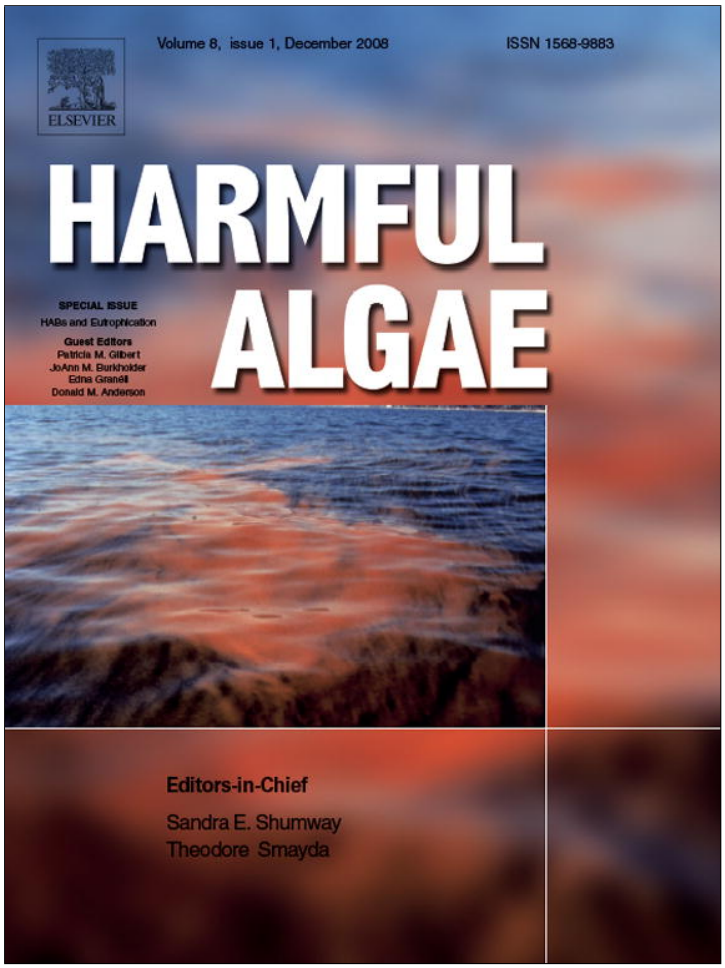

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Use of electrospray ionization (ESI) mass spectrometry to investigate complex dissolved organic matter (DOM) and its potential applications in phytoplankton research

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ABSTRACT

Organic nutrients are one of many factors considered to be important in the growth and proliferation of phytoplankton including many species that cause harmful algal blooms (HABs). Several studies have investigated the effects of known organic compounds on phytoplankton growth, however, the role of natural dissolved organic matter (DOM) in phytoplankton nutrition remains understudied at the compound level. This lack of research is due in part to analytical limitations for the characterization of DOM compounds. Electrospray ionization (ESI) mass spectrometry (MS) provides an unprecedented level of chemical information on thousands of organic compounds that comprise the bulk DOM pool. In this paper we provide a brief overview of some of the benefits and caveats of using ESI to investigate DOM in natural freshwater and marine systems and show an example of ESI-MS DOM characterization for a natural bloom of the raphidophyte *Chattonella* cf. *verruculosa*.

1. Introduction

Dissolved organic matter (DOM) is crucial to microbial production in aquatic systems. It accounts for greater than 97% of the total organic matter present in seawater and is one of the major reservoirs of carbon on earth, equaling the amount of CO_2 present in the atmosphere (Siegenthaler and Sarmiento, 1993; Benner, 2002). DOM is produced by all organisms and is used as a nutrient source by bacteria and some phytoplankton (Azam et al., 1983; Antia et al., 1991; Granèli et al., 1999; Berman and Bronk, 2003; Glibert and Legrand, 2006).

Numerous studies have investigated the uptake of individual known organic compounds such as glucose, urea and amino acids by various phytoplankton species and populations (examples include Carpenter et al., 1972; McCarthy, 1972; Wheeler et al., 1977; Flynn and Butler, 1986; Gobler and Sañudo-Wilhelmy, 2001). Although these studies have provided evidence that phytoplankton are capable of using specific fractions of DOM, known compounds generally only represent a small portion of natural DOM (Benner, 2002; Bronk, 2002). These known compounds may not reflect the bioavailability to phytoplankton of the thousands of organic compounds that comprise the bulk DOM pool.

