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An isotopic labeling method for determining production of volatile organohalogens by marine microalgae

Abstract—An isotopic method has been developed to establish the biological production of volatile organohalogens. The marine microalgae *Porphyridium purpureum* and *Dunaliella tertiolecta* were grown in seawater medium containing $NaH^{13}CO_3$, and volatile organohalogens were extracted and analyzed by gas chromatography-mass spectrometry (GC-MS). Isotopically labeled methyl halides were formed in each microalgal culture, and the $^{13}CHCl_3$ found in the *P. purpureum* culture confirmed the production of chloroform reported by Scarratt and Moore (Limnol. Oceanogr. 1999. **44:** 703–707). No incorporation of ^{13}C into dichloromethane was observed, even though slightly increased CH_2Cl_2 concentrations were detected in both algal cultures at the end of the incubation period.

Environmental concerns relating to the role of halogenated compounds in the destruction of stratospheric ozone have stimulated investigations into the biological production of volatile organohalogens. Many reports have implicated marine algae as a source of halogenated compounds (reviewed by Gribble 1996). For example, the giant kelp *Macrocystis pyrifera* (Manley and Dastoor 1987) and several species of phytoplankton (Scarratt and Moore 1998) produce methyl chloride, and a methyl chloride transferase has been detected in cell extracts of the red alga *Endocladia muricata* (Wuosmaa and Hager 1990). The mechanism for the production of polychlorinated volatiles is not available in the literature, but the production of several polychlorinated compounds by marine algae has been described. Nightingale et al. (1995) detected chloroform production by macroalgae, and Abrahamsson et al., (1995) reported the detection of perchloroethylene and trichloroethylene in two macroalgal cultures and in cultures of the red microalga *Porphyridium purpureum*. While attempting to corroborate the observations of Abrahamsson et al., Scarratt and Moore (1999) detected chloroform (but not perchloroethylene and trichloroethylene) in *P. purpureum* cultures. Since these latter two studies involved the measurement of extremely small amounts of the organochlorines that are normally present in the atmosphere (Khalil and Rasmussen 1999), it was critical that there was no contamination from external sources, a potentially serious problem in laboratory environments. Indeed, high blank values prevented Gschwend et al. (1985) from assessing the production of dichloromethane by marine macroalga, and the biological production of perchloroethylene and trichloroethylene by marine algae is an ongoing question (Marshall et al. 2000; Abrahamsson and Pedersén 2000). We now present a method that distinguishes between ^{13}C-enriched biological products and contaminants of normal isotopic abundance by measuring $^{13}C/^{12}C$ isotopic ratios for volatile organohalogens present in microalgal cultures grown in medium containing ^{13}C-enriched bicarbonate.

L-1 Seawater medium (Guillard and Hargraves 1993) (1.5 liter) was acidified with 1 M HCl (3.3 ml), then purged with ultra–high-purity air until no chloroform could be detected by gas chromatography-mass spectrometry (GC-MS) (*see below*). The medium was supplemented with sodium [^{13}C] bicarbonate (0.9 g, 99% ^{13}C; Cambridge Isotope Laboratories), and the pH was measured (7.9). The medium was autoclaved in an airtight container and aseptically transferred in 100-ml portions to sterile, narrow-neck glass ampoules using glass syringes fitted with long stainless steel needles to minimize gas exchange with the ambient atmosphere. Before the transfer of medium, the ampoules were purged for several minutes with ultra–high-purity air. The medium in the ampoules was inoculated with 100 μl of a growing culture of either the temperate red alga *Porphyridium purpureum* (CCAP 1380/3; culture collection of algae and protozoa, Dunstaffnage Marine Laboratory) or the temperate green alga *Dunaliella tertiolecta* (CCMP 1320; Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences). Two ampoules were not inoculated and served as controls. The ampoules were sealed with a Swagelok nut and a ceramic-filled Teflon ferrule (Supelco) to prevent gas exchange. The cultures were incubated at 25°C, with a 12 : 12 light : dark cycle, until there was significant growth (19 d). Prior to inoculation, the stock cultures were examined for axenicity by inoculating test tubes containing sterility test medium (5 ml), which consisted of L-1 seawater medium, bactopeptone (1 g L^{-1}), and methylamine HCl (0.675 g L^{-1}), with 50 μl of the culture. After 15 d, uninoculated sterile test medium and the tubes inoculated with *P. purpureum* remained clear, indicating that the algal culture was axenic. However, medium inoculated with raw seawater or *D. tertiolecta* was turbid, indicating that this alga culture was nonaxenic.

