Uptake, turnover and distribution of chlorinated fatty acids in aquatic biota

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Abstract

Chlorinated fatty acids (ClFAs) are the major contributors of extractable, organically bound chlorine in fish lipids. A known anthropogenic source of ClFAs is chlorine bleached pulp production. Additional anthropogenic sources may exist, e.g., chlorine-containing discharge from industrial and house-hold waste and they may also occur naturally. ClFAs have a wide geographic distribution. They have, for instance, been identified in fish both from Alaskan and Scandinavian waters. In toxicological studies of ClFAs, the most pronounced effects have been found in reproductive related processes. ClFAs have also been shown to disrupt cell membrane functions. The present study was carried out to further characterise the ecotoxicological properties of ClFAs and their presence in biota.

To investigate the biological stability of ClFAs, two experiments were carried out using radiolabelled chlorinated and non-chlorinated fatty acids. In both experiments, ClFAs were taken up from food by fish and assimilated to lipids. From the first experiment it was concluded that the chlorinated fatty acid investigated was turned over in the fish to a lower degree than the non-chlorinated analogue. In the second experiment, the transfer of a chlorinated fatty acid was followed over several trophic levels and the chlorinated fatty acid was transferred to the highest trophic level.

In samples with differing loads of persistent organic pollutants (POPs) from both fish and marine mammals, high concentrations and diversity of ClFAs were detected. This was also observed in samples with low POP concentration. Chlorohydroxy fatty acids made up a considerable portion of the ClFAs in certain samples, both from limnic fish and marine mammals.

ClFAs in fish were found to be bound in complex lipids such as triacylglycerols (storage lipids) and phospholipids, as well as in acyl sterols (membrane lipids). In the marine mammals investigated, high concentrations of ClFAs were mainly bound in phospholipids.

If ClFAs are assimilated like "normal" fatty acids, are incorporated into membrane lipids, and are recalcitrant to catabolism, they may thus give rise to ecotoxicological effects when released to the environment and accumulated in biota.

Key words

Chlorinated fatty acids, chlorohydrins, lipid, phospholipid, membrane, respiration, food chain, metabolism, fish, porpoise

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Chlorine is a nasty stuff
to the nose it's rather rough.
Reaching to the stratosphere
it kills the ozone present there.
When the chlorine goes organic
there is reason for you to panic.
Crawling through food's web and chain
it eventually reaches your membrane.
Where it sits and sits and sits and sits,
sits and sits and never quits.
Until the germs and worms consume you
and to the food-web then returns you.

Contents

List of papers

This thesis is based on the following papers, which are referred to by their Roman numerals. Paper I is reprinted with permission from the publisher.

I. Björn, H., Sundin, P., Wesén, C., Mu, H., Martinsen, K., Kvernheim, A.L., Skramstad, J. & Odham, G. 1998. Chlorinated fatty acids in membrane lipids of fish. *Naturwissenschaften*, **85**:229-232.

II. Björn, H., Ewald, G., Sundin, P., Wesén, C., Skramstad, J. & Frøyen, P. Metabolic fate of ^{14}C-labelled chlorinated and non-chlorinated fatty acids in goldfish (*Carassius auratus*). (*Submitted*)

III. Björn, H., Wesén, C. & Sundin, P. Food chain transfer of radio-labelled dichlorostearic acid and oleic acids. (*Manuscript*)

IV. Björn, H., Börjesson, P., Wesén, C., Agrell, C. & Sundin, P. Chlorinated fatty acids and persistent organic pollutants in muscle lipids of harbour porpoise (*Phocoena phocoena*). (*Manuscript*)

V. Vereskuns, G., Sundin, P., Wesén, C., Mu, H., Björn, H., Odham, G., Klavins, M. & Göransson, A. Chlorinated fatty acids and persistent organic pollutants in pike (*Esox lucius*) from Latvian lakes and from the Baltic. (*Manuscript*)