Several complex multi-compound DOM sources such as humic acids, river water concentrates, rainwater and storm water runoff have also been shown to increase phytoplankton abundance in both cultures and natural populations (Prakash and Rashid, 1968; Peierls and Paerl, 1997; Seitzinger et al., 2002; Boyer et al., 2006; See et al., 2006). Although it is known that phytoplankton can use some DOM compounds, the details of the relationship between phytoplankton and DOM in nature are not well known. This is not only due to complicated physiology and community structure, but also to a lack of analytical methods to characterize the DOM in complex samples at the compound level. A number of analytical techniques (e.g., nuclear magnetic resonance (NMR; Simpson et al., 2001), gas chromatography mass spectrometry (GC–MS; Rowland et al., 2001), direct temperature-mass spectrometry (DT-MS; Simjouw et al., 2004) provide insight into the "black box" of DOM; however, approximately 75% of natural DOM still remains uncharacterized at the individual compound level (Benner, 2002; Bronk, 2002). Here we describe several analytical methods using electrospray ionization (ESI) mass spectrometry to investigate the uncharacterized compounds that comprise the bulk DOM pool and we provide selected examples that illustrate the use of ESI mass spectrometry to investigate complex environmental questions and its potential use in the study of phytoplankton nutrition.

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1.1. Electrospray ionization – how it works

ESI mass spectrometry is a relatively new analytical tool for characterizing ionizable organic compounds. Electrospray is designed to convert liquids into aerosols using electricity, not gas, to form the droplets (described in Kebarle and Ho, 1997). Unlike many other types of inlet systems that fragment compounds, ESI is a soft ionization inlet system that can allow for the detection of complete non-fragmented compounds (Marshall et al., 1998). To convert liquids into aerosols a positive or negative charge is applied to a liquid sample via a capillary tip. The liquid sample is destabilized by increasing amounts of charge. When the sample reaches the point when no more charge can be held by the liquid it disperses forming an aerosol of highly charged droplets. As the droplets flow through the system the carrier solvent evaporates leaving behind the charged ions that can be detected using a variety of instruments including several different types of mass spectrometers (McEwn and Larsen, 1997; Chernushevich et al., 1997; Bier and Schwartz, 1997; Laude et al., 1997; These and Reemtsma, 2003). Compounds are detected with mass spectrometers based on mass to charge ratios. Singly charged compounds represent their molecular weight MW + 1 $(MW + H)^+$ in the positive ionization mode and MW − 1 $(MW - H)^-$ in the negative ionization mode (McEwn and Larsen, 1997). A number of more detailed reviews address the capabilities and caveats of ESI mass spectrometry including the use of ESI mass spectrometry to investigate both high molecular weight and low molecular weight compounds (Gaskell, 1997; Marshall et al., 1998; Kebarle, 2000; Kujawinski, 2002).

1.2. Pairing ESI with other analytical instruments

There are a number of different instrumental applications that use ESI as an inlet system. Here we briefly review three instrumental pairings: ESI with a single quadrupole detector (ESI-MS), ESI with tandem mass spectrometry (ESI-Tandem MS or ESI-MS/MS) including ion traps, and ESI with Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS). Other applications not reviewed in this paper include time-of-flight mass spectrometry (Chernushevich et al., 1997) and liquid chromatography paired with ESI-MS (Voyksner, 1997).

1.2.1. Single quadrupole (ESI-MS)

The ESI-MS with single quadrupole detection has unit mass resolution and provides molecular weight information of detected compounds represented as a mass to charge ratio (*m/z*). Ion abundance is used as a measure of concentrations. With unit mass resolution one mass may represent one or more compounds (Kebarle and Ho, 1997). ESI-MS can be used to process a large number of samples per day, to determine which masses (*m/z*) are present and track changes in each of those masses via changes in the ion abundance and can be used quantitatively using authentic standards. Furthermore, characteristics of the functional groups associated with each compound can be determined by the detection of a compound in one or both of the two ionization modes, positive and negative (Kebarle and Ho, 1997). A response in the positive ionization mode indicates basic functional groups (e.g., alcohols) while a response in the negative ionization mode indicates more acidic functional groups (e.g., carboxylic acids) as demonstrated by standards (Seitzinger et al., 2003). In order to further characterize individual compounds in terms of exact structure and chemical composition, ESI coupled with other detectors can be used.

