

# **Application of MALDI-MS to Identification of Phytoplankton in Ballast Water**

**SI-1248**

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# Acronyms

|                    |                                                               |
|--------------------|---------------------------------------------------------------|
| ACHC               | Alpha-cyano 4-hydroxycinnamic acid                            |
| ACN                | Acetonitrile                                                  |
| ASP                | Amnesic shellfish poisoning                                   |
| BW                 | Ballast water                                                 |
| DHB                | Dihydroxybenzoic acid                                         |
| DSP                | Diarrhetic shellfish poisoning                                |
| FA                 | Ferulic acid                                                  |
| FSS                | Filter sterilized seawater                                    |
| HA                 | Harmful algae                                                 |
| HAB                | Harmful algal blooms                                          |
| IS                 | Internal standard                                             |
| MALDI-MS           | Matrix-assisted laser desorption/ionization mass spectrometry |
| NaCl               | Sodium chloride                                               |
| NH <sub>4</sub> Cl | Ammonium chloride                                             |
| NWFSC              | Northwest Fisheries Science Center                            |
| NIS                | Non-indigenous species                                        |
| ODU                | Old Dominion University                                       |
| PI                 | Principle investigator                                        |
| PNNL               | Pacific Northwest National Laboratory                         |
| P/N                | Penicillin/neomycin                                           |
| Ppt                | part per thousand                                             |
| P/S                | Penicillin/streptomycin                                       |
| PSP                | Paralytic shellfish poisoning                                 |
| Rpm                | Revolutions per minute                                        |
| SA                 | Sinapinic acid                                                |
| SEM                | Scanning electron microscopy                                  |
| SERDP              | Strategic Environmental Research and Development Program      |
| SEED               | SERDP Exploratory Development                                 |
| TFA                | Trifluoroacetic acid                                          |
| TFE                | Tetrafluoroethylene                                           |
| TSB                | Tryptic soy broth                                             |

## 1.0 Executive Summary

Non-indigenous species (NIS) are increasingly evident in marine and estuarine environments throughout the world as invasions linked to ballast water transport continue to increase (Ruiz et al. 1997). More effective and quick screening tools are necessary to characterize the aquatic non-indigenous species that may be present in ballast water. This report describes the results of a short term project within the U.S. Department of Defense Strategic Environmental Research and Development Program's (SERDP) Exploratory Development Program (SEED) to provide a potentially innovative approach for microorganism analysis that is proving successful for other environmental applications. It outlines the work necessary for "proof-of-concept" of its application to the identification of phytoplankton in ballast water.

This project demonstrates "proof-of-concept" of using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) for detecting and monitoring non-indigenous species with representative phytoplankton, one component of NIS potentially found in ballast water. For this "proof-of-concept" we focused our efforts on target species of toxin-producing dinoflagellates (e.g. *Gymnodinium*) and diatoms (e.g. *Pseudo-nitzschia*) that have been documented as occurring in ballast water (Hallegraeff, 1998, Burkholder et al. 2005). Both *Gymnodinium* and *Pseudo-nitzschia* are known to cause serious risks to human health and economic impacts to the aquaculture industry. As such, they are of increasing concern because transport in ballast water is a frequent occurrence with potentially serious consequences.

This study provided the first indication that identification and screening of phytoplankton in ballast water is feasible with MALDI-MS techniques. Differences in the MALDI spectra of several phytoplankton genera and species were obtained during this study, demonstrating the potential of this method as an identification tool. Methods developed for bacterial species at the Pacific Northwest National Laboratory (PNNL) (Saenz et al. 1999, Wahl et al. 2002) were modified to optimize detection of phytoplankton. Similar to analysis of bacteria, exchange of sodium chloride, often used for storage of the marine species in the laboratory, with ammonium chloride was necessary. In addition, concentration of the cultured organisms by centrifugation significantly enhanced the MALDI-MS signal strength. MALDI mass spectra of phytoplankton such as *Rhodomonas sp.* and *Pseudo-nitzschia* exhibited several ions that may be useful for differentiation. In addition, *Rhodomonas sp.* was detectable when spiked into ballast water samples of varying salinities. Future work should include additional species for identification and replication to ensure ions observed are unique for identification of various life stages and species of phytoplankton.

Overall, we are encouraged that minimal sample preparation may be required for this type of analysis, and that differentiation is possible among phytoplankton species. In addition, recent advances in MALDI mass spectrometry instrumentation and tandem mass spectrometry capabilities can provide additional structural ion identification information. Future research utilizing this instrumentation could increase the sensitivity of this type of analysis by at least ten-fold.

## **1.0 Objective**

This project aimed to develop a rapid assay technique that was less time-consuming than more traditional methods of microorganism (specifically phytoplankton) species identification in ballast water samples. The basic technology to be applied to identifying phytoplankton in ballast water is matrix-assisted laser desorption/ionization mass spectrometry. Pacific Northwest National Laboratory has developed a MALDI-MS method and data algorithms for rapid identification of bacterial species (Saenz et al. 1999). Statistically-based algorithms were developed to discern the “fingerprints” of bacterial species in an automated system (Jarman et al. 1999). This technology has been demonstrated to identify bacterial species in multi-species mixtures (Jarman et al. 2000).

The specific objectives for this project were to:

- Modify MALDI-MS bacterial identification techniques for the analysis of phytoplankton.
- Determine the uniqueness of phytoplankton MALDI-MS fingerprints with a limited subset of phytoplankton.
- Initiate evaluations of known phytoplankton identification in spiked environmental/ballast water samples.

## 2.0 Background

Non-indigenous species invasions in marine and estuarine environments are becoming increasingly linked throughout the world to ballast water transport (Smith et al. 1996, Ruiz et al. 1997). Ballast water is known to transport a variety of organisms, including viruses and pathogenic bacteria, invertebrates, ichthyoplankton, zooplankton, and toxigenic phytoplankton (Hallegraeff 1998, Wiley 1997, Ruiz et al. 2000, California EPA 2002).

The management of NIS in ballast water is necessary to reduce proven threats to marine and aquatic ecosystems, and to food safety and public health. A key element in any management approach to this issue is the identification of organisms in the ballast water. Conventional approaches to identification of ballast water organisms are time-consuming and require substantial taxonomic expertise that is both expensive and in limited supply. Harmful algae (HA) identification is difficult because they are a minor component of the planktonic assemblage (Anderson 1995). Also, morphologically similar strains may be difficult to distinguish even for the expert and may require scanning electron microscopy (SEM) (Anderson 1995, Zhang and Dickman 1999).

Ballast water treatment technologies have been proposed by numerous entities throughout the world to reduce the number of microorganisms entering coastal waters including ballast water uptake/release practices, ballast water exchange, onshore treatment, filtration/separation systems, biocides, heat treatment, ultraviolet radiation, ultrasound, magnetic treatment, and ozone. A majority of these technologies are still in the developmental stages (California EPA 2002). Even fewer technologies have been adopted and tested onboard vessels. At this point in time, no single treatment option exists that will remove 100% of the microorganisms contained in ballast water (Dobbs and Rogerson 2005). However, whatever technologies are adopted, they must be compatible with the shipping industry and take into consideration ship design and vessel crossings.

Phytoplankton in ballast water can include harmful algae, such as certain diatoms and dinoflagellates, which pose problems to human health, commercial shellfisheries and the aquaculture industry (Hallegraeff 1998, Wiley 1997). Harmful algal blooms (HABs) have been known to cause mass mortalities of finfish and shellfish (Bold and Wynne 1985). In British Columbia, HABs led to losses of farmed salmon totaling about \$14 million between 1986 and 1991 (Taylor 1993). Toxins concentrated in shellfish from diatoms and dinoflagellates are known to cause amnesic shellfish poisoning (ASP), diarrhetic shellfish poisoning (DSP), and paralytic shellfish poisoning (PSP) (Todd et al. 1993). Monitoring for shellfish toxins in Canada has cost about \$3.3 million annually (Todd 1995). Algal toxins, specifically domoic acid produced by *Pseudo-nitzschia multiseries* and *P. australis*, have been implicated in the 1998 deaths of sea lions in California as well (Trainer et al. 2000).

Increased frequency and severity of HABs are deemed to be more closely related to harmful algal transport via ballast water than to increased awareness or increased frequency of blooms from coastal eutrophication (Hallegraeff 1998). For example, PSP outbreaks have spread from being confined to Europe, North America and Japan in the 1970s to being almost globally

distributed in the 1990s. PSP was unknown in Australia until the late 1980s when the first dinoflagellate blooms occurred in two Australian ports. Examination of the fossil record of dinoflagellate cysts and depth profiles of cysts in age-dated sediment cores provides convincing evidence that the toxic dinoflagellate *Gymnodinium catenatum* was introduced to Australian ports in 1972.

Some life history strategies of diatoms and dinoflagellates further complicate approaches to their monitoring and management of ballast water. In response to unfavorable environmental conditions, diatoms and dinoflagellates form resting stages (resting spores and cysts, respectively) (Bold and Wynne 1985). These resting stages provide a mechanism by which harmful algae can be transported long distances via ballast water and then establish themselves in the receiving ports (Hallegraeff 1998). Also, these resting stages sink such that sediment in ballast tanks and harbors becomes a reservoir that facilitates transport and is difficult to monitor and treat effectively (Hallegraeff 1998). Therefore, any monitoring or management system for ballast water needs to address the resting as well as vegetative stages of phytoplankton.

