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AN INVESTIGATION INTO THE RELEASE OF METHYL HALIDES BY PHYTOPLANKTON CULTURES

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ABSTRACT

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by Cristina Fiona Peckett

Methyl halides are known to contribute significantly to ozone destruction in the stratosphere and to play an important part in tropospheric chemistry. The oceans act as both a source and a sink for these methyl halides, and marine organisms have been shown to be a possible major oceanic source. This study describes the results of an investigation into the release of methyl halides by various marine phytoplankton species grown in batch cultures.

Phytoplankton cultures were grown in specifically designed, gas-tight glass vessels, from which the headspace was analysed every 1-3 days using a gas chromatograph linked to either an electron capture detector (GC-ECD) or a mass spectrometer (GC-MS). Phytoplankton growth was monitored in the cultures by determining cell counts, chlorophyll and nutrient concentrations on frequently sampled small volumes of the culture medium. Initial experiments were conducted using the following species: Isochrysis galbana, Phaeocystis globosa, Chaetoceros sp., Emiliania huxleyi and Thalassiosira gravida. Methyl chloride, methyl bromide and methyl iodide production was detected for most species. Some apparent removal of methyl halides from the culture vessels was also observed. Dimethyl sulphide (DMS) production was also detected.

Culture experiments were designed to examine i) the effect of different nutrient regimes and ii) the addition of grazers (Oxyrrhis marina) on methyl halide release from microalgal cells. Methyl chloride and methyl bromide production normalised to chlorophyll or cell counts generally increased with increasing initial nitrate concentration, and the introduction of grazers was shown to produce a short term increase in methyl halide release. Natural phytoplankton populations sampled from a Norwegian fjord in May 2000 were maintained in culture vessels for a few days at close to in situ conditions and methyl iodide production detected, which was apparently affected by the supplemented nutrient regime.

The extent to which this study has given further insight into the factors that affect methyl halide production by marine phytoplankton and the mechanisms involved in their release are discussed.

List of Contents

List of Tables

List of Figures

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1.0 INTRODUCTION

1.1 Methyl halides and ozone

Methyl halides contribute significantly to ozone destruction in the stratosphere, by acting as sources of halogen radicals through photolysis, which are in turn capable of catalytically destroying ozone (Albritton & Watson, 1992; Solomon *et al.* 1992). Atmospheric methyl bromide (CH_3Br) is the chief source of stratospheric bromine (Butler, 1995), and bromine is estimated to be about 40-100 times more effective than chlorine at removing ozone on a per-atom basis, thus methyl bromide is considered to be on a par with some of the chlorofluorocarbons (CFCs) in contributing to stratospheric ozone depletion (Butler, 1996). Chemical coupling between iodine and chlorine and between iodine and bromine species also may be of significant importance in stratospheric ozone destruction (Solomon *et al.* 1994).

1.2 Sources and sinks of methyl halides

Sources of atmospheric methyl halides include biomass burning (Lobert *et al.* 1991), production by fungi (Harper, 1985; Wuosmaa and Hager, 1990), soil fungi (Hoekstra *et al.* 1998), some terrestrial plants (Wuosmaa and Hager, 1990) and cattle (Williams *et al.* 1999) and the use of methyl bromide as an agricultural fumigant (the latter is now restricted under the Montreal Protocol). However, and most relevant to this study, the ocean is considered to be a major source of methyl halides (Cicerone *et al.* 1988).

1.2.1 Methyl bromide

The oceanic methyl bromide (CH_3Br) production rate has been estimated as between 0.3-1 Gmol yr^{-1} by Butler (1996) and 1.6 Gmol yr^{-1} by Lobert *et al.* (1995). Much of this is consumed by hydrolysis, chloride substitution reactions and microbial breakdown (King and Saltzman, 1997). Measurements of methyl bromide in the ocean and atmosphere have been used to justify a large oceanic flux to the atmosphere (Singh *et al.* 1983; Pilinis *et al.* 1996), a small oceanic net sink (Moore and Webb, 1996; Anbar *et al.* 1996) and a large oceanic net sink (Lobert *et al.* 1995; Lobert *et al.* 1997; Groszko and Moore, 1998). The latest estimates have been in favour of a

global, net oceanic sink of atmospheric methyl bromide of between –0.11 Gmol yr-1 (Groszko and Moore, 1998) and –0.22 Gmol y^{-1} (Lobert *et al.* (1997).

1.2.2 Methyl chloride

Methyl chloride (CH_3Cl) is the main natural chlorinated constituent of the atmosphere, for which the oceans are a predominant source (Singh *et al.* 1983; Liss, 1986). The estimated global production of methyl chloride is between 3-9 Gmol yr^{-1} (Moore *et al.* 1996a). A major production mechanism of methyl chloride may actually be chloride substitution. Moore *et al.* (1996a) have suggested the reaction of methyl bromide and methyl iodide with chloride ions in seawater may represent 40-75% of the oceanic source of methyl chloride, however this still leaves a large portion of the oceanic production unaccounted for. The only known significant chemical sink for methyl chloride in seawater is hydrolysis (Tait *et al.* 1994) but biological degradation may also occur (Moore *et al.* 1996a).

1.2.3 Methyl iodide

Methyl iodide (CH_3I) is thought to be the most dominant of the potential vectors of iodine from the ocean to the atmosphere (Moore and Tokarczyk, 1992 and 1993) and the oceans are believed to be the only significant source of methyl iodide to the atmosphere (Liss, 1986). The global oceanic source of methyl iodide was estimated by Singh *et al.* (1983) to be 3.5 Gmol yr^{-1} and more recently 1 Gmol yr^{-1} by Nightingale (1991).

Thus the world oceans are both a source and a sink for methyl halides.

1.3 Oceanic concentrations of methyl halides

Mean oceanic concentrations of the methyl halides discussed above range from 3.5 pmol l^{-1} for methyl iodide in the open ocean (Tait *et al.* 1994) to 56 pmol l^{-1} in coastal waters (Moore and Tokarczyk, 1993), 84 pmol l^{-1} for methyl chloride in the open ocean (Moore *et al.* 1996a) and 8.8 pmol l^{-1} for methyl bromide in the Southern Ocean (Comiso *et al.* 1993). Other important methyl halides include bromoform ($CHBr_3$), diiodomethane (CH_2I_2) and chloroiodomethane (CH_2ClI) and considerable concentrations of these have also been measured in Swedish coastal waters, Antarctic

open ocean waters (Klick and Abrahamsson, 1992) and the northwest Atlantic (Moore and Tokarczyk, 1992).

1.4 Biogenic production of methyl halides in the ocean

A biogenic mechanism is likely to be responsible for most oceanic methyl halide production. Many species of marine macroalgae have been shown to produce a wide variety of methyl halides in the laboratory (Gschwend *et al.* 1985; Manley and Dastoor, 1987 and 1988; Manley *et al.* 1992; Schall *et al.* 1994; Abrahamsson *et al.* 1995a and 1995b; Nightingale *et al.* 1995).

1.4.1 Production of methyl halides by phytoplankton

Phytoplankton are obvious candidates for marine methyl halide production since they are abundant in surface waters and globally distributed. Species of phytoplankton have been identified which produce a variety of methyl halides in laboratory culture experiments (Tokarczyk and Moore, 1994; Tait and Moore, 1995; Moore *et al.* 1996b; Scarratt and Moore, 1996 and 1998; Manley and de la Cuesta, 1997; Saemundsdóttir and Matrai, 1998) and those tested for methyl chloride, methyl bromide and methyl iodide release are summarised in Table 1. Phytoplankton cultures have also been shown to produce other methyl halides including $CHBr_2Cl$, CH_2Br_2, CH_2BrI, $CHBr_3$, $CHCl_3$, CH_2ClI and CH_2I_2 (Tokarczyk and Moore, 1994; Moore *et al.* 1996b; Scarratt and Moore, 1999). C_2H_5I has also been detected (Moore *et al.* 1996b). Abrahamsson *et al.* (1995b) has measured C_2Cl_4 and C_2HCl_3 production from axenic cultures of *Porphyridium purpureum*, although a subsequent study using the same clone of this species did not detect production of these compounds (Scarratt and Moore, 1999). Some in situ measurements have also been made which have revealed a correlation between the presence of a *Phaeocystis* sp. bloom and high levels of methyl bromide (Baker *et al.* 1999).

Species of phytoplankton	Production detected			Ref.
	CH_3Cl	CH_3Br	CH_3I	
Amphidinium carterae CCMP 1314	n.m	✓	n.m	6
Chaetoceros sp. CCMP 208	n.m	✗	n.m	6
C. atlanticus CCMP161	n.m	✓	n.m	6
C. calcitrans CCMP 1315 (axenic)	✓	✓	n.m	4
C. diversum A1299[a]	n.m	✓	n.m	6
C. neogracile CCMP 1317	n.m	n.m	✗	5
Coccolithus pelagicus CCMP 299	n.m	n.m	✗	5
*Crypthecodinium cohnii** CCMP 316	n.m	✓	n.m	6
Dunaliella parva UTEX 1983	n.m	n.m	✗	5
Emiliania huxleyi CCMP 373 (370, ref. 6) (axenic in ref. 5)	✓	✓✗	✗	4, 5, 6
Guillardia theta CCMP 327	n.m	✓	n.m	6
Hemiselmis rufescens CCMP 439	n.m	✓	n.m	6
Isochrysis sp. CCMP 1324 (axenic)	✓	✗	n.m	4
Isochrysis galbana CCMP 1323	n.m	n.m	✗	5
Navicula sp. CCMP 545	n.m	✗	✗	2
Navicula sp. CCMP 546	n.m	✗	✗	2
Navicula sp. CCMP 547	n.m	n.m	✓	5
Nitzschia sp. CCMP 580	✓	✓	✓	1, 2, 5
N. arctica CCMP 1116	✗	✓	✗	2
N. punctata UTEX 2041	n.m	n.m	✗	5
N. seriata (strain not reported)	✓	n.m	n.m	1
Odontella mobiliensis (strain not reported)	✓	✓	n.m	1
Pavlova sp. CCMP 617	n.m	✓	n.m	6
P. gyrans CCMP 608 (axenic)	n.m	✓	n.m	6
P. lutheri CCMP 1325	n.m	✓	n.m	6
Phaeocystis sp. CCMP 627 (axenic[b] in ref. 5)	✓	✓	✓	3, 4
Phaeocystis sp. CCMP 1521	n.m	n.m	✓	5

Table continued on next page.

Species of phytoplankton	Production detected			Ref.
	CH_3Cl	CH_3Br	CH_3I	
Phaeocystis sp. CCMP 628	n.m	✓	n.m	6
Phaeodactylum tricornutum CCMP 630 (axenic in ref. 5)	✓	✓	✓	1, 3, 4
Pleurochrysis carterae CCMP 645	n.m	✓	n.m	6
Porosira glacialis CCMP 651	✓	✓	✓	1, 2, 5
Porphyridium sp. UTEX 190	✓	✓	n.m	4
P. purpureum CCAP 1380/3 (axenic)	n.m	n.m	✓	7
Prorocentrum sp. CCMP 703	✓	✓	n.m	4
P. micans CCMP 1589	n.m	✓	n.m	6
Pycnococcus provasolii CCMP 1203	n.m	✓	✗	5,6
Skeletonema costatum UTEX 2308	n.m	n.m	✗	5
Synechococcus sp. (axenic) CCMP 1334	✓	✓	✗	4,5
S. bacillaris CCMP 1333	n.m	✗	n.m	6
Synedra minuscula CCMP 845	n.m	✗	n.m	6
Tetraselmis sp. CCMP 961 (axenic)	✓	✓	n.m	4
T. levis CCMP 896	n.m	✗	n.m	6
Thalassiosira pseudonana CCMP 1015	n.m	✗	n.m	6
T. weissflogii CCMP 1336	✓	✓	✓	1, 3

Table 1. Phytoplankton species previously tested under culture conditions for the production of methyl chloride, methyl bromide and methyl iodide.

n.m. = not measured. ✓ = production detected. ✗ = no production detected. ✓✗ = production detected by one author but not by another. * = heterotrophic dinoflagellate. CCMP = Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbour, Maine, U.S.A. UTEX = University of Texas at Austin Culture Collection. CCAP = Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, Oban, Scotland. [a] = Brand's culture collection (MBF/RSMAS), Univ. Miami. [b] = from J. Stefels, Univ. Groningen.

Reference numbers refer to: 1) Tait and Moore (1995); 2) Moore *et al.* (1996b); 3) Scarratt and Moore (1996); 4) Scarratt and Moore (1998); 5) Manley and de la Cuesta (1997); 6) Saemundsdóttir and Matrai (1998) and 7) Scarratt and Moore (1999).

Previous experiments have therefore shown some species of phytoplankton in culture to produce methyl halides. The levels of methyl halide production that have been measured are difficult to compare between studies by different authors due to the various ways in which the data have been calculated. For example, some authors have shown the amount of each methyl halide present in each culture flask, others have reported the maximum methyl halide production per cell and others the maximum methyl halide production per gram of chlorophyll. However, in general, species that are strictly coastal or more abundant in coastal areas have appeared to be stronger producers of some methyl halides than species that are more abundant in open ocean areas (Saemundsdóttir and Matrai, 1998) and *Phaeocystis* sp. has consistently been shown to be a high methyl halide producer compared to all the other species tested (Saemundsdóttir and Matrai, 1998; Scarratt and Moore, 1996 and 1998).

Yet to be addressed in any detail is the effect that changing environmental conditions may have on the production of methyl halides by phytoplankton. Higher levels of illumination have been found to increase concentrations of methyl halides produced by phytoplankton cultures (Moore *et al.* 1996b), suggesting the involvement of photosynthesis in the methyl halide production mechanism, however the actual mechanism remains unknown. The effect of nutrient concentration on phytoplankton methyl halide production was briefly considered by Scarratt and Moore (1996). These authors studied methyl halide production from batch cultures under a range of dissolved nitrate levels, but their data were inconclusive. The effect of zooplankton grazing on the release of methyl halides by phytoplankton has not previously been reported.

1.4.2 Global significance of oceanic biogenic production of methyl halides

By extrapolating measured production rates of methyl halides for a variety of species of phytoplankton it has been estimated that phytoplankton may be responsible for between 23 and 126 % of the annual global oceanic production of methyl chloride based on the estimates of Moore *et al.* (1996a) and between 28-93 % of the annual global oceanic production of methyl bromide based on the estimates of Butler (1996) or 26% for *Phaeocystis* sp. using Lobert *et al.*'s (1995) estimate (Scarratt and Moore, 1996 and 1998). Globally significant phytoplankton production estimates of 0.05–0.82 Gmol methyl bromide yr^{-1} in coastal and upwelling areas and 0.027–0.50 Gmol yr^{-1} in the open ocean have also been calculated from culture experiments

(Saemundsdóttir and Matrai, 1998). For methyl iodide estimates range from 6.7 x 10^{-6} Gmol yr^{-1} based on the production rates of *P. glacialis*, to 0.025 Gmol yr^{-1} based on the production rates of *Phaeocystis* sp., with a mean of 0.0085 Gmol yr^{-1} for all species tested (Manley and de la Cuesta, 1997), compared to an estimated production of 0.004 Gmol yr^{-1} by macroalgae (Manley *et al.* 1992). Using Nightingale's (1991) estimate, phytoplankton production may therefore only account for 0.85% of the annual global oceanic production of methyl iodide. Depending on which global production estimates such calculations are based on, and which combination of species are used to calculate the methyl halide production, the global importance can range from minor to very significant.

2.0 OBJECTIVES

An uncertainty remains regarding the importance of the contribution of marine phytoplankton to global methyl halide production. The initial objective of this study therefore, was to provide more data on the production of methyl halides by cultures of phytoplankton, from which more accurate estimates of the natural production of methyl halides from coastal and open ocean regimes could be achieved.

Subsequently this led to a more detailed study of the effects of changing environmental conditions on the production of methyl halides by phytoplankton. Data reporting the seasonal variability of biogenic methyl halide concentrations in the sea are scarce (Klick, 1992) and it has been speculated that production by phytoplankton may be strongly dependent on environmental conditions (Moore *et al.* 1996a). The secondary objectives of this study were therefore to better understand the roles of nutrients and zooplankton grazing pressure on the production of methyl halides by phytoplankton, possibly leading to further insight into the methyl halide production mechanism. Two hypotheses were tested:

a) Increased nitrate concentration will increase biomass-normalised methyl halide release from phytoplankton cultures.
b) Cell damage by grazing will increase biomass-normalised methyl halide release from phytoplankton cultures.

This study reports methyl halide production from monoalgal (but not axenic) batch cultures of species of phytoplankton isolated from differing ecosystems, and short-term cultures of natural populations of phytoplankton and zooplankton, grown under close to in situ conditions. Initial experiments were conducted to establish methyl halide production from a range of species and subsequent experiments developed using a) a range of initial nutrient concentrations and b) the addition of a protozoan grazer. Gas chromatography was used for methyl halide analysis, linked to either an electron capture detector (GC-ECD) or a mass spectrometer (GC-MS).

3.0 MATERIALS AND METHODS

3.1 Stock cultures

The stock cultures used in this study and where they were obtained from are summarised in Table 2.

Species	Strain	Source
Isochrysis galbana	PLY 565	PML, Plymouth
Emiliania huxleyi	PLY 92E	PML, Plymouth
Phaeocystis globosa	PLY 64	PML, Plymouth
Oxyrrhis marina	PLY 209B	PML, Plymouth
Oxyrrhis marina	CCAP 1133/5	M. Steinke, UEA.
Thalassiosira gravida	CCMP 986	CCMP, U.S.A.
Prochlorococcus marina	CCMP 1375	CCMP, U.S.A.
Chaetoceros sp.	-	isolated by author

Table 2. Stock cultures. PML = Plymouth Marine Laboratory, Plymouth, Devon. UEA = University of East Anglia, Norwich. CCMP = Provasoli-Guillard Center for the Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbour, Maine, U.S.A. CCAP = Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, Oban, Scotland.

The *Chaetoceros* sp. stock culture was originally isolated from the North Atlantic on RRS Discovery cruise 233, April-June 1998. During the cruise, individual cells were isolated from water samples in areas of high surface water methyl halide concentrations with the intention of identifying the methyl halide producers by culturing the species back in the laboratory. Cells of *Nitzschia* sp., *Rhizosolenia* sp. and *Ceratium* sp. were also isolated, but did not survive to become stock cultures.

All the stock cultures (apart from *P. marina*) were maintained in autoclaved artificial seawater medium prepared from Harrison *et al.*'s (1980) recipe (Appendix 1) and enriched using a slightly modified form of K medium (Keller *et al.* 1987) (Appendix 2), in cotton-stoppered 250ml Pyrex flasks. These were incubated at 15^0C, at approximately 40 μE m^{-2} s^{-1} irradiance, in a light:dark cycle of 14:10 (*T. gravida* at

4^0C, light:dark 16:8). *P. globosa* was initially kept in enriched seawater (modified K) medium, (Keller *et al.* 1987), due to problems with growing this strain in artificial seawater medium. Using artificial seawater increases the reproducibility of culture growth and allows more accurate nutrient manipulation. One disadvantage however is that some halide ion concentrations are elevated as AnalaR NaCl, for example, contains a 0.005% contamination of iodide (I^-), which at 20.8 g kg^{-1} gives artificial seawater of 1000 μg I^- l^{-1} compared to 13-25 μg I^- l^{-1} in surface ocean waters (Luther *et al.* 1988).

Stock cultures were sub-cultured every 4 weeks except for the fast growing *P. globosa* (every 2 weeks) and the slow growing *T. gravida* (every 2 months). *O. marina* CCAP 1133/5 was fed weekly with *Dunaliella tertiolecta* (also from UEA). *O. marina* PLY 209B did not require feeding as it contained a small naviculoid diatom which grew alongside the heterotrophic dinoflagellate. The identity of this diatom could not be established (J. Green, personal communication). None of the stock cultures were axenic.

Stock cultures of *P. marina* were maintained in a K medium specially modified to grow this species (recipe from CCMP, formulated by Maureen Keller, unpublished), in cotton-stoppered 10-25ml Pyrex flasks and incubated at 24^0C, 30 ± 5 μE m^{-2} s^{-1}, light:dark 13:11, considered by Moore *et al.* (1995) to be the optimum conditions to grow CCMP 1375. However, *Prochlorococcus* sp. is very difficult to maintain in culture (F. Partensky, personal communication), and the stock culture could not be sustained. It was hoped to be able to culture this species as a relationship was seen between prochlorophyte abundance and methyl iodide seawater concentrations on the North Atlantic RRS Discovery cruise 233 (D. Smythe-Wright and R. Davidson, personal communication) and it would be interesting to determine its methyl halide production status, especially due to its ubiquitous distribution in near surface oceanic waters.

3.2 Apparatus

Gas-tight culture vessels were designed using specially modified 2L or 5L glass flasks and dreschel heads, fitted together with ground glass seals wrapped in parafilm. The three arms were attached to 2-way Swagelok valves using Swagelok

fittings and Vespel ferrules. The Swagelok valves were dismantled prior to use and cleaned of all lubricating grease (which may have introduced contaminating volatile compounds into the culture vessels) by washing with acetone, then propan-2-ol and then distilled water. They were dried in a warm vacuum oven and carefully reassembled.

Figure 1 is a schematic illustration of a culture vessel. Sampling of gas phase methyl halides in the headspace could be achieved without atmospheric contamination using a gas-tight, oven-baked, ground-glass syringe (routinely 100 ml and occasionally 250 ml) with a male luer tap which fitted into the female luer on the end of arm 1. The syringe was filled with headspace gas until resistance was felt, then a clean air mixture from the cylinder attached directly to arm 2 was turned on and allowed to enter slowly into the culture medium. The slight increase in pressure allowed the syringe to be fully filled. The syringe was then closed. Just enough clean air was introduced to the flask to replace the sample extracted, thus the headspace remained at atmospheric pressure and the amount of gas replaced was assumed to be 100 ml. An increase in headspace pressure was occasionally noted when sampling, therefore <100 ml clean replacement air was needed. This may have led to slight over-estimation of methyl halide concentrations in the vessel on these sampling days.

As the vessels were gas tight, a headspace sample of 100 ml could not be taken without the addition of a small amount of clean air during sampling (the rest of the replacement air was added after sampling had occurred) otherwise a large negative pressure would build up in the vessel. In order to minimise any mixing of the clean air and the headspace sample, the clean air was added to the bottom of the vessel as the headspace sample was being taken from the top (Figure 1). The bubbles of clean air entered the culture medium slowly, and were large, with a small surface area (they entered through a glass tube of internal diameter 4mm) so that de-bubbling of methyl halides in the medium was kept to an absolute minimum. Ideally a reservoir holding 100 ml of the replacement clean air could have been attached to arm 2 so that the exact replacement volume would have been known. Initially a syringe of clean air was used, attached to arm 2 in this way, but sampling of one syringe whilst adding clean air from another proved an impossible job for one individual.

Samples of medium could be withdrawn without atmospheric contamination through arm 3, using a sterile plastic syringe with a male luer end (Figure 1). Usually

only 5-20 ml of medium was sampled each time so this was not replaced with clean air.

The 2L vessels held 1L of culture medium at the start of each experiment, leaving 1395 ml of headspace for gas exchange. The 5L vessels held 3L of medium leaving 2450 ml of headspace.