1.2.2. Tandem MS (MS/MS) or ion trap

While the ESI inlet system does not fragment compounds, it does allow for the intentional fragmentation of individual compounds through the addition of MS/MS or ion traps (Gaskell, 1997; McEwn and Larsen, 1997). MS/MS uses multiple mass spectrometers in line with each MS adding another level of fragmentation. The ion trap has only one analyzer but is able to focus in on, or trap, one ion/compound for additional fragmentation. While these two instruments are different in terms of mechanical design, they are similar in that both provide information on the chemical structure of a given mass through the fragmentation of the parent ion (Bier and Schwartz, 1997). Using the mass spectra obtained through either of these methods, one can begin to recreate the structure of a compound based on the functional groups cleaved during the fragmentation process (McLafferty and Turecek, 1993). While this method may not allow one to determine definitively if a compound contains elements like nitrogen, it can identify if a compound contains functional groups like nitrogen-containing amines or amides with well studied cleavage behavior (McLafferty and Turecek, 1993). This is important information when addressing dissolved organic nitrogen (DON) or potentially dissolved organic phosphorus (DOP) bioavailability. For information on the molecular formula of a compound, ESI paired with FT-ICR MS can be used.

1.2.3. FT-ICR MS

FT-ICR MS provides higher mass resolution than ESI-MS. ESI-MS has unit mass resolution in that it can differentiate between compounds with an *m/z* of 889 vs. an *m/z* of 890. The FT-ICR MS has the capability to obtain sub ppm resolution allowing for a 0.0001 *m/z* or better detection and thus can differentiate between components like CH_4 and O, a difference undetectable with single quadrupole (Marshall et al., 1998; Kujawinski, 2002; Koch et al., 2007). With increased mass resolution, FT-ICR MS also has increased mass separation. Unlike ESI-MS where each *m/z* can represent one or more compounds, with FT-ICR MS each *m/z* represents only one molecular formula. However, when trying to determine the molecular formula of that one compound, there may be multiple mathematical molecular formula possibilities. The higher the molecular weight of a compound, the greater the number of possible molecular formulas for that particular mass (Koch et al., 2007). Several papers have suggested "rules" for the accurate molecular formula assignment of natural DOM using ESI FT-ICR MS (Stenson et al., 2003; Koch et al., 2005, 2007; Kujawinski and Behn, 2006).

Due to the high mass resolution obtained by FT-ICR MS, it is a great tool for the investigation of specific unknown compounds (*m/z*'s). However, because of the large amount of data generated for each sample, even for each unit mass, and the large number of potential molecular formula assignments per each compound, ESI FT-ICR MS may not be the best tool to use for a first step assessment of a wide variety or larger number of samples. FT-ICR can not be used quantitatively and relatively few (10 or less) samples can be processed on the instrument per day. When instruments like ESI-MS or MS/MS are used prior to the use of ESI FT-ICR MS, it may reduce the amount of time required to process samples and data and provide additional levels quantitative and structural information.

1.3. Use of the ESI mass spectrometry to study DOM dynamics

ESI mass spectrometry has been used in a wide variety of research fields including complex environmental investigations (examples include Leenheer et al., 2001; Kujawinski et al., 2004; Persson et al., 2005; Koch et al., 2005; Seitzinger et al., 2005a; Kim et al., 2006). ESI-MS with single quadrupole has been used to compare and contrast DOM signatures of different sites and sources; for example, rainwater and suburban streams have

