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Organic Geochemistry 37 (2006) 1003–1018
Organic Geochemistry
www.elsevier.com/locate/orggeochem

Effects of mercuric chloride and protease inhibitors on degradation of particulate organic matter from the diatom *Thalassiosira pseudonana*

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Received 31 January 2006; received in revised form 23 May 2006; accepted 25 May 2006
Available online 8 August 2006

Abstract

Mercuric chloride ($HgCl_2$) and broad-spectrum protease inhibitors are often used to inhibit bacterial or enzymatic activity in environmental samples. In this study, we investigated their effects on degradation of particulate organic matter derived from a culture of the diatom, *Thalassiosira pseudonana,* and from sediment traps, with emphasis on compositional changes over one month. In control (untreated) samples, concentrations of particulate organic carbon (POC) and nitrogen (PN) and particulate amino acids (PAAs) decreased by 50%, dissolved combined amino acids (DCAAs) decreased by 34% and dissolved free amino acids (DFAAs) decreased by 64% relative to time zero. Production of γ-aminobutyric acid after one month indicated biological degradation. Fatty acids, but not neutral lipids, were also degraded substantially. Chlorophyll *a* (Chl-*a*) increased after one month by about 30%, suggesting the release of free Chl-*a* from Chl–protein complexes via decomplexation or degradation of proteins.

Concentrations and compositions of samples treated with protease inhibitor (PI) were similar to untreated controls over time, indicating that PI alone did not stop bacterial activity. For samples treated with $HgCl_2$, 10–20% of the initial organic carbon and nitrogen, PAAs and lipids were lost. DCAAs and DFAAs increased by about the same amount as PAAs decreased, suggesting that dissolution was a major mechanism for loss of particulate organic compounds. Particulate arginine, tyrosine and threonine were slightly enriched, and 16:3, 18:3 and 18:4 fatty acids depleted, in Hg-treated samples. Moreover, Chl-*a* decreased by a factor of 4 after one month, compared to controls. Simultaneously, a significant amount of Chl-*a* O-allomer was produced. Although Chl-*a* allomerization in oceanic sediment trap samples treated with $HgCl_2$ was not as great as in Hg-treated diatom cultures, we recommend summing Chl-*a* and the allomer peak areas to avoid underestimating Chl-*a* concentrations in natural samples, or reporting the allomer separately. $HgCl_2$ is an excellent agent for preventing microbial decomposition of marine particulate materials in both field and experimental use, but the changes in composition it causes must be considered in interpreting analytical results.
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1. Introduction

Preservation of the compositional integrity of marine organic matter (OM), either in dissolved or

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doi:10.1016/j.orggeochem.2006.05.013

particulate form, is required in laboratory and field studies. In the laboratory, preservatives or poisons are often added to samples as controls in OM decomposition experiments (e.g. Christian and Karl, 1995; Harvey et al., 1995). In the field, preservatives are used to prevent degradation of OM, for example in sediment traps used to collect sinking particles that are deployed for long time periods (Gardner et al., 1983; Ittekkot et al., 1984; Knauer et al., 1984).

Several studies have compared the effectiveness of different poisons or preservatives with respect to OM. Knauer et al. (1984) determined how C, N and trace metal compositions of sediment trap materials were affected by formalin, sodium azide and $HgCl_2$ during deployment and concluded that formalin and $HgCl_2$ were effective at stopping degradation, but sodium azide was not. In principle, treatment with $HgCl_2$ (hereafter termed HG) can stop microbial activity by reacting with protein sulfhydryl groups, thus denaturing most enzymes irreversibly (Royer, 1982). Lee et al. (1992) showed that 180 μM HG stopped essentially all bacterial activity (<1%) in particulate samples from sediment traps. Moreover, HG does not introduce any organic C into the sample, unlike formaldehyde and chloroform. On this basis, HG has been regularly used to preserve OM in sediment traps in field studies (e.g., Lee et al., 2000; Wakeham et al., 2002; Ingalls et al., 2003). However, it is not known exactly how exposure to HG affects the composition of marine OM. A systematic study comparing the effects of HG, antibiotic, azide, formalin and chloroform on sediment trap C, N and biochemicals was complicated by the presence of abundant "swimmers" (Lee et al., 1992; Hedges et al., 1993; Wakeham et al., 1993). The impact of "swimmers" may be minimized using special sediment trap technology (e.g., Peterson et al., 1993).

In the marine environment, bacteria release many extracellular enzymes (exoenzymes) into solution to cleave macromolecules into smaller compounds that can be directly absorbed (Azam et al., 1983; Chrost, 1991). Exoenzymes without sulfhydryl or other groups that react with HG may still degrade organic compounds in the presence of HG after the bacteria have been killed. Proteases are a major family of exoenzymes that catalyze the hydrolysis of peptide bonds. Instead of HG, protease inhibitors (PIs) can be used to prevent hydrolysis of specific proteins by blocking the active sites of proteases (Barrett and Salvesen, 1986). Most broad-spectrum PIs can inhibit serine, cysteine, and aspartic proteases and would thus prevent the hydrolysis of protein in marine particulate samples. The effectiveness of PIs in inhibiting exoenzymatic activities during degradation of marine particles has not been well studied.

In this study, we investigated the effects of HG and PI on the amount and composition of marine OM during a laboratory decomposition experiment (1 month) and compared the results to materials collected by sediment traps in the Mediterranean Sea. We used a common marine diatom *Thalassiosira pseudonana* to represent particulate OM. We measured organic C and N content and the amounts and compositions of AAs, pigments, fatty acids (FAs) and sterols. The goal was to provide information on how preservation of marine samples using HG or/and PIs affects OM decomposition in the field and laboratory and how to better determine the extent of degradation in field-preserved samples.

2. Materials and methods

2.1. Laboratory decomposition protocol

T. pseudonana 3H was obtained from Bigelow laboratory (CCMP1335). It was grown in culture under a 14:10 h (light:dark) cycle at 20 °C after axenic inoculation into 1000 ml of seawater supplemented with f/2 medium. After 10 d, cell counts showed that the culture had reached stationary phase. At this point, about 600 ml filtered (2 μm Nylon filter) seawater from Stony Brook Harbor, NY was added to the culture flask to introduce bacteria. The culture was incubated a further 3 d under the same conditions (light/dark cycle) to allow bacteria to grow.

After shaking well, the culture was split into 7 × 200 ml aliquots and each was placed into a 250 ml Nalgene bottle. We treated these sub-samples as follows: nothing was added to two control aliquots and $HgCl_2$ (Sigma) was added to two, with a final concentration of 180 μM. Additional treatments included 0.5 ml protease inhibitor (PI) in two aliquots and both $HgCl_2$ (180 μM) and 0.5 ml PI in one aliquot. For protease inhibition we used a broad-spectrum cocktail from Sigma (P8465), containing 4- (2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, E-64, bestatin and sodium EDTA. This mixture has a broad specificity for the inhibition of serine, cysteine, aspartic and metallo-proteases and aminopeptidases. Five ml is recommended for inhibition of proteases extracted from 20 g of *Escherichia*

coli; in our case, 0.5 ml PI should have been enough for 200 ml of culture.

