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Protein recycling in Bering Sea algal incubations

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ABSTRACT: Protein present in phytoplankton represents a large fraction of the organic nitrogen and carbon transported from its synthesis in surface waters to marine sediments. Yet relatively little is known about the longevity of identifiable protein *in situ*, or the potential modifications to proteins that occur during bloom termination, protein recycling and degradation. To address this knowledge gap, diatom-dominated phytoplankton was collected during the Bering Sea spring blooms of 2009 and 2010, and incubated under darkness in separate shipboard degradation experiments spanning 11 and 53 d, respectively. In each experiment, the protein distribution was monitored over time using shotgun proteomics, along with total hydrolyzable amino acids (THAAs), total protein, particulate organic carbon (POC) and nitrogen (PN), and bacterial cell abundance. Identifiable proteins, total protein and THAAs were rapidly lost during the first 5 d of enclosure in darkness in both incubations. Thereafter the loss rate was slower, and it declined further after 22 d. The initial loss of identifiable biosynthetic, glycolysis, metabolism and translation proteins after 12 h may represent shutdown of cellular activity among algal cells. Additional peptides with glycan modifications were identified in early incubation time points, suggesting that such protein modifications could be used as a marker for internal recycling processes and possibly cell death. Protein recycling was not uniform, with a subset of algal proteins including fucoxanthin chlorophyll binding proteins and Ru-BisCO identified after 53 d of degradation. Non-metric multidimensional scaling was used to compare the incubations with previous environmental results. The results confirmed recent observations that some fraction of algal proteins can survive water column recycling and undergo transport to marine sediments, thus contributing organic nitrogen to the benthos.

KEY WORDS: Protein recycling · Bering Sea · Amino acid · Nitrogen cycle · Tandem mass spectrometry · Diatom · Cell death · Preservation

INTRODUCTION

Diatoms typically dominate phytoplankton biomass in many regions of the global ocean, particularly the Bering Sea (Banahan & Goering 1986, Springer et al. 1996), which is also one of the most productive marine systems in the world (Sambrotto et al. 1986, McRoy et al. 1987). During ice retreat in spring, factors such as rapid diatom production, reduced impact by zooplankton grazing, and the shallow average shelf depth combine to allow a large fraction of new primary production to be transported to sediments (Chen et al. 2003, Lovvorn et al. 2005). Recent work

has documented that specific components of cellular material including intact phytoplankton proteins can be transported to shallow shelf and deeper basin sediments in the Bering Sea, confirming the link between primary production and inputs of organic nitrogen to the ocean floor (Moore et al. 2012a). With diatom aggregates estimated to sink at rates of 100 m d⁻¹ or greater (Smetacek 1985), this raises the question of the kinetics of water column recycling, how long identifiable phytoplankton proteins might be retained, and what fraction of protein material representing exported organic nitrogen is incorporated to sediments and benthic organisms.

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In a previous laboratory study, proteins from a pure culture of the marine diatom *Thalassiosira pseudonana* were observed over 23 d following exposure in darkness to a natural microbial community (Nunn et al. 2010). While this study demonstrated the potential for preservation of proteins, the greatest loss in the number of identifiable proteins took place during the initial 5 d darkness period of the 23 d degradation. This suggests that diatoms may restructure and recycle their proteome in order to acclimate to low light levels, and disable replication pathways during the initial stages of bloom senescence. An examination of the early stage of diatom cell death in natural communities is required to better understand protein cycling prior to microbially catalyzed degradative processes. Proteomic studies on plant programmed cell death (Chivasa et al. 2011, Choi et al. 2011) and algal cell stress (Jamers et al. 2009, Silvestre et al. 2012) have identified potential indicators and regulators of proteomic alteration. In the Bering Sea, ice retreat modulates the highly productive, but temporally constrained spring bloom, which facilitates tracking of potential algal population markers for cell death and subsequently bloom termination.

After bloom termination, sinking phytoplankton material accounts for much of the high organic matter export (Moran et al. 2012). A study of Bering Sea algal material, sinking sediment trap material, and surface sediments collected during the spring phytoplankton bloom found a statistically significant correlation between the number of identifiable proteins and the fraction of particulate nitrogen made up by total hydrolyzable amino acid nitrogen (THAA-N/PN; Moore et al. 2012a). In this study, shipboard incubation experiments using collected natural communities allow the timing of degradation to be observed, revealing which proteins/processes are involved in nitrogen export to sediments. The objectives were to use proteomic and bulk analysis to track changes of newly produced bloom material during its degradation to determine proteome changes, selective preservation, the longevity of individual proteins under more realistic environmental conditions, and thus the fate of a major organic nitrogen pool in the region.

MATERIALS AND METHODS

Incubations

Bering Sea water was collected during Bering Sea Ecosystem Study (BEST; Wiese et al. 2012) cruises on the outer shelf during the spring of 2009 (59.9037° N,

176.1278° W; sampling depth 5 m; water column depth 136 m) and 2010 (56.7272° N, 169.4271° W; sampling depth 36 m; water column depth 104 m) in areas which coincided with the developing spring bloom adjacent to the retreating ice. The phytoplankton community at the time of sampling was diatom dominated (Lomas et al. 2012, Moran et al. 2012). In each year, single 20 l carboys were filled from the CTD rosette taken from the chlorophyll maximum based on chlorophyll fluorescence at the time of sampling. In order to increase the amount of algal material for analysis throughout the incubation, 1 l of concentrated phytoplankton material was obtained by gently passing 10 l of CTD water from an additional bottle through a 10 µm mesh and combined with untreated seawater to make up the 20 l incubation. Macrozooplankton were excluded from the incubation by passing CTD water through a 1 mm plankton net before being added to the 20 l carboys.

Incubations were placed in shipboard –1°C cold rooms for 11 d (2009) and 53 d (2010) for the duration of the experimental period. Carboys were covered to exclude light throughout the incubations and aerated with filtered air. At regular time points, carboys were gently mixed until algal material was homogeneously distributed, and 1 l water samples were collected and filtered onto 25 mm pre-combusted glass fiber filters (GF/F) and 37 mm polycarbonate (0.2 µm) filters for analysis (Table 1). In addition, whole water samples were collected at each time point and filtered onto 0.2 µm filters, DAPI stained, and fixed onto microscope slides for bacterial counts. All incubation particles and bacterial slides were stored at –70°C until analysis or counting. Stained bacterial cells were counted on an Olympus BH2-RFCA fluorescent microscope.