There is a need for rapid and accurate detection and speciation of non-indigenous and/or harmful phytoplankton in ballast water. This project assessed the technique of matrix-assisted laser desorption/ionization mass spectrometry for detection of large, intact biomolecules from phytoplankton within ballast water samples. A laser-absorbing matrix material is co-crystallized with analyte molecules onto the sample support surface before insertion into the vacuum system of the MALDI mass spectrometer where a laser beam then pulses onto the sample. Ions representative of the sample are then ionized and desorbed from the surface and mass analyzed in the mass spectrometer. The resulting mass spectra can then be evaluated manually or with automated algorithms. Statistically-based algorithms developed at PNNL can be used to analyze the spectra to discern “fingerprints” of different organisms and to discern individual species from mixtures. Recently, MALDI-MS techniques have been developed to identify bacterial species at PNNL (Wahl et al. 2002). As part of this project, these methods were modified for phytoplankton analysis. MALDI-MS is particularly attractive as a screening tool because of the high speed of analysis, minimal liquids/consumables required and femtomole sensitivity, thus providing a streamlined, cost-effective approach to screening microorganisms transported in ballast water. Implementation of a rapid MALDI analysis system for phytoplankton identification in the field would involve automating sample collection and sample processing for delivery of aquatic (ballast water) samples to the MALDI mass spectrometer. Development of fieldable MALDI-MS instrumentation is in progress elsewhere (English and Cotter 2003).

### **3.0 Materials and Methods**

This research was designed to demonstrate a “proof-of-concept” to use MALDI-MS for monitoring non-indigenous phytoplankton species in ballast water, to begin development of statistically-based algorithms to discern “fingerprints” of specific phytoplankton species, and thereby provide an enabling technology for ballast water monitoring. In order to fully maximize all available resources, the strategy undertaken was to:

- Ensure that selected target phytoplankton species were determined early on and were relevant to the client’s interests. This was done in consultation with the other ballast water/non-indigenous species project PI’s.
- Leverage from past and on-going research at PNNL for development of bacterial species identification using MALDI-MS and data analysis algorithms to streamline the developmental time for phytoplankton analysis.
- Utilize available opportunities through complementary research programs, to acquire ballast water samples for testing. This included coordination with subcontractors (F. Dobbs) and other project PIs (E. Holm) that had major efforts focused on ballast water sample collection.

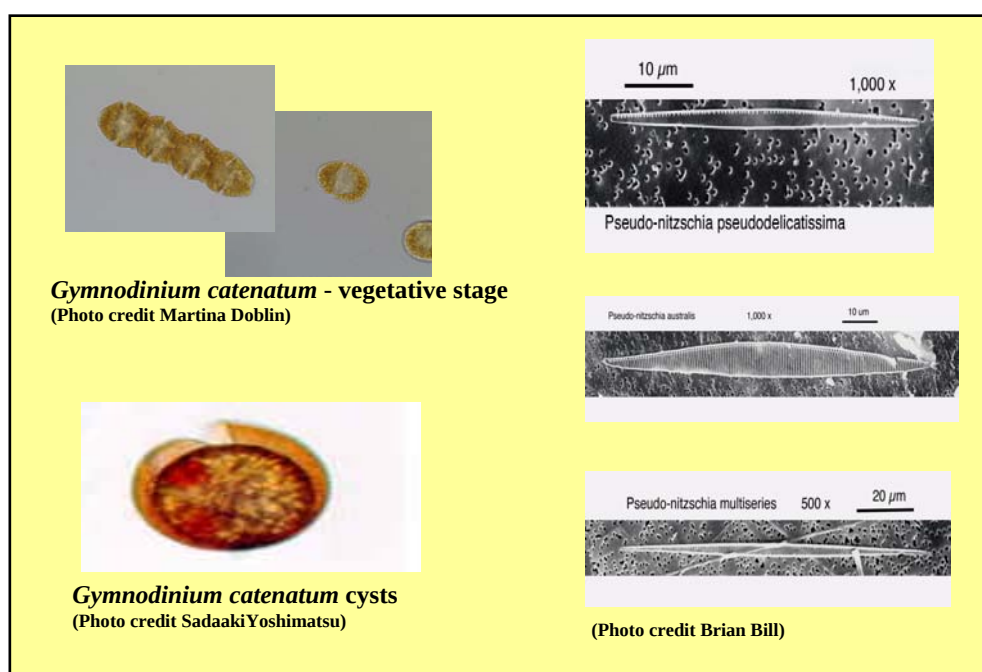
### 3.1 Phytoplankton Selection and Sample Preparation

Phytoplankton species were selected and in collaboration with Old Dominion University (ODU) (F. Dobbs, PI) and cultures prepared by ODU and PNNL. Table 1 lists the phytoplankton species and condition of sample that were included in this study.

**Table 1.** Phytoplankton samples used for MALDI-MS analysis.

| <b>Division/<br/>Class</b>      | <b>Species</b>                                 | <b>Type</b>              | <b>Holding<br/>Solution</b>   | <b>Holding<br/>State</b> |
|---------------------------------|------------------------------------------------|--------------------------|-------------------------------|--------------------------|
| Chrysophyte,<br>Prymnesiophyte  | <i>Isochrysis sp.</i>                          | Healthy,<br>Vegetative   | FSS                           | frozen                   |
| Chrysophyte,<br>Prymnesiophyte  | <i>Isochrysis sp.</i>                          | Healthy,<br>Vegetative   | FSS                           | lyophilized              |
| Chrysophyte,<br>Prymnesiophyte  | <i>Isochrysis sp.</i>                          | Healthy,<br>Vegetative   | NH <sub>4</sub> Cl<br>(2.0%)  | frozen                   |
| Chrysophyte,<br>Prymnesiophyte  | <i>Isochrysis sp.</i>                          | Healthy,<br>Vegetative   | NH <sub>4</sub> Cl<br>(2.0%)  | lyophilized              |
| Chrysophyte,<br>Prymnesiophyte  | <i>Isochrysis sp.</i>                          | Senescent,<br>Vegetative | FSS                           | frozen                   |
| Chrysophyte,<br>Prymnesiophyte  | <i>Isochrysis sp.</i>                          | Senescent,<br>Vegetative | FSS                           | lyophilized              |
| Chrysophyte,<br>Prymnesiophyte  | <i>Isochrysis sp.</i>                          | Senescent,<br>Vegetative | NH <sub>4</sub> Cl<br>(2.0%)  | frozen                   |
| Chrysophyte,<br>Prymnesiophyte  | <i>Isochrysis sp.</i>                          | Senescent,<br>Vegetative | NH <sub>4</sub> Cl<br>(2.0%)  | lyophilized              |
| Cryptophyte<br>(Cryptomonads)   | <i>Rhodomonas sp.</i>                          | Healthy,<br>Vegetative   | NaCl<br>(2.4%)                | frozen                   |
| Cryptophyte<br>(Cryptomonads)   | <i>Rhodomonas sp.</i>                          | Healthy,<br>Vegetative   | NH <sub>4</sub> Cl<br>(2.0%)  | frozen                   |
| Pyrrhophyte<br>(Dinoflagellate) | <i>Gymnodinium<br/>catenatum</i>               | Healthy,<br>Vegetative   | NaCl<br>(2.4%)                | frozen                   |
| Pyrrhophyte<br>(Dinoflagellate) | <i>Gymnodinium<br/>catenatum</i>               | Healthy,<br>Vegetative   | NH <sub>4</sub> Cl<br>(2.0%)  | frozen                   |
| Pyrrhophyte<br>(Dinoflagellate) | <i>Gymnodinium<br/>catenatum</i>               | Cyst                     | NaCl<br>(2.4%)*               | frozen                   |
| Pyrrhophyte<br>(Dinoflagellate) | <i>Gymnodinium<br/>catenatum</i>               | Cyst                     | NH <sub>4</sub> Cl<br>(2.0%)* | frozen                   |
| Chrysophyte<br>(Diatom)         | <i>Pseudo-nitzchia<br/>australis</i>           | Healthy,<br>Vegetative   | f/2Guillards<br>medium        | 12° C                    |
| Chrysophyte<br>(Diatom)         | <i>Pseudo-nitzchia<br/>pungens</i>             | Healthy,<br>Vegetative   | f/2Guillards<br>medium        | 12° C                    |
| Chrysophyte<br>(Diatom)         | <i>Pseudo-nitzchia<br/>pseudodelicatissima</i> | Healthy,<br>Vegetative   | f/2Guillards<br>medium        | 12° C                    |
| Chrysophyte<br>(Diatom)         | <i>Pseudo-nitzchia<br/>multiseriis</i>         | Healthy,<br>Vegetative   | f/2Guillards<br>medium        | 12° C                    |

Species were selected to represent phylogenetic differences in algal taxa, including several life cycle stages of development. In addition, two harmful algal genera were selected (*Pseudo-nitzschia* and *Gymnodinium*) (Figure 1) that have been identified previously in ballast water samples (Hallegraeff 1998). Every attempt was made to reduce contamination of the algal cultures, however axenic cultures were not available in all circumstances. To reduce bacterial contamination, *Gymnodinium* were cultured with either penicillin/streptomycin (P/S) antibiotics or penicillin/neomycin(P/N). *Pseudo-nitzschia* species culture stocks were obtained from the Northwest Fisheries Science Center (NWFSC). The following strains were used for this study: *P. multiseries* (NWFSC-010), *P. australis* (NWFSC-073), *P. pseudodelicatissima* (NWFSC-081). *Pseudo-nitzschia* were cultured and stored in enriched f/2 Guillard's medium (Guillard and Ryther 1962).



**Figure 1.** Harmful algal species and life stages included in the MALDI-MS study.

A common storage solution for the phytoplankton is sodium chloride (NaCl) which is problematic for MALDI-MS. Therefore, as a test, a sub-set of samples were shipped in ammonium chloride (NH<sub>4</sub>Cl) (see Table 1), a more compatible matrix for mass spectrometric analysis. There was some concern that the phytoplankton would be less stable and leak in a NH<sub>4</sub>Cl solution. However, what appears to be useful MALDI-MS was obtained from samples shipped in NH<sub>4</sub>Cl solution without detrimental effect. Samples shipped in NaCl were washed with NH<sub>4</sub>Cl prior to MALDI-MS analysis.