Treated samples were mixed well. Smaller subsamples from one HG-treated, one PI-treated and one control bottle were immediately filtered on to combusted GFF 47 mm filters, as time zero samples for particulate C and N, AA, pigment and lipid analyses. Filtrates were also collected for analysis of dissolved AAs. The remaining four treated subsamples were incubated in the dark at 9 °C; after 33 d, they were filtered in the same way as for time zero. All the filters and filtrates were frozen (−20 °C) immediately after collection. Dissolved oxygen was measured using a Unisense oxygen sensor and all samples were oxic after 33 d. For convenience, samples are referred to as Ctrl-0, HG-0 and PI-0 for the control, HG-treated and PI-treated samples at time zero, respectively. Ctrl-33, PI-33, HG-33, HG + PI-33 represent the control, PI-treated, HG-treated and both PI- and HG-treated samples at 33 d, respectively.

2.2. Sediment trap collection

Particulate material from sediment traps was collected from 200 m at the DYFAMED site in the Ligurian Sea (Mediterranean) in 2003 using two types of sampler (Peterson et al., 2005). Fresh, unpoisoned material was collected using a NetTrap device in May; its detailed organic composition is described by Goutx et al. (unpublished results); a fraction of this fresh material was also treated with HG for 5 d. Material preserved with HG was also collected using an IRS sediment trap deployed from March until May. Samples were filtered on to combusted GFF filters immediately after collection.

2.3. Analytical methods

2.3.1. POC and PN

POC and PN were measured using a Carlo Erba model 1602 CNS analyzer after acidifying filters under HCl fumes to remove inorganic C (Peterson et al., 2005); precision for N is ±5% and ±2% for C.

2.3.2. Amino acids (AAs)

AAs were measured using high performance liquid chromatography (HPLC) with fluorescence detection after acid hydrolysis and pre-column OPA derivatization of the samples (Lindroth and Mopper, 1979; Lee et al., 2000). For particulate amino acids (PAAs), particles on GFF filters were hydrolyzed under N_2 at 110 °C for 20 h with 6 N HCl (with 0.25% phenol) to release free AAs. Hydrolyzates were filtered through combusted glass wool, dried under N_2 and dissolved in water. For dissolved AAs (taken from the sample filtrate), total AA samples were first hydrolyzed using a microwave vapor-phase hydrolysis system (Kuznetsova et al., 2005) and then injected into a Shimazu HPLC instrument equipped with an Alltech Alltima C_{18} column (Ingalls et al., 2003), while free amino acid samples (DFAAs) were injected into the HPLC instrument directly without hydrolysis. Dissolved combined amino acid (DCAA) concentrations were calculated as the difference between total dissolved amino acids and DFAAs. Averaging analyses of the three time zero samples resulted in variations in individual AA concentrations of the order of 2–8% for most compounds, with 12% for methionine and 11% for lysine.

2.3.3. Chlorophyll

Chl-*a* was analyzed using HPLC with UV detection as described in Sun et al. (1991). Briefly, it was extracted twice from filters using 100% acetone under ultrasonification; extracts were combined and filtered through a 0.2-μm Zetapor membrane. Samples were injected into the HPLC instrument within 24 h of extraction. Chl-*a* retention times and concentrations were determined using an authentic standard (Turner Design). Averaging analyses for the control and PI time zero samples resulted in concentration variations of 4%. An unknown peak eluting 2 min before Chl-*a* was observed after 33 d in HG-treated samples. This peak was identified using HPLC-TOF-MS (electrospray ionization) according to Benotti et al. (2003). The HPLC-MS mobile phase consisted of 95% acetonitrile, and a 5% mixture of tetrabutyl ammonium acetate (0.5 mM) and ammonium acetate (10 mM). Other unknown peaks were not identified.

2.3.4. Lipids

Lipids were analyzed using gas chromatography (GC) according to Wakeham et al. (1997a). Briefly, they were extracted from GFF filters with CH_2Cl_2:MeOH (2:1) using an ultrasonic probe and partitioned into CH_2Cl_2 with 5% NaCl solution in a separatory funnel and dried over Na_2SO_4. The extracts were saponified and sequentially extracted at pH > 13 and pH < 2 to separate non-saponifiable lipids (hereafter neutral lipids) from fatty acids (FAs). FAs were methlyated with diazomethane to

produce methyl esters, while neutral lipids were derivatized with BSTFA-pyridine to form trimethysilyl ethers. Both FAs and neutral lipids were analyzed using GC and GC–MS. Cholestane and methyl nonadecanoate were used as internal standards. Averaging analyses for the three time zero samples resulted in variations of 4–15% for most FAs and sterols, but of 22% for 18:0 fatty acid.

2.4. Statistical analysis

Principal components analysis (PCA) is a multivariate regression analysis used here to investigate the compositional differences among samples with different treatments. In most cases, PCA reduces the number of variables into two major principal components, PC1 and PC2. PC1 explains most of the variance in a data matrix, while PC2 explains most of the residual variance. Site scores are the relative position of each sample along the axis of the principal component. Loadings (eigenvectors) of each variable contribute a certain amount to each principal component. PC1 has been successfully used as a degradation index for marine sedimentary material (Dauwe and Middelburg, 1998; Sheridan et al., 2002) or to differentiate sources of marine OM (Goni et al., 2000; Canuel, 2001; Ingalls et al., 2006). Using MATLAB 6.5 (The MathWorks Inc.), we performed PCA on a data matrix that included C and N concentrations, AA compositions (mole%), allomer and Chl-*a* ratios (HPLC peak areas), FA and neutral lipid compositions (weight%) after standardization by subtracting the means and dividing by the standard deviations.

3. Results

3.1. POC and PN

POC and PN in the 3 time zero samples (Ctrl-0, PI-0 and HG-0) agree within 5%, indicating the algal culture was split well. After 33 d, about 50% of the initial POC and PN were lost in both control and PI treated samples (Fig. 1 and Table 1), suggesting that PI did not slow degradation or solubilization of organic C and N. In contrast, only about 10% of the POC and PN were lost in HG-treated samples. The sample treated with both PI and HG lost about 16% of the initial POC and PN. Although half the total POC and PN were lost in Ctrl-33 and PI-33 samples, the C/N ratio in these samples was similar to initial values. HG-treated samples had slightly lower C/N ratios (7.7) than samples without HG treatment (8.6).

3.2. Amino acids

AAs are structural components of proteins, the largest reservoir of organic N in most organisms. They make up a major fraction of the characterized C in marine particulate matter and are useful indicators of decomposition and transport in the marine environment. In our incubation samples, PAA accounted for about 35% and 75% of organic C and N in all samples, respectively. In general, PAA showed the same overall pattern among treatments (Fig. 2) as for POC and PN. About 50% of PAA was lost in both the control and PI-treated samples after 33 d, while only 15% of PAA HG-treated and

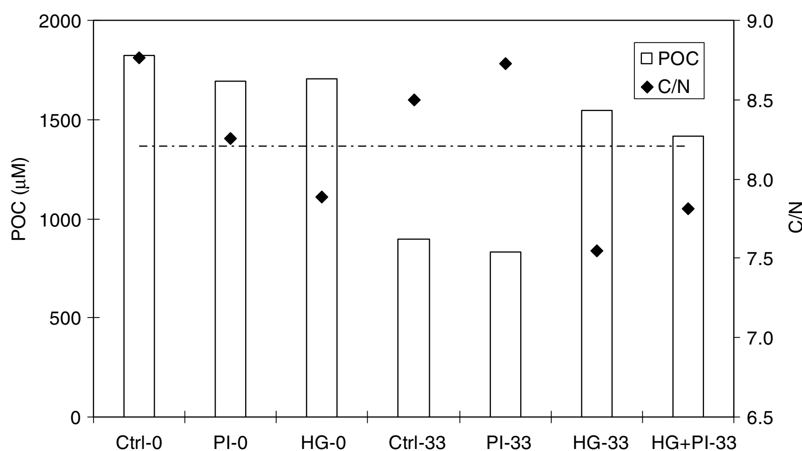

Fig. 1. POC concentrations and C/N ratios in control (Ctrl), protease inhibitor (PI)-treated, HG-treated or HG + PI-treated samples at time zero and 33 d. Dashed line = average (8.2) of all C/N ratios.