Amino acid and bulk analysis

Total hydrolyzable amino acids (THAAs) were identified and quantified by gas chromatography/mass spectrometry (GC/MS) using the EZFaast method (Phenomenex), which uses derivatization of amino acids with propyl chloroformate and propanol for detection (Waldhier et al. 2010). Briefly, suspended particles collected on GF/Fs were hydrolyzed for 4 h at 110°C (Cowie & Hedges 1992) with 6 M analytical-grade HCl and L-γ-Methylleucine as the recovery standard. Following hydrolysis and derivatization, amino acids were quantified using an Agilent 6890 capillary GC with samples injected at 250°C and separated on a DB-5MS (0.25 mm ID, 30 m) column with

Table 1. Results of shipboard degradation experiments from 11 d (2009) and 53 d (2010) incubations of phytoplankton collected from the Bering Sea: changes over time in the C/N ratio, total hydrolyzable amino acids (THAAs), total protein (Bradford assay), bacterial cell abundance, and total protein identifications (using trypsin, EndoGluC, and PNGase + trypsin). nd: not determined

11 d incubation					
Day	C/N	THAAs (mg l ⁻¹)	Total protein (mg l ⁻¹)	Bacteria (cells l ⁻¹)	Total IDs
0	3.99	1013.00	1067.53	6.03 × 10 ⁷	127
0.5	3.63	448.52	618.51	nd	48
1	3.77	612.73	545.53	5.60 × 10 ⁷	64
3	3.54	571.18	680.93	1.03 × 10 ⁸	41
5	3.58	539.73	568.45	1.62 × 10 ⁸	51
7	3.56	454.31	566.94	2.10 × 10 ⁸	37
9	3.59	424.91	386.81	2.73 × 10 ⁸	42
11	3.55	400.61	404.50	1.14 × 10 ⁸	39
53 d incubation					
Day	C/N	Amino acids (mg l ⁻¹)	Total protein (mg l ⁻¹)	Bacteria (cells l ⁻¹)	Total IDs
0	4.37	805.63	988.57	7.56 × 10 ⁷	95
5	4.57	439.98	453.59	1.63 × 10 ⁸	32
12	4.47	386.44	177.42	8.90 × 10 ⁷	31
22	4.62	337.93	233.63	7.94 × 10 ⁷	15
35	4.93	244.67	248.82	7.79 × 10 ⁷	13
47	4.96	200.72	185.55	8.04 × 10 ⁷	8
53	4.83	178.81	295.31	7.74 × 10 ⁷	13

H₂ as the carrier gas. The oven was ramped from an initial temperature of 110°C to 280°C at 10°C min⁻¹ followed by a 5 min hold. Amino acid identification was accomplished by an Agilent 5973N mass spectrometer run under the same conditions with helium as the carrier gas and mass spectral acquisition over the 50 to 600 Da range. The protein bovine serum albumin (BSA) was analyzed in parallel to correct for responses among individual amino acids and calculation of molar ratios. The analytical precision (% relative standard deviation) for amino acid analysis was ±5%. Amino acids were normalized to percent carbon and nitrogen present in bulk samples analyzed by standard combustion methods. Total protein content was also estimated by the Bradford assay (Bradford 1976).

Preparation for proteomic analysis

Incubation particles collected on polycarbonate filters were extracted for proteins with pulse sonication in 6 M urea with a Branson 250 sonication probe at 20 kHz for 30 s on ice. The extracts were then frozen at -80°C, thawed, and sonicated again for 30 s on ice. This was repeated for a total of 5 sonications and 4

freeze/thaw cycles. Filter extracts of each incubation time point were then digested in 3 replicate groups: (1) standard tryptic digestion with reduction and alkylation (Nunn et al. 2010); (2) digestion with Endoproteinase GluC (Endo GluC), which cleaves peptide bonds C-terminal to glutamic acid (Drapeau et al. 1972) and to a lesser extent aspartic acid (Birktoft & Breddam 1994), to increase the number of proteins identified; and (3), incubation with Peptide N-Glycosidase F (PNGase F), which hydrolyzes nearly all types of N-glycan chains from glycoproteins and glycopeptides (Maley et al. 1989), in order to observe potentially modified proteins prior to tryptic digestion. All digests were concentrated using a speedvac to a volume that gave a final protein concentration of 1 µg per 10 µl based on measured protein concentrations of filter extracts. The uniform 1 µg per 10 µl protein concentration ensured that results would not be biased by sampling, or protein concentration differences at different incubation time points.

Mass spectrometry and database searching

Protein identification of sample digests was performed via shotgun proteomic tandem mass spectrometry (MS²; Aebersold & Mann 2003). Digests were analyzed using full scan (specific mass to charge ratio [*m/z*] 350–2000), followed by gas phase fractionation with repeat analyses over multiple narrow, but overlapping, *m/z* ranges (Yi et al. 2002, Nunn et al. 2006). Mass spectra were evaluated and database searched with an in-house copy of SEQUEST (Eng et al. 1994, 2008). All searches were performed with no assumption of proteolytic enzyme cleavage (e.g. trypsin, Endo GluC) to allow for identification of protein degradation products due to microbial recycling. A fixed modification was set for 57 Da on cysteine and a variable modification of 16 Da on methionine resulting from alkylation and reduction steps, respectively. A variable 1 Da modification was set for asparagine on PNGase F + trypsin digested samples to account for the conversion of asparagine to aspartic acid after cleavage of glycan chains with the use of PNGase F (Plummer et al. 1984), which takes place specifically at the consensus sequence Asn-Xxx-Ser/Thr where Xxx can be any amino acid except proline (Bause & Hettkamp 1979).

Each tandem mass spectrum generated was searched against a protein sequence database to correlate predicted peptide fragmentation patterns with observed sample ions. Probabilistic scoring of protein identifications employed PeptideProphet and

ProteinProphet (Keller et al. 2002, Nesvizhskii et al. 2003) with thresholds set at 90% confidence levels on PeptideProphet and ProteinProphet for positive protein identifications from SEQUEST search results. Mass spectra from all samples were searched against a database containing the proteomes of *Thalassiosira pseudonana* (marine diatom), *Prochlorococcus marinus* (marine cyanobacterium), and *Candidatus Pelagibacter ubique* (marine bacterium belonging to the SAR11 clade). These proteomes were selected to follow protein degradation in a diatom dominated system with potential input of bacterial proteins (e.g. Nunn et al. 2010). Results of database comparison studies showed functional agreement for over 95% of identified peptides between the *T. pseudonana* - *P. marinus* - *Ca. P. ubique* database versus the larger NCBI non-redundant database containing over 11 million protein sequences (Moore et al. 2012b). To better compare shipboard incubations with previous field observations, non-metric multidimensional scaling (NMDS, theory and applications described in Borg & Groenen 2005), was performed using R statistical software to group incubation time points with Bering Sea water column particles and surface sediments from a previous study (Moore et al. 2012a), based on the distribution of identified proteins in each sample. Suspended water column particles, sinking sediment trap material, and surface sediments analyzed by Moore et al. (2012a) were collected before, during, and after the spring 2009 phytoplankton bloom and analyzed using the same proteomic methods as samples from experimental incubations.

RESULTS

Algal proteins, identified as originating from diatoms resembling those of the proteome from *Thalassiosira pseudonana*, were detected throughout both incubations for as long as 53 d (Table 1, Fig. 1A). In the 11 d incubation, there was a rapid loss among identifiable proteins in the first 0.5 d after the onset of darkness, followed by a slower rate of loss after 1 d. In the longer 53 d incubation, there was a rapid loss of identifiable proteins in the first 5 d. The rate of loss slowed thereafter and the number of identified proteins remained relatively constant after 22 d. Similar trends were observed in both incubations for THAAs (Fig. 1B) and total protein (Fig. 1C). The THAA distribution was fairly consistent among the 11 and 53 d incubations (Appendix 1), with a sharp drop in alanine from the start (0 h) to the 12 h time point in the