Background

Industrial activities during the last decades have resulted in increased load of chlorinated compounds in the environment (e.g., Walker *et al.* 1996). Among those compounds, the persistent, organochlorine pollutants such as PCBs, DDT and chlorinated dioxins have attracted considerable attention. This is mainly due to their ability to accumulate in biota and cause physiological disturbances (e.g., Walker *et al.* 1996). However, in fish and other aquatic organisms, only up to 5% of the total extractable, organically bound chlorine (EOCl) derives from known, persistent pollutants (Lunde *et al.* 1976, Södergren *et al.* 1988, Håkansson *et al.* 1991, Newsome *et al.* 1993). Instead, it has been shown that the major part of chlorine predominates in chlorinated fatty acids (ClFAs) (Wesén *et al.* 1990, Wesén *et al.* 1992, Wesén *et al.* 1995a, Mu *et al.* 1996b). Despite this, ClFAs have not played any major part in research the last few years. The reasons for this are connected to both the compound itself and the methods of analysis. ClFAs are hard to detect with the methods commonly applied in environmental research (Mu 1996). Because ClFAs are lipids they are usually destroyed when samples are prepared for the analysis of persistent pollutants. And the detection method most commonly used (gas chromatography with electron capture detection) is unsuitable for the detection of ClFAs (Sundin *et al.* 1992, Wesén *et al.* 1992).

The origin of ClFAs is still unclear, but many possible sources exist. One of the anthropogenic sources that has been documented so far is the production of chlorine bleached paper pulp (Leach & Thakore 1977, Voss & Rapsomatiotis 1984, Neilson *et al.* 1991). Unsaturated fatty acids are rapidly chlorinated over the double bond by for instance Cl_2, ClO_2 or HOCl and the incorporation of chlorine is often proportional to the number of double bonds (Ghanbari *et al.* 1980, 1982, Gibson *et al.* 1986, Onodera *et al.* 1992). Thus, it is likely that chlorination of drinking water, disinfection of food, and chlorine-containing discharges of industrial and house-hold waste can act as anthropogenic sources of ClFAs as well (Komo-Suwelack *et al.* 1983, Murphy & Perry, 1983, 1984, 1987, Omori *et al.* 1987, Schwack 1988,

Jernelöv 1989). However, the fate of chlorine in the environment includes a large natural production of chloroorganic compounds too (e.g., Grimvall 1995) and certain species of ClFAs are known to be synthesised by organisms (White & Hager 1977, Neidleman & Geigert 1986).

Besides having such a diffuse origin and being difficult to detect (with common methods), assessing the fate and behavior of ClFAs as pollutants for aquatic organisms has to be done at least partly in a different way than is usually done for assessing the uptake and toxicity of anthropogenic compounds. The reason for this being due to the qualities that ClFAs possess as lipids, thus not accumulating passively from water to organisms. These conditions might have limited previous ecotoxicological investigations of ClFAs. The present study is an attempt to further characterise of the ecotoxicological properties of ClFAs, as well as the presence and distribution in different aquatic organisms.

* In Papers I, IV & V the distribution of ClFAs between different lipid fractions of fish and mammals is examined.

*In Papers II & III the biochemical stability of certain species of ClFAs is studied.

*In Paper IV the amount and diversity of ClFAs as well as the quantity of certain persistent chlorinated pollutants in a species of a marine mammal (at a high trophic level) is accounted for.

*In Paper V the amount and diversity of ClFAs as well as the quantity of certain chlorinated pollutants in a species of limnic fish (at a high trophic level) are considered.

*

Methods

As implied above, methods used for analysing ClFAs are often more demanding than those used for identifying persistent anvironmental pollutants. The methods to determine ClFAs have been reviewed by Mu *et al.* (1997b). Below is a short summary and explanation of some of the methods used for the determination of ClFAs in Papers I-V.

Transesterification

After extraction of the lipid matrix from the samples under investigation, the fatty acids have to be converted to their corresponding methyl esters (FAMEs). This is done in order to release the fatty acids from the complex lipids in which they are bound, as well as to make them volatile and suitable for gas chromatographic analysis.

Enrichment

Often ClFAs are in low concentrations compared to unchlorinated fatty acids. The column in a gas chromatograph (GC) can be overloaded if too large a sample is injected to obtain a response for the ClFAs. Even if the detector selectively can trace the ClFAs, the retention of the sample compounds will be affected resulting in unreliable gas chromatograms. In order to avoid this, an enrichment procedure is normally employed (Mu *et al.* 1996b). By the enrichment procedure the polyunsaturated FAMEs as well as saturated, straight-chain FAMEs in the samples are removed in two steps. This results in an up to 30 times higher concentration of ClFAs in the sample. This method was also used in Paper III for radioactive samples. By enriching the samples it could be shown that the radioactivity in the samples was bound to ClFAs.

