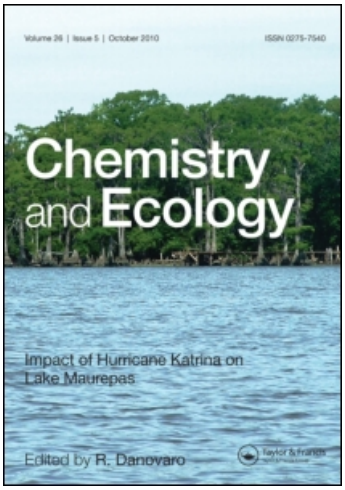

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Effect of Chlorination on the Gill Lipids of the Mussel *Mytilus edulis* (L)

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Effect of Chlorination on the Gill Lipids of the Mussel *Mytilus edulis* (L)

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Experiments are described which involve the chlorination of standard solutions of lipids and populations of the mussel, *Mytilus edulis* (L). The object was to test the hypothesis that chlorine will bind to unsaturated lipids. Evidence is presented that chlorine and aqueous chlorinated species rapidly and readily form addition compounds with unsaturated lipids *in vivo* as well as *in vitro*. The relevance of these findings to possible environmental effects of chlorination is discussed.

INTRODUCTION

Chlorination is a successful antifouling method, widely used by marine power stations for total control of soft fouling and limited control of hard fouling.

Introduction of chlorine into seawater initiates a series of reactions, the products of which have an unknown toxicity. Traditionally, chlorination products are measured by analysing for total oxidation potential of the water, although this method does not detect all of the substances produced as some do not have an oxidative capacity. In summary, an initial fast reaction produces hypobromous acid (Wong and Davidson 1977; Hostgaard-Jensen *et al.*, 1977) which then decays slowly to give a large number of different chemical species. In total these have a lower oxidation potential than the original chlorine. Decay may occur by volatilisation, reaction with ammonia, reduction with organic matter (Hostgaard-Jensen,

et al., 1977) and formation of bromate ions under conditions of bright sunlight (Macalady *et al.*, 1977).

Much of the work on chlorination product toxicity has taken the form of LD50 or LC50 tests on organisms in isolation. (Sung *et al.*, 1978; Whitehouse, 1975). Sub-lethal and behavioural effects (Capuzzo *et al.*, 1976, 1977; Capuzzo, 1977), changes in community structure (Dickson *et al.*, 1977), reduction in growth (White, 1966; Eppley *et al.*, 1976), and loss of primary productivity potential (Fox and Moyer, 1975) have also been shown.

Evidence for the physiological action of chlorine products is seen in disruption of bacterial enzyme systems (Green and Stumpf, 1946; Ingols *et al.*, 1953; Knox *et al.*, 1948) increased respiration rates (Bass and Heath, 1975, 1977; Block *et al.*, 1977) decreased ATP levels in phytoplankton (Erickson and Foulk, 1980) lowered haemoglobin counts in fish (Buckley, 1976; Buckley *et al.*, 1976; Zeitoun, 1977) and loss of osmoregulatory ability in crabs (Vreenegoor *et al.*, 1977). Much of this work (Capuzzo *et al.*, 1976; Capuzzo, 1977; Bass and Heath, 1975, 1977; Block *et al.*, 1977; Buckley, 1976) suggests that gills may be the primary site of action for chlorine products especially in fish. The functional activity of a membrane is likely to be determined, at least in part, by its fluidity and, in the case of selectively permeable membranes such as the gills, this will be especially important (Hazel and Prosser, 1974; Hazel, 1972, a,b; De Grier and Van Deenen, 1964). One obvious environmental factor which will alter membrane fluidity is temperature. Many poikilotherms appear to modify the lipid composition of their membranes in response to changes in water temperature. During adaption from high to low temperatures there is an increase in the unsaturated-saturated fatty acid ratio of the membrane phospholipids (Morris and Culkin, 1976; Sargent, 1976; Vernberg and Silverthorn, 1979; Somero and Hochachka, 1976; Chapelle, 1978; Hazel and Prosser, 1974; Caldwelle and Vernberg, 1970; Farkas, 1979).

A similar response to contamination by lipophilic material has been reported in the gills of *Gammarus duebeni* (Morris *et al.*, 1982a). The greater the degree of gill contamination, the greater proportion of 20:5 polyunsaturated fatty acid was found in the gill phospholipids. This was at the expense of 18:2 fatty acid, the metabolic precursor of the 20:5 acid. It was considered that one of the possible effects of such gill contamination would be a reduction in that membrane's fluidity. This would occur either because the unsaturated double bonds of the membrane fatty acids were affected by the formation of loose bonds with the alien material or because the unsaturated-saturated ratio of the membrane fatty acids was altered because of the incorporation of relatively saturated compounds. It appeared that *Gammarus* was adapting to the presence of alien materials in

its gill membranes by incorporating additional, highly unsaturated, fatty acids into the gill lipids (Morris *et al.*, 1982a).

The ability of halogens to add across double bonds such as those which occur in unsaturated fatty acids is well established, the order of reactivity being $Cl_2 > Br_2 > I_2$. Hypohalous acids (HOX) also react readily with double bonds in a trans addition reaction. The formation of such addition compounds *in vivo* will have the effect of reducing the degree of unsaturation, with concommitant physiological effects, but it appears to be a factor not covered by previous work on the effects of chlorination on ecosystems.