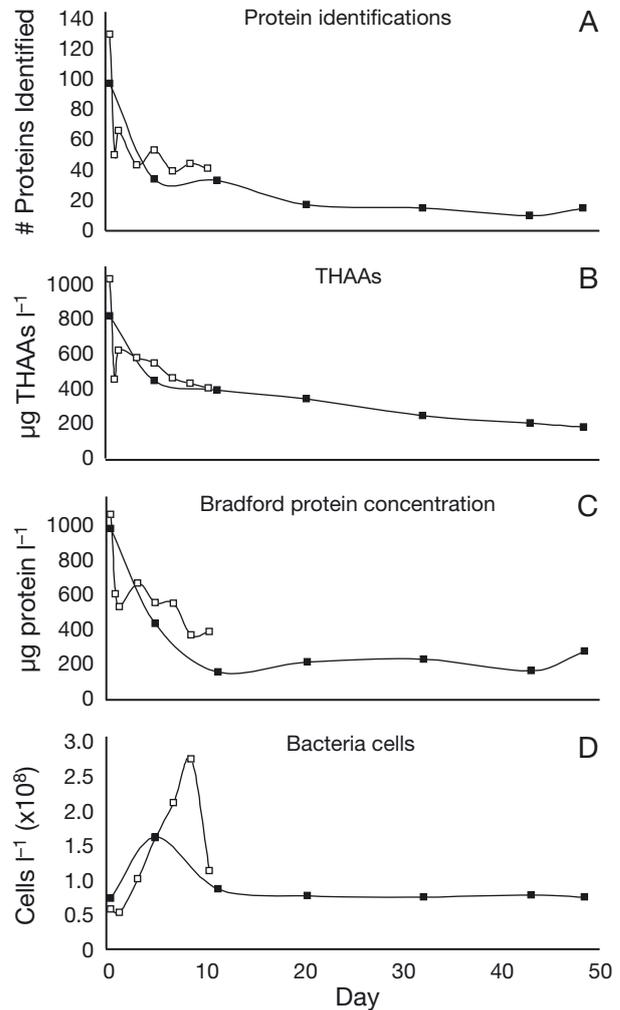


Fig. 1. Changes over time during (□) 11 d (2009) and (■) 53 d (2010) incubations of phytoplankton collected from the Bering Sea in (A) total protein identifications; (B) total hydrolyzable amino acids (THAAs); (C) total protein (Bradford assay); (D) bacterial cell abundance

11 d incubation, and a spike in glutamic acid/glutamine at Day 22 of the 53 d incubation. Total bacterial abundance peaked within the first 9 d of both incubations and declined thereafter (Fig. 1D). Despite the rapid increase in bacterial abundance, only 3 and 2 specific prokaryotic proteins were identified over the course of the 11 and 53 d incubations respectively.

Among the suite of proteins observed during the degradation sequence, chloroplast and secretory proteins were the 2 major cellular compartments represented by identified proteins in both incubations, with smaller contributions of proteins from the mitochondria, nucleus, ribosome, and unknown compartments (Fig. 2). As the incubation progressed, the sequence coverage of identified proteins generally decreased in tandem with the number of identified

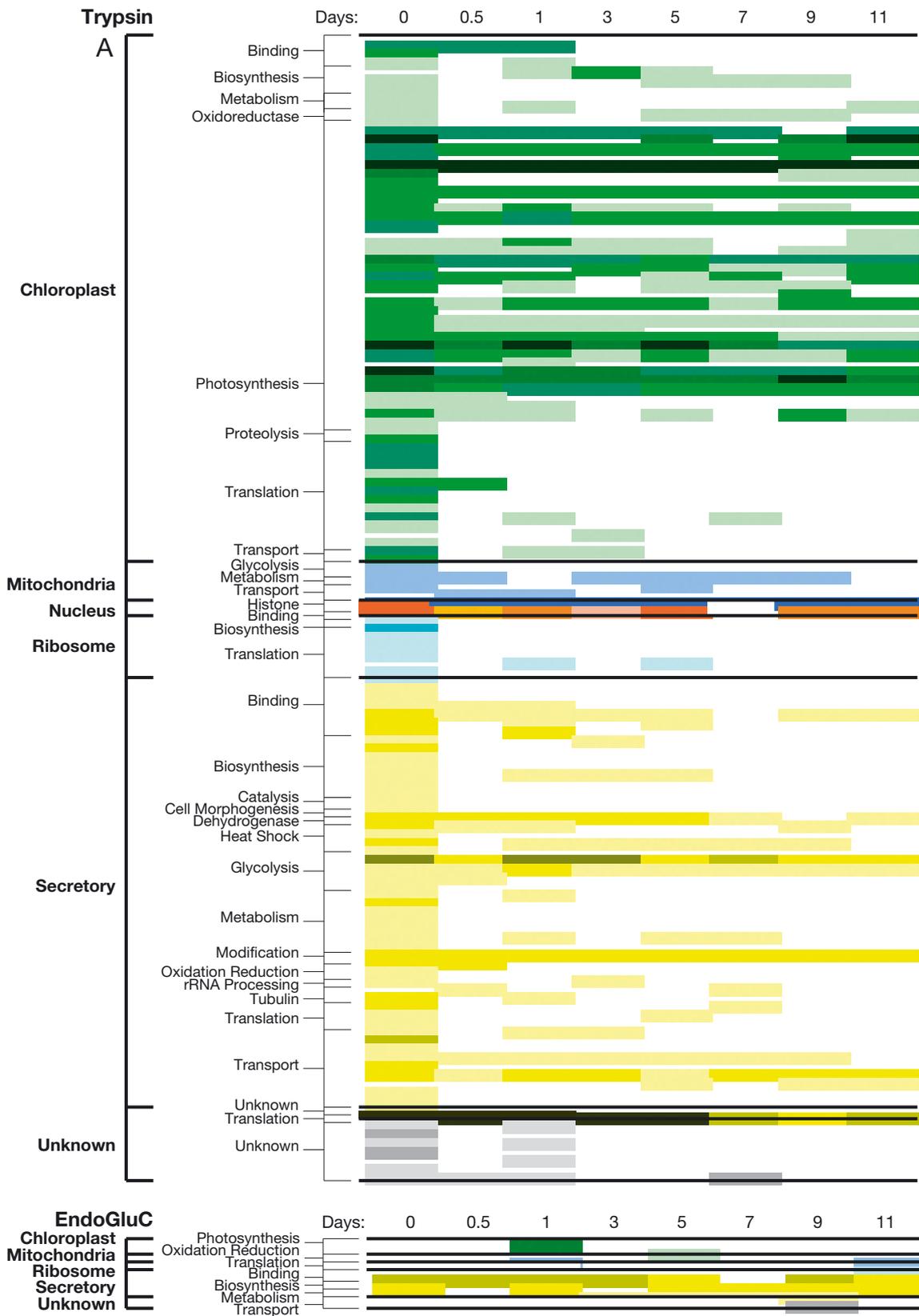


Fig. 2. Proteins identified in incubations at each time point analyzed, organized by compartment and function, showing trypsin and EndoGluC identifications for (A) 11 d and (B, next page) 53 d incubations. Cellular compartments are differentiated by color, with individual protein sequence coverage (%) represented by shading, as shown in the key

