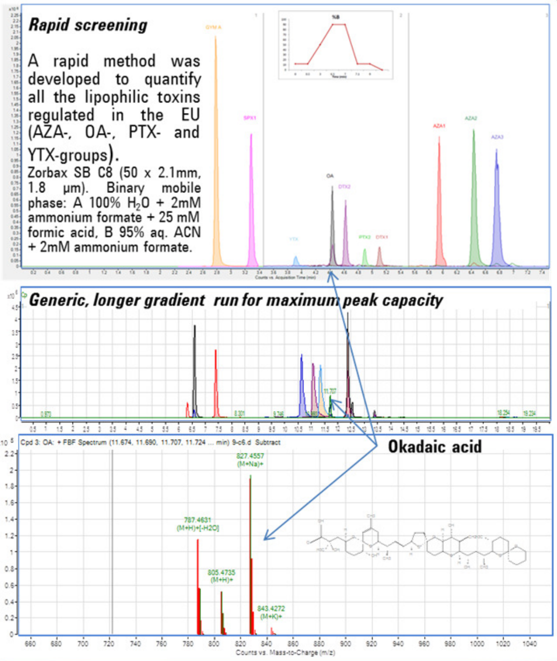
Toxicity evaluation of algal extracts using fly larval assay



LC – Q-ToF methodology

Full scan screening

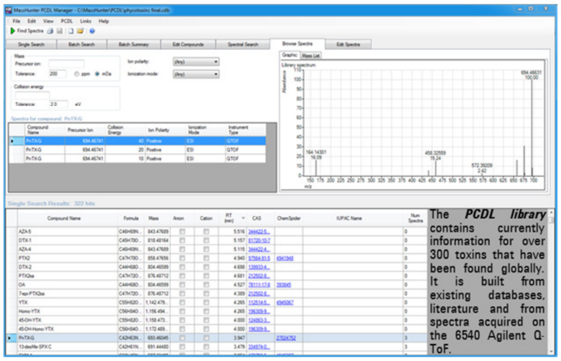
LC-high resolution mass spectrometry with a resolution of 35000 and mass accuracy of 1ppm allows for rapid quantitation of knowns or extensive screening of unknowns.



Rapid screening

A rapid method was developed to quantify all the lipophilic toxins regulated in the EU (AZA-, OA-, PTX- and YTX-groups). Zorbax SB C8 (50 x 2.1mm, 1.8 µm). Binary mobile phase: A 100% H_2O + 2mM ammonium formate + 25 mM formic acid, B 95% aq. ACN + 2mM ammonium formate.

Generic, longer gradient run for maximum peak capacity



The ***PCDL library*** contains currently information for over 300 toxins that have been found globally. It is built from existing databases, literature and from spectra acquired on the 6540 Agilent Q-ToF.

Different chromatographic approaches can be exploited using the same high resolution mass spectral library with elemental composition, accurate mass and individual or composite spectra acquired using three collision energies. The *Find-by-Formula* algorithm of Mass Hunter software automatically associates related ions (H^+, Na^+, NH4+, K^+ etc.) and removes chemical noise & column bleed.

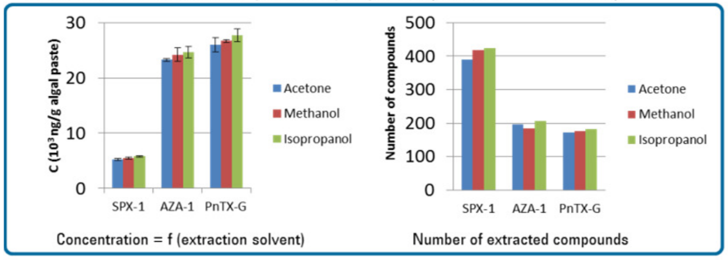
References

Jauffrais et al. (2012) Quantitative analysis of azaspiracids in Azadinium spinosum cultures. Anal. Bioanal. Chem 403: 833-846.
Munro MHG, Blunt JW (2007) MarinLit - Database, Version 13.5. Dept. Chemistry-Univ. Canterbury. Christchurch, New Zealand.