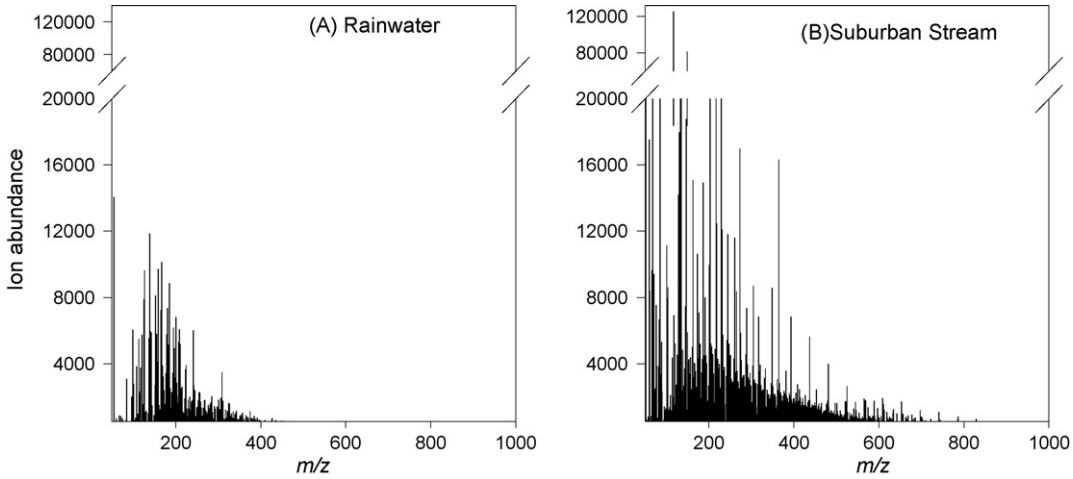

Fig. 1. ESI-MS positive mode spectra of (A) rainwater sample collected on June 6, 2002 in the New Jersey Pinelands (re-plotted from Seitzinger et al., 2005a) and (B) suburban stream sample collected on April 1, 1998 in New Brunswick, NJ (plot created using data from Seitzinger et al., 2005b).

different low molecular weight DOM signatures (Seitzinger et al., 2005a,b; Fig. 1). From these samples collected in New Jersey, almost twice as many masses (557 *m/z*'s) were present in the suburban stream sample compared to the rainwater sample (241 *m/z*'s). The rainwater sample also had a narrower molecular weight distribution with no masses greater than *m/z* 500 detected, while 24% of the masses detected in the suburban stream sample had molecular weights greater than 500 *m/z*.

As shown in the above example different sources, like the rainwater and suburban streams, have visibly different DOM signatures, sources with similar origins have similar signatures. In a comparison of two different suburban streams with similar land uses, 88% of masses detected in the positive mode occurred in both streams (Seitzinger et al., 2005b). ESI-MS is able to detect changes in the presence of individual *m/z*'s as well as the production and consumption of compounds based on changes in their ion