Detection

For gas chromatographic analysis of halogenated fatty acids, a GC with electrolytic conductivity detector (ELCD) can be used. This is suitable because the ELCD can be operated

in the halogen mode, responding only to halogenated compounds and giving a response directly proportional to the halogen content of the sample. When chlorinated fatty acids in a sample have been detected by ELCD, different mass-spectrometric techniques can be applied for their identification (Sundin *et al.* 1992, Mu *et al.* 1996a).

Distribution of chlorinated fatty acids

ClFAs have a wide geographic distribution. They have so far been detected in fish from Scandinavian, Alaskan and Baltic waters (Wesén *et al.* 1995a, Mu 1996, Papers I, V), bivalves from Scandinavian waters (Wesén *et al.* 1995b), lobster from the Canadian east-coast (Milley *et al.* 1997), and marine mammals from the Swedish west-coast (Paper IV, Clark *et al.* 1999).

The distribution of ClFAs throughout the body of fish may not differ significantly from that of unchlorinated fatty acids. In an autoradiography study with perch, radioactivity from dichlorostearic acid was taken up and incorporated in lipids of all organs studied, including brain tissue (Ewald *et al.* 1996).

ClFAs are present both in triacylglycerols (nonpolar storage lipids) and phospholipids (polar membrane lipids) of different fish species such as eel, salmon, pike and herring (Wesén 1995, Mu 1996, Paper I, Paper V) as well as in acyl sterols (nonpolar membrane lipids) of pike (Paper V). ClFAs have also been detected at various concentrations in triacylglycerols and phospholipids from muscle lipids and in triacylglycerols from blubber of porpoise (Clark *et al.* 1999, Paper IV). Migratory fish like salmon and eel store triacylglycerols in the muscles and adipose tissue. These lipid deposits also contain the major load of ClFAs, whereas the concentration of ClFAs seems to be higher in phospholipids than in triacylglycerols both in the studied fish and mammals (Papers I, V & IV).

Uptake, turnover and biochemical properties of chlorinated fatty acids

The bioconcentration factor for ClFAs is reported to be low (Craig *et al.* 1990). However, fatty acids are desirable biomolecules for heterotrophic organisms in that they are high in energy. The biochemical processing of ingested lipids during digestion leaves the fatty acids intact throughout the absorption in the gastrointestinal tract, and during transport to and into the target cells. The most common catabolism of a fatty acid takes place through ß-oxidation in the mitochondria, where the fatty acid is oxidised from the carboxyl end (Fig. 1) in steps of two carbon units being split off at a time, then entering the Krebs cycle as acetyl-coenzyme A. Fatty acids can also be catabolised at the terminal end through ω-oxidation, which takes place mainly with highly substituted molecules (Gurr & Harwood 1996).

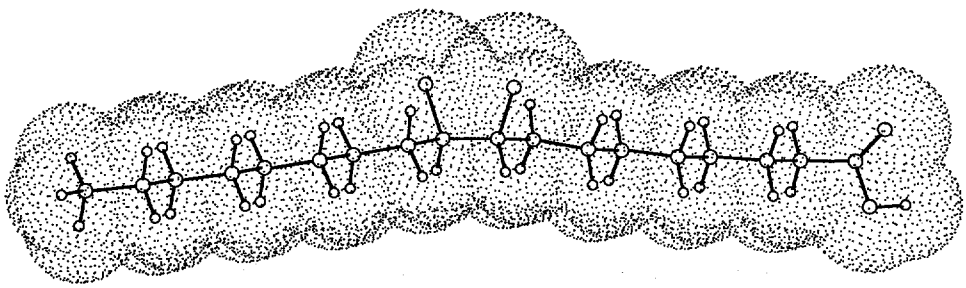

Figure 1. A space-filling projection of dichlorostearic acid, with the carboxyl end to the right.

Results of studies of uptake and assimilation of ClFAs in organisms suggest that ClFAs can participate in fatty acid biochemical pathways, and thus become incorporated into cellular lipids in a manner similar to that of unchlorinated species (e.g., oleic acid). Rats have been used in several uptake and assimilation studies, showing that chlorinated fatty acids are taken up (Cunningham & Lawrence 1976, Cunningham & Lawrence 1977a), transferred to foetuses in pregnant rats (Cunningham & Lawrence 1977b) and assimilated by suckling rats from milk (Cunningham & Lawrence 1977b). Murphy and Perry (1983, 1986) showed that certain