This paper presents details of experiments carried out on the chlorination of standard solutions of lipids, and populations of the mussel, *Mytilus edulis,* in order to test whether chlorine will bind to unsaturated lipids *in vivo* as well as *in vitro*.

METHODS

Experiments on standard lipid solutions

Standard solutions (50 ppm) of glycerol trioleate and cod liver oil in hexane, distilled water and sea water were prepared and sonicated in order to ensure complete dispersion. 2 ml aliquots of each standard solution were taken and placed in 5 ml capped tubes. One was used as a control and the second was briefly exposed to chlorine by bubbling the gas through the solution for 30 seconds. The tube was capped, shaken and allowed to stand for a further 30 seconds. The lipids from the control and chlorinated solutions were then immediately extracted into chloroform, methylated (Morrison and Smith, 1964) and analysed by gas liquid chromatography (GLC) and gas-liquid chromatography—mass spectrometry (GC-MS) (Morris *et al.*, 1982b).

Experiments with *Mytilus edulis*

Groups of animals taken from Highcliffe, Dorset and acclimatised in the aquarium at the Department of Oceanography, University of Southampton, were placed in three separate tanks containing 100% sea water (population 1), 100% sea water plus 10 ppm "chlorine"† (population 2) and 100% sea water and 60 ppm "chlorine" (population 3) respectively, for a period of 15 minutes. The "chlorine" concentrations were achieved by adding known amounts of sodium hypochlorite to 250 ml of filtered sea water. Oxidation potential was measured iodometrically using a photometric endpoint detector (Bryan *et al.*, 1976).

† oxidative capacity is conventionally expressed as equivalent to ppm "chlorine".

Normally under these conditions the animals in populations 2 and 3 would have closed their valves immediately. However in order to ensure a reasonable contact of the chlorinated water with the gills, all the experimental animals had their adductor muscles cut.

After the exposure period the animals' gills were carefully removed. The lipids of both the bodies and the gills were then extracted and analysed (Folch, Lees and Sloane Stanley, 1957) as described earlier.

RESULTS AND DISCUSSION

Chlorination of standard lipids *in vitro*

When the GLC analyses of the standard lipids were compared, it was apparent that in all the chlorinated sample solutions (hexane, distilled water, sea water) the chromatographic peaks corresponding to the monounsaturated or polyunsaturated fatty acids of the controls had disappeared (e.g., Figures 1 and 2). The peaks corresponding to the saturated fatty acids were not affected. A new set of peaks (Figure 1-A; Figure 2 A, B, C, D) were observed which by their number and relative peak areas, appeared to correspond to the monounsaturated fatty acids, having retention times approximately 10 minutes longer than those of the normal monounsaturates. GC-MS analysis of the compounds responsible for these peaks gave base peaks of 74 mass units suggesting a series of saturated fatty acids but apparent molecular ions which implied a series of diunsaturated fatty acids. (e.g., Figures 3a, 4a 5a). This is a very different pattern for that observed for the standard monounsaturated acids (e.g., Figures 3b, 4b, 5b). We interpret these data as follows: the chlorine is reacting with the unsaturated fatty acids to form fairly stable chlorinated compounds which withstand the extraction, methylation and GLC procedures without breakdown; these compounds have greatly increased retention times on the GLC column due to the increase in molecular weight and hence decreased volatility. Indeed the increase in boiling point and retention time of the polyunsaturated acids make it impossible to elute them from the GLC column which was used. These compounds do not, however, seem to be stable enough to produce a molecular ion during GC-MS analysis and fragment, possibly by loss of HCl, to give rise to an apparent molecular ion of a diunsaturated acid and the fragmentation pattern of a saturated acid.

In addition to the major series of unknown peaks, a new series of minor peaks are found in the analyses of the aqueous solutions. The minor peaks occur just after the major peaks (e.g., Figure 6). in the case of the glyceroltrioleate in sea water, the minor unknown gave both 55 and 74 as base peaks and an apparent molecular ion of 295 (Figure 7).

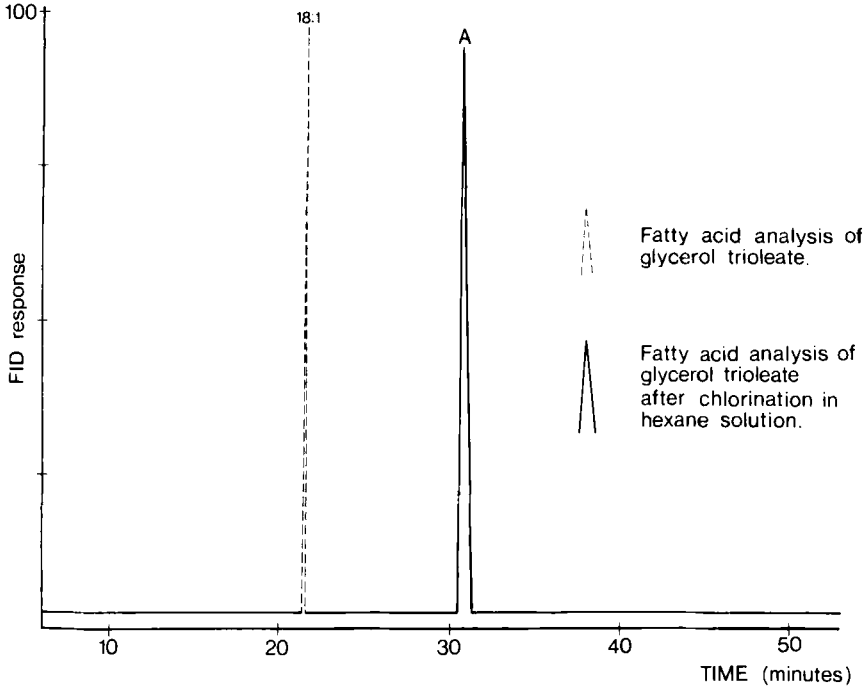


FIGURE 1 Fatty acid analysis of glycerol trioleate before and after chlorination in hexane.