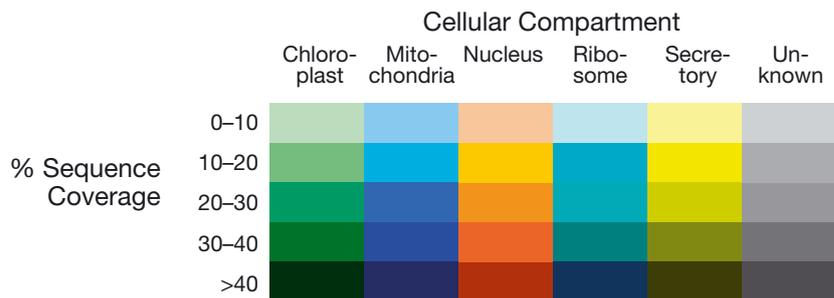
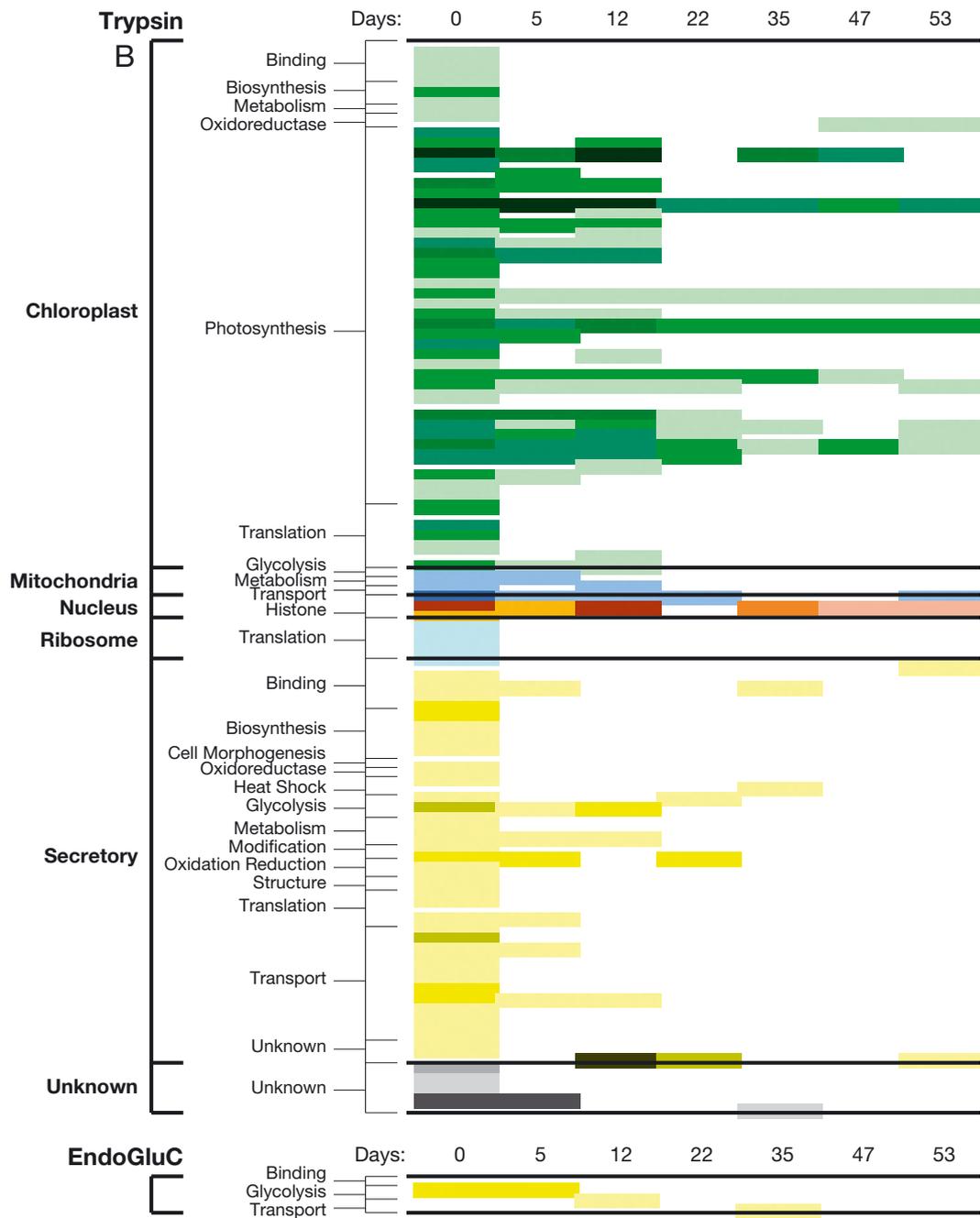


Fig. 2 (continued)

proteins. Chloroplast proteins were the most persistent, increasing in relative abundance from 44% and 48% of total proteins at Day 0 to 74 and 67% in the final time points of the 11 and 53 d incubations respectively. Conversely, the combined proportion of biosynthesis, glycolysis, metabolism, and translation proteins dropped from 38% and 27% at Day 0 to 15% and 0% percent at the final time points of the 11 and 53 d incubations, respectively.

The most dramatic loss of proteins from a specific functional group was observed among translational proteins. Of the 23 translation proteins identified in Day 0 of the 11 d incubation only 8 were identified in time points beyond Day 0, and none were identified after Day 7. Of the 15 translation proteins identified at Day 0 in the 53 d incubation only 3 were identified in time points after Day 0, and none were identified after Day 12. Furthermore, only 4 secretory proteins, which might be expected to be more available for microbial recycling than compartmentalized chloroplast proteins, were identified after Day 22. Of the 11 proteins identified at Day 53 of the long incubation, 7 were located in the chloroplast.

The use of PNGase F to lyse glycosidic bonds allowed the identification of several modified peptides, which appeared during the most rapid loss of total identifiable proteins. Two modified peptides were observed in the 11 d incubation, and 3 modified peptides were observed in the 53 d incubation. These peptides contained the consensus sequence of Asn-Xxx-Ser/Thr (Table 2). Such modified peptides were only observed in the first 5 d of both incubations. The unmodified tryptic version of the peptide from ATP synthase CF0 B chain subunit I, an important component in ATP production, was observed in the same PNGase F + trypsin digest as the modified form at Day 5 of the 53 d incubation (Table 2). Inspection of the MS² fragmentation spectra of each peptide

showed the mass change on b-ions that contained the altered asparagine for the modified peptide (Fig. 3A,B).

Digestion by EndoGluC also resulted in additional protein identifications: 8 in the 11 d incubation and 3 in the 53 d incubation (Fig. 2A,B). The use of EndoGluC on extracted protein yielded 4 additional identifications at Days 1, 5, 9, and 11 in the 11 d incubation. Over the course of the 53 d incubation no more than one additional protein was identified using EndoGluC at any time point. Secretory proteins made up the majority of additional protein IDs using the EndoGluC treatment. There were no common protein IDs made using both PNGase F and EndoGluC that were not already identified using trypsin alone.

In the NMDS analysis, early incubation time points (Days 0 to 11) cluster closely together, as do later incubation time points (Days 22 to 53). Chl-max particles and sinking sediment trap particles are positioned more closely to initial incubation time points than later time points in the 11 and 53 d incubations. The post-bloom shelf surface sediment is positioned closer to early incubation time points while post-bloom basin sediment and over wintered shelf sediment are equidistant to intermediate and later incubation time points. It should be emphasized that results of NMDS analysis of shipboard incubations and Bering Sea suspended particles, sinking sediment trap material and surface sediments are qualitative in nature. Samples that are ordinated closer together have protein distributions that are more similar than samples that are ordinated farther apart (Fig. 4). Similar ordinations between incubation time points and water particles or sediments could allow approximate time frame estimations to be assigned to samples in the field.