Results: extraction, microalgal metabolomes & chemotaxonomy

Choice of extraction solvent for metabolomic studies

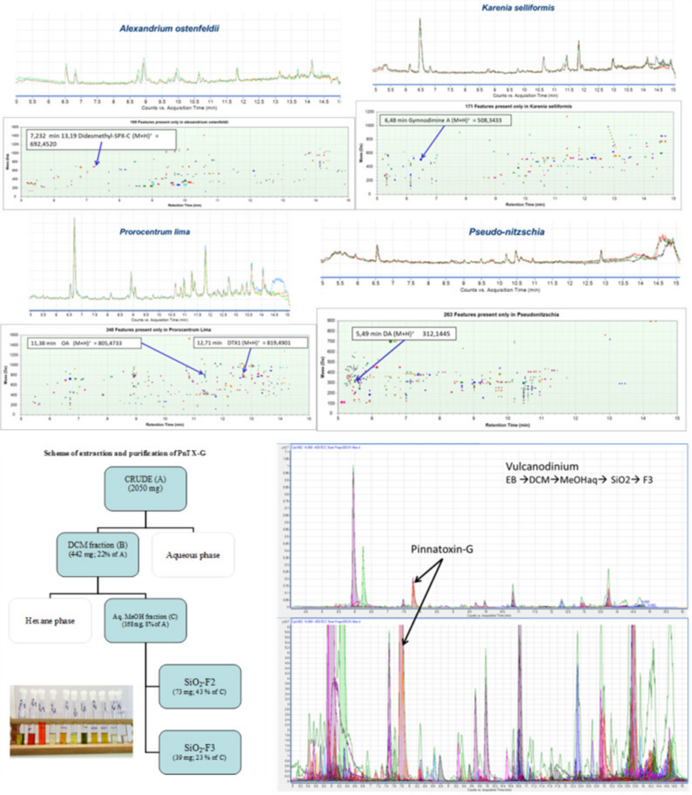
Extraction efficiencies, expressed as concentrations in extracts (figure 2) are not significantly different. Isopropanol extracts more compounds from the algae paste than the other solvents (noise cut-off 800 counts). Unambiguous identification of toxins specific to each algae in extracted algae profiles (e.g. PnTX-G for V. rugosum). Isopropanol was chosen as extracting solvent as it avoids artefact formation previously reported (Jauffrais et al., 2012).



Concentration = f (extraction solvent)

Number of extracted compounds

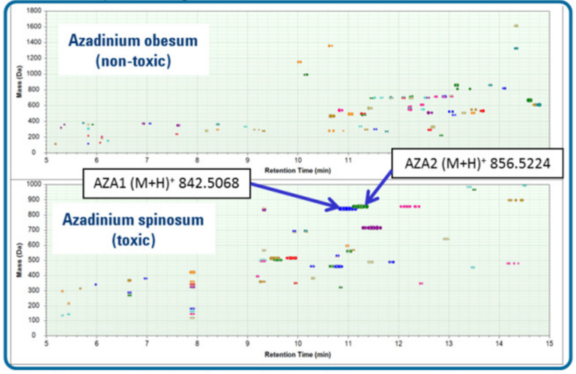
Metabolomes of indivdual species or fractions of extracts



Individual microalgal species can be rapidly assessed for their content of known natural products. Toxins can be rapidly identified using the PCDL library and the *Identify by database search* algorithm. Unknowns in fractions may be tentatively dereplicated using large natural products databases.

Comparative metabolomics

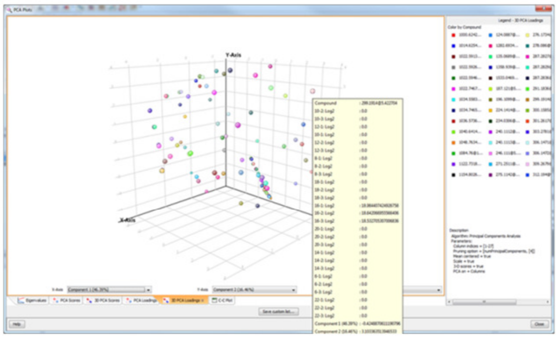
Comparison of closely related algal species was carried out using the long gradient and full scan HRMS, followed by data analysis using the Mass Profiler software:

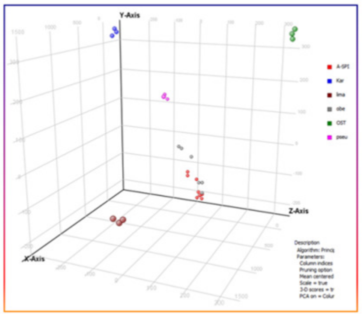


Replicate analysis showed that 77 compounds were only present in *A. obesum*, while 59 were only in *A. spinosum* including known toxins (i.e. azaspiracids); 95 compounds were common features in both algal species.

Chemotaxonomy

Principal Component Analysis carried out in Mass Profiler Professional software allowed for classification of species.