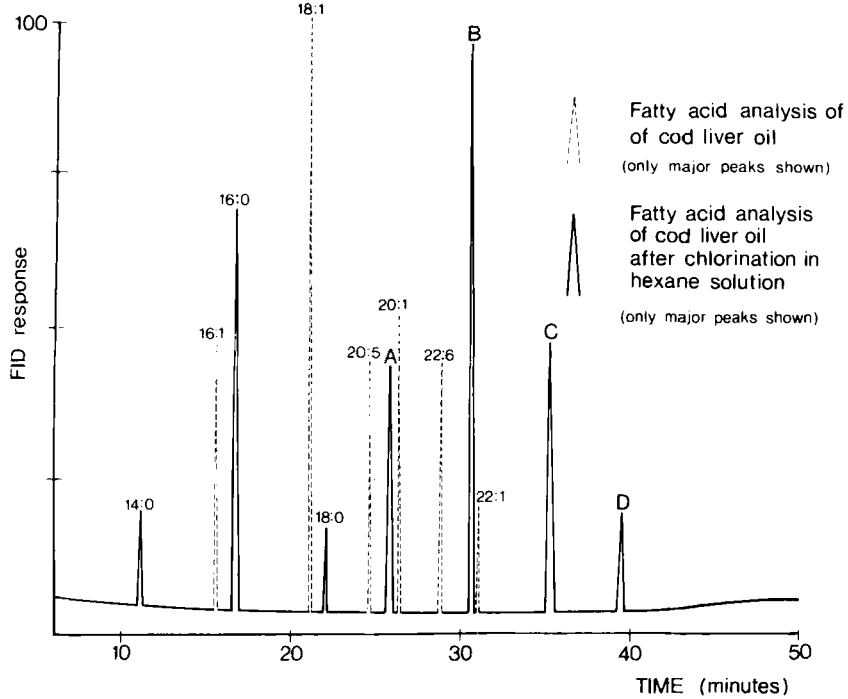


FIGURE 2 Fatty acid analysis of cod liver oil before and after chlorination in hexane.

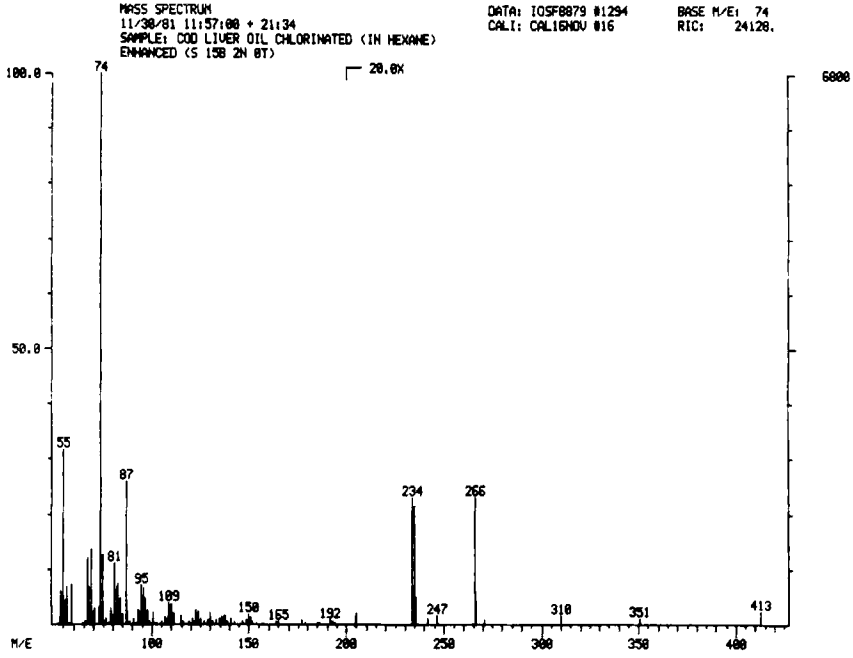


FIGURE 3a Mass spectral analysis of unknown A (Figure 2).