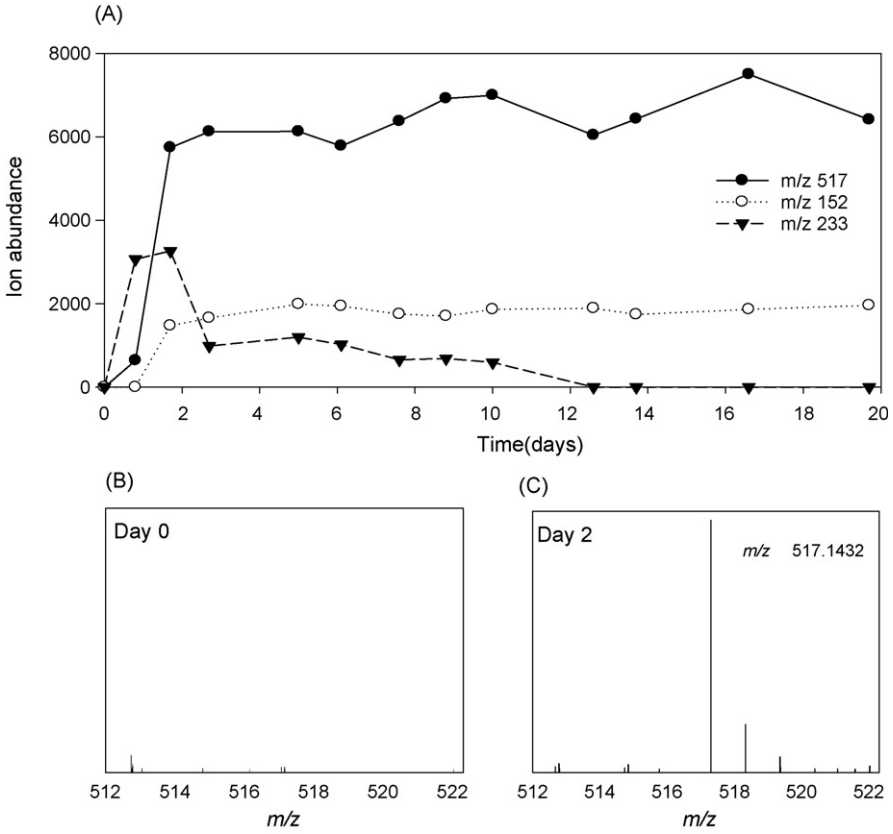

Fig. 2. Investigation of biological production of new organic compounds by freshwater bacteria; (A) time series plot of compounds produced by a single species of bacteria using ESI-MS (plot created using data from Gruber et al., 2006), (B) ESI FT-ICR MS spectra of day 0 sample and (C) ESI FT-ICR MS spectra of day 2 sample showing increase in *m/z* 517.1432.

abundance. It is this ability to observe differences and similarities among samples that enables us to compare and contrast DOM signatures from various sources and sites including potential comparisons of phytoplankton blooms.

ESI-MS has been useful in observing the biological production of new organic compounds by freshwater bacteria (Gruber et al., 2006). They observed that when a single organic compound, glucose, was initially supplied and consumed by a single species culture of freshwater bacteria (*Pseudomonas chlororaphis*), a number of new organic compounds were rapidly produced. Some of these compounds were consumed (e.g., *m/z* 233) while others persisted throughout the course of the experiment (e.g., *m/z* 517 and 152; Fig. 2A). In this experiment over 100 compounds were produced from a single organic precursor, glucose. The production of a large number of organic compounds by even a single microbial species provides insight into the complexities of natural DOM pools. While changes in general ion abundance (concentration) were observed for a number of *m/z*'s in this experiment, the exact molecular formula of the changing *m/z*'s could not be determined with ESI-MS. Compounds identified using ESI-MS that showed significant changes in ion abundance were further evaluated using ESI FT-ICR MS (9.4-T Fourier transform ion cyclotron resonance mass spectrometer equipped with an ESI source at the National High Magnetic Field Laboratory (NHMFL)) to determine the chemical composition. *m/z* 517 from Fig. 2A is used as an example for higher resolution FT-ICR MS investigations.

In this experiment with freshwater bacterial DOM, both data from ESI-MS and FT-ICR MS show negligible ion abundance for compounds represented by *m/z* 517 on day zero (Fig. 2B). By day two of the experiment both ESI-MS and FT-ICR MS data show a significant increase at m/z 517. FT-ICR MS validated the data obtained through the ESI-MS investigation and provided increased resolution, allowing for molecular formula assignment. The day two peak was identified as *m/z* 517.1432 (Fig. 2C). Using MIDAS Formula Calculator Software (v1.1), 144 molecular formulas were possible for *m/z* 517.1432. Following the suggested guidelines outlined by Koch et al. (2007), the list of 144 possible molecular formula assignments were reduced to seven possible molecular formulas for that one compound. With additional statistical and chemical evaluation or through the application of MS/MS or ion trap the number of possible molecular formulas could be further reduced to one and structural information obtained.

In addition to observing the production of unknown compounds from simple known compounds, the ESI-MS has also been used to investigate the bioavailability of complex land derived DOM from a suburban stream to natural assemblages of bacteria (Seitzinger et al., 2005b). Approximately 40% of the land derived DOM compounds supplied to the bacterial assemblage decreased in ion abundance (e.g., *m/z* 265) throughout the course of the experiment (Fig. 3), while other masses either increased (5%) or remained unchanged (55%). Duplicate flasks showed good replication in the ion abundance changes of specific compounds, indicating that equal amounts of the same compound(s) were consumed in both flasks.