To analyze for volatile organohalogens, samples (80 ml) were drawn from each of the cultures into a 100-ml glass syringe and passed through a glass fiber filter. The purge and trap system was flushed with the filtrate, and the gases were extracted from a 40-ml volume by a stream of helium flowing at 40 ml min^{-1}. After the gas stream had been dried by passage through a tube containing magnesium perchlorate, the volatile compounds were trapped at −150°C before being injected into a GC-MS system, which is described more fully by Moore et al. (1996*a*). All five compounds were determined in one run using the following temperature profile: 45°C for 1 min, increased to 140°C at 10°C min^{-1}, and held at that temperature for 4 min. The samples were run as duplicates in the following order: uninoculated medium, *P. purpureum* culture, *D. tertiolecta* culture, and standards. For each compound of interest, at least one ion (mass = *m*) typical of the C-12 compound was monitored, together with

the corresponding ion for the C-13 labeled molecule (mass $= m + 1$).

The method has greatest potential to provide evidence for production of a C-13 labeled compound when the $m + 1$ ion being monitored has a relatively low intensity in the mass spectrum of the unlabeled compound. For example, in the case of methyl iodide, ions at masses 142 ($^{12}CH_3I$) and 143 ($^{13}CH_3I$) are monitored. The mass 143 ion appears in standard methyl iodide (i.e., natural abundance C-13) at only 2% of the intensity of the mass 142 ion. Any synthesized C-13 methyl iodide will show up clearly against this low background. In the case of chloroform, the situation is somewhat less favorable: the fragment ion of mass 84 is about 5% as intense as the mass 83 fragment ion characteristic of the unlabeled compound.

Chloromethane, bromomethane, and iodomethane were produced in each culture, and the ratios of characteristic ions in the mass spectrum of each compound are shown (Figs. 1a–1c). In each case, the medium and standards show similar isotopic composition, whereas the algae showed patterns consistent with production of the methyl halide with some C-13 labeling. One possible exception is the *Dunaliella* cultures for which production of methyl iodide is less certain. The concentrations of $^{13}CH_3I$ were 50 times higher in the *Porphyridium* cultures, and it cannot be entirely ruled out that some carry-over from *Porphyridium* samples to the *Dunaliella* samples occurred during analysis, hence giving the isotopic composition indicated in Fig. 1c. However, carry-over would have a greater effect on the first of the two consecutively run *Dunaliella* samples; the slightly higher ratio measured for the second sample is inconsistent with carry-over.

It is seen that the highest ratios of ^{13}C to ^{12}C ions (Fig. 1) are associated with methyl halides, the compounds that are less likely to appear as contaminants. For example, methyl iodide is usually present in the atmosphere at only extremely low concentrations (ca. 1 part per trillion) because it is quickly lost from air by photolysis. Additionally, its high toxicity militates against its general use in the laboratory. A ratio of about 3 for CH_3Br in both cultures and CH_3I in the *Porphyridium* culture indicates a composition of approximately 75% $^{13}CH_3X$ and 25% $^{12}CH_3X$, and not the 99% C-13 of the bicarbonate added to the medium. The reduced level of C-13 in the methyl halides may be attributed to incomplete removal of ^{12}C bicarbonate from the seawater. An increase in the pH of the medium during purging may have led to CO_2 being removed too slowly, hence its incomplete elimination.

In the case of CH_3Cl production, the C-13/C-12 ratio is lower, at around 1.5. Among the possible explanations is isotopic fractionation occurring during the conversion of