Table 2. Peptides identified with the amidase PNGase F + the proteolytic enzyme trypsin during 11 d (2009) and 53 d (2010) incubations of phytoplankton collected from the Bering Sea. Each of these individual peptides contain the 1 Da mass increase on asparagine resulting in the 115 Da aspartic acid (indicated by: N[115]), which represents the presence of an N-glycan chain post translational modification on the peptide. The protein to which the peptides were correlated, and the first day of the incubation on which the protein was identified are also given

Protein	Peptide(s)	Day
11 d incubation		
RuBisCO large subunit	IHYLGDDVVLQFGGGTIGHPDGIQAGATAN <u>N[115]</u> R	1
Predicted protein	DLAEIWDN <u>[115]</u> SSPVIVQGGSLR	1
53 d incubation		
RuBisCO large subunit	TALDLWKDISFN <u>[115]</u> YTSTDTADFAETATANR	0
ATP synthase CF0 B chain subunit I	ALIN <u>[115]</u> ETIQKLEGDLL	5
Predicted protein	QVVELYTEDGLDRPFFAIVETPGSGN <u>[115]</u> JVVR	5

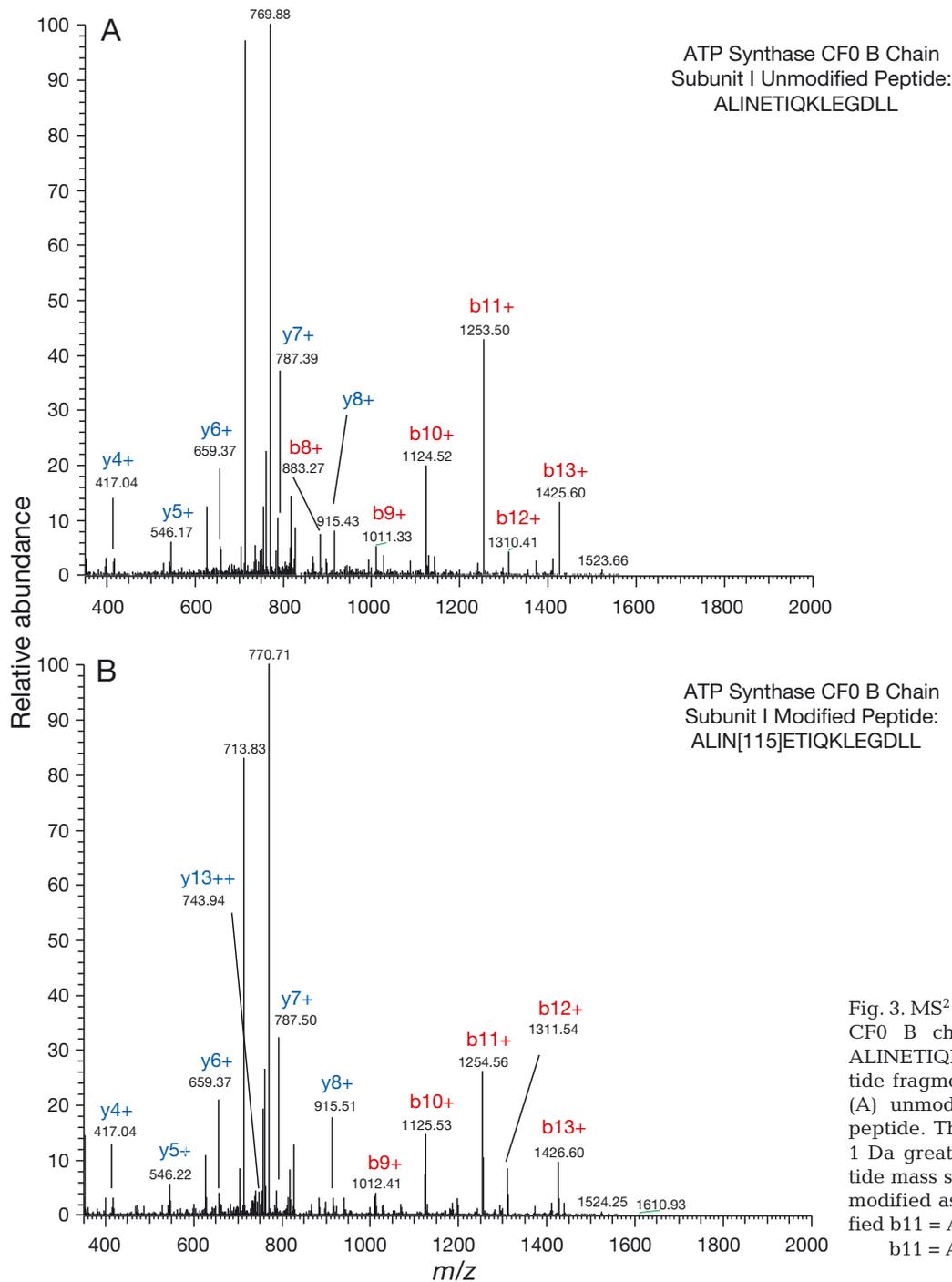


Fig. 3. MS² spectra of ATP Synthase CF0 B chain subunit I peptide ALINETIQKLEGDLL showing peptide fragment b- and y-ions of the (A) unmodified and (B) modified peptide. The b-ions 9 to 13 are all 1 Da greater in the modified peptide mass spectra, representing the modified asparagine, (e.g. unmodified b11 = ALINETIQKLE; modified b11 = ALIN[115]ETIQKLE)

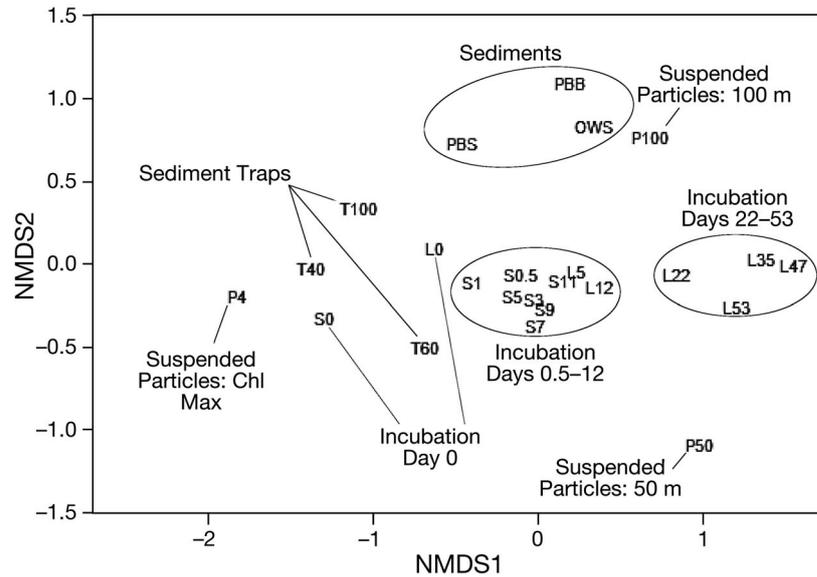
DISCUSSION

Differential recycling of algal proteins and preservation

The survival of algal proteins over 53 d of microbial recycling demonstrates that some fraction of intact proteins can survive microbial recycling long enough to be exported from the marine water column to sedi-

ments. At a sinking rate of up to 100 m d⁻¹ (Smetacek 1985), the incubation period of 53 d is more than adequate for algal proteins in a diatom-dominated system to transit the water column to Bering Sea shelf and basin sediments. The presence of such proteins in sediments was previously observed by Moore et al. (2012a), suggesting that other diatom-dominated ecosystems have the potential for export of identifiable protein from phytoplankton blooms to the sediment.