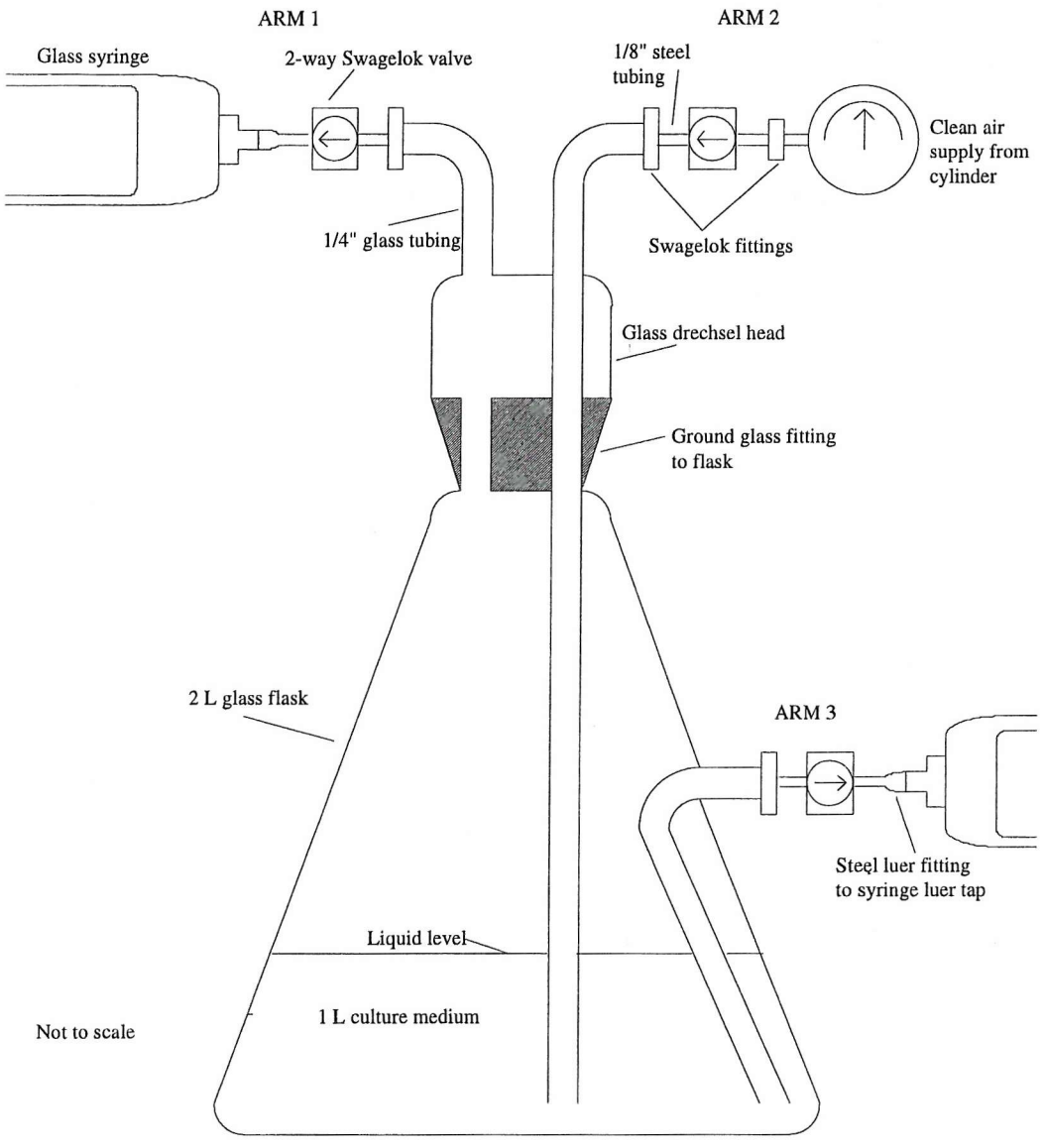


Figure 1. Schematic diagram of a 2L culture vessel.

3.3 Experimental cultures

3.3.1 Initial experimental cultures to determine methyl halide production

Experimental cultures using *I. galbana*, *P. globosa*, *Chaetoceros* sp., *E. huxleyi* and *T. gravida* were investigated for methyl halide release. As summarised in Table 1 (section 1.4.1), *I. galbana* has previously been used by Manley and de la Cuesta (1997) to investigate methyl iodide release and it was found to be a non-producer, although Scarratt and Moore (1998) detected methyl chloride but not methyl bromide release from *Isochrysis* sp. *Phaeocystis* sp. has been found to produce a range of methyl halides both in culture (Scarratt and Moore, 1996 and 1998; Manley and de la Cuesta, 1997; Saemundsdóttir and Matrai, 1998) (Table 1) and blooms of the species in situ in the coastal waters of the North Sea and in open ocean regions of the northeast Atlantic have been correlated with methyl bromide production (Baker *et al.* 1999). *P. globosa* was used in this study initially to check the integrity of the sampling and analytical system and also so that the resulting data would have a basis for comparison. *Chaetoceros calcitrans* has been found to produce methyl chloride and methyl bromide (Scarratt and Moore, 1998) but *Chaetoceros neogracile* did not produce methyl iodide (Manley and de la Cuesta, 1997) (Table 1). The species of the *Chaetoceros* sp. isolated in this study was not conclusively identified, but it was possibly *Chaetoceros mitra* (R. Davidson, personal communication). *E. huxleyi* has been found on separate occasions both to produce and not to produce methyl bromide, but to produce both methyl chloride and methyl iodide (Manley and de la Cuesta, 1997; Saemundsdóttir and Matrai, 1998; Scarratt and Moore, 1998) (Table 1). *Thalassiosira pseudonana* did not release methyl bromide when tested by Saemundsdóttir and Matrai (1998), but *Thalassiosira weissflogii* has been shown to produce methyl chloride, methyl bromide and methyl iodide (Tait and Moore, 1995; Scarratt and Moore, 1996) (Table 1). *T. gravida* was chosen in this study as its distribution in the North Atlantic was found to be correlated with seawater methyl halide concentrations (D. Smythe-Wright and R. Davidson, personal communication).

Enriched artificial seawater was used for all experiments in this study with the exception of the first *P. globosa* cultures where enriched K medium was used (section 3.3.2 describes further *P. globosa* experiments). Although the cultures were not axenic, they were regularly checked to be monoalgal. The autoclaved medium or seawater was kept in the dark and the enrichment solutions added aseptically. 1L of

medium was added to each culture vessel which was then assembled and made gas-tight. The medium was then sparged overnight with a clean, bottled air mixture (300 ppm carbon dioxide, 21% oxygen in nitrogen, from Air Products) introduced through arm 2, to remove naturally occurring methyl halides and to prevent carbon dioxide limitation. The bottled air was analysed on a number of occasions using both GC-ECD and GC-MS and was found to contain a high and varying concentration of CFC 113 but no other CFCs or methyl halides were detected. CFC 113 is present in some lubricants and it is possible that such a lubricant was used by the manufacturer in the cylinder head. The medium in each culture vessel was then inoculated through arm 3 using a sterile plastic syringe with between 3-10 ml of stock culture (each set of cultures were inoculated with the same volume). The stock culture providing the inoculum was always in the exponential phase of growth. Two culture vessels were set up for each experiment under the same conditions of nutrients, temperature and light. Table 3 summarises the experimental cultures and treatments in this section.

Strain	Species	Temp (^{0}C)	Irradiance ($\mu E\ m^{-2}\ s^{-1}$), L:D	NO_3:PO_4^{3-} (μM)
PLY 565	*I. galbana*	15	80 ± 20, 14:10	88:1
PLY 64	*P. globosa*	15	80 ± 20, 14:10	> 88>1
isolated by author	*Chaetoceros* sp.	15	80 ± 20, 14:10	88:1
PLY 92E	*E. huxleyi*	15	80 ± 20, 14:10	88:1
CCMP 986	*T. gravida*	4.0	80 ± 20, 16:8	88:1

Table 3. Summary of cultures, species and treatments for the initial methyl halide release experiments. L:D is the light/dark cycle in hours. NO_3^-:PO_4^{3-} is the dissolved nitrate : dissolved phosphate concentration at the start of the experiments. The *P. globosa* cultures were grown in enriched seawater and the nutrient concentrations were not measured in these cultures, hence the uncertainty of the exact NO_3^-:PO_4^{3-} concentrations.

3.3.2 Nutrient manipulation of experimental cultures

Three further culture vessels were constructed in order to examine the effect of different nutrient concentrations on methyl halide production. *P. globosa* was chosen for these experiments as it had been seen to reliably produce large amounts of methyl

halides thus a change in release due to nutrient manipulation would be more evident than for lesser producers. *P. globosa* was grown under four different nutrient regimes, firstly using a range of four nitrate concentrations from 8 μM – 88 μM and a phosphate concentration of 1 μM, and then repeated using two lower ranges of nitrate which were more representative of oceanic concentrations, between 6 μM – 48 μM and 4 μM – 32 μM, both using 1 μM phosphate (Table 4). 1L of medium containing the correct nutrient concentrations in each culture vessel was inoculated as for the previous experiments with 8 ml of stock culture.

Expt. no.	Strain	Species	Temp (^{0}C)	Irradiance (μE m^{-2} s^{-1}), L:D	NO_3:PO_4^{3-} (μM)
1	PLY 64	*P. globosa*	15	30 ± 10, 14:10	88:1, 44:1, 16:1, 8:1
2	PLY 64	*P. globosa*	15	30 ± 10, 14:10	48:1, 24:1, 12:1, 6:1
3	PLY 64	*P. globosa*	15	30 ± 10, 14:10	32:1, 16:1, 8:1, 4:1

Table 4. Summary of cultures, species and treatments for the nutrient manipulation experiments. Annotations as for Table 3.

A control was set up with each experiment; this was inoculated with enriched artificial seawater instead of stock culture. Any methyl halide release detected in this culture would clearly not be due to phytoplankton production. The nutrient concentrations used for the controls were the same as the highest concentrations being used in that experiment. The cultures were sampled every 2-3 days.

3.3.3 Natural populations from a Norwegian fjord

Short term experimental cultures (4-5 days) were set up at the Bergen Marine Biology Station using fjord water containing natural populations of phytoplankton and zooplankton. Methyl halide release was measured using a GC-MS for analysis. For these experiments 5L culture vessels were used, similar in every respect other than volume to the 2L flask, and shown in Figure 2. Larger culture vessels were used in an attempt to simulate natural conditions more effectively. The culture experiments were set up using the same nutrient regimes as those used in mesocosm experiments that were running simultaneously in the fjord, and it was anticipated that the results from both situations could be compared.

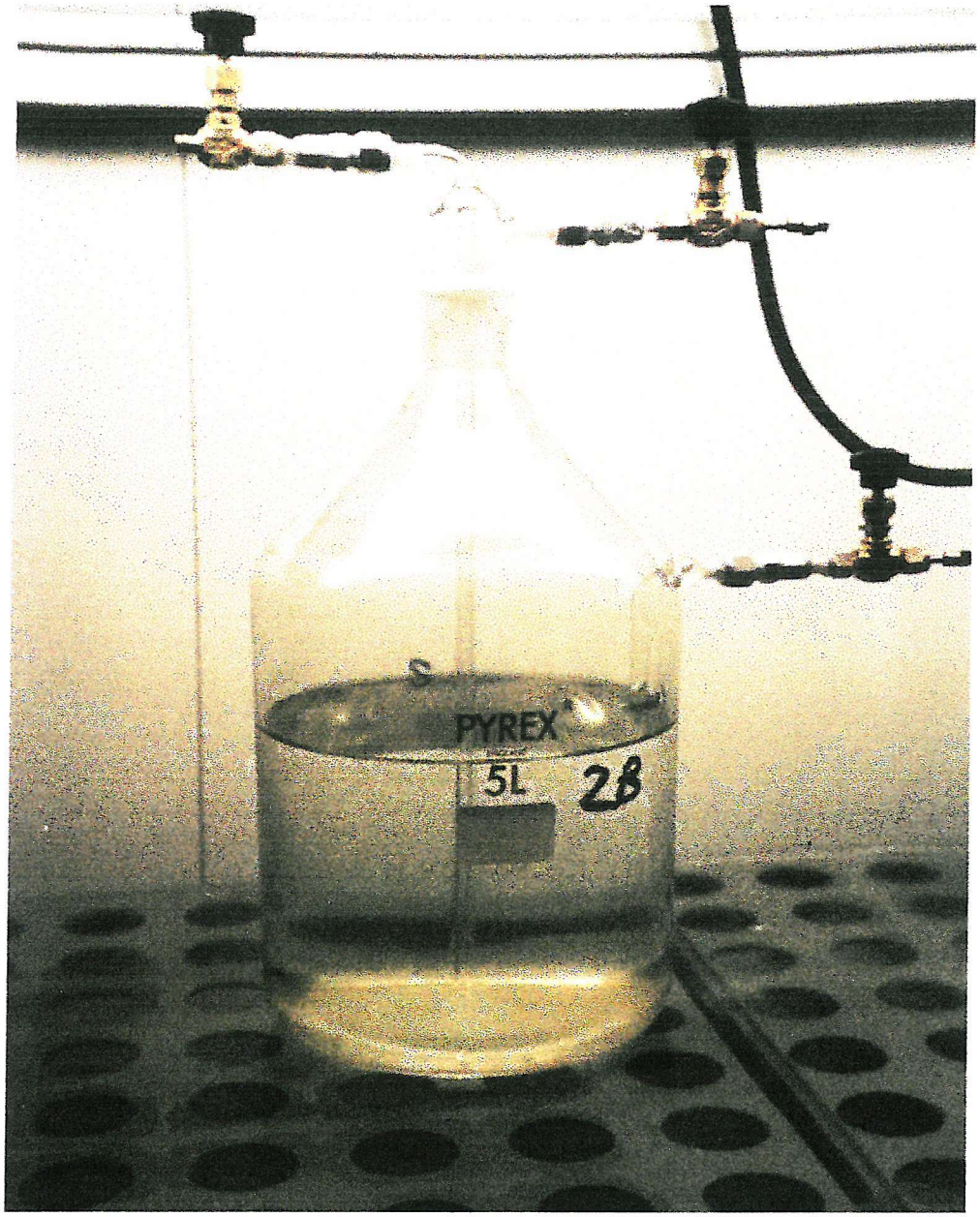


Figure 2. 5L culture vessel set up in the culture room at the Bergen Marine Biology Station.

For the first set of experiments, water was used from 1 m depth from each of the five mesocosm experiments that had already been set up and spiked with nutrients (Table 5) two days previously. The nutrient concentrations therefore had already decreased slightly in these. In the second set of experiments, water was used from 6 m depth in the fjord itself and each vessel was individually spiked with nutrients as shown in Table 5.

Nutrient treatment	Nitrate (μM)	Phosphate (μM)	Silicate (μM)	Iron (μM)
high phosphate	15	1	no addition	no addition
additional Si	15	1	5	no addition
additional Fe	15	1	5	0.1
low phosphate	15	0.1	no addition	no addition
control	no addition	no addition	no addition	no addition

Table 5. Nutrient treatment of experimental cultures grown at University of Bergen Marine Biology Station, May 2000.

The culture vessels were placed in a culture room which was kept at the temperature in the fjord at 1 m depth. This was monitored daily and ranged between 10 – 12.5^{0}C. The average irradiance was 45 (±15) μE m^{-2} s^{-1}. The cultures were sampled daily and lasted for 4-6 days.

3.3.4 Grazing in experimental cultures

E. huxleyi and *P. globosa* were used as prey in two separate grazing experiments to determine the effect of grazing on methyl halide production. The heterotrophic dinoflagellate *Oxyrrhis marina* (typically 16-20 μm) was used as the grazer as it grows well in culture, and although it is not a species representative of open coastal or oceanic waters, it is unlikely that its general predatory behaviour is unique (Flynn *et al.* 1996). The dinoflagellate swims in a random manner, and on encountering a prey, captures the cell with its transverse flagellum and ingests it with the help of trichocysts which cover its body (Tarran, 1992). *O. marina* has previously been shown to use *E. huxleyi* as prey (Wolfe *et al.* 1994; Wolfe and Steinke, 1996), preferring calcified over non-calcified cells (Hansen *et al.* 1996), and to use *P. globosa* as prey (Kadner, 2000) – it is capable of growing at a rate of 0.37 d^{-1} by feeding on *P. globosa* alone (Hansen *et al.* 1993).

In contrast to other grazing experiments using *O. marina* which have been performed in the dark (Goldman *et al.* 1989) or in very low light conditions (Hansen *et al.* 1996), a similar light:dark cycle was used in this study as in the previous experiments, allowing growth of the phototrophic prey to continue after the grazer was added. Establishing the grazing rates was not important, and as photosynthesis

may be involved in the process of methyl halide release from phytoplankton, the main criterion was not to interfere with these processes in any other way than the introduction of grazers. It was quite possible that the products of photosynthesis were also responsible in part for affecting the palatability of the prey.

The predator:prey ratios used in this study and in others are shown in Table 6. Prey numbers were high to emulate bloom conditions, under which the grazing process would potentially have the greatest effect on methyl halide release.

Reference	Predator:prey ratio	
	O. marina : E. huxleyi	*O. marina : P. globosa*
Wolfe and Steinke, 1996	1:10 – 1:70	-
Hansen *et al.* (1996)	1:8 – 1:15	-
Hansen *et al.* (1993)	-	Not stated
Kadner (2000)	1:10 – 1:40	1:10 – 1:40
This study	1:200	1:400

Table 6. Predator:prey ratios used in grazing experiment studies.

1) *E. huxleyi*

The five culture vessels were set up with 1L of medium as for previous experiments. The effect of grazing was monitored in two of the vessels, two vessels contained only *E. huxleyi* for comparison against the grazed cultures, and one vessel contained only *O. marina* as a control to determine if methyl halide production was detectable from this dinoflagellate alone. 8 ml of prey culture was added to the relevant vessels at the start, and a similar volume of enriched artificial seawater to the vessels without prey. The *O. marina* was grown to the required density in order to create a predator:prey density of 1:200, then kept in the dark for 4 days to ensure complete removal of the diatoms prior to its use. 50 ml of the starved *O. marina* stock culture was added to the relevant vessels on day 11 once the *E. huxleyi* were well established in the exponential phase of growth. An equivalent volume of enriched artificial seawater was added to the other vessels. The vessels were kept in an incubator under the conditions shown in Table 7 and sampled every 2-3 days.

2) *P. globosa*

Five culture vessels were inoculated with 1) *P. globosa* alone, 2) *O. marina* alone, 3) and 4) *P. globosa* and *O. marina* and 5) artificial seawater (control), as described below. 8ml of *P. globosa* stock culture was used to inoculate the relevant vessels. The *O. marina* stock culture was grown to the required density in order to create a predator:prey density of 1:400. The *O. marina* was then starved as outlined for the previous experiment and subsequently 60 ml was added to the relevant vessels after sampling on day 11 when the cultures of *P. globosa* were well established in the exponential phase of growth. The vessels were kept in an incubator under the conditions shown in Table 7 and sampled every 2-3 days.

Strain	Species	Temp (^{0}C)	Irradiance ($\mu E\ m^{-2}\ s^{-1}$), L:D	NO_3:PO_4^{3-} (μM)
PLY 92E PLY 209B	*E. huxleyi* and *Oxyrrhis marina*	16	30 ± 10, 14:10	88:1
PLY 64 CCAP 1133/5	*P. globosa* and *Oxyrrhis marina*	16	30 ± 10, 14:10	88:1

Table 7. Summary of cultures, species and treatments for the grazing experiments. Annotations as for Table 3.

3.4 Culture sampling

The culture medium was sampled at frequent intervals for analysis of nutrients, chlorophyll *a* and for cell population density. Samples of 5 – 20 ml were taken directly following the extraction of the headspace samples. The medium was gently swirled prior to sampling as the phytoplankton cells tended to settle. Arm 3 (Figure 1) was first cleared of the residual culture medium it contained and this discarded before the actual sample was taken. Part of the sample was used for determination of the cell density (cell count) using a Sedgewick-Rafter chamber or a hemocytometer, depending on the cell density, using samples stained with Lugol's iodine and/or formalin. Ten fields were counted under a microscope and the results averaged. To obtain ten fields the hemocytometer was filled five times and the

Sedgewick-Rafter chamber was filled once. The remaining sample was filtered in duplicate (10 ml) through 25 mm GF/F filters for later chlorophyll *a* analysis, and the filtrate used for nutrient analysis. Filters and filtrate were stored frozen prior to later analysis.

Chlorophyll *a* and phaeo-pigment concentrations were determined as follows by the method of Parsons *et al.* (1984). The loaded filters were extracted for 24 hours in 5 ml of 90% acetone in the dark in the fridge. An Aminco fluorometer was used to measure the fluorescence of the acetone extract, calibrated daily using a 1:100 dilution of an approximately 2 mg l^{-1} chlorophyll *a* standard (Sigma). The exact concentration of the pre-diluted standard was determined spectrophotometrically (Cecil CE292 Digital UV spectrophotometer series 2) by measuring the absorbance at wavelengths of 664, 647, 630 and 750 nm and then at 667 and 750 nm after adding 2 drops of 10% hydrochloric acid (Lorenzen 1967; Jeffrey and Humphrey, 1975). Chlorophyll *a* and phaeo-pigment concentrations were calculated using the Lorenzen (1967) equations. Phaeo-pigment data are not shown in this study.

Dissolved nitrate and dissolved phosphate concentrations were determined using an autoanalyser (Burkard Scientific SFA-2) according to the methods outlined by Hydes (1984). Nutrient samples were frozen for between 2-8 months prior to analysis. Three mixed standards were prepared covering the range of concentrations used. The data were calculated using the calibrations and accounting for drift through each run, using Microstream software.

3.5 Culture vessel headspace sampling

The headspace was sampled at the time of inoculation and then every 1-3 days as stated for each experiment. The medium in the culture vessel was gently swirled to ensure the gases were in equilibrium between the medium and the headspace prior to sampling. The gas line from the clean air cylinder was attached to arm 2, the syringe attached to arm 1 and the valve on arm 1 opened to allow sampling (Figure 1). The valve on arm 2 was opened once resistance to sampling was felt in the syringe, the syringe filled, the valve on arm 1 closed and the syringe tap closed. Once the vessel was back to its original pressure (determined when the syringe piston did not move when the tap was quickly reopened), valve 2 was closed. The syringe was transported to another laboratory within 5 minutes for headspace analysis. A duplicate was taken

for some cultures on the last day of sampling to assess the reproducibility of gas sampling. Methods to enable more frequent analysis of duplicates were tested but were not satisfactory. Attempts were made to sample a larger volume of gas (200 ml) then inject half the volume into the GC, waiting for the run to finish and injecting the second half. However these were not successful as the syringes were not gas-tight enough over the period of time it took for the first injection to run (approximately 30 minutes, see section 3.6.1), and some lab air was shown to have entered the second sample. Taking two separate syringe samples from one culture vessel was not an option as the same treatment would need to be done for each culture, and time constraints prevented duplicates being taken from each vessel in one day. The replication errors are stated in the results for each experiment where duplicates were taken.

3.6 Gas analysis

3.6.1 Gas chromatography

Headspace analysis was performed using a GC-ECD or a GC-MS, dependent on the availability of either instrument due to their periodic requirement on research cruises for sea-going analysis. The instrument used is stated in the results for each experiment. The GC-ECD consisted of a sample loop (4.838ml) and cryotrap coupled to a Shimadzu GC-14A fitted with two megabore columns (DB-624, 70m x 0.53mm id, 3μm film thickness, with a 1.5m pre-column) and a pair of electron capture detectors. The carrier gas was helium (5.0 grade) through the injection unit, trap and chromatography column, and the make-up gas through the ECD was 5% methane/balance argon (ECD grade). The 100 ml headspace sample was injected into the loop to flush it through, then the loop volume was passed onto the trap held at -196^{0}C in liquid nitrogen. The trap was then heated to 150^{0}C using a Bunsen heater and the sample injected into the GC. The column oven temperature was 40^{0}C and the detector temperature was 300^{0}C. A chromatographic analysis time of 40 minutes was used initially to determine which compounds were present. This was reduced to 25 minutes during later experiments once it was established that no compounds of interest were being released from the cultures (or were able to be detected) later than methyl iodide. Data collection and peak integration was performed using Borwin

software, the valve control software was written in Shimadzu BASIC (Boswell and Smythe-Wright, 1995). Example chromatograms are shown in Appendix 4.

