Title: Biochemical Composition of Dissolved Organic Matter Released During Experimental Diatom Blooms

Popular Summary:

Experiments were conducted with a common and dominant algae (the diatom *Skeletonema costatum*) from the mid-Atlantic coast. A bacteria-free culture of the algae was grown in the laboratory to study the size and chemical composition of the algal-excreted dissolved organic matter (carbohydrates, proteins and fats) in the absence of actively growing bacteria. A second culture was grown in a large outdoor tank and then placed in darkness for a period of 51 days to examine the impact of the algal bloom and bacterial decomposition on dissolved and particulate organic composition. Because bacteria play an important role in consuming and changing the material released by algae, the growth of algae was examined in the presence and absence of bacteria. Samples from each experiment were collected and filtered to obtain dissolved organic matter (DOM) which was subsequently separated into three nominal size fractions: LDOM (low), HDOM (high) and VHDOM (very high). In each experiment, the algae excreted 28-33% of primary production as dissolved organic carbon (DOC). In the bacteria-free culture, HDOM and LDOM each comprised about half of the algal-excreted DOC with <1% as VHDOM. Algae from both experiments released carbohydrate-rich DOM. Most of the DOM released from the bacteria-free culture was identified (61% of HDOM and 78% of VHDOM), consisting primarily of carbohydrates (45% of HDOM and 55% of VHDOM). Substantial amounts of protein (16% of HDOM and 22% of VHDOM) and small amounts of fats (<1%) were also released. The presence of bacterial fats and bacterial cell wall compounds within HDOM and VHDOM from the outdoor tank experiment revealed that bacteria contributed a variety of molecules to DOM during the growth and decay of the algal bloom. Release of significant amounts of DOC by algae demonstrates that DOM excretion is an important component of algal primary production and thus the global carbon cycle.

Significant findings:

The diatom, *Skeletonema costatum*, which is the dominant algal species in the mid-Atlantic coastal ocean and the Chesapeake Bay and Delaware Bay excreted up to 33% of its production as dissolved organic matter (DOM). Bacteria rapidly decomposed algal material with most of the algal remains found as dissolved organic matter. Bacteria impacted the size and composition of algal-released DOM and also contributed to the DOM compounds examined during the growth and decay of the diatom blooms. The study demonstrated that primary production would be substantially underestimated if extracellular release of DOM by algae is not taken into account. The fact that algal material in the form of DOM persists after 50 days of microbial decomposition illustrates the importance of algal-derived DOM to the ocean's carbon cycle and its possible role in global climate change.

Biochemical Composition of Dissolved Organic Matter Released During Experimental Diatom Blooms

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Abstract

An axenic culture of *Skeletonema costatum* was grown to late-log phase to examine the molecular weight distribution and the biochemical composition of high molecular weight dissolved organic matter released in the absence of actively growing bacteria. A second culture was grown in a 5 m^3 mesocosm and placed in darkness for a period of 51 days to examine the impact of phytoplankton bloom dynamics and microbial decomposition on dissolved (DOM) and particulate organic matter (POM) composition. DOM was separated using tangential-flow ultrafiltration into three nominal size fractions: LDOM (< 1 kDa DOM), HDOM (1-30 kDa) and VHDOM (30 kDa-0.2 μm) and characterized. Both axenic and mesocosm diatom blooms released 28-33% of net primary production as dissolved organic carbon (DOC). In the axenic culture, HDOM and LDOM each comprised about half of the diatom-released DOC with <1% as VHDOM. Diatoms from both experiments released carbohydrate-rich high molecular weight DOM. Much of the axenic diatom-released high molecular weight DOC could be chemically characterized (61% of HDOM and 78% of VHDOM) with carbohydrates as the primary component (45% of HDOM and 55% of VHDOM). Substantial amounts of hydrolyzable amino acids (16% of HDOM and 22% of VHDOM) and small amounts of lipids (<1%) were also released. Proportions of recognizable biochemical components in DOM produced in the mesocosm bloom were lower compared to the axenic culture. The presence of bacterial fatty acids and peptidoglycan-derived D-amino acids within high molecular weight fractions from

the mesocosm bloom revealed that bacteria contributed a variety of macromolecules to DOM during the growth and decay of the diatom bloom. Release of significant amounts of DOC by diatoms demonstrates that DOM excretion is an important component of phytoplankton primary production. Similarities in high molecular weight DOM composition in marine waters and diatom cultures highlight the importance of phytoplankton to DOM composition in the ocean.

Keywords: dissolved organic matter, phytoplankton extracellular release, diatoms, D-amino acids, bacteria, lipids

1. Introduction

Primary production is the principal source of dissolved organic matter (DOM) to aquatic systems either directly through extracellular release by phytoplankton or indirectly through sloppy feeding by algal grazers, lysis of phytoplankton cells, microbial hydrolysis, and the physical transport of allochthonous organic matter. The release of DOM by phytoplankton accounts for ~12% of the total carbon fixed (range of 2-50%), with the amount of extracellular DOM increasing with particulate primary productivity (Baines and Pace, 1991 and references therein). As a result, primary production can be substantially underestimated if extracellular release by phytoplankton is not taken into account (e.g. Karl et al., 1998). Despite the importance of this fraction, the composition and distribution of biochemical components released by phytoplankton remain poorly defined. Experiments with algal cultures and field measurements indicate that phytoplankton release a variety of organic molecules which include polysaccharides (Ittekkot et al., 1981; Biddanda and Benner, 1997), proteins (Hellebust, 1965) and small molecules such as free saccharides (Ittekkot et al., 1981; Biddanda and Benner, 1997), free amino acids (Sellner, 1981) and glycolic acid (Hellebust, 1965). Previous research also suggests that phytoplankton release predominantly low molecular weight compounds (<1000 Daltons; e.g., Jensen, 1983; Lancelot, 1984; Biddanda and Benner, 1997), but comparative analyses of the molecular weight range and biochemical composition of released DOM are scant.

Given that 40 to 60% of primary production may be cycled through bacteria (Cole et al., 1988; Hoch and Kirchman, 1993), including DOC released by algae (Chrost and Faust, 1983; Jensen, 1983), microbial processes likely have a dramatic affect on DOM composition. Bacterial assimilation of excreted DOM occurs rapidly, with much of the DOC taken up within a few hours to several days (Lancelot, 1979; Amon and Benner, 1994; Chen and Wangersky, 1996; Cherrier et al., 1996). Because only small compounds (<600 Da) can be transported directly through bacterial cell membranes (Gottschalk, 1986), the most labile components of DOM are those compounds which can be taken up without hydrolysis, such as glucose and dissolved free amino acids (DFAA). The rapid turnover times for glucose (1.8 to 20 hours; Rich et al, 1996; Keil and Kirchman, 1999; Skoog et al., 1999) and DFAA (<0.5 to 26 hours; Fuhrman, 1987; Coffin, 1989; Keil and Kirchman, 1999) is reflected in their low concentrations typically observed in natural waters. Because hydrolysis of dissolved polysaccharides is considered the major source of free glucose (Ittekkot et al., 1981; Skoog et al., 1999), high molecular weight DOM can support much of the bacterial carbon demand. Incubations of bacteria with size-fractionated DOM from diverse environments revealed consistently greater bacterial utilization rates, growth rates and respiration rates for >1 kDa DOM (UDOM) than <1 kDa DOM (Amon and Benner, 1996), supporting the hypothesis that more labile DOM is found in UDOM, excluding highly labile DFAA and monosaccharides which had likely been utilized prior to experimentation. Because bacteria rapidly utilize algal-

excreted DOM (Lancelot, 1979; Amon and Benner, 1994; Chen and Wangersky, 1996; Cherrier et al., 1996), the actual amount of DOC released and its composition must be examined in the absence of actively growing bacteria and grazers. Protozoan and metazoan grazing activities also release substantial amounts of DOC (see review by Nagata, 2000).

As the major primary producers in coastal environments, diatoms release substantial portions of primary production as DOM (24-32% of total organic carbon; Jensen, 1983; Biddanda and Benner, 1997). *Skeletonema costatum* represents the dominant bloom forming diatom in U.S. mid-Atlantic coastal waters (Pennock and Sharp, 1986; Marshall and Alden, 1993). To determine the molecular weight distribution and biochemical composition of DOM released by algal blooms prior to bacterial consumption, *S. costatum* was grown within an aseptic large volume culture to late-log phase and sampled over time. The impact of phytoplankton bloom dynamics, with accompanying microbial decomposition, on high molecular weight DOM and POM composition was examined with a mesocosm experiment with *S. costatum* to simulate the growth and decay of a diatom bloom. For the mesocosm, DOM and POM were collected during exponential growth, stationary phase, and at several time points as the phytoplankton decayed. The goals were twofold. First, to provide comprehensive information on the chemical composition of high molecular weight DOM produced in coastal waters by diatoms prior to bacterial modification. Secondly, to follow the production and removal of macromolecules with moderate turnover

rates and thus the impact of microbial processing on the chemical signature of DOM. Such experiments can enhance our understanding of DOM and POM cycling within algal blooms, especially the changes in biochemical composition and retention time of algal organic carbon in the marine water column.

2. Methods

2.1. Axenic Culture

The axenic culture of *Skeletonema costatum* (CCMP1332) was grown over a 14 day period in a 42 L glass vessel containing f/2 nutrient media plus Si and diluted seawater which was ultrafiltered prior to inoculation to remove DOM >1 kDa. The sterile estuarine media was prepared with filtered (0.2 μm filter) coastal seawater (30 psu) which was then diluted to 15 psu with UV-oxidized Nanopure water, ultrafiltered to remove the >1 kDa nominal fraction and autoclaved. The culture was grown under a 12 h light:12 h dark cycle over 14 days to a cell density of 7.6×10^8 cells L^{-1}. The culture was aerated with sterile air and maintained in suspension by gentle mixing with a magnetic stirrer. Samples for particulate organic carbon (POC) and nitrogen (PN) and DOC analysis were collected at various time points during the growth of the diatom culture. DOC samples were obtained by filtering whole water through GF/F filters and also from 0.2 μm filters (Mannino and Harvey, 1999). The entire culture was filtered during late log phase (day 14 of growth) for DOM and POM chemical characterization. Bacterial abundances were monitored by epifluorescence microscopy using

acridine orange (Hobbie et al., 1977). Low numbers of bacteria ($<4x10^6$ cells L^{-1}) were observed when the entire culture was sampled, an abundance 1000 to 10,000 times less than typically seen in coastal waters (e.g. Hoch and Kirchman, 1993). Such low abundances of bacteria in the absence of bacterivores but within a nutrient-rich media suggests that bacterial activity was minimal. Hence, the culture is referred to as "axenic" for the purpose of our discussion.

2.2. Mesocosm culture

An outdoor fiberglass mesocosm measuring 5 m^3 (2.44 m by 1.07 m depth) was filled with coastal seawater (30.6 ‰) and allowed to settle for six days. This water was filtered (30 μm) into to a second pre-cleaned mesocosm (~5000 L) and diluted to 20.2‰ with filtered groundwater. Nutrients were added to the mesocosm corresponding to concentrations typically observed during spring in the Delaware estuary where *S. costatum* grows (e.g. Sharp, 1995), ~100 μM nitrate (42.5 g $NaNO_3$), ~50 μM silicate (75 g Na_2SiO_2) and ~1.25 μM phosphate (0.87 g NaH_2PO_4) prior to inoculation of the seed cultures. Additional nutrients were present in seawater and the inoculum. A series of *S. costatum* cultures were grown in the laboratory to provide a sufficiently large inoculum (~320 L) for rapid growth within the mesocosm. Initial cultures of *S. costatum* were grown in filtered (0.2 μm) and diluted seawater (20 psu) amended with f/2 nutrient media plus Si and Se. Phosphate limitation in the mesocosm was a likely consequence since N:P of added nutrients exceeded the Redfield ratio. The mesocosm was

screened to reduce photoinhibition and potential contamination from large airborne particles and animals. Maximum light level of 1270 $\mu E\ m^{-2}\ s^{-1}$ was measured in surface waters (190 $\mu E\ m^{-2}\ s^{-1}$ at the bottom) at noon on a sunny day in late September (10 days prior to inoculation) of the screened and water-filled mesocosm.

Two 25.4 cm air stones were used to aerate and maintain the culture in suspension. Samples for bulk measurements including POC, PN and DOC. Chlorophyll, algal and bacterial abundances were also collected frequently during the growth of the diatom bloom to monitor growth status of the culture. Once the culture reached late stationary phase (day 11 of growth), the mesocosm was covered the to prevent further photosynthetic activity and the diatom bloom permitted to decay for 51 days. Large volume samples for ultrafiltration of DOM and for POM filtration were collected one day prior to mesocosm inoculation (day -1), during exponential growth (day 6), during late stationary phase (day 11), and during the 51 day decay sequence.