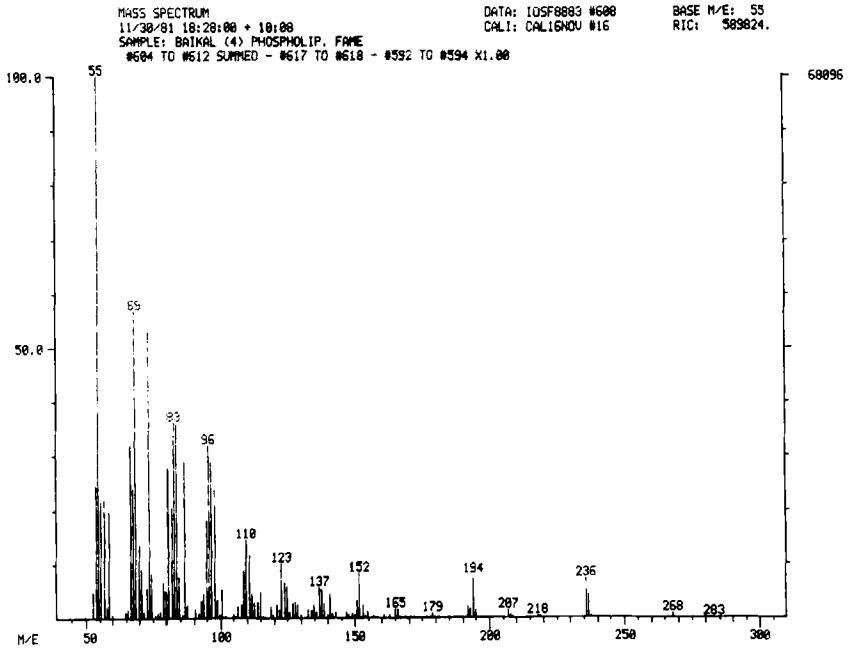


FIGURE 3b Mass spectral analysis of 16:1 ω 7 methyl ester.

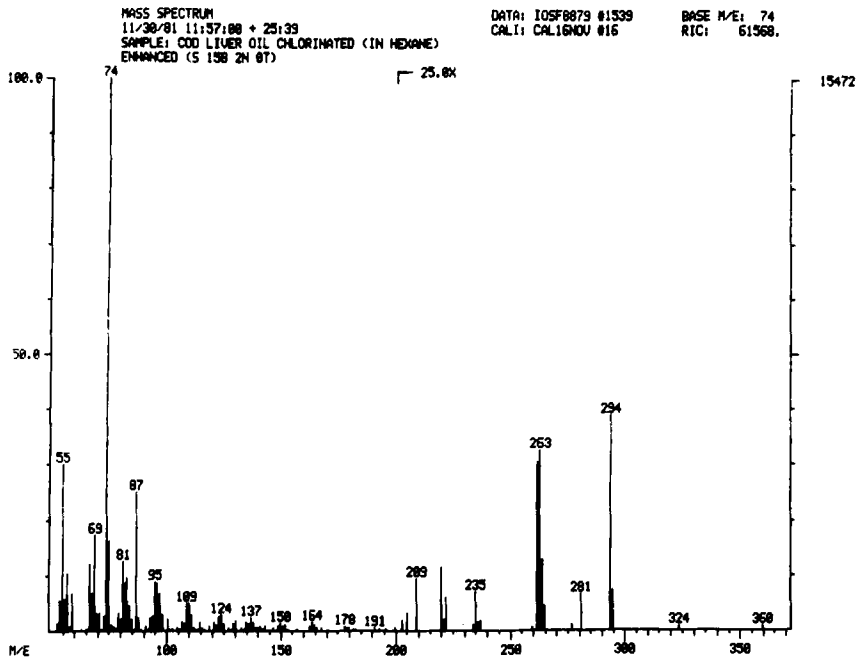


FIGURE 4a Mass spectral analysis of unknown B (Figure 2).

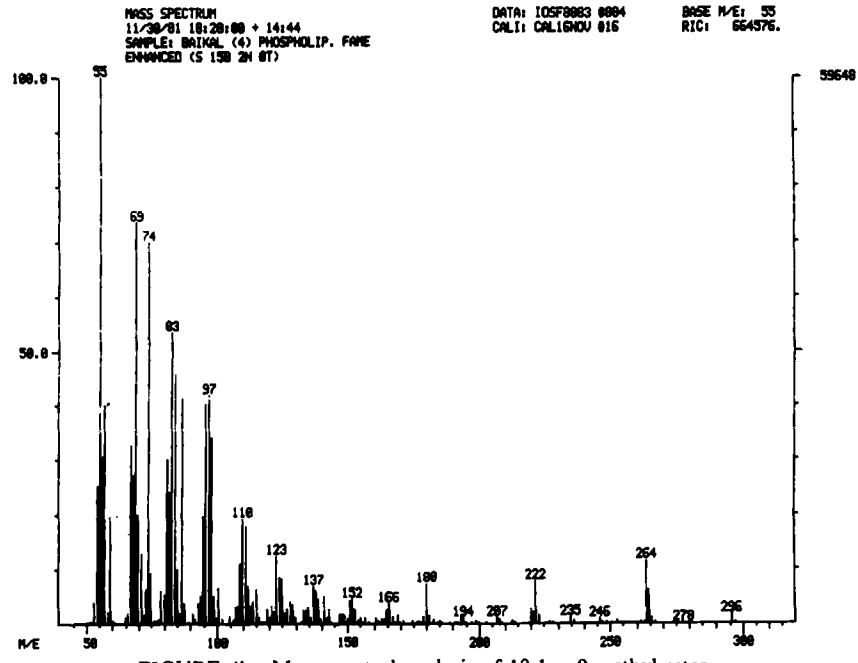


FIGURE 4b Mass spectral analysis of 18:1 ω 9 methyl ester.