### 3.2 Ballast Water Samples

Ballast water samples were obtained from several collaborators on this project. We had arranged early in the project to obtain samples in collaboration with SERDP project CP-1245 (E. Holm, PI), which focused on fleet oilers and cargo vessels operated by the Military Sealift Command, and lighters operated by the U.S. Army. (Holm et al. 2005). Due to unforeseen delays in timing of collections, we only received one sample from this source which we used early on in our study. This sample was collected July 1, 2003 from a fleet oiler, on the east coast. A 100% empty-refill exchange had occurred 5 days prior in the western central Atlantic, hence this represented a “clean” ballast sample. The temperature at collection time was 26.4°C. (Holm et al. 2005). The sample was shipped to PNNL in late July 2003.

A second source of samples used for a majority of our work involved commercial vessels sampled in the Great Lakes and Chesapeake Bay. These samples were collected and archived by F. Dobbs (ODU). The samples described below (Table 2) were shipped to PNNL in August 2003.

**Table 2.** Ballast water samples collected in the Great Lakes and Chesapeake Bay from commercial vessels.

| Sample ID | Collection Location | Date of Collection | Salinity (ppt) |
|-----------|---------------------|--------------------|----------------|
| CB #1     | Chesapeake Bay      | Feb 26, 2003       | 32             |
| CB #2     | Chesapeake Bay      | May 16, 2003       | 37             |
| CB #3     | Chesapeake Bay      | July 17, 2003      | 42             |
| CB #4     | Chesapeake Bay      | July 30, 2003      | 3              |
| GL #5     | Great Lakes         | June 6, 2002       | 34             |
| GL #6     | Great Lakes         | Aug 7, 2002        | 10             |
| GL #7     | Great Lakes         | Sept 13, 2002      | 60             |
| GL #8     | Great Lakes         | Sept 13, 2002      | 78             |
| GL #9     | Great Lakes         | Nov 12, 2002       | 28             |
| GL #10    | Great Lakes         | Oct 11, 2002       | 0              |

### 3.3 Phytoplankton Sample Preparation for Mass Spectrometric Analysis

Due to the competitive ionization of MALDI-MS, there is potential for interference by the background solution on the analysis of the desired phytoplankton components. Of particular concern with these marine species is the potential interference from the salt content of the water environment. While MALDI-MS is compatible with some salt and low level detergents and other soluble components, there is known degradation of mass spectral signals with increased salt and non-volatile buffer components (Kussman et al 1997).

Several different quick preparation methods to clean up samples prior to mass spectrometric analysis have been used successfully for removal of sodium salts and certain detrimental buffers (Kussman et al 1997). The primary goal of these methods is to clean up potential interferences such as high salt content from the samples prior to MALDI-MS analysis. Several of these methods for sample cleanup were tested for potential application to samples on this project. Spot washing is a simple application of water to the dried sample on the MALDI sample plate with the outcome of solubilizing salts away from the phytoplankton components such as proteins that will preferentially stick on the surface of the sample plate relative to the salts. Other sampling

cleanup methods utilizing bead-based chemistry such as ion exchange beads, and hydrophobic Zip Tips (Millipore Corp. Billerica, MA), where C-18 resin was packed within a small pipette tip were briefly tested for enhancement of the protein related signal with no significant improvement.

In addition, the use of tetrafluoroethylene (TFE) membranes (Zitex, Saint-Gobain Performance Plastics, Mickleton, NJ) were tested as a way of desalting based on methods suggested in the PerSeptive Biosystems MALDI-MS Training Manual. In this case, a TFE filter was floated on top of Milli-Q water in a Petri plate. Then 10  $\mu\text{L}$  of the sample was pipetted on the floating TFE membrane. A 1  $\mu\text{L}$  droplet of acetonitrile (ACN) was pipetted onto the sample drop. After 45-60 minutes at room temperature, the sample drop was pipetted back off the membrane and 0.5  $\mu\text{L}$  was pipetted onto the MALDI sample plate. The matrix solution was then layered over the sample spot.

MicroCon filters (Millipore Corp) were tested for potential sample cleanup and signal enhancement. The MicroCon filter was prewashed with MilliQ water twice with approximately 25 minutes centrifugation each time. The sample was then placed in the filter and used as described in the manufacturer's instructions. C4 Zip Tips were used following the manufacturer's instructions (without guanidine hydrochloride) with the following solutions:

**Table 3.** Solutions used with C4 Zip Tips.

|                         |                     |
|-------------------------|---------------------|
| Wetting solution:       | 50% acetonitrile    |
| Equilibration solution: | 0.1% TFA            |
| Wash solution:          | 0.1% TFA            |
| Elution solution:       | 50% ACN in 0.1% TFA |

The MicroCon filters were used following manufacturer's instructions. The sample spot washing on the MALDI plate was obtained from the PerSeptive Biosystems MALDI-MS Training Manual.

Ultimately it was determined that the methods tested above provided very little additional value in terms of sample cleanup. Sample concentration appeared to be more critical to successful MALDI-MS analysis than cleanup of the samples.

### 3.4 Sample Concentration

Pre-concentration of the sample prior to MALDI-MS analysis was necessary in this early stage to ensure that sufficient material was present in the small microliter sample used for mass spectrometric analysis. From previous experience with bacterial samples, centrifugation was determined to be an effective way of concentrating microorganism samples prior to MALDI-MS analysis. One mL of the various phytoplankton cultures was centrifuged at 12,000 to 14,000 rpm for 2 to 5 minutes. There was no evidence that this caused any negative effect on the samples or the analyses. Samples were then washed 2 times with sterile Milli-Q water. The pellets were resuspended in 100  $\mu\text{L}$  of sterile water. Samples (0.5  $\mu\text{L}$ ) were spotted onto a stainless steel MALDI plate and 0.5  $\mu\text{L}$  of ferulic acid (FA) matrix solution (or other matrix solutions tested) to each sample spot. A variety of matrices were tried to determine which would be the most

effective for ionization of these samples (Section 4.5). Samples were allowed to air dry before analysis by MALDI-MS.

### 3.5 MALDI- MS Analysis of Phytoplankton Samples

A key component in the development of the MALDI mass spectrometric analytical techniques for marine phytoplankton was the evaluation of various steps in the process relative to procedures developed previously for bacteria (Jarman et al. 2000). Some of the challenges for phytoplankton included the physical structure and larger size compared to bacteria, and the generally higher salt concentration of the samples. The processes evaluated for optimization included the concentration of the sample, the relative ratio of the sample to the matrix, the choice of the matrix compound and the choice of the matrix solution.

There are several different commonly used MALDI matrices including sinapinic acid (SA) and ferulic acid, primarily used for proteins, and alpha-cyano 4-hydroxycinnamnic acid (ACHC) and dihydroxybenzoic acid (DHB) used primarily for peptides and smaller analytes. Typically, the matrix solvent is a mixture of acetonitrile and water with the addition of trifluoroacetic acid (TFA) to help aid in ionization (Cohen and Chait 1996). Acid content has been shown to influence the extraction of proteins from bacteria and thus several different concentrations were briefly evaluated to try to optimize the MALDI-MS signals (Wang et al. 1998, Dickinson et al. 2004, Warscheid and Fenselau 2003).

Nine matrix solutions listed below were evaluated for most effective analysis of the phytoplankton samples as determined by intensity and number of sample related ions.

- 10 mg/mL of ACHC in 70:30 v:v 1% TFA:acetonitrile
- 10 mg/mL of ACHC in 70:30 v:v 0.1% TFA:acetonitrile
- 20 mg/mL of ACHC in 70:30 v:v 1% TFA:acetonitrile
- 10 mg/mL of FA in 70:30 v:v 1% TFA:acetonitrile
- 20 mg/mL FA in 70:30 v:v 1% TFA:acetonitrile
- 10 mg/mL DHB in 70:30 v:v 1% TFA:acetonitrile
- 10 mg/mL SA in 70:30 v:v 1% TFA:acetonitrile
- 10 mg/mL SA in 70:30 v:v 2% TFA:acetonitrile
- FA<sup>2</sup> - FA in 17% formic acid (7mg FA, 123μL – 65% formic acid, 155μL acetonitrile, 192μL H<sub>2</sub>O) (Madonna et al. 2000).

To evaluate each solution, a 0.5 μL droplet of prepared sample was spotted onto a MALDI stainless steel plate followed by a 0.5 μL droplet of the test matrix solution and air-dried. The plate was then inserted into the vacuum system of the mass spectrometer for MALDI-MS analysis.

Protein standards, horse heart cytochrome *c* and angiotensin I, used for mass axis calibration of the mass spectrometer, were purchased from Sigma (St. Louis, MO). Ferulic acid and TFA were purchased from Aldrich (Milwaukee, WI). Acetonitrile was obtained from J.T. Baker (Phillipsburg, NJ). The water was from a Milli-Q Plus purification system (Millipore Corp., Bedford, MA).

Two different sample deposition methods were briefly tested: the previously adopted layering method of sample deposition (Wahl et al. 2002) followed by matrix deposition onto the sample spot and the dried droplet method (Karas and Hillenkamp 1998) where a sample is premixed with the matrix prior to spotting onto the MALDI plate. As with the previous work in our laboratory with bacterial samples, the premixed spotting did not produce any noticeable benefits for these samples and therefore the layered method of sample spotting was adopted for the phytoplankton research.

Mass axis calibration of the instrument was performed daily with the  $(M+H)^+$  ions of angiotensin I and cytochrome *c* at  $m/z$  1,297 and 12,361 respectively. The calibration solution was prepared by adding three  $\mu\text{L}$  of 1 mg/mL cytochrome *c* and 0.5  $\mu\text{L}$  of 1 mg/mL angiotensin to 197.5  $\mu\text{L}$  matrix solution. The matrix solution (0.5  $\mu\text{L}$ ) with internal standard was spotted over the dried sample for internal calibration or in close proximity for external calibration. MALDI analysis was performed on a PerSeptive Biosystems Voyager-DE RP MALDI time-of-flight mass spectrometer with a nitrogen laser (337 nm) operated in the linear, delayed extraction mode with 25 KV accelerating voltage. Each spectrum was collected in the positive ion mode as the average of 128 laser shots. Replicate spectra were collected from the sample. Ideally, a minimum of three replicate spectra of each sample were collected, but in some cases weak signal prevented successful collection for all matrix and solvent combinations. The *Rhodomonas sp.* data collected with different ratios and concentrations of TFA were internally calibrated with the cytochrome *c* and angiotensin added to the matrix spotted on the sample. The raw data files were then transferred to the data analyst for automated peak extraction and analysis.