The GC-MS consisted of three sample loops of 2.918 ml and 0.746 ml (which were used together to inject 3.664 ml) and a larger loop of 49.99 ml. Two different cryotraps were used on separate occasions; one was filled with a combination of Carboxen 1000 and 1003 in a 19 gauge (1.1 mm id) tube and held at -50^0C using cryocooled propan-2-ol. The other was filled with glass beads and was held at -196^0C in liquid nitrogen. The loops and trap were coupled to a Hewlett Packard (HP) 6890 GC fitted with a CB Sil-5 column (30 m, 0.32 mm id, 3 μm film thickness) and a HP 5973 MS. The carrier gas was helium (5.0 grade) throughout. The MS oven temperature rose from 35^0C up to a final temperature of 200^0C through an analysis time of 14.25 minutes. Data collection and analysis was performed using HP Chemstation software. A larger range of methyl halides could be detected using the GC-MS than with the GC-ECD and it allowed much easier identification of compounds eluting at similar times, although it was not quite as sensitive as the GC-ECD (Table 8).

The lowest detectable peaks for methyl chloride, methyl bromide, methyl iodide, CFC 11 and DMS using the GC-ECD and GC-MS were calculated as amount injected in pmol and are shown in Table 8.

	Injected amount (pmol)				
Instrument	CH_3Cl	CH_3Br	CH_3I	CFC 11	DMS
GC-MS	0.05	0.01	0.01	0.004	0.04
GC-ECD	0.04	0.01	0.008	0.002	n.d

Table 8. Lowest detectable injection of compounds of interest into the GC-MS and GC-ECD. n.d = not detected, as DMS could not be quantified using the GC-ECD.

3.6.2 Calibration

The instrument to be used was calibrated prior to each initial experiment with known concentrations of a number of methyl halides (CH_3Cl, CH_3Br, CH_3I, CH_2Cl_2, CH_2BrCl, $CHBrCl_2$, $CHCl_3$, CCl_4, $CHBr_3$), trichlorofluoromethane (CFC 11) and dimethyl sulphide (DMS), from a Kin-Tek gas standards generator (model 491). Initially the potential release of all these compounds from batch cultures of

phytoplankton were being investigated, so all these calibrations were necessary. However it became clear that no compounds of interest were being released from the batch cultures after the elution of methyl iodide (using the GC-ECD) and DMS (using the GC-MS). Therefore subsequent experiments were calibrated for CH_3Cl, CH_3Br, CH_3I, CFC 11 and DMS. Permeation tubes of each compound were used (manufactured by Kin-Tek Laboratories, Inc.) which allowed the diffusion of vapours through a polymeric membrane in order to establish a very small, but stable and reproducible flow of component compound vapour. Whilst not in use, the tubes were stored in stainless steel canisters in the fridge to slow their loss by diffusion. The tubes were placed in the generator at least 12 hours before use, at 40^0C, in a glass tube holder within a gas flow of either nitrogen (primarily) or helium (for initial experiments and in Bergen). By varying the gas flow through the generator and by varying the dilution of the resulting gas, it was possible to inject many, different, known concentrations of each compound, into the GC-ECD or GC-MS, from which calibration curves showing moles injected against peak area were constructed. Calibration curves for methyl chloride, methyl bromide, methyl iodide, CFC 11 and DMS for both the GC-ECD and GC-MS are shown in Appendix 3.

During the analysis of headspace samples, Kin-Tek injections were run on a regular basis to check the sensitivity of the system. In addition, blank injections of the helium carrier gas were run regularly in order to determine any background noise in the chromatograms. Compressed air collected at Niwot Ridge, Colorado and analysed to NOAA calibration standards by the Climate Monitoring and Diagnostics Laboratory, Boulder, Colorado, was used as a second standard to check on the precision of the system.

3.6.3 Leak detection

CFC 11 concentrations in the headspace were measured in order to detect any leaks in the culture vessels; the concentration should decrease over time with sampling, as CFC 11 was shown not to be present in the clean replacement bottled air. Any detected increase in CFC 11 in the headspace would be indicative of an influx from the laboratory air, which contained high concentrations of CFC 11.

3.7 Data analysis

Using the sampling procedures and analytical technique described, the concentrations of methyl halides in the headspace were obtained. As discussed previously, methyl halide data were only obtained for methyl chloride, methyl bromide and methyl iodide. Concentrations of CFC 11 were also calculated and for some experiments also dimethyl sulphide (DMS). These values were converted to total methyl halide amounts in each vessel using the following empirical formulae to determine Henry's Law constants (Hc):

$Hc = 4000e^{-2750/T}$ (CH_3Cl)

$Hc = 3860e^{-2859/T}$ (CH_3Br)

$Hc = 3930e^{-2889/T}$ (CH_3I)

$\log Hc = -1736/T + 4.806$ (DMS)

where T is the temperature in the headspace in degrees Kelvin (Elliot and Rowland, 1993; Wong and Wang, 1997). Using Henry's Law assumes that the compounds are in equilibrium between the headspace and the medium. Corrections were made for temperature, headspace and culture volume (due to medium being extracted for sampling), and amount of headspace removed by sampling. Methyl chloride, methyl bromide and methyl iodide concentrations in the medium were corrected for losses due to hydrolysis using the temperature dependent reaction rate constants reported by Moelwyn-Hughes (1938). Methyl bromide and methyl iodide concentrations were also corrected for losses due to nucleophilic chloride substitution using the temperature dependent reaction rate constants reported by Elliot and Rowland (1993). The total chemical degradation (hydrolysis and chloride substitution) represented 0.03%, 3.7% and 1.5% d^{-1} for methyl chloride, methyl bromide and methyl iodide, respectively, at 15^0C and 0.004%, 0.6% and 0.2% d^{-1} at 4^0C.

Microbial methyl halide consumption may also have occurred, as reported for methyl bromide by King and Saltzman (1997). Loss of methyl bromide in seawater by bacterial consumption may be as much as 5-10% d^{-1}, independent of its concentration (King and Saltzman, 1997), but this was measured at higher temperatures (21-29^0C) than used in the present study. There are no published rates for the bacterial consumption of methyl halides at the temperatures used in this study, apart from those requiring the knowledge of bacterial biomass which apply to the degradation of

methyl bromide by methanotrophic bacteria (Stirling and Dalton, 1980; Oremland *et al.* 1994). Methyl halide production has not been corrected for any losses due to microbial degradation and therefore may be underestimated.

All methyl halide data has been expressed as the total amount in the entire culture vessel at the time of sampling in pmol.

4.0 RESULTS

4.1 Gas-tight integrity of culture vessels

CFC 11 concentrations in the headspace decreased throughout the duration of the experiments, shown in Figure 3 for two sets of *P. globosa* cultures. These indicated the culture vessels were gas-tight over the long term since CFC 11 is present in the lab air. During preliminary experiments there was some evidence for small influxes of lab air on occasional days, but this was solved by using parafilm as a sealant round every joint on the culture vessels for subsequent experiments.

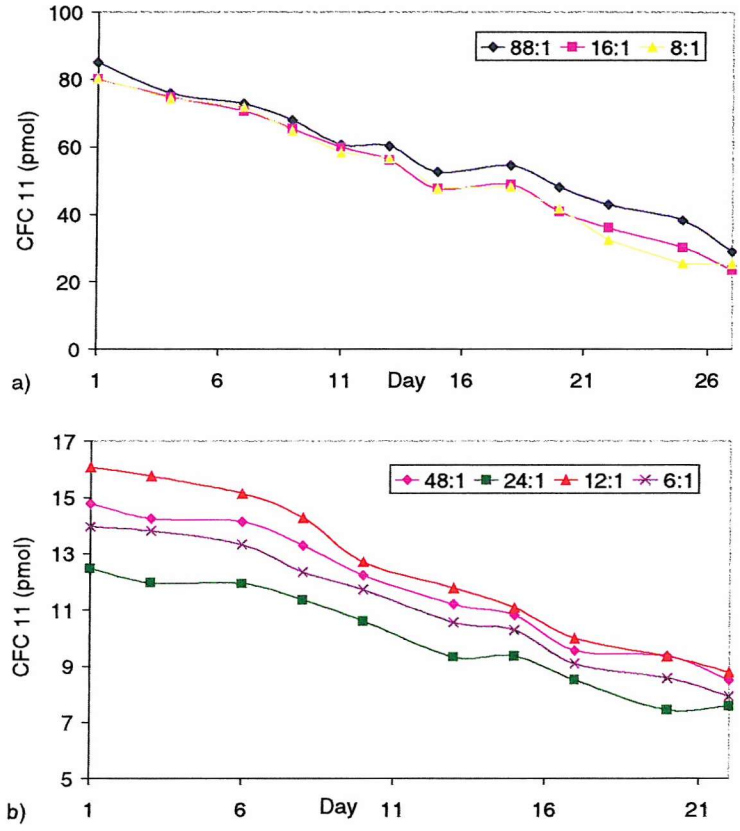


Figure 3. CFC 11 in the headspace of *P. globosa* cultures during nutrient manipulation experiments a) 1 and b) 2.

4.2 Initial experimental cultures to determine methyl halide production

4.2.1 *Isochrysis galbana*

This was the first culture to be grown and tested in this study for methyl halide release. The GC-ECD was used for methyl halide analysis. The culture grew well, but no methyl halide release was detected.

4.2.2 *Phaeocystis globosa*

Phaeocystis sp. is a colonial organism which lives in large (1-10 mm) spherical colonies bound in a mucopolysaccharide matrix (Rousseau *et al.* 1994). Such colonies were evident in these cultures, forming during the end of the exponential phase of growth. The PLY 64 strain used had not previously been known to form colonies (M. Jutson, PML, personal communication). A prolonged stationary phase was not detected, possibly due to nitrate limitation, and the cultures were followed through to senescence (Figure 4). A few days prior to senescence the *P. globosa* cells became enlarged, possibly due to nutrient stress, then disappeared due to cell lysis. There was no nutrient data for this experiment.

The GC-ECD was used for methyl halide analysis. The total methyl chloride and methyl bromide in the culture vessels increased rapidly from zero at the end of the exponential phase (day 7) (Figure 4). Methyl iodide production started a two days later and was detected at a much higher level in one of the cultures. Methyl halide release occurred for only a few days and then started to disappear from the headspace – this being most evident for methyl iodide.

Since the sampling and analytical system was deemed to be working and methyl halide release had been detected for *Phaeocystis* sp., a number of other species were tested for methyl halide production in culture.

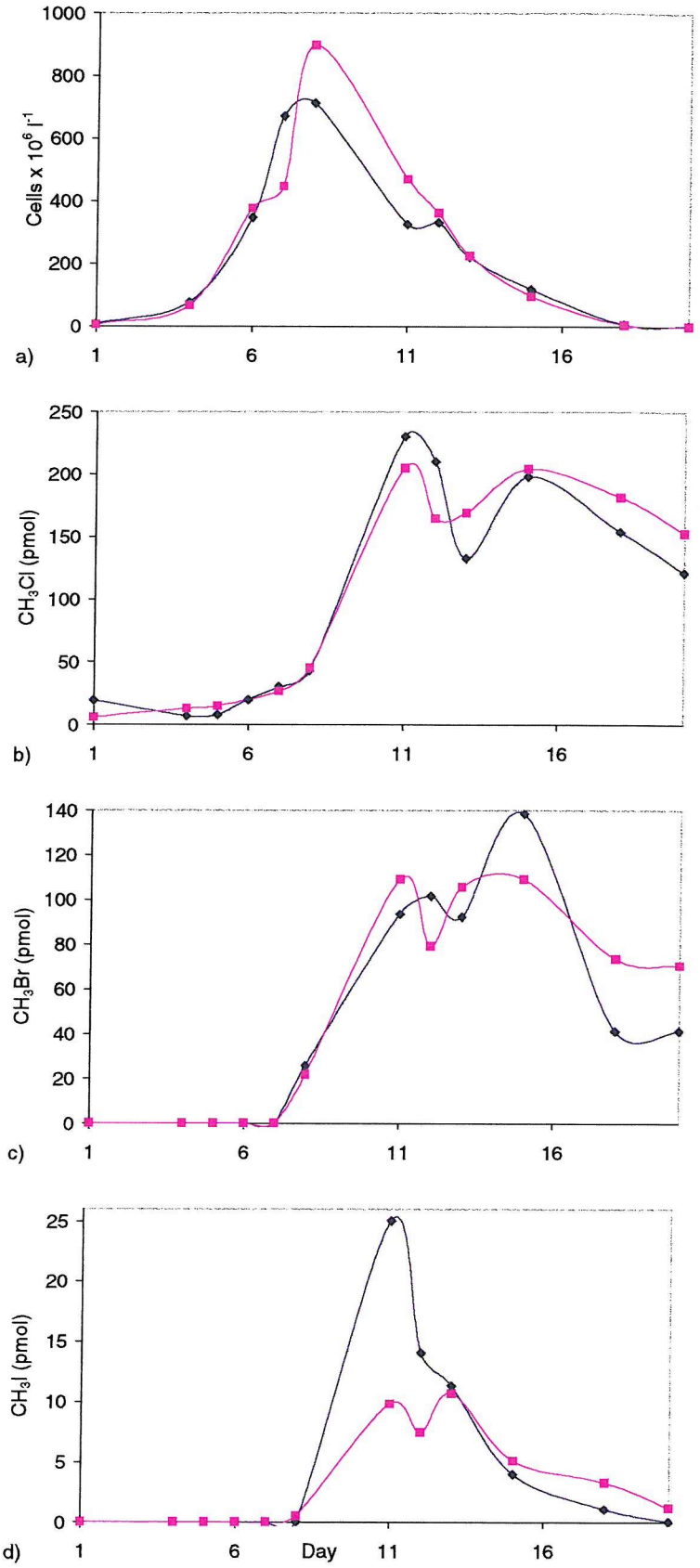


Figure 4. Two *P. globosa* cultures grown under identical conditions, showing a) cell growth b) total methyl chloride c) total methyl bromide and d) total methyl iodide.

4.2.3 *Chaetoceros* sp.

The *Chaetoceros* sp. cultures were shown to produce methyl chloride, methyl bromide and methyl iodide, detected using GC-ECD, during the exponential phase of growth (Figure 5). Methyl bromide is not shown as it could not be quantified due to interference from an unknown compound eluting at the same time in the GC-ECD blank. This unknown compound displayed an inconsistent peak and therefore could not be accounted for. A similar pattern of methyl chloride release occurred for both cultures although the release started earlier in one culture despite their similar growth curves. Methyl iodide was released erratically, and showed some evidence of uptake once having been released, both in the middle of the experiment and at the end. Analytical error was considered to explain the drop in methyl iodide in both cultures on day 11, but no decrease was detected for any other compound, including CFC 11, so the reason for this decrease remains unknown. Methyl chloride also decreased in the vessels towards the end. Methyl iodide release has not previously been reported for a *Chaetoceros* species in culture. Nutrient concentrations were not measured during this experiment.

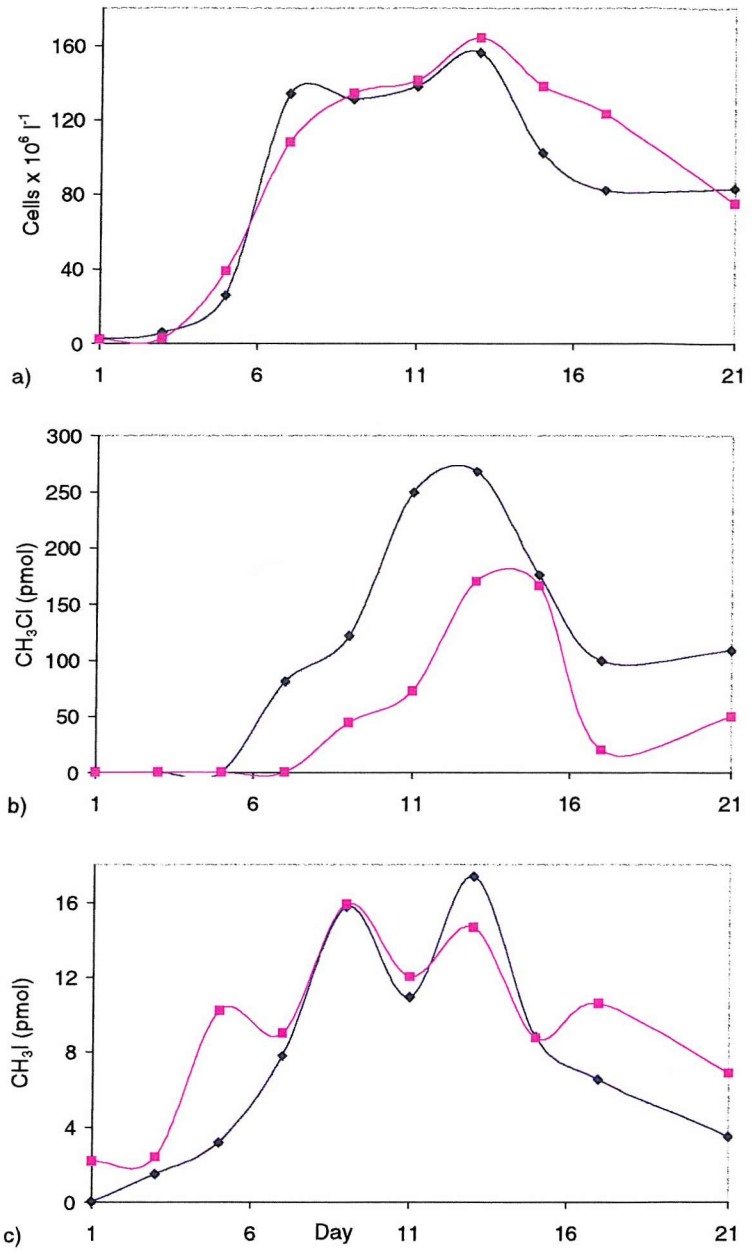


Figure 5. Two *Chaetoceros* sp. cultures grown under identical conditions, showing a) cell growth b) total methyl chloride and c) total methyl iodide.

4.2.4 *Emiliania huxleyi*

The GC-ECD was used for methyl halide analysis. Figure 6 shows the growth of the *E. huxleyi* cultures and their methyl halide release. Variation in the cell population during the stationary phase may have been due to cell clumping, increasing the error involved in counting, although it is possible that populations of cells were growing and dying in such a manner over this period. Methyl chloride was

released earlier than methyl iodide, as seen for the *P. globosa* cultures. Methyl bromide was also released but could not be quantified due to interference from an unknown compound present in the blank. Nutrient concentrations were not measured during this experiment.

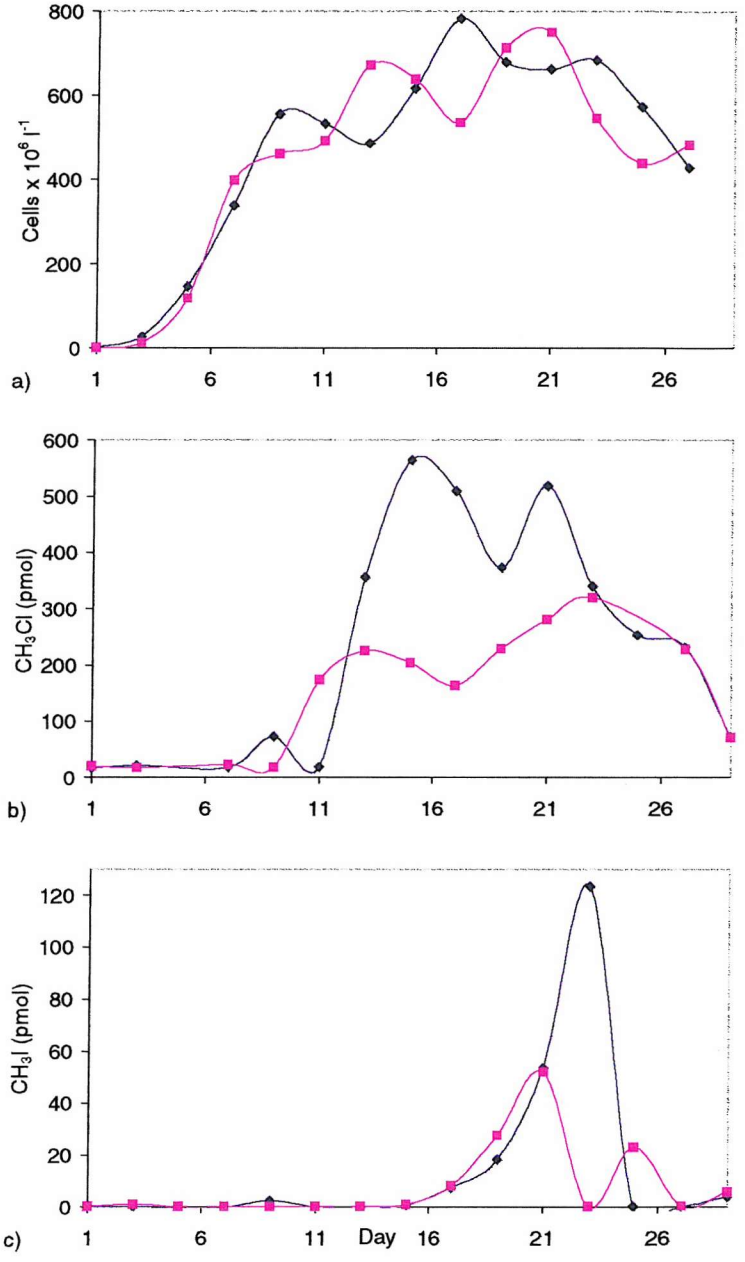


Figure 6. Two *E. huxleyi* cultures grown under identical conditions, showing a) cell growth b) total methyl chloride and c) total methyl iodide.

4.2.5 *Thalassiosira gravida*

Of all the cultures grown, this species showed the lowest growth rate. It is a cold water species and was kept at 4^0C (Table 3). The cultures were slow to reach the stationary phase and remained in this phase for 25 days, showing no signs of senescence when the experiment was terminated (Figure 7). After 17 days phosphate was undetectable although nitrate was present. Some apparent regeneration of phosphate was seen on day 52, which coincided with a peak of DMS in one of the cultures (data not shown).

The GC-MS was used for methyl halide analysis (hence DMS release could be detected). The *T. gravida* cultures released methyl bromide although levels were low and production erratic throughout the exponential and stationary phases. Methyl iodide and DMS were also produced (not shown), a total of 3.75 pmol of methyl iodide and 45 μmol of DMS was detected in one culture vessel, but methyl chloride was not detected. The amounts of each methyl halide released were about an order of magnitude lower than those seen for the warm water species tested. The percentage error involved in sampling and analysis was calculated from a duplicate taken on the last day of sampling, and was found to be 2.5% for methyl bromide and 3.1% for methyl iodide.