2.3. Sample Collection, Microscopy and Elemental Analyses

Phytoplankton abundance was determined by light microscopy on 1% lugol-stained samples using a hemocytometer. Water samples were filtered through 24 mm GF/F for chlorophyll *a* determination. Chlorophyll *a* was extracted with 90% acetone for 24 hours in the dark at 4°C and quantified by absorbance measurements (Jeffrey and Humphrey, 1975). Bacteria preserved in

1% gluteraldehyde were filtered onto black 0.2 μm Osmonics polycarbonate filters and enumerated by epifluorescence microscopy using DAPI (Porter and Feig, 1980; Turley, 1993).

Large volume water samples were initially filtered through 3 μm and 0.2 μm cartridge filters to remove particles. The DOM filtrate was then separated into three nominal size fractions: 30 kDa-0.2μm (VHDOM; Very High Molecular Weight DOM), 1-30 kDa (HDOM; High Molecular Weight DOM) and <1 kDa (LDOM; Low Molecular Weight DOM) by sequential filtration and desalting using an Amicon DC-10L tangential flow ultrafiltration unit as previously described (Mannino and Harvey 1999, 2000). Samples for DOC analysis were collected from the <0.2 μm filtrate and each DOM size fraction, and analyzed in triplicate (standard deviations ≤5%) by high temperature combustion using a Shimadzu TOC 5000 (Benner and Strom, 1993). Summation of the three DOC size fractions generally accounted for 90-103% of total DOC, which compared well to previous results using similar instrumentation and procedures (81-128% DOC; Guo and Santschi, 1996; Benner et al., 1997; Mannino and Harvey, 1999). Additional DOC samples were collected at time points between large volume sampling and processed by filtering whole water through pre-combusted GF/F filters. Variability between 0.2 μm filtered and GF/F filtered DOC measurements was <3%. Total dissolved nitrogen, nitrate+nitrite, nitrite and ammonium, were measured for total DOM and each dissolved size fraction solely from the axenic culture using standard protocols (D'Elia et al., 1997).

Remaining HDOM and VHDOM concentrates were stored frozen and concentrated further by rotary evaporation and subsequently lyophilized to dry powders. Carbon and nitrogen content was measured using an Exeter Analytical CHN analyzer from GF/F filters for POM and on vapor acidified lyophilized DOM (Hedges and Stern, 1984). Because of the high levels of DOC present within the LDOM fraction of the seawater and F/2 nutrient media (267 μM C for axenic culture and 82 μM C in the mesocosm), LDOM was analyzed for DOC only, and no further characterization was made on the LDOM fraction.

2.4. Lipids

Lipids from the HDOM and VHDOM subsamples (~2 mg of organic carbon) were extracted by sonication (15 min) in a mixture of dichloromethane:methanol (1:1) followed by overnight extraction at 4°C, similar to that previously described (Mannino and Harvey, 1999). Internal standards for polar and neutral fractions (nonadecanoic acid and 5α-cholestane) were added to lipid extracts. Extraction was repeated twice, and the solvents combined. Particulate lipids were extracted from filters with 15 ml of dichloromethane:methanol three times and processed similarly to DOM fractions. Procedural blanks were processed simultaneously with samples. Small amounts of contaminants found in the procedural blanks (e.g., 14:0, 16:0 and 18:0 fatty acids) were subtracted from the respective samples. Neutral and polar lipids were quantified by capillary gas chromatography (DB-5MS, 60 m column, 0.32 mm

internal diameter, 0.25 μm film thickness; J & W Scientific, Inc.) with flame ionization detection and hydrogen as the carrier gas (2 ml min^{-1}) and a temperature ramp of 10°C min^{-1} from 50°C to 120°C and 4°C min^{-1} thereafter to 300°C (isothermal for 5 or 15 minutes for polar and neutral fractions, respectively). Structural identification was made utilizing gas chromatography-mass spectrometry (HP-5890-II GC coupled to a HP-5970B) operating at 70 eV with mass range acquisition of 50-600 amu using a similar column, with helium as the carrier gas and temperature program as described previously. Identifications of select compounds were confirmed by GC-MS using positive chemical ionization with CH_4 as the ionizing gas (HP-6890 GC coupled to a 5973N mass selective detector). Double bond positions of monounsaturated fatty acids were determined by GC-MS analysis of dimethyl disulfide adducts following the protocol described by Nichols et al. (1986). Analytical variability of lipids were based on two arbitrary sets of duplicates for each of the three sample sets (POM, VHDOM and HDOM) and were generally <25%.

2.5. Carbohydrates

Polysaccharide content was measured using a modification of the MBTH method (Pakulski and Benner, 1992; Mannino and Harvey, 2000). Duplicate subsamples of lyophilized DOM or dried filters for POM (select duplicates) were acidified in 50-ml serum vials with 1 ml of 12 M H_2SO_4 for 2 hours at room temperature, diluted with 9 ml of deionized water and hydrolyzed at 100°C for 3

hours. After cooling, the pH of the hydrolysis solution was neutralized with NaOH. Triplicate aliquots of hydrolysis products (and triplicate blanks) were placed in test tubes, and the aldehydes were reduced to their respective alditols, oxidized to formaldehyde and reacted with MBTH. Glucose standard curves were generated concurrently, and total carbohydrate concentration was calculated as glucose equivalents. Coefficients of variation for carbohydrate measurements were <9% for POM and VHDOM samples and <5% for HDOM samples.

2.6. Total Hydrolyzable Amino Acids

Duplicates of lyophilized DOM, dried POM or culture subsamples were hydrolyzed with 1 ml of 6 N sequanal-grade HCl at 150°C for 2 hours (modified from Cowie and Hedges, 1992a; Mannino and Harvey, 2000). Hydrolyzed amino acids were derivatized to form their respective trifluoroacetyl isopropyl esters following the protocol described by Silfer et al. (1991). THAA were analyzed by capillary gas chromatography with flame ionization detection (GC-FID; HP-5890II) using a 60 m DB-5MS column (0.32 mm I.D., 0.25 μm film thickness). Hydrogen served as the carrier gas (2 ml min^{-1}), and a temperature program of 10°C min^{-1} from 50°C to 85°C followed by 3.5°C min^{-1} to 200°C and 10°C min^{-1} to 280°C was used. Individual amino acid standards and mixtures of amino acids were derivatized as described above and analyzed by GC-FID and GC-MS (HP-5890II GC coupled to a HP-5970B MSD) in parallel for confirmation of amino acid identity in DOM and POM samples. Helium served as the carrier gas for

GC-MS, and the temperature program above was used. The MSD was operated in electron impact mode at 70 eV with acquisition over 50-600 a.m.u. range. L-γ-methyl-leucine or N-methyl-L-leucine was added to samples prior to hydrolysis to serve as an internal standard for THAA quantitation. Reagent blanks processed simultaneously with each sample group indicated no contamination from reagents or handling. Responses of individual amino acids relative to the internal standards were corrected using commercially available amino acid mixtures. Analytical variability was <6% for total amino acids within each size fraction.

Enantiomeric ratios (D/L) for derivatized THAA were analyzed by GC-FID and GC-MS using a 50 m Chirasil-Val column (Alltech; 0.25 mm I.D., 0.16 μm film thickness) and a temperature program of 15°C min^{-1} from 45°C to 70°C followed by 2°C min^{-1} to 120°C, then 3°C min^{-1} to 200°C and maintained at 200°C for 15 min (Silfer et al., 1991). Lysozyme and RuBisCo proteins were hydrolyzed to free amino acids and derivatized as described above to monitor racemization due to acid hydrolysis.

3. Results

3.1. Diatom production and DOC release

During log-phase growth, the axenic *Skeletonema costatum* culture increased from 78.9×10^6 cells L^{-1} to 757×10^6 cells L^{-1} between days 7 and 14 of growth (Fig. 1A). Growth rate of diatoms was highest between days 7 and 8 (1.25

d^{-1}) and slowed to 0.074 d^{-1} during late-log phase (day 12 to 14). Chlorophyll *a* attained a maximum concentration of 303 μg L^{-1} on day 14. Algal excreted DOC increased in concentration rapidly during growth, reaching 349 μM C by day 14 (Fig. 1A). Although maximal cellular carbon production rates were seen during the early-log phase (days 7 to 9), the release rate of DOC per diatom was highest late in the growth period at 0.2 pmol DOC $cell^{-1}$. Diatoms from the axenic culture released DOC at a rate of 7.5 to 59 μM C d^{-1} from days 7 to 8 and days 9 to 12 of growth, respectively. Between days 9 and 14 of growth, the DOC released accounted for 44-48% of total organic carbon (TOC) produced. Bacteria were virtually absent (<$4x10^6$ cells L^{-1}; 1 bacterium per 200 diatoms) in the axenic culture on day 14.

In the outdoor mesocosm, *S. costatum* density increased more slowly than in the axenic culture, from $75.3x10^6$ cells L^{-1} after inoculation (day 0) to 395 x 10^6 cells L^{-1} on day 5 (Fig. 1B). Chlorophyll *a* attained a maximum concentration of 221 μg L^{-1} on day 5, and diatoms exhibited slower growth rates thereafter (0.26 d^{-1} from days 0 to 6). Bacterial abundance initially paralleled primary production within the mesocosm, reaching a peak of $22.9x10^9$ bacterial cells L^{-1} on day 6, but declined much more rapidly than POC between days 6 and 11 ($3.4x10^9$ cells L^{-1}; Fig. 2). Despite the high bacterial numbers, a substantial amount of net DOC was released in the mesocosm culture, especially within the first six days of growth. DOC peaked on day 5 (352 μM C) and declined to 225 μM C by day 62. The most rapid release of DOC occurred during exponential growth of *S. costatum*

between days 3 and 5 (116 μM C), resulting in a net release of 58 μM DOC day^{-1}, comparable to the 51 μM DOC day^{-1} released by the axenic culture. Despite the large amount of cells produced between days 0 and 3, DOC declined by 28 μM C and can be attributed to microbial activity (Fig. 2). The proportion of net DOC released to net TOC produced ranged from 9.5% (day 3) to 52% (day 9; 36% days 5-6) in the mesocosm bloom which is comparable to the DOC released in the axenic culture. Beyond day 14, a net loss of DOC was observed in the mesocosm bloom.

HDOM and LDOM comprised equivalent amounts of total DOC released by the axenic diatom culture with the VHDOM fraction contributing only 0.8% (2.64 μM C; Fig. 3). Molecular weight distributions of dissolved organic nitrogen (DON) were similar to DOC with HDOM and LDOM comprising 49.7% and 47.6% of total DON (2.8% for VHDOM; Table 1). Measurements of DON are based on the difference between total dissolved nitrogen and measurements of nitrate+nitrite, nitrite and ammonium, and are subject to higher analytical variability. Nevertheless, mass balances of the dissolved inorganic components and total dissolved nitrogen among size fractions suggest excellent recovery and separation by ultrafiltration, with the exception of ammonium due to its low concentration (Table 1).

In contrast to the axenic culture, HDOM comprised only 23% of the DOC released during diatom growth in the mesocosm and 7 to 11% of the total organic carbon produced. The amount of VHDOM released was considerably higher, with

the maximal net release of VHDOM-C (3.96 μM C) by day 14 (Fig. 3). LDOM released comprised only 12% of TOC in the axenic culture but 25-38% of TOC produced during growth in the mesocosm bloom. Concentrations for all three DOM fractions peaked after 3 days of darkness (day 14; Fig. 3) which is the approximate time of diatom cell death based on loss of RuBisCo activity once placed in darkness (Harvey et al. 1995).

3.2. Biochemical Composition of Diatoms and Excreted Macromolecular DOM

A smaller fraction of POC and DOC from the mesocosm bloom could be chemically characterized compared to the axenic culture. About 48% of the POC was identified from the mesocosm bloom, compared to 73% of POC from the axenic diatoms (Fig. 4). Much of the excreted high molecular weight DOC from the axenic diatom culture was characterized (61% of HDOM-C and 78% of VHDOM-C; Fig. 4). Carbohydrates and proteinaceous matter (THAA) were the major biochemical components in diatoms and in released high molecular weight DOM. Both DOM fractions were enriched in carbohydrates and depleted in THAA and lipids compared to particles. Although particulate carbohydrate content was similar, THAA and lipids comprised a smaller fraction of POC in the mesocosm bloom than in the axenic culture (each 9 to 15% less; Fig. 4). Axenic diatoms contained similar amounts of lipids (14%) and carbohydrates (17%) and over twice as much THAA (42%). In the mesocosm bloom, lipid and THAA content of POC increased during diatom growth and declined during decay. For

much of the mesocosm bloom, a greater proportion of VHDOM-C was characterized than POC or HDOM-C (52-73% of VHDOM-C; Fig. 4). Only 22 to 36% of HDOM-C (17% prior to inoculation) consisted of identifiable biochemical components during the growth and decay of the mesocosm bloom compared to over 60% for the axenic culture (Fig. 4). Carbohydrates comprised most of the identifiable HDOM-C (15-31%) followed by THAA (2-6%).