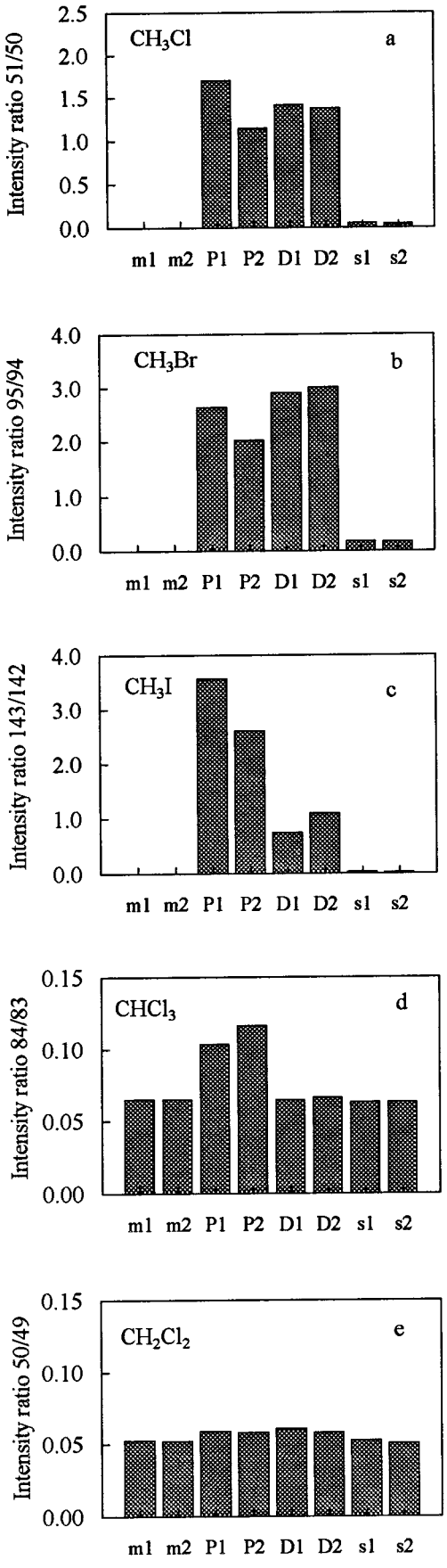

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Fig. 1. Ratios of intensities of characteristic ions ($m + 1 : m$) in the mass spectra of (a) methyl chloride, (b) methyl bromide, (c) methyl iodide, (d) chloroform, and (e) dichloromethane in duplicate samples of the medium controls (m1 and m2), the *P. purpureum* cultures (P1 and P2), the *D. tertiolecta* cultures (D1 and D2), and the corresponding standards (s1 and s2).

both CH_3Br and CH_3I to CH_3Cl by nucleophilic reaction with Cl^-.

The contamination problem is more acute with common laboratory solvents and compounds that have higher background concentrations in the atmosphere. Chloroform occurs at concentrations of about 18 parts per trillion in the atmosphere (Khalil and Rasmussen 1999), is abundant in chlorinated tap water, and has relatively high aqueous solubility.

In these experiments, chloroform was present at significant levels in the medium (46 pmol L^{-1}), presumably due to contamination entering the system from the laboratory atmosphere. Modest increases in concentration of unlabeled $CHCl_3$ were seen in both *Porphyridium* and *Dunaliella* cultures: 19 and 7%, respectively. Comparison of the ratios of the intensities of the fragment ions at masses 84 and 83 for each sample (Fig. 1d) shows that the isotopic composition of chloroform was the same in all of the cultures and standards with the exception of the two *Porphyridium* samples, which had significantly increased 84/83 ratios (98% confidence level in the standard *t*-test) consistent with the biosynthesis of $^{13}CHCl_3$. In the *Porphyridium* cultures, the background levels of chloroform were higher than those of the methyl halides, and this yields the lower 0.12 ratio of the isotopic intensities (84/83). The mechanism of chloroform production is unclear. A study of the enzymes in *P. purpureum* extracts was unable to detect chloroperoxidase activity (Murphy et al. 2000), perhaps due to the extremely low levels of enzyme needed to account for the small amounts of chloroform produced over the 19-d incubation period.

The four culture samples had higher levels of dichloromethane than the medium: by 4.5% in the case of the *Porphyridium* cultures and 18% for the *Dunaliella* cultures. Figure 1e shows that there is a slight increase in the ratio of the ions at masses 50 and 49 for the four algal samples compared with the media and standards, but the change is small and would not be consistent with higher production of CH_2Cl_2 in the *Dunaliella* cultures. These results are not regarded as providing significant evidence (<90% confidence level in the *t*-test) that CH_2Cl_2 was produced by these algae rather than being a contaminant.

An isotopic method has been developed to verify the production of volatile, carbon-containing compounds by photosynthetic organisms. The key objective of this study, to provide confirmation that *P. purpureum* is a source of chloroform, was achieved. This organism is the first example of a marine microalga that has been shown to produce chloroform. At the same time, this experiment has provided evidence for its production of methyl chloride, bromide, and iodide. By analogy with studies (e.g., Moore et al. 1996*b*) that have shown microalgal production of dibromomethane together with bromoform, it might be anticipated that a producer of chloroform would yield dichloromethane as well. However, this work did not provide evidence for dichloromethane production by *P. purpureum.*

The value of the method in distinguishing between biotic increases in concentration and contamination during the incubation period is illustrated by the contrasting labeling results obtained for chloroform and dichloromethane. The method does not demand complete elimination of unlabeled bicarbonate from the initial culture medium. Although the method has the advantage that it is not necessary to eliminate all contamination from the system, its usefulness is ultimately limited by the relative amounts of contaminant and material synthesized in the culture.