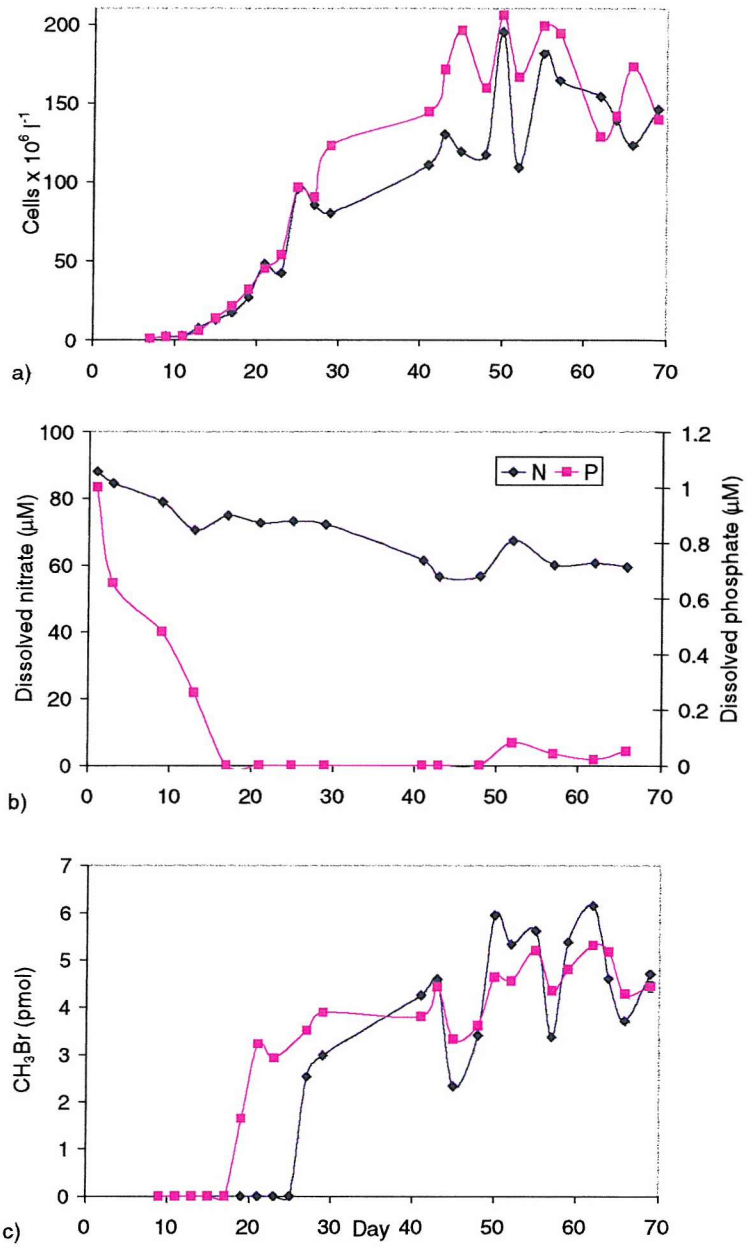


Figure 7. Two *T. gravida* cultures grown under identical conditions, showing a) cell growth b) dissolved nitrate and phosphate and c) total methyl bromide.

4.3 Nutrient manipulation of experimental cultures

4.3.1 *Phaeocystis globosa*

In these experiments microscopic examination revealed the *P. globosa* cells existed as motile cells throughout. The exponential growth rates increased with increasing nutrient levels for each individual experiment, however on comparing between experiments it can be seen that the culture at 48 μM nitrate in experiment 2 actually reached higher densities than the 88 μM nitrate culture in experiment 1 (Figures 8, 9 & 10). The growth patterns were also different between experiments, the exponential phase was shorter in experiment 1 and longer in experiments 2 and 3, and the latter cultures lasted longer before senescence. The chlorophyll *a* concentration was also followed in these culture experiments and this showed similar growth patterns to the cell growth curves. At lower cell population densities and in colony-forming cultures the measurement of chlorophyll *a* is probably a more accurate way of determining culture biomass. Dissolved nitrate and phosphate decreased in all the cultures as they grew, but the largest reduction in nitrate was seen in the culture with the highest starting value in each experiment. None of the cultures appeared to become entirely nitrate or phosphate depleted (phosphate data not shown).

GC-MS was used for methyl halide analysis in experiment 1 and GC-ECD in experiments 2 and 3. Methyl chloride, methyl bromide and methyl iodide were shown to increase as expected (Figures 8, 9 & 10). DMS was also detected in experiment 1 (using the GC-MS) with the pattern of DMS release similar to that of methyl halide release. In experiment 1 methyl iodide decreased in the control after day 4 but the levels remained high in the other cultures prior to showing a more subsequent increase (primarily in the culture with the highest nitrate concentration), suggesting methyl iodide production at this stage of growth (Figure 8). In experiment 2, methyl iodide release started earlier than in the other two experiments. It is evident that for each experiment the cultures produced higher quantities of methyl halides at higher initial nitrate concentrations. The percentage error involved in sampling and analysis for these cultures (calculated from the duplicate taken at the end of the culture) was 0.04% for methyl chloride, 6.3% for methyl bromide, and 18% for methyl iodide.

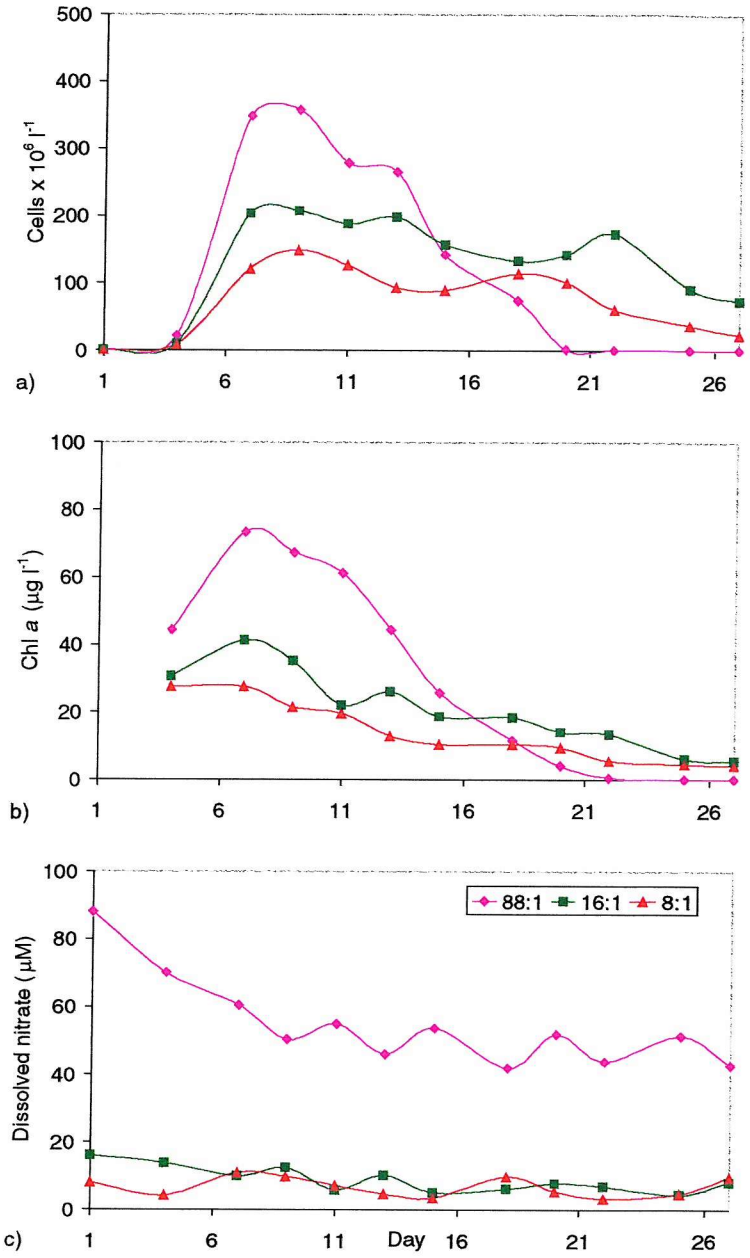


Figure 8. Nutrient manipulation experiment 1. *P. globosa* cultures under different nutrient conditions, showing a) cell growth b) chlorophyll *a* and c) dissolved nitrate.

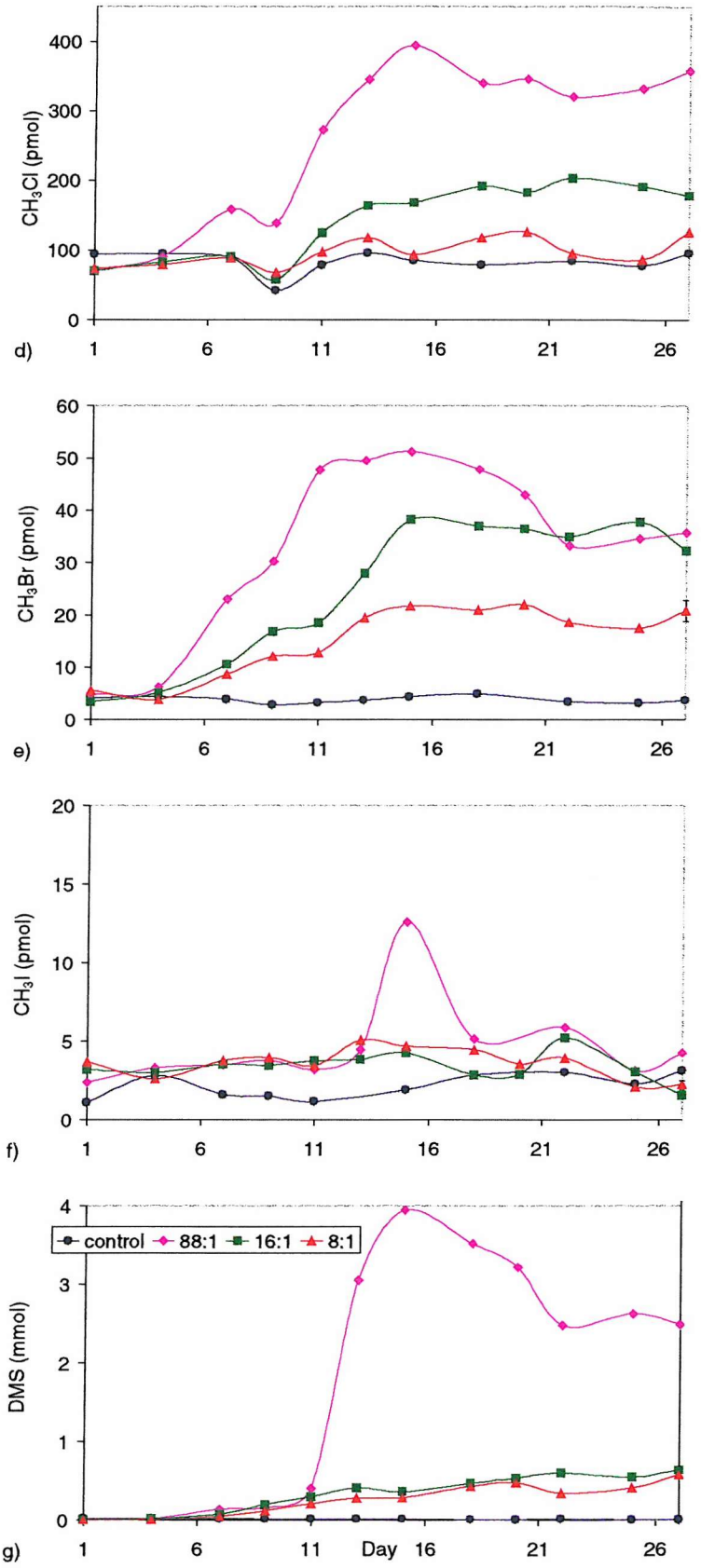


Figure 8. Nutrient manipulation experiment 1. *P. globosa* cultures under different nutrient conditions, showing d) total methyl chloride e) total methyl bromide f) total methyl iodide and g) total DMS.

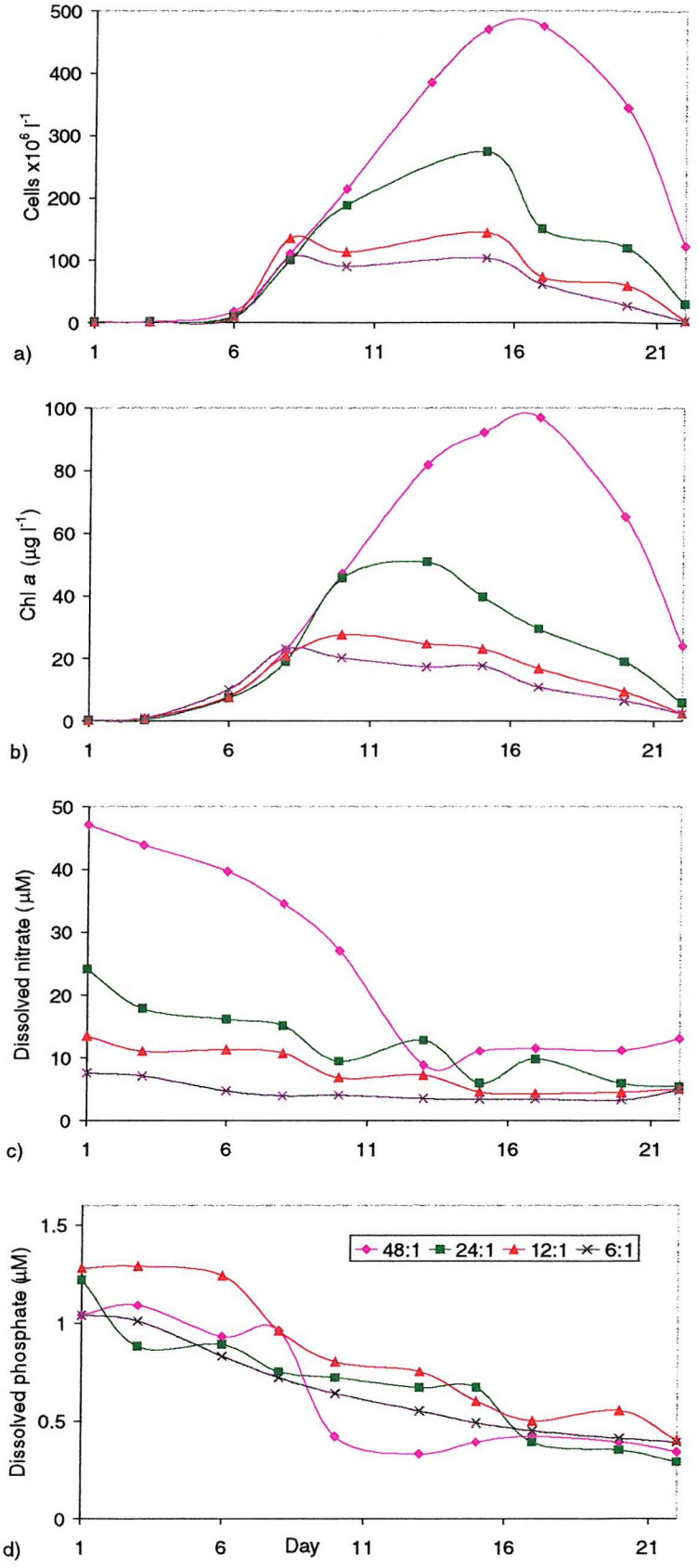


Figure 9. Nutrient manipulation experiment 2. *P. globosa* cultures under different nutrient conditions, showing a) cell growth b) chlorophyll *a* c) dissolved nitrate and d) dissolved phosphate.

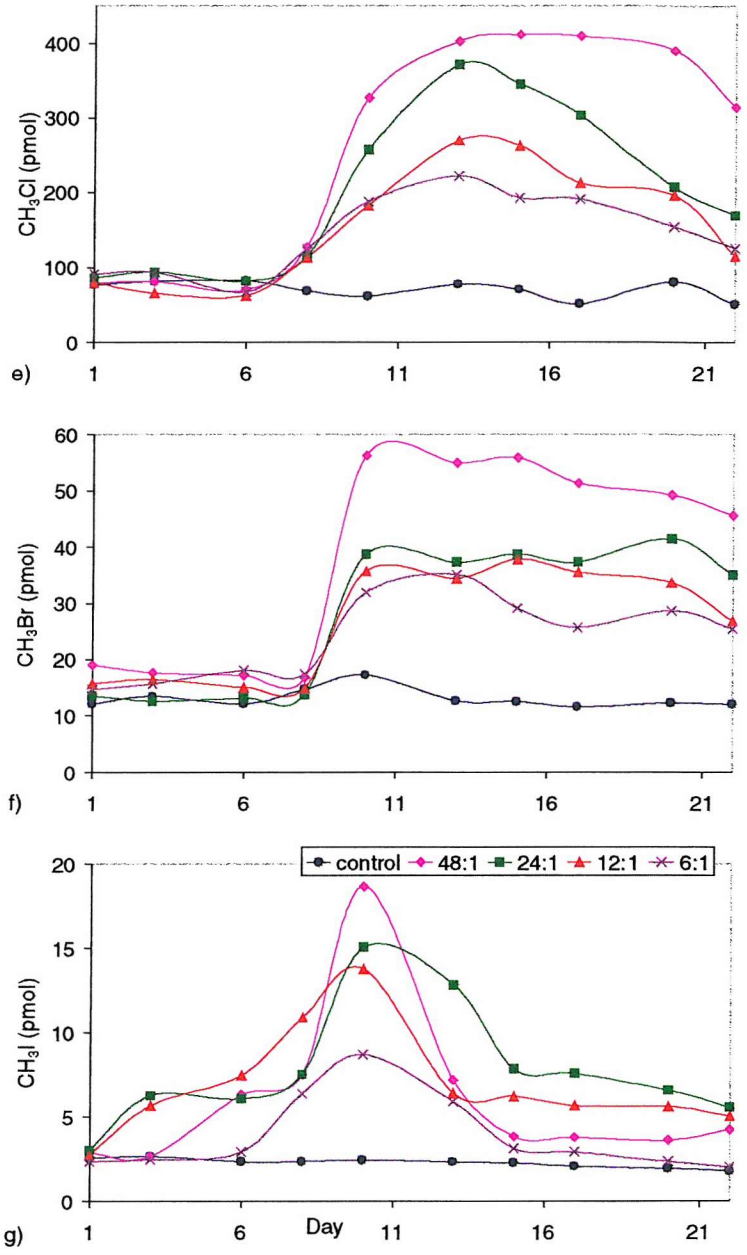


Figure 9. Nutrient manipulation experiment 2. *P. globosa* cultures under different nutrient conditions, showing e) total methyl chloride f) total methyl bromide and g) total methyl iodide.

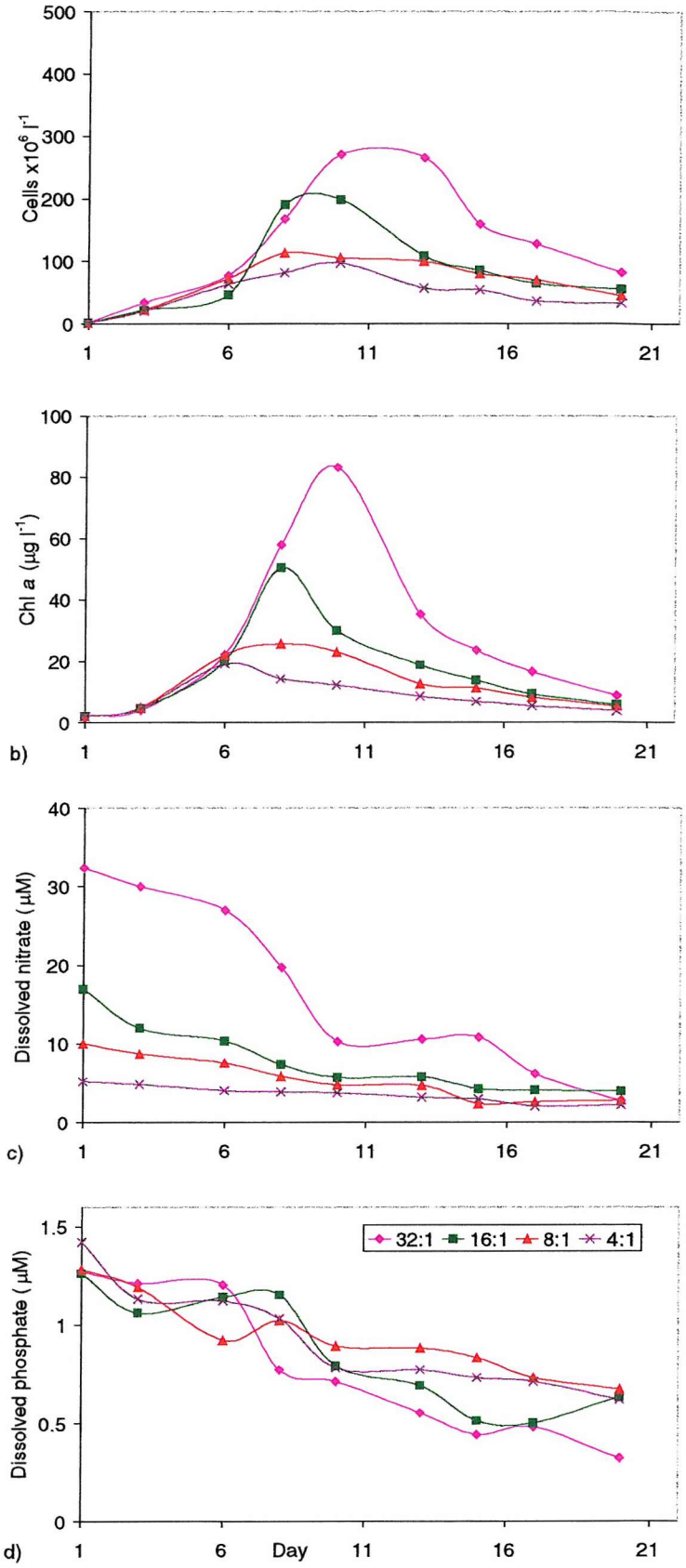


Figure 10. Nutrient manipulation experiment 3. *P. globosa* cultures under different nutrient conditions, showing a) cell growth b) chlorophyll *a* c) dissolved nitrate and d) dissolved phosphate.