microorganisms are able to distribute chlorinated fatty acids specifically into cellular phospholipids. In salmon (*Oncorhynchus nerka*), ClFAs are transferred to the developing roe together with other lipids and do not seem to be discriminated against in this process (Mu 1996). A few experiments using fish have shown that chlorinated fatty acids can be taken up with little obstruction via food (Håkansson *et al.* 1991, Ewald *et al.* 1996). In an assimilation experiment with perch, radiolabelled dichlorostearic acid was compared to stearic and oleic acids (Ewald *et al.* 1996). No significant discrimination was observed with respect to the chlorinated fatty acid in comparison to a non-radiolabelled compound. Moreover, dichlorostearic acid was incorporated into complex lipids and distributed in the bodies similarly to the unchlorinated fatty acids. Also in the experiments described in Papers II & III, dichlorostearic acid was taken up to a similar extent as oleic acid and incorporated into lipids by fish (Paper II) and taken up by chironomid larvae (Paper III).

As described earlier, ClFAs are usually bound to a higher degree in polar than in neutral lipids. Part of the reason for this may be that most polar lipids are components of membranes and once incorporated into the membrane, the turnover of ClFAs may be lower than that of other fatty acids. This has been implied earlier in a study of migrating sockeye salmon (*Oncorhynchus nerka*) in Alaska (Mu 1996). The ClFAs in triacylglycerols from muscle lipids were released to the same extent as the unchlorinated ones during a 400 km spawning migration. However, the total amount of chlorinated fatty acids increased in roe and in muscle phospholipids. The finding indicates a lower turnover of chlorinated fatty acids in phospholipids, since the total mass of muscle phospholipids decreased concurrently.

The structural configurations of ClFAs are similar to those of "normal" fatty acids, but with the difference that chlorine atoms are substituted for hydrogen atoms at certain positions in the carbon chain (Fig. 1). Among the many species of chlorinated fatty acids identified so far, dichlorostearic acid is one of those most commonly found (Wesén 1995, Wesén *et al.* 1995a,

Mu *et al.* 1996ab, 1997a, Papers I, V). In 9,10-dichlorostearic acid, the relatively large chlorine atoms are placed in the middle of the molecule (Fig. 1).

Ewald & Sundin (1993) suggested that the chlorine atoms in a chlorinated fatty acid introduced a bulkiness to the molecule that could sterically hinder the enzyme involved in ß-oxidation. Dichloromyristic acid (of 14 carbon atoms chain length) was found to be the main metabolite when rats were fed dichlorostearic acid (18 carbon atoms) (Conacher *et al.* 1984). Recent results also confirm that no metabolites shorter than dichloromyristic acid are found when human cells are exposed to dichlorostearic acid (Gustafson-Svärd *et al.* 1999). In fish, there are indications that the relative composition of chlorinated fatty acids changes with the distance to polluted areas. In fish sampled near discharge of chlorine bleached pulp mill effluents, long-chain (16 to 18 carbons) chlorinated fatty acids predominated. Further away from the polluted area, dichloromyristic acid dominated (Wesén 1995). One reason for this may be that fish can not easily degrade dichloromyristic acid. In Paper II an experiment was done to test the "biological stability" of dichlorostearic acid. ^{14}C-labelled chlorinated fatty acids or oleic acids were fed to goldfish and the respiration of $^{14}CO_2$ and incorporation of lipid bound radioactivity was measured. The results imply that dichlorostearic acid was partly degraded to at least 16-carbon chain length, and that the turnover of dichlorostearic acid was lower than that of oleic acid (Fig. 2 and Fig. 3 in Paper II), possibly because ß-oxidation is impeded by the chlorine substituents in the molecule. Also the experiment described in Paper III addresses the biological stability of dichlorostearic acid by testing its transfer through a food-chain in comparison to oleic acid. A food-chain consisting of chironomid larvae, goldfish and and pike was set up and the chironomids were fed either dichlorostearic acid or oleic acid. Again, dichlorostearic acid had a lower turnover, and was transferred throughout the food-chain to a higher extent than oleic acid (Fig. 1 in Paper III). Thus, dichlorostearic acid may possess a certain biological stability.

Although the experiment described in Paper II shows that dichlorostearic acid was partly degraded to a chain length of at least 16-carbon, in the food-chain experiment (Paper III) no metabolites could be detected by GC (Fig. 3 in Paper III). There may be several explanations for this. Goldfish were used in both experiments and they were being fed the compounds under investigation for a comparable period of time. However, the food-chain experiment was carried out at a lower temperature than the experiment done in Paper II, which might be a reason for not detecting any metabolites. The pikes in the food-chain experiment were being fed for a considerable longer time (about five months), but still no metabolites were detected. Pike is a leaner fish than goldfish and has very low concentrations of triacylglycerols (Henderson & Tocher 1987). Instead, a high amount of the lipids are in the form of phospholipids (membrane lipids), and again the incorporation of ClFAs into membrane lipids may slow down the turnover of the molecules, and thus only low concentrations of metabolites occur. Also, only muscle samples from the pike were investigated in comparison to whole fish (except intestines) being extracted in case of goldfish.