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Evidence of trace metal limited photosynthesis in eutrophic estuarine and coastal waters

Abstract—Based on field observations and incubation experiments, this study provides evidence that trace metal limited (e.g., Fe) photosynthesis may function in eutrophic coastal waters. In the Zhujiang (Pearl River) Estuary and the adjacent coastal area, analyses of water samples indicate that phytoplankton photosynthesis is apparently phosphorus limited in the freshwater and upper estuary and nitrogen and/or silica limited in the coastal waters farther offshore. The incubation experiments using in situ water samples show, however, that in both cases photosynthesis can be enhanced by addition of trace elements (e.g., Fe and Cu). This is presumably because exogenous influx of major plant nutrients has skewed the primary production toward trace-element limitation, which in turn limits the increase in chlorophyll biomass and full consumption of major nutrients in eutrophic coastal waters as compared to the nutrient-poor oligotrophic open ocean.

Recent studies have shown that addition of trace amounts of iron may dramatically increase photosynthesis in the open ocean, at both the equator and high latitudes (e.g., Martin and Fitzwater 1988; De Baar et al. 1995; Coale et al. 1996*a*,*b*; Takeda 1998), and in coastal high nutrient and low chlorophyll (HNLC) upwelling regions (Hutchins and Bruland 1998). If such an Fe-limited photosynthesis is an important mechanism that regulates the marine autotrophic production and associated draw-down of atmospheric CO_2, a fundamental challenge is to characterize the variability of Fe limitation along the nutrient gradients between the eutrophic coast (including estuaries) and the interior of the ocean.

The coastal ocean is nutrient enriched and has altered nutrient ratios as a result of changed riverine input due to land-use change and anthropogenic nutrient emission over the last century (Nixon 1995; Paerl 1997). Coastal water ecosystems respond to such changes in nutrient budgets by shifting population dynamics of phytoplankton and hence of the rest of the food web, leading to changes in top-down as well as bottom-up control of community structures (Landry et al. 1997). Current research on coastal eutrophication largely concerns the response to the increased plant nutrient (N, P, and Si) loadings from land, and trace metals (e.g., Fe) are usually considered to be in excess in coastal waters due to natural weathering and, most recently, pollution drainage from land source (De Jonge et al. 1994; Pelley 1998). In eutrophic coastal waters, photosynthesis is traditionally believed to be limited by one of the major plant nutrients (e.g., N or P) due to a distorted nutrient ratio and enhanced influx (Turner and Rabalais 1994; Humborg et al. 1997). Trace metal limitation (e.g., Fe) in coastal waters has only received attention in recent years (cf. Sunda and Huntsman 1995; Schmidt and Hutchins 1999; Wells 1999). Evidence from the Zhujiang (Pearl River) Estuary and South China Sea indicates, however, that trace-metal-fertilized photosynthesis could be an important feature of eutrophication in coastal waters. Our conceptual model of coastal eutrophication should consider the potential impacts of trace elements as additional regulators of phytoplankton production and community composition.

The Zhujiang (Pearl River) is the largest river draining South China. It discharges 350×10^9 m^3 yr^{-1} of freshwater with a sediment load of 85×10^6 tons yr^{-1} into the South China Sea (Fig. 1). The land use of the drainage area is very complex, including agriculture over the whole watershed plus urban activities in the lower reaches and delta region (e.g., Guangzhou, Hong Kong, and Macao). The adjacent coastal area (21.5–23°N, 112.5–114.5°E) is eutrophic, with elevated riverine nutrient inputs, i.e. 60–100 μM for DIN (DIN = $NO_3^- + NO_2^- + NH_4^+$), 0.5–1.0 μM for phosphate, and 100–150 μM for dissolved silica (H_2SiO_3), respectively, into the South China Sea via eight channels/streams of the Zhujiang (Zhang et al. 1999).

The estuary of the Zhujiang (the Lingdingyang) is characterized by a microtide: the tidal range is 1–2 m with current of 1–2 m s^{-1}. The high salinity water enters the Lingdingyang from the southeastern side (i.e., Hong Kong), whereas the freshwater effluents from various channels move southward mainly via the western part of Lingdingyang (Fig. 1). The residual current shows a counterclockwise pattern, with a surface velocity of 10–30 cm s^{-1} (Zhang et al. 1999).

This study attempts to increase understanding of nutrient dynamics in the Zhujiang Estuary in relation to photosynthesis and eutrophication of the South China Sea. The knowledge was gained from cruises in the wet (August–September 1996) and dry (January–February 1997) seasons and in situ nutrient additions (August–September 1996) in the Zhujiang Estuary and the adjacent shelf region.