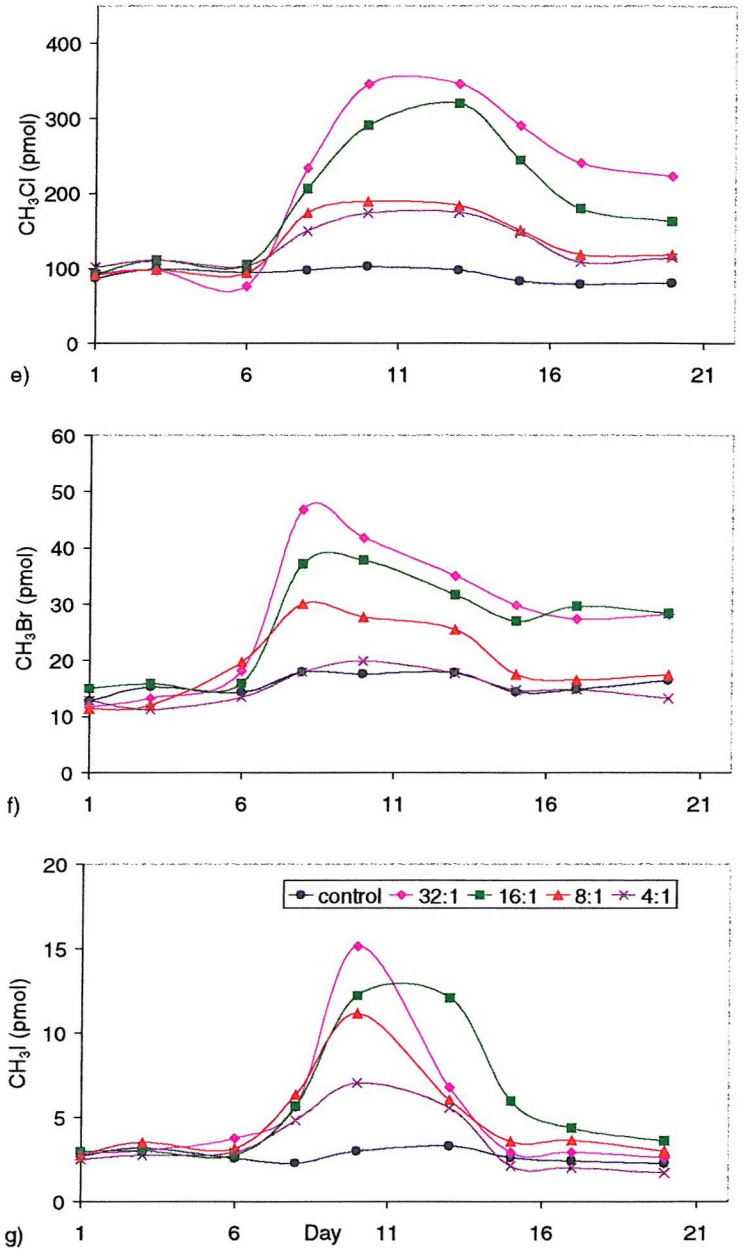


Figure 10. Nutrient manipulation experiment 3. *P. globosa* cultures under different nutrient conditions, showing e) total methyl chloride f) total methyl bromide and g) total methyl iodide.

The effect of the nutrient regimes on methyl halide production can only be truly compared, however, if the methyl halide data are normalised to phytoplankton biomass, so the methyl halide production per cell or per gram of chlorophyll can be compared over the varying nutrient regimes. Maximum production rates were calculated for each culture using the steepest part of the slope in the methyl halide release graphs, then adjusted to production over 24 hours and subsequently biomass normalised using the mean chlorophyll concentration or mean cell density during that period of time (Figures 11, 12 & 13). For each experiment it can be seen that in general for methyl chloride and methyl bromide the maximum biomass normalised methyl halide production rate increased with increased nitrate concentration, thus at higher nitrate concentrations, the amount of methyl halide produced per cell or per gram of chlorophyll was higher. The data for methyl iodide was not as reproducible.

The biomass normalised production rates for DMS for experiment 1 (not shown) were much increased at the highest nitrate concentration (24.2 mmol g Chl a^{-1} d^{-1}) compared to the lower concentrations (1.77 mmol g Chl a^{-1} d^{-1} at 16 μM nitrate and 2.33 mmol g Chl a^{-1} d^{-1} at 8 μM nitrate).

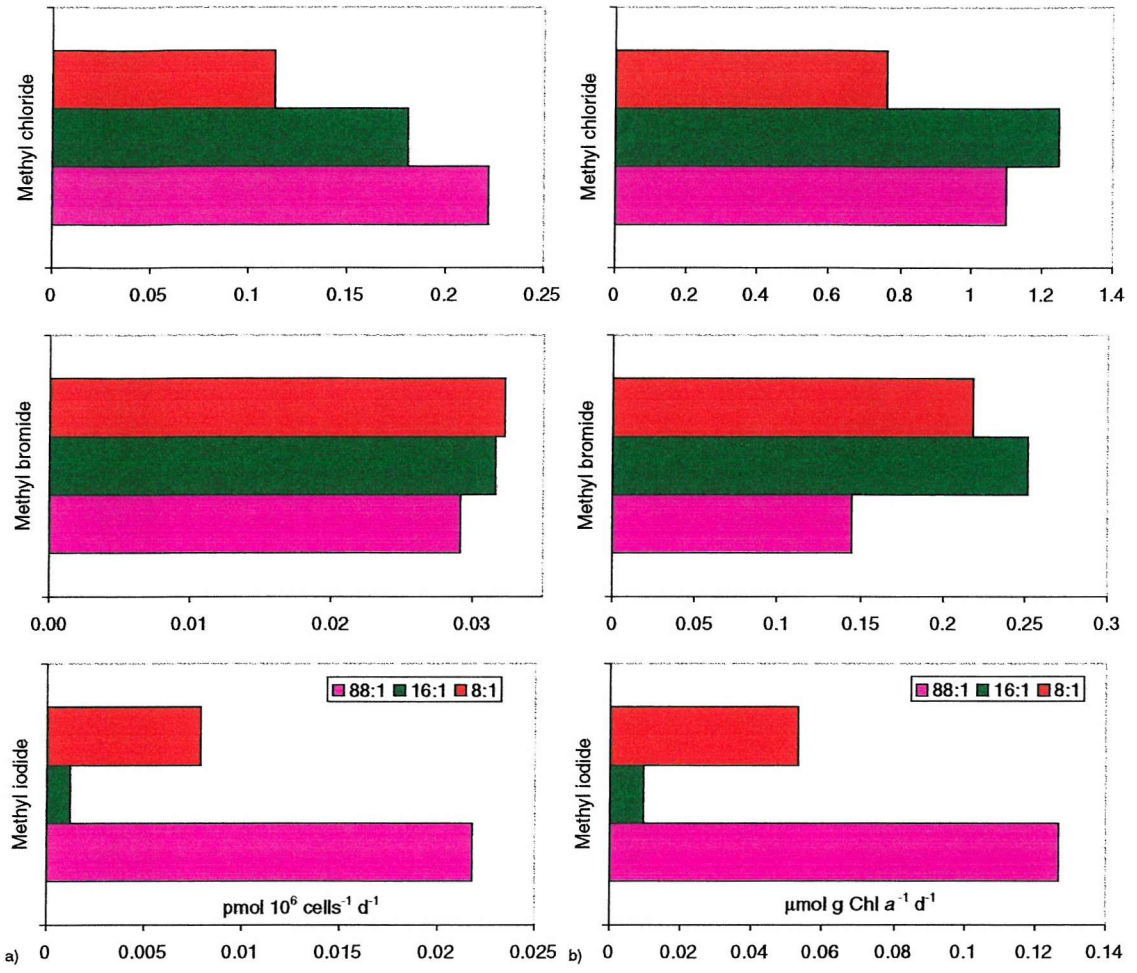


Figure 11. Maximum biomass normalised methyl halide production rates for nutrient manipulation experiment 1 a) normalised to cell density and b) normalised to chlorophyll *a*.

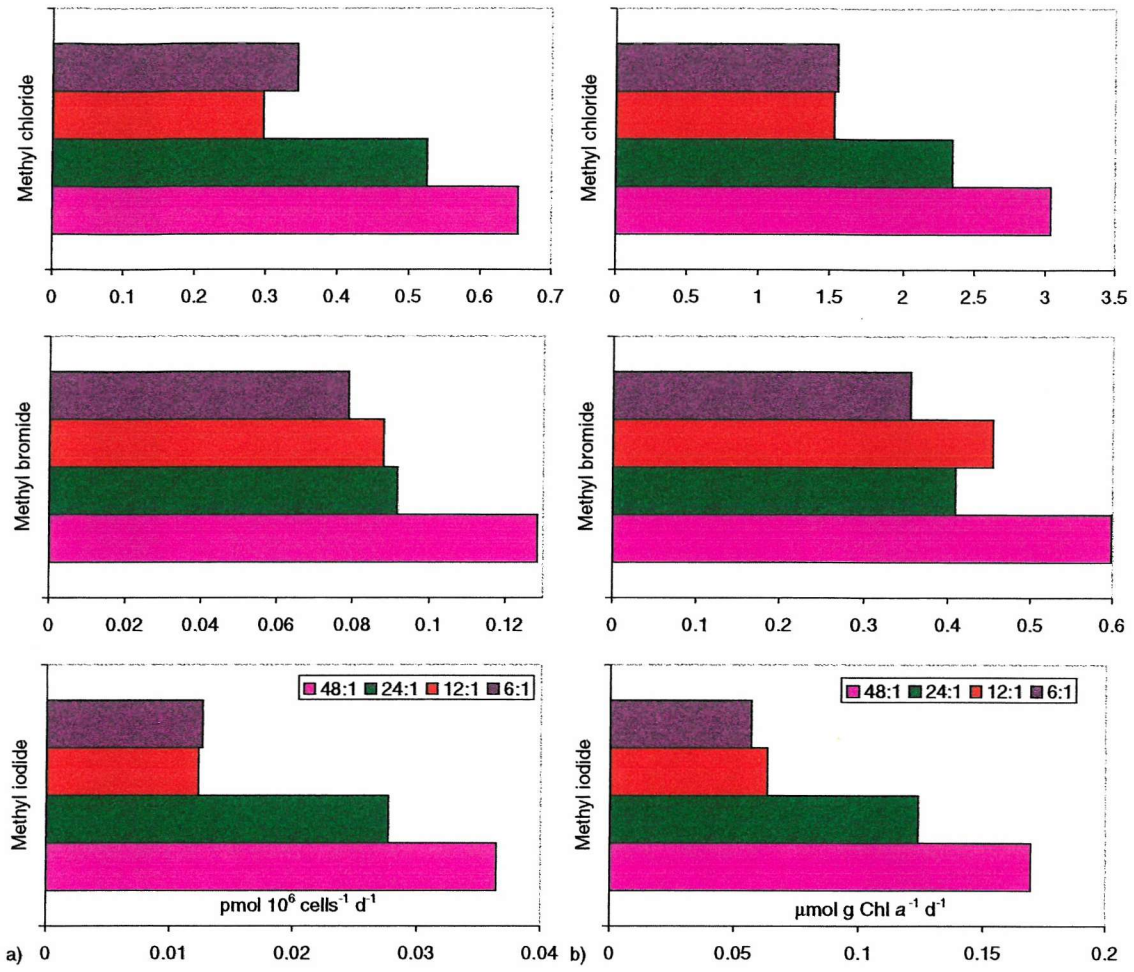


Figure 12. Maximum biomass normalised methyl halide production rates for nutrient manipulation experiment 2 a) normalised to cell density and b) normalised to chlorophyll *a*.

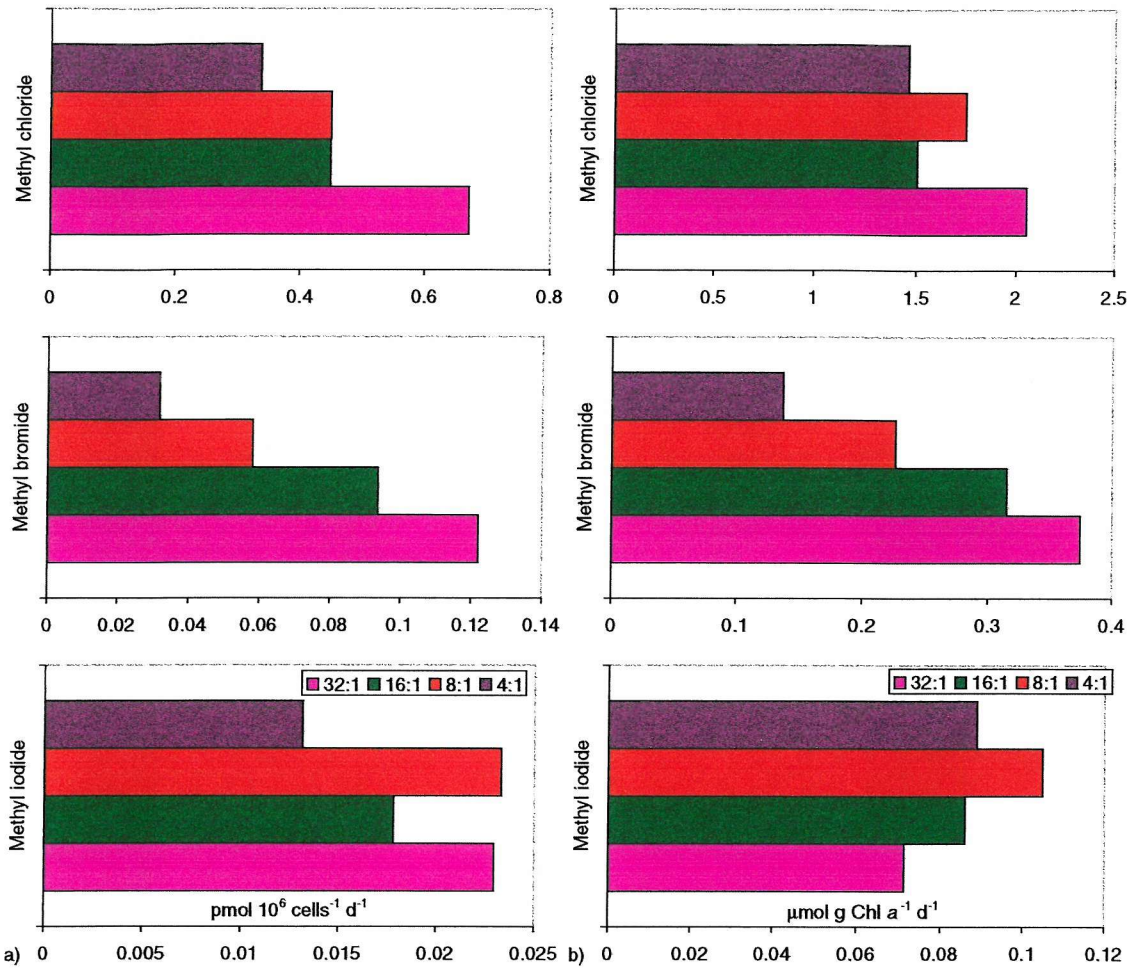


Figure 13. Maximum biomass normalised methyl halide production rates for nutrient manipulation experiment 3 a) normalised to cell density and b) normalised to chlorophyll *a*.

4.3.2 Natural populations from a Norwegian fjord

In experiment 1 the cultures did not grow and the chlorophyll decreased from day 1 (Figure 14). This may have been because the original nutrients had been depleted or possibly due to zooplankton grazing as a disproportionate number of zooplankton may have been collected into the culture vessels. When the vessels were filled with fjord water from 6 m and spiked with nutrients individually (experiment 2) phytoplankton growth was improved (Figure 15). The chlorophyll concentrations in both experiments were highest for those cultures with high phosphate and those with added silicate or silicate and iron and lowest for the cultures with low phosphate or no added nutrients. The nitrate concentrations decreased in accordance with their growth. However the chlorophyll concentrations in the mesocosms reached considerably higher levels (not shown) than those seen in the culture vessels, even though the vessels were kept under similar conditions of temperature. The dominant species in all the vessels were *Rhizosolenia fragillissima* and *Emiliania huxleyi*.

GC-MS was used for methyl halide analysis. Methyl chloride, methyl bromide and methyl iodide were released from all the vessels, but the former two were close to their detection limits thus only methyl iodide is shown (Figures 14 & 15). As methyl iodide was produced in both experiments, this suggests that it is produced both during the exponential phase of growth (experiment 2) and senescent phase of growth (experiment 1). Even the vessel with no added nutrients produced small amounts of methyl halides, but it is clear that methyl iodide release was higher in those vessels with higher nitrate concentrations. The total methyl iodide amounts detected in the vessels using natural populations from the fjord were high compared to pure culture experiments as the vessels used were 5L compared to the smaller 2L. Also, due to delivery problems resulting from strike action in Norway, the clean air cylinder was not available when the experiments were being set up in Norway, thus the vessels were not sparged with clean air prior to inoculation, leading to a high background level of methyl iodide (and the other methyl halides not shown) being present in the vessels at the start of the experiment.

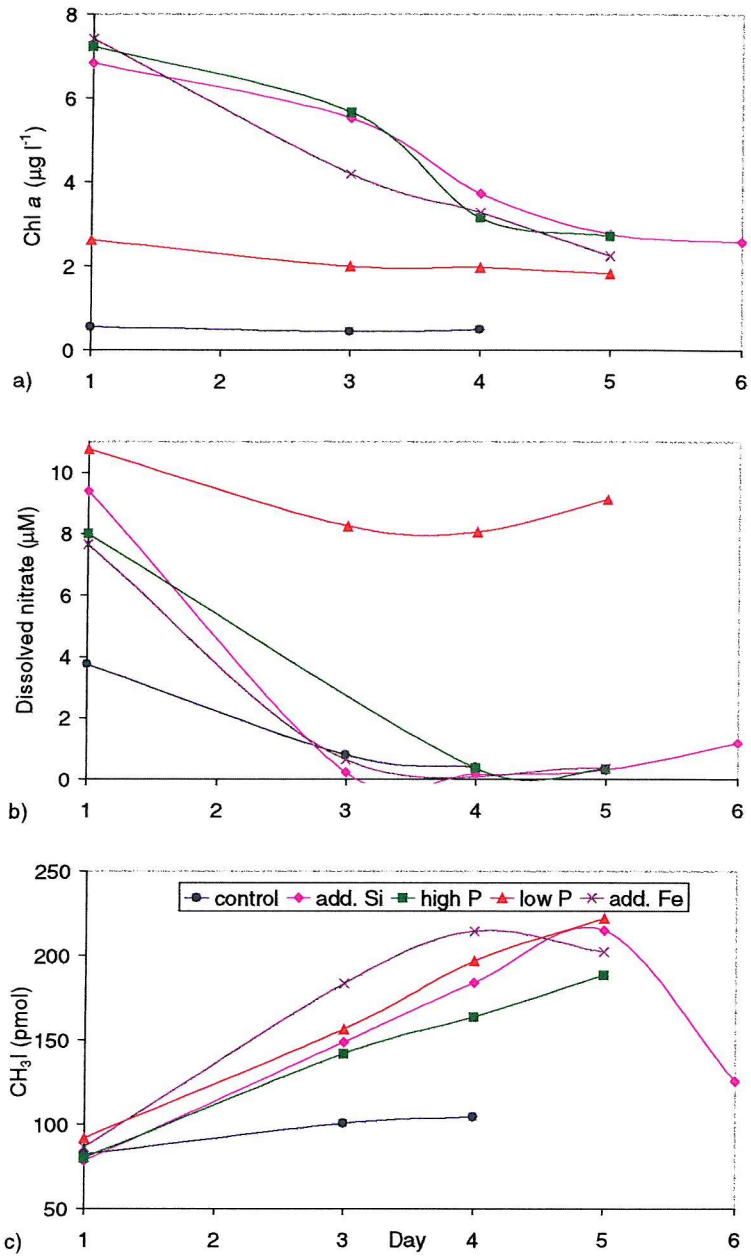


Figure 14. Cultures of natural phytoplankton populations collected from 1 m in mesocosms in a Norwegian fjord, experiment 1, showing a) chlorophyll *a* b) dissolved nitrate and c) total methyl iodide. See Table 5 for treatment details.

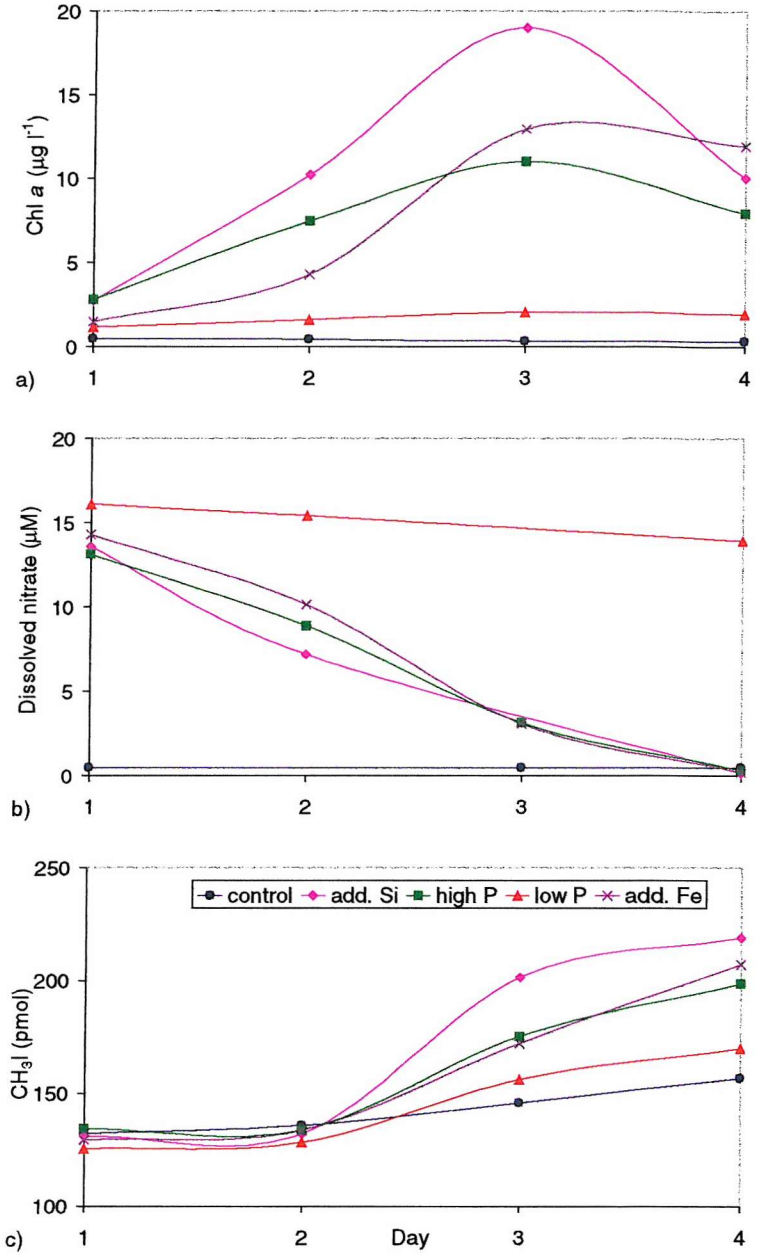


Figure 15. Cultures of natural phytoplankton populations collected from 6 m in a Norwegian fjord, experiment 2, showing a) chlorophyll *a* b) dissolved nitrate and c) total methyl iodide. See Table 5 for treatment details.

The maximum biomass normalised production rates were calculated as for the nutrient manipulation experiments and shown for the cultures with added nutrients (Table 9). The rates were similar for the three cultures which grew well (add. Si, add. Fe and high P). The highest rates were for the low phosphate cultures, which still contained high levels of nitrate but did not grow as well due to phosphate limitation; these cultures produced similar amounts of methyl iodide to the cultures with higher

phosphate in experiment 1, but not as much in experiment 2. The results show, however, that release of methyl halides by natural mixed phytoplankton populations are influenced by the nutrient concentrations available.

Experiment (growth phase of cultures)	Methyl iodide maximum production rates (μmol g Chl a^{-1} d^{-1})			
	add. Si	high P	low P	add. Fe
1 (senescent)	1.9	1.6	4.7	2.8
2 (exponential)	1.6	1.5	5.1	1.5

Table 9. Maximum chlorophyll *a* normalised production rates for methyl iodide in the natural population experiments.

4.4 Grazing in experimental cultures

4.4.2 *Emiliania huxleyi*

Figure 16 shows the growth of an *E. huxleyi* culture followed by the decrease in cells and chlorophyll *a* when the protozoan grazer *O. marina* was added to one of the cultures. Replicates for each treatment were placed in a separate incubator due to lack of space on this occasion, and they did not grow well, thus the data could only be compared for one type of each treatment. GC-ECD was used for methyl halide analysis. Methyl chloride was released by both cultures, and despite the considerably lower cell density seen in the grazed culture, it released much more methyl chloride than the ungrazed culture, most of the release being on day 15 when the cells were being heavily grazed.