Distributions of carbohydrate, THAA and lipid revealed substantial differences in POM and high molecular weight DOM throughout the growth and decay of the mesocosm bloom (Fig. 5). Particles contained much higher concentrations of THAA than other components until day 41 when they equaled carbohydrates. This was not seen in DOM where carbohydrate concentrations were higher throughout the growth and decay period. Lipids remained low for all three size fractions, especially in DOM. Peak concentrations for all biochemical components were observed in particles on day 6, but differed for dissolved components. With the exception of carbohydrates in POM and HDOM, concentrations of all biochemical components were at least one order of magnitude greater in POM than either HDOM or VHDOM. A substantial amount of the carbohydrates observed in HDOM and VHDOM during the growth and decay period was present in the mesocosm prior to inoculation (Fig. 5). Nearly all of the POM produced was lost during decay (>90% of POC). The concentrations of carbohydrates and lipids in VHDOM actually increased between days 41 and day 62 which suggest a net release of these materials from decomposing particles.

Organic matter in the axenic culture was more abundant than in the mesocosm for both POM and HDOM but similar for VHDOM. Axenic cells contained over twice as much THAA (9806 $\mu g\ L^{-1}$) than carbohydrates (4423 $\mu g\ L^{-1}$) with lipids contributing 2113 $\mu g\ L^{-1}$. In contrast, the HDOM and VHDOM released in the axenic culture contained nearly three times as much carbohydrates (1958 and 43.5 $\mu g\ L^{-1}$, respectively) as THAA (675 and 16.3 $\mu g\ L^{-1}$) and much less lipid content (1 and 0.3 $\mu g\ L^{-1}$).

3.3. Total Hydrolyzable Amino Acids

Individual amino acids (on a mole% basis) remained relatively constant in POM, but varied for DOM fractions during the growth and decay of the mesocosm bloom (Table 2). Shifts in amino acid composition for all three size fractions were most apparent during log phase growth in the mesocosm (between days -1 and 6). As diatom decay progressed, alanine, glycine, threonine and serine comprised a greater portion of VHDOM-THAA than during growth, whereas aspartic acid and glutamic acid comprised a smaller portion (Table 2). The HDOM content of alanine and glycine also increased during the decay sequence, and glutamic acid declined. The non-protein amino acid β-alanine, found only in HDOM, increased on a mole% basis between days 6 to day 27 with lower amounts thereafter (Table 2).

Differences in THAA composition among size fractions was also evident throughout the growth and decay sequence. Particles were enriched in aromatic

and neutral amino acids (valine, leucine, isoleucine and proline) and depleted in basic, acidic and the neutral amino acids alanine and glycine (Table 2). VHDOM was most enriched in the hydroxyl amino acids (threonine and serine). The HDOM fraction was most deplete in aromatic and neutral amino acids, but enriched in alanine, glycine, acidic and basic amino acids (Table 2).

Bacteria and abiotic racemization are the major sources of D-amino acids to aquatic ecosystems (Lee and Bada, 1977). Enantiomeric ratios (D/L) for THAA from Lysozyme and RuBisCo were similar to values observed in POM, VHDOM and HDOM from the axenic *S. costatum* culture with the exception that in VHDOM D-valine and D-leucine were more abundant (Table 3; Fig. 6). Due to the virtual absence of bacteria in the axenic culture and the fact that abiotic racemization proceeds slowly under natural conditions (Bada, 1985), the similarity in D/L values between the reference proteins and THAA from all three size fractions of the axenic culture demonstrates that these ratios can be used as a measure of racemization due to analytical procedures (Fig. 6). D/L values of leucine in POM, VHDOM and HDOM from the mesocosm bloom were not much greater than the analytical racemization values (Table 3). With the exception of valine at day -1, glutamic acid, phenylalanine (POM only) and alanine, D/L values in POM and VHDOM were comparable to the analytical racemization values. In contrast, enantiomer ratios for amino acids in HDOM (except for leucine) from the mesocosm diatom experiment were well above the analytical racemization values indicating a bacterial contribution (Table 3; Fig. 7).

3.4. Lipids

Lipid classes for the axenic *S. costatum* culture and the mesocosm bloom differed among particles and dissolved fractions. Lipids in POM were 1 to 2 orders of magnitude higher than observed for VHDOM or HDOM with fatty acids as the major lipid component (Fig. 8). The VHDOM fraction showed a different pattern in lipid class distributions during the growth and decay of the mesocosm bloom (Fig. 8). On a carbon basis, POM was progressively depleted in all lipid classes after day 20 of the mesocosm decay.

Sterols and chlorophyll-derived phytol were the major components of neutral lipids from particles for both the axenic and mesocosm cultures. The two major diatom sterols, 24-methylcholesta-5,24(28)-dien-3β-ol ($28\Delta^{5,24(28)}$) and 24-methylcholest-5-en-3β-ol ($28\Delta^{5}$) dominated the composition of total sterols for the axenic diatoms and in POM throughout the growth and decay of the *S. costatum* mesocosm bloom (50% to 86% of total sterols; Table 4). Initially, $28\Delta^{5,24(28)}$ comprised only 6.7% of the sterols from POM present in the mesocosm prior to inoculation (day -1), with cholesterol as the dominant sterol (42%). Only trace amounts of sterols were observed in HDOM with the $28\Delta^{5,24(28)}$ and $28\Delta^{5}$ observed on day 6 of the mesocosm bloom. In contrast, sterols were an important component of VHDOM neutral lipids. Similar to POM, the $28\Delta^{5,24(28)}$ and $28\Delta^{5}$ were the dominant sterols for VHDOM in both cultures after growth commenced (42% to 83%; Table 4). Sterol composition shifted as the diatom bloom decayed with relatively smaller contributions of $28\Delta^{5,24(28)}$ and greater proportion of $28\Delta^{5,22}$

(Table 4).

Fatty acid composition differed between diatoms in the axenic culture and for POM throughout the growth and decay of the diatom bloom. The axenic culture was dominated by polyunsaturated fatty acids (PUFAs; 87 μg (mg POC)$^{-1}$; 51% of total fatty acids), primarily C_{16} PUFAs (25.7%) and the 20:5 acid (11.7%; Fig. 9). The 14:0 and 16:1$^{\Delta 9}$ acids were the most abundant saturated and monounsaturated acids for the axenic culture, respectively (21.6% and 14.8%). Bacterial fatty acids (saturated branched odd-chain and monounsaturated odd-chain acids; Kaneda, 1991) were not detected in POM or HDOM from the axenic culture and comprised 2% of the fatty acids in VHDOM (0.19 μg (mg OC)$^{-1}$; Fig. 9). Fatty acid composition for mesocosm POM on day 11 was most similar to the axenic culture except for high amounts of bacterial fatty acids (8.1%) and lower amounts of the 14:0 and 16:1$^{\Delta 9}$ acids (14% and 6.9%, respectively) in the mesocosm bloom.

During the initial growth period, mesocosm POM contained high amounts of monounsaturated acids (22.4 μg (mg POC)$^{-1}$), mostly 18:1$^{\Delta 11}$ and 16:1$^{\Delta 9}$. As the mesocosm bloom decayed, saturated and monounsaturated acids increased in contribution between day 11 and day 62, and PUFAs decreased substantially during that same period (Fig. 9). Saturated fatty acids, primarily the 16:0 acid, dominated the composition of dissolved fatty acids for both the axenic and mesocosm cultures (Fig. 9). Monounsaturated and polyunsaturated fatty acids were more abundant in VHDOM than HDOM, although PUFAs comprised <3%

of VHDOM fatty acids. The dominant monounsaturated acids included $16:1^{\Delta 9}$, $18:1^{\Delta 9}$ and $18:1^{\Delta 11}$ for both dissolved fractions. Bacterial fatty acids were found in VHDOM and HDOM with higher amounts on day 6 for HDOM (0.05 μg (mg OC)$^{-1}$) and day 11 for VHDOM (0.39 μg (mg OC)$^{-1}$; Fig. 9)).

4. Discussion

4.1. DOC Production

Experimental and field observations highlight the importance of diatoms in excreting substantial portions of primary production as DOM. In our experiments, diatom growth resulted in substantial release of DOC, equivalent to 28.5% of net primary production (POC + DOC) for the axenic *S. costatum* culture and 33% of net ecosystem production in the mesocosm bloom (day 6). The growth rates of our *S. costatum* cultures (0.32 d^{-1} for the axenic culture and 0.26 d^{-1} for the mesocosm bloom) were similar to growth measurements of *S. costatum* in previous laboratory (0.22 d^{-1}; Biddanda and Benner, 1997) and field experiments (0.6 d^{-1}; Eberlein et al., 1983). In previous experiments, the DOC released from various phytoplankton (*S. costatum*, *Phaeocystis sp.*, *Emiliania huxleyi* and *Synechococcus bacillaris*) comprised 10 to 32% of TOC with the highest release occurring within a culture of *S. costatum* (CCMP 775; Biddanda and Benner, 1997). A similar range was observed in other diatom cultures (24-32%) and during the collapse of a diatom bloom in the Randers Fjord (10-28%; Jensen, 1983). Such studies illustrate the significance of algal-released DOM to

both the physiology of phytoplankton and the carbon cycle of aquatic ecosystems. It also demonstrates that underestimation of primary production can be substantial if extracellular release of DOM by phytoplankton is not taken into account.

The mechanism for this DOM release is uncertain, particularly for macromolecular DOM. Previous research suggests that low molecular weight DOM constitutes the majority of DOC released during phytoplankton growth and decay (e.g. Jensen, 1983; Biddanda and Benner, 1997). In our axenic *S. costatum* culture, HDOM and LDOM accounted for equal portions of the total DOC and DON released. Bacterial utilization of HDOM within non-axenic blooms may explain the higher amounts of LDOM observed in natural phytoplankton blooms, our mesocosm bloom, and previously reported experiments. These results imply that microbial processing can rapidly alter the distribution of DOM after algal release.

4.2. Biochemical composition of diatoms and released DOM

Only 40-50% of mesocosm POC was amenable to chemical characterization as amino acids, carbohydrate or lipid compared to 73% for axenic diatoms, with lower amounts of each biochemical component observed as the mesocosm bloom progressed (Fig. 4). A similar reduction of structurally distinct compounds have been observed by Harvey et al (1995) under controlled degradation studies of algae by microbial consortia. Based on bacterial abundance alone (i.e. Lee and Fuhrman, 1987), bacteria were an important component of

POM in the mesocosm, comprising up to 13% of POC on day 6. Differences in fatty acid and THAA content between the axenic and mesocosm diatoms (Figs. 4 and 8) may be related to different nutrient conditions, physiological state of the algae and perhaps the presence of bacteria and protozoa. Perhaps the higher growth rate and richer nutrient conditions, especially phosphate, in the axenic culture promoted greater phospholipid content, even though sterol content was similar in both cultures (Table 4; Fig. 9). While fatty acid and sterol composition were similar between axenic diatoms and mesocosm POM on day 11, bacterial fatty acids were common in mesocosm particles (Table 4; Fig. 9).

Phytoplankton are a major source of dissolved polysaccharide in marine environments. Perhaps it should be no surprise that carbohydrates dominated the composition of VHDOM and HDOM in both *S. costatum* cultures, whereas POM contained mostly THAA; this implies that dissolved protein is degraded more rapidy than carbohydrates (Fig. 4). In a series of culture experiments with diverse phytoplankton species, carbohydrates comprised 23-80% of DOC (<10 to 35% for *S. costatum*) and 56-74% of >1 kDa DOM (74% for *S. costatum*) (Biddanda and Benner, 1997; Biersmith and Benner, 1998). Other culture experiments have also demonstrated that high molecular weight DOM excreted by *Thalassiosira weissflogii* and *E. huxleyi* is dominated by carbohydrates (~64% of C; Aluwihare and Repeta, 1999). Smith et al. (1995) have suggested that a portion of diatom excreted DOM arises from hydrolysis of algal surfaces by attached bacteria. This could explain the predominance of carbohydrates in seawater DOM (e.g. Benner

et al., 1992) since the mucus layer which surrounds the cell walls of diatoms and other phytoplankton are composed primarily of polysaccharide (Hoagland et al., 1993). A second source of carbohydrates to the high molecular weight DOM in our axenic *S. costatum* culture could be diatom excretion of polysaccharide or sloughing off of mucopolysaccharides surrounding the cell walls of diatoms. As diatoms shift from log phase growth to stationary phase, the production of extracellular polysaccharide increases (Hoagland et al., 1993 and references therein) which would enhance aggregation of diatom chains. We observed that aggregation of diatoms in the mesocosm bloom coincided with increases in carbohydrate content of POM and HDOM between days 6 to 11 (Fig. 4) which suggest an increase in extracellular polysaccharide production by diatoms. During this period, diatoms were decreasing in size as seen by the relatively constant diatom abundances and a decline in POC (Fig. 1B). Although one might first suspect nutrient depletion, Myklestad (1977) observed that the N:P ratio of media did not influence extracellular production of polysaccharide by *S. costatum.* Hence, nutrients may not have impacted polysaccharide production

In addition to carbohydrates, diatoms in both experiments also released significant amounts of proteinaceous matter to HDOM and VHDOM (Figs. 4 and 5). Structural diatom detritus such as cell wall or cell membrane material may be more refractory than non-structural molecules which would enrich VHDOM in proteins and lipids derived from structural material. The higher amounts of VHDOM components released in the mesocosm compared to the axenic culture

could be related to algal decay, since maximum concentrations of VHDOM-C, THAA and carbohydrates were found on day 14 (Figs. 3 and 5). Diatom autolysis, viral lysis, grazing and bacterial hydrolysis may have promoted higher release of VHDOM-C and greater proportion of THAA and lipids in VHDOM. A greater proportion of VHDOM-C was identified from the axenic culture as THAA and carbohydrates than the mesocoms bloom, in part due to the unidentified VHDOM-C present in the seawater media (day -1) which presumably persisted throughout the experiment. Differences in THAA composition for VHDOM among the two cultures is in part related to differences in POM amino acid composition (Table 2). Similar distributions of THAA among size fractions between the *S. costatum* cultures and samples from the Delaware Estuary indicate that algal exudation contributes to differences in amino acid distributions between size fractions observed in organic matter from the Delaware Estuary (Table 2; Mannino and Harvey, 2000). Amino acid content in HDOM was much lower for the mesocosm culture than the axenic culture and is likely due to microbial degradation in the mesocosm (Fig. 4).