Table 1
Fatty acids, phytol, sterols and Chl-*a* concentrations (μg/L), organic C and N (μM), AAs (mole%) and PAA (μM)

Experiment[a]	14:0	15:0	16:3	16:1	16:0	18:4	18:3	18:1ω9	18:1ω7	18:0	20:5	22:6	TFA[b]	phytol	24MS[c]	24ES(E)[c]	24ES(Z)[c]	TNL[d]	Chl-*a*
Ctrl-0	681	34	276	946	849	77	28	46	211	31	348	37	3564	140	143	9	6	298	662
PI-0	668	33	255	958	820	95	33	48	183	48	408	48	3593	188	1 08	10	7	311	699
HG-0	620	30	298	928	788	98	28	43	155	35	445	50	3515	170	123	10	6	309	557
Ctrl-33	80	15	20	133	118	10	1	10	88	5	60	13	551	123	75	25	11	233	873
PI-33	14	5	1	38	45	3	4	5	18	8	15	3	157	215	73	28	19	334	897
HG-33	703	0	10	633	818	10	8	22	188	38	235	35	2697	170	100	9	5	284	171
HG + PI-33	695	30	55	713	873	38	13	40	153	53	265	43	2968	178	113	25	13	328	274
	OC	N	ASP	GLU	HIS	SER	ARG	GLY	THR	ALA	TYR	GABA	MET	VAL	PHE	ILE	LEU	LYS	PAA
Ctrl-0	1825	208	10.5	14.5	1.4	10.2	4.3	13.3	6.6	10.1	2.8	0.0	0.4	5.6	4.2	4.3	7.1	4.7	130
PI-0	1691	205	11.2	15.2	1.4	8.7	4.3	12.3	6.4	10.5	2.8	0.0	0.4	5.8	4.3	4.4	7.3	5.0	139
HG-0	1703	216	10.6	13.0	1.4	9.4	4.4	13.3	6.5	10.7	3.0	0.0	0.3	5.6	4.5	4.4	7.5	5.5	139
Ctrl-33	897	106	11.3	12.7	1.9	9.0	4.3	13.5	6.4	10.5	3.0	0.2	0.1	5.6	4.9	4.5	7.8	4.2	58
PI-33	830	95	10.8	11.7	1.9	9.4	4.4	13.9	6.6	10.7	2.8	0.3	0.3	5.9	5.1	4.7	7.8	3.6	66
HG-33	1547	205	10.7	12.6	1.5	9.8	4.7	13.2	6.8	10.5	3.1	0.0	0.1	5.6	4.5	4.3	7.6	4.8	117
HG + PI-33	1418	182	12.5	12.5	2.0	7.7	4.6	12.5	6.5	10.4	3.0	0.0	0.2	6.0	4.7	4.9	8.0	4.5	110

[a] For experiment description see text.
[b] TFA = total fatty acids (μg/L).
[c] 24-MS: 24-methylcholesta-5,24(28)-dien-3β-ol; 24-ES(E): 24-ethylcholesta-5,24(28)*E*-dien-3β-ol; 24-ES(Z): 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol.
[d] TNL = total neutral lipids (μg/L).

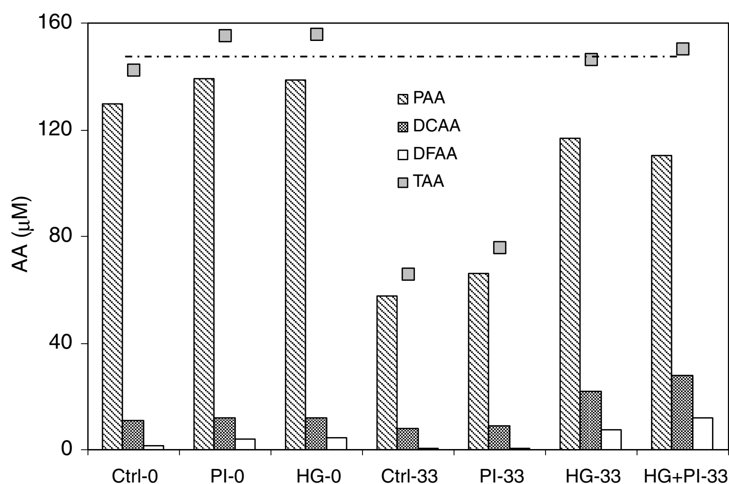

Fig. 2. Amino acid concentrations in control (Ctrl), PI-treated, HG-treated or HG + PI-treated samples at time zero and 33 d. Concentrations of TAA, total amino acids; PAA, particulate amino acids; DCAA, dissolved combined amino acids; and DFAA, dissolved free amino acids. Relative standard deviation of the 5 TAAs (other than Ctrl-33 and PI-33) agrees within 4% and dashed line = average.

HG + PI-treated samples was lost. Although DCAA concentrations were about the same in time zero samples, DFAA concentrations were 3–4 times greater in HG-0 and PI-0 than in Ctrl-0 samples. After 33 d, DCAA in both Ctrl- and PI-treated samples had decreased by only 30%, while DFAA decreased by a factor of 3–4 in these samples. Concentrations of DCAA and DFAA in HG-33 and HG + PI-33 increased, however, by about a factor of 2, relative to time zero samples. The amounts of PAA lost were about the same as the amount of DCAA and DFAA gained in these HG-treated samples (within ±4%).

PAA compositions (mole%) of all the samples were quite uniform (Table 1) and were dominated by acidic and neutral AAs, including aspartic acid, glutamic acid, serine, glycine and alanine, consistent with the composition of diatoms and other phytoplankton (Cowie and Hedges, 1992; Lee et al., 2000; Sheridan et al., 2002). Slightly higher mole percentages (2–3%) of glutamic acid in both Ctrl-0 and PI-0 than other samples were probably due to the freshness of these two samples. Glutamic acid was not enhanced in the particulate HG-0 sample, but was higher in the dissolved AAs of HG-treated samples (data not shown). This suggests that HG might lyse the diatom cells, releasing intracellular components enriched in glutamic acid (Lohrenz and Taylor, 1987). GABA (γ-aminobutyric acid) was present in Ctrl-33 and PI-33 samples (0.2% and 0.3%, respectively), but was not detected in any other samples.