Significant release of neither methyl bromide nor methyl iodide was detected, in contrast to the earlier *E. huxleyi* cultures (Figure 6). However, methyl iodide was not released until day 16 in the previous *E. huxleyi* cultures (Figure 6) so may have been produced in this experiment if it had continued longer.

This experiment therefore gave initial indications that grazing may have an effect on the rate of methyl halide release. For further grazing experiments however it was decided to use *P. globosa* which was a more reliable producer of methyl halides, thus any effect from grazing would be more easily seen.

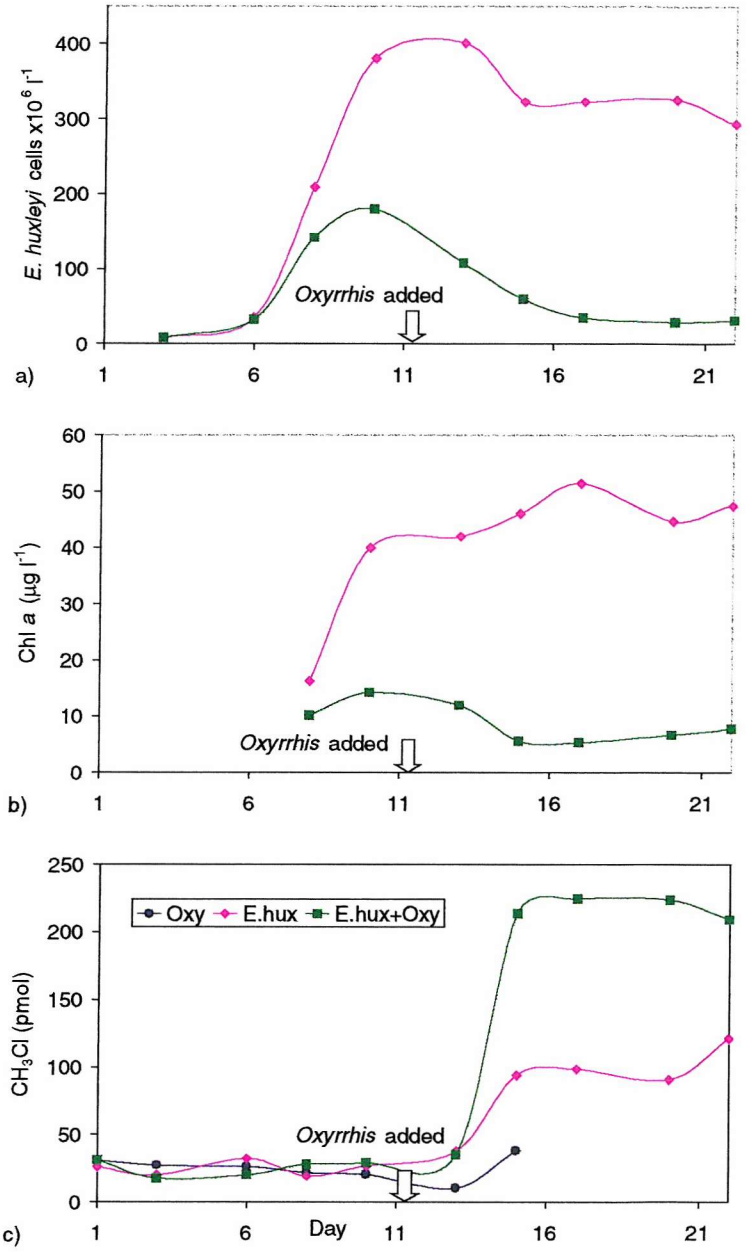


Figure 16. Grazing experiment *E. huxleyi* cultures showing a) cell growth b) chlorophyll *a* and c) total methyl iodide.

4.4.3 *Phaeocystis globosa*

The three cultures containing *P. globosa* exhibited very similar growth until the addition of *O. marina* to two of them on day 11. The *P. globosa* cells were grazed completely within 7 days, and the *O. marina* cells grew well with *P. globosa* as their prey (Figure 17). Dissolved nitrate and phosphate decreased in the cultures showing growth of *P. globosa* to day 11, but showed little change in either the control culture or in the culture containing *O. marina* alone. The grazed cultures showed a small increase in both dissolved nitrate and phosphate after the majority of the *P. globosa* cells had been grazed, compared to in the ungrazed culture where the dissolved nutrients continued to decrease as the culture maintained growth.

GC-MS was used for methyl halide analysis. Methyl chloride, methyl bromide and methyl iodide were produced in the cultures containing *P. globosa* but not in the control or the *O. marina* culture. After the addition of the grazer, the grazed cultures continued to release methyl halides for the first 2 days at similar (methyl chloride and bromide) or even higher (methyl iodide) rates than the ungrazed culture, despite their decrease in cell numbers through grazing (Figure 17). The cell numbers fell sharply in the grazed cultures and the levels of methyl halides stabilised or decreased, whereas release continued in the ungrazed culture as it reached the stationary phase of growth.

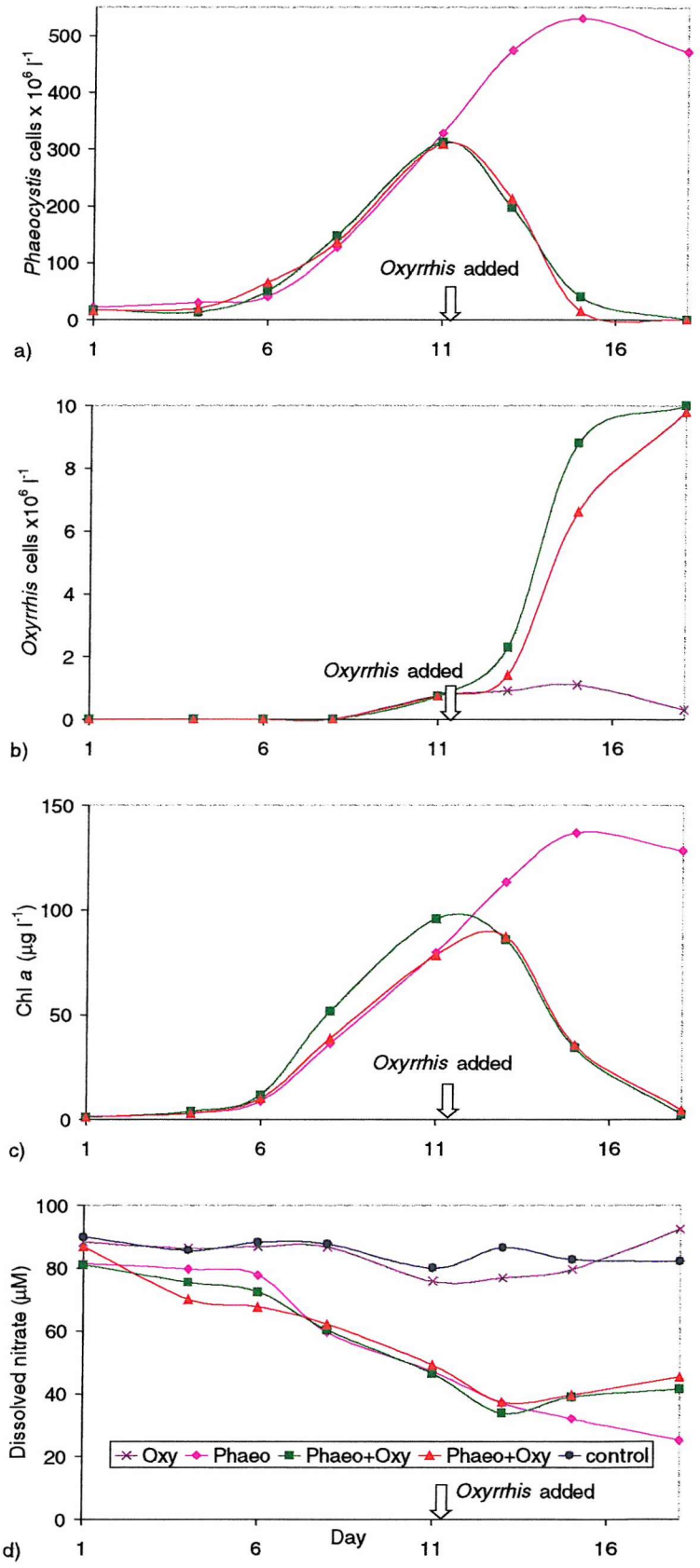


Figure 17. Grazing experiment *P. globosa* cultures, showing a) *P. globosa* cell growth b) *O. marina* cell growth c) chlorophyll *a* and d) dissolved nitrate.

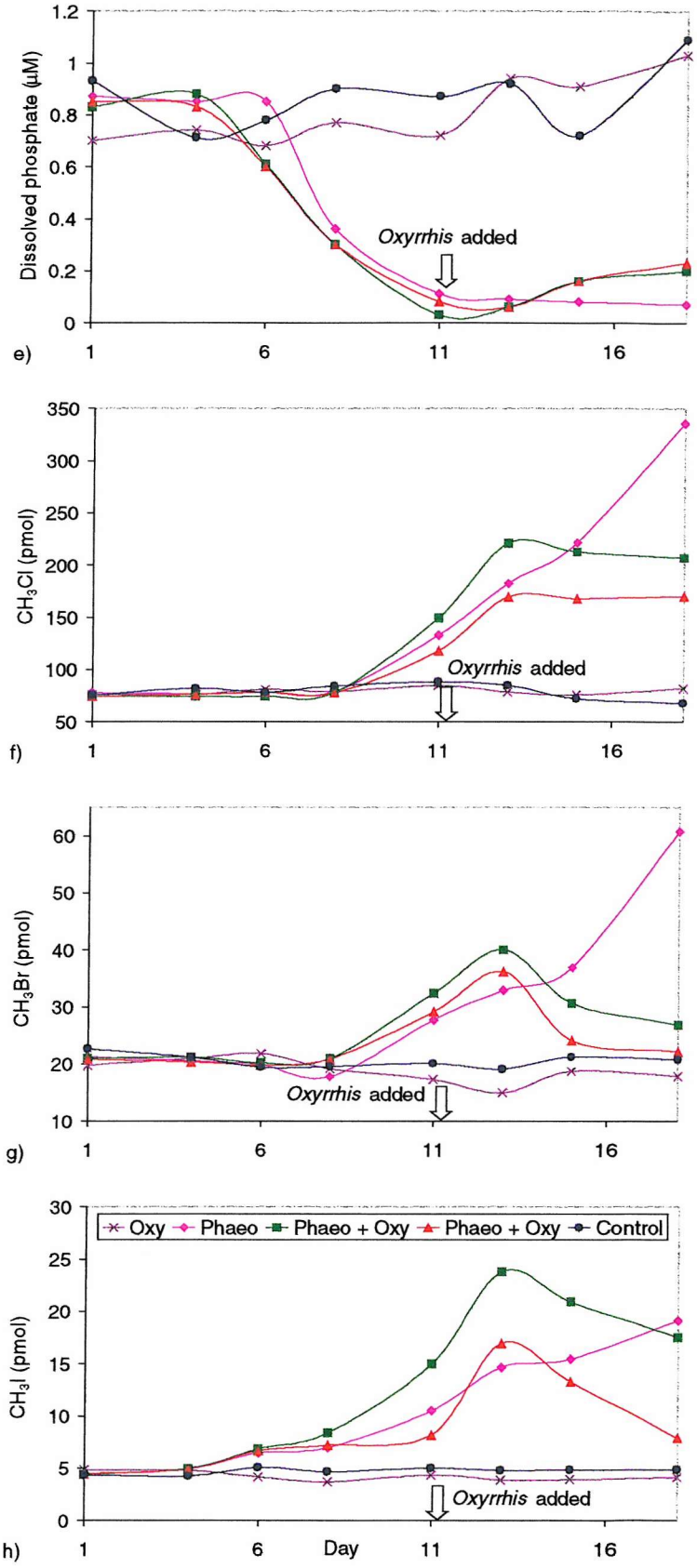


Figure 17. Grazed and ungrazed *P. globosa* cultures, showing e) dissolved phosphate f) total methyl chloride g) total methyl bromide and h) total methyl iodide.

To determine the actual difference in methyl halide production between the grazed and ungrazed cultures, biomass-normalised production rates were calculated over the 2 days after the addition of the grazers. The rates were calculated as previously for the nutrient manipulation experiments and were normalised to mean cell numbers and to mean chlorophyll concentration over that period (Figure 18). These data clearly show that for each methyl halide the production rates after the addition of the grazers were higher in the grazed cultures than in the ungrazed culture.

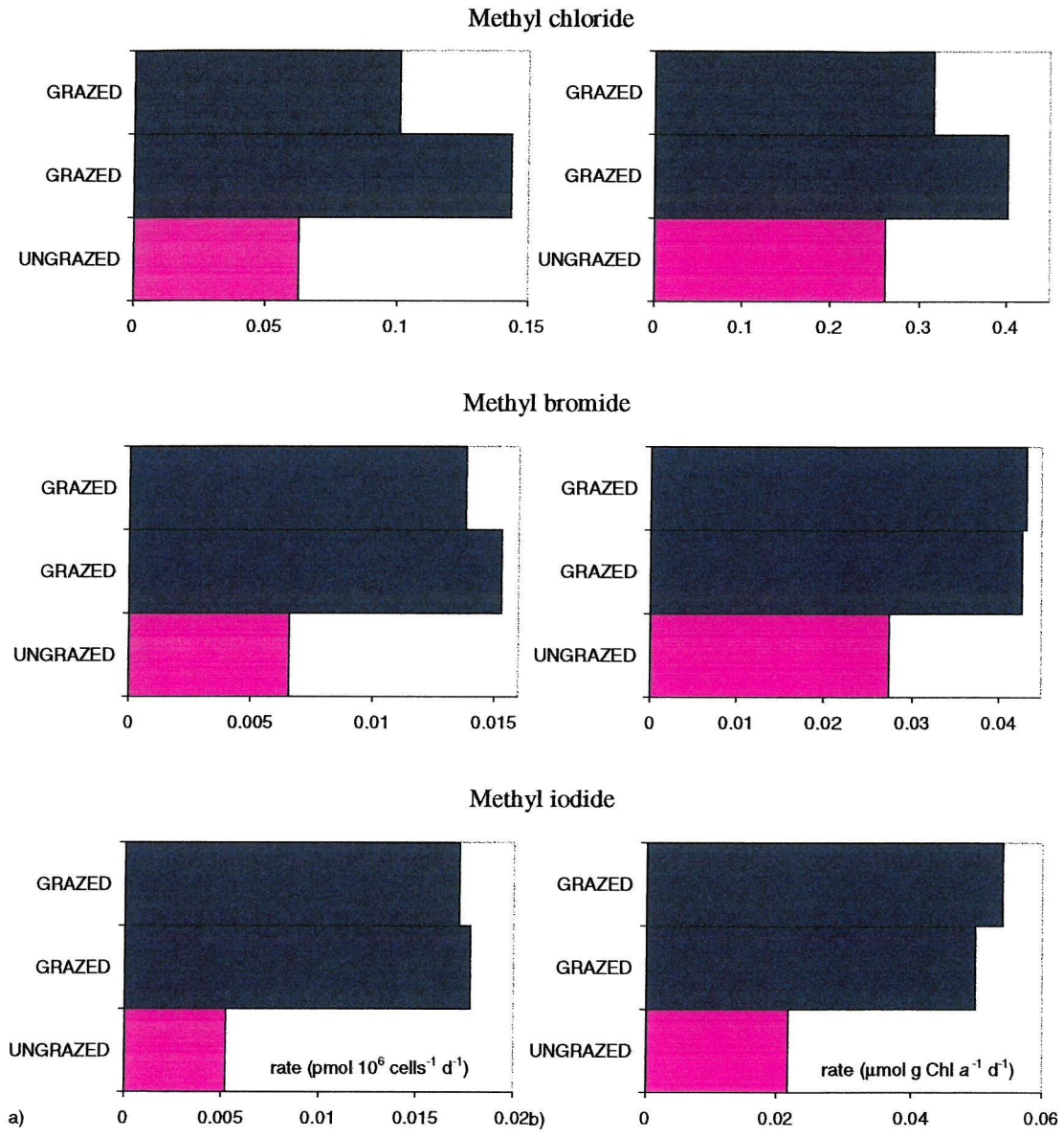


Figure 18. Biomass normalised methyl halide production rates after grazers were added, showing a) normalised to cell density and b) normalised to chlorophyll *a*.

5.0 DISCUSSION

5.1 Methyl halide release

It is now widely established that many species of phytoplankton produce methyl halides, especially methyl chloride and methyl bromide (Tokarczyk and Moore, 1994; Tait and Moore, 1995; Moore *et al.* 1996b; Scarratt and Moore, 1996 and 1998; Manley and de la Cuesta, 1997; Saemundsdóttir and Matrai, 1998). This study has shown this to be the case for 4 out of the 5 species tested.

T. gravida has not previously been tested for methyl halide production, and was shown in this study to produce methyl bromide and methyl iodide, but not methyl chloride. *T. weissflogii* is known to produce all three compounds (Tait and Moore, 1995; Scarratt and Moore, 1996) but *T. pseudonana* does not produce methyl bromide (methyl chloride and iodide were not tested) (Saemundsdóttir and Matrai, 1998), so it is clear that methyl halide production is not necessarily ubiquitous throughout a genus. This species variation within a genus has previously been observed for *Nitzschia* sp. and between different strains of *Navicula* sp. (see Table 1).

It is also possible that one strain of a species may not produce the same methyl halides when tested more than once, as for *E. huxleyi* (PLY 92E) in this study which produced methyl chloride, methyl bromide and methyl iodide in an initial experiment (section 4.2.4) but only methyl chloride in a further experiment (section 4.4.2). The cultures were illuminated with 80 ± 20 μE m^{-2} s^{-1} at 15^0C and 30 ± 10 μE m^{-2} s^{-1} at 16^0C, respectively, and the first cultures grew to almost double the cell densities seen in the second experiment. Moore *et al.* (1996b) found that higher levels of illumination increased amounts of polyhalogenated methanes released per cell by *Nitzschia* sp. and *Porosira glacialis* cultures; this was probably due to increased hydrogen peroxide availability – a product of photosynthesis and used by bromoperoxidases to form polybrominated compounds, however monohalogenated compounds are not formed by haloperoxidases (see section 5.7). Similarly the same strain of *E. huxleyi*, CCMP 373, has been shown to produce methyl bromide in one study (Scarratt and Moore, 1998) but not in another (Saemundsdóttir and Matrai, 1998). The cultures were however grown at 25^0C in 30 μM nitrate and 22^0C in 550 μM nitrate, respectively. Clearly the experimental conditions are very important when

comparing methyl halide release data and must play an integral part in the methyl halide production process.

This study has shown for the first time that *Chaetoceros* sp. is capable of producing methyl iodide, and also a considerably larger amount of methyl chloride than seen previously for this genus. Scarratt and Moore (1998) measured 9 pmol methyl chloride in a *C. calcitrans* culture at 18^0C and 45 μM nitrate in a 1L vessel, compared to this study in which 150-250 pmol was detected in a *Chaetoceros* sp. culture at 15^0C and 88 μM nitrate in a 2L vessel.

Methyl chloride and methyl bromide production rates, normalised to chlorophyll *a*, were compared to those reported by other authors for *Phaeocystis* sp. (Table 10). The production rates for *Phaeocystis* sp. covered a wider range than those reported by Scarratt and Moore (1996 and 1998) probably due to the different nitrate concentrations used, but were still comparable, and the rates for methyl bromide were similar to that found by Saemundsdóttir and Matrai (1998).

Reference	Treatment	Maximum production rate (μmol g Chl a^{-1} d^{-1})	
		Methyl chloride	Methyl bromide
Scarratt and Moore (1996)	18^0C, > 50 μM nitrate	1.0	0.14
Scarratt and Moore (1998)	15^0C, 25 μM nitrate	0.70-0.95	0.105-0.115
Saemundsdóttir and Matrai (1998)	22^0C, 850 μM nitrate	-	0.48
This study	15^0C, 4 μM – 88 μM nitrate	0.3-3.0	0.06-0.60

Table 10. Comparison of maximum methyl halide production rates for *Phaeocystis* sp. in culture. Table 1 shows the strain of *Phaeocystis* sp. used by these authors.

The ratio of methyl chloride:methyl bromide production rates ranged from 3.3-10.5 (mean 5.7) in this study for all the *P. globosa* cultures compared to Scarratt and Moore's (1998) mean ratio of 7.4 for culture experiments and an estimate of

between 8.5-9.6 derived from production rates reported for Pacific Ocean waters (Lobert *et al.* 1995; Moore *et al.* 1996a).

All the cultures used in this study were non axenic i.e. contained bacteria. Therefore it cannot be fully ascertained whether the phytoplankton were entirely responsible for the release of the methyl halides, or whether bacteria played some part in their production. However, many axenic cultures have been shown to release methyl halides (Abrahamsson *et al.* 1995b; Scarratt and Moore, 1996 and 1998; Saemundsdóttir and Matrai, 1998) and methyl halide release in this study was clearly related to phytoplankton cell growth, and not with the bacterial increase which usually occurs towards the end of a culture.

5.2 Global significance

To determine the global significance of methyl halide production by phytoplankton, chlorophyll normalised maximum production rates for all the cultures grown of the 'high producer' *P. globosa* and the 'low producer' *T. gravida* were scaled to give a global estimate of phytoplankton methyl halide production which was then calculated as a percentage of estimated total oceanic production (Table 10). The calculations were based on an average oceanic phytoplankton standing stock of 30 mg m^{-2} (Morel and Berthon, 1989), an ocean area of 3.6 x 10^{14} m^2, and the calculation for each species assumed that it alone comprised the entire chlorophyll *a* standing stock. The percentages of total oceanic production were based on 3-9 Gmol methyl chloride y^{-1} estimated by Moore *et al.* (1996a), 1.6 Gmol methyl bromide y^{-1} estimated by Lobert *et al.* (1995) and 1 Gmol methyl iodide yr^{-1} estimated by Nightingale (1991). The results show that phytoplankton may be responsible for the entire oceanic production of methyl chloride and methyl bromide and 74% of methyl iodide, or as little as none of the methyl chloride production (*T. gravida* did not produce methyl chloride), 5% of methyl bromide and 1.5% of methyl chloride. Thus for methyl chloride, phytoplankton production and chloride substitution together could easily account for the total oceanic source strength.

The global production values previously published are similar to the lower values within the ranges calculated here (Scarratt and Moore, 1996 and 1998; Saemundsdóttir and Matrai, 1998; Manley and de la Cuesta, 1997 – see section 1.0),

thus some of the production rates for *P. globosa* are higher than have been calculated before.

	Global production (Gmol y^{-1})			% of total oceanic production		
Species	Methyl chloride	Methyl bromide	Methyl iodide	Methyl chloride	Methyl bromide	Methyl iodide
T. gravida	0	0.075-0.138	0.11-0.28	0	4.7-8.6	11-28
P. globosa	3-11.8	0.552-2.37	0.039-0.67	33-394	34-147	3.9-67

Table 11. Extrapolations of phytoplankton methyl halide production to a global scale.

Data calculated in such a way, using only one species and extrapolated from culture experiments, are obviously not ideal; phytoplankton assemblages will contain some species which do not produce methyl halides and those that do will only release methyl halides during certain growth phases and seasons, however taking *T. gravida* as a low producer of methyl halides and *P. globosa* as a high producer (most other species produce at lower rates (Scarratt and Moore, 1998)), it is likely that the actual percentages of total oceanic production from phytoplankton lie somewhere between the two calculated values above, for each of the methyl halides.