Previously developed algorithms for peak extraction and replicate averaging were implemented where feasible (Jarman et al. 1999, Jarman et al. 2000). In the first step of data processing, mass spectrometry peaks were extracted from the resulting spectrum using an automated spectral peak detection algorithm developed at PNNL (Jarman et al. 2003). Statistically based automated data-analysis algorithms provide a non-biased interpretation of the data and circumvent the need for highly trained scientists for data evaluation. Employing a histogram-based model for spectral intensity, peaks are detected by comparing the estimated variance of observations (the x-axis of the spectrum) to the expected variance when no peak is present inside some window of interest. Once peaks are extracted, statistically-based algorithms are used for specific biomarker identification and pattern recognition. Additional replicate cultures of the phytoplankton samples are needed in future studies to develop the phytoplankton fingerprints following our previously developed fingerprinting protocol. A preliminary table of ions extracted from the different phytoplankton on different days of analysis is provided in Appendix A. Until additional replicates and analyses are obtained, no further data processing or averaging of replicates for distinct species fingerprints is feasible.

### **3.6 Bacteria Spiked into Ballast Water**

*Serratia marcescens*, an environmental bacterium that occurs naturally in soil and water, was selected for use in ballast water spiking studies due to ease of culture and familiarity with MALDI-MS response for this bacterium. *S. marcescens* was cultured in tryptic soy broth (TSB) and incubated overnight in a shaker incubator at 26°C at 120 rpm. The cells (~1.5 mL per tube) were then centrifuged at 14,000 rpm for 2 minutes, decanted, and washed once with 1 mL of 2%  $\text{NH}_4\text{Cl}$  to remove the growth media. (Wahl et al. 2002). The cells were centrifuged again,

decanted and then mixed with 400  $\mu\text{L}$  of 0.1% TFA. After 1 minute of sitting at room temperature, 200  $\mu\text{L}$  of the cell suspension was centrifuged at 14,000 rpm for 2 minutes and decanted off. The cell pellet was then washed two times with corresponding test ballast water (add 100  $\mu\text{L}$  of ballast water, centrifuged, decanted). This was meant to ensure that substantially all of the liquid in the cells was ballast water rather than culture or laboratory solutions for best practical simulation. The final additional of 100  $\mu\text{L}$  of ballast water was then used as the spiked sample. A 10  $\mu\text{L}$  aliquot of the ballast water spiked solution was removed and diluted with an additional 10  $\mu\text{L}$  of the ballast water for a 1:2 dilution. This diluted sample was analyzed by MALDI-MS. An aliquot of *S. marcescens* was processed for each of 10 different ballast water samples tested with bacterial spiking and treated as described above.

### **3.7 Phytoplankton Spiked Ballast Water**

The phytoplankton spiked ballast water experiment was conducted with *Rhodomonas sp.* A 15  $\mu\text{L}$  aliquot of *Rhodomonas sp.* was washed with the addition of 200  $\mu\text{L}$   $\text{NH}_4\text{Cl}$ . This solution was centrifuged, pelleted, decanted and then resuspended in 20  $\mu\text{L}$  of ballast water. For this specific test, the fleet oiler ballast water was used. The spiked ballast water sample was spotted onto the MALDI sample plate along with FA MALDI matrix. Once dried, the sample plate was inserted into the vacuum system for MALDI-MS analysis.

Due to funding constraints, minimal effort was placed on spiking other species of phytoplankton into ballast water primarily due to the weaker direct signals obtained from other phytoplankton species at the time. Rather, effort was focused on spiked bacterial experiments into a variety of ballast water types (Section 4.6 and 5.4).

## 4.0 Results and Accomplishments

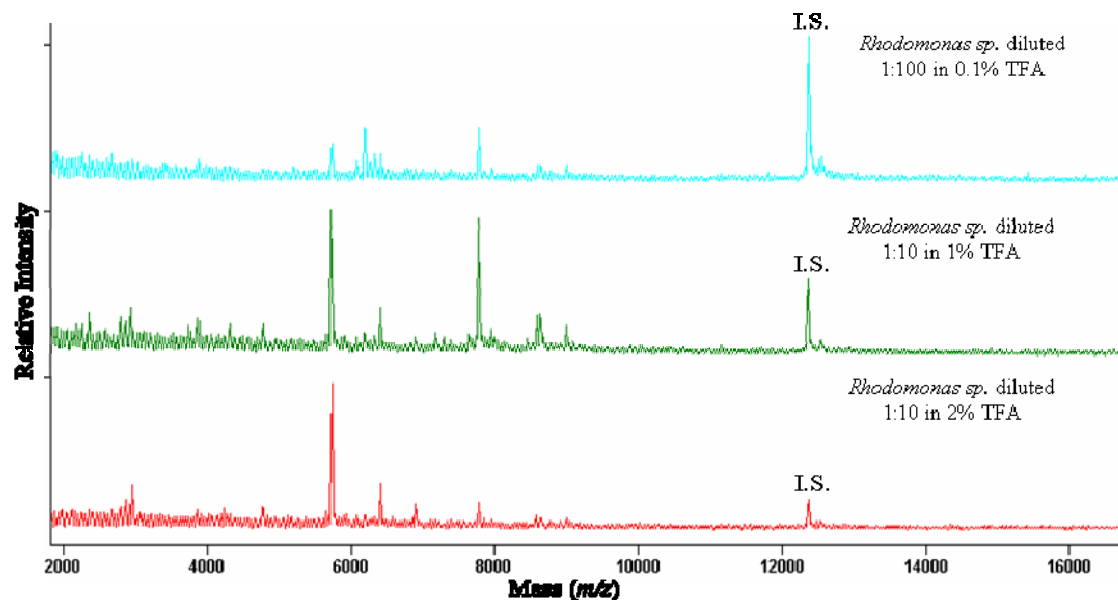
The results of preliminary studies to determine the ability of MALDI-MS to analyze phytoplankton in ballast water are presented here. The objectives were to modify the MALDI-MS bacterial identification techniques previously developed at PNNL for the current analysis of phytoplankton, determine the uniqueness of phytoplankton MALDI-MS fingerprints with a limited subset of phytoplankton, and to initiate evaluations of known phytoplankton identification in spiked environmental/ballast water samples.

### 4.1 MALDI-MS Spectra of Phytoplankton

One key challenge to address for analysis of a new type of sample, phytoplankton, is the amount of material necessary and the sample preparation that may be required for successful analysis by MALDI-MS. Previous experience with bacterial cells suggested that preconcentration of biological sample may be helpful for direct MALDI-MS analysis of the phytoplankton and exchange of NaCl salts used to maintain structure may need to be replaced with the more volatile  $\text{NH}_4\text{Cl}$  for mass spectrometric analysis.

As with any new sample type, several different MALDI matrices and application methods were tested with the phytoplankton samples to determine the optimum analytical conditions for these samples (Kusmann et al. 1997). As noted in Section 3.3 there was no significant difference between whether samples were pre-mixed with the MALDI matrix prior to application to the MALDI sample plate or the matrix and sample were layered in different orders. The method of layering the sample onto the slide followed by application of the matrix solution was chosen and used for all results presented in Figures 2 through 10. This is consistent with our previously optimized method for MALDI-MS analysis of bacteria (Wahl et al. 2002). MALDI matrices tested include FA,  $\text{FA}^2$ , DHB in 0.1% TFA, and SA in 2% TFA. As with our previous bacterial analysis (Saenz et al. 1999), FA matrix gave better results consistently than obtained with other matrices tested for the phytoplankton samples. Ions from most phytoplankton were typically observed in the  $m/z$  4,000 to 7,000 range, consistent with the small acid soluble putative proteins obtained in the direct MALDI MS analysis of bacteria (Arnold and Reilly 1999). Therefore, the majority of the data collected and presented here is from MALDI-MS analysis with FA matrix.

Representative spectra from *Rhodomonas sp.* in 2%  $\text{NH}_4\text{Cl}$  (frozen) with FA matrix prepared in acetonitrile/TFA water were obtained with relatively strong ion signals (Figure 2). The ratio of TFA concentration to *Rhodomonas sp.* sample was varied to determine if there was an optimal concentration or ratio. The TFA serves several purposes in this experiment including improved protonation during the ionization step as well as a potential aid in extraction/solubilization of the small acid soluble proteins from the organism.



**Figure 2 MALDI-MS of *Rhodomonas* sp. with various concentrations of trifluoroacetic acid. (I.S.=internal standard cytochrome c)**

Several different cleanup and concentration methods often used in conjunction with MALDI-MS were briefly evaluated with only minor differences in the resulting MALDI mass spectra. Ion exchange beads, Zip Tip cleanup, and TFE membrane cleanup did not prove effective with the samples tested to date. Also it was determined that spot washing for the majority of the samples as tested was unnecessary as remaining salts from samples or ballast water tested here did not seem to interfere with the MALDI analysis. The most effective method for improving the MALDI-MS results for the samples analyzed during this study was centrifugation of the phytoplankton samples to pellet and concentrate the cells. Centrifugation did not seem to adversely affect the phytoplankton, but rather concentrated them, yielding better MALDI-MS results. Therefore, the phytoplankton were centrifuged as described in the experimental section prior to mass spectrometric analysis. Figure 3 and Figure 4 show the advantage of centrifugation to concentrate a pellet of phytoplankton to improve signal-to-noise obtained by MALDI-MS. Ferulic acid was used as the matrix in both cases. In Figure 3 the supernatant slurry from the *Gymnodinium catenatum* (vegetative stage) exhibits very little meaningful data whereas the centrifuged pellet from the sample shows improved ion signal and the need for sample concentration. Additional signal enhancement could be obtained with more sensitive newer MALDI-MS instrumentation. Figure 4 shows representative MALDI mass spectra of the same *Pseudo-nitzschia australis* sample spotted directly onto the MALDI sample plate two times (top) and after centrifugation to concentrate the sample (bottom). In both cases the preferred FA matrix were used. Clearly the centrifuged sample (bottom) is more effective for MALDI-MS analysis.

