

High field NMR Spectroscopy and FTICR Mass Spectrometry: Powerful Discovery Tools for the Molecular Level Characterization of Marine Dissolved Organic Matter from the South Atlantic Ocean

N. Hertkorn^{1*}, M. Harir¹, B. P. Koch², B. Michalke¹, P. Grill¹, Ph. Schmitt-Kopplin¹

¹ Helmholtz Zentrum Muenchen, German Research Center for Environmental Health, Research Unit Analytical Biogeochemistry (BGC), Ingolstaedter Landstrasse 1, D-85764 Neuherberg, Germany

*Corresponding author. Tel: +4989-3187-2834; fax: +4989-3187-2705.

E-mail address: hertkorn@helmholtz-muenchen.de (Norbert Hertkorn);

² Alfred Wegener Institute, AWI, Am Handelshafen 12, D-27570 Bremerhaven, (Building Co-5), Germany

Summary response to reviewers #1 and #2

High-resolution organic structural spectroscopy by use of NMR spectroscopy and FTICR mass spectrometry allows discovery and attribution of molecular features within complex unknowns not available by any other means at present. This readily applies to marine organic matter, one of the least characterized and likely most complex organic mixtures on earth.

During the assembly of this manuscript it became clear that the unprecedented sensitivity and resolution of 800 MHz NMR spectroscopy with latest electronic circuits and cryogenic detection not only allowed to reveal previously intractable structural features of marine SPE-DOM but also demonstrated susceptibility of NMR spectra to physical characteristics of marine SPE-DOM. Primary examples included differential relaxation in 2D NMR spectra (which ultimately derives from variance in individual *atom* mobilities) and selective excitation and decoupling in ¹³C NMR spectra which primarily affect NMR resonance lineshape and amplitude. The latter reflected a complex interplay between disposable excitation bandwidth, high-quality (Q) NMR probehead ring down characteristics and energy transfer between molecules in complex mixtures and had to be mentioned in the manuscript (as desired now in the methods section) because it was only overcome by use of specific equipment (3 mm ¹³C NMR cryogenic observe probehead with a very short excitation ¹³C-90-degree pulse of 5 μs). These clearly recognizable effects from differential NMR relaxation, even when not fully understood at present, will nevertheless apply to all future NOM analyses using high-field NMR spectroscopy. Therefore they deserve description and, wherever applicable, tentative explanation. Analogous effects were not readily observed in own NMR analyses of natural products and complex mixtures of (small) biomolecules/metabolites using identical instrumentation; hence, these effects appear characteristic of marine SPE-DOM.

- 1) All specific remarks of reviewers #1 and #2 have been addressed. However the original chapter 3.3 has been kept in original sequence because it refers to critical fundamentals of NMR computation which are largely unknown yet indispensable for understanding.
- 2) The four selected SPE-DOM are naturally at first site-specific because of sampling location but primarily they were selected to represent oceanic water masses of general significance (open-ocean SPE-DOM with very marginal to absent terrestrial contributions). In general, surface marine SPE-DOM composition and structure will be more influenced by local compositional and temporal fluctuations (from algal blooms and other bioactivity, variance in sunlight irradiation and terrestrial input, if applicable) than deep water SPE-DOM. We have adjusted the text to accommodate for referees comments.
- 3) Any currently conceivable isolation of marine DOM from seawater will impose larger extents of molecular (compositional and structural) selectivity/alteration/discrimination from the very beginning of sampling than errors imposed by any other downstream analytical characterization (when methods are performed correctly). This often marginalized issue cannot be over emphasized. Most obviously, SPE-DOM obtained by PPL-extraction of marine waters is likely depleted in CHNO compounds as shown by its large C/N ratio near 25 : 1. Quantification of marine SPE-DOM by means of NMR spectroscopy refers to all SPE-DOM *atoms* present which resonate within a certain window of NMR relaxation, whereas quantification of SPE-DOM molecules via FTICR mass spectrometry is a complex and ill-defined issue in comparison (a dedicated chapter addressing transfer functions in isolation and characterization has been added to the manuscript to clarify these issues in a single place). Elaborations on isolation selectivity can be drawn from comparison with marine organic matter isolated by alternative methods. Elution of spent PPL cartridges after standard methanol elution of SPE-DOM with acetone-d₆ (and subsequent NMR analysis; data not shown) provides limited amounts of aliphatic-rich lipids (< 1 % of otherwise isolated SPE-DOM). Marine organic matter isolated by combined reverse osmosis/electrodialysis (ROED) shows competitive yields in the range of 65-90 % of carbon and higher proportions of carbohydrates than found in PPL-derived SPE-DOM. In contrast, marine DOC isolated by means ultrafiltration (UF-DOM) appears to favor isolation of peptides/proteins and carbohydrates and to discriminate against hydrophobic constituents (cf. new chapter 3.2). Marine SPE-DOM isolated by means of PPL cartridges show exceptional resolution in their NMR spectra. For instance, meaningful COSY NMR