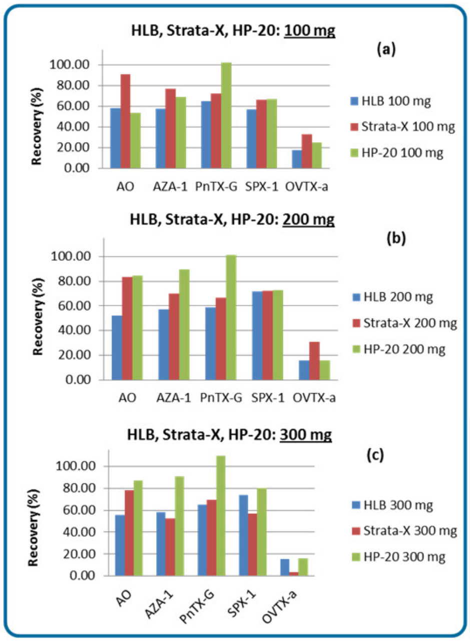




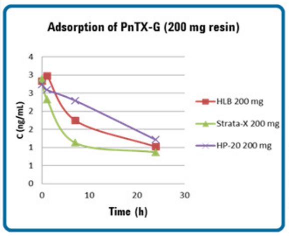
The loadings plot above indicates the compounds that are responsible for the separation in a multi-dimensional data space. The 3D-score plot on the right illustrates the classification of algal species into different groups in a 3D projection of this space.

Results: passive sampling

Absorption behavior of different passive sampling phases

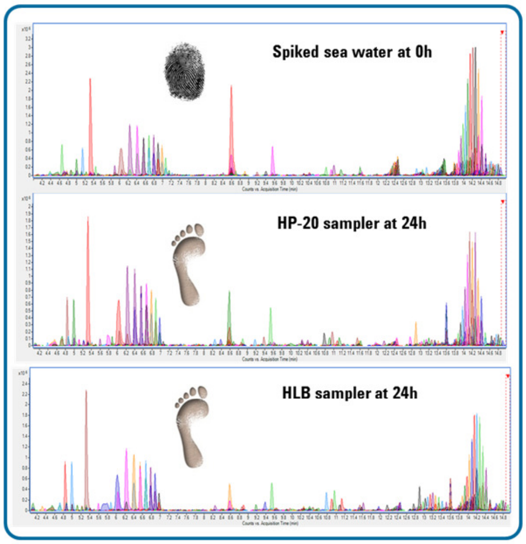


Passive samplers (above) were immersed in sea-water spiked with algal extracts and gently shaken for 24h to allow for adsorption. Different resins have different adsorption rates (below).



Toxin recoveries from passive samplers after 24h adsorption: (a) 100 mg resin, (b) 200 mg resin, (c) 300 mg resin. Note that even though HP20 adsorbs toxins most slowly, it reaches highest recoveries within 24h (a, b and c).

Fingerprints versus footprints of micro-algae



Comparison of metabolomic profiles of passively sampled compounds to those of 5 algal extracts spiked into seawater: HP-20 and HLB samplers yield similar profiles. Both resins adsorbed compounds of large range of lipophilicity present in the algal extracts. However, compared to HLB, it seems easier for HP-20 resin to adsorb compounds of medium lipophilicity (RT 8-10 min).

Conclusions

- ✓ **Different chromatographic approaches allowed for either rapid quantitative analysis of regulated marine biotoxins or extensive screening of unknowns through extended gradient runs for maximum peak capacity.**
- ✓ **Development of a personal compound database and library with accurate mass, retention time and mass spectral information allows for rapid and unequivocal identification of over 300 known toxins worldwide.**
- ✓ **Acetone, methanol and isopropanol give similar extraction yields for three different micro-algae, with isopropanol showing highest values both for quantitative extraction yield and number of compounds extracted.**
- ✓ **Metabolomes of microalgal strains were acquired and compared between each other. After data reduction with the *Molecular Feature Extraction* algorithm, comparison of 6 strains led to a chemotaxonomic classification using principal component analysis carried out with *Mass Profiler Professional* software.**
- ✓ **Analysis of purified fractions of Vulcanodinium rugosum led to the tentative dereplication of over 20 natural metabolites using the *MarineLit*™ database (Blunt and Munro, 2007).**
- ✓ **Comparison of three different resins used as passive samplers for microalgal metabolites in seawater showed that recovery of the algal metabolites is somewhat dependent on the amount of resin used but overall the HP20 resin recovered higher concentrations and more metabolites than HLB or Strata-X. However, HP20 showed slower adsorption over a 24h period. Both HP20 and HLB appear to be appropriate passive samplers for foot-printing (tracing of toxins in seawater).**

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