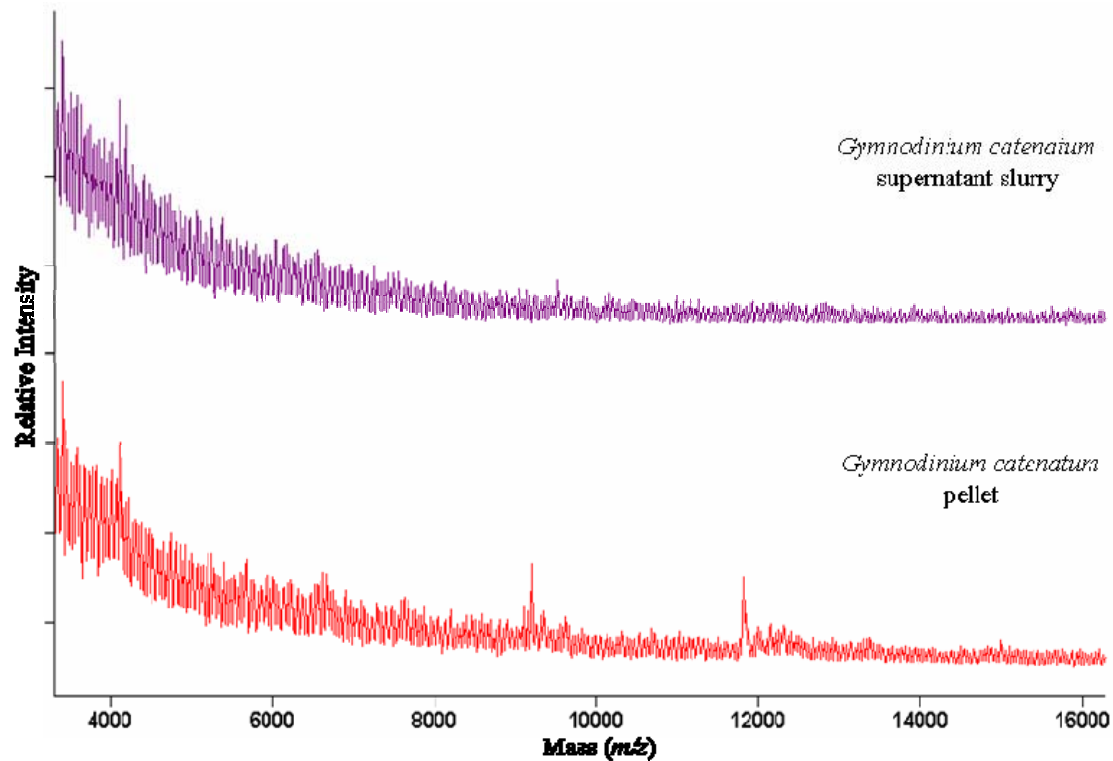


Figure 3 Comparison of MALDI-MS spectra of supernatant from antibiotic treated (P/S) *Gymnodinium catenatum* vegetative cell sample with pellet from centrifugation of the sample.

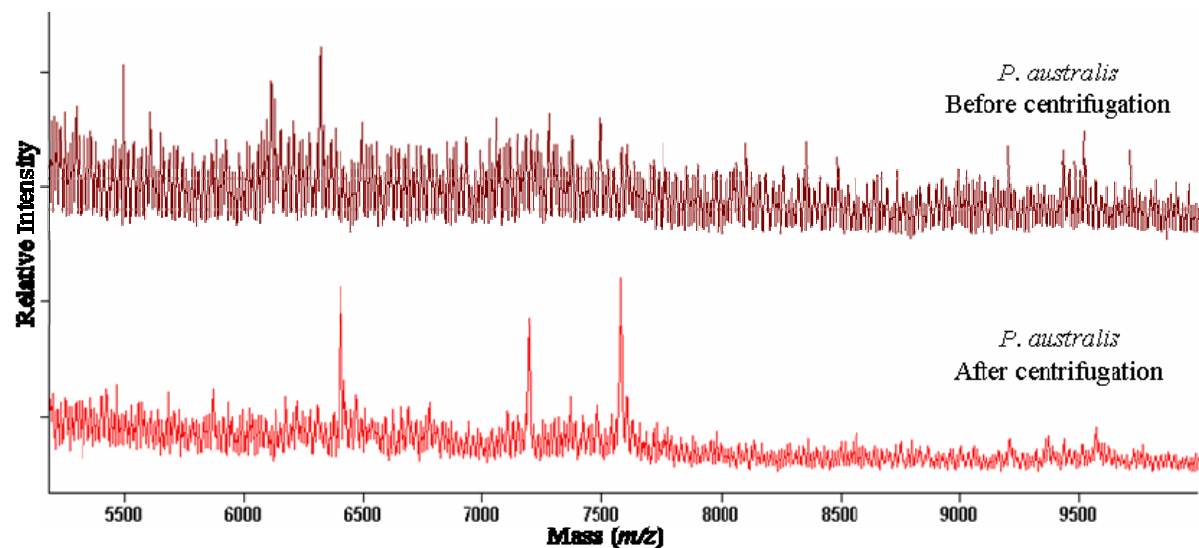


Figure 4 Comparison of MALDI-MS analysis of *Pseudo-nitzschia australis* before and after centrifugation to concentrate the organism.

To the extent possible, replicate cultures of the same organism were obtained and analyzed to capture some of the known biological variability by the mass spectrometric analysis. From

previous mass spectrometric analysis of other microorganisms, we have observed that the largest variability in our MALDI-MS analysis of organisms occurs as a result of inherent variability due to culturing of the microorganisms.

## 4.2 Phytoplankton Differentiation

There is preliminary evidence to suggest that, similar to other bacteria (Krishnamurthy et al. 1996, Holland et al. 1996, Claydon et al. 1996, Demirev et al. 1999), the MALDI-MS analysis of different phytoplankton genera and species within a single genera do produce distinctly different MALDI mass spectra that may be sufficient for differentiating between them as a first response screening method. Figure 5 shows representative MALDI mass spectra for several different genera of phytoplankton and two species of the diatom *Pseudo-nitzschia*. Figure 6 shows distinctly different spectra of four species of *Pseudo-nitzschia*.

Additional specimens would be required to verify the reproducibility of the ions. Useable spectra were obtained from *Pseudo-nitzschia multiseries*, *P. pseudodelicatissima*, *P. australis*, *Gymnodinium catenatum* (P/N and P/S) vegetative cells, *Isochrysis* senescent cells, and freeze dried healthy *Isochrysis* cells. The *Gymnodinium catenatum* cysts in NaCl and  $\text{NH}_4\text{Cl}^+$  with sodium polytungstate proved problematic for MALDI-MS analysis. The spectra from the cysts containing sodium polytungstate indicated the ions were related to the sodium tungstate, and likely not related to the cysts. Cysts received in NaCl holding solution also gave minimally useful MALDI-MS spectra, probably due in part to the limited concentration of cells received. Repeatability of the preliminary results presented here need to be verified by additional replicate cultures with an emphasis placed on reproducibility in culture collections and taking advantage of increased sensitivity of the MALDI-MS instrumentation that is currently available.

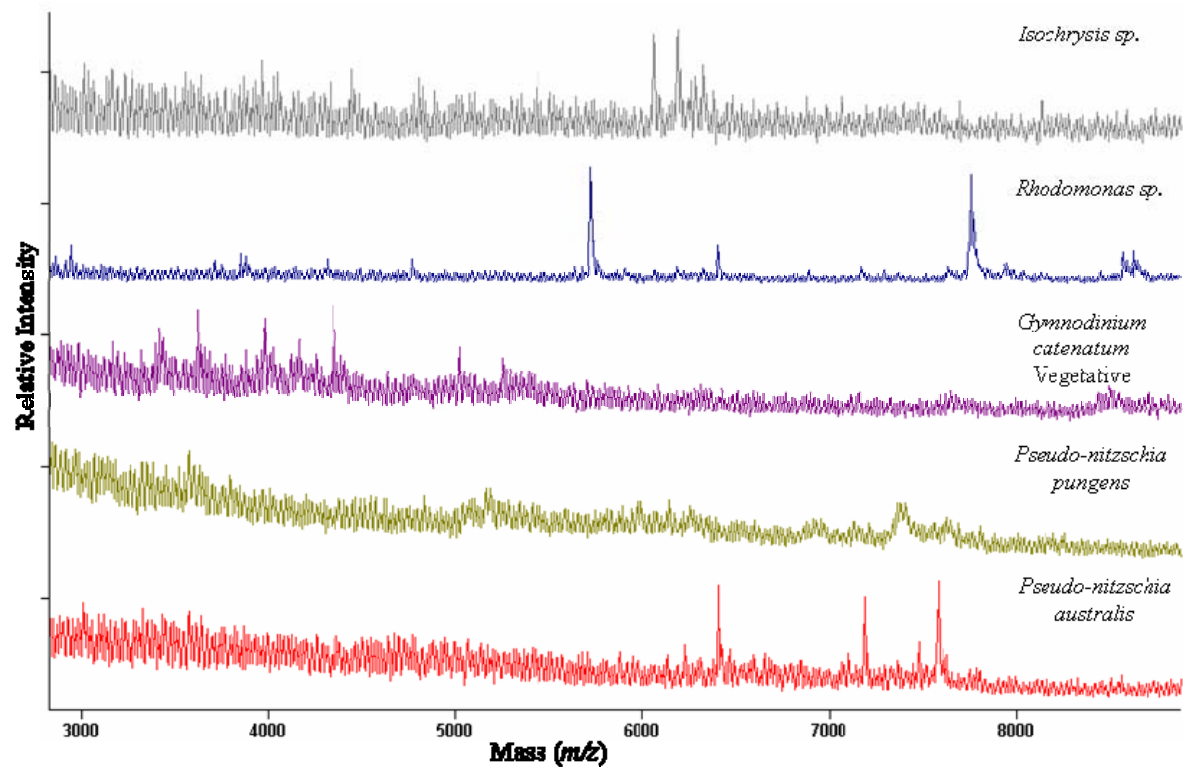


Figure 5 MALDI-MS of different classes of phytoplankton.