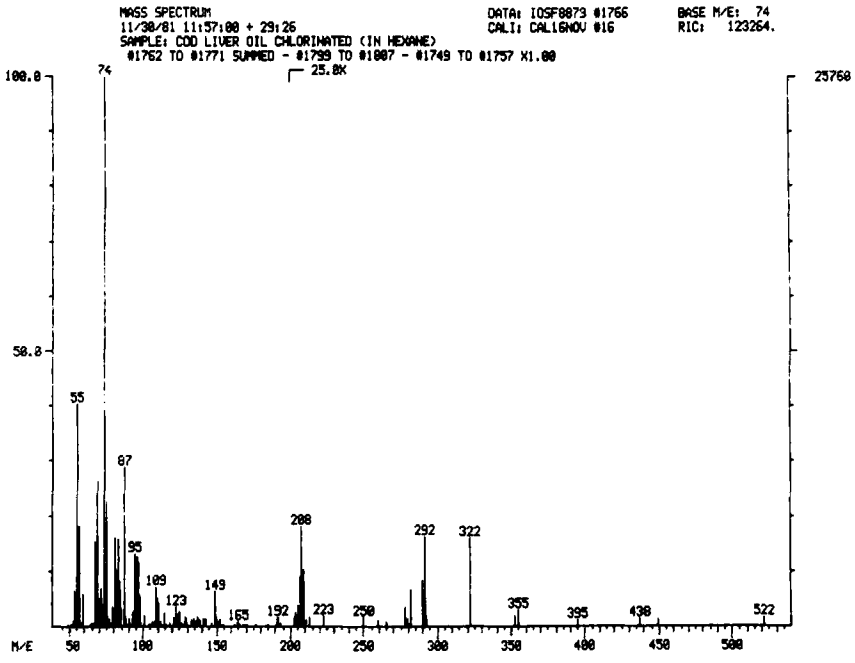


FIGURE 5a Mass spectral analysis of unknown C (Figure 2).

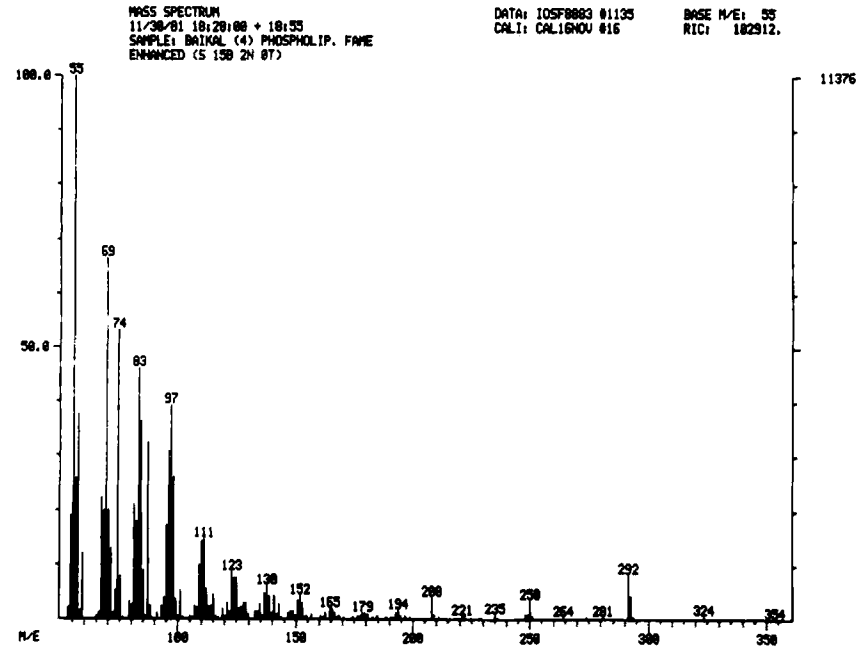


FIGURE 5b Mass spectral analysis of 20:1 ω 11 methyl ester.

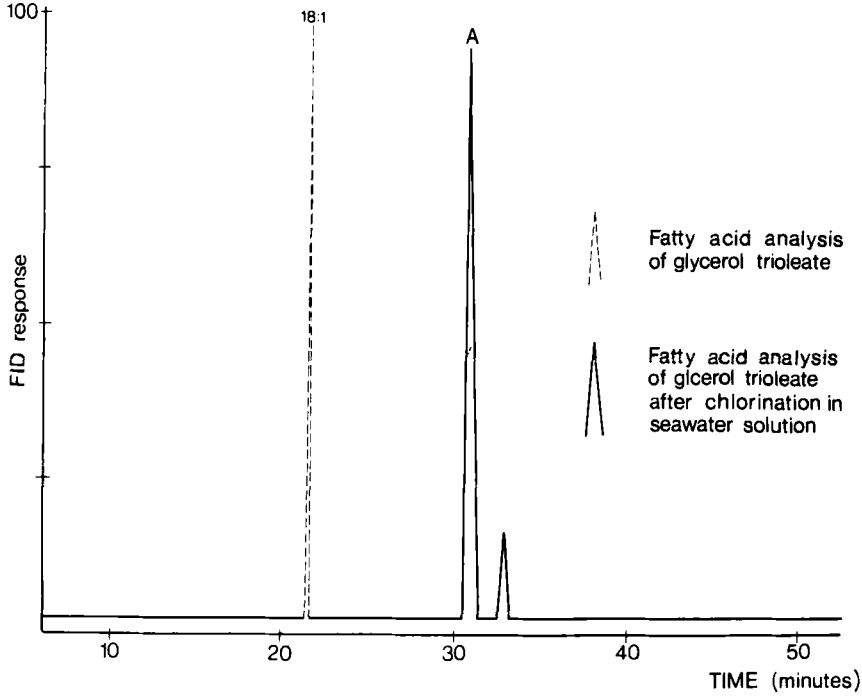


FIGURE 6 Fatty acid analysis of glycerol trioleate before and after chlorination in sea water.