On a carbon basis, lipids comprised a similar proportion of diatoms as carbohydrates, but only a small proportion of HDOM and VHDOM (Fig. 4). During log phase growth, only a small portion of lipids, primarily fatty acids and sterols, were released into high molecular weight DOM (Figs. 5 and 8). The sterol composition of diatoms and VHDOM from the axenic culture and mesocosm bloom were nearly identical, demonstrating that diatoms were the source of

VHDOM sterols (Table 4). The fact that sterols were observed in VHDOM but not HDOM (except on day 6 of mesocosm bloom) suggests that these compounds were associated with large fragments of diatom membrane debris. Sterol content was much higher in mesocosm VHDOM in comparison to the axenic culture with some differences in composition such as higher cholesterol content in the mesocosm may be due to the presence of protozoa (Table 4). Moreover, the VHDOM fraction was enriched in sterols, but depleted in PUFAs, compared to POM (Figs. 8 and 9). Selective release of sterols to VHDOM and retention of PUFAs by diatoms would yield the results observed. Perhaps the greater hydrophobicity of sterols, relative to phospholipid bound PUFAs, enhanced their aggregation to VHDOM. Alternatively, protozoan grazing on decaying diatoms in the mesocosm may have selectively removed unsaturated fatty acids and enriched the VHDOM fraction with sterols from diatoms and cholesterol from protozoa through the egestion of food vacuoles (Nagata, 2000 and references therein). At this point, the mechanisms that influence the release and distribution of lipids in high molecular weight DOM are uncertain. The presence of taxonomically specific sterols in VHDOM after an algal bloom has decayed or otherwise dissipated (grazed or sinks below the surface mixed layer) can be exploited to provide a short-term history of phytoplankton taxa which have formed algal blooms in the ocean weeks to perhaps months prior to sampling. Muhlebach and Weber (1998) have used this approach to estimate a half-life of 3 weeks for phytoplankton-derived dissolved sterols in the Weddell Sea.

Similarities in the biochemical composition of high molecular weight DOM released by both *S. costatum* cultures imply that such macromolecules are typically released during growth and leads us to question why diatoms retain proteinaceous matter and lipids and release primarily carbohydrates and unidentifiable organic matter. As discussed previously, the extracellular architecture of diatoms may promote the release of carbohydrates from their polysaccharide-rich outer coating. Another possibility is the conservation of limiting nutrients and vital cellular pools of N and P bound within proteins and phospholipids, and the physiological mechanisms to minimize their exudation. The release of excess photosynthetic capacity as carbohydrates may thus permit phytoplankton to maintain the cellular machinery in high gear even though nutrients are limiting. Possible explanations of excessive release of polysaccharide as a consequence of surplus light or as a protective sunscreen mechanism (Wood and Van Valen, 1990) are unlikely for this set of experiments as would be the possible release of compounds as a mechanism to obtain dissolved inorganic nitrogen or essential trace metals.

4.3. Bacterial contributions to DOM

The presence of D-amino acid isomers (McCarthy et al., 1998; Amon et al., 2001), amino sugars originating from bacterial cell walls (Boon et al., 1998; Kaiser and Benner, 2000), fatty acids from bacterial cell membranes (Mannino and Harvey, 1999) and a bacterial porin protein (Tanoue, 1995) within the ocean's

macromolecular DOM pool all validate the importance of bacteria as contributors to DOM. In this study, bacterial-derived fatty acids found in mesocosm HDOM and VHDOM likewise show a contribution of bacterial cell membrane fragments to high molecular weight DOM. Several D-amino acids commonly found in the peptidoglycan of bacteria (D-Ala, D-Asp, D-Glu and D-Ser; Gottschalk, 1986) and other D-amino acids of unknown origin (D-Val, D-Ile, D-Leu, and D-Phe), were present in HDOM during the growth and decay sequence of the mesocosm bloom (Table 3; Fig. 7). Furthermore, higher alanine (D+L) and glycine content found in mesocosm HDOM compared to the axenic culture could be due to inputs of bacterial peptidoglycan which is enriched in alanine and glycine. Only D-Ala and D-Phe were measured at values above racemization levels in POM from the mesocosm bloom on day 6 and 20, respectively (Table 3). Enantiomeric amino acid ratios for mesocosm VHDOM were not above racemization values and so are equivocal in their interpretation. Greater D/L amino acid values in HDOM versus POM in the mesocosm bloom revealed that as the bacterial population decreased following the bloom, bacterial cell wall remnants within HDOM resisted degradation.

4.4. Diagenetic Trends

Over 90% of the POC and 80% of TOC produced during *S. costatum* growth was degraded by day 62 of the mesocosm bloom. Much of the POM produced by diatoms was rapidly utilized by the microbial community with only

minor portions observable in DOM size fractions. The TOC remaining on day 62 consisted mostly of DOC (86%), primarily LDOM (64% of DOC). Norrman et al. (1995) have observed that bacteria preferentially assimilated freshly excreted DOC during growth and senescence of an experimental diatom bloom with little or no utilization of pre-bloom DOC (also Meon and Kirchman, 2001). Nevertheless, a portion of the DOM released was not utilized, even after nutrient additions and a one year incubation period, indicating that phytoplankton and/or microbial processing of algal detritus produced refractory DOM (Norrman et al., 1995; Meon and Kirchman, 2001). A substantial amount of DOM produced during diatom growth and early decay in this study was present at the end of the mesocosm experiment (100 μM DOC; Fig. 3). Higher concentrations of all biochemical components remained on day 62 for mesocosm HDOM and VHDOM compared to initial conditions, and suggests that a portion of diatom-derived DOM produced was resistant to microbial attack (Fig. 5). Even as POC, particulate THAA, carbohydrates and lipids continued to decrease after day 27 of the mesocosm bloom, the concentrations of biochemical components in high molecular weight DOM did not decline. In fact, DOC, carbohydrate and lipid (mostly sterols) concentrations in VHDOM increased between days 41 and 62 (Figs. 3, 5 and 8). Increases seen in LDOM-C during decay (e.g. between days 27 to 41) indicated that some portion of the remnant POC pool was hydrolyzed to small molecules, but not utilized immediately (Fig. 3).

Excluding lipids, the biochemical composition of POM showed only

minor variation during diatom growth and decay. Chlorophyll *a* was the most labile component of POM followed by other lipids, THAA and carbohydrates. Differences in lipid composition among size fractions can be viewed as varying stages of diagenesis with the relative amounts of PUFAs and sterols declining with size from POM to HDOM (Figs. 8 and 9). As POM from the mesocosm bloom decayed, the contribution of saturated and bacterial fatty acids to POM increased, and the contribution of PUFAs decreased, indicating preferential utilization of PUFAs by the microbial community (Fig. 9). Differential lability of individual lipids promotes selective release of particulate lipids to the high molecular weight DOM pool. The composition of membrane lipids and high THAA-N content in mesocosm VHDOM (29 to 47% of VHDOM-N) indicated prevalence of recently produced diatom and bacterial detritus. Sterol content in VHDOM was elevated in the mesocosm compared to the axenic cultures implying that senescence and bacterial processes enhance the transfer of algal organic matter to VHDOM. Grazing by protozoa on bacteria and decaying diatoms may have also contributed to the lipid composition observed in VHDOM through the egestion of macromolecular lipid complexes (Nagata and Kirchman, 1992). Biochemically VHDOM more closely resembled POM in terms of higher THAA and lipid content and lipid composition than HDOM, but VHDOM was enriched in carbohydrates compared to POM and HDOM (Fig. 4). As observed in coastal waters (Mannino and Harvey, 2000), the amount of organic carbon and nitrogen identifiable as hydrolyzable amino acids in the *S. costatum* cultures decreased

along a size gradient from POM to HDOM (Fig. 4; 33-68% of PN, 29-56% of VHDOM-N and 10-28% of HDOM-N). The decline in compositional lability of organic matter along the size gradient from POM to HDOM is consistent with the size-reactivity continuum hypothesis (Amon and Benner, 1996).

Some portion of the unidentified HDOM-C was present in the mesocosm prior to inoculation (Fig. 4). The presence of a refractory and conservative fraction of HDOM is consistent with the findings of Norrman et al. (1995), who observed preferential uptake of fresh phytoplankton-derived DOM and limited utilization of pre-bloom DOM. If we assume that the unknown HDOM-C present on day -1, i.e. this HDOM is also refractory and conservative in its properties, then the remaining 44-66% of HDOM-C can be attributed to biochemical components versus the 23-40% of total HDOM-C identified. The carbohydrate and THAA content on a carbon basis would then be similar for HDOM released in the axenic and mesocosm cultures during diatom growth.

Amino acids can provide insight into the degradation status of the mesocosm bloom, for example β-alanine has been proposed to gauge the extent of degradation for specific fractions of POM and DOM (Lee and Cronin, 1982; Cowie and Hedges, 1992b; Hedges et al., 1994). The presence of β-alanine in mesocosm HDOM, but not in VHDOM and POM, suggests that the THAA pool in HDOM was more degraded than in other size fractions (Table 2; and Mannino and Harvey, 2000). β-alanine was not observed in POM or DOM from the axenic diatom culture. Bacterial decarboxylation of the C_{α} on aspartic acid is the

proposed mechanism for β-alanine production (Bada, 1998). Since cleavage of the peptide chain at the aspartic acid C_α or decarboxylation of aspartic acid moieties at the C-terminus of polypeptide chains is necessary to produce β-alanine, the high contribution of β-alanine in HDOM results from bacterial accessibility to aspartic acid moieties which in turn requires hydrolysis and/or unfolding of particulate proteins or large dissolved proteins. The greater abundance of aspartic acid in HDOM compared to POM or VHDOM (Table 2) may also have contributed to the presence of β-alanine in HDOM. Although β-alanine concentrations increased during diatom growth and early decay in the mesocosm, a 20 nM decline in β-alanine between days 27 and 62 suggest bacterial uptake. Heterotrophic uptake of free β-alanine was observed in estuaries and oceanic waters but at rates 10 to 32 times slower than alanine (Cole and Lee, 1986). Although β-alanine cannot be used as a rigorous diagenetic chronometer, it provides a comparative measure for the degradation state of organic matter. The increasing contribution of serine, which is enriched in diatom cell wall proteins relative to cytoplasmic amino acids (Hecky et al., 1973; Swift and Wheeler, 1992), within POM from days 14 to 62 during diatom decay despite a constant composition for other amino acids suggests preferential preservation of diatom cell wall material (Table 2).

For all three size fractions, a higher portion of axenic culture organic nitrogen consisted of THAA-N than in the mesocosm, presumably due to the absence of actively growing bacteria. Although the low amounts of dissolved

THAA-N can result from the composition of molecules released by algae, these results illustrate that much of the high molecular weight DON, especially for HDOM, is not in the form of hydrolyzable amino acids. Lara et al. (1997) observed that in addition to excreting amino acids, diatoms also excrete significant amounts of other uncharacterized hydrophilic nitrogenous molecules. The higher concentrations of amino acids released from vapor-phase hydrolysis of dissolved combined amino acids compared to the more prevalent liquid-phase hydrolysis procedure suggest the presence of modified amino acids (up to 50% of THAA) not recognizable as proteins or THAA by standard methods (Keil and Kirchman, 1993). McCarthy et al. (1997) observed that THAA comprised only 8-9% of macromolecular DON in the oligotrophic ocean but other amide nitrogen (protein or chitin like polymers) distinguishable by ^{15}N-NMR constituted 65-86% of the remaining nitrogen. Chitin, a polymer of N-acetyl glucosamine, originating from diatoms such as *S. costatum* or amino sugars and non-hydrolyzable amino acids from bacterial cell walls, may contribute to some of this unidentified nitrogen.