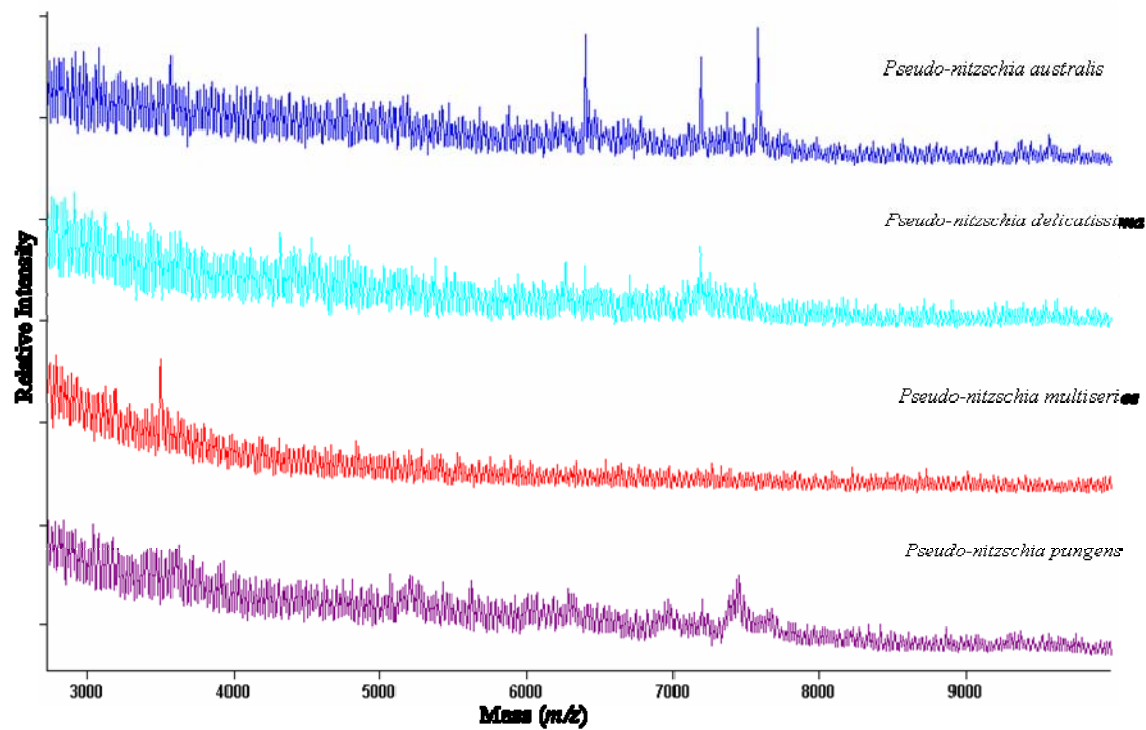


Figure 6 Representative MALDI-MS of different species of *Pseudo-nitzschia*.

Efforts were made to develop reproducible fingerprints of several phytoplankton similarly to previous work with bacterial samples (Jarman et al. 1999). However, it was difficult to obtain replicate phytoplankton cultures within the life span of this project. A table of ions observed from replicate MALDI-MS analysis of several different phytoplankton are provided in Appendix A. While there is some indication of ions common to the few replicate analyses, there are currently insufficient replicate cultures to state the significance of these ions for identification purposes. This table of ions serves as a reference for future expansion of the MALDI-MS analysis of phytoplankton.

### **4.3 Ballast Water Background Effect**

The third objective of the study was to assess the ability to detect phytoplankton in a complex mixture such as ballast water. Of initial concern was the potential for background interferences within the ballast water and/or potentially high salt content that could negatively impact successful MALDI-MS detection of phytoplankton. Due to the variability in replication observed in the phytoplankton samples, and based on our previous successful work with MALDI-MS analysis of bacteria, we chose to use a known bacterium to test a variety of ballast water samples, followed by preliminary tests with *Rhodomonas sp.* spiked into ballast water. This allowed us to determine whether a variety of ballast water samples had any significant deleterious effect on the ability to perform MALDI-MS analysis of organisms present.

#### **4.3.1 Bacteria for Replicate Testing of Ballast Water Interference**

In order to assess the effect of ballast water on the MALDI mass spectra, a bacterium readily cultured at PNNL was used to spike into the different ballast waters at different concentrations. This allowed quick assessment of the potential challenges of the ballast water with a readily available microorganism. *Serratia marcescens*, was selected for baseline experiments of organisms spiked into ballast water due to prior experience at PNNL with MALDI-MS analysis of this bacteria. Based on past culturing experience, it was easier to control the culture and concentration of this organism for spiking into a wide range of ballast waters. The ratio of *S. marcescens* and the different ballast waters were tested for effective MALDI-MS analysis. Figure 7 shows a comparison of the MALDI-MS analysis of ballast water alone, *S. marcescens* cells alone and then in 1:2 and 1:10 dilution of cells in the ballast water Sample #1. Additional spiking of *S. marcescens* in the variety of ballast water available is shown in Figure 8 and Figure 9. It must be noted that relative intensity of ions in the MALDI-MS of organisms is not as significant as the presence or absence of ions. Thus even though relative intensities of ions vary significantly in these representative spectra of the spiked ballast water, the majority of the ions are consistently observed across the ballast water samples. There is no evidence of significant adverse effect of most ballast water samples on the analysis of microorganisms spiked into the samples.

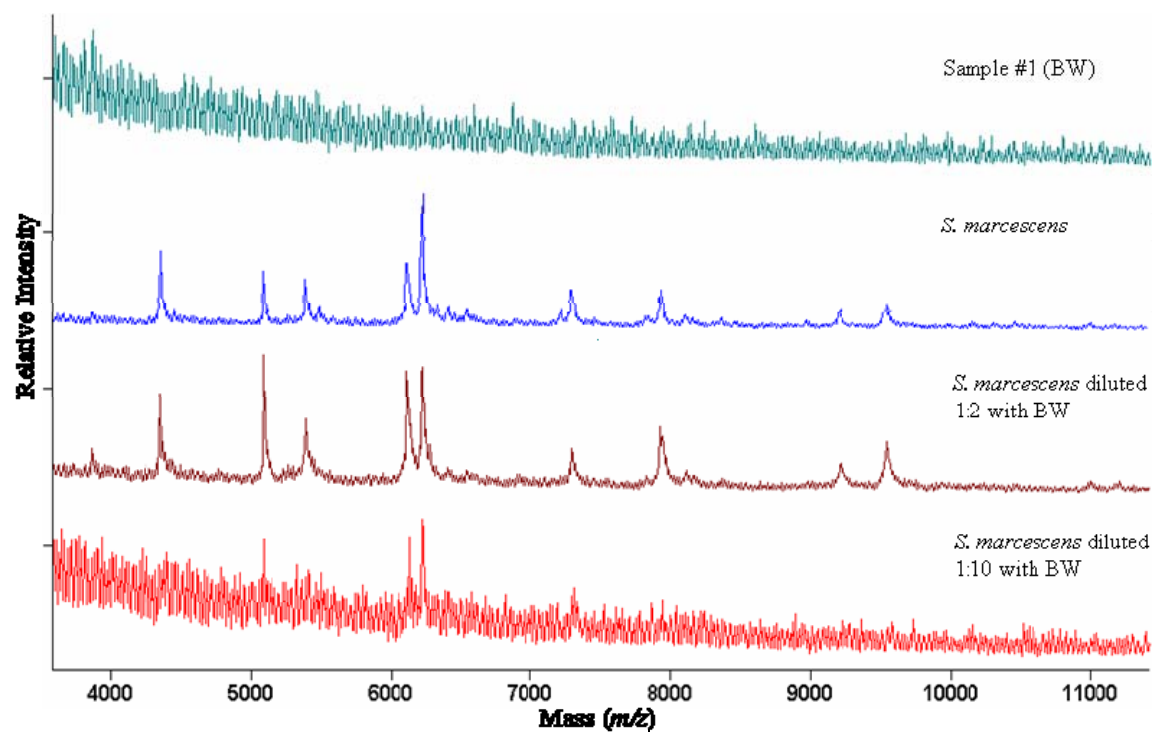


Figure 7 MALDI-MS of ballast water, *Serratia marcescens*, diluted with ballast water sample #1, 1:2 and 1:10.