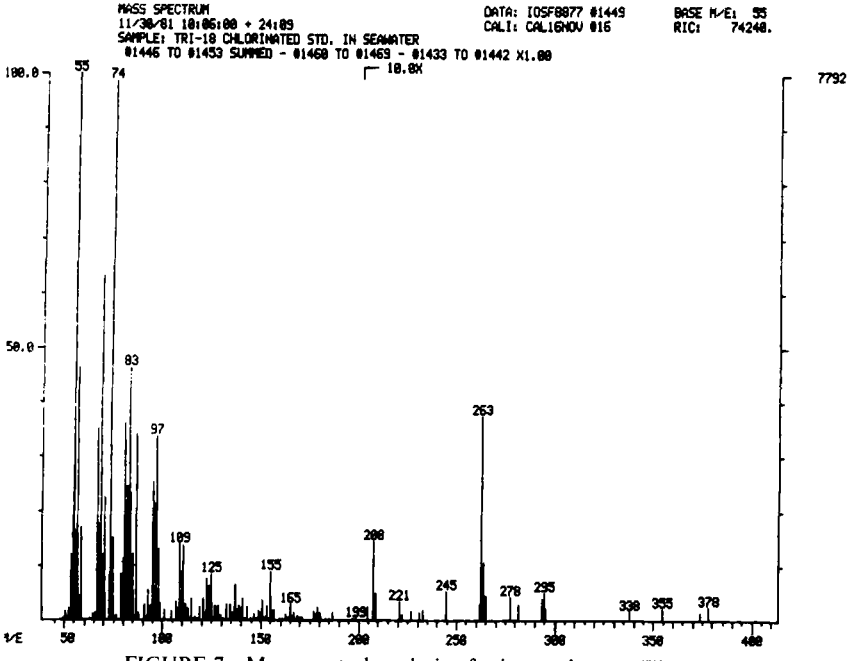


FIGURE 7 Mass spectral analysis of minor unknown (Figure 6).

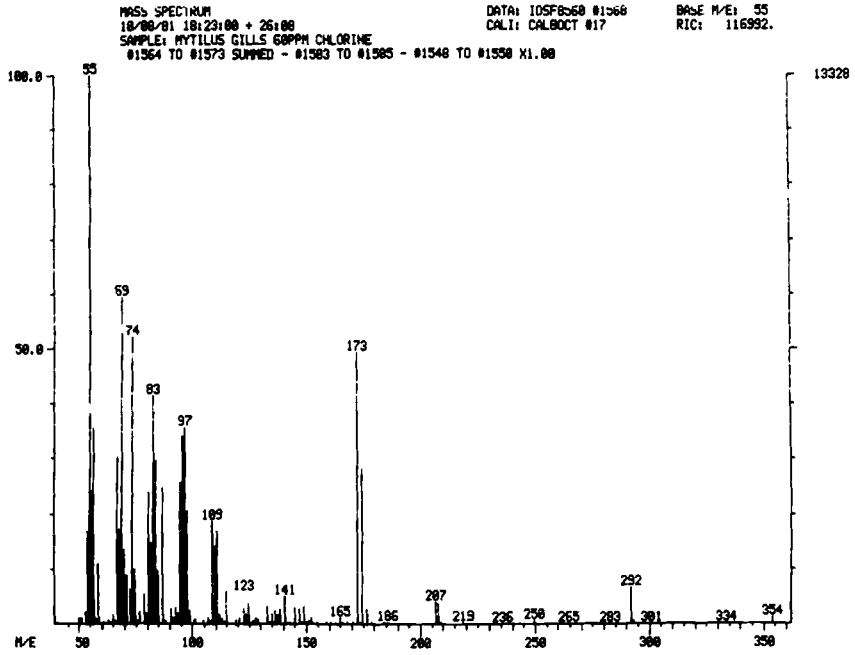


FIGURE 8 Mass spectral analysis of unknown in 60 ppm Cl_2 *Mytilus* gills (same retention time as unknown A, Figure 1).

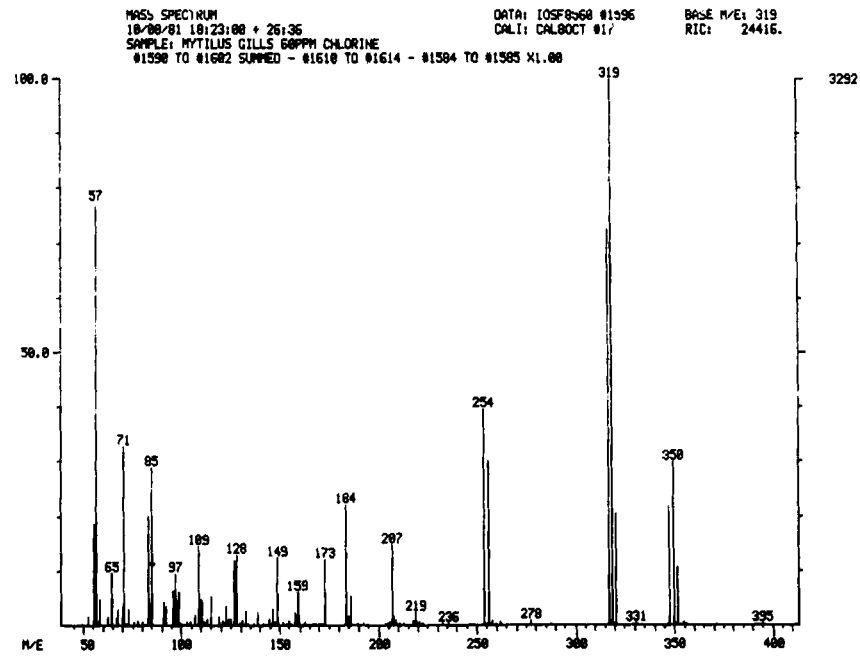


FIGURE 9 Mass spectral analysis of unkown in 60 ppm Cl_2 *Mytilus* gills (same retention time as minor unknown Figure 6).