The PIs used include the AA-containing compounds pepstatin A and bestatin, so the AA composition might have been affected if there were any PI sorption to the particles. However, the overall pattern of AA composition was similar regardless of PI addition, suggesting that these components did not affect the composition of particulate samples. As might be expected, much higher mole percentages of leucine were observed in DCAA measured in the incubation flasks with PI added, since leucine is an important structural component of bestatin. Concentrations of leucine decreased from 25 mole% at PI-0 to 7% in later samples and eventually to background levels after 33 d (data not shown). Lysine in PI-0 samples made up almost 50 mole% of the DFAA and, like leucine, decreased to background levels after 33 d. These results show that the PI probably decomposed over time, one possible reason being its inefficacy.

3.3. Chlorophyll

Chlorophyll is best known for its role in harvesting light energy during photosynthesis and is present in all plants. Although it contributes a tiny fraction of the total C and N in marine particulates, its unique source in surface waters makes its decomposition rate a useful indicator of diagenesis. Even though the diatom cultures were incubated in the dark, after 33 d the Chl-*a* concentration in the control sample had increased about 24% relative to initial values (Fig. 3). This is well above the analytical

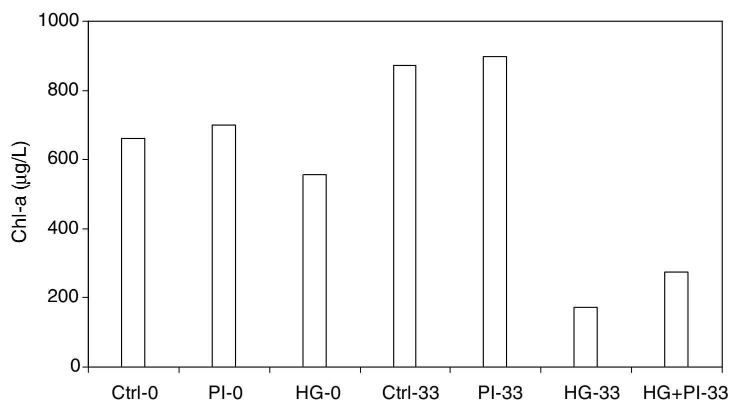

Fig. 3. Chl-*a* concentrations in control samples (Ctrl), PI-treated, HG-treated or HG + PI-treated samples at time zero and 33 d.

error of 10%. The same increase in Chl-*a* was also observed in the PI-treated samples.

In HG-treated and HG + PI-treated samples, Chl-*a* concentrations decreased by as much as a factor of 4 after 33 d compared to initial values (Fig. 3). Even at time zero, HG-treated samples had lower levels of Chl-*a* than other time zero samples. While Chl-*a* was lower in HG-treated samples, an "unknown" peak was higher by a factor of 3 compared to time zero samples. This peak had a retention time of 28.6 min (compared to 30.4 for Chl-*a*; Fig. 4). HPLC-MS (TOF) in positive ion mode showed that the major ion $[M + H]^+$ of the unknown peak at 28.6 min was at *m*/*z* 887, suggesting that it was the O-allomer of Chl-*a*, which usually has a retention time just before Chl-*a* (Harris et al., 1995; Furuya et al., 1998). Another small unidentified peak occurred at 39.5 min. Clearly, HG affected Chl-*a* concentration and probably transformed Chl-*a* to its allomer under our experimental conditions (dark, 9 °C).

3.4. Lipids

3.4.1. Fatty acids (FAs)

FAs are important components of wax esters, triacylglycerols, steryl esters and phospholipids in organisms. Assuming lipids are made up of 85% organic C (Wakeham et al., 1997a), FAs accounted for 12–15% of total POC in all samples except Ctrl-33 (4%) and PI-33 (1%). FAs in the control samples decreased by a factor of 6 after 33 d and those in the PI-treated sample by even more – a factor of 23 (Table 1). In contrast, those in samples treated with HG or HG + PI decreased only 17–23% after 33 d. Consistent with total FAs, most individual FAs were much lower in Ctrl-33 and PI-33 samples than at time zero, but were well preserved in HG-33 or HG + PI-33 (Table 1). FAs following this pattern included 14:0, 16:0, 18:1ω7, 20:5ω3; 16:1ω7, 18:1ω9, 18:0, 22:6ω3. Some showed other patterns, however: after 33 d, 16:3, 18:3 and 18:4 FAs were poorly preserved for any treatment, although HG + PI-samples were preserved better than others, and 15:0 was well preserved for HG + PI, 50% lower in Ctrl and PI samples, and completely gone in HG-samples.

FA compositions (wt%) in the three time zero samples were dominated by 14:0 and 16:0, 16:1ω7 and polyunsaturated 20:5ω3 (Fig. 5), the major FAs in *T. pseudonana* and many other diatoms (Volkman et al., 1980; Volkman et al., 1989), and these 4 compounds represented about 80% of the total FAs; 15:0 and 18:0, 18:1ω7 and 18:1ω9, and 18:4, 18:3, 16:3 and 22:6 FAs were minor components, accounting for only 20% of FAs. Compositions of the 3 time zero samples agree within ±0.8%, except for 22:5 (±1.5%). To compare compositional changes over time among different treatments, we subtracted the initial wt% of each individual FA (averaged for the three time zero samples) from the wt% in samples from each treatment after 33 d (Fig. 6). After 33 d, the 14:0, 16:3, 16:1 and 16:0 FAs in control samples decreased by about 2–4%, while 18:1ω7 increased by 11%. Other FAs remained about the same. A different pattern was observed for PI-treated samples: 14:0 and 16:3 decreased by 7.5% and 9.5%, respectively, and 16:0, 18:1ω7 and 18:0 increased by 4–6%. For HG + PI and HG-treated samples, 16:3 decreased 7.4%, 16:1, 18:4 and 20:5 were 2–3% lower, while 14:0 and 16:0 increased about 7% after 33 d.

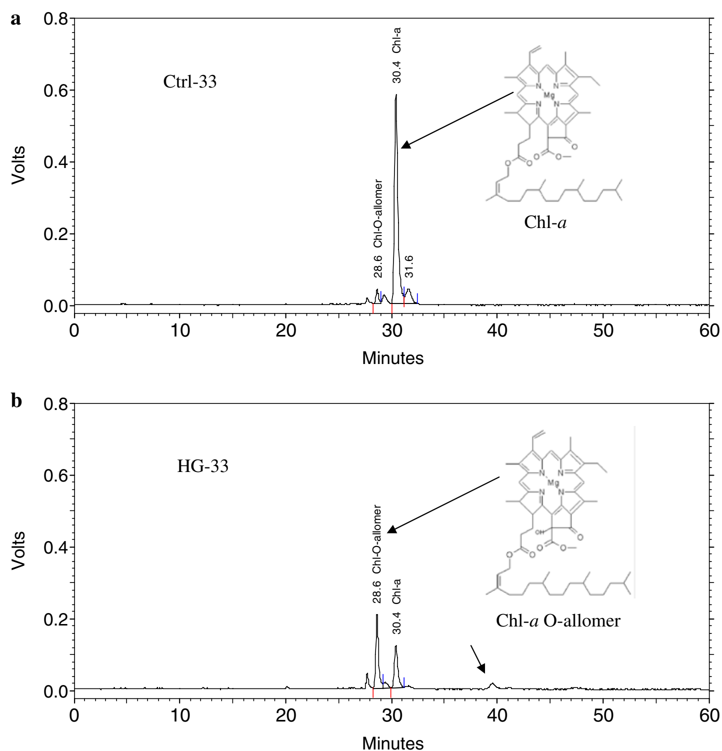

Fig. 4. HPLC chromatograms showing allomerization of Chl-*a*: (a) control sample at 33 d and (b) HG sample at 33 d. Peaks with arrows are unknowns (peak at 28.6 min is Chl-*O*-allome; peak at 39.5 min is unidentified).