Using methyl iodide production data from the experiments with natural populations from a Norwegian fjord (section 4.3.2), the maximum production rate for the culture in experiment 2 with added Si was 1.6 μmol g Chl a^{-1} d^{-1}, which is an order of magnitude higher than the maximum methyl iodide production rates seen in the unialgal culture experiments in this study. Using the calculations as above, this would lead to a vast global production of 6.3 Gmol y^{-1} which is 630% of the estimated total global production. However, these production rates would only be expected under the bloom conditions which were being simulated with added nutrients in these natural population cultures. Phytoplankton production of these three methyl halides is therefore clearly important, but whether it is important on a global scale is still in question, as the maximum production rates detected in this study are only likely to be seen during bloom events and in areas of nutrient input/upwelling.

If we look therefore at purely coastal production of methyl halides, assuming an average coastal chlorophyll concentration of 100 mg m^{-2} (Morel and Berthon, 1989) and a coastal ocean area equal to 10% of the total, coastal phytoplankton could still account for as much as 100% of the total oceanic production of methyl chloride, 50% of methyl bromide and 22% of methyl iodide based on *P. globosa* production rates or 200% of methyl iodide if based on the natural population production rates.

5.3 DMS release

The production of DMS by phytoplankton, especially *Phaeocystis* sp., is well established (Keller *et al.* 1989). DMS carries most of the flux of sulphur from the oceans to the atmosphere where it is oxidised to form acidic species including sulphur dioxide and sulphuric acid, which in turn affect the acidity of atmospheric aerosols forming acid rain. Sulphuric acid derived from marine DMS in areas of the open ocean is also the main source of aerosol particles which act as cloud condensation nuclei, which affect the scattering of solar radiation and ultimately the radiation balance of the atmosphere. The DMS production rates by *P. globosa* in this study are a little lower than field observations (2-87 nmol 10^6 $cells^{-1}$ (Liss *et al.* 1994)).

5.4 Effect of nitrate concentration

The data from the nutrient manipulation experiments suggests that the dissolved nitrate available to a cell during growth has an effect on the methyl halide production process such that increased nitrate availability increases the amount of methyl halide each cell can release during the period of maximum production. The data appears to support this for methyl chloride and methyl bromide, and also DMS, but possibly not for methyl iodide.

The nature of the relationship between dissolved nitrate and methyl halide production can only be speculated from these preliminary experiments. The relationship is not proportional, as a doubling of the nitrate concentration did not effect a doubling in the methyl halide production rate, and subsequent doublings of the nitrate concentration did not effect similar sized increases in methyl halide production rate. This would suggest that nitrate concentration does not have a direct relationship with methyl halide release, rather an indirect relationship.

The maximum production rates for each methyl halide, normalised to biomass, have been summarised for all three nutrient manipulation experiments, and also for the ungrazed culture from the *P. globosa* grazing experiments (Figure 17), and plotted against initial dissolved nitrate concentration (Figures 19 and 20). These figures show that the range of production rates were lower for experiment 1 than for experiments 2 and 3 (the latter two experiments were more comparable). During the period of maximum production, the *P. globosa* cells in experiment 1 produced less of each methyl halide per cell than the *P. globosa* cells in experiments 2 and 3. This could have been due to a difference in analytical technique, as experiment 1 was analysed using the GC-ECD and experiments 2 and 3 were analysed using the GC-MS, but this is unlikely. However it can be seen that the cells in experiment 1 initially grew much faster than the cells in experiments 2 and 3, and released methyl halides in the stationary and senescent phases of growth, whereas methyl halides were released from experiments 2 and 3 mainly in the exponential and stationary phases (Figures 8-10); so it is possible that cells in the later stages of the growth cycle produce less methyl halide per cell. The ungrazed culture which had an initial dissolved nitrate concentration of 88 μM (Table 7), also had a much lower biomass-normalised maximum production rate for each methyl halide (Figures 19 and 20). As for the nutrient manipulation experiment 1, a large proportion of each methyl halide was released from this ungrazed culture during the stationary phase, just before the experiment was terminated. The pattern of methyl halide production was relatively steady in this ungrazed culture (Figure 17 f-h), compared to the nutrient manipulation experiments where production occurred over a short period of time. If the methyl halide production rate per cell changes according to the stage of growth the cell has reached, then this introduces another variable into the debate, and the relationship between nitrate and methyl halide production may change according to the growth cycle. Further experiments should therefore be careful only to compare cultures with identical growth patterns.

Looking at the nutrient manipulation experiments 2 and 3 together, there is some evidence of a correlation between the maximum methyl halide production rates, normalised to biomass, and the initial dissolved nitrate concentration (Figures 19 and 20). This is evident for methyl chloride and methyl bromide, but not so clear for methyl iodide. However, this correlation between production rate and nitrate concentration is much clearer within each experiment than between experiments,

probably due to the slightly different patterns of cell growth and methyl halide release between the experiments.

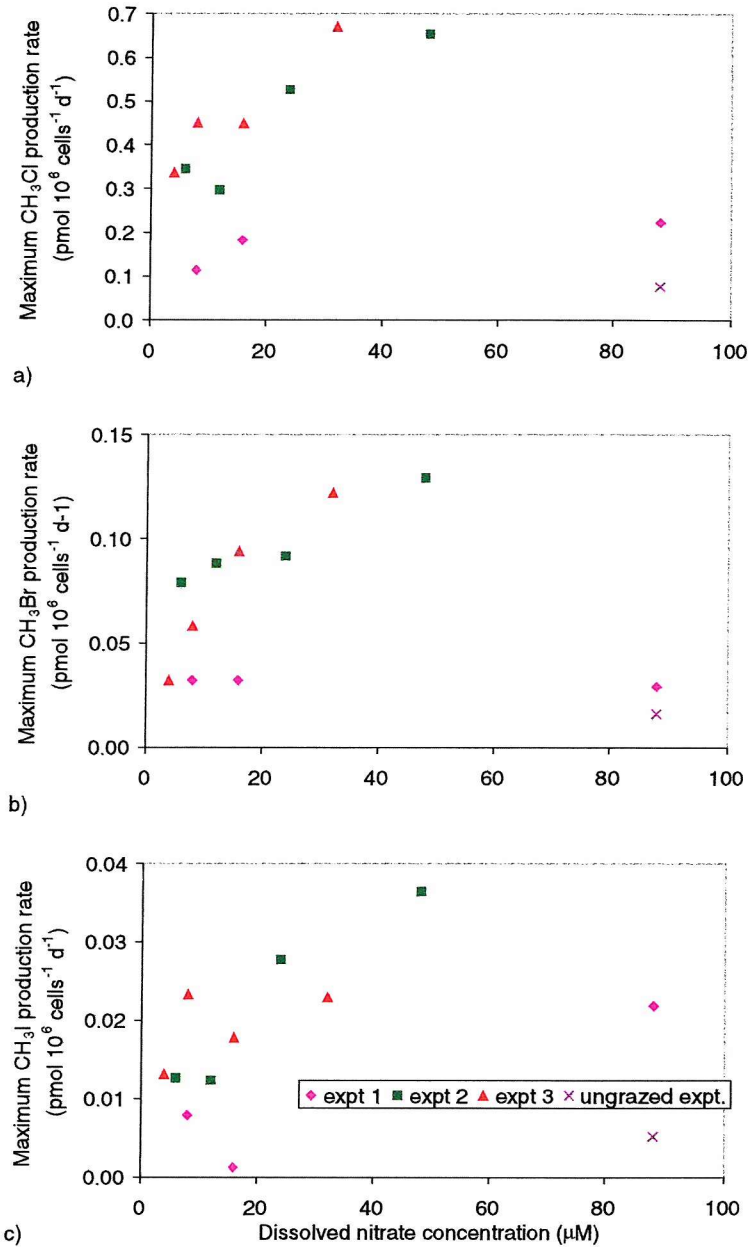


Figure 19. Summary of Figures 11-13. Maximum methyl halide production rates normalised to cell counts, for the dissolved nitrate concentrations used in nutrient experiments 1, 2 and 3 (expts. 1-3 in legend) and in the ungrazed culture from the *P. globosa* grazing experiments (ungrazed expt. in legend), showing a) methyl chloride; b) methyl bromide and c) methyl iodide.

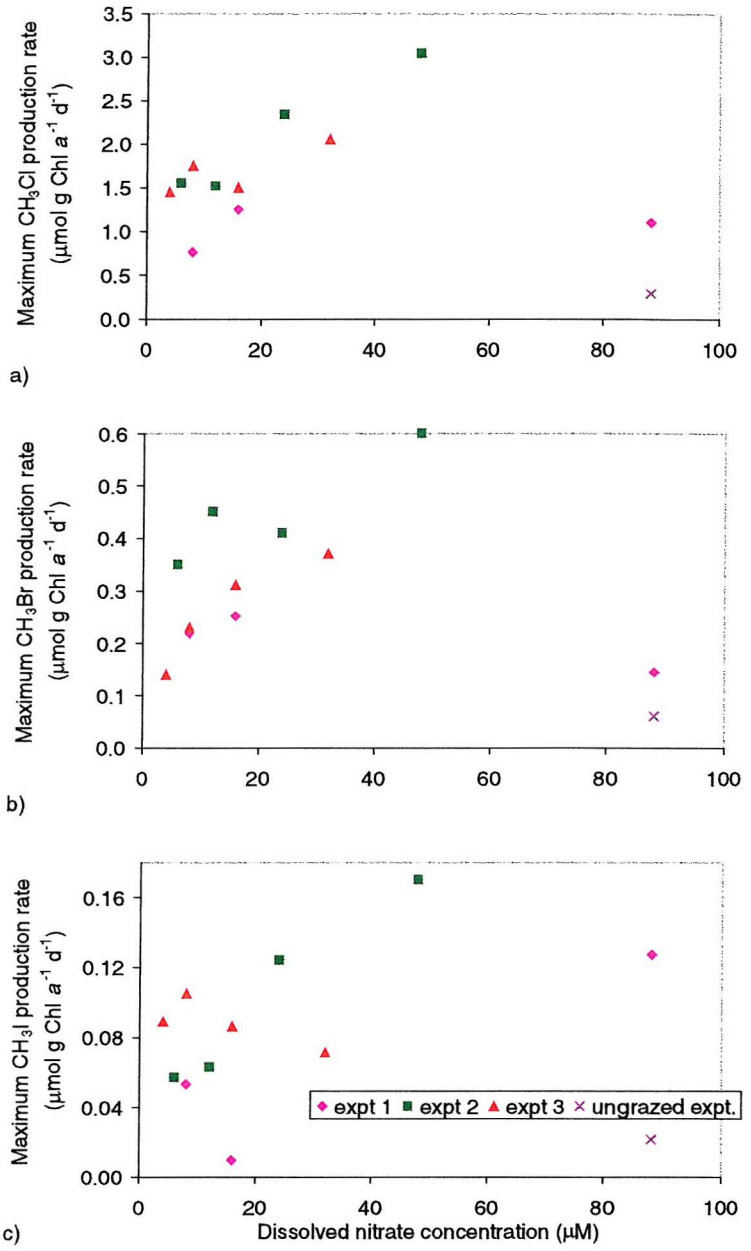


Figure 20. Summary of Figures 11-13. Maximum methyl halide production rates normalised to chlorophyll *a*, for the dissolved nitrate concentrations used in nutrient experiments 1, 2 and 3 and in the ungrazed culture from the *P. globosa* grazing experiments, showing a) methyl chloride; b) methyl bromide and c) methyl iodide.

As discussed previously, cells in the exponential phase of growth may be able to produce more methyl halides per cell than cells in the stationary phase. It may therefore follow that faster growing cells are able to produce more methyl halides per cell. To examine this further the exponential phase cell growth rates (μ) were calculated for the cultures from experiments 2 and 3 (as expected the growth rates increased with increasing initial dissolved nitrate concentration, see Table 12) and plotted against the maximum methyl halide production rates, normalised to cell counts (Figure 21). Data from experiment 1 could not be used in this comparison as the maximum production rates were measured in the stationary phase of growth. Within each experiment a correlation can be seen between growth rate and biomass normalised methyl halide production rate (Figure 21), so faster growing cells are able to produce more methyl halides per cell, suggesting that methyl halide release is a metabolic process. However, the growth rates for experiment 3 were slightly higher than for experiment 2 (Table 12), and the data from the two experiments are not well correlated in Figure 21. Growth rate therefore does not appear to be a direct indicator of the methyl halide production rate.

Expt. 2		Expt. 3	
Nitrate concentration (μM)	Growth rate (d^{-1})	Nitrate concentration (μM)	Growth rate (d^{-1})
6	0.90	4	0.92
12	0.91	8	0.98
24	0.94	16	1.06
48	0.96	32	1.07

Table 12. Exponential phase cell growth rates for the cultures in nutrient manipulation experiments 2 and 3.

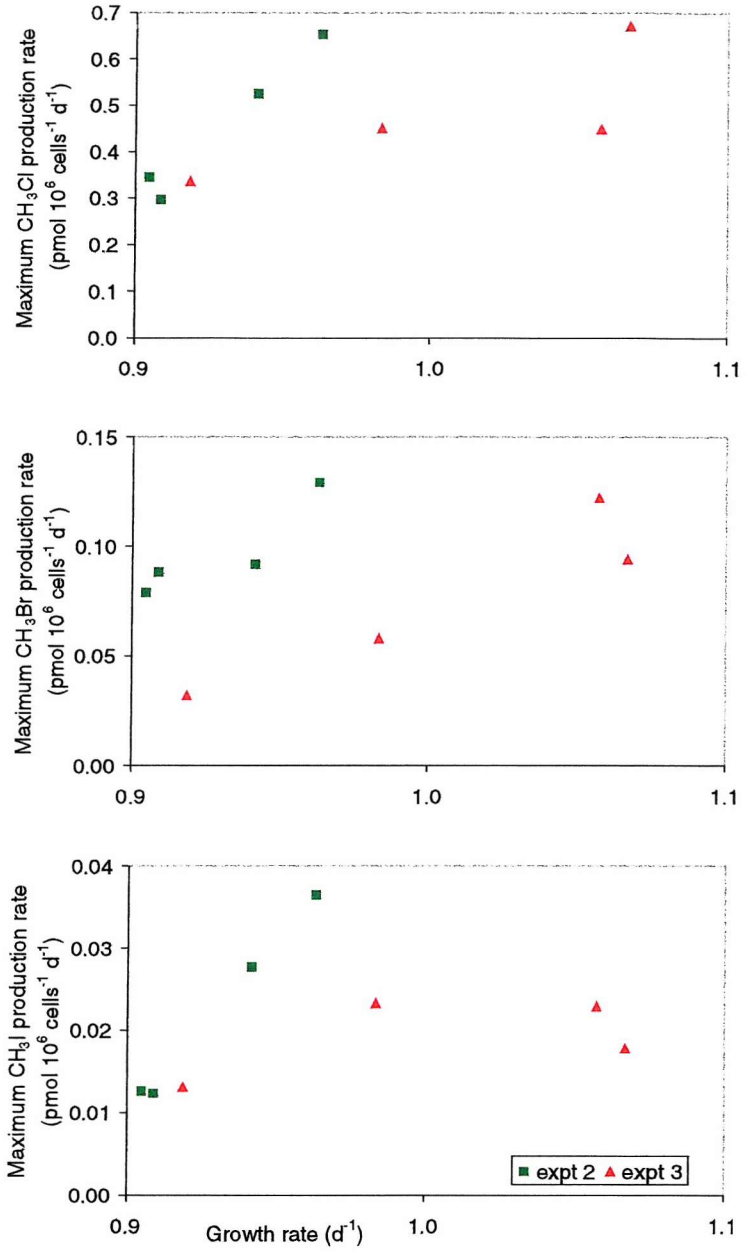


Figure 21. The maximum methyl halide production rates, normalised to cell counts, against the exponential phase growth rates (μ) for the nutrient manipulation experiments 2 and 3, showing a) methyl chloride; b) methyl bromide and c) methyl iodide.

Only one previous study has investigated the effect of nutrient concentration on methyl halide release from phytoplankton. *Phaeodactylum tricornutum* and *Thalassiosira weissflogii* were cultured under a range of nitrate concentrations, from 45 μM to 230 μM, but no conclusive effect on methyl halide production was seen (Scarratt and Moore, 1996). These nitrate concentrations were considerably higher

than those used in this study, so it is possible that an effect is only seen at the lower nitrate concentrations used in this study, which are more representative of open ocean conditions.

5.5 Methyl halide production from natural populations

Natural populations of phytoplankton and zooplankton have rarely been tested under culture conditions for methyl halide release. Ice microalgae (predominantly either *Nitzschia stellata* or *Porosira pseudodenticulata*) which were stored in carboys under suitable conditions for growth for between 0-3 days were found to have released methyl bromide and other brominated and chlorinated methyl halides (Sturges *et al.*, 1993). All other studies have used unialgal cultures either from phytoplankton stock cultures or isolated by the authors, although macroalgae have been studied as natural populations, for example in situ in rock pools (Ekdahl *et al.* 1998). The release of methyl iodide from cultures of natural populations of phytoplankton has not previously been reported, and this study shows high production rates over short periods of time, suggesting the potential for high levels of methyl iodide production under bloom conditions in the fjord. *E. huxleyi* was present in the vessels, and has already been shown to produce methyl iodide in culture in this study, although not in Manley and de la Cuesta's (1997) study. The other dominant species was *Rhizosolenia fragillissima* which has not previously been tested for methyl halide release. Considerably higher chlorophyll biomass was detected in the mesocosm experiments in the fjord than measured in the culture experiments described here, suggesting the potential for even higher levels of methyl iodide in the fjord, with the higher cell densities present.

Measuring methyl halide release from cultures of natural populations is clearly limited as they are not able to accurately simulate natural conditions. As for these experiments, even when placed under the same conditions of light, temperature and nutrients as in the mesocosms, there was a discrepancy in the maximum chlorophyll biomass produced.

The phosphate limited cultures produced the highest amounts of methyl iodide per gram of chlorophyll, which was possibly an anomaly due to the low chlorophyll biomass in the cultures, but could be worth further investigation. The other cultures showed higher production rates in experiment 1 at the beginning of the senescent

phase, which is in contrast to the unialgal cultures in this study which tended to show maximum production rates at the end of the exponential phases of growth. Compared to the high P culture (15 μM nitrate, 1 μM phosphate) the cultures with additional Si (add. Si) and additional Si and Fe (add. Fe) showed higher production rates (Table 9) suggesting an effect due to these added nutrients on the methyl iodide production mechanism.

5.6 Effect of grazing

In this study it was seen that biomass normalised methyl halide production was increased over the 2 days after the addition of the grazers, compared to production in the ungrazed culture. This may therefore suggest that grazing has a positive effect on methyl halide production from phytoplankton. It is possible this is due to the breaking up of the cells leading to the increased release of intracellular methyl halides, or alternatively that methyl halides are released as a defence mechanism or a stress response. The former explanation is possible; an increased release of methyl halides has not been seen during cell lysis in the senescent phases of growth in any of the cultures in this study, but those cells were already dying. Lysis of growing cells, containing compounds which are important in the production of methyl halides (compounds which may be subsequently broken down at cell senescence), may bring these compounds together more quickly than would otherwise occur without grazing, possibly causing a synergistic release of methyl halides. A stress response also cannot be ruled out - macroalgal stress in the form of grazing, darkness, nutrient limitation and temperature elevation has been shown to increase methyl halide production (Gschwend *et al.* 1985; Klick, 1993; Nightingale *et al.* 1995; Laturnus *et al.* 1998).

A grazing effect is difficult to both determine and express unless it is very obvious. Wolfe and Steinke (1996) looked at the effect of *O. marina* grazing on *E. huxleyi* DMS production and this effect was obvious as presence of the grazer lead to DMS production, whereas no DMS was released in the grazer's absence. *E. huxleyi* produces dimethylsulfoniopropionate (DMSP) and DMSP lyase which are segregated intracellularly, requiring cell lysis for these compounds to react to form DMS (Wolfe and Steinke, 1996). This reaction deters protozoan herbivores and thus DMS release is possibly a defence mechanism (Wolfe *et al.* 1997). These authors could therefore

express their data quite simply, however their study would have been more difficult to interpret had they used *Phaeocystis* sp. as this species produces large quantities of DMS during growth (Stefels and van Boekel, 1993). Similarly *Phaeocystis* sp. produces large quantities of methyl halides during growth, the release of which are not always entirely reproducible in amount or pattern, so any grazing effect would have to be seen in many replicates to truly confirm the effect.

5.7 Methyl halide degradation and release due to factors other than phytoplankton

The reduction seen in total methyl halides in some of the culture vessels after methyl halide production had ceased, in some cases down to zero, suggests either a leak in the vessels or some form of degradation. The gas-tight integrity of the culture vessels has been discussed (section 4.1) and they were gas-tight for CFC 11 (MW 137.5), but it is possible the vessels were not as gas tight for the lighter, smaller molecules of methyl chloride (MW 50.5) and methyl bromide (MW 95), although it is likely they were gas tight for methyl iodide (MW 142). However it was methyl iodide that showed the greatest decrease in the vessels after its initial increase. Chemical degradation has been accounted for, leaving the possibility of microbial degradation. Using King and Saltzman's (1997) estimate of 5-10% loss d^{-1} of methyl bromide in seawater by bacterial consumption at 21-29^{0}C, and assuming the same for methyl chloride and methyl iodide, between half and all of most of the losses can be accounted for. For example, in experiment 2 of the nutrient manipulations, the culture initiated with 24 μM nitrate on day 13 the vessel contained 371 pmol methyl chloride, but by day 22 this had decreased to 169 pmol (Figure 9). A 10% decrease per day from day 13 would leave 144 pmol on day 22. Assuming this amount of microbial degradation leads to the possibility that production was continuing along with degradation, but determining the proportions of each would not be possible at this stage. Thus the data for methyl halide release in this study may have been underestimated and gross production might have continued for longer in some of the experiments.

The degradation or disappearance of the methyl halides in the culture vessels was most evident for methyl iodide, with large amounts of methyl iodide disappearing very quickly. In the atmosphere methyl iodide is rapidly broken down by photolysis,

possibly within 4 days (Zafiriou, 1974) so the methyl iodide released into the headspace of the culture vessels may have been broken down within this timescale. This might explain the discrepancy in methyl iodide release seen between similar cultures in the initial *P. globosa* and *E. huxleyi* methyl halide release experiments (Figures 4d and 6c). With sampling occurring every 2-3 days it is possible that large releases of methyl halides were not detected if they had already been degraded before the next sampling time.

Methyl iodide can be formed by UV photo-production. This phenomenon occurs in seawater, requiring a source of iodide ions, methyl radicals and sunlight or UV light, and can produce methyl iodide at rates ranging between 16.8-33.6 pmol l^{-1} d^{-1} (Moore and Zafiriou, 1994). There is evidence of methyl iodide production from open ocean areas with relatively low biological activity, which has led to the hypothesis that photochemical production may indeed be the dominant pathway for CH_3I production in the open ocean (Moore and Zafiriou, 1994; Happell and Wallace, 1996). However the vessels were illuminated with artificial light alone throughout this study, thus there was no source of UV light and none of the methyl iodide release seen in this study should have been due to photo-production.