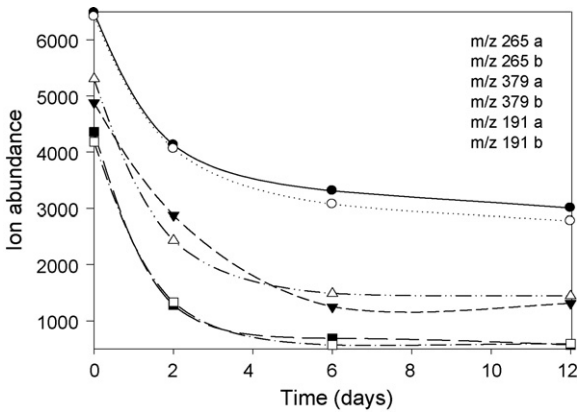

Fig. 3. Examples of the use of ESI-MS to study the consumption of land derived DOM by a natural population of bacteria (re-plotted from Seitzinger et al., 2005a,b) (a) and (b) are results from duplicate flasks.

ESI FT-ICR MS has been used in similar experiments to investigate the biodegradability of DOM from temperate and tropical streams (Kim et al., 2006). Stream water concentrates were supplied to natural microbial communities as a nutrient source. There was a 22% decrease in bulk dissolved organic carbon (DOC) concentrations in the tropical stream sample compared to a 42% decrease in DOC in the temperate stream sample. This decrease in DOC concentration correlated with a shift to a lower mass region after being exposed to the microbial population based on the FT-ICR analysis. Molecular formulas were also evaluated, the microbial community in this experiment preferentially degraded oxygen-rich molecules while hydrogen deficient molecules were generally refractory, thus providing insight into the properties that make a compound bioavailable.

2. Application of salt removal method for use with ESI mass spectrometry

The examples presented for the use of ESI mass spectrometry in molecular level characterization and studying DOM dynamics have thus far been in freshwater environments. As with many other techniques, the salts in brackish and seawater samples tend to interfere with the detection of compounds (Gaskell, 1997). As much as 70% of DOC can be recovered from saline samples when ultra filtration (UF) and solid phase extraction (SPE) are used in tandem (Simjouw et al., 2005). Both UF and SPE have been used independently to recover approximately 30% of DOC in saline systems. This new tandem approach to salt removal has doubled the recovery and allows for the investigation of a larger portion of the DOM pool. While SPE has been used to extract DOM for analysis using ESI FT-ICR MS (Koch et al., 2005), the tandem UF/SPE salt removal method has not been used to date with ESI mass spectrometry to our knowledge.

The analysis of two replicate field samples of a natural *Chattonella cf. verruculosa* bloom was used to demonstrate the precision of the salt removal method when used with ESI-MS. Bloom samples were collected from Russell canal, a tributary of Jefferson Creek (38°31.250′N, −75°3.668′W) in Bethany beach, Delaware, on August 25, 2006, and had a salinity of 28.1. This bloom was identified and monitored by the Citizens Monitoring Program, affiliated with the University of Delaware, Lewes. While cell counts of *C. verruculosa* are not available for the date sampled, the bloom abundance was 1.11×10^6 *C. verruculosa* cells l^{-1} at the same site the previous day, August 24, 2006.

DOM from duplicate *C. verruculosa* samples was extracted using the UF and SPE salt removal method (Simjouw et al., 2005). The DOM of these samples was then characterized using ESI-MS (Agilent 1100 liquid chromatograph/mass spectrometer with ESI source) in the positive ionization mode (see Seitzinger et al. (2005b) for details of instrument operation conditions). Good replication between *C. verruculosa* samples was found, with 95% of the masses present in both replicates. Of the remaining masses, 3% were unique to replicate 1 and 2% were unique to replicate 2. The ion abundance also showed a high degree of similarity; of the 95% of masses present in both samples, only 11% were statistically different (95% CI) in ion abundance (concentration). The data from the *C. verruculosa* duplicate samples demonstrate the reproducibility in both the