3.4.2. Neutral lipids

Neutral lipids, including phytol and sterols, accounted for only 8–10% of total lipids in all samples, except Ctrl-33 and PI-33, where they accounted for 30% and 68%, respectively. Concentrations of neutral lipids were similar throughout the 33 d incubation (Table 1). Only neutral lipids in Ctrl-33 were significantly lower (22%) than initial values. Due to its unique origin from Chl-*a*, phytol has been used as an indicator of phytoplankton bio-

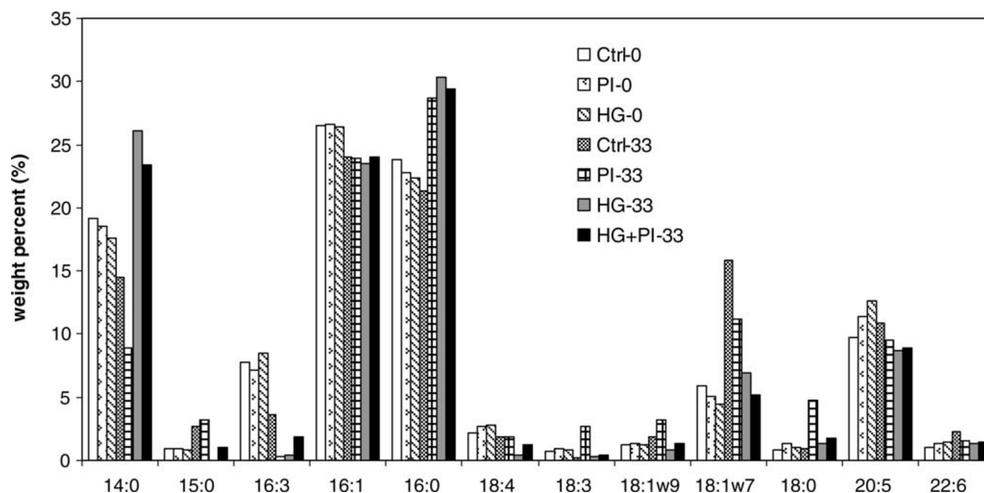

Fig. 5. Wt% FAs in control samples (Ctrl), PI-treated, HG-treated or HG + PI-treated samples at time zero and 33 d.

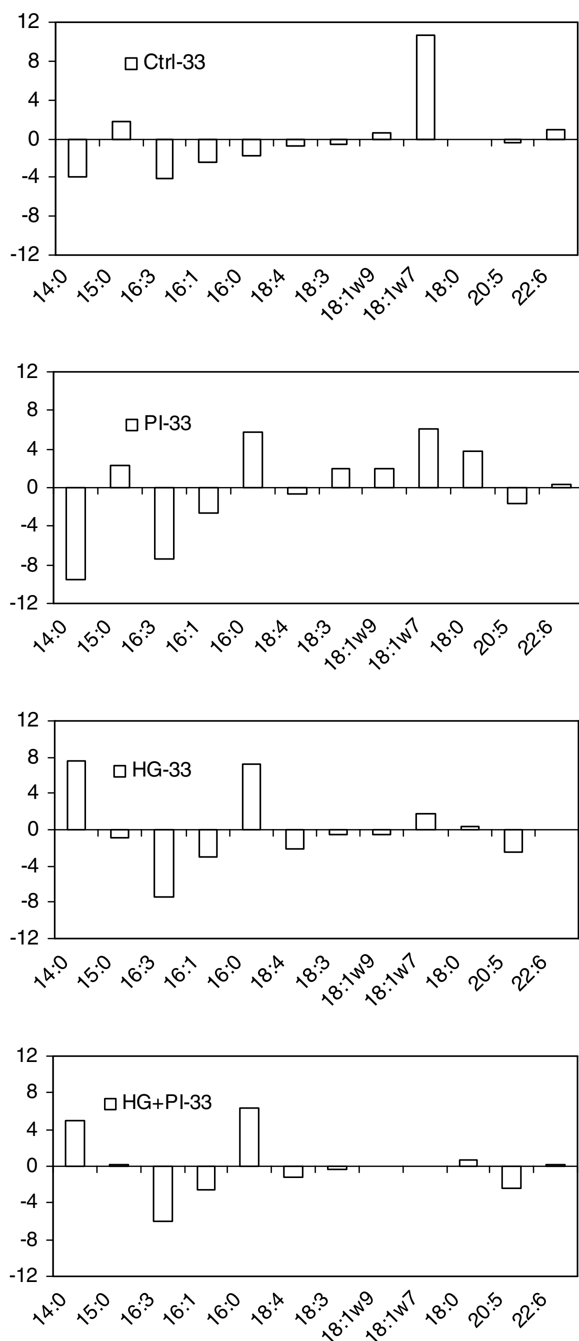

Fig. 6. Wt% Individual FAs in each treatment after 33 d minus average wt% of the three time zero samples.

mass. Differences in initial concentrations of phytol among the treatments were significant, with the order PI-0 > HG-0 > Ctrl-0. Interestingly, phytol concentrations were in the same relative order after 33 d.

Sterols are ubiquitous lipids in marine organisms as components of cell membranes and metabolic regulators; diatoms primarily synthesize C_{28} sterols (Libes, 1992). In this study, sterols were dominated by 24-methylcholesta-5,24(28)-dien-3β-ol, the major sterol in *Thalassiosira* spp. (Volkman et al., 1998). This compound accounted for 90% of total sterols in the time zero samples. Also present were 24-ethylcholesta-5,24(28)*E*-dien-3β-ol and 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol. After 33 d, concentrations of 24-methylcholesta-5,24(28)-dien-3β-ol in the control and PI-treated samples decreased by 47% and 32%, respectively; concentrations in HG-samples decreased by 18% relative to time zero and HG + PI-samples did not change much (Fig. 7). There were slight increases in the concentrations of 24-ethylcholesta-5,24(28)*E*-dien-3β-ol and 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol in Ctrl-33, PI-33 and HG + PI-33 by day 33 relative to time zero, but these increases are probably not significant given the small samples, low concentrations and analytical errors involved.