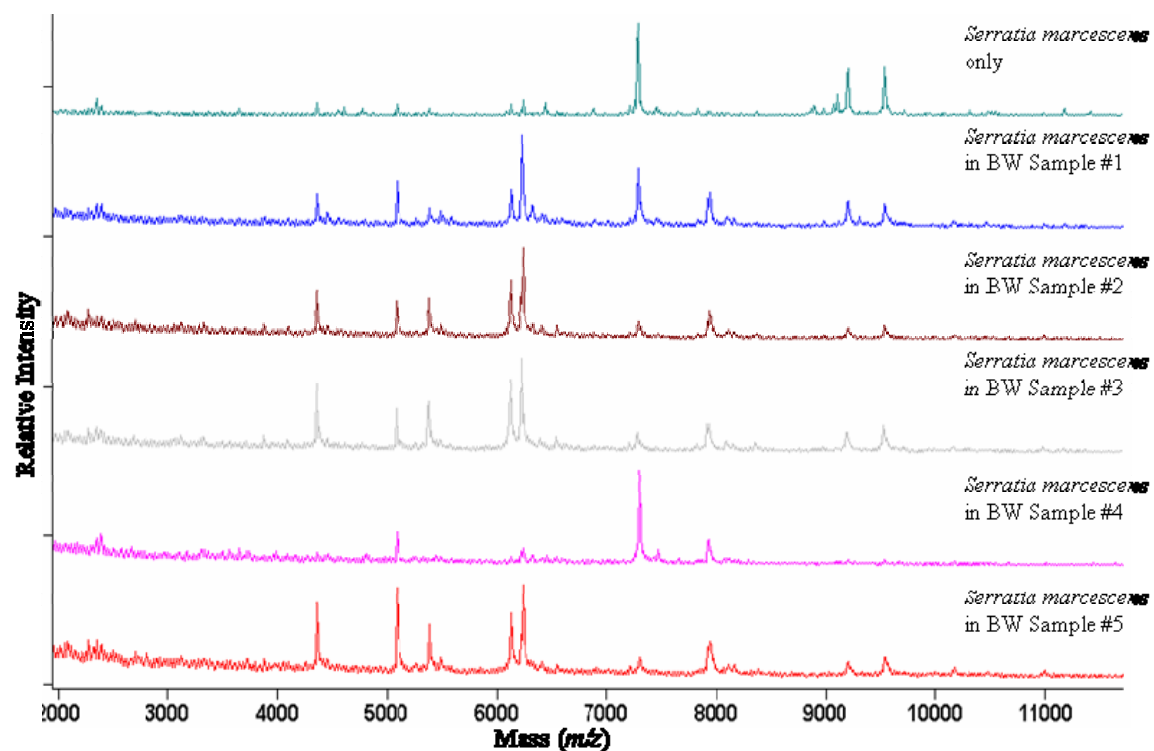


Figure 8 MALDI-MS of *Serratia marcescens* spiked into a variety of ballast water samples #1 through 5 collected from commercial vessels in the Chesapeake and the Great Lakes.

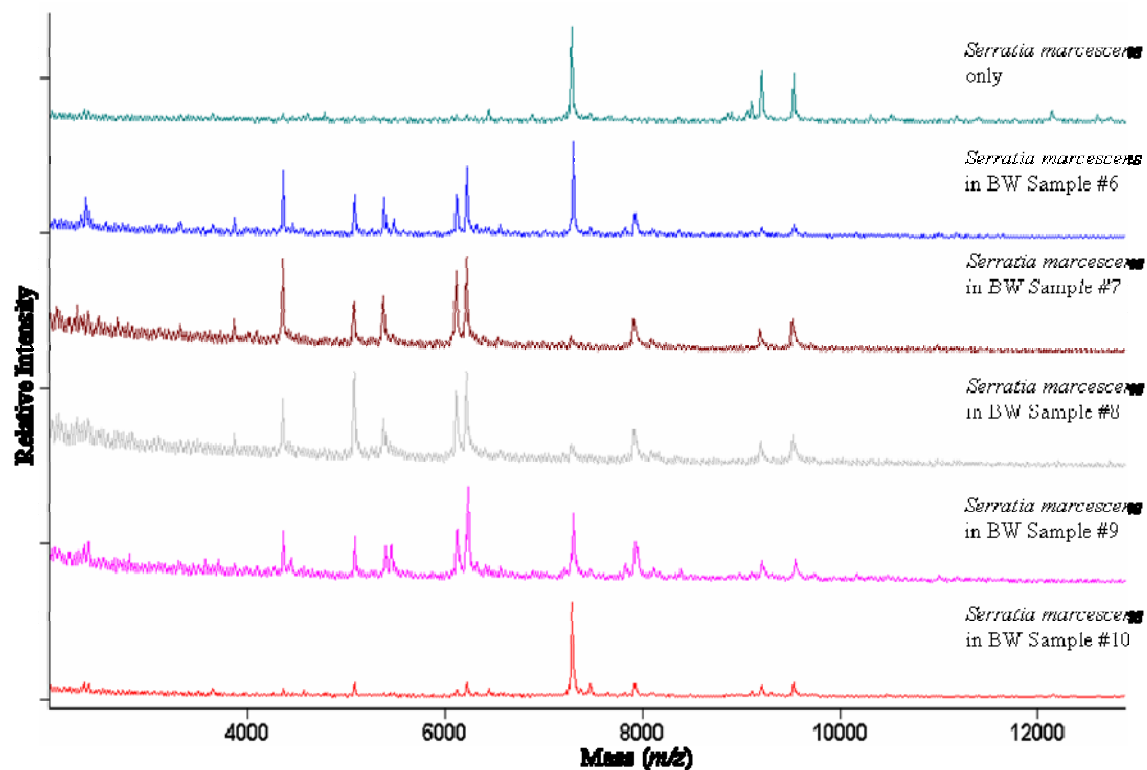


Figure 9 Additional MALDI-MS of *Serratia marcescens* spiked into ballast water samples #6 – 10 obtained from commercial vessels in the Great Lakes.

#### 4.3.2 Spiking Phytoplankton into Ballast Water Samples

Ballast water spiked with phytoplankton gave similar results to phytoplankton prepared in the holding solution,  $\text{NH}_4\text{Cl}$ . The ballast water and the background salt did not interfere with the MALDI-MS analysis. Figure 10 shows a comparison between *Rhodomonas* sp. in  $\text{NH}_4\text{Cl}$  and *Rhodomonas* sp. spiked into a ballast water sample. There is minimal difference between the MALDI-MS analysis indicating that ballast water does not adversely affect the MALDI-MS analysis. These findings are consistent with the bacterial spiked samples experiments discussed in Section 5.3.1.

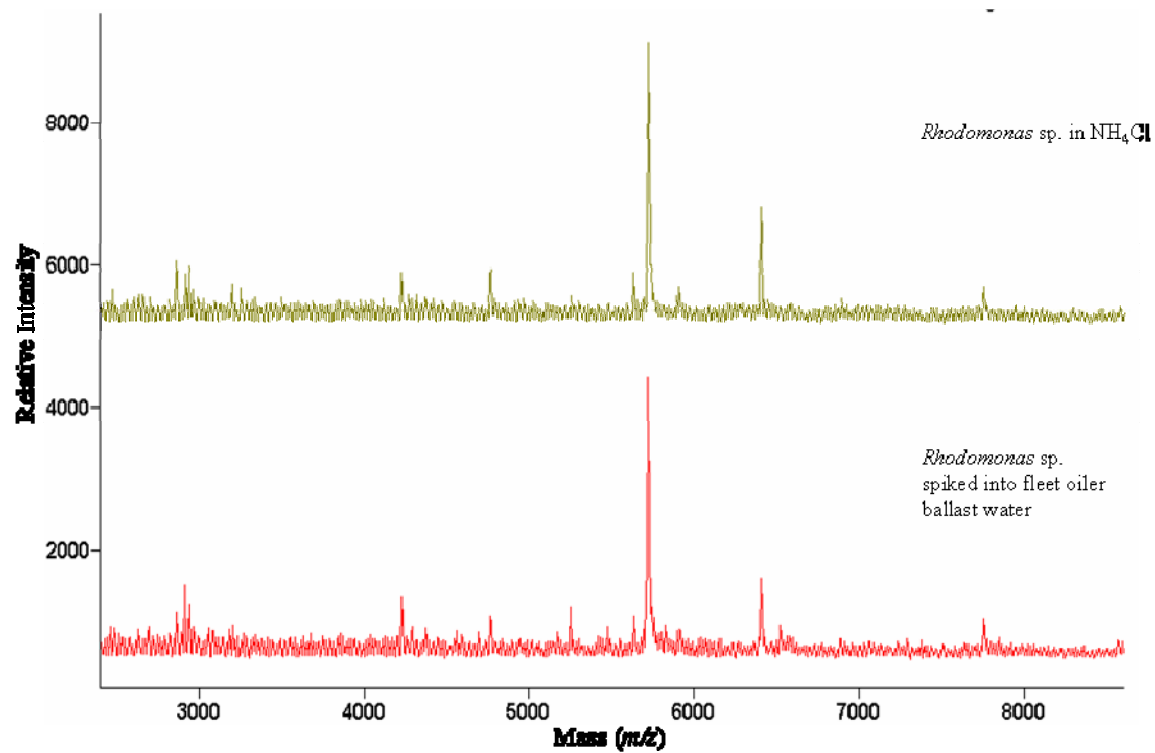


Figure 10 MALDI-MS of *Rhodomonas* sp. in ammonium chloride compared to *Rhodomonas* sp. spiked into ballast water

## 5.0 Conclusions

This “proof-of-concept” study provided the first indication that identification and screening of phytoplankton is feasible with MALDI-MS. Preliminary results from the MALDI-MS analysis of several different phytoplankton genera and species were obtained during this “proof-of-concept” study and indicate that ions representative of different phytoplankton classes are observed by MALDI-MS. It was determined that minimal sample preparation may be necessary for this analysis and that sample concentration via centrifugation was sufficient to concentrate and prepare most of the samples tested prior to MALDI-MS analysis. In addition, it was demonstrated that MALDI-MS analysis of phytoplankton can be successful in the presence of background ballast water with minimal sample preparation. This is particularly encouraging for the ultimate goal of detecting phytoplankton and other non-indigenous species in ballast water. Increased sensitivity with new MALDI mass spectrometer and tandem mass spectrometry capability for additional structural information of ion identification is now available and would increase sensitivity of the analysis at least 10 fold. In future research activities, additional samples and replication are needed to ensure ions observed are unique for identification of phytoplankton to genus and species level. Verification of the identity of some of the key ions of interest for the different species is also important during the next phase of research.

Since the time of initiation of this project, several other researchers have started to evaluate the use of MALDI-MS for phytoplankton and toxin analysis. Sleno and Volmer (2005) used MALDI-MS to quantify the amount of spirolide toxins present in a phytoplankton sample. Others have used MALDI-MS for the proteomic study of phytoplankton involved in harmful algal blooms (Chan et al. 2004, Chan et al. 2005) and the differentiation between toxic and non-toxic strains. These studies indicate an interest in mass spectrometric methods for phytoplankton research at the fundamental level, but to date, this is the first study where MALDI-MS analysis of phytoplankton has been directly applied to ballast water research.