Fig. 4. Non-metric multidimensional scaling (NMDS) ordination based on the protein distribution of Bering Sea water column and sediment samples (Moore et al. 2012a) and the time points of the 11 d and 53 d incubations: P4: Chl Max suspended particles; P50: 50 m suspended particles; P100: 100 m suspended particles; T40: 40 m sediment trap; T60: 60 m sediment trap; T100: 100 m sediment trap; PBS: Post-bloom shelf sediment; PBB: Post-bloom basin sediment; OWS: Overwintered shelf sediment; S0, S0.5, S1, S3, S5, S7, S9, S11: 11 d incubation on Days 0, 0.5, 1, 3, 5, 7, 9 and 11, respectively; L0, L5, L12, L22, L35, L47, L53: 53 d incubation on Days 0, 5, 12, 22, 35, 47 and 53, respectively



The path of changes to the suite of identifiable algal proteins seen over time appears to start with proteome changes within the cells, followed quickly by cell lysis and then microbial recycling. After Day 0, in the 11 d incubation, the decrease in the total number of identified proteins and in the proportion of biosynthetic, glycolysis, metabolism, and translation proteins may represent the shutdown of cellular activity or onset of lysis among algal cells. This is supported by results showing that the biosynthetic, metabolism, and translation proteins in the chloroplast were lost much more rapidly than photosynthetic proteins in the chloroplast, despite the same level of membrane protection. These changes took place early in the incubation, before the rapid rise in bacterial abundance at Day 9 (of the 11 d incubation), which is consistent with the pattern of diatom cellular activity and decline observed by Harvey et al. (1995) in laboratory incubations. This initial drop in total protein IDs, THAAs, and total protein may also be due to cell lysis and/or extracellular enzymes, which would release cytoplasmic proteins and particulate organic matter into the dissolved phase (Amon & Benner 1996, Vetter & Deming 1999). Particulate sampling, coupled with potential heterogeneity of carboxy material, would then miss this newly dissolved material as observed at 0.5 d.

The observed loss of identified translation proteins at early time points likely indicates the shutdown of protein synthesis, as algal cells stop growth under simulated bloom termination, and undergo internal protein recycling. The overall loss of identified cytoplasm proteins was more rapid than the loss of

chloroplast proteins, but identified translation proteins located in the chloroplast were lost just as rapidly as translation proteins located in the ribosome or cytoplasm. In previous studies it has been observed that proteins encapsulated in organic matter or cellular compartments are preferentially preserved during microbial recycling (Nguyen et al. 2003, Nunn et al. 2010, Moore et al. 2012a). Cell lysis may have played a role in the loss of cytoplasmic translation proteins, but the rapid loss of identifiable translation proteins with additional membrane protection in cellular compartments indicates that some loss was due to internal recycling within the cell in tandem with microbial activity.

After initial cellular recycling, further changes to protein distribution were observed during microbial recycling as biosynthesis, glycolysis, metabolism, and translation proteins continued to decline until Day 22 and were no longer detectable at the conclusion of the 53 d incubation. However abundant photosynthetic proteins persisted and remained detectable after 53 d of degradation (Fig. 2). After 53 d, 5 and 3 peptides were identified from the RuBisCO large and small subunits respectively, contrary to previous findings that RuBisCO was degraded rapidly and not retained in a lab based degradation of *Thalassiosira pseudonana* (Nunn et al. 2010). Two fucoxanthin chlorophyll binding proteins (FCPs) were identified throughout the 53 d incubation in agreement with findings that these proteins remain identifiable for extended periods in lab degradation (Nunn et al. 2010), Bering Sea shelf sediments (139 m), and Bering Sea basin sediments (3490 m) (Moore et al. 2012a).

Microbial input

At early time points in both incubations, protein IDs, THAAs, and total protein decreased followed by increases in bacterial cell numbers. The increase in bacterial abundance was first apparent at Day 3 of the 11 d incubation and continued until Day 9, indicating that there is a slight lag in cell replication after the initial recycling of particulate THAAs, total protein measured spectrophotometrically, and identifiable proteins (Fig. 1). Protein IDs, THAAs, and total protein subsequently decrease more slowly after bacterial cells return to original levels (Fig. 1). Grazing by bacterivores, such as flagellates, protists, and viral lysis (Steward et al. 1996, Vaque et al. 2008) may have decreased bacteria cell counts after initial bacterial proliferation. This would limit overall bacteria-driven protein recycling in later incubation time points. Despite this, bacteria in these incubations appear to be the primary protein recyclers given that macrozooplankton were excluded in these incubations. Using an average bacterial protein content per cell of 24 fg cell⁻¹ (Zubkov et al. 1999), the estimated bacterial fraction of total protein and THAAs was highest at or near the peak bacteria cell abundance in both the 11 and 53 d incubations (Table 1), but never exceeded 2% of total protein or THAAs throughout the incubations. This low value for what is also expected to be a large suite of different bacterial protein types may explain why identifiable proteins were dominated by diatom sources and few prokaryotic proteins were observed.

Identifying modified proteins

While difficulties are common in recognizing protein modifications, identification of such modifications by proteomic methods has the potential to more fully characterize degraded and modified proteins in environmental samples. Non-enzymatic glycation is a major cause of spontaneous damage to cellular and extracellular proteins in physiological systems (Ahmed & Thornalley 2007). Glycation of RuBisCO has been shown to decrease the enzyme's activity, and increase its susceptibility to proteases (Yamauchi et al. 2002). Various mechanisms have been observed in higher plants (Kim & Kim 2003, Sultana et al. 2009) and the microalga *Chlorella zofingiensis* (Sun et al. 2011) to prevent and repair protein glycation. Thus, the potential glycation of RuBisCO and other proteins (Table 2) in the early stages of recycling may represent protein turnover within the cell.

Glycation has also been hypothesized as a mechanism for extra-cellular protein preservation and the formation of sedimentary geopolymers (Collins et al. 1992, Burdige 2007). Mass spectral analysis with InsPecT (Tanner et al. 2005) was used to identify recurring modification masses on precursor ions, revealing potential sugar modifications to proteins from degraded phytoplankton (Nunn et al. 2010). All modified peptides in this study were observed during the early stages of both incubations while the number of observed biosynthesis, glycolysis, metabolism, and translation proteins decreased. While the 5 d time period over which modified peptides were seen does not allow unequivocal evidence that the observed peptide modifications and proteome changes were directly linked to cell death, (e.g. Peters & Thomas 1996), such changes may be the initial steps that take place before dormancy and eventually cell death (Bidle & Falkowski 2004, Nunn et al. 2010).

Identifiable protein correlates to organic matter degradation proxies

The ratios of THAA/OC and THAA-N/PN can be useful proxies for degradation status of organic matter (Cowie & Hedges 1994 and references therein). Plotting the number of identified proteins against the ratio of THAA/OC and THAA-N/PN (Fig. 5), reveals that proteins throughout the 11 d incubation and the 3 earliest time points of the 53 d incubation (Days 0, 5, 12) have greater numbers of protein IDs and higher ratios of THAA/OC and THAA-N/PN than samples over longer incubation times of the 53 d incubation (Days 22, 35, 47, 53), which cluster together more closely. The shift in the number of identified proteins in the 11 d incubation and early time points of the 53 d incubation to the cluster of 4 final time points of the 53 d incubation suggests that, after initial rapid degradation, identifiable protein material degrades much more slowly.