3.5. Statistical analysis

PCA allowed us to compare quantitatively the effect of various treatments on the composition of diatoms used in the incubation experiment. PC1 explained 43% of the variance in the data matrix and PC2 a further 25% of the residual variance (Fig. 8). For PC1, the 3 time zero samples had the most positive site scores (3.2–4.6), and Ctrl-33 (−3.8) and PI-33 (−7.0) the most negative site scores. PC1 site scores for HG-33 and HG + PI-33 were intermediate at 1.4 and −1.7, respectively. For PC2, site scores for the HG-treated samples were negative, with −5.4 and −3.9 for HG + PI-33 and HG-33 respectively, while the rest of samples had similar site scores, in the range 1.2 to 2.2. The loadings for different variables on PCs 1 and 2 correspond to the site scores on PCs 1 and 2, respectively. In general, samples with positive site scores were enriched in variables with positive loadings and vice versa. In Fig. 8, samples were clearly separated into 3 groups based on their relative positions: Ctrl-0, HG-0 and PI-0 belonged to group 1, HG-33 and HG + PI-33 to group 2 and Ctrl-33 and PI-33 to group 3. Along the *x*-axis (PC1), group 1 was enriched in variables having the most positive loadings (0.15–0.25), including POC and PN, LYS, and GLU, 14:0, 16:1, 16:3 and 24–MS; group 3 was enriched in variables having negative loadings

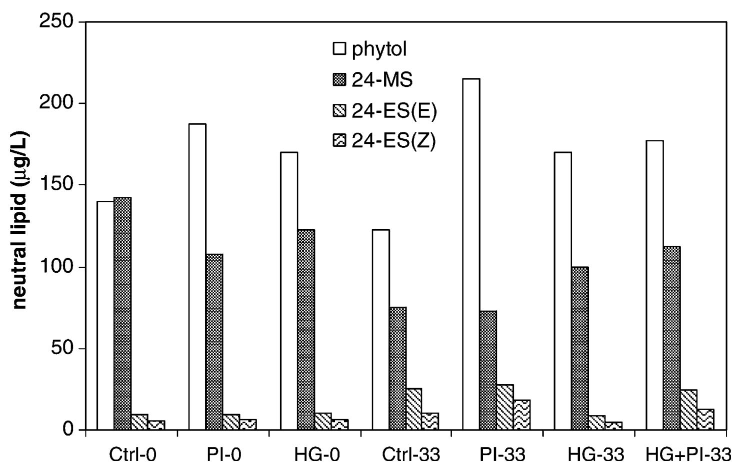

Fig. 7. Neutral lipid concentrations in control samples (Ctrl), PI-treated, HG-treated or HG + PI-treated samples at time zero and 33 d; 24-MS: 24-methylcholesta-5,24(28)-dien-3β-ol; 24-ES(E): 24-ethylcholesta-5,24(28)*E*-dien-3β-ol, 24-ES(Z): 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol.

(−0.17 to −0.25), including PHE, GABA, HIS and LEU and ILE, 15:0, 18:1ω7, 18:0, 18:1ω9, 22:6 FAs, and 24–ES(E) and 24–ES(Z). Along the *y*-axis (PC2) group 2 was mostly enriched in ARG, TYR, THR, and 16:0 and 14:0 fatty acids, while the rest of samples were mostly enriched in allomer/Chl-*a*, MET, 18:4, 20:5 and 16:1 FAs.

4. Discussion

4.1. Degradation in control (and PI-treated) samples

Samples with and without protease inhibitor had similar degradation rates (PI vs. Ctrl and HG vs. HG + PI) and generally similar compositions

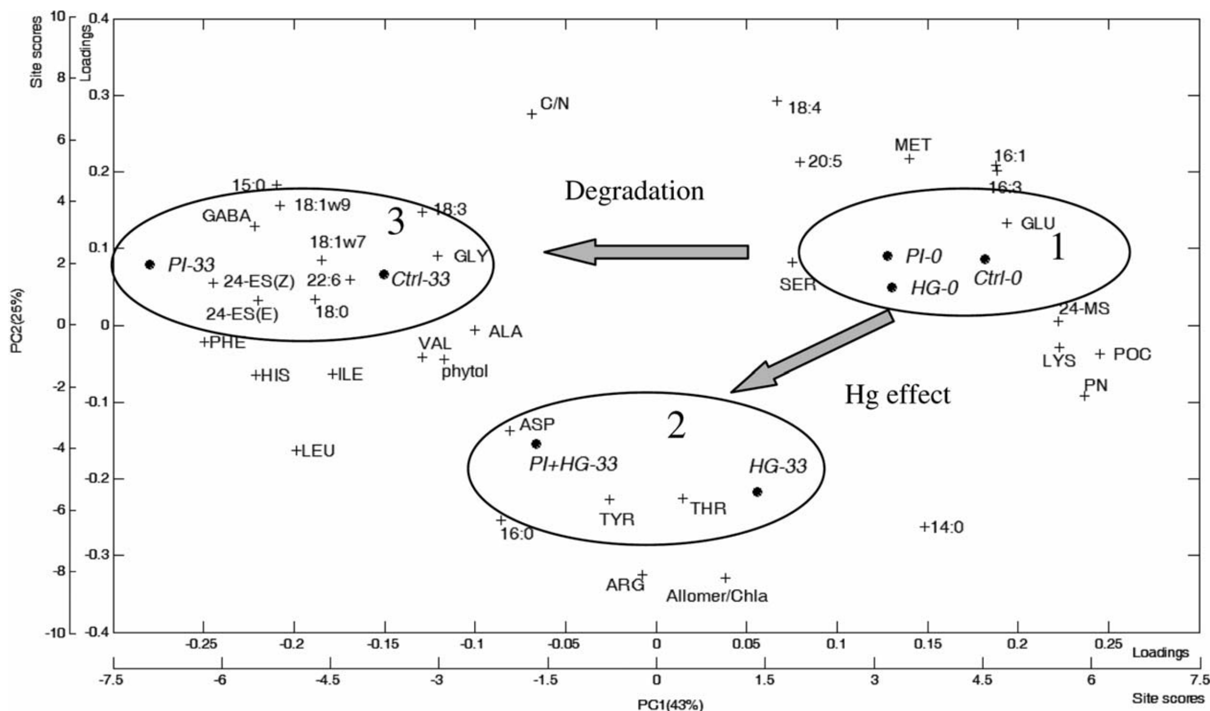

Fig. 8. PCA. Variance accounted for by each PC is listed on axis label. Two sets of axes were used for loadings and sites scores, respectively. Arrows represent direction of degradation and HG effect; 24-MS: 24-methylcholesta-5,24(28)-dien-3β-ol; 24-ES(E): 24-ethylcholesta-5,24(28)*E*-dien-3β-ol, 24-ES(Z): 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol.

(Fig. 8), suggesting that the protease inhibitor used had little effect on degradation, or that they themselves decomposed quickly over the long term. Although it is possible that the protease inhibitor might not have functioned under the experimental conditions used, other studies have used similar cocktails successfully to inhibit protease activity (Haley and Wessel, 1999; Bidle et al., 2003). While we used a broad-spectrum inhibitor to inhibit serine, cysteine, aspartic and metallo-proteases and aminopeptidases, these proteases may be a small fraction of the exoenzymes present in the environment. Other proteases, and cellulases, amylases, lipases, reductases, etc., might exert more influence over degradation in general. Thus, we discuss below our results assuming that the control and PI treatments are replicates and that HG and HG + PI-treatments are replicates.