Methyl iodide release often occurred slightly later or in a different pattern to methyl chloride and methyl bromide release, and in the nutrient manipulation experiments it was clear that the production rate data were not as reproducible for methyl iodide as for methyl chloride and methyl bromide. In the natural population experiments, methyl iodide release was evident but methyl chloride and methyl bromide production was very low. It is possible that there is a separate production pathway for methyl iodide than for methyl chloride and methyl bromide. Indeed brominated and chlorinated compounds have a separate pathway to iodinated compounds released from macroalgae. A diurnal variation in the macroalgal production of chlorinated and brominated methyl halides connected to photosynthesis, photorespiration and respiration has been found, which was not the case for iodinated compounds (Ekdahl *et al.* 1998). The enzymes involved in the separate processes have been identified, these are haloperoxidases which catalyse the destruction of hydrogen peroxide and have been found in species of both macrophytes (Wever *et al.* 1991) and phytoplankton (Moore *et al.* 1996b). Bromoperoxidases catalyse iodination and bromination reactions and form polyhalogenated (iodinated or brominated) products, but iodoperoxidases are able to oxidise only iodide. Methyl halide

compounds produced by *Nitzschia* sp. (CCMP 580) and *Navicula* sp. (CCMP 545) reflect that the former contains bromoperoxidases whereas the latter contains solely iodoperoxidases (Moore *et al.* 1996b). However phytoplankton production of monohalogenated compounds (i.e. methyl chloride, methyl bromide and methyl iodide) does not require the presence of haloperoxidases (Moore *et al.* 1996b) and so it follows that the production mechanism for monohalogenated compounds is independent of that for polyhalogenated compounds. However the presence of these separate haloperoxidases means it may be just as likely that similar enzymes are involved in the separate production of methyl iodide from methyl chloride and methyl bromide. Experiments by Manley (1994) have suggested the possible involvement of methylcobalamin in the production of methyl iodide.

5.8 Comparison of the GC-ECD and GC-MS data

The two different systems of gas chromatography analysis used in this study complimented each other reasonably well. Identical experiments were not performed using each system, but similar *P. globosa* cultures were analysed for methyl halides using both systems (the initial *P. globos*a cultures and the 88 μM nitrate *P. globosa* culture in experiment 1 of the nutrient manipulation experiments). There were differences between the culture conditions as enriched seawater was used in the initial *P. globosa* experiments which probably had higher dissolved nitrate and phosphate concentrations than the artificial seawater used in the nutrient manipulation culture. Higher cell densities were therefore achieved in the initial cultures and colonial growth was seen, whereas the nutrient manipulation cultures consisted of only motile cells. Despite this, the GC-MS methyl halide data for the *P. globosa* cultures were quite similar to the GC-ECD *P. globosa* data, with maximum methyl halide amounts in the vessels of 350 pmol, 50 pmol and 12 pmol (initial cultures) and 230 pmol, 140 pmol and 25 pmol (nutrient manipulation culture) for methyl chloride, methyl bromide and methyl iodide, respectively.

6.0 CONCLUSIONS

Methyl chloride, methyl bromide and methyl iodide have been shown to be released by certain species of phytoplankton. It has been ascertained that methyl halide production by phytoplankton may be important on a global scale, although the validity of these calculations remain in dispute.

Higher initial dissolved nitrate concentrations have been shown to lead to increased maximum methyl chloride and methyl bromide production rates (normalised to biomass), consistent with the hypothesis. The stage within the cell growth cycle of the maximum methyl halide production rate has been shown to be important in this relationship, and it has been suggested that exponential phase cells may have an increased potential for methyl halide production compared to stationary phase cells. For the cultures that released most of their methyl halides in the exponential of growth, the growth rate was correlated with the maximum methyl halide production rate, suggesting that methyl halide release is a metabolic process. Therefore on extrapolation to a global scale, biomass normalised methyl halide production may be relatively higher in areas of increased nitrate in the oceans.

The importance of the nutrient regime has also been shown using natural populations of phytoplankton which were cultured and shown to produce methyl halides, the release of which was affected by the concentration of nutrients added.

Increased methyl halide production was detected soon after the addition of a grazer, consistent with the hypothesis. This may be important under bloom conditions where grazing could release large amounts of methyl halides to the atmosphere over a short period of time.

APPENDIX 1

Recipe for artificial seawater base media from Harrison *et al.* (1980).

	Mr	g/kg solution	mmol l^{-1}
Solution 1 - *Anhydrous salts*			
NaCl	58.44	20.758	362.661
Na_2SO_4	142.04	3.477	24.993
KCl	74.56	0.587	8.038
$NaHCO_3$	84.00	0.170	2.066
KBr	119.01	0.0845	7.249×10^{-1}
H_3BO_3	61.83	0.0225	3.715×10^{-1}
NaF	41.99	0.0027	6.570×10^{-2}
Solution 2 - *Hydrated salts*			
$MgCl_2.6H_2O$	113.33	9.395	47.176
$CaCl_2.2H_2O$	147.03	1.316	9.139
$SrCl_2.6H_2O$	266.64	0.0214	8.200×10^{-2}

Composition of artificial seawater base (S= 30.5 psu).

Anhydrous and hydrous salts are dissolved and autoclaved separately, then left to stand for 48 hours before combining. For units of concentration (mmol l^{-1}), Sp. Gr. = 1.021 at 20^0C.

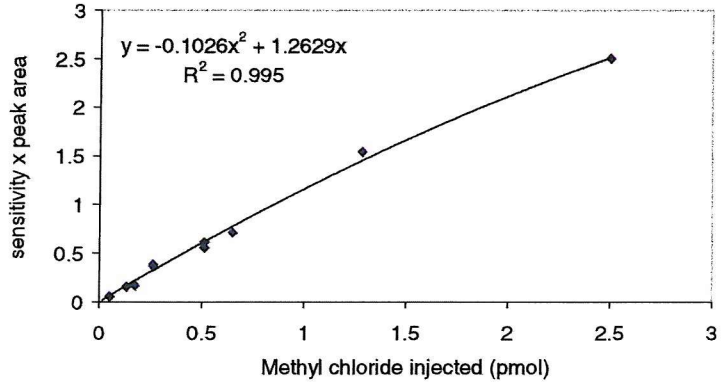
APPENDIX 2

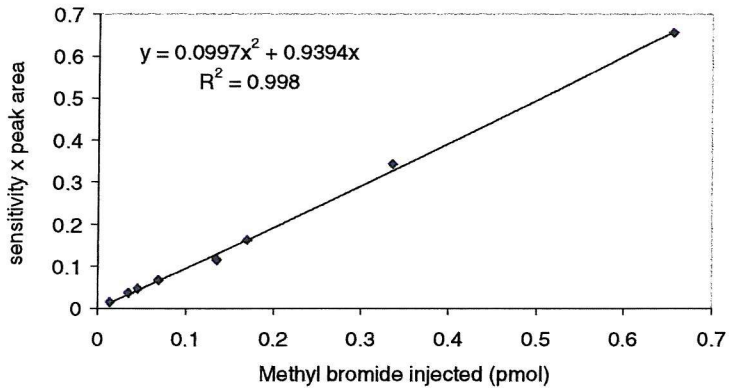
Adaptation of K medium (Keller *et al.* 1987).

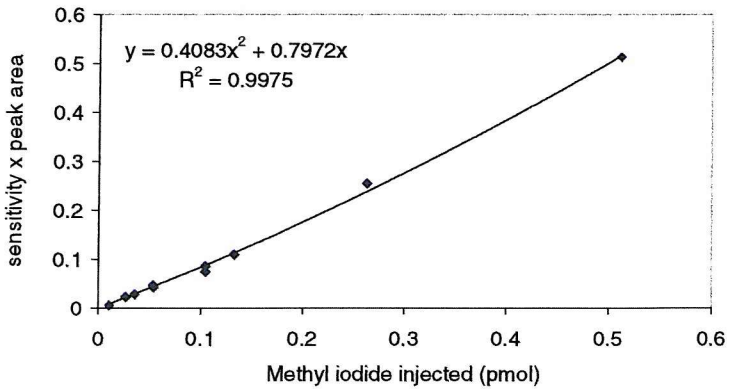
Compound	Mr	1^0 stock (100ml Milli-Q)	Working stock (100ml Milli-Q)	To 1L artificial seawater base	Molarity in final media (μM)
$NaNO_3$	85		0.75g	1ml	88
$NaH_2PO_4.2H_2O$	156		0.0158g	1ml	1
H_2SeO_3	129	0.013g	1ml	1ml	0.01
$Na_2O_3Si.9H_2O$	284		3.04g (heat)	0.5ml	53.5
FeNaEDTA	367		0.440g	1ml	12
Na_2EDTA	372		0.372g	1ml	10
Trace metals:		(mixed)			
$CuSO_4.5H_2O$	250	0.1g	1ml	1ml	0.04
$ZnSO_4.7H_2O$	287.5	0.22g			0.075
$CoCl_2.6H_2O$	238	0.11g			0.047
$MnCl_2.4H_2O$	198	1.8g			0.91
$NaMoO_4.2H_2O$	242	0.065g			0.026
Vitamins:		(separate)	(mixed)		
Biotin		0.01g	1ml	0.5ml	0.002
B_{12}		0.01g	1ml		0.0004
Thiamine HCl			0.02g		0.3

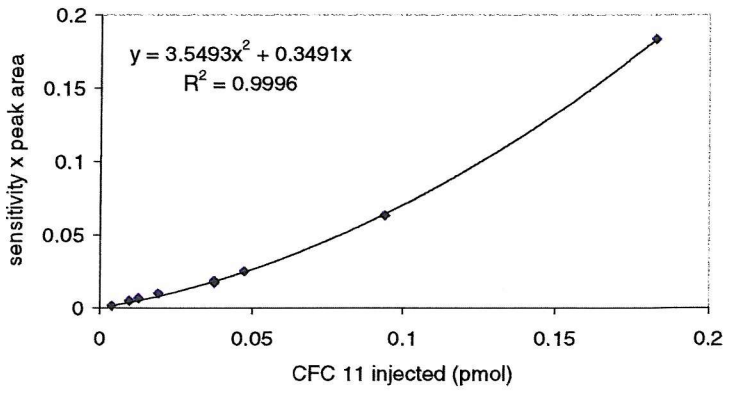
APPENDIX 3

Calibration graphs used for one set of experimental cultures for the GC-ECD.

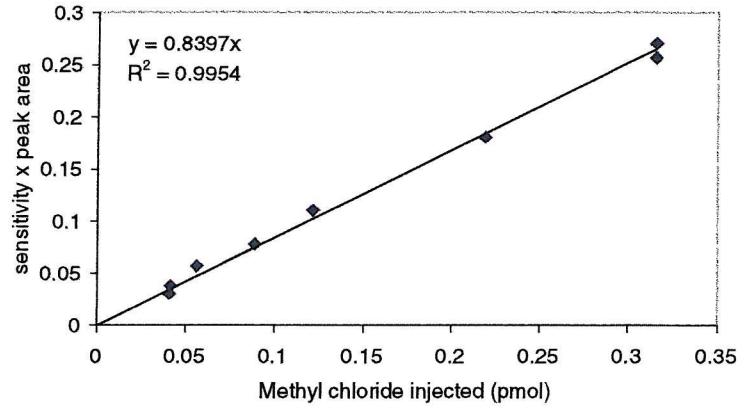


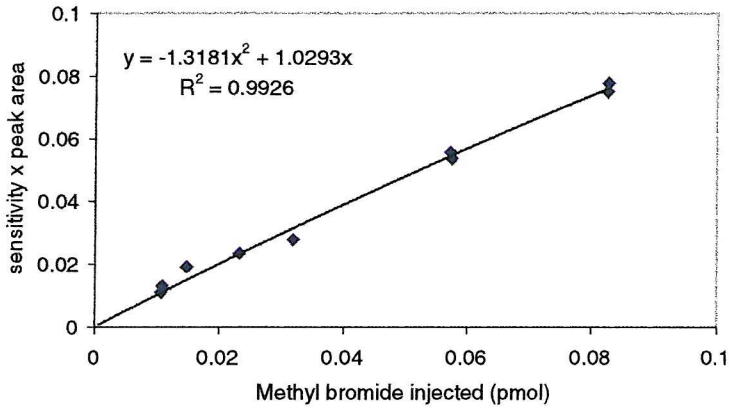


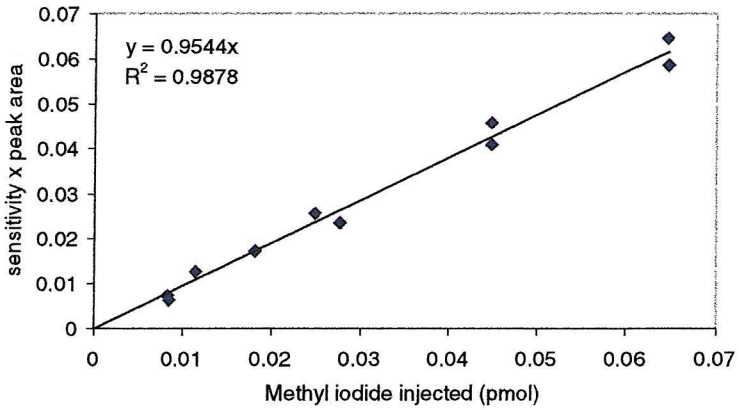


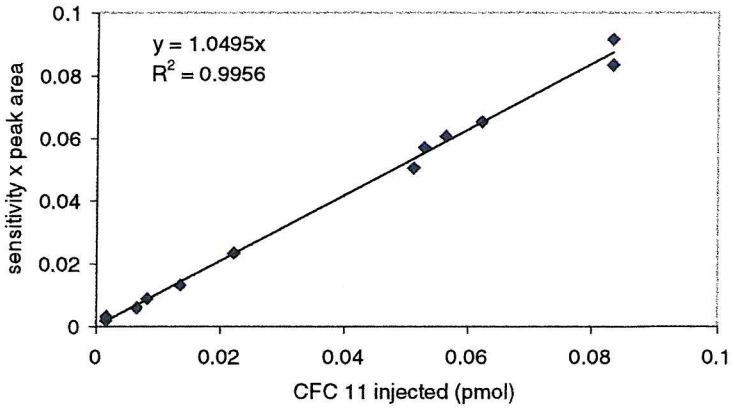


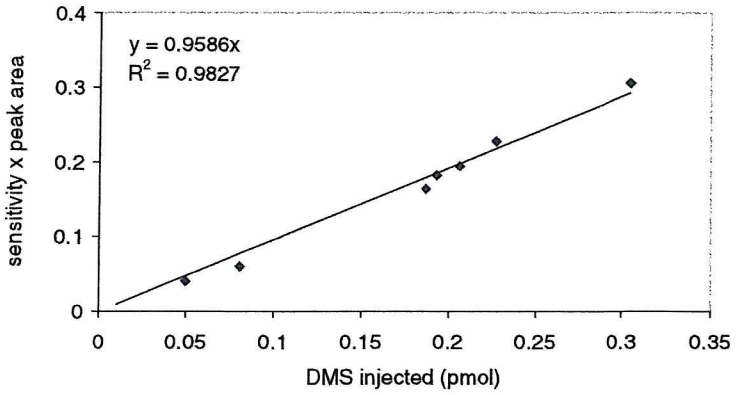
Calibration graphs used for one set of experimental cultures for the GC-MS.





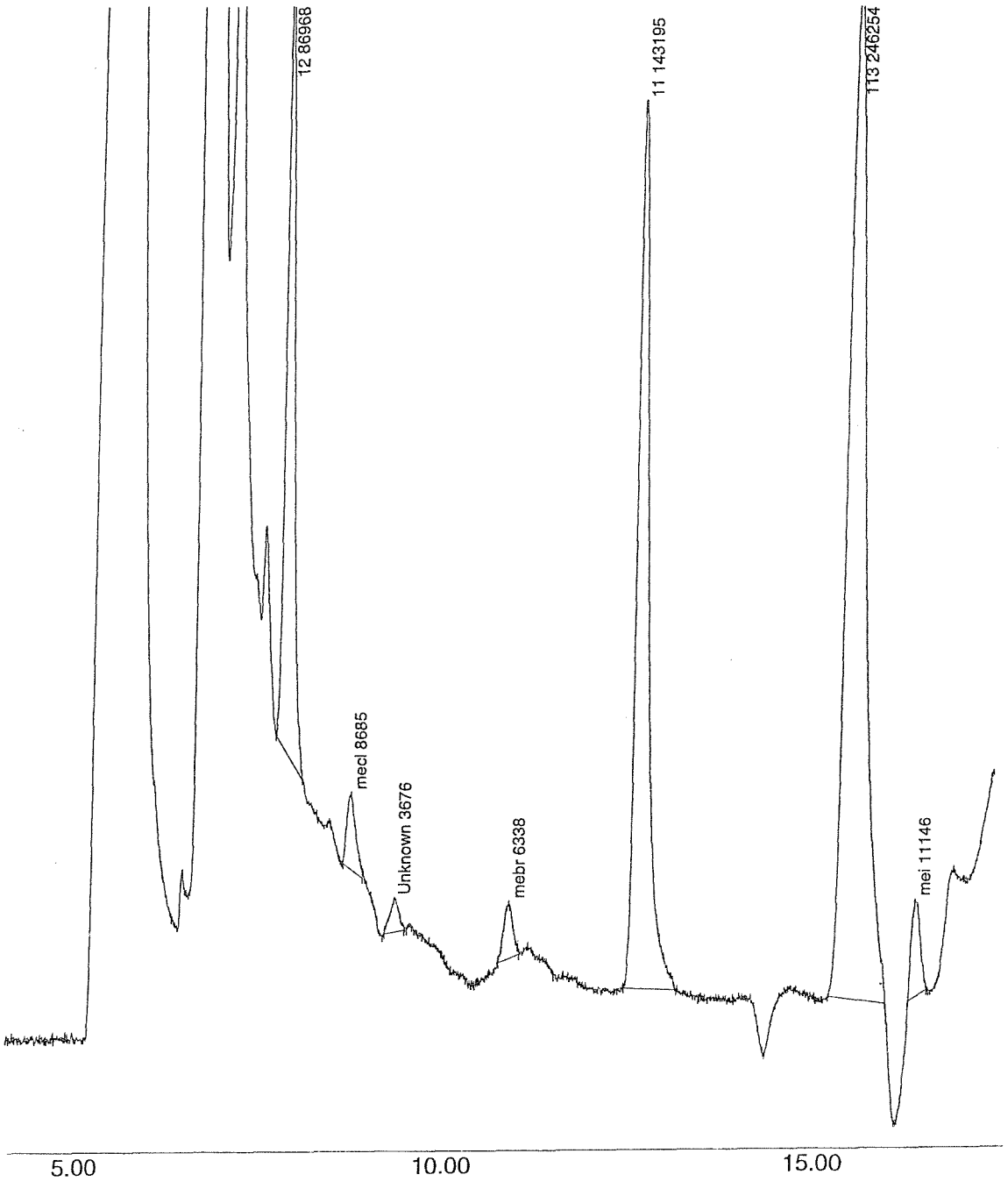





y = 0.9586x
R2 = 0.9827
sensitivity x peak area
DMS injected (pmol)
0.4
0.3
0.2
0.1
0
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35

APPENDIX 4

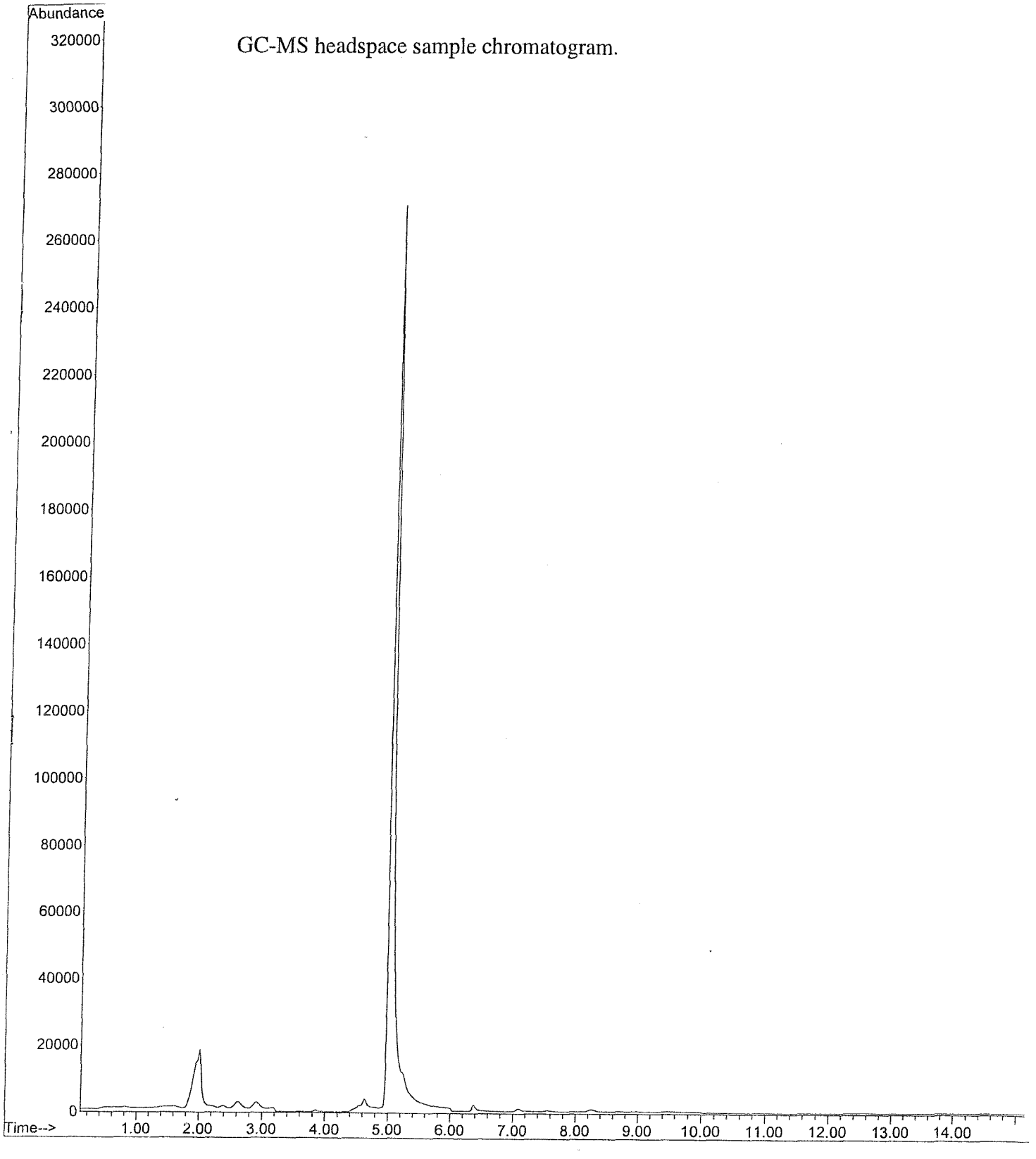
GC-ECD headspace sample chromatogram.



#	Name	Rt	Area	Quantity
1	12	7.98	86967.900	0.000
2	mecl	8.85	8685.200	0.000
3	Unknown	9.45	3676.500	0.000
4	mebr	10.98	6338.200	0.000
5	11	12.74	143194.850	0.000
6	113	15.61	246254.300	0.000
7	mei	16.43	11146.200	0.000

Total Area of Peak = 506263.15

GC-MS headspace sample chromatogram.



Internal Standards	R.T.	QIon	Response
Target Compounds			
1) ???	0.00	65	0
2) Vinyl Chloride ?	0.00	62	0
3) HCFC-22	0.00	51	0
4) CFC-114	2.60	85	0
5) CFC-12	2.62	85	101825
6) MeCl	2.92	50	111643
7) MeBr	3.85	94	9476
8) CFC-11	4.63	103	109922
9) MeI	5.03	142	5334
10) methylene chloride	5.10	84	16661
11) dimethyl sulphide	5.01	62	12463545

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