spectra of marine organic matter have not been observed prior to this study. SPE has removed certain constituents of yet unknown origin and structure from seawater which otherwise would have accelerated NMR relaxation. Plausible candidates include high-molecular weight marine gels, which commonly show high carbohydrate content. Entanglement of SPE-DOM components within large oligomers and polymers is a likely cause for fast transverse NMR relaxation. Efficient removal of metal ions and organic metal coordination compounds which could mediate chemical exchange and atom mobility in solution is another likely reason for the exceptional resolution displayed in certain 2D NMR spectra of marine SPE-DOM.

4) The mass spectrometry section has been entirely rewritten and data derived from positive ESI FTICR mass spectra have been included in the discussion.

Unfortunately, only a few labs working in environmental and ocean sciences worldwide operate organic structural spectroscopy with adequate resolution and sensitivity to cope with the intrinsic molecular diversity of natural biogeochemical mixtures. Consequently, the current scientific approach to evaluate organic molecular complexity rather refers to the assessment of biological/biochemical mixtures in which clearly resolved patterns (and their alterations) are readily observed. The vastly more complex biogeochemical mixtures carry far more intrinsic averaging within any analytical method employed for investigation and necessarily produce less resolved signatures. Even the exceptional mass peak resolution commonplace in FTICR mass spectrometry is deceptive, because the ultimate resolution of the C,H,O-compositional space within a sizable mass range is readily observed in high quality FTICR mass spectra. Then, any mass peak in FTICR mass spectra of marine SPE-DOM will likely represent thousands of isomers irrespective of the ionization method used; recognition of ionization selectivity will even enhance this extent of signal superposition in case of common ions.

A mass shift $\Delta m \geq 10$ m/z for the intensity weighted average of SPE-DOM mass peaks from surface to deep waters across a bandwidth of m/z: 150 – 800 as observed in negative and positive ESI FTICR mass spectra had to overcome this massive intrinsic averaging and should not be considered insignificant. In an analogy, mass peaks can be thought to represent age groups within a nation's entire population (with any person contributing an equal intensity increment), and mass spectra will represent the age distribution. Then, the observed displacement Δm might be compared with an extension of the individual-related average life-span within a nation's entire population by more than one year. Because Δm and compositional/structural similarity are inversely related, any MS-displacement will represent major structural changes.

Given the wealth of new data (and effects to be considered in their interpretation) the authors have thought seriously how to arrange the manuscript. However, given the individuality of each NMR spectral behavior (note, that NMR reflects *atom-specific* properties) there was no other realistic choice than sorting this contribution according to methods. Nevertheless, the original conclusion section has now expanded to improve overlap with common perception of marine DOM molecular complexity.

Markedly improved detection selectivity and sensitivity will unavoidably result in recognition of novel influencing variables. Non-target organic structural spectroscopy of marine organic matter is a key aspect of this special issue dedicated to the molecular analysis of dissolved marine organic matter from a North-South transect in the Atlantic Ocean. While recent progress in MS instrumentation and performance is acknowledged by many scientists, respective knowledge referring to quite comparable NMR improvements within the last decade is far less common. This contribution would have met marginal interest in a dedicated NMR journal, because of the polydispersity of marine organic matter – an alien subject to common NMR research which preferentially investigates isolated natural products, pure biopolymers and industrial polymers with rather uniform composition. The accelerating growth of disposable data and knowledge in any single scientific topic carries the intrinsic distress of fragmenting modern science into individual topics, each for itself ostensibly self-sustainable and rewarding. Hence, interdisciplinary research will remain of supreme importance for conceptual scientific advance, especially in ocean sciences, where physics, chemistry and biology jointly define the scenery. The journal *Biogeosciences* strives to “*disseminate knowledge of interacting biological, chemical and physical processes with an objective to cut across boundaries of established sciences and achieve an interdisciplinary view of these interactions. Experimental, conceptual and modelling approaches are welcome*”. According to these goals, we consider this contribution both timely and suitable to appear in the journal *Biogeosciences*.

The authors acknowledge that the abstract provided is rather expansive. However, the range of novel findings and the individual behavior of NMR and mass spectra have to be mentioned summarily outside the main text – we nevertheless have condensed the abstract as far as found suitable. We have avoided double mentioning of items in the abstract and in the conclusion sections, they are designed for sufficient complementarity.