In both Papers IV & V many of the ClFAs found were monochlorohydroxy fatty acids, most likely in the form of chlorohydrins (one chlorine and one hydroxy group introduced over the double bond). Chlorohydrins are among the species of ClFAs that have been suggested to occurr naturally (White & Hager 1977), but they are also formed during treatment of drinking water with HOCl (Gibson *et al.* 1986, Onodera *et al.* 1992). Chlorohydrins may also be formed in the mammalian cellular defense system (Neidleman & Geigert, 1986). Certain types of leukocytes that are stimulated upon infections, are known to produce hypochlorous acid, which in turn can react with unsaturated fatty acids and produce for instance chlorohydroxy fatty acids. This is thought to be of importance both in inhibiting microorganisms (by impaired membrane function) in addition to cytotoxicity towards tumour cells (Winterbourn *et al.* 1992, Carr *et al.* 1996). Whether the origin of the chlorohydroxy fatty acids detected (Papers IV, V) is connected to any kind of infection in the individuals analysed is not known.

Physiological impact of chlorinated fatty acids

Because ClFAs are bound in complex lipids such as triacylglycerols and phospholipids (Paper I) they, theoretically, have a potency to disturb any biological process involving lipids. This includes, for instance, the roles lipids have as barriers and as carrier molecules but also their importance in metabolic control and as chemical messengers (e.g., Stryer 1988). The acute toxicity of dichlorostearic acid is relatively high in fish (Leach & Thakore 1977), but low in rat (Cunningham 1980), and the studies done so far indicate that chlorinated fatty acids mainly exert toxicity in reproductive related processes (Cherr *et al.* 1987, Håkansson *et al.* 1991, Björn *et al.* 1998). In an *in vitro* experiment, free fatty acids released from lipid fractions with a high chlorine content caused a reduction of the arachidonic acid-stimulated testosterone production in goldfish testes (Björn *et al.* 1998). Wilson *et al.* (1983) showed in an experiment with rats that *brominated* fatty acids altered the ratio between the natural, essential fatty acids in a similar manner as DDT (Tinsley & Lowry 1971). The changed ratio of essential fatty acids was proposed to cause disturbances in the production of arachidonic acid (Wilson *et al.* 1983). Arachidonic acid is a precursor for the formation of both leukotrienes and prostaglandins, and it has been proposed that chlorohydroxy fatty acids may inactivate leukotrienes and prostaglandins, both being important biomolecules in the body (Winterbourn *et al.* 1992).

Ewald and Sundin (1993) suggested that chlorine atoms not only create a bulkiness in the chlorinated fatty acids (Fig. 1), but also alter the conformation of the molecules. Chlorination of an unsaturated fatty acid normally occurs over the double-bond. The chlorine atoms in the resulting dichlorinated fatty acid give rise to a restriction in the rotation around the -CHCl-CHCl- bond, which gives the carbon chain a low-energy conformation resembling that of a monounsaturated fatty acid. After exposing mammalian cells to mono- and dichlorostearic acids, ATP leakage increased, which possibly resulted from an impact on the membrane

functions. The ratio between saturated and unsaturated fatty acids in phospholipids is an important factor that determines membrane fluidity and permeability, and a change in this ratio or incorporation of bulky fatty acids may cause increased leakage of cellular components, as discussed by Ewald & Sundin (1993). Thus, because dichlorostearic acid seems to be assimilated similarly to unchlorinated analogues, membrane-related, physiological malfunctions may result from exposure to chlorinated fatty acids.

Although ClFAs may have adverse effects on organisms, they do not seem to elicit elimination efforts by organisms. Common ways of assessing the physiological impact of traditional pollutants are to investigate the detoxifying hepatic enzyme system, cytochrome P_{450}, and the activity of the hepatic enzyme EROD (7-ethoxyresurofin O-deethylase). Neither of these systems are activated by ClFAs (given as fish lipids with a high chlorine content or as dichlorostearic acid), suggesting that chlorinated fatty acids are not recognised by organisms as xenobiotics (Håkansson *et al.* 1991, Goksøyr & Larsen 1993).