The compositional shift from POM to dissolved fractions suggest that as particles are hydrolyzed to DOM and metabolized by microbial consumers, a greater portion of dissolved carbohydrates are inherently more resistant to bacterial degradation. Nevertheless, the axenic diatom experiment shows that phytoplankton are the fundamental factors in determining the carbohydrate-rich composition of VHDOM and HDOM. Structural modification of dissolved

polysaccharide could explain their apparent resistance to microbial degradation compared to particulate polysaccharide. Ogawa et al. (2001) proposed that microbial processes are more likely to produce refractory organic matter than abiotic processes. Pakulski and Benner (1992) observed that 9-28% of ^{13}C-NMR measurable carbohydrates from high molecular weight DOM were not quantified with the MBTH procedure. The MBTH procedure is not as efficient in quantifying uronic acids and amino sugars (only 86% recovery) from polymers such as chitin or peptidoglycan (Pakulski and Benner, 1992). Boon et al. (1998) observed high polysaccharide content in >1 kDa DOM consisting of neutral carbohydrates and N-acetyl amino sugars originating from bacteria. Substantial contributions of amino sugars and non-hydrolyzable amino acids in high molecular weight DOM would help resolve the perplexity of the unidentified organic carbon and nitrogen. The unidentified HDOM from the axenic and mesocosm cultures had a lower C:N than the characterized fraction (C:N difference of 1.3 to 3.5). Because actively growing bacteria were absent in the axenic culture and the C:N for the unidentified HDOM was 3.77 (unidentified VHDOM C:N=4.3), a substantial portion of the uncharacterized HDOM would presumably consist of non-hydrolyzable modified proteins (C:N of proteins=3.6). Modification of proteins or polypeptides by diatoms (and possibly bacteria and grazers in the mesocosm bloom) may remove such macromolecules from our analytical window and artificially reduce the measurable proteinaceous excretions from phytoplankton to DOM. In contrast, the C:N for the unidentified HDOM

from the mesocosm culture (8-9.1 between days 6 and 62) points to an unidentifiable mixture of amino sugars and other carbohydrates as well as modified polypeptides.

5. Conclusions

During growth diatoms release substantial amounts of primary production as DOC, up to 28.5% of TOC in the absence of bacteria and 33% of TOC as net ecosystem production in the mesocosm bloom. Most of the high molecular weight DOM released by diatoms was characterized as carbohydrates and hydrolyzable amino acids with small amounts of lipids. The release of diatom-derived organic matter to VHDOM and HDOM, including fatty acid and sterol biomarkers, was observed in both axenic and mesocosm blooms. Compositional differences in amino acids and lipids among POM, VHDOM and HDOM in both experiments reveal that such distinctions are inherent properties of each size fraction. Release of polysaccharide by diatoms and other algae during growth appears a fundamental factor in determining the carbohydrate-rich composition of high molecular weight DOM in natural waters. The contribution of bacterial-derived fatty acids and D-amino acids from cell membrane and cell wall constituents to high molecular weight DOM in the mesocosm bloom demonstrate the importance of bacterial inputs to DOM during its utilization. Even though DOC accumulation occurred during diatom growth, changes in DOC concentrations during late stationary phase and decay were often slow, suggesting

that as POM was degraded most was utilized on time scales of hours to days. In fact, much of the POM produced during diatom growth was not observed in any of the DOM size fractions, but remineralized rapidly by the microbial community. The higher amounts HDOM-C (50% of DOC) released in the axenic culture compared to the mesocosm bloom (22% of DOC) indicated that bacteria utilized freshly produced high molecular weight DOC preferentially to LDOM (with the likely exception of free amino acids and monosaccharides). Even in these short time frames, the microbial community can efficiently remove a large portion of the identifiable biochemical fractions (as THAA, carbohydrate, and lipids) while leaving traces of their activities (bacterial fatty acids and amino acids). The remainder includes an increasing fraction of organic matter that continues to elude structural identification.

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Figure Legends

Figure 1. *Skeletonema costatum* cell density, Particulate organic carbon (POC) and dissolved organic carbon (DOC) distributions during growth in the (A) axenic and (B) mesocosm blooms. Initial DOC present in the axenic culture media was subtracted, and DOC data represent the net DOC released. The lag phase in the axenic culture indicates extremely slow growth of diatoms during the first 6 days following inoculation.

Figure 2. Distributions of (A) bacteria and (B) organic carbon during the growth and decay of the *Skeletonema costatum* mesocosm bloom. Day -1 is the day prior to inoculation of diatom culture. Mesocsosm was placed in darkness on day 11 as shown in bar.

Figure 3. Molecular weight distribution of the net DOC released in the axenic culture of *S. costatum* and during the mesocosm bloom. Initial DOC present in the axenic culture media and the mesocosm seawater media (day -1) were subtracted to obtain the net DOC released. Axenic diatom culture (Ax), VHDOM (30 kDa to 0.2 μm DOM), HDOM (1-30 kDa DOM), LDOM (<1 kDa DOM).

Figure 4. Biochemical composition of (A) POM, (B) VHDOM and (C) HDOM from the *S. costatum* blooms. Total hydrolyzable amino acids (THAA), total carbohydrates (TCHO).

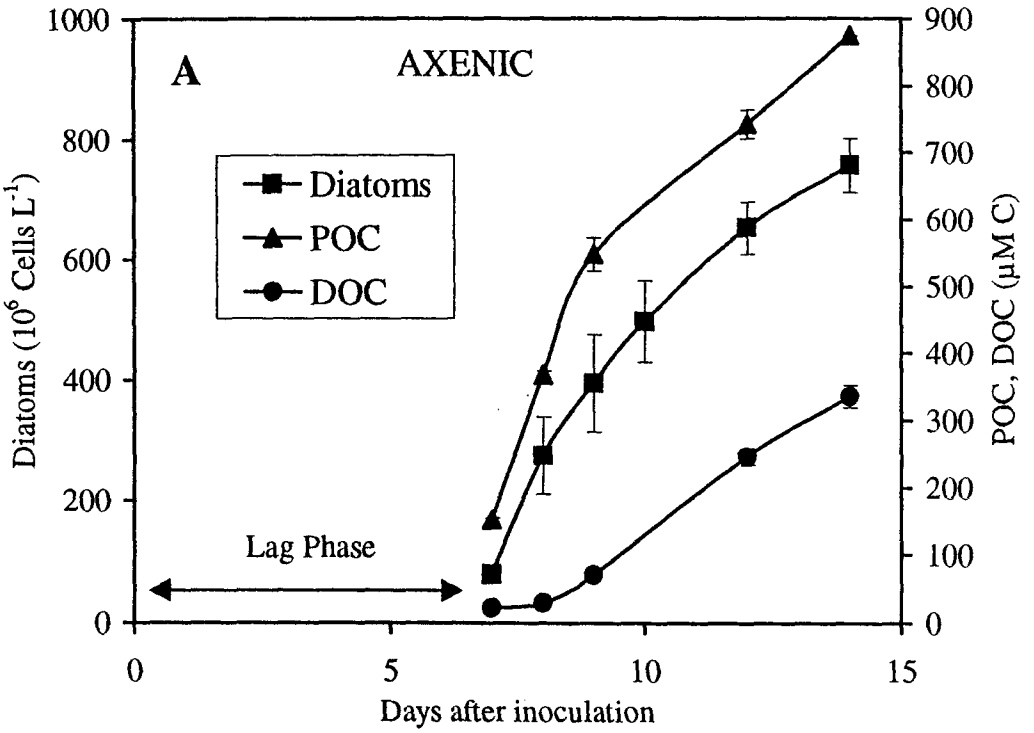
Figure 5. Distributions of carbohydrates (TCHO), total hydrolyzable amino acids (THAA) and lipids in (A) POM, (B) VHDOM and (C) HDOM from the *S. costatum* mesocosm bloom.

Figure 6. Enantiomeric ratios (D/L) of amino acids formed through racemization during acid hydrolysis in proteins (Lysozyme and RuBisCo), axenic *S. costatum* culture (average from POM, VHDOM [except leucine and valine] and HDOM) and literature values (Lee and Bada, 1977; McCarthy et al., 1998).

Figure 7. Enantiomeric ratios (D/L) of THAA in HDOM during the growth and decay of the *S. costatum* mesocosm bloom. Racemization due to acid hydrolysis (racemization). Legend indicates day of sampling.

Figure 8. Lipid composition in (A) POM, (B) VHDOM and (C) HDOM fractions from the axenic and mesocosm *S. costatum* blooms. Values for POM for the axenic diatoms are reduced to 50% of actual values to fit within the scale shown. Axenic diatom culture (Ax).

Figure 9. Fatty acid distributions in (A) POM, (B) VHDOM and (C) HDOM from the axenic and mesocosm *S. costatum* blooms. Bacterial - branched odd-chain saturated and odd-chain monounsaturated fatty acids, PUFA - polyunsaturated fatty acids, MUFA - monounsaturated fatty acids. Values for POM for the axenic diatoms are reduced to 25% of actual values to fit within the scale shown.