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## APPENDIX A

Table A.1 contains a preliminary list of  $m/z$  values for ions observed from the MALDI-MS analysis of phytoplankton. These ions were from automated peak detection and extraction algorithms developed at PNNL primarily for bacterial analysis. This is the first step in developing a list of signature ions for the different species of phytoplankton. Some ions are close in  $m/z$  and with more replicate culturing and MALDI-MS analysis this list of ions will become more robust as to the unique ions for different species. This data should not be used at this time to differentiate different phytoplankton and is only presented as preliminary information.

**Table A.1 List of  $m/z$  Values of MALDI-MS Analysis of Eight Different Phytoplankton Species. Colors denote date of MALDI-MS analysis.**

| <i>P. australis</i> | <i>P. pseudodelicat.</i> | <i>P. multiseriis</i> | <i>Rhodomonas</i> | <i>Isochrysis</i> | <i>Gymnod. cysts</i> | <i>G. caten. veg P/N</i> | <i>G. caten. Veg P/S</i> |
|---------------------|--------------------------|-----------------------|-------------------|-------------------|----------------------|--------------------------|--------------------------|
|                     |                          |                       |                   |                   | 843                  |                          |                          |
| 919                 |                          |                       |                   |                   |                      |                          |                          |
| 990                 |                          | 991                   |                   |                   | 994                  |                          |                          |
|                     | 1016                     |                       |                   |                   |                      |                          |                          |
|                     | 1039                     |                       |                   |                   |                      |                          |                          |
|                     |                          |                       |                   |                   |                      |                          | 1065                     |
|                     | 1105                     |                       |                   |                   |                      |                          |                          |
| 1138/1140           | 1141                     |                       |                   |                   | 1144                 |                          |                          |
| 1166                |                          |                       |                   |                   |                      |                          |                          |
| 1185                | 1185                     |                       |                   |                   |                      |                          |                          |
|                     | 1233                     |                       |                   |                   |                      |                          |                          |
|                     | 1273                     |                       |                   |                   |                      |                          |                          |
| 1290/1297           | 1296/1297                |                       |                   | 1304              | 1301                 |                          |                          |
| 1331                | 1321                     |                       |                   |                   |                      |                          |                          |
|                     | 1342                     |                       |                   |                   |                      |                          |                          |
| 1446                | 1446                     |                       |                   |                   |                      |                          |                          |
|                     | 1464                     |                       |                   |                   |                      |                          |                          |
| 1565                |                          |                       |                   |                   |                      |                          |                          |
| 1591                |                          |                       |                   |                   |                      |                          |                          |
|                     |                          |                       | 1625              |                   |                      |                          |                          |
| 1849                |                          |                       |                   |                   |                      |                          |                          |
|                     |                          |                       |                   | 1933              | 1937                 |                          |                          |
|                     |                          |                       |                   |                   |                      |                          |                          |
|                     |                          |                       |                   | 1942              |                      |                          |                          |
|                     |                          |                       |                   |                   |                      | 2093                     |                          |
| 2180                |                          |                       |                   |                   |                      |                          |                          |
| 2364                |                          |                       |                   |                   |                      |                          |                          |
|                     |                          |                       |                   |                   | 2455                 |                          |                          |
|                     |                          |                       |                   |                   |                      | 2934                     | 2934                     |
|                     |                          |                       | 2942              |                   |                      |                          |                          |
|                     |                          |                       |                   |                   | 2969                 |                          |                          |
|                     |                          |                       |                   |                   | 2985                 |                          |                          |
|                     |                          |                       |                   | 3022              |                      |                          |                          |
|                     |                          |                       |                   | 3180              |                      |                          |                          |
|                     |                          |                       |                   | 3197              |                      |                          |                          |
|                     |                          |                       |                   | 3226              |                      |                          |                          |
|                     |                          |                       |                   | 3301              |                      |                          |                          |
|                     |                          |                       |                   |                   | 3372                 |                          |                          |
|                     |                          |                       |                   |                   |                      | 3393                     |                          |
|                     |                          |                       |                   |                   | 3418                 |                          |                          |

|           |                         |  |      |           |      |      |      |
|-----------|-------------------------|--|------|-----------|------|------|------|
| 3498      |                         |  |      | 3498      |      |      |      |
|           |                         |  |      |           | 3551 |      |      |
|           |                         |  |      |           | 3568 |      |      |
| 3569      |                         |  |      |           | 3568 |      |      |
|           |                         |  |      |           |      | 3617 |      |
|           |                         |  |      |           | 3756 |      |      |
|           |                         |  |      |           | 3809 |      |      |
| 3821      |                         |  |      |           |      |      |      |
|           | 3862                    |  | 3853 |           |      |      |      |
|           |                         |  |      |           |      | 3984 |      |
|           | 4066                    |  |      |           |      |      |      |
| 4103      |                         |  |      |           |      | 4098 | 4107 |
|           | 4119                    |  |      |           |      |      |      |
| 4204      |                         |  |      |           |      |      |      |
|           |                         |  | 4228 |           |      |      |      |
| 4277      |                         |  |      |           |      |      |      |
|           | 4312                    |  |      |           |      |      |      |
|           |                         |  |      |           |      | 4347 |      |
| 4405      | 4403                    |  |      |           |      |      |      |
|           | 4514                    |  |      |           |      |      |      |
|           | 4532                    |  |      |           |      |      |      |
| 4547      |                         |  |      |           |      |      |      |
| 4594/4583 |                         |  |      |           |      |      |      |
|           |                         |  |      |           |      | 4635 |      |
|           | 4689                    |  |      |           |      |      |      |
|           |                         |  | 4766 |           |      |      |      |
|           | 4789                    |  |      |           |      | 4787 |      |
|           | 4863                    |  |      |           |      |      |      |
|           |                         |  |      | 4901      |      |      |      |
|           | 5012                    |  |      |           |      |      |      |
|           |                         |  |      |           |      | 5024 |      |
| 5118      |                         |  |      |           |      |      |      |
|           | 5132                    |  |      |           |      |      |      |
|           | 5143                    |  |      |           |      |      |      |
|           | 5189                    |  |      |           |      |      |      |
| 5207      |                         |  |      |           |      |      |      |
|           |                         |  |      |           |      | 5253 |      |
|           | 5269                    |  |      |           |      |      |      |
| 5314      |                         |  |      |           |      | 5305 |      |
| 5431      |                         |  |      |           |      |      |      |
| 5573      |                         |  |      |           |      |      |      |
|           |                         |  | 5633 |           |      |      |      |
|           |                         |  | 5720 |           |      |      |      |
|           |                         |  | 5733 | 5738/5732 |      |      |      |
|           |                         |  |      |           |      |      | 5761 |
|           | 5815                    |  |      |           |      |      |      |
| 5879      | 5879                    |  |      |           |      |      |      |
|           |                         |  | 5910 |           | 5914 |      |      |
|           |                         |  |      |           | 5924 |      |      |
|           |                         |  |      |           | 5932 |      |      |
|           | 6027                    |  |      |           |      |      |      |
|           |                         |  | 6062 |           |      |      |      |
| 6083      |                         |  |      |           |      |      |      |
| 6110      |                         |  |      |           |      |      |      |
|           | 6137                    |  |      |           |      |      |      |
|           |                         |  | 6183 | 6184      |      |      |      |
| 6202      |                         |  |      |           |      |      |      |
|           | 6260/6263/<br>6260/6267 |  |      |           |      |      |      |

|           |                         |  |      |       |      |       |       |
|-----------|-------------------------|--|------|-------|------|-------|-------|
| 6293      |                         |  |      |       |      |       |       |
|           |                         |  | 6321 |       | 6324 |       |       |
| 6337      |                         |  |      |       |      |       |       |
| 6400      | 6400                    |  | 6405 |       |      |       |       |
| 6535      |                         |  |      |       |      |       |       |
|           |                         |  |      |       |      |       | 6625  |
| 6667      |                         |  |      |       |      | 6669  | 6670  |
|           |                         |  |      |       |      |       | 6678  |
| 6706      | 6706                    |  |      |       |      |       |       |
| 6783      |                         |  |      |       |      |       |       |
|           |                         |  | 6890 |       |      |       |       |
|           | 6936                    |  |      |       |      |       |       |
|           | 7095                    |  |      |       |      |       |       |
| 7149      |                         |  |      |       |      |       |       |
| 7165      |                         |  |      |       |      |       |       |
| 7186/7190 | 7178/7187/<br>7173/7191 |  |      |       |      |       |       |
| 7472      |                         |  |      |       |      |       |       |
| 7570/7562 |                         |  |      |       |      |       |       |
| 7576      |                         |  |      |       |      |       |       |
|           |                         |  | 7757 |       |      |       |       |
| 8089      | 8089                    |  |      |       |      |       |       |
|           |                         |  |      |       |      |       | 8396  |
|           |                         |  |      |       |      | 8514  |       |
|           |                         |  | 8574 | 8576  |      |       |       |
|           |                         |  | 8630 |       |      |       |       |
|           | 8668                    |  |      |       |      |       |       |
|           |                         |  |      |       | 8854 |       |       |
|           |                         |  |      |       |      |       | 8902  |
|           |                         |  |      |       |      | 8924  | 8921  |
|           |                         |  | 8994 |       |      |       |       |
|           |                         |  |      |       |      | 9204  | 9204  |
|           |                         |  |      |       |      | 9332  |       |
|           |                         |  |      |       |      |       | 9343  |
| 9574      |                         |  |      |       |      |       |       |
|           |                         |  |      |       |      | 9604  | 9610  |
|           |                         |  |      |       |      | 9815  | 9816  |
|           |                         |  |      |       |      | 10052 |       |
|           |                         |  |      |       |      | 10454 |       |
|           |                         |  |      |       |      |       | 10684 |
|           |                         |  |      | 11268 |      | 11259 |       |
|           |                         |  |      |       |      | 11827 | 11820 |

#### Dates of MALDI-MS Data Collection

10/24/02, 10/28/02, 11/02 (ACHC matrix used)

All other data collected with Ferulic Acid matrix)

2/11/03, 2/28/03

2/21/03,

2/28/03

3/17/03

4/29/03