In control samples (Ctrl and PI), about 50% of POC and PN were degraded, suggesting no preferential loss of N over C, or PAA over total C and N, as might be expected since they are all bulk parameters. Consistently, $t = 0$ and $t = 33$ samples had similar C/N ratios (Fig. 1). FAs turned over 3–5 times faster, but sterols turned over more slowly compared to bulk C and N. The overall order of lability of organic compound classes is generally FAs > AAs ≈ POC ≈ PN > sterols. This is consistent with the order for sinking particles in the water column (Wakeham et al., 1997b). Assuming a simple first-order decay (Westrich and Berner, 1984; Harvey et al., 1995), we can calculate rate constants for the different organic classes using time points at time zero and 33 d. Our decay constants for POC ($0.02\ d^{-1}$), PAA ($0.02\ d^{-1}$) and lipids (0.06–$0.1\ d^{-1}$) generally agree with other studies, but are at the lower end of the range (e.g., Otsuki and Hanya, 1972; Westrich and Berner, 1984; Harvey and Johnston, 1995; Harvey et al., 1995). Our decomposition experiment was conducted at 9 °C, while other studies were generally warmer, around 20 °C. This 10 °C difference would likely reduce the microbial activity in our experiment by a factor of 2–3 (Westrich and Berner, 1988), leading to lower apparent degradation rates. Our decay constants were still much higher than those reported for anoxic decomposition of algal materials. For example, Harvey and Macko (1997) reported that anoxic decay constants for POC and FAs for the diatom *T. weissflogii* were 0.008 and $0.02\ d^{-1}$, respectively. Decay constants of major FAs, including 14:0,16:0, 16:1ω7 20:5ω3, were in the range 0.05 (20:5ω3) to 0.06 (14:0) d^{-1}, and there was no obvious relationship between degradation and degree of saturation.

PCA clearly showed that control samples after 33 days of decomposition were enriched in γ-aminobutyric acid (GABA) and 18:1ω7 FA. GABA is a product of decarboxylation of glutamic acid (Lee and Cronin, 1982; Ittekkot et al., 1984), so its appearance is consistent with the degradative loss of about 50% of the C, N and PAA. We did not detect β-alanine, which is also often observed in sinking particles due to decarboxylation of aspartic acid (Lee and Cronin, 1982), possibly due to differences in the OM or microbial communities present. Degradation also led to enrichment in HIS and PHE after 33 d, as these two AAs have negative PCA loadings. The wt% 18:1ω7 FA, also a potential indicator of microbial processing of OM (Volkman et al., 1980; Wakeham et al., 1997b) was significantly greater after 33 d.. The observation that the three sterols of *T. pseudonana* also group in PCA with the microbial indicators at the end of the decomposition experiment probably results from their relative stability towards microbial decomposition (Wakeham et al., 1997b).

Because of its high reactivity, Chl-*a* concentrations in particulate and sedimentary material usually decrease quickly with depth in the water column or sediment (Lee et al., 2000). In our study Chl-*a* concentrations in control samples were, however, significantly greater after 33 d (Fig. 3). We did not detect phaeophytin, a major product of bacterial degradation of Chl-*a* (Bianchi et al., 1988; Sun et al., 1993), suggesting again that Chl-*a* was not degraded over the course of the experiment. In phytoplankton chloroplasts, Chl-*a* is non-covalently bound to proteins in several types of pigment–protein complexes (Larkum and Barrett, 1983) and the rate limiting step in Chl-*a* degradation may be the cleavage of these Chl–protein complexes (Ziegler et al., 1988). Sun and co-workers (Sun et al., 1991; Sun et al., 1994; Sun and Wakeham, 1994) operationally defined Chl-*a* as both "free" and "bound" forms, based on the release of Chl-*a* from sediment by freeze–thawing and solvent extraction techniques. They hypothesized that bound Chl-*a* must be released from a particle or cellular matrix before it can degrade. Our results are consistent with this idea. At time zero, intact diatom cells, which would contain bound Chl-*a*, were present in the incubation samples. Acetone alone is not strong enough to penetrate the chloroplasts and break

down the Chl–protein complexes, so Chl-*a* would escape extraction or detection. After 33 d, decomposition of protein decomposed (as the PAA results showed, Fig. 2) might cleave the Chl–protein complex and release free Chl-*a*; this would result in an increase in "apparent" concentration of Chl-*a*.

4.2. Effectiveness of mercuric chloride (and HG + PI)

Compared to the time zero samples, POC, PN, PAA, FA and sterol concentrations were all about 10–20% lower after 33 d in HG-treated samples. At a concentration of 180 μM, HG can stop bacterial activity in marine particulate samples (Lee et al., 1992). Thus, there should have been no bacterial degradation of OM in HG-treated samples; the absence of γ-aminobutyric acid from HG-treated samples after 33 d would confirm this. Loss of PAA over time was about the same as the increase in dissolved AA concentration (DCAA + DFAA), suggesting that solubilization was a major mechanism leading to loss of particulate material. Addition of HG may cause cell lysis, such that intracellular components or soluble storage compounds like carbohydrates might be released into solution. Indeed, DFAA increased immediately after HG was added in HG-0, suggesting cell lysis and release of DFAA is rapid. Solubilization might also explain why C/N ratios in HG-treated samples were lower. For example, certain soluble carbon-rich sugars might dissolve quickly after lysis of the diatom cell, which could decrease the C/N ratio in the residual particulate phase.

Many individual compounds were selectively preserved in HG-treated samples. For example, 14:0 and 16:0 FAs were enriched (on a relative abundance scale) in HG-treated samples (Figs. 5 and 8). On the other hand, 15:0, 18:3, 18:4 and 16:3 FAs were almost completely lost after 33 d. Abiotic oxidation reactions due to the presence of HG might degrade these compounds. Alternatively, these FAs might be present in the cells in a soluble form. Although FAs are generally not soluble in water, they can attach to soluble compounds, such as acylated proteins (McKee and McKee, 1999) and form micelle-like aggregates. In sediment trap samples poisoned by NaN_3 (Körtzinger et al., 1994), for example, dissolution of individual FAs ranged from 16% to 68% of the total. The AAs, ARG, TYR, and THR, LEU and ASP are relatively enriched in the two HG-preserved samples as seen in the PCA results (Fig. 8). Organic materials collected by NetTrap and later preserved by HG also showed similar enrichment in ARG, TYR and LEU (data not shown). Because TYR and LEU are hydrophobic AAs and thus less soluble than others, their enrichment in residual particulate matter could occur if they were not easily dissolved or hydrolyzed while other AAs were preferentially dissolved.

The major compositional change that occurred in HG-treated samples over time was the rapid loss of Chl-*a* and the concurrent formation of what is thought to be a Chl-*a* O-allomer. Allomerization of Chl-*a* can occur by either enzymatic or chemical oxidation, leading to a complex mixture of compounds (Pennington et al., 1967). In our experiment, Hg^{2+} might act as an oxidant; in addition, dissolved oxygen and absence of light have been reported as important allomerizing conditions (Kuronen et al., 1993; Woolley et al., 1998) and may have contributed to this process in our samples. However, an authentic standard of Chl-*a* in seawater treated with HG did not allomerize within 7 days, suggesting that allomerization might occur during the release from the Chl–protein complex present in cellular material. Phytol, the side chain of Chl-*a* can not only be released from it by enzymatic hydrolysis due to zooplankton grazing, but is also produced during analysis by alkaline hydrolysis during laboratory processing of samples (Wakeham et al., 2002). The similar concentrations of phytol in all treatments suggest that alkaline hydrolysis during analysis was the only source, a likely scenario in the absence of zooplankton grazing in the experimental system. The fact that phytol was not lost as quickly as Chl-*a* in the HG-treated samples is consistent with allomer formation, since the transformation of Chl-*a* to its allomer occurs within the chlorin macrocycle and need not affect the phytyl side chain. Although we have no experimental proof that allomerization of Chl-*a* occurs during release from the bound state, this process is consistent with the results from our sediment trap samples (Lee et al., 2000; Sheridan et al., 2002; Ingalls et al., 2006), where little allomer is present in sinking particles treated with HG. Table 2 shows the degree of allomerization among different sample types, including fresh diatoms, sinking particles collected from 200 m depth with a NetTrap (Peterson et al., 2005) and HG-treated sinking particles collected from 200 m using a time-series sediment trap. Fresh diatoms had a low ratio of allomer to Chl-*a* until they were treated with HG. The ratio in fresh