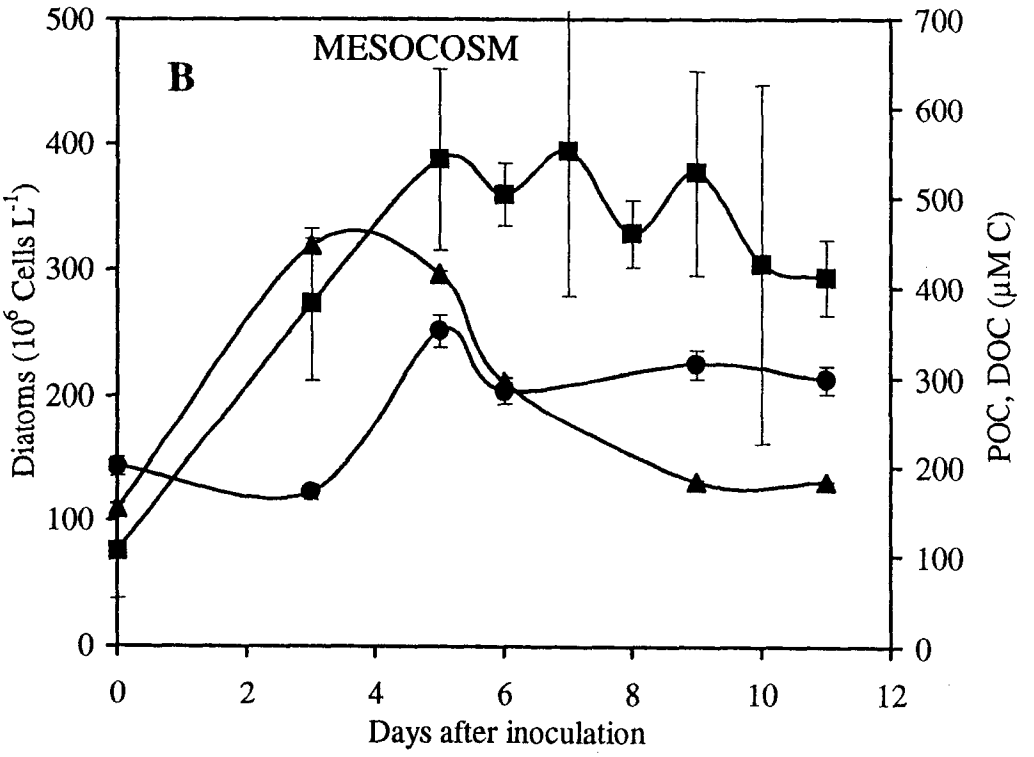


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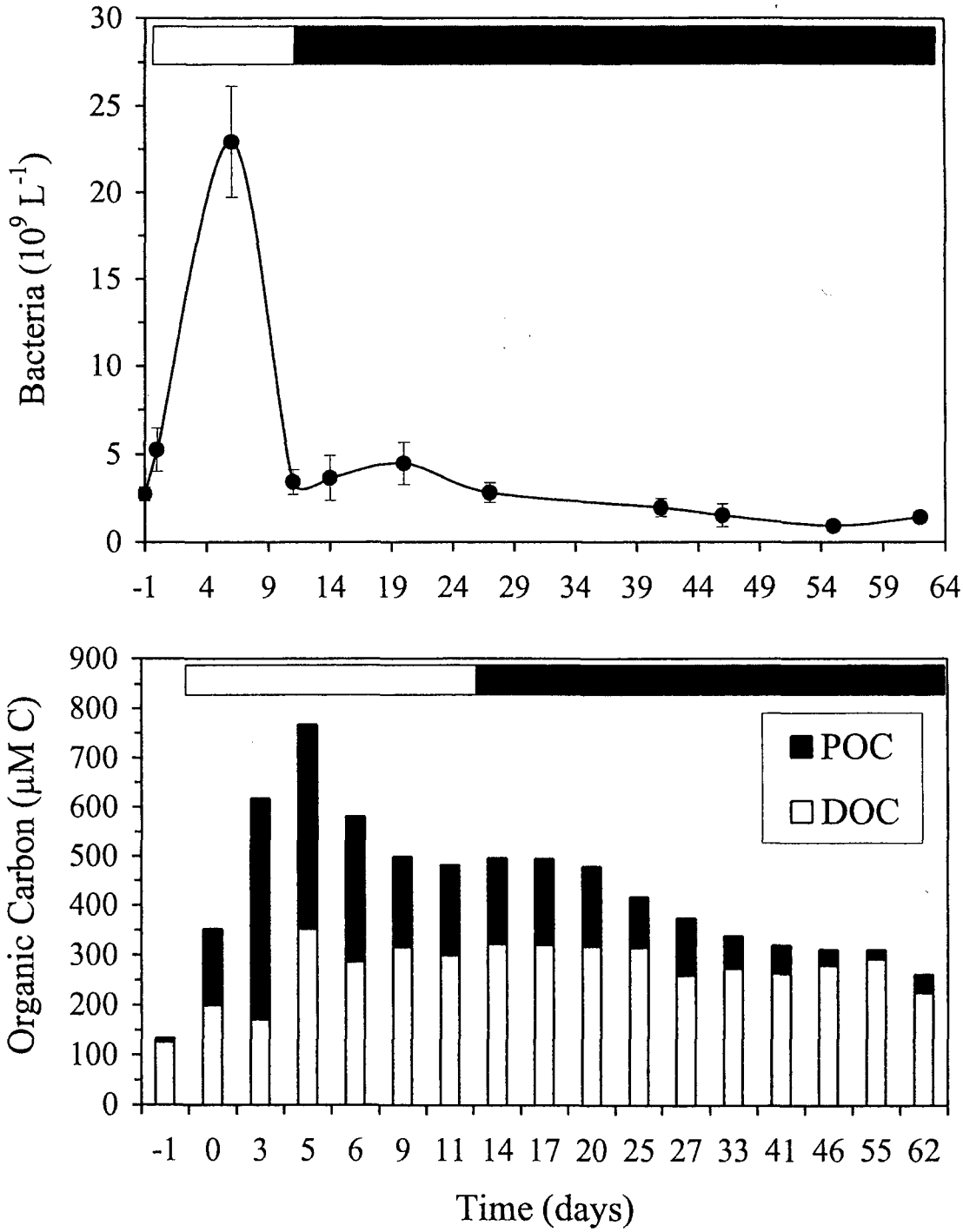
Figure 1



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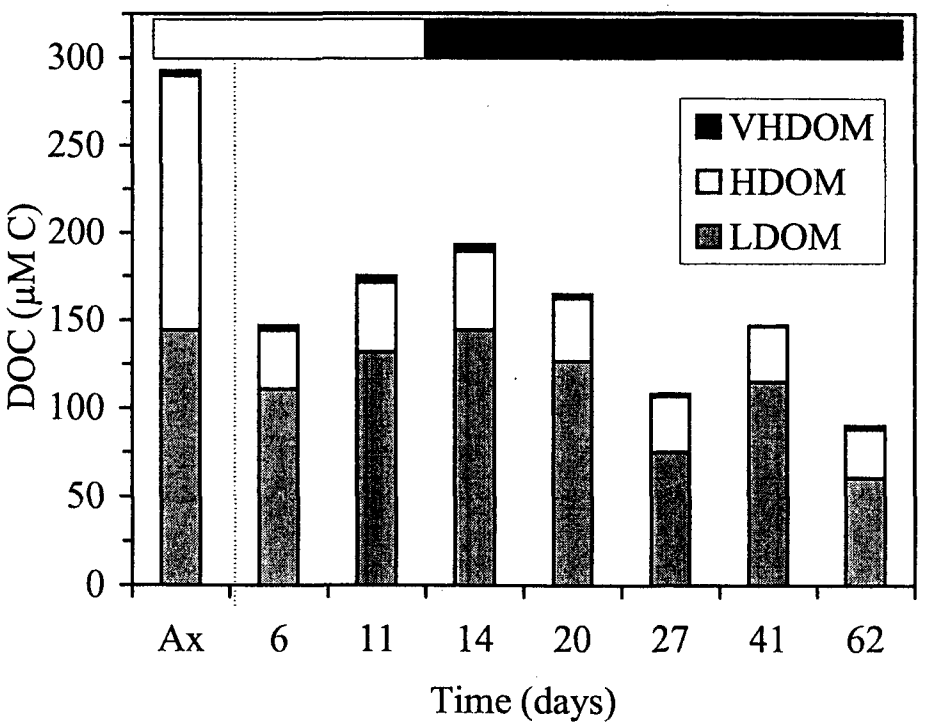

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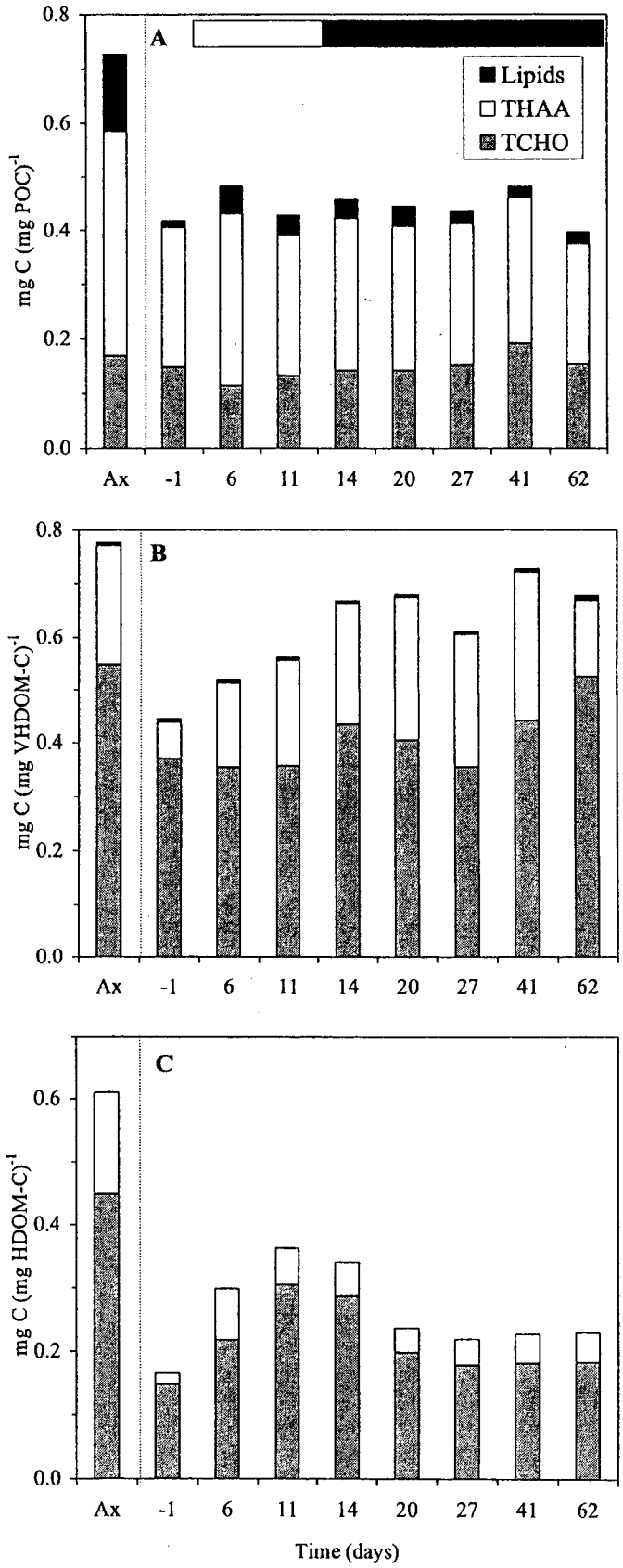

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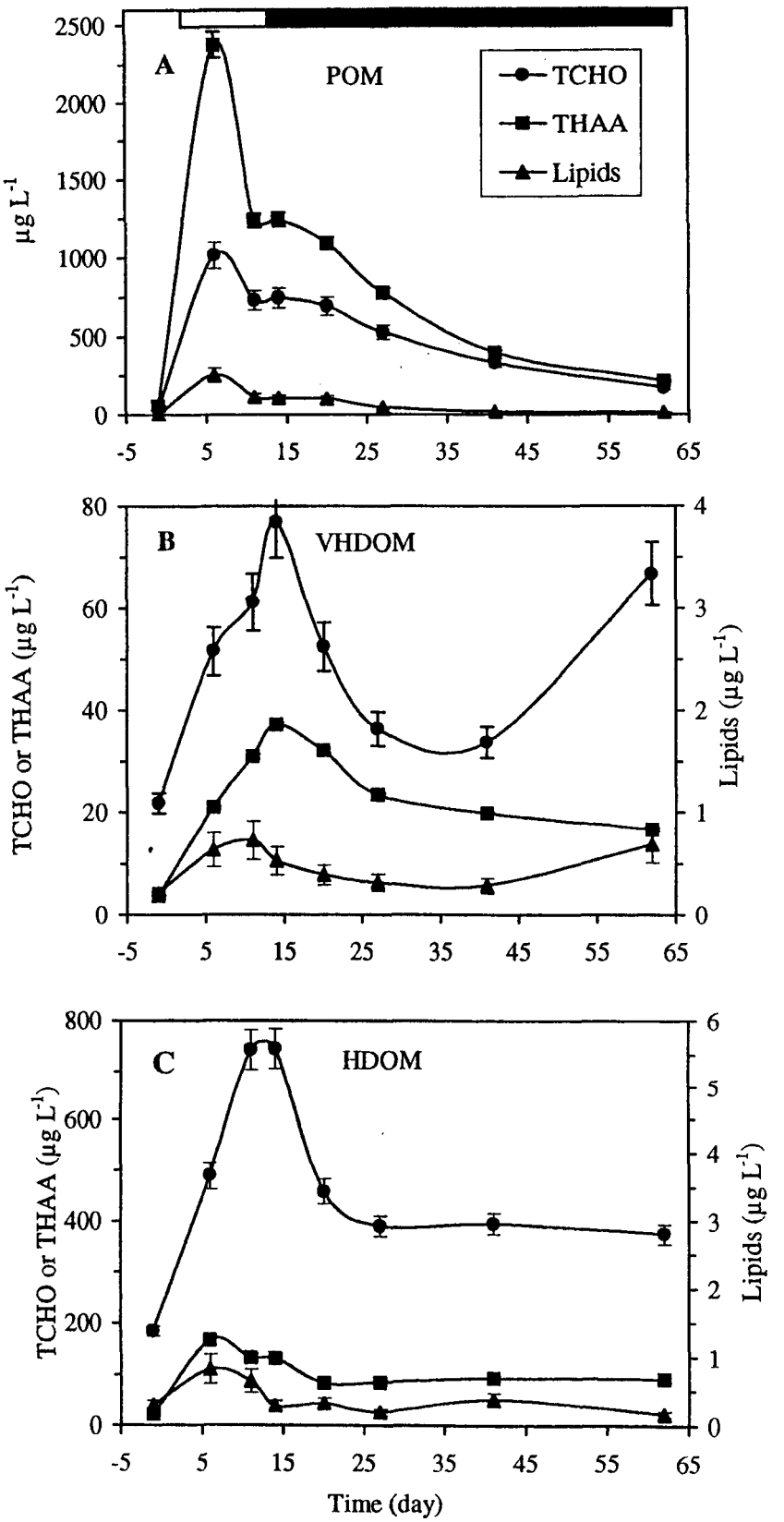