ClFAs and persistent organochlorine pollutants seem to possess much of the same qualities from an ecotoxicological point of view (i.e. resistance to degradation, potential for concentration in biota and toxicity to biota). Nevertheless, there is an important difference in that ClFAs do not seem to be recognised by biological systems as xenobiotics, despite the adverse effects they might have on the organism. If chlorinated fatty acids are broken down only to some degree and remain in the body as biochemically active molecules, they may cause implications up to the ecosystem level due to altered fitness of species.

Conclusions

*ClFAs are incorporated into membrane lipids, often to higher concentrations than in triacylglycerols.

*Dichlorostearic acid is taken up by certain benthic invertebrates from food, and taken up and assimilated by fish from food.

*Dichlorostearic acid may be recalcitrant to a complete biological degradation and possess a certain biological stability and thus has the potency of being bioaccumulated.

*A high diversity of ClFAs can be found in marine mammals differing in load of persistent organic pollutants.

*A high diversity of ClFAs can be found in fish from waters relatively unpolluted by persistent organic pollutants, though with a different gas chromatographic pattern than in fish from waters with a relatively high load of persistent organic pollutants.

*Chlorohydroxy fatty acids can make up a considerable portion of ClFAs.

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The following is the list of Doctoral theses from the Department of Chemical Ecology and Ecotoxicology, University of Lund, Sweden.

1. ANDERS SÖDERGREN, Transport, distribution and degradation of organochlorine residues in limnic ecosystems (defended at the Dept. of Limnology). May 23, 1973.
2. GÖRAN BENGTSSON, Ecological significance of amino acids and metal ions, a microanalytical approach (defended at the Dept. of Zooecology). May 24, 1982.
3. CARITA BRINCK, Scent marking in mustelids and bank voles, analyzed of chemical compounds and their behavioral significance (defended at the Dept. of Zooecology). May 17, 1983.
4. ANDERS TUNLID, Chemical signatures in studies of bacterial communities. Highly sensitive analyses by gas chromatography and mass spectrometry. October 3, 1986.
5. ANDERS THURÉN, Phthalate esters in the environment: analytical methods, occurrence, distribution and biological effects. November 4, 1988.
6. PETER SUNDIN, Plant root exudates in interactions between plants and soil microorganisms. A gnobiotic approach. March 16, 1990.
7. ANDERS VALEUR, Utilization of chromatography and mass spectrometry for the estimation of microbial dynamics. October 16, 1992.
8. HANS EK, Nitrogen acquisition, transport and metabolisation in intact ectomycorrhizial assocoations studied by ^{15}N stable isotope techniques. May 14, 1993.
9. ROLAND LINDQUIST, Dispersal of bacteria in ground water—mechanism, kinetics and consequences for facilitated transport. December 3, 1993.
10. ALMUT GERHARDT, Effect of metals on stream invertebrates. February 17, 1995.
11. OLOF REGNELL, Methyl mercury in lakes: factors affecting its production and partitioning between water and sediment. April 21, 1995.
12. PER WOIN, Xenobiotics in aquatic ecosystems: effects at different levels of organization. December 15, 1995.
13. GÖRAN EWALD, Role of lipids in the fate of organochlorine compounds in aquatic ecosystems. October 18, 1996.
14. JOHAN KNULST, Interfaces in aquatic ecosystems: Implications for transport and impact of anthropogenic compounds. December 13, 1996.
15. GUDRUN BREMLE, Polychlorinated biphenyls (PCB) in a river ecosystem. April 25, 1997.
16. CHRISTER BERGWALL, Denitrification as an adaptive trait in soil and groundwater bacteria. November 14, 1997.
17. ANNA WALLSTEDT, Temporal variation and phytotoxicity of Batatasin-III produced by Empetrum hermaphrodytum. November 27, 1998.
18. DARIUS SABALIUNAS, Semipermeable membrane devices in monitoring of organic pollutants in the aquatic environment. April 28, 1999.
19. CECILIA AGRELL, Atmospheric transport of persistent organic pollutants to aquatic ecosystems. May 21, 1999.
20. OLOF BERGLUND, The influence of ecological processes on the accumulation of persistent organochlorines in aquatic ecosystems. Sepetember 17, 1999.
21. HELENA BJÖRN, Uptake, turn-over and distribution of chlorinated fatty acids in aquatic biota. October 1, 1999.