Table 2
Allomer/Chl-*a* ratios in NetTrap and sediment trap samples from DYFAMED site (Peterson et al., 2005)[a]

Sample type	Conditions	$HgCl_2$	No $HgCl_2$
Diatom (this study)	9 °C, dark, 33 d	1.32	0.06
NetTrap 200 m	13 °C, dark, 5 d	0.14 ± 0.04 ($n = 8$)	0.06 ± 0.02 ($n = 56$)
Sediment trap 200 m	13 °C, dark, 60 d	0.33 ± 0.11 ($n = 11$)	N/A

[a] Particles collected by NetTrap without $HgCl_2$ added (deployment 2–3 d); $HgCl_2$ (37 μM)was then added for 5 d (in the dark, 13 °C). Samples collected by time series trap treated with $HgCl_2$ (180 μM) in situ (deployment 2 months).

untreated sinking materials collected by NetTrap was similar to that in diatoms, but increased when treated with HG. Time-series sediment trap samples are HG-treated during collection and are collected for a longer time; ratios in these samples were higher than in NetTrap samples, but much lower than in HG-treated diatoms. Sinking particles from this site (northwestern Mediterranean) contained degraded diatom material (Goutx et al., unpublished results). Perhaps much of the Chl in the diatoms had already been released from the Chl–protein complex before sinking, hence the lower trap ratios. Alternatively, since phytoplankton present in the Mediterranean are mixtures of diatoms, prymnesiophytes and cyanobacteria (Marty et al., 2002), Chl-*a* in these plankton might allomerize differently from the *T. pseudonana* strain we used.

Clearly, considering only concentrations of Chl-*a* in HG-treated samples would significantly underestimate actual amounts originally present. Adding allomer and Chl-*a* concentrations is possible if we assume they have the same HPLC response factor. However, in the diatom samples treated with HG, the allomer and Chl-*a* calculated in this way account for only about 50–60% of the original Chl present in control time zero samples, suggesting that a substantial fraction of Chl-*a* is also lost in other ways, and indeed other unidentified peaks appeared in HG-treated samples (Fig. 4). At this point, we recommend including allomer concentrations in reports of Chl-*a* in HG-treated samples, or reporting it separately. More work is clearly needed to know the exact mechanism and pathways of Chl-*a* allomerization by HG.

4.3. Compositional changes due to degradation vs. $HgCl_2$

As particulate OM decomposes or dissolves, both the amount and composition of the material change, with different classes of compounds having different decay or dissolution rates. As indicated above, HG treatment of samples also caused unexpected compositional changes. PCA allowed us to investigate the relative importance of biological degradation vs. treatment with HG for the composition of particulate material. The greatest variation in the composition between samples from group 1 to group 3 was along the *x*-axis (PC1; Fig. 8). The loadings of individual degradation markers (e.g., GABA) suggest that variance along PC1 is mostly due to biological degradation, in agreement with past work (Dauwe and Middelburg, 1998; Sheridan et al., 2002). However, the HG treated samples had PC1 site scores intermediate between group 1 and group 3 along PC1 as well as being more negative along PC2. Minimal biodegradation should have occurred in HG treated samples (Lee et al., 1992), so compositional changes due to dissolution and chemical oxidation may have driven these samples left along the *x*-axis and downward along the *y*-axis. Since group 1 ($t = 0$) and group 3 (biologically degraded) samples are almost inseparable for PC2 and group 2 (HG treated) is well separated from groups 1 to 3 on PC2, we hypothesize that compositional changes along PC2 were caused only by the HG. Such a HG effect includes mainly enrichment in ARG, TYR, THR, 16:0 and 14:0, depletion in Chl-*a*, 18:4 and decrease in C/N ratios. Using PCA of samples treated by $HgCl_2$, it may be possible to quantify the effect of HG on compositional information for field or laboratory samples.

5. Summary and conclusions

This study demonstrates the effects of $HgCl_2$ (HG) and protease inhibitors (PIs) on a diatom culture under conditions that resembled the in situ conditions of a 1–month sediment trap deployment. Both quantitative and compositional information were obtained. In general, PI did not appear to stop degradation or dissolution of diatom OM over 33 d. HG preserved much of the OM, yet did affect the composition of several classes of compounds. These changes are likely due to chemical oxidation and dissolution.

1. Decomposition of (bulk) POC and PN was effectively inhibited by HG, only 10% being lost due to dissolution compared to 50% for samples without HG added; PI, on the contrary, did not prevent loss of POC or PN.
2. PAAs were well preserved from biological decomposition by HG, yet 15% were lost due to dissolution; AA composition was also well preserved, but mole percentages of ARG, TYR, and THR increased slightly, possibly due to selective dissolution. PI did not prevent changes in composition due to decomposition.
3. Chl-*a* was extensively allomerized by HG in the diatom degradation experiments, but much less so in sediment trap samples. We recommend summing Chl-*a* and allomer concentrations, or reporting the allomer separately to minimize underestimation of Chl-*a*. Chl-*a* in control and PI treated samples increased over 20% in a month, probably due to the release of Chl-*a* from Chl–protein complexes as proteins were degraded.
4. Total FAs were fairly well preserved in HG treated samples, since only 20% were lost compared to 80% in the control. Most individual FAs followed this general pattern, but some showed different behavior. For example, 16:3, 18:3 and 18:4 were not well preserved.
5. Sterols were well preserved by HG; 24-methylcholesta-5,24(28)-dien-3β-ol in PI-33 and Ctrl-33 was 30–40% less than initial values; 24-ethylcholesta-5,24(28)*E*-dien-3β-ol and 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol changed little over time in any of the treatments.

Therefore, in studies using $HgCl_2$ to preserve marine particles, both quantitative and compositional effects of $HgCl_2$ on OM need to be considered. POC, PON, AAs and sterols are well preserved with minimal compositional changes. FAs and chlorophyll pigments are degraded and their composition altered.

Acknowledgements

We thank N. Fisher for kindly helping us to grow *T. pseudonana* 3H. We thank M. Benotti and B. Brownawell for helping to identify Chlorophyll allomer. We also thank M. Peterson and the crew of R/V *Seward Johnson II* for helping with the Net-Trap deployment. Many thanks go to M. Goutx, C. Guigue and L. Abramson for sample collection. J. Xue helped with PCA. We are grateful to Dr. F. Prahl and an anonymous reviewer for constructive comments. The research is part of the MedFlux program supported by the National Science Foundation Chemical Oceanography Program, and is Contribution No. 1317 from the Marine Science Research Center, Stony Brook University and MEDFLUX Contribution No. 6.

Associate Editor—**J. K. Volkman**

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