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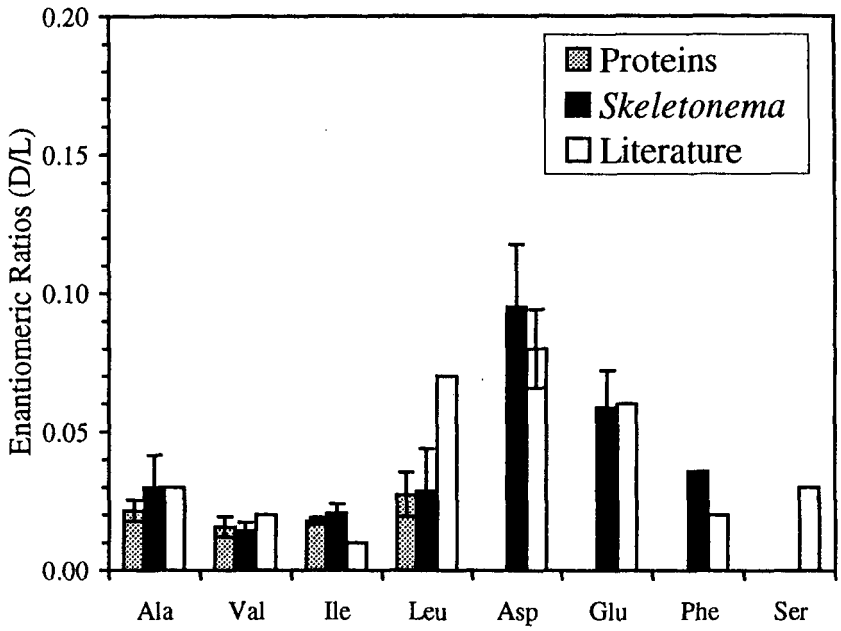

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Fig. 6

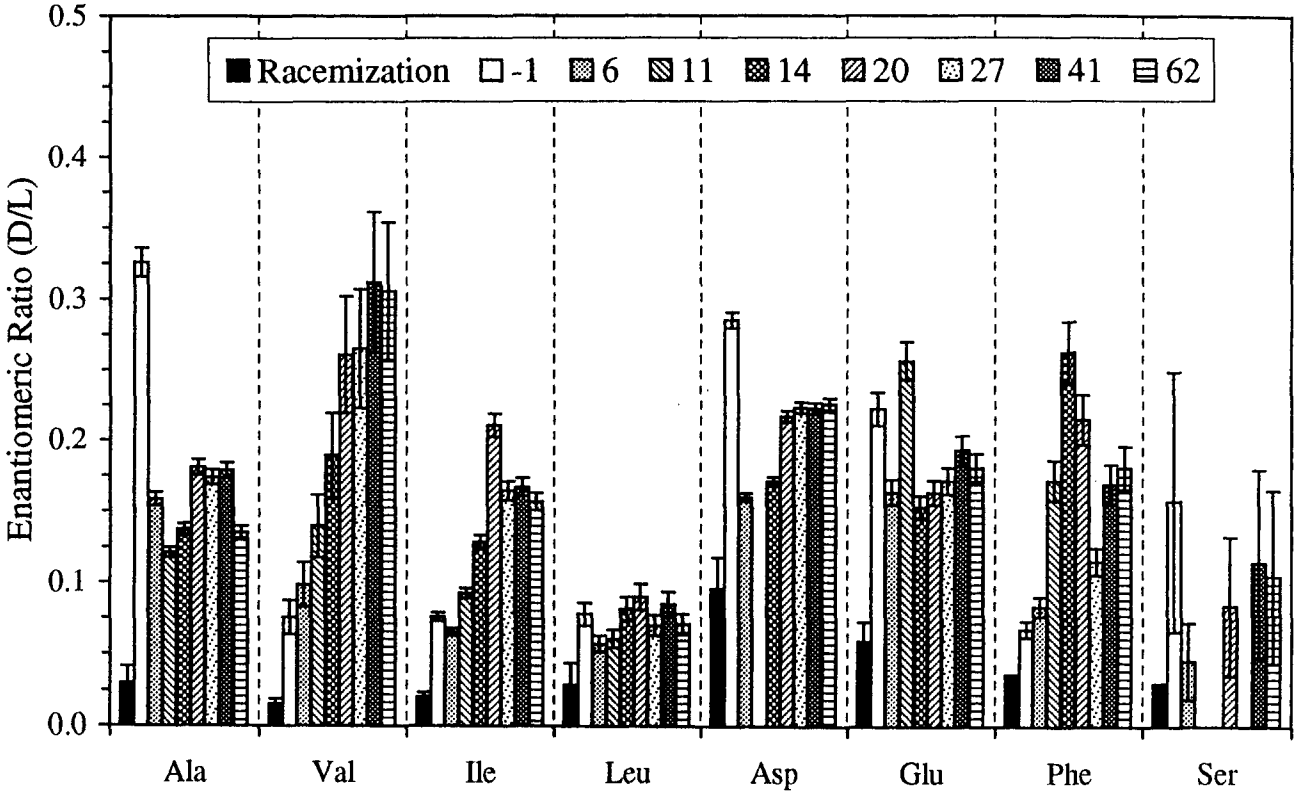

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Fig. 7

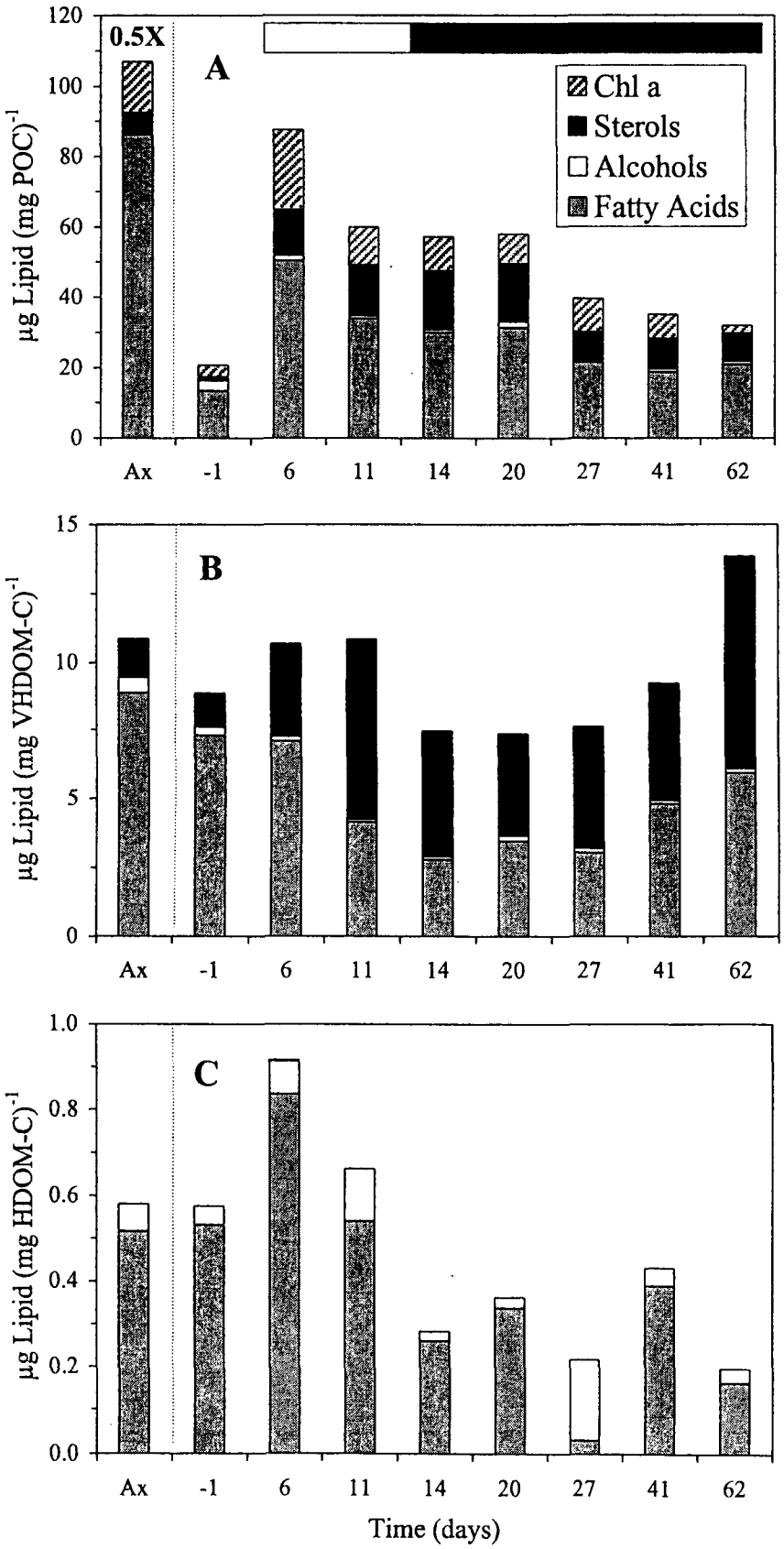

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Figure 8

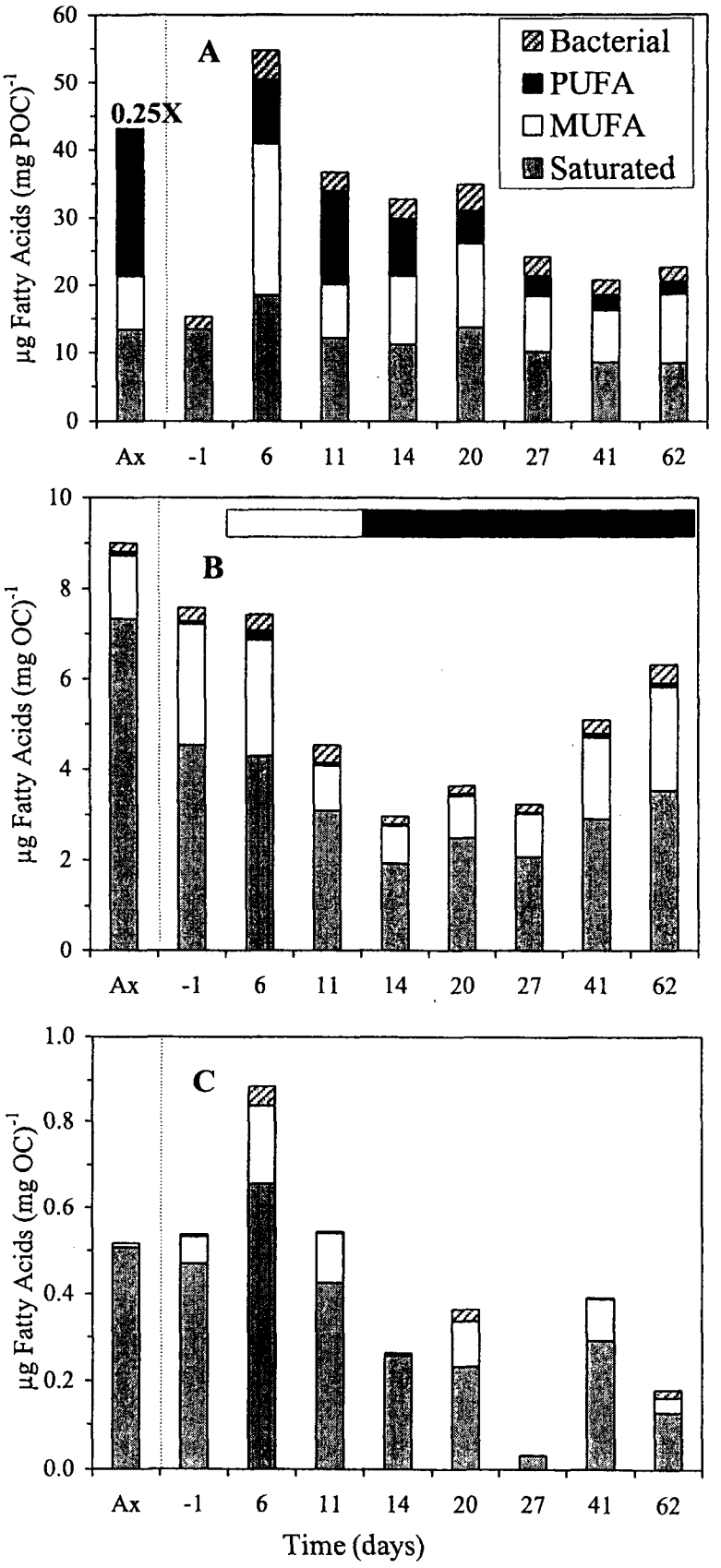

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Figure 9

Table 1. Molecular weight distributions of dissolved nitrogen species (μM N) in the axenic *Skeletonema costatum* culture at the conclusion of log-phase growth (day 14).

Nitrogen Species	Total (<0.2 μm)	VHDOM	HDOM	LDOM	SUM (% N)
NO_3	639	0.093	0.454	626	98.1
NO_2	5.29	0.0023	0.015	5.19	98.3
NH_4	0.29	0.012	0.033	0.36	140.8
TDN	679	1.06	17.4	616	93.5
DON	34	0.949	16.9	16.2*	na
TDIN (%)		0.016	0.074	93.1	

SUM: SUM = [(VHDOM + HDOM + LDOM)/total DOM]*100 for each N species

TDN: total dissolved nitrogen

TDIN: total dissolved inorganic nitrogen

*: LDOM = (Total - VHDOM - HDOM)

na: not applicable

Table 2. Total hydrolyzable amino acid distributions (mole%) during the growth and decay of the S. costatum cultures.