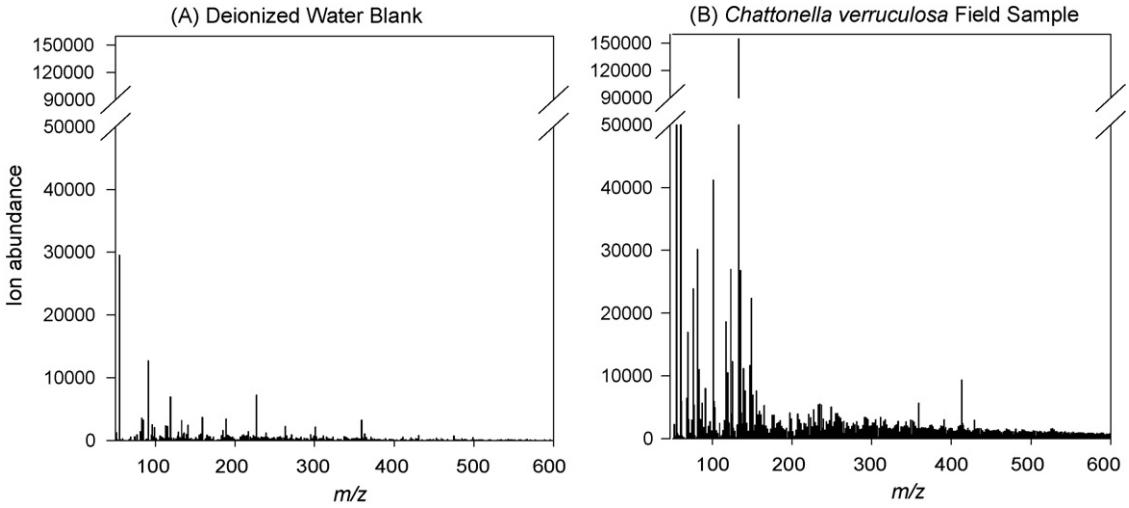

Fig. 4. Ion abundance of masses (*m/z*) detected by ESI-MS in the positive ionization mode after salt removal using UF/SPE: (A) deionized water and (B) field sample from a *C. verruculosa* bloom.

presence and ion abundance of the compounds retained during the salt removal process.

To ensure that the similarity of the *C. verruculosa* replicates was not due to contamination, procedural blanks from the salt removal process were run using deionized water (DI). DOC concentrations were determined via high temperature combustion using a Shimatzu 5000 total organic carbon analyzer (Sharp et al., 1993). The DI water blank had a DOC concentration of less than 5 μM C and the *C. verruculosa* sample had a DOC concentration of 735 μM C, indicating a relatively small contribution by contaminants (Fig. 4). Therefore, the masses observed represent natural compounds present in samples taken during this *C. verruculosa* bloom.

3. Future applications of the ESI in phytoplankton research

Understanding the nutritional requirements and preferences of different phytoplankton species is essential to understanding growth dynamics. To fully investigate phytoplankton nutrition and the bioavailability of a DOM source, we need to know more than simply that DOM can be used but what compounds are used. ESI coupled to mass spectrometry is a step forward in determining which compounds play a role in phytoplankton growth.

Although ESI is not yet widely used in the natural sciences, there is growing evidence that it is well suited for complex environmental investigations (examples include Leenheer et al., 2001; Kujawinski et al., 2004; Koch et al., 2005; Seitzinger et al., 2005b; Kim et al., 2006). ESI has a variety of instrumental applications and each application provides different information and varied levels of resolution for molecular weight determination, molecular formula assignment and structural composition assessment.

ESI mass spectrometry has the potential to provide new insights into the role of DOM in phytoplankton dynamics. Although organic nutrients appeared to play an important role in phytoplankton growth, prior to the use of ESI mass spectrometry, there was no direct way to asses which specific compounds from complex natural mixtures were produced and consumed by phytoplankton. The salts in saline samples also made analysis difficult. Through the use of tandem UF/SPE we can investigate a larger portion of the natural DOM pool. This ability to investigate brackish and saline samples is essential to understanding marine microbial dynamics.

In this paper we have briefly reviewed the use of ESI mass spectrometry to investigate both fresh and saline systems from field and laboratory samples and have used ESI-MS to characterize the DOM of duplicate *C. verruculosa* bloom samples, which had not previously been characterized. The tandem UF/SPE salt removal method paired with ESI mass spectrometry has the potential to compare and contrast DOM signatures from various bloom sites and species, evaluate which DOM compounds are being used and produced throughout the course of a bloom, explore the origin of bioavailable compounds, assess the role of DOM in species progression, and investigate DOM cycling within microbial communities. ESI mass spectrometry with other analytical techniques will help us look deeper into the black box of uncharacterized DOM.

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