There are significant correlations between the number of protein identifications and both the ratios of THAA/OC and THAA-N/PN (Fig. 5) as observed in suspended particles, sinking material, and surface sediments in the Bering Sea (Moore et al. 2012a). In comparing our shipboard incubations to field collected material from the same location (Moore et al. 2012a), it is striking that the number of identified proteins and ratios of THAA/OC and THAA-N/PN for incubations times of 22, 35, 47, and 53 d were very similar to those seen in deep basin sediments (3490 m), over wintered shelf sediments (101 m), and

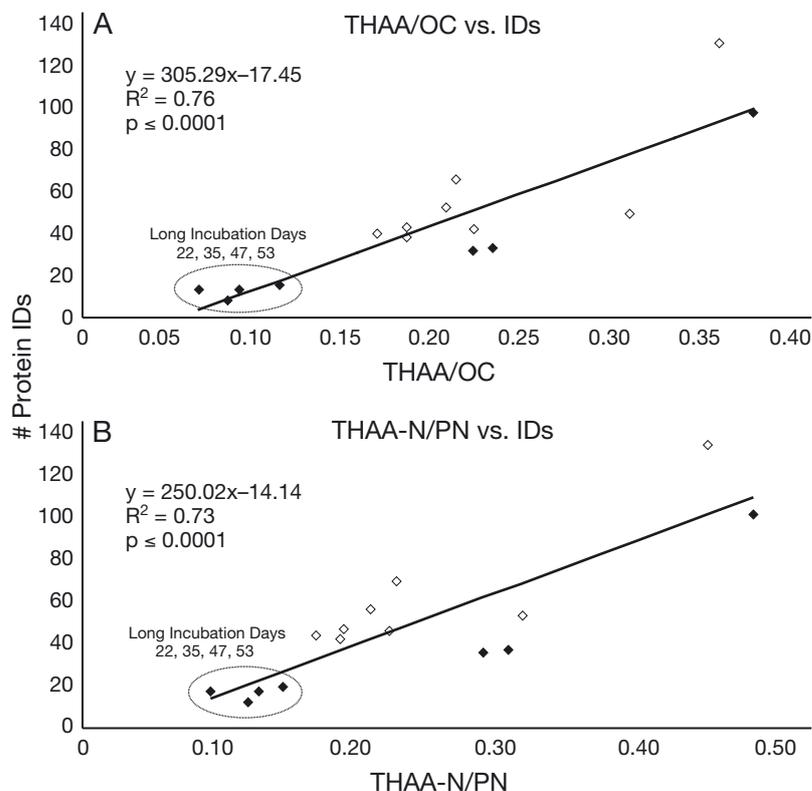


Fig. 5. Plots of total protein identifications plotted against the organic matter degradation status parameters, for (\diamond) 11 d and (\blacklozenge) 53 d incubations, showing fraction of (A) organic carbon made up by total hydrolyzable amino acids (THAA/OC); and (B) particulate nitrogen made up by total hydrolyzable amino acid nitrogen (THAA-N/PN). Linear regressions represent the correlation between identifiable proteins and organic matter recycling

suspended particles (50 m, 100 m) of the Bering Sea. Although this finding suggests that the rapid protein losses seen in shipboard incubations are realistic, it also supports findings that intact proteins contribute to a fraction of marine sedimentary amino acids (Pantoja & Lee 1999, Nguyen & Harvey 2001, Nunn et al. 2010, Moore et al. 2012a). The distribution of remaining proteins observed after 22 d also indicates that abundant chloroplast proteins make up the majority of identified preserved proteins.

Amino acids are known to be important contributors to particulate and sedimentary marine nitrogen pools (Burdige & Martens 1988, Lee et al. 2000). Analysis by ^{15}N nuclear magnetic resonance (NMR) has also shown that most organic nitrogen present in dissolved and particulate organic matter is amide linked, like the bonds that connect amino acids in proteins (McCarthy et al. 1997, Zang et al. 2001). The correlations between protein IDs, THAA/OC and THAA-N/PN, and the longevity of identifiable protein observed in this study, and in findings by Moore et al. (2012a) identifying algal proteins in Bering Sea sinking sediment trap material and sediments, suggest that diatom dominated systems are important regions of organic nitrogen and organic carbon export. These studies also show promise for proteomic characterization of amide linked nitrogen or protein

material measured as THAAs throughout sediments of the global ocean.

Statistical analysis of incubations with environmental samples

Inspection of the identified protein pool among sediment samples and later incubation time points with NMDS, taking into account protein function, shows evidence that the distribution of proteins identified in post-bloom shelf sediment (Moore et al. 2012a) matches more closely with incubation time points from 0.5 to 12 d than incubation time points after 22 d (Fig. 4). Proteins identified at later time points of the 53 d incubation were also identified in post bloom shelf sediment, but they make up only 23% of identifications. The distribution of identified proteins at time points after 22 d of the 53 d incubation matches more closely with post-bloom basin sediment and overwintered shelf sediment in which proteins identified after 22 d make up 38% and 52% of identified proteins, respectively. The distribution of common proteins between later time points, post-bloom basin, and overwintered shelf sediment includes primarily chlorophyll binding proteins, photosystem II proteins, and DNA binding histones. This

provides further evidence that protein material which lasts beyond the apparent 22 d threshold represents material that can be preserved on seasonal timescales and be available as a food source to the benthic community, not only during the productive spring bloom, but the ice-covered winter months as well. The observation that 50 and 100 m suspended particles are positioned closer to later time points than post-bloom shelf sediment illustrates the importance of export to sediments towards removal from water column recycling and protein preservation.

Implications for benthic communities and the nitrogen cycle

Given the reduced impact of microbial recycling following 22 d of incubation on protein IDs, THAAs, total protein, and the similar ratios seen in THAA/OC and THAA-N/PN after 22 d with Bering Sea sediments, it is reasonable to consider that incubation material after 22 d is representative of primary production arriving at the sea floor after bloom termination. Approximately 42% of THAAs remain after 22 d of microbial recycling compared to initial amounts in the 53 d incubation. This is a substantial fraction of organic nitrogen available to fuel benthic activity. Therefore, the transport of algal proteins from the water column to sediments indicates that primary production is an important organic nitrogen source to benthic communities. Recycling by consumers may reduce the amount of identifiable proteins in sinking sediment trap material compared to bloom material as observed by Moore et al. (2012a), but grazing impact is reduced during periods of high production (Chen et al. 2003, Lovvorn et al. 2005). This agrees with previous findings that production from the water column supports high benthic faunal density in the Bering Sea (Grebmeier et al. 1988).

The contribution of intact protein to marine sediments may also explain why similar $\delta^{15}\text{N}$ values have been observed between water column material and surface sediments from the Bering Sea shelf during ice covered winter months (Lovvorn et al. 2005), and in time series samples from coastal upwelling regions of the NE Pacific (Altabet et al. 1999). Furthermore, the longevity of exported phytoplankton proteins to the continental shelf and basin may be important in providing a food source to benthic filter feeders during the ice-covered low production winter months when new material is not being actively exported to sediments. This suggests that protein preservation plays a role in the yearlong ecosystem support of

Bering Sea, and potentially other high latitude coastal, benthic communities.