Chlorination of *Mytilus* gill lipids *in vivo*

The results of the fatty acid analyses on the *Mytilus* gills and bodies are shown in Table I. There is no change in the component fatty acids of the bodies, but in the gills there appears to be a definite loss of unsaturated acids, particularly monounsaturates, relative to the saturated acids after chlorination. This is more noticeable in population 3 (60 ppm "chlorine") than in the population 2 (10 ppm "chlorine").

TABLE I

Fatty acid analysis of *Mytilus edulis* (L) exposed to various levels of "chlorine"

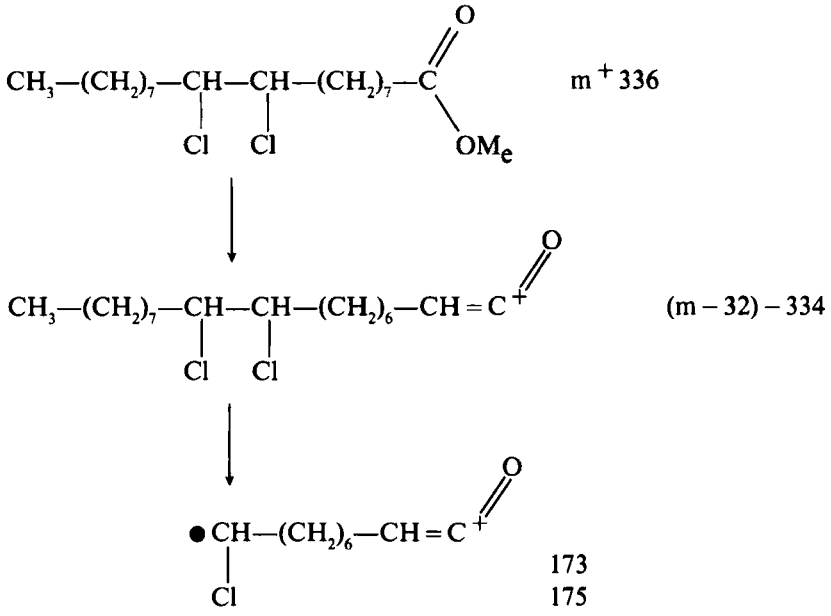
	Control body	10 ppm body	60 ppm body	Control gills	10 ppm gills	60 ppm gills
14:0	4.9	3.8	2.4	4.3	3.1	3.5
14:1	—	T	0.8	1.4	0.7	1.0
16:0	19.6	20.7	20.7	16.1	19.2	27.8
16:1 ω 7	12.7	12.7	11.2	5.6	5.6	5.6
17:0	1.5	1.6	2.4	3.2	4.2	3.6
17:1	T	—	—	1.0	T	T
18:0	2.9	3.2	5.2	8.0	9.0	7.0
18:1 ω 11)[a] ω 9)	9.2	8.5	8.6	8.4	6.1	4.9
18:2 ω 6	3.7	4.2	4.7	2.8	3.3	2.9
18.3 ω 3	7.9	7.1	6.3	2.4	2.6	3.4
18:4 ω 1	T	T	T	1.8	0.4	0.7
20:1 ω 11	2.5	2.8	2.4	3.8	3.7	3.1
20:4 ω 6	2.0	2.2	2.0	4.6	3.9	2.7
20:5 ω 3	18.4	20.1	20.6	16.1	17.8	18.7
20:1 ω 11)[a] ω 13)	2.0	2.2	2.0	5.6	5.6	3.6
22.6 ω 3	9.7	10.0	10.3	15.6	15.0	13.1
Saturated	29.9	30.3	31.5	32.8	35.4	41.9
Monounsaturated	27.4	26.8	25.5	25.5	22.2	18.6
Polyunsaturated	42.7	42.9	43.0	41.7	42.4	39.5

[a] mixture of isomers

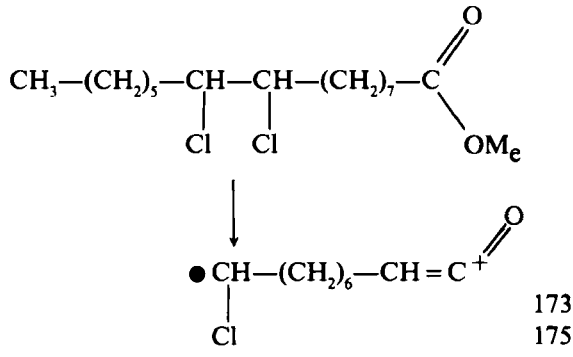
A range of minor unknown peaks appeared during the GC analysis of the chlorinated gills. Some of their retention times corresponded to those found for the unknown compounds in the chlorinated glycerol trioleate and cod liver oil. GC-MS analysis of these peaks indicated the presence of relatively stable chlorinated molecular ions or mass fragments. The unkown which has the same retention time as the postulated chlorinated 18:1 ω 9† acid (see