Day	Size	Neutral							Hydroxyl			Acidic		Aromatic		Basic		THAA
	Fraction	Ala	Gly	Val	Leu	Ile	Met	Pro	H-Pro	Thr	Ser	Asp	Glu	Phe	Tyr	Lys	ß-ala	(µM)
Axenic	POM*	13.6	13.1	6.6	8.8	5.0	0.27	4.1	0.12	5.8	8.1	11.0	10.4	4.2	2.4	4.5	-	78.26
	VHDOM	13.1	12.2	7.5	6.6	3.8	-	4.5	1.06	11.3	14.5	11.4	8.4	3.1	1.49	1.2	-	0.14
	HDOM	8.9	11.3	5.5	5.4	3.8	0.41	3.7	0.30	5.3	5.6	29.7	14.8	2.2	0.44	2.7	-	5.15
-1	POM	14.2	8.8	9.0	12.2	8.2	-	2.3	-	9.8	16.5	6.1	4.0	7.5	1.45	-	-	0.46
	VHDOM	22.7	16.5	8.7	6.9	4.4	-	1.8	1.02	12.0	13.6	6.4	2.4	2.7	0.95	-	-	0.033
	HDOM	17.7	26.5	5.0	3.3	1.9	0.96	4.7	0.42	2.9	5.5	13.3	8.5	2.3	-	1.3	5.7	0.20
6	POM	14.2	12.8	10.3	12.0	8.9	-	2.7	0.11	8.4	9.2	6.3	4.8	7.4	2.47	0.5	-	19.8
	VHDOM	17.9	13.5	10.4	6.9	6.4	-	3.5	0.12	7.9	6.8	12.4	7.6	4.0	1.42	1.1	-	0.175
	HDOM	15.9	16.1	7.7	5.6	4.4	1.43	4.7	0.36	3.3	5.4	14.4	13.4	3.0	0.41	3.0	1.0	1.36
11	POM	14.2	13.0	9.4	11.7	8.0	-	2.8	0.16	7.6	9.5	8.2	6.1	6.3	2.16	0.8	-	10.2
	VHDOM	18.2	14.0	10.2	7.6	6.4	-	3.4	0.12	7.2	6.8	11.9	7.4	4.3	1.13	1.2	-	0.259
	HDOM	16.1	17.3	7.7	5.3	4.1	1.38	4.8	0.45	4.9	7.3	13.9	10.2	2.9	0.22	2.2	1.3	1.11
14	POM	13.6	12.4	9.7	12.6	8.5	-	2.5	0.15	8.5	10.6	6.0	4.2	8.2	2.69	0.4	-	10.3
	VHDOM	17.2	13.3	9.0	6.1	5.4	-	3.0	0.12	9.9	8.8	12.0	7.5	4.2	2.15	1.3	-	0.311
	HDOM	16.2	19.3	7.0	4.5	3.7	1.61	4.6	0.49	4.4	6.9	12.8	9.4	2.9	0.56	3.0	2.8	1.11
20	POM	13.1	12.1	9.4	12.4	8.5	-	2.6	0.17	8.6	11.7	6.3	3.9	8.4	2.79	0.2	-	9.1
	VHDOM	19.1	14.6	9.7	5.8	5.7	-	2.7	0.10	10.1	9.1	10.8	6.1	3.7	1.51	0.8	-	0.275
	HDOM	16.3	21.3	7.8	4.5	3.8	1.39	4.5	0.24	2.8	5.2	13.4	10.0	2.5	0.23	2.9	3.2	0.71
27	POM	13.9	12.3	9.2	12.1	8.1	-	2.6	0.17	8.4	12.1	6.5	4.1	8.0	2.39	0.1	-	6.5
	VHDOM	19.4	15.0	9.2	5.8	5.5	-	2.7	0.09	9.4	8.4	11.0	6.6	4.0	1.68	1.1	-	0.200
	HDOM	14.2	20.0	6.9	4.5	3.7	-	4.5	0.42	3.5	7.3	13.9	10.4	2.7	0.54	3.3	4.3	0.72
41	POM	13.7	11.0	9.9	12.3	8.5	-	2.5	0.18	8.9	12.4	6.4	4.1	8.0	2.15	-	-	3.3
	VHDOM	19.8	15.4	9.2	5.7	5.5	-	2.6	0.12	9.8	9.1	10.2	6.1	3.7	1.52	1.1	-	0.170
	HDOM	17.5	22.2	8.6	4.3	3.6	1.19	4.3	0.22	2.5	4.5	13.4	9.3	2.3	0.29	2.7	3.1	0.81
62	POM	13.5	12.0	9.7	12.3	8.2	-	2.5	0.17	9.7	13.0	5.7	3.3	8.1	1.81	-	-	1.8
	VHDOM	24.7	18.5	8.2	6.5	4.3	-	2.7	0.21	8.7	9.5	8.2	4.3	3.3	1.02	-	-	0.150
	HDOM	17.3	21.2	8.6	4.5	3.5	1.39	4.7	0.20	3.3	4.6	14.2	9.7	2.4	0.20	2.7	1.4	0.77
S.D.	POM	0.06	0.17	0.39	0.11	0.26	-	0.11	0.03	0.28	1.11	0.50	0.46	0.51	0.08	0.13	-	0.42
	VHDOM	0.26	0.12	0.19	0.09	0.09	-	0.06	0.003	0.51	0.08	0.37	0.24	0.07	0.08	0.21	-	0.0036
	HDOM	0.38	0.60	0.39	0.11	0.14	0.05	0.07	0.05	0.62	0.21	0.26	0.50	0.14	0.05	0.14	0.71	0.066

S.D.: 1 standard deviation which is based on two duplicate analyses per size fraction

* Arg detected, 1.9 mole%; -: not detected.

Table 3. Enantiomeric ratios of amino acids (D/L ratios) from *Skeletonema costatum* cultures. Lysozyme and RuBisCo proteins were analyzed along with THAA samples from the axenic *S. costatum* culture to determine racemization of amino acids due to acid hydrolysis.

	Proteins			Axenic		Mesocosm								
	D/L	S.D.		D/L	S.D.	-1	6	11	14	20	27	41	62	%CV
Ala	0.021	0.004	POM	0.023	0.006	0.038	0.101	0.047	0.054	0.059	0.043	0.046	0.025	2.6
			VHDOM	0.024	a	0.115	0.057	0.055	0.062	0.069	0.063	0.067	0.081	6.5
			HDOM	0.040	0.013	0.326	0.159	0.121	0.137	0.181	0.174	0.179	0.135	3.1
Val	0.016	0.004	POM	0.013	0.000	0.080	0.022	0.024	0.024	0.025	0.024	0.026	0.025	9.6
			VHDOM	0.101	a	0.022	nd	0.022	0.020	0.011	nd	0.012	0.017	8.1
			HDOM	0.016	0.004	0.075	0.098	0.140	0.189	0.261	0.265	0.312	0.305	15.8
Ile	0.018	0.001	POM	0.021	0.006	nd	0.026	0.034	0.030	0.033	0.029	0.038	0.029	5.8
			VHDOM	nd		0.026	0.027	0.029	0.030	0.029	nd	0.035	0.031	4.6
			HDOM	0.020	0.001	0.075	0.065	0.092	0.129	0.211	0.164	0.167	0.157	3.9
Leu	0.027	0.008	POM	0.019	0.013	nd	0.041	0.033	0.036	0.040	0.030	0.036	0.035	23.6
			VHDOM	0.089	0.066	0.037	0.042	0.042	0.041	0.045	0.043	0.045	0.049	19.1
			HDOM	0.038	0.013	0.077	0.057	0.060	0.081	0.089	0.070	0.085	0.071	10.2
Asp	nd		POM	0.106	0.019	nd	0.120	0.100	0.118	0.097	0.101	0.089	0.047	10.5
			VHDOM	0.077	0.015	0.137	0.135	0.128	0.125	0.139	0.121	0.125	nd	12.0
			HDOM	0.102	0.032	0.285	0.160	nd	0.171	0.217	0.223	0.223	0.226	1.9

	Proteins			Axenic		Mesocosm								
	D/L	S.D.		D/L	S.D.	-1	6	11	14	20	27	41	62	%CV
Glu	nd		POM	0.053	0.008	nd	0.093	0.082	0.081	0.085	0.094	nd	nd	5.8
			VHDOM	0.046	a	0.044	0.071	0.069	0.070	0.082	0.048	0.047	0.104	8.8
			HDOM	0.071	0.011	0.222	0.164	0.256	0.153	0.163	0.172	0.193	0.181	5.3
Phe	nd		POM	0.036	a	nd	0.077	nd	0.076	0.141	0.078	nd	nd	nd
			VHDOM	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd
			HDOM	nd		0.067	0.082	0.172	0.262	0.215	0.114	0.169	0.181	8.3
Ser	nd		POM	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd
			VHDOM	nd		nd	nd	nd	nd	nd	0.017	0.016	0.020	12.0
			HDOM	nd		0.157	0.045	nd	nd	0.083	nd	0.113	0.104	58.2

S.D.: 1 standard deviation
%CV: maximum coefficient of variation for mesocosm D/L ratios from duplicate analyses per size fraction
nd: D amino acid not detected either from absence or coelution

Table 4. Sterol composition of particles and VHDOM in *Skeletonema costatum* experimental cultures.

Compound	Axenic	-1	6	11	14	20	27	41	62
POM (% of total)									
Cholesta-5,22-dien-3β-ol	-	15.7	0.3	1.0	1.6	2.4	2.4	3.0	2.9
Cholest-5-en-3β-ol	1.4	41.8	5.7	6.2	7.8	8.3	7.8	7.4	5.8
Cholesta-5,24-dien-3β-ol	4.4	-	-	-	-	-	-	-	-
24-Methylcholesta-5,22-dien-3β-ol	-	11.5	1.4	5.3	5.8	7.2	8.7	11.9	14.7
24-Methylcholesta-5,7,22-trien-3β-ol	-	-	3.3	4.7	5.9	6.8	7.4	5.0	4.3
24-Methylcholesta-5,24(28)-dien-3β-ol	59.2	6.7	56.5	40.4	35.6	32.2	28.5	26.6	20.4
24-Methylcholest-5-en-3β-ol	26.3	-	24.0	27.9	28.7	27.3	30.0	33.0	29.7
24-Methylcholest-24(28)-en-3β-ol	2.5	-	0.5	1.8	1.6	1.3	1.7	1.6	1.1
24-Ethylcholesta-5,22-dien-3β-ol	-	7.2	0.5	4.3	4.2	3.4	3.3	2.6	4.4
24-Ethylcholest-5-en-3β-ol	6.2	17.2	6.7	8.6	8.9	10.1	9.6	8.9	16.8
Total Sterols (μg (mg OC)$^{-1}$)	11.9	1.03	13.0	14.3	16.6	16.5	8.1	8.5	7.9
VHDOM (% of total)									
24-nor-cholesta-5,22-dien-3β-ol	1.9	2.5	0.43	0.17	0.11	0.14	0.44	7.8	2.7
27-nor-24-cholesta-5,22-dien-3β-ol	-	-	-	-	0.10	0.15	0.22	0.73	1.7
Cholesta-5,22-dien-3β-ol	-	6.0	1.5	1.7	2.0	3.5	4.0	3.9	3.5
5α-cholest-22-en-3β-ol	-	0.55	0.22	0.31	0.22	0.15	0.20	0.44	0.39
Cholest-5-en-3β-ol	3.8	38.2	9.3	6.6	7.3	9.4	8.7	8.0	6.2
5α-cholestanol or 27-nor-24-methylcholestanol	0.19	1.4	0.95	1.4	1.2	1.0	0.80	0.68	0.71
24-Methylcholesta-5,22-dien-3β-ol	0.8	6.6	1.2	7.7	8.9	11.0	11.4	14.3	15.7
24-Methylcholesta-5,7,22-trien-3β-ol	0.2	1.1	0.3	1.0	2.2	1.8	2.8	3.7	2.2
24-Methylcholesta-5,24(28)-dien-3β-ol	45.4	6.4	42.7	42.0	35.1	27.1	21.9	18.8	17.7
24-Methylcholest-5-en-3β-ol	37.3	2.3	27.2	16.5	19.3	25.1	24.6	22.9	23.8
24-Methylcholest-24(28)-en-3β-ol	1.8	1.0	4.7	2.6	2.3	1.5	1.2	1.1	0.8
24-Methylcholestanol	0.26	3.3	1.4	1.8	1.5	1.5	1.5	1.6	1.5
24-Ethylcholesta-5,22-dien-3β-ol	-	12.8	1.3	6.7	7.0	5.9	5.2	4.4	6.0
24-Ethylcholest-5-en-3β-ol	8.3	17.9	8.8	11.5	12.7	11.8	16.9	11.6	16.9
Total Sterols (μg (mg OC)$^{-1}$)	1.39	1.17	3.43	6.06	4.30	3.53	4.20	3.99	7.21