Global calculations of marine nitrogen fixation and denitrification have estimated a fixed nitrogen deficit of roughly 200 Tg N yr⁻¹ (Mahaffey et al. 2005, Codispoti 2007). Sedimentary $\delta^{15}\text{N}$ records however, have suggested global N cycle homeostasis during the Holocene (Deutsch et al. 2004, Altabet 2007). A more recent study by Großkopf et al. (2012) has shown that a widely used method for measuring N₂ fixation rates underestimates the contribution of nitrogen fixing diazotrophs compared to a newly developed method. This results in a nitrogen fixation increase of 103 to 177 ± 8 Tg N yr⁻¹ leaving a remaining fixed nitrogen deficit of 23 to 97 Tg N yr⁻¹. The 2 yr spring summer study by Horak et al. (2013) suggested that denitrification rates in Bering Sea shelf sediments are higher than previously thought, and that sediment regenerated NH₄⁺ is an insignificant contribution to water column dissolved inorganic nitrogen (DIN) and primary production. Coupled nitrification-denitrification observed by Granger et al. (2011) has also indicated that N loss occurring in Bering Sea sediments is greater than other regions of the ocean and that off-shelf waters potentially contribute nitrate to the outer shelf. This study presents evidence suggesting that exported protein material from the Bering Sea water column to sediments, where it is denitrified, may also be an important potential pathway for loss of fixed nitrogen transported to the Bering Sea from off shelf, thus contributing to the fixed nitrogen deficit.

CONCLUSIONS

The persistence of algal proteins during both short-term and extended shipboard incubation degradation experiments indicates that some fraction of this material can survive water column recycling in time-frames that are amenable to being transported to marine sediments. The initial changes to the distribution of identified translation, biosynthesis, glycolysis, and metabolism proteins indicate that internal cellular recycling plays an important role in protein cycling during the early time points of simulated bloom termination. Glycation modifications were only observed during the first 5 d of the 2 incubations, suggesting these modifications may be linked with the shutdown of cellular machinery. Continued rapid loss of secretory proteins during the period of bacterial cell population growth represents the microbial recycling of bioavailable proteins that are not encapsulated in cellular compartments and re-

leased during cell lysis. The number of identifiable proteins, THAAs, total protein, and the ratios of THAA/OC and THAA/PN declined more slowly after 22 d. Identifiable proteins that survived after the observed 22 d threshold appear to represent material that can be preserved over seasonal timescales in sediments, and be available as a food source to the benthic community. The large fraction of remaining THAAs after initial microbial recycling, and correlations between identifiable proteins and the ratios of THAA/OC and THAA/PN, suggest that identifiable algal proteins make up an important fraction of marine sedimentary organic nitrogen. The observed longevity of identifiable proteins during simulated bloom termination and microbial recycling suggests that diatom dominated systems are important regions for organic nitrogen export and potential preservation in sediments.

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Appendix 1. Amino acid distribution (mole %) of phytoplankton derived particulate material from each time point of 11 d and 53 d shipboard incubations of phytoplankton collected from the Bering Sea. Values are means with standard error in parentheses.

11 d incubation	Day							
	0	0.5	1	3	5	7	9	11
Alanine	14.92 (0.33)	9.80 (0.18)	9.63 (0.12)	8.55 (0.46)	8.52 (0.17)	9.48 (0.06)	10.08 (0.77)	9.60 (0.16)
Glycine	7.37 (0.09)	7.83 (0.05)	8.35 (0.13)	8.42 (0.13)	8.30 (0.19)	8.96 (0.12)	8.86 (0.17)	9.48 (0.11)
Valine	6.93 (0.33)	5.42 (0.11)	5.37 (0.08)	5.84 (0.15)	5.69 (0.08)	5.83 (0.10)	5.55 (0.08)	5.74 (0.12)
Leucine	6.56 (0.17)	6.32 (0.05)	6.59 (0.01)	6.64 (0.09)	6.48 (0.10)	6.83 (0.04)	6.69 (0.01)	6.62 (0.09)
Isoleucine	4.35 (0.16)	4.60 (0.05)	4.49 (0.30)	4.98 (0.10)	4.65 (0.21)	4.35 (0.06)	4.11 (0.16)	4.30 (0.03)
Threonine	3.66 (0.25)	7.87 (0.54)	7.29 (0.30)	7.43 (0.35)	8.35 (0.42)	5.93 (0.14)	5.59 (0.32)	6.06 (0.04)
Proline	4.42 (0.09)	4.17 (0.03)	4.22 (0.02)	3.81 (0.04)	3.58 (0.10)	4.34 (0.12)	4.21 (0.03)	4.16 (0.07)
Aspartic acid/asparagine	5.56 (0.24)	9.53 (0.13)	9.53 (0.10)	10.25 (0.30)	10.07 (0.25)	9.45 (0.05)	9.37 (0.28)	9.37 (0.15)
Phenylalanine	2.33 (0.04)	3.13 (0.06)	3.32 (0.01)	3.69 (0.13)	3.49 (0.08)	3.27 (0.06)	3.26 (0.02)	3.25 (0.11)
Glutamic acid/glutamine	10.18 (1.40)	12.32 (1.01)	11 (0.16)	10.47 (0.17)	12.22 (0.59)	10.21 (0.87)	11.01 (1.22)	10.31 (0.97)
Lysine	3.94 (0.72)	3.18 (0.12)	3.21 (0.11)	3.45 (0.08)	4.53 (0.31)	2.94 (0.38)	3.46 (0.27)	2.81 (0.47)
Tyrosine	3.65 (1.92)	1.99 (0.25)	1.22 (0.03)	1.16 (0.13)	2.28 (0.14)	0.68 (0.18)	0.91 (0.13)	0.70 (0.07)
53 d incubation	Day							
	0	5	12	22	35	47	53	
Alanine	17.93 (1.79)	15.30 (0.51)	15.75 (1.52)	12.87 (0.70)	15.43 (0.27)	15.45 (0.16)	15.30 (0.72)	
Glycine	13.57 (0.16)	13.24 (0.55)	15.99 (2.47)	11.62 (0.51)	15.37 (0.72)	16.90 (0.29)	17.14 (0.63)	
Valine	6.18 (0.44)	6.91 (0.39)	5.96 (0.63)	7.72 (0.25)	10.68 (0.68)	11.66 (0.03)	11.26 (0.76)	
Leucine	9.12 (0.35)	9.26 (0.60)	9.24 (0.92)	7.81 (0.32)	9.21 (0.44)	9.80 (0.18)	9.26 (0.58)	
Isoleucine	4.37 (0.13)	3.56 (0.23)	4.23 (0.19)	4.28 (0.12)	3.89 (0.03)	3.72 (0.16)	3.53 (0.22)	
Threonine	3.42 (0.44)	4.18 (0.49)	4.75 (0.67)	7.21 (0.39)	6.70 (0.74)	6.79 (0.48)	6.99 (1.44)	
Proline	6.04 (0.62)	5.99 (0.20)	7.06 (1.57)	4.51 (0.08)	3.02 (0.49)	2.62 (0.27)	3.13 (0.55)	
Aspartic acid/asparagine	10.18 (0.88)	10.48 (0.46)	11.39 (0.95)	10.18 (0.59)	4.97 (0.98)	4.88 (0.86)	7.41 (2.08)	
Phenylalanine	3.69 (0.41)	3.46 (0.28)	3.96 (0.12)	4.16 (0.10)	5.31 (0.21)	5.32 (0.33)	4.98 (0.13)	
Glutamic acid/glutamine	12.74 (0.57)	14.34 (0.80)	13.63 (3.72)	19.57 (1.41)	12.16 (0.96)	10.61 (0.77)	10.11 (0.28)	
Lysine	5.43 (0.57)	5.88 (0.44)	4.18 (1.18)	5.11 (0.18)	5.48 (0.71)	4.54 (0.65)	3.63 (0.64)	
Tyrosine	7.47 (0.35)	7.41 (3.37)	3.87 (1.54)	4.99 (0.38)	7.78 (0.56)	7.69 (1.52)	7.27 (0.95)	