† The number preceding the colon gives the number of carbon atoms in the chain. The number of double bonds is indicated by the number following the colon. The number following the Greek letter omega (ω) denotes the number of carbon atoms between the terminal methyl group and the middle of the double bond nearest the terminal methyl group.

earlier) unknown A Figure 1; unknown B Figure 2) gave a stable isotopic mass fragment (173^+ 175^+, ratio 2:1) (Figure 8). This is believed to originate from the following fragmentation and re-arrangement of the dichlorinated 18:1 ω methyl ester:



A similar isotopic mass fragment was found in a peak whose retention time corresponded to the postulated chlorinated 16:1ω7 in the cod liver oil. The proposed fragmentation and rearrangement given above would give the same 173/175 fragment from this molecule



Mass spectrometric scanning of the area corresponding to the minor unknowns (see Figure 6) gave an isotopic molecular ion 350^+ 352^+ (ratio 3:1) with a base peak at 319 (Figure 9). The molecular ion would correspond to a hydrated (H_3O^+) chlorine containing 18:1 methyl ester, the base peak arising from the common m-31 fragmentation (loss of OMe).

It would appear that in the *Mytilus* experiments more stable chlorine containing compounds were formed than in the simple experiments with standard solutions. This could however merely be the result of different times of exposure, or the method of chlorination used (i.e., Cl_2 gas vs hypochlorite). Certainly pH would be expected to differ according to which of these treatments was utilized. The explanation of these apparent differences in the stability of the chlorinated species is not clear and must be the subject of more detailed study.

CONCLUSION

We believe this present work has demonstrated that chlorine and aqueous chlorinated species rapidly and readily form addition compounds with unsaturated lipids *in vivo* as well as *in vitro*. In organic solutions the dichlorinated compound is formed, but in aqueous solutions chlorine hydrins are also present. The ability of chlorine to react with bromide in sea water has been observed (Wong and Davidson, 1977). However, we did not see any evidence for the formation of brominated compounds in the *Mytilus* gills during these present short-term experiments.

Although chlorine addition reactions are found, in the present work, to occur at relatively high levels of chlorination, the periods of exposure were short, and in the case of the *Mytilus* experiments significant changes to the unsaturated-saturated lipid pattern of the gills is evident. Such changes must produce a change in the physical properties of the gill membrane and this is likely, we believe, to affect the function of these membranes.

In terms of the relevance of these findings to the possible environmental effects of chlorination, it is necessary to distinguish between discharges of chlorinated fresh water and discharges of chlorinated sea water. Chlorinated fresh water will contain a range of reactive chlorine species. Therefore in the receiving waters of such discharges, the unsaturated lipids of the indigenous organisms may well form addition compounds with the chlorine species.

In the case of the chlorinated sea water discharges normally associated with coastal power stations, the levels of chlorination are generally low enough (0.4–1 ppm at the condensers) to ensure that all of the chlorine in the cooling sea water has exchanged with the bromide (Hostgaard-Jensen *et*

al., 1977; Wong and Davidson, 1977; Wong, 1982) to give various bromine species prior to its discharge. The present work is not relevant to such situations. However, although not used in continuous chlorination, relatively high levels of chlorine dosing are used when "slugging" a power station cooling system intermittently. Levels of up to 5 ppm against mussels and crustaceans and 12 ppm for eels and jellyfish are reported by Mclean (1973). Even higher doses are reported by Straughlan (1972) where 20 ppm allowed control of fouling organisms in a tropically sited power station. When such chlorine "dosings" are employed the capacity of the bromide in the sea water to exchange with the chlorine may become saturated (Wong, 1982) and chlorine species will be discharged with the potential to form addition compounds with unsaturated lipids. This is more likely to occur in estuarine situations where salinity and therefore amount of bromide ion is reduced.

As already discussed, many aquatic organisms have a biological mechanism for coping with loss of membrane fluidity by the increase in the unsaturated lipid content of the affected membranes. Whilst this mechanism may be able to compensate for the effects of short-term exposure to chlorine species on membrane lipids, the position regarding long-term exposure is very different. In this situation, we believe the adaptive biochemical process of increasing the levels of unsaturated fatty acids to compensate for loss of fluidity would be to no avail, as the chlorine would complex with the newly-formed unsaturated acids as quickly as they were laid down in the membranes.

There are some behavioural observations which tend to confirm these hypotheses. During other experiments with *Mytilus*, similar in nature to those described here, the gill cilia are seen to stop rapidly (within 3 minutes) when the animal is placed in chlorinated water (20 ppm). The gills appear to recover after 24 hours in clean water if the initial chlorination period was short. Repeated dosing of the animals with chlorine however causes a complete cessation of gill action.

The apparent ability of chlorine to "saturate" membrane lipids could conceivably be of direct relevance to the effects of chlorine/chlorination on humans if the discomforture which many people suffer from irritated and dry nasal throat and corneal membranes after bathing in chlorinated swimming pools is also due to such an effect. Recovery from such ailments normally takes some hours.

We believe that further work on interaction of chlorine and natural product lipids is clearly necessary. The effect of long term exposure of aquatic organisms to low levels of chlorine species must be studied in relation to the lipid composition of the organisms' membranes. In addition the possibility of the bromine species (originating from the reaction of bromide with chlorine) reacting with unsaturated lipids should be investigated.

Acknowledgements

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